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List of Abbreviations

aa	amino acid	**ACAID**	Anterior Chamber Associated Immune Deviation
ADCC	Antibody-dependent cell-mediated cytotoxicity	**ADCP**	Antibody-dependent cellular phagocytosis
ADP	Adenosine diphosphate	**AML**	Acute myeloid leukemia
AMP	Adenosine monophosphate	**AMPc**	cyclic AMP
APC	antigen presenting cells	**Arg**	Arginine
ARG1	Arginase-1	**ATP**	Adenosine triphosphate
BART	BamHI-A rightward transcripts	**Bat3**	human leukocyte antigen B (HLA-B)-associated transcript 3
BCG	Bacillus Calmette–Guérin	**Bcl2**	B cell lymphoma 2
BCR	B cell receptor	**Bim**	Bcl-2 interacting mediator of cell death
CCDR	C terminal carbohydrate recognition domain	**CCL17**	chemokine ligand 17
CCL2	chemokine ligand 2	**CCL22**	chemokine ligand 22
CCR4	C-C Motif Chemokine Receptor 4	**CCR8**	C-C Motif Chemokine Receptor 8
CD	cluster of differentiation	**CDC**	Complement-dependent cytotoxicity
cDNA	complementary DNA	**Cflip**	Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein
CIA	collagen induced arthritis	**CML**	chronic myelogenous leukemia
COX2	Cyclooxygenase	**CRC**	colorectal cancer
CRD	Carbohydrate recognition domains	**CRISPR**	Clustered regularly interspaced short palindromic repeats
CSF	Colony stimulating factor	**CST**	complementary strand transcripts
CTL	cytotoxic T lymphocytes	**CTLA**	cytotoxic T-lymphocyte-associated protein
CXCR4	C-X-C chemokine receptor type 4	**DC**	Dendritic cell
DDR	DNA Damage Response	**DNA**	Deoxyribonucleic acid
DNAM 1	DNAX accessory molecule	**EBER**	EBV-encoded RNAs
EBi-3	Epstein-Barr virus induced gene 3	**EBNA**	EBV nuclear antigens
ECA	Ecalactin	**Egr-2**	early growth response gene 2
ELISA	enzyme-linked immunosorbent assay	**EMT**	Epithelial mesenchymal transition
ESCRT	endosomal sorting complex required for transport	**Evs**	extracellular vesicles
Fc-region	fragment crystallizable region	**FCRL3**	Fc receptor like 3
FDA	Food and Drug Administration	**Fgl2**	fibrinogen- like protein 2
FLICE	FADD-like IL-1β-converting enzyme	**FNAC**	nasopharyngeal biopsies and fine needle aspiration
FOXN1	Forkhead box protein N1	**Foxp3**	forkhead box protein 3
Gal-9	Galectin 9	**Gal-9L**	Galectin-9 large isoform
Gal-9M	Galectin-9 medium isoform	**Gal-9NULL**	Galectin-9 synthetic truncated isoform
Gal-9S	Galectin-9 small isoform	**GARP**	glycoprotein A repetitions predominant

G-CSF	Granulocyte colony-stimulating factor	**gfp**	green fluorescent protein
GITR	glucocorticoid-induced TNFR-related protein	**GM-CSF**	Granulocyte-macrophage colony-stimulating factor
GrzB	Granzyme B	**GST**	Glutathione S-transferases
HCC	Hepatocellular carcinoma	**HCV**	Hepatitis C virus
hGal-9	human Gal-9	**HHV-4**	human herpesvirus 4
His	Histidine	**HLA**	human leukocyte antigen
HNSCC	Head and neck squamous carcinoma	**HPV**	human papilloma virus
HSV	Herpes simplex virus	**iTregs**	Induced regulatory T cells
ICAM-1	Intercellular Adhesion Molecule 1	**ICOS**	Inducible T-cell costimulator
ICOSL	Inducible T-cell costimulator ligand	**IDO**	Indoleamine 2,3 dioxygenase
IF	Immunofluorescence	**IFN**	Interferon
IFNα/beta	Interferon α/beta	**IFNγ**	Interferon γ
Ig	Immunoglobulin	**IGF-1**	insulin-like growth factor-1
IgG1	Immunoglobulin G1	**IHC**	Immunohistochemistry
IL	Interleukin	**iNos**	inducible Nitric oxide synthases
IPEX	'immune dysregulation, polyendocrinopathy, enteropathy, X-	**IRF3**	interferon regulatory factor 3
ITIM	immunoreceptor tyrosine-based inhibition motif	**ITIM**	immunoreceptor tyrosine-based inhibitory motif
iTreg	induced Treg	**ITT**	immunoglobulin tail tyrosine
KO	Knockout	**LAG-3**	Lymphocyte-activation gene 3
LAP	Latency associated peptides	**LMP**	latent membrane proteins
LPS	Lipopolysaccharides	**LSECTin**	liver and lymph node sinusoidal endothelial cell C-type lectin
M1	Macrophages	**Mab**	Monoclonal Antibodies
MAGE	Melanoma antigen gene	**MAGE-C1**	Melanoma antigen gene protein CT7
MCA	Methylcholanthrene	**MDSC**	Myeloid-derived suppressor cells
Met	Methionine	**MHC**	major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A	**MICB**	MHC class I polypeptide-related sequence B
MIP-1	macrophage inflammatory protein	**miRNA**	micro RNA
MMP3	matrix metalloproteinase-3	**mRNA**	messenger RNA
mTOR	mammalian target of rapamycin	**Muc1**	Mucin 1, cell surface associated
MVB	multivesicular body	**Mves**	multivesicular endosome
Mvs	Macrovesicles	**NAPDH**	Nicotinamide adenine dinucleotide phosphate
NCDR	N terminal carbohydrate recognition domain	**NF-AT**	Nuclear factor of activated T-cells
NF-κB	nuclear factor-κB	**NK**	Natural Killer
NKG2D	Natural Killer Group 2D	**NKT**	Natural killer T cells
NO	Nitric oxide	**NPC**	nasopharyngeal carcinoma
NRP1	Neuropilin 1	**NSCLC**	non-small cell lung carcinoma
nTregs	thymus derived naturally occurring regulatory T cells	**NY-ESO-1**	New York-esophageal cancer-1
ori lyt	lytic origin of DNA replication	**ori p**	origin of plasmid replication
OS	overall survival	**OSCC**	oral cavity squamous cell carcinoma
OX40	Tumor necrosis factor receptor	**PAP**	prostatic acid phosphatase

	superfamily, member 4		
PBMC	peripheral blood mononuclear cell	**pDCs**	Plasmacytoid dendritic cells
PDI	Protein disulfide isomerase	**PDL1**	programmed cell death ligand 1
PFS	progression free survival	**PGE2**	Prostaglandin E2
PGE2	prostaglandin E2	**PI3K**	phosphatidylinositol 3-kinase
PKR	Protein kinase R	**PMA**	phorbol 12-myriastate 13-acetate
PML	promyelocytic leukemia	**PRR**	Pattern recognition receptors
PS	Phosphatidylserine	**PTEN**	Phosphatase and tensin homolog
RA	Rheumatoid arthritis	**RAG2**	Recombination activating gene 2
RNA	Ribonucleic acid	**RISC/ago**	RNA-induced silencing complex argonaute
ROS	Reactive oxygen species	**RSV**	respiratory syncytial virus
siRNA	small interfering RNA	**Smad7**	Mothers against decapentaplegic homolog 7
SNAPE	soluble NSF attachment protein	**SNARE**	SNAP Receptor
snoRNA	small nucleolar RNA	**STAT3**	signal transducer and activator of transcription
TAAs	tumor-associated antigens	**TAM**	tumor associated macrophage
Tconv	conventional T cells	**TCR**	T cell receptor
TCRs	T cell receptors	**Teff**	Effector T cell
T_{FH}	follicular helper T cells	**TGF-β**	transforming growth factor β
Th	T helper cell	**TIGIT**	T cell immunoreceptor with Ig and ITIM domains
TILs	tumor-infiltrating lymphocytes	**Tim-3**	T cell immunoglobulin and mucin domain containing 3
TLR	Toll-like receptor	**TME**	tumoral microenvironment
TNFα	Tumor necrosis factor α	**Tr1**	type I regulatory T cell
TRAF	TNFR-associated factors	**TRAIL**	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell	**TSAs**	tumor specific antigens
VEGF	vascular endothelial growth factor	**WHO**	world health organization

Introduction

I. Immunology

Every organism currently existing on the planet needed to develop mechanisms to defend itself against an almost limitless number of potential invaders. These invaders can be opportunistic parasitic organisms ranging in size from 30 nm viruses which can cause polio, to relatively huge worms that can grow inside the human body to over 100 cm in length, not to mention all the different bacteria in between. These organisms are considered as pathogens whenever they cause dysfunction in the host and start using it as the means to dissipate. This sort of interaction is a perfect example of competition driving evolution which leads the hosts to create defense mechanisms. For this purpose, organisms evolved "immunity" to protect themselves. While at one point it was thought that only complex multicellular organisms had immunity, now we understand that it exists in most life forms. Immunity could be something as simple as the peptidoglycan or cell wall in bacteria, cellulose fibers in plants or the epithelial layers in mammals. These act like borders preventing pathogens from gaining access to the more vital parts of the organisms. More specific immunity designed against a specific set of invaders also exists in varying complexities across different species. Bacteria employ the clustered regularly interspaced short palindromic repeats/Caspase (CRISPR/Caspase) complexes to edit out foreign DNA (Deoxy-ribonucleic acid), plants have the RNA-induced silencing complex/argonaute (RISC/ago) complexes to take care of foreign ribonucleic acid (RNA) while mammals use the T cell receptor/ major histocompatibility complex (TCR/MHC) complexes capable of recognizing almost every possible foreign protein. Admittedly, immunity across different species is interesting to study and understand, yet here we are interested in human immunity.

In general, it is admitted that the more complex the organism is, the more complex their defense mechanisms have to be. In humans, the immune system is crucial for survival. Given the almost infinite number of potential pathogens, it has had to evolve highly complex and dynamic networks of interconnected organs, cells, molecules and pathways. These had to be strong enough to protect the organism from external threats yet specific enough to not damage the organism itself or the beneficial commensal flora that exists in it (1).

The following manuscript will describe the human immune response to cancer focusing on specific regulatory pathways involved in cancer's ability to escape the immune system. In these escape mechanisms, special focus will be given to regulatory T cells (Tregs), exosomes and Galectin-9 (Gal-9) as immunosuppressive actor. Then we will discuss recent advances in cancer immunotherapy. Finally, the manuscript will discuss nasopharyngeal carcinoma (NPC) as the cancer model used in this thesis.

II. Oncogenesis and Immunosurveillance

Cancer is unlike any other pathology encountered by the immune system. First of all, it could not be considered as a single disease but rather as a result of an accumulation of conditions which ultimately lead to a failure in the control of cell proliferation, differentiation and survival. Typically, cancer requires an accumulation of multiple genetic or epigenetic mutations in DNA repair, cell survival, proliferation and motility mechanisms. A heathy cell being exposed to a mutagen would typically undergo DNA repair or apoptosis. However, in some cases, this cell, by accumulating various mutation will eventually form a neoplasmic tumoral mass from which cells could with time detach to form distant metastasis. At this point tumor spreading would prove fatal to the patient (2). Since different tissues exhibit different selective pressures on the mutating cells, cancers arising in different origins tend to accumulate different mutations. Even though certain mutations in key genes *(P53, RAS, MYC, PTEN, and RB)* are fairly common between different tumors they are often accompanied by other mutations which tend to be more unique to the specific tumor (3).

A. Escape from immune response

The hallmarks of cancer represent a description of the totality of events involved in oncogenesis. Although all of these hallmarks are crucial for an effective tumorigenesis, here we are interested in cancer's ability to escape the immune response (Figure 1).

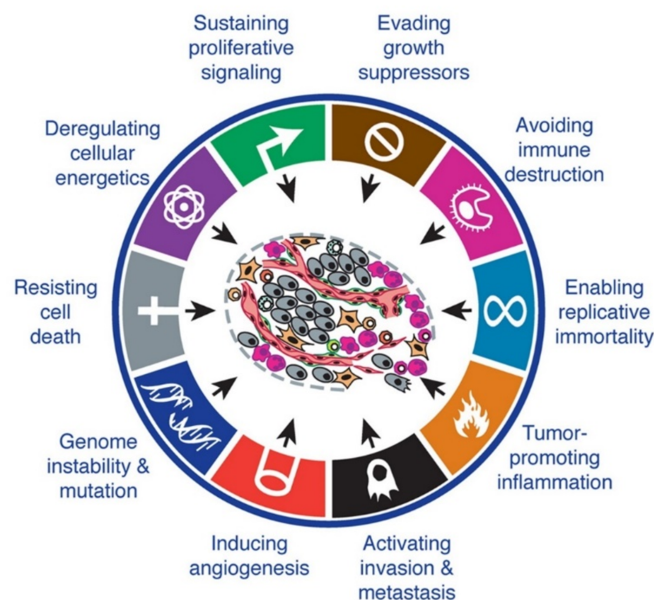


Figure 1 - The next generation of Hallmarks of cancer
Schematic representation of the key features of tumors representing the mechanisms enabling cancerogenesis. (4)

1. Historical background

The link between inflammation and cancer had been observed since 1863 when Rudolf Virchow found leucocytes in neoplastic tissues and correlated inflammation to cancer. He proposed that the "*lymphoreticular infiltrate*" mirrored the origin of cancer at locations of chronic inflammation (5). In 1909, Paul Ehrlich hypothesized that modifications in tumor cells would make them recognized and permit their elimination by the immune system and that it is thanks to the immune system that there is not an overwhelming frequency of carcinomas (6). The work of Peter Medawar in the 1950s on the importance of cellular immunity in allograft rejection was crucial in understanding the importance of antigenic determinants in inducing tumor rejections. Yet proving the presence of such tumor antigens required the development of inbred mice strains in which tumors could be induced either by viral or chemical carcinogens. Methylcholanthrene (MCA)-Induced Sarcomas in inbred mice was the major model at the time (7). Between the years of 1957 and 1965, it was proven that these mice could be immunized against syngeneic transplants of such tumors giving strong evidence supporting of the existence of tumor antigens (8). Very simply, immunosurveillance would not exist if the tumors were indistinguishable from the rest of the body cells. This concept was first suggested by Burnet who in 1957 said: *"It is by no means inconceivable that small accumulations of tumor cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumor and no clinical hint of its existence."* (9)

Despite these notions, the scientific community was far from achieving consensus on the concept of anti-tumoral immunity. Towards the end of the 20th century several key pieces of evidence arose to further defend the concept of anti-tumoral immunity. Thierry Boon and his colleagues showed that tumor antigens exist by using mouse teratocarcinoma cell lines obtained following mutagen exposure. These lines called Tum- were not only incapable of forming a progressive tumor in syngeneic mice but also provided protection against the original parental cell lines which were originally non immunogenic (10). They also showed the interplay between the immune system and cancer by using antigen specific CTLs. This revolutionary work showed that these CTLs would almost completely eradicate the tumor before it would progress again. These results showed that the few tumoral cells which survived the CTLs transferred lost their expression of the targeted antigen leading to their escape from the immune response (11). Later work showed that it was mouse Cluster of Differentiation 8 (CD8+) T lymphocytes which could recognize distinct MHC class I peptide antigens on syngeneic 3-methylcholanthrene (MCA) induced sarcomas. Such peptides were capable of activating a cytotoxic T lymphocyte (CTL) response mainly characterized by Tumor necrosis factor α (TNFα) secretion (12). Moreover, immunocompromised nude mice; knockout (KO) for Forkhead box protein N1 gene FOXN1 were more susceptible to (MCA) induced tumors (13). In the same line of work, Recombination-activating gene 2 (RAG2) KO mice lacking T cells, B cells, and natural killer (NK) T cells were more susceptible to MCA induced sarcomas (14). Many more crucial features of the anti-tumoral immune response; such as perforin expression by T and NK cells, Interferon gamma (IFNγ) secretion and

receptor binding as well as Interferon alfa/beta (IFNα/β) and many other; were discovered using the same MCA induced sarcoma model in different KO mice stains (15-19). Finally, on the other hand, it has been shown that depleting immunosuppressive Tregs improved anti-tumoral immunity (20).

2. The concept of immunoediting

Nowadays, our understanding of the capacities of the immune system to stop cancer has greatly grown and with it our understanding of exactly how cancer manages to subvert the immune response and promote its own survival. In fact, in most healthy individuals, the immune system is actively preventing cancer on a daily basis. For example, the immune system:

- Protects against viruses and virus induced cancers
- Stops prolonged and chronic inflammation caused by certain pathogens and physical traumas which has been shown to be favorable for cancer development
- Directly recognizes mutating and potential cancer cells (21)

However, cancers manage to escape this immune control and even exploit the natural regulatory homeostatic mechanisms of the immune response to further enhance its survival. Hence the theory of immunoediting arose, which illustrates the "dance" between the developing cancer and the immune system. A crucial part in the immune system's ability to stop tumor development is its ability to recognize tumor antigens. Even though the term immunoediting was used for the first time by (22), the basis of the concept of tumor immune escape was laid down by Boon's team. A more recent work done by *Dupage et al*. did elegantly illustrate the theory of immunoediting, along with the importance of the T cell mediated immune response in tumoral control. Briefly they employed model for immunocompetent and immuno-deficient mice for the recombination-activating gene (Rag-/-) engineered to constitutively express activated, oncogenic form of Kras with a floxed p53 tumor suppressor gene. Then they injected the mice with a lentivirus encoding the cre-recombinase plus strong class I model epitopes (SIINFEKL and SIYRYYGL). This led to the outgrowth of SIINFEKL and SIYRYYGL expressing sarcomas in the Rag KO mice. On the other hand, in the immunocompetent mice, the appearance of sarcomas was delayed and when they grew they lacked expression of the model antigens. SIINFEKL and SIYRYYGL expression was edited out due to a T cell dependent mechanism that resulted in the epigenetic silencing of the exogenously introduced genes encoding the model antigens (23). On a more clinical level, a recent study was published on a melanoma patient whose tumor was initially positive for New York-esophageal cancer-1 (NY-ESO-1), Melanoma antigen gene (MAGE) protein CT7 (MAGE-C1) and Melan-A antigens. However, following treatment with a NY-ESO-1 targeting vaccine, ensuing tumoral growth lacked NY-ESO-1 expression but not MHC Class I, MAGE-C1 nor Melan-A. Numerous studies of this kind exist and they show that immunoediting is a process which occurs in both mice and humans and that successful cancer immunotherapy would require the targeting of multiple tumor antigens at the same time (24). The importance of the innate immune system in the immunoediting was also shown by comparing the

frequency of MCA induced tumors between Rag2 KO mice, which was lower than Rag2$^{-/-}$ × γc$^{-/-}$ mice. This mutation in common chain of the cell surface receptor of the 6 members of the Interleukin (IL) family (IL-2, 4, 7, 9, 15, and 21) means that these mice do not have an innate immune response nor an adaptive one (25).

Immune editing as we understand is divided into 3 phases (21, 22, 24, 26-30):

3. Elimination

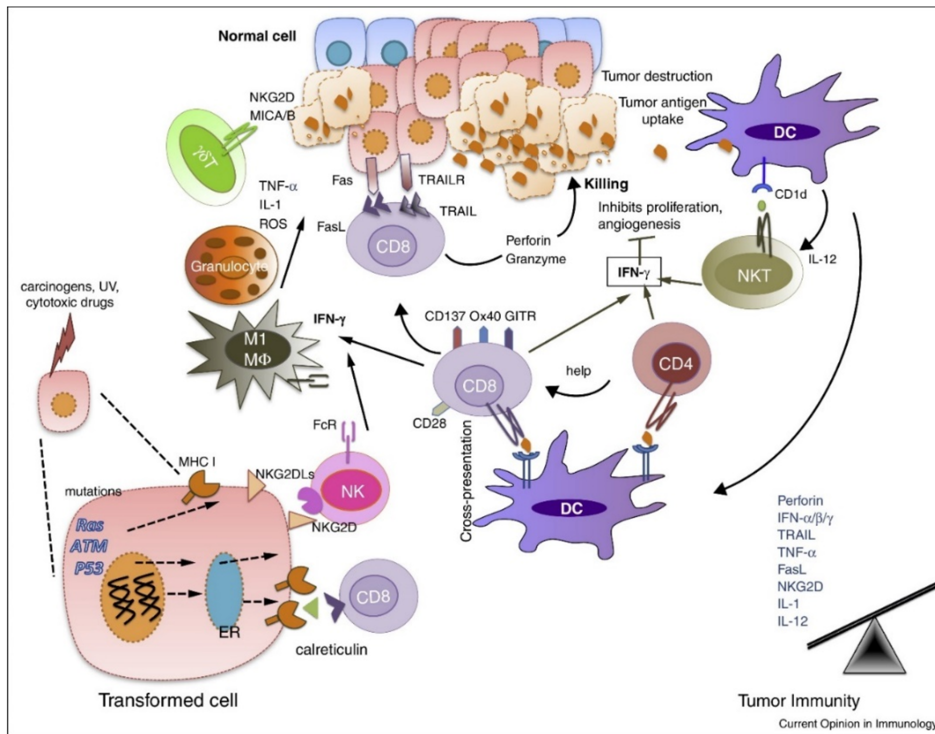


Figure 2 - Cancer immune editing: Elimination
Elimination in the cancer immunoediting phase during which both sides of immune system work together to find and eliminate early tumors before they become clinically visible. (24)

The process begins with normal cells which transform into tumor cells by carcinogens and other genotoxic factors coupled with the failure of intrinsic tumor suppressor mechanisms (e.g. p53, ATM). These tumoral cells express stress-induced molecules (calreticulin), tumor antigens in the form of MHC class I molecules, and/or Natural Killer Group 2D (NKG2D) ligands which are recognized by CD8^{+} effector cells and NK cells, respectively. Dendritic cells (DCs can also take-up tumoral proteins and cross-presents them as antigens to T cells including Natural Killer T cells (NKT) (glycolipid antigens presented *via* CD1d). These activated effector cells (Teff) release IFNγ which can directly inhibit tumor proliferation and angiogenesis. CD8^{+} T cells possess several mechanisms with which they can induce apoptosis of tumoral cells. They interact with Fas and TNF-related apoptosis-inducing ligand (TRAIL) receptors on tumor cells. They can also release perforin and granzymes. Teff will maintain their

activation, promote their proliferation and enhance their survival *via* the expression of co-stimulatory molecules such as CD28, CD137, glucocorticoid-induced TNFR-related protein (GITR), OX-40. γδ T cells have also been shown to participate in anti-tumoral immunity, for example they can detect and kill tumoral cells expressing NKG2D ligands MHC class I polypeptide-related sequence A/B (MICA/B) in humans. Innate immune cells such as macrophages (M1) and granulocytes also participate in the anti-tumor immune response *via* the secretion of TNFα, IL-1, IL-12 and Reactive oxygen species (ROS). In the Elimination phase, the anti-tumoral immune response outweighs the tumor's defenses this is mainly mediated by death receptors (Fas and TRAIL) on the tumors, the abundance of tumoral antigens (MHC class I) as well as the different activating factors secreted by the immune cells (IFNα/β/γ, IL-1, IL-12, TNFα) which control the tumoral microenvironment (Figure 2).

The fact that immunodefficient humans and mice are more likely to develop spontaneous tumors is direct evidence of the potency of this elimination step. This step relies a lot on secreted factors starting with interferons. While IFNγ targets mostly tumor and hematopoietic cells, IFNα/β act on host immune cells to stimulate them. A recent study has shown that type I interferons are crucial for the initiation of an early anti-tumoral immune response by activating CD8α+/CD103+ DCs and enhancing their ability to cross present antigens to CD8+ T cells (31).

One particular study on spontaneous tumor regression came from using the Eμ-myc mouse model for B cell lymphomas. The authors have shown that these tumors despite being highly tumorigenic sharply regress in the periphery of mice between 41 and 65 days of age. This regression was dependent on CD4+, CD8+, NK1.1+ cells as well as on the DNA Damage Response (DDR). When DNA damage occurs certain ligands are presented on the cell surface. In this mouse model, blocking the DNAX accessory molecule-1 (DNAM-1) receptor impaired spontaneous tumor regression (32)

Another example involves the innate immune response mediated by NK cells. It has recently been shown that senescent p53 expression in senescent tumor cells induced the secretion of Chemokine ligand 2 (CCL2) which recruited NK cells to mediate the elimination of these cells. NK cells recognize such cells *via* their NKG2D receptor which binds its ligand expressed on the tumoral cells. NKG2D ligands also known as MICA/B in humans used by NK cells to recognize cell induced antigens on diseased cells which are expressed following genotoxic stress, stalled DNA replication, poorly regulated cell proliferation and certain viral infections (33, 34).

The role of macrophages is far from fully elucidated. However, given that all cancer cells express CD47 also known as the "don't eat me signal" for macrophages would indicate a potential role for macrophages in spontaneous tumor elimination (35).

4. Equilibrium phase

There are many hurdles when it comes to studying the exact molecular mechanisms of the equilibrium phase. The first and foremost is the lack of mouse models capable of replicating a dormant

tumor. During the equilibrium phase of cancer immunoediting, the tumor is kept in a functionally dormant state. The constant pressure from the immune system will push the tumor to undergo and accumulate genetic and epigenetic modifications, till the moment a tumor cell variant that is resistant to the immune systems control emerges. This resistance can be loss in antigen presentation and/or the expression of immunosuppressive ligands "Programmed cell death Ligand (PDL1)." Again due to the lack of animal models, our understanding of the molecular mechanism of the equilibrium phase is still lacking. In a landmark study done in 2007 on MCA induced sarcomas, the researches selected mice developed a stable tumoral lump at the injection site instead of a progressively growing sarcoma after being injected with MCA. The tumor in these mice would remain stable until injected with antibodies which disturbed the immune system, such as anti-CD4 or anti-CD8 depleting antibodies (36). The equilibrium of immune-stimulating IL-12 vs immune-suppressive IL-23 seems to play an important role in this phase (37). In fact, different cytokine balances seem to be in play.

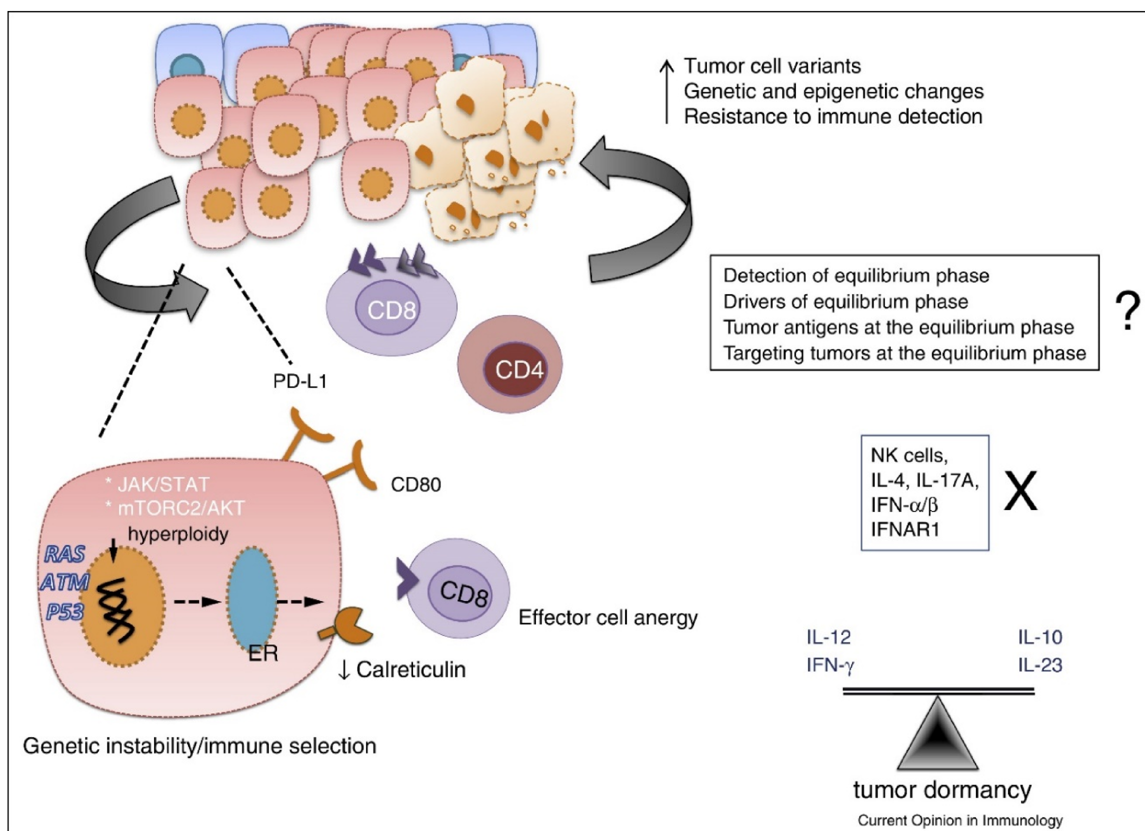


Figure 3 - The Equilibrium phase of cancer immunoediting
The immune response maintains the tumor in a dormant state. Some tumor cells undergo genetic and epigenetic changes which will eventually grant them a proliferative advantage against the immune system's control. This pressure means that some tumoral cells will eventually undergo a genetic or epigenetic mutational advantage against the immune system (24).

5. Escape

There are numerous paths which the tumoral cell can take in order to turn the balance on the equilibrium phase and escape the immune system's control: reduced immune recognition (decrease in expression of immunogenic antigens, inhibition of MHC class I), increased survival and resistance (increased expression of anti-apoptotic molecule like B cell lymphoma 2 (Bcl2) or of signal transducers and activators of transcription 3 (STAT-3)). It can also alter its immediate surrounding *via* development of an immunosuppressive tumor microenvironment that includes the production of cytokines such as vascular endothelial growth factor (VEGF), transforming growth factor β (TGF-β), immunoregulatory molecules such as indoleamine 2,3 dioxygenase (IDO), programed cell death 1/ PDL-1 (PD-1/PD-L1), T cell immunoglobulin and mucin domain containing 3 (Tim-3)/galectin-9, Lymphocyte-activation gene 3 (LAG-3)). As shown in (Figure 4), these discovered mechanisms are too numerous to describe.

The following thesis will address the mechanisms most relevant to the work accomplished during my PhD work along with a chapter on the existing immunotherapeutic approaches to overcome the aforementioned obstacles.

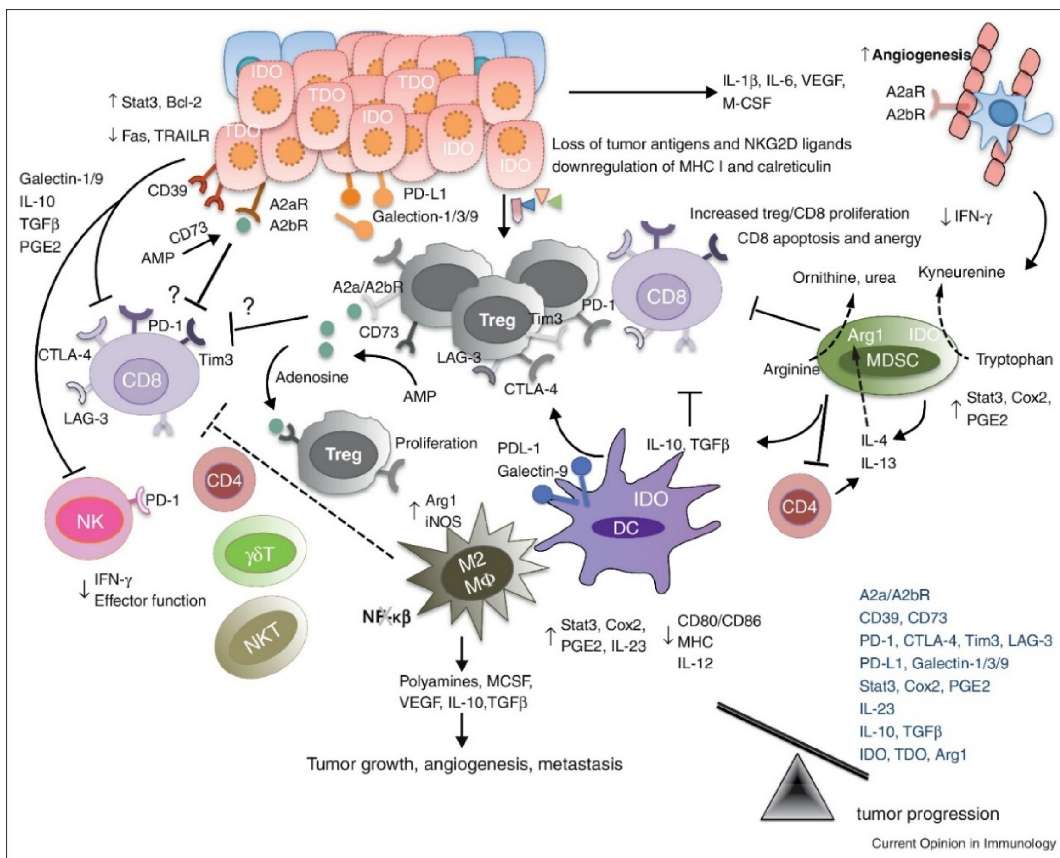


Figure 4 - The Escape phase of cancer immunoediting
The immune system fails to restrict tumor outgrowth and tumor cells emerge causing clinically apparent disease (24)

6. Infiltration of immune cells in solid tumors

Before going into details of how cancer escapes the immune system, this part will briefly present the different functions of different immune cells in the anti-tumoral immune response. Various types of immune cells can be found in various parts of the tumor. In fact, a strong immune cell infiltration of the tumor is often beneficial for the patient. However, the scientific community is starting to realize that it is not simply the quantity but the quality of this infiltrate which really matters. The different types and statuses of immune cells found in the tumor have varying impacts on the patient's prognosis. Often, high density of CD3+ T cells, CD8+ cytotoxic T cells and CD45RO+ memory cells is correlated with longer disease free and overall survival (38, 39). CTLs are considered essential for the success of an anti-tumoral immune response. Numerous studies have shown that tumor-infiltrating lymphocytes (TILs) containing an abundant level of CTLs with an ability to target tumoral cells correlated with better prognosis for the patient. Naturally, for the T cell mediated response to be effectively induced it does require recognition of tumor antigen by specific T cells (40, 41). Tumor antigen presentation to naïve T lymphocytes might be mediated by tumor cells after they have migrated to tumor draining lymph nodes (direct presentation) or *via* cross-presentation by antigen presenting cells (APCs) (42). When an APC cross-primes naïve CD8+ T cells, it induces their activation into to tumor specific CTLs which can proliferate and migrate to the tumor where they ultimately attack and hopefully successfully destroy tumor cells (43). CTLs can use various pathways to kill tumor cells such as granzymes, perforin (44, 45)], and ligands of the TNF superfamily such as Fas ligand (46). The anti-tumoral effect is also achieved by cytokine secretion such as IFNγ (47) and TNFα (48) from activated $CD8^+$ T cells.

Our understanding of the role of CD4+ T cells in the anti-tumoral immune response has immensely grown over the past 2 decades. While at one time the function of a CD4+ T cell in the tumor was being doubted as being inhibitory or stimulatory to tumoral growth, now we know that this completely depends on which subset of T cells exist in the tumor. When adequate antigen presentation and costimulatory signals are found, naive $CD4^+$ T cells are activated, polarized, and differentiated into distinct subsets including T helper cell 1 and 2 (Th1/Th2), Tregs, Th17, Th9, Th22 and follicular helper T cells (T_{FH}). Among these different $CD4^+$ T cells subpopulations, the Th1 has the most crucial role in the anti-tumoral immune response as it coordinates and amplifies the cell-mediated response against the cancer cells (49). First and foremost, Th1 cells, produce large quantities of IFNγ and various chemokines, to enhance the expansion, priming and infiltration of $CD8^+$ T cells into the tumor site (50). This IFNγ also exerts anti-proliferative, pro-apoptotic actions and inhibit angiogenesis in tumor cells in a $CD8^+$ T cells-independent manner (51). Th1 cells also recruit and activate inflammatory cells (macrophages, granulocytes, eosinophils and NK cells) in around the tumor (52). Moreover, Th1 cells can directly kill $MHC\text{-}II^+$ tumor cells *via* their proper mechanisms such as through perforin and granzymes, TRAIL receptor and Fas/FasL ligand pathways (53).

Just exactly how important NK cells are in the anti-tumoral immune response has been becoming more evident over the past few years. These cells do possess a vast arsenal with which they can attack tumor cells. They can induce tumor cell apoptosis first by secretion of cytoplasmic granules, perforin and granzymes or by inducing the expression of death receptor-mediated apoptosis and lastly by the secretion of TNF-α and through antibody dependent cellular cytotoxicity *via* CD16 expression. Furthermore, NK cells also indirectly boost the innate and adaptive antitumor activity by producing cytokines, chemokines and growth factors. One of these cytokines is IFNγ which is crucial for the anti-tumoral response. Last but not least, NK cells also induce inflammatory responses that can modulate monocytes, DCs and granulocyte growth and differentiation (54).

The role of macrophages in the tumoral microenvironment is more particular. On one hand they can play the very important role of phagocytoses of apoptotic tumor cells *via* their ability to recognize special molecules on these cells such as but not limited to lipid phosphatidylserine, oxidized Phosphatidylserine (PS), oxidized low-density lipoprotein and the multi-functional protein calreticulin. On the other hand, numerous reports have documented that an increase in macrophage infiltration into the tumor does correlate with a poor prognosis in cancer patients. The type 2 macrophages promote metastasis and progression by transferring tumor cells into the blood stream and secreting Th1 inhibitory cytokines. (55)

Other immune cells correlate with a negative prognosis for patients. For example; CD68+ tumor associated macrophage (TAM) infiltration is a negative prognostic marker in classic Hodgkin's lymphoma (55). TAMs are recruited to the tumor by CCL2 and result in the progression of colorectal cancer (CRC) (56). Infiltration of macrophages into invasive breast carcinoma correlated with increased vascular grade and reduced relapse-free survival (57). The figures below represent a summary of the innate (Figure 5) and adaptive (Figure 6) immune responses.

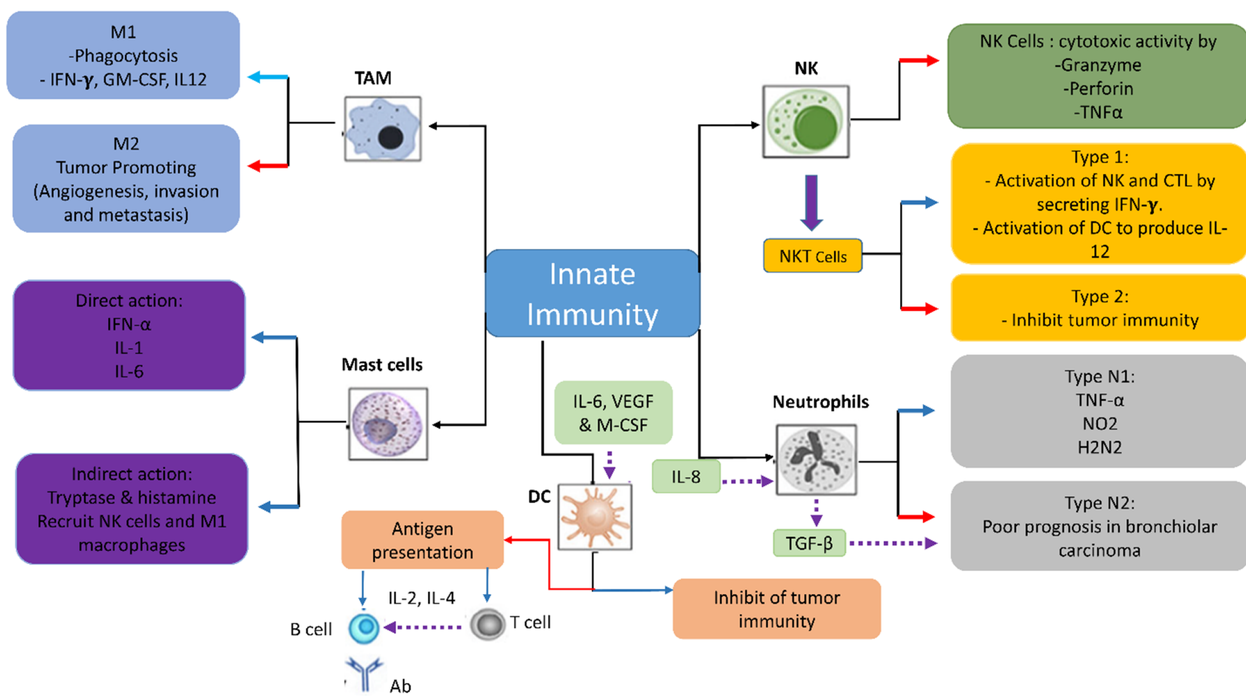


Figure 5 - Innate anti-tumoral immune response
A summary of the innate actors in the anti-tumoral immune response. Edited (56).

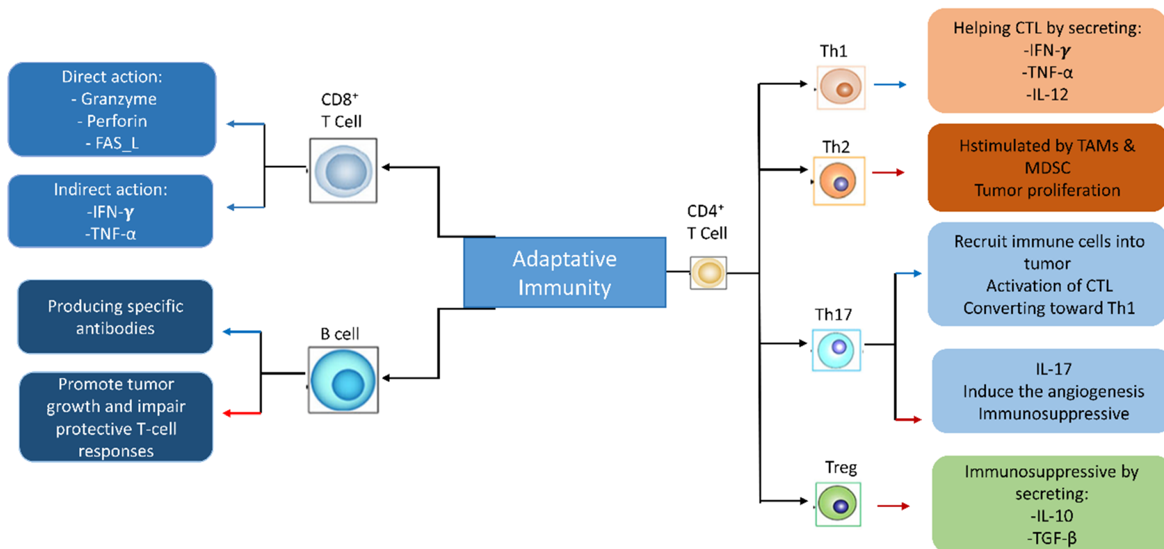


Figure 6 - Adaptive anti-tumoral immune response
A summary of the adaptive cellular actors in the anti-tumoral immune response (56)

B. Tumor Immune Escape Mechanisms

Researchers have established the importance of immune system induced tumor elimination and have proven that a tumor before establishing itself into a clinically detectable disease must escape the anti-tumoral responses of the various safeguards of the immune system. Despite what was previously

accepted, the method by which the tumor achieves this escape is not simply through the selection of non-antigenic tumoral clones. Even though the immune system can eliminate the majority of tumor various variants, a few “unique” clones will arise. These clone could have the ability to hide from immune detection (*via* changes to their antigenic presentation) and/or could possess mechanisms which actively suppress the immune system. It is these clones that would give rise to a tumor. Tumors after achieving a certain volume establish an immunosuppressive tumoral microenvironment (TME) by elements secreted by the tumor cells themselves or by recruiting immunosuppressive cells into their surroundings. One classic example of TME immunosuppression described in multiple tumors, including but not limited to renal cell carcinoma (RCC),CRC and oral carcinoma, is the loss of CD3-ζ chain of T cells and thus the loss of their functional capacity (57, 58).

In this thesis we will mainly focus on 4 mechanisms of tumor escape and their proposed therapeutic approaches. First, we will discuss co-inhibitory molecules which have led to the development of immune-checkpoint inhibitors. Then we will detail Tregs along with their capacities to inhibit the anti-tumoral immune response and the difficulties in the field of Treg targeting therapeutics. After that, there will be a chapter on tumoral antigens which lead to the idea of tumoral vaccinations. Finally, we will see the role of tumor derived exosomes and Galectin-9 in the escape from the immune system.

1. Tumor driven immune evasion

1.1. Overview

Apoptosis induction is a major weapon in the arsenal of immune cells against tumoral cells. One of the reasons behind that is tumors’ ability to escape this apoptosis is its expression of anti-apoptotic proteins such as survivin and Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (cFLIP) making them resistant to death receptor-mediated apoptosis. Tumor cells also downregulate their expression of Fas which protects them from apoptosis by FasL expressing immune cells. This has been seen in melanoma, colon carcinoma and lung carcinoma (59).

Moreover, we have seen that the adaptive immune response is key in tumor elimination yet it is defective in tumors. This is because tumors take advantage of regulatory mechanisms in the body to achieve that. It seems that the adaptive anti-tumoral immune response is controlled by the same regulatory mechanisms which prevent chronic inflammation following long term exposure to the same antigen. We have come to understand that these mechanisms which are designed to protect the body from an excessive inflammatory state following repeated interactions with viral antigens govern the anti-tumoral immune response. The profile of tumor infiltrating lymphocytes often resembles that of T cells responding to viral infection, after a while they become exhausted expressing a distinct set of inhibitory receptors such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and PD-1 which aim to prevent an immune pathology (60). Tumors will also express ligands designed to activate these

exhaustion pathways such as the ligands of PD-1 (PD-L1 and PD-L2) which induce T cell anergy after receptor binding. Moreover, tumors secrete various immunosuppressive and tumor promoting cytokines such as VEGF, IL-10, TGF-β, Prostaglandin E2 (PGE2), soluble Fas and FasL (61) Last but not least, tumor induce oxidative stress which inhibits anti-tumor immunity (62, 63).

1.2. T cell exhaustion

Recognition of a specific antigen by the TCR of T lymphocyte is not sufficient for its activation a second co-stimulatory signal from the APC has to present. This signal is antigen independent and binds to co-receptors on the T cell called the B7/CD28 family. Depending on which receptor is binding the signal on the APC, this signal can be stimulatory or inhibitory. The CD28 co-stimulatory receptors binding would result in activation of the T cell however binding the CTLA-4 or PD-1 would result in an inhibitory signal. These inhibitory signal also called immune checkpoints are crucial in physiological conditions to prevent a T cell anti-self-reaction, or an over-activation by inhibiting T cell function. In cancer, the activation of these pathways would result in a suppression of the anti-tumoral immune response and tumoral growth. In fact, we will later see that using antibodies to block these pathways have been very successful recently and revolutionized cancer therapy.

CTLA-4

Early on, due to the fact that CTLA-4 was found to be expressed on T cells only following activation, it was believed to simply be another co-stimulatory receptor. It was only in 1994 that its suppressive function was determined whereby CTLA-4 binds to the co-stimulatory molecules on APCs and inhibits the activation of T cells (64, 65). *"This was the first stepping stone leading to presumably one of the most important papers in the history of cancer immunotherapeutics where in 1996, James P. Alison and his team showed that using a CTLA-4 blocking antibody could inhibit tumor growth in mice and even decrease the size of already established tumors (66). Fast-forward 15 years to the future, in 2011 an anti-CTLA-4 antibody became the first immune checkpoint inhibitor approved for treatment of a cancer, in this case metastatic melanoma."* In the early phase following T cell activation CTLA-4 expression is triggered and it translocates to the plasma membrane. CTLA-4 binds, at a much higher affinity than CD28, members of the B7 family, where it downregulates the function of activated T-cells. CTLA-4 downregulates activated T-cell function not only through preventing co-stimulation by outcompeting CD28 for its ligand, B7, but also by inducing T-cell cycle arrest (67, 68). CTLA-4 is over expressed on tumor-specific activated T cells. Moreover, CTLA-4 is expressed on Tregs which can also inhibit the immune response by competing to binding the co-stimulatory molecules

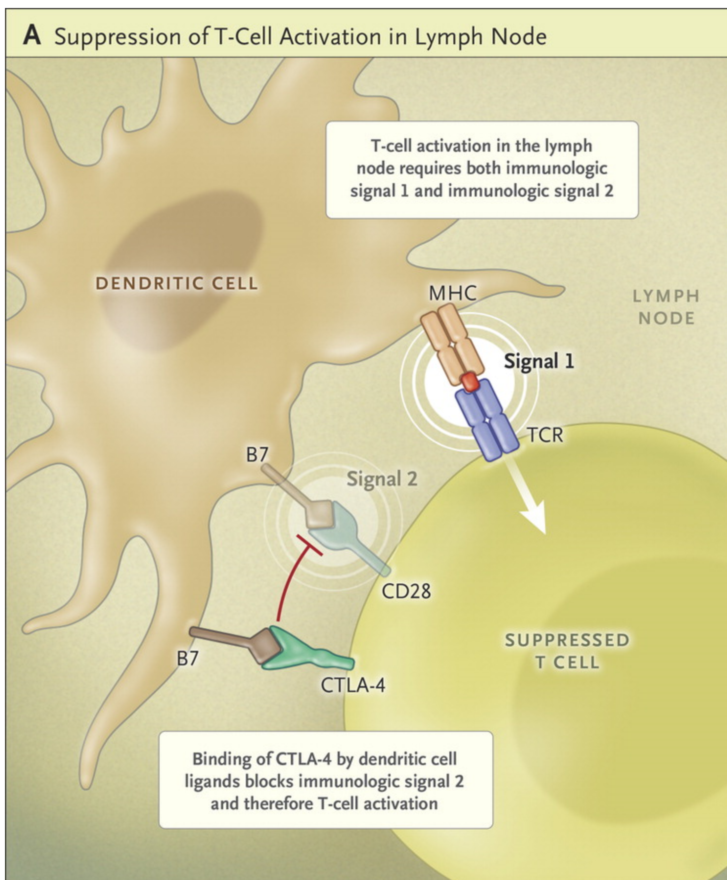


Figure 7 - Basic mechanism of CTLA-4 function
Figure representing the inhibition of T cell activation by CTLA-4 (69)

PD-1

PD-1/PDL-1 pathway exist as a negative feedback loop where cytokines secreted by activated T cells mainly IFNγ and IL-4 stimulate the expression of PD-L1 in order to attenuate the immune response. PD-1's expression depends on the activation status of the immune cells. It becomes up-regulated on activated NK, T and B cells, monocytes and DCs, with an especially high expression on CD4+ T lymphocytes and follicular T helper cells. It has two ligands, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273), which belong to the B7 protein family (70). Each ligand seems to induce inhibition in different sites. PD-L1 functions primarily in non-lymphoid organs and could be important in protecting immune-privileged sites such as the eye, placenta or testis from an unnecessary T-cell immune responses, while PD-L2 functions mainly in lymphoid organs (71). Substantial evidence shows that the PD-1/PD-L1 pathway is critical in human malignancies as PD-1 is overly expressed on tumor specific T cells indicating the functionally exhausted state of these cells. At the same time PD-L1 is expressed by numerous tumor cells mainly under IFNγ induction (which is released during an active immune response) suggesting the employment of this checkpoint pathway as an escape mechanism from anti-tumoral immunity to facilitate tumor progression (72, 73).

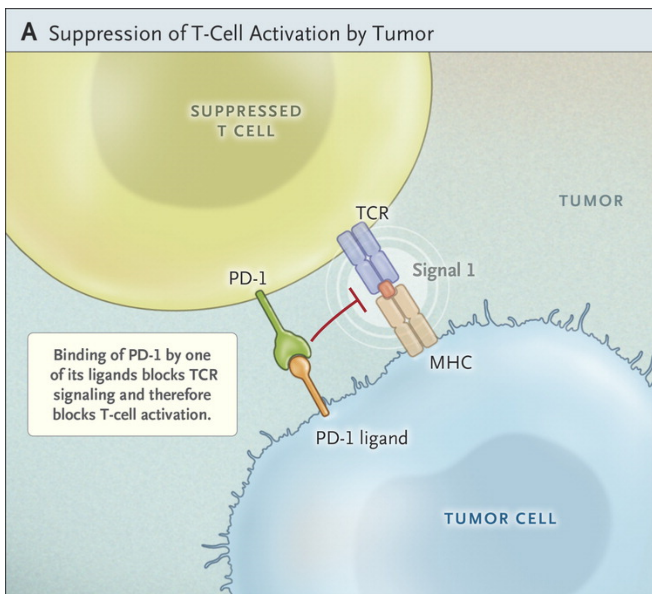


Figure 8 - Basic mechanism of PD -1/PD-L1 function
Figure representing the inhibition of T cell activation by PD-1/PD-L1 pathway (69)

1.3. Tumor recruited immune suppressive cells

Most tumors are associated with chronic inflammation which leads to the recruitment of specific subsets of immune cells whose main function is to inhibit the immune response and stop inflammation. In normal responses to pathogenic events, the immune system induces inflammation to enable the recruitment of different cell types to clear the pathogen. Once this has been accomplished and inflammation is no longer necessary, Tregs and Myeloid-derived suppressor cells (MDSC) are recruited to stop the immune response. Unfortunately, in malignant immune responses this mechanism is abused by the tumor to facilitate its immune escape.

MDSC are recruited to the tumor microenvironment *via* chemokines and other tumor-secreted factors including IL-1β, CSF colony stimulating factor and PGE2. This population is well defined in mice, mainly expressing CD11b and Gr1 cell surface proteins and is suppressive in nature. While in humans the term MDSC refers to a highly heterogeneous population having a variety of cell surface markers but is generally immunosuppressive. Much like Tregs they employ several immuno-suppressive mechanisms. They secrete immno-suppressive cytokines such as IL-10 and TGF-β. They are able to produce Arginase-1 (ARG1) which catabolizes L-arginine to urea and ornithine, limiting its availability to T cells which require this amino acid to proliferate. MDSCs can also produce inducible Nitric oxide synthases (iNOS) that converts L-arginine to citrullin and nitric oxide (NO) further depleting L-arginine from the microenvironment. NO which is mostly converted into radical peroxynitrite can lead to the nitrosylation of T cell receptors (TCRs), reducing their function. Reactive oxygen species are known as potent mediator of immune dysfunction. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits generate super oxide upon MDSC activation and induce immunosuppression in a direct cell contact dependent mechanism (74). In addition to these mechanisms prostaglandins acts as a suppressive signals to effector T cells and further induce the recruitment and generation of MDSC (75)

Tregs have an essential role in peripheral tolerance, and when recruited to the tumor microenvironment, they are able to significantly inhibit anti-tumor immune responses. The exact nature of Treg induced immune suppression will be detailed in the following chapter.

2. Regulatory T cells

2.1. History

The possible existence of immune cells whose function is purely to inhibit the immune response in order to maintain homeostasis had been debated for several decades. Even though it is now widely accepted that Tregs are a fundamental part of the immune response this has not always been the case as it took over 20 years from the moment that these cells were discovered to the moment when their existence would become accepted. In 1970, Gershon and Kondo, showed for the first time the existence of lymphocytes that can suppress an antigen specific response (76). They showed that the interplay between thymus and immunity depends on thymus dependent bone marrow derived cells. They also discovered that induction of tolerance and immunity in thymus dependent bone marrow derived cell population seems to require the co-operation of thymus derived cells. Moreover, they observed that transferring antigen encountered T cells to naïve mice can lead to antigen specific tolerance by arresting activity of T cells (76, 77). These were the first indication that the immune system, *via* these "suppressor cell", was at the same time not only responsible of eliminating antigens but also preventing auto-immunity. It took over 20 years for researcher to be able to exactly define this population which is why, their discovery despite its great significance remained un-noticed. In 1995, Sakaguchi and colleagues rekindled the fire of suppressor cell research. They showed that when CD4+ cells isolated from Lymph nodes and spleen of BALB/c nu/+ were depleted of cells expressing the α chain of the IL-2 receptor (CD25) by antibodies and then injected to BALB/c athymic nude (nu/nu) mice, all the recipient mice developed autoimmune disease symptoms (78). They continued to show that these CD4+CD25+ cells maintained tolerance to self and nonself-antigens in an antigen non-specific manner. This made the CD25 molecule the phenotypic marker defining suppressive cells which were later named as thymus derived naturally occurring regulatory T cells (nTregs). Approximately 10% of peripheral CD4+ T lymphocytes cells and less than 1% CD8+ T cells in normal unimmunized adult mice expressed the CD25 molecule (78). By 2003, forkhead box protein 3 (Foxp3) was confirmed as an essential transcription factor in the development of nTregs with several groups showing that Foxp3 deficient mice lacked the regulatory T cell population and developed fatal autoimmune lesions and lymphoproliferative disease or that ectopic Foxp3 expression confers suppressive function to peripheral CD4+CD25- T cells (79-81). Later the phenotypic definition of this population was defined with the use of flow cytometry, it was proven that the Treg CD4+Foxp3+ population expressed CD25 at a high level. Whereas the CD25 low expressing cells were Foxp3- (82). nTregs express high affinity hetero-trimeric receptor for IL-2 composed of CD25, CD122 and CD132 chains. The fact that Tregs are in fact a dedicated lineage of cells in humans came mostly from evidence from people suffering

mutations in the Foxp3 as these mutations were the cause of fatal human autoimmune disorders 'immune dysregulation, polyendocrinopathy, enteropathy, X-linked' (IPEX). However unlike mice, human nTregs having the CD4+ CD25+ profile are not alone in expressing FoxP3. TCR stimulated CD4+CD25- human conventional T cells (Tconv) also express FoxP3 and can acquire Treg suppressive function when foxp3 expression is induced in them (80, 83, 84). The importance of Foxp3 in mature human Treg suppressive capacity was confirmed by knocking down Foxp3 *via* small interfering RNA (siRNA) in Tregs. These Tregs had decreased suppressive potential, an impaired phenotype and were non-responsiveness. They downregulated the expression of function molecules, and reduced the production of suppressive cytokines (85). Another marker which became a staple for Treg characterization and discrimination from Tconv is the IL-7R α-chain (CD127). Most Tregs either did not express CD127 or expressed it at very low levels whereas Tconv in an activated or resting state were positive for CD127 (86) . All these discoveries sped up the research in Treg biology and it eventually became one of the glamorous fields of study in immunology.

2.2. Treg subtypes

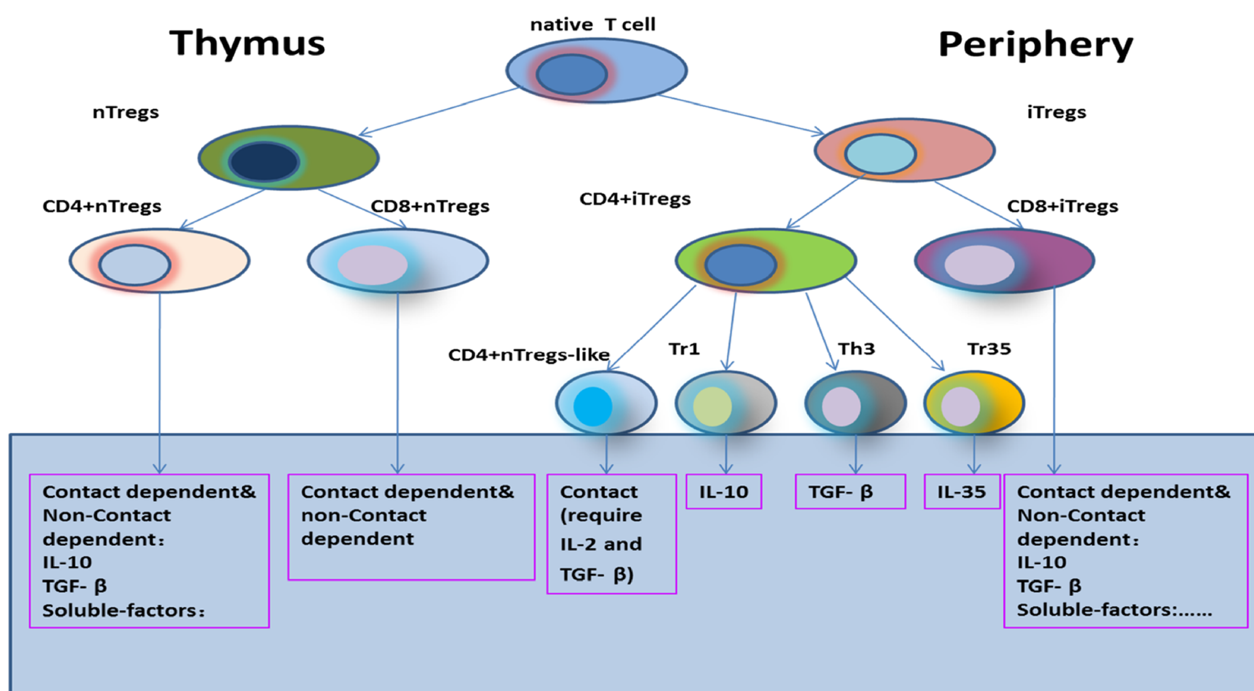


Figure 9 - Treg subtypes and origins
A diagram representing the different subtypes of Tregs along with their differentiation site and basic suppressive programs (87)

Even though Tregs are a subset of T lymphocytes, they can be divided into different subsets themselves. The major difference between the 2 populations being that nTregs acquire the suppressive identity from naïve T cells in the thymus while induced Treg (iTregs) acquire it in the periphery (Figure

9). nTregs, constitutively expressing Foxp3 and CD25, and comprising about 1-2% of circulating T cells in humans represent a mature functionally suppressive population responsible, in non-pathogenic conditions, for maintaining self-tolerance and immunological homeostasis (88). Studies have shown that FOXP3 suppresses the transcription of Il-2, Il-4, and IFNγ genes *via* direct interactions with NF-κB and nuclear factor of activated T-cells (NF-AT). This complex is crucial for nTreg suppression and biogenesis as well as their upregulation of CD25, CTLA4 and GITR (89, 90). Numerous epigenetic modifications and receptor ligand interactions play crucial roles in nTreg lineage commitment. Genome wide analysis from multiple researchers has determined nTreg specific DNA methylation or histone modifications that differ from Tconv (91-94). One of the more stable and crucial epigenetic changes in Tregs is the hypomethylation region in the Foxp3 gene (92). Receptor ligand interactions such IL-2/CD25 have been shown to be crucial for nTreg development and function despite the fact that Tregs do not produce IL-2. Interaction with IL-2 receptor mediates STAT5 activation which is crucial in the development of nTregs (95). Another crucial factor is CD28 ligation by CD80/CD86 (96). Numerous other factors are involved and, in short, the lineage commitment of nTregs is complicated and is not the main subject of this thesis.

The search for nTreg specific markers which can differentiate them from iTregs has been ongoing since the days of Gershon. Several markers have been proposed based on results obtained from mice Tregs for example Neuropilin 1 (NRP1) and the Ikaros zinc finger transcription factor, Helios, allowed the differentiation between mouse iTreg and nTregs but not so much in humans. Still Helios expressing human Tregs have been associated with higher suppressive capacities (97).

iTregs unlike nTregs, regulate the immune response mostly for microbial and tissue antigens. They develop from naïve T cells that fail to properly activate due to insufficient antigenic presentations. Such antigens include commensal bacteria and tumoral neo-antigens. CTLA-4 binding seems to play an important part. The presence of high levels of TGFβ, IL-10 and IL-2 also do promote the induction of these Tregs especially when immature DCs are present (98). iTregs appear in the mesenteric during the induction of oral tolerance (99), and following organ transplantation (100). More importantly, tumors can induce the expression or secrete iTreg stimulating factors such as the Cyclooxygenase 2 (COX2), IDO, IL-10 and TGFβ (101, 102). iTregs are divided into different defined subpopulation. Of special mention is the CD4+ CD25-/low FoxP3-/low type I regulatory T cell (Tr1) population (103). This population has been well described in Hodgkin's lymphoma, Head and neck squamous carcinoma (HNSCC), Hepatocellular carcinoma (HCC) and CRC (104-107). These cell are considered highly suppressive and mainly function *via* IL-10, TGFβ secretion. Even though these cells are very potent immune suppressors their role in human tumors has not been sufficiently investigated, one small clinical trial found that the ratio of Tr1 to Foxp3+ nTregs is a predictive factor for increased survival in four recurrent ovarian cancer patients that had undergone adoptive T cell transfer (108).

Th3 cells are similar to Tr1 in their role in maintaining oral tolerance. They have been studied in mouse models, their contribution and relevance to human cancers is not clear (109).

Throughout this thesis we focused on CD4+ Tregs, yet there exists a different group known as CD8+ Tregs. The identification of CD25 as a marker for CD4 Tregs had completely exploded the interest in CD4+ Tregs leaving research on CD8+ lagging behind. They represent less than 1% of circulating peripheral cells. This population is increased in the intestines. Several markers have been proposed including CD28low, CD122+ and CD8αα expression instead of the normal CD8αβ (110). Similar to their CD4 counterparts they can develop in the thymus or in the periphery. CD8+CD25+FoxP3+CTLA-4+ nTregs have been identified in several studies in mice and humans having contact dependent suppressive activity as well (111). they seem to be less suppressive than CD4+ Tregs (112). They have been found in prostate and colorectal cancer patients (113, 114).

2.3. Some Treg markers and regulatory mechanisms

After establishing that Tregs are essential for peripheral tolerance, researchers started looking deeper into their mechanisms of action as well as specific markers for their identification. In fact, most phenotypic markers that define the Treg population also play a functional role in Treg induced immuno-regulation. The suppressive mechanisms used by an nTregs or an iTreg depend on the pathogenic conditions currently facing the body. Depending on whether the Treg cell is confronted with a Th1, Th2 or Th17 immune to suppress, it will use a different weapon in its arsenal. Still these suppressive mechanisms based on their mode of action can be divides into four groups:

- Suppression by inhibitory cytokines
- Suppression by cytolysis
- Suppression by metabolic disruption
- Suppression by contact dependent mechanisms

Here we will focus on the suppressive mechanisms already described to be used by Tregs in TME.

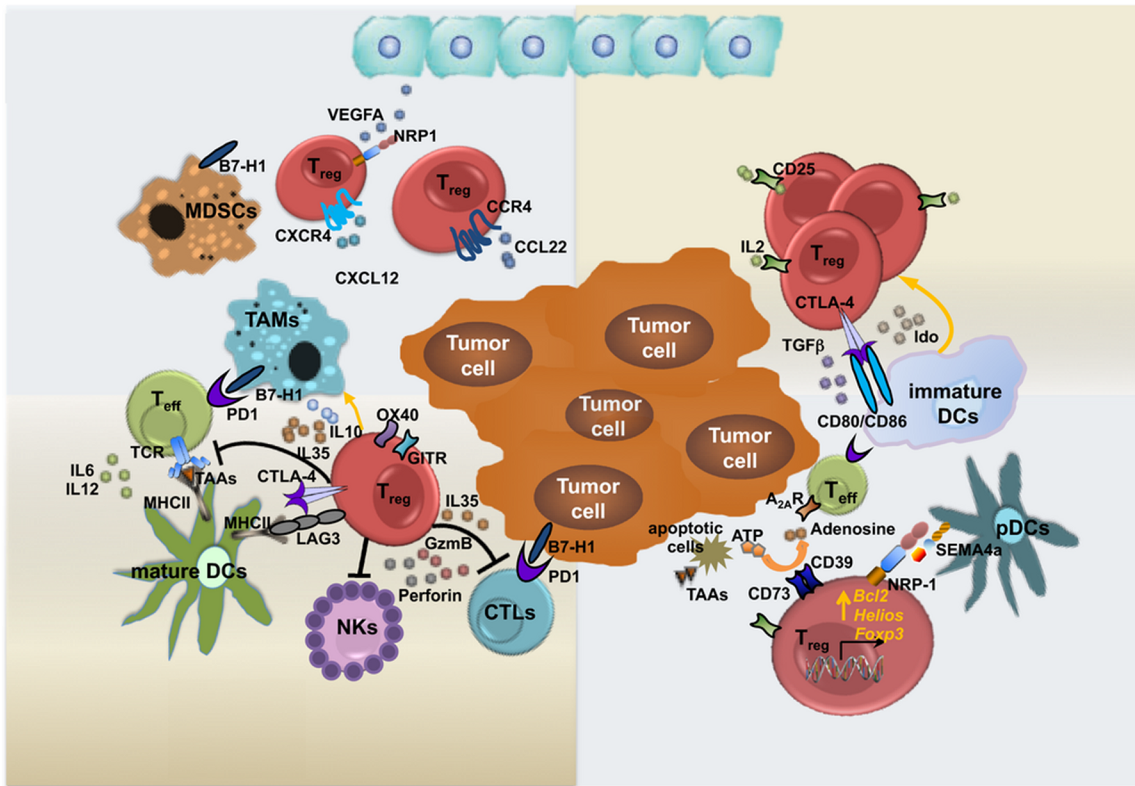


Figure 10 - -Suppressive mechanisms of Tregs in the TME
Figure detailing the most well characterized mechanisms of Treg suppression in the tumoral microenvironment along with the various interactions occurring between Tregs, other immune cells and cancer cells (115)

Suppressive cytokines:

Inhibitory cytokines such as IL-10 and TGF-β have considerable impact as mediators of Treg cell induced suppression. IL-10 has potent immunoregulatory functions highlighted by the fact that IL-10 or IL-10 receptor knock out mice developed inflammatory bowel disease-like pathology (116, 117). While IL-10 does not seem to be essential to Treg mediated suppression *in-vitro* (118) it does play a crucial role in Treg enforced restrain of the immune response at environmental interfaces, especially in the intestines, and in the control of systemic tolerance (119). It should be noted that while the role of IL-10 in strict foxp3 positive nTreg induced suppression is not fully established, it is clear that IL-10 is a major immuno-regulatory cytokine used by iTregs. Chronic activation of human and murine Tconv does eventually induce a decrease in proliferation, Th1 cytokine secretion and an increase in IL10 secretion (103, 120-122). IL-10 can exert several immuno-regulatory effects including the direct inhibition of Th1 and Th2 T cell proliferation (123). IL-10 can also influence the function of DCs, monocytes and macrophages by inhibiting production of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-12, IL-18, Granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF, and IL-12. It also blocks their maturation and ability to express co-stimulatory molecules (124). The role of IL-10 in cancer is very interesting. On one hand, it is a major mediator of Treg induced suppression of the anti-tumoral Th1 response. On the other hand, it inhibits the tumor promoting inflammatory Th17 response. IFN

signaling and proper antigen presentation seem to be important in determining the exact function of IL10 in the tumoral microenvironment (125-127). Currently there is no IL-10 blocker in clinical or preclinical trials.

The transforming growth factor β (TGF-β) family of proteins are known regulators of the immune response (128). TGF-β1 has the predominant immuno-regulatory function and has been proven to play vital roles in the Treg suppressive function (129). In fact, similar to Foxp3, any disruption in the TGF-β signaling pathway results in fatal lymphoproliferative disease in mice (130). Is has been proven that TGF-β is essential for the development and the maintenance of both thymic and peripheral Tregs (131, 132), in fact TGF-β enhances Treg suppressive potential *via* a positive feedback loop where it induces Foxp3 which in turn inhibits TGF-β receptor antagonist Mothers against decapentaplegic homolog 7 (Smad7) (133). The Latency associated peptides (LAP)/TGF-β complex expressed on Tregs plays a role in the conversion of Tconv into iTregs. Moreover, TGF-β is also capable of inducing IDO on DCs which can also induce iTregs (134). In line with its role in nTreg contact dependent inhibition, TGFβ is expressed on the membrane Tregs. Cell surface TGF-β on Treg is capable of binding to TGF-β receptor on effector T cell (Teff), monocytes and NK cells to inhibit them (135, 136). Membranous TGFβ on Tregs was shown to downregulate NK cell function by downregulating the NKG2D receptor expression. Mice receiving Tregs but not TGFβ KO Tregs suffered decreased NK cytotoxic function and an accelerated tumor growth (137). TGFβ was found to be also secreted by cancer cells to promote its progression by establishing a Treg rich immunosuppressive microenvironment (138). Moreover, TGFβ was shown to inhibit the M1 response and promote the M2 macrophage response. It should be noted that TGFβ is probably one of the most important if not the most important cytokine present in the tumoral microenvironment. A huge quantity of research exists on its tumoral promoting, or in a few cases, inhibiting functions. These functions are too pleiotropic to be detailed in this thesis. The general consensus is that inhibiting TGFβ in the patient with solid tumors should provide a survival advantage for the patients and in fact there are numerous TGFβ antagonistic molecules currently in Phase I/II clinical trials. However, the fact that TGFβ is crucial in controlling inflammation at the environmental interfaces does make inhibiting it a risky approach (139).

IL-35 is another addition to the set of regulatory cytokines used by Tregs (140). Similarly to IL-10, IL-35 is not constitutively expressed by nTregs in human but might play a role in specific regulation (141-143). IL-35 was shown to expand Foxp3+ Tregs as well as inhibit the proliferation of Tconv and decrease their IFNγ secretion (144). A recent study on the role of IL35 in various mouse cancer models has shown that Treg derived IL-35 inhibits TILs, their effector function and memory T cells. The same work showed that IL-35 inhibition by Monoclonal Antibodies (Mab) limited tumoral growth in these mice (145).

Mediated Cytolysis

Cytolysis induction *via* the Granzyme/perforin pathway is well established in various cells of the immune system mainly CD8+ CTLs and NK cells. Granzyme A is produced by activated human nTregs which *via* the action of perforin is released into the cytoplasm of the target cell. The serine protease function of these proteins triggers the caspase cascade which leads to apoptosis (146). The mechanism of cytotoxicity dependent Treg cell suppression has been proposed in several studies. Granzyme B (GrzB) production in Tregs is mediated by CD28/TCR ligation *via* phosphatidylinositide 3-kinases (PI3K)/ mammalian target of rapamycin (mTOR) pathway (147). Tregs can induce cytolysis in CD8+ T cells, CD4+ T cells, NK cells, DCs, and B cells either *via* the Grz perforin pathway or *via* the Fas-FasL pathway (148, 149). GrzA perforin cytolysis depended on the expression of the adhesion molecule CD18 (149). In the tumoral context Tregs suppress the NK cell and CD8+ CTLs anti-tumoral function *via* GrzB-perforin pathway (150). Foxp3+ induced Tregs from tumor bearing mice also express perforin and GrzB which they used to induce apoptosis of MHC class II expressing APC. This was an indication that Tregs could also inhibit anti-tumoral immunity by stopping CD8+ T cell priming by lysing DCs. Another study found that Tregs isolated from cancer patients expressed the FasL CD95 and used it to mediate apoptosis of autologous CD8+ CTLs, which was not the case for Tregs isolated from healthy donors (151)

Metabolic disruption

Upregulation of the IL-2 receptor α chain (CD25) was the first phenotypic surface marker suggested as a Treg marker (78). CD25 plays an essential part in the suppressive function of Tregs as it allows them to deplete local IL-2. This results in starved Tconv which undergo apoptosis or become anergic (152-154).

