THESIS

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Effect of Nasopharyngeal Carcinoma-derived Exosomes on the induction of tolerogenic dendritic cells

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CNRS UMR8161 Mechanisms of Tumorigenesis and Targeted Therapy
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Science is a way of life. Science is a perspective. Science is the process that takes us from confusion to understanding in a manner that's precise, predictive and reliable - a transformation, for those lucky enough to experience it, that is empowering and emotional

Brian Greene

I am among those who think that science has great beauty

Marie Curie

To Nanny and Grandad,

To my family,

To Skander & Jelly

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Oral communications

- *23-25 May 2018:* 11th Annual Scientific days of the **Canceropôle Nord-Ouest**, Deauville, France
  Awarded prize for 3rd best oral presentation
- *21 March 2018:* 7th Annual **HerPAs** Conference, Tours, France
- *14 September 2017:* 17th Edition of the "Journée André VERBERT" , **EDBSL**, Lille, France
- *29 March 2017:* 6th Annual **HerPAs** Conference, Lille, France
- *27 oct. 2016 :* Seminar for **Master 2 Immuno-Cancérologie de Rennes-Nantes**, Roscoff, France
- *21-26 August 2016:* 19th International Immunology Conference by the **ICI** and **SFI**, Melbourne, Australia
- *4-7 May 2016:* Annual **ISEV** conference, Rotterdam, Netherlands
- *21-22 April 2016:* 2nd Summer Meeting of Immunotherapy and graft oncology, **Labex IGO**, Nantes, France
- *23 March 2016:* 5th Annual **HerPAs** Conference, Paris, France
- *10-12 June 2015:* 8th Annual Scientific days of the **Canceropôle Nord-Ouest**, Deauville, France

Written communications

- *2-6 May 2018:* Annual **ISEV** Conference, Barcelona, Spain
- *17-18 April 2018:* 3rd Summer "IGO Meeting" by **Labex IGO**, Nantes, France
- *10-14 June 2018:* 15th International Symposium on Dendritic cells, Essen, Germany
- *10-12 May 2017:* 10th Annual Scientific days of the **Canceropôle Nord-Ouest**

  Deauville, France
- *19-20 September 2016:* Annual **CFCD** Conference, Paris, France
- *21-26 August 2016:* 19th International Immunology Conference by the **ICI** and **SFI** Melbourne, Australia
- *18-20 May 2016:* 9th Annual Scientific days of the **Canceropôle Nord-Ouest**, Deauville, France
- *4-7 May 2016:* Annual **ISEV** Conference, Rotterdam, Netherlands
- *23 March 2016:* 5th Annual **HerPAs** Conference , Paris, France
- *7-8 December 2015*: Annual **CFCD** Conference, Paris, France
- *4-6 November 2014:* Annual **SFI** and **AFC** Conference, Lille, France
  Took part in the organisation of the conference

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Abbreviations

Numbers

5-FU	Fluorouracil

A

Ago2	Argonaute 2
AIDS	Acquired Immune Deficiency Syndrome
AJCC	American Joint Committee on Cancer
Akt	Serine/Threonine-specific protein kinase
Alix	ALG-2 interacting protein X
AML	Acute Myeloid Leukaemia
ANKL	Aggressive Natural Killer leukaemia
ANOVA	Analysis Of VAriance
AP-1	Activator Protein-1
APCs	Antigen Presenting cells
ARF6	ADP-Ribosylation Factor 6

B

BART	BamHI-A region without Rightward Transcripts
Bcl-2	B-cell lymphoma 2
BCR	B Cell Receptor
BDCA	Blood Dendritic Cell Antigen
BHRF1	BamHI fragment H rightward open Reading Frame 1
BL	Burkitt Lymphoma
BSA	Bovine Serum Albumin
BTK	Bruton's Tyrosine Kinase
BZLF1	Basic Leucine Zipper Nuclear Factor 1

C

CAEBV	Chronic-Active EBV infection
CAR-T cells	Chimeric Antigen Receptors-T cells
CBS	Chromosome Binding Sites
CCC	Covalently Closed Circle
CCL	C-C motif Chemokine Ligand
CCR	C-C motif chemokine Receptor
CCRT	Cisplatin CRT
CD	Cluster of Differentiation
cDC	conventional or classical DC
cDNA	complementary DNA
CDP	Dendritic Cell Progenitor
CLPTM1L	Cleft-Lip and Palate TransMembrane Protein-1-Like
CLR	C-type lectin receptors
cMoP	common Monocyte Progenitor

CMV	CytoMegaloVirus
cpm	counts per minute
CR2	Complement Receptor type 2
CRT	Chemo-Radio Therapy
CSC	Cancer Stem Cell
CTAR1/2	Cterminal Activating Reion 1 and 2
Cter	C-Terminal
CTL	Cytotoxic T lymphocytes
CTLA4	Cytotoxic T-lymphocyte-Associated protein 4
CXCL	C-X-C motif Chemokine Ligand
CXCR	C-X-C motif Chemokine Receptor

D

DAMPs	Damage Associated Patterns
DC	Dendritic cell
DC-CIK	Dendritic Cell – Cytokine Induced Killer cells
DEX	Dendritic cell derived Exosomes
DKK1	Gene encoding Dickkopf-related protein 1
DLBCL	Diffuse Large B-Cell Lymphoma
DNA	DeoxyriboNucleic Acid
dNTP	deoxyribose Nucleoside TriPhosphate
DRiP	Defective Ribosomal Products
dsRNA	double stranded RNA
DTT	DiThioThreitol

E

EBERs	Epstein-Barr virus-encoded small RNAs
EBNA	Epstein-Barr Virus Nuclear Antigen
EBV	Epstein-Barr virus
EBV-1/2	Epstein Barr Virus type 1 and 2
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immuno Assay
EMT	Epithelial-Mesenchymal Transition
ER	Endoplasmic Reticulum
ER	Estrogen Receptor
ERK	Extracellular signal-Regulated Kinases
ESCRT	Endosomal Sorting Complexes Required for Transport
EVs	Extracellular vesicles

F

Fas-L	Fas Ligand
FCS	Foetal Calf Serum
FDA	Food and Drug Administration
Foxo3a	Forkhead Box O3a

FoxP3	Forkhead box P3
Fra-1	Fos-related antigen-1

G

Gal-9	Galectin-9
GAPDH	Glyceraldehyde-3-Phosphate DeHydrogenase
GILZ	GC-induced leucine zipper
Gli1	Glioma-associated protein-1
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GMP	Granulocyte Macrophage Progenitor
GR	Glycine Alanine
GSTM1	Glutathione S-Transferase Mu1
GTP	Guanosine-5'-TriPhosphate
GVHD	Graft Versus Host Disease

H

HCl	HydroChloric acid
HCV	Hepatitis C Virus
HHV-4	Human Herpes Virus-4
HIF1α	Hypoxia-Inducible Factor 1α
HIV	Human Immunodeficiency Virus
HL	Hodgkin Lymphoma
HLA	Human Leucocyte Antigen
HLH	Hemophagocytic Lymphoistiocytosis
hnRNPA2B1	Heterogeneous Nuclear RiboNucleoProtein A2/B1
HPLC	High Performance Liquid Chromatography
HPRT	Hypoxanthine guanine PhosphoRibosyl Transferase
Hrs	Hepatocyte growth factor-regulated tyrosine kinase substrate
HSC	Hematopoietic Stem Cell
HSP	Heat Shock Protein
Huh7	Human liver cell line

I

ICAM1	InterCellular Adhesion Molecule1
ICOS	Inducible T-cell COStimulator
ICOS-L	Inducible T-cell COStimulator Ligand
iDC	immature Dendritic Cell
IDO	Indoleamine 2,3-dioxygenase
IEA	Immediate Early Antigens
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin Growth Factor-1
IL	Interleukin
ILT	Immunoglobulin-Like Transcript

ILVs	IntraLuminal Vesicles
IM	Infectious Mononucleosis
INM	Inner Nucleus Membrane
IR1/2/3/4	Internal Repeats 1/2/3/4
IRF	Interferon Regulatory Factor
IRX-2	Iroquois-class homeodomain protein
ISEV	International Society of Extracellular Vesicles
ITAM	Immunoreceptor Tyrosine-based Activation Motif

J

JAK	Janus Kinase
JNK	c-Jun N-terminal Kinase

K

KITL	KIT Ligand
KRAS	Kirsten Rat Sarcoma oncogene
Kyn	Kynurenine

L

LAMP-1	Lysosomal-Associated Membrane Protein-1
LCL	Lymphoblastoid Cell Line
LCV	Lymphocryptovirus
LF3	4-thioureido-benzenesulfonamide derivative compound
LFA1	Lymphocyte Function-associated Antigen 1
LMP1/2	EBV Latent Membrane Protein1/2
LN	Lymph Nodes
LncRNA	Long non-coding RNA
LPD	Lympho Proliferative Disorder
LPS	Lipopolysaccharid

M

mAb	monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinases
MCP-1	Monocyte Chemoattractant Protein-1
mDC	mature Dendritic cell
MDP	Macrophage and Dendritic cell Progenitor
MDSCs	Myeloid-Derived Suppressive Cells
mfi	median fluorescence intensity
MHC	Major Histocompatibility Complex
MICB	MHC Class I chain-related protein B
miRNA	microRNA
MLP	Multi-Lymphoid Progenitor
MLR	Mixed Leucocyte Reaction

MMPs	Matrix MetalloProteinases
MoDC	Monocyte-derived Dendritic Cell
MoMac	Monocyte-derived Macrophage
MRI	Magnetic Resonance Imaging
MSCs	Mesenchymal Stem cells
Mtb	*Mycobacterium tuberculosis*
MVB	MultiVesicular Body
MVE	MultiVesicular Endosomes
MyD88	Myeloid Differentiation primary response 88

N

NCL	Nucleolin
ND10	Nuclear Bodies 10
NF-KB	Nuclear Factor-KB
NHL	Non-Hodgkin Lymphoma
NHP	Non-Human Primates
NK	Natural Killer
NKG2	Killer cell lectin-like receptor 2
NLR	NOD-Like Receptor
NOD	Non-Obese Diabetic
NONO	Non-POU domain-containing Octamer binding protein
NPC	NasoPharyngeal Carcinoma
Nter	N terminal
nTL	Naïve T cells

O

ONM	Outer Nuclear Membrane
OPD	Ortho-Phenylenediamine Dichloride
OriP	Origin of replication
OS	Overall Survival

P

$p27^{kip1}$	Cyclin-dependant kinase inhibitor1B
p38	MAP Kinase 14
p53	Tumor protein 53
PAMPs	Pathogen Associated Molecular Patterns
PAP	Prostatic Acid Phosphatase
PAX5	Paired box Protein-5
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PD-1	Programmed cell Death-1
pDC	plasmacytoid Dendritic Cell
PD-L1	Programmed Death- Ligand 1
PDT	PhotoDynamic Therapy
PFCs	Poly Functional T Cells
pg350/220	glycoprotein 350/220

PI3K	Phosphatidylinosito- 3-kinase
PKR	Protein Kinase RNA acivated
PLD2	PhosphoLipase D2
PM	Plasma Membrane
PML	ProMyelotic Leukemia
POU3F3	POU classe 3 homeobox 3
PRR	Pattern Recognition Receptor
PTA	PhosphoTungstic Acid
PTX	Paclitaxel
PUMA	P53 Up-regulated Modulator of Apoptosis

R

RA	Retinoic acid
RBM14	RNA Binding Motif Protein 14
RCC1	Regulator of Chromosome Condensation 1
RIG-I	Retinoic acid Inducible Gene-I
RIPK	Receptor-Interacting Protein Kinase
RISC	RNA-Induced Silencing Complex
RLR	RIG-I-Like Receptor
RNA	RiboNucleic Acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RTqPCR	Real Time quantitative Polymerase Chain Reaction

S

SATB1	Special AT-rich sequence Binding protein1
SCC	Squamous Cell Carcinoma
SCID	Severe Combined ImmunoDeficiency
SHP-1	Src Homology region 2 domain-containing Phosphatase-1
Smad2	SMA and MAD-related protein2
SOCS3	Suppressor of Cytokine Signalling 3
Sp1	Specificity protein 1
Src	Sarcoma Tyrosine Kinase
STAM1	Signal Transducing Adaptor Molecule 1
STAT	Signal Transducer and Activator of Transcription

T

TAP	Transporter associated with Antigen Processing
TAM	Tumour Associated Macrophages
TCR	T Cell Receptor
tDC	tolerogenic Dendritic Cells
TDO	Tryptophan 2,3-Dioxygenase
TEM	Transmission Electron Microscopy
TERT	TElomerase Reverse Transcriptase

TEX	Tumour Exosomes
TGF-β	Transforming Growth Factor-β
TGF-βR	Transforming Growth Factor-β Receptor
TGN	Trans-Golgi Network
Th	T helper
TILs	Tumor Infiltrating Lymphocytes
Tim3	T-cell Immunoglobulin and mucin domain-containing-protein-3
TLR	Toll-Like Receptor
TME	Tumour MicroEnvironment
TNBC	Triple Negative Breast Cancer
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TNM	Tumor, lymph Node and Metastasis
TRADD	Tumor necrosis factor Receptor type 1-Associated Death Domain protein
TRAF	TNF Receptor Associated Factor
Treg	Regulatory T cells
Trp	Tryptophane
TSG101	Tumor Susceptibility Gene 101

U

U1/2/3/4	Unique Region 1/2/3/4/
U_L	Long Unique Region
ULBP1/2	UL16 Binding Protein 1/2
U_S	Short Unique region
USP7	Ubiquitin-Specific-processing Protease 7

V

VCA	Viral Capsid Antigen
VEGF	Vascular Endothelial Growth Factor
Vps	Vacuole protein sorting

W

WHO	World Health Organisation

Z

Zta	Z Epstein Barr replication activator protein

Preamble

Dear readers,

This thesis will be divided in four major parts: introduction, Material & methods, results and a general conclusion/discussion regarding my thesis work.

The introduction describes the four major actors of my thesis subject: the Epstein Barr Virus, nasopharyngeal carcinoma, exosomes and dendritic cells. For each part, the general Biology and implication in cancer is described.

A detailed material and methods of each experiment presented in this thesis follows.

Then, we present the results obtained during my thesis. First the phenotypical study of exosomes and exosome-treated dendritic cells which is completed the findings by functional studies. Lastly, we investigate molecular pathways suspected to be involved as well as other methods of DC maturation.

Finally, we discuss the results and summarize them in a synthetic schema followed by general conclusion.

Introduction

I. The Epstein Barr Virus

A. EBV Discovery

In 1958, Burkitt Lymphoma (BL) was described for the first time by a British surgeon of the same name (Burkitt, 1958). Denis Burkitt was posted to Uganda to practise medicine and it is during this time that he was often confronted to children that showed aggressive tumours in which a type of white blood cell – B cells- proliferated uncontrollably. This disease showed a particular geographical distribution that seemed to depend on the climate, much like malaria. Therefore, Burkitt and his colleagues thought that this cancer was due to a virus carried by insects. He gave a talk in 1961 in London where a young researcher named Antony Epstein, who mostly worked on electron microscopy and the Rous sarcoma virus that caused cancer in chickens attended. A collaboration between Burkitt and Epstein led to the study of the African children's tumour biopsies by Epstein and his student Yvonne Barr. Three years later they showed that some of the cancer cells were indeed carriers of viral particles (Epstein et al., 1964), as shown in figure 1. The following year, Werner and Gertrude Henle showed that the infected B cells were capable of infecting healthy B cells and transform them into cancer cells (Epstein et al., 1965). Thus, the Epstein-Barr virus (EBV) was the first human oncovirus discovered as this last study showed that EBV was responsible for the development and promotion of BL. Later on, EBV was found to be associated with other cancers such as Hodgkin Lymphoma (HL) and non-Hodgkin lymphoma (NHL) (Levine et al., 1971), some types of gastric cancers (Shibata et al., 1991), lymphoproliferative disorders and nasopharyngeal carcinoma (NPC) (zur Hausen et al., 1970; de Schryver et al., 1969).

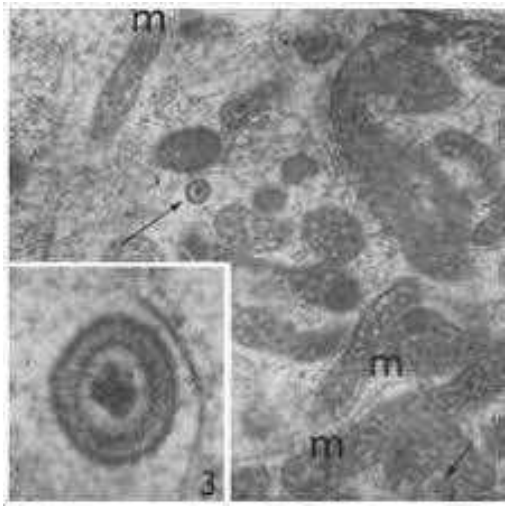


Figure 1: EBV particles observed by electron microscopy in BL cell lines. m: mitochondria, arrow pointing to mature EBV particles. Inset: the viral particle is clearly composed of a dark nucleoid surrounded by a double layered membrane also darker (Epstein et al., 1964).

B. Epidemiology

It has been shown that EBV is currently present in over 95% of the world's populations. Most primary infections happen before adulthood. In Africa and other developing countries, the first infection occurs during childhood whereas this happens later during adolescence in developed countries (Evans et al., 1972). The primary infection is usually asymptomatic but can sometimes manifest itself as infectious mononucleosis (IM) (Evans, 1972). This benign illness is due to an increase in the number EBV+ B cells in peripheral blood accompanied by a strong proliferation of EBV-specific CD8+ T cells. Transmission of the virus is generally through oral secretion as the saliva contains many viral particles. In addition to this, the transmission of EBV has also been noted after transplantation of organs or hematopoietic stem cells (Orazi et al., 1997; Paya et al., 1999). It is noteworthy to mention that contracting EBV-associated-IM during young adulthood is linked with a threefold increased risk of developing EBV-associated HL (Hjalgrim et al., 2000). However, there seems to be no link between IM and other EBV-associated malignancies, such as NPC (Goldacre et al., 2009).

C. General characteristics

1) EBV structure

EBV is part of the *gammaherpesvirinae* family and is also known as Human Herpes Virus-4 (HHV-4). Like all member of this family, it is a double stranded DNA virus. The double helix DNA contains 85 genes and is in the nucleoid. The nucleus-like region is bound by an icosahedral nucleocapsid that measures 100-120nm in diameter and is made up of 162 capsomeres. The space between the nucleocapsid and the outer envelope is lined with the tegument, a protein-rich matrix. This envelope contains proteins and surface glycoprotein projections that originate from the cell's nuclear membrane. The projections contribute to the binding of the virus to the target cell (Figure 2).

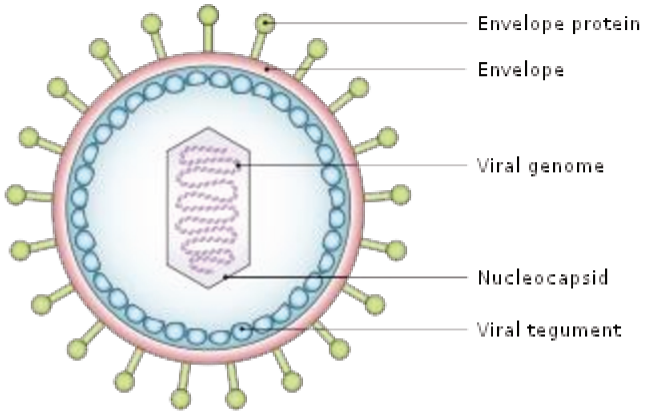


Figure 2: Structure of the Epstein Barr virus. This illustration of an EBV virion shows, inwards to outwards: a viral genome held within a nucleocapsid. The tegument lines the viral envelope that has anchored proteins (study.com).

2) EBV DNA organisation

The full sequencing of the EBV genome by Baer and collaborators in 1984 has given new insight into its organisation and function (Baer et al., 1984) (Figure 3). The double stranded linear DNA of EBV is around 172kpb and can be divided in two major regions: Long Unique Region (U_L) and Short Unique Region (U_S). The U_S is also referred to as U1 (Unique Region 1), whereas the U_L region is divided into 4 unique sub-regions (U2/3/4/5). Between these unique regions are found repeated sequences in the EBV genome named Internal Repeats (IR1/2/3/4) of around 3kpb each. The end regions are also made up of terminal repeats (TR) that are 500pb tandems sequences. These TR allow the viral DNA to have a circular form called Covalently Closed Circle (CCC) or episome. The episome structure is key for the virus to stably maintain its genome in the nucleus of the infected cell without having to integrate it into the host cell's genome.

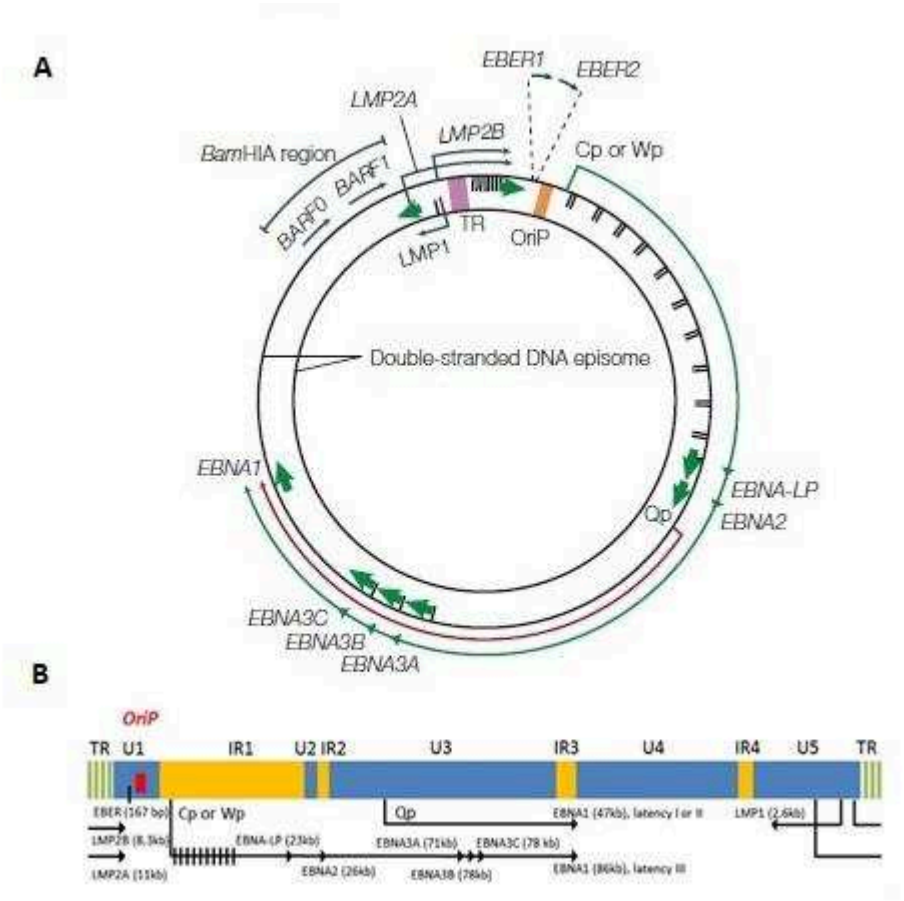


Figure 3: Schematic diagrams of the EBV genome. A. EBV circular double stranded DNA or episome. The arrows show the direction of transcription, the origin of plasmid replication (OriP) is shown in orange, the larger green arrows indicate the exon coding regions for the latency proteins. Cp or Wp are the promoter for EBNA2, EBNA3A/B/C and EBNA1, the long outer green arrows represents the primary transcript that is spliced differently to produce the different EBNA proteins. The inner long red arrow shows the EBNA1 transcript, originating from the Qp promoter. BARF0/1 are promoters for the LMPs and Terminal Repeats (TR) region of the LMP proteins is shown in pink. The TR region is formed during the circulisation of EBVs viral DNA. The blue arrows represent the highly transcribed RNAs of EBER1/2 (Young and Rickinson, 2004). B. Diagram of EBV's linear DNA. Unique regions (U1-5) are shown in blue whilst Internal Repeats (IR1-4) are in yellow, the OriP is coloured in red and the two Terminal Repeat (TR) regions in green link together during the circulisation of the genome to give the episome. The promoter of latency genes Cp, Wp and Qp are shown at the top of the black arrows showing the direction of transcription (Ok et al., 2013).

The origin of replication (OriP), found in U1, is only functional during the latency phase of EBV. Two other origins of replication named OriLyt are found in IR2 and IR4 and are active during the lytic phase. These two phases are determined by the expression state of the Epstein-Barr Nuclear Antigens (EBNA) proteins. Their expression depends on four promotors: Cp, Wp, Fq and Qp.

D. EBV life cycle

After infection of a cell by EBV, this virus is generally in a lytic phase where it reproduces quickly and spreads to other targets by producing many virions. However, the virus can also enter into a low activity latency phase that can last anywhere from a few days to a whole lifetime. This phase of "hibernation" will last until the virus is reactivated and enters a lytic phase once again.

1) Lytic phase

The lytic phase can occur either after a primary infection or after a reactivation. This is the replication phase of the virus allowing it to infect and propagate to other epithelial or B cells. The Z Epstein Barr Replication Activator protein (Zta or EB1), encoded by the gene *BZLF1*, is thought to be the key player for the transition of a latency phase into a lytic phase (Grogan et al., 1987). Zta induces the expression of other early proteins such as Rta (or EB2) and Mta respectively encoded by the genes *BRLF1* and *BMLF1*. These transactivators are expressed very early on during the lytic phase and are named immediate Early Antigens (IEA). These IEA genes activate the expression of proteins involved in the DNA replication machinery (BALF2 and BAFL5) as well as structural viral proteins expressed further along the lytic phase such as the Viral Capsid Antigen (VCA) and Membrane Antigens (MA) found on the viral envelope.

Indeed, it is during this lytic phase that new virions are produced and secreted into the extracellular media in order to infect new cells (Figure 4). For this process, the viral DNA is cleaved into linear form so it can be packed in the nucleocapsids. Then, the nucleocapsids exit the nucleus by nuclear egress: the capsid buds into the Inner Nucleus Membrane (INM) to form a primary envelope and buds outwards into the cytosol via the Outer Nuclear Membrane (ONM). By then, the tegument-coated nucleocapsid is taken up by cis-Golgi vesicles that finish by fusing with the cell plasma membrane (PM) to secrete the newly formed virions into the extracellular space.

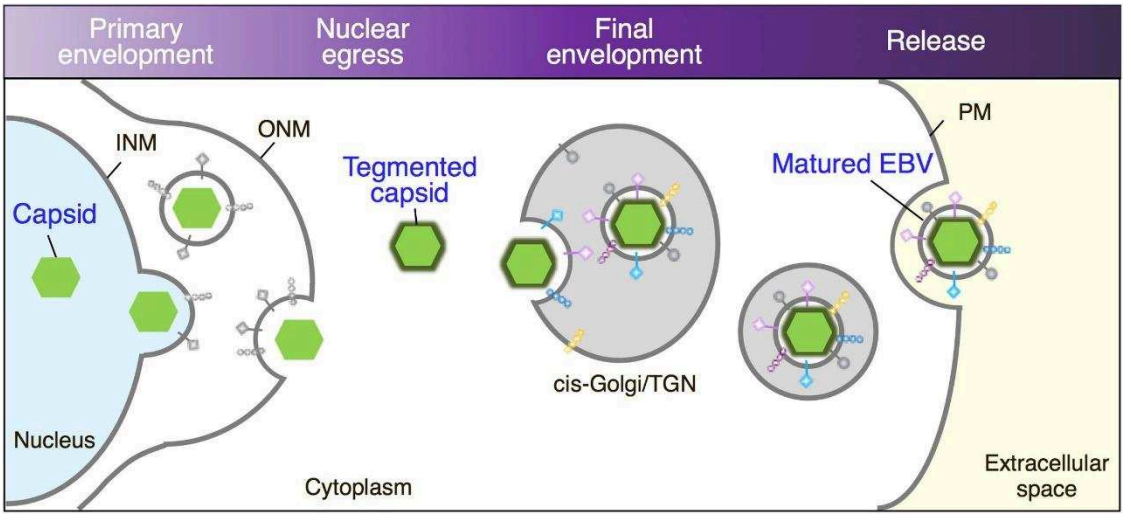


Figure 4: Maturation and release of new EBV virions. The circular DNA episome, found in the host cell's nucleus, is linearized and packed into capsids. Nucleocapsids use the Inner Nuclear Membrane (INM) to form the primary envelope. This envelope will then fuse with the Outter Nuclear Membrane (ONM) to release the tegument-coated nucleocapsid into the cytosol: this is nuclear egress. The tegumented capsid will then be enveloped by budding into the intracellular compartments derived from the cis-Golgi/Trans-Golgi Network (TGN). The virions will mature in these compartments and be transported to the cell surface where the vesicles will fuse with the plasma membrane (PM) to release the virions into the extracellular space (Nanbo et al., 2018).

In vitro, Lymphoblastoid Cell Lines (LCLs) are commonly found in latency phase and only a small part (2-10%) are in a lytic cycle. This is because the latency genes are actively suppressing the lytic program. Nevertheless, it is possible to induce a lytic phase with exogenous stimuli such as Transforming Growth Factor-β (TGF-β), surface immunoglobulins or vectors containing proteins for instance Zta or EB2 (Gradoville et al., 2002).

2) Latency phase

The latency phase is used by EBV to ensure its survival within the host. This phase is characterised by the expression of a low number of genes. Indeed, only a few genes are responsible for the set up and maintenance of latency. Three main categories of latency genes have been described, namely the genes coding for: the EBNA proteins, the Latency Membrane Proteins (LMP) and the small RNA EBV Encoded RNAs (EBERs) and BamHI-A region without Rightward Transcripts (BARTs). Different types of latencies have been described and can be distinguished by the expression profile of only a few latency genes. Table I shows the expression of viral proteins in latencies type 0, I, II and III. Furthermore, each latency is associated with a type of malignancy (see Table 1).