Tregs have been also shown to generate extra-cellular adenosine as a suppressive mechanism. CD39 and CD73 are two ecto-enzymes highly expressed on Tregs that can hydrolyze Adenosine triphosphate (ATP), Adenosine diphosphate (ADP) or Adenosine monophosphate (AMP), releasing adenosine which then binds to adenosine receptor 2A on Teff cells as well as APCs, thus inhibiting them. Tregs from CD39 KO mice had reduced suppressive potentials. Similar effect could be obtained by used of a CD39 antagonist (155-158). Adenosine binding to APCs changes the cytokine secretion profile from pro-inflammatory to inhibitory (IL-12, TNFα to IL10) and lowers the expression of co-stimulatory molecules (159).The effects on Teff range from general inhibition of proliferation to induction of an anergic state and even the generation of iTregs (160). This effect is mainly mediated by the elevation of intra-cellular Cyclic AMP (cAMP). In fact it has been shown that Tregs can even transfer cAMP to Teff *via* gap junctions to induce their inhibition (161).

Cell to cell contact dependent suppression: Involvement of co-stimulatory and co-inhibitory signals

As we have seen before, antigen recognition is initiated through TCR engagement but its intensity is determined by the ensuing balance of co-stimulatory and co-inhibitory signals strength (162). As we have also seen, tumors can deregulate this balance in favor of immunosuppression and one of the ways they do that is *via* recruiting Tregs. Tregs suppress Teff and DC function by the expression of co-inhibitory molecules on their surface. The *in-vitro* suppressive assay using, Tregs KO or using antagonistic antibodies, has allowed the identification of a large number of such molecules. CTLA-4 which was discussed before is one of these molecules. It binds with high affinity; 20- to 100-fold higher affinity than CD28; to costimulatory molecules on both Tconv as well as APC (158, 163). CTLA-4 binding to APC causes a down-regulation of B7 and B7.1 (164, 165) as well as activation of the immuno-regulatory enzyme IDO which catabolizes tryptophan to kynurenine (158, 166). IDO is crucial in immuno-suppression, it has even been shown that it is expressed by cancer cells (167). On one hand, tryptophan depletion alone has been shown to directly inhibit T cell proliferation (168). On the other hand the combined effect of tryptophan depletion and accumulation of its metabolites has been shown to promote the proliferation of Tregs and the de-novo induction of iTregs (169-171). Whether CTLA-4 is constitutively expressed in human nTregs or whether it is only upregulated under Treg activation conditions has been debated (172). In either way, CTLA-4 is crucial for Treg mediated immunosuppression as CTLA-4 KO Tregs lose their ability to maintain homeostasis in mice models. These mice suffer from spontaneous systemic lymphoproliferation, fatal T cell-mediated autoimmune disease, hyperproduction of immunoglobulin E in mice, and lowered effective tumor immunity (164).

Ivars and colleagues first reported that Tregs down-regulated the expression of co- CD80 and CD86 on APCs in vitro (173). Lymphocyte-activation gene 3 (LAG-3) which is a transmembrane CD4 related protein is another surface molecule that takes part in the Treg suppressive repertoire (174). LAG-3 is also capable of inhibiting Teff by inhibiting APCs. LAG-3 binding to MHC class II on DCs results in inhibition of their maturation (165, 175). While LAG-3 has been proven as a marker of nTreg in mice, its constitutive expression on human nTregs is controversial and may occur only after activation (174, 176). However LAG-3 is a marker of iTreg in humans (177). The expression of early growth response gene 2 (Egr-2), which is important in anergy induction seems to control the expression of LAG-3 in iTregs having CD4+CD25-LAG3+ profile and secreting high levels of IL-10 (178). A CD4+Foxp3+LAG3+ subset from circulating peripheral blood mononuclear cell (PBMCs) of melanoma and colorectal cancer patients was shown to be highly suppressive secreting large amounts of IL-10 and TGFβ. Interestingly *in-vitro* induced suppression by this population was still reliant on contact dependent mechanisms (176).

PD-1 is also expressed in Tregs. The interaction of PD-1 with PD-L1 expressed on APCs seems to be important for iTreg generation as well as maintenance of their suppressive potential. PD-1/PD-L1 binding enhances the expression of the Foxp3 and suppressive potential *via* down regulation of

phosphorylation of Akt, mTOR and ERK2 and upregulation of the phosphatase and tensin homolog (PTEN) which are key signaling molecules in the development of Tregs (179, 180)

T cell immunoreceptor with Ig (Immunoglobulin) and ITIM (immunoreceptor tyrosine-based inhibition motif) domains (TIGIT) expression is under the direct control of Foxp3 (181). TIGIT+ Treg cells further express Foxp3, CD25, and CTLA-4 at higher levels. Engagement of TIGIT on Tregs induces the expression of fibrinogen- like protein 2 (Fgl2) (involved in the cytokine production by Th2) which can inhibit pro-inflammatory responses by Th1 and Th17 (182). It is also observed now that Helios+ memory Tregs expressing TIGIT and Fc receptor like 3 (FCRL3) are highly suppressive Tregs (183, 184). Numerous other markers for Tregs have been discovered summarized in Table 1. The reason behind discussing CTLA-4, Lag-3, PD-1, and TIGIT is the fact all these molecules as we will see represent actual functioning anti-tumoral immune checkpoint inhibitors or highly promising candidates.

Table 1 - Treg suppressive mechanisms

Level	Marker	Comment	Ref
High	**CD25**	Only 1~2% of $CD4^+$ T cells with highest CD25 expression are Tregs with suppressive function in human peripheral blood	(78, 185, 186)
	CTLA-4	The expression level of CTLA-4 is correlated with suppressive function of Tregs. CTLA-4 is also rapidly induced in Teffs upon activation	(164, 187)
	ICOS	The majority of mouse Tregs express ICOS. $ICOS^+$ Tregs possess superior suppressive activity than $ICOS^-$ Tregs in mice. Both human $ICOS^+$ Tregs and $ICOS^-$ Tregs have strong suppressive capacity	(188-190)
	TNFR2	The majority of human and mice nTregs are TNFR2 expressing cells. Mouse $TNFR2^+$ Tregs have the maximally suppressive function. Mouse $TNFR2^-$ Tregs, even if they are $FoxP3^+$cells, only have minimal or no suppressive activity	(191-193)
	GITR	High surface expression of GITR is detected on Tregs. It is also detectable on Teffs and the function of Tregs are not dependent on GITR	(190, 194, 195)
	OX40 **4-1BB**	4-1BB and OX40 are preferentially up-regulated on the surface of Tregs by TNF stimulation and can promote the proliferation or survival of Tregs. However, OX40 and 4-1BB also inhibit the development and suppressive activity of Tregs	(196-198)
	LAG-3	LAG-3 defines an active Tregs subset and contributes to their function. All T cells upon activation express LAG-3	(174, 176, 199)
	LAP	LAP expression is high on the surface of activated Tregs and induces TGF-β secretion. LAP^+Tregs have higher suppressive abilities	(200, 201)
	GARP	GARP is highly expressed on activated Tregs and contributes to their suppressive activity by aiding in TGFβ secretion	(202) (203)
Low/ negative	**CD127**	Tregs are generally characterized by low surface expression of CD127. However, down-regulation of CD127 is also observed on activated Teffs. Activation of mouse Tregs also results in up-regulation of CD127 expression in vivo and vitro	(204, 205)
	CD6	Low/negative expression of CD6 is reported on human nTregs	(206)
	CD26	Human Tregs are characterized by low/negative CD26 expression	(207)
	CD49d	CD49 is present on proinflammatory effector cells but absent on Tregs. The combination of CD127 and CD49d identifies highly pure population	(208)
Intra cellular	**Foxp3**	Foxp3 is the most specific marker for Tregs, but Foxp3 expression is found in human activated Teffs. Foxp3 expression in Teffs confers neither a regulatory phenotype nor suppressor function	(84, 209)
	Helios	Helios is expressed exclusively in mouse nTregs but not in human nTregs	(210)

3. Tregs and Cancer

As we have previously discussed, numerous studies have correlated an increase in CD8+ TILs with a positive prognostic indicator (211, 212). However, as we have also seen, Tregs are not normal TILs. In fact, while numerous studies have correlated an increase in tumoral Tregs with a poor prognosis, other have observed the inverse. These contradictory results are exactly why a better deciphering of the clinical significance of tumoral Tregs is required. For example, studies for hepatocellular carcinoma, lung carcinoma, and renal cell carcinoma have clearly correlated a poor prognosis with an increase in tumoral Treg density (40, 213, 214). Whereas data from other trials sometime on the same type of cancer have yielded contradictory results. While differences in detection techniques, statistical methodology or data interpretation could justify some of the difference, it does not seem to be the major reason. For example when studying the overall number of tumoral Foxp3+ cells in colorectal cancer it shows that Tregs promote a favorable response (215) studying the ratio of CD8+ to Foxp3+ cells in the TME does correlate Tregs with a poor prognosis in the same cancer (216). These apparently contradictory results may be attributed to several biological factors. Tumor etiology could be an important factor, where in most cases intratumoral Tregs correlate with a bad prognosis except in inflammation driven and maintained tumors where the ability of these Tregs to inhibit inflammation could be beneficial for the overall outcome for the patient (217). The tumoral stage during which Tregs were detected could be important. Inflammation in the early stage has been shown to be a driving pressure for tumors to break the equilibrium stage and Tregs in these early stages could be a positive prognostic factor with their ability to inhibit this inflammation (115). Another question is the validity of only using Foxp3 as a marker for Tregs in these studies. Not all Foxp3 expressing cells in human are suppressive. A simultaneous expression of either suppression, tumor homing markers or both is essential in defining a tumor infiltrating suppressive Treg. As it was observed in a study on oral squamous cell carcinoma, where only Foxp3+CCR4+ Tregs correlated with a poor prognosis whereas the total Foxp3+ population did not have any prognostic value (218). A recent study looked at circulating Tregs by flow cytometry from different cancer patients including pancreatic and metastatic colorectal cancer. They could not find any difference when looking at Foxp3+ and Helios positive cells between the cancer patients and the healthy donor controls. However when adding LAP/ glycoprotein A repetitions predominant (GARP) into the phenotype they found a significant increase in Treg with this phenotype in cancer patients than the controls (219)

One noteworthy study on the impact of Foxp3 in cancer prognosis is 2015 meta-analysis. This study analyzed 76 publications encompassing 17 different cancer types including over 15 thousand patients. While the overall results suggested that Foxp3+ Tregs were associated with a negative prognosis with respect to overall survival, this results was stratified varying in function of tumor type, molecular subtype, site and stage (220).

3.1. Treg migration into the tumor

In order for Tregs to inhibit the anti-tumoral immune response, they have to be able to find the tumor. The ability of Tregs to migrate into the TME using multiple pathways has been well documented. One of the more common examples is *via* the CCL17 and CCL22 which are produced by plasma cells and macrophages to attract T helper cells into the TME also attracts Tregs. Other sources of these cytokines include other TME associated suppressive cells such as tolerogenic DCs, cells and M2 macrophages. For example, using a C-C Motif Chemokine Receptor 4 (CCR4) antagonist (CCR4 being the receptor for CCL22) in tumor bearing mice targeted the Tregs expressing CD44 high and Inducible T-cell costimulator (ICOS+) and increased the accumulation of antigen specific CD8+ T cell (221). In humans, CCR4 and CCR8 positive Tregs seem to have a better response to the CCL17 and CCL22 produced by APCs as they were able to compete with Tconv over APC binding in response to these cytokines (222). CCR8 and C-X-C chemokine receptor type 4 (CXCR4) were also found to be important for trafficking of the Tregs to the tumors (223). Another study showed that using an anti-CCL1 led to the reduced number of the tumoral Tregs while increasing effector CD8+ T cells and NK cells improving the anti-tumoral response in mice (224). In pancreatic mouse models, disruption of the CCR5/CCL5 pathway reduced Treg migration into the tumors and resulted in controlled tumoral growth (225).

Tumoral IL-6 and IL-8 upregulated CXCR1 on Tregs and increased their tumoral migration mainly in lung cancer mouse models (226). Plasmacytoid dendritic cells (pDCs) expressing Inducible T-cell costimulatory ligand (ICOSL) were shown to expand suppressive intra-tumoral Tregs in mouse breast cancer models (227). Table 2 represents various clinical trials that studied Tregs in cancer patients indicating the recruitment method when studied

3.2. Treg expansion and activation in the TME

Treg accumulation in the TME is not only due to migration of Tregs but also due to their expansion. In the TME, several cues are responsible for the differentiation and activation of Tregs which differ from the TCR/CD28 signaling. The TME promotes the expansion of nTregs as well as the generation of iTregs in situ due to the abundance of IL-10, TGF-β and adenosine which is produced by both tumor cells and MDSC (228). CD40 and IL-4 produced by MDSC also help the recruitment and proliferation of Tregs in tumor microenvironment (229). Up-regulation of IDO by APC has been reported to activate Tregs and promote their proliferation (230). Ligation of CD80 and CD86 by CTLA-4 constitutively expressed on Treg increases the functional activity of IDO by DC forming a positive feedback loop. IL-2 produced by NK cells and T cells in tumor microenvironment seems to feed Tregs and their expansion in tumor microenvironment (231). In IL-2-/- or CD25-/- foxp3 green fluorescent protein (GFP) knock-in allele mice, it was observed that IL-2 signaling was required for maintenance of the expression of genes involved in regulation of Treg growth and metabolism (232). Treatment with IL-2 commonly used for melanomas was found to expand the ICOS+ Treg expansion (233). TGF-β, has

been observed to play a central role for peripheral expansion of Tregs (234). Tumor cells are capable of producing TGF-β themselves and can also modulate MDSC's and immature DC to produce it (235).

3.3. Treg and cancer therapy

Aside from the prognostic value of Tregs in untreated tumors, Tregs have been found to hinder other anti-cancer therapies. The success of high dose IL2 treatment for metastatic melanoma and renal cell carcinoma is hindered by the fact that it results in the generation of Tregs (214, 233). The EGFR-targeted antibody Cetuximab in HNSCC has been shown to induce intra-tumoral CD4+ Foxp3+ Tregs expressing CTLA-4, CD39 and TGFβ (236). Moreover, Tregs have been shown to not only be resistant to a radiotherapy but also to benefit from it. Radiotherapy enhanced the proliferation, and suppressive function of Tregs via multiple pathways which include an increase in TGFβ secretion (237).

Table 2 - Summary of diagnostic clinical trials for the impact of Tregs in various cancers

Cancer	Histological type	Phenotype	Stage	N°	Prog.	comment	Ref
HCC	non-metastatic/non-treated/after resection	Tumor Foxp3+ IHC	NS	302	Poor		(40)
RCC	Following IL-2 treatment	Tumor Foxp3+ IHC/CD3+/CD4+/CD8+	NS	100	poor		(214)
	Clear cell Renal cell carcinoma	Tumoral and peri-tumoral Foxp3+ by IHC. COX2 also	I to IV	125	NA/ Poor	NA for tumoral. Poor for peri-tumoral.	(238)
	Renal cell carcinoma	FC for circulating and tumoral CD25+Foxp+		30 P 20 HD	Poor	Tumoral Treg were more suppressive	(239)
Lung	NSLC newly diagnosed	Tumor Foxp3+/Tim3+ by FC	NS	51	Poor		(240)
	NSLC	Foxp3+ IHC	NS	87	poor		(213)
	NSLC	Tumor Foxp3+ IHC	I	64	poor		(241)
	NSLC	ratio Tumor Foxp3+ to CD8+	III,IV	159	poor		
Cervical	HPV+	Tumor Foxp3+ IHC	NS	107	Poor	CXCR4/CXCl12	(242)
	non treated	ratio Tumor CD8+ to Foxp3+ IHC and IF	I, II	115	poor		(243)
Prostate cancer	non-treated	Tumor Foxp3+ IHC/ PBMC GITR, ICOS, and FOXP3 by FC	Ic, II	15	poor	CCR4/CCL22	(244)
	treated or non-treated	foxp3+ IHC	II to IV	2002	Poor		(245)
Melanoma	non-treated vertical growth phase primary cutaneous melanomas	Tumoral CD25+ Foxp3+ IHC	I to III	66	poor		(246)
	Non-treated intermediate thickness or think melanoma	Tumoral Foxp3+ IHC	I to IV	97	None		(247)
Breast cancer	Breast tumors	Tumoral Foxp3+ IHC	NS	191	poor	CCL22/CCR4	(248)
	non-metastatic. Treated or non-treated breast cancer	Tumoral Foxp3+/CXCR4. CXCL12 IHC	NS	491	poor	CXCR4/CXCL12	(249)

Breast cancer	operable invasive breast carcinomas	Tumoral Foxp3+ IHC	I to III	1445	NS	Correlated with stage	(250)
	HER2-overexpressing breast carcinoma treated with neoadjuvant chemotherapy	Tumoral Foxp3+ IHC	I to III	103	Good (OS)		(251)
	locally advanced breast cancer receiving neoadjuvant chemotherapy	Tumoral Foxp3+ IHC	NS	101	Good (OS)		(252)
	oestrogen receptor negative breast cancer	Tumoral Foxp3+ IHC	Advanc ed	175	Good (RFS)		(253)
Ovarian Cancer	untreated malignant ovarian epithelial cancers	CD4+CD25+Foxp3+ FC for malignant ascites. Tumoral CD4+CD25+ by IF	II to IV	104	Poor (OS)	CCR4/CCL22	(254)
	Ovarian carcinoma	Foxp3 expression by QPCR. Foxp3+ IHC	I to III	99	poor		(255)
	serous ovarian cancer	Ratio of CD8+ to CD4+CD25+Foxp3+ by IF	NS	52	Poor		(256)
Head and neck cancer	Non-treated primary head and neck squamous cell carcinoma	CD4+Foxp3+ IF	I to IV	84	Good		(257)
	Non-treated HNSCC	PBMC FC CD4+CD25+/highCD 127-	I to IV	39	poor	Correlated with stage.	(258)
	non treated oral squamous cell carcinoma	ratio of Foxp3+CCR4+ by IHC /CD8+	I to IV	87	poor	CCR4/CCL22	(218)
Lymphoma	DLBCL	PBMC FC CD4+CD25highFox3 +	NS	27	Good		(259)
	DLBCL	Tumoral Foxp3+ IHC	NS	96	Good		(260)
	Non-treated follicular lymphoma	Tumoral Foxp3+ IHC	NS	97	Good		(261)
	Classical Hodgkin Lymphoma	Tumoral Foxp3+ IHC	I to IV	87	Poor		
Colorectal cancer	colorectal cancer stratified by mismatch-repair	Tumoral Foxp3+ IHC	NS	1420	Good (DFS)		(215)
	Colon cancer	Tumoral and peripheral CD4+ CD25high Foxp3+ by FC	NS	25	Poor	CCR5/CCL5	(262)
	Colon cancer	Tumoral and normal adjacent mucosa Foxp3+ IHC	II, III	967	Good	Mucosal Foxp3 poor	(263)
	CRC	Ratio CD8+/Foxp3+	I to III	94	Good (OS)		(216)

C. Tumor antigens

As previously described anti-tumor immunity can be mediated by many ways. One of the most potent pathways is mediated by the adaptive immune response. However, for this pathway to function, the immune system needs to be able to recognize tumor antigens. Tumors in general express 2 types of antigens, those unique to the tumor and those shared with the healthy immune cells. Tumor antigens have been classified into two categories, 1) tumor-associated antigens (TAAs) which are expressed by more than one type of tumor cells as well as normal tissues and, 2) tumor specific antigens (TSAs) also known as neo-antigens, on the other hand, are products of random somatic point mutations induced by physical or chemical carcinogens and therefore expressed uniquely by individual tumors and not by any normal tissue, representing the only true tumor-specific antigens.

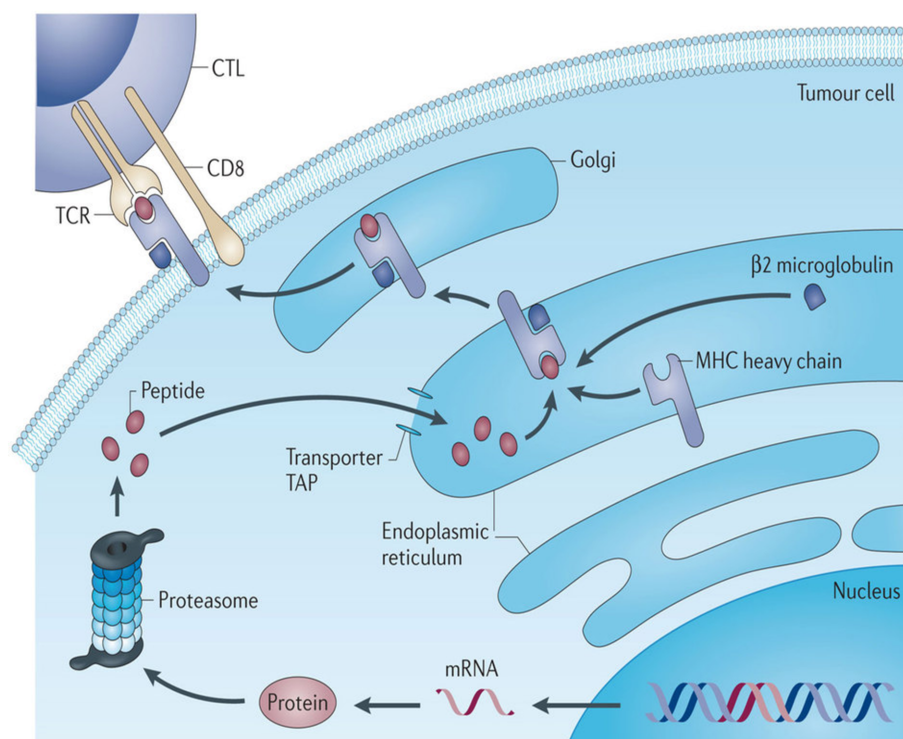


Figure 11 - Processing of tumor antigens that are recognized by CD8+ T cells.
Most antigenic peptides presented to CTLs by major MHC class I molecules are produced through the degradation of intracellular proteins by the proteasome. Then a transporter associated with antigen processing (TAP) transports the resulting peptides into the ER, where they coupled to newly created MHC class I molecules and travel through the Golgi to the cell surface. Different antigenic peptides are created by other proteases (264)

Tumor unique antigens are ideal targets for the adaptive immune system since they can be usually recognized with high affinity by the TCR and B cell receptor (BCR) without the problem of central tolerance. This mostly applies to tumors with high mutational rates which often end up creating

a unique immune-peptidome. The new epitopes are generated due to mutations which alter a specific amino acid, shift the reading frame or extend the protein beyond the stop codon. The best example for this is melanoma where the induced T cell response is dominated by neo-antigen specificity and as we will see the mutational load and neo-antigen profile can determine the therapeutic outcome in melanoma patients following immune-checkpoint treatment (265, 266) . In fact, the effectiveness of the anti-tumoral immune response, and the patient's prognosis, is determined by the immunogenicity of the mutations, as immunogenic mutations increase so does the survival probability of the patient (267). Of special mention are viral infection driven tumors, such as: head and neck, cervical, adult T cell leukemia, hairy-cell leukemia and Kaposi's sarcoma all which can present highly immunogenic viral antigens which are unique to the virus and can be exploited in vaccination strategies. Prophylactic vaccination against the human papilloma virus (HPV), the virus behind head and neck as well as cervical cancer, has already proven its effectiveness (268). Unique tumor antigens represent an interesting class of targetable antigens, particularly the viral antigens (264, 269).

Becoming less antigenic in order to evade detection by T cells is one of the most crucial mechanisms employed by tumor to avoid immune elimination. Downregulation of MHC class I occurs in several tumors. Changes to MHC class I can be structural, transcriptional or post transcriptional. Human leukocyte antigen (HLA) class I β2 side chain mutation exists in 21% of CRC and 15% of melanomas while haplotype loss is found in 36% of head and neck squamous cell carcinomas (270). In addition to loss of MHC, tumors lose their expression of their antigens making them less antigenic and protecting them from TAA specific T or B cell responses.

Even when the immune cells are capable of recognizing a tumoral antigen and mounting an immune response, often they are not capable of inducing full tumor regression.

One of the immunotherapeutic approaches benefiting from tumor antigens to treat cancer is vaccination which we will see late on.

D. Exosomes

Exosomes are small extra-cellular vesicles “EVs” produced by virtually all body cells and found in all body fluids. In fact, cells release different types of EVs of differing sizes. These include apoptotic bodies having a size of “1000-5000nm”, macrovesicles “MVs” of intermediate size “200-1000nm” and the smallest being exosomes “30-120nm”. In addition to being smaller in size, exosomes differ from other vesicles in their production method, molecular content as well as their function (271, 272). Apoptotic bodies are the remnants of dead cells. MVs bud of the cytoplasm acquiring their cytosolic contents are random. However, exosome biogenesis involves controlled mechanisms by the endosomal compartment indicating that they play important roles in homeostasis (273). In fact, when Stahl and Johnstone in the 1980s showed that 50 nm vesicles were released from maturing reticulocytes carrying and externalizing the transferrin receptor, they believed they were witnessing a process of cellular waste disposal instead of that of receptor recycling. It took several decades for exosomes to be acknowledged for their role as information carrying vesicles rather than “sanitation workers.” (274). Now we know that exosomes are produced by bacteria, plants and animals making them an evolutionary conserved communication system. The fact that it exists in all these lifeforms would indicate that it is necessary for survival. While the world of exosomes is definitely new and exciting, here we will focus on cancer exosomes. In cancer, exosomes play an important role in tumoral growth, metastasis and immune escape. In fact, certain literatures refer to tumor derived exosomes as oncosomes owing to their great potential in oncogenesis.

Despite the recent advances in the field, much work is still required before we can fully decipher the code of cancer exosomal communication.

Table 3 - Overview on different types of EVs

EV type	Origin	Diameter (nm)	Density (g/ml)	Marker proteins
Exosomes	Endosome/ MVBs	30–120	1.13–1.19 (sucrose)	CD63, CD81, Hsp70, Alix, TSG101
Ectosomes	Membrane shedding	50–200	Unknown	CR1, C1q
Microvesicles	Membrane shedding	100–1000	Unknown	Integrins, selectins, CD40 ligand
Oncosomes	Membrane shedding	1000–10,000	1.10–1.15 (iodixanol)	CK18
Apoptotic bodies	Apoptotic blebbing	1000–5000	1.16–1.28 (sucrose)	Histones, TSP, C3b

1. Exosome biogenesis in cancer

Exosomes unlike other EVs originate from multivesicular endosome "MVEs". During endocytosis, early endosomes start by an inward invagination of the plasma membrane. As it becomes a late endosome its membrane undergoes multiple inward invaginations which close off to form multiple intra-luminal vesicles. At this point the endosome is called a multivesicular body (MVB) containing numerous vesicles each with its own enclosed part of the cytosol and carrying its own cargo. If the MVB fuses with the plasma membrane, these vesicles are released as exosomes. Most MVBs do end up fusing with the lysosomes for degradation, only certain MVBs whose membrane associated lipids contains high levels of ceramide escape lysosomal degradation and fuse with the plasma membrane (271)(Figure 12). Each exosome is generally considered as a representation of its parent cell containing nucleic acids, proteins, enzymes, lipids, cytokines and other soluble factors. Due to its endosomal origin, it is normal that exosomes contain endosomal components mainly the endosomal sorting complex required for transport (ESCRT) and various ESCRT-associated molecules (275, 276).

Tumor cells produce more exosomes than normal cells. This is coupled with an increase in circulating exosomes in the plasma of cancer patients (277). While the exact reason behind this is not fully understood, it has been attributed to several reasons(278):

- Increased stress in the tumoral microenvironment especially hypoxia (279).
- Increased P53 expression (280).
- The Rab GTPase proteins, especially Rab27a and Rab27b which play a role in exosome production and are deregulated in some tumors (281).

As for the content of tumor exosomes, well it is highly variable and reflects that of the parent cell. Tumor exosomes will carry the classical exosomal markers described above along with various tumor associated molecules mainly immune suppressive ones. These include FasL or TRAIL, PD-L1, IL-10 and TGF-β1, as well as prostaglandin E_2 (PGE_2) and CD39 and D73. On the other hand, tumor exosomes do tend to carry tumoral antigens, co-stimulatory molecules and MHC components.

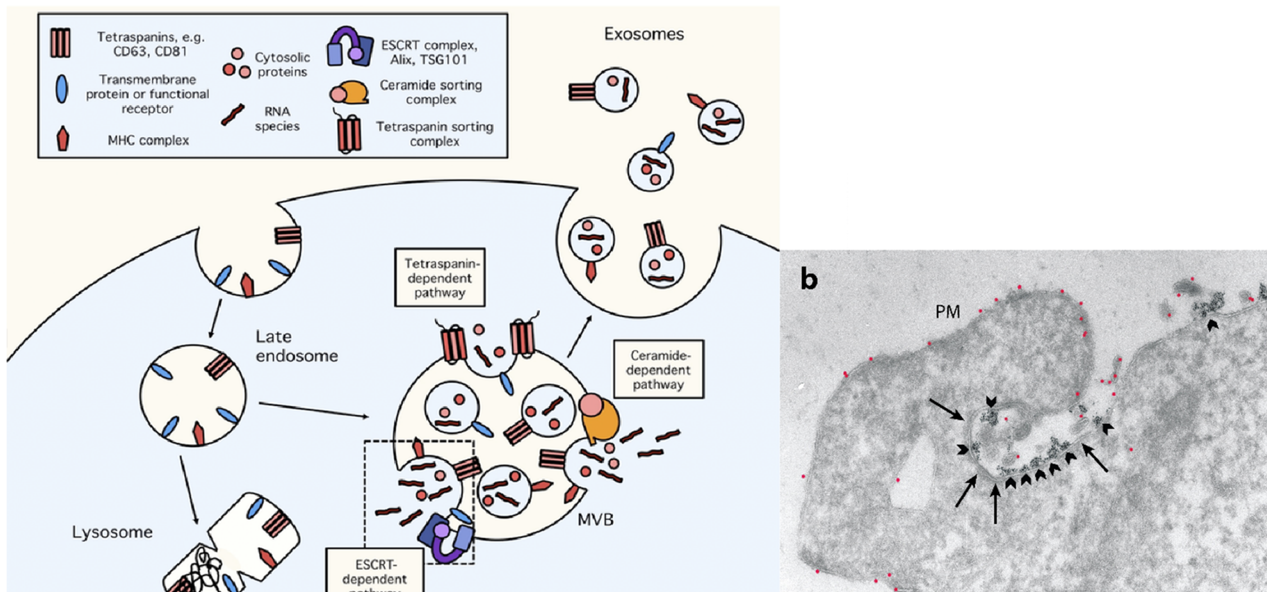


Figure 12 - EVs release from eukaryotic cell

a. Is a schematic representation of the synthesis and release of exosomes following endocytosis, formation of MVB and then fusion with plasma membrane (282). b. “*Electron microscopy image of fusion of a MVB with the PM (arrows) in an EBV–transformed B cell. BSA-gold (small particles) was internalized into MVB (clusters of BSA-gold are indicated by arrowheads); large gold particles (pseudocolored in red) label MHC class II at the cell PM and on internal vesicles of the MVB.”* (282) (283)

2. Exosomal components in general and in cancer in specific.

The exosomal membrane carries the same content as the MVEs but due to the second set of invaginations these components have the same orientation as the parent cells. Hence the exosomal membrane contains fusion proteins, such as Annexins, flotillin, or GTPases, and lipids such as ceramides, sphingomyelin, and cholesterol (284). Exosomal membranes have been shown to have endosome specific tetraspanins (CD9, CD63, CD81, and CD82) which seem to play important roles in exosome biogenesis (285, 286). The vesicular and membrane content of the exosome is similar but does not exactly duplicate that of the parent cell. In fact, a recent study found significant differences in the protein and nucleic acid relative quantities between exosomes and parent cells (287). This further supports the idea that trafficking content to the exosomes is a regulated process which unfortunately is still poorly understood. Last part of the classic exosomal content arises from the actors involved in its release from the parent cell. This multistep process seems to be highly regulated and requires the corporation of numerous elements including the cytoskeleton components “actin and microtubules” along with associated kinesins and myosins, Rab GTPases, and molecules which can drive MVB fusion with the cell membrane, including the SNAP (Soluble NSF Attachment Protein) REceptor (SNARE) complex (273).

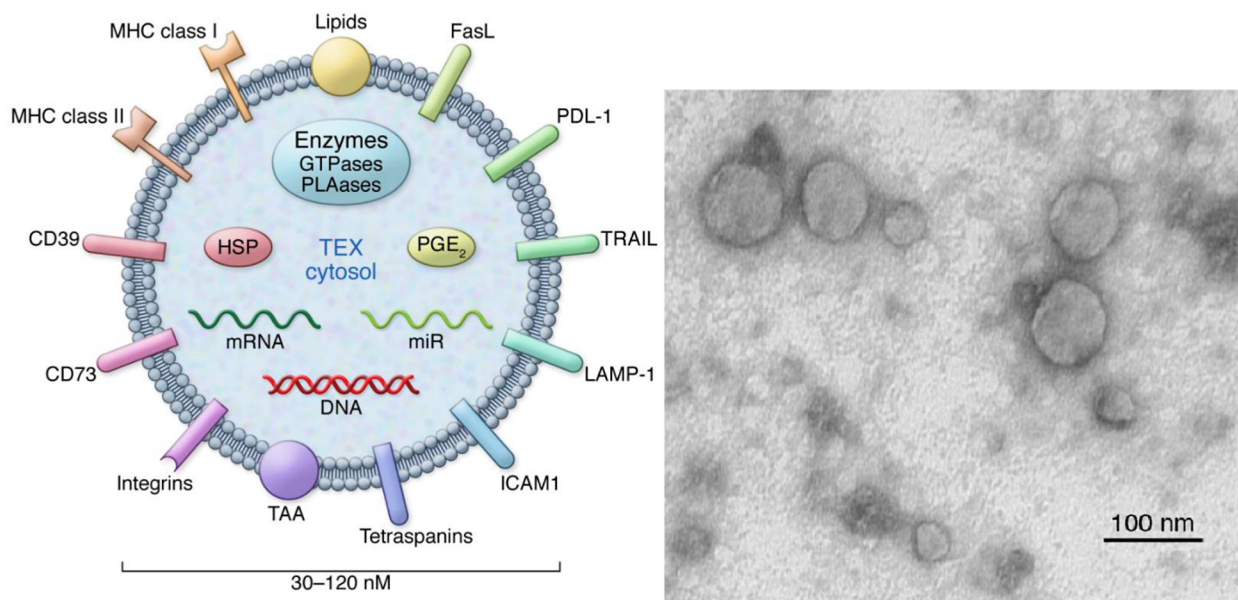


Figure 13 - Tumor derived exosome
Illustration of a tumor derived exosome with the classic exosomal components along with components commonly found on tumoral exosomes. Electron micrograph of tumoral exosome produced by a human head and neck cancer cell line (288)

Again, given that exosomes tend to mimic the contents of their parent cell, it is only logical to imagine that tumor exosomes would contain tumoral molecules. As we have seen, tumor display an arsenal of immunosuppressive molecules which is why a great amount of literature shows tumor derived exosomes as being immunosuppressive (Figure 14). The list of molecules that have been found to be carried by patient exosomes and capable of inducing immune suppression is immense. A few examples include the exosomes released from melanoma cells which are capable of inducing T cell apoptosis by carrying the FasL (289). While other exosomes from cancer patients carried MICA/B which inhibited NK cytotoxicity (290). In our team, we have given special interest to exosomes derived from nasopharyngeal carcinoma in which we have found the presence of Galectin-9 (Gal-9) and CCL20. These tumoral exosomes had the ability to inhibit CD4+ T cell proliferation and induce their transformation into regulatory T cells. Moreover, these exosomes could recruit Tregs into the tumor and induce their proliferation (291) (Figure 14).

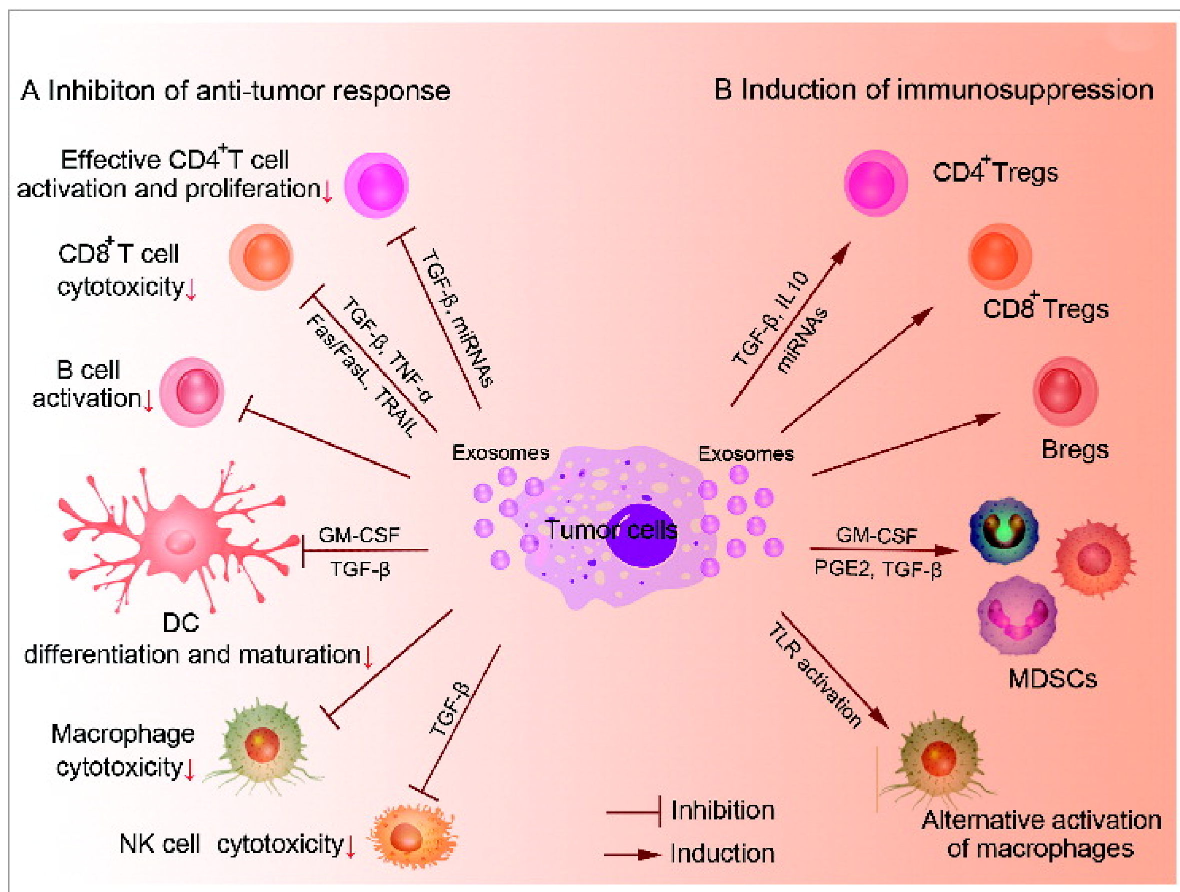


Figure 14 - Exosome-mediated immunosuppression of tumor immunity
Tumor cells use exosomal intercellular communication to transfer their immunosuppressive potential to the necessary target immune cell (292)

3. Exosomal communication with their targets.

In-order to fulfill their function of inter-cellular communication, exosomes need to be able to communicate with their target cell. Numerous publication proved the existence of exosomal content uptake by target cells. For example, after mouse proteins were found in animal cells after exosomes from mouse origin transferred the RNA of mouse to the human mast cells (293). More recent techniques involve the use of fluorescent lipophilic dyes that label the exosomes as they leave the parent cell and then this dye is transferred to the target cell following contact with the exosome. Exosomes are equipped with the necessary molecules and employ different mechanisms to transfer their information from RNA, micro RNA (miRNA, DNA), proteins or lipids to the target cells. These mechanisms are summarized in (

Figure 15). An exosome carrying the necessary ligand can bind and activate its cognate receptor on the target cell. Fusion of the exosome with the target cell is highly possible and has been observed, exosomal lipid rafts and an acidic microenvironment seem to be important for fusion to happen (294, 295). Phagocytosis was observed when the dye carried on the exosome was found in the phagosome of the target dendritic cell and not on its membrane (296). Last but not least is exosomal

uptake *via* endocytosis. Cytochalasin D which can depolymerize the actin filament network inhibiting endocytic pathways has been shown to significantly decrease exosomal uptake (297, 298). This pathway depends on multiple proteins including Intercellular Adhesion Molecule 1 (ICAM-1) whose blockade inhibited exosome uptake by dendritic cells (297).

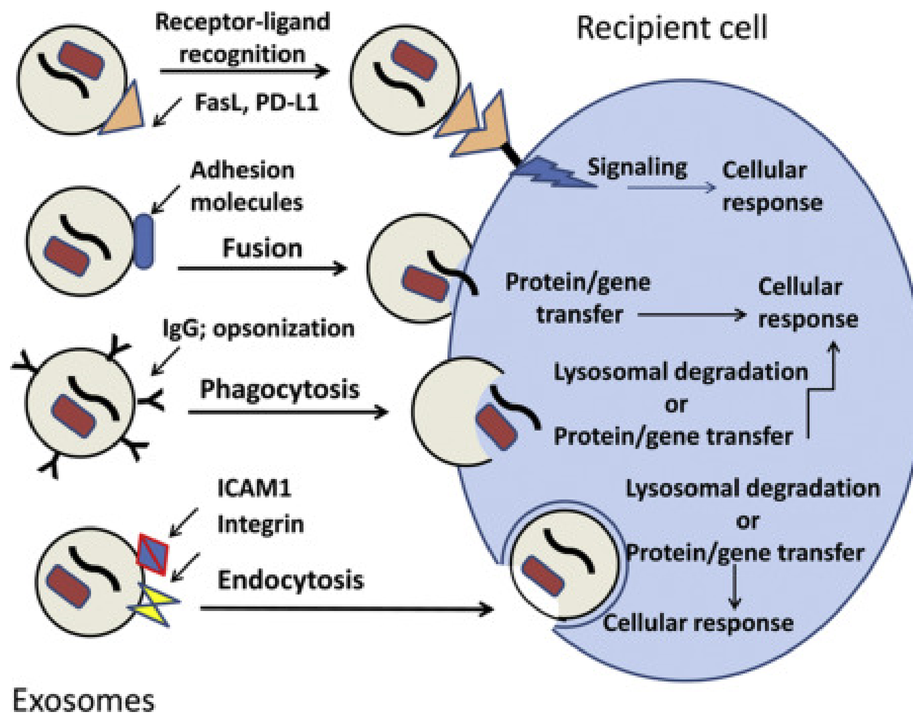


Figure 15 - Exosome uptake by the cell
Schematic representation of the different pathways mediating exosomal uptake (273)

E. Cancer stem cells

Cancer stem cells are tumoral cells with unique phenotypes with the capabilities of self-renewal, and tumor initiation. This subset has been identified in many tumors and has become of particular interest due to its resistance to anti-cancer treatments thus predisposing patients to relapse (299). This has been observed cancer cells in patients with minimal residual disease or patients with relapse express CSC-related gene signatures (299-301). Similarly, high expression level of CSC markers in cancer patients has been correlated with poor overall survival (302). Moreover, recent data suggests that CSCs can have a direct impact not only on classical anti-cancer therapeutics such as chemo- and radiotherapy but also on new generation immunotherapeutics and immunecheckpoint inhibitors (303, 304). The niche in which the CSCs reside has been known to be an important factor in the immunosuppressive TME. For these reasons and other the field of CSCs has exploded over the last few years.

Previously, CSCs were thought to be limited to hierarchical model in which CSCs represent a biologically distinct tumor cell subset. In this model carcinogenesis occurs when a normal body stem cell escapes regulation and becomes cancerous with long-term clone propagating capacity. This model was under major debate since it contradicts with the more accepted stochastic model of carcinogenesis and is not capable of explaining the heterogeneity found within a tumor. Meaning that the cells in one tumor are too different to have originated from a single progenitor. Nowadays, CSCs are no longer being limited to the hierarchical model but are being included in the plasticity model. This model reconciles the hierarchal and stochastic model. In this model cancer cells have the capacity to change between stem-like or differentiated states (305). This transition is dependent on signals from the TME which leads to importance of understanding the CSC niche.

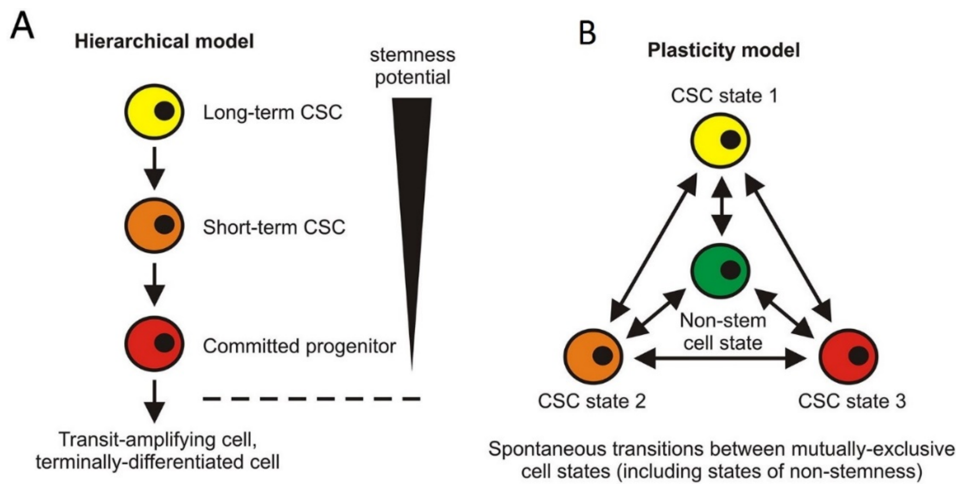


Figure 16 - Cancer Stem cell concept
(A) Depicts the hierarchical where a single progenitor gives rise to terminally differentiated cells. (B) Shows the plasticity model in which cancer cells interchange between different states. Edited from (306)

1. The CSC niche

The niche is a distinct microenvironment within the TME in solid tumors whose main function is to stimulate CSC self-renewal, stemness, and survival. The cells in this niche therefore produce numerous factors to achieve that including angiogenic factors, metastasis stimulating factors and immuno-modulatory factors. Tumor associated fibroblasts are one of the main cells in the niche which stimulate both tumoral growth and migration via the secretion of numerous factors such as CXCL12, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and hepatocyte growth factor (HGF) (307). MSCs are one of the cell populations which can be found in the CSC niche. MSCs as explained before promote angiogenesis and tumor immune evasion. They can also promote the stemness of cancerous cells via secreting certain cytokines (CXCL12, IL6 and IL8) which promote the activation NF-κB pathway (308). MSCs also secrete BMP antagonist Gremlin 1 which promotes an undifferentiated state (309). The cancer stem cell niche is also rich in type II macrophages and MDSCs both of which release TGFβ and thus can promote the Treg population. Tumor associated macrophages have also been shown to produce efflux transporter containing exosomes which promote multidrug resistance in CSCs (310). This niche also breaks down the ECM leading to evasion and metastasis of cancer cells. Finally, hypoxia has also been shown to be an important factor in the niche where it promotes the immunosuppressive microenvironment and protects from classic cancer therapeutics via ROS-activated stress response pathways (311). For these reasons and numerous others, understanding and subsequent targeting of CSCs and their niche has become essential in order to insure full remission in cancer patients.

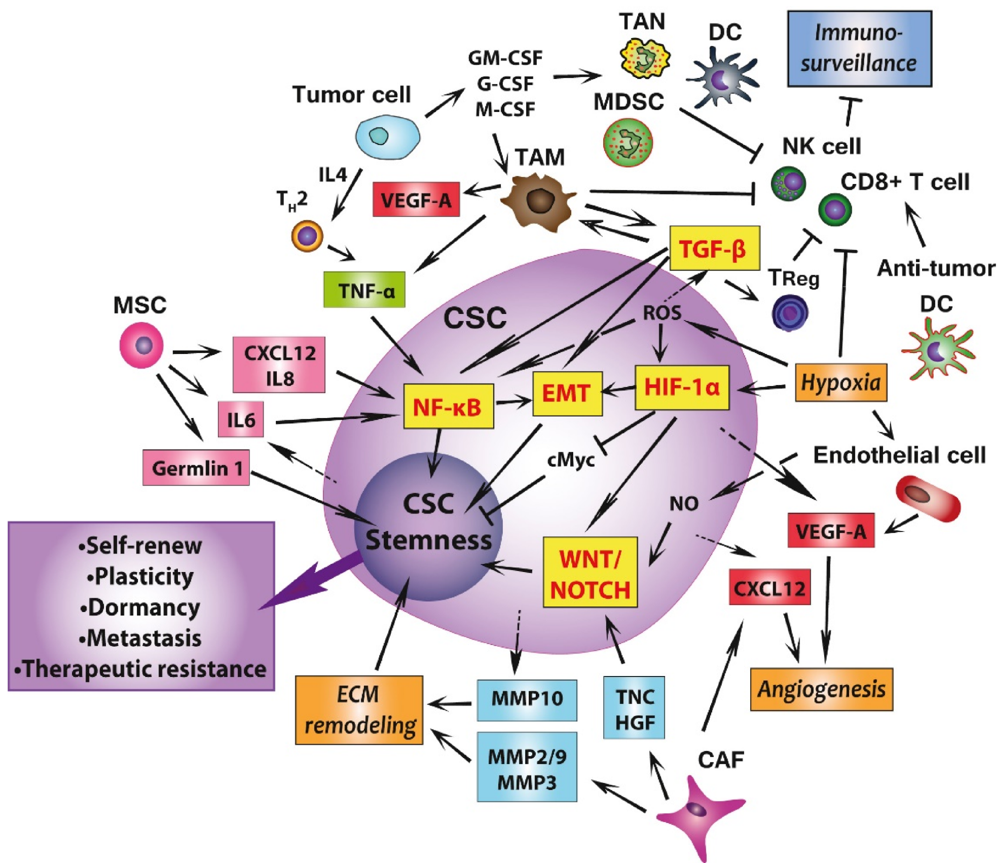


Figure 17 - The Cancer Stem Cell Niche (305)

III. Galectins

Before presenting galectins, a small introduction into glycobiology. The most common understanding of molecular biology revolves around the dogma of DNA to RNA to protein. This concept with its precision of template to product has led to the belief that understanding one of these actors will inevitably reveal the rest eventually leading to a complete explanation of all cellular structures and functions. However, it is important to remember that cells require two other major classes of molecules to function which are Lipids and carbohydrates. These molecules can act as intermediates in both energy generation and signaling, recognition, and structure. These two molecules also include a major part of proteins' posttranslational modifications. Lipids and carbohydrates help to explain how the relatively small number of genes in the typical genome can generate the enormous biological complexities which are essential in the development, growth, and functioning of intact organisms.

These carbohydrates which are bound to proteins can be recognized by a special class of evolutionary conserved proteins which include the Lectin family. Lectin binding to a protein can activate, inhibit or even change the known function of the protein making the understanding of the Lectin family crucial. Carbohydrate recognition domains (CRDs) define most members of the Lectin family. These give the lectins their ability to bind their target and have apparently evolved from common ancestral genes (312).

Lectins have been known for over 100 years. In 1888, a German microbiologist Peter Hermann Stillmark found that castor bean extracts contained proteins which could agglutinate red blood cells. These proteins were named agglutinins. Interest in these proteins came with the Second World War as their specificity to different blood groups was discovered and they started to be used in blood typing. Their name changed to "lectins," derived from the Latin word "legere," meaning "to select". The first lectin from animal cells was the hepatic asialo-glycoprotein receptor, a C-type lectin (313). Now when the carbohydrate target of a lectin is a β-galactoside this lectin is known as a Galectin.

A galactoside is a glycoside containing galactose. The H of the OH group on first carbon of galactose is exchanged for an organic moiety. When the glycosidic bond exists above the plane of the galactose, it is called a β-galactoside. The most commonly recognized and used β-galactoside in biochemistry is lactose (Figure 18).

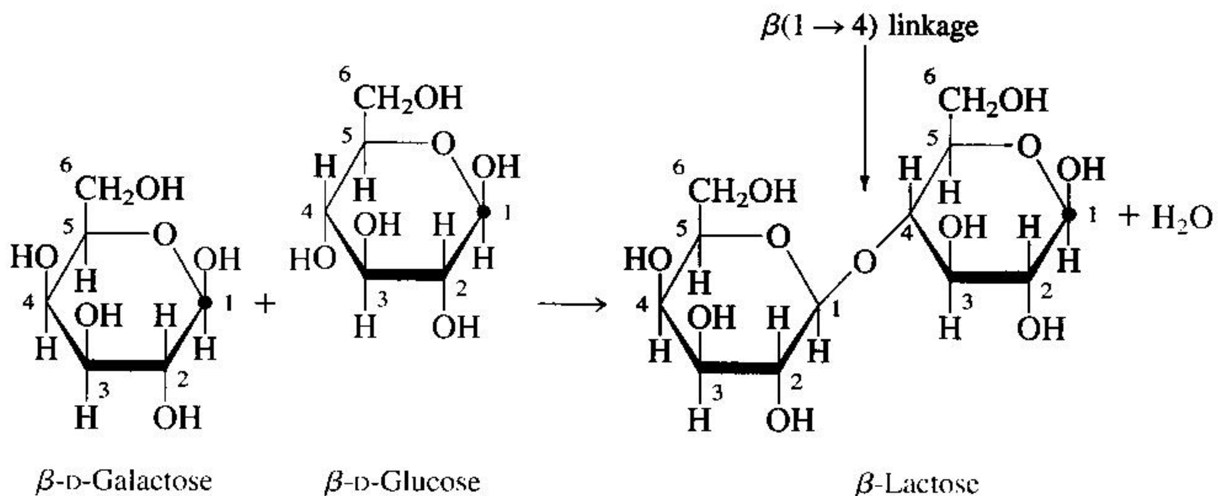


Figure 18 - Beta-galactosidic bond formation

Galectins are an evolutionary conserved family of β-galactoside-binding lectin, ubiquitously found in eukaryotic taxa, including the sponges and both protostome and deuterostome lineages of metazoans, and fungi.

The naming of Galectins first appeared in 1994 as follows : "*Membership in the galectin family requires fulfillment of two criteria: affinity for β-galactosides and significant sequence similarity in the carbohydrate-binding site, the relevant amino acid residues of which have been identified by X-ray crystallography* (314)". Using this definition helped unite numerous previously discovered proteins from different species into one family and greatly simplified the communication of future discoveries in this family.

Galectins were discovered based on the hypotheses that cell surface carbohydrates take part in cell adhesion. Thus, tissue extracts were analyzed for their ability to agglutinate erythrocytes and/or fractionated on affinity columns with immobilized β-galactosides, a common cell surface carbohydrate that at the same time was reasonably accessible in quantities enough for biochemical experiments (e.g. in lactose, and asialofetuin)(315)

The next lectin found in animals was the protein now recognized as the first galectin. It was originally described in 1975 during studies on the possible presence of lectins in the electric organs of the electric eel (316) The protein, termed electrolectin, had hemagglutination activity with trypsinized rabbit erythrocytes that was inhabitable by β-galactosides and could be isolated by affinity chromatography on β-galactoside supports. In 1976, the first mammalian Galectin now known as Galectin-1 was purified from calf heart by affinity chromatography on asialofetuin-Sepharose. It also required reducing conditions to maintain activity. The calf heart/lung galectin-1 is about 15 kD in size

and exists as a noncovalent dimer.(317). Later the same year the same Galectin was purified from embryonic chick muscles by two separate groups (315)

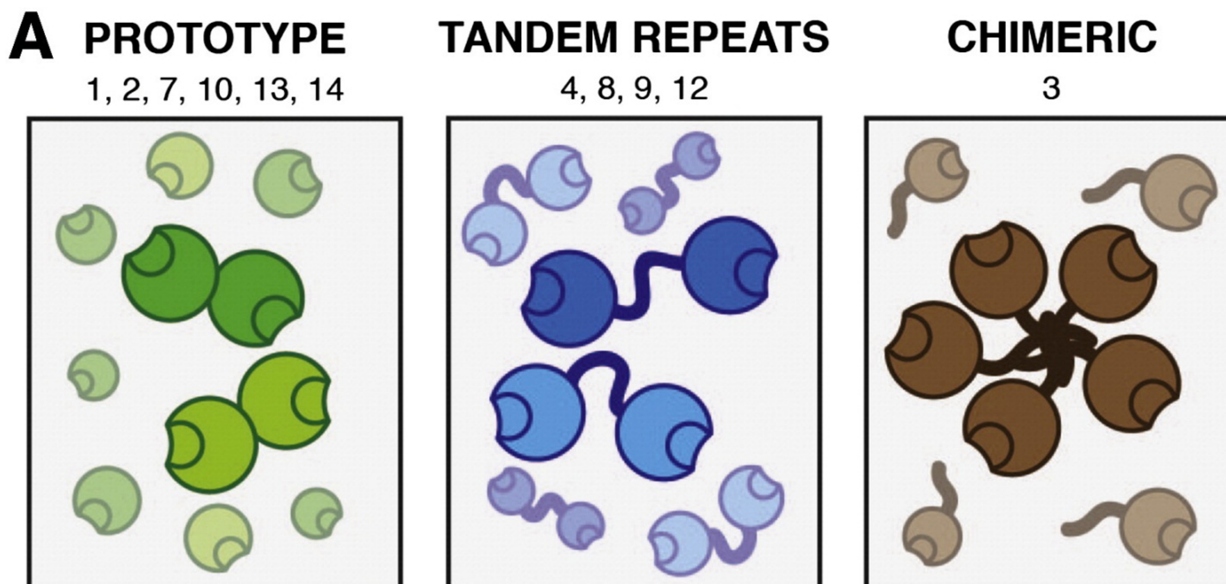


Figure 19 - Different types of the human Galectin family
The 11 members characterized by the presence of a conserved CRDs. Base on their structure they are divided of three subgroups. Prototype galectins are formed of one domain which can homodimerize. Tandem repeat galectins are formed of two domains which are connected by a flexible linker of variable lengths due to alternative splicing. Chimeric galectins are formed of a one domain fused to a N-terminal 'tail' of a short sequence proline, tyrosine and glycine-rich tandem repeats (318).

Based on their structure, Galectins are classified into three types “proto”, “chimera”, and “tandem-repeat”. Proto-type galectins have a single subunit form homodimer *via* non-covalent links. The chimera type galectins have one C-terminal CRD with a proline and glycine rich N-terminal domain. Tandem repeat galectins have two CRDs linked by a functional linker sequence. The different galectin subtypes were given numbers by the order of their discovery. Currently there are 15 galectins described in mammals. Galectins 1, 2, 5, 7, 10, 11, 13, 14, and 15 are proto-type. Galectins 4, 6, 8, 9, and 12 are tandem repeat. Galectin 3 is the only chimera type (Figure 19).

IV. Galectin 9

A. History

Galectin-9 was originally identified in 1995 *via* SEREX technique (serological identification of antigens by recombinant expression cloning) while attempting to find tumoral antigens for use as potential vaccines from the tumoral tissue of Hodgkin's lymphoma patient's (319). The newly discovered protein at the time named HOM-HD-21 was found to have 2 domains with conserved motifs implicated in the carbohydrate binding capabilities of galectins. Moreover, the recombinant protein was found to have galactose and lactose binding activity (320). In 1997 the new protein was identified as Galectin-9. Independently and during the same time period Gal-9 was identified by 2 other groups. One group identified and cloned mouse Galectin-9 from mouse embryonic kidney (321). The second group of Hirashima identified a secreted protein in the culture medium of a T-lymphocyte cell line that had been immortalized following an infection of human T-cell lymphoma-leukemia virus. This secreted Galectin which they originally called ecalactin (due to its eosinophil chemoattractant capabilities (ECA)) had 5 amino-acid variations from the already cloned Gal-9 (322). Later the team published that ecalactin was in fact Gal-9 and the observed difference were probably due to sequencing or cloning artifacts (323). The expression of Gal-9 in various mouse tissues was then studied by northern blotting and it was found to be widely expressed at different levels in adult tissue with spatiotemporal expression in developing embryo (324). Interestingly the team which identified the human Gal-9 in the Hodgkin's lymphoma tumoral tissue went on to determine by northern blotting that Gal-9 expression was limited to lymphatic tissue and peripheral blood leukocytes and was unable to determine its presence in breast, kidney, brain, skeletal muscle, skin, testis, and stomach. After that another group found Gal-9 while attempting to find autoantigens in autoimmune diabetes by screening cDNA expression library of human pancreatic islets and screening it for reactivity with diabetic patients' sera. They found that the 2 isoforms of Gal-9 clones were highly reactive with the sera and then moved on to determine the presence of anti-Gal-9 autoantibodies not only in the sera of patients but also in healthy donor sera. This suggested the ubiquitous presence of natural autoantibodies to Gal-9 and its isoforms. It is important to note that this interaction between the in vitro produced Gal-9 and the serum antibodies was not inhibited by high concentrations of lactose indicating that it was not due to an aspecfic interaction between Gal-9 CRD domains and the antibodies. This team which later cloned human Gal-9 also looked to determine its expression in different human tissue and unlike previously obtained results, they determined by PCR the presence of Gal-9 transcripts in multiple human tissues such as liver, lungs, tonsils and islets. They also detected Gal-9 in Jurkat and HeLa cell lines. Gal-9 was not detected in brain tissue (325). The presence of these auto-antibodies does indicate the importance of the regulation of Gal-9 in humans.

B. Gal-9 isoforms.

The first detection of the small isoform of Gal-9 was by RT-PCR in HUVEC cell lines. This isoform corresponded to a deletion of exon-6 of the medium isoform which at the time was the accepted isoform of Gal-9. The large isoform at the time was considered an insertion of exon-5. This made the HUVEC endothelial cell line the first ever cell line where all 3 isoforms of Gal-9 were clearly detected(326) . Using cDNA from Jurkat cells, the team of Hirashima fully identified the 3 isoforms and discovered that they only differ in the length of their linker peptide. This linker peptide between the 2 CRD domains had a length of 14, 26 and 58 amino acids (aa) in the small, medium and large isoform respectively. The resulting estimated molecular weight of each of the proteins was 34.7, 35.9 and 39.5 kDa respectively (327) (Figure 20). Prolines are common in beta turn and unlikely form an extended structure indicating a low probability of a configurational difference between the N and C terminus domains between the 3 isoforms (328).

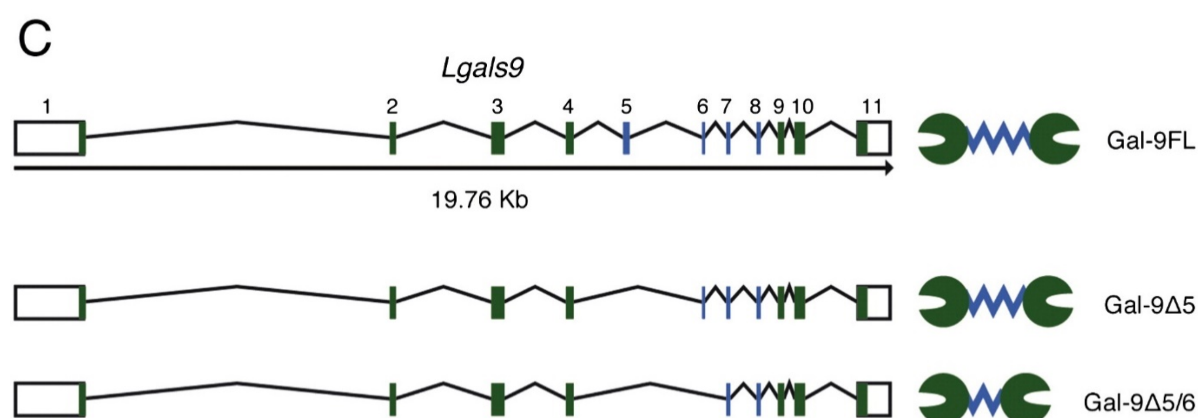


Figure 20 - Three naturally occurring isoforms for Gal-9
Graphical representation mRNA of the three Gal-9 isoforms. Edited from (329)

The 3 isoforms carrying the 4KD hexa-histidine sequence (His-tag) were generated as recombinant proteins in *Esherichia coli* by Hirashima's team. The large isoform had a much lower yield probably due to reduced solubility or stability. These proteins as well as the previously produced Glutathione *S*-transferases (GST)-tag Gal-9M showed comparable hemagglutination capabilities, eosinophilic chemoattractant activity and betagalactoside binding potential (328).

Interestingly, it was later shown that Gal-9 L was sensitive to thrombin digestion. Thrombin which is a key enzyme in blood coagulation had a recognition site in the linker sequence of Gal-9L. This recognition site which is at the C-terminal of Arginine (Arg) 175 in Gal-9L is specific to this isoform since it exists in the protein sequence coded by exon 5. The group found that thrombin greatly reduced Gal-9L ECA activity while it did not affect that of Gal-9M (330).

Another difference in the function of the three isoforms was found while transfecting different isoforms into human colon carcinoma cell lines. The Gal9L increased the expression of E-selectin while the Gal9M didn't. Only when transfecting the Gal-9S or Gal-9M did they observe an increased adhesion of the cell lines to umbilical vein endothelial cells. This gave direct evidence the fact that these different isoforms can have different functions (331).

A truncated Gal-9 was produced by Hirashima's team (Gal-9NULL). This form of Gal-9 lacking the linker sequence was produced in an attempt to increase the stability of Gal-9 for eventual use in clinical and research efforts. The form was produced in E-coli (BL21 DE3) without a His Tag and then purified by affinity chromatography. This Gal-9NULL recombinant protein had its linker region substituted by a Histidine-Methionine (His-Met) residue. Early tests found this form to be 100 times more stable to different proteases such as matrix metalloproteinase-3 (MMP3)/stromelysin or elastase than Gal-9M or Gal-9S. Interestingly even the native forms of Gal-9 were highly stable against trypsin probably due to the lack of arginine and or lysine residues in the linker peptide. This mutant form was at least 2 times more apoptotic to the MOLT-4 cell line than the native forms (332).

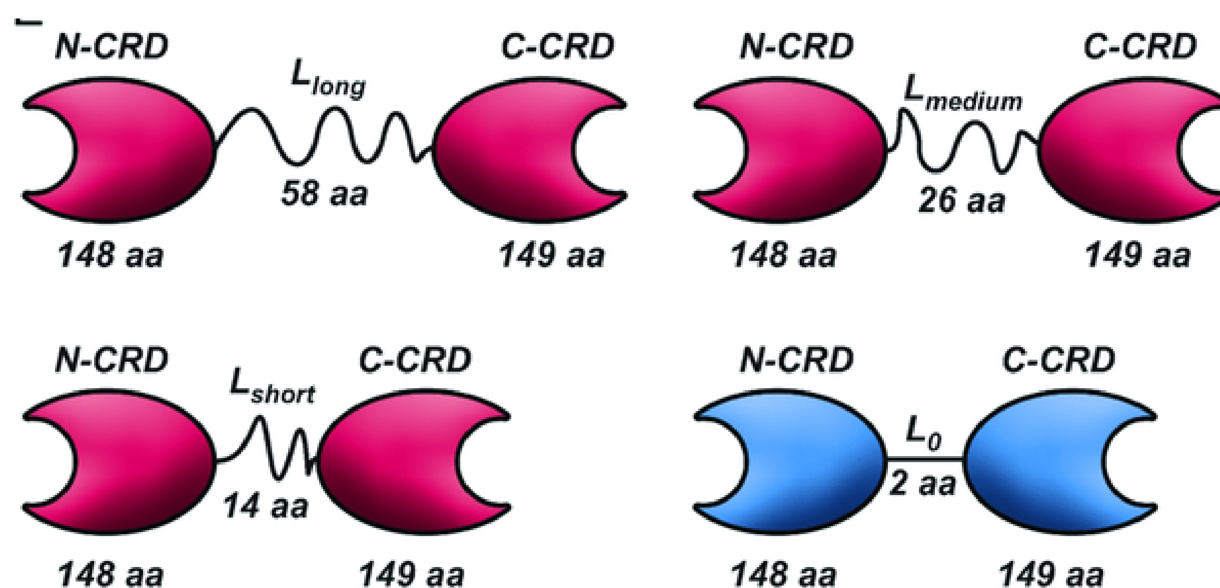


Figure 21 - Illustration of Gal-9 isoforms natural and synthetic
This illustration shows the naturally occurring isoforms of Gal-9 the Gal-9L, Gal-9M and Gal-9S with their linkers of 58, 26, 15 amino acids respectively. It also shows in blue the Gal-9NULL isoform having the 2aa linker (333).

A very interesting study on the importance of the linker sequence in tandem repeat Gal-9 was published by Lesley et al. The team hypothesized that tandem repeat Galectins such as Gal-9 were more potent than proto-type Galectins in ligand binding and apoptotic potential due to the presence of linker sequences which facilitated the dimerization and crosslinking of glycoprotein receptors thus improving the downstream signaling cascade (334). To prove this, the team took 2 identical Gal-1 CRDs and linked them using different types of linker sequences one of which was the 14aa linker sequence of Gal-9S

creating a tandem repeat Galectin. They found that even though the linker did not change the Galectin glycan binding specificity of Gal-1, using the Gal-9S linker added the cell death induction potential by 20 folds. They moved on to show that having a linker sequence did facilitate dimerization and the formation of galectin-glycoprotein lattices. (335). Mentioning this form is very important. As this manuscript will show, this form was used to generate most of the currently accepted "common knowledge" on Gal-9. The fact that this form does not exist in nature would indicate that results obtained from using it should be taken with a grain of salt especially with the recent discoveries of the importance of the linker sequence in Gal-9 functions (336).

C. Gal-9 molecular function

The generation of Gal-9 recombinant proteins consisting of only the C or N terminal CRD without a linker chain or proteins of two N-terminal CRD or two C-terminal CRD connected by the medium size linker sequence showed that both CRD have similar affinities to closely related sugar chains with a few minor differences. Moreover, in the same work by using frontal affinity chromatography on a panel of 33 oligosaccharides versus these protein, Hisrashima's team determined that Gal-9 binds to eosinophil surface glycoprotein(s) *via* a tri and or tetraantennary N-linked glycans with N-acetyllactosamine unites. This interaction might result in dimerization or multimirization of the cell surface glycoproteins or receptors and could therefore initiate intracellular signaling pathways linked to the chemotactic process (328)

Using affinity chromatography and surface plasmon resonance, human Gal-9 molecules were found to be able to interact with each other forming multimers *via* their CRD domains (337). Another study reported on the crystal structure of mouse and human Gal-9 N terminal carbohydrate recognition domain (NCDR) and found that while mouse NCRD formed dimers, human Gal-9 NCRD did not. This was interesting since both CRDs have high sequence identities. This indicates that the dimerization potential arises from the C terminal carbohydrate recognition domain (CCRD). The same team continued to find that human and mouse NCRDs have different carbohydrate binding affinities, for example hGal-9 NCRD had a 100-fold higher affinity to Forssmann pentasaccharides than mGal-9 NCRD. The study continued with functional tests and showed that hGal-9 exhibited a much higher capability of inhibiting Jurkat cell proliferation than mGal-9 (338, 339)

D. Gal-9 secretion:

Gal-9 is a secreted protein. For example, both medium and large isoforms were detected in the supernatant of Jurkat cells (327). Galectins do not have a signal peptide for secretion and at this time, their secretory pathway is still unknown (340). A small hint regarding the secretion was obtained when

it was shown that the secretion of the medium isoform was decreased by the use of tissue inhibitor of metalloproteases BB-94 (327).

E. Galectin-9 expression

Human Gal-9 was detected by RT-PCR in human kidney cells and in HUVEC all isoforms, HMC, U937 and HL60 only medium and large (326). Gal-9 expression in HUVEC was induced by double stranded RNA and mediated by Toll-like receptor (TLR) 3, phosphatidylinositol 3-kinase (PI3K) and interferon regulatory factor 3 (IRF3) (341).

Gal-9 medium and large isoform were found to be expressed by Jurkat cells by RT-PCR and western blotting. Their expression was shown to be upregulated by phorbol 12-myriastate 13-acetate (PMA). The team then used flow cytometry to successfully detect Gal-9 on the surface of the cells. Interestingly though the levels of expression of Gal-9 was very similar between the intra and cell surface staining (327)

Gal-9 RNA and protein was detected in the fibroblast cell line HFL-1 this expression was upregulated by IFNγ but not by IL-4. IFNγ treated HFL-1 cells induced stronger adhesion of eosinophils which was dependent on their betagalactoside binding capabilities since it was inhibited by Lactose and anti-Gal-9 antibody(342)

Gal-9 expression was induced by IFNγ in human primary fibroblasts isolated from nasal polyps. This induction passed by the mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3 phosphate kinase (PI3K) pathways. (343)

Gal-9 RNA was shown to be constitutively expressed in mouse isolated cornea Immunofluorescent staining showed that Gal-9 protein was localized to the corneal epithelium and endothelial cells but not the corneal stroma. The protein was also expressed in the iris-ciliary body. This is one example of Gal-9 induction of homeostasis at environmental interfaces. (344).

Gal-9 expression was found to be upregulated by HUVEC endothelial cells following IFN-γ stimulation. HUVEC which express the Gal-9M under steady state conditions upregulated this expression and started expressing the Gal-9L following IFNγ stimulation. Interestingly IL-4 another potent stimulant of endothelial cells to induce eosinophil recruitment had no effect on Gal-9 expression (345).

Similar results were obtained with fibroblasts. This IFNγ induced expression was regulated by histone deacetylase 3 (346)

F. Gal-9 binding partners

1. Tim-3

T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) was identified as a marker for Th-1 cells following the immunization of rats with differentiated mouse Th1 cells. The resulting 20 thousand antibodies were screened and resulted in the discovery of the anti-Tim-3 antibody which stained Th1 and cytotoxic CD8+ cells but not Th2 cells. (347) Tim-3 induction on primary human T cells either by TCR stimulation CD3/CD28 or by gamma chain cytokines such as IL-2. IL-7. IL-15 and IL-21 but not IL-4 (348)

<u>Gal-9 and Tim-3</u>

Gal-9 was discovered as a ligand for Tim-3 *via* the use of Tim-3-Ig fusion proteins. These proteins stained TK-1 CD8+ cell lines. Downstream precipitation assay and analysis determined the protein as Gal-9 large isoform. Further tests determined that all Gal-9 isoforms could bind Tim-3. This binding was dependent on Gal-9 beta-galactoside binding capabilities as it was inhibited in a dose dependent manner by lactose. It was previously determined that 2 crucial sites for carbohydrate binding activity existed in the CRDs of Gal-9 (349). Site mutagenesis of either one of the arginine residues R64 and R238 in the CRDs greatly lessened Gal-9 Tim-3 binding. When both sites were mutated to alanine this binding was abolished. The authors also proved that Gal-9 Tim-3 binding induced calcium influx and apoptosis of Th-1 cells. Interestingly, Gal-9 mediated cell death was not completely abolished in Tim-3 knockout Th1 cells indicating that Gal-9 has another apoptosis inducing receptor. Gal-9 induced cell death seemed to be preceded by cell aggregation and involved both apoptotic and necrotic processes coupled with calcium influx. Finally they showed that *in-vivo* injection of Gal-9 in Experimental autoimmune encephalomyelitis mice only depletes activated antigen specific Th1 cells and not all peripheral T cells (350)

Gal-9 binding to Tim-3 was shown to increase the phosphorylation of the Tyrosine 265 in the intra cellular domain of Tim-3. This phosphorylation was mediated by inducible T cell kinase, which is known to be upregulated in allergies. It should be noted that Y265 Tim-3 mutants continues to be phosphorylated following Gal-9 binding but at a much lower level indicating the existence of other phosphorylation sites. (351) In an elegant set of experiments on primed allo-tolerance mouse splenocytes challenged with donor irradiated splenocytes treated with Tim-3-Ig which blocks soluble Gal-9 had a strong decrease in the TGF-β secretion without affecting Foxp3 expression. (352).

Co-immunoprecipitation and site directed mutagenesis have shown that mouse Tim-3 binds human leukocyte antigen B (HLA-B)-associated transcript 3 (Bat3) at the 252-270 residues of Tim-3 and inhibits its function. Gal-9 binding and induced phosphorylation to Tim-3 releases Tim-3 from Bat-3. Bat-3 was shown to be immunostimulatory as it played a crucial role in the induction of autoimmunity. (353) Another study on PBMCs isolated from 87 multiple sclerosis patients showed that

increased Bat-3 expression correlated with a more progressive disease. Moreover, when Bat-3 was overexpressed by patient cells, the blocking of Tim-3 and Gal-9 did not affect IFNγ or IL-17 production of the pathogenic T lymphocytes stimulated in *in-vitro* assays. (354)

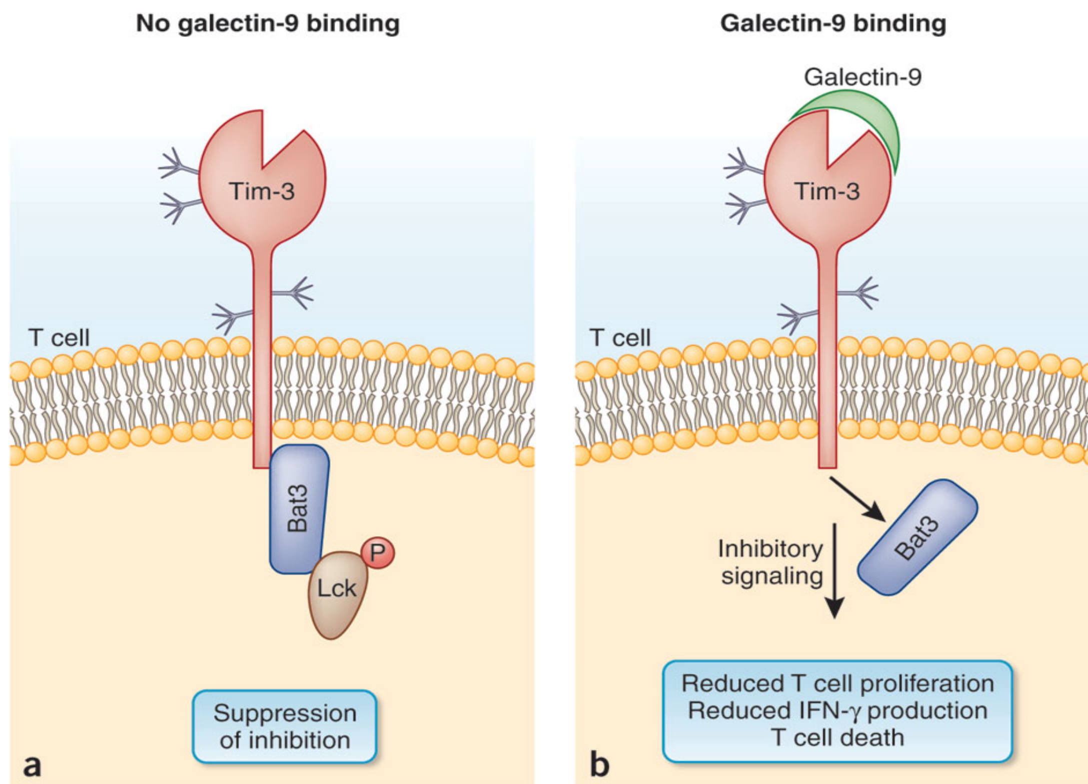


Figure 22 - Illustration of the Tim-3/Gal-9/Bat-3 interactions
Bat-3 acts a negative regulator for Gal-9 induction of the Tim-3 suppressive pathway (355)

2. Gal-9 and CD44

While demonstrating the therapeutic potential of the Gal-9NULL as a treatment for bronchial asthma in a mouse model, Hirashima's team found that Gal-9 was capable of binding CD44 preventing its binding to hyaluronic acid. The CD44 antigen is a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration. It had been shown that this CD44-hyaluronan interaction was crucial for the extravasation and egress of activated T lymphocytes on the inflamed vascular endothelium (356). This is possibly one of the mechanisms by which Gal-9 inhibits metastasis. The gal-9 CD44 interaction was also shown in different contexts. This interaction leads to formation of complexes with other receptors leading to receptor aggregation and phosphorylation of different SMAD family members. So far this mechanism has been shown in osteoblasts for the GMB receptor and in CD4+ T cells for the TGFβ receptor in osteoblast differentiation as CD44 binding induced phosphorylation of Smad1/5/8 and nuclear translocation of Smad4 where it bound a promoter (357).

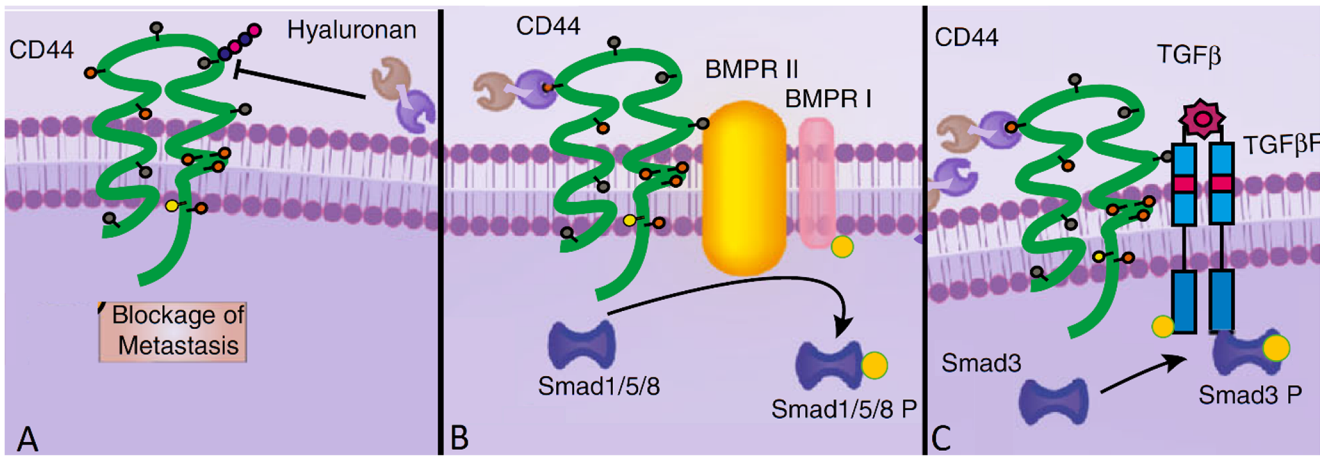


Figure 23 - Gal-9 CD44 interaction
A. Gal-9 interacts with CD44 inhibits its binding to HA and leads to blocking of metastasis. B. Gal-9/CD44 interaction forms a complex with BMPRII which phosphorylates Smad 1/5/8 complex leading to osteoblast proliferation. C. Gal-9/CD44 interaction leads to complex formation with TGFβR and with TGFβ binding induces phosphorylation of Smad3. Edited (336)

3. Gal-9 and CD40

In mice Gal-9 was shown to interact with CD40 preventing the proliferation of CD4lowCD40+ cells and inducing their cell death in a Tim-3 independent pathway. Gal-9 also co-immunoprecipitated with CD40 from BALB/c CD4lowCD40+ T cells (358)

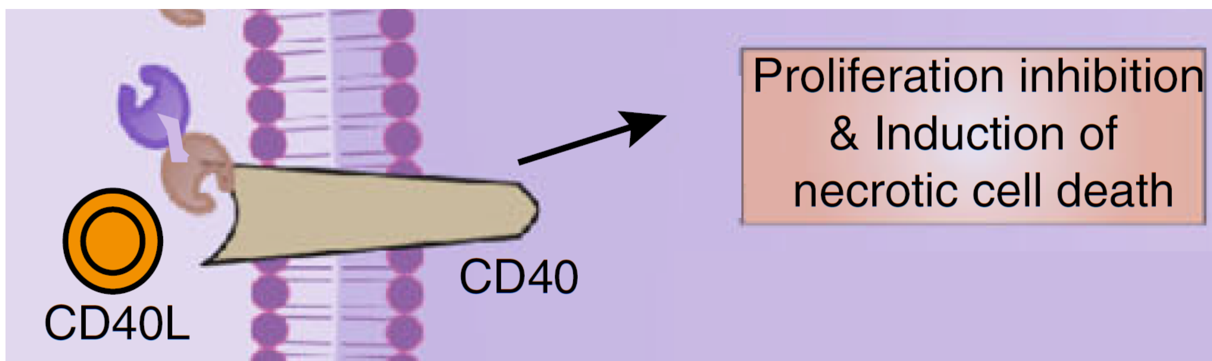


Figure 24 - Gal-9/CD40 interaction
Gal-9 interaction with CD40 is an additional mechanism by which Gal-9 inhibits the immune response. Edited (336)

4. Gal-9 independent Tim-3 ligand

Another elegant set of experiments showed that the IgV region of Tim-3 possess 2 two noncanonical disulfide bonds that change the folding of the of this region creating a unique ligand binding surface unrelated to Gal-9. This binding was shown to be distinct from the Gal-9-Tim-3 interaction. Furthermore, murine Tim-3 IgV tetramers were capable of binding primary CD4+ T cells, CD8+ T cells, Tregs, B cells, dendritic cells and macrophages.(359)

Hirashima's team showed that Gal-9 binds both human and mouse IgE, highly glycosylated antibody, preventing its ability to induce degranulation of mast cells. Additionally, the expression of Gal-9 in activated mast cells suggests that Gal-9 can regulate allergic activity *via* this pathway. Binding of Gal-9 to IgE depends on Gal-9's beta-galactoside binding ability since it was inhibited by lactose. (360)

Gal-9 was shown to induce death on cells which did not express neither Tim-3 nor CD44 which was mediated by cytochrome c release from the mitochondria and was blocked by over expression of Bcl-2 (334). Gal-9 affinity chromatography of solubilized membranes from Tim-3 negative cells showed that Gal-9 can bind some of the Protein disulfide isomerase (PDI) family of proteins via Gal-9 CRD as it was inhibited by lactose. Interestingly, PDI was significantly expressed on mouse TH-2 but not TH-1 cells which could explain the resistance of TH-2 cells to Gal-9 induced death. Gal-9 can anchor PDI to the cell membrane, and binding of Gal-9 to PDI increased cell migration by increasing PDI disulfide reductase activity at the cell membrane of TH-2 cells (361)

In-vitro treatment with truncated Gal-9 of TCR stimulated CD4+ cells isolated either from normal or from Tim-3 KO mice induced similar levels of apoptosis in both cell types showing that Tim-3 was not the only TH-1 death receptor for Gal-9 (362)

G. Galectin-9 functions:

1. Apoptosis

The high expression levels of Gal-9 in the developing thymus indicated a possible role in thymocyte stromal cell interaction during embryonic development in mice. This lead to the production of a recombinant Gal-9 protein with a c-myc-$(His)_6$-tag. This protein which retained its lactose binding activity was shown to be capable of inducing apoptosis of mouse thymocytes at a dose of 2.5μM. The observed apoptotic potential was inhibited by lactose (324). This work is the earliest work indication an immunosuppressive potential for Gal-9. The authors of the paper related their discoveries to a potential role of Gal-9 in the clonal deletion of self-reactive cells during embryonic development. Galectin 9 induced apoptosis of several immune cell lines MOLT-4 (T cells) Jurkat (B cells) BALL-1 (B cells) and THP-1 (monocytes) following a 24 hour culture with Gal-9 at 1μM (363). This was blocked by 30nM of lactose indicating that it was dependent on β-galactoside binding. This apoptosis was mediated by Caspase-1 *via* the calpain-caspase-1 pathway. In fact, the authors of this paper determined that Gal-9 treatment induces Ca^{2+} influx into the MOLT-4 cells. Interestingly primary isolated T CD4+ and CD8+ cells were sensitive to the same Gal-9 induced apoptosis but only in activated conditions. Later Tim-3 was determined to be the receptor inducing this type of apoptosis (350). Similar work also showed that the apoptotic function of the Gal-9S on Jurkat cells did not completely pass *via* the calapin-caspase-1 pathway as the use of caspase inhibitors only partially inhibited Gal-9S induced DNA fragmentation and

phosphatidylserine cell surface exposure. These findings are very interesting because they further prove that the apoptotic function of Gal-9 is not limited to the Tim-3 Caspase pathway. In the same study, the authors studied several mutants of human Gal-9S that either contained one CRD domains or 2 N/C domains joint by a linker sequence. They showed that the presence of 2 CRD domains was crucial for the induction of apoptotic function regardless of which domains formed the dimer (364).

2. Chemoattractant

Gal-9 secretion was found to be upregulated by T lymphocytes following stimulation. The Gal-9 recombinant protein was found to be a strong eosinophil chemoattractant assessed by *in-vitro* chemotaxis tests (322). N or C terminal only recombinant Gal-9 proteins showed 100 fold less chemoattractant potential despite retaining their affinity for lactose even when used together suggesting the importance of have both terminals linked *via* the linker sequence for Gal-9 function. Moreover, site directed mutagenesis of positions Arg65 of Gal-9-NT and Arg239 of CCRD to an aspartic acid residue resulted in the loss of both lactose-binding and ECA activities indicating the importance of the galactoside binding activity for the chemoattractant function (349).

3. Induces proliferation

Unlike its anti-proliferative function, human Gal-9 (hGal-9) was found to induce proliferation of human osteoblasts *via* interactions with lipid rafts. This proliferation was not shared with other tested galectins. hGal-9 at doses from 0.1 to 100 nM induced clustering of lipid rafts and c-Src phosphorylation activating the ERK signaling pathway and inducing proliferation. This is very interesting since Gal-9 is generally considered as an apoptotic factor (365)

H. Gal-9 and pregnancy

Pregnant women have higher levels of circulating Gal-9 with respect to non-pregnant ones. These levels increase as the pregnancy proceeds (366). Again Gal-9 plasma levels from 20 normal pregnancies and 35 cases of unexplained recurrent spontaneous abortion patients were higher than that in the plasma of healthy non pregnant women. Moreover, women suffering from spontaneous abortions had a decrease in Gal-9 levels (367). Gal-9 seems to be crucial in downregulating the immune response at feto-maternal interfaces preventing the mother's immune system from attacking the fetus (368-370). A very interesting study on the expression of Tim-3, Gal-9 and PD-1 in the immune cells at the fetomaternal interfaces in BALB-c mice showed an increased expression of both receptors. Gal-9 expression was also increased in the placenta. Decidual NK and γ/δ T cells had an increased expression of the exhaustion receptors. Tim-3 expression on these cells correlated with a decrease in cytotoxic activity (371).

I. Gal-9 and grafting

Gal-9 seems to be a candidate for a new immunosuppressor to be used in graft tolerance. In a study of skin grafts on mice, Tim-3 was found to be expressed on activated alloreactive CD8+ cytotoxic T cells but not on naïve CD8+ T cells. Gal-9 treatment (isoform not specified) at 5ug/ml induced apoptosis of these cells. Gal-9 treated mice had a prolonged allograft survival as well. This increase survival was accompanied with a decrease in the CD8+CD44highCD62Llow fraction and a decrease in IFNγ levels (372). The same team later using the same model determined that Gal-9 inhibited proliferation of mouse T cells under TCR stimulation (373). In another experiment, Gal-9 injections prolonged the survival of mismatched cardiac allografts in mice by reducing the number of graft infiltrating CD4+ and CD8+ T cells as well as decreasing the overall mRNA expression of IFNγ and IL-17 while increasing that of Epstein-Barr virus induced gene 3 (EBi-3) and Gal-9 (374). Human recombinant Gal-9 treatment was also used in mice to ameliorate acute graft vs host disease in mice having received a miss matched bone marrow transplant. This effect was mediated by inducing Ca2+ mediated apoptosis of Th1 CD4+ cells (375). Numerous other studies determined the importance of Gal- in graft acceptance (376, 377).

One example of special interest to the project is the effect of Gal-9 in corneal transplants and tissue. Analysis of Gal-9 expression analyzed by western blot showed that it was downregulated in rejected mouse corneal grafts (378). Mouse corneal graft rejection following a treatment of either an anti-Tim-3 or an anti-Gal-9 antibody was increased. This corresponded with an increase in graft infiltrating CD8+ and CD4+ T lymphocytes. The most interesting part about this work is that Gal-9 blocking did not affect the eye associated immunotolerant environment also known as Anterior Chamber Associated Immune Deviation (ACAID). The researchers, using a model for alloantigenic – specific tolerance in mice that involved the injection of allo-splenic cells in the mice eyes with or without pre-sensitization, showed that Gal-9 blocking did not inhibit ACAID in non-pathogenic mice. (344).

J. Gal-9 and viruses

An increase of Gal-9 levels has been documented in several viral infections.

1. Gal9 and hepatocellular carcinoma linked to HCV infection

Circulating Gal-9 levels in HCV were shown to be high at 841pg/ml and even higher in Hepatitis C virus (HCV) HCC patients 1376pg/ml vs almost 0 in healthy controls. Immunohistochemistry (IHC) and Immunofluorescence (IF) analysis of liver biopsies showed that Kupffer cells from these patients were the origin of Gal-9 secretion as most of them stained positive for Gal-9 whereas the hepatocytes were Gal-9 negative. Finally, Gal-9 induced apoptosis of HCV specific CTLs in vitro (379). Similarly, cells

from patients suffering from HBV induced HCC were isolated and checked for Gal-9 expression. In these patients, Kupffer cells expressed the highest levels of Gal-9 followed by myeloid dendritic cells and plasmocytoid dendritic cells (380). In another study, circulating serum levels of Gal-9 in HBV infected patients were much higher than in healthy controls. The *in-vitro* use of a combination of Tim-3 and PD-1 blocking antibodies on isolated exhausted CD8 cells from HBV patients rescued their effector phenotype (IFNγ and TNFα secretion as well as cytotoxic potential) in a synergistic non redundant fashion (381). Yet another report from *in-vivo* infected human primary hepatocytes showed that the HCV infected hepatocytes produced high amounts of Gal-9 and TGFβ and were capable of inducing Foxp3+ Tregs in vitro as well in a co-culture assay (382). Another study on 90 patients who had undergone liver resection showed that Gal-9 mRNA expression in tumor adjacent tissue was much higher than that found in the tumoral hepatic tissue further showing the discrete localization of Gal-9 expression (383). In contradictory results, treatment of 2 cell lines of HCC with Gal-9 null induced apoptosis. (384).

The role of Gal-9 in HCV infected patients was further elucidated by another study comparing serum Gal-9 levels between patients with chronic HCV infection and patients that underwent spontaneous resolution of the infection with the results showing higher Gal-9 levels in the chronically infected patients(385)

2. Gal-9 and Influenza virus

Circulating serum level of Gal-9 as detected by enzyme-linked immunosorbent assay (ELISA) were significantly higher in patients suffering from influenza virus infection than healthy donors or patients suffering from pneumococcal pneumonia. (386) Gal-9 KO mice had a much better acute and memory response to influenza virus. (387) similar work was done on Herpes simplex virus (HSV) infection (388)

Gal-9 was shown to be highly upregulated in patients with Dengue virus infection and declined during the recovery phase (389).

In a mouse model for respiratory syncytial virus (RSV) infection which is characterized with a strong inflammatory pathogenesis Gal-9 treatment *via* a recombinant adenoviral plasmid administered intranasally into the RSV infected mice was able to decrease disease severity (390).

Another work on HSV infection in mice further showed the immunoregulatory role of Gal-9 in these mice as Gal-9 knockout mice had a better acute and memory response. Similar results were obtained with lactose treatments of these mice (391).

K. Gal-9 and autoimmunity.

Gal-9's role in autoimmunity has been shown in various human and mice studies. The common theme was showing that Gal-9 is upregulated in the circulation of patients suffering from autoimmunity and that rGal-9 injections into mice models for autoimmunity helped relieve the symptoms

Multiple studied investigated the role of Gal-9 in Rheumatoid arthritis (RA). In humans, one study detected increased levels of Gal-9 in the synovial fluid of Rheumatoid arthritis patients with respect to osteoarthritis patients. Gal-9 was able to induce apoptosis of fibroblast like synoviocytes (which are one the major inflammatory cells in RA) in a Tim-3 independent manner since these cells did not express Tim-3 (392). Another patient study looked at the PBMC expression of Gal-9 mRNA between RA patients with active or inactive and healthy donors. They found that Gal-9 mRNA was highest in RA patients with inactive disease while healthy donors had nearly undetectable levels. Interestingly Tim-3 expression did not differ between patients and healthy donor PBMCs (393).

Gal-9NULL injection in mice models for collagen induced arthritis was able to reduce disease severity. These mice had decreased levels of IL12, IFNγ and IL17 in their joints while the levels of IL-4 and IL-10 were not affected. This work showed that the circulating CD4+Tim-3+ in mice decreased following treatments while that of CD4+CD25+Foxp3+ increased (392, 394). A separate study using a different arthritis model showed that Gal-9NULL injection relieved disease symptoms by shifting macrophage functions from pro-inflammatory to anti-inflammatory. (395)

Another study looked at Gal-9 in juvenile dermatomyositis using patient plasma and multiplex immunoassay. Gal-9 was shown to be significantly increased in patients suffering from the disease and it correlated with a poor prognosis (396).

Gal-9 Serum levels were shown to be significantly elevated in plasma of patients suffering from atopic dermatitis a Th-2 polarized autoimmune disease. Gal-9 levels decrease after treatment (397).

Gal-9 at the same time was shown to protect from autoimmune diseases in mice. One study showed that over expressing Gal-9 in diabetic mice was enough to protect mice from diabetes (398). Another study found that Gal-9NULL injections reduced the disease severity of a lupus mouse model in part by inducting apoptosis of plasma cells. This study was the first to show that Gal-9 can induce apoptosis of CD19-/lowCD138+ cells (399). Finally, Gal-9 treatment of mice glomerulonephritis protected the mice from the disease by inhibiting the TH1 and Th17 responses (400).

These patient and mice results combined would indicate that increase in Gal-9 levels especially in the serum is common in patients suffering from immune disease. The fact, mouse models went on to show that exogenous Gal-9 injections would relieve the symptoms would indicate that increase in Gal-9

is a regulatory response from the immune system trying to control inflammation in pathological conditions.

L. Gal-9 in hyper sensitivity

Similar to autoimmunity, Gal-9 seems to be expressed in several human inflammatory diseases and rGal-9 seems to have a therapeutic effect on treating mice inflicted with such diseases.

Gal-9 was over expressed at the RNA level and at the protein level in periodontal ligament cells following LPS treatment. galectin-9 mRNA expression was detected in the mRNA from PDLs of patients with periodontal disease when compared with healthy donors (401).

Gal-9 expression was increased in nasal polyps from patients with chronic sinusitis than in control polyps. This was detected by immunohistochemistry and RNA expression. This increase of Gal-9 positive cells correlated with an increase in EG2-positive cells (402)

Gal-9 expression was detected in *in-vitro* dermal fibroblasts following IFNγ stimulation. Moreover, immunohistochemical staining of lesioned skin of psoriasis vulgaris a Th1-polarized skin disease showed a high expression of Gal-9. (403)

Gal-9NULL was used to treat mice contact hypersensitivity and psoriatic reactions which are TH1 and Th17 mediated skin inflammations indicating further the immunosuppressive role of Gal-9. It induced apoptosis of CD4, CD8 and NK cells, decreased IFNγ, IL17 and IL22 production (404). Th17 and TH1 cells from psoriasis patients had a lower expression of Tim-3 upon stimulation with respect to controls from healthy donors. These cells showed higher inflammatory cytokine secretion (405).

In a model of mouse lung inflammation, Gal-9NULL was shown to expand immunosuppressive macrophages having the CD11b+Ly-6Chigh profile thus decreasing the levels of IL-1, IL-6, IL-17 and IFNγ ameliorating the lung inflammation. The immunosuppressive potential of these cells was shown in an MLR with TCR stimulated CD4+ cells. As expected in this model Gal-9 treated mice had less Tim-3+ cells and showed an increase of CD4+CD25+Foxp3+ splenic cell population (406).

Gal-9 treatment in mice model for chronic asthma induced by double stranded RNA suppressed the symptoms (407).

Crohn's disease patients' PBMCs and Th cells isolated from intestinal mucosa expressed less Tim-3 upon stimulation than healthy donor cells. This indicates that the Gal-9/Tim-3 pathway could play a role in the etiology of IBDs. (408)

Taken together these results along with the data obtained from the role of Gal-9 in autoimmunity and viral infection suggest a strong anti-inflammatory role for Gal-9. In fact, the exact

role of Gal-9 on the various cells of the immune system has been studied under different healthy or pathogenic conditions and the results are summarized in the following paragraph.

M. Gal-9 and different immune cells

The function of Gal-9 on the various immune cell population has been proven by ex-vivo culture of human primary immune cells with rGal-9, by injecting Gal-9 into mice suffering from immune related pathologies or *via* the use of various transgenic mice (overexpressing or suppressing Gal-9). The ensemble of the published does indicate that Gal-9 has an overall immunosuppressive role (372).

1. Gal-9 and Th1 and CTLs

In a study of skin grafts on mice, Tim-3 was found to be expressed on activated alloreactive CD8+ cytotoxic T cells but not on naïve CD8+ T cells. Gal-9 treatment (isoform not specified) at 5ug/ml induced apoptosis of these cells. The same team later using the same model determined that Gal-9 inhibited proliferation of mouse T cells under CD3 CD28 stimulation (373). While this is only one example, the previously cited works clearly shows that Gal-9 inhibit Th1 CD4+ and activated CD8+ T cells.

2. Gal-9 inhibits M1 and promotes M2 macrophages

Human peripherally derived macrophages differentiated in vitro with mCSF produced basal levels of Gal-9. IFNγ stimulation induced strong Gal-9L and Gal9-M production at 48 hours (379). In model of mice induced arthritis, truncated Gal-9 injections were shown to decrease macrophage functions by decreasing TNFα and IL-1β production while increasing IL-10. In vitro treatment of mouse macrophages upregulated FcγRIII expression as well (395). In a mouse model of lung inflammation, truncated Gal-9 was shown to expand immunosuppressive macrophages having the CD11b+Ly-6Chigh profile thus decreasing the levels of IL-1, IL-6, IL-17 and IFNγ as well as ameliorating the lung inflammation. The immunosuppressive potential of these cells was shown in an MLR with TCR stimulated CD4+ cells (406)

3. Gal-9 inhibits B lymphocytes and plasma cells

The study done by Moritoki et al, was the first to show that Gal-9 can induce apoptosis of CD19-/lowCD138+ cells. The authors found that Gal-9NULL injections reduced the disease severity of a lupus mouse model in part by inducting apoptosis of plasma cells. (399). A separate study confirmed these results in humans as they showed that Gal-9 could inhibit the proliferation of primary stimulated human B lymphocyte as well as inhibit their IgG production as shown by proliferation and Elispot tests respectively (409). Another study showed that the immunosuppressive potential of Gal-9 extends to B cells as injection of Gal-9NULL into mouse model of asthma greatly suppressed the Th-2 response.(356)

4. Gal-9 enhances MDSC

Gal-9/Tim-3 pathway induced the generation of CD11bLyc6G+ myeloid suppressor cells as Tim-3 or Gal-9 transgenic mice designed to over express either Tim-3 or Gal-9 had an increase in this population coupled with a decrease in their effector and memory immune response (410). Moreover, another study showed that it expands and improves the chemotaxis of suppressive CD14-CD11b+Gr1+ (by increasing expression of CCR 1, 2 and 5) plasmocytoid DC like macrophages *via* downregulation of TLR2 and TLR4. This lead to the suppression pro-inflammatory response in a mouse model of acute lung injury induced by intranasal LPC inoculation. Mice treated with Gal-9NULL had decreased secretion of TNF-a, IL-6, and IL-1β and increased IL-10 secretion (411). The use of an anti-Tim-3 antibody did not inhibit the Gal-9 mediated affect suggesting that Gal-9 induction of MDSCs is Tim-3 independent. Gal-9 KO mice had an exacerbated response to LPS induced lung injury.

5. Gal-9 and NK cells

A study on both human and mouse NK cells showed that Gal-9 could regulate NK activation in a Tim-3 independent manner. Human isolated NK cells when treated with Gal-9 decreased their expression of crucial for their immune response such as perforin and TRAIL. Moreover, Gal-9 inhibited IFNγ secretion by IL12/IL15 stimulated NK cells. These results were confirmed by cytotoxicity assays where Gal-9 treatment decreased the lymphocyte associated killing by NK cells. This suppressive effect was Tim-3 independent since the use of an anti-Tim-3 antibody did not change the suppressive functions of Gal-9 on human NK cells. Moreover, Tim-3 negative NK cells responded in similar way to Gal-9 treatment as Tim-3 positive NK cells (412). Another study on human NK cells in pregnancy found that an increase in Gal-9 or Tim-3 expression correlated with a decreased activity of NK cells. These results are interesting since Tim-3 knock down of these NK cells resulted in a decreased expression of maturation markers (CD94 and CD69) as well as decreased cytotoxicity. However, Gal-9 knock down did the inverse as in it increased NK activity. These results give a notion towards a possible diverging role of Tim-3 and Gal-9 in NK cells (370). However, another study on Tim-3 expression on NK cells in adenocarcinoma found that Tim-3 expression correlated with poorer NK cytotoxicity and that Tim-3 blocking increased NK function (413). This further increase the possible functional complexity of this pathway in NK cells.

6. Gal-9 inhibits the Th17 lymphocytes

Tim-3 was expressed on *in-vitro* stimulated mice Th-17 cells which were sensitive to Gal-9 induced apoptosis. Gal-9 treated TH-17 cells inhibited IL-17 secretion both *in-vivo* and *in-vitro*. Equally Gal-9 administration in pneumoniae infected mice accelerated the infection (414). The effect of Gal-9 on the inhibition of the Th17 response in favor of a Treg has been extensively studied as it will be explained in the Gal-9 Tregs part.

7. Gal-9 inhibits Neutrophils

One study showed that Gal-9 treatment of pneumoniae infected mice decreased neutrophil activation (414).

8. Galectin 9 and Dendritic cells:

Via the use of transcript analysis by hybridization to cDNA immobilized on microarrays, Gal-9 transcript was found to be expressed in both immature and mature human dendritic cells with double the level of expression in the mature DCs. Immature DCs were obtained following a 7-day incubation with GM-CSF and IL-4, while mature DCs were obtained by a follow up culture of the immature DCs in a cytokine cocktail of IL-6, TNF-α, IL-1β and prostaglandin E2. (415). Another study analyzed the effect of recombinant Gal-9 on the differentiation of human CD14+ monocytes and maturation of dendritic cells. They found that while recombinant Gal-9 at a concentration of 10ug/ml induced apoptosis in about 30 percent of the cultured cells with or without GMCSF and IL-4 it did induce the maturation phase DCs without affecting the differentiation of monocyte to immature DC. They found that Gal-9 did not have any significant effect on the expression of markers associated with immature DCs mainly CD80, CD14 and CD86. However, they found that Gal-9 induced to a lesser extent than LPS the maturation of DCs increasing the expression of different maturation markers such as CD40, CD54, CD80, CD83, CD86 and HLA-DR. They did also found that these Gal-9 matured DCs were capable of stimulating the proliferation allogenic CD4+ T lymphocytes again to a lower level than LPS matured DCs. Lymphocytes activated by Gal-9 matured DCs secreted activation cytokines to a similar level to T cells activated by LPS matured DCs (IFNγ, TNFα and IL-2). Interestingly T cells stimulated by Gal-9 matured DCs however they did have a much higher secretion of IL-10 than LPS matured DCs. The fact that these T lymphocytes do secrete much higher levels of IL-10 does pose a question regarding their identity, did they generate iTregs using their protocol? The most interesting part of this study was that this stimulatory effect was not blocked with lactose and hence was proven to be independent of the galactoside binding function of Gal-9. The researchers did prove that Gal-9 induced phosphorylation of MAPK p38 and ERK1/2 in the maturation of DCs but did not determine the exact binding partner of Gal-9 which would have been interesting especially since the binding happens regardless of galactoside binding activity (416).

Two independent studies later showed that these monocyte derived DCs following Gal-9 treatment were tolerogenic as the CD4+ T cells activated by these cells were in fact iTregs (417). Human monocyte derived dendritic cells that were treated with 1ug/ml of Gal-9 during their maturation phase and then co-cultured with naïve T cells were much efficient in generating Tregs than non-Gal-9 treated dendritic cells. (418).

9. Gal-9 and astrocytes

Using RT-PCR and western blotting, the medium form Gal-9 was found to be expressed by astrocytes following il-1β stimulation. This expression was inhibited by dexamethasone. Interestingly

protein was only found intracellularly and was not secreted. (419). Gal-9 expression from astrocytes seems to be induced by TNFα *via* the JNK MAP kinase pathway which plays a crucial role in the inhibition of neuro-inflammation (420). It was not expressed in steady state. IL-6 IL-10 and IL13 did not affect Gal-9 expression. Another study found that Galectin-9 functions as an astrocyte-microglia communication signal and promotes cytokine production from microglia in a Tim-3 independent manner (421).

10. Gal-9 and multipotent mesenchymal stromal cells.

The MSCs are another immunosuppressive cell type in the immune system that have been shown to suppress the immune response and inflammatory reaction. Gal-9 was shown to be constitutively expressed by human MSCs and upregulated following stimulation by proinflammatory cytokines such as TNFα and IFNγ. Interestingly, the researchers could not detect membranous Gal-9 on these cells and the flow cytometric intra-cellular staining intensity of Gal-9 did not increase following stimulation only the secreted Gal-9 detected by ELISA did. The researchers used siRNA to knock down Gal-9 in MSCs. These MSCs were shown to secrete less Gal-9 and when co-culture with autologous PBMCs had lowered suppressive potential (422). Gal-9 blocking using lactose in co-cultures of MSCs with T lymphocyte or B lymphocytes restored the proliferation of the reporter cells. Overexpression of Gal-9 increased the immunosuppressive function. (409)

11. Gal-9 and Tregs

The relation of Gal-9 and Tregs has been studied by several teams. Most of the data has arisen from mouse models for viral infection of inflammatory disease, but some data has been obtained on human Tregs. The current literature shows that Gal-9 can affect both nTregs and iTregs with most of the data showing an effect on iTregs found in pathogenic conditions.

Gal-9 and nTregs

The first work that only focused on the role of Gal-9 in nTreg induced suppression came from F.Wang et al in 2009. They proved that mouse Tregs expressed Gal-9 by RT-PCR and western blotting. Moreover, the use of an anti-Tim-3 blocking antibody inhibited the suppressor function of Tregs in an MLR with Tconv cells. They continued to show that Tim-3 blocking in MLR conditions restored IFNγ production without affecting IL-2 or IL-4. They validated their results *in-vivo* by showing that allogenic-Treg transfer to induce mismatched graft acceptance could be inhibited by the anti-Tim-3 antibody. (423). Another study showed that Gal-9 induced invitro proliferation of cultured healthy mouse splenic Tregs under TCR stimulated conditions with an anti-CD3 anti-CD28 and Il-2 (424).

Gal-9 and iTregs

The first work showing the relation between Gal-9 and iTregs came from Hirashima's team. While they were attempting to study the role of Gal-9 in a mouse model for induced arthritis, they found that Gal-9 deficient mice showed a decrease in the percentage of CD4+CD25+Foxp3+ cells in the total

splenocytes. They further demonstrated that Gal-9 was important for Treg differentiation since adding Gal-9 to a culture of CD4+ CD62L+ naïve T cells cultured in stimulated conditions with anti-CD3, anti-CD28, Il-2 and TGFβ1 increased expression of Foxp3. (394). These results have been confirmed in rheumatoid arthritis patients as a study positively correlated an increase Gal-9 mRNA expressed in PBMCs from RA patients with Foxp3 expression (393).

These results were also found by another study that showed that Gal-9 also synergizes with TGFβ to induce foxp3+ Tregs from CD4+CD25- mouse splenocytes under TCR stimulation conditions. This study also showed that Gal-9 could induce Tr1 cells when added to an APC and CD25- T cells co-culture. Again these Tregs were suppressive as shown by suppressive tests. This study showed that Gal-9 when synergized with TGFβ increase the activation of the smad2/3 pathways which was shown by increased phosphorylation of both proteins. It also increased the association of Smad2/3 and Smad4. Moreover, Gal-9 further induced the activation of the PIK3 pathway *via* an increase of Erk1/2 phosphorylation (417).

In a key study by Kuchroo's team, the researchers found that Gal-9 deficient mice have a decreased iTreg population. These mice had a decrease in their neoropilin-1 positive Foxp3+ population in the intestinal *lamina propria*. These results were confirmed by *in-vitro* differentiation assays as recombinant Gal-9 with TGFβ was able to restore Foxp3 expression. Smad3 was shown to transcriptionally regulate Gal-9 expression. iTregs differentiated in vitro from Gal-9 KO mice had lower suppressive capabilities measured by MLR and a decreased expression of the suppressive markers CTLA-4, ICOS and GITR. Gal-9 was also found to be necessary for maintaining the stability of the iTreg population. This study showed that Gal-9 binding to CD44 in the presence of TGFβ induces the clustering of CD44 and TGFβ receptor which synergizes and amplifies the kinase activity of the TGFβ receptor. Smad3 which is activated by the TGFβ receptor not only activated the Gal-9 promoter but also binds the Foxp3 non coding region 1 (CNS1) and enhances foxp3 expression suggesting a feed forward loop for Gal-9 in iTregs. Interestingly the ability of Smad3 to access CNS1 does not exist in nTregs which means that Gal-9 does not regulate Foxp3 expression in mice nTregs (425) (Figure 25) .

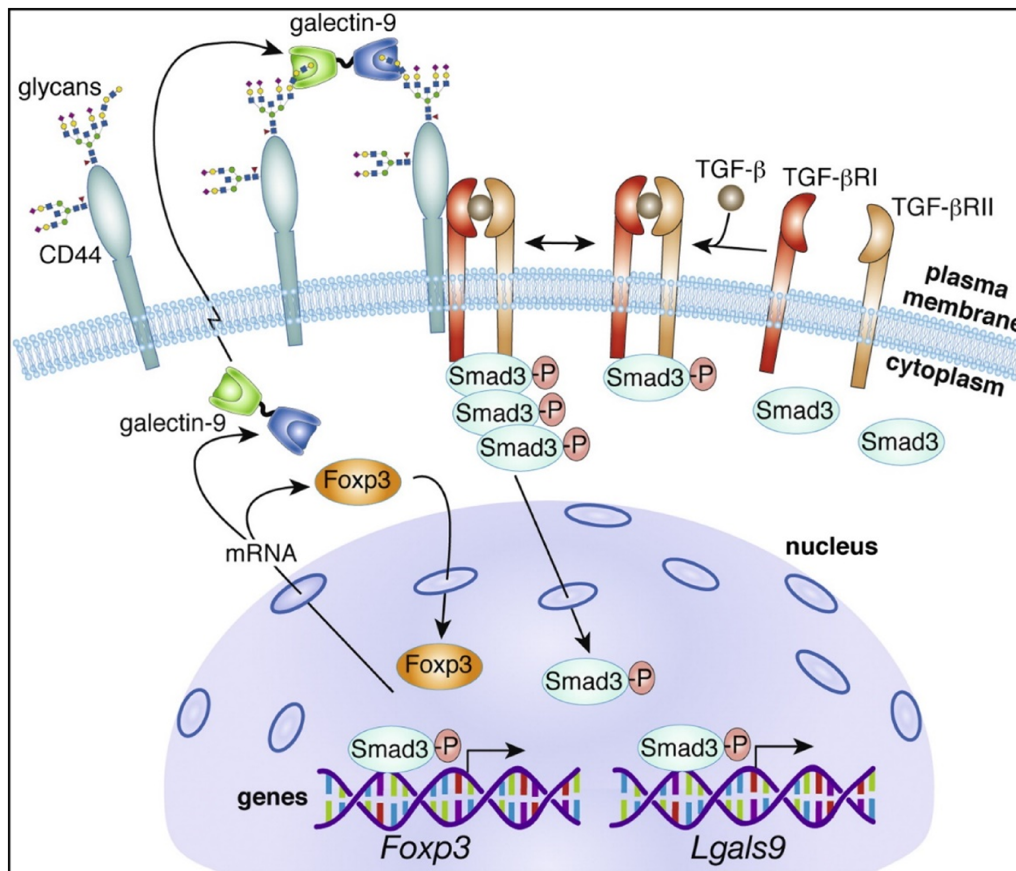


Figure 25 - Illustration of Gal-9/CD44/TGFβ interactions in iTregs
Gal-9 binding to CD44, coupled with TGFβR binding to TGFβ and receptor aggregation leads to Smad3 phosphorylation and Foxp3 and Gal-9 induction of transcription which trigger a forward feed loop since Foxp3 can equally induce TGFβ (426).

Finally, human monocyte derived dendritic cells that were treated with 1ug/ml of Gal-9 during their maturation phase and then co-cultured with naïve T cells were much more efficient in generating Tregs than non-Gal-9 treated dendritic cells. (418)

Gal-9 and Tregs in pathological conditions

More evidence into the function of Gal-9 in Tregs came from work on the therapeutic potential of Gal-9 in controlling chronic immuno-inflammatory diseases. Researchers showed that Gal-9 was able to control the ocular lesions induced by HSV infection and this control passed in part *via* Tregs. The use of Tim-3 blocking antibodies as expected aggravated the lesions mainly by increasing frequency of Tim-3 positive IFNγ and TNFα producing CD4+ T cells. They proved that in fact most CD4+ cells in the HSV infected mice became Tim-3+ whereas the non-infected mice only had a small percentage of Tim-3+ cells and that Gal-9 treatment did act by reducing the number of these cells by apoptosis. The researchers were the first to show the resistance of Foxp3+Tim-3+ cells to Gal-9 induced apoptosis. Naturally Gal-9 treatment of their mouse model increased the splenic population of Foxp3+ cells. (424). Moreover, further supporting the importance of Gal-9 in Tregs, splenocytes from the lactose treated HSV infected mice had a lower number of foxp3+ cells and isolated Foxp3+ cells from the lactose treated mice had a lower level of expression of foxp3 as determined by their MFI measured by flow cytometry and a lower suppressive potential determined by MLR suppression assay (391).

Gal-9 treated of viral myocarditis suffering mice following coxsackievirus B3 infection had a decreased Th1 response coupled with an increase in their CD4+CD25+Foxp3+ population and the CD11b+ or Gr1+ population in the cardiac tissue with myocarditis (427)

Again similar results were observed in a mouse model for encephalomyelitis. Gal-9 KO of the mice suffering from the inflammatory condition induced an increase in the splenic percentage of TH17 cells and a decrease in the CD25+Foxp3+ cells. As usual healthy Gal-9 KO mice did not have the same condition. Treatment of naïve mouse CD4+ cells with Gal-9 in Th17 skewing conditions inhibited Th17 cell development and prevented IL-17 secretion by suppressing the expression of RORγT, inducing that of Foxp3 and without affecting that of Tbet nor GATA3. This inhibition of TH-17 development seemed to be Tim-3 independent. It is true that Tim-3 is expressed on activating TH-17 cells and Gal-9 binding to it induces apoptosis of these cells. However, developing TH-17 cells express very low levels of Tim-3 and the use of a Tim-3 blocking antibody did not affect the Gal-9 induced inhibition of Th-17 development. The authors further continued that unlike Gal-9 induced apoptosis which depends on N-glycans, the inhibition of Th17 development depended on O-linked beta-galactosides (428)

They also showed that Gal-9 *in-vitro* can induce conversion of splenocytes from DO11.10 RAG-/- Treg lacking mice into foxp3+ cells in the presence of TCR stimulation with IL-2 and with or without TGFβ. The effect was much higher and seemed to be synergistic with TGFβ. In a similar experiment, Gal-9 inhibited the generation of TH17 cells (424).

Gal-9 treatment of PBMCs from healthy of HCV infected patients at 5ug/ml increased the percentage of CD4+CD25+CD127lowFoxp3+ fragment after 5 days of culture. This was mediated by TGFβ since a blocking antibody decreased the effectiveness of the conversion (379). Moreover, another study on human PBMCs co-cultured with Gal-9 expressing HCV infected human hepatocytes showed that these cells could induce the generation of foxp3+ suppressive Tregs from CD25- cells. This induction could be achieved either by Gal-9 alone or by TGFβ alone but the synergistic effect was much higher when used together. This study did use the Gal-9 null and did show that Tim-3 blocking inhibited part of this conversion (382) .

Circulating Gal-9 increased the Treg population in HCV infected patients as demonstrated by Foxp3 staining of PBMCs, these Tregs also expressed CTLA4 and CD39. This study further determined that CD39+ Tregs had a higher Gal-9 expression by QPCR than CD39- Tregs from the same patients. They also correlated the intensity of Foxp3+ staining by flow cytometry with Gal-9 expression. (385)

One study using Gal-9 treated mice in a model for ConA induced hepatitis showed that only a small fraction of CD25+Foxp3+ positive cells were apoptotic following Gal-9 treatment (10%) compared to the 90% of the CD4+CD25-/low cells (429). Another study in the same model showed that Con-A treated mice hepatic Tregs had a higher Tim-3 expression. Tim-3 blocking restored IFNγ secretion from reporter cells in a co-culture. (430)

Isolated PBMCs patients suffering from ulcerative colitis another types of inflammatory bowel disease showed a decrease in their mRNA Gal-9 expression coupled with a decrease in the mRNA expression of Foxp3 and an increase in that of IL17. (431)

Invitro culture of PBMCs obtained from atherosclerosis with Gal-9 medium isoform under TCR stimulation resulted in the expansion of the Foxp3+ population in a dose dependent manner (432)

Gal-9 injections in a mouse model for respiratory syncytial virus (RSV) lead to a decrease in the TH17 population coupled with an increase in the Treg Foxp3+ positive population in the mice lungs. This was correlated with an increase in IL-10 production (390).

N. Galectin-9 and cancer

Following is discovery in Hodgkin's lymphoma patients' tumoral tissue (320), the study of expression of Gal-9 in various tumoral tissues, cells lines or patient sera became of great interest especially taking into consideration its apoptotic potential as well as its immunoregulatory properties. The expression of the Gal-9 transcript was detected early on in Jurkat and Hela cells (325). Another early study found the expression of Gal-9 transcripts again by PCR at various levels in established human colon epithelial cell lines showing for the first time the possibility of the presence of a frame shift mutation resulting in truncated version of a protein without the C-terminal; something which had not been previously seen for any Galectin (433). Results on the expression of Gal-9 by tumoral cells have been controversial. On one hand based on all its suppressive potential, Gal-9 is a primer target for being an immunoregulatory molecule in the tumoral microenvironment, however, its characterization as an apoptotic factor has led some to believe that it would be downregulated in tumor cells in order to improve survival.

1. Gal-9 increase as a positive prognostic factor

One early study by PCR examined the transcript expression level of Gal-9 in 61 human tumor cell lines of different origins. Interestingly they found the transcript only in part of the cell lines of colon tumor origin. Meaning that cell lines of breast tumors, lung tumors showed no expression. The expression was occasionally detected in certain breast and ovarian cancer cell lines (434).

Gal-9 and myeloma.

Gal-9NULL was used to inhibit the proliferation of different myeloma cell lines as well as patient derived myeloma cells in vitro. This inhibition passed through the JNK and p38 MAPK signaling pathways followed by h2AX phosphorylation. Human Myeloma tumor growth in Nude mice was also controlled by the Gal-9NULL injections. (435)

Gal-9 and breast cancer

The role of Gal-9 in breast cancer has been linked to metastasis. MCF-7 cell lines with overexpression of Gal-9 exhibited higher aggregation potential in vitro. These cells when injected into the mammary tissue of nude mice formed well defined round-margined tumors with large nests. However, cells with native expression of Gal-9 exhibited scattered growth when injected in mice. Cells over expressing Gal-9 S or L exhibited reduced adhesion to type 4 collagen, fibronectin, vitronectin or laminin in vitro as well. In the same study IHC analysis of breast cancer tissue for Gal-9 expression from 84 patients revealed a correlation between the expression of Gal-9 and the decrease of distant metastasis. The results showed that 50 percent of the breast cancer patients had tumors positive for Gal-9. These patients were followed over an average period of 14 years and patients with Gal-9 positive tumors had a much better disease free survival with respect to Gal-9 negative patients (436).

Another study *via* IHC analysis on patient biopsies of inverted papilloma or squamous cell carcinoma correlated an increase in Gal-9 expression in tumoral epithelial tissue with respect to healthy tissue (437)

Gal-9 and Gastric cancer

Decreased Gal-9 expression has been related to poor prognosis in gastric cancer patients. This study was done patient biopsies following surgical intervention without any previous treatment. Gal-9 expression was assayed by tissue microarray immunohistochemistry on 305 cancer gastric cancers and comparted to 84 paired adjacent normal samples. (438). On a study on 44 gastric patient tissue obtained following surgery Gal-9 expression at the RNA and protein level was decreased with respect to adjacent non tumoral tissue or distant normal tissue. Decreased Gal-9 expression correlated with clinical staging, tumor pathological stage lymph node metastasis and survival rate (439). On the contrary. Another study analyzed Tim-3 expression either on PBMCs or tumor infiltrating NK cells from 62 gastric cancer patients that had no received any treatment except surgical intervention vs 32 healthy controls. They did find an increase expression of Tim-3 only on the NK cells from the total PBMC population of the patients with respect to the healthy subjects. Moreover, an increase in Tim-3 expression on NK cells correlated with a poor with clinical stage of the disease and a poor prognosis for the patient. (440)

Gal-9 and non-small cell lung cancer

Gal-9 expression was studied at the transcriptomic level on tumoral tissue from 87 stage 1 or stage 2 NSCLC patients. Statistical analysis correlated a decrease in overall survival with a decrease in Gal-9 expression specially the Gal-9 M and L isoforms. However, IHC analysis of 45 paraffin embedded tissue samples did show that while tumors did often stain negative or very slightly for Gal-9, the tumoral endothelium and stromal tissue had a much stronger signal. (441)

2. Gal-9 increase as a poor prognosis

Gal9 and acute myelogenous leukemia

A study mainly focusing on myeloid leukemia stem cells found a significantly higher level of Gal-9 in the plasma of 15 AML patients (398 pg/ml on average) than 12 healthy controls or 5 non-Hodgkin's lymphoma patients. By reconstituting mice with blast from human AML patients they were able to determine that it was the leukemic stem cells that secreted the majority of the Gal-9. This study determined that Tim-3 was expressed on the CML leukemic stem cells and that Gal-9 Tim-3 signaling was important for maintaining the function of these cells (442). Another work confirmed their results as they found that AML stem cells from patients were positive for Tim-3 that the Gal-9/Tim-3 pathway is crucial for the maintaining the development of AML. They determined that unlike its classical inhibitory pathway, Gal-9 binding to Tim-3 in these cells triggered the NF-κB and β-catenin pathway which is known to promote LSC-self renewal (443) (Figure 26)

In another work on acute myelogenous leukemia AML in immunocompetent C57BL/6 mice researchers showed the importance of the Gal-9 pathway in treating hematological malignancies in parallel with the blocking of the PD1/PDL1 pathway. The AML cell line expressed luciferin to follow *in-vivo* cancer development. The researchers showed that in fact a much bigger percentage of the CD8+ T cells in the AML mice had the Tim-3+ profile and were exhausted secreting far less IFNγ and TNFα but equal amounts of IL-2 compared to the Tim-3- cells. Furthermore, the Tim-3 expression seem. The researchers then showed that the tumor cell line used did express Gal-9 and in fact most of the mice immune cells tested positive for intra cellular Gal-9 with the highest intensity obtained in liver immune cells. Furthermore, the researchers found that the percentage of foxp3+ cells in AML mice were much higher than in AML Gal-9 knockout mice even though the tumor cells were not Gal-9 knockout. Furthermore, Gal-9 knockout AML mice were more resistant to the cancer development with the difference in the obtained signal being statistically significant at days 14 and 21 but not at days 28 post AML injection. Finally, apart Gal-9 KO of the mice not the cancer cells or anti-PDL1 treatment did not completely cure the AML mice however combination of both results in 100 percent disease regression (444).

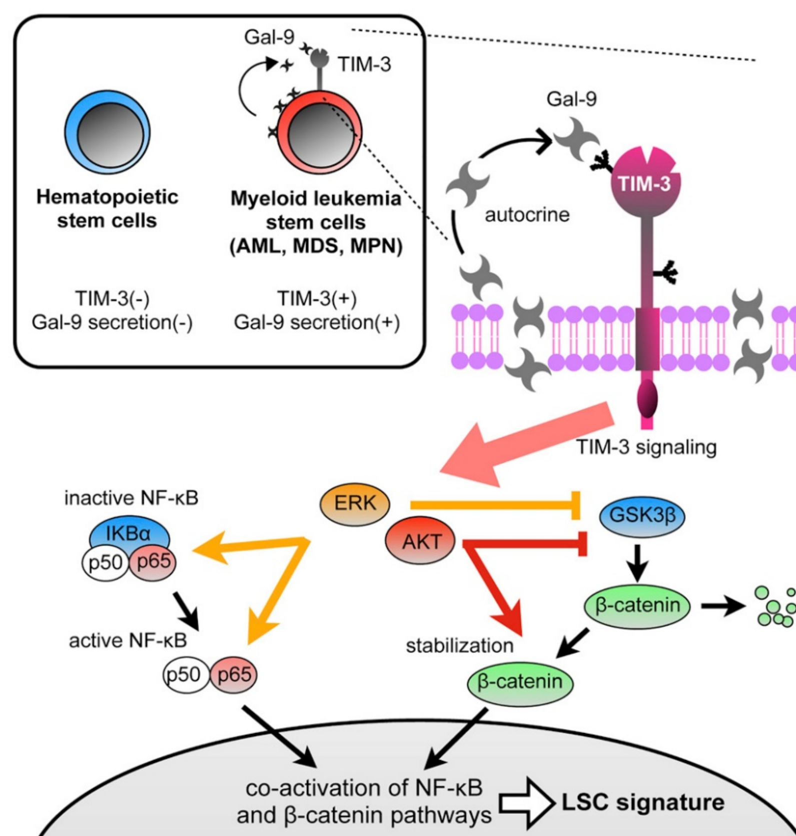


Figure 26 - Gal-9/Tim-3 pathway is crucial for AML development
Gal-9 binding to Tim-3 activated the NK-κB and β-catenin pathway which triggers a feed forward loop and maintains AML (443).

Gal-9 and squamous cervical cancer

In a study on cancerous tissue obtained 160 patients suffering from squamous cervical cancer following surgery and without having received prior anti-cancer therapy Gal-9 expression was correlated with a better prognosis. However, again Gal-9 expression was only studied in the tumoral and para-tumoral tissue by immunofluorescence, ignoring the levels of circulating Gal-9. This study did find as usual that Gal-9 expression is decreased in the centers of tumors but when expressed it was expressed at high levels in the invasive border and stroma. Most of the Gal-9 expressing cells where CD163+ cells. Yet most of CD163+ cells did not express Gal-9. No correlation was found with angiogenesis. (445)

Gal-9 and squamous cell carcinoma

On a study which included 93 cases of oral lesions including 40 patients suffering from oral cavity squamous cell carcinoma (OSCC), 40 cases of potential malignant disorders and 13 normal histopathological profiles Gal-9 expression was found to be increased in the OSCC patient tissue with respect to the controls. IHC analysis was done on lesions from the 40 OSC patients which presented malignant epithelial cells. The results showed that Gal-9 had a significantly higher expression in these lesions than in the healthy controls. (446)

Gal-9 and renal cell carcinoma

A retrospective study involving 196 patients undergoing nephrectomy for clear cell renal cell carcinoma studied the expression of Gal-9 by immunohistochemistry. The patients had not receive any adjuvant therapy. Out of the patients 48 died and 61 suffered recurrence. The authors correlated high levels of Gal-9 expression with a decrease in overall survival and an increase in recurrence (447).

Gal-9 and NPC

Gal-9 was shown to be expressed by NPC cell lines and in NPC patient tumoral tissue at the protein level by western blots and IHC. Gal-9 was found to interact with LMP-1 in lipid rafts (448). Gal-9 producing exosomes were found in serum of both NPC patients and mice xenografted with human NPC. Exosomes conveyed protection against proteo-lysis to Gal-9 while it still retained it Tim-3 binding capabilities. Recombinant Gal-9 induced apoptosis of EBV-specific cytotoxic CD4+ T-cell clone with an ID50 of 100pg/ml which was blocked by an anti-Tim-3 antibody or an anti-Gal-9 antibody (449).

In Osteosarcoma.

Tim-3 expression on CD4+ and CD8+ T cells in the periphery of 82 osteosarcoma patients was shown to increase with tumoral stage and pathology. This expression was significantly upregulated when compared to healthy controls. The increase of this expression negatively regulated with the circulating levels of IL-2 IFNγ and TNFα. This work shows the importance of this pathway in downregulating the antitumoral immune response in cancer patients (450).

In human lung adenocarcinoma

Tim-3 expression on peripheral NK cells from 85 human adenocarcinoma patients showed an increase in Tim-3 expression with respect to healthy controls. This increase correlated with metastatic state as well as tumoral stage. Tim-3 blocking of these NK cells increased their activity (413).

In gliomas.

Gliomas are known to induce a strong local and systemic TME. A study carried out on peripheral blood and tumoral tissue from 53 glioma patients' revealed crucial roles for this pathway. Tim-3 expression on both peripheral and tumor infiltrating CD4+ and CD8+ T cells correlated with disease stage and severity. Moreover, an increase Gal-9 expression detected by immunohistochemical analysis in the tumoral tissue also correlated with a disease stage and severity. These results indicate an important role of the Gal-9/Tim-3 pathway in glioma induced immune suppression (451).

Gal-9 in para-tumoral tissue

Gal-9 expression in tumoral endothelia was compared to that in normal tissue endothelium for 4 different types of cancerous tissue. Gal-9 expression was clearly higher in the tumoral endothelium that in the normal tissue. Transcriptomic analysis of HUVEC cells showed that in fact it was the Gal-9M isoform which was the most abundantly expressed. (452).

3. Controversial results

Gal-9 and melanoma

A study focused on the expression of Gal-9 in human melanomas, it included both melanoma cell lines as well as melanoma tumoral tissue from patients. Unlike the PCR study, they found Gal-9 transcript and protein expression at different levels between the different cell lines and patient tumoral tissues depending on the stage of the cancer. Using flow cytometry, they detected higher cytoplasmic and cell surface expression of Gal-9 protein in colony forming proliferating cell lines than in non-colony forming proliferating cell lines indicating a role for Gal-9 in cell aggregation. Regarding the patient tissue, the researchers used IHC staining to determine the level of Gal-9 positivity. They interpreted their work by deducing that higher Gal-9 expression correlates with a better prognosis since they discovered that high Gal-9 expression in the melanocytic nevi of primary melanomas but was downregulated with the evolution of the disease especially in the metastatic lesions. Patients with high Gal-9 expression in the tumor showed significantly lower lymph node metastasis recurrence and death than those with lower expression. Moreover, primary lesions with high Gal-9 expression were associated with a better disease free and survival time over a 5-year period. In the same study Gal-9 at 1 µM following a 72hrs culture induced apoptosis of almost all the melanoma cells in vitro. Smaller concentration of Gal-9 over 12hrs induced aggregation of the non-colony forming cell lines(453). A different role of Gal-9 in melanoma was recently determined. Gal-9 was increased in the plasma of melanoma patients by 3.6 folds with respect to healthy controls. At the same time Gal-9 was expressed in the 57% of the tested tumors. Increase plasma levels were correlated with decreased 2-year survival.

Gal-9 levels in these patients promoted M2 macrophages and a Th2 response while inducing apoptosis in of Th1 cells (454).

Gal-9 and colon cancer

Various studies have revealed contradictory results on the role of Gal-9 in colon cancer and hence the clinical significance of its expression level. In a 2016 paper (455), the authors studied the expression level of Gal-9 in tumoral or para-tumoral tissue from patients histologically diagnosed with colon cancer by immunohistochemistry. The authors correlated an increase in Gal-9 expression with a better clinical outcome for the patient. They based their conclusion on the fact that patients who were diagnosed at TNM 1 or 2 meaning early stages of colon cancer without lymph node metastasis often had high levels of expression of Gal-9 and this correlated Gal-9 expression to better clinical outcome. Conversely patients, with low Gal-9 expression had a lower overall survival. The authors moved to associate this survival state with NK cells. They found that para-tumoral tissue in 38 colon cancer patients had a higher NK cell infiltration and expressed higher levels of Gal-9 than the tumoral tissue of the corresponding patient. They moved on to show that recombinant human Gal-9 at 100ng/ml induces chemotaxis of primary NK cells at a level comparable to that of IL-12. Another study found Gal-9 expression by colon cancer cells as well as Tim-3 expression on tumor infiltration CD8+ T cells from 7 patients. They went to show that these Tim-3+ cells were had efficient IFNγ secretion yet were more apoptotic. The authors continued to show that Tim-3 blocking by monoclonal antibody was able to inhibit tumoral growth in colon cancer bearing mice (456).

V. Immunotherapy

Now that the various mechanisms in tumoral immune evasion which are relevant to this dissertation have been introduced we will discuss the different approaches which exist to counteract them.

A. Origins of immunotherapy

William B. Coley is considered as the godfather of cancer immunotherapy. He had noticed that some of his sarcoma patients who suffered from severe infections following surgery experienced tumor regression. So he started to infect sarcoma patients with Streptococcus pyogenes which proved difficult to dose and lead to sepsis related fatalities. This led him to switch to inoculation with killed S. pyogenes which resulted in total disease regression; one of this patients died 26 years following the sarcoma removal surgery of a heart attack. "Coley's toxins" were used in many different malignant diseases with varying degrees of success, but with the lack of well documented studies along with the start of chemo and radiation therapy "Coley's toxins" quickly vanished. (457). Over the past decade the world of cancer immunotherapy has exploded. The major problem facing the overwhelming amounts of emerging research is the transferring from the animal mouse model to the human patients. The human system is far too complicated. Trying to restore the balance to the human immune response is the equivalent to balancing an acrobat walking a tight wire, with cancer weighing in on one side and immunologists and oncologists trying to decipher the minuscule nudges required to help him maintain its equilibrium and reach the end of the line "in this case tumor elimination". However, as we will see, often one push too far and the outcome can be hyper reactivity, autoimmunity and death.

Immunotherapies as they exist today are classified either into active or passive

B. Passive immunotherapy

Passive immunotherapy basically is about providing the patients with components of the immune system to fight the cancer such as cytokines, monoclonal antibodies or adoptively transferred cells.

1. Cytokine therapy

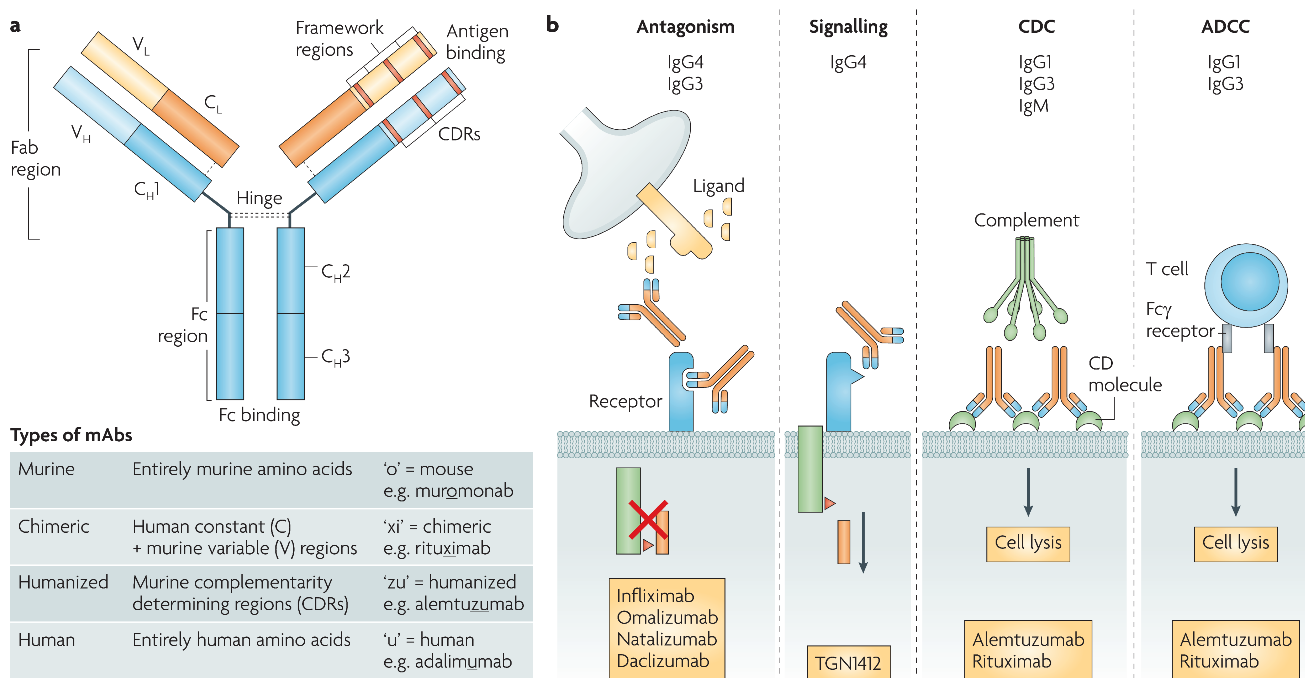
Cytokines mediate communication between the different subset of the immune system and regulate the immune response whether it is by activating or inhibiting it. These were some of the earliest immunotherapeutics used to treat cancer patients. The fact that they were capable of activating the immune response made them perfect candidates to reverse the TME induced immunosuppression.

Moreover, they are relatively easy to synthesize and administer. At this time, three cytokines are licensed by regulatory agencies worldwide for use as immunostimulatory agents in cancer patients. These are recombinant IFN-α2a (Roferon-A), recombinant IFN-α2b (Intron A→) and recombinant interleukin (IL)-2 (aldesleukin, Proleukin). Recombinant IFNα2a is used to treat hairy cell leukemia and chronic phase, Philadelphia chromosome-positive chronic myelogenous leukemia (CML), upon minimal pretreatment (within 1 year of diagnosis); recombinant IFNα2b was approved for follicular lymphoma, multiple myeloma, hairy cell leukemia, AIDS-related Kaposi's sarcoma, melanoma, genital warts (Condyloma acuminata) and cervical intraepithelial neoplasms. Recombinant IL-2 is being administered to patients suffering from metastatic forms of melanoma and RCC. Three different cytokines have been approved worldwide but not by the FDA as cancer therapeutics due to their oncotoxic and immuno-reconstituting potential rather than immuno-stimulating role. These are recombinant granulocyte colony-stimulating factor (G-CSF, also known as Filgrastim, Lenograstim or Neupogen), recombinant granulocyte monocyte colony-stimulating factor (GM-CSF, also known as Molgramostim, Sargramostim, Leukomax, Mielogen or Leukine), and recombinant TNF. The major problem with cytokines is that they are far too potent as stimulators having a wide range of activities often leading to lethal side effects. There numerous side effects have led to them being replaced by safer approaches (458)

2. Antibody therapy

Using antibodies in therapies became possible once the technology behind creating monoclonal antibodies was mastered. The first monoclonal antibodies against CD3 (OKT3) were generated in 1975 by hybridomas and were used in treatment in preventing kidney transplant. Since then, technical advances have permitted the evolution from mouse, *via* chimeric and humanized, to fully human mAbs thus reducing the potentially immunogenic mouse components. This has resulted in mAbs having marked clinical success. Anti-tumoral monoclonal antibodies can function either by directly binding tumor antigens, or by targeting antigens in the tumor micro-environment with the hopes that it leads to an indirect anti-tumor effect. Antibody based therapies have different modes of actions which include: fragment crystallizable region (Fc-region) induced activation of cytotoxicity through Antibody-dependent cell-mediated cytotoxicity (ADCC), Antibody-dependent cellular phagocytosis (ADCP) and Complement-dependent cytotoxicity (CDC), the blocking of target protein signaling, the activation of the signaling pathway of the targeted receptor, the activation of TRAILR2 or other apoptotic receptors, or the delivery vehicle for toxins or radionuclides (459) (Figure 27). Unconjugated antibodies used for therapy include Rituximab, an anti-CD20 expressed by non-Hodgkin lymphoma and chronic lymphocytic leukemia (460), Trastuzumab, an anti-HER2 on breast cancer (461), Cetuximab, an anti-EGFR for colorectal cancer (462) having a human Immunoglobulin G1 (IgG1) Fc region for inducing Fc based cytotoxicity. These are only some of the antibodies which were based on directly targeting the tumor (463) . The recent trend in antibody therapies has been the targeting of tumor microenvironment instead of the tumor itself. Immune checkpoint inhibitors being the new stars of the

world of oncology. These antibodies block the inhibitory pathways of the immune system allowing for a more efficient anti-tumoral immune response. This strategy of “easing of the breaks pedal” has had much more success than previous strategies of “stepping on the accelerator”



Murine	Entirely murine amino acids	‘o’ = mouse e.g. muromonab
Chimeric	Human constant (C) + murine variable (V) regions	‘xi’ = chimeric e.g. rituximab
Humanized	Murine complementarity determining regions (CDRs)	‘zu’ = humanized e.g. alemtuzumab
Human	Entirely human amino acids	‘u’ = human e.g. adalimumab

Figure 27 - Therapeutic antibodies: Structures and functions.
A representation of the structure of antibodies along with the different types of therapeutic antibodies, their nomenclature and different modes of action (464)

3. Immune checkpoint inhibitors.

Anti-CTLA4 and anti-PD1

Ipilimumab “a fully human IgG1” and Tremelimumab (a fully human IgG2), both target CTLA4 which expressed on activated T cells and inhibits its ligand binding. CTLA4 competes with binding to the co-stimulatory ligands CD80 and CD86 on APC leading to decreased activation of the T cell. Pembrolizumab and Nivolumab “both fully human IgG4”, both bind PD-1 which is also expressed on activated T cells and block its interaction with its cognate ligands PD-L1 and PD-L2. These ligands are expressed on a wide variety of cells including tumors and the engagement of PD1 to its ligand leads to T cell inactivation and exhaustion (465, 466). CTLA-4 blockade by Ipilimumab in stage III or IV melanoma increased overall survival to 10 months compared to 6.4 months for patients receiving only gp100 peptide vaccines. Even more surprisingly, the group receiving both gp100 plus Ipilimumab had similar overall survival to those only treated with Ipilimumab (467). Pembrolizumab (anti-PD1) treatment had strong response rate in a metastatic melanoma cohort (468), a later study using Pembrolizumab on patients with ipilimumab-refractory advanced melanoma demonstrated an overall response rate of 26% (469). Nivolumab, in parallel, was shown to increase the OR and OS of metastatic melanoma patients, previously untreated and without BRAF mutation, when compared with Dacarbazine

chemotherapy (Objective response rate (ORR) 40.0% vs 13.9% respectively) in a phase III study (470). Nivolumab has proven its higher effectiveness against other classical chemotherapeutics in various cancers and has since been approved by the FDA for treating them. For example, Nivolumab has been approved for previously treated advanced or metastatic non-small cell lung carcinoma (NSCLC) after a study has shown that patients with advanced NSCLC that had progressed during or after platinum-based chemotherapy, treated with Nivolumab had a better overall survival than those treated with docetaxel (471, 472). Nivolumab anti-tumoral with minimal side effects in metastatic renal cell carcinoma was shown in a phase II clinical trial with a total of 168 patients and has since been approved for treatment of this cancer (473). It is important to note that Nivolumab treatment did not have an objective response in patients with colorectal cancer nor castration-resistant prostate cancer (474). The success story of these 2 inhibitors does not end here as researcher and oncologists have moved to using combination therapy of both. This would seem logical as the targeted pathways are not overlapping and are even complementary (475). In fact, numerous *in-vivo* data from mouse models does support the concept of this combination (476, 477). When it comes to patients, the combination of Ipilimumab plus Nivolumab treatment for melanoma in a phase I and a phase II clinical trials and an ongoing phase III clinical trial have shown very promising results with the combination therapy being more effective that each of the mono-therapies. In the phase I clinical trial 53% of the 53 patients had an objective response, all with tumor reduction of 80% or more and the 3 year overall survival is at 68% (478). The results of the phase II trial of 142 patients were recently published and do correlate with the phase I results showing an overall survival of 63.8% at 24 months (479). The median for progression free survival in the phase III which targeted 945 previously untreated melanoma patients was 11.5 months for the combination therapy compared to 2.9 and 6.9 months for Ipilimumab and Nivolumab monotherapies respectively. This study was also important in showing that the efficacy of anti-PD1 was dependent on the expression of PDL1 by the tumor cells (480). In fact, the possibilities of combination therapies do not stop here. The use of immune checkpoint inhibitors with other therapeutics such as vaccinations and radiotherapy has been tested. For example, the combination of anti-PD1 treated mice together with multi-peptide based vaccine in a mouse model for breast cancer (481). Combining anti-PD1 or anti-PDL1 with fractionated radiotherapy also in a mouse breast cancer model increased tumor control in an IFNγ T cell dependent manner (482). Similar increased efficiency was obtained when combining radiotherapy with anti-CTLA-4 antibody in syngeneic mouse models for lymphoma and lung carcinoma (483). The combination of immune checkpoint inhibitors with classic chemotherapeutics has already been proven successful in a phase III clinical trial. Ipilimumab plus Dacarbazine in 502 previously untreated metastatic melanoma patients showed higher survival rates than Dacarbazine alone (484, 485). Last but not least, follow up analysis on patients who received from CTLA-4 blockade treatment have shown that the best responders were patients with a high mutational load in their tumors which also expressed neoepitope landscape capable of activating CD4 response (266). This observation could apply to other immune checkpoint inhibitors. One example is a study on the differential effect of Pembrolizumab therapy on colorectal cancer patients that were divided into 2

groups which both received the same treatment. The criteria for the division was the miss-match repair machinery in the cancer. One group was miss-match repair proficient while the other was deficient. The idea is that miss-match repair deficient tumors will accumulate more somatic mutations eventually leading to the expression of more non-self-antigens which could be recognized by the immune response. The difference between the outcomes was clear as miss-match repair deficient patients had a much high ORR "40% vs 0%" and progression free survival (PFS) "78% vs 11%" than the proficient patients (486). While this summary is far from exhaustive, it does show the amazing potential of immune checkpoint inhibitors in cancer therapy. In fact, the outstanding success of these 2 blockade mechanisms has led to the development of other antibodies capable of targeting other inhibitory pathways.

Other noteworthy checkpoints currently in clinical phase

Lag-3 and Tim-3 are the new targets in the world of immune checkpoint inhibitors which are currently being translated to the clinic.