Table I: EBV latencies

Type of latency	Latency proteins and RNAs expression	Associated malignancies	References
Type III	EBERS, LMP1, LMP2, EBNA1, EBNA2, EBNA 3	Post-transplant Lymphoproliferative disorders Diffuse large B cell Non-Hodgkin lymphoma	(Gaidano and Dalla-Favera, 1995; Hamilton-Dutoit et al., 1993; Sakamoto et al., 2017)
Type II	LMP1, LMP2, EBERs, EBNA1	Nasopharyngeal Carcinoma NK/T Lymphoma Hodgkin Lymphoma Diffuse large B cell Non-Hodgkin lymphoma T cell NHL	(Hamilton-Dutoit et al., 1993; Harabuchi et al., 1990; Hu et al., 2016; Levine et al., 1994; Niedobitek et al., 1997)
Type I	EBERs, BARTS, miRNA of BARTs, EBNA1	Burkitt Lymphoma Gastric cancer	(Marquitz et al., 2014; Onnis et al., 2012; Sivachandran et al., 2012)
Type 0	EBER1, EBER2, LMP2A transcripts, BARTS	-	(Babcock and Thorley-Lawson, 2000; Shaknovich et al., 2006; Thorley-Lawson, 2001)

a. Latency type III

Latency type III is the growth program found in naive B cells after primary infection or EBV reactivation. In this pattern, all viral latency proteins are expressed: EBNA1-6, LMP1, LMP2a/b, EBERs and BARTs. Such viral proteins and RNAs are found in lymphoproliferative disorders due to Acquired Immune Deficiency syndrome (AIDS) or transplantation but also in some types of NHL (Tse and Kwong, 2015) (see references in Table I). The infected B cells are driven into a proliferating state. This pattern is also known as the immortalisation phase of EBV. LCLs are commonly found in this type III phase (Iwakiri et al., 2006). However, this highly immunogenic state results in the destruction of infected B cells by cytotoxic CD8+ T lymphocytes. This leads to a positive selection of B cells capable of switching to the default pattern: latency type II.

b. Latency type II

Also known as the “default program”, latency type II is characterised by the expression of EBNA1 and the LMP proteins (LMP1, LMP2A and LMP2B). The type II pattern of expression is linked to many EBV-associated malignancies such as nasopharyngeal carcinoma, HL, NK/T cell lymphomas and T cell

NHL (see references in Table I). LMP1 and LMP2a initiate the differentiation of the infected B cells into resting memory B cells (Young and Rickinson, 2004). In turn, these cells can switch to two types of immunologically silent latency profiles: latency type I or 0.

c. Latency type I

Latency type I has been described in BL and some types of gastric cancers (see references Table I). The only viral protein expressed in this pattern is EBNA1. However, other viral actors such as the small RNAs EBERS, BARTs and miRNAs of BARTs are also present (see ref Table 1). This low protein expression profile is found in replicating memory B cells and allows the virus to merely maintain itself, primarily due to EBNA1.

d. Latency type 0

The Type 0 latency pattern shows a complete shutoff of all viral proteins expression. Only the small RNAs EBER1/2 and BARTs are found in resting B memory cells (see references Table I). Immunologically invisible, the virus is maintained in infected cells by hiding amongst the host's cell's nucleus. No pathologies have yet been associated to latency type 0.

3) Latency type II viral proteins and small RNAs

My thesis work focuses on nasopharyngeal carcinoma, that's why I choose to only describe the latency type II-associated proteins (EBNA1, LMP1, LMP2a/b), small RNAs EBERs and BARTs.

a. EBNA1

EBNA1 was the first EBV viral protein discovered (Reedman and Klein, 1973) and has since been shown to be involved in both the lytic and latency phases. It is the only EBV viral protein found in all EBV-associated malignancies which demonstrates its vital function for EBV. EBNA1 binds to the OriP but also to chromosomal DNA. This binding allows the protein to act as a transcriptional activator for viral and cellular genes. Furthermore, ligation to the hosts cells chromatin enable EBNA1 to maintain and ensure correct viral DNA segregation within the infected cell (Mackey et al., 1995). However, the exact elements that allow EBNA1 to bind to host chromatins have yet to be identified. N-terminal (Nter) AT-hooks are an interesting new lead in this search. The hypothesis is that AT hooks present in EBNA's structure bind to the AT-rich DNA. Indeed, AT-hooks have been found in the Chromosome Binding Sites 1-3 region (CBS1-3) of EBNA1 and are thought to be responsible for the episome's chromatin binding (Kanda et al., 2013; Sears et al., 2003) (Figure 5). An argument in favour of this idea is the use of netropsin, a drug that binds to the AT-rich DNA sites, which leads to the loss of EBV viral

genome in epithelial and lymphoid cells (Chakravorty and Sugden, 2015). But the deletion of AT regions on the Nter EBNA1 sequence does not stop the binding of EBNA1 to the cell chromatin all together (Hodin et al., 2013). Indeed, Deschamps also showed that the AT regions, although important, were not essential for binding to mitotic chromatins during interphase. They found that EBNA1 also binds to the cellular Regulator of Chromosome Condensation 1 (RCC1), a guanine-nucleotide releasing factor for the Ran GTPase enzyme that is key for the early condensation of chromosomes during S phase. This novel partner directly binds to EBNA1 during metaphase via the same domains that tether with mitotic DNA: CBS1/3 and the Glycine-Arginine (GR) motifs (Deschamps et al., 2017). Expectedly, these mechanisms are good targets for future treatment of EBV-associated malignancies but much still remains unclear (Figure 5 in red).

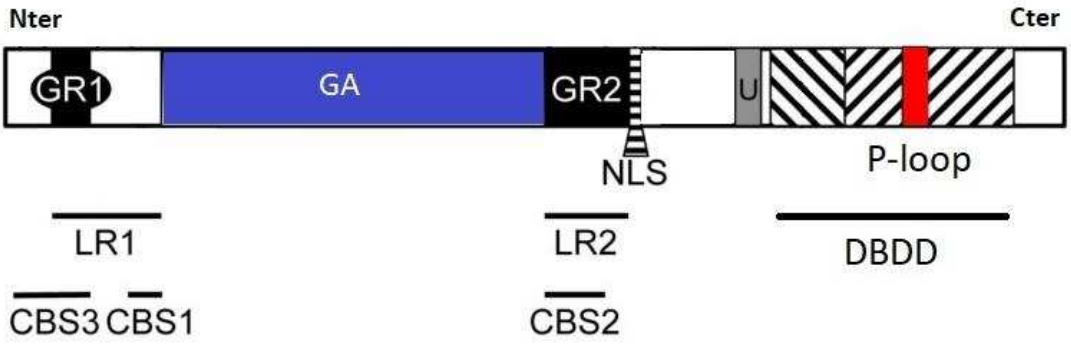


Figure 5: Linear representation of EBV's latency protein EBNA1. GR (black box): Gly/Arg rich domains (GR1/2), the blue zone is a region of Gly/Ala repeats (GA); Nuclear Localisation Signal (NLS) is shown in the horizontally crossed area; the grey box shows the ubiquitin-specific protease 7 (USP7) core binding domain (U); the diagonal cross-hatching is DNA binding and dimerization domain (DBDD), the proline-rich loop (P-loop) is marked in red and the linking regions 1 and 2 (LR1/2) are indicated within the GR1/2 regions as well as the chromatin binding sites 1, 2 and 3 (CBS1/2/3) (adapted from Wilson et al., 2018)

EBNA1 also plays a key role in the immortalisation of the infected cells as it has a potent anti-apoptotic effect (Frappier, 2012). Indeed, it favours survival and blocks apoptosis through multiple pathways. Nagy and Klein showed that EBNA1 blocks the interaction of tumour Protein 53 (p53) to one of its partners Ubiquitin Specific protease 7 (USP7) (Holowaty et al., 2003; Nagy and Klein, 2010). Indeed, p53 and EBNA1 compete to bind to the same Nter domain named Tumor Necrosis Factor (TNF) Receptor Associated Factor (TRAF), EBNA1 has a higher affinity than p53 for USP7 (Saridakis et al., 2005) (Figure 5). Other USP7-independent disturbances of p53 functions have also been described in CNE2 NPC cells lines. Indeed, EBNA1 was able to impair DNA repair and apoptosis by disrupting promyelocytic leukemia (PML) nuclear bodies 10 (ND10s) (Sivachandran et al., 2008). Moreover, EBNA1 can also increase oxidative stress resulting in the accumulation of toxic Reactive Oxygen Species (ROS) as well as non-functional uncapped telomeres involved in apoptosis and DNA damage recognition (Gruhne et al., 2009; Kamranvar and Masucci, 2011; Lassoued et al., 2008). Additionally,

high expression of survivinin human B cell lymphomas was described by Lu and his collaborators. It was shown in this study that EBNA1 can link to the survivin promoter, Specificity protein 1 (Sp1). A knockdown of both survivin and EBNA1 showed greater apoptosis in infected cells (Lu et al., 2011). Finally, EBNA1 is implicated in the disruption of other cell proliferation and apoptosis pathways. Amongst these, EBNA1 increases the expression and phosphorylation of Signal Transducer and Activator of Transcription 1 (STAT1), a protein involved in cell death (Wood et al., 2007). Wood also showed that EBNA1 decreases the expression of TGF-β1 by lowering the levels of its transcription factor SMA- and MAD- asscociated protein 2 (Smad2) (Wood et al., 2007). In addition, it was shown in NPC biopsies by Valentine et al. that EBNA1 can block Nuclear Factor-K B (NF-KB) activity by hindering its binding to DNA. NF-KB was no longer nuclear but cytoplasmic as its p65 subunit was not phosphorylated (Valentine et al., 2010).

Finally, another role of EBNA1 that remains elusive is EBNA1's capacity to promote immune evasion. For this, EBNA1 blocks antigen presentation by two mechanisms (i) blockade of the proteasomal mechanism responsible for degrading and charging the Major Histocompatability Complex type I (MHC I) with the EBNA1-specific peptide (ii) but also by silencing its own expression, allowing the virus to remain immunologically invisible. Using its Glycine-Alanine rich domain, it was shown by Letviskaya in 1995 that EBNA1 inhibits its recognition by Cytotoxic T Lymphocytes (CTL). This ground-breaking study showed for the first time that viruses are able to escape immune surveillance (Levitskaya et al., 1995a). Nevertheless, if this was the only machinery at play, an accumulation of undegraded-EBNA1 would be expected in infected cells. But this is not the case as Yin et al showed that EBNA1 is also capable of auto-silencing its expression in order to escape immune surveillance (Yin et al., 2003). It is thought that by inhibiting its expression, EBNA1 limits the possibilities of producing Defective Ribosomal Products (DRiPs) as they are suspected to be the main source of peptides charged on to the MHC I molecule (Yewdell et al., 1996).

Notably, NPC patients show high levels of anti-EBNA1 Immunoglobulin (Ig) G antibodies and increasing anti-EBNA1 IgAs during the progression of their illness. Although healthy carriers of EBV also show circulating IgGs against EBNA1, these levels remain low throughout life. In Asia, the use of an anti-EBNA1 IgA as a tool of NPC prediction for high-risk patients is well underway (Coghill et al., 2014; Liu et al., 2012). We can even hope that one day EBNA1 will help stratify patients in order to administer appropriate treatments and predict their efficacy.

b. LMP1

LMP1 is a transmembrane viral protein with a short half-life (around 3 hours). It aggregates at the membrane and is tightly linked to the cell's cytoskeleton. It is made up of 3 domains: a cytoplasmic Nter domain (24 aa), a transmembrane domain made up of 6 α helixes (162 aa) and a C-terminal (Cter) cytoplasmic domain (200 aa) (Figure 6).

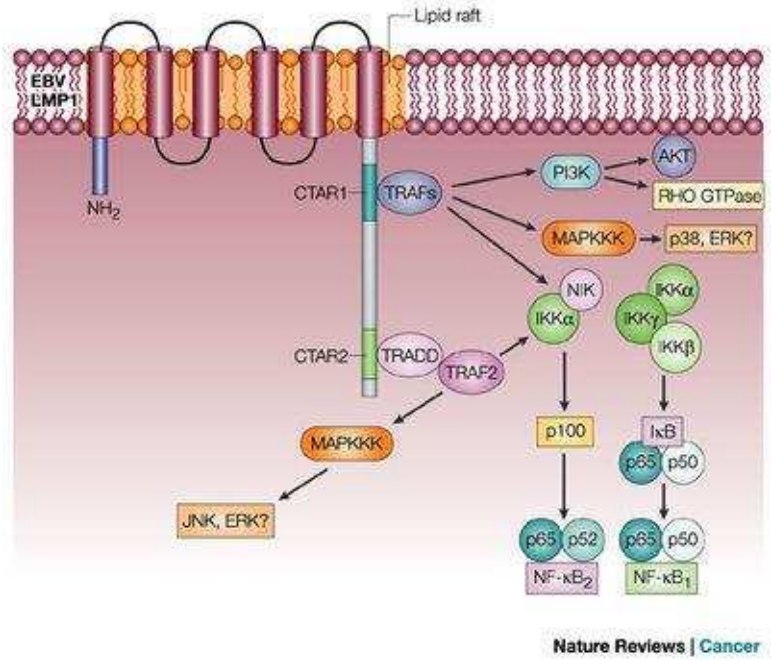


Figure 6: Structure of LMP1 viral protein. LMP1 has 6 transmembrane domains, two cytosolic domains and two regions of binding to members of the Tumour Necrosis Factor Receptor (TNFR) family (CTAR1/2) (Adapted from Young and Rickinson, 2004).

The two first domains are mainly involved in the anchoring of the protein in the membrane. However, the Cter domain is responsible for the activation of several cellular pathways. It contains two distinct domains named Cter Activating Region 1 and 2 (CTAR1/2). LMP1 is a functional homologue of TNFs and thus activates this pathway by recruiting members of the TNF receptor (TNFR) family: TNFR type I Associated Death Domains (TRADDs) and TNF Associated Factors (TRAFs) (Kaye et al., 1996). By interacting with TRADD and TRAF, LMP1 disrupts many pathways such as Mitogen-Activated Protein Kinases (MAPK), c-Jun Nter Kinase (JNK), Extracellular signal-regulated kinase (Erk) and NF-KB in infected cells, thus uncontrollably increasing their proliferation and survival capabilities (Eliopoulos et al., 1999a, 1999b; Greenfeld et al., 2015; Kieser et al., 1997). Rickinson's team showed the importance of LMP1 for immortalisation of infected cells. Indeed, when the EBV genome was deleted of its LMP1, it was no longer able to immortalise B cells (Garibal et al., 2007).

Additionally, Morris founds that LMP1 is involved in cellular adhesion mechanisms by activating TGF-β and integrin A signalling (Morris et al., 2016). A more recent study by the same team, showed

that LMP1 can induce an Epithelial-Mesenchymal Transition (EMT) phenotype in epithelial cell lines (MDCK) via its CTAR1 domain. It was found that although the LMP1-mediated EMT does not depend on the established EMT contributor TGF-β, LMP1 does induce kinase-dependant mechanisms involved in integrin activation. As commonly found in EMT, ERK-MAPK, Phosphatidylinositol 3-Kinases (PI3K)/ Serine/threonine Kinase (Akt) and Sarcoma Tyrosine kinase (Src) pathways are all overactivated by LMP1 which leads to enhanced cell survival, invasiveness and metastatic potential (Morris et al., 2018).

Necroptosis is an alternate cell death mechanism that is induced when apoptosis is blocked or when cells are infected by a pathogen (Vandenabeele et al., 2010). To escape cell death, LMP1 inhibits necroptosis factors by promoting the ubiquitinylation and subsequent degradation of Receptor-Interacting Protein Kinase 1 and 3 (RIPK1/3) (Liu et al., 2018).

Interestingly, LMP1 has been detected on EBV+ cell-derived nanovesicles names exosomes. Hurwitz found that CD63 is required for the LMP1-mediated increase in exosome secretion and its packaging onto exosomes. However, CD63 does not seem involved in LMP1 trafficking to lipid rafts which mediates signalling pathways such as NF-KB, PI3K/Akt and MAPK/ERK (Meckes et al., 2013; Yasui et al., 2004). The LMP1+ exosomes increase the proliferation, migration and invasiveness of tumour cells (Hurwitz et al., 2017). They also favour immune evasion of cancer by enhancing the immunosuppressive tumour microenvironment through the recruitment and exacerbation of regulatory T cells (Tregs) in a NPC model (Mrizak et al., 2015).

c. LMP2

LMP2 is a transmembrane protein that has two isoforms: LMP2A and a shorter LMP2B. Both proteins are coded by the same gene but their expression is regulated by different promoters.

LMP2A has an extra cytosolic Nter domain rich in Tyrosines containing an Immunoreceptor Tyrosine-based Activation Motifs (ITAM). This motif can seize two tyrosine-kinases, Lyk and Syk, that are necessary for the function of the B Cell Receptor (BCR) of infected B cells (Winberg et al., 2000). This disruption of BCR function allows the virus to stay in a latency state and blocks its entry into the lytic phase (Merchant et al., 2001). In contrast, as LMP2A shows a homology with the BCR, it is also capable of activating it. This prolonged minimal activation of the BCR ensures the survival of the infected cells and stops them from dying (Merchant et al., 2001). It has also been shown in BL that LMP2A can accelerate the development of lymphomas and inhibit apoptosis by a translocation of the c-myc proto-oncogene (Bultema et al., 2009). A study by Fish showed *in vivo* that LMP2A thrives on the involvement of c-myc in the cell cycle to promote a hyperproliferation of B cells and tumorigenesis. Indeed, it was demonstrated that LMP2A increases c-myc expression and thus favours c-myc-

dependant degradation of the tumour suppressor $p27^{kip1}$, a cyclin-dependant kinase inhibitor (Fish et al., 2017).

In epithelial cancer cell lines such as gastric cancer, LMP2A also plays a key role in the transformation of cells (Fukuda and Longnecker, 2007). For this, the PI3K/Akt and β-catenin pathways are disrupted (Morrison and Raab-Traub, 2005) and LMP2A increases the cancers capabilities to form new metastases (Pegtel et al., 2005). It was also shown that LMP2A could increase invasiveness of cancer cells by modulating the expression of Matrix Metallo-proteinases (MMPs), and notably MMP9. The expression of MMP9 depends on the transcription factors. Activator Protein 1 (AP-1), Fos-related antigen 1 (Fra-1) and the Erk1/2 pathway, all of which are regulated by LMP2A (Lan et al., 2012). Incrocci also showed that LMP2A was able to enhance the production of the immunosuppressive cytokine Interleukin 10 (IL-10) (Incrocci et al., 2013). He then later determined that the phosphorylation of STAT3, a major IL-10 activating factor, by Bruton's Tyrosine Kinase (BTK) was activated by LMP2A (Incrocci et al., 2017). LMP2A is also seemingly implicated in an immune evasion strategy which involves the downregulation of the Human Leucocyte Antigen (HLA). Indeed, in gastric cancer cells and HEK293 LMP2A+ cells, LMP2A downregulates HLA expression via the Hedgehog signalling pathway, notably via the Glioma-associated oncogene 1 (Gli1) (Deb Pal and Banerjee, 2015).

Unlike LMP2A, LMP2B lacks the first exon at the cytosolic terminal region that contains supposed binding sites. Although, not much is known about the role of LMP2B, many EBV-associated malignancies such as NPC, HL and gastric carcinoma express both LMP2 isoforms. Lennette has even shown that 5% of tested NPC patients only express LMP2B (Lennette et al., 1995). It has been suggested that LMP2B contributes to the transformation of epithelial cells to favour metastatic invasion of EBV-associated tumours (Allen et al., 2005). Also, LMP2B negatively regulates the expression of LMP2A (Rechsteiner et al., 2008a). It re-establishes the function of the BCR and thus favours the reactivation to the lytic phase (Rechsteiner et al., 2008; Rovedo and Longnecker, 2007).

d. EBERs

EBERs are small, nuclear, non-polyadenylated and non-coding RNAs found in all the types of latencies. EBERs are transcribed by the host's RNA polymerase III (Rymo, 1979) into EBER-1 and EBER-2. It has been described by Lerner that they aggregate into very stable ribonucleic protein complexes (Lerner et al., 1981). EBERs are found in high amounts in infected cells and can inhibit apoptosis to ensure the maintenance of the virus. For this, EBERs bind to double stranded RNA-activated Protein Kinases (PKRs) and have a key role in the mediation of anti-viral effect via interferons (Clemens et al., 1994; Nanbo and Takada, 2002; Sharp et al., 1999). Also, it was shown that EBERs can induce the

expression of immunosuppressive cytokines such as IL-10 (Kitagawa et al., 2000) in EBV+ BL cells or IL-9 in EBV+ T cells (Yang et al., 2004). However, EBER2 can regulate LMP1/2 expression as well by indirect interaction with the cellular transcription factor Paired Box Protein 5 (PAX5). Lee later revealed that the direct binding factor to EBER2 and PAX5 are Splicing Factor Proline and Glutamine rich protein (SFPQ), RNA Binding Motif Protein 14 (RBM14) and Non-POU domain-containing Octamer-binding protein (NONO). This modulation of LMP1/2 expression by EBERs allows them to indirectly control lytic replication of EBV (Lee et al., 2016).

Recently, a metanalysis carried out on patients with Diffuse Large B-Cell Lymphoma (DLBCL) revealed that EBERs expression was significantly correlated with worse clinical outcomes (overall survival (OS) and progression-free survival) (Gao et al., 2018).

e. BARTs and miRNAs

The EBV genome encodes miRNAs from two regions: *BamHI* A Region Rightward Transcripts (BARTs) and *BamHI* fragment H Rightward open reading Frame 1 (BHRF1). BART-miRNAs are also found in all types of latencies whereas BHRF1-miRNAs have a restricted distribution (Imig et al., 2011; Marquitz et al., 2014; Pratt et al., 2009). The highest levels of BARTs are found in infected epithelial cells including NPC and gastric cancer cells (Hitt et al., 1989; Marquitz and Raab-Traub, 2012). BARTs are a group of heterogeneously spliced RNAs that vary from 4 to 8kpb in size (Sadler and Raab-Traub, 1995). These mi-RNAs of around 22 nucleotides long and can modulate gene expression.

It has been shown that the disturbance in the production of these miRNAs can act in favour of tumour progression by promoting cell proliferation, survival and migration. For example, miR-BART5 can inhibit the expression of p53 by modulating one of its partners P53 Up-regulated Modulator of Apoptosis (PUMA) and thus promotes cell survival (Choy et al., 2008). Mir-BHRF1-3 can block the C-X-C motif Chemokine Ligand 11 (CXCL11) (Xia et al., 2008). CXCL11 binds to C-X-C motif Chemokine Receptor 3 (CXCR3) during the Interferon (IFN) immune response led by T lymphocytes allowing the virus to fight against the host's immune response. Moreover, it has been shown that BART16 can downregulate LMP1 expression by targeting its untranslated 3' region. Also, BART16 inhibitors caused hyperproliferation of cells and no change in apoptotic activity (Zhang et al., 2018c). Although it has been shown that EBV-miRNAs play a role in the development of cancer, they do not seem to be the main driver and act as a reinforcement mechanism (Feederle et al., 2011; Seto et al., 2010; Wahl et al., 2013).

E. Physiopathology of EBV

1) EBV Tropism

It was long thought that EBV tropism was restricted to B cells and epithelial cells but later studies have shown that a wide range of cells can be infected by the virus. Indeed, EBV is now associated with several diseases ranging from epithelial NPC, to T/NK lymphomas or B cell lymphomas.

a. B lymphocytes

The first step of viral infection is the attachment of the virus to the target cell. For this, the viral envelope glycoprotein 350/220 (pg350/220) binds with high affinity to the Complement Receptor type 2 (CR2/CD21) on B cells (Fingeroth et al., 1984; Frade et al., 1985; Tanner et al., 1988). Along with CD19, CD81 and CD225, CD21 forms the B cell co-receptor complex that lowers the activation threshold of the BCR. Although important, the pg350/220-CR2/CD21 interaction does not seem to be the only mechanism of viral attachment (Janz et al., 2000). Once this step achieved, the B cell will endocytose the virus leading to the fusion of the cell membrane with the viral membrane (Tanner et al., 1987). This fusion is led by a complex of 3 viral glycoproteins: gH, gL, gB. This complex is made up of a gB trimer and a heterodimer of gH and gL. These three viral glycoproteins are also referred to as the core fusion machinery. This complex can act on its own but can also benefit from an extra co-factor to ensure viral fusion with the host cell. For B cells, the MHC II molecule interacts with a viral protein named gp42. The latter binds directly to gH (Wu and Hutt-Fletcher, 2007) through a non-covalent bond (Li et al., 1997; Wang and Hutt-Fletcher, 1998). The fusion is also triggered by the interaction of gp42 with the MHC II (Li et al., 1997; Mullen et al., 2002).

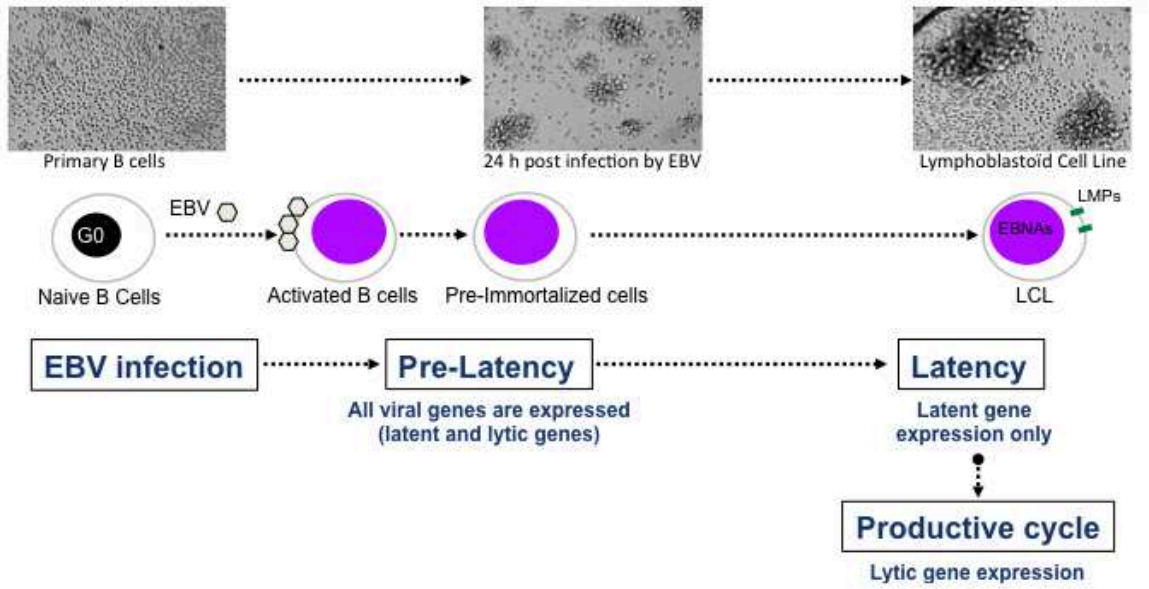


Figure 7: B cell immortalisation into LCLs *in vitro*. Primary B cells are infected with EBV. Then, they are activated and enter a pre-latency phase, these are known as pre-immortalized cells. Finally, the cells become immortalised LCLs that express only latency or lytic viral genes. As shown above, the cells grow in clumps once infected by EBV (Ciri.inserm.fr).

EBV infects B cells and drives their immortalisation into LCLs. During this process, the phenotype of the B cell is firstly that of an activated cell, as CD21 is a co-stimulatory marker. Then the virus establishes a pre-latency programme by expressing all viral genes which gives rise to pre-immortalised B cells. This pattern triggers the transformation of cells into immortalised LCLs and the virus induces a latency type II pattern. After this, the cells enter a lytic phase where only the viral lytic genes are expressed. *In vitro*, LCLs are characterised by their CD23hi/CD58+ phenotype. In culture, the cells will grow in clumps as their adherence changes. The expression of adherence molecules such as CD54, InterCellular Adhesion Molecular 1 (ICAM1) and Lymphocyte Function-associated Antigen 1 (LFA1) are all increased (Figure 7). Additionally, a recent review by Kamranvar and Masucci summarises how EBV can tamper with telomere homeostasis during B cell immortalisation (Kamranvar and Masucci, 2017). Indeed, EBV seems to be capable of activating telomerase activity (Terrin et al., 2008), inducing chromosomal instability (Kamranvar et al., 2013) and oxidative stress that leaves telomeres unprotected. All of this genetic instability ultimately leads to oncogenesis (Chen et al., 2016; Opresko et al., 2005).

b. Epithelial cells

In vitro attempts of epithelial cell infection with EBV proved more difficult than with B cells which left many wondering if epithelial cells could directly be infected by EBV. But, it was shown that co-cultivating infected lymphoblastoid cells (Akata cells) with epithelial cell line resulted in the infection of the latter (Imai et al., 1998). A more recent study found that EBV infection could not only be via the lysis and release of virions but also by a cell-to-cell contact-dependant mechanism. Indeed, supernatant alone of infect B cells was not as effective as a co-culture of infected B cells and epithelial cells. The hypothesis put forward by the authors is that EBV can bind to the surface of B cells to simultaneously infect B cells by endocytosis and epithelial cells by cell-to-cell-contact (Shannon-Lowe et al., 2006). This contact allows EBV to attach to epithelial cells, but this occurs via a different pathway than that used to infect B cells. For instance, epithelial cells only express low levels, if not any, CR2. It has since been suggested that IgAs specific to viral capsid proteins gp350/220 can bind to a polymeric IgA receptor (Sixbey and Yao, 1992). This study showed that Sixbey and his team were able to successfully infect a colon adenocarcinoma cell line using IgAs purified from IM patients. Unlike B cell fusion with the virus, epithelial cells do not use the gp42/MHC II pathway as they do not express MHC II molecules. The glycoprotein complex only includes gH, gL and gB. Interestingly, gp42 hinders EBV fusion with epithelial cells (Kirschner et al., 2006; Wang et al., 1998). Infected B cell virions are devoid of gp42 but are more efficient to infect epithelial cells. EBV alternates between a dimeric (gH/gL) or trimeric (gH/gL/gp42) complex to best infect its target cell (Borza and Hutt-Fletcher, 2002).

c. Natural Killer cells (NKs)

Although EBV mainly targets B lymphocytes and epithelial cells, other populations have been identified as EBV+. In healthy donors, after a first infection, EBV+ NKs have occasionally been found in tonsillar tissues (Hudnall et al., 2005; Trempat et al., 2002). However, infected NKs have been identified in NK lymphomas showing a latency type II pattern (Chiang et al., 1996), but also in lymphoproliferative disorders (Kanegane et al., 1996a, 1996b). Kasahara found EBERs in a CD16+ cell population which was identified as NKs (Kasahara et al., 2001). In a study by Trempat and Tabiasco, two distinct types of patients with Chronic-Active EBV infection (CAEBV) were described, in one of which EBV+ NKs were found (Trempat et al., 2002). It still remains unknown how EBV infects NKs as they do not express CD21. But a recent study showed that viral proteins or DNA could be transferred to NK cells without cell-to-cell contact (Lee et al., 2018). Hence, the next major theory put forward is that EBV+ exosomes transfer viral mRNAs or miRNAs to target cells.