Lag-3

Lag-3 was discovered 25 years ago. Lag-3 is upregulated on activated CD4+ CD8+ and a subset of NK cells. It can bind MHC class II with higher affinity than CD4. It can also bind liver and lymph node sinusoidal endothelial cell C-type lectin (LSECTin) which is expressed in many tumors. When it engages its receptor, Lag-3 crosslinks CD3 and inhibits T cell proliferation, cytokine production and calcium flux (487, 488). Moreover, Lag-3 is expressed on and is crucial for the suppressive function of nTregs and iTregs (174). Lag-3 blocking inhibits the suppressor activity of Tregs whiles it's over expression confers Tconv with suppressive potential. Taken together it seems that Lag-3 plays a crucial role in the tumoral suppressive microenvironment which is why several preclinical mouse trials have used Lag-3 blocking antibodies alone or in combinations mainly with anti-PD1 antibodies to treat different cancers (489). The earliest clinical trials involving Lag-3 were based on the use of a soluble Lag-3 Ig (IMP321) which was designed to bind to MHC class II and improve APCs maturation. The results obtained from the use of this molecule were not as satisfactory and the shift is now towards the use of anti-Lag-3 antibodies which block its interaction with MHC class II (490, 491). Currently the BMS-986016 (anti-Lag3 from Bristol-Myers Squibb) is being tested in A Phase I/2a study clinical trial with or without Nivolumab in advanced solid tumors.

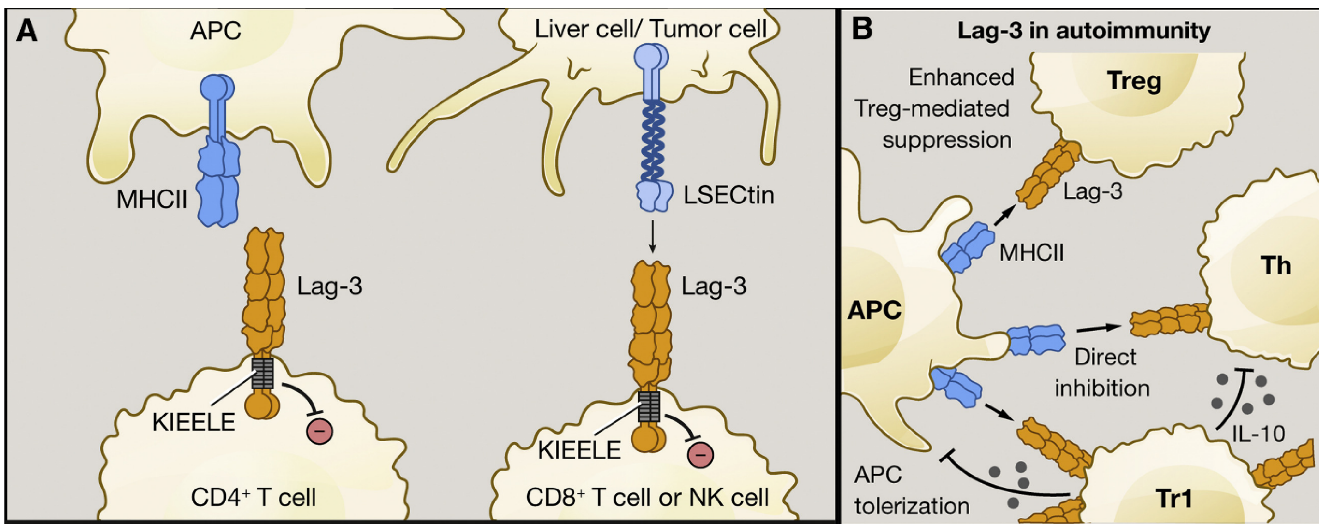


Figure 28 - Lag-3 function in physiological conditions and autoimmunity
Lag-3 protects from autoimmunity by controlling the Th cell responses directly or through engagement of MHCII. Moreover, it indirectly inhibits effector T cell responses by promoting Treg mediated suppression (491).

Tim-3

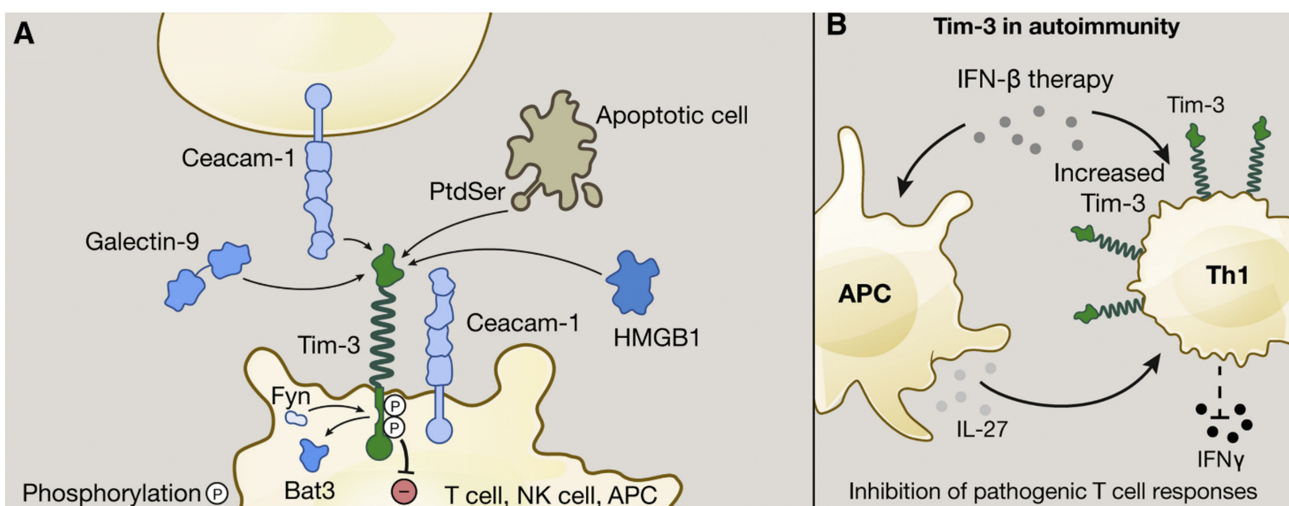


Figure 29 - Tim-3 function in physiological conditions and autoimmunity
Tim-3 is found on T cells, NK cells, and some APCs. Tim-3 binds soluble ligands galectin-9 and HMGB1 and cell surface Ceacam-1 and Phosphatidyl serine. Ligand binding dissociates of Bat-3 from the cytoplasmic tail of Tim-3, which allows Fyn to bind and activate the inhibitory function of Tim-3 (491).

Tim-3 was first identified in 2002 as a molecule selectively expressed on IFN-γ-producing CD4+ Th1 cells and CD8^{+} cytotoxic T cells (347). Since then Tim-3 has now been found on Tregs and on DCs, NK cells, monocytes. Its gene family has been associated with immune-related diseases such allergies and asthma. Subsequent animal model studies on Tim3 KO mice, Tim-3 over expressing mice or mice treated with Tim-3 Ig have shown that it is crucial for induction of antigen specific tolerance and inhibiting autoimmunity (492). Several ligands have been found for Tim-3 (Galectin-9, Ceacam-1,

HMGB-1, phosphatidyl serine) all of which seem to mediate its inhibitory function. Tim-3 has been found to be upregulated on exhausted patient CD8+ cells in different cancers including non-small-cell lung cancer, metastatic melanoma and follicular B cell non Hodgkin lymphoma. Its co-expression with PD-1 seems to marker a fully dysfunctional Tils and a co-blockade was effective in restoring function (493). Currently TSR-022 an anti-Tim-3 antibody from Tesaro is in a phase 1 study for its effectiveness alone or with Nivolumab in patients with advanced solid tumors.

TIGIT

The T cell immunoreceptor with Ig and ITIM domains (TIGIT also known as Vstm3/WUCAM/VSIG9) has recently immerged as an important player in immune regulation (expressed by T and NK cells). The TIGIT/CD226 pathway has been shown to function between Tregs and DCs in a similar function to the CD28/CTLA-4 pathway (494). TIGIT is a type 1 transmembrane protein containing an IgV extracellular domain and an immunoglobulin tail tyrosine (ITT)-like phosphorylation motif followed by an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic tail. It can bind with high affinity receptor CD155 (also known as PVR/Necl-5/Tage4) on monocytes and CD11c+ human DCs as well as on certain cancer cells and CD112 (495-497). TIGIT competes with CD226 (also known as DNAM-1) for the binding of this receptor. CD155 recognition by CD226 potentiates CD8+ CTLs and NK cells anti-tumoral response (498, 499). CD226 co-stimulatory function seems to be important when antigen presentation is achieved by non-professional APCs such as B cells or tumor cells (500). CD226-deficient mice have impaired anti-tumor and antiviral T cell responses (501, 502) as their CD8+ CTLs have decrease cytotoxic ability due to its decreased efficiency in establishing the synapse (503). CD226 also regulates CD4 T cell expansion and cytokine production as its blockade decreases TH1 differentiation (504). It has been observed that TIGIT engagement with CD155 on DC leads to the IL-10 production by DC and diminished production of IL-12p40 rendering them tolerogenic (505). TIGIT is highly expressed on exhausted human and murine TILs in the tumoral micro-environment. TIGIT is highly expressed on CD8+ TILs co-expressing PD-1 in melanoma, (NSCLC) and (CRC) patients as well as several mouse tumor models (491, 506). TIGIT was also found to be overexpressed on peripheral blood CD8+ cells in acute myeloid leukemia (AML) patients (507). In cancer patients TIGIT seems to be concentrated in the TME and not in the peripheral lymphoid organs (508). Combined blockade of TIGIT with PD-1 improved exhausted CD8+ TILs proliferation, cytokine secretion and degranulation (506, 509). Interestingly, TIGIT KO mice do not develop spontaneous autoimmunity but still do produce a stronger response to foreign antigens (494). TIGIT blocking has been shown to improve the anti-viral and anti-tumoral response in several mouse model. That and other reasons have lead the development of an anti-TIGIT (MTIG7192A, RG6058) fully human monoclonal antibody designed to bind to TIGIT and prevent its interaction with CD155 which is currently in a Phase Ia/Ib open-label, dose-escalation Study in locally advanced or metastatic tumors.

1. Treg targeting strategies

As we have seen, Tregs constitutively express CD25+ hence targeting this receptor seemed to be a promising strategy to effect Treg number and function. Two main strategies were developed, the first was with the use of an anti-CD25 monoclonal antibody and the second with the use of diphtheria-toxin conjugated IL-2 (Denileukin difitox, ONTAK).

Using the anti-CD25 antibody (Daclizumab) seemed promising in numerous mouse preclinical models, yet failed when it came to clinical trials. The major problem with such technique is the fact that CD25 expression is shared with activated Tconv cells making these strategies largely non-specific (510-512). More recently the combination of Daclizumab with a breast cancer vaccine in metastatic breast cancer patients lead to a decrease in Tregs in the patients (513). In melanoma a combination of a DC vaccine with Daclizumab was able to decrease circulating Tregs without affecting progression free survival (514).

Denileukin difitox is another approach designed to deplete Tregs based on the expression of CD25. The cells expressing the receptor would internalize the diphtheria-toxin conjugated IL-2 and die. This approach was originally US Food and Drug Administration (FDA) approved for lymphoma and was found effective in renal cell carcinoma (515). However, the drug failed at producing positive results in melanoma patients (516). Again the problem was the specificity, and the fact that it did not affect CD25lowFoxp3+ iTregs. This drug had little clinical efficacy and elevated toxicity (517). Denileukin is currently in clinical testing in parallel with various other immunotherapies.

Many new antibodies have shown their capabilities to inhibit Tregs as a secondary function to their immune-checkpoint inhibitor capabilities both in mouse models as well as in-vitro tests.

Given what is known about Tregs and the fact that they express numerous molecules involved in the tumor suppressive microenvironment including co-inhibitors and cytokine receptors, there are many speculations owing the success of many “new age” immunotherapeutics to their ability to target Tregs. Of course little clinical evidence in human of such effect exists. Figure 30 represents the actual and possible Treg targeting strategies that are either FDA approved, in clinical testing or still in the pre-clinical stage.

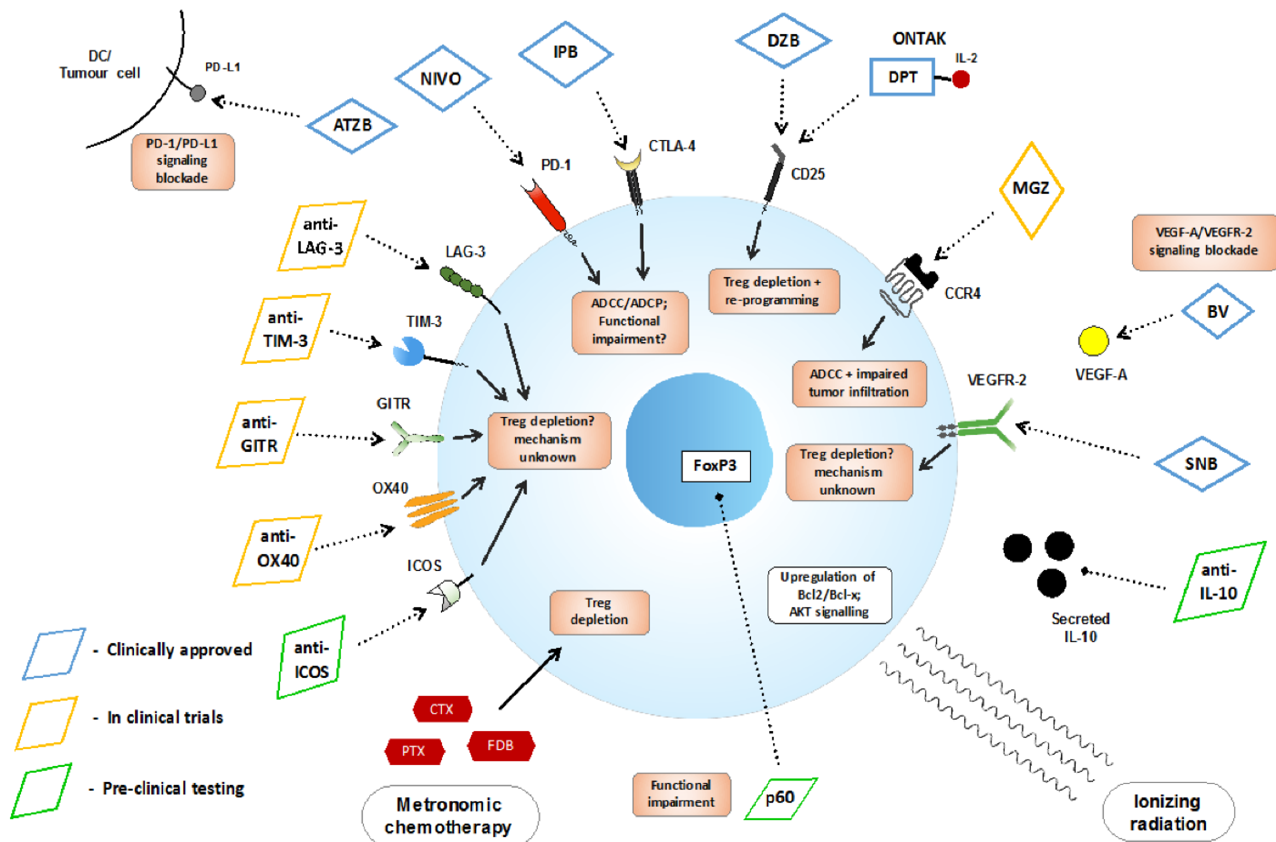


Figure 30 - Summary of all actual and possible Treg targeting approaches.
ADCC: antibody-dependent cell-mediated cytotoxicity, ADCP: antibody-dependent cellular phagocytosis, ATZB: atezolizumab, BV: bevacizumab, CTX: cyclophosphamide, DC: dendritic cell, DZB: daclizumab, CCR4: C-C motif chemokine receptor 4, DPT: diptheria toxin, FDB: fludarabine, IPB: ipilimumab, MGZ: mogamulizumab, NIVO: nivolumab, ONTAK: denileukin difitox, PTX: paclitaxel, SNB: sunitinib.

The evidence for the actual Treg targeting effect of these antibodies in patients still lacking and controversial. In fact, patient data suggests that treatment with neither anti-CTLA4 antibodies nor anti-PD1 antibodies has had an effect on the Treg population as tested by the researchers. These results are summarized in Table 4.

Table 4 - Clinical effect of immune checkpoint inhibitors on Tregs. From (518)

Treatment	**Cancer**	**Treg Markers**	**PB/ TILs**	**Functional Analysis**	**Expanded?**	**Survival**
Ipilimumab	Resected stage IIIc/IV melanoma (n = 75)	CD25+	PB	Suppressive; no effect after treatment	No change	N/A
	Unresectable stage III/IV melanoma (n = 80)	CD25hi/ CD127low/ FoxP3+	PB	N/A	No change at weeks 4 & 12	N/A
Ipilimumab	Stage IV malignant melanoma	CD25+/ FoxP3+	PB	N/A	Decreased	No statistical link

	Bladder cancer patients prior to radical cystectomy (n = 6)	CD25+/ FoxP3+	PB	Suppressive pre/post-treatment	Overall decrease; variable initial response	N/A
	Bladder cancer patients prior to radical cystectomy (n = 6)	CD25+/ FoxP3+	TILs	NA	Increase in ICOS+ Teff : FoxP3+ Treg ratio	N/A
	Bladder cancer (n = 12)	CD25+LAP+ /FoxP3+/ CD127lo	PB	CD25+LAP+, but not CD25+CD127lo, suppressive post-treatment	CD25+LAP+increased in patient subset	N/A
	Metastatic RCC or metastatic melanoma (n = 10)	CD25+/ FoxP3+	PB	Suppressive pre/post-treatment	No change; increase in activated T cells	N/A
	Progressive metastatic hormone-refractory prostate cancer (n = 24)	CD127low/ CD25hi	PB	Suppressive post-treatment	Increased, and Ki67+	N/A (study* ongoing)
	Stage III/IV melanoma (n= 37)	CD25HI/ Foxp3^{+}	PB	N/A	Increased at 6 weeks post-treatment	Associated with improved PFS
	Stage III/IV melanoma (n= 10)	CD25HI/ Foxp3^{+}	TILs	N/A	Variable	Inverse trend between Treg & clinical benefit
	Stage IV melanoma (n = 82)	CD127low/ CD25+/ FoxP3+	PB	N/A	Increased over 14 weeks of treatment	Higher than median Tregs associated with better survival
Tremelimu-mab	DTIC-treated stage IV melanoma (n = 10)	CD25+/ CD127−or FoxP3+	PB	Suppressive pre-treatment; transient resistance to Treg suppression post-treatment	Increase in absolute Treg count, but not proportion	Treatment-induced transient Treg resistance associated with better survival
	Stage III/IV melanoma, combined with IFN-α2b (n = 37)	CD25hi/ FoxP3+or CD25hi/ CD39+	PB	N/A	Both subsets Increased	N/A
	Metastatic melanoma (n = 7)	CD25+/ FoxP3+	TILs	N/A	Variable	N/A
Nivolumab	Unresectable stage III/IV melanoma (n= 90); IPB-naive (n = 34) or IPB-refractory (n = 56)	CD25+/ CD127low/ FoxP3+	PB	N/A	Decreased in responders & stable patients; increased in non-responders	Increased Tregs associated with progression at 12 weeks
	Stage IIIc/IV melanoma (n = 33)	CD127low/ FoxP3+	PB	N/A	Expanded in PB at 12 & 24 weeks	Trend towards lower Tregs in non-relapsing patients

C. Active Immunotherapy

Active immunotherapy is based on inducing an *in-vivo* response to antigens. This is mainly achieved *via* different types of vaccinations.

1. Vaccination

Dendritic cells are at the basis of most vaccination strategies. These cell are the primary drivers of the anti-antigen immune response. Ever since they have been discovered by the late Nobel Prize winner Ralph Steinman in 1973, they have been heavily studied. To date, there is only FDA approved dendritic cell based cancer vaccine. Sipuleucel-T is used for minimally symptomatic metastatic castration-refractory prostate cancer. Even though it is considered a dendritic cell vaccine, it includes an ex-vivo DC maturation step. The DCs or DC precursors are extracted then washed and pulsed with a fusion protein consisting of prostatic acid phosphatase (PAP) and GM-CSF for 40 hours before being washed and infused back into the patient (519, 520). The main DC vaccine based anti-cancer interventions are:

- Treating DCs or *in-vitro* generated DCs with activating stimuli
- Exposing DCs ex-vivo to an enrichment of tumor associated antigens "TAAs"
- *In-vivo* loading of DCs with TAAs
- Using DC derived exosomes to activate the immune response

Regarding EX-vivo loading of DCs, it can be achieved by several techniques:

- Adding autologous tumor cell lysates or recombinant TAAs to a culture of immature DCs iDCs with
- transfecting DCs with TAA-coding vectors, TAA-coding RNAs or bulk RNA isolated from neoplastic cells
- generating fusions between DCs and inactivated cancer cells "dendritomes"

In-vivo loading of DCs can be achieved by:

- Fusing TAAs to DC targeting monoclonal antibodies
- Encapsulating TAAs in DC-targeting immunoliposomes
- Encoding TAAs in DC-targeting vectors

It is important to remember than any of these techniques would have to be accompanied by the proper stimuli (TLR agonist and stimulatory cytokines) to induce DC maturation since iDCs are potent tolerogenic agents of the immune system and can easily inhibit an anti-tumoral immune response.

Numerous clinical trials are currently under way based on DC vaccinations. These phase I or phase II clinical trials all seem to show that DC based cancer intervention is well tolerated and induce an objective response in a fraction of the patients (520).

Peptide Vaccination

Peptide vaccinations rely upon the knowledge of which antigens are expressed by the tumors. All different classes of tumor-associated antigens have been targets of vaccination therapies with the most impressive results being generated in peptide or protein vaccine approaches using non-self, tumor specific epitopes. Vaccination against nonself peptides evades the central tolerance trimming of potential anti-tumor specific T cells. HPV16-induced gynecological carcinomas are a prime target for this mode of vaccination. These develop from the premalignant states that are caused by high-risk types of HPV. When patients in this premalignant state are vaccinated with overlapping 25-35-mer peptides covering HPV16 E6 and E7 proteins 9 out of 19 patients had complete remissions of their disease (171). When long-peptide vaccination was used in advanced gynecological carcinomas, T cell responses were elicited, but these were restrained by tumor mediated immune suppressions so while it was possible to elicit a response no direct clinical effect was noted (172). Melanoma, and melanoma-associated antigens have also been used for peptide based vaccination. TAA gp100 has been used as a target of peptide vaccination. In a clinical trial commenced after the discovery of this TAA, two 9-mer peptides were mixed with incomplete Freud's adjuvant (IFA) and patients were vaccinated thrice in combination with high dose IL-2. Surprisingly, 42% of 31 patients had objective responses (173). In a follow-up phase-III trial, comparing IL-2 with vaccination using gp100 peptides combined with IL-2, a less stellar response was noted with only 16% response rate. This was still significantly higher than the treatment with IL-2 alone which had a response of 6% with 11.1 months overall survival compared to 17.8 with peptide vaccination (174). In RCC, another immunogenic tumor, vaccination with a multi-peptide vaccine IMA901 which contains epitopes for antigens such as Cyclin D1 and Mucin 1, cell surface associated glycoprotein (MUC1) was able to generate responses to multiple epitopes (175). This vaccine, in combination with cyclophosphamide, elicited immune responses that were associated with clinical benefit, particularly in patients with low suppressive cell populations (176). While peptide vaccines get to the heart of vaccination they must overcome hurdles that other modes of vaccine do not need to overcome. As DCs remain the lodestone of vaccine strategies, injection with peptide alone would generate a weak or possibly tolerogenic response, hence the requirement of adjuvants to generate "danger signals" in DCs. These include adjuvants such as montanide, IFA, monophosphoryl lipid A, Bacillus Calmette–Guérin (BCG) as well as imiquimod that are able to activate the Pattern recognition receptors (PRRs) through various mechanisms and activate DCs (177). Additionally, fusion protein-peptides that contain DC ligands have been developed to ensure that the peptide is presented by APCs which is optimally effective to deliver antigen (178). A MUC1 fusion protein based vaccine approach targeting mannose receptor in the setting of stage II breast cancer has shown remarkable effect. Out of 16 patients vaccinated only two had a recurrence, while in the placebo group the expected

recurrence rate of 60% in 15 patients was noted (179). Further augmenting epitope responses can be achieved through modification of single or multiple amino acids in the peptides used for vaccination. This was indeed already the case for the gp100 vaccinations where a threonine was replaced with a methionine led to increase overall responses in patients (173). Modification of other melanoma antigens has led to increased binding to MHC and directly led to increased levels of IFN-γ production in T cells and increased anti-tumor immune response (180).

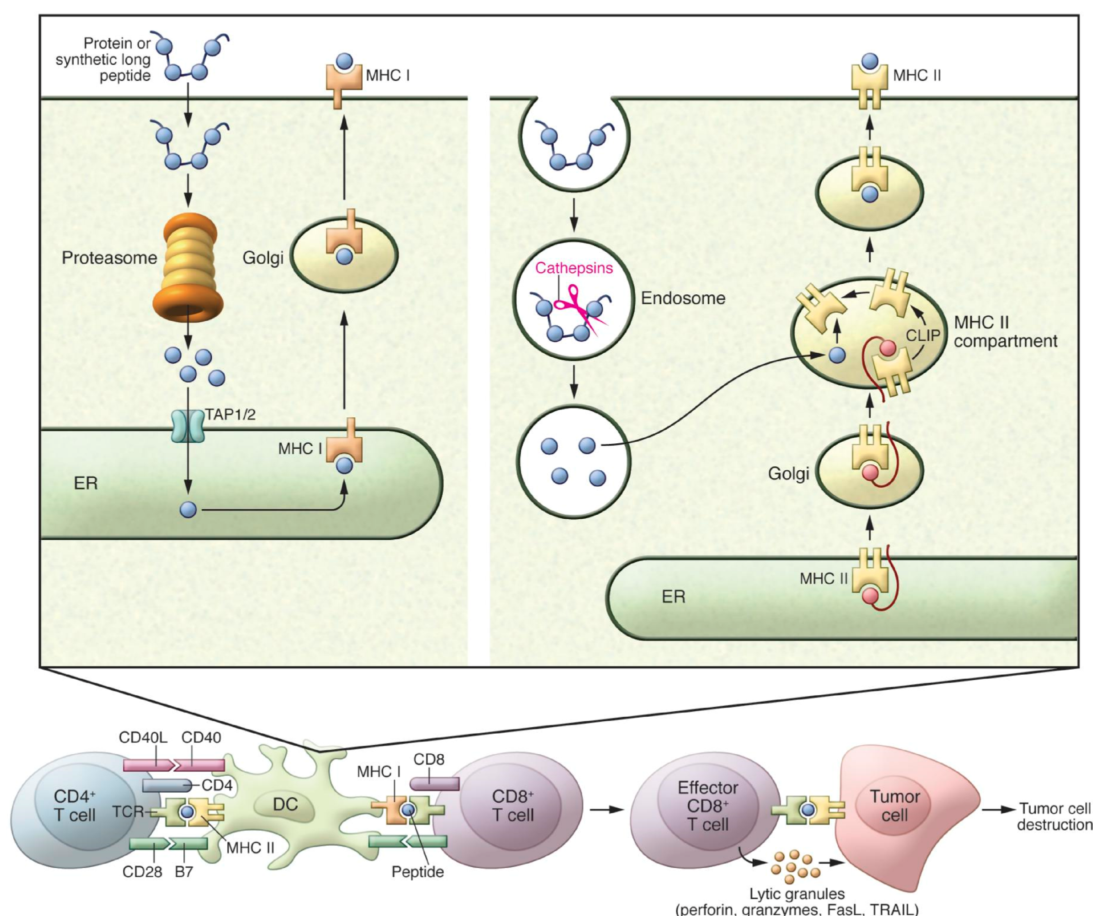


Figure 31 - Vaccine antigen processing

Loading antigens into HLA class I requires that it enters DCs cytoplasm so that it is processed by the proteasome complex. Long antigens are broken into smaller 9 to 15 aa and sent through the TAP giving them access to the ER where they are loaded to HLA class I and then transported to the cell surface. Loading into HLA class II requires that the antigen passes the endosomal system, in which it is digested by cathepsins, then loaded to HLA class II as fragments of 12 to 15 aa at low pH and, following DC maturation, and finally transport to the cell surface. Once DCs have fully matured, they interact with CD8+ and CD4+ T cells by stimulating the TCR with antigen presented by HLA class I or II molecules, respectively, and costimulatory molecules such as CD28. Activated, primed CD8+ T cells are then capable of killing tumor cells *via* ligation of the TCR with antigen presented by HLA class I molecules (521).

VI. EBV related cancers as a tumoral model

According to a 2012 review, over 50% of cancers around the world are associated with preventable causes including infections (522). The prevalence of infection related preventable cancers in much higher in developing parts of the planet. In 2008, out of the 12.7 million new cancer cases reported, 2 million were related to infections and 2 thirds of those were related to well-known viral infections such as human T lymphotropic virus type 1 (HTLV-1), HBV, HCV, HPV, Kaposi sarcoma-associated herpesvirus (KSHV), and EBV (523).

Table 5 - infectious agents and cancer. Edited from (524)

Infectious Agents	**Cancer Sites**
Epstein-Barr virus	Nasopharyngeal carcinoma, Burkitt lymphoma, immune-suppression-related non-Hodgkin lymphoma, extra nodal natural killer/T-cell lymphoma (nasal type), Hodgkin lymphoma
Hepatitis B virus	Hepatocellular carcinoma
Hepatitis C virus	Hepatocellular carcinoma, non-Hodgkin lymphoma
Kaposi sarcoma herpes virus	Kaposi sarcoma, primary effusion lymphoma
Human immunodeficiency virus type 1	Kaposi sarcoma, non-Hodgkin lymphoma, Hodgkin lymphoma, carcinoma of the cervix, anus, conjunctiva
Human papillomavirus type 16	Carcinoma of the cervix, vulva, vagina, penis, anus, oral cavity, and oropharynx and tonsil
Human T-cell lymphotropic virus type 1	Adult T-cell leukemia and lymphoma
Helicobacter pylori	Non-cardia gastric carcinoma, low-grade B-cell MALT gastric lymphoma
Clonorchis sinensis	Cholangiocarcinoma
Opisthorchis viverrini	Cholangiocarcinoma
Schistosoma haematobium	Urinary bladder cancer

In this thesis we used nasopharyngeal carcinoma (NPC) as a tumoral model to study our proposed immunotherapies. NPC is a virus induced cancer as it is almost always associated with Epstein Bar virus (EBV) infection. This chapter will start by introducing EBV and its role in NPC oncogenesis. The role of EBV in the immune response against NPC will be explained followed by a detailing of the current available therapeutics for NPC treatment.

A. EBV and associated pathologies

1. Historical background

In 1958, Denis Burkitt (1911-1993) an English surgeon reported an increased proliferation in B lymphocytes in African children with a superior maxillary tumor in African children. In 1961, Burkitt was presenting his work on, what later became known as, Burkitt's lymphoma at a London medical school and theorized the possibility that this lymphoma was being spread by a virus. In the audience of that meeting was Anthony Epstein a young cancer research and expert in the technique of electron microscopy who became determined to discover the first cancer causing virus in humans. With the help of Achong and Barr in 1964, the Epstein Barr virus was identified (Figure 32). In 1968, an epidemiological study showed that EBV infected 95% of the population and was the causative agent of infectious mono-nucleuses (525). A few years later, the virus was detected in nasopharyngeal carcinoma tissue (526). EBV's ability to immortalize cell lines in vitro as well as its ability to induce lymphomas in primates further proved its oncogenic capabilities. Since then, EBV was shown to be implicated in numerous benign and malignant pathologies of lymphoid or epithelial origin. In 1984, EBV became the first human virus whose genome was completely sequenced (527).

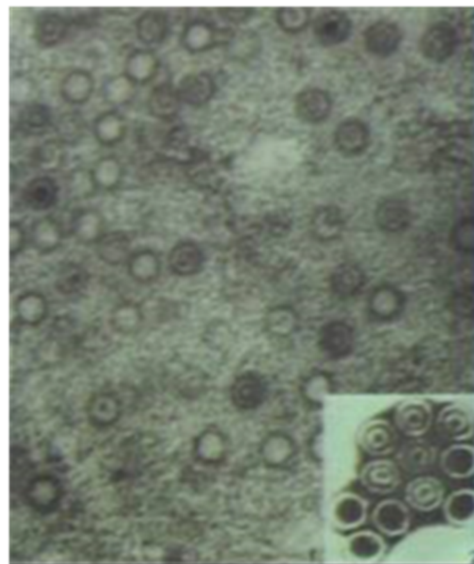


Figure 32 - Viral particle found in Burkitt's Lymphoma cell lines in 1964
(Henle L, Rickinson AB, EBV Symposium 2004, Regensburg)

2. Structure, genome and organization

EBV or human herpesvirus 4 (HHV-4), is one of eight known viruses in the herpes family. Similar to other herpesviruses the EBV virion is approximately 200 nm in diameter and consists of four common elements, a toroid-shaped protein core containing the linear double-stranded DNA genome, an icosahedral capsid, an amorphous protein tegument and an outer lipid envelope

The EBV genome is a linear, double-stranded DNA, approximately 172 kilo base pair (kbp) in length, and with a 60% Guanine (G) + Cytosine (C) content (528). Upon infection, the genome circularizes into a double stranded episome. The EBV genome consists of four components common to all herpesviruses, including a large unique region flanked by series of 0.5 Kb terminal direct repeats (529). In addition, two origins of replication: lytic origin of DNA replication (oriLyt) and origin of plasmid replication (oriP) are found within large unique region. The EBV wild-type (EBVwt) strain was created by assembling sequences of EBV strains B95.8 and Raji (530).

Like other herpesviruses, EBV's life cycle consists of 2 phases, a lytic replicative phase and a latency phase. A unique expression of various latency genes gives EBV its oncogenic properties and it ability to immortalize B lymphocytes *in-vitro*. EBV preferentially targets resting (naive) B cells, although epithelial cells and resting memory B cells can be infected as well (531, 532). Moreover, under certain conditions T cells, NK cells, smooth muscle cells and monocytes can also be infected (533). In general, EBV undergoes brief lytic replication in epithelial cells of the oropharynx (531). Subsequently, EBV remains latent in memory B lymphocytes (534).

3. EBV LATENT INFECTION

B lymphocytes are the main reservoir of latent EBV (528). After the initial infection of resting primary B cells, the linear EBV genome circularizes and is maintained as an episome within the nucleus. About 10-100 self-replicating episomes are maintained within the latently infected B cell. During the latent phase of the viral infection, a limited number of genes is expressed without viral production. The few expressed genes are important for inducing cell proliferation, inhibiting apoptosis, stopping the lytic replication, and ensuring correct division of episomal viral genomes to daughter cells (535). The small subset of genes expressed during latency include:

- Six EBV nuclear antigens (EBNA's) 1, 2, 3A, 3B, 3C and LP
- Three latent membrane proteins (LMP's) 1, 2A and 2B.
- Small non-coding RNAs are actively expressed during B cell transformation in vitro (536), including:
    - the highly abundant EBV-encoded RNAs, EBER-1 and EBER-2 (EBER's),
    - A set of highly spliced non-coding BamHI-A rightward transcripts (BART's), also known as complementary strand transcripts (CSTs),
    - One small nucleolar RNA (snoRNA) (537),
    - 44 mature microRNAs (miRNAs) derived from 25 precursor-miRNAs, the functions of which may control viral latency (538, 539)

3.1. The different latency programs

Depending on the profile of latency genes expressed there are 4 distinguishable type of EBV latency programs (0, I, II, III). The EBER 1 and 2 RNA are expressed in each of the programs.

The type I latency is the one which is found in Burkitt's lymphoma. It is characterized only by the presence of the EBNA 1 protein and a particular cellular phenotype. The infected B cells express CD10 and CD77 on their surface and little or no activation antigens or adhesion molecules (540).

The type II latency, also called the default program is characteristic of nasopharyngeal carcinoma, Hodgkin's disease, and certain NK/T cell lymphomas. It is characterized by the expression of the proteins EBNA1, LMP1, LMP2A and LMP2B.

The type III latency which permits the expression of all the latency genes EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, LMP1, LMP2A et LMP2B. It is found in B cells from lymphoproliferative diseases arising in immunocompromised patients.

These 3 types of latencies are typically found in certain malignant pathologies associated to EBV. In individuals with a persistent and asymptomatic infection, memory B cells are the major reservoir for the virus where the EBV virus is maintained in the type I latency program during which the EBER are transcribed but not translated (541).

For this thesis we used peptides from the type II latency program which is typically associated with NPC. Which is why for the following chapters will only introduce Type II latency genes.

VII. Nasopharyngeal carcinoma

Nasopharyngeal carcinomas develop in the back of the nasal cavity in the epithelial mucosa at the upper part of the pharynx. In the typical form, it consists of very immature malignant epithelial cells and has an inflammatory nature.

Based on the World health organization (WHO) classification, NPC has three different histological types. Type I NPC consists mostly of well-differentiated cells that are keratinized. Type II NPC is non-keratinizing squamous carcinoma, which varies in its extent of cellular differentiation. Type III NPC is undifferentiated and nonkeratinizing carcinoma. In areas where NPC is endemic, the non-keratinising subtypes constitute most cases (>95%). These are always associated with EBV infection, whereas type I disease is more common the rest of the world (542).

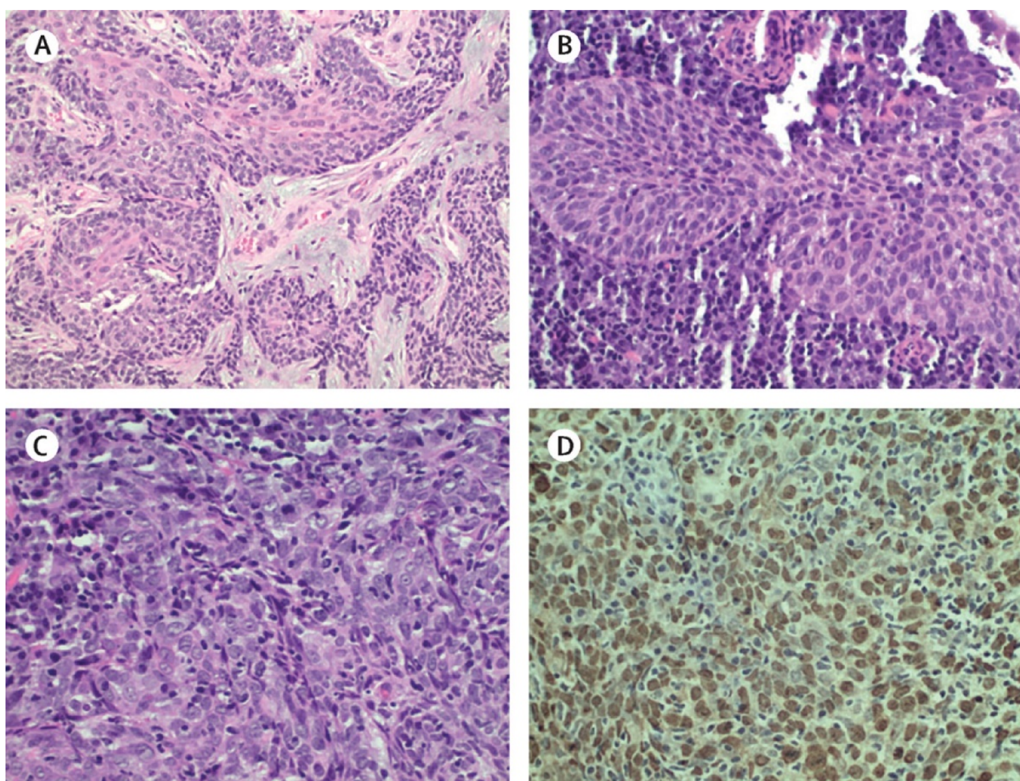


Figure 33 - Light microscopic images of nasopharyngeal carcinoma

"(A) Keratinising squamous cell carcinoma; haematoxylin and eosin (H&E) stain, magnification 200×. (B) Non-keratinising carcinoma, differentiated subtype; H&E stain, magnification 400×. (C) Non-keratinising carcinoma, undifferentiated subtype; H&E stain, magnification 400×. (D) Detection of Epstein-Barr virus-encoded small RNA by in-situ hybridisation." (542)

A. Epidemic distribution

With respect to other cancer types, NPC is uncommon and has a unique geographical distribution. In 2012, 86 500 cases of NPC were reported worldwide which accounts for 0.6% of all cancers diagnosed that year. 71% of new cases were in east and southeast parts of Asia, with south-central Asia, and north and east Africa accounting for the remainder. Regarding age-standardized incidence rates (ASRs), countries in South-eastern Asia (Malaysia, Singapore, Indonesia and Vietnam) and Northern/Eastern Africa (Algeria and Kenya) had the highest ASRs globally (3-7.2 cases per 100,000 people a year). Throughout China the ASR is only 1.9 cases per 100,000 people a year, however in the Southern and Eastern parts of the country (Guangdong, Hainan, Guangxi, Hunan, Fujian and Hong Kong) ASRs is higher than 15 cases per 100,000 people a year for males. Elsewhere in the world, ASRs for NPC are typically less than 1 per 100,000 people a year.

In addition to geographical variation, some ethnic groups also seem to have a predisposition for nasopharyngeal carcinoma (the Bidayuh in Borneo, Nagas in northern India, and Inuits in the Artic, have ASRs which are higher than 16 per 100 000 people a year in men). Demographically, men are two to three times more likely than women to develop NPC. Peak age of disease development is between 50 and 60 years (542).

B. Risk factors of NPC

Clinical risk factors for NPC include genetic variation, EBV infection, and environmental factors.

1. Genetic

The accumulation of genetic and epigenetic anomalies is necessary for tumorigenesis. Despite multiple breakthroughs over the last 20 years, defining the events crucial to NPC initiation and progression is still challenging for researchers. Yet some studies have elucidated molecular event implicated in the appearance of NPC. For example, telomerase activation and Bcl2 over expression are always present in the dysplastic lesions and invasive carcinoma (543). Polymorphisms in the gene coding for the Cytochrome P4502E1 enzyme or HSP70-2 have been linked to an increased to develop NPC (544). Several immune system genes have been associated with an increased NPC risk. For example, modification in the expression of some cytokines were associated with an increased NPC risk "IL-1A, IL-1B, IL-2, IL-8, IL-10, IL-12, IL-16 et IL-18." (543).

2. EBV related factors

The presence of EBV in the epithelial cells is an important factor for the formation of this tumor. Type I and II NPCs are systematically associated with EBV. For type I the association is frequent in the endemic regions but rare outside. This association to the virus has made NPC an excellent model

for the study of viral induced cancers. Moreover, even though viral particles are not found in the tumor, the viral DNA can be found in the nucleus with some viral genes being expressed.

A set of EBV latency genes have been identified that play an important role in NPC development, and multiple mechanisms including the restriction of cell homeostasis, the improvement of cell mobility, and the initiation of stem-like cancer cells were proposed.

A group of EBV latency genes have been found to be crucial for NPC development.

2.1. EBNA1

EBNA1 is found in all EBV-related tumors. It is one of the most crucial viral proteins that promote NPC and it helps maintain the viral latency in NPC (545, 546). It interacts with EBV oriP to maintain stable maintenance of EBV episomes in dividing cells (387). EBNA1 also induces the expression of other EBV-encoded genes through transcriptional activation of the Cp and LMP promoters. Transfecting EBV-negative cells with EBNA1 enables them to grow more rapidly as well as increases their metastatic potential in immunodeficiency mice (547). EBNA1 has various mechanisms of function which include promoting the expression of tumor angiogenic cytokines (548); downregulating STAT1 which improves proliferation by altering the TGFβ signaling (549) disrupting of promyelocytic leukemia (PML) nuclear bodies, which are related to p53 activation, DNA repair and cell apoptosis (550); reducing p53 levels by preventing the deubiquitination and consequent stabilization of p53; inhibiting the anti-oncogenesis canonical p65 nuclear factor-κB (NF-κB) pathway (551); and inducing the metastatic potential proteins (552) as well as epithelial–mesenchymal transition (553). Therefore, EBNA1 play crucial role in NPC oncogenesis and maintenance (554)

2.2. LMP1

LMP1 is another important viral oncoprotein. It functions mostly regarding epithelial transformation (555, 556), angiogenesis (557) and hypoxia response (558, 559). LMP1 is mainly found in pre-invasive lesions, but not in later stage of NPC, suggesting that it play a role in the initiation of NPC (560). LMP1 ability to promote tumor invasiveness and metastasis by remodeling actin filament (561-563), promoting epithelial mesenchymal transition (564) and inducing matrix metalloproteinases Moreover, LPM1 has anti-apoptotic properties (565-567). Finally LMP1 has been shown to induce cancer stem/progenitor-like cells in nasopharyngeal epithelial cell lines (568, 569). LMP1 has several tumor promoting properties. It mimics the TNF-α receptor family members TNFR1 and CD40. Unlike the TNFRs, LMP1 does not require ligand binding and thus is maintained in a constitutively activated state where it modulates several TNF-responsive signaling proteins such as TNFR-associated factors (TRAFs) and the TNFR-associated death domain protein. The activation of these pathways triggers multiple signaling pathways which lead to tumor survival and progression (570)

Early studies have shown that LMP1 could inhibit the differentiation in human epithelial cell (571). LMP1 increases NPC cell proliferation and survival by inducing expression of mitogenic

receptors, such as EGFR and c-Met, as well as activating growth promoting and antiapoptotic mediators, such as survivin, NF-κB, PI3K/Akt, and MAPK pathways. LMP1 can also decrease p16 expression by cytoplasmic sequestration of E2F4/5 and Ets2 transcription factors (572). LMP1 promotes the metastasis of NPC cells by inducing epithelial-mesenchymal transition and increasing the expression and release of matrix metalloproteinases through activation of activator protein 1, NF-κB, specificity protein 1, and ERK–MAPK pathways. LMP1 seems to also be capable of promoting angiogenesis in the NPC microenvironment by inducing HIF1α and VEGF. High LMP1 expression in primary NPC samples has been correlated with regional and distant metastasis (570, 573). LMP1 can also induce epigenetic modifications in NPC. Specifically, LMP1 can induce the expression and activity of DNA methyltransferases 1, 3a, and 3b, resulting in promoter hyper methylation of tumor-suppressor genes, such as E-cadherin (574)

2.3. LMP2A/B

LMP2A is almost always found in all NPCs, whereas LMP2B expression is only detected in about 40% of the samples. Even though, these 2 proteins cannot transform normal cells by themselves, their almost ubiquitous expression does indicate that they play an important role in the oncogenic development of NPC (575). LMP2A transfected epithelial cells showed increased proliferation, epithelial-mesenchymal transition, invasion, and migration by activating multiple pathways, including Notch, ERK, Syk, PI3K/Akt, and Wnt/β-catenin. LMP2B could also activate the PI3K/Akt signaling pathway, and promote cell motility by disrupting cellular adhesion (576). Finally, both LMP2A and LMP2B provide resistance to antiviral IFN signaling in NPC cells by promoting the degradation and turnover of IFN- receptors, IFN-α/β receptor (IFNAR) and IFN-γ receptor (IFNGR) (577). LMP2A is important in maintaining the latency phase as it blocks BCR signaling.

2.4. EBERs

EBER1 and EBER2 encode small RNAs (167 and 172 nucleotides in length,) which are highly expressed in NPC cells, up to 1 million copies detected per cell (578). Studies suggest that EBERs promote NPC growth, inhibit apoptosis and play a role in controlling host immunity. Studies on the C666-1 cell line have shown that EBERs promote cell growth *via* an autocrine mechanism by inducing the expression and release of insulin-like growth factor-1 (IGF-1), this expression of IGF-1 is also frequently detected in NPC biopsies

2.5. BARF1, BARTs and miRNAs

BARF1 is encoded in the BamH1-A fragment of EBV and is a homolog of the human colony stimulating factor 1 (CSF-1) receptor with known tumorigenic potential mainly *via* its effect on apoptosis inhibition by inducing Bcl-2 (579). The BARTs are multi-spliced RNAs transcribed from the BamH1 A region of EBV. BARTs can interact with notch receptor to promote the expression of LMP1 in the absence of EBNA2 (580). EBV genome encodes a minimum of 44 miRNAs transcribed from the

BHRF1 and BART regions (581). These RNAs have different tumorigenic functions mediated by their ability to inhibit expression of different gene. For example they can target the tumor suppressor gene DICE1 (582), the pro-apoptotic protein Bim and the E-Cadherin gene (583, 584).

2.6. Lytic phase proteins

Reactivation of lytic phase seems to occur in NPC. EBV reactivation induces NPC proliferation, migration and tumorigenicity (585). The existence of this reactivation in patients has been confirmed by the strong presence of high titers of antibodies against structural and lytic antigens in NPC (586). Moreover, some lytic cycle proteins have been detected in NPC patients. The EBV protein BZLF1 has been occasionally detected in NPC samples (587). BamHI-G leftward frame 5 (BGLF5), and BamHI-N leftward frame 2a (BNLF2a) have also been detected in patient biopsies along with antibodies against them (588, 589). EBV DNase (BGLF5) is an alkaline nuclease encoded by the BGLF5 open reading frame of EBV and is important in the generation and processing of linear viral genomes (590). BGLF5 involved in repressing DNA repair, inducing genomic instability in human epithelial cells (591). BHRF1 has been shown to inhibit NPC apoptosis as it acts as a homolog to Bcl-2 (592).

Table 6 below provides a more detailed function of the various proteins and their role in oncogenesis.

Table 6 - Table of the transformation potential of EBV proteins in NPC

General mechanisms	Viral products	Molecular mechanisms	Ref
Promotion of transformation and angiogenesis	**EBNA1**	Mediates AP-1 to upregulate IL-8, VEGF, HIF-1α	(548)
	EBNA1	downregulating STAT1 which improves proliferation by altering the TGFβ signaling	(549)
	LMP1	Upregulates the phosphorylation of histone H3; inhibits the LKB1-AMPK pathway	(555, 556)
	LMP1	Mediates the NF-κB, MEK-ERK, and JNK pathways to induce endocan; mediates the degradation of prolyl hydroxylases 1 and 3 to upregulate HIF1-α	(557-559)
	LMP1	Mimics the TNFα receptor family members TNFR1 and CD40	(570)
	LMP2A/B	Transformation of epithelial cells *via* PI3K/Akt signaling pathway	(576)
	EBERs	Upregulate IGF-1	(593)
	BARF1	Increases the cell growth rate	(594)
	miR-BART1	Upregulates PSAT1 and PHGDH	(595)
	miR-BART3	Inhibits DICE1 tumor suppressor	(582)
	BGLF5	Represses DNA repair inducing genomic instability	(591)
Inhibition of apoptosis	**EBNA1**	Disrupts PML nuclear bodies	(550)
	LMP1	Inhibits Chk1 to impair the G2 checkpoint; increases p53-mediated survival; mediates EGFR and STAT3 to induce cyclin D1	(565-567)
	EBERs	Upregulate Bcl-2 and downregulate caspase-3 and PARP	(596)
	BARF1	by inducing Bcl-2	(579)
	miR-BART5	Inhibits PUMA	(597)
	BART	Inhibits Bim	(584)
	BHRF1	homolog to Bcl-2	(592)
Induction of stem cell-like phenotype	**LMP1**	Induces the CSC/CPC-like phenotype and self-renewal; activates the hedgehog pathway to induce CD44v6, NGFR (p75NTR), and CXCR4	(568, 569)
	LMP2A	Activates hedgehog to induce CD133 and CXCR4; induces stem-like cells and self-renewal	(569, 598)
Enhancement of cell mobility	**EBNA1**	Upregulates stathmin 1, aspin, and Nm23-H1	(552)
	EBNA1	Mediates TGFβ1/miR-200/ZEB to induce EMT	(553)
	LMP1	Activates the PI3K/Akt pathway to promote actin stress-fiber formation; interacts with FGD4 to activate Cdc42; mediates the NF-κB pathway to upregulate TNFAIP2	(561-563)
	LMP1	Downregulates E-cadherin to induce EMT	(564)
	LMP1	Upregulates MMPs (e.g., MMP1, 3 and 9)	(599-601)
	LMP2A	Induces epithelial mesenchymal transition	(598)
	LMP2A	Mediates the ERK/Fra-1 pathway to induce MMP9	(602)
	miR-BART7	Enhances migration and invasion and inhibits PTEN to induce EMT	(603)
	miR-BART9	Inhibits E-cadherin to induce EMT	(583)

C. EBV and the host immune response

1. Innate responses against EBV

NPC is characterized by the abundant presence of infiltrating leukocytes in the tumoral stroma. These different cells including neutrophils, NK cells, monocytes, macrophages, DCs are the first defense against EBV (604-606).Using flow cytometry, EBV was proven to bind to the neutrophil surface *via* gp350 and then induce the production of antiviral cytokines and chemokines, including IL-1α, IL-1β, IL-8, and macrophage inflammatory protein (MIP)-1 (607, 608).

DCs and pDCs sense EBV products through Toll-like receptors. They produced IFNα when challenged with live EBV virions or EBV DNA (609). Moreover, treatment of DCs with EBERs lead to the production of IFNβ, IFNγ, and TNFs (610).

EBV can potentially infect NK cells *via* the gp85-gp25-gp42 complex which can directly bind the HLA class II molecules on NK cells (611). NK cells can also play an important role in the lytic infection phase since the downregulation of HLA class I is accompanied by a sensitization of NK cells (612).

EBV has been shown to activate macrophages (613). This is induced *via* the dUTPase of EBV which promotes the secretion of TNFα, IL1β, and IL6 by macrophages in a MyD88-dependent activation of NF-κB (614). Regarding monocytes, EBV infection induces their secretion of several cytokines some are shared with macrophage while others include IFN-inducible protein-10 (IP-10), MIP-1, monocyte chemotactic protein-1 (MCP-1), and IL-8 at the mRNA (615, 616).

2. Antibodies detected during EBV infection

EBV-specific antibodies, mainly IgG and IgA, can be found in NPC patients' sera. Several EBV specific targets are recognized by these antibodies including EBV structural antigens (glycoproteins gp350/220, gp78), viral capsid antigen-proteins (VCA-p18 and VCA-p40), lytic antigens (BRLF1, BZLF1 and EBV-DNase), and latent antigens (EBNA1 and LMPs) (586). A study focusing on studying the humoral immune response in NPC patients which included a large number of patients found abundant IgG antibodies against lytic antigens and EBNA1 but not against LMP1, LMP2, nor BARF1 (617). The marginal immunogenicity of LMPs and BARF1 to humoral immune responses may be due to their intrinsic properties (for example, rapid and complete secretion of BARF1 leaves little protein within or on the surfaces of cells for detection (618)) and to their limited expression on the plasma membrane.

3. Cellular responses to EBV infection

The cellular immune response is crucial in controlling EBV during the primary and persistent phase. Its importance in NPC can be elucidated from the fact that CTLs are detected in NPC patients (619, 620), and that EBV-specific memory CTLs can be reactivated *in-vitro* following isolation from patients (621). Interestingly, CTLs against EBNA1 have been shown to decrease significantly in NPC

patients (622). Similarly, LMPs, and BARF1 specific CD4+ T cells have been detected in NPC patients at low levels (620, 623)

D. Evasion of innate immune responses

The fact that EBV can establish lifelong persistence infection in over 90% of the world's adults is a clear indication of its ability to evade the immune response. This is often not only mediated by the latent viral genes but also by a part of the lytic proteins which can be detected in NPC due to viral reactivation (624, 625).

1. Modulation of phagocyte apoptosis and maturation

After binding neutrophils, EBV penetrates them and localizes to their nuclei and induces their apoptosis by modulating the Fas/FasL pathways. This in achieved by increasing both membranous Fas/FasL expression as well as soluble FasL (626).

It has also been proven that EBV inhibits PKC activity in monocytes thus impairing their phagocytic activity (627). Moreover, EBV's contact with monocytes during their maturation induces their apoptosis which results in a decrease in the quantity of mature DCs (628). EBV can also inhibit the functions of pDCs reducing their secretion of TNFα (629). Without the support of properly functioning DCs, the antiviral immune response, mainly its T cell fraction is severely inhibited.

NKG2D ligands MICA and ULBP4 are downregulated by LMP2A (630) while MICB is downregulated by BARTs (631).

2. Blockade of antiviral cytokines

EBERs and LMP2 are capable of inhibiting the type I IFN responses by both disrupting IFN-stimulated transcription and by targeting IFN receptors for degradation (632). EBV can also stimulate the production of a soluble IL-1 receptor antagonist to further block the function of neutrophils (633).

EBV interferes with the activation of the NF-κB pathway in monocytes, which inhibits COX-2 which is a crucial enzyme for PGE2 biosynthesis (634). The interference with the same pathway seems to inhibit TNFα secretion by monocytes (635, 636)

EBERs can counteract the effects of anti-viral IFNs by inhibiting the activation of the downstream pathways of IFN signaling (637). EBERs inhibit the phosphorylation of the cellular substrate of PKR, which signals a translational block of protein synthesis which protects EBV-infected cells from Fas-mediated apoptosis induced by IFNs (638).

E. Evasion of adaptive immune responses

1. Switching off immunodominant viral antigen expression

EBV has established multiple tactics to escape cellular immune responses during its latency phase. To escape the cellular immune response, EBV, much like other herpesviruses, turns off the expression of most of its viral genes during the latency phase. This mainly applies for highly immunogenic viral proteins thus hiding from the adaptive immune response. For example, latent viral factors such as EBNA3 and EBNA2 with high immunodominance are never expressed in NPC patients (639). However, ex-vivo culture of patient CTLs with autologous EBV-transformed lymphoblastoid cell lines does induce an anti-EBNA3 reaction (619).

2. Impairment of the antigen-presenting HLA I or HLA II pathway

NPC patient biopsies do show a downregulation in HLA class I antigen presentation machinery. This could be due to the fact that LMP1 can induce c-Myc which is known to downregulate HLA class I antigen presentation (640). LMP1 can also undergo self-aggregation mediated by the first transmembrane domain stopping the cis-presentation of an LMP1-derived epitope (641).

EBNA1 is resistant to proteasomal digestion which protects it from being endogenously presented by MHCI meaning that it is mainly targeted by CD4+ cells (642). Exogenous EBNA1 can be presented by MHC class I *via* a TAP independent pathway (643). A deletion in the glycine-alanine repeat domain of EBNA1 enables its endogenous presentation on MHC class I and its recognition by CD8+ cells (644).

Among the detectable EBV lytic antigens in NPC patients, BZLF1, BGLF5, and BNLF2a have been shown to interfere with the adaptive immune response. BGLF5 can induce a host shutoff function to block the synthesis of host HLA I, hence inhibiting $CD8^{+}$ T cell recognition (645). BZLF1 can suppress MHC class II expression by inhibiting the transcription of the MHC class II transactivator, (CIITA) which is an essential transcriptional coactivator of MHC class II expression (646). BNLF2a binds TAP and inhibits its function affecting the presentation of early proteins to the HLA class I (647, 648)

3. Immuno-inhibitory molecules

IL-10 has been shown to be upregulated in EBV positive NPC where it is responsible for the inhibition of CTL response (649). Various EBV proteins promote IL-10 expression in NPC. LMP1 was the originally recognized by its ability to induce IL-10 *via* the p38/stress-activated protein kinase 2 pathway (650). EBER1 and EBER2 are related to an increased IL-10 mRNA expression (651). BCRF1 is another lytic cycle protein. It has 70% structural homology with IL-10 and has been shown to inhibit PBMC IFNγ synthesis in vitro (652)

Decoy receptor 3 (DcR3) has immune regulatory functions as it has been shown to induce DC apoptosis and downregulate MHC class II expression on TAMs. LMP1 has be shown to upregulate DcR3 expression *via* the NF-κB PI3K pathway. (653-656)

4. Tregs and NPC

The importance of Tregs in EBV infection was first identified as an explanation for the very low immunogenicity against LMP1 in Hodgkin's Lymphoma (107, 657). Another team found that EBNA1 stimulated PBMCs developed both a TH1 response coupled with an induction of Tregs which were shown to be suppressive *in-vitro* (658)

Tregs are constantly detected in NPC patients either circulating or in the TME. In fact, 12% of the TILs were CD4+CD25+Foxp3+ (659). LMP1 has been shown to induce IL-10 secretion by Tregs (657). Treg depletion of NPC patient PBMCs *via* an anti-CD25 antibody restored the function of defective EBNA-1 and LMP2- specific CD8+ T cells. Further indicating the importance of the immunosuppressive role of Tregs in NPC (660).

5. Exosomes

Finally, NPC derived exosomes expressing LMP1, Gal-9 and CCL20 with immunosuppressive capacities have been found to be elevated in NPC patients (449). Our team has found that these exosomes have been shown to recruit, induce and expand Tregs in the TME of NPC (661)

F. NPC and EBV-based screening and diagnosis

NPC is currently diagnosed by histopathological examination of biopsies and fine needle aspiration (FNAC) of neck lumps. Whenever it is clinically difficult to diagnose the patient by the classic techniques, due to the presence of occult primaries or submucosal disease, the detection of EBV DNA (by PCR) and RNA (EBER-ish) is used only as a confirmation step. Radiotherapy is currently the most effective treatment option if the disease is diagnosed at an early stage, having a 5-year survival probability of up to 90%. Unfortunately, at diagnosis 75–90% of patients with NPC have already have developed local or regional spread (662). Therefore, early disease screening in asymptomatic individuals may potentially improve NPC treatment outcomes. In endemic regions, the presence of EBV is a strong indicator of NPC presence. Due to this high association with EBV infection, the potential of using antibodies specific for EBV capsid antigen (IgA/VCA), early antigen (IgA/EA) and plasma DNA for early NPC screening has been extensively studied. Data from both endemic and non-endemic regions indicates that plasma DNA can be used for screening, diagnosis, staging, follow-up and prognostication (663-665). A recent study on 1318 healthy volunteers showed that plasma EBV DNA analysis is useful for early detection of NPC before clinical signs appear (666). Another study has provided convincing evidence that detection of plasma EBV DNA is a more sensitive and specific marker than the serum

IgA/VCA titer for the diagnosis and follow up of NPC patients (667). Other potential serological biomarkers for diagnosis and prediction of treatment efficacy include the circulating EBV miR-BART7 and miR-BART13 (668). A study published earlier this year, showed that EBV-encoded mir-bart1-5p detection *via* nasopharyngeal brush sampling, a less invasive technique, can diagnose early-stage NPC with 93.5% sensitivity and 100% specificity (669). However, all miRNA detections techniques still require large patient cohorts across multiple centers for confirmation.

G. Treatments

At the moment, NPC treatment options are based on the disease stage. The general rule is to administer intensity modulated RT for stage I disease, IRT with or without chemotherapy for stage II and stage III till IVB with IRT and concurrent chemotherapy. In general, NPC patients respond well to first line radiotherapy and chemotherapy. Excellent local control of the tumor is achieved however, distant recurrence is highly frequent and the current therapies for distant metastasis are underwhelming. Moreover, high toxicity remains and issue especially for patients with intracranial extension of the tumor. Therefore, there is a continuing need to develop new therapeutic approaches for NPC (670).

1. Immunotherapy

The fact that EBV is present in almost all NPC cells does provide unique treatment opportunities. The fact that NPC cells have preserved their antigen presentation potential should make these viral antigens (*via* virus-restricted MHC class I) a great target for the immune system to eliminate the cancer cells harboring them (671). Nevertheless, this is not the case and the situation is more complicated. Due to downregulation of MHC class I expression in NPC tumors, coupled with low percentage of EBV specific T cells capable of recognizing these low immunogenic proteins and the presence of a suppressive tumor microenvironment designed to inactive T cells the development of NPC based immunotherapeutic approaches has been slow and challenging (672, 673)

Adoptive transfer of EBV specific CTLs was one of the used strategies. Two trials were done by Roonie's team. The first included 10 patients: 6 with a refractory disease and 4 with an advanced disease that was in remission at the time of CTL injection. 2 patients had a complete response, 1 had a partial response, 1 presented a stable disease and 2 were non-responders. The 4 patients that were on remission stayed that way (674). The second more recent trial included 23 patients: 8 were in remission and 15 were diagnosed with the active disease. Out of the 8, 5 remained in remission while 3 suffered relapse at (2, 14 and 60 months). Of the remaining 15, 5 had a complete response, 2 only partial, 3 presented a stable disease and 5 were non-responders (675).

In 2004, a preliminary study by Comoli's team of an NPC patient with LMP2 specific allogenic CTLs. He experienced a temporary disease stabilization which was coupled with an increase in TILs

(676). The same group continued with a study on 10 stage IV NPC patients who progressed after radio and chemotherapy. The CTLs were reactivated and expanded ex-vivo by means of contact with autologous B-lymphoblastoid cell lines (LCL). These cells which were mostly CD3+CD8+ T lymphocytes had the potential to kill autologous NPC cells and other LMP2 expressing cells. Following injections of these cells, 2 patients had partial response while 4 stabilized the disease (677). Recently the same team recruited 11 EBV+ NPC patients who did not respond to classical therapy. Autologous CTLs were administered in 2 doses following nonmyeloablative lymphodepleting. Tumor regression over a period of more than 4 months was observed in 6 patients similar to the previous results indicating that lymphodepletion did not have any effect (678).

A phase II clinical trial included 21 patients with pretreated metastatic NPC. These patients received nonmyeloablative peripheral blood stem cell transplantation. Seven patients showed partial response and three achieved stable disease. Four patients were alive at 2 years and three presented prolonged disease control for 344, 525 and 550 days each. The median PFS was 100 days and median (overall survival) OS was 209 days. Interestingly the patients who experienced chronic GVHD had better OS 426 vs 143 days (679). Another adoptive CTL transfer trial using anti-CD45 antibodies for lymphodepletion prior to CTL transfer achieved similar results (680).

Another CTLs transfer involved adenoviral vector-based vaccine that encodes EBNA1 fused to multiple CD8+ T-cell epitopes from LMP1 and LMP2. 24 metastatic NPC patients were included in the study. The vaccine successfully expanded EBV specific T cell in 16 of the patients. OS increase from 220 days to 523 with the treatment. (681)

Dendritic cell vaccination strategy was less successful. *In-vitro* matured monocyte derived DCs pulsed with LMP2 restricted epitopes and then reinjected into patients induced a CD8+ T cell response in 9 of the 16 patients suffering from local recurrence and distant metastasis. 2 of these patients experienced tumor size reduction (682). Another phase II clinical trial on metastatic patients of a DC vaccination strategy used autologous DCs infected with an adenovirus expressing truncated LMP1 and full-length LMP2. Even though the treatment was well tolerated only 1 out of 12 patients had a partial response so the efficacy was negligible (683)

More successful approaches were obtained with non-cell based vaccinations. A recent study tested the effectiveness of a new recombinant modified *vaccinia Ankara, "MVA-EL",* which encodes a C-terminal EBNA1/full length LMP2 fusion proteins. The vaccine was given 18 NPC patients from Honk Kong in remission 12 weeks following the classic therapy. Different doses were given and the immunogenicity of patient cells to the proteins was tested 1 year after vaccine administration with positive results obtained in 15 out of the 18 patients. (684) No advert toxic effects were observed. The result of this trial were confirmed in another trial on 16 patients from the UK suffering EBV positive NPC. The vaccine was well tolerated and induced an immunity in 8 out of the 14 patients (685). The success of these 2 studies lead to the start of a phase II clinical trial for the effectiveness of this vaccine

in patients with persistent, recurrent or metastatic NPC (NCT01094405 clinical trial which is currently recruiting).

Immunecheckpoint inhibitors in NPC are a definite possibility. PDL1 expression on tumoral cells and tumor infiltrating macrophages is common in virus induced malignancies (686). An increase in PD-1 expression on CD8+ has been correlated with a poor prognosis in NPC patients (687). Moreover LMP1 with IFNγ has been shown to increase PD-L1 expression in EBV infected cells (688). These observations have opened the door for an ongoing multicenter phase II using Nivolumab in previously treated Patients suffering from recurrent and metastatic NPC (NCT02339558).

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Objectives

As it is described in the introduction, the immune system is capable of identifying and eliminating cancer cells but is often inhibited by certain regulatory mechanisms. We have also seen that anti-cancer immunotherapy existed for a while; however, real success in these field is relatively recent.

Several studies have clearly confirmed that Tregs are crucial down regulators of the anti-tumoral immune response and that the means of effectively targeting them is still lacking. We have also seen that vaccination approaches could be an effective approach for treating viral induced cancers.

In this context, we propose in this work to evaluate two different anti-cancer therapeutic approaches in the model of NPC; a cancer whose treatment by vaccinations is still lacking due to rather particular suppressive mechanisms (Tregs and Gal-9 containing exosomes).

At the beginning of my thesis, I was associated with a project which had the main objective of determining the role of Gal-9 containing exosomes on human regulatory T lymphocytes (Tregs). This work has been published in the Journal of National Cancer Institute (Mrizak et al, JNCI 2015) and has been complemented by publishing a Letter to the Editor in the same journal (Delhem N. & Mustapha R. JNCI 2015).

The second objective aimed to demonstrate the capacity of a vaccination strategy based on EBV type II latency peptides to induce a successful CD4+ and CTL mediated anti-NPC immune response. We also wanted to prove that in the presence of such adequate CD4+ promiscuous antigens, the induced cells were resistant to NPC suppressive effects. We also aimed to prove the *in-vivo* therapeutic potential of this approach in a humanized SCID mice model of NPC and *ex-vivo* on NPC patient immune cells.

Regarding my main thesis project, the first objective was to confirm the role of Gal-9 in human Treg-mediated-immuno-suppression. Then we wanted to test the capabilities of an anti-Human-Gal-9 antibody (mouse IgG1) developed by Busson's team to block Gal-9 suppressive function and the effect of this blocking on Treg function and subsequently the anti-tumoral immune response. The ultimate hope is to use Gal-9 antagonistic monoclonal antibodies as a clinically viable therapeutic approach to treat cancer. This project was the subject of a patent (published in December 2015) with which I am associated as co-inventor

Article 1: Effect of nasopharyngeal carcinoma-derived exosomes on human regulatory T cells.

Journal of the National Cancer Institute. 2015;107(1):363-.

Mrizak D, Martin N, Barjon C, Jimenez-Pailhes A-S, **Mustapha R**, Niki T, Guigay J, Pancre V, de Launoit Y, Busson P, Morales O, Delhem N.

I. Context

One of the paradoxes in the pathogenesis of nasopharyngeal carcinoma is the persistence of a virus coupled with the emergence of a malignant process all in a local inflammatory context rich in leukocyte infiltration into the primary tumor and associated with the production of several immunogenic viral proteins. As we have seen in the introduction, the existence of numerous immune suppressive mechanisms is clearly described in pathologies associated with Epstein Barr Virus (EBV) and particularly in NPC. Furthermore, Pierre Busson's group has recently described in NPC, a major production of tumor exosomes carrying Galectin-9 a ligand of the Tim3 receptor found primarily on Th1 cells inducing their death by apoptosis. In addition, a strong recruitment of regulatory T cells (Tregs) has been described in NPC patients up to 12% of tumor infiltrating lymphocytes. Given their suppressive potential, it is only natural that Tregs and exosomes greatly contribute to the formation of the immunosuppressive microenvironment associated with NPC and the potential failure of therapeutic approaches. The exact interaction of these two elements in NPC had not been described.

This study aimed to explore the *in-vitro* immunosuppressive potential of NPC exosomes especially in the context of their impact on the phenotype, expansion, recruitment and function of Tregs. We used exosomes isolated from NPC cell lines called her NPC-Exo, from NPC patient's sera called here patient-Exo and exosomes from healthy donors here called HD-Exo.

II. Methodology and principle results:

First of all, this study showed that only NPC-Exo and not HD-Exo had the ability to inhibit the proliferation of CD4+CD25- T cells (Tconv) following a 120-hour culture at concentration higher than 1 µg/ml.

Then we showed that CCL20 known for its Treg chemoattractant potential is expressed in NPC cell lines. *In-vivo*, the role of CCL20 in Treg recruitment was studied in a humanized NPC mouse model.

Briefly, a human NPC tumor was xenotransplanted into SCID mice. These mice were then reconstituted with human PBMC enriched with 10% Tregs that had been stained with the VT680 fluorescent dye. Mice were treated or not with intra-peritoneal injections of anti-CCL20 antibody. The fluorescent signal was analyzed *in-vivo via* the IVIS Lumina-XR machine to study the effect of the CCL20 blocking. The results revealed a decrease in the fluorescent signal emitted from the tumor due to Treg infiltration following antibody treatment. This results were confirmed by immuno-histo-fluorescent analysis showing a decrease in tumor infiltrating Tregs as well as by flow cytometry showing an increase in circulating Tregs CD4+CD25+CD127low in the periphery. The study goes on to show by western blot that CCL20 is expressed in NPC-exo at a concentration of about 300 pg/ml determined by using a semi-quantitative gradient of the recombinant protein. This result was confirmed by electron microscopy coupled with immunogold staining not only on NPC-Exo but also on patient-Exo. Finally, the CCR6 expression (CCL20 receptor) determined by QPCR increased in Tregs following exposure to NPC-exo. Moreover, *in-vitro* chemo-attraction tests done *via* a Boyden chamber showed that NPC-exo and patient-Exo specifically attracted Tregs and this attraction was inhibited with an anti-CCL20 antibody.

Interestingly, we observed that the tumoral-exosomes had the capacity to moderately recruit Tconv. So we wondered if these exosomes would have the ability to induce the conversion of these Tconv into iTregs. A 72 or 120 h culture with NPC-Exo increased their expression of fraction of CD25+/high which was accompanied with high levels of TGFβ secretion. This was not observed following a culture with HD-Exo. Moreover, Tconv following a culture with NPC-Exo and not HD-Exo acquired the ability to inhibit the proliferation of autologous PBMCs in a mixed leukocyte reaction suppression test.

Furthermore, NPC exosomes induced the *in-vitro* proliferation of Tregs and increased their expression of suppressive markers (Lselectin, ICAM1, OX40, Tbet, and Granzyme B). Finally culturing Tregs with NPC-exo was shown to increase their expression of Foxp3 which was not observed when culturing Tregs with HD-Exo. This increase in suppressive potential was confirmed by MLR suppression tests as Tregs pre-cultured with NPC-Exo or patient-Exo but not HD-Exo could more effectively inhibit the proliferation of autologous PBMCs.

III. Conclusion:

This work showed for the first time that NPC-Exo potentiate the suppressive functions of human Tregs by inducing their expansion, differentiation, and increasing their chemoattraction at tumor site. This corporation between Tregs and exosomes could be crucial in inducing the suppressive tumoral micro-environment present in NPC patients and could be at the origin of NPC's ability to escape the immune system and resist current therapeutic approaches.

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ARTICLE

Effect of Nasopharyngeal Carcinoma-Derived Exosomes on Human Regulatory T Cells

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Abstract

Background: Regulatory T cells (Treg) and tumor-exosomes are thought to play a role in preventing the rejection of malignant cells in patients bearing nasopharyngeal carcinoma (NPC).