Regardless of how NKs are infected, they can be involved in carcinogenesis. It was found in a new *in vivo* murine model of Aggressive NK-cell Leukemia (ANKL) that the injection of primary EBV+ CD16+ CD56+ CD3- and CD19- cells infiltrated several organs (spleen, liver and bone marrow) and strongly contributed to the worsening of the disease (Kinoshita et al., 2018). In contrast, NKs can be impaired by the virus to allow it to infect and proliferate more freely without NK immune surveillance. This has been found in endemic BL during co-infection with malaria and EBV (Forconi et al., 2018).

d. T lymphocytes

T-cell infection by EBV is unusual but is increasingly studied as it seems to underline a viral immune evasion mechanism. Indeed, T cells mount a large immune response against EBV so it can be assumed that the virus infects T cells to impair or divert them. EBV+ T cells have been described in EBV+ diseases such as T cell Lympho Poliferative Disorders (LPD) (Chiang et al., 1996; Kanegane et al., 1998), hemophagocytic lymphohistiocytosis (HLH) (Beutel et al., 2009; Kasahara and Yachie, 2002) and in CAEBV (Cohen et al., 2011).

But the infection of mature T cells *in vitro* by the EBV type 1 strain (EBV-1) has often failed unlike the EBV-2 strain that can infect mature T cells and lead to the expression of latency genes, modify its cytokine secretion and activate T cells (Coleman et al., 2015).

e. Monocytes, monocyte-derived dendritic cells and plasmacytoid dendritic cells

Savard and his team showed that EBV is capable of infecting approximately 20% of freshly isolated human monocytes (Savard et al., 2000). This work also established that EBV infection of monocytes leads to the activation of viral replication as infectious viral particles were found in culture supernatant. Functionally, the infected monocytes showed a less efficient phagocytic activity. Another study went further and demonstrated that EBV infected monocytes and monocyte-derived dendritic cells (MoDCs) via the same trimeric complex as B cells (gH/gL/gp42) (Guerreiro-Cacais et al., 2004). The EBV infection of monocytes led to immature tolerogenic dendritic cells (DCs) as their differentiation from monocytes was blocked. Moreover, it has also been shown that EBV can infect plasmacytoid DCs (pDCs) by binding to HLA-DR. The infected pDCs express latency genes, show an impaired TNF-α secretion, are incapable of mounting a proper T cell response and express regulatory molecules Inducible T-cell Costimulator (ICOS-L) and Programmed Death-Ligand 1 (PD-L1) (Severa et al., 2013). Taken together, these studies show infecting monocytes, MoDCs and pDCs allows EBV to promote immune silencing and evasion in order to maintain itself in the host.

2) EBV infection

a. EBV primary infection

Primary EBV infection is most common among children but can also happen during young adulthood. Usually, the virus persists asymptomatically in the host but can sometimes lead to LPDs, lymphomas and epithelial malignancies. Infected B cells can be found in peripheral blood but it is though that only the presence of EBV in the pool of memory B cells leads to life-long EBV persistence. Thus, two hypotheses are put forward: either EBV infects naive B cells and pushes them towards a memory B cell phenotype or EBV directly infects memory cells (Khanna, 2015). Either way, the presence of the virus in the B cell memory compartment is the key to its latent and long-term presence in the host.

However, the question of how primary infection occurs remains unanswered. Is it B cells that are first targeted by saliva bound virions, which then proliferate in the tonsils and lead to the infection of the underlying tonsillar epithelium? Or is it the epithelial cells of the oropharynx that are first infected that transmit the virus to infiltrating B cells? To further elucidate these questions and have a better understanding of EBV, *in vivo* models are being developed.

b. EBV infection in vivo models

The interest in how EBV infects cells is growing and new methods of studying this mechanism are rising, especially in humanised mouse models that are getting better at reproducing EBV infection, tumorigenesis and resistance to the human immune response (Fujiwara, 2018; Münz, 2017; Rongvaux et al., 2013). Generally, persistence of EBV in the body is asymptomatic but patients with acquired or innate immunodeficiency show an increased proliferation of B cells that can lead to epithelial malignancies or lymphomas. In this context, three main approaches have been developed: **(i)** the use of old-world non-human primates (NHPs) that are infected by a virtually identical EBV-related Lymphocryptovirus (LCV) and are refractory to human-associated herpesvirus infection (Rangan et al., 1986; Rivailler et al., 2002), **(ii)** infection of new-world NHPs with EBV (Epstein et al., 1975; Shope et al., 1973) or **(iii)** immunodeficient mice that are engrafted with a functional human immune system are infected with EBV. The third option is the most attainable for most laboratories, but the graft and the underlying murine immune system were the first obstacles. Firstly, mice were grafted with human Peripheral Blood Mononuclear Cells (PBMCs), but this approach only lasts 3-4 weeks as the mouse eventually rejects the human cells. It was then found that implanting the mice with human foetal thymic and liver tissue before reconstitution with CD34+ hematopoietic progenitor cells leads to the proper mounting of an adaptive immune response (Melkus et al., 2006). As these humanised models

improve, our knowledge of EBV physiopathology grows (Yajima et al., 2008). And as we know more about EBV infection and how it drives oncogenesis, new EBV targets are thus starting to emerge from these *in vivo* models (Antsiferova et al., 2014; Wilson et al., 1996; Zumwalde et al., 2017). Moreover, new treatments for EBV-associated malignancies will also be better assessed by novel *in vivo* models (Ahmed et al., 2018; Yuen et al., 2015).

F. Immunity and EBV

1) Innate immune response against EBV

a. Natural Killer cells

Patients that show functionally compromised NKs show an increased susceptibility to infection by EBV and other herpesviruses (de Vries et al., 1996). This first study led researchers to believe that NKs play an important role in anti-EBV immunity. *In vitro*, it was found that NKs limit the transformation of EBV+ B cell if they are added to the culture within the first few days post-infection. NKs notably carry out the anti-viral immune response by secreting large amounts of IFNγ (Lotz et al., 1985). Further evidence shows that high NK numbers can be detected in IM patients for over a month after the initial diagnosis which is also correlated with reduced levels of viral load (Williams et al., 2005). Pappworth showed that lytic EBV+ cell express lower levels of MHC I and are more easily recognised by NKs. Thus they stop the virus in a latency phase from progressing into a lytic phase (Pappworth et al., 2007). Interestingly, the regulatory enzyme indoleamine 2,3 dioxygenase (IDO) is produced by EBV+ B cells which inhibits the killer cell lectin-like receptor (NKG2D) on NKs via the JNK signalling pathway (Song et al., 2011).

A specific subpopulation of NKs restricted to tonsillar tissue are CD56hi and NKG2A+ can restrict the infection of B cell by EBV via IFN-γ (Jud et al., 2017), as shown in figure 8. It was put forward by Strowig that tonsillar tissue is most likely the first to be infect by EBV and NKs confine the infection to that tissue until a specific adaptative immune response can be mounted (Strowig et al., 2008).

b. Monocytes

Toll-Like Receptor 2 (TLR2) found on monocytes recognises EBV and signals a large secretion of cytokines IFN and IL-10, chemokines such as Monocyte Chemotactin Protein-1 (MCP-1) (Fiola et al., 2010; Gaudreault et al., 2007) and activate NF-KB via the Myeloid Differentiation primary response-88 (MyD-88) signalling pathways (Ariza et al., 2009) which are all key in anti-EBV immunity. EBV also

activates another parallel mechanism via TLR9. Indeed, antiviral cytokines IFNα and IL-8 are more abundantly secreted when monocytes are activated with viral DNA (Fiola et al., 2010).

c. Dendritic cells

Dendritic cells are able to recognise danger signals and patterns through many receptors. Among these, TLR3 and TLR9 have been linked to EBV sensing. TLR3 recognises double stranded RNA (dsRNA) in the endosomal compartment and EBERs (Iwakiri et al., 2006) activating the DCs to prime T cells (Figure 8).

Moreover, pDCs also seem able to initiate a T cell immune response. Although the exact mechanism remains unclear, it is though that the unmethylated linear viral DNA found in non-cell bound virions is sensed by TLR9 (Fiola et al., 2010) (Figure 8). pDCs play a more important role than initially thought in the anti-EBV response. In a study led by Lim and collaborators, a humanised NOD-SCID mouse model of LPD showed that when EBV+ PBMCs were depleted of their pDC compartment, the EBV infection could thrive. Whereas when PBMCs were enriched in pDCs the EBV infection was better controlled (Lim et al., 2007b). Notably, pDC-dependant IFN-α secretion was only effective for a short time after the initial viral detection (24h) (Lotz et al., 1985). pDC-enhanced PBMCs eventually show a greater NK and T cell response. This suggests that pDCs are only initially effective until the suitable adaptive response is mounted. Nonetheless, it is unlikely that pDCs prime T cells. In fact, it has been shown *in vitro* that MoDCs can prime an EBV-specific T cell response (Bickham et al., 2003) as TLR3 (Iwakiri et al., 2009) and TLR2 (Fiola et al., 2010) induce MoDC maturation as above-mentioned. Additionally, a cross-presentation mechanism has also been identified where MoDCs prime T cells with EBV-infected B cell fragments. MoDCs can prime either CD4+ T cells with EBNA1 (Münz et al., 2000) or CD8+ T cells with EBNA3A and LMP2 (Subklewe et al., 2001).

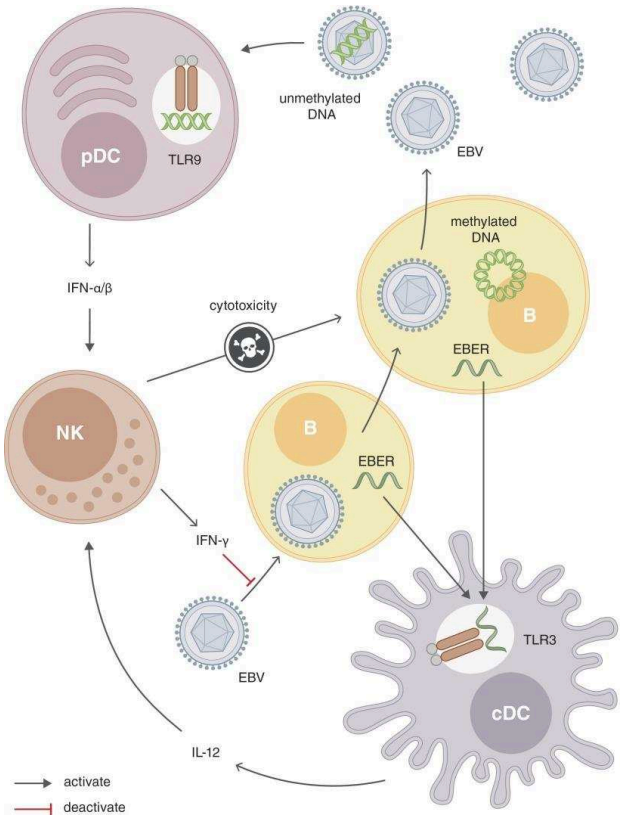


Figure 8: EBV immune response lead by innate immunity. TLR9 is found on pDCs and recognise EBV's unmethylated DNA. Additionally, TLR3 expressed by cDCs is activated by the viral EBER RNAs. Both pathogen-associated signals allow the activation of pDCs and cDCs, which in turn can activate NK cells by cytokine secretion (IFN-α/β and IL-12). NK activation results in the death by lysis of EBV infected cells or enhances resistance to EBV-led immortalisation of B cells via IFN-γ (Chijioke et al., 2013).

2) Adaptive immune response

Once the initial innate immune response is fighting the virus, the adaptative immune response is building itself to target more specifically all whilst creating a memory pool, helping it to anticipate future infections by the same pathogen. Viral antigens are taken up by antigen presenting cells (APCs) to activate the adaptive cellular immune response.

a. CD8+ T cells

The primary infection by EBV is accompanied by a hyperlymphocytosis of mainly EBV-specific CD8+ T cells clones. Indeed, during the acute phase of IM very high levels of CD8+ T cells have been observed (Callan et al., 1996). Two explanations have been put forward to explain this, either CD8+ T cells undergo a non-specific expansion (McNally and Welsh, 2002) or the clonal expansion is a response to a superantigen encoded by EBV (Sutkowski et al., 2001). It was however, shown that the CD8+ T cells express low levels of b cell lymphoma-2 (bcl-2) which makes them more sensitive to apoptosis if there is no antigenic stimulation (Akbar et al., 1993; Moss et al., 1985). The nature of the epitope on

EBV-specific CD8+ T cells is heterogenous and can be classified based on their immunodominance. Among these, are lytic cycle proteins that can represent up to 40% of total CD8+ T cells (Steven et al., 1997). These are mainly IEAs (BZLF1 and BRLF1) and then Early Antigens (BMLF1, BMRF1 BALF2 and BALF5) of the lytic cycle (Hislop et al., 2002; Steven et al., 1997). However, latency protein antigens are also found, but only make up 1-5% of the CD8+ T cells pool. EBNA3A/B/C give the strongest immune response and can sometimes be accompanied by an immune response towards EBNA1 but never LMP1 nor EBNA2. Additionally, structural proteins can also be used as anti-EBV antigens (gp 350, gp 85, gp 110) although they only elicit a weak immune response (Callan et al., 1998; Steven et al., 1996). However, Steven showed that the frequencies of epitope populations in the memory compartment are not the same as in the primary populations. Indeed, dominant epitopes were much less found than in the primary population and less frequent latency epitopes were more frequent in the memory compartment (Steven et al., 1996)

Ex vivo, the isolated CD8+ T cells express perforins which shows cytotoxic capabilities (Callan et al., 2000) as well as expression of the activation marker CD45RO+. The phenotype of different EBV-CD8+ T cells varies during the development of IM. Expression of CD45RO+ shows the activation of CD8+ T cells. Latency epitopes always maintain the expression of CD45RO activation marker whereas lytic epitopes finish by losing it. During the acute phase, lytic protein-specific CD8+ T cells make up 20-50% of the pool, but after 1-2 years they only account for 0,5-2%. However, latency epitopes are not always detectable during the acute phase of IM but always appear later on (Hislop et al., 2002). In fact, a study by Ouyang used a lytic EBV epitope tetramers and found that older people (>87 years old) have higher levels of anti-EBV CD8+ T cells than younger people (20-40 years old). Nevertheless, these cells were not functionally effective. This suggests that the elderly's CD8+ T cells undergo clonal expansion and thus could translate a lack of T cells ready for novel antigens (Ouyang et al., 2003). These results seem to agree with hypothesis that the "immunological space" is filled by the dysfunctional CD8+ T cells put forward by Franceschi (Franceschi et al., 1999). Taken together with the age-related lack of new T cells available for novel antigens, the study suggests that this could account for the greater incidence of infectious diseases in elderly people.

b. CD4+ T cells

Unlike EBV-CD8+ T cells, in IM there is no increase in EBV-CD4+ T cells during the acute phase. This population makes up 1-2% of total circulating CD4+ T cells (Amyes et al., 2003). This was explained by the lack of detectable clonal expansion of EBV-CD4+ T cells (Maini et al., 2000). However, it was described that after 17-28 days of disease onset the responses to EBNA2 and lytic antigens were

already quickly decreasing. So, it was thought that there could be an initial clonal expansion that happened very early on after infection, even before the patients show symptoms. Indeed, Piriou showed in a case report that during primary EBV infection after a renal allograft, $CD4^+$ T cells responded strongly to EBV lysate, 1-2 weeks before the $CD8^+$ T cell response peaked (Piriou et al., 2006).

Multiple studies have used either peptides, viral proteins or infected cell lysates to detect the viral antigen recognised by the CD4+ T cell population (Amyes et al., 2003; Lam et al., 2018; Münz et al., 2000; Piriou et al., 2004; Precopio et al., 2003; Sohn et al., 2015; Woodberry et al., 2005). As the frequency of $CD4^+$ T cells epitopes are 10 folds lower than $CD8^+$ T cells, it is very difficult to study this population. Using a MHC II tetramers to nine different EBV-epitopes, Long showed that EBNA2 was the strongest epitope-specific responses attaining up to 1.5% of $CD4^+$ T cells but other latency and lytic cycle antigens clones were also amplified during primary infection (Leen et al., 2001; Long et al., 2005, 2011). Nevertheless, it is interesting to mention the atypical kinetics of IgG-EBNA1 response in IM patients. Indeed, the EBNA1 clones are not detected until weeks or months after the infection and remain present throughout the life of the patient (Henle et al., 1987; Hille et al., 1993; Long et al., 2013; Rickinson et al., 2014). *In vitro*, Long found that EBNA1 is very little released by EBV-infected cells explaining the late EBNA1 response due to its limited availability.

After the symptoms fade, 0.1% of circulating T cells are EBV-specific (Amyes et al., 2003), and a CD4+ memory T cell population can be detected. Interestingly, this subpopulation shows a $CD45RA^+$, $CCR7^+$, $CD62L^+$, $CD28^+$, $PD1^-$ phenotype that generally defines naive T cells (nTL) (Sallusto et al., 1999a). This population has not been detected in EBV-free donors suggesting that this could be a *bona fide* memory T cell population. Harari et al. described three different subtypes of EBV-memory CD4+ T cells depending on the antigen persistence. The IL-2+ cells are derived from an immune response that led to antigen clearance. However, if the antigens were only present in small amounts, this would generate a weak immune response with long-life cells that are IL-2+ and IFN-γ+. And if the antigen response was intense due to large amounts of antigen, then the memory cells are effectors and only IFN-γ+ (Harari et al., 2005).

Additionally, it was shown by different teams that the EBV-CD4+ T cell can have helper cytotoxic functions. *In vitro*, EBV-CD4+ T cells specific of EBNA1, EBNA2 or EBNA3C epitopes were capable of killing autologous LCLs (Khanna et al., 1997; Long et al., 2005; Paludan et al., 2002). The cytolytic effect is mediated by the granzyme B enzyme (Moralès et al., 2012). But Nikiforow also found that in EBNA1-specific CD4+ T cells, the cytolytic effect seems to act via a Fas/Fas-Ligand (Fas-L) mechanism (Nikiforow et al., 2003). They also showed that the EBV-specific CD4+ T cells can alter infected B cell infection, transformation and proliferation (Nikiforow et al., 2001). This could explain

how the CD8+ T cells and CD4+ T cells have complementary functions to fight against EBV infection by initially blocking B cell infection and transformation followed by a mass eradication of infected cell by the highly cytotoxic CD8+ T cells.

c. CD4+ and CD8+ Polyfunctional cells

Unlike classical single-function T cells, Polyfunctional T cells (PFC) have many roles ranging from the production of multiple cytokines simultaneously (IL-2, IFN-γ and TNF-α) to degranulation of cytotoxic proteins. PFCs are more commonly studied in cases of Human Immunodeficiency Virus (HIV) infection but are also associated to a number of other chronic infections such as hepatitis C virus (HCV), Cytomegalovirus (CMV) and EBV (Casazza et al., 2006; Ciuffreda et al., 2008; Duvall et al., 2008; Lam et al., 2018). Identifying this population is done by assessing functional parameters such as IFN-γ, TNF-α, IL-2, perforin secretion and CD107a expression (Harari et al., 2006; Makedonas et al., 2010; Pantaleo and Harari, 2006; Precopio et al., 2007). After stimulation, if T cells displays three or more of these functions they are considered PFCs. A recent study by Lam assessed PFCs during IM from the acute phase to the long-term chronic stage. The most immunodominant EBV-antigens found in patients were latency proteins EBN3A/B/C which correlates with a previous study (Ning et al., 2011). But others showed lytic IEAs (BZLF1 and BRLF1) and early antigen (BMLF1) as the most prominent EBV-PFC population. In relation to the previous chapter, a low frequency of EBNA1-T CD4+ T cells was detected at both the early and later stages of the disease unlike earlier findings (Henle et al., 1987; Hille et al., 1993; Long et al., 2013; Rickinson et al., 2014).

Cytotoxic CD8+ PFCs were easily detectable due to their greater numbers but effector cytolytic CD4+ PFCs were also found. Clinically, CD8+ PFCs have been preferentiality associated to non-progressor patients with HIV who have a better clinical outcome (Betts et al., 2006).

d. Regulatory T cells

Tregs' tolerogenic capacities are put to use by the EBV in order to ensure viral persistence within the host. Baumforth showed that in HL, EBV can recruit Tregs via the C-C motif Chemokine Ligand 20 (CCL20) as Tregs express its C-C motif Chemokine Receptor 6 (CCR6) (Baumforth et al., 2008). Wingate found high levels of immunosuppressive cytokines IL-10 and TGF-β in patient plasma during the acute phase of IM. However, it was hypothesized that Treg levels would be higher at the site of infection (tonsils), but the distribution of Tregs showed no difference whether it was tonsils from IM patients or healthy EBV-seropositive donors. Moreover, the levels of a CD4+ CD25hi T cell population was reduced in IM patients during the acute phase (Wingate et al., 2009). So, the increase in the release

of immunosuppressive cytokines IL-10 and TGF-β seems be due to another cell population. Indeed, it has been shown that LMP1 induces IL-10 secretion via p38 kinase and PI3K signalling pathways (Lambert and Martinez, 2007) and also TGF-β release to favour integrin A activity during the promotion of EMT (Morris et al., 2016). But, the Treg markers used in the Wingate study are quite limited and further investigations using more recent and precise Treg makers are still needed to validate this finding.

e. B lymphocytes

B cells can detect viral factors using TLR9 much like classical DCs (cDCs) and pDCs. In the same way, TLR9 recognises unmethylated linear viral DNA and stops the infected cell to reactivates itself into a lytic phase (Figure 9). For this, it blocks key lytic gene BZLF1 and activates NF-KB. Interestingly, TLR9 expression is downregulated in infected B cells during lytic replication by the viral protein BGLF5 (van Gent et al., 2011). And during the latency phase, LMP1 down regulated the transcription of the *TLR9* gene by activating NF-KB (Fathallah et al., 2010). And finally, tampering with the BCR, as previously described via LMP2B, re-establishes BCR function and activates the lytic gene BZLF1 otherwise repressed by TLR9. This leads to the reactivation of the infected cell into a lytic phase and allows the virus to escape elimination by impairing its recognition by innate immunity receptors.

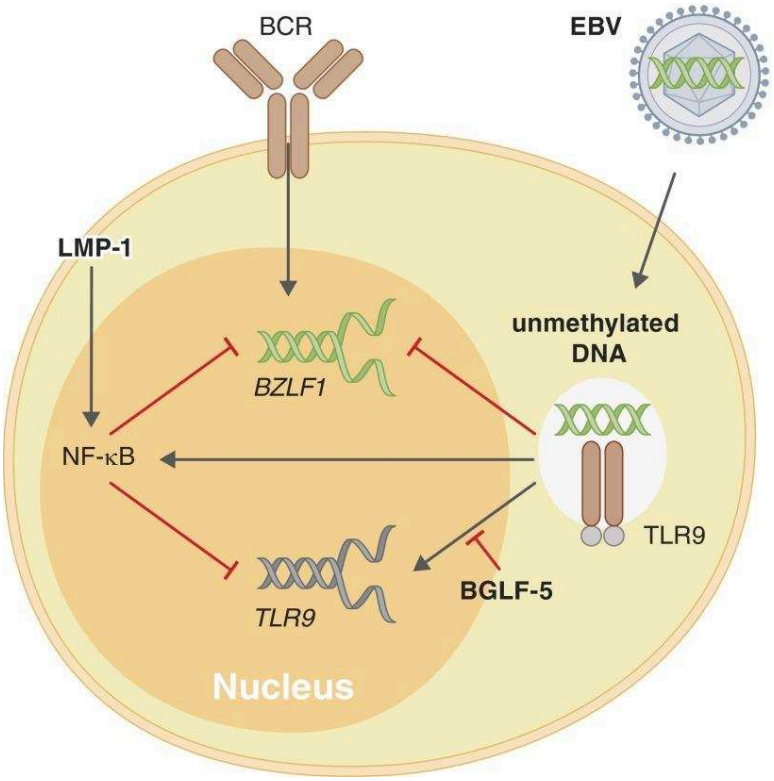


Figure 9: B cell EBV recognition. TLR9 identifies unmethylated viral DNA, which inhibits the lytic reactivation of EBV by suppressing the transcription of a key lytic gene, BZLF1, directly and indirectly via NF-KB induction. But TLR9 expression is hindered by LMP1-dependant NF-KB and BGLF5 activation. Moreover, the activation of the BCR also helps the infected cell move onwards into its lytic phase.

3) Immune evasion led by EBV in cancer

As it has been shown that EBV can induce and enhance carcinogenesis, it seemed logical to find immune evasion mechanisms led by EBV that could favour the progression of cancer. Although strong antiviral immunity, EBV manages to replicate and maintain itself over a long period of time in the host by evading the immune response.

a. Lytic phase

Innate antiviral immunity is mainly mediated by IFNs and danger signal recognition receptors such as TLRs that allows the host to detect and eliminate the virus. We previously discussed the important role of TLRs in viral detection. However, evidence shows that EBV has evolved to counter such antiviral mechanisms. This is mainly accomplished through destabilisation of host mRNA to alter the expression of key antiviral factors, this is known as a shutoff. Lytic genes such as BGLF5 downregulates the expression of TLR2/9 by degrading their mRNA (Gaudreault et al., 2007; van Gent et al., 2011; Ressing et al., 2015). It is noteworthy to state that the expression of TLR4, a receptor not involved in EBV detection, is not modulated which shows the specificity of the shutoff (Gaudreault et al., 2007; Ressing et al., 2015). In addition, the interference with the host's IFN-regulatory factors (IRFs)

by various EBV lytic factors has also been described. To name a few, BZLF1 interacts with IRF7 (Hahn et al., 2005), BRLF1 hinders IRF3/7 expression (Bentz et al., 2010), LF2 binds to IRF7 (Wu et al., 2009) and the BGLF4 kinase inhibits by phosphorylation IRF3 (Wang et al., 2009). All of these mechanisms lead to a reduction in IFN expression and activity leaving the antiviral immune response considerably weakened. Other ways in which EBV interferes with innate antiviral cytokine signalling is via the Immediate-Early lytic protein BZLF1. This protein can limit the expression of the IFN and TNF receptors (Bristol et al., 2010; Morrison et al., 2001) but also promote a non-responsiveness to IFN type I by inducing the Suppressor Of Cytokine Signalling 3 (SOCS3) (Michaud et al., 2010).

Additionally, a potent adaptive immune response is also mounted against EBV via antigen presentation pathways. EBV evades CD8+ T cell recognition by downregulating its HLA type I molecules. For this, three mechanisms have been described, **(i)** BGLF5 degrades mRNA encoding HLA I thus blocking peptide presentation (Rowe et al., 2007; Zuo et al., 2008), **(ii)** BNLF2a inhibits the transporter associated with antigen presentation (TAP), therefore blocking the loading of HLA I (Hislop et al., 2007; Horst et al., 2009) and **(iii)** BILF1 increases the lysosomal degradation of HLA I by increasing the turnover from the cell surface (Zuo et al., 2009). It is thought that the use of having three different mechanisms to evade HLA I recognition by CD8+ T cells lies in the differential expression of BGLF5, BNLF2a and BILF1 during the lytic cycle of EBV. This enables the virus to evade HLA I recognition during all of its lytic cycle, when it is the most exposed to the host's immune system.

The other main player in adaptive anti-EBV immunity are the CD4+ T cells that recognise antigens mounted on the HLA type II molecules. We have previously discussed that HLA II/peptide complex is involved in the infection of B cells by its interaction with viral pg42 receptor. This interaction is associated with a blockade of the T-cell receptor (TCR) thus inhibiting T-cell activation (Ressing et al., 2003). Other lytic proteins are also involved in CD4+ T cell evasion: BGLF5 degrades the mRNA encoding for HLA II (Rowe et al., 2007), BZLF1 impairs HLA II presentation blocking the IFNγ signalling and modifies the invariant chain of HLA II molecules (Morrison et al., 2001; Zuo et al., 2011). Moreover, BCRF1 is a viral homologue for the human IL-10 immunosuppressive cytokine. BCRF1 can thus inhibit CD4+ T cell priming and functions of effector T cells in the same ways human IL-10 can. It negatively regulates the immunostimulatory cytokines IL-12 and IFNγ but also blocks T helper 1 (Th1) and Th2 immune responses (Brooks et al., 2006; Zdanov et al., 1997). Likewise, it was described that the IL-10 homologue blocks the expression of co-stimulatory molecules on human monocytes (Salek-Ardakani et al., 2002). Interestingly, a protein expressed late in the lytic cycle, BDLF3, can impair both the MHC I and II antigen presenting pathways by targeting them for ubiquitinylation and subsequent proteasomal degradation (Quinn et al., 2016).

b. Latency phase

It is during EBV's lytic phase that it is most immunologically visible. EBV remains perceptible by the immune system during latency but much less than in its lytic phase. So, EBV uses its latency proteins to promote immune evasion.

EBNA1

The viral maintenance protein EBNA1 is a latency protein found in all latencies except latency type 0. It is virtually undetectable by the immune system as its β sheet structure protects it from proteasomal degradation (Levitskaya et al., 1995b). In fact, the EBV+ population shows very little EBNA1-specific CD8+ T cells but it has been successfully engineered *in vitro* (Lee et al., 2004; Tellam et al., 2004; Voo et al., 2004). EBNA1 also ensures its immune evasion by limiting its own expression via its interaction with the host's nucleolin (NCL) and the EBNA1 encoding mRNA at the Glycin-Alanine rich site (GR domain) (Lista et al., 2017). By limiting its transcription and degradation rate, EBNA thus limits its presentation as an antigenic peptide via the MHC I pathway. This low immune visibility allows the virus to evade immune surveillance whilst enabling life-long persistence in the host's cells. This makes EBNA1 a very appealing yet difficult target for EBV-associated malignancies.

EBNA2

EBNA2 modulates IFNα/β antiviral immune responses by interfering with the activity of STAT3. Muromoto showed that EBNA2 cooperates with LMP1 to enhance STAT3 transcription (Muromoto et al., 2009). STAT3 is constitutively activated in transformed EBV+ human immune cells (Chen et al., 2001; Weber-Nordt et al., 1996). Moreover, EBNA2 can act as a transcriptional activator for cyclinD2 (Sinclair et al., 1994) and proto-oncogene c-myc (Kaiser et al., 1999) which promotes cell growth and survival that are key for cell transformation.

LMP1

LMP1 is a major viral oncogene that favours carcinogenesis by multiple pathways but notably via immune evasion (Middeldorp and Pegtel, 2008). The latency protein is essential for B cell immortalisation and proliferation (Kaye et al., 1993). LMP1 mimics CD40 as they share functional homology, they both activate the NF-KB signalling pathway involved in immune regulation (Gires et al., 1997; Lalmanach-Girard et al., 1993). LMP1 is constitutively active and does not depend on a receptor-ligand interaction unlinke the CD40 and CD40-Ligand interaction. Thus, in transgenic mice LMP1 expression induced B cell lymphomas (Kulwichit et al., 1998). In fact, when CD40 was constitutively expressed it induced lymphomagenesis (Hömig-Hölzel et al., 2008).