Methods: Treg recruitment by exosomes derived from NPC cell lines (C15/C17-Exo), exosomes isolated from NPC patients' plasma (Patient-Exo), and CCL20 were tested in vitro using Boyden chamber assays and in vivo using a xenograft SCID mouse model ($n = 5$), both in the presence and absence of anti-CCL20 monoclonal antibodies (mAb). Impact of these NPC exosomes (NPC-Exo) on Treg phenotype and function was determined using adapted assays (FACS, Q-PCR, ELISA, and MLR). Experiments were performed in comparison with exosomes derived from plasma of healthy donors (HD-Exo). The Student's *t* test was used for group comparisons. All statistical tests were two-sided.

Results: CCL20 allowed the intratumoral recruitment of human Treg. NPC-Exo also facilitated Treg recruitment (3.30 ± 0.34 fold increase, $P < .001$), which was statistically significantly inhibited ($P < .001$) by an anti-CCL20 blocking mAb. NPC-Exo also recruited conventional $CD4^{+}CD25^{-}$ T cells and mediated their conversion into inhibitory $CD4^{+}CD25^{high}$ cells. Moreover, NPC-Exo enhanced ($P = .0048$) the expansion of human Treg, inducing the generation of $Tim3^{Low}$ Treg with increased expression of CD25 and FOXP3. Finally, NPC-Exo induced an overexpression of cell markers associated with Treg phenotype, properties and recruitment capacity. For example, *GZMB* mean fold change was 21.45 ± 1.75 ($P < .001$). These results were consistent with a stronger suppression of responder cells' proliferation and the secretion of immunosuppressive cytokines (IL10, TGFB1).

Conclusion: Interactions between NPC-Exo and Treg represent a newly defined mechanism that may be involved in regulating peripheral tolerance by tumors and in supporting immune evasion in human NPC.

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Nasopharyngeal carcinoma (NPC) is a malignant epithelial tumor, consistently associated with the Epstein-Barr virus (EBV) (1,2). Although NPC is rare worldwide, it is endemic in several areas, particularly in Southeast Asia, suggesting a multifactorial etiology involving EBV infection, genetic predisposition, and environmental and other factors still unknown (3–8).

An important biologic feature of NPC is the presence of a massive lymphoid infiltrate in the primary tumor, which is likely favored by inflammatory cytokines produced by tumor cells (9–12). Several studies support the idea of a local immunosuppressive environment inhibiting proper effector cell function even after correct homing. The presence of abnormal quantities of regulatory T cells (Treg) within the tumor site and the peripheral blood is a clear indication of this immune suppression (13). Recent findings confirm the consistent production of galectin-9 (LGALS9) by NPC cells (14), in association with exosomes (15). LGALS9 is a β-galactoside binding lectin; its expression can be induced by different stimuli, such as proinflammatory cytokines (16) and probably EBV infection (17). The nature of LGALS9 receptors on the membrane of target cells is still controversial. HAVCR2, also known as Tim3 (T-cell immunoglobulin domain and mucin domain 3), might be one of them (18). Some of us have reported the detection of NPC tumor exosomes containing LGALS9 in the plasma of NPC patients or mice xenotransplanted with NPC tumor lines. There is evidence that these LGALS9-positive tumor exosomes can induce apoptosis of EBV-specific Th1 lymphocytes through LGALS9/ HAVCR2 interaction. These findings support the idea that NPC exosomes exert Th1 suppressive functions (19).

The specific mechanisms of Treg recruitment within NPC tumors and the putative interaction between NPC-Exo and Treg have not yet been addressed. Therefore, we intended here to investigate the existence of intercellular cross talk between the tumor and immune cells that might regulate antitumor immune response. We showed, for the first time, that CCL20 plays a crucial role in the recruitment of human Treg into NPC tumor. We also reported that NPC-Exo have unique immunoregulatory properties. They induce Treg expansion and upregulate their suppressive functions. They also promote the conversion of conventional $CD4^+CD25^-$ T cells (Tconv) into Treg and enhance Treg chemoattraction via CCL20. Interactions of NPC-Exo with Treg represent a newly defined mechanism that might be involved in regulating peripheral tolerance by tumor cells and in supporting immune evasion of human NPC.

Methods

Isolation of T-Cell Subsets

Human blood samples were collected from healthy adult donors with informed consent obtained in accordance with approval of the Institutional Review Board at the Institut de Biologie de Lille. Mononuclear cells (PBMC) were isolated from peripheral blood samples by density gradient centrifugation using Ficoll (GE healthcare, Uppsala, Sweden). Treg and Tconv were isolated from PBMC using Treg isolation kits (Miltenyi Biotech, Germany). Obtained purity was over 95%.

Exosome Isolation

Patient-derived EBV-positive xenografted tumors (C15 and C17) were permanently propagated by subcutaneous passage in nude or SCID mice as previously described (19). In accordance with institutional guidelines, homozygous CB-17 scid/scid (SCID) mice were purchased from animal facility of Institut Pasteur de Lille (IPL) (Lille, France). Animals were housed under specific pathogen-free conditions at the animal facility of the IPL.

C15 and C17 tumor exosomes were isolated from in vitro conditioned culture media or plasma samples from NPC patients (patient-Exo). Control exosomes were isolated from plasma samples given by healthy donors (HD-Exo). Conditioned culture media were prepared by collagenase dispersion of cells from the C15 and C17 xenografts and incubation of these cells for 48 hours in low serum conditions (allowing collection of C15 and C17-Exo). NPC plasma samples were obtained at the Institut Gustave Roussy (Villejuif, France) from seven patients bearing nonkeratinizing undifferentiated NPC with high tumor mass prior to any treatment. All these donors gave informed written consent before the sampling procedure according to the legal provisions (French Huriet Law).

Exosome isolation from C15 and C17 xenograft culture media (C15/C17-Exo), or from NPC plasma was done by differential centrifugation and flotation on a D_20/sucrose cushion, as previously reported (19). Plasma and serum sample were initially diluted at a ratio of 1:50 and 1:2, respectively, in phosphate buffered saline medium (PBS).

Western Blot Analysis

Different cell subsets and exosomes were lysed (10 minutes on ice) in PY buffer consisting of 20 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.02% sodium azide, and a cocktail of proteases inhibitors (Roche, Basel, Switzerland). After centrifugation (20 000 g, 15 minutes, +4°C), cell debris were removed and supernatants were collected. Protein concentrations were measured using Bio-Rad Protein Assay according to manufacturer's instructions (Bio-Rad, Marnes la Coquette, France). Exosomes and total cell extracts were then analyzed by western blotting. Further details are available in the Supplementary Methods (available online).

Cell and Exosome Functionality Assays

Proliferation was measured after [^{3}H]thymidine (1µCi/well) (PerkinElmer, Courtaboeuf, France) incubation for the last 18 hours before harvesting. Radioactivity was determined using a β-counter (1450 Trilux, Wallac, Finland). To study the effects of exosomes on T cell proliferation and Treg suppressor activity, reactions were set up in contact or not of increasing concentrations of exosomes (0.01, 0.1, 1, and 10 µg/mL). Each proliferation assay was carried out in triplicate and estimated in count per minute (cpm), and results were normalized compared with nontreated condition. Further details are available in the Supplementary Methods (available online).

Real-Time Quantitative Polymerase Chain Reaction Assays

Total RNA from C15 pellets (3.10^6 cells) was extracted using the TRIzol reagent (Invitrogen) method according to the manufacturer's instructions. Pellets of inferior concentration (Treg) were isolated using the RNeasy minikit II (Qiagen) according to the manufacturer's instructions. RNA concentration and purity were measured by spectrophotometric methods (Ultrospec 3000, Pharmacia Biotec). Total RNA were stored at -80°C until further use. Further details are available in the Supplementary Methods (available online).

Article 2: Tumor-derived Exosomes do not Disturb a Novel EBV Peptide-based Immunotherapy for Nasopharyngeal Carcinoma

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Article submitted to Cancer Discovery

I. Context and objective:

The Epstein Barr virus (EBV) is implicated in numerous malignancies which are distinguished by the expressed latency program. The type II latency is commonly associated with Naso-pharyngeal carcinoma (NPC). The expression of viral antigens by tumoral cells has made NPC a tempting target for cancer vaccinations. Numerous approaches attempted to use CD8+ T cell or dendritic cell inducing vaccines to treat NPC with little success. A great amount of research shows that CD4+ T cells play a crucial role in the anti-tumoral immune response against NPC which is why our team has opted for a CD4+ stimulating approach as an NPC vaccination strategy. Using the TEPITOPE program has allowed the team to define 6 promiscuous peptides derived from type latency II proteins. The team had shown that these peptides are capable of inducing a strong Th1 skewed immune response against the viral antigens. We have also published that NPC cells *via* their tumoral exosomes can suppress Th1 cells by inhibiting their proliferation and inducing their apoptosis. In this context, these NPC-Exo could pose an obstacle for future NPC immunotherapeutics approaches.

We aimed in this study to determine the efficacy of using an EBV latency type II peptide vaccine therapeutic approach against NPC while at the same time determining the effect of tumoral exosomes on the anti-tumoral immune response in the context of this vaccine.

II. Methodology

CD4 + T cells were isolated from human PBMC and CD4 + T cell lines specific for EBV are generated by repeated presentations of EBV peptides. Both the CD4 + and EBV specific CD4+ T cell lines, were cultured with increasing doses of tumoral exosomes. These exosomes are isolated from

xenotransplanted NPC in immunodefficient mice or from patient sera and purified on sucrose cushion. We performed (i) proliferation assays by measuring the 3H-thymidine incorporation (ii) cytotoxicity tests by measuring the NPC cells lysis and (iii) cytokine secretion by ELISA. The efficacy of peptides vaccination was assessed in an immunodefficient SCID mice model xenotransplanted with NPC cell lines (C666.1 -Luc), and reconstituted with human PBMC. The evolution of the tumor has been appreciated, both by measuring the tumor volume and the bioluminescence emitted by NPP-Luc cells. ELISPOT was used to study IFNγ secretion by NPC patient (at different disease stages) PBMCs ex-vivo following stimulation with the peptide cocktail.

III. Results:

First of all, we showed that exosomes obtained from NPC patients' sera have the proper form under electron microscopy. Moreover, we checked that they have the capacity to inhibit CD4+ and PBMCs proliferation. This is an indication of the potential inhibitory effect that exosomes could have on our vaccination approach.

We have previously selected 6 peptides derived from the 3 EBV latency II antigens, with a highly promiscuous capability of binding on HLA II molecules. We show here that all these peptides used in cocktail are recognized by human PBMC, even in a tumoral context, after pre-incubation with NPC cell lines, inducing a broad IFNγ cytokine secretion and proliferation of these PBMCs. Furthermore, peptide-specific CD4+ T cell lines have been generated, and their cytotoxic potential assessed by specifically lysing different EBV+ NPC cell lines, even in the presence of autologous exosomes or recombinant galectin-9. This indicates that the peptide induces proper activation of the CD4+ cell which became resistant to tumor induced suppression. Nevertheless, these cells remained sensitive to cyclosporine A suppressive effect.

We also report for the first time the ability of the cocktail to restrain tumoral growth in an original NPC xenotransplanted humanized SCID mice. The SCID following splenectomy were xenotransplanted with a luminescent human NPC tumor and reconstituted with human PBMCs. The mice also received 3 shots of the peptide cocktail in an adjuvant. Tumor growth was followed over the course of the treatment with manual and luminescent measurements. The peptide adjuvant treatment slowed the growth of the tumor significantly with respect to the control.

Finally, we confirmed the ability of the cocktail to reactivate NPC patients' memory T cells and drive a Th1 response. Patient PBMCs were cultured ex-vivo with the cocktail and the results showed reactivation of their memory cells evident by increase in IFNγ secretion studied by ELISPOT.

IV. Conclusion:

Our data clearly suggest that EBV latency II-derived peptides could be used as tumor vaccine for immunotherapy of nasopharyngeal carcinoma, mainly in refractory patients or to combat residual disease and prevent relapse in high risk patients after classical treatments.

Tumor-derived Exosomes do not Disturb a Novel EBV Peptide-based Immunotherapy for Nasopharyngeal Carcinoma

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Running Title: EBV-Peptide Immunotherapy in NPC

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Key Words: Exosomes, EBV, NPC, Immunotherapy, SCID mice, Patients

We declare that "The authors disclose no potential conflicts of interest."

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ABSTRACT

Background: Epstein-Barr virus (EBV) is constantly present in nasopharyngeal carcinoma (NPC). Despite the abundant presence of immunosuppressive NPC exosomes, we propose a peptide immunotherapy able to induce a $CD4^+$ Th1-response specific of EBV antigens

Methods: Human EBV-specific CD4+ T cell lines were generated from autologous $CD4^+$ T lymphocytes. The function of both cells was evaluated with NPC cell- and patient-derived exosomes. Immunotherapy was assessed in humanized NPC xenotransplanted SCID mice. NPC Patients' PBMC were challenged with EBV peptides.

Results: EBV peptides stimulation induced PBMC proliferation and IFNγ secretion after pre-incubation with NPC cells. EBV-specific $CD4^+$ T cell lines induced lysis of NPC cell lines, even in the presence of autologous exosomes. EBV peptides restrained *in vivo* tumor growth. All NPC patients' PBMC recognized the EBV peptides, eliciting a strong Th1 response.

Conclusion: EBV peptides could be used as tumor vaccine for immunotherapy of nasopharyngeal carcinoma, regardless the immunosuppressive properties of NPC exosomes.

STATEMENT OF SIGNIFICANCE

These results demonstrate that a single peptide cocktail can elicit a strong protective antitumor Th1 response able to resist to deleterious immunosuppressive microenvironment. Therefore, these findings have critical implications for the development of a new immunotherapeutic approach for NPC treatment.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a malignant epithelial tumor, consistently associated with the Epstein-Barr virus (EBV) (689). Although NPC is rare worldwide, it is endemic in several areas, particularly in Southeast Asia suggesting a multifactorial etiology involving EBV infection, genetic predisposition, the environment and other factors still unknown (690, 691). The regular detection of Epstein Barr virus (EBV) in the malignant cells of virtually all cases of NPC Type II and Type III (692) suggests that the virus is a determinant factor in oncogenesis.

EBV is a γ-herpes virus that causes asymptomatic life-long persistent infections in >90% of adults worldwide. EBV infects resting B cells in the oropharynx either directly or *via* epithelial cells (693). EBV is associated with several malignancies worldwide ranging from gastric (GC) and nasopharyngeal carcinoma (NPC), about 98,900 and 84,000 cases per year respectively, to Hodgkin (HL) and Burkitt lymphomas (BL) with about 28 000 and 7 000 cases each year respectively (694). All these malignancies can be distinguished according to the set of EBV latency antigens expressed within tumor cells. Among these genes, Epstein Barr Nuclear Antigen (EBNA)-1, Latency Membrane Protein (LMP)-1 and LMP-2, the hallmark of the EBV latency-2 program, are known to play a key role not only in EBV latency but also in EBV associated malignancies as they allow infected cells to transform, proliferate and resist apoptosis (695-697).

An important biologic feature of NPC is the presence of a massive lymphoid infiltrate in the primary tumor, which is likely favored by inflammatory cytokines produced by tumor cells (698-701). Several studies support the idea of a local immuno-suppressive environment inhibiting proper effector cell function even after correct homing. The presence of abnormal quantities of regulatory T cells (Treg) within the tumor site and the peripheral blood is a clear indication of this immune suppression (702). Moreover, we have recently reported that NPC Exosomes (NPC-Exo) have unique immunoregulatory properties. They induce Treg expansion and up-regulate their suppressive functions. Interactions of NPC-Exo with Treg represent a newly defined mechanism that might be involved in regulating peripheral tolerance by tumor cells and in supporting immune evasion of human NPC (291).

Recent findings confirm the consistent production of galectin-9 (LGALS9) by NPC cells (703), in association with exosomes (704). Gal-9 is a β-galactoside binding lectin, whose expression can be induced by different stimuli, such as pro-inflammatory cytokines (705) and probably EBV infection (706). The nature of Gal-9 receptors on the membrane of target cells is still controversial. HAVCR2, also known as Tim3 (T-cell immunoglobulin domain and mucin domain 3), might be one of them (350). Some of us, have reported the detection of NPC tumor-exosomes containing, LGALS9 in the plasma of NPC patients or mice xenotransplanted with NPC tumor lines. There is evidence that these LGALS9 positive tumor-exosomes can induce apoptosis of EBV-specific Th1 lymphocytes through LGALS9/ HAVCR2 interaction. These findings support the idea that NPC exosomes exert Th1 suppressive functions (707).

Radiotherapy is the traditional treatment of non-metastatic NPC, achieving local control in 50–90% of cases (708). Recently, on the basis of randomized trials confirmed by two meta-analysis, concurrent platinum-based chemo- radiotherapy became the new standard of care, especially in patients with T3–T4 primary tumors and/or lymph node involvement (N1–N2–N3) (670, 709). Despite improvements in therapeutic results, local regional failure and distant metastasis are still occurring in a significant number of patients. Moreover, radiotherapy and chemotherapy often result in both acute side effects and long-term sequelae, with the latter being even more severe in younger patients (710). Therefore, it seems necessary to develop novel approaches, with the aim of improving outcome for refractory disease and, eventually, deescalating? Conventional cytotoxic therapies. There is now compelling evidence that cytotoxic T lymphocytes- based immunotherapy is effective in another entity of EBV-linked malignancies, namely post-transplant lymphoproliferative disorders (PTLD) (711-713). Our team had recently developed a strategy which allows the definition and selection of $CD4^+$ “promiscuous” peptides (714), and their immunogenicity was tested in the EBV tumor model; Hodgkin Lymphoma (715).

These promising results encouraged us to develop similar strategies for other EBV-positive tumors, such as NPC. Hence, in this present study, we confirm the immunogenicity of the peptide cocktail in front of T cells pre-conditioned with NPC cell lines, and the ability of EBV-specific $CD4^+$ T cell lines to

lyse different NPC cell lines, even in the presence of autologous exosomes. We also report for the first time the ability of the cocktail to restrain tumor growth in an original humanized SCID (Severe Combined Immunodeficiency) mouse model. And finally, the capacity of the peptide cocktail to recall CD4+ memory T cell and elicit a strong Th1 response in a variety of NPC patients. All together our results strongly nominate our EBV peptide cocktail as a good vector for the development of immunotherapeutic strategies against NPC.

RESULTS

Phenotypic and functional characteristics of exosomes

The size of exosomes extracted from C15 culture (C15-Exo) was assessed by electron microscopy. Results indeed showed a homogenous diameter ranging between 30 and 60 nm (Fig. 1A). The protein content of C15-Exo was then analyzed by western blotting and compared to that of exosomes extracted from the sera of healthy donors Patients-exo (Fig. 1B). As expected, C15-Exo contained the oncoprotein EBV protein LMP-1, HLA-Drα, tetraspanin CD63 and Gal-9 whereas Grp94 was not detected (Fig. 1B).

The function of C15-Exo and Patient-Exo was assessed by their ability to inhibit the proliferation of either PBMC or $CD4^+$ T cells in *in-vitro* proliferation assays. Both exosome types showed inhibitory capacities. C15-Exo induced a significant suppression of the proliferation of both PBMC and CD4 T cells following 120 hours of incubation at concentrations of 1 and 10 μg/ml (Fig. 1C1 and 1C3). Whereas Patient-Exo induced a significant suppression of the proliferation of only CD4 T cells after 48 and 120 hours of culture at a concentration of 1μg/ml (only at 48 hours) and 10μg/ml (Fig. 1C2 and 1C4).

Peptide cocktail immunogenicity

It was essential to test the immunogenic capacity of the peptide cocktail in the tumoral context to mimic the immunosuppressive NPC microenvironment. To that end, we first tested the effect of peptide stimulation on the sensitivity of PBMC to the immunosuppressive effect of either exogenous Gal-9, C15-Exo or Patient-Exo *in-vitro*. PBMC were cultured for 6 days with or without peptide stimulation following which they were cultured in proliferative conditions with or without an immunosuppressive factor for 72 hours during which their proliferation was assessed. Surprisingly, the stimulated PBMC unlike non-stimulated PBMC showed a reduced sensitivity to the suppressive effect of gal-9, C15-Exo as well as Patient-Exo (Fig. 2A).

Furthermore, we tested the capacity of the peptide cocktail to induce the proliferation of pre-conditioned PBMC that had been previously co-cultured with C15 EBV-positive NPC tumor cell line for 5 days. The proliferation of these cells after the co-culture phase was assessed during a 72 or 144 hours of culture with or without peptide stimulation (Fig. 2B). The results clearly show that stimulation by the peptide cocktail induced a significant increase in the proliferation of both pre-conditioned PBMC and PBMC that had not had contact with NPC cells.

Moreover, the immunogenic potential of the cocktail was further demonstrated in the same tumoral context by studying its ability to induce IFN-γ secretion in pre-conditioned PBMC. ELISA analysis showed a significantly higher production of IFN-γ in cocktail stimulated pre-conditioned PBMC with an average of 35 and 22 pg/ml at 72 and 144 hours post C15 contact respectively than in non-

stimulated pre-conditioned PBMC which produced on average 5 pg/ml of IFN-γ at the same time points (Fig. 2C).

Peptide cocktail CD4+ T cell line cytolysis capacity

As a following step, we investigated the ability of generated EBV-specific CD4+ T cell lines (effector) to induce the lysis of various NPC cell lines (targets). A comparison of the induced lysis of 3 different EBV positive target NPC cell lines (C15, C666-1, C17) to an EBV negative NPC target (CNE1) at increasing Effector:Target (E:T) ratios revealed a ratio dependent induction of lysis which was more potent in the EBV positive cell lines than in the EBV negative one. Regarding the C15 cell line, compared to the CNE1 cell line, a significant or highly significant increase in induced lysis was observed at all tested E:T ratios; with this increase ranging from 10%; at a ratio of 0.312:1; to 30% at a ratio of 20: 1. Similarly with the C666-1 cell line, a significant or highly significant increase in induced lysis ranging from 15% to 40% was observed at effector to target ratios ranging from 1.25:1 to 20:1. Finally with the C17 cell line as well a significant increase in induced lysis was observed ranging from 17% to 45% between the ratios of 2.5:1 and 10:1 (Fig. 3A).

The ability of the generated EBV-specific CD4+ T cell lines to resist the immunosuppressive activity of NPC tumors was then assessed. To achieve this, we added either C15-Exo or recombinant Gal-9 to the cytotoxic assay of the generated cell lines (Effector) with C15 cells (Target) to observe any variation in specific lysis. Even though a significant decrease in specific lysis was observed upon the addition of 1μg/ml of Gal-9S at low E:T ratio (less than 2.5:1), there was basically no significant change with C15-Exo nor with Gal-9S at higher E:T ratios. (Fig 3B).

Effect of tumor micro-environment on EBV peptide cocktail CD4+ T cell-line

Next we studied the direct effect of C15 exosomes and recombinant Gal-9 on the proliferation of the EBV-specific CD4+ cell lines. Interestingly even in the presence of 10μg/ml of C15 exosomes or 1μg/ml of recombinant Gal-9, there was no significant change in proliferation neither at 48 nor 120 hours (Fig. 4A). Given the fact that the EBV-specific cell line was generated following a step of depletion of CD8+ cells, it was necessary to insure that the inability of C15 exosomes or Gal-9 to inhibit the cell lines was not due to this depletion step. To this end, PBMC depleted of CD8+ cells were chosen as a control and the impact of C15 exosomes on their proliferation was assayed. Unlike the cell lines, the results clearly show that in fact C15 exosomes could inhibit the proliferation of these CD8 depleted PBMC with an inhibition being highly significant at a concentration of 1μg/ml and 10μg/ml of C15 exosomes at 48 hours and only at a concentration of 10μg/ml at 120 hours (Fig. 4B).

Furthermore, resistance of the EBV-specific cell lines to Patient-Exo was confirmed by testing the effect of these exosomes on the proliferation of the cell lines. Even though the Patient-Exo were

shown to suppress the proliferation of PBMC (Fig 1C), they had no significant effect on the proliferation of the cell lines (Fig 4C).

Finally, to verify that the resistance of the EBV-specific $CD4^+$ T cell line to suppression was specific to exosomes, we studied the effect of Cyclosporine A (CsA), a highly potent immune-suppressor, on their proliferation. Interestingly CsA could inhibit the proliferation of the $CD4^+$ cell line. This significant or highly significant decrease in proliferation ranged between 60 to 70 % at both 48 and 120 hours at the tested concentrations 300, 500 and 1000 nM of CsA. (Figure 4D)

SCID-Hu-NPC mice model

Following the *in-vitro* experiments on primary cells we tested the effectiveness of the peptide cocktail in an *in-vivo* model. To that end, we created an original splenectomised SCID mouse model that has been xenotransplanted with C666-1-Luciferase cells and reconstituted with human PBMC. Mice reconstituted with PBMC from one donor were either non-treated, injected with just the adjuvant or a mix of the adjuvant with the peptide cocktail. A control group of non-reconstituted PBMC was done for every experiment. Tumor growth was followed over a period of 28 days by measuring the luminescence signal emitted by luciferase expressing tumor cells following luciferin injection as well as the actual size of the tumor obtained by manual measurements. (Fig. 5A).

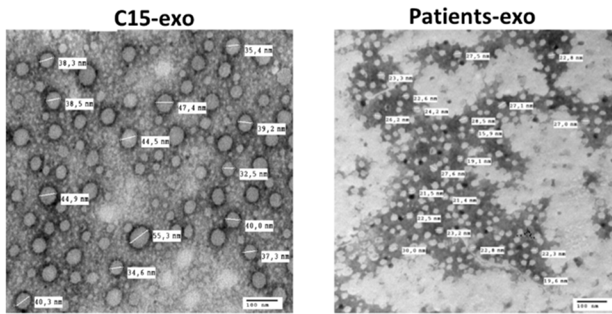
Comparing the four groups of mice we observed that mice only receiving tumor cells without being reconstituted with human immune cells demonstrated the fastest tumoral growth which was comparable to the group only receiving the adjuvant, as assessed by direct manual measurement (Fig. 5B) or by luminescent signal (Fig. 5C). Mice receiving PBMC had a reduced tumoral growth with respect to the control but it was still higher than the group receiving both PBMC and the cocktail. Results are further demonstrated in the figure 5D where images of the luminescent signal over mice, visibly indicate a reduction of tumor growth in mice under peptide treatment compared to the control ones.

Therapeutic potential of the EBV peptide cocktail for NPC

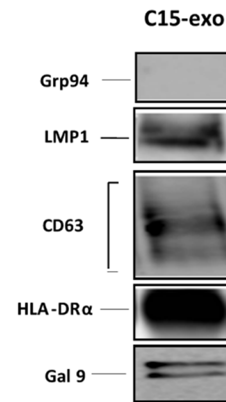
As a final step, we tested the immunogenic potential of the peptide cocktail on immune cells derived from NPC patients. PBMC were isolated from NPC patients at different stages of disease progression, either at diagnosis or under treatment with radio and chemo therapy (Table I). These cells were stimulated or not during 6 hours with the peptide cocktail after which, IFNγ secreting cells frequencies were determined using ELISPOT assay. A marked increase in IFNγ secretion was observed in peptide stimulated cells with all of the 10 donors. The response to the cocktail was more potent in cells obtained from patients at diagnosis ranging from 150 to 650 spot-forming cells per million (spc/10^6) of PBMC, whereas patients under treatment tend to reach 200 spc/10^6 PBMC. Type 2 patients seems to be less reactive than those from type 3 either for patients at diagnosis or under radio-chemo

therapy treatments, with a number of responding cells going from 25 to 350 spc/10^6 PBMC against a range from 150 spc to 650 spc/10^6 PBMC respectively.

A.



B.



C.

1)

Normalized values of proliferation
1.2
1
0.8
0.6
0.4
0.2
0
0 | 0,1 | 1 | 10 | 0 | 0,1 | 1 | 10
48h | 120h
PBMC + C15 Exo (µg/mL)

2)

Normalized values of proliferation
1.2
1
0.8
0.6
0.4
0.2
0
0 | 10 | 0 | 10
48h | 120h
PBMC + Patient-Exo (µg/mL)

3)

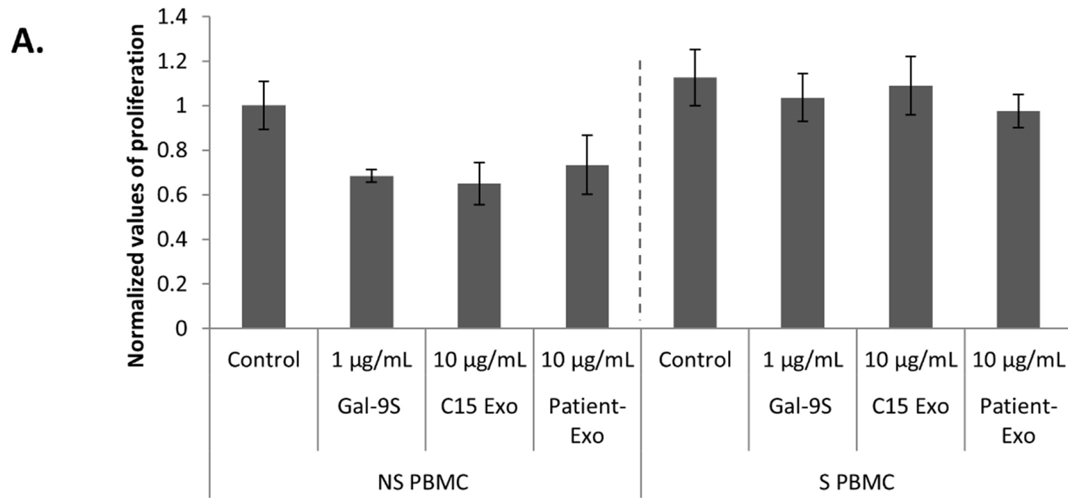
Normalized values of proliferation
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 | 0,01 | 0,1 | 1 | 10 | 0 | 0,01 | 0,1 | 1 | 10
48h | 120h
T CD4 + C15 Exo (µg/ml)

4)

Normalied values of proliferation
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 | 0,1 | 1 | 10 | 0 | 0,1 | 1 | 10
48h | 120h
TCD4 + Patient-Exo (µg/mL)

Figure 1

A.



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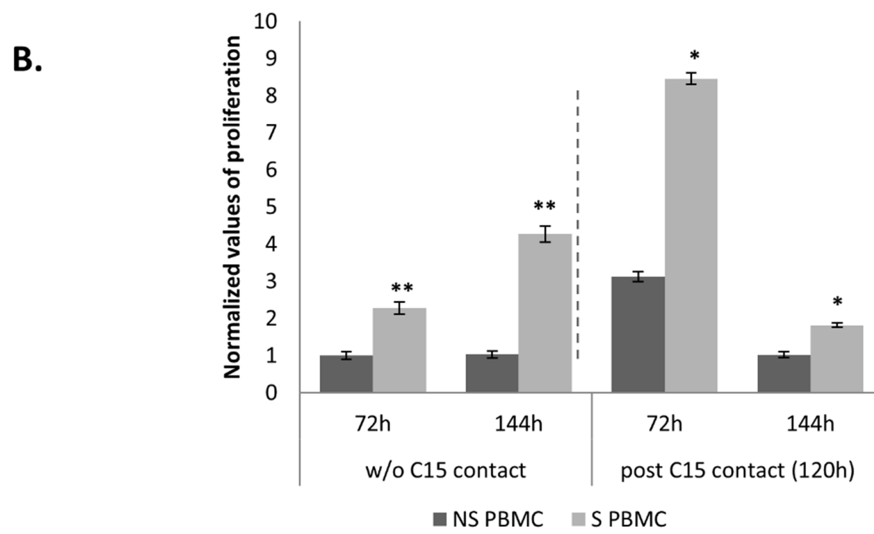


C.

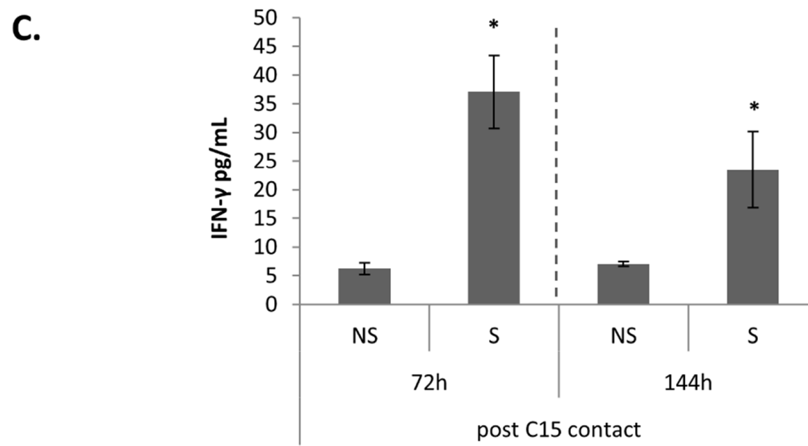
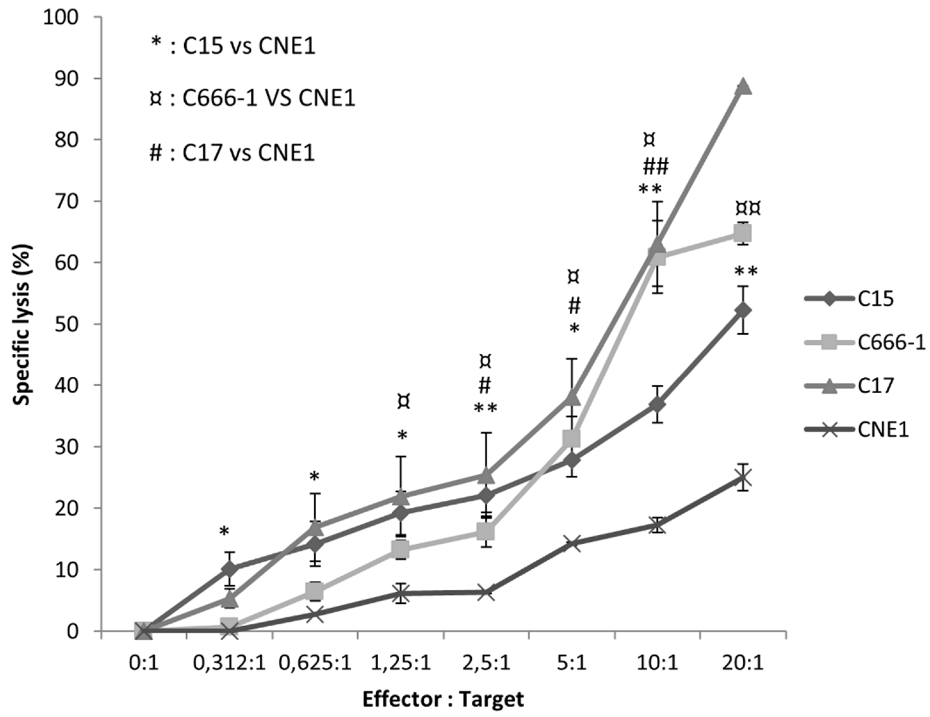


Figure 2

A.



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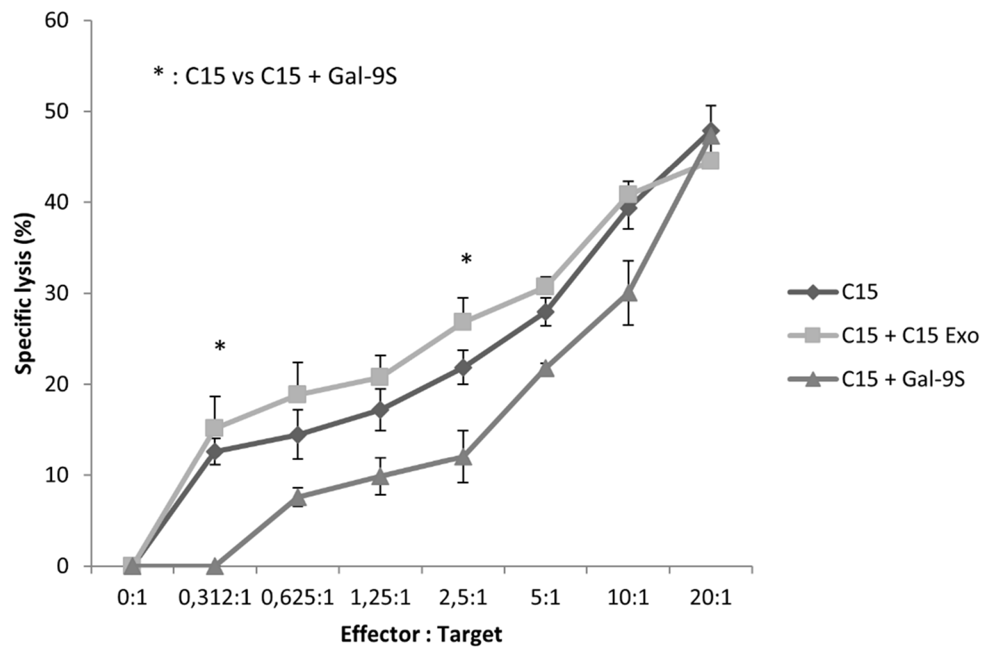


Figure 3

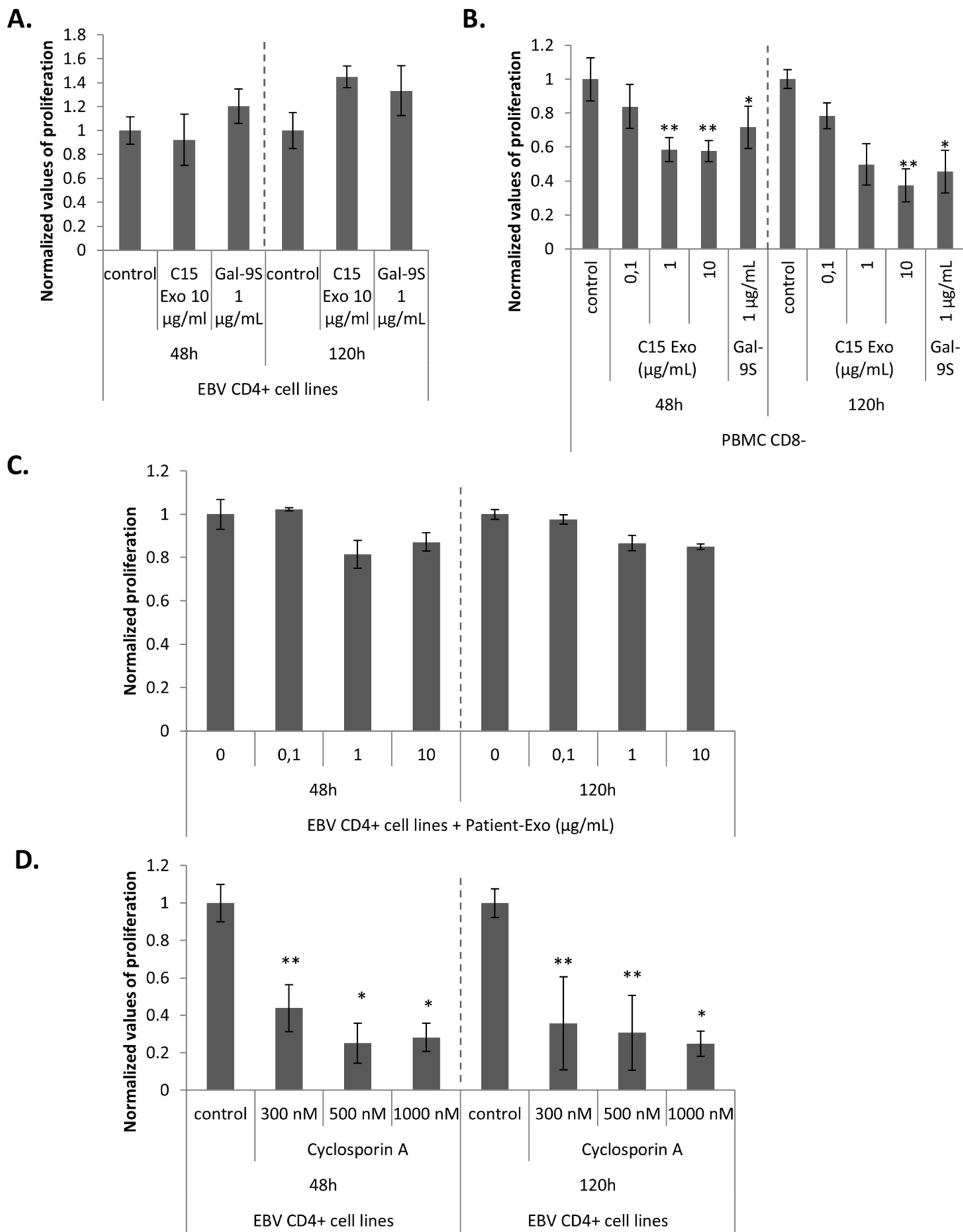


Figure 4

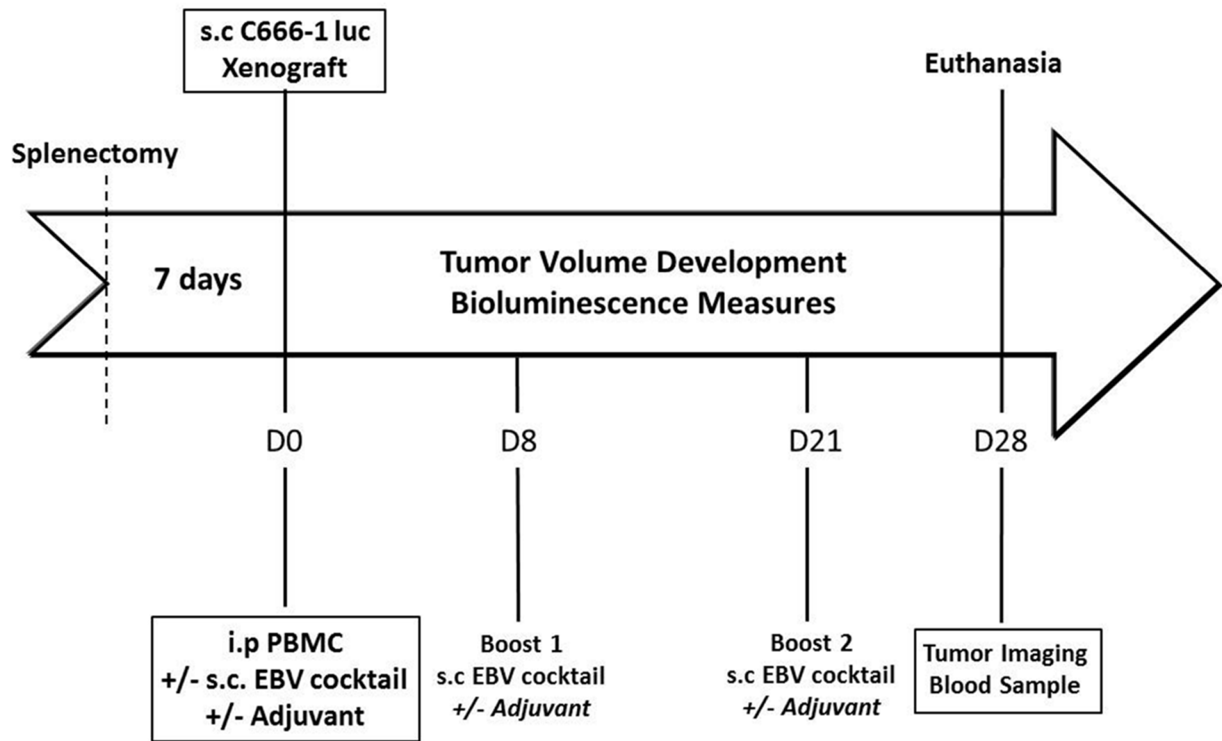
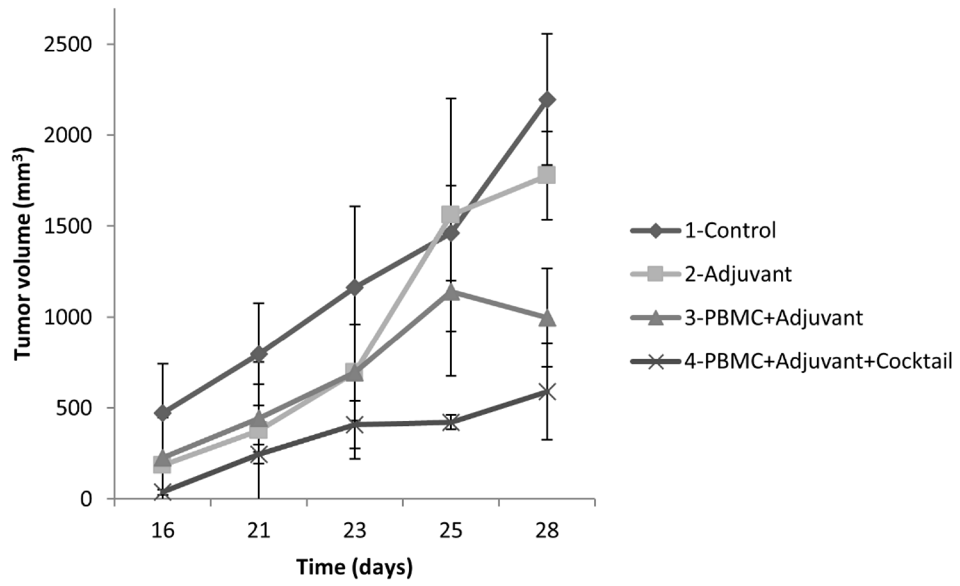


Figure 5-a

B.



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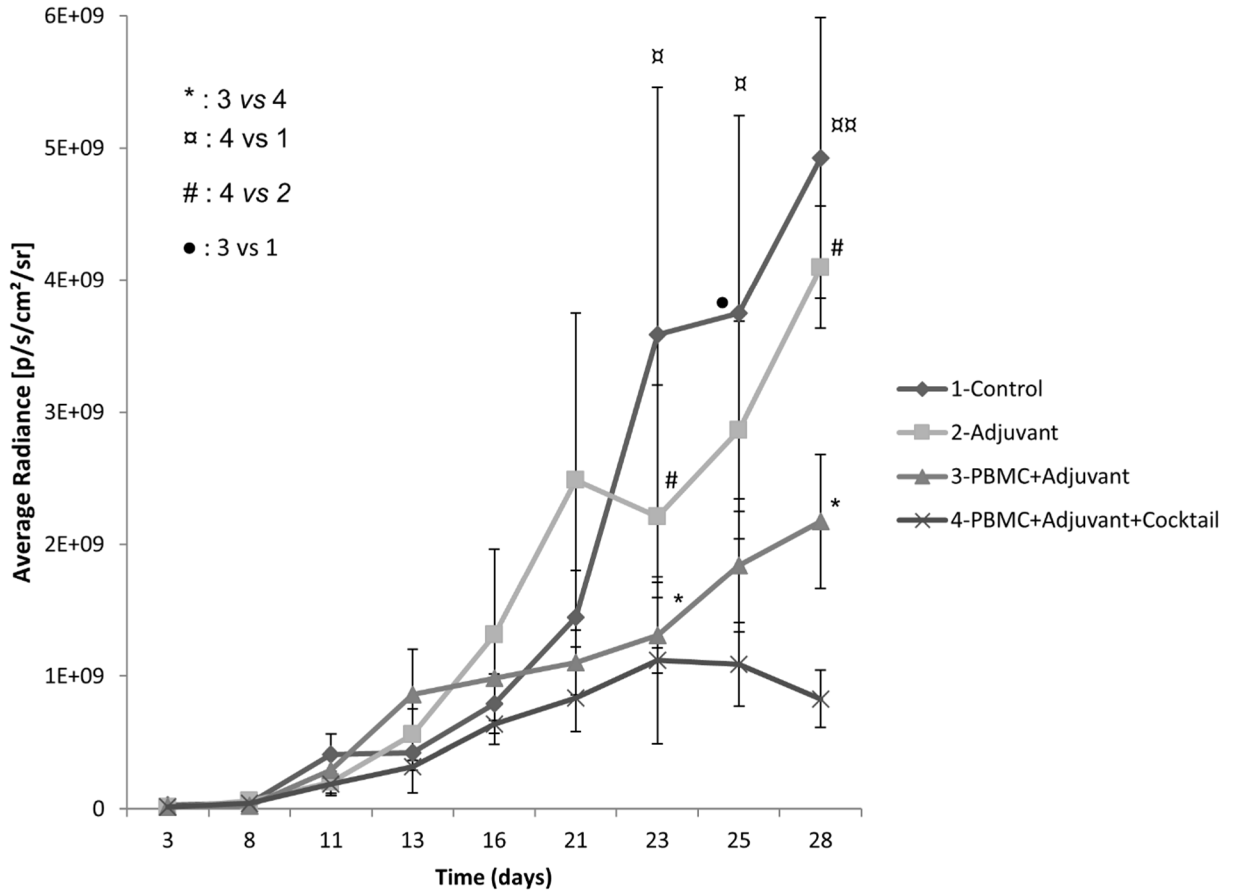


Figure 5-b

D.

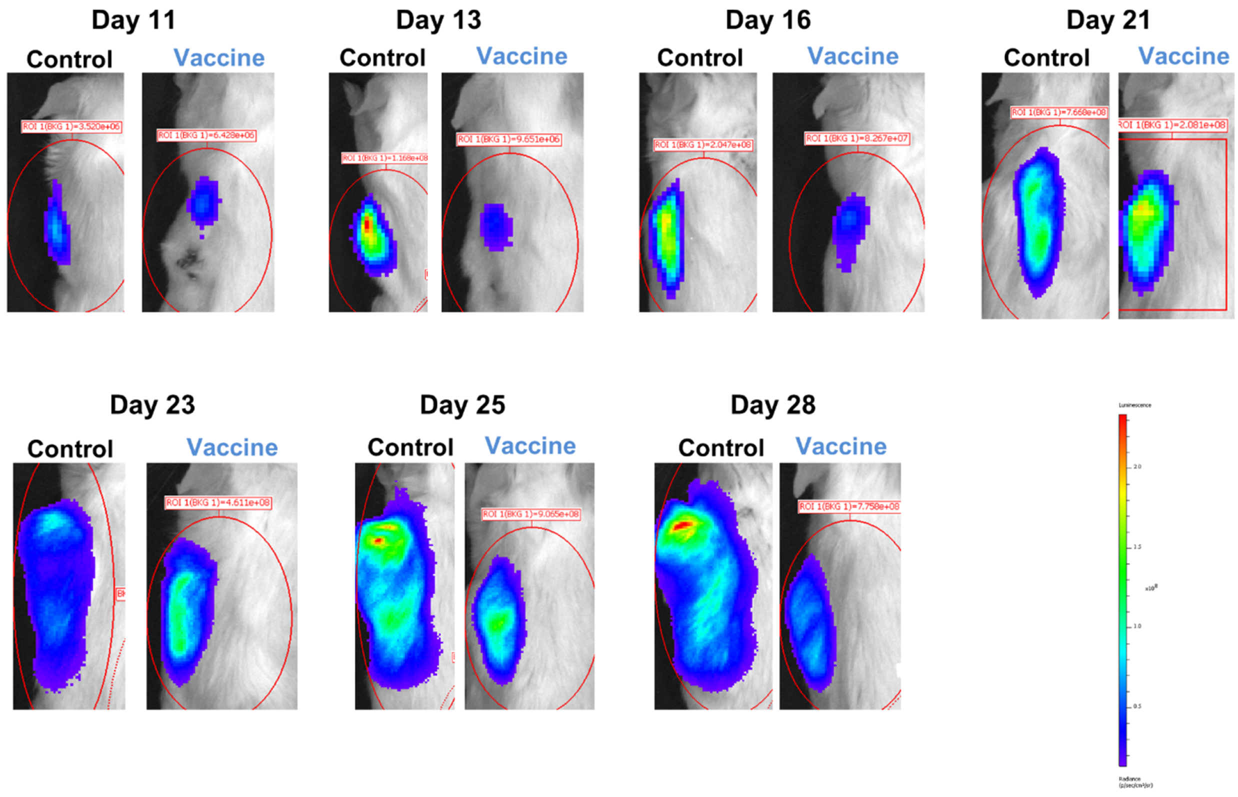
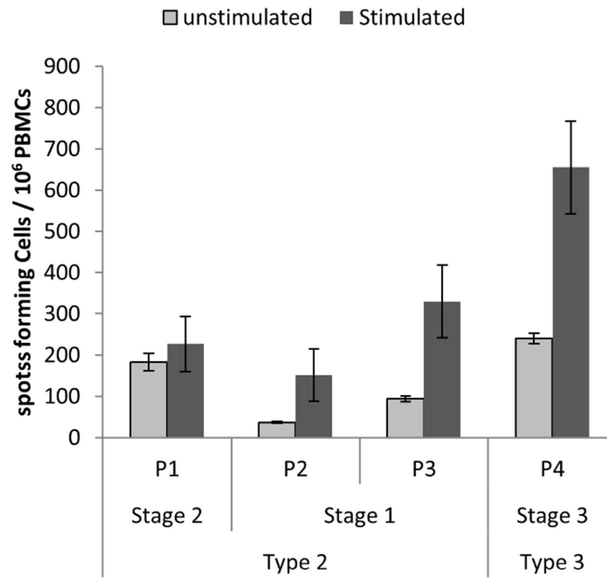


Figure 5-c

A.



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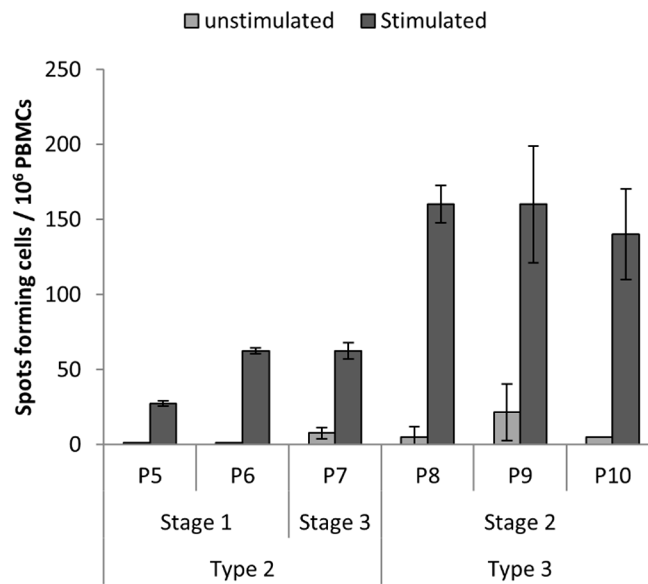
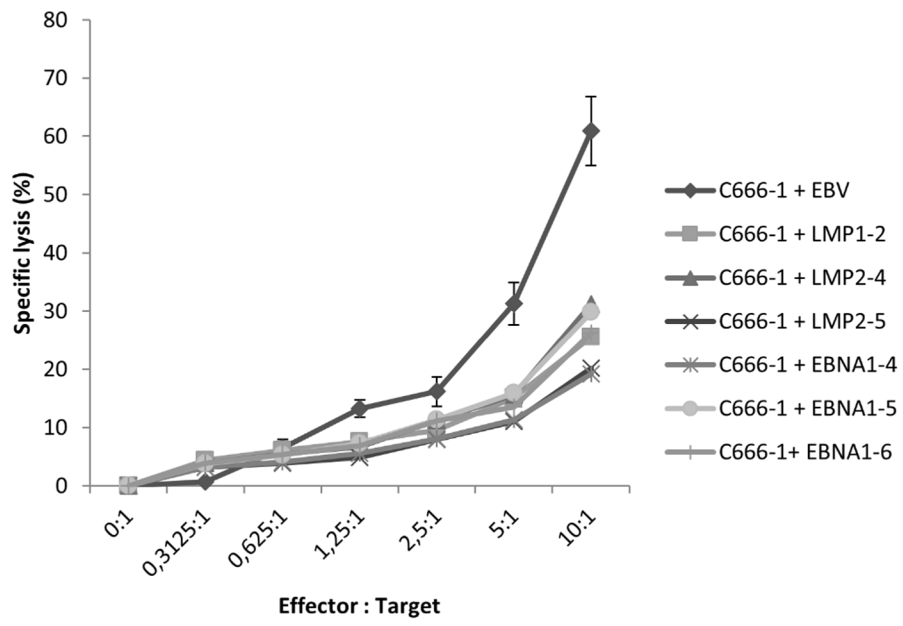


Figure 6

Table I

Patients	Sexe	Age	NPC type	NPC stage	Treatment	RT dose (Gy)
P1	M	53	2	T2	at diagnosis	-
P2	M	41	2	T1	at diagnosis	-
P3	M	35	2	T1	at diagnosis	-
P4	M	42	3	T3	at diagnosis	-
P5	F	45	2	T1	RT	50
P6	M	57	2	T1	RT	50
P7	M	60	2	T3	RT & CT	70
P8	M	48	3	T2	RT	50
P9	F	43	3	T2	RT	70
P10	M	59	3	T2	RT	70

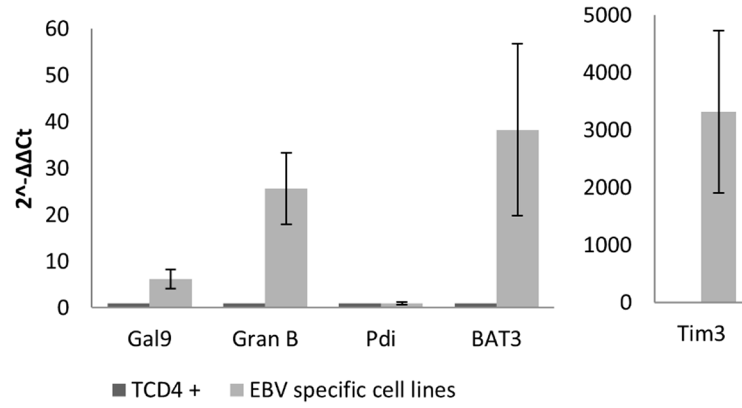
P= patient; M= male; F= female; RT = radiotherapy (5 sessions); CT = chemotherapy (Bleomycin, Epirumicin and Cisplatin)



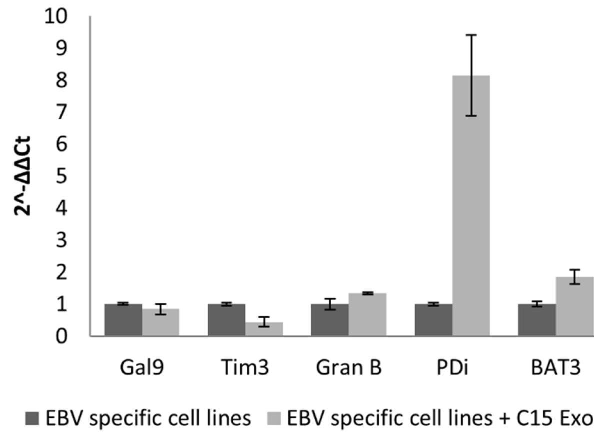
Supplementary Data 1

A.

1)



2)



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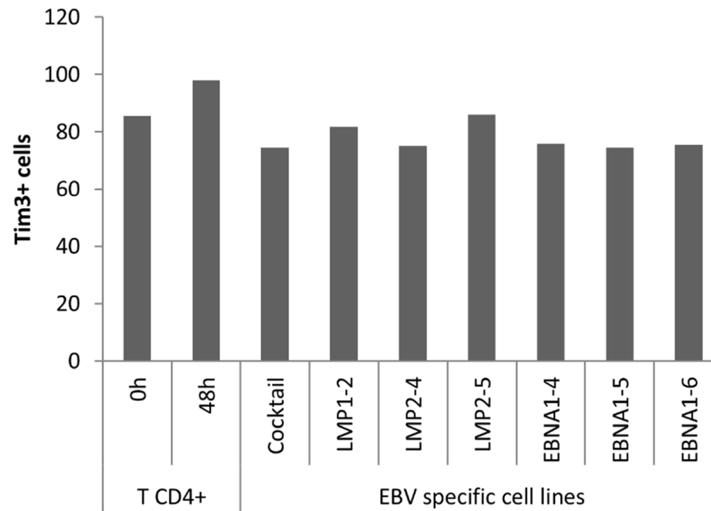

Supplementary Data 2

Table I: Nasopharyngeal carcinoma patient's characteristics.

Patients	**Sex**	**Age**	**NPC type**	**NPC stage**	**Treatment**	**RT dose (Gy)**
P1	M	53	2	T2	at diagnosis	-
P2	M	41	2	T1	at diagnosis	-
P3	M	35	2	T1	at diagnosis	-
P4	M	42	3	T3	at diagnosis	-
P5	F	45	2	T1	RT	50
P6	M	57	2	T1	RT	50
P7	M	60	2	T3	RT & CT	70
P8	M	48	3	T2	RT	50
P9	F	43	3	T2	RT	70
P10	M	59	3	T2	RT	70

P= patient; M= male; F= female; RT = radiotherapy (5 sessions); CT = chemotherapy (Bleomycin, Epirumicin and Cisplatin)

DISCUSSION

To date, more than 30 epitopes derived from the latency antigens have been identified (716). The $CD4^+$ T cell response to latency II antigens was less frequently described (717, 718), in part due to a lack of interest in this field which mainly focused on the significant $CD8^+$ T cells expansion during primary infection, the small size of the CD4 memory compartment, and obviously the paucity of defined $CD4^+$ EBV epitopes. In a previous work, we had developed original technical approaches to identify a promiscuous peptide cocktail within the $CD4^+$ T cell epitope, and proposed it as a potential vaccine against EBV latency II malignancies (715). Therefore, we chose to stimulate $CD4^+$ T cells with peptides derived from EBV latency II proteins LMP-1, LMP-2 and EBNA-1. The immunogenicity of these peptides was previously validated (i) in transgenic mice for the human HLA-DR1 (719), (ii) on isolated PBMC from healthy donors (720) and (iii) on isolated PBMC from HL patients (719). The objective pursued, here, is mainly to extend the use of the peptide cocktail to nasopharyngeal carcinoma; another EBV latency II associated malignancy, despite its highly immunosuppressive microenvironment.

Therapeutic strategies related to EBV latency type II associated malignancies, such as NPC, have been developed targeting mainly the activation of $CD8^+$ T cells as effector cells (721), or using polyclonal autologous or allogeneic $CD8^+$ T cells, specific to EBV or only LMP proteins. The results were relatively disappointing because they had led to the development of transient and non-specific partial responses. Unfortunately, even if a decrease of viral load was achieved, it was not accompanied by a significant clinical effect. This lack of clinical effect coupled with a lack of specificity makes these therapies ineffective against NPC. In this context, we proposed here to develop an immunotherapeutic strategy based on specific stimulation of $CD4^+$ T lymphocytes by EBV latency II derived peptides. Indeed, it's well described that the helper $CD4^+$ T lymphocytes play an important role in the establishment of the adaptive immune defense in EBV latency II malignancies. These cells are essential (i) during the process of isotype switching during antibody formation by B cells, (ii) they play a leading role in the activation and growth of the CD8 T cells, and (iii) they increase the activity of phagocytes such as macrophages. Thus $CD4^+$ T cells play a central role in activating the immune system. It is recognized that any immune system without proper activation and maturation of $CD4^+$ helper lymphocytes (Th1 cytokine secreting effectors) and memory T lymphocytes (against reinfection) is incomplete and ineffective. Furthermore, it is described that the activation of $CD4^+$ T cells may in some infectious conditions produce itself cytotoxic effects (722).

NPC is characterized by a specific immunosuppressive microenvironment associated with increased infiltration of $CD4^+$ $Foxp3^+$ $CD25^{high}$ natural regulatory T lymphocytes (nTreg), which can

represent up to 12 % of total tumor infiltrating lymphocytes (702). In addition, this environment is compounded by the massive presence of NPC tumor exosomes containing the Gal -9 – a specific ligand of death receptor Tim-3 expressed on activated CD4 Th1 (449) . Thus exosomes, by inhibiting both local and systemic anti-tumor immune response, are the main actors of this immunosuppression. In this context, these tumor exosomes may represent a potential barrier to the use of a peptide cocktail, aiming to develop a specific $CD4^+$ Th1 response, in the treatment of NPC. To investigate this hypothesis, we first purified and characterized tumor exosomes from xenotransplanted NPC tumors. As described, the isolated exosomes achieved the expected phenotypic characteristics, both in terms of size and expression of specific markers as they carry LMP-1, Gal-9 and CD63 (449). In addition, exosomes obtained were functionally active because they conserved their immunosuppressive properties (449). Moreover, the fact that these tumor exosomes are able to inhibit the proliferation of PBMC, $CD8^+$ depleted PBMC and isolated total $CD4^+$ T cells, validates the hypothesis that they may represent a potential barrier to the use of the peptide cocktail as a vaccine. In contrast, the induction of the $CD4^+$ T cells proliferation by exosomes from healthy donors is not surprising. Indeed, the work of L. Zitvogel shows that exosomes isolated from dendritic cells pulsed with synthetic peptides can activate immune cells suggesting that these non-tumor exosomes could have some therapeutic potential (723).

We first validated the immunogenicity of the EBV peptides in the context of NPC. Indeed, the results clearly showed an activation of the human PBMC by the cocktail, as evident by the induction of their proliferation and IFN-γ secretion, a main Th-1 and anti-tumoral cytokine. More importantly, we obtained similar results with PBMC pre-conditioned with NPC cell line, suggesting that we may recall a memory response. This response is very significant at 72 h incubation and remained at 144 h, although declining. This could be explained by the fact that it is an *in vitro* system and it is difficult to maintain long primary cells in culture, and also the activation induced cell death of stimulated T cells. This response is presumably more sustained *in vivo*, in a true physiological context. Moreover, the difference in proliferation obtained for each donor is related to individual variability. Thus, the results confirm and support the idea that the peptide cocktail is not only capable of activating CD4 isolated from HL (719), but can also activate PBMC previously sensitized by EBV^+ NPC cell lines, confirming also its ability to target latency 2 antigen commonly presented in both tumor cells.

The next step was to ensure that the EBV-specific $CD4^+$ T cell lines generated were able to lyse NPC tumor cells. So, we performed cytotoxic assays which allowed us to prove that each of the oligoclonal T cell lines were able to induce a cytotoxic lyses of NPC cell lines. We also observed that these T $CD4^+$ cell lines could induce a weak lysis of the EBV-negative CNE1 cell, but without exceeding more than 20% of lysis, which represented probably the basal cytotoxic activity generally observed within oligoclonal T cell lines enriched in $CD4^+$ T cells population in a heterologous system. Moreover, it

could also be explained by the EBV status of the CNE1 cell line which is still controversial as evidenced by Chan et al, who published that CNE1, usually described as EBV negative cells, could express LMP1 protein (724). Very interestingly, when we repeat the cytotoxic assays in the presence of exosomes isolated from two NPC cell lines (C15 and C666 -1), the presence of tumor exosomes does not seem to impair the ability of EBV-specific $CD4^{+}$ T cell lines to lyse their target. To investigate this unexpected result, we evaluated the direct impact of tumor exosomes on EBV-specific $CD4^{+}$ T cell lines, and the presence of tumor exosomes did seem to adversely affect neither the viability (data not shown) nor the proliferation of the EBV cell lines, while autologous $CD4^{+}$ T cells are very sensitive to the immunosuppressive effect of exosomes. In the same way, recombinant Gal- 9S had no significant effect on the specific EBV cell lines. However, we show that the EBV-specific cell lines are indeed sensitive to other immunosuppressants such as cyclosporine A. So, it would be interesting later to determine at the molecular level, how these lines which nevertheless express Tim3 are resistant to Gal-9 and exosome induced suppression. Indeed, we could suggest that sub-expression of Tim3 can explain this insensitivity to Gal-9^{+} exosomes. However, the preliminary results we obtained, by flow cytometry, showed that EBV-specific cell lines express the Tim3 marker at the same level (Data not shown) as conventional $CD4^{+}$ T cells. In addition, we observed, by quantitative real-time PCR, an up-regulation of the gene encoding *GRANZYME B* (*GZMB*) in EBV-specific lines (Supplementary Fig. 1A). Very interestingly, it has been recently reported that the over-expression of Perforin and Granzyme B in $CD8^{+}$ cytotoxic T cell promoted their resistance to Gal-9 (725). Obviously, this would need to be evaluated by further experiments. The molecular adaptor human leukocyte antigen B (HLA-B)-associated transcript 3 (*BAT3*) is able to bind to the intracellular tail of Tim-3, hampering its activity and protecting the cell from Tim-3 induced apoptosis. Here, *BAT3* is overexpressed in EBV specific cell lines and its expression increases after exposition to C15-Exo. Finally, in the presence of C15-Exo, *Pdi* (transcripts are over-expressed (Supplementary Fig. 1B) which in turn is able to retain Gal-9 at the membrane, avoiding any cell signal from its receptors (726).

After graduating *in vitro* evidence to support the use of peptides «vaccine» in the context of NPC despite the presence of immunosuppressive exosomes, we wanted to validate the in *vivo* efficacy of the cocktail as a direct peptide vaccine. Insofar as the C666-1 cells were used in the animal model of NPC, they were chosen to test whether the cocktail of EBV proteins as a whole was necessary to obtain the desired potency of CD4+ induced lysis or whether a specific protein of this cocktail would be sufficient. Seven CD4+ T cell lines (effectors) were produced for each specific EBV peptides. The results clearly show that at a ratio of 1.25 to 1 and above the EBV "whole" specific CD4+ cells were clearly more potent in inducing lysis of the NPC cell line than each of the single peptide specific CD4+ lines (Supplementary Fig. 2). The peptide cocktail as a whole was more effective than of the individual peptides in generating CD4+ T cell lines with higher capacity to induce specific lysis of the C666-1 cell lines

So, we evaluated the anti-tumor effect of vaccinating with EBV peptides in the C666-1 mice model by two approaches, the first was to manually measure the tumor volume and the second was to measure the bioluminescence emitted by the NPC tumor cells (C666.1-Luc). It is important to note that the mass of the mice was not affected by the various treatments whether it was PBMC reconstitution or vaccination (data not shown). Indeed, by analyzing the curves, a change in weight was observed over time, corresponding to the normal weight gain for a SCID mouse. In contrast, the direct measurement of tumor volume showed a significant difference between the average tumoral volume of the vaccinated and PBMC reconstituted group of mice, with respect to the average of the other groups, with an overall decrease of 50%. Thus, immune reconstitution coupled with vaccination allows the control of tumoral growth over time as tumor volumes remain well below the other groups. Interestingly, but not surprisingly, we also observed that the simple addition of PBMC in SCID-Hu-NPC mice can decrease tumor growth. This result is probably due to the induction and activation of an immune response in human PBMC against the NPC in this heterologous system. Note also, that to a lesser extent, the injection of EBV peptides coupled to the adjuvant seems to be favorable. One can imagine that this response is obtained by the presence of the adjuvant which could induce the activation of a residual natural killer cells (NK) response in the mouse, insofar as these SCID mice have functional NK (722), and that this adjuvant is known to favor Th1 anti-tumor effects of peptide vaccine (727).

Data from tumor bioluminescence analysis consolidated these previous results. We noted, in particular, in the group of reconstituted and vaccinated mice, a reproducible decrease in the bioluminescence. The data is different to the other groups, in which a steady increase in bioluminescence was observed. It should be noted that in the last days of the experiment, tumor necrosis was observed. Tumor necrosis may cause a dramatic decrease in bioluminescence. Moreover, as we have observed previously with direct measurements of the tumor, simply adding human PBMC or EBV peptides in the SCID-Hu-NPC mice can reduce tumoral growth. It would be interesting to complete this study by immunohistochemical and immunofluorescence analysis, in order to determine the presence of an inflammatory infiltrate in the human tumor.

In conclusion, taken together our results suggest that the six EBV latency II–derived peptides could be used in therapy for nasopharyngeal carcinoma. Indeed, the use of the peptide cocktail causes beneficial effects both *in vitro* (cell-specific lysis NPC) and especially *in vivo* (decreased bioluminescence and tumor volume) despite the presence of NPC-derived exosomes. The main focus of this vaccine is not to replace the protocols and therapies used in the primary tumors of NPC, but to act in conjunction with standard treatment protocols (728, 729). This peptides vaccine could be used in refractory patients to combat residual disease, and in patients with relapses, which is unfortunately very common and still fatal for NPC patients.

MATERIALS AND METHODS

Synthetic Peptides

As previously described (715), we used TEPITOPE, a T-cell epitope prediction software that allows the identification of HLA-DR-binding ligands (730, 731) to detect peptides containing HLA class II-restricted epitopes within the sequences of the EBV latency II antigens: EBNA1 (NCBI accession number NP_039875), LMP1 (CAA26023) and LMP2 (AAA45887). Peptides corresponding to the predicted sequences were synthesized using standard Fmoc chemistry by Neosystem (Strasbourg, France). Their purity was approximately 90% as indicated by analytical high-performance liquid chromatography. The synthesized peptides were submitted to HLA-DR and HLA-DP4 specific-binding assays, and separated out into active and inactive peptides. Six peptides were selected according to their highly promiscuous capacity (active for more than 7 HLA II molecules):1 derived from LMP1 ($LMP1_{68-83}$) was called *LMP1-2*, 2 derived from LMP2 ($LMP2_{224-244}$ and $LMP2_{372-388}$) were called *LMP2-4* and *LMP2-5,* and finally 3 derived from EBNA1 ($EBNA1_{475-500}$, $EBNA1_{514-539}$, $EBNA1_{526-552}$) were called *EBNA1-4, -5 and -6* respectively. These 6 peptides were used alone or combined in a peptide cocktail containing equal quantities of each peptide called *EBV-cocktail*.

Donors, Cell lines and Culture conditions

Donor's cells

Cells from healthy donors were isolated from whole blood bags provided by the "Etablissement Français du Sang – Nord de France (EFS)", in accordance with the official ethics agreement between the latter and the "Centre National de la Recherche Scientifique" (CNRS) – "Délégation Nord Pas-de-Calais et Picardie". The study was approved by the "Institut de Biologie de Lille", (CNRS) and EFS Institutional Review Boards Written informed consent was obtained from each donor.

Cells were isolated from NPC patient's blood provided by the Maillot Hospital (Algiers, Algeria), in accordance with the Institutional Review Board of the hospital. All these donors gave informed written consent before the sampling procedure according to the Huriet Law. Patients' information is summarized in table 1.

NPC Target cell-lines

The C15, C17 and C666-1-luc tumor cell lines are derived from EBV-positive xenotransplanted NPC and continuously subcutaneously propagated in SCID mice every 4-6 weeks. All animal procedures were performed by certified personnel, in accordance with the French and European regulations in the animal facility of the "Institut Pasteur de Lille" (France). The CNE1 tumor cell lines are derived from EBV-negative NPC and were kindly provided by Dr Pierre Busson. CNE1 cells were cultured in standard medium culture described below.

Cell culture conditions

RPMI 1640 medium (Invitrogen, Paisley, UK) supplemented with 10% human AB serum (BioWest, Nuaillé, France) for donor's cells or 10% fetal calf serum (GIBCO, Invitrogen) for NPC target cell lines, 2 mM L-Glutamine, 1 mM sodium pyruvate, 10 mM non-essential amino acids, 10 mM HEPES, 50 U/mL streptomycin, 50 µg/mL gentamycin and 50 µM β-mercaptoethanol was used as standard culture medium. Cells were incubated at 37°C under controlled atmosphere (5% CO2 and 95% humidity) in a Hera Cell 150 incubator (Thermo Electron, Cergy Pontoise, France). When appropriate, PBMC were activated using PHA (5µg/mL). For $CD4^+$ T cells activation, anti-CD3 antibody (1,5 µg/mL) (Clinisciences, Montrouge, France) and anti-CD28 antibody (100 ng/mL) (Clinisciences) were used.