LMP1 was found on EBV+-tumour-derived exosomes and could be up taken by the host's uninfected immune cells (Flanagan et al., 2003). Indeed, LMP1 is bound to exosomes to exert its

immunosuppressive effects. It was shown that LMP1+ exosomes strongly inhibited effector T cell's proliferation and NK's cytotoxicity (Dukers et al., 2000). In a NPC humanised mouse model, LMP1+ tumour exosomes favour immune evasion enhancing the immunosuppressive tumour microenvironment through the recruitment and exacerbation of Tregs (Mrizak et al., 2015). Moreover, Pioche-Durieu found in NPC cell line C15, that LMP1 directly interacts with Galectin-9 (Gal-9), an immunomodulatory factor also found on NPC-derived exosomes (Klibi et al., 2009a; Pioche-Durieu et al., 2005).

A study by Bi and his collaborators showed that the high expression of the checkpoint inhibitor PD-L1 in EBV+ cells can be correlated with LMP1 expression (Bi et al., 2016). PD-L1 is currently a poor prognosis factor in NK/T cell lymphoma (Jo et al., 2017), EBV-associated gastric cancer (Seo et al., 2017) and NPC (Zhou et al., 2017).

LMP2A/B

Stewart showed that LMP2A inhibits IL-6 secretion by blocking NF-KB activity, this leads to hindered signalling of the Janus Kinase (JAK)/STAT pathway in carcinoma cell lines (Stewart et al., 2004). However, it has also been shown in a transgenic mouse model that LMP2A can activate NF-KB to increase bcl-2 and block apoptosis of EBV+ B cells (Swanson-Mungerson et al., 2010). IFN responsiveness can be compromised by LMP2A/B by increasing the turnover of IFN receptors (Shah et al., 2009).

EBERs and BARTs

Immunologically, mi-BARTS contribute in many ways to the immune evasion of cancer. EBV-BART6-3p and miR-197 decrease the expression of the IL-6 receptor in BL (Zhang et al., 2017). BART6-3p also inhibits Pathogen C-type Lectin Receptors (CLR), viral RNA detector Retinoic acid-Inducible Gene I (RIG-I) which ultimately blocks IFN type I signalling (Lu et al., 2017). Tagawa and his team showed that mi-RNAs can inhibit the secretion of immune-stimulatory cytokines such as IL-12 in infected B cells (Tagawa et al., 2016). More importantly, EBV mi-RNAs can block the differentiation of naive CD4+ T cells into Th1 cells. They can also interfere in the antigen presentation process on MHC class II. All this leads to immune escape mechanisms meant to help the virus maintain itself in cells without being detected (Tagawa et al., 2016).

In parallel to this work, another study focused on the EBV-specific CD8 T cells. Indeed, mi-RNAs were shown to limit the recognition and processing of the major EBV antigen: EBNA1. For this, the expression of MHC II, EBNA1 and peptide transporter TAP2 were all significantly decreased (Albanese et al., 2016). The expression of a T-cell attracting chemokine, CXCL11, was downregulated by BHRF1-3 miRNAs (Xia et al., 2008). MHC class I Chain-related protein B (MICB), a stress-induced NK ligand, is

targeted by BART2-5p miRNA and allows the EBV+ cell to escape recognition by NKs and subsequent killing (Nachmani et al., 2009).

Moreover, it has been shown that exosomes can carry miRNAs. Indeed, EBV encoded miRNAs held on exosomes can be transferred to uninfected cells (Meckes et al., 2010; Pegtel et al., 2010). Once the exosome is taken up by the target cell, it can modulate gene expression. For example, Higuchi has shown in a humanise LPD mouse model using Akata-lymphoblastoid cell lines that Akat-derived exosomes increases the severity of the LPD. The most interesting is that the miRNA found in these exosomes induced *in vitro* a regulatory immune response with IL-10, arginase 1 and TNFα secreting macrophages (Higuchi et al., 2018). Clinically, the levels of EBV-encoding miRNAs seem strongly linked to clinical outcome of elderly patients with DLBCL opening the possibility that EBV-encoding miRNAs could be biomarkers or even diagnostic markers of B-cell lymphomas.

The involvement of EBERs in immune evasion remains unsure. It is thought that EBER1-2 can bind to dsRNA-dependant PKR to inhibit IFNα-induced apoptosis (Clarke et al., 1991; Nanbo et al., 2002). However, these findings remain uncertain as the direct role of EBERs in the anti-apoptotic effect via PKR inhibition has been questioned (Ruf et al., 2005).

Thus, EBV is a common virus worldwide that generally maintains itself in the host asymptomatically but can also manifest itself by IM or cancer. EBV is now a very interesting target that could be the key to curing the many EBV-associated malignancies. More importantly, an anti-EBV vaccine could prevent the emergence of EBV-associated malignancies all together.

II. Nasopharyngeal carcinoma

Nasopharyngeal carcinoma is a malignant tumour of the head and neck that originates in the epithelial tissue that lines the nasopharynx. Every year, over 80 000 new cases of NPC are diagnosed and 50 000 NPC-deaths are recorded worldwide. Patient's 5-year survival rate remains low as it is often diagnosed at late stages (30-40%). Although radiosensitive, new treatments are needed to lower the mortality associated to NPC.

A. NPC classification

The World Health Organisation (WHO) has classified NPCs into three subtypes: (i) type I keratinizing carcinoma or Squamous Cell Carcinoma (SCC), (ii) type II non-keratinizing carcinoma which includes differentiated and undifferentiated subtypes (iii) and basaloid SCC (AK et al.). Type III NPC is the undifferentiated non-keratinizing carcinomas also referred to as lymphoepithelioma and show a high T cell infiltration (Vokes et al., 1997).

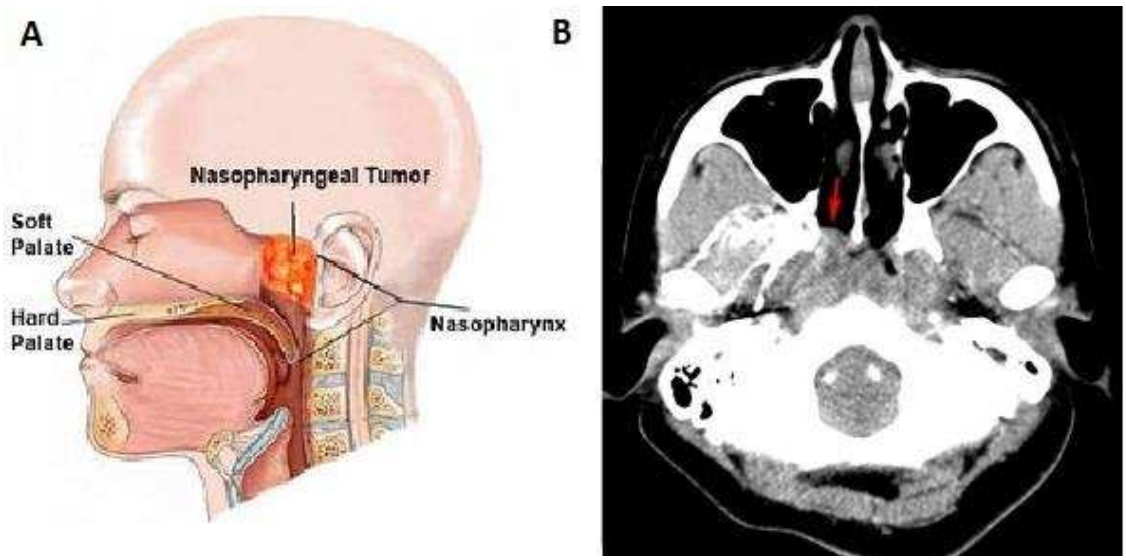


Figure 10: Nasopharyngeal carcinoma anatomy. A. Illustration of the pharynx and nasopharynx anatomy. B. A Magnetic resonance imaging (MRI) scan of a Low-grade NPC patient. The tumour is indicated by the red arrow (Petersson, 2015).

NPC typically develops on the mucosa that lines the nasopharynx' epithelium, as shown in Figure 10. The most common forms of NPCs are those that originate from the epithelial tissue lining the nasopharynx which represent 75–95% of NPCs in the low-risk populations and almost 100% of NPCs in high-risk populations (IARC, 1997).

B. Epidemiology

In most parts of the world, NPC is a rare disease affecting less than 1 out of 100 000 people per year. Nevertheless, a few regions or populations show a higher incidence of NPC. For instance, NPC has a rare incidence of <1/100 000 among Caucasians and in Europe. But, some parts of South China,

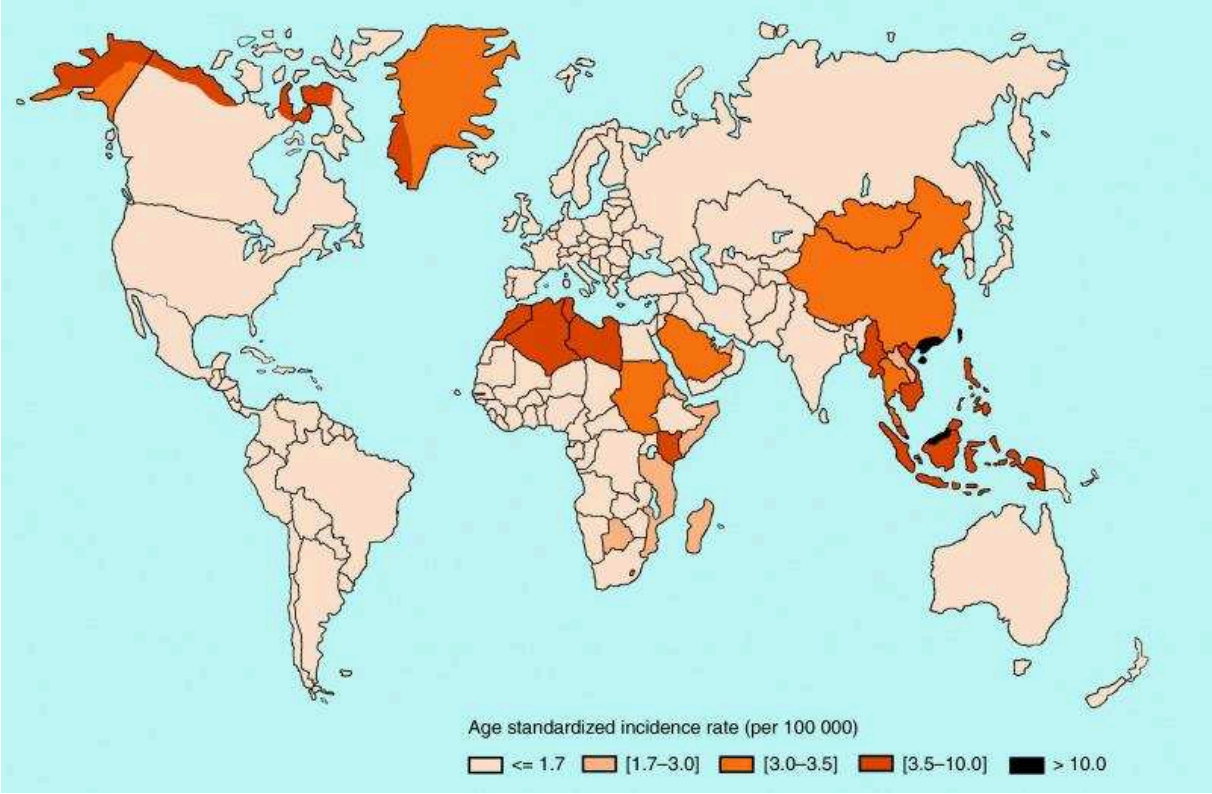


Figure 11: Incidence of EBV-associated nasopharyngeal carcinoma worldwide. The incidence rate was calculated using an age-standardisation. The darker the colour the higher the NPC incidence (Busson et al., 2004).

notably Canton and Hong Kong in the Guang-dong province have the highest levels of incidence in the world (15-50/100 000). The rest of South-East China has an intermediate incidence much like Indonesia and Vietnam (3-8/100 000 people) (Figure 11). It is noteworthy to mention that Chinese people that immigrate to North America still show a higher NPC incidence than non-Chinese North Americans. Some North African countries also have a high incidence of NPC, notably Tunisia, Libya, Algeria and Morocco (3-8/100 000). NPC is also significantly more common in Artic regions such as Greenland and Alaska with an incidence of 3-8/100 000, mostly in people that have Inuit or Aleut heritage (IARC, 1997).

Interestingly, it has been described that men are three times more likely to develop NPC than women (1997; Yu and Yuan, 2002). It was also found that in low-risk groups, the incidence increases with age which is the usual distribution risk for epithelial cancers. However, in the moderate-risk and high-risk populations, there is a peak of incidence respectively at young adulthood and at 45-54 years

old followed by a decline in incidence at older age. It is thought that the early-age peak is due to early-life exposure to an etiologic factor that varies between cultures (IARC, 1997).

C. Etiology

Like all cancers, NPC is multifactorial and is caused by a number of etiologic factors. Other than its clear association to the Epstein-Barr virus, other environmental and genetic factors have been linked to higher risks of developing NPC.

1) Non-viral factors

a. Genetic predispositions

As previously mentioned, when Chinese people at high risk of developing NPC migrate from China they show a lower incidence. However, they still have more risks than the average population to develop NPC. This key information confirms that environment and genetic factors both contribute to the emergence of NPC. Indeed, families with many members developing NPC have been studied yet there are no clear genetic markers that fully elucidate the seemingly strong predisposition for NPC in these cases (Zeng and Jia, 2002). However, studies of the HLA locus have led to understand it is important in NPC carcinogenesis. Some specific HLA haplotypes (A2, B17 and Bw46) show higher risks of NPC (Lu et al., 1990) whereas other haplotype like A31, B13, B27, B39 and B55 actually protect the person from NPC (Hu et al., 2005). Moreover, now that the technology is sufficiently advanced, genome-wide searches for other susceptibility loci have revealed the chromosome 3p21 (Xiong et al., 2004) as well as the D4S405 and D4S3002 markers on the chromosome 4 (Feng et al., 2002).

Another reported susceptibility locus for NPC is the TElomerase Reverse Transcriptase (TERT)/Cleft-Lip and Palate TransMembrane protein-1-Like (CLPTM1L) encoding region. A 2016 meta-analysis study carried out in over 15 000 people of Chinese descent identified that a variation in the TERT/CLPTM1L locus (5p15.33) was linked to an increased NPC risk (Zhang et al., 2016b). Indeed, the TERT is a subunit of the telomerase complex. A default in telomerase activity is associated to many cancers, including NPC (Wen et al., 2002). Viral protein LMP1 has also been linked to abnormally long telomerases in NPC cell lines (Du et al., 2005). Additionally, CLPTM1L is known to be involved in Ras-dependant oncogenic transformation in lung cancer (James et al., 2014). Thus, further studies are still needed to determine the exact mutation site and underlying mechanisms that contribute to NPC onset.

Other loci already identified in carcinogenesis are studied for their potential involvement in NPC. When deleted, the Glutathione S-Transferase M1 (GSTM1) is linked to increased NPC susceptibility in all high-risk populations (Liu et al., 2015). Studies have also shown mutated genes

encoding for cytokines or their promoters such as IL-1α (Cheng et al., 2014), IL-16 (Qin et al., 2014), and IL-18 (Guo and Xia, 2013; Li et al., 2015) can increase the risk of NPC.

Finally, it is well described that men are 2-3 times more likely to develop NPC than women. Thus, the involvement of X chromosome variations has been hypothesized. However, the study of the X chromosome is generally left out in genome studies as it is far more complex to study than autologous chromosomes. The X-inactivation phenomenon in females helps balance out the allele dosage between genders and silences one of the two copies of the gene (Carrel and Willard, 2005). This makes studying potential susceptibility loci on sexual chromosomes very difficult. Nevertheless, the difference in NPC risk observed between genders could also be explained by the culturally unequal exposure to environmental contributing factors.

b. Environmental factors

Dietary habits

Dietary habits were first put forward as an etiological factor for NPC by John Ho (Ho, 1972). He suggested that prolonged and repetitive consumption of Cantonese-style salted fish contributed to NPC onset. Indeed, this was later confirmed in a study on rats as Cantonese-style salted fish enhanced nasal cavity carcinomas and NPC (Huang et al., 1978; Yu et al., 1989; Zheng et al., 1994). Indeed it was found that this food contains nitrosamines and their precursors that are highly carcinogenic (Huang et al., 1981; Zou et al., 1994) and are also EBV-activating factors (Shao et al., 1988). Similar results were obtained on salted fish consumed in Greenland (Poirier et al., 1989; Shao et al., 1988). The high incidence rate can also be explained by dietary habits in North African countries where nitrosamines precursors and EBV-activating substances were found in salt-preserved foods, harissa, qaddid and touklia (Bouvier et al., 1995; Jeannel et al., 1990; Poirier et al., 1989; Shao et al., 1988)

Lifestyle

The risk of developing cancer in the upper respiratory tract is known to be linked to smoking cigarettes (Centers for Disease Control (CDC), 1989). However, studies have been inconsistent regarding the impact of smoking on NPC incidence (Cheng et al., 1999; Lin et al., 2015; Yu et al., 1990; Yuan et al., 2000). As the nasopharynx traps primarily medium-size particles (5-10µm), maybe the nasopharynx epithelium is less sensitive to cigarette smoke-induced carcinogenesis. But studies carried out on British, North American and Chinese workers that are regularly exposed to coal combustion smoke and wood dust showed greater risks of NPC (Armstrong et al., 1983; Demers et al., 1995; Henderson et al., 1976; Hildesheim et al., 2001; Yu et al., 1990). Moreover, alcohol consumption was not initially thought to contribute to NPC (Cheng et al., 1999; Yuan et al., 2000), but after re-evaluation

it was concluded that substantial use of alcohol significantly increase NPC risks (Nam et al., 1992; Vaughan et al., 1996).

2) Viral factors: EBV

NPC is widely recognised as an EBV-associated malignancy. Nevertheless, as EBV infects most of the world's population, it was hypothesised that certain strains of EBV were responsible for specific NPC endemic regions. Indeed, some EBV variant are significantly correlated with high incidence of NPC in endemic regions (Khanim et al., 1996; Tzellos and Farrell, 2012). Feng found that a single nucleotide polymorphism in the EBV genome (locus155391: G>A) can be correlated to NPC endemic region in South China (Feng et al., 2015). Furthermore, infection with multiple strains of EBV has also been described using heteroduplexes (Rey et al., 2008). Evidence has shown a selective presence of different EBV strains in the saliva and peripheral blood of the same patient (Sitki-Green et al., 2003). This variation is thought to occur during the high replication phase of the virus. The different strains, that vary in their EBNA1 expression, might affect its recognition by the host's immune system (Bell et al., 2008). This brings an evolutionary advantage to EBV as this diversity increases the number of targets thus, making its eradication much more strenuous for the host.

D. NPC stages

Before treating the patient, the stage of the cancer has to be determined using the AJCC Cancer Staging Method. This classification system takes into consideration three main factors: (T) the characteristics of the main tumour mass, (N) the status of cancer spread in the lymph nodes (LNs) and (M) the status of metastasis outside the head and neck. This grading system is named the Tumour, Nodes, Metastasis (TNM) classification and patient are assessed according to the table II chart. T and N are determined by precise guidelines shown in figure 12 and 13.

Table II: TNM classification of NPC *(http://headandneckcancer.org)*

		Characteristics
T Stage	**T1**	The tumour is within the nasopharynx, or it has grown into the oropharynx and/or nasal cavity, but there is no extension into the parapharyngeal space (soft tissue space behind and to the side of the pharynx).
	T2	The tumour extends into the parapharyngeal space.
	T3	The tumour has grown into the bone of the skull base and/or the sinuses.
	T4	The tumour has grown into the skull and/or involves the cranial nerves, hypopharynx, eye socket. Alternatively, it has extended to the infratemporal fossa or masticator space.
N Stage	**N0**	No evidence of cancer spread to LNs in the neck or retropharyngeal space
	N1	Presence of cancer in the LNs on one side of the neck (largest is 6cm or less) and all the LNs are above the clavicle (supraclavicular fossa). The LNs at this stage should be found in the retropharyngeal space (6cm or less in size, one side or both).
	N2	Presence of cancer in the LNs on both sides of the neck (biggest LN is 6cm or less, all the LNs are above the supraclavicular fossa).
	N3a	Presence of a LN with cancer that is bigger than 6cm.
	N3b	There is a LN of any size that is far down the neck, just above the clavicle.
M Stage	**M0**	No evidence of distant spread outside the head and neck
	M1	Evidence of spread outside the head and neck

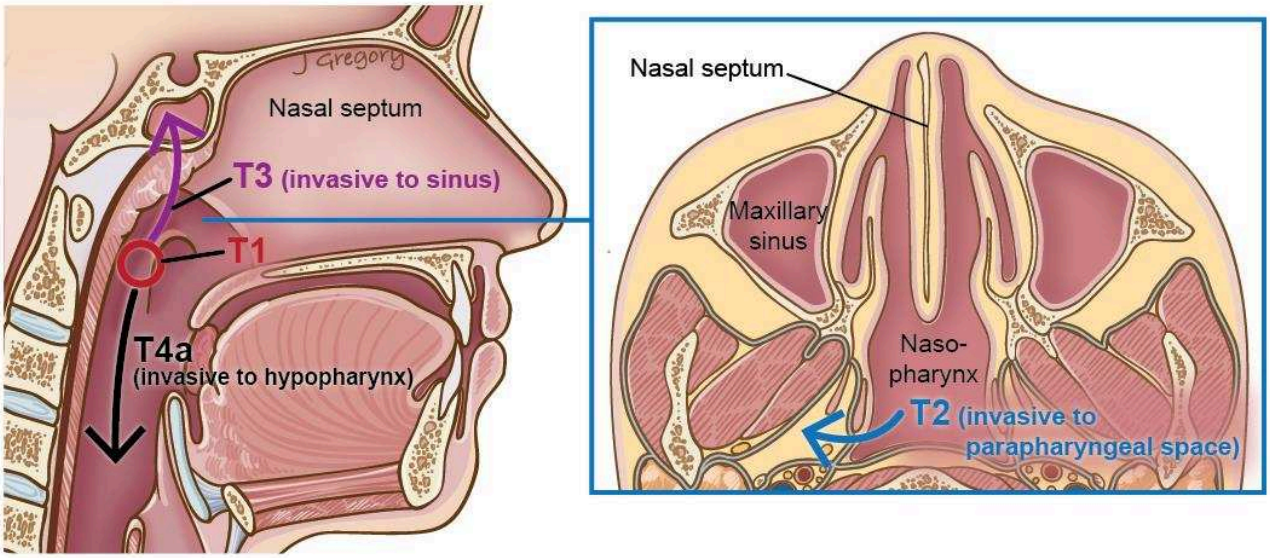


Figure 12: Illustration of the different T stages of NPC. T1 (red) remains localised in the nasopharynx, T2 (blue) spreads to the parapharyngeal space, T3 (purple) invades the sinuses and/or base of the skull and T4 (black) invades the lower part of the neck (hypopharynx) and/or the skull, cranial nerves and eye socket (http://headandneckcancer.org).

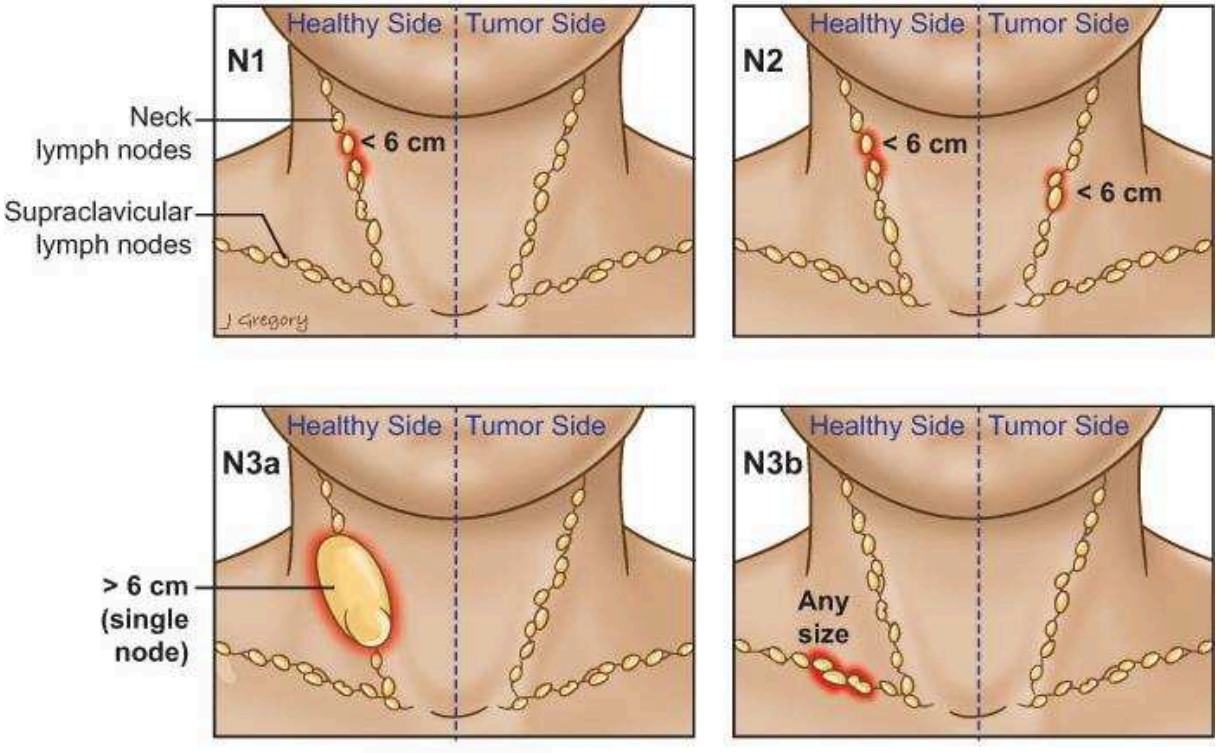


Figure 13: Illustration of the different N stage of NPC. The neck lymph nodes are represented in yellow and the cancerous regions in red. N1 shows cancerous LNs on one side of the neck (smaller than 6cm) and above the supraclavicular fossa, N2 shows the cancer has spread to LNs on both sides of the neck and remain above the supraclavicular fossa, N3a is when any LN is bigger than 6cm in size and N3b shows cancerous LNs of any size further down the neck just above the clavicle (http://headandneckcancer.org)

Table III: NPC stage chart – TNM classification

Stage 0	Tis	N0	M0
Stage 1	T1	N0	M0
Stage 2	T1	N1	M0
	T2	N0	M0
	T2	N1	M0
Stage 3	T1	N2	M0
	T2	N2	M0
	T3	N0	M0
	T3	N1	M0
	T3	N2	M0
Stage 4a	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage 4b	Any T	N3	M0
Stage 4c	Any T	Any N	M1

Thus, the patient's stage can be best determined using the TNM classification enabling the doctors to adopt the adequate treatment.

E. Tumour microenvironment

NPC is highly linked to the host's immune system. It has been shown that EBV favours carcinogenesis by evading the immune response. Although the patient shows a strong antiviral response coupled with a high tumour infiltration with leucocytes, this is still not enough to fight the tumour. This immune infiltrate is mainly made up of tumour-infiltrating T cells (TILs), B cells, DCs, monocytes and eosinophils. It has been found that the infiltrating immune cells are lead to the tumour site by chemokine-dependant mechanisms (Hu et al., 2004; Mrizak et al., 2015; Tang et al., 2001). Indeed, it is now known that an immunosuppressive tumour microenvironment gives immunological space for the tumour to grow. This local tolerance is mediated by cytokines and regulatory immune cells that are diverted from their original purpose (Busson et al., 1987; Duffield et al., 2018; Tang et al., 2001). It was also discovered that tumour-derived exosomes (TEXs) also contribute to maintain tumoural immune tolerance by favouring Tregs (Mrizak et al., 2015) amongst other mechanisms (Zhou et al., 2018). EBV contributes to this immunosuppressive tumour microenvironment as it thrives on it. This is mediated by viral proteins such as EBNA1 and LMP1 (Ai et al., 2017; Yoshizaki et al., 2018).

F. NPC Treatments

1) Conventional treatments

Due to the deap-seated localisation of NPC, surgery to remove the tumour is generally not carried out. When diagnosed at early stages, NPC is classically treated with either radiotherapy and/or chemotherapy with over 90% 5-year survival rate (Lee et al., 2005). In a phase III clinical trial (INT-0099), it was found that coupling fluorouracil (5-FU) and cisplatin Chemotherapy with RadioTherapy (CRT) increased OS by 31% (Al-Sarraf et al., 1998). However, if NPC has locally spread, the 5-year survival rate falls to 50-70% and plummets even lower in cases of distant metastases. Although palliative chemotherapy does have an initial 80% response rate, the patient eventually stops responding to treatment after 12-18 months (Lee et al., 2005). Unfortunately, patients that no longer benefit from CRT find themselves at a therapeutic impasse. But efforts are currently underway to develop new treatments.

2) Novel therapies

a. Targeted therapy

Targeted therapies were being tested for all types of cancers at the turn of the century. These molecular agents target the increased cell growth and resistance to cell death attributed to tumour cells. Monoclonal antibodies are used to target the Epidermal Growth Factor Receptor (EGFR) pathway and block multiple key effector Tyrosine-kinases. In lung cancer, they are now the gold standard treatment for patients. However, clinical trials have shown only modest advantages for NPC patients. Major toxic side-effects were recorded including some cases of grade 5 tumour haemorrhaging with Pazopanib (Lim et al., 2011) and Sunitinib (Hui et al., 2011) leading to the premature stop of this last trial. Thus, the multi-kinase inhibitors' lack of effectiveness explains why they are not used for the treatment of NPC at this day.

b. Immunotherapy

Given the strong immunosuppressive tumour microenvironment and the EBV-linked nature of NPC, when the first immunotherapies were tested in clinical trials, NPC posed as a good candidate for this novel treatment. Immunotherapy engulfs many types of approaches from EBV vaccines, adoptive immune cell therapy and immune checkpoint inhibitors (Jain et al., 2016).

EBV-based strategies

We have previously discussed how the expression of viral proteins in NPC contribute to its progression. The aim is to target essential viral proteins or RNAs to weaken the viral driver of NPC (Cao, 2017; Teow et al., 2017).

It is well established that NPC expresses viral proteins, mainly EBNA1-3 and LMP1-2 that are involved in carcinogenesis. However, evidence shows that the best strategies are the ones that target multiple EBV proteins simultaneously. Thus, an EBV vaccine would increase the availability of viral antigens and ultimately enhance the EBV-specific immune response. Phase I studies using highly immunogenic fusion protein expressed by a recombinant virus showed an increase in the EBV-T cell population (Hui et al., 2013), whereas the loading modified LMP1-2 on autologous DCs did not lead to any change in the number of EBV-T cells (Chia et al., 2012).

Viral RNAS are also interesting targets as they are expressed in all EBV-latencies. However, their exact functions remain elusive and their diversity makes them complex targets. But, RNA-targeting drugs such as Ribavirin, a nucleoside inhibitor, are already being used to successfully treat other viral diseases like Hepatitis C and viral haemorrhagic fever. Thus, this gives us promise that viral RNA could be a faisable lead for the treatment of EBV-associated malignancies. Nonetheless, it must be mentioned that these are diseases caused by RNA viruses, whereas EBV is a DNA virus. To our knowledge, no major development of EBV-RNA-based treatments are currently underway.

Cell therapy

Given the strong T-cell response mounted towards EBV, it seems logical to try and enhance such an immune response. The two main leads are using autologous dendritic cells or T cell that are enhanced *in vitro* and then reinjected into the patients (Chia et al., 2012; Lin et al., 2002; Lutzky et al., 2014; Smith et al., 2017). A Phase III trial currently underway, aims to treat patients with enhanced EBV-specific cytotoxic T cell after completing a first course of chemotherapy (NCT02578641) (Chia et al., 2014). Out of the 35 patients tested, 3 showed a complete response and 22 patients partially responding. The overall response rate being 71.4% including 5 patients that did not require further chemotherapy treatment. Another group used autologous T cells that were presented with EBV antigens, notably LMP2, by autologous EBV+ LCLs. The cells obtained were indeed cytotoxic CD3+ CD8+ T cells that showed specific killing of EBV-LCLs. After reinjection into the stage IV NPC patients, 6 out of 10 patients showed a control of disease progression (2 with partial response and 4 with stable disease). Moreover, the treatment was generally well tolerated with grade 1 and 2 toxicities observed in two patients (Comoli et al., 2005). The same group hypothesised that a lymphodepletion before reinjecting the cells would help enhance the adoptive cell therapy. Unfortunately, this was not the case

as administering lymphodepleting chemotherapy beforehand did not improve clinical benefit (Secondino et al., 2012).

Nevertheless, it is noteworthy to mention that although cell therapy has shown promising results, it still remains very costly and technically difficult. Thus other, cheaper and more accessible therapies are also being developed.

Checkpoint inhibitors

Given that most key immune regulatory checkpoints are expressed in NPC cells, targeting them using checkpoint inhibitors seems to match the rational. Programmed death-1 (PD-1) is found on the surface of activated B and T cells and is an inhibitory molecule that favours immune tolerance. PD-1 interacts with members of the B7 family : PD-L1 and PD-L2 (Keir et al., 2008). But other immune regulatory checkpoint molecules are also gaining interest including cytotoxic T lymphocyte-associated protein 4 (CTLA4) (Walunas et al., 1994), that is expressed on activated T cells and blocks activating molecules (CD80-CD86) found on APCs. Indeed, it is even suggested that the expression of PD-1, PD-L1 and CTLA-4 could be used as biomarkers for prognosis and to better stratify NPC patients (Ahmadzadeh et al., 2009; Chen et al., 2013; Hsu et al., 2010b; Huang et al., 2016). The following table summarizes all the completed and ongoing clinical trials testing immunotherapy in NPC.

Table IV: Summary of completed or ongoing clinical trials involving immunotherapy in NPC

Phase	Status	Treatment tested	Patient details	Aim of the study	Reference
I	Completed	EBV-specific adoptive T cell immunotherapy	28 relapsed or metastatic NPC patients	To determine the safety of EBV-based adoptive transfer immunotherapy in NPC	NCT00431210
I	Active, not recruiting	EBV-specific T cells (2 antigens) that have an extra T cell receptor named DNT +/- chemo lymphodepletion beforehand (Cyclophosphamide and fludarabine)	14 participants with advanced NPC	To examine efficacy of EBV-specific T cells in NPC patients and determine if lymphodepleting chemotherapy before T cell infusion increases treatment efficacy	NCT02065362
I	Recruiting	CAR-T cells (recognise EpCAM)	30 NPC and breast cancer patients	Aim is to determine if treatment is well tolerated, at what dosage and what the adverse effects are.	NCT02915445
I	Completed	Using two variants of LMP2 peptide vaccine	99 patients with a high-risk of NPC recurrence	Aim is to evaluate the immunologic effectiveness of peptide immunization in adjuvant settings in NPC patients.	NCT00078494
I/II	Recruiting	LMP1-CAR-T cells	20 patients with EBV associated malignant tumours (Nasopharyngeal neoplasms)	The purpose of this study is to evaluate the safety and efficacy of the designed LMP1-CAR-T cells in the treatment of EBV associated malignant tumours.	NCT02980315
I/II	Recruiting	High-activity NKs	20 NPC patients with small metastases	Assessment of the safety of high activity NKs on NPC patients.	NCT03007836
I/II	Completed	Cancer stem cell vaccine	40 metastatic NPC patients	To demonstrate *in vitro* that CTL generated after Cancer Stem Cell (CSC) vaccination are capable of specific killing of CSCs and conferring antitumour immunity.	NCT02115958 (Ning et al., 2012)
II	Active, not recruiting	EBV-specific adoptive T cell immunotherapy	20 relapsed or metastatic NPC patients	To determine efficacy and safety of EBV-based adoptive transfer immunotherapy in NPC.	NCT00834093
II	Recruiting		200 patients with treatment-	Aim is to investigate the efficacy of chemotherapy with DC-CIK and	NCT03047525

		Combinations of DCs and Cytokine-induced Killer Cells (DC-CIK) treatment in solid tumours	refractory solid tumours: Colorectal cancer Renal cell Carcinoma NPC Lung cancer	CIK treatment in patients with treatment-refractory solid tumours.	
II	Recruiting	Cisplatin chemotherapy and CRT (CCRT) +/- TILs	116 patients with locoregionally advanced high-risk NPC	The phase I results showed that TILs following CCRT resulted in sustained anti-tumour activity, anti-EBV immune responses and good overall tolerance.	NCT02421640
II	Recruiting	(cisplatin) CRT +/- nivolumab	40 stage II-IVB NPC patients	Establish how well nivolumab and chemotherapy work to treat advanced NPC.	NCT03267498
II	Not yet recruiting	Pembrolizumab	63 patients with detectable levels of EBV DNA in plasma after CRT. No residual disease and/or metastases	Examine efficacy and safety of pembrolizumab on NPC patients.	NCT03544099
II	Recruiting	Ipilimumab and nivolumab	35 patients with advanced NPC	Test a combination of ipilimumab and nivolumab in EBV+ NPC patients	NCT03097939
III	Recruiting	Chemotherapy (Gemcitabine and IV carboplatin) + autologous EBV-specific cytotoxic T cells	330 participants with advanced NPC	Assess the efficacy of CTL following first line chemotherapy in prolonging OS of NPC patients	NCT02578641 Phase II complete trial (Chia et al., 2014)

III. Exosomes

A. Discovery

Exosomes were described for the first time over thirty years ago in 1983 (Harding et al., 2013). Two papers, that were released within a week of each other, both showed that reticulocytes' transferrin receptors were externalised into the extracellular space (Harding and Stahl, 1983; Pan and Johnstone, 1983). Harding, Heuser and Stahl showed for the first time that a type of late endosome dubbed "multivesicular endosomes" or MVEs were responsible for the recycling of the transferrin receptor. To their surprise, they found that the MVEs fused with the PM which led to the release of small vesicles, under 100nm in diameter, found within MVEs (Harding et al., 1983) (Figure 14).

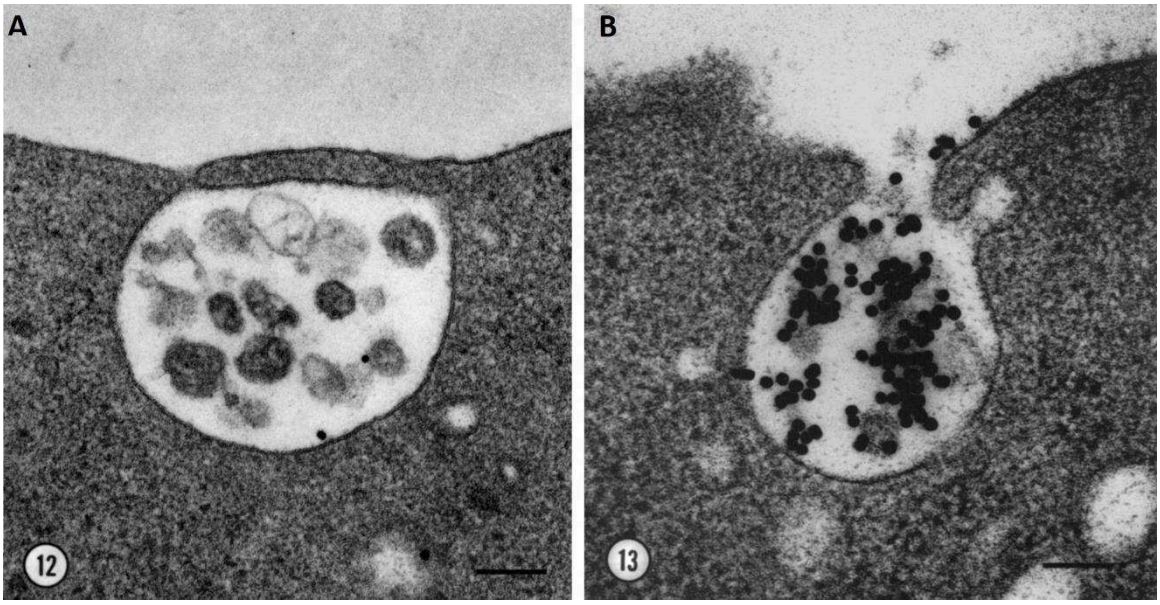


Figure 14: Exocytosis of MVEs and release of exosomes. Electronic images of quick-frozen fixed reticulocytes labelled with AuTf showing A. fusion of MVE membrane with the plasma membrane B. and the release of transferrin receptor-loaded exosomes into the extracellular space. Scale bar 100nm, Harding et al., 1983.

At the same time, Pan and Johnstone proved in sheep reticulocytes that the transferrin receptor is externalized via vesicles. Although they did not visualize the phenomenon by electron microscopy, they used anti-transferrin-receptor antibodies to follow the trafficking of the protein (Pan and Johnstone, 1983).

This discovery showed that PM recycling did not only occur for molecules bound to early endocytic compartments, as was the view at the time, but also molecules present on late endosomes such as MVEs. Indeed, other molecules found deeper in the endocytic system were also recycled to the PM. For example, processed antigens seemed to be recycled from lysosomes to endosomes in order to bind to MHC II. This complex is then expressed on the cell surface for T cell presentation (Harding et al.,

1991). Also, Lippincott-Schwartz and Fambrough found that the Lysosome-Associated Membrane Protein-1 (LAMP-1), then named LEP 100, was addresses to the PM (Lippincott-Schwartz and Fambrough, 1987).

Exosomes are secreted by virtually all cell types, but most notably by immune cells. Their immunomodulatory functions were first demonstrated by Raposo in B cells. They found that MHC II-enriched MEVs released exosomes that could present MHC II/peptide complexes to T cells and induce an immune response (Raposo et al., 1996). Knowing this, other studies then looked at DCs and found they could also secrete T cell activating exosomes that are potent enough to eradicate tumours in an *in vivo* mouse model (Théry et al., 1999, 2001; Zitvogel et al., 1998). However, dendritic cell-derived exosomes (DEX) seem to be the only ones capable of activating naive T cells (Théry et al., 2002). It was further described that naive CD4+ T cells need the presence of APCs for this activation to take place. This led us to believe that the exosomes are bound to the APC membrane during this process (Denzer et al., 2000; Vincent-Schneider et al., 2002). But a potent activation of naive CD8+ T cell has been described in the absence of APC and with the stimulation of IL-12 only (Li et al., 2017a; Sprent, 2005).

Moreover, macrophage-derived exosomes can also present antigens to T cells in a cell-surface-bound exosomes system (Ramachandra et al., 2010). It is noteworthy to mention that in this study the cells were infected with *Mycobacterium tuberculosis* (Mtb) and this alone enhances the production of exosomes. It has also been described in many virus-associated pathologies that exosomes favour the expansion of viral infection. For instance, HIV has been known to hijack the exosomal packing and/or release pathways to its advantage (Ellwanger et al., 2017). It is suggested that HIV can charge its viral RNA into the infected cells' exosomes and intensify their release to facilitate the infection of neighbouring cells.

B. Biogenesis

Exosomes differ to other secreted vesicles notably by their biogenesis. They arise in late endosomal compartment also dubbed multivesicular bodies (MVBs), and when exosomes are not-yet secreted they are referred to as intraluminal vesicles (ILVs). Although unknown for a long time after their discovery, new light has been shed on exosome formation (Hessvik and Llorente, 2018). Molecules that are found on MVB membrane are either degraded after fusion of the MVB with lysosomes or secreted in the extracellular compartment after fusion with the PM (Luzio et al., 2000; Mathivanan et al., 2010) (Figure 15). In 2001, a paper by Katzmann showed that the sorting of ubiquinylated MVB cargo was dependant on a 350kDa complex dubbed Endosomal Sorting Complex Required for Transport-I (ESCRT-I) (Katzmann et al., 2001; Raiborg and Stenmark, 2009). Previous work identified

ESCRT proteins as the major player in protein sorting of the vacuole in yeast (Babst et al., 1997). The ESCRT machinery is made up of vacuolar protein sorting (Vps) subunits and 4 different protein complexes have been reported: ESCRT-0, I, II and III, but can also be associated to the AAA ATP Vps4 complex (Henne et al., 2013). ESCRT is able to sequester and sort cargo and deform the MVEs membrane to bud inwards to give rise to ILVs (Colombo et al., 2013).

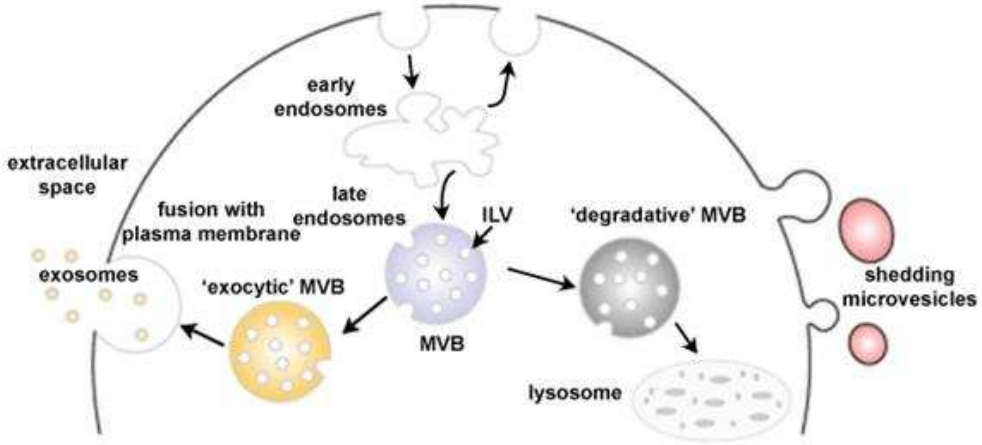


Figure 15: Schematic representation of exosome biogenesis. Early endosomes mature into late endosomes named MVBs in which ILVs have formed after inwards budding of the membrane. The MVBs have two possible fates: lysosome degradation by membrane fusion or release of the exosomes into the extracellular space by fusion with the plasma membrane (Adapted from Mathivanan et al., 2010).

In the case of exosomes, elucidating the pathway leading to the fusion of the MVE membrane with the PM is key. Many proteins are involved in exosome biogenesis, from the inward budding of the MVE to the trafficking and subsequent fusion with the PM. The sphingolipid ceramide contributes to the transfer of specific exosome-associated cargo into the lumen of exosomes as well as their release. But ceramide has also been shown to trigger the curving of the MVB membrane and subsequently bud (Kajimoto et al., 2013; Trajkovic et al., 2008). It has also been suggested that calcium variations are important in the regulation of exosome biogenesis (Baietti et al., 2012; Savina et al., 2005).

But other ESCRT-independent pathways of exosome biogenesis have been reported (Stuffers et al., 2009). Ghossoub et al. found that the GTAPase ADP Ribosylation Factor 6 (ARF6) and PhosphoLipase D2 (PLD2) control exosome biogenesis via an ESCRT-independent pathway involving ALG-2 interacting protein X (Alix) (Ghossoub et al., 2014). Blocking Alix did not lead to a decreased secretion of exosomes but rather a change in their composition. Meaning that Alix is more likely to be involved in cargo sorting than loading. However, silencing of Signal Transducing Adaptor Molecule 1 (STAM1) or Tumor Susceptibility Gene 101 (TSG101) led to no or partial reduction of exosome secretion but mainly to a variation in protein composition (Colombo et al., 2013; Tamai et al., 2010). Hoshino even showed that an increase in exosomes secretion after knocking down a member of the ESCRT-0 complex, Hepatocyte

growth factor-regulated tyrosine kinase substrate (Hrs), led to a more invasiveness cancerous behaviour (Hoshino et al., 2013).

The release of exosomes into the extracellular domain depends on the fusion of the MVE membrane with the PM (Colombo et al., 2014). For this, MVE docking involves multiple Guanosine-5'-TriPhosphate (GTP)-binding proteins such as Rab4, Rab5, Rab11, Rab27, Rab35 and ARF that are all known for their contribution to exosome secretion (Hsu et al., 2010a; Ostrowski et al., 2010; Savina et al., 2002; Vidal and Stahl, 1993). Other regulators include the cortical actin regulator (cortactin) (Sinha et al., 2016) and the fusion regulator synaptotagmin-7 (Hoshino et al., 2013).

It has recently been suggested that exosome biogenesis is not necessarily ESCRT-dependant or independent but rather that the two pathways could act synergistically or be compensatory (Maas et al., 2017). For example, Alix is known as an ESCRT accessory protein but is also involved in ESCRT-independent pathways. Moreover, a third pathway of exosome biogenesis involving the tetraspanin CD63 has been described as both ESCRT-dependant and independent (van Niel et al., 2011). The idea is that the pathway of exosome biogenesis defines the nature, composition and content of the exosome.

C. General characteristic

Exosomes are nanovesicles of 30-120nm in diameter and are limited by a lipid bilayer. Unlike other extracellular vesicles (EVs), their origin is endocytic. As a result, exosomes are rich in endosome-associated proteins such as tetraspanins, which are often the gold standard for exosome identification. Although other molecules such as heat shock proteins and MHC I and II are recognised as “exosome markers” they have also been found on other non-exosomal EVs in a study carried out on DEXs (Kowal et al., 2016). Much work has gone into deciphering the protein, lipid and RNA content as well as the functions of exosomes. Databases such as EVpedia, Vesiclepdia and Exocarta all contribute to understanding this.

1) Functions

a. Cell-to-cell communication

Exosomes can be considered as “mini cells” in that they have the same membrane orientation as the cell itself, RNA can be contained in their lumen, they carry ligands, receptors and other various signalling molecules. Many ways of communication with the target cell have been described. Exosomes can release their cargo in the extracellular space near the target cell, they can also be endocytosed,

they can fuse with the PM, work in a “kiss and run” manner or simply release their content into the cells’ cytoplasm before targeting another cell (Mulcahy et al., 2014) (Figure 16).

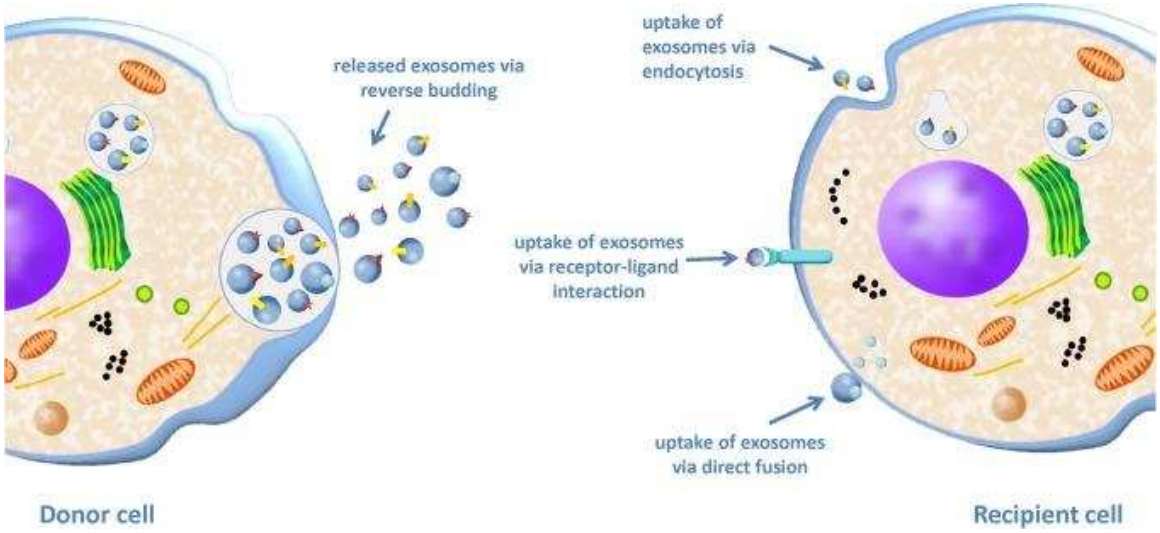


Figure 16: Exosome uptake from a donor cell to a recipient cell. The donor cell releases exosomes by fusion of the MVB membrane with the PM and subsequent secretion of ILVs into the extracellular space giving rise to exosomes. The exosomes then interact with the recipient cell via three possible mechanisms: uptake by endocytosis, ligand/receptor interaction or by direct fusion of the exosome membrane with the PM (Steinbichler et al., 2017).

Nevertheless, how exosomes target recipient cells still remains largely unknown. We are only just scratching the surface but some studies are starting to decipher this (Costa-Silva et al., 2015; Hoshino et al., 2015). Targeting could be led by either ligand-receptor affinity or by chemokines found on exosomes. The latter would explain the recruitment of exosomes to more distant sites (Mrizak et al., 2015).

An interesting study by Heusermann also found that exosomes can bind to the active regions of filopodia and lamellipodia to then enter at endocytic hot spots of human liver cell lines (huh7) (Heusermann et al., 2016). Moreover, this study demonstrated that exosomes taken up by the cell remain intact and are seemingly addressed to the ER where the exosomal cargo is released before subsequent degradation by fusion with lysosomes. It has interestingly been discovered that siRNA and miRNA loading into RNA-Induced Silencing Complex (RISC) takes place in the endoplasmic reticulum (ER) membrane (Li et al., 2013; Reid and Nicchitta, 2015; Stalder et al., 2013). Thus, the authors put forward the hypothesis that this would allow an efficient entry of the exosomal RNAs into the cell’s translational machinery.

The study of miRNAs has been gaining interest as they are now seen as a possible biomarker for many diseases, including cancer (Skog et al., 2008). Part of the circulating miRNA is found in exosomes (Gallo et al., 2012) although other studies have found that most of the miRNA is linked to the Argonaute

2 (Ago2) protein (Turchinovich et al., 2011). It was shown that miRNA enrichment on exosomes can be regulated by the oncogene Kirsten Rat Sarcoma (KRAS) (Cha et al., 2015) and the ribonucleoprotein A2B1 (hnRNPA2B1) (Villarroya-Beltri et al., 2013). Regardless, exosomes do carry miRNA and these have potent biological effects ranging from regulating translational activity to stability and localisation of molecules in recipient cells (Batagov and Kurochkin, 2013).

Exosomes have the advantage of being stable in the extracellular media notably due to its lipid bilayer, unlike free single molecules that are more exposed to degradation. Moreover, exosomes can travel virtually anywhere in the organism, even passing through the cells and nucleic membrane via microtubes (Osswald et al., 2016) and nanotubes (Connor et al., 2015).

b. Immune modulation

It is known that exosomes are secreted in larger amounts by immune cells compared to other cell types. Indeed, it was shown that exosomes alone can mediate antigen presentation and thus are able of inducing a potent immune response (Robbins and Morelli, 2014; Roche and Furuta, 2015) (Figure 17). MHC II αβ dimers found on the PM of APCs can be incorporated into ILVs. After this, the charging of the antigen onto the MHC II happens within the MVBs (Buschow et al., 2009). And the subsequent release of exosomes charged with MHC II-antigen complexes into the extracellular media leads to the activation of naive CD4 T cells (Théry et al., 2002). It was shown that the major release of these exosomes happens at the immunological synapse site between APCs and T cells. DCs can also induce a T cell response by releasing exosomes that express co-stimulatory molecules CD80 and CD86 or ICAM-1 (Segura et al., 2005a, 2005b; Théry et al., 2002). A study by Qazi, showed *in vivo* that antigen-charged exosomes alone can induce a potent Th1 response (Qazi et al., 2009). Thus, exosomes secreted at the immunological synapse site allow intercellular communication between the different immune cell populations. Indeed, DEXs are taken up by T cells but the latter can also release exosomes, containing miRNAs and/or TCR capable of regulating gene expression and cell signalling in DCs (Choudhuri et al., 2014; Mittelbrunn et al., 2011). Monteclavo also found that mice DCs could exchange functional miRNA via exosomes (Montecalvo et al., 2012) (Figure 17).

Likewise, exosomes can also be used by regulatory immune cells to block the immune response and induce “infectious tolerance” (Chatila and Williams, 2014). In fact, mice Tregs were found to secrete exosomes containing miRNA let-7d that is taken up by $CD4^+$ $TNF\gamma^+$ Th1 cells and reduced their immunogenicity (Okoye et al., 2014). Other studies have shown that exosomes derived from mice Tregs can induce seemingly tolerogenic DCs via miR-150-5p and miR-142-3p (Tung et al., 2018). But the exosome-mediated tolerance of Tregs goes beyond miRNAs. Indeed, Okoye found that other

factors such as non-coding RNA, chemokines, interleukins, collagen and matrix proteins found on mouse Tregs-derived exosomes could induce immune tolerance (Figure 17). The immunosuppressive effect of Treg-derived exosomes seems so potent that it is even being considered as a possible therapeutic for autoimmune diseases and transplant patients (Agarwal et al., 2014; Monguió-Tortajada et al., 2014).

Moreover, immunosuppressive Mesenchymal Stem Cells (MSCs) can use exosomes to inhibit the macrophage response, by impairing TLR recognition (Phinney et al., 2015), but also to induce Treg differentiation after a TGF-β and IFNγ stimulation (Zhang et al., 2018b). Other *in vivo* murine studies show that exosomes derived from tolerogenic DCs and Myeloid-Derived Suppressor Cells (MDSCs)

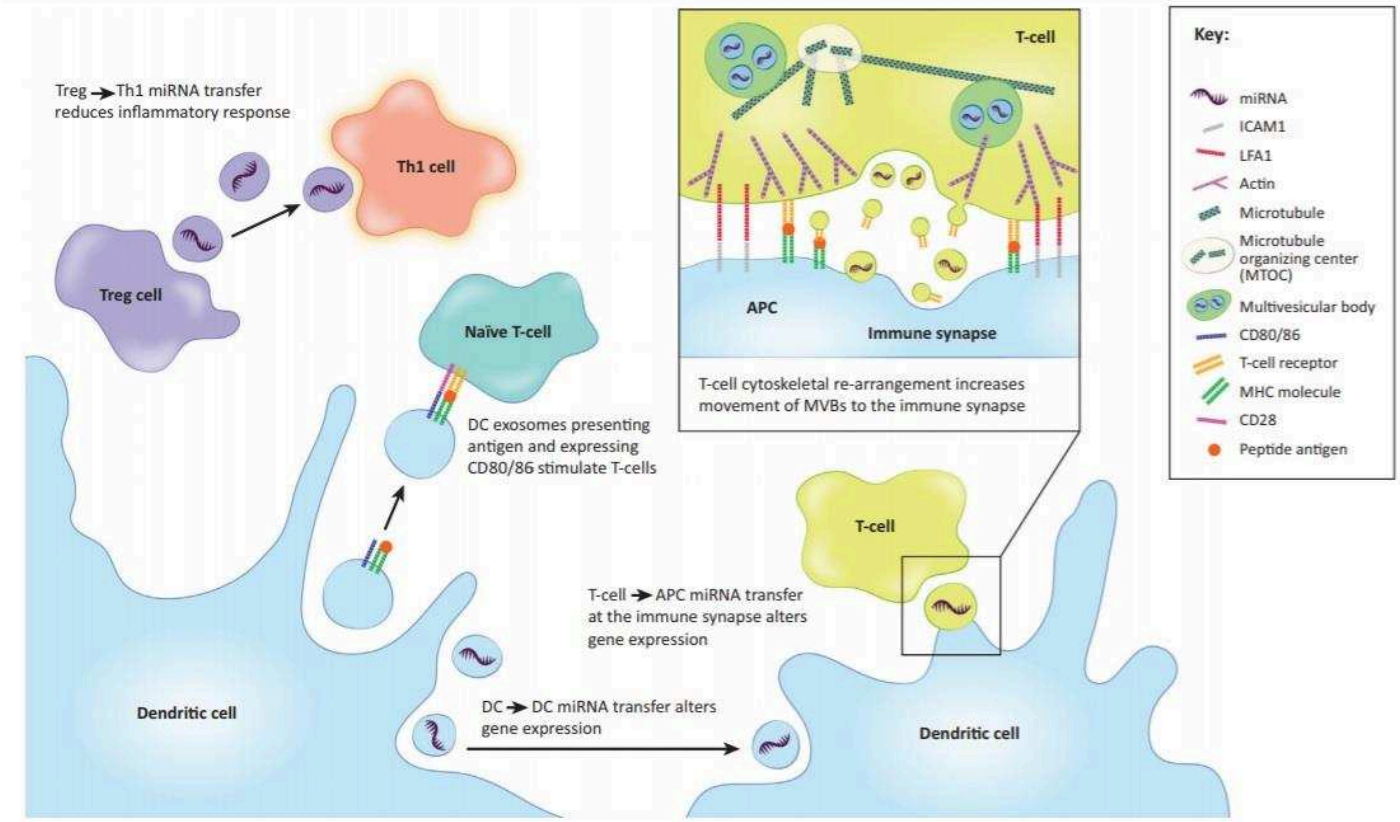


Figure17: Immune modulation by exosomes. DEX carry miRNA, co-stimulatory molecules (CD80/86) and antigen charged MHC molecules that can be transferred to (i) naive T cell to subsequently induce anti-tumour Th1 T cells, (ii) other DCs (iii) or T cells at the site of the immunological synapse to modulate the immune response. T cells can in return also secrete exosomes that will influence recipient DCs via miRNAs or the TCR that they carry. Exosomes derived from Tregs can block the immune response by hindering Th1 T cells via exosomal miRNA (Maas et al., 2017).

favour Treg expansion and an inhibition of the Th1 response (Wang et al., 2016b; Yang et al., 2010). Although these results are very encouraging, we must bear in mind that most of these studies are carried out on murine Treg-derived exosomes and further studies on human cells need to be done.