Generation of EBV-cocktail or single peptide specific cell-lines

$CD4^+$ T-cell lines were generated according a protocol adapted from Voo (732). Briefly, CD8-depleted PBMC were stimulated *in-vitro* in the standard culture medium supplemented with 10% human AB serum at 2.10^5 cells per well in a flat-bottomed 96-well plate with or without the presence of either EBV-peptide cocktail or only one of the peptides at 60 µg/mL. On days 7 and 14, cells were stimulated again with autologous gamma-irradiated (5 000 rad) PBMC pulsed with the same peptide or EBV-cocktail. On day 8 and 15, 300 U/mL of Interleukin IL-2 (Peprotech) were added to the cultures. On day 19, all wells showing marked T-cell growth were tested for peptide-specific reactivity using autologous LCLs or autologous PBMC pulsed for 2 hours with the cognate peptide and/or the cocktail, or with a non-related one (20 µg/mL). The peptide-specific cell lines were considered positive as they released large amounts of interferon (IFN)-γ and low quantities of IL-10 after ELISA assay as described below, in a target dependent way.

Human Immune Cells Isolation

PBMC isolation

PBMC from healthy donors were isolated by standard density gradient centrifugation using Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden).

$CD4^+$ T cell isolation

$CD4^+$ T cells were isolated from PBMC using a negative selection protocol according to manufacturer's instructions (Miltenyi Biotec, Berlin, Germany). Flow cytometry analysis showed that more than 98% of the isolated cells are $CD4^+$ cells.

Flow Cytometry Analysis

Cell immunophenotype was analyzed by flow cytometry using FACSCalibur flow cytometer powered by CellQuest Pro software. Cell staining and data analysis was done as previously described (291). MAbs anti-human -CD4-flurescein isothiocyanate (FITC), -CD25-FITC (BD Pharmingen, San

Diego, CA), -CD25-PE (Miltenyi Biotech, Germany), -CD127-FITC or -CD127-PE (Clinisciences, Montrouge, France), -CD8-PE (Beckman Coulter, USA), -CD20-PE, -CD16-FITC and CD56-PE ((BD Pharmingen, San Diego, CA) were used for detection of cell surface antigens.

Cell Proliferation Assay

Proliferation was measured by [^{3}H] thymidine incorporation (PerkinElmer, Courtaboeuf, France). 10^5 cells (PBMC, CD8$^+$ depleted PBMC, CD4$^+$ T cells or EBV-specific CD4$^+$ T cell lines) were incubated in 96 well plates with [methyl-^{3}H]-thymidine (1 µCi/well) during the last 18 hours. of culture and harvested on fiber glass filter (Printed Filtermat A, Wallac, Turku, Finland) using Tomtec harvester (Wallac). The filter was then sealed in a sample bag after drying and addition of scintillation liquid (Beckman Coulter). Proliferation was measured by [^{3}H] thymidine (1 µCi/well) (PerkinElmer, Courtaboeuf, France) incorporation during. Radioactivity was determined using a β-counter (1450 Trilux, Wallac, Finland). Each proliferation assay was carried out in triplicate and estimated in count per minute (cpm) and results were normalized and compared to non-stimulated condition (NS). Some proliferation assays were also performed in the presence of the short form of recombinant Gal-9 (Gal-9S) at 1µg/mL kindly provided by Dr Toshiro Niki (Galpharma, Japan).

Cytokine Detection (ELISA)

Five hundred thousand PBMC were pre-incubated for 5 days with C15 cells and then cultured with or without EBV specific peptide cocktail for 72 or 144h in standard culture medium supplemented with 10% human AB serum, in a 48-well plate. Cells were activated Supernatants were tested for IFN-γ cytokine production by Enzyme-Linked ImmunoSorbent Assay (ELISA) as previously described (733). Results were expressed as the mean of duplicate wells after subtraction of the background.

Cytotoxicity Assays

Effector (E) EBV-specific CD4$^+$ T-cell lines as described above were added to different targets (T) NPC cell lines at known E:T ratios in a 6 hour assay; and in the presence or not of Gal9-S or C15-Exo. Cell lysis was assessed using CytoTox-Glo Cytotoxicity assay according to manufacturer's instructions (Promega, Madison, WI, USA). Results are expressed in percentage of specific lysis as means of triplicate wells.

Exosomes Isolation

EBV-positive NPC tumor cell lines (C15) were xenografted and permanently propagated by subcutaneous passage in SCID mice as previously described (449). In accordance with institutional guidelines, homozygous CB-17 scid/scid (SCID) mice derived from breeding stocks provided by J.P Decavel ("Institut Pasteur de Lille: IPL"), were housed under specific pathogen-free conditions at the animal facility of IPL (Lille, France). Exosomes from the culture media of C15 xenografts and exosomes

from NPC patients' sera were obtained as previously described (449). The exosomes were prepared and imaged as previously described (291).

Western Blot Analysis

Exosome western blots were done as previously described (291) primary antibodies used were : Grp94 1:1000 (ADI-SPA-850-F, Enzo Life Sciences), HLA-Drα 1:1000 (sc-53449, Santa Cruz Biotechnology), CD63 1:1000 (ab59479, Abcam, UK), LMP1 1:4 (S12 hybridoma), Galectin-9-CT-L1 1:100 (was kindly provided by Galpharma, Japan).

NPC Humanized SCID Mice Model

Anesthetized SCID mice were previously splenectomised and subcutaneously xenotransplanted (7 days later) with NPC tumors induced by C666-1-Luciferase cells (C666-1-Luc). The C666-1-Luc is an EBV-positive human nasopharyngeal cancer cell line expressing the EBV latency II proteins and the luciferase gene (734). This NPC cell line has been obtained from a subclone of xenotransplanted human tumor from South China. The protocol was approved by the local Ethical Committee of the IPL performed with required permission and approval of the regional governing ethical board (approval number CEEA 152010). At the time of xenotransplantation, mice were reconstituted or not with 20.10^6 PBMC isolated from healthy donor blood by an intraperitoneal injection. At the same time, the mice also received or not, a sub-cutaneous injection of 120 µg of EBV peptides (20µg of each peptide), with or without chemical Montanide adjuvant (ISA 720 VG Montanide TM, SEPPIC, France). Two recall vaccines were performed on days 8 and 21 The animals were sacrificed on day 28 for subsequent analysis.

Measurements and Data Analysis

Measurements of tumor bioluminescence were acquired at different times: day 1, 3, 8, 11, 15, 21, 23, 25 and 28 to monitor tumor growth, after i.p. injection of 100 µL of D-Luciferin (30 mg/mL, Perkin Elmer) into each mouse before analysis. The IVIS-Lumina XR® (Caliper, Life Sciences, MA, USA) was used to measure the bioluminescence. Images were then analyzed under the Living Image 4.1 software (Caliper Life Sciences). Moreover, the tumor length and width were measured with manual caliper at day 18, 23 25 and 28.The tumor volumes were then determined by considering that they have an elliptical form (X, Y and Z axis, with X = length/2, Y =Z = width/2), thus establishing the same value for Y and Z. The following formula was used: *Volume of the Tumor* = $4/3 * \pi * X * Y * Z$.

Interferon gamma (IFNγ) Enzyme-linked Immuno-spotAssay (ELISPOT)

We used a human IFNγ ELISPOT assay (Diaclone) to detect and qualify the response of 6 NPC patients PBMC after a 60 µg/mL peptide cocktail stimulation. Briefly, 2.10^5 PBMC were cultured in standard culture medium supplemented with 10% human AB serum in a 6-hour assay. Positive control of IFNγ consisted in activated PBMC using 1 ng/mL of phorbol 12-myristate 13-acetate (PMA) and 500 ng/mL of Ionomycin (Sigma-Aldrich, Saint Quentin, France). Spots were then revealed according to the manufacturer's instructions based on sandwich immune-enzyme technology. Plates were read in a specific plate reader (AID ELISPOT Robot HR powered by AID ELISPOT software 3.2.3, AID Germany) at the Institut Cochin (Paris, France). Experiments were realized in triplicate wells and are expressed in spot-forming cells (SFC).

Real-Time Quantitative PCR assays

Total RNA from $CD4^+$ T cells and EBV specific cell lines had been extracted using the TRIzol reagent (Invitrogen) method according to the manufacturer's instructions. RNA concentration and purity were measured by spectrophotometric methods (Ultrospec 3000, Pharmacia Biotec). Total RNA were stored at -80°C until further use. RT-qPCR was performed as previously described (15).

Statistical Analyses

SigmaStat software was used for data treatment and statistical analysis. All quoted *P*-values are two-sided, with $P< 0.01$ (*or # or ¤) and $P<0.001$ (**or ## or ¤¤) being considered statistically significant and highly significant respectively. Student t test was applied for all analyses; all statistical tests were two-sided.

Acknowledgments:

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LEGENDS OF FIGURE

Figure 1: Phenotypical and functional characterization of NPC derived-exosomes.

A. Electron microscopy of C15-Exo and Patients-Exo suspensions contrasted with 2% phosphotungstic acid (PTA). The diameter of some vesicles was measured. Scale bar represents 100 nm. The images are representative of 4 independent experiments. B. C15 exosomes were analyzed by Western blotting for HLA DR, CD63 and Grp94 as common markers and for LMP1 and galectin-9 as NPC-specific markers. C. PBMC and $CD4^+$ T cells were isolated from blood of healthy donors, then activated by anti-CD3 and anti-CD28 mAbs and incubated with increasing amounts of purified C15-Exo (1 and 3) and Patient-Exo (2 and 4). Proliferation was measured using [^{3}H]-thymidine incorporation assay during the last 18 hours of culture and values were obtained as counts per minute (cpm). Assays were performed after 48 and 120 h of culture. The results represent normalized cpm values of triplicate wells of 3 independent experiments from 3 different donors ± standard deviation (SD) bars.

Figure 2: Immunogenicity of EBV peptides on human PBMC

A. PBMC were isolated from blood of healthy donors, then activated by anti-CD3 and anti-CD28 mAbs and incubated with 1 µg/mL of Gal-9S, 10 µg/mL of C15 or Patients Exo, PBMC were either previously stimulated (right panel) or not (left panel) with 60 µg of EBV peptides (10 µg per each peptide) B. PBMC were co-cultured or not with C15 cells, then activated by anti-CD3 and anti-CD28 mAbs and stimulated with 60 µg of EBV peptides. Proliferation was measured using [^{3}H]-thymidine incorporation assay during the last 18 hours and values were obtained as counts per minute (cpm). Assays were performed after 72 and 144 h of culture. The results represent normalized cpm values, compared to the non-stimulated condition at 72h, of triplicate wells of 5 independent experiments from 5 different donors ± standard deviation (SD) bars. C. Time course of IFN-γ secretion. PBMC from 5 healthy EBV^+ adult donors were pre-cultured with NPC C15 cells and activated with EBV peptide cocktail at a concentration of 60 µg/ml in 25 cm^3 culture flasks (2.10^6 cells/ml). Culture Medium was

recovered after 72 and 144 hours of incubation and analyzed for the cytokine release. The values shown here represent the means of 5 separate experiments performed on PBMC extracted from 5 different donors.

Figure 3: Cytotoxic activity of the specific $CD4^+$ T cell lines against NPC cell targets

Specific cell lines can lyse EBV carrying targets. A. Assessment of cytolytic activity of EBV-specific $CD4^+$ T cell lines. Cell lysis was assessed using CytoTox-Glo Cytotoxicity assay. Assays were conducted over 6 hours on C15 cell lines co-cultured with 10 µg/mL of NPC derived exosomes (C15 Exo) or 1 µg/mL of Gal-9S. B. Assessment of cytolytic activity of EBV cocktail-specific $CD4^+$ T cell lines. Assays were conducted over 6 hours on EBV^+ NPC cell lines (C15, C666-1 and C17) and EBV^- cell lines (CNE1).

Figure 4: Impact of NPC-derived exosomes on EBV-specific $CD4^+$ T cell lines.

A. Proliferation assays on non-activated EBV-specific $CD4^+$ T cell lines co-cultured with 10 µg/mL of C15-derived exosomes or 1µg/mL of recombinant Gal-9S. B. Proliferation assays of $CD8^+$ depleted PBMC activated by anti-CD3 and anti-CD28 mAbs and incubated with increasing amounts of purified C15-derived exosomes. C. Proliferation assays on non-activated EBV-specific $CD4^+$ T cell lines co-cultured with increasing concentrations of Patients Exo. D. Proliferation assays of non-activated EBV-specific $CD4^+$T cell lines cultured with or without increasing amounts of CsA. The proliferation was measured using [^{3}H]thymidine incorporation assay during the last 18 hours and values were obtained as counts per minute (cpm). Assays were performed after 48 and 120 h of culture. Results are expressed in normalized cpm values of triplicate wells of 5 independent experiments ± standard deviation (SD) bars. Data represents the results of 5 independent experiments from 5 different donors. The cpm values were normalized and presented as relative proliferation ± standard deviation (SD) bars.

Figure 5: *In vivo* EBV peptides vaccination in a humanized SCID mice model of NPC

A. Schematic representation of the protocol used to establish the humanized NPC mouse model. four groups of C-666-1-Luc xenografted SCID mice were created: (i) A control group only xenotransplanted with C666-1-Luc (n=9); (ii) A group only receiving Montanide adjuvant (n=9); (iii) A group only receiving PBMC and Montanide and (iv) A group receiving both PBMC, EBV peptides and Montanide (n=9). B. *Measure of the tumor volume*. The tumor length and width were measured with manual caliper at days 16, 21, 23, 25 and 28. The tumor volumes were then calculated. The average tumoral volume from a maximum of 9 different mice for each condition at each time point was calculated and plotted. C. Tumor growth curves obtained by Bioluminescence measurements with the IVIS-Lumina XR® (Caliper, Life Sciences, MA, USA). Images were analyzed under the Living Image 4.1 software (Caliper Life Sciences) to quantify the intensity of bioluminescence. The average of these values for every measurement from a maximum of 9 different mice is represented over the course of 28 days. PBMCs of 3 different donors were used. 3 mice per condition were reconstituted with PBMCs from one donor. D. Representative images of bioluminescence emitted by the tumors in mice. The control (PBMC) and vaccinated (PBMC +Vaccine) groups are compared at days 11, 13, 16, 21, 23, 25 and 28. The images are representative of 3 independent experiments.

Figure 6: Immunogenicity of EBV peptides on NPC patients PBMC

A. IFN-γ Elispot assay. Freshly isolated peripheral blood mononuclear cells (PBMC) were isolated from 6 NPC-patients at diagnosis (1) or under treatment (2). PBMC were stimulated or not (control) with 60 µg/mL of peptide cocktail at 2.10^5 per well and the result is the mean of triplicate wells ± standard deviation (SD) bars.

Supplementary Figure 1: Transcriptomic analysis of EBV specific cell lines

A. Transcriptomic analysis of T $CD4^+$ and EBV specific cell lines by QPCR. Results were presented as relative expression 2^-ΔΔCt of *Gal9, Gran B, Pdi, BAT3*, and *Tim3* in EBV specific cell line with respect to T CD4+ cells.

B. Transcriptomic analysis of EBV specific cell lines with or without contact with C15-Exo by QPCR. Results were presented as relative expression 2^-ΔΔCt *of Gal9, Tim3, Gran B*, *Pdi* and *BAT3* in EBV specific cell line post CD15-Exo with respect to the EBC specific cell lines that had not had contact with C15-exo. Data for both graphs represents the mean of 3 independent experiments from cells for 3 donors.

Supplementary Figure 2: Cytolytic analysis of EBV specific cell lines

Assessment of *in vitro* cytolytic activity of EBV peptide cocktail or EBV single peptide-specific $CD4^+$ T cell lines on C666-1(EBV positive) cell lines. All assays were performed at different Effector-to-Target (E:T) ratios. The capacity of each $CD4^+$ T cell lines to kill EBV infected cell lines was evaluated by determining the leak of a cytoplasmic marker from lysed cells. Data shown here represent the means of 5 separate experiments performed on PBMC from 5 different donors. Results are represented in percentage of specific lysis.

Principle article: Evaluation of Galectin-9 blocking monoclonal antibodies as novel immune-checkpoint inhibitors *via* the targeting of regulatory T cells in cancer.

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I. Introduction

The immune system has the potential to recognize and eliminate cancer cells but is held back by certain inhibitory pathways. These pathways could induce direct inhibition of the immune response such as PD-L1/PD-1 pathway, or could recruit immune-suppressive cells to the tumoral microenvironment such as Regulatory T cells (Tregs). Tregs are T lymphocytes whose role is to inhibit the immune response in order to maintain immunological homeostasis. The Treg population is often upregulated in cancer patients where it promotes tumor development. Tumoral Tregs can either be natural or induced Tregs. nTregs of thymic origin are recruited by various paths into the tumor where they suppress the anti-tumoral immune response *via* their numerous inhibitory mechanisms. By contrast, iTregs are created from naïve T cells in the tumor due to the presence of the certain factors in the tumoral micro-environment. The role of Tregs in tumors is aggravated by their resistance to typically employed radio- and chemo-therapeutics. These tumor promoting functions have made Tregs an appealing target for immunotherapeutic approaches such as anti-CD25 which focused on inhibiting Treg surface molecules. However, current approaches against Tregs have proven unspecific and ineffective in improving the clinical outcome. A new approach in the field of cancer therapies is the targeting of specific immunosuppressive molecules and pathways by antibodies known as immune-checkpoint inhibitors, for example, anti-PD-1, is having great success in clinical trials. Gal-9 is a β-galactoside binding lectin with immunosuppressive capabilities expressed by both cancer cells and various types of immune cells including Tregs. The study of numerous viral infections, autoimmunity diseases, and inflammatory disorders has revealed a potential role for Gal-9 in Treg function and iTreg induction. At the same time, an increase in circulating Gal-9 levels in the serum of cancer patients has been correlated with a poor prognosis in multiple cancers including nasopharyngeal carcinoma (NPC). Finally, at the moment, there is very little research studying the function of Gal-9 in physiological conditions by its specific blocking with an antibody, most of the current data has been obtained from the use of recombinant Gal-9 in pathological conditions.

In this article, we first aimed to confirm the role of Gal-9 in human Treg-mediated-immuno-suppression. Then we tested the capabilities of an anti-human-Gal-9 antibody (mouse IgG1) developed by Pierre Busson's team to block Gal-9 suppressive function and the effect of this blocking on Treg function and subsequently the anti-tumoral immune response. Finally in-line with the development of a potential therapeutic agent for patient use, fully humanized anti-Gal-9 antibodies (human IgG1) were produced and their antagonistic capabilities were tested.

II. Results and methodology

Following Treg isolation from healthy donors, we have proven *via* QPCR, ELISA and flow cytometry that Gal-9 is expressed and secreted by Tregs at a higher level than conventional T cells. This expression was never found on the membrane but always intracellularly. The secretion of Gal-9 by Tconv was upregulated following TCR activation but it never attained the level of Tregs. The anti-Gal-9 antibody was able to inhibit the anti-proliferative function of recombinant rGal-9 on human PBMCs as test by multiple proliferation tests. Moreover, using this antagonist we were able to augment the Th1 response induced by TCR activated PBMCs by promoting the secretion of Th1 cytokines TNF-α and IFN-γ as well as decreasing that of TGFβ without inducing their hyper proliferation *in-vitro*. Furthermore, the anti-Gal-9 antibody significantly inhibited the immuno-suppressive function of Tregs in mixed leukocyte reaction proliferation tests. At the same time it prevented Gal-9 ability to promote iTreg. We also successfully used the anti-Gal-9 antibody to inhibit the suppressive function of hNPC derived exosomes. *In-vivo*, anti-Gal-9 antibody limited the growth of xeno-transplanted hNPC tumors in immuno-deficient mice that were previously reconstituted with human PBMCs. The success of our strategy and methodology was also determined *via* a breast cancer mouse model as a counter example. In this model as per the literature, the presence of Tregs controlled tumoral growth while the use of anti-Gal-9 restored it. Finally, we proved that the humanized anti-bodies could antagonize the anti-proliferative function of Gal-9 at an equal or slightly higher level than their mouse counterpart.

III. Conclusion

The current results demonstrate that Gal-9 targeting could be a viable and effective cancer immuno-therapeutic approach. The advantage of Gal-9 blocking *via* monoclonal antibodies is the fact that we are capable or reversing the immunosuppressed state observed in cancer patients *via* targeting of a secreted marker responsible for immune suppression instead of targeting directly the Treg population. Since Gal-9 is over expressed only in pathological inflammatory conditions, this could mean that the monoclonal antibodies would have minimal side effects on the patient.

Introduction

Regulatory T cells or Tregs are a subpopulation of T lymphocytes that play a crucial role in maintaining immunological homeostasis, preventing chronic inflammation and autoimmunity (735, 736). Several studies have confirmed the presence of elevated levels of Tregs in cancer patients both tumor infiltrating and circulating and have correlated these elevated levels with a poor prognosis (737-739). Several cancer immunotherapies affecting Tregs were then developed and some are currently in the clinical trials phase. Some studies aimed deplete Tregs (e.g. anti-CCR4) while others such as the immune-checkpoint inhibitor Ipilimumab (anti-CTLA4) and anti-PD-1 were proven to also target Tregs by yet unknown mechanisms (740). The current approaches which focus on depletion or inhibition of function have not been completely successful in all cancers due to their lack of specificity as all of the targeted pathways are shared to a certain extent with activated Tconv (741). These current approaches target cell surface molecules shared with other immune cells currently actively participating in the anti-tumor immune response. Galectin-9 (encoded by *Lgals9*) is a member of the β-galactoside-binding lectin family with multiple biological functions such as chemoattraction, cell aggregation, and apoptosis (742, 743). It is localized on the cell membrane, in the cytoplasm and nucleus (744). Galectin-9 can also be secreted to the extra-cellular environment *via* its carbohydrate binding activity (745). In vivo treatment with galectin-9 leads to the suppression of pro-inflammatory cytokines and an increase of Treg cells (395). Moreover, recent studies also indicate that administration of exogenous galectin-9 can regulate Th17 and Treg cell development (385, 394). Gal-9 secretion is upregulated in inflammatory conditions where it plays a role in the down regulation of the activated immune response by inducing apoptosis of activated Th1 T cells or cytotoxic CD8+ T cells. Gal-9 has also been proven to be secreted by different types of tumors and correlated with a poor prognosis of the patient due to its capability of inhibiting the anti-tumoral immune response (746). Galectin-9 has been identified as a ligand for Tim-3, binding of galectin-9 to Tim-3 causes an inhibitory signal resulting in apoptosis of Th1 cells (350). The Tim-3-Galectin-9 pathway has been shown to play an important role in mouse Tregs suppressive function since the use of Tim-3 blocking antibody resulted in a decrease in Treg suppressive function (747). More recently, galectin-9 has been shown to play a crucial role in the generation of induced mouse Tregs from CD4+CD25- T cells (Tconv) *via* binding to the CD44 receptor in the presence of TGF-β (425). Gal-9 has been also proven to interact with numerous other receptors indicating a crucial immunoregulatory role. Even though, the research on Gal-9 has immensely increased over the past year some crucial points are still lacking. First of all most of the research has taken place on murine Gal-9 in virus, autoimmunity or hyper sensitivity models. In fact, it has now been confirmed that mouse Gal-9 has significantly lower apoptotic ability than human Gal-9 (339). More importantly, till now no research has studied the function of human Gal-9 *via* its direct inhibition by specific blocking antibodies.

In this paper, we show expression of Gal-9 by human Tregs at the transcriptomic and the proteomic level. We continue to show that Gal-9 is constitutively secreted by Treg at a significantly higher level than Tconv which only increase their secretion following activation. Moreover, we prove

that the 1g3 an anti-Gal-9 antibody is has antagonistic potential capable of inhibiting Gal-9 induced suppression of proliferation. Using this antagonist we were able to augment the Th1 response induced by TCR activated PBMCs without inducing their hyper proliferation *in-vitro*. We were able to use this antibody to inhibit nTreg induced suppression *in-vitro* as well as iTreg induction both tested by *in-vitro* suppression test (MLR). Furthermore, knowing the importance of Gal-9 and Tregs in NPC pathogenesis we decided to test the anti-Gal-9 antibody as an anti-cancer immunotherapeutic. First we showed that anti-Gal-9 inhibited the suppressive effect of NPC-Exo on TCR activated PBMC and Tconv proliferation. Then we moved to an NPC humanized mouse model in which we showed the potential of the antibody to enhance the anti-tumoral immune response by inhibiting the suppressive function of Tregs leading to controlled tumoral growth. Finally we used a breast cancer mouse model as a counter example. In this model as per the literature, the presence of Tregs controlled tumoral growth while the use of anti-Gal-9 restored it.

Materials and methods

Isolation of T-Cell Subsets

Human blood samples were collected from healthy adult donors with informed consent obtained in accordance with the approval of the Institutional Review Board at the "Institut de Biologie de Lille". Mononuclear cells (PBMCs) were isolated from peripheral blood samples by density gradient centrifugation using Ficoll (GE healthcare, Uppsala, Sweden). Tregs (CD4+CD25+) and Tconv (CD4+CD25-) were isolated from PBMC using Treg isolation kits (Miltenyi Biotech, Germany) according to manufacturer's instructions. Obtained purity was over 95%.

Anti-Galectin-9 antibodies

Galectin 9 blocking antibodies: Mouse anti-human Galectin-9 blocking antibody 1g3 (748) kindly provided by "Pierre Busson institute Institut Gustave Roussy" was used for all the cell culture and proliferation experiments with or without exosomes or recombinant human Galectin-9, the Treg suppressive assays, and the NPC humanized SCID mice model. LEAF purified mouse IgG1, kappa. Isotype control (401405 Biolegend U.S.) was used as a control. Unless indicated otherwise, the anti-body were used at 3 µg/ml. Flow cytometry antibody: APC anti-human Galectin-9 Antibody (348907) was used for cell surface staining with the appropriate isotypic controls APC Mouse IgG1, κ Isotype Ctrl (FC) Antibody (400122) for membrane staining isotypic control and APC Mouse IgG1, κ Isotype Ctrl (ICFC) Antibody (400142) for intra-cellular staining (Biolegend U.S). Western blot and Immunofluorescence: mouse anti-human Galectin-9-CT-L1 mAb 1:100 9CT (was kindly provided by Galpharma, Japan)

Exosome Isolation

Patient-derived EBV-positive xenografted tumors (C15) were permanently propagated by subcutaneous passage in nude or SCID mice as previously described ((449)). In accordance with institutional guidelines, homozygous CB-17 scid/scid (SCID) mice were purchased from animal facility of Institut Pasteur de Lille (IPL) (Lille, France). Animals were housed under specific pathogen-free conditions at the animal facility of the IPL.

C15 tumor exosomes were isolated from in vitro conditioned culture media. Conditioned culture media were prepared by collagenase dispersion of cells from the C15 xenografts and incubation of these cells for 48 hours in low serum conditions (allowing collection of C15-exo). Exosome isolation from C15 xenograft culture media (C15-Exo) was done by differential centrifugation and flotation on a D20/sucrose cushion, as previously reported (749). Exosomes from healthy donors (HD-Exo) were obtained from healthy donor plasma.

Western Blot Analysis

Different cell subsets were lysed (10 minutes on ice) in PY buffer consisting of 20mM Tris-HCl, 50mM NaCl, 5mM EDTA, 1% Triton X-100, 0.02% sodium azide, and a cocktail of proteases inhibitors (Roche, Basel, Switzerland). After centrifugation (20000g, 15 minutes, +4°C), cell debris were removed and supernatants were collected. Protein concentrations were measured using Bio-Rad Protein Assay according to manufacturer's instructions (Bio-Rad, Marnes la Coquette, France). Total cell extracts were then analyzed by western blotting.

Briefly, proteins were separated by SDS PAGE electrophoresis using gradient pre casts gels (4 12% gradient, Bis Tris, Invitrogen) in standard conditions. Then proteins were transferred on nitrocellulose membranes (Hybon dTM-C Extra, Amersham Biosciences, UK). The latter was blocked for 1 hour at room temperature in blocking buffer containing 0.2% AuroraTM blocking reagent (MP Biomedicals, Illkirch Graffenstaden, France), 0.1% Tween20 (Sigma Aldrich) and PBS (1X), and incubated overnight at 4°C with primary antibodies directed against: mouse anti-human Galectin-9-CT-L1 mAb 1:100 (was kindly provided by Galpharma, Japan). Human recombinant Galectin-9 for the small Gal-S or the medium isoform Gal-M (kindly provided by Galpharma, Japan).

Membranes were washed with blocking buffer, then incubated for 1 hour at room temperature with peroxydase conjugated secondary antibodies (anti mouse or anti-rat or anti rabbit, 1:10000) (GE Healthcare, Wauwatosa, USA) and washed again with blocking buffer. Specific protein signals were visualized using SuperSignal™ West Dura Extended Duration Substrate (34076 Thermo Fisher Scientific France)

Cell proliferation assays.

Proliferation was measured after [3H]thymidine (1µCi/well) (PerkinElmer, Courtaboeuf, France) incubation for the last 18 hours before harvesting. Radioactivity was determined using a β-counter (1450 Trilux, Wallac, Finland). PBMC cultures and PBMC/Treg co-cultures were done in RPMI 1640 Medium (21875-034) supplemented with sodium pyruvate 1mM (11360-039), MEM Non-Essential Amino Acids Solution 1x (11140-035), HEPES 25mM (15630-056), 2-Mercaptoethanol 50µM (31350-010), gentamicin 10µg/ml (15710-049) (34076) Thermo Fisher Scientific France) and 10% fetal calf serum (GIBCO, Invitrogen).CD25- cultures and CD25-/Treg co-cultures were done in AIM V® Serum Free Medium (12055091 Thermo Fisher Scientific France) PBMC or CD25- cells were cultured at 100 thousand cells per 100 µl of medium in round bottom 96-well plates (353077 Corning France) with or without Gal-S (2 µg/mL), 1g3 or IgG1 (3 µg/mL) for the proliferation tests. Each proliferation assay was carried out in triplicates and estimated in count per minute (cpm), and results were normalized compared with non-treated condition.

Mixed leukocyte reactions

Suppressive activity of Treg was measured by their ability to inhibit the proliferative response of autologous PBMCs in a MLR. Either PBMCs or CD25- cells (100 thousand) were co-cultured with autologous Tregs (50 thousand) with or without 1g3 or IgG1 (3 µg/mL) for 72 hours in round bottom 96-well plates or 24 flat bottom well plates. Cells were activated with plate-bound anti-CD3 (1 µg/mL) mAb, incubated at 37°C 2 hours before the culture and soluble Anti-Human CD28 Functional Grade Purified (1 µg/mL) mAb (16-0289-81 eBioscience USA) added at the time of the culture. When used, 1g3 or IgG1 was pre-cultured with Tregs in the necessary medium for 2 hours before adding the Treg antibody mix to the culture without a washing step

Toxicity Tests

Toxicity tests for the effects of 1g3 and IgG1 antibodies on PBMC and CD25- cells were achieved by CyQUANT® Direct Cell Proliferation Assay (C35011 Thermo Fisher Scientific France) and CellTiter-Glo® Luminescent Cell Viability Assay (G7571 Promega, Madison, WI, USA) according to manufacturer's instruction.
Briefly proliferation of cells cultured with or without the antibodies or Gal-9 96 Well Flat Clear Bottom White Polystyrene TC-Treated Microplates (3610 Corning US) was measured by CyQUANT assay which is based on a cell-permeant fluorescent DNA-binding dye in combination with a background suppression dye reagent. The masking dye blocks staining of dead cells or cells with compromised cell membranes resulting in only healthy cells being stained. Both components were mixed in PBS at usable concentration just before being added to the wells. Following a 1 hour incubation at 37°C. The plates were read at an excitation wavelength of 485nm and emission wavelength of 520nm by FLUOstar Optima microplate bottom reader (BMG LABTECH France). Data was presented as normalized index of proliferation.
Regarding the viability assay, briefly the amount of ATP produced by cells cultured with or without the antibodies or Gal-9 was determined by adding a single CellTiter-Glo reactive which induces lysis of cells and at the same time contains a thermostable luciferase which produces a luminescent signal proportional to the quantity of ATP present in the culture. This signal was measured by Centro LB 960 plate luminometer (Berthold, Thoiry, France) powered by MikroWin 2000 Software. Results were normalized for every donor and presented as relative percentage of viable cells.

Real-Time Quantitative Polymerase Chain Reaction Assays

RNA was isolated using the RNeasy minikit II (Qiagen) according to the manufacturer's instructions. RNA concentration and purity were measured by spectrophotometric methods (Ultrospec 3000, Pharmacia Biotec). Total RNA were stored at -80°C until further use.

mRNA Reverse Transcription

2 µg of total RNA were supplemented with 5 µL of a master mix of 1 µL oligo dT (Roche Diagnostic, Meylan, France) and 0.1 µL RNAsin (40 U/µL, Promega, Charbonnières, France) and then incubated at 70°C for 5 to 10 min. After 5 minutes at room temperature, 10 µL of the following mix were added: 6 µL buffer 5X (Invitrogen) + 1 µL DTT 0.1 M (Invitrogen) + 2 µL dNTPs 10 mM (Amersham) + 0.1 µL RNAsin 40 U/µL (Promega) + 1 µL Superscript (Invitrogen). The reaction was then followed by an initial incubation step at 45°C for 45 to 60 minutes, a second incubation at 95°C for 5 minutes and treatment for 20 minutes at 37°C with RNase H (Promega). Finally, ultrapure distilled water (GIBCO-Life Technologies) was added to obtain a final concentration of 10 ng total DNA/µL and stored at -20°C until further use.

Mx3005PTM Sequence Detection System

Transcripts were quantified using real-time quantitative RT-PCR with the Mx3005PTM sequence detection system (Agilent technologies, France), in optical 96 well reaction plates (Eurogentec S. A., Belgium). In each well, 10 µL of a specific couple of primers, designed for real-time PCR and purchased from MWG-Biotech (Germany), were deposed at a final concentration of 10 pg/mL and then stored at -20°C. The housekeeping genes (HKG) β-actin, Glyceraldehyde-3-Phosphate DeHydrogenase (G3PDH), ubiquitin and Hypoxanthine guanine PhosphoRibosyl Transferase (HPRT) were used as controls in each plate. PCR reactions were performed according to the manufacturer's instructions, in a final volume of 20 µL, using 2X MESA GREEN qPCR MasterMix Plus for SYBR® 258 Assay (Eurogentech) containing Meteor Taq DNA polymerase, MgCl2 (4 mM final concentration), dNTPs (including dUTP), SYBR® 260 Green I, stabilizers and passive references required for signal normalization and optimized buffer components, for 1 µL of cDNA (equivalent to 10 ng total RNA/µL). The PCR program included initial denaturation and Meteor Taq activation for 5 minutes at 95°C, followed by 40 standard amplification cycles as follows: 15 seconds at 95°C (denaturation), 1 minute at 60°C (annealing and elongation). Fluorescent products were detected at the last step of each cycle. A melting curve analysis was carried out immediately after amplification, in accordance with the manufacturer's instructions.

Data Expression

Quantitative PCR reactions were used to quantify gene expression of Galectin-9. The housekeeping genes: b-actin, G3PDH, ubiquitin and HPRT were used as controls. All primers were designed for real-time PCR and purchased from MWG-Biotech (Germany).Quantitative analysis was achieved based on the cycle threshold (CT) value for each well and calculated using MxPro software.

Each individual value was normalized using the mean of the 4 HKG according to the standard ΔC_T method (733): $\Delta C_T = C_T - C_{THKG}$. For comparisons between groups, the relative gene expression was expressed in $2^{-\Delta\Delta C_T}$ leading to arbitrary value of 1 for the reference group.

Immunofluorescence

700 thousand cells (CD25- or Tregs) were treated with human FcR blocking reagent for 15 minutes at room temperature (130-059-901 Miltenyi Biotech, Germany) and then fixed with Paraformaldehyde solution 4% in PBS (Sc-271692 Santa Cruz Biotechnology U.S.A) before being plated on slides by centrifugation (Cytospin 4 ThermoShandon, France). Cells were then washed 3 times with PBS (20012-019 Thermo Fisher Scientific France) and permeabilized with 0.5% Triton X-100 (T8787 Sigma France) in PBS. Following 3 wash steps, blocking was done with PBS, bovine serum albumin 2% (A7906 Sigma France) 0.3% Triton X-100. Both primary and secondary antibodies incubations were carried out in PBS containing 0.5% BSA, 2 hours at room temperature. Primary antibody was 9CT used at 4 µg/ml and secondary antibody was a Goat anti-Rabbit IgG (H+L) coupled to Alexa Fluor® 555 used at 8 µg/ml (A21429 Thermo Fisher Scientific France). Nucleus was stained with 4'6'-diamidino-2-phenylindole (Sigma Aldrich, USA) for 5 min at room temperature. Slide and blade assembly was done with Mowiol® 4-88 (81381 Sigma Aldrich, USA) and the slides were kept in the dark at 4°C. A control was done without primary Ab to eliminate background noise due to nonspecific bindings of the secondary Ab. Slide analysis was done under Zen software (Carl Zeiss, France) *via* confocal microscopy LSM780 (Carl Zeiss)

Flow Cytometric Analysis

Cell immunophenotype was analyzed by flow cytometry using BD FACSCANTO II flow cytometer powered by BD FACSDiva™ (BD Biosciences, France). Data were analyzed with FlowJo software (Flowjo, Ashland, USA). Results were expressed as delta median of fluorescence intensity (ΔMFI=MFI antibody-MFI isotypic control) with representative overlays of dot blots or histograms. Dead cell exclusion was based on 7AAD staining and cell size and granularity. After their harvest, cells were washed with Phosphate-Buffered Saline (PBS) (GIBCO-Life technologies) and treated with human FcR blocking reagent for 15 minutes at room temperature (130-059-901 Miltenyi Biotech, Germany) and labeled with fluorochrome-conjugated antibodies according to manufactures instructions. Mouse anti-human: CD4-vioblue (130-094-153), CD25-VioBright FITC (130-104-274), CD127-PE-Vio770 (130-099-719) were used for cell surface staining with the appropriate isotypic controls (130-094-671, 130-104-575, 130-096-638 respectively) (Miltenyi Biotech, Germany). APC anti-human Galectin-9 Antibody was used for cell surface staining with the appropriate membrane staining isotypic control APC Mouse IgG1, κ Isotype Ctrl (FC) Antibody (400122 Biolegend U.S.A). Intracellular staining was achieved with the FoxP3 staining Buffer kit (130-093-142 Miltenyi Biotech, Germany), according to the manufacturer's

instructions with the use of Anti-FoxP3-PE (130-093-014), its appropriate isotype control (130-092-212 Miltenyi Biotech, Germany), the same mouse anti-human Gal-9-APC antibody and APC Mouse IgG1, κ Isotype Ctrl (ICFC) Antibody (400142 Biolegend U.S.A) for intra-cellular staining isotypic control.

Cytokine detection (ELISA)

Supernatants from different cell culture or co-culture conditions were collected at the specified time points and frozen for subsequent analysis. Supernatants were tested for cytokine (IL-10, Transforming Growth Factor (TGF-β1), interferon gamma, IFNγ and tumor necrosis factor alpha TNFα) production by Enzyme-Linked Immuno Sorbent Assay (ELISA) method as previously described (733). Results were expressed as the mean of duplicate wells after subtraction of the background. Galectin-9 secretion was detected by an ELISA Kit for Galectin 9 (GAL9) (SEA309Hu Euromedex France) according to manufacturer's instructions. Supernatants either from Treg, CD25- culture or PBMC/Treg co-culture were gathered at the specified times following a centrifugation at 300g for 10 minutes to pellet the cells and frozen at -80°C till the analysis.

iTreg induction

Tconv as in CD4+CD25- cells depleted of CD25+ Tregs were cultured in TCR stimulating conditions for 5 days alone, with 1g3 2µg/ml, GalS or GalM 30nM, or a mixture of 1g3 and the rGal-9. Following the 5 days the living cells were counted, washed and placed in co-culture for 48 hours with autologous PBMCs (ratio 1:1) in TCR stimulating conditions. Proliferation was assessed by [3H] thymidine incorporation.

Humanized SCID Mice C666-1 Model

All procedures were approved by the local Ethical Committee of the IPL performed with required permission of the regional governing ethical board (approval number CEEA 152010). Anesthetized SCID mice were previously splenectomised and then subcutaneously xenotransplanted (7 days later) with NPC tumors induced by C666-1-Luciferase cells (C666-1-Luc). The C666-1-Luc is an EBV-positive human nasopharyngeal cancer cell line expressing the EBV latency II proteins and the luciferase gene [34]. This NPC cell line has been obtained from a subclone of xenotransplanted human tumor from south China. Mice (n =40) were reconstituted or not by intraperitoneal injection of $50x10^6$ PBMC isolated from a healthy donor (n=9) and enriched or not by 7% of autologous Treg. Mice also received, or not 4 injections of 1g3 or IgG1 (20µg) at days 0, 7, 14 and 21 post xenotransplant. All mice received CpG ODN 1826 (130-100-103 Miltenyi Biotec France) to activate the PBMCs.

Measurements and Data Analysis

Measurements of tumor bioluminescence were acquired at different times: day 5, 7, 9, 12, 14, 16, 19, 21 and 24 post xeno-grafting to monitor tumor growth. The IVIS-Lumina XR® (Caliper, Life Sciences, MA, USA) was used to measure the bioluminescence following intra-peritoneal injection of 100 µL of D-Luciferin (30 mg/mL, 122799 Perkin Elmer) into each mouse. A series of images was acquired at 2 minute intervals till a decrease in total signal was observed. Images were then analyzed under the Living Image 4.1 software (Caliper Life Sciences) and the maximum value of total flux for every mouse was acquired. Moreover, the tumor length and width were measured with manual caliper at days 9, 12, 14, 16, 19, 21 and 24 and the tumor volumes were then determined by considering that they have an ellipse form (X, Y and Z axis, with X = length/2, Y and Z = width/2), thus establishing the same value for Y and Z. The following formula was used: Volume of the Tumor = $4/3 * \pi * X * Y * Z$. After sacrificing the mice the tumors were photographed on scale paper before being prepared for immunohistochemical analysis.

Data presentation:

The obtained value (luminescence or volume) for every time point for every mouse in every group was calculated. Results from the same group as in the same donor were normalized with respect to the measurement of the group PBMC+Treg+anti-Gal-9 antibody obtained at the last day (day 24). This value became the reference point equal to 1. The rest of the values were presented as relative curves with respect to this point. When all the values from all the donors were normalized, these values were averaged together to form the final graphs presented.

Immunohistochemical analysis.

Briefly, tumors were frozen in Neg-50™ Frozen Section Medium (6502 Thermo Fisher Scientific France) and cut on CryoStar™ NX70 Cryostat (Thermo Fisher Scientific France) into 4um slices and placed on previously gelatinized slides. The slides were fixed with cold 100 % acetone for 10 mins and left to dry for 1 hour at RT followed by 3 washes with PBS. Deactivation of endogenous peroxidase was done by a 0.3% hydrogen peroxide in methanol for 20 mins. Blocking followed 5% PBS 5% goat serum (S-1000 Vector Labs U.S.A) for 2 hours. Then followed by avidin and biotin blocking for 15 minutes each (SP-2001 Vector Labs U.S.A). After a single rinse step with PBS, the primary antibody Rabbit monoclonal anti-human CD-4 (ab133616) was added in 2% BSA 2% goat serum PBS solution at 1/100th dilution overnight at 4°C under agitation. Following 3 washes in PBS for 5 mins the secondary antibody was added, biotinylated Goat Anti-Rabbit IgG Antibody (BA-1000 Vector Labs U.S.A) at 1/250 dilution in PBS, 2% BSA 2%serum for 1 hours. Following 3 wash steps, amplification was done by VECTASTAIN Elite ABC Kit (PK-6100 Vector Labs U.S.A). 3 wash steps were done before the revelation by ImmPACT AEC Peroxidase (HRP) Substrate (SK-4205 Vector Labs U.S.A) for 4 minutes before washing with water. Counter staining was done by Mayer's Hematoxylin Solution (CL64040 LABOMODERNE France) for 2 minutes. Following a water bath for 15 mins the slides were fixed with 0.2% NH_3 solution for 1 min.

The slides were then rinsed in water. Slide mounting was done by Dako glycergel mounting medium (C0563 Dako france.)

Humanized SCID Mice MDA-MB-231 Model

Similarly to the C666-1 model. The MDA-MB-231 cell lines were non-luminescent so tumoral growth was only analyzed by manual measurements.

Chimeric and humanized antibodies

These antibodies were synthesized by Biotem, France. 3 chimeric antibodies were synthesized using an IgG1, IgG2, and an IgG4. 8 IgG1 isotype humanized antibody variants were synthesized. The antibodies were purity controlled by Coomassy blue staining and their Gal-9 binding capacity was verified by Biacore.

Statistical Analysis

Graphpad software was used for data treatment and statistical analysis All quoted P-values are two-sided, with P< 0.05 (*or # or ¤) and P<0.01 (**or ## or ¤¤) being considered statistically significant and highly significant, respectively. The Student's t test was applied for all analyses while Mann Whitney was used for the mice results.

Results

Galectin-9 expression by Tconv and Tregs Freshly isolate or after culture.

First of all the level of expression of Gal-9 by either Tregs or Tconv was determined at the transcriptomic level by QPCR and at the proteomic level by immuno-cyto-fluorescence, flow cytometry, and western blot and finally at the secretory level by ELISA. Briefly, primary human Tregs and Tconv were isolated from PBMCs obtained following ficoll gradient centrifugation of whole blood of healthy donors. The obtained cells were either directly studied for the expression of Gal-9 or placed in culture for 24, 48 or 72 hours in stimulated (anti-CD3 anti-CD28 1μg/ml) or non-stimulated conditions and then recovered and analyzed for Gal-9 expression. The results show that at the RNA level Tregs constitutively express higher levels of Gal-9 than Tconv. These levels progressively increased with culture in stimulated conditions for both cell types with Tregs maintaining a higher level of expression (Figure. 1). At the proteomic level, immunofluorescence and Western blot analysis showed a comparable level of expression of Gal-9 between freshly isolated Tregs and Tconv (Figure 2 A, B). Flow cytometry results obtained using anti-Gal-9 antibody showed that Gal-9 is not expressed on the surface of both the cells either fresh or following culture for 24, 48 or 72 hours (Figure 2C). Intra-cellular flow cytometric staining showed comparable levels of expression of Gal-9 between Tregs and Tconv with fresh cells (Figure 2 D). Gal-9 intra cellular expression between Tregs and Tconv was studied following a co-culture of PBMC and Tregs (2:1 ratio) for 72 hours. After the culture, the intracellular expression of Gal-9 was compared by flow cytometry between the CD4+Foxp3+ cells and the CD4+Foxp3- cells. The results were presented as normalized values for delta MFI (mean fluorescence intensity) (Figure 2 E). These results show that Gal-9 was expressed at a significantly higher level in the Foxp3+ cells than in the Foxp3- cells. Finally Gal-9 secretion was studied by ELISA. Results show a significantly higher level of secretion of Gal-9 by Tregs than by Tconv at all studied time points. This secretion was irrelevant of the activation status of the Tregs. Regarding the Tconv, even though their secretion of Gal-9 was almost negligible barely at the detection limit of the commercial ELISA kit, we did observe an increase in the secretion in stimulated conditions following a 48 and 72 hour culture with respect to the non-stimulated condition (Figure 3 A). Gal-9 secretion was also studied between a PBMC and a PBMC+Treg (2:1) co-culture. Adding Tregs to a PBMC culture significantly increased the quantity of secreted Gal-9 (2 fold) (Figure 3 B). Therefore, these results prove that Gal-9 expression and secretion by Tregs is constitutive and relatively high indicating a possible essential role of Gal-9 in Treg function.

Anti-Gal-9 antibody (1g3) antagonizes rGal-9

Thanks to our collaborator (Pierre Busson Gustave Roussy) we obtained an anti-Gal-9 antibody targeted against a 13 aa sequence in the C-terminal domain of Gal-9 (aa 210 to 223). The ability of this antibody to detect Gal-9 was proven by ELISA tests. In order to test if this antibody could be used as a Gal-9 antagonist we tested the anti-Gal-9 ability to inhibit the immunosuppressive potential of recombinant Gal-9 in both proliferation and apoptosis assays. Adding 60nM of recombinant Gal-9 to a

PBMC or Tconv culture in TCR activated conditions inhibited their proliferation. This suppressive effect was almost completely inhibited with a pretreatment of the recombinant Gal-9 with the anti-Gal-9 antibody at a concentration of 3µg/ml for 2 hours. Treatment at 1 µg/ml did not have any effect, whereas treatment at 5 µg/ml did not have a significant advantage over 3 µg/ml. Moreover, a mouse IgG1 was used as an isotypic control for the antibody to ensure specificity of the observed effect. This isotypic control did not affect Gal-9 induced suppression. Lactose was used as a control. Lactose can bind the CRDs of Gal-9 and has been shown to inhibit its suppressive activity. In our tests, lactose at 5 mM inhibited Gal-9 function whereas sucrose was used as a non-specific sugar did not have any effect (Figure 4). Similarly recombinant Gal-9 was shown to induce apoptosis of PBMCs studied by annexin-V flow cytometry; pretreatment with the anti-Gal-9 antibody inhibited this apoptotic effect (result not shown). These results were a clear indication that our antibody was in fact a Gal-9 antagonist.

Gal-9 blocking induces a Th1 immune response

Then we decided to study the effect of Gal-9 blocking by the anti-Gal-9 antibody on the immune response elicited by stimulated PBMCs. So we cultured human PBMCs alone, with anti-Gal-9 antibody or with its isotype and in TCR activated conditions for 72 hours after which the supernatant was recovered and the secreted cytokines were studied by ELISA. We observed a significant increase in the secretion of INFγ (8 folds on average) and TNFα (2 fold on average) coupled with a decrease in the secretion TGFβ (half). These changes in the cytokine secretion profile are indicative of a potent TH-1 response such as that observed in an anti-tumoral immune response (Figure 5).

Gal-9 blocking does not have a toxic effect on the cells of the immune system

The possibility that the anti-Gal-9 antibody would induce toxic hyper-proliferation of PBMCs and Tconv was tested by viability and proliferation tests. PBMCs and Tconv were cultured for different times under activating conditions with the anti-Gal-9 antibody or its isotype at varying doses. Adding the antibody to a culture of activated PBMCs or Tconv at concentration up to 12 µg/ml did not have a significant effect on the proliferation nor the viability tests respectively at any time point (Figure 6).

Anti-Gal-9 inhibited nTreg suppressive activity in MLR

Based on the early results of Gal-9 constitutive expression by Tregs we wanted to see if Gal-9 was essential for the Treg immuno-suppressive function. We tested that by seeing if the anti-Gal-9 antibody could inhibit the function of Tregs. Treg function is studied by the Tregs ability to inhibit the proliferation of autologous reporter immune cells in an *in-vitro* co-culture assay. Briefly, culturing Tregs with Tconv or PBMC from the same donor in stimulated conditions decreases the proliferation of these cells. This change in proliferation can be studied *via* the incorporation of radioactive thymidine by these cells since the Tregs are by nature anergic in vitro. Indeed we did find that adding anti-Gal-9 to a Tconv/Treg or PBMC/Treg co-culture did partially restore the proliferation levels of the reported cells

negating in part the suppressive functions of Tregs. These results were a clear indication that Gal-9 played a role in the immuno-suppressive function of Tregs and that by blocking Gal-9 in the culture medium with the anti-Gal-9 antibody we could inhibit the suppressive function of Tregs (Figure 7 A B).

Anti-Gal-9 antibody inhibits Gal-9 induced iTregs

Gal-9 has been shown to induce suppressive Tregs *in-vitro* (424). We wanted to test if the anti-Gal-9 can inhibit this function. We created a Treg induction protocol in which Tconv depleted of Tregs were cultured for 120 hours under TCR stimulation conditions. To this culture we either added the anti-Gal-9 antibody (3 μg/ml), Gal-9S (1 μg/ml), Gal-9M (1 μg/ml) or a mix of the antibody and the rGal-9. The control was Tconv cultured only in activating conditions. Following the pretreatment (PT), the cells were harvested, washed, counted and put in a co-culture with autologous PBMCs (ratio 1:1) again in TCR stimulating conditions for 72 hours. At the end proliferation was assessed by radioactive thymidine incorporation. The results show that the co-culture with the control or with the anti-Gal-9 PT cells increased the observed proliferation values. PT with rGal-9S or rGal-9M resulted in suppressive cells which inhibited the proliferation following the co-culture. This was expected as in line with the literature. However, the use of the anti-Gal-9 antibody during the pre-treatment phase inhibited both isoform's ability to induce suppressive cells as the proliferation in this co-culture was at the same level as the control (Figure 7 C).

All these results when taken together show that the anti-Gal-9 antibody could inhibit the suppressive function of nTregs and prevent the Gal-9 induction of iTreg

Anti-Gal9 as an anti-tumoral therapeutic

Given the known importance of Tregs in suppressing anti-tumoral immunity in cancer patients we wanted to test the potential use of anti-Gal-9 as a potential cancer immune therapeutic. We shows the NPC model which is known for its high Treg infiltrate and its consistent production of galectin-9 free or (448, 659) in association with exosomes (750). We have also recently published on the importance of NPC derived exosomes in inhibiting the anti-tumoral immune response by Treg recruiting and activating exosomes. These could inhibit also the proliferation of immune cells (291).

Anti-Gal9 inhibits NPC exosomes

First, we tested if the anti-Gal-9 antibody to inhibit the suppressive function of NPC derived exosomes. We culture PBMC or Tconv for 72 or 120 hours with 10 μg/ml of either NPC-exosomes or exosomes from healthy donors (HD-exo). The exosomes were pre-incubated for 2 hours with or without the anti-Gal-9 antibody or its isotype. Following culture, cell proliferation was analyzed. We found that using the anti-Gal-9 we could block the suppressive function of these exosomes by reversing their effect on the proliferation of immune cells. The antibody did not have any effect on HD-exo which were used as a control. The isotype did not change the NPC-exo induced suppression indicating that the effect

observed by the antibody was specific (Figure 8). The same results were observed using viability tests (results not shown).

Next we decided to test the antibody in an *in-vivo* cancer mouse model. Given that the entirety of our results were based on human immune cells. Also since the antibody is designed to recognize human Gal-9 we needed to develop a humanized mouse model carrying a human tumor whose growth could be easily followed and at the same time containing human immune cells to mimic a human immune response seen in cancer patients.

Anti-Gal9 controls *in-vivo* tumoral growth

The developed model was based on SCID mice. These mice were splenectomised one week before they received a subcutaneous graft of a human luciferase expressing C-666-1 NPC tumor and an injection of 50 million human PBMCs enriched or not with 7% Tregs. The tumoral growth in these mice was followed over a period of 24 days. The mice were divided into groups some received the anti-Gal-9 antibody while others received the isotypic control and others were not treated. The treatment regimen was one injection of 20 (µg/mouse) at the moment of the human immune cell reconstitution followed by booster injections at one week intervals till the sacrifice of the mice at the end of the experiment. With every injection of the anti-bodies starting at day zero, the mice received injections of CpG-ODN which is a TLR-9 agonist in order to activate the reconstituted PBMCs. (Figure 9).

As a first step, the human immune cell reconstitution was tested. Using immuno-histochemical analysis of tumor frozen cuts, we were able to identify human CD4+ tumor infiltrating cells which could not be found in the non-reconstituted mice. These results were a clear confirmation of the success of the reconstitution (Figure 10).

The presence of human PBMC reconstitution was be able to control the tumoral could decrease the growth rate of the human tumor. Enriching the PBMCs with Tregs almost completely negated the positive effect of the PBMCs meaning that the tumoral growth was restored. Treating the mice with the isotypic control had no observed effect. Treating the mice which had received the Treg enriched PBMCs with anti-Gal-9 had a striking effect on tumoral growth effectively capable of inhibiting tumoral growth to a higher level than that observed in mice having only received non-enriched PBMCs. The difference in the size of the tumor between the PBMC+Treg+iso group vs the PBMC+Treg+1g3 group was highly significant and significant respectively for both manually measured volume and luminescence starting from day 14. The difference between the PBMC+Treg group vs the PBMC+Treg+1g3 group was significant at day 21 and 24 for the manual measurements and significant or highly significant starting from day 16 using the bioluminescence data. Finally the different between the PBMC+Treg+1g3 group and the nothing group was highly significant or significant starting from day 16 for the manual measurements and from day 19 for the bioluminescence (Figure 11)

Finally, we showed the follow up of 2 mice one of which was treated by the anti-Gal-9 antibody and the second by the isotype (Figure 11 A.). Photographs showing the luminescence of the tumors allows its follow up in size and signal intensity with respect to the mice. The results clearly show that the growth of the tumor in the anti-Gal-9 treated mouse was significantly slowed down with respect to the isotype treated mouse. Figure 2 B and 2 C shows photos of the tumors following resection and mice sacrifice. The photos reveal a clear difference between the tumors. For every figure, the 2 tumors originate from 2 mice receiving the same immune cells of the same donor. The tumor from the treated mouse in both cases was clearly smaller (Figure 12).

Proving the Humanized mouse model in a breast cancer model

In an attempt to prove the effectiveness and specificity of our therapy, we decided to use a triple negative breast cancer model. We chose the breast cancer model since in this cancer the increase in Tregs or Gal-9 have been independently associated with a better prognosis (253, 436). In theory this makes this model basically the opposite of our NPC model. Similarly to the NPC model we created humanized mice that received a xenograft of a MDA 231 cell line and the PBMCs enriched or not with Tregs as well as the anti-Gal-9 Mab. The results showed that the PBMCs alone did not affect the growth of the tumor, however the Treg enrichment was able to control tumoral growth. Treatment with the antibody was able to reverse the positive effect observed with the Tregs and tumoral growth was restored (Figure 13.A). We also noted the mass of these tumors following sacrifice and we found the results were coherent. The tumor coming from the mouse which had received Treg enriched PBMCs without treatment has the lowest mass whereas the other tumors had very similar masses.

Chimeric and humanized anti-Gal-9 antibody

These promising results pushed the development of the project towards the creation of humanized antibodies with lowered immunogenicity for the eventual use in clinical trials. The first step involved the generation of 3 chimeric antibodies which yielded results in vitro regarding their ability to block both recombinant Gal-9 and Treg induced immuno-suppression. The chimeric antibodies using the IgG1 or the IgG2 were able to reverse the suppressive effect of both soluble Gal-9 as well as Tregs. This effect was comparable to that observed using 1g3 (Figure 14). We continued by generating IgG1 humanized antibodies. 8 clones of humanized antibodies were obtained and as first step their ability to antagonize Gal-9 was tested in both proliferation and viability tests (Figure 15.A.). All the tested hAbs had the ability to antagonize Gal-9 suppressive effect on Tconv. Then we tested their possible toxicity and none was observed (Figure 15.B.)

Figures

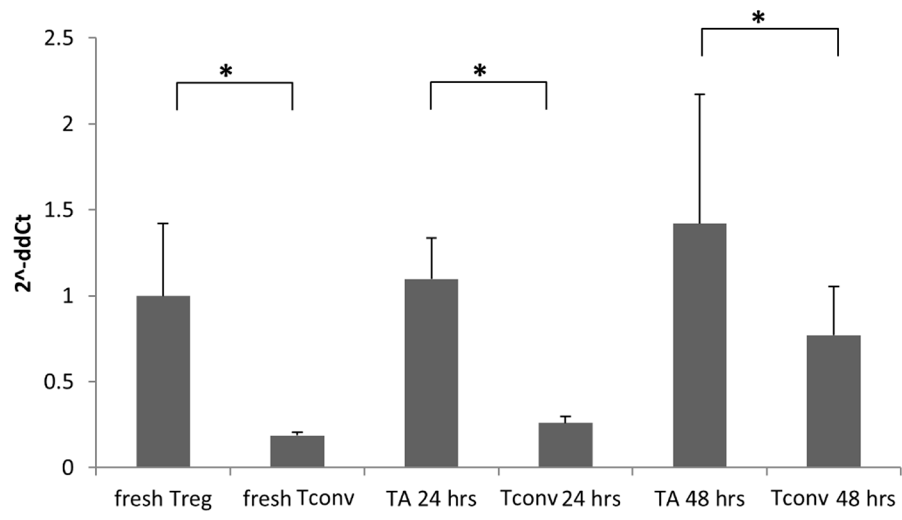
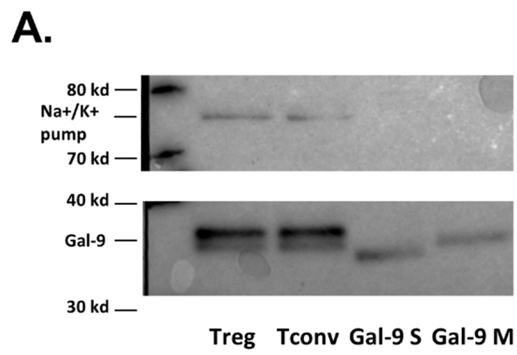


Figure 1

A.



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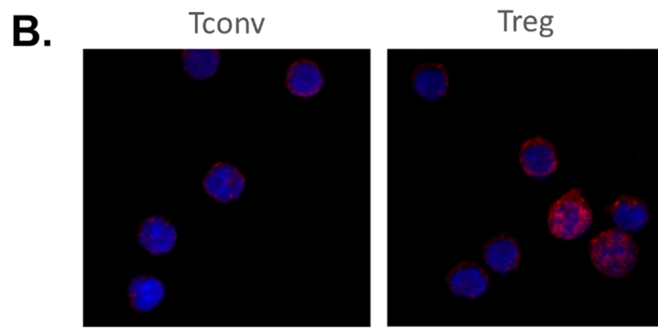

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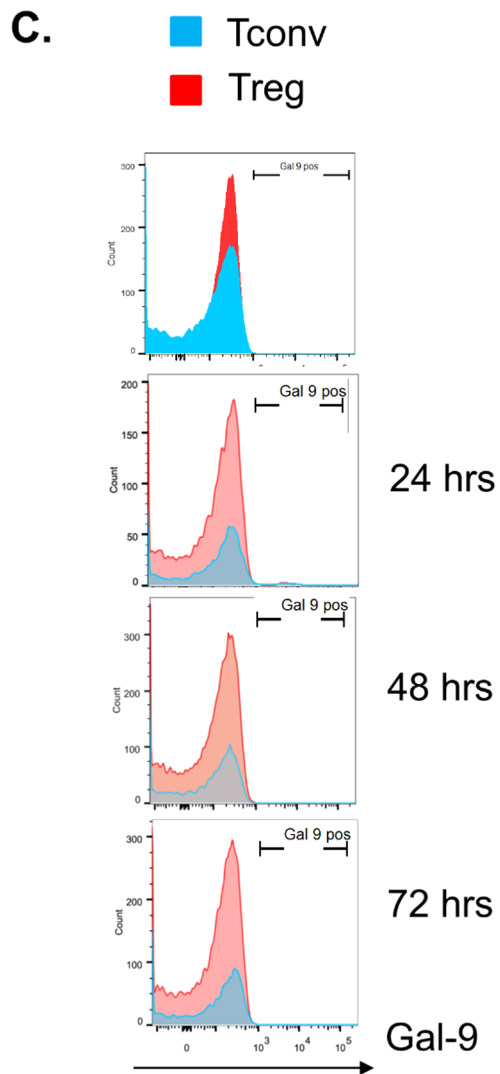

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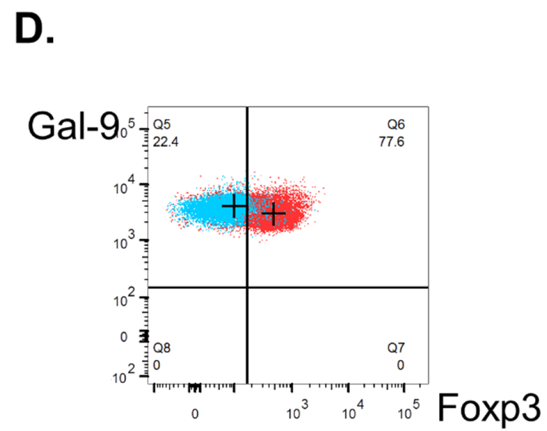

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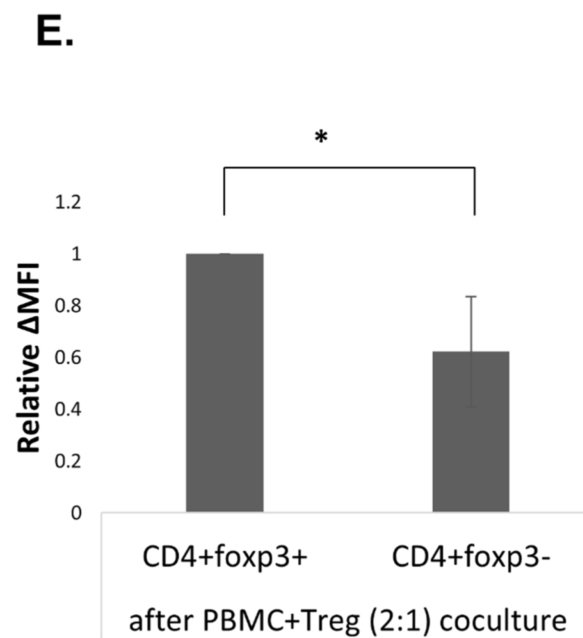
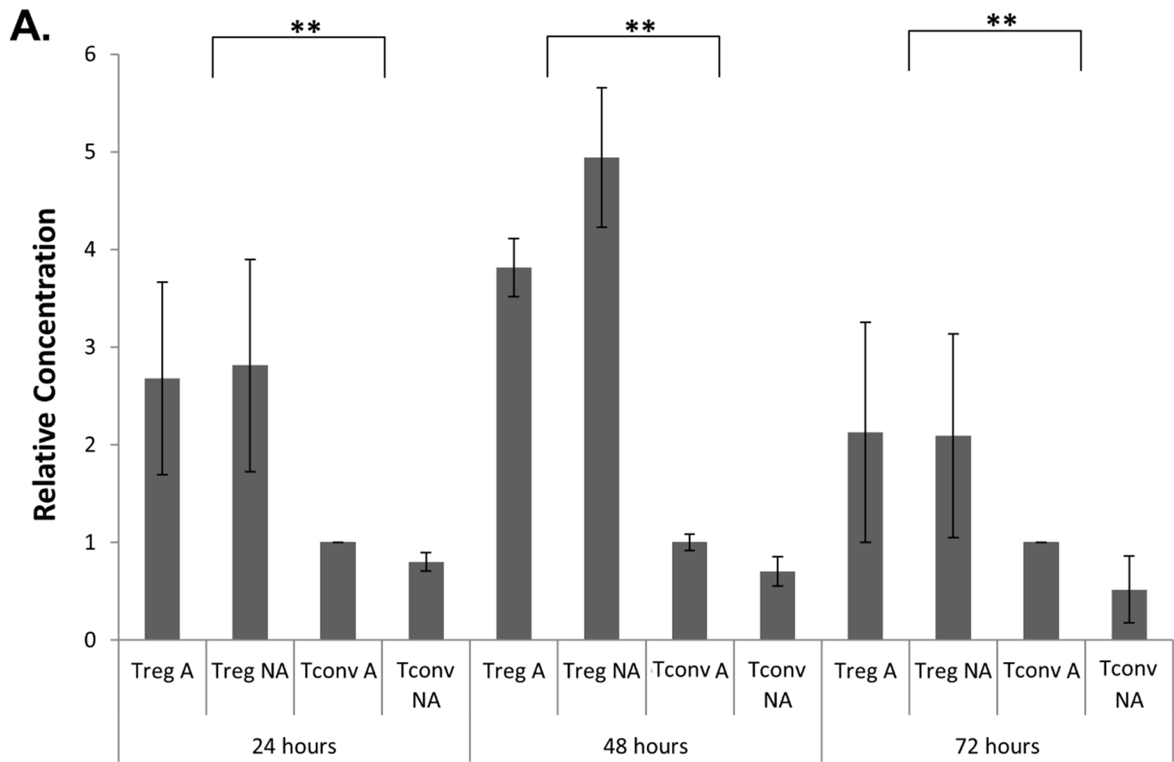
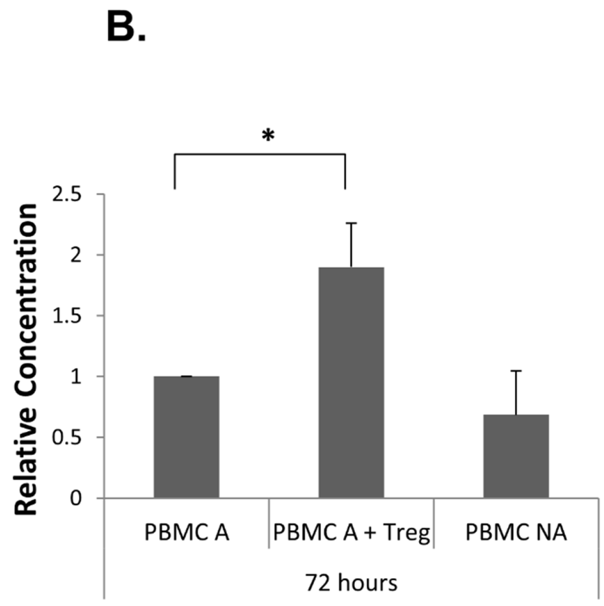


Figure 2





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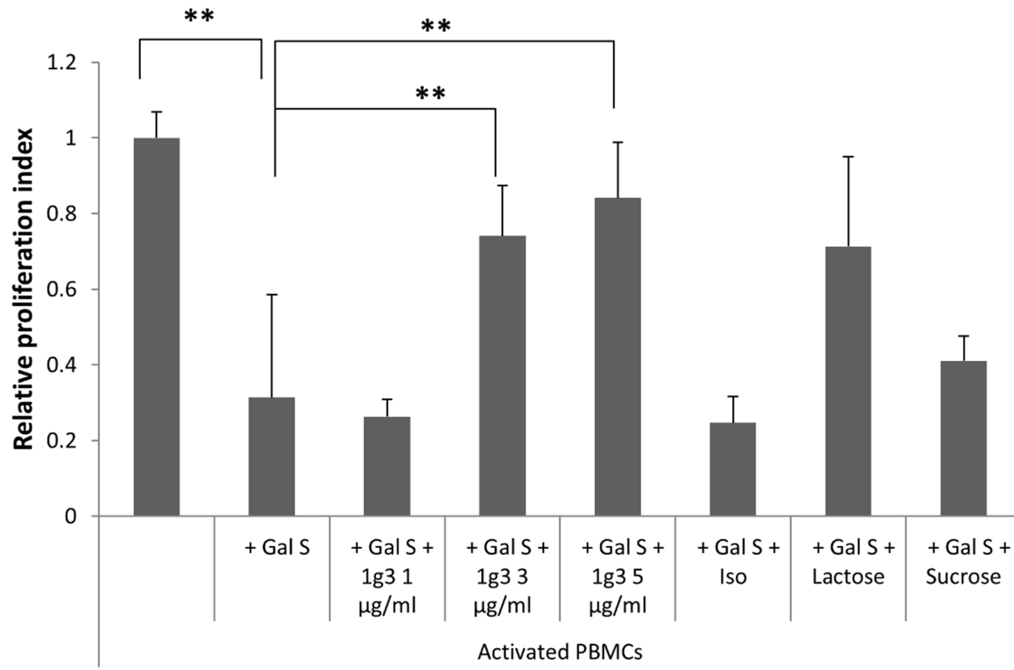


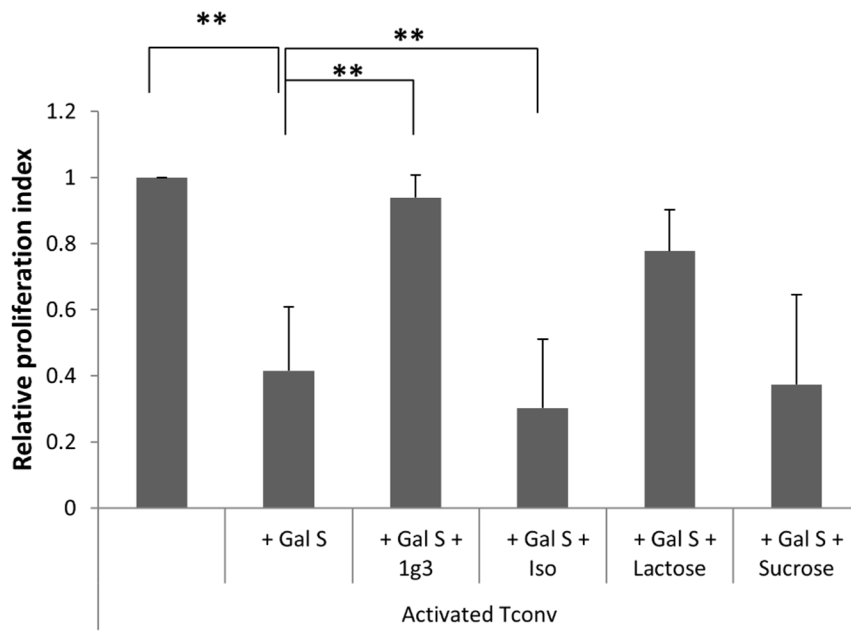


Figure 4

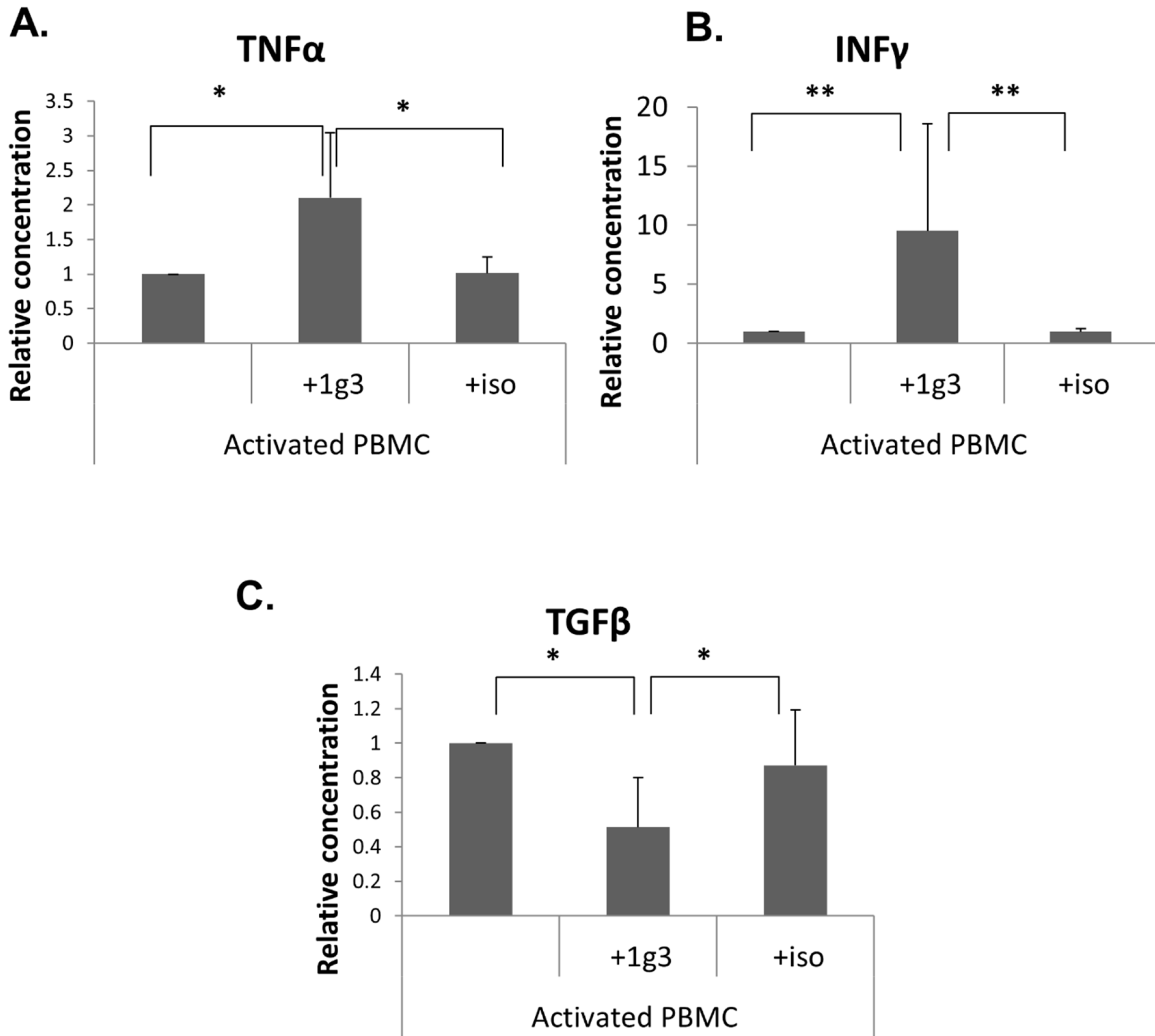


Figure 5

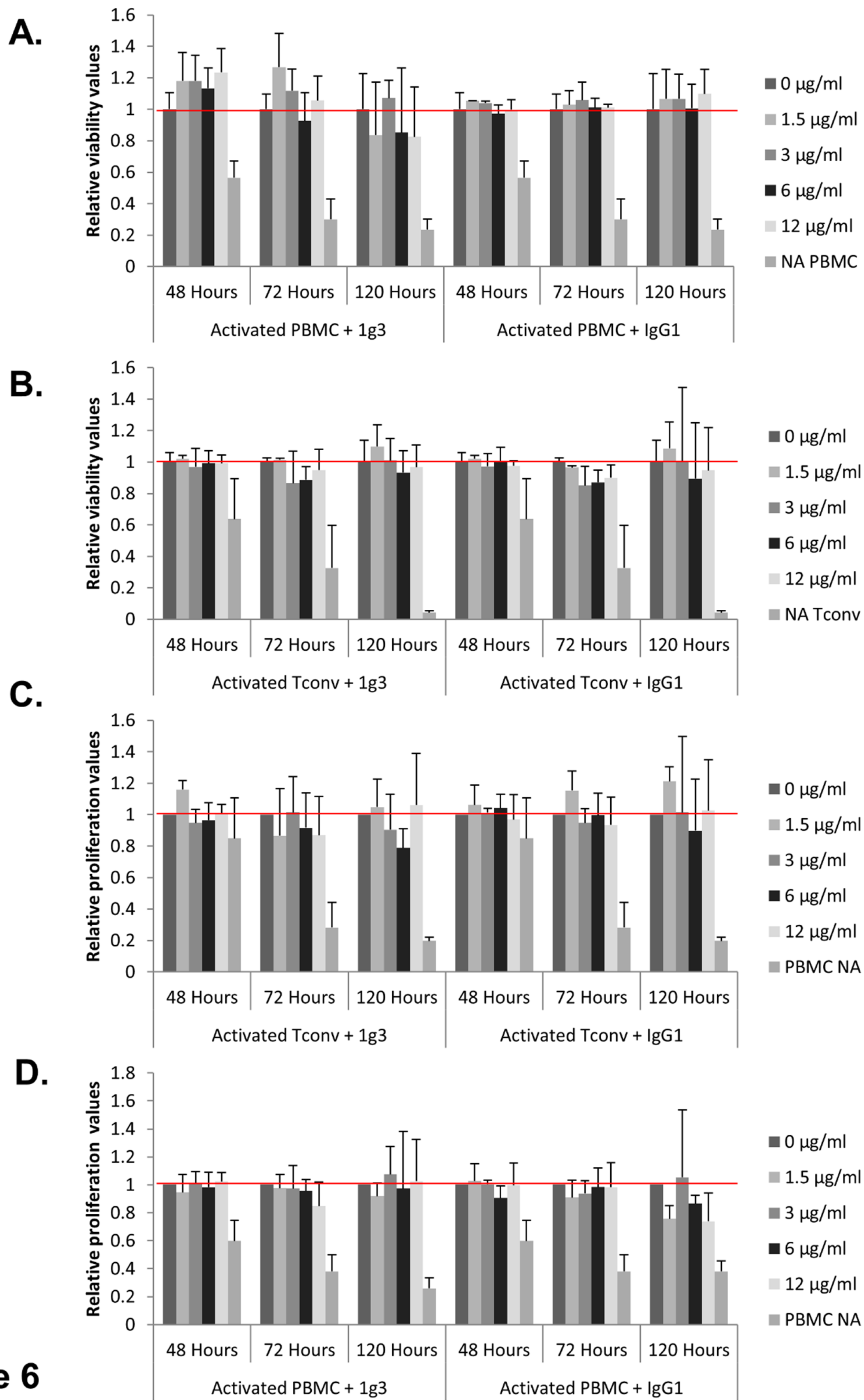
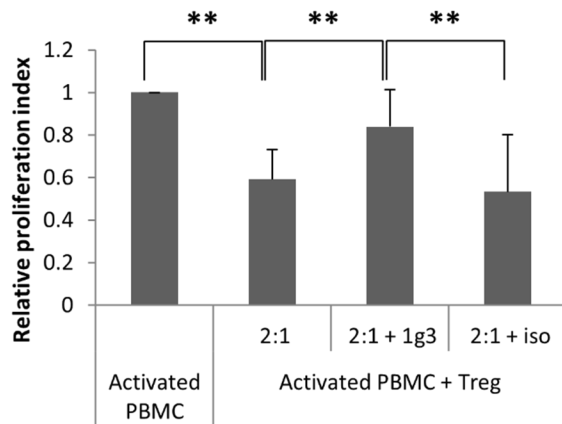


Figure 6

A.