Thus, the use of exosomes in a systemic response such as the immune response makes perfect sense as they are highly itinerant and efficient. They most probably contribute to the fine-tuning of the immune balance in an energy-efficient and systemic manner.

2) Isolation methods

Exosomes can be isolated via two main methods: a series of high speed centrifugation with the use of a gradient (sucrose or iodixanol flotation) (Théry et al., 2006) or by immunoaffinity-based capture. Both methods have proven highly efficient and with a good yield (Li et al., 2017b; Yamashita et al., 2016). However, it must be pointed out that the immunoaffinity-based capture can only be used for characterisation of exosomes as functional tests would be hindered by the binding agent (György et al., 2011; Kalra et al., 2013). And ultracentrifugation could also damage the exosomes by the strong pressure the exosomes are put under (Jeppesen et al., 2014). Other methods have also been developed such as exosome precipitation using water-excluding polymers (polyethylene glycol) (Kim et al., 2016a; Zeringer et al., 2015) or microfluidics-based isolation (Davies et al., 2012; Lee et al., 2015). The following table summarises the advantages and disadvantages of the above-mentioned techniques (Table V).

Table V: Comparison of exosome isolation techniques (Li et al., 2017b)

Isolation technique	Isolation principle	Potential advantage	Potential disadvantage
Ultracentrifugation-based techniques	Density, size and shape based sequential separation of particular constituents and solutes	Reduced cost and contamination risks with separation reagents. Large sample capacity and yields large amounts of exosomes	High equipment cost, cumbersome, long run time, and labor intensive low portability – not available at point-of-care, high speed centrifugation may damage exosomes thius impending downstream analysis. (Jeppesen et al., 2014)
Size-based techniques	Exosome isolation is exclusively based on the size difference between exosomes and other particulate constituents	Ultrafiltration: fast, does not require special equipment, good portability, direct RNA extraction possible SEC: high-purity exosomes, gravity flow preserves the integrity and biological activity; superior reproductability, moderate sample capacity	Ultrafiltration: low equipment cost, moderate purity of isolated exosomes, sheer stress induced deterioration, possibilit of clogging and vesicle trapping, exosome loss du to attaching to the membrane. SEC: moderate equipment cost, requires dedicated equipment, not trivial to scale up, long run time.
Exosome precipitation	Altering the solubility or dispensibility of exosomes by the use of water-excluding polymers	Easy to use, does not require specialized equipment, large and scalable capacity	Co-precipitation of other non-exosomal contaminants like proteins and polymeric metarials. Long run time. Requires pre-and post-cleanup.
Immunoaffinity capture-based technique	Exosome fishing based on specific interaction between membrane-bound antigens (receptors) of exosomes and immobilized antibodies (ligands)	Excellent for the isolation of specific exosomes. Highly purified exosomes – much better than those isolated by other techniques, high possibility of subtyping.	High reagent cost, exosomes tags need to be established, low capacity and low yields, only works with cell-free samples, tumor heterogeneity hampers immune recognition, antigenic epitope may be blocked or masked. (Batrakova et Kim, 2015)
Microfluidics-based technique	Microscale isolation based on a variety of properties of exosomes like immunoaffinity, size and density.	Fast, low cost, portable, easy automation and integration, high portability.	Lack of standardization and large scale tests on clinical samples, lack of method validation, moderate to low sample capacity.

Each method has its pros and cons, but what is essential is to develop a technique that will lead to widespread use of exosome, hopefully for the clinic. This must be a cost-effective and easily mastered isolation method.

D. Tumour exosomes

1) Exosomes and the tumour microenvironment

Even though exosomes are involved in the progression of many diseases, notably viral dissemination, neuropathies and others, we will solely focus on exosomes secreted by tumour cells also named TEXs. TEXs act at many levels of cancer and at all stages of the disease. Cell cycle, angiogenesis and metastasis initiation are all commonly enhanced during cancer and are now associated with TEXs (Hu et al., 2018).

a. Proliferation

In an acute myeloid leukemia (AML) blasts model, exosomes secreted by AML cells blocked normal hematopoiesis and osteogenesis as they induced the expression of Dickkopf-related protein 1 (DKK1) in bone marrow stromal cells. DKK1 is a known suppressor of these mechanisms and thus leads to a progression of AML. In addition, AML-derived exosomes are able to downregulate the expression of many other factors involved in normal hematopoiesis such as CXCL2, KIT Ligand (KITL) and Insulin Growth Factor-1 (IGF-1). Indeed, when the release of AML-exosomes was blocked or DKK1 was specifically inhibited, it was shown that AML growth and OS of AML-engrafted mice were significantly increased (Kumar et al., 2018).

Moreover, a study by Hong and colleagues found that in colorectal cancer, tumour microvesicles contain mRNA capable of interfering with cell cycle, mostly during the M phase (Hong et al., 2009). The viral oncoprotein LMP1 is also known for its ability to drive oncogenesis by interfering with many vital cell functions including proliferation. Hypoxia inducible factor 1 α (HIF1α) also shares this disruptive function as it enhances migration, invasiveness and overall tumorigenicity. Interestingly, a study by Aga in NPC not only showed that LMP1 upregulates HIF1α, but also that exosomes carry HIF1α which maintains its DNA-binding activity to upregulate its expression in recipient cells (Aga et al., 2014). This data strongly suggests that EBV also plays a part in NPC metastasis formation.

b. Angiogenesis

Enhanced angiogenesis is a common characteristic amongst all solid tumours. This allows the tumour to better access nutrients in order to grow. But tumour blood vessels are scruffy and leaky

which partly explaining why treatments don't always reach the tumour and lack efficacy (Nishida et al., 2006). A study by Lang found that exosomes derived from glioma cells contain long non-coding RNAs (LncRNA) of the POU Class 3 homeobox 3 (POU3F3) transcription factor. The upregulation of POU3F3 is correlated to late stages of glioma and the Linc-POU3F3 expressing TEXs increased the expression levels of pro-angiogenic factors such as TGF-β, TGF-β receptor (TGF-βR) and Vascular Endothelial Growth Factor-A (VEGF-A) (Lang et al., 2017). Moreover, as hypoxia is a known inducer of angiogenesis, and miR-210 were found in exosomes derived from hypoxic tumour cells. Indeed, miR-210 levels are very high within the Tumour Micro-Environment (TME) and can modulate VEGF expression leading to a recruitment of endothelial cells for tumour angiogenesis (Jung et al., 2017).

c. Metastasis

Involvement of exosomes in metastasis has long been suspected, and evidence has now shown just how much exosomes contribute to this mechanism (Steinbichler et al., 2017; Subramanian et al., 2016). It has been found *in vitro* that TEXs can educate tumour-supporting cells to prepare for metastasis. For example, melanoma-derived exosomes incite proangiogenic signalling program and condition the LNs to respond better to melanoma cells. TEXs are recruited to the LNs and will in turn attract melanoma cells to the LNs in order to set up a secondary tumour site (Hood et al., 2011). A similar study conducted in a rat model of adenocarcinoma by Rana also showed it was mediated by TEX microRNAs (Rana et al., 2013). They found that TEXs modified the tissue phenotype by silencing the expression of structural molecules such as matrix metalloproteinases and cadherin-17 thus favouring the setup of future metastases. It was even suggested that more aggressive tumour cells could secreted exosomes to educate the lesser aggressive tumour cells to become invasive and itinerant via the microRNA-200 family (Epstein, 2014). Thus, these circulating TEXs show great potential as diagnostic and predictive relapse biomarkers.

Moreover, two studies found that NPC tumour cells could be modified by MSC-derived exosomes. Indeed, they promote the proliferation, migration and tumorigenesis of NPC cells (Shi et al., 2016). A study by Aga found that HIF1α actively participates in the transmission of the pro-metastatic effect of TEXs to recipient cells (Aga et al., 2014). And finally, TEXs in hepatocarcinoma carry the chemokine CXCR4 to promote cancer cell migration, invasivess and lymphatic angiogenesis. All of this helps prepare for the premetastatic niche and thus future metastases implementation (Li et al., 2018).

2) Immune evasion in cancer

Exosomes play a key role in promoting and maintaining the TME as they are potent intercellular communicators. Immune modulation by exosomes in cancer is gaining interest as it has been shown that exosomes are often put to use by the tumour to favours its progression and immune escape (Graner et al., 2018; Greening et al., 2015; Seo et al., 2018) (Figure 18). Although, in some specific types of inflammatory cancers, such as Triple Negative Breast Cancer (TNBC), the tumour and thus, TEXs aim to induce a sustained immune response, mainly by acting on APCs such as macrophages and DCs (Chow et al., 2014; Greening et al., 2015). However, we will focus here on cancers that seek to suppress the anti-tumour response, such as NPC.

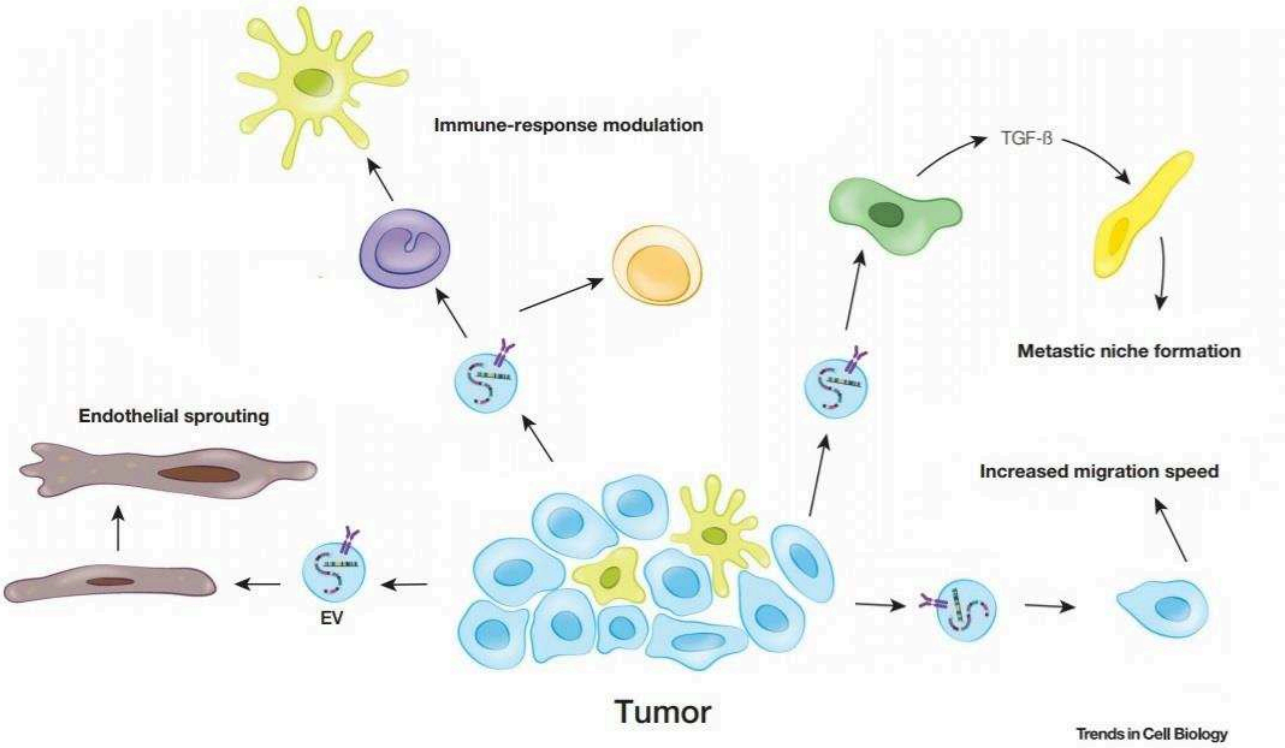


Figure 18: Tumour exosomes and cancer. Exosomes contribute to the progression of cancer via multiple mechanisms: (i) they promote angiogenesis via endothelial sprouting, (ii) modulate the immune response to favour immune evasion of cancer, (iii) increase migration capacities of tumour cells (iv) and contribute to the setup of premetastatic niches and development of secondary tumours (adapted from Maas et al., 2017).

a. Innate immunity

NK cells are one of the first lines of defence against tumour cells. Thus, TEXs are able to downregulate the activating NKG2D receptor that NKs use to recognize their targets (Clayton et al., 2008). TEXs express NKG2D ligands such as MICA/B and UL16 Binding Protein 1 and 2 (ULBP1/2) to prevent tumour cell killing by acting as decoys (Ashiru et al., 2010; Clayton and Tabi, 2005). Thus,

impairing NK sensitivity and subsequent cytotoxic capabilities (Chitadze et al., 2013; Mincheva-Nilsson and Baranov, 2014). *Ex vivo*, exosomes isolated from patients with AML notably contain high levels of TGF-β1 and MICA/MICAB. They were also capable of decreasing NKG2D expression and NK cytotoxicity which was counteracted by the NK homeostatic cytokine IL-15 (Szczepanski et al., 2011).

b. Adaptive immunity

Regulatory T cells

Tregs are major contributors to immune evasion as their natural ability to inhibit the immune response is hijacked in cancer. It is now known that TEXs are one of many mechanisms used by the tumour in order to profit from Tregs (Szajnik et al., 2010; Wieckowski et al., 2009). TEXs promote Treg induction, expansion and suppressive function. For example, Mrizak et al showed that NPC TEXs favour the recruitment of Tregs but also conventional T cells in order to then induce Tregs and enhance their suppressive functions (Mrizak et al., 2015). TEXs can contain, TGF-β, Fas-L, CD73 and galectines which are all involved in immune regulation (Abusamra et al., 2005; Clayton et al., 2011; Klibi et al., 2009a; Schuler et al., 2014).

Effector T cells

Other mechanisms of immune evasion by TEXs involve targeting T cells. Immunosuppressive molecules found on TEXs contribute to T cell impairing or killing, notably via Fas Fas-L and Gal-9 (Andreola et al., 2002; Keryer-Bibens et al., 2006; Klibi et al., 2009a). The study by Andreola and colleagues showed that Fas-L was accumulated in melanoma cells and thus secreted Fas-L-enriched TEXs. This leads to the Fas-L-mediated death by apoptosis of antitumoural T cells. CD8 T cell death by the same mechanism was shown by Wiechowski the same year (Wieckowski et al., 2009). As previously discussed, Gal-9 and LMP1 enriched exosomes found in NPC promote Tregs but also inhibit effector T cells. Indeed, Gal-9 kills T cells by binding to the cell death receptor T cell Immunoglobulin and mucin domain containing protein 3 (Tim3) found on T cells, leading to their death by apoptosis. Moreover, they also convert effector T cells into Tregs, further contributing to the immunosuppressive TME (Mrizak et al., 2015). Physiologically, Gal-9 helps prevent prolonged inflammation as it is damaging for tissues. Similarly to the NK impairing, the expression of NKG2D by TEXs also disrupts the activity of CD8 T cells. Indeed, Clayton and Tabi found that incubation of PBMCs with TEXs containing NKG2D ligands leads to a drop in the number of CD3+ CD8+ cells and the remaining cells showed reduced killing abilities (Clayton and Tabi, 2005).

Furthermore, TEXs also have the ability to disrupt cytokine-mediated pathways that play a major role in the fine-tuning of the immune response. This tempering by TEXs can take many forms.

For example, IL-2 sensitivity of lymphocytes can be impaired by TEXs (Clayton et al., 2007). IL-2 is a key cytokine for effector T cell and NK function but also supports Treg immunosuppression. Indeed, TEXs show a "double hit" mechanism by selectively skewing IL-2 responsiveness of effector T cell and NK cells whilst favouring Treg function. This study by Clayton and colleagues found that not only did TEXs preferentially distribute IL-2 to Tregs but also enhanced their function by carrying TGF-β. Another example of cytokine disruption, is the blocking of MoDCs by TEXs which leads to the increased secretion of regulatory cytokines and lesser anti-tumoural cytokines. These suppressive signals release by tempered DCs leads to a blockade of the T cell anti-tumour response (Yu et al., 2007). Similarly, TEXs are also responsible for the induction of MDSCs which also give rise to Tregs (Valenti et al., 2006). This unbalanced immune environment subsequently leads to an overall pro-tumoural immunosuppressive TME (Figure 18).

E. Clinical applications of exosomes in cancer

1) Tumour exosomes and drug resistance

Studies have described that TEXs can contribute to drug resistance in cancer (Azmi et al., 2013; Bach et al., 2017; Yu et al., 2015). For instance, exosomes derived from estrogen receptor (ER)-positive breast cancer favour resistance to tamoxifen. For this, TEXs transfer miR-221/222 which downregulates tamoxifen targets, p27 and ERα, in recipient cells (Wei et al., 2014). And in lung cancer, TEXs were found to worsen resistance to cisplatin chemotherapy by transferring miR-100-5p (Qin et al., 2017). A recent study in TNBC found that TEXs were able to confer drug resistance to recipient cells (Ozawa et al., 2018). In fact, a large study carried out on over 60 different cell lines showed a correlation between exosomes and resistance to drugs in cancer (Shedden et al., 2003). Also, exosomes seem to aid the tumour in sequestering and eliminating drugs which leads to drug resistance (Chen et al., 2006; Safaei et al., 2005). This knowledge could help better stratify cancer patients and anticipate drug resistance, or even target TEXs during treatments to limit drug resistance.

Exosomes are increasingly appealing in the field of cancer theranostics. Their easy access makes them a target of choice. Yet, the isolation and characterisation of exosomes remains either costly and/or time consuming. And a common method is still lacking as technical variants could lead to conflicting results (Panagiotara et al., 2017).

2) Exosomes as diagnostic and prognostic biomarkers

It is well known that the earlier the cancer is diagnosed, the better the outcome is for the patient. Liquid biopsies are gaining interest, as they are non-invasive, quickly obtained and cost-effective. Exosomes are found in most bodily fluids, notably urine and serum. Thus, new light is being shed on the specificity of TEXs and their possible use as potent new diagnostic and prognostic biomarkers.

TEXs can drive tumorigenesis by multiple means but notably via transcriptionally interfering miRNAs. Distinctive miRNAs are found in each type of TEXs and can even help in determining the nature of the cancer. For example, in non-small cell lung cancer, TEX miRNAs such as miR-181-5p, miR-30a-3p, miR-30e-3p and miR-361-5p are biomarkers for adenocarcinoma. Whereas squamous cell carcinoma can be diagnosed with other distinct miRNAs like miR-10b-5p, miR-15b-5p and miR-320b (Jin et al., 2017; Rabinowits et al., 2009). Moreover, Wu and colleagues found that a positive correlation between the levels of miR-96 and the stage of lung cancer. With highest levels being associated to highly aggressive, metastatic and drug-resistant lung cancer (Wu et al., 2017).

Furthermore, in pancreatic cancer which shows a high mortality rate, miR-191, miR-21 and miR-451a can serve as early diagnostic and prognostic biomarkers (Goto et al., 2018). Another study found seven miRNAs (let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a) associated with colon cancer, even at an early stage, compared to healthy donors (Ogata-Kawata et al., 2014).

In NPC, detection of TEXs in liquid biopsies have also been shown as good diagnostic and prognosis biomarkers. EBV-BART-miRNAs contribute to tumorigenesis and are found in high levels in NPC patient serum (Lo et al., 2012). Generally, EBV-linked proteins and DNA, whether they are contained in exosomes or not, are of good prognostic value (Gurtsevitch et al., 2017; Yip et al., 2014; Zhang et al., 2016a). Also, the concentration of exosomes in NPC-patient serum is higher in cases of LNs metastasis, thus associated with poorer prognosis (Ye et al., 2016). This study found that TEXs are enriched with miR-24-3p compared to healthy donor exosomes, making it a very appealing biomarker. Other molecules found specifically in high content in NPC TEXs are HLA-II (Keryer-Bibens et al., 2006) and Gal-9 (Klibi et al., 2009a). HLA-II being too ubiquitous, Gal-9 could also be a potentially prominent new biomarker.

3) Exosomes-based treatments

The growing knowledge surrounding exosomes in cancer biology has shown their importance and it comes as no surprise that novel cancer treatment encompasses them. Their small size, easy transfer

to cells as well as their protective lipid bilayer structure makes them very interesting tools for therapeutic use (Tai et al., 2018).

a. Use of immunostimulatory effects

DEXs have shown great therapeutic promise as they are potent immune activators (Pitt et al., 2014). Pioneers in the field first showed the potential of antigen-pulsed DEXs as anticancer treatments in 1998 (Zitvogel et al., 1998). Indeed, DEXs can prime a specific T cell anti-tumour response as well as induce NK functions.

Early phase clinical trials have shown encouraging results as the DEX-based treatments are safe, well tolerated and allow longer progression free survival (Besse et al., 2016; Dai et al., 2008; Escudier et al., 2005; Morse et al., 2005).

b. Targeting of tumour exosomes

As we have previously discussed how exosomes contribute in many ways to tumorigenesis, targeting TEXs is also envisioned as a novel cancer treatment. Targeting molecules that are specific to TEXs, and thus, the tumour cells they derive from, is an appealing possibility. For example, Gal-9 is specific to NPC-derived exosomes and is also predominantly found on tumour cells and Tregs. A drug that blocks Gal-9 could significantly hinder NPC progression (Klibi et al., 2009; Mrizak et al., 2015, patent US20170283499A1). As previously mentioned, TEXs are enriched in Fas-L which leads to effector T cell apoptosis. This mechanism can be blocked using Iroquois-class homeodomain protein (IRX-2), a primary cell-derived biologic agent consisting of cytokines produced by Th1 cells. In fact, clinical trials testing IRX-2 have shown promising results with other phase II trials still underway (Freeman et al., 2011; Wolf et al., 2018). Immunomonitoring of immune cell populations during IRX-2 treatment shows no difference in CD4+ CD25high Treg levels but a reduction of B cells, NKT cells and naive T cells. This is thought to be the result of a redistribution of these cells to tissues (Whiteside et al., 2012). In fact, the same team also found that in head and neck cancer, IRX-2 treated patients showed an increase in tumour infiltrating T cells and macrophages, presumably to restore immune responsiveness vis-à-vis the cancer (Berinstein et al., 2012). However, it should be noted that activated T cell also naturally express Fas-L, in a protein Kinase D1/2-dependant manner, in order to kill other activated T cell to prevent the arise of autoimmune diseases (Mazzeo et al., 2016; Monleón et al., 2001). Although no severe toxicities have been noted in the first clinical trials, immunological balance is likely to be at risk with this drug and the development of autoimmune diseases must be avoided.

c. Exosomes as nanocarriers of drugs

One innovative use for exosomes in therapeutics, is as a drug nanocarrier. Indeed, their endogenous nature makes them more tolerated by the body, unlike other synthetic drugs. Charging drugs as cargo into exosomes allows their protection and efficient access to all areas of the body (Ha et al., 2016). Interestingly, exosomes were even shown to deliver anticancer drugs across the blood-brain barrier, which is often hermetic to drugs, this is promising for treating brain tumours or even neurodegenerative diseases (Yang et al., 2015).

Exosomes can be charged with drugs by different techniques including electroporation, lipofection, sonication or simple incubation with the drug (Johnsen et al., 2014). Electroporation is generally very successful but the major downside is that siRNA precipitates and DNA is poorly charged into exosomes (Kooijmans et al., 2013; Lamichhane et al., 2015). Moreover, incubation of the drug with exosomes can lead to its uptake but only if it is small enough to penetrate through the exosome's lipid bilayer membrane (Johnsen et al., 2014). In addition, lipofection is an effective, non-viral and reproducible method for introducing foreign DNA, siRNA and drugs into exosomes. However, lipofection is only restricted to cells that have a high division rate (Gresch et al., 2004). A recent study aimed to determine which method was best for loading paclitaxel (PTX) into exosomes to treat multi drug resistance in cancer. The authors tested incubation of PTX with exosomes, electroporation or sonication. The greatest loading capacity was obtained by mild sonication of exosomes (Kim et al., 2016b). Other findings in this study correlated well with results by Saari and colleagues in that PTX is more efficiently targeted to tumour and showed increased cytotoxicity (Saari et al., 2015). Furthermore, these results highlight another advantage of exosomes, their specific organotropism which is mediated by integrins (Hoshino et al., 2015). Tian and colleagues were able to engineer exosome membranes to express given integrins. And indeed, the modified exosomes that carried doxorubicin, delivered the drug to specific tissues leading to a inhibition of tumour growth with less overall toxicity (Tian et al., 2014). The deep-seated delivery of drugs via exosomes to a given tissue allows for better and more specific treatments with less toxicity.

Thus, exosomes have now been revealed as major players in intercellular communication and the immune response. Therefor exosomes are an ideal target for cancer in order to disrupt tissue homeostasis and allow tumour growth. The varied physiological functions of exosomes make it hard for the body to counteract when they are hijacked by cancer. Hence, exosomes are now novel targets for the treatment of cancer and theranostics. Nevertheless, much remains unknown about exosome biology making it one of the most fast-growing and interesting fields in oncology yet.

IV. Dendritic cells

DCs are key regulators of the fine-tuning of the immune system. They play a critical role in the induction of an adaptive immune response but also promote tolerance when needed. Depending on the nature of the antigen taken up, DCs will then orchestrate an appropriate immune response. It was Ralph Steinman who discovered DCs in 1973 in mice, and was later awarded with a Nobel Prize for this major contribution (Steinman and Cohn, 1973; Steinman et al., 1974, 1975, 1979). He found cells in mice spleen that were distinct from macrophages, had poor phagocytic capacity and showed long branch-like structures. The term *dendritic cell* comes from the Greek *dendron*, which means tree in reference to their morphology. It was only a few years later that DCs were attributed their potent immune properties. Mixed Leucocyte Reactions (MLRs) testing showed that they were the instigators of both the cellular and humoral immune responses (Inaba et al., 1983; Nussenzweig et al., 1980). After this, studies of DC migration patterns showed that antigen-loaded DCs travelled to the spleen and LNs in order to deliver the antigen to naive T cells and subsequently activate them for a specific immune response (Austyn et al., 1988). Although other cell can present antigens, DCs are professional APCs (Sprent, 1995). What defines DCs is their high expression of MHC II, the expression of the CD11c integrin, their ability to migrate from the periphery to lymphoid organs and their capacity to activate naive T cells. Nevertheless, it must be noted that individually, each trait is not specific to DCs, it is the concomitance of these features that define DCs.

A. Dendritic cell classification

1) Ontogeny

To this day, choosing a nomenclature that best classifies DCs remains a subject of debate. It is uncertain whether DCs should be classified depending on their cellular precursors, functions, phenotype or organotropism. So far, the most accepted classification is based on ontogeny and organ residence (Guilliams et al., 2014; Schraml and Reis e Sousa, 2015).

Since the turn of the century, the field of DC ontogeny has been fast expanding but many questions remain unanswered. The difficulty lies in the multiple types of DC described, which could also be due to cell plasticity and their challenging location within the body (Hashimoto et al., 2011). This is why murine DCs are often studied to help elucidate DC biology. Although mice and human DCs differ on some aspects, studying mice DCs has brought us valuable knowledge on human DCs. The following chart illustrated DC lineage in humans and mice, which also enable a clear comparison of murine and human DCs subsets (Figure 19). My thesis work was solely carried out on human MoDCs, thus we will

here only focus on human DC ontogeny. Firstly, all DCs originate from a common bone marrow-derived Hematopoietic Stem Cell (HSC) progenitor. HSCs can either give Multi-Lymphoid Progenitors (MLPs) or Granulocyte Macrophage Progenitors (GMPs). Both can become Macrophage and DC Progenitor (MDP) (Fogg et al., 2006) or a Common DC Progenitor (CDP). Then DCs develop into either pDC, pre-DCs or a common Monocyte Progenitor (cMoP). All three progenitors then migrate to peripheral tissues to further differentiate. cMoP become monocytes that will migrate to peripheral tissues and give rise to either monocyte-derived macrophages (MoMac) or MoDCs (Guilliams et al., 2014; Onai et al., 2007, 2010). Furthermore, pre-DCs differentiate into peripheral classical or conventional DCs (cDC), and cDC subsets are generally dependant on the expression of phenotype markers. For example, Blood Dendritic Cell Antigen 1 (BDCA1) is a universal myeloid DC marker but not all DCs are BDCA3+, although they come from the same pre-DC progenitor. BDCA3+ cells are considered to be a different subtype of cDCs than the BDCA3- cells (Jin et al., 2014). And although this is the currently accepted model of DC ontogeny, new research is giving us new information regarding the different progenitors and DCs subsets, but also about what drives them to specific differentiation patterns (Heidkamp et al., 2016).