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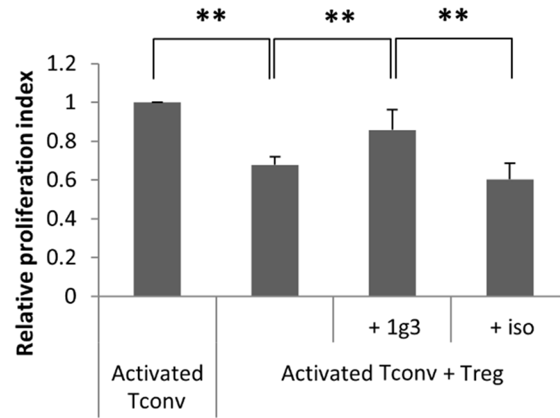

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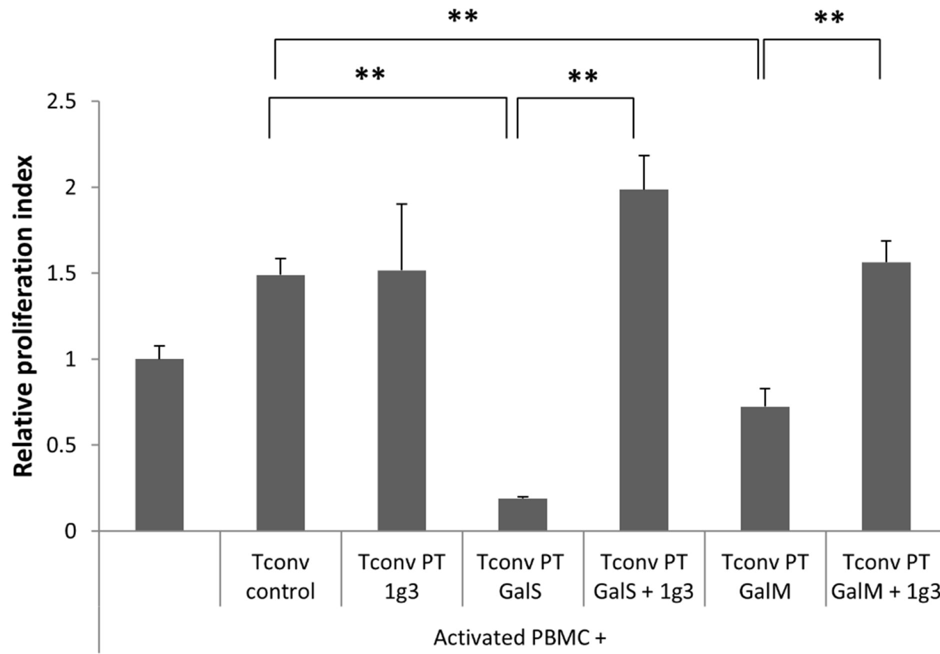


Figure 7

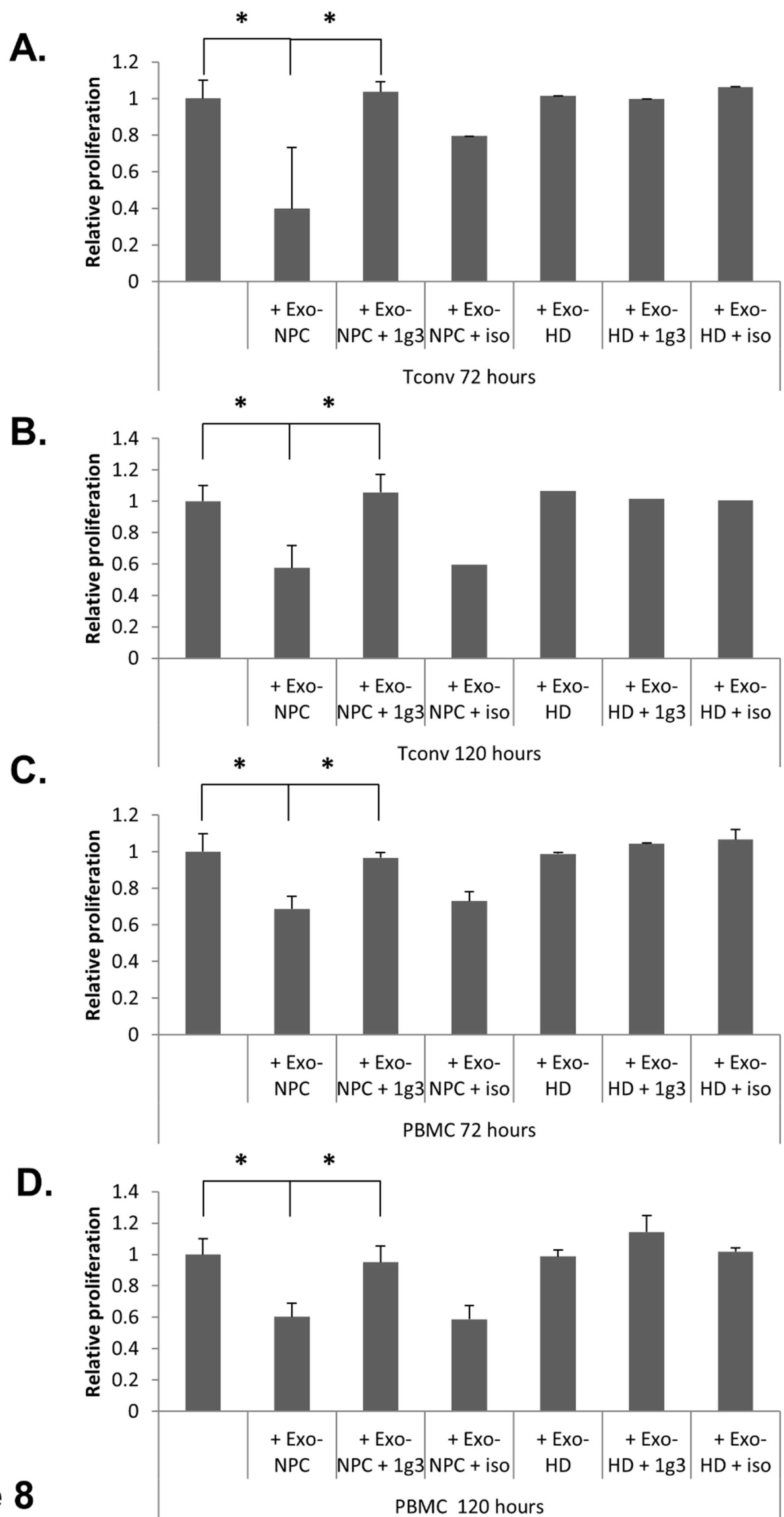


Figure 8

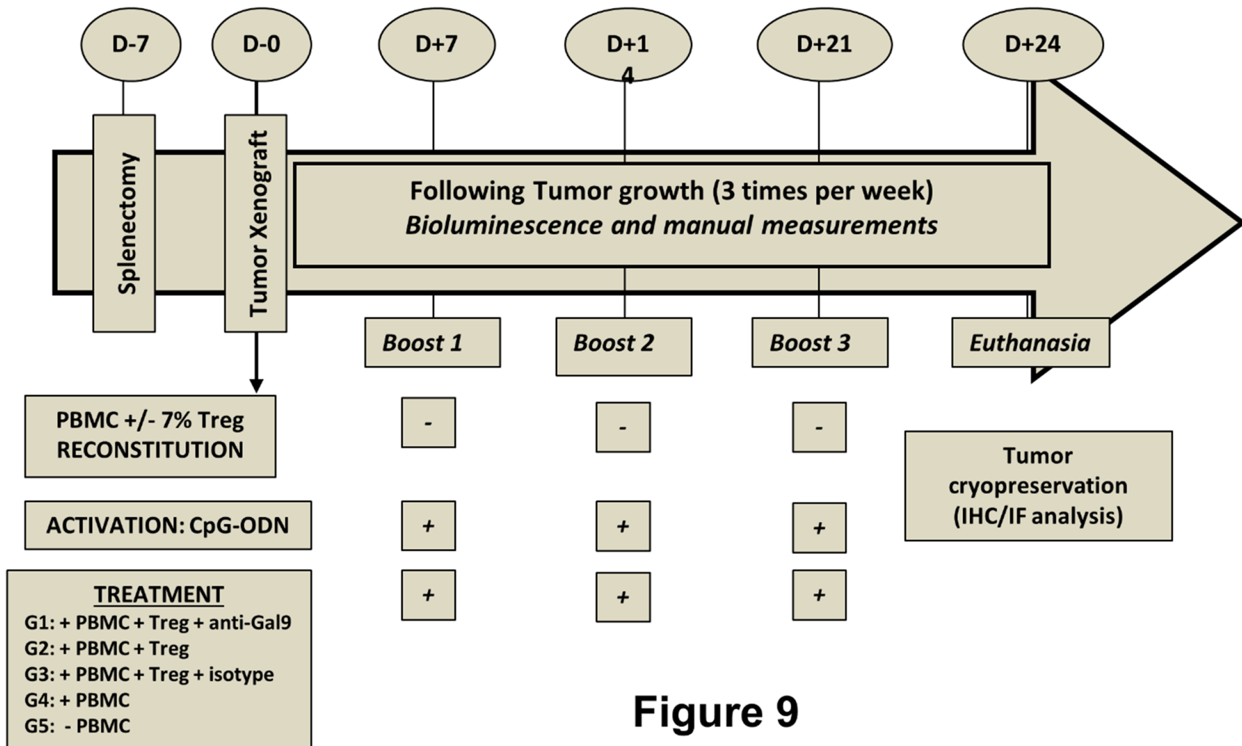
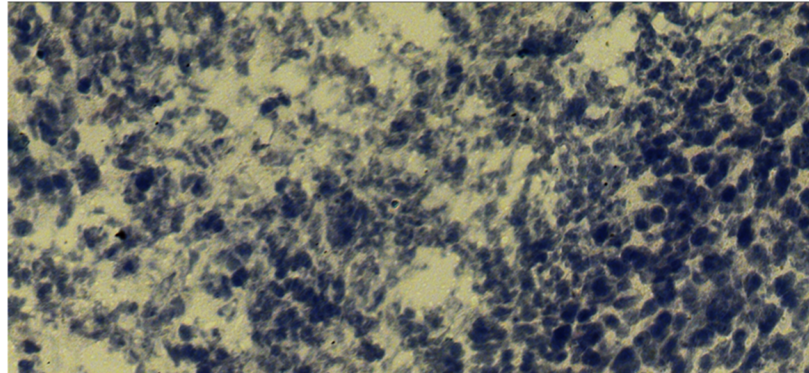


Figure 9

A.



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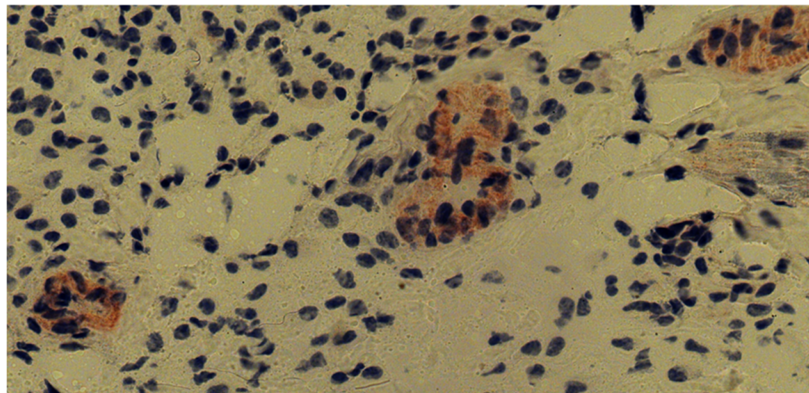
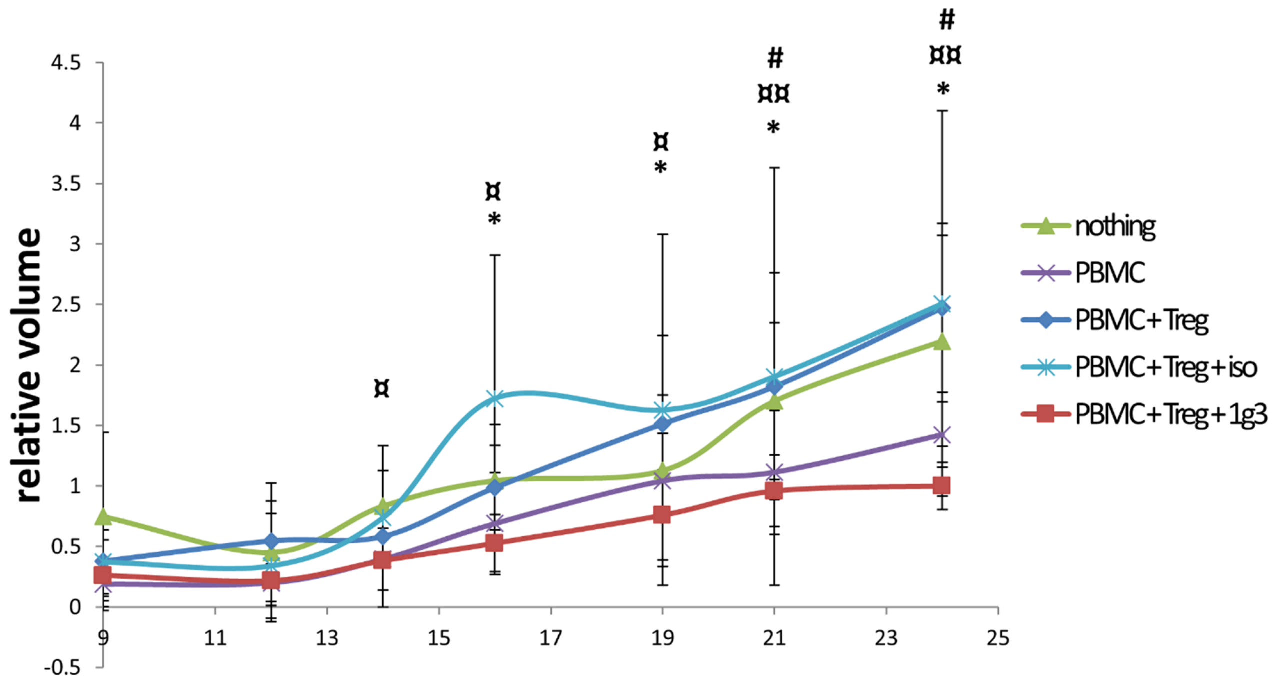


Figure 10



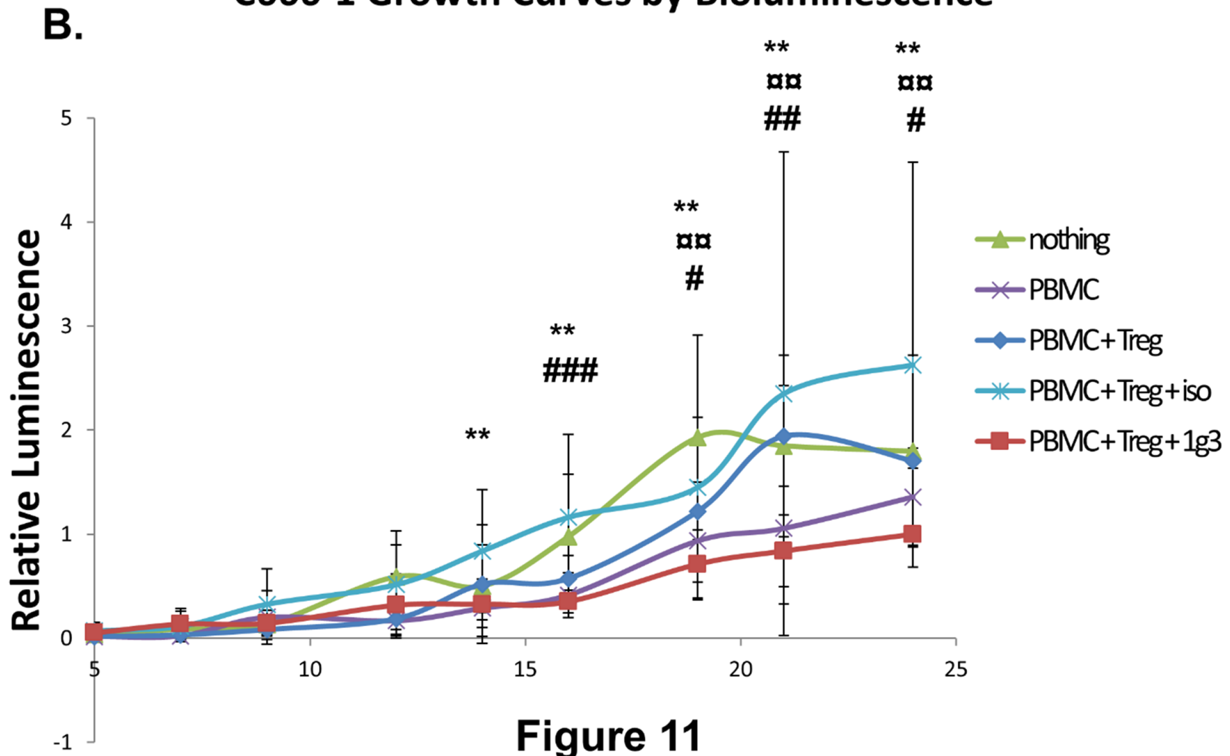


Figure 11

A.

J5 J7 J9

1g3 Iso 1g3 Iso 1g3 Iso

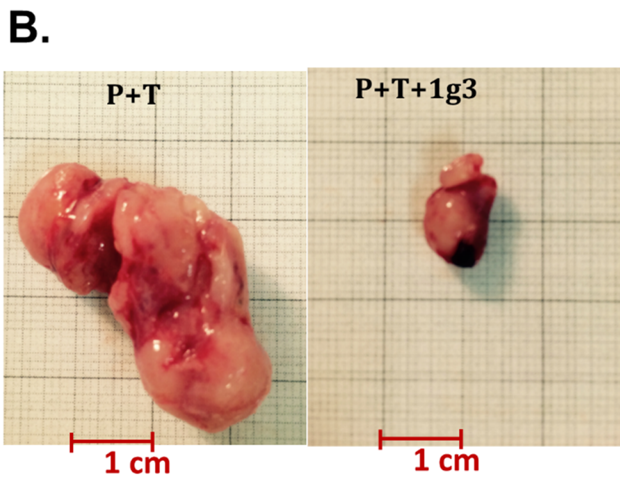
J11 J14 J16

1g3 Iso 1g3 Iso 1g3 Iso

J19 J21 J24

1g3 Iso 1g3 Iso 1g3 Iso

B.



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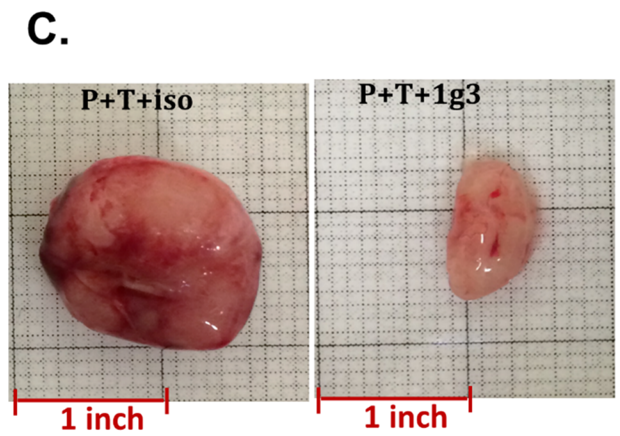
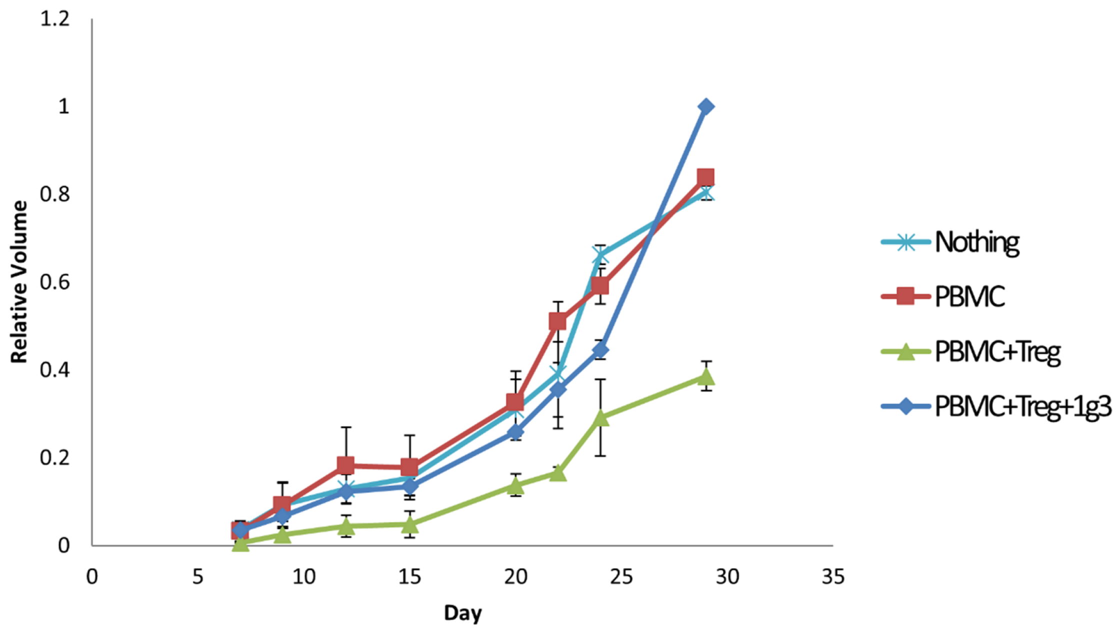
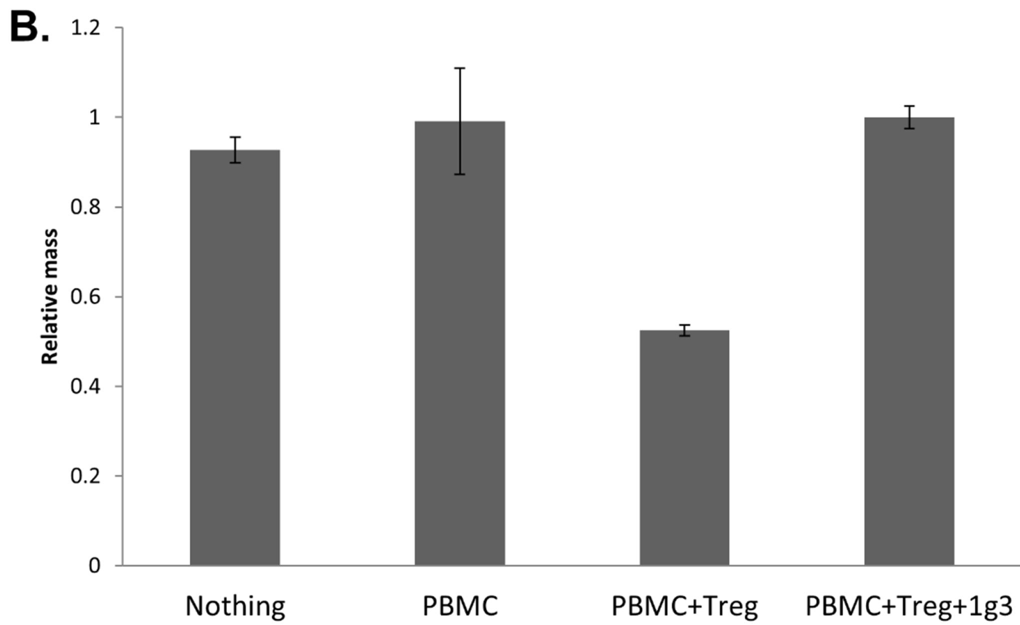


Figure 12





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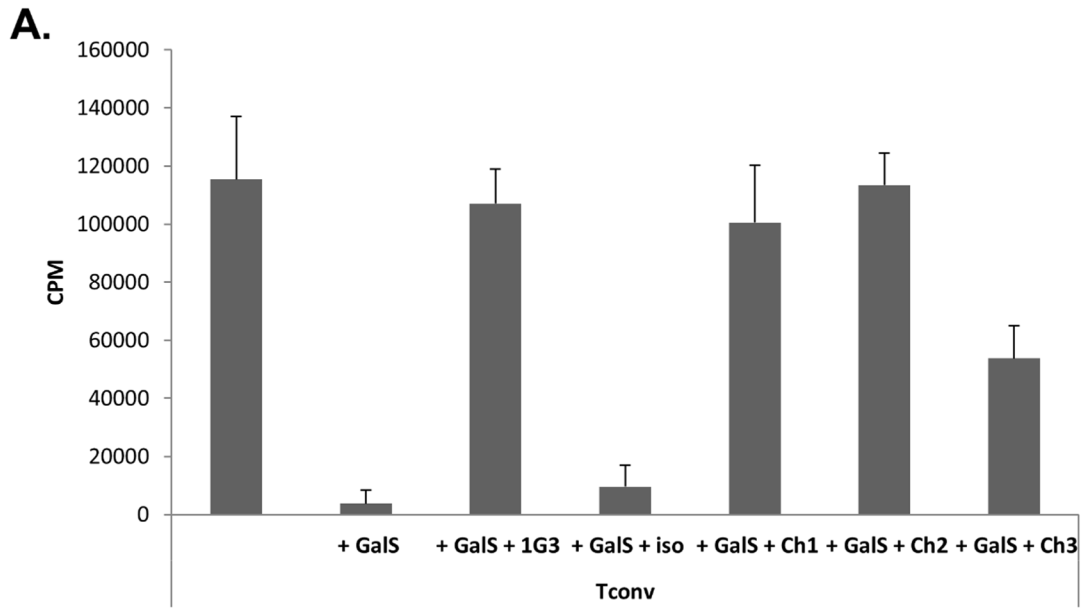

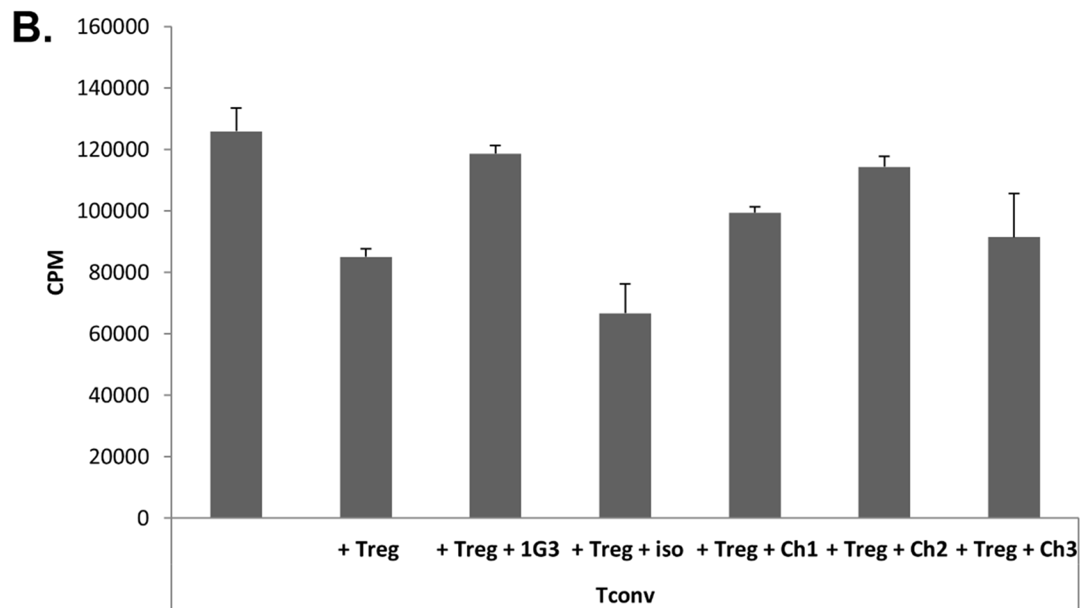

Figure 14

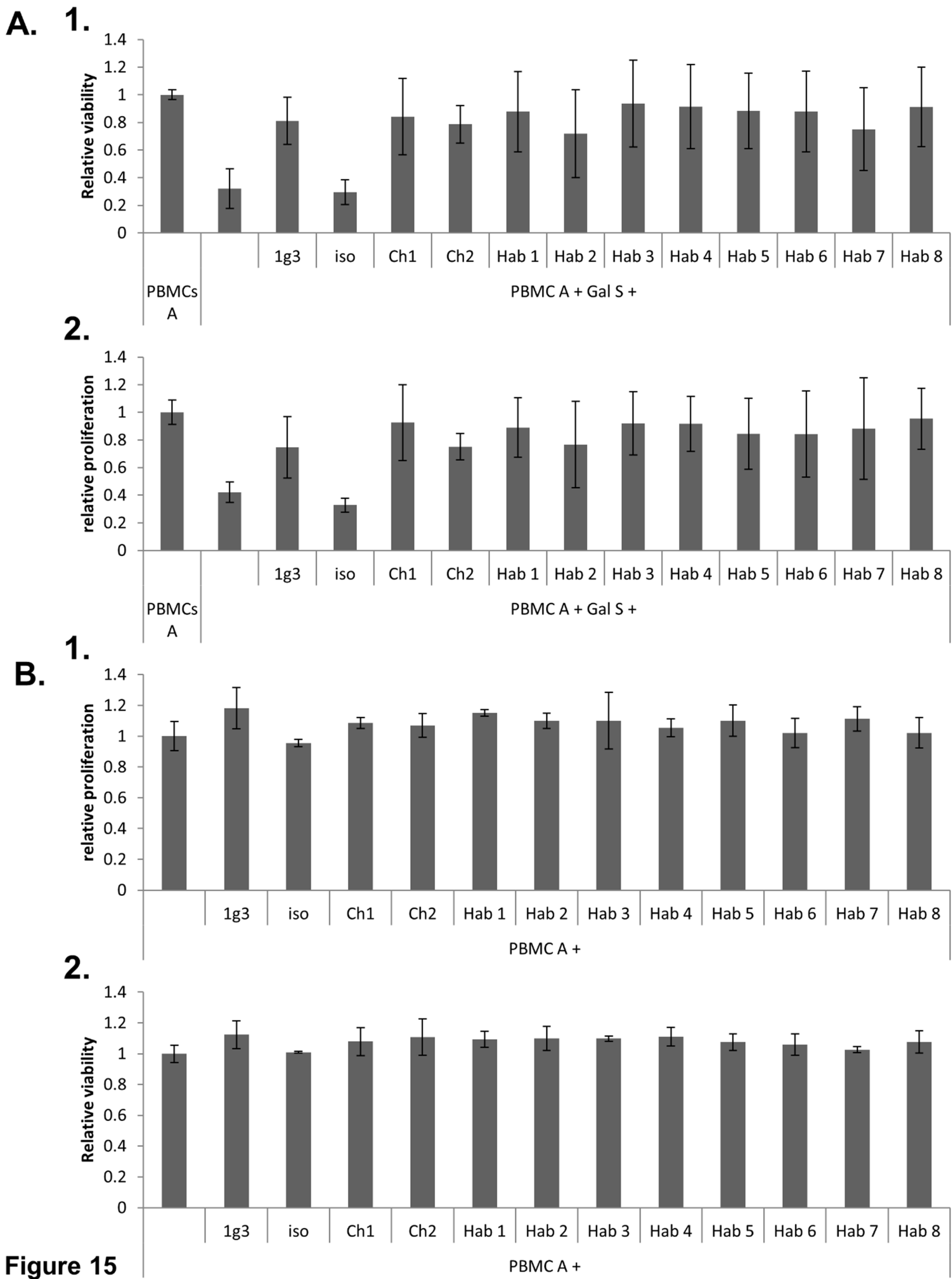

Figure 15

Discussion

In this work we show that Gal-9 is expressed in a constitutive manner by Tregs which is higher than that found in Tconv. This was best shown by the transcriptomic and secretory results showing that Tregs expressed Gal-9 at a higher level than Tconv regardless of activation conditions. The fact that Gal-9 expression and secretion in Tconv increased in strong stimulated conditions using 1µg/ml of anti-CD3 and anti-CD28 is expected and comforts results observed in the literature (336). The expression level of Gal-9 protein between fresh Tconv and Tregs is very interesting since it shows that there is no clear difference. However, a difference was observed at the transcriptomic and secretory. These type of results are not uncommon to Gal-9 expression. A recent study established that Gal-9 expression in multipotent mesenchymal stromal cells increased following IFNγ treatment however this increase was only detected at the RNA and secreted level and not by intra cellular flow cytometry (422). This most likely means that the secretory pathway used by Gal-9, which is still not discovered, does not allow for the accumulation of the Gal-9 protein inside the cell.

An important biologic feature of NPC is the presence of a massive lymphoid infiltrate in the primary tumor, which is likely favored by inflammatory cytokines produced by tumor cells (542). Several studies support the idea of a local immuno-suppressive environment inhibiting proper effector cell function even after correct homing. The presence of abnormal quantities of regulatory T cells (Treg) within the tumor site and the peripheral blood is a clear indication of this immune suppression (659).

It is very interesting to see that Gal-9 using multiple antibodies (including the 1g3 antibody results not shown), could not be detected on the membrane of the Tregs nor Tconv regardless of culture conditions. Numerous reports did indicate that Gal-9 can be detected on the membrane (346). It is possible that the antibodies tested in our staining conditions simply could not detect the membranous Gal-9 but this does not completely negate the possibility of the anti-body staining surface Gal-9. It is worthy to note that there are other researchers who failed to detect Gal-9 on the surface of activated CD4+ and CD8+ cells by flow cytometry but did manage to detect in the lysate containing the membrane fraction as well as on the intracellular part of the plasma membrane. This same work detected Gal-9 using the same staining protocol on the surface of dendritic cells (751).

Here were reported for the first time that Gal-9 is constitutively secreted by human nTregs at a significantly higher level than Tconv. We also report that using our anti-Gal-9 antibody we were able to significantly inhibit to a certain extent the suppressive function of Tregs. These results are very interesting. It is generally accepted that Treg mediated suppression in *in-vitro* suppression assays (MLR) is mediated by contact dependent mechanisms (752). So the fact that our antibody could inhibit Treg induced suppression and that Gal-9 is constitutively expressed and secreted by Tregs does indicate that Gal-9 is not only important as a suppressive mechanisms for nTregs but that it also plays a role in maintaining their overall suppressive potential. The discovery that Gal-9 is constitutively

expressed by human nTregs does open new opportunities to fully understanding the intricate mechanisms governing Treg suppressor function.

Here we show that Gal-9 blocking in a culture of human PBMCs increases the secretion of IFNγ and TNFα and decreases that of TGFβ. These results combined with what has been shown in the literature further prove that Gal-9 acts as a regulatory mechanism to inhibit the over activation of the immune response. This is confirmed by the substantial data both from mice models and human patients that shows that Gal-9 expression is increased in viral infections and acts to downregulate the immune response (753). Showing that the anti-Gal-9 antibody could be used to revert these suppressive effects inducing a Th1 response is an indication of this antibodies potential use as an immune checkpoint inhibitor. One particular concern is the potential toxicity of this antibody. Especially whether or not it will lead to an over activation of the immune system. While this cannot be fully assessed in vitro, the current data that we have does show that blocking Gal-9 in a PBMC or Tconv culture did not induce hyper proliferation of these cells. When we take this into consideration along with other results showing that Gal-9 KO in healthy mice does not seem to induce autoimmune disease (425) we would obtain hints regarding the potential future toxicity of the anti-Gal-9 antibody. However, more tests are required.

NPC is a malignancy that is almost always associated with EBV. NPC patients suffer from a strong immunosuppressive microenvironment in which the Treg population and tumoral exosomes are key players (542). We have chosen to study the potential of the anti-Gal-9 Mab as an anti-cancer immunotherapeutic in this cancer model. Others along with our team have demonstrated that NPC derived exosomes could inhibit the Th1 immune response by inhibiting the proliferation of CD4+ T cell (291). First we were pleased to find that the Mab inhibited the suppressive function of the NPC-Exo. These results were very motivating and pushed us to test the potential of using the anti-Gal-9 Mab in an *in-vivo* model. Since the basis of our work on the Mab is that it is capable of inhibiting human Gal-9 and boost the anti-tumoral immune response of human immune cells by blocking Tregs we could not use syngeneic mouse models. Therefore we created our humanized mouse model. This model is based on SCID mice which have a deficiency in their adaptive immune response losing their ability to induce an adaptive B and T cell immune response. These mice received a xenograft of a human NPC tumor expressing luciferase. This tumor has been shown to express the EBV latency type II proteins of EBV (754). We also reconstituted these mice with human PBMCs which had been enriched or not with Tregs. The Tregs allows us to mimic the immunosuppressive environment observed in NPC patients. Using this model we were able to prove the immunoregulatory role of Tregs in NPC pathogenesis as mice that had received the Treg enriched experienced significantly faster tumoral growth. Moreover, using the anti-Gal-9 Mab we were able to significantly hinder the growth of the tumor to a level which was even better than non Treg enriched PBMCs. This means that our therapeutic can induce an anti-tumoral

immune response which is mediated not only by Treg inhibition but also by the activation of a Th1 response.

The effectiveness of the humanized model used and the ability of 1g3 to inhibit Treg functions is clearly seen by comparing the breast cancer model with the NPC model. These models are the exact opposite in terms of prognostic value for in breast cancer an increase in the Foxp3+ Treg population or Gal-9 are independently associated with a better prognosis (253, 436). Hence proving that the anti-Gal-9 as well as the Treg enrichment could have opposite effects in this model with respect to the NPC model is the ultimate evidence of the success of our experimental protocol and results. We were very pleased to find that in fact Tregs in this model were able to control tumoral growth while the Gal-9 treatment restored it.

Humanization is a crucial step in the development of an antibody for human therapeutic use (464). This reduces the potential toxicity of the antibody by removing most of the antigenic mouse part. The 1g3 antibody was humanized by an external company. The first step involved the creation of 3 chimeric antibodies based on three isoforms (IgG1, IgG2 and IgG4) which we tested for their ability to inhibit the suppressive functions of both rGal-9 and Tregs. We obtained promising results from these tests which lead us to the development of humanized antibodies based on the IgG1 isoform. The rational was that we wanted to provide the antibody with cytolytic potential. So far the tests indicate that the humanized antibodies are capable of neutralizing the anti-proliferative function of Gal-9. The preliminary data also demonstrates no hyper proliferative effect. In the following months one of clone of the humanized antibodies will be chosen for testing against Treg induced suppression and ultimately in humanized mouse models.

Legends of figures

Figure 1: Expression of LGALS9 in Tregs and Tconv by QPCR

LGALS9 expression was studied at the mRNA level by QPCR. Each individual value was normalized using the mean of the 4 HKG according to the standard ΔC_T method (733): $\Delta C_T = C_T - C_{THKG}$. For comparisons between groups, the relative gene expression was expressed in $2^{-\Delta\Delta C_T}$ leading to arbitrary value of 1 for the reference group. The expression of Gal-9 by freshly isolated Treg was used as the reference point n=10 Error bars represent standard deviations. P values from Student's t-test. All statistical tests were two-sided.

Figure 2: Expression of Gal-9 protein in Tregs and Tconv freshly isolated or following culture

The expression of Gal-9 at the protein level was analyzed by different techniques. A. Western blot from Gal-9 between Tconv and Tregs showing 2 bands which correspond to the medium and the large isoform as determined by comparison to the bands obtained from running the recombinant Gal-9S and Gal-9M in the same gel. Equal loading was verified by the sodium potassium pump. B. Immunofluorescence of Tconv and Tregs freshly isolated showing the expression of Gal-9 in red. C D E. Blue represents Tconv and Red represents Treg. C. Intensity expression histogram showing the membranous expression of Gal-9 between freshly isolated Tregs and Tconv or following culture in TCR stimulated conditions for 24, 48 or 72 hours. D. Dot blot showing the expression of Foxp3 and Gal-9 following intracellular staining by flow cytometry of freshly isolated Tregs and Tconv. E. Histogram showing the delta mean fluorescent intensity of intra cellular Gal-9 staining between Foxp3 positive and Foxp3 negative cells following a PBMC+Treg coculture (ratio 2:1) for 72 hours (n=4) Error bars represent standard deviations. P values from Student's t-test. All statistical tests were two-sided.

Figure 3: Gal-9 secretion following culture studied by ELISA

Secreted Gal-9 concentration was studied by ELISA. A. Tregs or Tconv were placed in culture in TCR activating condition or not for 24, 48 and 72 hours. Results are presented as relative concentrations with respect to the value obtained from Tconv activated condition for every time n=10. B. PBMCs alone or with Tregs were cultured for 72 hours in TCR activating conditions or not. Results are presented a relative concentrations with respect to the PBMC activated condition (n=3) Error bars represent standard deviations. P values from Student's t-test. All statistical tests were two-sided..

Figure 4: anti-Gal9 antibody inhibits rGal-9 induced suppression.

Proliferation of PBMCs or Tconv was analyzed following 72 hour culture by incorporation of radioactive thymidine. Results were presented as relative proliferation with respect to the PBMC or Tconv TCR stimulated conditions. n=5. Error bars represent standard deviations. P values from Student's t-test. All statistical tests were two-sided.

Figure 5: Cytokine secretion profile following treatment of treatment of PBMCs with anti-Gal9

PBMCs were culture for 72 hours with or without the anti-Gal9 antibody or its isotype. The secretion of IFNγ (A.), TNFα (B.) and TGFβ (C.) in culture supernatant were analyzed by ELISA. Results are presented as relative concentrations with respect to the PBMC TCR stimulated condition n=4 Error bars represent standard deviations. P values from Student's t-test. All statistical tests were two-sided.

Figure 6: Effect of anti-Gal9 on potential toxicity of PBMCs and Tconv

PBMCs or Tconv were cultured for 48, 72 or 120 hours with or without varying concentrations of ant-Gal9 antibody (1.5, 3, 6, 12 μg/ml) under TCR activated conditions or no. Potential toxic effect of the anti-Gal-9 antibody was assessed by Cyquant®. For every time point, results were expressed as relative proliferation values with respect to the non-activated control. (n=3) Error bars represent standard deviations.

Figure 7: Suppression tests using MLR. Effect of anti-Gal9 on Treg suppression

A B. PBMCs or Tconv were culture for 72 hours alone or in co-culture with autologous Tregs (ratio 2:1) in TCR stimulating conditions with or without the anti-Gal-9 antibody or the isotypic control. Proliferation of reporter cells was assessed by radioactive thymidine incorporation following 72 hours of culture. C. PBMCs were culture with Tconv that had been pretreated (PT) for 5 days before the co-culture in the conditions as indicated. The PT conditions were either just TCR activation or TCR activation with: anti-Gal9 ab, Gal-9S, Gal-9S + anti-Gal-9 ab, Gal-9M, Gal-9M + anti-Gal-9 ab. The proliferation was assessed after a 48 hour co-culture by incorporation of radioactive thymidine Proliferation results obtained are presented as relative proliferation following normalization with the respect to the PBMC activated or Tconv activated respectively. The differences are significant as indicated. A B n=5. C n=3 Error bars represent standard deviations. P values from Student's t-test. All statistical tests were two-sided.

Figure 8: Effect of anti-Gal-9 on the suppressive function of NPC-Exo.

PBMC or Tconv were cultured in TCR activating conditions for 72 or 120 hours with nasopharyngeal carcinoma derived exosomes (Exo-NPC) or healthy donor serum derived exosomes (Exo-HD) with or without the anti-Gal-9 antibody or its isotype. Proliferation was measured by the Cyquant® test at the end of the culture. Results are presented as normalized proliferation values following normalization with the PBMC or Tconv TCR activated condition for every donor. Differences were significant as indicated (n=5) Error bars represent standard deviations. P values from Student's t-test. All statistical tests were two-sided.

Figure 9: Graphical representation of the humanized *in-vivo* mouse model.

Anesthetized SCID mice were splenectomised and then subcutaneously xenotransplanted (7 days later) with C666-1-Luciferase cells (C666-1-Luc NPC cell line). Mice (n=40) were reconstituted or not by intraperitoneal injection of PBMC isolated from healthy donors (n=9) and enriched or not with 7% of autologous Treg. Mice also received, or not 4 injections of 1g3 or IgG1 (20µg) at days 0, 7, 14 and 21 post xenotransplant. All mice received CpG ODN 1826 (130-100-103 Miltenyi Biotec France) to activate PBMCs. 5 groups in total were obtained: Nothing: Mice which only received the tumor without PBMCs not antibodies. PBMC: Mice which received the tumor with 50 million PBMC. PBMC+Treg: Mice which received the tumor with 50 million PBMCs enriched with 7% Tregs. PBMC+Treg+iso: Mice which received the tumor with 50 million PBMCs enriched with 7% Tregs with injections of the isotypic control of the antibody. PBMC+Treg+1g3: Mice which received the tumor with 50 million PBMCs enriched with 7% Tregs with injections of the anti-Gal9 antibody. At the end of the experiment tumors were collected for IHC analysis.

Figure 10: Immuno-histochemical analysis.

Following mice sacrifice, the infiltration of CD4+ human lymphocytes was analyzed by immunohistochemical analysis. A. Microscopic photographs of a tumor from a non-reconstituted mouse clearly on the blue counter stain is observed without the presence of human CD4+ cells (400x). B. Photo of a tumor slide from a PBMC reconstituted mouse showing clearly in brown the presence of tumor infiltrating CD4+ cells (400x).

Figure 11: Tumor growth curves NPC tumors

The tumoral growth was followed by bioluminescence and manual measurements throughout the experiment. Tumoral growth curves are presented as relative growth following normalization. The value obtained for the Group of PBMC+Treg+anti-Gal-9 ab at the 24th day was used as the reference point 1. The relative tumor size or bioluminescent signal at every measurement point was calculated for every group of mice corresponding to a different donor. The results were averaged between the different groups to generate the following graphs. The statistical difference are presented and as follow. *: PBMC+Treg+iso vs PBMC+Treg+anti-Gal-9. ¤: PBMC+Treg+1G3 vs Nothing. #: PBMC+Treg+1G3 vs PBMC+Treg. A. represents the relative tumoral growth calculated using manual measurements. B. Represents the relative tumoral growth calculated using bioluminescence measurements. Total of 9 healthy donors and 40 mice Error bars represent standard deviations. P values Mann-Whitney test.

Figure 12: visual representation of tumor growth and size

A. Overlays of photographic and luminescent images following a representative group of mice throughout the 24 day test. Both mice received Treg enriched PBMC reconstitution. The mouse on the left (1g3 had been treated with the anti-Gal-9 antibody while the mouse on the right represents the mouse which had received the isotype. B and C. Tumor photographs following mice sacrifice and tumor resection. The tumors in every figure originated from the same group. The four tumors in the 4 photos were obtained from mice that had received the Treg enriched PBMCs. The mouse on the left only received PBMC+Treg without any antibody while the mouse on the right received PBMC+Treg+anti-Gal-9 ab. The tumors are posed against a measuring paper where 1 square is 1 square cm. The mouse on the left only received PBMC+Treg+isotype while the mouse on the right received PBMC+Treg+anti-Gal-9 ab. The tumors are posed against a measuring paper where 1 square is 1 square inch.

Figure 13: Tumor growth in MDA mice

A. The tumoral growth by manual measurements was followed during the experiment. Tumoral growth curves are presented as relative growth following normalization. The value obtained for the Group of PBMC+Treg+anti-Gal-9 ab at the 29th day was used as the reference point 1. The relative tumor size or at every measurement point was calculated for every group of mice corresponding to a different donor. The results were averaged between the different groups to generate the following graphs. Total of 8 mice were done for 2 different donors. B. Following mice euthanasia the recovered tumors mass

was recorded. The results were normalized for every donor and presented as relative tumoral mass. Error bars represent standard deviations.

Figure 14: Chimeric antibody test

Proliferation of Tconv following 72 hours of culture measured by incorporation of radioactive thymidine and presented as (counts per minute). A. The ability of the chimeric antibody to antagonize rGal-9 inhibitory function was demonstrated. Gal-9 exhibited strong inhibition of Tconv proliferation. This effect was inhibited by either 3 μg/ml of 1g3, chimeric IgG 1 (Ch1) or chimeric IgG2 (Ch2). The isotype had no effect. Chimeric IgG4 (Ch3) had a lower antagonistic function. B. The ability of the chimeric antibody to antagonize Treg inhibitory function was demonstrated by MLR proliferation tests. Tregs suppressed the proliferation of Tconv. The use of 1g3 or the chimeric antibodies was able to antagonize this suppressive. The isotypic control had no effect. N=2 donors. Error bars represent standard deviations.

Figure 15: Humanized antibody test

Proliferation or Toxicity of Tconv was measured following a 72 hour culture. All the tested antibodies, 1g3, chimeric of humanized (Hab1-8) were able to antagonize rGal-9 induced suppression measured by both techniques, the isotypic control had no effect. Toxicity was tested by the same techniques and the antibodies did not have any significant effect. N=2 donors Error bars represent standard deviations.

Patent: Antibody Which is Directed against Galectin-9 and is an Inhibitor of the Suppressor Activity of Regulatory T Lymphocytes

The invention relates to an antibody which is directed against galectin-9 and functions an inhibitor of the suppressor activity of regulatory T lymphocytes, and also to the use of this antibody for the treatment of diseases associated with the suppressor activity of regulatory T lymphocytes, in particular the treatment of cancer.

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(54) Title : ANTIBODY WHICH IS DIRECTED AGAINST GALECTIN-9 AND IS AN INHIBITOR OF THE SUPPRESSOR ACTIVITY OF REGULATORY T LYMPHOCYTES

(54) Titre : ANTICORPS DIRIGÉ CONTRE LA GALECTINE 9 ET INHIBITEUR DE L'ACTIVITÉ SUPPRESSIVE DES LYMPHOCYTES T RÉGULATEURS

(57) Abstract : The invention relates to an antibody which is directed against galectin-9 and is an inhibitor of the suppressor activity of regulatory T lymphocytes, and also to the use of this antibody for the treatment of diseases associated with the suppressor activity of regulatory T lymphocytes, in particular the treatment of cancer.

(57) Abrégé : L'invention concerne un anticorps dirigé contre la galectine 9 et inhibiteur de l'activité suppressive des lymphocytes T régulateurs, ainsi que l'utilisation de cet anticorps pour le traitement de maladies associées à l'activité suppressive des lymphocytes T régulateurs, notamment le traitement de cancer.

(12) International Application Status Report

Discussion

EBV peptide vaccine

Cancer vaccinations are a crucial step in cancer prevention and therapy. In fact, during his one year report for the "*Moonshot Initiative*", the vice president of the U.S.A insisted on the importance of cancer vaccines as a *"as a safe and effective strategy for combatting various types of cancers".* In the report he mentioned to expand the use of HPV vaccines as a preventative method. EBV vaccines on the other hand are lagging behind. At the current time, there is one EBV based NPC vaccine that has shown promising results and has currently moved into phase II clinical trials. The effectiveness of this vaccine will be determined with time. One interesting fact is that for this virus vaccination strategy, the researchers chose to use the full length EBNA1/LMP2 fusion protein. The use of full length proteins has been proven to not be the optimal approach in vaccinations. These proteins could contain weak antigenic sites which are detrimental for an effective immune response (617, 755).

One deterrent to previous vaccines designs is that the strategies focused on stimulating a strong CD8+ CTL response without paying attention to the activation of CD4+ TH1 cells. The importance of CD4+ cells in the vaccine response has been known for a while CD4+ cells are crucial for a successful vaccine response (756). Nevertheless, multiple vaccination strategies have used short, exact HLA class I–binding peptides only capable of inducting a short termed CD8+ response which is quickly exhausted (521). Peptide vaccinations using short peptides HLA class I binding peptides which are small enough to not require processing by professional APCs will randomly bind to all cells having surface HLA class I. Therefore, these molecules will be presented in large numbers on various APCs especially the semi-mature ones without the necessary costimulatory molecules. This sequence of events is exactly the sequence which leads to tolerance, exhaustion and iTreg generation (757-759). This is one of the more crucial advantages of the use of our peptide strategy. By using HLA class II promiscuous peptides we are able to insure a strong activation of the Th1 response which can maintain the following CTL response.

Another important aspect of vaccination strategies is the ability of inducing a strong memory response. In fact, the memory T cells have been shown to be more effective than transiently activated CTLs in controlling tumor growth (760). Using ex-vivo patient PBMCs the peptide vaccine has shown its ability to stimulate IFNγ from patient memory cells. This is a starting step in the showing the possible success of such a peptide vaccination strategy in the treatment of NPC.

Moreover, even though virus based cancer vaccines do not suffer from the main problem of having to use TAA which could be of low antigenic potential due their expression in the thymus, they still have to overcome the other immunosuppressive challenges imposed by the TME. These include anergy and exhaustion due to chronic exposure to viral antigens. Different suppressive cells such as Tregs and everything else we saw in the introduction. Of special mention in the case of NPC are tumoral

exosomes and Gal-9. We had previously published that these exosomes have the strong immuno suppressive potential. In particular they were especially effective in inhibiting the proliferation of Th1 cells. The same was shown for Gal-9. These exosomes could also expand and recruit Tregs. In the vaccine article we were pleasantly surprised to find out that the CD8 depleted PBMCs that were pulsed with the peptide cocktail were resistant to both exosomes and Gal-9 mediated suppression. The exact molecular changes that made this possible are still to be determined. However, in more global context we could theorize that the peptide cocktail was able to generate a strong and persistent immune response that overcame classic exhaustion mechanisms which would have made the activated cells susceptible to suppressive effects of exosomes and Gal-9.

Blocking Tregs is cancer is as important as it is difficult.

To date, the role of Tregs in cancer is still of great debate. This is understandable as whenever the subject of Tregs in cancer is evoked it is associated with the prognostic value of Foxp3+ T cells in cancer. As the introduction has shown this value is inconsistent and different studies have shown contradictory results sometimes in the same cancer. This problem is partly due to the fact that Tregs cannot be narrowed to Foxp3 expression. It is completely true that Foxp3 is the master regulator of suppressive activity in mice Tregs but this is far from true for human (172). In fact, hardly anything in the human immune response is as black and white as is it is in the mouse immune system (761). This is to be expected, since the general rule is, as the complexity of the organism increases, as do intricacies of the immune system put in place to protect it (762, 763). Back to Foxp3 in cancer; a human effector CD4+ or even CD8+ T cell can express Foxp3 and maintain its full effective antitumoral activity. So does this mean that all the results that looked at Foxp3 expression in tumors are irrelevant? Not really, these have provided the basis of the data required to push the understanding of the role of regulatory T cells in cancer.

A recent diagnostic clinical study failed correlate the expression of Foxp3+CTLA4+ cells in different cancer with a prognosis value however when they looked at LAP/GARP expression, these Tregs were found to correlate with a poor prognosis (219). This does not mean that we should question every cancer study that correlated an increase in Foxp3 expression with a positive prognosis. Certain types of cancer simply profit from the presence of inflammatory environments as drivers for their growth, hence in these tumors targeting Tregs would not beneficial. In other cancer types which, once established, actively induce an immunosuppressive environment to support their growth, Tregs pose a major hurdle against recovery that the medical and scientific community still has to overcome (764). By Tregs in this discussion I mean T cells expressing an ensemble of regulatory markers and who have as a function the suppression of the anti-tumoral immune response. The first step in overcoming the Treg hurdle is to identify which Tregs are predominant in the cancer in question. This characterization of cancer specific Tregs is already on its way. One example was found in COX-2 expressing HNSCC. Patients with this kind of tumor suffer from an increase in the PGE2 expressing infiltrating iTregs which are

correlated with a poor prognosis (101). Nowadays, researchers have identified a rather significant number of suppressive makers which could be expressed by Tregs and could play a part in its suppressive activity. So the obvious starting point would be to identify the predominant Treg type or fraction in different tumors. Technological advancement has reached a point where such studies are feasible. Technologies exist which allows to look at the transcriptome of immune cells extracted from the tumor one cell at a time all (765). At the microscopy and cytometry level, the world of fluorochrome conjugated antibodies with the tools required to observe them has exploded over the past few years. We can simultaneously stain and detect most of the known Treg suppressive markers at the same time (766). The challenge now is to actually start employing these tools in creating databases of the Treg subtypes most likely to be found in various cancer at different stages in the hopes of identifying the necessary targets to block in order to go from immune suppression to immune promotion.

Identification is only the first step, inhibition is more complicated.

CD25 has been identified as a marker of regulatory T cells since 1997, however over 30 years later we cannot say that we managed to inhibit Tregs *via* CD25 in an efficient and safe manner. Clearly something has gone wrong. So exactly how to inhibit a regulatory T cell? There are 4 approaches. Depleting Tregs, inhibiting their function, preventing their trafficking to the tumor and using their own plasticity against them (115).

Depleting Tregs

The most common approach in humans would be to target cell surface antigens using antibodies which can either induce ADCC by themselves (IgG1) or by coupling the antibodies to certain toxins. History has shown that this is not the best approach. The anti-CD25 antibody which successfully depleted Tregs also induced autoimmune reactions and inflammatory diseases in the targeted patients (464). This is to be expected. Tregs are crucial in holding the immune system under control and the idea of depleting without any side effect is too farfetched. One argument behind the failure of this technique could be the fact that the target was too generalized. CD25 is expressed on activated T cells and Tregs alike. So maybe with the choice of a better marker would be a better choice. The problem is that all the currently known suppressive markers which are expressed on Tregs are equally expressed on activated/exhausted conventional T cells (767). This makes the use of depleting antibodies a very dangerous proposal since it may lead to the depletion of the same cells we are trying to save from immune suppression.

Transitioning to Ipilimumab. A reminder that Ipilimumab is human igG1 antibody against CTLA4. Some have theorized that part of the success of Ipilimumab is due to its ability to deplete Tregs in tumors. However the data supporting this claim are scarce, controversial and inconclusive. The few clinical trials which investigated this effect have shown that the patient peripheral Foxp3+CD25+ population following treatment did not changed and was still suppressive (518). This result is to be

expected since an anti-CTLA4 antibody would not deplete CTLA-4 negative Tregs. nTregs in the periphery upregulate their CTLA-4 expression following stimulation (172). Assuming that the antibody did have a depleting role and it was capable of depleting CTLA-4 positive cells. This would mean that it also depleted exhausted Tconv cells and iTregs at environmental interfaces that are known to be CTLA-4 positive (768). This could explain the toxic immune reactions that are being observed with certain patients being treated with Ipilimumab (474). Regardless of all this, the antibody is working. Not at the same efficiency that was observed in pre-clinical studies but it is working. It is possible that the antibody simply blocked the CTLA-4 pathway in both Teff and Treg. We have seen that Tregs require the engagement of the CTLA-4 pathway to maintain their activation. Exactly why it worked in some patients and not others is starting to be explored. Regardless the reason, the therapeutic value of Ipilimumab is impressive when compared to chemo, radio and targeted therapy but is still lacking when compared to PD-1 blockers. Not only have non-depleting anti-PD-1 antibodies shown higher effectiveness, they have also proven less toxic. Simply put, using antibodies capable of depleting targeted immune cells is not the best option especially that we now know that we can simply revert the exhausted state in Tconv and, due to Treg plasticity, push a Treg cell into a pro-inflammatory pathway (769).

Targeting Treg induced suppressive mechanisms.

This can either be done by blocking certain inhibitory receptors on the Tregs (latent TGFβ, CTLA-4, Lag-3) or by blocking immuno-suppressive molecules secreted by Tregs (TGFβ, IL-10, IL-35). Unlike a depleting antibody, an antagonistic one does not have the capacity to induce ADCC. Instead, it blocks the signaling cascade of a certain pathway by binding one of its elements at a key site which is essential for the activation of this pathway. One example is the anti-Lag3 antibody which is considered an antagonist since it binds the Lag-3 receptor at its interaction site with costimulatory molecules. This negates the receptors ability to bind its target. Which means that this suppressive function is no longer available for Tregs to use and hence Tregs have one less tool in their arsenal. Similarly the anti-TGF-β anti-body is capable of binding the TGF-β soluble ligand and preventing its interaction with its target. One of the problem with this technique could be that the receptor has redundancies with respect to activating signals. This means that sometimes the receptor can be activated by multiple signals hence making its ability to be antagonized by a single antibody impossible. This brings me to Tim-3. Tim-3 is a very large receptor. The current anti-Tim-3 antibody is designed to target the Gal-9 binding site on Tim-3. This should result in the inhibition of the Tim-3 induced death/exhaustion pathway on T cells. While this is impressive, it might not be enough, recent data suggests that Tim-3 has multiple potential ligand binding sites all of which are capable of triggering the signaling cascade leading to its activation (359, 770). This means that this approach can prove to be lacking as despite antibody fixing the receptor, the pathway would still activate. Targeting soluble ligands in immuno-regulatory pathways could prove more interesting, unlike receptors, ligands tend to be small molecules much more easily sequestered by antibody binding strategies. The only problem when targeting ligands is finding the correct ligands to

target which are important for Treg induced suppression. There are not that many soluble targets involved in Treg induced suppression. The main ones are TGFβ, IL-10 and IL-35. The problem is that these cytokines are crucial for immune system homeostasis and inhibiting them would inevitably lead to immune related disease in treated targets mainly at the level of immunological interfaces. By proving that Gal-9 is an immune-suppressive molecule used by human Tregs we were able to find another target for a new antagonistic approach. We also showed that blocking Gal-9 with a Mab was able to hinder to a certain extent Treg induced suppression. Of course, an anti-TGFβ antibody has been shown to do the same thing so why opt for blocking Gal-9. Well TGFβ KO mice usually die from lymphoproliferative disease, but Gal-9 KO mice are healthy. Meaning that unlike TGFβ, Gal-9 is not crucial for maintaining homeostasis in non-pathological conditions. In fact the only steady state expression of Gal-9 was found in the peripheral cells of the cornea of mice. As the introduction has shown, Gal-9 in humans is mostly used to regulate the immune response in viral infections. As we have seen, the down regulation of the immune system which is observed in cancer, *via* Tregs and exhaustion pathways, is very similar to that seen in viral infections. Therefore, not only does Gal-9 have important immune suppressive functions in the TME, these functions are specific to the site of the chronic inflammation. So it is fairly possible that Gal-9 blocking would be an efficient immunotherapeutic approach with minimal toxic effects. Nevertheless, many more tests are required before this can be confirmed.

Targeting Treg plasticity and stability.

The scientific community is only starting to understand the role of Induced Tregs also known as peripheral Tregs in cancer. It seems that these cells carry strong suppressive mechanisms and play very important roles in controlling the tumoral microenvironment. Admittedly the mechanisms of generation of induced Tregs *in-vitro* are not fully deciphered but we know that they depend on TGFβ for the generation of these cells (771). Even more, new data suggests that TGFβ is important for maintaining the Foxp3 expression in Tregs and hence their suppressive potential. Again the importance of antagonizing TGFβ to reverse the immunosuppressive microenvironment are even more evident. Yet as we previously explained this is too dangerous (772, 773). Fortunately for us, Gal-9/CD44 interaction has been shown to enhance TGFβ signaling in Tregs. As explained before this interaction, *via* Gal-9 ability to dimerize, clustered the TGFβ receptor and enhanced its downstream activity which was shown to induce stronger Foxp3 expression. With stronger Foxp3 expression we can expect a more powerful activation of the iTreg suppressive program as it was shown by Kuchroo's team. The most important part in this process at least for the anti-Gal-9 project is the fact that this Foxp3 stabilization function does not seem to be important in nTregs at least in the mouse model studied by Kuchroo's team. Whether this is the case for human Tregs is still to be validated. Hypothetically speaking and assuming that the model works in the same way in a cancer patient. We expect that administering the Gal-9 antibody would be able to destabilize this suppressive population of iTregs that has developed in the tumoral microenvironment by interfering with Foxp3 expression. This again in theory would decrease the immunosuppressive load in the TME. Now the fact that the Mab does not affect nTreg

Foxp3 expression we would expect the nTreg population which is precious for homeostasis to remain intact and functional where it needs to be mainly at environment interfaces. Going further, this would mean lower overall toxicity in the patient. However, this does mean that tumoral Mab does not touch tumoral nTregs. Our experimental protocol clearly shows that nTreg induced suppression was inhibited by the Mab. This is more likely due to the fact that Gal-9 secreted by nTregs is by itself suppressive capable of inhibiting the proliferation of other immune cells mainly Th-1 and CD8+ cells. Meaning that the Gal-9 antibody would affect the TME by "attacking on two fronts" both the nTreg and iTreg population at different levels. The major question now is exactly how important is Gal-9 induced suppression by nTregs at environment interfaces and what kind of toxic effect can we expect? Well, according to mouse models, not so much, but before we get to human trials; much like with every other Immunecheckpoint inhibitor on the market; we will not know.

Why develop anti-Gal-9 antibody when an anti-Tim-3 antibody is in clinical trials?

This question has arisen several times during this thesis. This discussion provides a perfect opportunity to detail an answer. As we have seen before, Tim-3 is a large receptor which can be activated by multiple ligands and not just Gal-9 (359). In fact recent data has shown that the simply the presence of the cytoplasmic tail of Tim-3 without Gal-9 binding is enough to inhibit the activation of TCR activated human CD8+ T cells (774). Hence using an antibody which simply blocks Gal-9 binding to Tim-3 will not inhibit the brood range of Tim-3 functions. On the other hand Gal-9 is a small molecule with numerous targets. Using Gal-9 antibodies to sequester soluble Gal-9 should in theory provide a much broader array of biological effects. We have already discussed the importance of the potential inhibition of the Gal-9/CD44 pathway to inhibit iTregs yet there are other important pathways still less defined. Going back to the original set of experiments by Kuchroo's team, they found that Tim-3 KO Th1 cells did not fully escape Gal-9 induced apoptosis. Indicating the potential of a second Gal-9 death receptor (350). More evidence of the presence of a second death receptor was shown by treatment of Jurkat cells with Gal-9S, as explained before these cells underwent Tim-3/Caspase-1 independent cell death (364). Another set of experiments done on mice Th1 cells showed that Gal-9 could induce apoptosis even without Tim-3 expression (775). This has even been validated by even other experiments (334). Outside its apoptotic ability, Gal-9 immunomodulatory potential of other immune cells did not always depend on Tim-3. Gal-9 enhancement of MDSCs seems to pass by both Tim-3 dependent and Tim-3 independent pathways (410, 411). Also part of Gal-9's inhibition of NK cell cytotoxicity seems to be Tim-3 independent (412). Taken together, these results would indicate that blocking Gal-9 rather than Tim-3 would most likely have a much greater impact on the immunosuppressive TME by inhibiting Tim-3 dependent/independent apoptosis, CD44 dependent iTreg induction, Tim-3 independent MDSC induction and Tim-3 dependent/independent NK exhaustion. An upcoming step in this project will be to determine exactly which Gal-9/receptor binding is being inhibited by the anti-Gal-9 antibody using Biacore.

A second point to discuss is the advantage of blocking a soluble protein vs a receptor. Nowadays, several techniques are employed to ensure that antibodies do not induce CDC nor ADCC. The current anti-Tim-3 antibody is an IgG4 antibody which means that it is unlikely to induce CDC nor ADCC. However, it is becoming more and more evident that the simple act of cross linking a receptor on a cell can induce a signaling cascade leading to cell death. This pathway involves cytoskeleton reorganization, lysosomal activation, and production of reactive oxygen species (776, 777). As explained before the point of immune-checkpoint inhibitors is to reverse the exhausted state of the anti-tumoral TILs and not kill them. Given that Tim-3 is expressed on activated anti-tumoral immune cells, the risk of cell death simply due to antibody binding does increase. This is why targeting a soluble factor in this case Gal-9 would be a more prudent approach.

Controversy of Gal-9 membranous expression.

Does Gal-9 exist in a membranous form? If yes will the humanized IgG1 antibody risk inducing lysis of cells expressing Gal-9 on their surface? In response to the first question, yes Gal-9 does exist on the membrane. Disregarding flow cytometry data, most teams that worked on purifying Gal-9 by ultra-centrifugations were able to detect Gal-9 in the membrane fraction. This is to be expected. Gal-9 probably exists as an intermediate during its secretory process which could be found on the membrane. Whether this intermediate is functional we do not know yet. This would also mean that Gal-9 probably does exist on the surface of Tregs. Does this mean that the anti-Gal9 antibody can induce ADCC. Well this is where the controversy starts. Several teams were able to use anti-Gal9 antibodies in flow cytometry to detect Gal-9 by cell surface staining. Yet this detection with the available antibodies was never homogenous on all the cells in culture. Meaning that some cells stained positive for cell surface Gal-9 while other stained negatively. Taking Jurkat cells into question. When I stained Jurkat cells for cell surface Gal-9 I obtained a positive staining which was fairly similar to that obtained by Hirashima's team. However, I took the experiment one step further and found that all the cells which stained positive for cell surface Gal-9 stained positively for 7AAD or propidium iodide both of which are used of the detection of dead cells. Does this mean that Gal-9 can be used to detect dead cells? No it most probably means that since the cells are dead, the antibody gave a false positive signal due to non-specific binding (778) . In fact, the results that we demonstrated in the article on the membranous expression of Gal-9 on nTregs and Tconv were obtained after removal of 7AAD positive cells. Since had we kept the 7AAD positive cells, then we would have observed a population with false membranous Gal-9 staining (data not shown). So Gal-9 exists in the membrane yet we cannot detect with the antibody, why? Two explanations are possible the first being that the theorized Gal-9 intermediate during secretion exists mostly on the inner side of the membrane which is why we cannot detect it. The second explanation is that whatever protein is carrying Gal-9 blocks its ability to bind antibodies. The second explanation is less likely since we attempted to use 4 different antibodies each targeting different fractions of Gal-9 and none of them were able to positively stain known Gal-9 secreting cells whether it were Jurkat

cells or Tregs. Will the Gal-9 IgG1 Mab generate ADCC. Given that the mouse IgG1 that we used was not able to bind cell surface Gal-9 then we can expect that the humanized version also can not bind cell surface Gal-9 which would mean that ADCC is not likely. This is not necessarily bad news. Since again as we explained before ADCC is often not the best approach when it comes to immune cells, especially since Gal-9 was found in the membranous fraction of monocytes in their maturation process and killing these cells would be detrimental to a successful anti-tumoral reaction. Having said that, at this point in time we have not tried to stain pDCs, which we can differentiate in our team for membranous Gal-9. This will be a crucial part in the upcoming tests of the antibodies and does present part of the perspectives for the project. With any luck we should be able to obtain these results by the time of the thesis defense.

A few thoughts on the potential blocking MDSCs and MSCs

It is becoming more and more evident that MDSCs are important immuno-regulatory cells in the TME (779). It is true that this population is better described in mice but there is enough evidence of its existence in humans. As we have seen in the introduction, Gal-9 has been found to be expressed by mouse MDSCs (410). Overexpression of Gal-9 in mice lead to the expansion of this population with higher suppressive potential. Another study found that MDSCs treated with rGal-9 had higher suppressive potential. Interestingly, knocking out Tim-3 did not affect the suppression promoting effect of Gal-9 on MDSCs. This means that Gal-9 expanded and activated suppression induced by this population in a Tim-3 independent manner giving the anti-Gal-9 Mab a new potential function in reversing the suppressive TME which would not be observed by using an anti-Tim-3 (411).

With regards to MSCs, this population is more interesting since current data on the role of Gal-9 in this population is obtained from human donors. MSCs much like Tregs became known for their regulatory potential *via* the MLR test. Also much like the results we obtained on Tregs, researchers found that this population secretes Gal-9. They also showed that knocking down Gal-9 in these cells by siRNA decrease its suppressive potential in MLR tests. Since this population is also known to play an important regulatory role in cancer it could be another target to be tested against the Gal-9 Mab (409, 422).

Determining whether or not the use of the anti-Gal-9 Mab could inhibit the suppressive potential of these two immune cell population is another future perspective which could help push our understanding of the potential pharmacological effects of anti-Gal-9 Mab.

Gal-9 expression in cancer.

As we saw in the introduction, more articles correlated an increase of Gal-9 expression by the tumor with a better cancer prognosis rather than the other way around. In fact, the view of Gal-9 as an apoptotic factor had dominated the literature for a long time (329). Taking into consideration the given information at the time and before understanding its immunoregulatory role, it was normal to imagine

that Gal-9 as a factor capable of inducing cell death would be down-regulated by cancer cells. It is true that Gal-9 induced deaths requires the expression of the correct receptor on the cancer cell for Gal-9 to induce its apoptosis but since the scientific community has not identified all of the Gal-9 death receptors, this remains a possibility. So studies on the intra tumoral expression of Gal-9 by IHC in breast-cancer, non-small cell lung cancer and gastric cancer showing that increase in Gal-9 expression in the tumor correlates with a better prognostic value for the patient with a link to decreased metastasis (434) (436). On the other hand we see that in every cancer where Gal-9 circulating serum levels were studied, an increase in Gal-9 levels correlated with a poor prognosis. Melanoma is one particular example. One study concluded that an increase in the Gal-9 intratumoral expression at the early stages correlated with a better prognosis as it decreased metastases, another study found intratumoral Gal-9 to exist in about half the studied tumors yet, the circulating levels of Gal-9 correlated with a poor prognosis (453). These are interesting results since at the first glance they would indicate contradictory results in 2 types of cancer yet with closer observations we find that these studies are complementary. A potential explanation would be that tumors use Gal-9 in two ways to favor their growth. Inside the tumor, Gal-9 is suppressed as it is an apoptotic factor, however in the tumoral peripheries where Gal-9 can be directly secreted to interact with the immune cells in TMEs it is upregulated explaining the increase in circulating levels of Gal-9. This theory is in fact supported by work in NSCLC, the authors found a decrease in intra tumoral Gal-9 levels which was coupled with an increase of Gal-9 in the paratumoral tissue thus favoring the theory of localized expression of Gal-9 in tumors (441). One possible factor behind this location based control of Gal-9 is hypoxia. Currently we are testing the effect of hypoxia on the expression of Gal-9 by tumoral and immune cells in relation with the expression of HIF-1α.

Tregs in vaccinations: the two projects converge

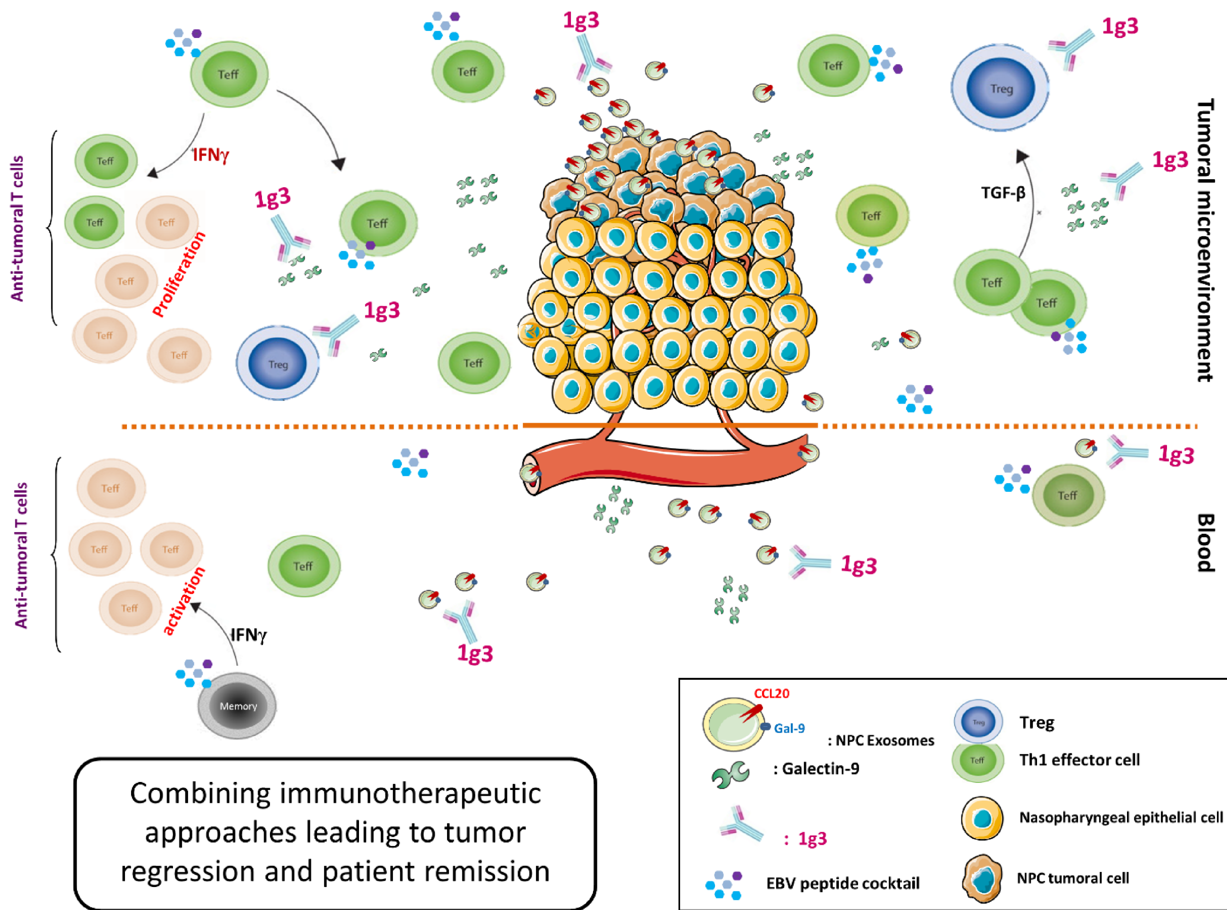
One important notion in the study of Tregs which has not fully matured is that of antigen specificity of Tregs. Recent data suggests that nTregs are developing thymic cells that have a certain affinity for TCR engagement yet are not selected for cell death. This expression of intermediate TCR affinity by Tregs has been elegantly shown in GFP transgenic mice designed to vary their GFP signal strength as a function of TCR engagement. This model has shown that nTregs had a stronger signal than Tconv yet weaker than the threshold for thymic induced cell death as part of the negative selection process (780). The duration of the TCR engagement seems to be the key factor in inducing the epigenetic hypomethylation of the Foxp3 promoter region which are crucial for nTreg development (92). Similar processes seem to govern the generation of iTregs in the periphery (781). This is important since vaccine antigen specific Tregs are becoming more and more recognized as a major obstacle for vaccination efficacy. One study in a melanoma vaccination clinical trial using MHC class I restricted antigens or melanoma tumor lysate-loaded APCs associated the failure of the vaccination approach with the expansion of the Treg population (782). A more recent study on an HIV vaccination attempt found that it was in fact the HIV antigen specific Tregs co-expressing

CD4+CD134+CD25+CD39+FoxP3+ and not the total Treg pool that were responsible for inhibiting an effective vaccine response (783). Given that our vaccination approach used highly promiscuous HLA class II antigens, in theory, we should not experience the problem of iTreg generation but this is still to be determined by future experiments. If this is not the case and the strategy does ultimately generate Tregs, then a combination of the anti-Gal-9 Mab with the peptide vaccines would be interesting to test. The idea of combining Treg targeting approaches with vaccination strategies is not new as it has been tried without much success for breast cancer and melanoma (513, 514). However, we hope that in this case the specificity of the Gal-9 blocking strategy in a Gal-9 rich tumor would synergize well enough with the peptide vaccine to have a significant effect of tumor size. In our in-vivo model the proposed therapeutic approaches apart have shown their efficacy in controlling the tumoral growth but not in eliminating the tumor. In the future, we hope that by administering a combined treatment of vaccine and anti-Gal-9 Mab in humanized mice where the tumor has been allowed to grow beforehand we can not only limit tumoral growth but effectively reduce tumoral size and maybe induce its complete elimination.

Conclusion

In this thesis we defined a new mechanism by which tumoral exosomes regulate the immune response in the tumoral microenvironment of NPC. Then we moved on to show that using a proper vaccination strategy based on HLA class II promiscuous EBV peptides we can induce a successful anti-tumoral immune mediated by Th1 cells, CTL and memory cells and that this response is also resistant to the suppression induced by tumoral exosomes. In parallel given that we had also published that Galectin-9 carrying tumoral exosomes induce Tregs in the tumoral microenvironment. We moved on to demonstrate the importance of Gal-9 in Treg function and showed that specific anti-Gal-9 antibodies can inhibit Treg induced suppression. Via various in-vitro and in-vivo tests we arrived at the conclusion that Gal-9 targeting could be a viable and effective cancer immuno-therapeutic approach. The advantage of Gal-9 blocking *via* monoclonal antibodies is the fact that we are capable or reversing the immunosuppressed state observed in cancer patients *via* targeting a secreted marker responsible for immune suppression instead of targeting directly the Treg population. Since Gal-9 is over expressed only in pathological inflammatory conditions, this could mean that the monoclonal antibodies would have minimal side effects on the patient.

These two strategies between vaccination and inhibition of immune checkpoint affect the tumoral micro-environment on two fronts. The vaccine activates the immune response against viral antigens on the tumor and the anti-Gal-9 Mab helps negate all the breaks which hinder the anti-tumoral response.


Teff
IFNγ
Anti-tumoral T cells
Proliferation
1g3
Treg
TGF-β
Tumoral microenvironment
Blood
activation
Memory
Combining immunotherapeutic approaches leading to tumor regression and patient remission
CCL20
Gal-9
: NPC Exosomes
: Galectin-9
: 1g3
EBV peptide cocktail
Treg
Th1 effector cell
Nasopharyngeal epithelial cell
NPC tumoral cell

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List of Publications

Published

Mrizak D, Martin N, Barjon C, Jimenez-Pailhes AS, **Mustapha R**, Niki T, Guigay J, Pancre V, de Launoit Y, Busson P, Morales O, Delhem N. Effect of Nasopharyngeal Carcinoma-Derived Exosomes on Human Regulatory T Cells. Jnci-Journal of the National Cancer Institute. 2015;107(12):1

Delhem N, **Mustapha R.** RE: Effect of Nasopharyngeal Carcinoma-Derived Exosomes on Human Regulatory T Cells Response. Jnci-Journal of the National Cancer Institute. 2015;107(1):13

Morales O, Mrizak D, Francois V, **Mustapha R,** Miroux C, Depil S, Decouvelaere AV, Lionne-Huyghe P, Auriault C, de Launoit Y, Pancre V, Delhem N. Epstein-Barr virus infection induces an increase of T regulatory type 1 cells in Hodgkin lymphoma patients. British Journal of Haematology. 2014;166(6):875-90

Submitted

Mrizak D*, **Mustapha R***, Renaud S, Rafa H, Jerraya H, Martin N, Liu F, Niki T, Kwok-Wai Lo, Pancré V, Morales O * and Delhem N *. Tumor-derived Exosomes do not Disturb a Novel EBV Peptide-based Immunotherapy for Nasopharyngeal Carcinoma. *Waiting for editor response from Cancer Discovery*

In preparation

Mustapha R*, Mrizak D*, Renaud S, Barjeon C, Pancré V, Busson P, Moralès O and Delhem N. Evaluation of Galectin-9 blocking mono-clonal antibodies as novel immune-checkpoint inhibitors via the targeting of regulatory T cells in cancer.

Patents:

Abstract: Antibody which is directed against galectin-9 and is an inhibitor of the suppressor activity of regulatory T lymphocytes
International Application Number: PCT/FR2015/051498
Publication Number: WO/2015/185875
Filing Date: June 5th, 2015
Publication Date: December 10th 2015
Authors: Delhem N., Busson P., Morales O., Barjon C., Mrizak D., Lhuillier C., **Mustapha R**.

Annexes

I. Article 4: Epstein–Barr virus infection induces an increase of T regulatory type 1 cells in Hodgkin lymphoma patients

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Context and objectives

In about 50% of patients with Hodgkin's Lymphoma (HL), the Epstein-Barr virus (EBV), an oncogenic herpes virus, is present in the neoplastic cells. An immunosuppressive environment is currently described in HL patients; however, very little is known concerning regulatory mechanism induced by the expression of EBV proteins in tumoral cells. So, we intended here to investigate an association between regulatory T cells and EBV tissue positivity in HL patients.

In this study, our principle objective was an eventual association between the EBV status of an HL patient and the presence of Tregs.

Methodology and results

After transcriptomic analysis of tumoral RNA samples of both EBV-positive and EBV-negative HL tumors, we found that EBV infection significantly increased gene expression of Tr1-related markers (CD4+, CD49b, CD18, LAG3) and associated-immunosuppressive cytokines (IL10, TGF-β). This up-regulation was associated with an over-expression of several chemokines known to attract Th2 and regulatory T cells (CCL17, CCL22, CCL4 and CCL2) and may evade immune surveillance by Th1 cells. Moreover, recruitment of Tr1 cells in EBV-positive HL was confirmed by immunohistochemical analysis on frozen nodes biopsies and by flow cytometry on PBMCs of EBV-positive HL patients. Finally, using both ELISA and QPCR we showed that IL-10 production, a characteristic cytokine of the Tr1 subset, was significantly enhanced in tumors and blood of EBV-positive HL.

Conclusion

In HL, an increased number of Treg is associated with the loss of EBV-specific immunity. Our results identify a mechanism by which EBV preferentially recruits Tr1 cells to the microenvironment of HL by inducing the expression of several chemokines implicated in Treg recruitment. Our data suggest that EBV expression in tumoral cells enable the escape of EBV-infected HL cells from the virus-specific CTL response.

bjh research paper

Epstein–Barr virus infection induces an increase of T regulatory type 1 cells in Hodgkin lymphoma patients

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Summary

Epstein–Barr Virus (EBV) is present in the neoplastic cells of around 20–30% of patients with Hodgkin Lymphoma (HL). Although, an immunosuppressive environment is currently described in HL patients, little is known concerning the regulatory mechanism induced by EBV proteins expression in tumour cells. This study aimed to investigate an association between regulatory Type 1 cells (Tr1) and EBV tissue positivity in HL patients. Transcriptomic analysis of both EBV-positive and EBV-negative tumours showed that EBV infection increased gene expression of Tr1-related markers (*ITGA2, ITGB2, LAG3*) and associated-immunosuppressive cytokines (*IL10*). This up-regulation was associated with an over-expression of several chemokine markers known to attract T-helper type 2 (Th2) and regulatory T cells thus contributing to immune suppression. This Tr1 cells recruitment in EBV-positive HL was confirmed by immunohistochemical analysis of frozen nodes biopsies and by flow cytometric analysis of peripheral blood mononuclear cells of EBV-positive patients. Additionally, we showed that IL10 production was significantly enhanced in tumours and blood of EBV-positive HL patients. Our results propose a new model in which EBV can recruit Tr1 cells to the nodes' microenvironment, suggesting that the expression of EBV proteins in tumour cells could enable the escape of EBV-infected tumour cells from the virus-specific CTL response.

Keywords: Epstein–Barr Virus, Hodgkin lymphoma, Tr1, IL10, chemokines.

The Epstein–Barr virus (EBV) is associated with the development of several human malignancies, including Hodgkin lymphoma (HL) and EBV-positive undifferentiated nasopharyngeal carcinoma (NPC) (Young & Rickinson, 2004). HL is characterized by the presence of the malignant Hodgkin and Reed-Sternberg (HRS) cells, which constitute only a minority of the total tumour mass and are surrounded by a rich background of T cells, B cells, macrophages, and other inflammatory cells (Depil *et al*, 2004). In approximately 20–30% HL patients, EBV can be detected in the HRS cells, in which the virus expresses a limited subset of viral genes including the Epstein–Barr nuclear antigen (EBNA)-1 and the latent membrane proteins (LMP)-1 and LMP2 (Jarrett *et al*, 2005).

Although EBV-specific cytotoxic T cells (CTLs) can be detected in HL patients and have been shown to kill LMP1 and LMP2 expressing cells *in vitro*, they are unable to eliminate EBV-infected tumour cells *in vivo* (Chapman *et al*, 2001). Several hypotheses have been made to explain the apparent inefficiency of the antitumoural immune response in HL patients. Some studies suggest that $CD4^+$ T lymphocytes producing Th2 cytokines and chemokines could probably contribute to the local immunosuppression of Th1 cellular immune response (Poppema *et al*, 1999; Skinnider & Mak, 2002). On the other hand, this failure could be caused by an increase in the recruitment of regulatory T cells (Tregs). Tregs are described as specialized immunosuppressive $CD4^+$ T cells that play an essential role in controlling

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II. Article 5: Letter to the Editor

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Nadira Delhem, **Rami Mustapha**

Context

Following a comment from Theresa L. Whiteside (University of Pittsburgh) on the JNCI article.

OXFORD

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RESPONSE

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We thank Prof. Whiteside for her interest in our work and for recognizing the quality of our studies. Unfortunately, because of *JNCI* space constraints, it was impossible to cite every publication related to our work. However, we did acknowledge the work of Prof. Whiteside and colleagues on microvesicle-induced tumor immunosuppression and cited their review (1).

We also did not claim to have discovered the effect of exosomes on regulatory T-cells. We stated in our *JNCI* paper, "Several recent publications have revealed that tumor exosomes can exert a broad array of detrimental effects on the immune system (2) ranging from apoptosis of activated cytotoxic T cells and impairment of dendritic cell maturation to induction of myeloid-suppressive cells and Treg" (1,3,4). Thus, we did not fail to credit original reports and acknowledged previously published work.

After clarifying this point, I would like to discuss Prof. Whiteside's view that our work is merely an extension of her team's work. In fact, her excellent work (5) related the effect of tumor microvesicles on Treg, but it was exclusively carried out on microvesicles derived from ovarian cancer cell lines or ascites of ovarian cancer patients. This original work cannot and should not be generalized to all cancer types. Generalizing this mechanism to all cancers could be dangerous because of the well-known differences between cancers cells. Notably, our current work on hepatocellular carcinoma-derived exosomes clearly indicates that not all tumor exosomes exhibit similar effects on Treg.

Our *JNCI* article was the culmination of many years of our work on Treg (6), as well as our collaboration with experts on exosomes (7). In this regard, we claim its originality in showing, for the first time, the effect of nasopharyngeal carcinoma (NPC)–derived exosomes on human Treg expansion and function. Note that these exosomes were obtained not only from NPC cell lines but also from NPC patients' blood. Furthermore, the novelty of our work was not only the type of cancer studied, but also that we showed for the first time that NPC exosomes carry the CCL20 chemokine, which is able to preferentially recruit human Treg into the tumor. We also used a unique humanized NPC mouse model to show the importance of blocking the CCL20 pathway to limit Treg recruitment to the tumor. Thus, the fact that NPC exosomes promote Treg immunosuppression and specifically recruit human Treg using a CCL20-dependent mechanism had never been previously published, giving us the right to claim that, "Interactions of NPC-Exo with Treg represent a newly defined mechanism that might be involved in regulating peripheral tolerance by tumor cells."

We regret that we could not cite all of Prof. Whiteside's work. However, we feel that the *JNCI* editors assessed and approved the novelty of our work carefully and fulfilled their responsibility towards their readership.

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Oral and Poster Communications

- 8 September 2016: 1st Symposium of Anti-Tumoral Immunity, Lille, France. **Oral presentation. Prize of Immune InsighT society for the Best Oral Presentation**

- 21 to 26 August 2016: 19th International Congress of Immunology (ICI), Melbourne, Australia. **Oral Presentation**

- 18 to 20 May 2016: 9ème journée scientifique du Canceropôle Nord-Ouest, Deauville, France. **Poster Presentation**

- 21 and 22 April 2016: 2nd Summer Meeting of immunotherapy and graft oncology Labex IGO, Nantes, France. **Oral presentation.**

- 14 Septembre 2015: 15th Journée André VERBERT. **Oral presentation**

- 6 to 9 September 2015: 4th European Congress of Immunology Vienna 2015. **Poster presentation**

- 10 to 12 Juin 2015: 8ème journée scientifique du Canceropôle Nord-Ouest, Deauville, France. **Poster Presentation**

Training

- **September 2014**: Formation for animal experimentation Level 2 "From genetic modification to functional exploitation.

- **2013-2014**: French courses validated the C1 level.

- **November 2013**: Flow-cytometry formation at BICEL imaging platform.

French Summary

TRAVAIL PRINCIPAL

Evaluation d'anticorps monoclonaux bloquant la Galectine-9 en tant que nouveaux immune checkpoint inhibitor-*via* le ciblage des cellules T régulatrices dans les cancers.

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Introduction

Les lymphocytes T régulateurs (Treg) sont une sous-population de cellules immunitaires jouant un rôle crucial dans la maintenance de l'homéostasie immunologique, la prévention des inflammations chroniques et l'apparition de réponses auto-immunes. De nombreuses études ont confirmé la présence d'un niveau élevé de Treg, circulant et localement infiltrés, chez des patients atteints de cancer. Ces niveaux élevés de Treg sont associés à un mauvais pronostic. C'est pourquoi plusieurs immunothérapies anti-tumorales ciblant les Treg ont été développées ; certaines sont aujourd'hui en phase clinique de développement. Parmi ces études, certaines cherchent à réduire le taux de Treg (eg. anti-CCR4) tandis que d'autre tels que l'IpilimuMab (anti CTLA-4) et anti PD-1 (Nivolumab) inhibent un check-point immunitaire et ciblent les Treg.

Les approches couramment utilisées, qui se concentrent sur une déplétion ou une inhibition de la fonction des Treg, n'ont pas montré un réel succès dans tous les types de cancer dû à un manque de spécificité étant donné que les voies de signalisation ciblées sont similaires jusqu'à un certain degré à celle des lymphocytes T conventionnels (Tconv) activés. Ces approches ciblent, en effet, des molécules exprimées au niveau de la membrane plasmique qui sont présentes à la surface d'autres cellules immunitaires et qui participent activement à la réponse immune anti-tumorale.

La Galectin-9 ou Gal-9 (que code le gène *Lgals9*) est un membre de la famille des lectines associées aux β-galactosides. Cette famille est impliquée dans de nombreuses fonctions

biologiques telles que la chemoattraction, l'agrégation cellulaire et l'apoptose. La Gal-9 peut-être cellulaire, elle est alors localisée au niveau de la membrane (notamment celle des exosomes), du cytoplasme et dans le compartiment nucléaire. Cependant, elle peut également être secrétée en association ou non avec des exosomes, et retrouvée dans le compartiment extracellulaire.

L'injection de Gal-9 chez la souris immunocompétente conduit à la suppression des cytokines pro-inflammatoires et à l'augmentation du taux de Treg circulants. Par ailleurs, des études récentes indiquent également que l'administration de Gal-9 (exogène) peut réguler le développement des Th17 et des Treg. La sécrétion de Gal-9 est régulée positivement dans les conditions inflammatoires ou elle joue un rôle dans l'inhibition de la réponse immunitaire par l'induction de l'apoptose des T CD4+ Th-1 et des cellules T CD8+ cytotoxiques. Il a également été prouvé que la Gal-9 est secrétée par différents types de cellules tumorales et est associée à un mauvais pronostic.

La Gal-9 a été identifié comme un ligand de Tim-3. L'association Gal-9/Tim-3 conduit à la génération d'un signal inhibiteur conduisant à l'apoptose des Th-1 et la régulation négative de l'immunité médié par les th-1. Il a par ailleurs été montré que cette voie de signalisation Gal-9/Tim-3 joue un rôle crucial dans la fonction suppressive des Treg. Car l'utilisation d'anticorps anti Tim-3 conduit à une diminution de l'activité suppressive des Treg.

Plus récemment, il a été prouvé que la Gal-9 joue un rôle clé dans la génération des Treg induit (iTreg) à partir des lymphocytes T CD4+CD25- à la suite d'une liaison au CD40 en présence de TGFβ. Il a également été montré que la Gal-9 interagit avec de nombreux récepteurs ce qui semble indiquer que la Gal-9 présente un rôle important dans la régulation immunitaire. Bien que le nombre d'articles de recherche concernant la Gal-9 aient considérablement augmenté ces dernières années, certains points de compréhension sont manquants. Premièrement la majorité des études cherchant à déterminer la fonction de la Gal-9 ont eu lieu sur des modèles murins d'infection viral, présentant une hyper sensibilité ou une réponse auto-immune. Il a cependant été prouvé que la Gal-9 murine présente une activité apoptotique moindre que la Gal-9 humaine. De manière plus importante, jusqu'à maintenant aucune étude ne s'est concentrée sur la fonction de la Gal-9 humaine *via* son inhibition directe utilisant des anticorps bloquants.

Dans ce papier nous montrons l'expression de la Gal-9 par les Treg humains au niveau transcriptomique et protéomique. Nous montrons que la Gal-9 est constitutivement secrétée par les Treg à un taux significativement plus élevé que celui des Tconv, qui eux ne présentent une augmentation de leurs sécrétions de Gal-9 qu'à la suite d'une activation. En outre, nous avons prouvé que 1G3 un anticorps anti-Gal-9 est un potentiel antagoniste capable d'inhiber la suppression de la prolifération induit par la Gal-9. L'utilisation de cet antagoniste nous a permis d'augmenter la réponse Th-1 induit par les PBMC activé *via* leurs TCR sans pour autant induire leur hyper-prolifération in vitro. Nous avons été capables d'induire l'inhibition de l'activité suppressive des nTreg et des iTreg in vitro lors d'expériences de MLR.

Connaissant l'importance de la Gal-9 et des Treg dans le carcinome du nasopharynx (CNP) nous avons décidé d'étudier les fonctions de cet anticorps anti-Gal-9 en tant que potentiel traitement immuno-thérapeutique anti-tumoral. Dans un premier temps nous avons montré que l'anti-Gal-9 a la capacité d'inhiber l'effet suppressif des exosomes dérivés du CNP sur la prolifération des PBMC et des Tconv activés. Par la suite nous avons travaillé sur un modèle de souris humanisées, nous avons alors montré le potentiel de l'anticorps à exacerber la réponse anti tumorale *via* l'inhibition de la fonction suppressive des Treg conduisant au contrôle de la croissance tumorale. Pour finir, nous avons utilisé un modèle de murin humanisé du cancer mammaire comme contre-exemple. Dans ce modèle en accord avec la littérature, la présence des Treg empêche en effet la croissance tumorale tandis que l'utilisation de la Gal-9 restaure la croissance.

Résultats

Expression de la Galectine-9 par les Tconv et Treg fraîchement isolés ou après culture.

Le degré d'expression de la Gal-9 par les Treg ou par les Tconv a tout d'abord été apprécié au niveau transcriptomique par RT-qPCR, puis au niveau protéomique par immuno-cyto-fluorescence, cytométrie en flux et Western Blot, finalement le niveau de sécrétion de Gal-9 a été testé par ELISA. Brièvement, les Treg et Tconv humains ont été premièrement isolées à partir des PBMC obtenues par centrifugation sur gradient de ficoll à partir de sang total de donneurs sains. Les cellules ainsi obtenues ont été soit directement utilisées afin de déterminer le niveau d'expression de Gal-9, soit mises en culture pendant 24, 48 ou 72 heures dans des conditions de stimulation antigénique (par l'anti-CD3 et l'anti-CD28 à 1 pg / ml) où non stimulées pour être ensuite récupérées afin d'analyser le d'expression de Gal-9.

Les résultats montrent que l'expression constitutive de la Gal-9 par les Treg se fait à des niveaux plus élevés en comparaison aux Tconv au niveau transcriptomique. Nous avons observé que cette expression augmente dans les conditions de stimulation pour les deux types cellulaires avec le maintien d'un niveau d'expression plus élevé par les Treg.

Au niveau protéomique, les résultats obtenus par les techniques d'immunofluorescence et de cytométrie de flux utilisant deux différents anticorps anti-Gal-9 ont montré que Gal-9 n'est pas exprimé à la surface des deux types cellulaires. Alors que le marquage intracellulaire a mise en évidence des niveaux d'expression comparables entre Treg et Tconv fraîchement isolées. Comme escompter, le Western blot a également montré un niveau d'expression de Gal-9 comparable entre les deux types cellulaires fraîchement isolées.

Ensuite, nous avons étudié l'expression intracellulaire de Gal-9 par les Treg et Tconv suivant une co-culture de PBMC/Treg (avec un ratio de 2 : 1) de 72 heures. Nous avons comparé l'expression par cytométrie en flux entre les cellules CD4+ Foxp3+ et les cellules CD4+ Foxp3-. Les résultats, exprimés en delta de la moyenne de l'intensité de fluorescence (δMFI), ont été présentés sous forme de valeurs normalisées. Ils indiquent que la Gal-9 est exprimée à un niveau significativement plus élevé dans les cellules Foxp3 + que dans les cellules Foxp3-.

Enfin, la sécrétion de Gal-9 a été étudiée par ELISA. Les résultats montrent un niveau significativement plus élevé de sécrétion de Gal-9 par les Treg que par les Tconv pour tous les temps étudiés. Ainsi, cette sécrétion est indépendante de l'état de stimulation des lymphocytes T régulateurs. En ce qui concerne les Tconv, bien que leur sécrétion de Gal-9 fût presque négligeable, à peine à la limite de détection du kit ELISA vendu dans le commerce, nous avons observé une augmentation de la sécrétion de Gal-9 dans les conditions de stimulations après 48 et 72 heures de culture par rapport à l'état de non stimulation.

La sécrétion de Gal-9 par les PBMC ou lors d'une co-culture de PBMC/Treg (avec un ratio de 2:1) a également été étudiée. L'ajout des Treg à une culture de PBMC a augmenté de manière significative la quantité de Gal-9 sécrétée (doublement du taux). Ces résultats prouvent que l'expression de Gal-9 et sa sécrétion par les Treg est constitutive et relativement élevé nous permettant de suspecter, un rôle essentiel delà Gal-9 dans la fonction des Treg.

Le fait que l'expression et la sécrétion de la Gal-9 par les Tconv ait augmenté dans des conditions d'activation forte (utilisant 1 µg/ml d'anticorps anti-CD3 et anti-CD28) était attendu et conforte les résultats observés dans la littérature.

Il est très intéressant de constater que la Gal-9 ne pouvait être détecté sur la membrane des Treg et des Tconv en utilisant plusieurs anticorps (y compris l'anticorps 1G3) et cela dans toutes les conditions de culture. Cependant, de nombreux rapports ont indiqué que Gal-9 pouvait être détectée sur la membrane cellulaire. Il est possible que les anticorps testés dans nos conditions de marquage ne puissent tout simplement pas détecter la Gal-9 membranaire, mais cela ne dénigre pas totalement la possibilité que les anticorps anti-Gal-9 ont marqué la surface membranaire. Enfin, nous présentons pour la première fois la sécrétion constitutive de Gal-9 par la cellule Treg qui est un bon indicateur de l'importance de Gal-9 dans la fonction de ces cellules.

Les anticorps anti-Gal-9

Notre collaboration avec l'équipe de Pierre Busson (IGR), nous a permis d'obtenir un anticorps anti-Gal-9 ciblé contre une séquence de 13 aa dans le domaine C-terminal de Gal-9 (aas de 210 à 223) commune à toutes les isoformes de la Gal-9.

La capacité de cet anticorps à détecter la Gal-9 a été mise en évidence par ELISA. Afin de tester le potentiel de cet anticorps à être utilisé comme antagoniste de Gal-9, nous avons testé la capacité de cet anticorps à inhiber le potentiel immunosuppressif de la Gal-9 recombinante (rGal9) par des expériences de prolifération et d'apoptose.

L'ajout de 60nM de rGal-9 à une culture cellulaire de PBMC ou de Tconv, inhibe, dans des conditions d'activation antigénique, la prolifération de ces cellules étudiées par incorporation de thymidine radioactive. Cet effet suppressif peut être presque complètement inhibé par un prétraitement de la rGal-9 avec l'anticorps anti-Gal-9 à une concentration de 3 µg/mL pendant 2 heures. Le traitement à 1 µg/mL n'a pas eu d'effet alors que le traitement à 5 µg/mL avait un avantage significatif sur celui avec 3 µg/mL.

De plus, un anticorps IgG1 de souris a été utilisé comme contrôle isotypique de notre anticorps afin de s'assurer la spécificité de l'effet observé. Ce contrôle isotypique n'a pas affecté la suppression induite de Gal-9.

Le lactose a été utilisé comme témoin, il peut se lier aux CRD de Gal-9 et a montré un effet inhibiteur de l'activité suppressive de la rGal-9. Dans nos conditions expérimentales, le lactose à 5 mM a inhibé la fonction de la rGal-9 alors que le saccharose, utilisé comme un sucre non spécifique, n'a pas eu d'effets. D'une manière similaire, la rGal-9 a montré un effet

inducteur de l'apoptose chez les PBMC étudiées par annexin-V en cytométrie en flux ; Le prétraitement avec l'anticorps anti-Gal-9 inhibe cet effet apoptotique. Ces résultats sont une indication claire que notre anticorps est bien un antagoniste de la Gal-9.

L'effet du blocage de Gal-9 sur la réponse immunitaire

Par la suite, nous avons décidé d'étudier l'effet bloquant de la Gal-9 sur la réponse immunitaire provoquée dans les PBMC stimulées. Nous avons donc réalisé une culture de PBMC humains, dans laquelle nous avons additionné l'anticorps anti-Gal-9 ou son isotype contrôle dans différentes conditions d'activation (par anti-CD3 et anti-CD28 à 1 µg/mL). Après quoi le surnageant a été récupéré et les cytokines sécrétées ont été étudiées par ELISA. Nous avons observé une augmentation significative de la sécrétion d'IFNy (8 fois en moyenne) et de TNFα (2 fois en moyenne) couplée à une diminution de la sécrétion de TGFβ (de moitié). Ces changements dans le profil de sécrétion cytokinique sont indicatifs de la restauration d'une puissante réponse Th-1, similaire à la réponse observée dans la réponse immunitaire anti tumorale.

La possibilité que l'anticorps anti-Gal-9 induise une hyper-prolifération toxique des PBMC ou des Tconv a été testée par des tests de viabilité et de prolifération. Les PBMC et les Tconv ont été cultivés dans différentes conditions d'activation avec l'anticorps anti-Gal-9 ou son isotype contrôle, à des doses variables et pour différentes durées. L'ajout de l'anticorps à une culture de PBMC activées ou de Tconv à une concentration allant jusqu'à 12 µg/ml n'a pas eu d'effet significatif sur la prolifération et la viabilité testée respectivement par les tests Cyquant® et CelltiterGlo® aux différents temps.

Anti-Gal-9 et nTreg

Sur la base des premiers résultats de l'expression constitutive de Gal-9 par les Treg nous voulions voir si la Gal-9 était essentielle à la fonction immunosuppressive de ces cellules. Nous avons testé cela en déterminant si l'anticorps anti-Gal-9 pouvait inhiber la fonction des Treg. Nous avons étudié la fonction des Treg par leur aptitude à inhiber la prolifération des cellules immunitaires autologues dans un essai de co-culture in vitro. En bref, la mise en culture des

Treg avec les Tconv ou les PBMC activés provenant du même donneur, diminue, la prolifération de ces cellules. Cette modification de la prolifération peut être étudiée par l'incorporation de thymidine radioactive par les Treg étant donné que ces cellules sont, par nature, anergiques in vitro. En effet, nous avons trouvé que l'ajout d'anti-Gal-9 à une co-culture de Tconv / Treg ou à une co-culture de PBMC / Treg restaurait partiellement les niveaux de prolifération de ces cellules annulant en partie la fonction suppressive des Treg. Ces résultats indiquent clairement que Gal-9 joue un rôle dans l'activité des Treg et qu'en bloquant la Gal-9 dans le milieu de culture avec l'anticorps anti-Gal-9 nous avons pu inhiber la fonction suppressive des Treg.

Anticorps anti-Gal-9 et iTreg

Il a été montré que la Gal-9 avait la capacité médié l'activité suppressive des Treg in vitro. Nous avons voulu tester la capacité de l'anticorps anti-Gal-9 à inhiber cette fonction.

Nous avons créé un protocole d'induction dans lequel les Tconv, dépléter en Treg, ont été cultivées pendant 120 heures dans des conditions de stimulation (anti-CD3 et anti CD28 à 1 µg/mL). A cette culture, nous avons ajouté soit les anticorps anti-Gal-9 à 3 µg /mL, Gal-9S à 1 µg/mL et Gal-9M à 1 µg/mL ou un mélange associant l'anticorps et la rGal-9. Nous avons utilisé les Tconv cultivés en conditions stimulante en tant que contrôle. À la suite du prétraitement, les cellules ont récupéré, lavées, comptées et mises en co-culture avec des PBMC autologues (rapport 1 : 1) dans des conditions d'activation antigénique pendant 72 heures.

Finalement, la prolifération a été évaluée par l'incorporation de la thymidine radioactive. Les résultats montrent une augmentation de la valeur de prolifération pour la co-culture avec le contrôle ou les cellules ayant été prétraitée avec l'anti-Gal-9. Les cellules prétraitées avec rGal-9S ou avec rGal-9M ont donné lieu à des cellules suppressives puisqu'elles inhibent la prolifération après la co-culture. Ces résultats étaient attendus, conformément à la littérature. Cependant, l'utilisation de l'anticorps anti-Gal-9 pendant la phase de prétraitement a inhibé la capacité des deux isoformes à induire des cellules suppressives en effet le niveau de prolifération de cette co-culture était au même niveau que le contrôle.

L'ensemble de ces résultats montre que l'anticorps anti-Gal-9 pourrait à la fois inhiber la fonction suppressive des nTreg et empêcher l'activité inductrice d'iTreg par la Gal-9.

Utilisation de l’anticorps anti-Gal-9 comme agent thérapeutique anti-tumoral

Connaissant l’importance des Treg dans la suppression de l'immunité anti-tumoral, nous avons voulu tester la potentielle utilisation de l'anticorps anti-Gal-9 en tant qu'agent immuno-thérapeutique contre le cancer. Une caractéristique biologique importante du carcinome du Nasopharynx (CNP) est la présence d'un infiltrat lymphoïde massif dans la tumeur primaire, qui est probablement favorisé par les cytokines inflammatoires produites par les cellules tumorales et leur stroma. Plusieurs études soutiennent l'idée d'un environnement immunosuppressif locale inhibant le bon fonctionnement des cellules effectrices même à la suite d’un homing correct. La présence de quantités anormales de Treg au sein de la tumeur et dans le sang périphérique est une indication claire de cette suppression immunitaire. De récents résultats confirment la production constante de Gal-9 par les cellules du CNP, notamment en association avec les exosomes. Notre équipe a déjà établi la présence d'un environnement immunosuppresseur puissant chez les patients atteints de CNP médié par les exosomes recrutant et activant les Treg. Ces exosomes immunosuppressifs étant produits par des cellules du CNP, ils peuvent induire une inhibition de la prolifération des cellules immunitaires.

L’anticorps anti-Gal-9 inhibe les exosomes des cellules CNP.

Nous voulions voir si nous pouvions utiliser l’anticorps anti-Gal-9 pour inhiber la fonction suppressive des exosomes dérivés des cellules du CNP. Pour cela, nous avons cultivé des PBMC ou des Tconv pendant 72 ou 120 heures avec 10 µg/ml d’exosomes provenant soit de cellules du CNP (CNP-exo), soit de donneurs sains (HD-exo). Les exosomes ont été pré-incubés pendant 2 heures avec ou sans l’anticorps anti-Gal-9 ou son isotype contrôle. À la suite de la culture, la prolifération cellulaire a été analysée par Cyquant®.

Nous avons constaté que l'utilisation de l'anticorps anti-Gal-9 avait la capacité de bloquer la fonction suppressive des exosomes en inversant leurs effets sur la prolifération des cellules immunitaires. L'anticorps n'a eu aucun effet sur le HD-exo qui ont été utilisés comme témoins. L'isotype contrôle n'a pas modifié la suppression induite par les CNP-exo indiquant que l'effet observé par l'anticorps était spécifique. Les mêmes résultats ont été observés en utilisant des tests de viabilité par CelltiterGlo® (résultats non montrés).

Par la suite, nous avons décidé de tester l'anticorps dans un modèle in vivo de souris humanisées. Étant donné que l'ensemble de nos résultats étaient basés sur des cellules immunitaires humaines, et vu qu'en outre, l'anticorps est conçu pour reconnaître la Gal-9 humaine, nous avions besoin de développer un modèle de souris humanisé portant une tumeur humaine dont la croissance pouvait être facilement suivie et qui contiendrait des cellules immunitaires humaines afin d'imiter une réponse immunitaire humaine la plus proche de celle observé chez les patients atteints de cancer.

L'anticorps anti-Gal9 contrôle la croissance tumorale in vivo

Le modèle développé a été basé sur des souris SCID. Ces souris ont été splénectomisées une semaine avant qu'elles aient reçu une greffe sous-cutanée d'une tumeur humaine du CNP exprimant la luciférase (tumeur C-666-1 obtenue de nos collaborateurs Fei-Fei LIU (Toronto)) ainsi qu'une injection de 50 millions de PBMC humaines enrichies ou non avec 7% de Treg. L'enrichissement en Treg imite l'augmentation de la prévalence de la population de Treg chez les patients atteint du CNP. La croissance tumorale chez ces souris a été suivie sur une période de 24 jours. Les souris ont été divisées en plusieurs groupes. Certains groupes ont reçu l'anticorps anti-Gal-9 alors que d'autres ont reçu le contrôle isotypique et d'autres n'ont pas été traités. Le traitement consistait en une injection 20 µg/souris au moment de la reconstitution en cellules immunitaires humaines suivie par des injections de rappel à des intervalles d'une semaine jusqu'au sacrifice de la souris à la fin de l'expérience. A chaque injection d'anticorps à partir de J0, les souris ont reçu des injections de CpG ODN, qui est un agoniste du TLR-9 afin d'activer les PBMC reconstituées et d'orienter la réponse vers une voie Th1.

<u>Les groupes étaient distribués de cette façon :</u>

1) Rien: souris qui n'ayant reçu que la tumeur sans cellules PBMC ni anticorps.
2) PBMC: souris ayant reçu la tumeur avec 50 millions de cellules PBMC.
3) PBMC + Treg: Les souris ayant reçu la tumeur avec 50 millions de PBMC enrichies à 7% de Treg.
4) PBMC + Treg + iso : souris ayant reçu la tumeur avec 50 millions de cellules PBMC enrichies à 7% de Treg avec des injections de contrôle isotypique de l'anticorps

5) CMSP + Tregs + 1G3: Les souris ayant reçu la tumeur avec 50 millions de PBMC enrichies à 7% de Tregs avec des injections de l'anticorps anti-Gal-9.

La première étape consistait à vérifier le succès de la reconstitution en cellules immunitaires. Par la suite, des analyses immuno-histochimiques de coupes tumorales congelées, nous a permis d'identifier les cellules T CD4+ humaines infiltrées sur le site tumoral qui n'étaient pas retrouvées dans les souris non reconstituées. Ces résultats furent une confirmation claire de la réussite de la reconstitution.

La croissance tumorale a été mesurée 3 fois par semaine tout au long de l'expérience par des mesures manuelles et par bioluminescence. Les mesures manuelles ont été effectuées par un pied à coulisse et furent facilités par la localisation sous-cutanée de la tumeur. Le volume tumoral ainsi calculé a été ensuite utilisé pour générer les courbes de croissance tumorale. D'autre part, les mesures par bioluminescence ont été effectuées à l'aide d'un appareil, l'IVIS Lumina-XR, à la suite d'une injection de 30 mg de luciférine par souris. Après l'injection, les souris ont été anesthésiées en utilisant de l'isoflurane, puis placées dans la machine. Nous avons obtenu un signal luminescent pour chaque souris, enregistré à des intervalles de 2 minutes jusqu'à diminution du signal. La valeur maximale obtenue à partir des tumeurs de chaque souris à chaque session de mesure a été calculée. Par la suite, les valeurs obtenues ont été normalisées pour chaque donneur et moyennées entre les donneurs avant d'être présentées sous forme de courbes de croissance relative.

En conclusion, la seule reconstitution en PBMC humains semble être capable de diminuer le rythme de croissance tumoral. Enrichir les PBMC en Treg réduit presque totalement l'effet positif des PBMC et ainsi conduit au rétablissement de la croissance tumorale. Le traitement des souris avec le contrôle isotypique n'a eu aucun effet observé. Le traitement anti-Gal-9 sur les souris ayant reçu les PBMC enrichies en Treg a eu un effet saisissant sur la croissance tumorale : le traitement a conduit à une inhibition efficace de la croissance tumorale à un niveau supérieur à celui observé chez les souris ayant reçu les PBMC, non enrichies. La différence dans la taille de la tumeur entre le groupe iso + Treg + PBMC et le groupe PBMC + Treg + 1g3 a été hautement significative, respectivement pour les deux volumes mesurés manuellement et par luminescence, à partir de J14.

La différence entre les groupes PBMC + Treg et PBMC + Treg + 1g3 était significative à J21 et J24 pour les mesures manuelles, et était significative ou très significative à partir de J16 en utilisant les données de bio-luminescence. Enfin, la différence entre le les groupes PBMC + Treg + 1g3 et le groupe « rien » était hautement significative ou significative à partir de J16 pour les mesures manuelles et de J19 jours pour la bioluminescence.

Les résultats montrent clairement que la croissance de la tumeur chez les souris traitées par l'anticorps anti-Gal-9 a été significativement ralentie par rapport aux souris traitées par l'isotype. Les photographies obtenues révèlent une nette différence entre les tumeurs. Les comparaisons ont été faites sur des tumeurs différentes qui proviennent de souris recevant les mêmes cellules immunitaires du même donneur. La tumeur de la souris traitée était, dans les deux cas, nettement plus réduite.

Ces résultats prometteurs ont poussé au développement d'un projet cherchant à la mise au point d'anticorps humanisés qui pourraient être utilisés dans des essais cliniques. La première étape implique la génération de 3 anticorps chimériques. Nous avons alors sélectionné ceux ayant donné de bons résultats in vitro en ce qui concerne leur aptitude à bloquer la Gal-9 recombinante et l'immunosuppression induite par les Treg. En utilisant l'un des isotype des anticorps chimériques, huit anticorps humanisés ont été obtenus et seront testés in vitro et in vivo jusqu'à ce que nous déterminions le meilleur candidat pour de futurs travaux.

Discussion

Nous avons montré que la Gal-9 est exprimée de manière constitutive par les Treg, à un taux plus haut que par les Tconv indépendamment des conditions d'activation. Ces résultats ont été des plus concluants au niveau transcriptomique et sécrétoire. Comme escompter, les Tconv montrent une augmentation de l'expression et de la sécrétion de la Gal-9 en réponse à une forte stimulation (1 µg/ml d'anti CD3 et anti CD28) ce qui est en accord avec la littérature. Le niveau d'expression protéique de la Gal-9 étudié par marquage intracellulaire, entre Treg et Tconv fraichement isolé est très intéressant car il ne montre pas de différence. Toutefois, nous avons observé une différence au niveau transcriptomique et sécrétoire. Ce type de résultat concernant l'expression de la Gal-9 n'est pas rare.

L'expression de la Gal-9 par les cellules stromales mésenchymateuses multipotentes augmente à la suite d'un traitement à l'IFNγ. Toutefois cette augmentation a été uniquement détectée au niveau transcriptionel et sécrétoire et non pas *via* marquage intracellulaire utilisant la cytométrie en flux. Ceci signifie probablement que la voie de sécrétion de la Gal-9, qui reste jusqu'aujourd'hui inconnue, n'implique surement pas l'accumulation de la Gal-9 à l'intérieur de la cellule.

Il est intéressant de constater que malgré l'utilisation de différents anticorps, nous n'avons pas réussi à détecter la Gal-9 au niveau de la membrane cellulaire des Treg ou des Tconv quelque soient les conditions de culture alors que de nombreux rapports indiquent l'inverse. Il est possible que les anticorps que nous avons utilisé n'étaient pas capables de détecter la Gal-9 membranaire dans nos conditions de marquage. Il est tout de même intéressant de noter que l'absence de marquage à la Gal-9 à la surface des CD4+ et CD8+ par cytométrie en flux a également été notée dans d'autres études. Dans ces études les auteurs ont cependant réussi à détecter la Gal-9 dans le lysat cellulaire total.

Nous avons également montré, et ce pour la première fois, que la Gal-9 est constitutivement sécrétée par les nTreg humains à un niveau significativement plus élevé que les Tconv. De plus nous avons mis en évidence que l'utilisation de l'anticorps anti-Gal-9 permet

d'inhiber de manière significative la fonction suppressive des Treg jusqu'à un certain degré. Ces résultats sont très intéressants car il est communément admis que la fonction suppressive des Treg testé in vitro par MLR est médiée par un mécanisme dépendant du contact.

Ces résultats mis ensembles indiquent que la Gal-9 n'est pas seulement impliquée dans le mécanisme de suppression des Treg mais aussi dans la maintenance de leur potentiel suppressif global. La découverte de la sécrétion constitutive de la Gal-9 par les nTreg ouvre de nouvelles opportunités dans la compréhension complète des mécanismes complexes qui gouverne la fonction suppressive des Treg.

Nous montrons ici que le blocage de la Gal-9 dans une culture de PBMC humains induit l'augmentation de la sécrétion d'IFNγ et TNFα et la diminution de la sécrétion de TGFβ. L'association des données trouvés dans la littérature avec ces résultats, semble mettre en évidence l'implication de la Gal-9 dans un mécanisme de régulation qui viserait à inhiber la réponse immunitaire lors d'une sur-activation de celle-ci. Cette hypothèse semble être confirmée par l'accumulation des données provenant des modèles murins et des patients humains qui montrent une augmentation de l'expression de la Gal-9 lors d'infection virales qui agit de manière à réguler négativement la réponse immunitaire.

Mettre en évidence le fait que l'anticorps anti-Gal-9 pouvait inhiber l'effet suppressif et induire une réponse Th1, est une indication quant à la potentielle utilisation de cet anticorps en tant qu'inhibiteur de check-point immunitaire. Une inquiétude particulière est la toxicité de l'anticorps et notamment la possibilité qu'il induise une sur-activation du système immunitaire. Alors que cette question ne peut être totalement évaluée in vitro, la donnée que nous possédons actuellement montre que le blocage de la Gal-9 dans une culture de PBMC ou de Tconv n'induit pas une hyper prolifération de ces cellules. Prenant ces résultats en considération avec les résultats montrant que des souris saines ayant la Gal-9 mise sous silence (Gal-9 KO) ne présentent pas de maladie auto-immune, nous donne des indications concernant la potentielle toxicité de l'anticorps anti-Gal-9. Néanmoins des tests standardisés de cytotoxicités seront nécessaires.

Le carcinome du nasopharynx est généralement associé à une infection par le virus d'Epstein Barr. Les patients atteints du CNP présentent au site tumoral un fort microenvironnement suppressif dans lequel la population des Treg et les exosomes tumoraux jouent un rôle primordial. Nous avons décidé d'étudier le potentiel de l'anticorps anti-Gal-9 en tant qu'outil immuno-thérapeutique contre le cancer dans ce modèle. Plusieurs équipes, y compris la nôtre, ont montré que les exosomes dérivés du CNP pouvaient inhiber la réponse immunitaire de type Th-1 *via* l'inhibition de la prolifération des lymphocytes T CD4+. Dans un premier temps nous étions satisfaits de prouver que l'anti-Gal-9 inhibait la fonction suppressive des exosomes du NPC. Ces résultats se sont montrés motivant et nous ont poussées à utiliser le potentiel de l'anticorps dans un modèle in vivo. Etant donné que la base de notre travail repose sur la capacité de l'anticorps à inhiber la Gal-9 humaine et de renforcer la réponse immune anti-tumorale en bloquant l'activité des Treg, nous ne pouvions pas utiliser un modèle murin syngénique. C'est pourquoi nous avons créé un modèle murin humanisé. Ce modèle se base sur les souris SCID qui présentent une déficience au niveau de leur réponse immunitaire adaptative notamment médiée par les lymphocytes B et T.

Ces souris ont reçu une xénogreffe de tumeur du CNP humain exprimant la luciférase. Ce type de tumeur est connue pour exprimer la protéine de latence de type II d'EBV. Nous avons reconstitué les souris avec des PBMC humains enrichis ou non en Treg. Les Treg nous permettent d'imiter les conditions retrouvées dans un CNP concernant le microenvironnement suppressif. L'utilisation de ce modèle nous a permis de mettre en évidence le rôle des Treg dans la régulation immunitaire du CNP. Les souris enrichis en Treg ont en effet montré une croissance tumorale significativement plus rapide. De plus l'utilisation de l'anticorps nous a permis de considérablement réduire la croissance tumorale, ce résultat était exacerbé lorsque les souris n'étaient pas reconstituées en Treg. Cela semble indiquer que notre molécule thérapeutique a la capacité d'induire une réponse anti-tumorale non seulement médiée par l'inhibition des Treg mais également par l'activation de la réponse Th-1.

L'efficacité du modèle humanisé utilisé ainsi que la capacité du 1G3 à inhiber la fonction des Treg est clairement observable lorsque nous comparons les résultats obtenus dans le modèle CNP au modèle du cancer du sein. Ce dernier est en complète opposition en terme de pronostic, en effet dans ce cancer, une augmentation de la population en Treg Foxp3 + et du taux de Gal-9 sont de manière indépendante associés à un meilleur pronostic. Nous étions

satisfaits de voir que dans ce modèle, les Treg étaient capable de diminuer la croissance tumorale et que l'anticorps avait la capacité d'annuler cet effet. Par conséquence, la mise en évidence d'un effet opposé sur ce modèle mammaire témoigne du succès de notre protocole expérimental et de nos résultats.

Conclusion

Ces résultats montrent que le ciblage de la Gal-9 dans le cancer pourrait être une approche immuno-thérapeutique viable et efficace. L'avantage du blocage de la Gal-9 par un anticorps monoclonal est la possibilité d'inhiber l'état immunosuppressif observé chez les patients atteints du cancer en ciblant un marqueur secrété et responsable de la suppression immunitaire au lieu de cibler directement les Treg. De plus étant donné que la Gal-9 est surexprimée uniquement dans des conditions pathologiques inflammatoires, l'inhibition de la Gal-9 par l'anticorps ne devrait induire que des effets secondaires minimes.

Articles

ARTICLE 1 :

Les exosomes dérivés du carcinome du nasopharynx recrutent les lymphocytes T régulateurs humains, favorisent leur prolifération et augmentent leur activité régulatrice.

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Contexte et objectifs de l'étude

La pathogenèse du CNP présente un des paradoxes évidents dans la mesure où la persistance du virus et de la production de plusieurs protéines virales immunogènes, est contemporaine à l'apparition d'un processus malin dans un contexte inflammatoire locale associé à une infiltration leucocytaire massive au sein de la tumeur primaire. Comme nous l'avons vu dans l'introduction, les pathologies associées à EBV et plus particulièrement le CNP, présentent des mécanismes importants permettant l'échappement de la tumeur au système immunitaire. Par ailleurs, chez les patients atteints d'un CNP, il existe une production élevée d'exosomes tumoraux porteurs de la Galectine-9 (Exo-CNP) ayant la capacité de se lier au récepteur Tim3 présent principalement sur les lymphocytes T CD4+ Th1, ce qui entraine alors leur mort par apoptose. Il a également été décrit un recrutement massif de lymphocytes T régulateurs (Treg) chez les patients ayant un CNP, pouvant alors représenter jusque 12% des lymphocytes infiltrant la tumeur. L'implication à la fois des exosomes tumoraux et des Treg contribue à l'élaboration d'un microenvironnement immunosuppresseur persistant dans le CNP, constituent un frein à la mise en place de différentes stratégies d'immunothérapie.

Cependant, et à ce jour, l'existence d'interactions potentielles entre ces deux facteurs immunosuppresseurs n'a jamais été décrite.

Dans ce contexte, les objectifs centraux de cette étude ont été (i) de mieux connaître le potentiel immunosuppresseur des exosomes de CNP et (ii) d'étudier l'influence des exosomes sur le phénotype, l'expansion, le recrutement et la fonction des Treg.

Méthodologie et Principaux résultats

Des expériences de qPCR nous ont permis de confirmer la surexpression de CCL20, connue comme attractant des Treg, dans des lignées CNP. Afin d'évaluer *in vivo* le rôle joué par cette chimiokine dans le recrutement des Treg au sein de la tumeur, nous avons mis en place un modèle murin immunodéficient et humanisé secondairement xénotransplanté par un CNP humain (SCID-hu-CNP). Ces souris ont été reconstituées par des PBMC enrichies avec 10% de Treg préalablement marqués au VT680. Nous avons par la suite neutralisé, le CCL20 par injection intra-péritonéale d'un anticorps monoclonal bloquant cette chimiokine. Les expériences de mesure de la fluorescence *in vivo*, ont montré que les animaux ayant reçu l'anti-CCL20 présentaient une diminution de recrutement des Treg au niveau du site tumoral. De même, des expériences d'immuno-histo-fluorescence ont confirmé et ce de manière significative cette diminution du recrutement des Treg à la suite du traitement. Par ailleurs, des analyses en cytométrie de flux (FACS) ont montré, une élévation du taux de Treg humains dans le sang des souris du même groupe. Validant ainsi les mesures in-vivo.

Dans une seconde partie de l'étude, nous avons montré pour la première fois que les exosomes de CNP (Exo-CNP) expriment la CCL20 qui par ailleurs est absente des exosomes de donneurs sains (Exo-DS). L'utilisation d'une gamme de CCL20 recombinante nous a permis d'évaluer la quantité de cette chimiokine à moins de 300 pg/µg d'exosomes. Nous avons confirmé ces résultats en microscopie électronique par un marquage immunogold. Enfin, nous avons mis en évidence, par RT-qPCR, une augmentation de l'expression du CCR6 (récepteur de la CCL20) sur les Treg en présence des exosomes de CNP. Ce qui évoque un rétrocontrôle de l'attraction des Treg par les exosomes.

Nous avons également dévoilé que ces exosomes recrutent préférentiellement les Treg, d'une manière dépendante de CCL20. Des expériences de chimioatraction utilisant la chambre

de Boyden ont en effet montré que la CCL20 recombinante et les Exo-CNP recrutent fortement les Treg et que l'ajout d'un anticorps anti-CCL20 bloquant inhibe cette attraction de manière significative. Les Exo-DS, quant à eux, n'attirent que faiblement les Treg et le traitement par l'anti-CCL20 demeure sans effets.

Nous avons également montré que les Exo-CNP peuvent exercer une attraction modérée sur les Tconv. Dans ce contexte, nous avons évalué leur capacité à convertir ces Tconv en Treg. Une analyse FACS a révélé un « shift » ou « conversion phénotypique » d'une fraction des CD4+CD25- en CD4+CD25high après 72 h et 120 h de traitement avec les Exo-CNP, et non avec les Exo-Ds. Nous avons mis en évidence (i) par ELISA que la conversion des Tconv en Treg était dépendante du TGF-β, et (ii) que les Treg « converties » présentaient de propriétés immunosuppressives de la prolifération de PBMC autologues lors d'un test de suppression.

La littérature décrit les Treg comme anergiques *in vitro*, or l'ajout des exosomes de CNP stimule significativement la prolifération des Treg de manière dose-dépendante ; alors que les exosomes de DS ont un effet opposé et semblent même potentiellement inhiber l'expansion des Treg. De même, des analyses FACS montrent une augmentation significative et dose-dépendante de la fraction CD4+CD25high parmi les Treg en présence des exosomes de CNP, phénotype généralement correspondant à une population fortement suppressive. Finalement, nous avons étudié l'effet des Exo-CNP sur l'expression du FoxP3, facteur de transcription majeur des Treg, et avons prouvé que son expression augmente significativement chez les Treg en co-culture avec les Exo-CNP. A l'inverse, les exosomes de DS n'ont aucune conséquence sur la fraction CD4+CD25high ou sur l'expression du FoxP3.

Et enfin, nous avons estimé par MLR (PBMC autologues), l'effet des Exo-CNP sur la fonction immunosuppressive des Treg. Nous avons montré, une augmentation significative de cette activité qui n'est pas observé en présence des Exo-DS. Cette activité fut accompagnée d'une augmentation de la sécrétion des cytokines immunosuppressives majeures : l'IL-10 et le TGF-β.

Conclusion

Ce travail est le premier à montrer, que les exosomes de CNP potentialisent le phénotype suppresseur des Treg, en favorisant l'expansion et la différenciation des Treg, en augmentant leur capacité suppressive et en induisant leur chimioattraction au site tumoral. La mise en évidence d'une synergie entre les exosomes de CNP et les Treg est primordiale car elle pourrait être impliquée dans la régulation de la tolérance tumorale et constituer alors un nouveau mécanisme d'échappement du CNP au système immunitaire.

ARTICLE 2 :

Evaluation *in vitro* et *in vivo* d'une vaccination avec des peptides dérivés d'antigènes de latence II du virus d'Epstein-Barr dans le Carcinome du Nasopharynx

Article envoyé

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Contexte et objectifs de l'étude :

Le virus d'Epstein Barr (EBV) est engagé dans divers pathologies tumorales, dont le carcinome du nasopharynx (CNP) qui est caractérisé par une latence de type II. Les lymphocytes T CD4+ jouent un rôle fondamental dans le contrôle des tumeurs. C'est pourquoi, notre équipe a favorisé le développement d'une immunothérapie basée sur la stimulation d'une réponse T CD4 spécifique. Un logiciel de prédiction d'épitopes a mis en évidence 6 peptides *promiscues* issus des antigènes de la latence II d'EBV. Nos avons déjà montré que ces peptides étaient capables d'induire une forte réponse CD4+ cytotoxique de type Th1. Au même moment, les travaux de l'équipe de P. Busson ont montré que les cellules du CNP sécrétaient des quantités importantes d'exosomes contenant de la galectine-9, un ligand du récepteur membranaire Tim-3 ayant la capacité d'induire l'apoptose dans les lymphocytes CD4+ Th1 matures. Dans ce contexte, on pouvait soupçonner que ces exosomes tumoraux seraient un obstacle potentiel à l'utilisation du cocktail peptidique.

Dans ce contexte, les objectifs de cette étude ont consisté à (i) parcourir les moyens de promouvoir l'expansion et l'efficacité des effecteurs anti-tumoraux, particulièrement des lymphocytes CD4+ Th1 anti-EBV et (ii) d'estimer l'impact des exosomes dans la stratégie vaccinale proposée.

Méthodologie :

Nous avons d'abord isolé les lymphocytes T CD4+ à partir des PBMC humains et des lignées TCD4+ spécifiques d'EBV ont été générées par présentations répétées du cocktail peptidique. Nous avons mis les T CD4+ isolés ainsi que les lignées T CD4+EBV spécifiques, en

culture avec des doses croissantes d'Exo-CNP. Pour (i) des tests de prolifération (incorporation de thymidine radioactive), (ii) des tests de cytotoxicité de lignées cellulaires de CNP (Cytotox Glow) et (iii) la sécrétion de cytokine par ELISA sandwich. Nous avons également apprécié l'efficacité des peptides in vivo par la mise en place d'un modèle murin immunodéficient (SCID), xénotransplanté par des cellules de CNP exprimant la luciferase (C666.1-Luc) et reconstitué par des PBMC humains. L'évolution du volume tumoral a été déterminée, à la fois par mesure manuelle et par mesure de la bioluminescence.

Résultats :

Les exosomes obtenus présentaient les caractéristiques phénotypiques attendues, à la fois par leur taille et par l'expression des marqueurs des Exo-CNP (LMP-1, Gal9, CD63). Ils étaient fonctionnellement actifs dans la mesure où ils avaient la capacité de diminuer la aussi bien la prolifération des T CD4+ que des PBMC. Nous avons validé, dans un test in *vitro,* l'immunogénicité spécifique des peptides d'EBV lors d'un test de rappel, sur des PBMC ayant été pré-cultivées avec des cellules d'une lignée de CNP (C15). Dans ce contexte, les peptides ont toujours été capables d'induire une prolifération des PBMC associée à une production d'IFNγ. Les résultats que nous avons obtenus montrent également que les lignées T CD4+ spécifiques d'EBV conduisent à la lyse spécifique des cellules du CNP EBV positives. De manière imprévue, la présence des Exo-CNP ou de la rGal-9, ne semble pas perturber ces capacités de lyse, ni la prolifération de ces lignées EBV- spécifiques. Ces lignées sont sensibles à l'immunosuppression induites par la ciclosporine A. Enfin, nous avons pu valider l'efficacité des peptides dans un modèle *in vivo*. Nous avons alors testé l'effet anti-tumoral de la vaccination par les peptides EBV par 2 approches décrites préalablement afin de déterminer l'évolution de masses tumorales. Par ces 2 approches, nous montrons que la reconstitution immunitaire de la souris SCID-CNP, couplée à un programme de vaccination peptidique permet de contrôler le volume tumoral.

Conclusion :

Pour conclure, l'ensemble de nos résultats permet de renforcer l'hypothèse selon laquelle le cocktail de peptides dérivés des antigènes de latence II d'EBV, pourraient être utilisés chez les personnes atteintes d'un CNP. L'objectif principal n'étant pas de remplacer les protocoles et les thérapies utilisés à ce jour, mais d'apporter un complément aux protocoles thérapeutiques standards. Ces peptides vaccinaux pourraient ainsi être utilisés chez les

patients réfractaires, afin de combattre la maladie résiduelle, mais pourraient aussi être utilisés chez les patients présentant des rechutes, malheureusement très fréquentes et souvent fatales dans le CNP.

ARTICLE 3

Impact de la présence du virus d'Epstein-Barr sur le recrutement des lymphocytes T régulateurs dans la maladie de Hodgkin.

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Contexte et objectif de l'étude

Le virus d'Epstein Barr (EBV) est présent dans les cellules néoplasiques chez environs 50% des patients atteints d'un lymphome de Hodgkin (LH). L'existence d'un environnement immunosuppressif chez les patients atteints a été clairement décrite avec notamment une abondance de lymphocytes T régulateurs naturels (nTreg) ou induits (iTreg ou Tr1), cependant très peu de choses sont connues concernant le mécanisme régulateur induit par l'expression des protéines d'EBV dans les cellules tumorales.

Dans cette étude, notre principal objectif était de mettre en évidence une éventuelle association entre le statut EBV positif chez les patients avec un LH et la présence des lymphocytes T régulateurs.

Méthodologie et résultats

Une analyse par RT-qPCR réalisée sur des ARN isolés des biopsies tumorales de LH EBV-positives et EBV-négatives, nous avons montré que l'infection par EBV augmente de manière significative (i) l'expression des marqueurs associés aux nTreg (CD4+, CD25, FoxP3, GITR, CTLA4), (ii) l'expression des marqueurs associés aux Tr1 (CD4+, CD49b, CD18, LAG3), ainsi que (ii) l'expression des cytokines immunosuppressives majeures (IL10, TGF-β).

Cette surexpression est associée à une augmentation de l'expression de plusieurs chimiokines (CCL17, CCL22, CCL4 and CCL20) attirant les Th2 et les Tregs en générale concourant de fait à l'échappement immunitaire. De même, une analyse en immunohistochimie sur des biopsies de ganglions associée à une analyse en cytométrie de flux (FACS) sur des

PBMC isolés de sang de patients à mis en évidence le recrutement des Tr1 dans le LH EBV-positif.

Finalement, nous avons montré par des expériences de Q-PCR, d'ELISA, d'immunohistochimie et de FACS, que la sécrétion de l'IL-10 était significativement plus élevée dans les tumeurs et le sang des patients atteints d'un LH EBV-positif.

Conclusion

Nos résultats proposent ainsi un nouveau schéma dans lequel l'EBV recrute les Tr1 au niveau du microenvironnement ganglionnaire en induisant l'expression de plusieurs chimiokines impliquées dans le recrutement des lymphocytes T régulateurs. Nos données suggèrent que l'expression des protéines de latence d'EBV au sein des cellules tumorales favoriserait l'échappement des tumeurs à la réponse adaptative spécifiques du virus.

Abstract

Introduction and objectives: The immune system has the potential to recognize and eliminate cancer cells but is held back by certain inhibitory pathways. These pathways could induce direct inhibition of the immune response such as PD-L1/PD-1 pathway, or could recruit immune-suppressive cells to the tumoral microenvironment such as Regulatory T cells (Tregs). Tregs are often upregulated in cancer patients and promotes tumor development making them an appealing target for immunotherapeutic approaches such as anti-CTLA-4. Using such antibodies known as immune-checkpoint inhibitors represents a passive immunotherapeutic approaches. Gal-9 is a β-galactoside binding lectin with immunosuppressive capabilities expressed by both cancer cells and various types of immune cells including Tregs. Increase in circulating Gal-9 levels in the serum of nasopharyngeal carcinoma (NPC) patients (and other cancers) has been correlated with a poor prognosis. A second branch of immune therapeutics known as active therapies involves the use of vaccine strategies to activate the immune response against tumoral antigens. NPC which is a malignant epithelial cancer represents a prefect target for such strategies due to the fact that it is almost always associated with Epstein Bar Virus (EBV) and expresses several viral proteins. Numerous DC or CD8+ T cell activating vaccines which targeted different EBV peptides have been tested with limited success in clinical trials. We had already proven that NPC derived tumoral exosomes induce suppress the immune response either by directly inhibiting Th1 cells or by recruiting, activating and expanding Tregs. Hence these exosomes are a hurdle to overcome in future NPC therapeutic approaches.

First objective: CD4+ T cell response seems to be essential in managing NPC. The use of a CD4+ T cell response inducing peptide cocktail as a vaccination strategy has never been tested.

First Results and methodology: 6 peptides derived from the 3 EBV latency II antigens, with a highly promiscuous capability of binding on HLA II molecules were generated. We show here that these peptides used in a cocktail together are recognized by human PBMC inducing a broad IFNγ cytokine secretion. Furthermore, peptide-specific CD4+ T cell lines were generated, with high cytotoxic potential against various NPC cell lines and resistant to the suppression induced hNPC exosomes or rGal-9. In vivo administration of the cocktail with an adjuvant restrained growth of xeno-transplanted hNPC tumors in immuno-deficient mice that were previously reconstituted with human PBMCs. Finally, the cocktail reactivated NPC patients' memory T cells ex-vivo by inducing IFNγ secretion.

Second Objective: First we aimed to confirm the role of Gal-9 in human Treg-mediated-immuno-suppression. Then we tested the capabilities of an anti-human-Gal-9 monoclonal antibody (Mab) (mouse IgG1) obtained from Pierre Busson's team (principle collaborator on the project) to block Gal-9 suppressive function. We then studied the effect of this blocking on Treg function and subsequently the anti-tumoral immune response. Finally in-line with the development of a potential therapeutic agent for patient use, fully humanized anti-Gal-9 antibodies (human IgG1) were produced and their antagonistic capabilities were tested.

Second Results and methodology: Following Treg isolation from healthy donors, we proved *via* QPCR, ELISA and flow cytometry that Gal-9 is expressed and secreted by Tregs at a higher level than conventional T cells. The anti-Gal-9 Mab was able to antagonize the apoptotic and anti-proliferative function of recombinant Galectin-9 (rGal-9) on human PBMCs as test by annexin V staining and multiple proliferation tests. Moreover, the anti-Gal-9 Mab significantly inhibited the immuno-suppressive function of Tregs in mixed leukocyte reaction proliferation tests. At the same time, Gal-9 blocking in PBMC culture promoted the secretion of Th1 cytokines IFNγ and TNFα as well as decreases that of TGFβ without inducing toxic hyper proliferation of the cells. We also successfully used the anti-Gal-9 Mab to inhibit the suppressive function of hNPC derived exosomes. *In-vivo*, anti-Gal-9 antibody limited the growth of xeno-transplanted hNPC tumors in immuno-deficient mice that were previously reconstituted with human PBMCs and Tregs. Finally we proved that the humanized anti-bodies could antagonize the anti-proliferative function of Gal-9 at an equal to their mouse counterpart.

Conclusion: Inducing a successful anti-tumoral immune response with minimal toxic effect requires the combination of multiple approaches. Here we show that using the EBV peptide cocktail we could activate a specific CD4+ immune response which is crucial for successful anti-tumoral effect. However, simply activating the response is not enough as it has been proven that tumors can inhibit anti-tumoral immunity even after successful recognition of tumoral antigens. Hence the importance of the anti-Gal-9 approach. By blocking Gal-9 we inhibited Treg induced immune suppression and with it a major actor in the tumoral immunosuppressive environment ensuring maintained anti-tumoral function.