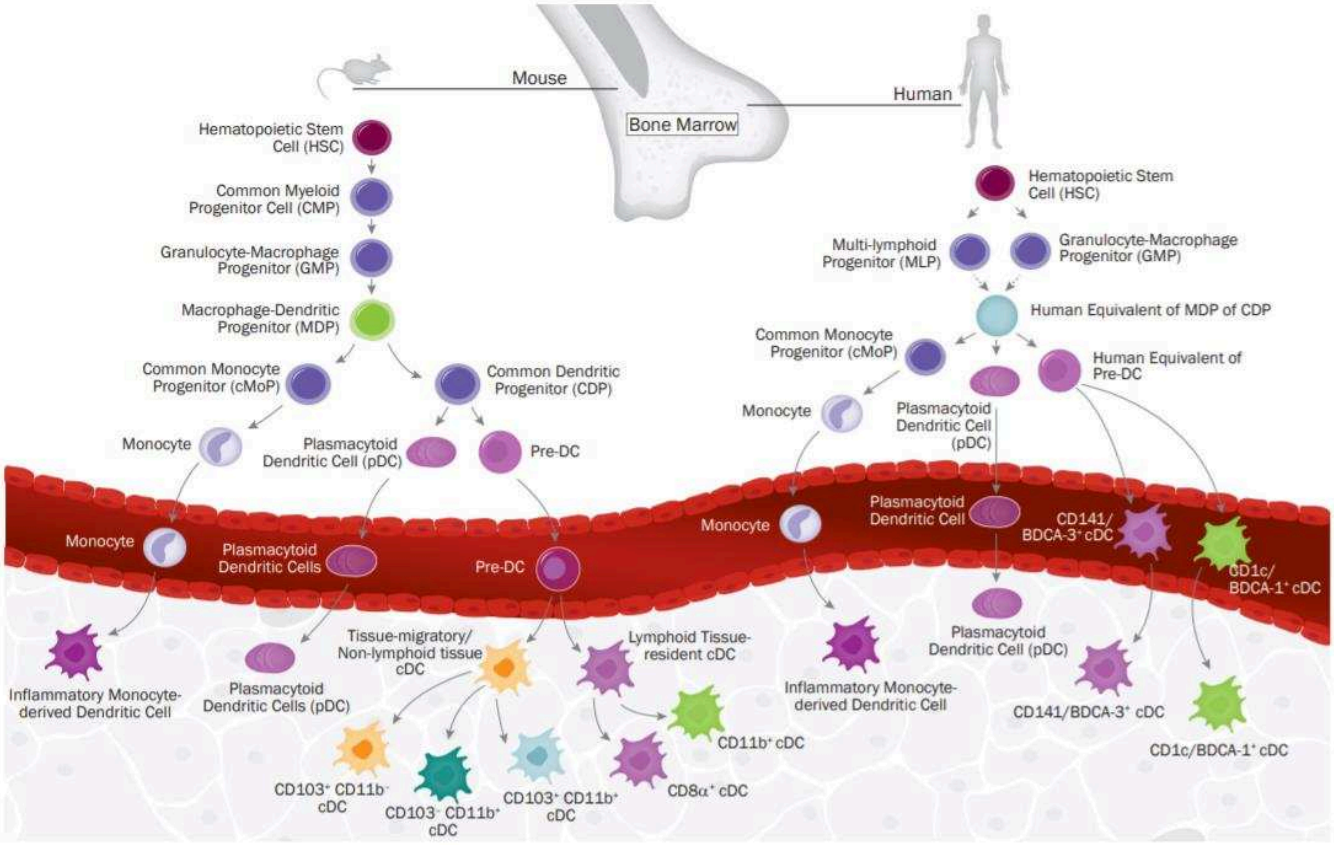


Figure 19: Human and mice DC lineage. Mice and human DCs ontogeny share many similarities, the equivalent cells are shown in the same colour in human and mice. After the HSC stage, progenitors between the two species differ, but the three main subsets of DCs are conserved: monocyte-derived DCs, plasmacytoid DCs and DCs that arise from a common pre-DC progenitor. The major difference are phenotypical markers used to identify each DC population (R&D Biotechne: https://www.rndsystems.com/pathways/dendritic-cell-lineage-development-pathways).

2) *In vitro* cultured human DCs

The study of human DCs is challenging, as they are not easily available. Thus, two major populations of human DCs are studied *in vitro*: pDCs and MoDCs.

a. Plasmacytoid DCs

pDCs are found in the peripheral blood (<0.4% of PBMCs) and peripheral lymphoid organs. Discovered in 1997, pDCs were unlike the other previously described DCs as they express CD4 and CD45RA but not CD3 nor CD11c. The same CD4+ CD11c- cells found in blood were also identified in tonsillar tissue, but with a higher apoptosis rate. This shows that they belong to a lymphoid lineage but remain *bona fide* DCs as they express high levels of MHC II (Grouard et al., 1997). pDCs show little antigen presentation abilities but secrete high levels of type I IFN allowing them to steer the immune response (Villadangos and Young, 2008). Although pDCs are found in peripheral blood, the study of

pDCs remains costly and difficult as they are a rare population amongst PBMCs. One of the advantages is that they are already differentiated, unlike MoDCs.

b. Monocyte derived DCs

The study of MoDCs has the advantage that monocytes are more common amongst PBMCs (15-25%). However, they need to be differentiated into DCs which is more time consuming and costly. The protocol of MoDC differentiation using granulocyte/macrophage colony stimulating factor (GM-CSF) and Interleukin 4 (IL-4) is now a well-established method (Sallusto and Lanzavecchia, 1994). It must be noted that iDCs generated *in vitro* from peripheral monocytes using GM-CSF and IL-4, seem to naturally express high levels of HLA II and co-stimulatory molecules compared to steady-state cells found *in vivo*. Thus, iDCs derived from human monocytes seem to be more advanced along the developmental stages than peripheral human iDCs. Nevertheless, *in vitro* generated DCs are still able to induce effector T cell anergy and the development of Tregs (Jonuleit et al., 2000). Interestingly, once the effector T cells were anergized, Jonuleit and colleagues were not able to reverse this effect with fully mature DCs.

B. DC subtypes

A decade after the initial discovery of DCs, Steinman and Schuler put forward the notion that DCs undergo a maturation process that it critical for their function (Schuler and Steinman, 1985). They found that immature DCs have a potent antigen capturing capacity but do not activate T cells. For this, DCs must undergo maturation that is both phenotypically and functionally distinguishable to an immature state. But the maturation process is not so binary, there are also semi-mature DCs or even steady-state DCs that show an intermediate level of maturation, subsequently influencing their function (Dudek et al., 2013).

1) DC maturation

Immature DCs (iDCs) are peripheral tissue resident cell that act as sentinels and constantly scour the body for potential threats, whether they are non-self or self. Physiologically, iDCs exist in an immature non-stimulated state and are also named steady-state. Unlike other APCs such as macrophages or B cells, DCs are professional APCs. This means they have a highly efficient antigen presentation capacity and are seemingly the only cells that can induce a T cell response from a naive CD4 T cell (Itano and Jenkins, 2003; Sprent, 1995). iDCs main goal is to uptake antigens to eventually present them to T cells in order to induce an immune response. For this, iDCs express a wide spectrum of antigen receptors that act as sensors and detect danger signals. They capture antigens by

phagocytosis, micropinocytosis or via the cell surface receptor which are then endocytosed. iDCs highly express a plethora of PRRs that distinguish Pathogen Associated Molecular Patterns (PAMPs) and Damage Associated Patterns (DAMPs). There are two subgroups of PRRs, membrane-bound PRRs and cytoplasmic-bound PRRs. The first engulfs TLRs and CLRs such as DC-SIGN (Diebold, 2009; Visintin et al., 2001). The latter includes NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). All of these are potent detectors of foreign pathogens and danger signals which promote the immune response. Interestingly, a study found distinct PRR expression patterns that depend on the DC subtype. Lundberg described a difference between CD1c+, CD141+ and CD16+ myeloid DCs and CD123+ pDCs, which would explain the specific aptitudes of each subtype (Lundberg et al., 2014). Regardless of the nature of the antigen, the detection an uptake of the antigen triggers signalling that leads to maturation in order to induce an immune response. Maturation is accompanied by profound phenotypical and functional changes (McIlroy et al., 2005; Türeci et al., 2003). During the maturation process, iDCs will migrate to T cell rich region in lymphoid organs. This migration is largely dependent on chemotaxis. Indeed, DCs express varying patterns of chemokine receptors in response to the chemotactic gradients (Lukacs-Kornek et al., 2008; Sallusto et al., 1998, 1999b; Sozzani, 2005; Tiberio et al., 2018). CCR6 is expressed by iDCs and determines their localisation in the body via its ligand chemokine CCL20. The secretion of CCL20 is induced by inflammatory cytokines or microbial factors. Thus, at these regions iDCs are recruited via the CCR6/CCL20 axis in order to initiate a specific immune response (Cook et al., 2000). In the case of MoDCs, CCR1, CCR3 and CCR5 all contribute to the migration of the DCs to the inflamed tissue (Beaulieu et al., 2002; Sato et al., 1999). Typically, during the migration/maturation process, maturing DC no longer express CCR6 but increase the expression of CCR7 which leads to the migration to LNs.

Moreover, during maturation DCs break down and process the antigen so it can be charge onto the HLA molecules in order to form the MHC, which will later interact with the TCR. Mature DCs (mDCs) interact with T cells to form an immunological synapse in order to prime T cells and induce a T cell response. For correct T cell priming, three essential signals have been described. Signal 1 is the interaction of the MHC with TCR. At the immature state, DCs are not immunogenic, they have not yet taken up the antigen and express little or no MHC II molecules (Lee et al., 1993; Mommaas et al., 1995). However, it has been described that iDCs are able to induce Tregs *in vivo* (Dhodapkar and Steinman, 2002; Gad et al., 2004). The second signal leads to the concomitant activation of DC and T cell via the expression of co-stimulation molecules (CD80, CD83, CD86 and OX40L). mDCs highly express these co-stimulatory molecules that consolidate the MHC-TCR interaction to induce an effector T cell response. The third signal is when naive T cells are overwhelmed by a DC-induced cytokine storm that also contributes to T cell activation. Depending on the nature of the antigen, DCs will either secrete Th1,

Th2 or Th17 cytokine combinations. In the case of a Th1 response, mDCs largely secrete IL-12 and IFNγ. To orientate the immune response towards a Th2 response, DCs will notably secrete IL-4 and IL-2. The secretion of IL-6, IL-21, IL-23 and TGF-β mainly characterizes the Th17 immune response. Furthermore, DC can also induce regulatory T cell by the concomitant secretion of TGF-β and IL-10 (Figure 20). All three signals participate in the activation of both the DC and the T cell, which will subsequently lead to a potent and appropriate immune response.

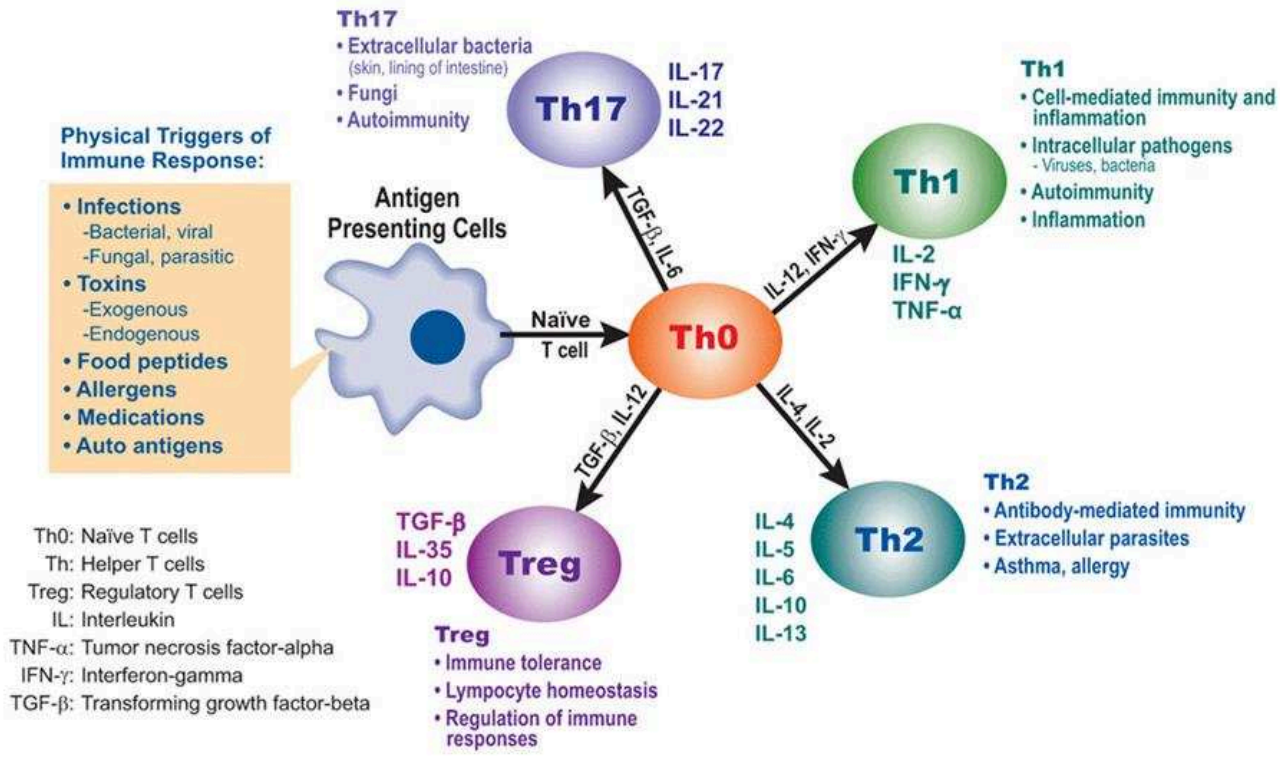


Figure 20: Dendritic cell cytokine secretion upon T cell priming. DC will interact with a naive T cell (Th0) and induce an appropriate immune response depending on the nature of the antigen. This will determine the cytokine profile expressed by the APC in order to steer the T cell immune response (http://varuncnmicro.blogspot.com).

2) Tolerogenic DCs

Tolerogenic DCs (tDCs) are regulatory DCs that promote immune tolerance (Steinman et al., 2003). In the same way that mDCs promote an immune response, tDCs trigger the induction and enhance the functions of Tregs whilst inhibiting effector T cells. Their physiological role is to maintain immune homeostasis and without them, autoimmune disorders soon arise.

a. Characteristics

The maturation state of tDCs is considered intermediate and thus, tDCs are also referred to as semi-mature DCs. Indeed, phenotypically, they are neither immature nor mature. A clear example of this is perceived by the partial expression of co-stimulatory molecules at their surface (CD40, CD80, CD83 and CD86). However, the line between iDCs and tDCs is often blurred so it is important to properly distinguish them. Although iDCs do not induce a T cell response, tDCs actively promote tolerance. Indeed, iDCs do not interact with T cell whereas tDCs induce effector T cell anergy and induce regulatory T cells. For this, tDCs upregulate the expression of inhibitory molecules (Fas-L, PD-L1 and PD-L2) and secrete high levels of immunosuppressive cytokines (IL-10 and TGF-β) whilst decreasing the secretion of effector cytokines (IL-6, IL-12 and TNFα). Moreover, the enzyme IDO is often found highly expressed in tDCs as it favours an immunosuppressive microenvironment in which

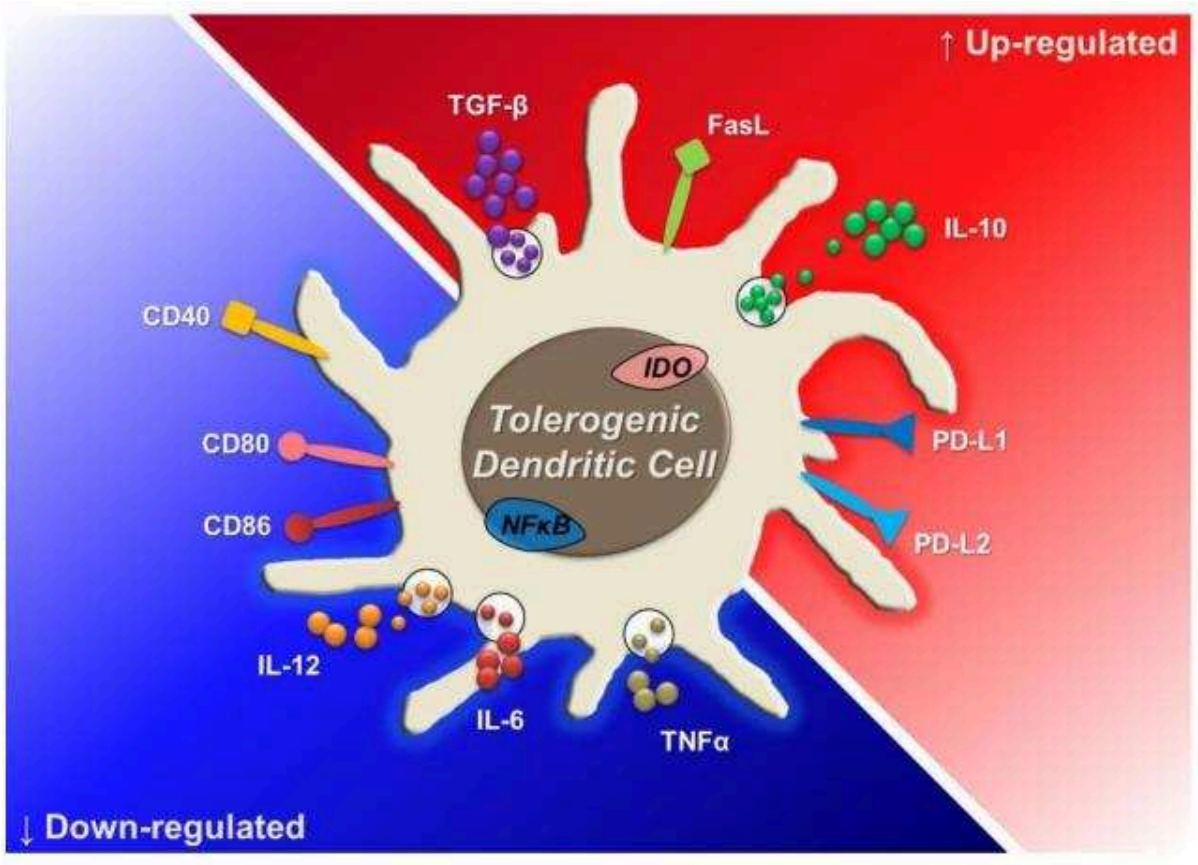


Figure 21: Characteristic profile of tolerogenic DCs. Co-stimulation markers are typically all or partly downregulated (CD40, CD80 and CD86), the secretion of effector cytokines IL-12, IL-6 and TNFα is decreased. The expression of the transcription factor NF-KB is also commonly found compromised in tDCs. An upregulation of inhibitory molecules at the cell surface such as PD-L1, PD-L2 and Fas-L has also been described in tDCs. The secretion of immunosuppressive cytokines IL-10 and TGF-β is generally increased which leads to a suppressive microenvironment. And finally, high expression of the immunoregulatory enzyme IDO is sometimes described in tDCs (Yoo et al., 2016).

Tregs thrive and effector T cells can no longer proliferate and eventually die. All of these characteristics

show how well tDCs are equipped to inhibit the effective immune response to promote tolerance (Figure 21).

b. In vitro tDC generation

The study of tDCs has led to the development of many protocols of generating tDCs (Kim et al., 2018b; Yoo and Ha, 2016). The use of dexamethasone, a commonly used steroid drug, is now a classical method of generation *in vitro* IL-10 secreting tDCs (Bosma et al., 2008). Moreover, organic molecules such as vitamin D3 can be used to generate tDCs (Farias et al., 2013). Yet, the DCs induced in both cases were phenotypically semi-mature but functionally not very effective mediators of tolerance. Nevertheless, it was found that the most effective method of tDC generation *in vitro* is by coupling both compounds (Nikolic and Roep, 2013). In this case, induced tDCs show a stable resistance to maturation with a low expression of co-stimulatory molecules, no or very little production of IL-12 and a high secretion of IL-10. However, they can more importantly induce effector T cell apoptosis and give rise to antigen specific Tregs. Other techniques include DC exposure to cytokines such as IL-10, CCL18, IFNγ etc. (Azzaoui et al., 2011; De Smedt et al., 1997; Eljaafari et al., 2009; Haase et al., 2002). What is now complicated is that all these different protocols seem to generate different tDCs that do have a common immunosuppressive function but show phenotypical differences. Indeed, a general tDC phenotype still needs to be clearly defined, as this would considerably aid in the study and understanding of tDC biology. Although this seems to be an increasingly utopic goal as tDC phenotype depends on origin and generation conditions (Navarro-Barriuso et al., 2018).

c. T cell immune suppression

tDCs are commonly thought of as DCs that have a blocked maturation. The direct consequence of this is that as the cell has not fully matured, and thus cannot induce an effective immune response. tDCs not only fail to induce an immune response, they have immunosuppressive properties that actively favour immune tolerance (Hattori et al., 2016).

Effector T cell impairing

As previously mentioned, tDCs upregulate their expression of inhibitory molecules PD-L1 and PD-L2. They bind to PD1 on the T cell which leads to its anergy or inactivation (Brown et al., 2003; Freeman et al., 2000; Latchman et al., 2001; Martin-Orozco et al., 2006). Moreover, Fas-L, which is also highly expressed on tDCs is a known inducer of effector T cell death (King and Ashwell, 1993; Kurts et al., 1998; Süss and Shortman, 1996). Moreover, the cytokine secretion profile of tDCs also contribute to effector T cell blockade. Indeed, the lower levels of IL-12 disrupt T cell function and limits the IL-12-

dependant stimulation of TNFα and IFNγ that are key for NK activation (Brunda, 1994). Furthermore, an increased secretion of immunosuppressive cytokines such as IL-10 and TGF-β leads to a blockade of effector cytokine secretion (IL-2, IL-12, TNFα and IFNγ) as well as the impairing of effector T cell function (Spits and de Waal Malefyt, 1992; Taga and Tosato, 1992; de Waal Malefyt et al., 1991). Another major contributor to T cell response blockade is the enzyme IDO1. Indeed, in most tDCs it is highly expressed. This enzyme metabolises the essential amino acid Tryptophan (Trp) into kynurenine (Kyn). The lack of Trp leads to effector T cell anergy and kyn is itself toxic for the cells and leads to their death (Harden and Egilmez, 2012; Hwu et al., 2000; Munn et al., 2002; Terness et al., 2002). Moreover, it has been found that Tregs thrive on the effects of IDO (Baban et al., 2009; Xie et al., 2015; Yan et al., 2010).

Inducers of Tregs

tDCs produce factors that are key for immune tolerance and setp up a favourable microenvironment for Tregs (Kushwah and Hu, 2011; Maldonado and von Andrian, 2010). tDCs can directly induce various types of regulatory lymphocytes in mice. For example, tDCs can induce CD4+ CD25hi Forkhead box P3 (FoxP3)+ Tregs from naive and effector T cells (Huang et al., 2010; Turnquist et al., 2007), CD8+ Tregs (Hsu et al., 2014) and even regulatory B cells (Qian et al., 2012). IL-10 is one of the main players for tolerance across the board but notably for Tregs (Akbari et al., 2001; McGuirk et al., 2002; Wakkach et al., 2003). Indeed, IL-10 contributes to Treg differentiation and proliferation (Sabat et al., 2010), but is also essential for the maintenance of Tregs' suppressive functions (Murai et al., 2009). Indeed, it was demonstrated that IL-10 released from tDCs maintain FoxP3 expression in Tregs, in a paracrine manner (Murai et al., 2009). Additionally, a study showed that IL-10 signalling supersedes co-stimulatory signalling via Src Homology region 2 domain-containing Phosphatase-1 (SHP-1) that dephosphorylates the cytoplasmic tails of CD28, Inducible T-cell COStimulator (ICOS) and CD2, subsequently disrupting their costimulatory activity (Akdis et al., 2000; Taylor et al., 2007, 2009). Furthermore, tDCs can also secrete the pleiotropic cytokine TGF-β. Even though it is involved in both inflammation and tolerance, it is an important cytokine in Treg biology. For example, TGF-β is capable of inducing the expression of FoxP3 (Chen et al., 2003) by activating upstream transcription factors Forkhead box 03a (Foxo3a) and Foxo1 (Harada et al., 2010), which leads to the differentiation of FoxP3+ Tregs (Chen and Konkel, 2010; Rubtsov and Rudensky, 2007). A study by Yamazaki et al., found that a subtype of mice DCs were able to induce Tregs in a TGF-β and retinoic acid (RA)-dependant manner (Yamazaki et al., 2008). In fact, RA inhibits maturation of DCs and induces tDCs (Jin et al., 2010). But tDCs also use RA as it can bind to FoxP3, enhance TGF-β signalling to favour Treg differentiation, inhibit the secretion and function of effector cytokines IL-6 and IL-23 (Xiao et al., 2008). Physiologically,

RA is used by tDCs to induce Tregs and maintain immune homeostasis in tissues (Manicassamy and Pulendran, 2009).

As previously mentioned, tDCs can express inhibitory molecules PD-L1 and PD-L2 that control effector T cell activation via PD-1. However, this pathway can also induce Tregs and participate in the maintenance of Treg suppressive function (Francisco et al., 2009; Riley, 2009; Wang et al., 2008).

Moreover, the tDC enzyme IDO inhibits effector T cells by altering the composition of the microenvironment around them. But what is deleterious for effector T cells is beneficial for Tregs. Indeed, Tregs thrive on the effects of IDO, Trp depletion and increased levels of Kyn enhances Tregs' suppressive function (Yan et al., 2010). IDO activity promotes the differentiation of Tregs notably via the Kyn its produces and an IFNγ auto-amplification loop, allowing the regulation of IDO expression (Belladonna et al., 2009; Katz et al., 2008; Mellor et al., 2017; Munn, 2011). Interestingly, tDCs and Tregs engage in cross talk, IDO expression can be induced by Tregs via factors such as IL-10, TGF-β and IFNγ (Fallarino et al., 2003; Janikashvili et al., 2011), this is known as "infectious tolerance". For instance, a study by McGuirk showed that conditioning DCs with Tregs led to functional tDCs that were able to induce Tregs in an IL-10-dependant manner (McGuirk et al., 2002).

C. tDCs and cancer

In many cancers, the patients' immune system has been subverted in order to profit the tumour. In the case of NPC, the tumour seeks to either impair the antitumour response by eliminating effector actors or favour immune tolerance. Thus, just as Tregs, tDCs are gaining interest in the field of anticancer immunotherapy. It is noteworthy to mention that tDCs are also being considered as novel cell treatments in autoimmune diseases and organ transplantation notably to prevent graft-versus-host disease (GVHD) (Marín et al., 2018; Moreau et al., 2012; Zhang et al., 2018a). In immunosuppressive cancers, tDCs are also targets but in that context are sought to be inhibited. Figure 22 gives an overall view of the role DCs play in cancer.

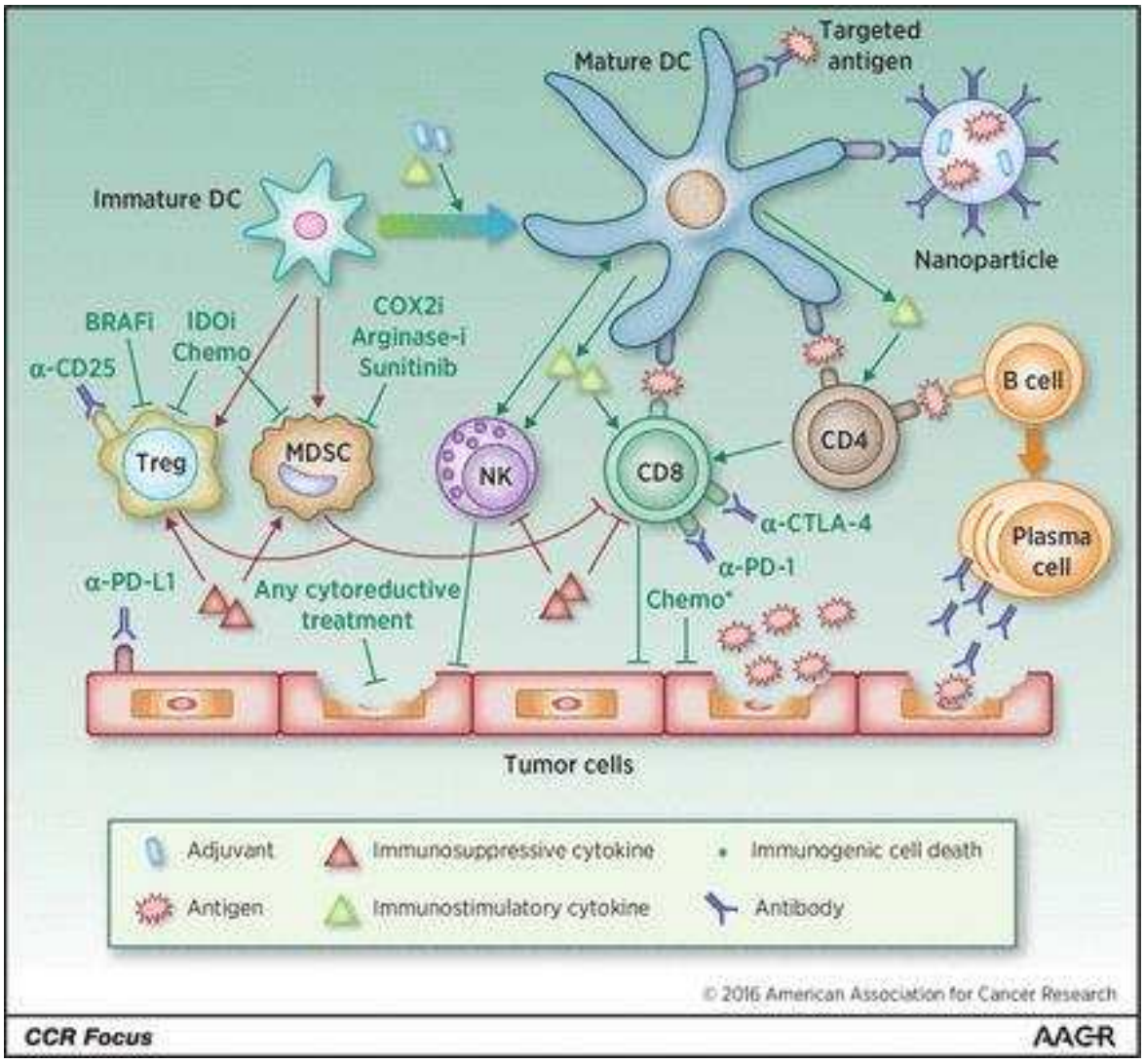


Figure 22: Cross talk between DCs, immune cells and tumour cells and possible targets of anti-cancer immunotherapies. Green arrows indicate immunostimulatory interactions while red arrows indicate suppression mechanisms. The green and blue arrow shows DC maturation, during which inflammatory cytokines are released and adjuvant therapy could enhance DC's anti-tumour functions. mDCs (right) present tumour antigens to B cells, CD8 T cells and CD4 T cells during anti-tumour response, with each of these adaptive immune cells actively fighting against cancer. Endogenous and exogenous tumour antigens can be brought to DCs via nanoparticles. DCs can also activate NK immune response. All of these interactions lead to the secretion of effector cytokines, contributing to anti-tumour immune response. iDCs induce MDSCs and Tregs which contribute to tumour progression via the release of suppressive cytokines. A number of therapies are aimed at these cells, including IDO inhibitors, CD25 blocking antibodies, BRAF inhibitors and COX2 inhibitors. The tumour cells express regulatory molecules such as PD-L1 and secrete immunosuppressive cytokines to induce suppressive cells and inhibit the function of effector cells (Kalijn et al., 2016).

1) Tumour induction of tDCs

DC maturation can be altered by tumour cells which have a double hit effect as this prevents the anti-tumour response but also induces immune tolerance (Vicari et al., 2002). Conferring tolerogenic properties to DCs enables the tumour to intensify local immune suppression in order to escape the

immune response (Gabrilovich, 2004). Multiple mechanisms are developed by the tumour to induce tDCs such as IL-10 and TGF-β secretion (Yang and Carbone, 2004), VEGF is secreted by tumour cells and stromal cells (Mo et al., 2018; Yang et al., 2018) and hyaluronan fragment secretion (Janco et al., 2015; Kuang et al., 2008).

2) Clinical use of DCs in cancer

DCs are now known to be the main instigators of the antitumour response by presenting tumoural antigens to adaptive immune cells. Administering soluble antigens alone has no effect on tumour growth whereas antigen-pulsed DCs are able to induce an antigen specific immune response (Dhodapkar et al., 1999). Since then, the use of DCs is now a promising new lead for anticancer immunotherapy.

a. Use of DCs as vaccines

The use of DCs in cancer is most commonly based on DCs that are pulsed with tumour antigens that act as a vaccine. Thus, as other vaccines, DCs will educate the patients' immune system and give them the tools necessary to better fight back against cancer. Indeed, DC-based vaccination has shown that it prevents metastasis and recurrence of cancer (Jung et al., 2018). However, although it does induce a systemic response, DC vaccinations shows no major effect on solid tumours as it secretes large amounts of TGF-β which impairs most crucial DC functions such as maturation, antigen presentation and migration (Kobie et al., 2003; Lim et al., 2007a; Lyakh et al., 2005). The use of DCs in clinical trials has also been coupled with effector cytokines that are intended to enhance DC response. Clinical studies involving systemic administration of IL-2 gave disappointing results, although preclinical work showed great promise (Shimizu et al., 1999). A Phase 1 study in advanced melanoma showed that patients tolerated well the treatment with no major adverse events though only partially efficient (Escobar et al., 2005). Another phase 2b study, also in advanced melanoma, showed that autologous tumour pulsed-DCs coupled with IL-2 failed to induce a clinical response in patients (Redman et al., 2008).

The most common protocol for *ex vivo* DC vaccination is based on MoDCs (O'Neill and Bhardwaj, 2005). This is mainly due to bio-availability of monocytes in peripheral blood of patients. However, other protocols have also been developed using CD34+ precursors from bone marrow. A study claimed that this approach stimulates a greater T cell response than MoDCs vaccination (Ratzinger et al., 2004). Nonetheless, this points remains controversial and needs further assessment (Romano et al., 2011).

The treatment of hormonal-refractory prostate cancer by Provenge (Sipuleucel-T), was the first Food and Drug Administration (FDA) approved cell-based therapy protocol (Kantoff et al., 2010). It is a vaccine made up of DCs, B cells, monocytes and NK cells that are cultured *ex vivo* with a recombinant fusion protein containing prostatic acid phosphatase (PAP) and GM-CSF. The *ex vivo* DCs are activated and after administering the vaccinal cocktail patients do indeed develop a PAP-specific immune response (Small et al., 2000). The phase 3 IMPACT trial displayed an overall improved survival of 4.1 months, an antigen-specific T-cell activation and the increase of T cell infiltration of the TME (Fong et al., 2014; Sheikh et al., 2013). Naturally, Provenge is now currently being tested in combination with other effective immunotherapies such as checkpoint inhibitors α-CTLA4 (NCT01804465) and α-PD-L1 (NCT03024216) to determine whether these associations would further enhance their effectiveness.

b. Photo Dynamic Therapy and DC-base immunotherapy

Photodynamic therapy (PDT) is a novel, well-established cancer treatment based on the use of a tumour-specific photosensitising drug and subsequent illumination that will activate the drug and trigger tumour cell destruction. Studies have shown that after PDT treatment, tumour cells express high levels of heat shock proteins (HSP) (Korbelik et al., 2005; Song et al., 2013). This is interesting because HSPs are molecular chaperons that interact with APCs during the transfer of antigenic peptides to CD8 T cells but also activate DC maturation via TLR signalling (Berwin et al., 2003; Srivastava, 2002). In fact, a study by Jun showed that loading DCs with lysate of PDT-treated tumour cells led to a greater inhibition of tumour growth (Jung et al., 2012). As PDT induces tumour cell death, a number of molecules released could be possible targets for future DC-base vaccines that would complement PDT treatment of cancer.

c. Targeting of DCs in cancer

Clinical use of tDCs stretches far beyond cancer, autoimmune diseases such as rheumatoid Arthritis, multiple sclerosis (Harry et al., 2010; Healy et al., 2008; Jaen et al., 2009; Ning et al., 2015), primary Sjögren's syndrome (Volchenkov et al., 2013), diabetes (Adorini, 2003) to name a few. All these diseases could benefit from tDC adoptive transfer and clinical trials are currently underway. In transplantation, adoptive tDC transfer clinical trials have proven to be safe, efficiently ease the acceptance of the transplanted organs and limit GVHD (Marín et al., 2018). Much of the knowledge surrounding tDCs in autoimmune diseases and transplantation can be transposed to cancer. Indeed, in cancer, tDCs favour immune evasion, which makes them compelling targets for future immunotherapies.

Anti-IDO

As previously mentioned, IDO is an immunosuppressive enzyme secreted almost exclusively by tDCs that favours immune tolerance. The downstream effects of IDO are multiple, but all are in favour of inhibiting effector immune cells and enhancing Treg suppressive function. In this new era of immunotherapy based on checkpoint inhibitors, all eyes were on IDO inhibitors that held great promise. Preclinical studies exhibited encouraging results and phase 1 trials showed IDO inhibitors were well tolerated and induced very little toxicities. However, recent data from the Epacadostat/Keytruda phase 3 clinical trial (Keynote-252/ECHO-301 - NCT02752074) has shown no major benefit of inhibiting IDO in melanoma, although the rational seemed to predict otherwise. Thus, IDO biology needs to be further elucidated to (i) understand why anti-IDO therapies failed and (ii) develop more efficient IDO-based anti-cancer treatments (Muller et al., 2018).

tDC reprogramming

Targeting of tDCs in cancer is based on two approaches: tDCs inhibition or reversing tolerogenic activity of tDCs. This second approach has been explored by the team of Conejo-Garcia for over a decade. Promising *in vivo* results in ovarian cancer orthotopic models show that CD11c and PD-L1 expressing tDCs preferentially engulfed nanoparticles encapsulating siRNA. This complex triggers TLR3, TLR5 and TLR7 activation and subsequent secretion of effector cytokines which seems to reverse the tolerogenic phenotype (Cubillos-Ruiz et al., 2009). Additionally, the silencing activity of RNA cargo also contributes to the reprogramming of tDCs into immunogenic “effector” DCs (Cubillos-Ruiz et al., 2012). This boost leads to a significant increase in OS in mice with an aggressive form of ovarian cancer. Furthermore, when they restored the expression of miR-155, this altered the transcriptional profile of tDC and further increased the OS of treated animals. Other studies of tDC reprogramming have shed light on the new-found plasticity of DCs like Tesone et al that specifically silenced the expression of Special AT-rich sequence-binding protein 1 (Satb 1) (Tesone et al., 2016).

tDC targeting using pathogens

The unlikely use of a parasite like *Toxoplasma gondii* has taken advantage of its preferential tropism for DCs and macrophages. A non-virulent strain of *T. gondii* has shown substantial increased survival in an ovarian cancer mouse model. Indeed, the parasite targets tDCs and allows for an expansion of tumour specific effector T cell, all of which overturns the immunosuppressive TME (Baird et al., 2013). The elegant approach has opened new perspectives of anti-cancer immunotherapy using pathogens.

Clinical trials involving DCs

Currently, there are only a handful of clinical trials that are evaluating the use of tDCs for the treatment of autoimmune and inflammatory diseases, notably rheumatoid arthritis, multiple sclerosis and transplantation (Table VI).

Table VI: Clinical trials involving tDCs in autoimmune diseases

Reference	Phase	Condition or disease	Nature of the treatment	Status
NCT02622763	Phase 1	Crohn's Disease	Autologous differentiated *in vitro* tolerogenic DCs	Underway
NCT01352858	Phase 1	Rheumatoid arthritis	Autologous differentiated *in vitro* tolerogenic DCs	Underway
NCT02618902	Phase 1	Multiple sclerosis	Myelin-derived peptide-pulsed tDCs	Underway
NCT02283671	Phase 1	Multiple sclerosis Neuromyelitis optica	tDCs loaded with myelin peptides	Underway
NCT02903537	Phase 1	Multiple sclerosis	Autologous monocyte derived tDCs, generated with vitamin D3, loaded with myelin peptides	Underway
NCT03337165	Phase 1	Autoimmune diseases	Autologous monocyte-deived tDCs	Underway
NCT02252055	Phase 1/2	Renal transplant recipients	Autologous tDCs	Underway
NCT01711593	Phase 1	Asthma Allergies	*In vitro* generated tDCs	Completed

With clinical trials involving tDCs in autoimmune diseases still being in the first stages of testing, one can only speculate on their outcome. First data displays a good tolerance and no adverse effects in patients (Flórez-Grau et al., 2018; Phillips et al., 2017; Suwandi et al., 2017). In transplantation, tDCs seem to considerably contribute to the proper acceptance of the transplanted organ (Marín et al., 2018). Further data is still needed to confirm the first line of promising results.

Unlike autoimmune disorders, anti-cancer cell-based therapy uses mature DCs to rebuild a tumour-specific immune response via DC vaccination. The following table summarises the current DC-vaccinations being tested in clinical trials in NPC or head and neck cancer (Table VII).

Table VII: DC-based clinical trials in nasopharyngeal carcinoma or head and neck cancer

Reference	Phase	Cancer type	Nature of the treatment	Status
NCT03282617	Phase 1	Nasopharyngeal carcinoma	Vaccine: DC pulsed with EBV peptides (CD137L-DC-EBV-VAX)	Underway
NCT03047525	Phase 1/2	Nasopharyngeal carcinoma Renal cell carcinoma lung cancer colorectal cancer	Cytokine-induced killer cells (CIK) or DC-CIK	Underway
NCT01821495	Phase 2	Nasopharyngeal carcinoma	DC-CIK treatment	Unknown
NCT00589186	Phase 2	Nasopharyngeal carcinoma	Autologous DCs transfected with EBV peptides (LMP1 and LMP2) + Celecoxib	unknown
NCT00404339	Phase 1	Head and Neck cancer	Vaccine: DCs loaded with wild type p53 ± T helper peptide	Completed

Anticancer immunotherapies based on autologous DC vaccination show little toxicity and have the advantage of being highly personalised. The downside is that their effect is not potent enough to efficiently eradicate the tumour with first clinical trials showing only modest or no improvement of patient's disease. Moreover, cell-based treatments remain costly and lack the off-the-shelf quality most pharmaceutical compagnies find so appealing. The possibility of using healthy donor derived DCs that come from myeloid precursors have been envisaged. The idea is that the mismatch in donor and recipient HLA would potentiate the allogenic immune response and give a stronger overall response (Wells et al., 2007).

Whether DCs are used in the treatments of cancer or autoimmune disorders, it is key to keep in mind that the immune balance of the body is very fragile and tipping this balance back to a state of immune homeostasis has to be carefully monitored. We are still discovering how immunotherapy works and we do not have all the answers but we do know that we are on a promising and exciting new road towards curing cancer.

V. Objectives

We have described in the introduction that NPC is an EBV-associated cancer that escapes the antitumoural immune response. For this, the highly immunosuppressive TME plays an essential role in the development and progression of the tumour. Two major actors govern this TME: tumour-derived nanovsicles names exosomes and Tregs. Indeed, these two key players interact and help sustain the strong immunosuppressive TME. In addition, one well-known mechanism of Treg induction is via tDCs. They are highly immunogenic APCs that convert naive T cells into Tregs and inhibit the proliferation of effector T cell. Thus, tDCs contribute to immune evasion by impairing the immune response.

In this context, the aim of my thesis was to first determine if tumour exosomes block the maturation of DCs to give rise to semi-mature tDCs. For this, we firstly assessed the maturation status and immunosuppressive functions of the cells icultured with NPC exosomes. But the only way to undeniably prove that tumour exosomes induce tDCs is to determine the effect of these cells on T cells. Then, we wondered whether NPC exosomes could attract monocytes or tDCs to the tumour site, in a CCL20-dependant manner, just as they attract Tregs in previous findings of the team.

Material

&

Methods

VI. Materials and methods

A. Mouse model and tumour exosomes generation

Patient-derived EBV-positive xenografted tumours (C15) were permanently propagated by subcutaneous passage in Severe Combined ImmunoDeficient (SCID) mice as previously described (Klibi et al., 2009a). In accordance with institutional guidelines, homozygous CB-17 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 the Insitut Pasteur de Lille (Lille, France).

C15 tumour exosomes were isolated from *in vitro* conditioned culture media (C15exo). Control exosomes were isolated from plasma samples given by healthy donors (HDExo). Conditioned culture media were prepared by collagenase and DNAse dispersion of cells from the C15 xenografts and incubation of these cells for 48h in low clarified serum conditions (allowing collection of C15exo).

B. Exosome isolation

Isolation of exosomes from healthy donors, C15 xenografts or from NPC plasma was done by differential centrifugation and flotation on a D_20/sucrose cushion (1.185 <d <1.20). Plasma and serum sample were initially diluted at a ratio of 1:50 and 1:2 respectively in Phosphate Buffered Saline (PBS) medium (Life Technologies, UK).

All steps of exosome purification are carried out at 4°C. The C15 cell culture supernatant was first centrifuged at 300g for 10 minutes and then at 1,900g for 15 minutes, while the plasma of healthy donors was centrifuged only once at 2,000g for 30 minutes. From this step, all centrifugation steps are identical to recover the exosomes from healthy donors or tumour samples. In order to remove the apoptotic bodies and the high molecular DNA, the supernatant was centrifuged at 12,000g for 35 minutes using a JLA 10.500 rotor (Beckman Coulter, France). The supernatant was then recovered and subjected to ultracentrifugation at 37,500g for 48 minutes using a Ti50.2 rotor (Beckman Coulter, France). The exosomes and microvesicles are contained in the pellet, exosomes and purified by flotation on a sucrose cushion (Tris 20 mM and sucrose) (D_2O, Sigma-Aldrich Chimie, Lyon, France). This gradient of two discontinuous phases was subjected to an ultracentrifugation at 25,000g for 75 minutes on a SW41 Ti rotor (Beckman Coulter, France). Microvesicles form a pellet whereas exosomes are contained in the cushion that is carefully collected without disturbing the pellet. Exosomes were

diluted in sterile PBS and pelleted by ultracentrifugation at 29,800g in a SW41Ti rotor (Beckman Coulter, France) for 90 minutes. Finally, the exosomes were washed twice by two ultracentrifugations of 55 minutes at 55,000g using a Beckman TLA100 rotor (Beckman Coulter, France). After resuspension in PBS, exosomes are then subjected to a protein colorimetric Bradford assay (BIORAD Laboratories, Germany) and stored at -80°C until further use.

C. Exosome protein dosage

Isolated exosomes were diluted 1:100 and total protein concentration was quantified according to the manufacturers' instructions (Biorad, USA) based on a Bradford dye-binding method and using Ascent™ Software. Exosomes were then added to cell culture at 5µg/mL.

D. Exosome characterization using Electron Microscopy

Around 2µg of Exosomes were diluted in PBS and placed on formvar-coated 200 mesh copper grids rinsed once with PBS and then fixed with glutaraldehyde and contrasted with 2% PhosphoTungstic Acid (PTA). Images were obtained with a Hitachi H7500 Transmission Electron Microscope (TEM) equipped with a wide-field 1024x1024-pixel digital camera from AMT Advantage HR (Elexience, France).

E. Isolation of Peripheral Blood Mononuclear Cells

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. PBMCs were isolated from peripheral blood samples by density gradient centrifugation using Ficoll (GE healthcare, Uppsala, Sweden) and leucosep™ according to the manufacturer's instructions (Dutscher, France).

F. Exosome characterization using NanoSight Ltd

Exosomes were characterized using a NanoSight NS300. The number of particles, the concentration as well as the average size of exosomes was determined using 5µg of exosomes diluted in PBS. The NanoSight is powered by NanoSight NS300 software.

G. NPC patient's exosomes

NPC patient's exosomes were collected from plasma samples obtained at the Mustapha-Bacha Hospital (Alger, Algeria) with the help of Dr. Rafa. Some patient samples were collected before treatment and others after, all treated patients received cisplatin-based chemotherapy. All the donors gave informed written consent before the sampling procedure according to the legal provisions (French Huriet Law).

H. Immune cell isolation

Monocytes were isolated from PBMCs using a positive selection CD14+ isolation kit (Miltenyi Biotec, Berlin, Germany) according to the manufacturer's instructions with an average purity of 95%. The CD4+ naive T cells were also isolated by a negative selection using a CD4+ naive T cell isolation kit (Miltenyi Biotec, Berlin, Germany) with an average purity of 90%. After co-culture with DCs, T cells were isolated using a CD3+ selection kit (Miltenyi Biotec, Berlin, Germany) with an average purity of 75%.

I. Dendritic cell generation

$1x10^6$ monocytes were cultivated in complete Roswell Park Memorial Insitute (RPMI) 1640 medium (Life Technologies, UK) with 10% Foetal Calf Serum (FCS) (Life Technologies, UK) that was previously clarified, as well as 2mM L-glutamine and 50µg/mL of gentamycine Life Technologies, UK).

Seeding of monocytes was done in 6-well plates (Nunc, Denmark) containing 10^6monocytes/mL in 4 conditions: monocytes only, with 25ng/mL GM-CSF and 10ng/mL IL-4 (PeproTech Inc, Rocky Hill, USA) for negative and positive controls, and 5µg/mL of exosomes from either healthy donors or C15 cells for testing. On the 5th day of culture, the media was replaced with new medium containing ± IL-1β and TNFα as mautirng agents. An alternate maturating agent also used at times was an *E.Coli* LipoPolySaccharid (LPS) (Sigma Aldrich, St.Louis, USA) at 100ng/mL. As a tDC control, using Vitamin D3 (Life Technologies, UK) and Dexamethasone (Sigma Aldrich, St. Louis, USA) were added to the fresh media at day 5 of culture for 48 hours respectively at 39ng/mL and 393ng/mL. After 7 days of culture the maturation state of the DCs were evaluated. Cells were recovered and assessed by flow cytometry (BD FACS Canto II) for cell surface maturation

markers. Supernatants were recovered at day 5 and 7 of the culture and stored at -80°C until further use.

J. Mixed Leukocyte Reaction (MLR) - DC/T cell co-culture

CD4+ CD45RA naive T cells were obtained from PBMCs exclusively from healthy donors by positive selection isolation kits (Miltenyi Biotec, Berlin, Germany). Heterologous mature DCs were co-cultured with naive T cells at a 1:5 ratio, at 10^6 cells/mL in complete RPMI for 5 days. At day 5 of the co-culture supernatants were firstly recovered and frozen at -80°C until further use. Then, dead cells were eliminated using a Dead Cell Removal kit and afterwards T cells were purified with a positive CD3 selection kit with an average purity of 95%. Both kits were used accordingly to the manufacturer's instructions (Miltenyi Biotec, Berlin, Germany).

The same co-culture procedure was also carried out using the conditioned DCs and total CD4+ T cells. CD4+ cells were isolated using a positive selection kit (Miltenyi Biotec, Berlin, Germany). Cells were co-cultured for 2-3 days with a ratio of 1:20 (DC:CD4+), at 10^6 cells/mL in complete RPMI. DCs were irradiated at 50Gy before the culture. T cell proliferation was then tested using radioactive thymidine and supernatant was stored at -80°C until further use.

K. Mixed Leukocyte Reaction (MLR) - suppression assay

Suppressive activity of T cells after co-culture with DCs was measured by their ability to inhibit the proliferative response of autologuous PBMCs in a MLR test. Assays were set up with a mixture of T cells: PBMCs (1:4; 1:2 and 1:1) in a round bottom 96-well plate (Corning Costar) and cultured for 48h and 72h. Cells were activated with plate-bound anti-CD3 (10ng/mL) mAb (Miltenyi Biotec, Berlin, Germany), incubated at 37°C for 2 hours before the culture and soluble anti-CD28 (10ng/mL) mAb (Clinisciences, Montrouge, France) was added at the time of the culture. There are three types of T cells: $T_{IL1\beta TNF\alpha \text{ or } LPS}$, T_{HDexo} and T_{C15exo}. 50,000 irradiated (5000 cGy) autologous PBMCs were used as APCs. Culture with freshly isolated autologous Tregs, without irradiated PBMCs, activated PBMCs, T cells or activating agents were also done as proliferation controls.

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). Each proliferation assay was carried out in triplicates and

estimated in count per minute (cpm) and results were normalized comparatively to the positive control.

L. PBMC and exosomes co-culture

Exosomes' immunomodulatory properties were tested by a proliferation assay. They were set up with cultures of 100,000 PBMCs in contact with exosomes at 5µg/mL in a round bottom 96-well plate and cultured for 120 hours. The cells were activated with plate-bound anti-CD3 (1µg/mL) mAb, incubated at 37°C for 2 hours before the culture and soluble anti-human CD28 mAb (100ng/mL) as added at the time of the culture. 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). Each proliferation assay was carried out in triplicates and estimated in cpm.

M. Flow cytometry analysis

Cell immunophenotype was analysed by flow cytometry using a BD FACSCanto II flow cytometer powered by FACS DIVA software. After their harvest, cells were washed with PBS (GIBCO-Life technologies) and labeled with fluochrome-conjugated mAbs. For each assay, the appropriate isotypic control mAbs were used for positive signal settings. Finally, median fluorescence intensity (mfi) data were analysed with FlowJo software.

Phenotypical analysis of DCs and T cells

DC phenotype was assessed by flow cytometry (BD FACS Canto II) for cell surface intracellular markers. The state of maturation was determined using monoclonal mouse anti-human CD11c-BioBlue, CD14-VioGreen, CD40-PE, CD80-APC, CD83-PE-Cy7, CD86-FITC, HLADR-PerCP and DC-SIGN-APC-Cy7 (Miltenyi Biotech, Berlin, Germany).

After DC-LT co-culture, the expression of iTreg surface antigens was tested using monoclonal mouse anti-human antibodies CD4-VioBlue, CD18-FITC, CD49b-PE-Cy7, LAG3-PE and CTLA4-APC. Monoclonal mouse anti-human CD4-BioBlue, CD25-FITC, CD127-PE-Cy7 (Miltenyi Biotech, Berlin, Germany) were used for detection of cell surface antigens on Tregs. FoxP3-APC intracellular staining was achieved with FoxP3 staining Buffer kit (Miltenyi Biotech, Berlin, Germany).

N. Western Blot analysis

Different cell subsets and exosomes were lysed (15-20 minutes on ice) in a lysis 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 protease inhibitors (Roche, Basel, Switzerland). After centrifugation (14,000rpm, 30 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.

Briefly, proteins were separated by SDS-PAGE electrophoresis using gradient pre-casts gels (4-12% gradient, Bis-Tris, Invitrogen) in standard conditions except when planning detection of CD63 and CD81 which require non-reducing conditions. Then proteins were transferred on PVDF membranes (Hybon d™-C Extra, Amersham Biosciences, UK). The latter was blocked for 2 hours at room temperature in blocking buffer containing 2% casein, 0.1% Tween20 (Sigma-Aldrich) and PBS (1X), and then incubated overnight at 4°C with primary antibodies directed against: rat anti-human Grp94 mAb 1:500 (ADI-SPA-850-F, Enzo Life Sciences), mouse anti-human HLA-Drα mAb 1:200 (sc-53449, Santa Cruz Biotechnology), mouse anti-human CD63 mAb 1:100 (ab59479, Abcam, UK), mouse anti-human LMP1 mAb 1:4 (S12) (Kerafast), mouse anti-human CD81 mAb 1:500, rabbit anti-IDO 1:1000 (Cell Signaling Technology, USA), mouse anti-cyclophilin B mAB 1:400 (Provided by Dr. Fabrice Alain's team), mouse anti-human Gal-9-CT-L1 mAb 1:200 (was 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 Western Lightning® Plus-ECL, Enhanced Chemiluminescence Substrate kit (PerkinElmer, Boston, MA, USA) and read in luminescent BioRAd ChemiDoc XRS+ machine using the ImageLab software.

O. High Performance Liquid Chromatography (HPLC)

The substrate and product i.e. L-Trp and Kyn of IDO were quantified in the supernatant of all the culture conditions at day 5 and 7. Supernatants were immediately frozen at -80°C until further analysis. Samples were tested by Professor Delphine Allorge's Laboratory of Toxicology at University Hospital of Lille, France. Concentrations of Trp and Kyn were assayed using an analytical procedure based on electrospray ionization liquid chromatography-tandem mass spectrometry (LC-ESI/MS/MS). This procedure was developed according to previously published methods, with slight modifications

(Zhu et al., 2011). One 100µL of culture medium were analyzed after the addition of 100µl acetonitrile containing Trp-D5 at 50,000 nM, as an internal standard. The samples were mixed and centrifuged and the supernatant (100µl) was added to deionized water (500µl). 15µL of this mixture were injected onto an UPLC-MS/MS system (Xevo TQ-S Detector, Waters, Milford, USA) equipped with an Acquity HSS C18 column (Waters, Milford, USA). Ions of each analysed compound were detected in a positive ion mode using multiple reaction monitoring. MassLinks software (Waters) was used for data acquisition and processing.

P. Enzyme-Linked Immuno Assay (ELISA)

In order to dose the cytokine secretion of the DCs and T cells at day 5, 7 and 12 of culture, the supernatants were tested for the secretion of the following cytokines: IL-10, TGF-β, IL-12p70, IL-6, TNFα and IFNγ (BD Pharmingen™, USA) and IDO (Uscn Life Science). The Enzyme-Linked ImmunoSorbent Assay (ELISA) method was carried out according to the manufacturer's recommendations (BD Pharmingem™). Results were expressed as the mean of normalized values for each well of a duplicate.

	Purified antibodies	**Biotinylated antibodies**
Anti-IL-10	IgG1 rat	IgG2a rat
Anti-IL-12p70	IgG1 mouse	IgG1 mouse
Anti-TGFβ	IgG2aRat	IgG2aRat
Anti-TNFα	IgG1 mouse	IgG1 mouse
Anti-IL-6	IgG1Rat	IgG2aRat
Anti-IFNγ	IgG1Mouse	IgG1Mouse

Briefly, purified primary antibodies were fixed in 96-well plates (MaxoSorb, NUNC, Denmark) overnight at 4°C for coating. After 4 washes in PBS 1X (Euromedex, France)-0.05% Tween (Sigma Aldrich, USA), plates were saturated with PBS-Bovine Serum Albumin (BSA) 3%, Sigma-Aldrich®, USA) for 2 hours at room temperature. Then, they were washed 3 times with PBS-Tween 0.05% and culture supernatant were added to the plate and incubated overnight at 4°C.

After 3 washes with PBS-Tween 0.05%, anti-cytokine biotinylated secondary detection Ab (1µg/mL), were incubated for 90 minutes at room temperature. Followed by 3 washes og PBS-Tween 0.05%, the reaction was amplified by adding streptavidin-peroxidase to 1/10000th (Interchim, UK) for 45 minutes at room temperature. After 4 washes, the plates were revealed by the addition of a solution of H_2O_2 (1/1000th) and Ortho-Phenylenediamine Dihydrochloride (OPD) at 1mg/mL (Sigma-Aldrich®, USA) in development buffer. This reaction was stopped by addition of Hydrochloric Acid (HCl) (VWR, USA). The plates were then read at 492nm on the spectrophotometer (Multiskan EX, ThermoLabsystems, France) using Ascent™ Software.

Q. Migration assay

The chemotaxis protocol was performed as previously described (23) using Boyden chambers (Neuroprobe, USA) and 8µm pore polycarbonate filters (Nucleoprobe, USA). The various populations of DCs were harvested and suspended in RPMI 1640 at a concentration of 10^6cells/mL. RPMI only was used as a negative control. Tumour exosomes (5µg/mL) were harvested in the lower chamber either in the presence or absence of a blocking anti-CCL20 mAb (PeproTech, USA) at a concentration of 20µg/mL. For this, tumour exosomes were pre-incubated with the anti-CCL20 for 2h at 37°C in 5% CO_2. The chemotaxis assay was performed in triplicates. The cells having migrated through the pores and into the lower well after 3h of incubations at 37°C in 5% CO_2, were then counted three independent times.

R. Real-Time Quantitative Polymerase Chain Reaction Assays (RTqPCR)

1) mRNA extraction

Total RNA from cultured cells (10^6 cells) was extracted using the TRIzol® reagent (Life Technologies, UK) method according to the manufacturer's instructions. Briefly, 10^6 cells were resuspended in 1mL of Trizol and stored at -80°C until further use. For RNA isolation, 200µL of chloroform were added to samples and gently homogenized for a few minutes, then cells were centrifuged at 12,000g for 15min at 4°C. The upper transparent phase is taken up and total RNA is precipitated with 500µL of isopropanol and stored at 4°C overnight. The next day, RNA are centrifuged for 12,000g, 15min at 4°C and the pellet of total RNA is washed with ethanol 70% which is then discarded and samples are left to dry at room temperature. Then samples are

centrifuged at 7,500g, 15min at 4°C and the pellet is resuspended in 15µL of RNAse free water (Life Technologies, UK).

RNA concentration and purity were measured by spectrophotometric methods using the NanoDrop (Thermo Scientific, USA). Total RNAs were stored at -80°C until further use.

2) mRNA Reverse Transcription

2µg of total RNA were supplemented with 5µL of a master mix: 1µL oligo dT (8nmol) (Roche Diagnostic, Meylan, France), 4µL of RNAse free water and 0.1µL RNAsin (40U/µL, Promega, Charbonnières, France). Then, samples were incubated at 70°C for 10 min, followed by 5 min at room temperature. After this, 10µL of the reaction mix were added to samples: 6µL buffer 5X (Tris_HCL, KCl, MgCl2) (Invitrogen, UK) + 1µL DiThioThreitol (DTT) (0.1M) (Invitrogen, UK) + 2µL deoxyribose Nucleoside TriPhosphates (dNTPs) (10mM) (Amersham Biosciences, UK) + 0.1µL RNAsin (40U/µL) (Promega, USA) + 1µL Transcriptase Reverse Superscript™ (200U/µL) (Life Technologies, UK). Samples are first incubated at 45°C for 60 minutes, and then a second incubation of 5 minutes at 95°C. Finally, ultrapure distilled water (GIBCO-Life Technologies) was added to obtain a final concentration of 10ng total complementary DNA (cDNA)/µL and stored at -80°C until further use.

3) Mx3005PTM Sequence Detection System

Transcripts were quantified using real-time quantitative RT-PCR with the Mx3005P™ sequence detection system (Agilent technologies, France), in optical 96 well reaction plates (Eurogentec S. A., Belgium). In each well, 10µL of a specific couple of primers (Sigma-Aldrich, USA) and 1µL of cDNA sample (equivalent to 10ng total RNA/µL). PCR reactions were performed according to the manufacturer's instructions, in a final volume of 20µL, using 2X MESA GREEN qPCR MasterMix Plus for SYBR® 258 Assay (Eurogentech). The PCR program included initial denaturation for 5 minutes at 95°C, followed by 40 standard amplification cycles as follows: 15 seconds at 95°C (denaturation) then 1 minute at 60°C (annealing and elongation). Fluorescent products were detected at the last step of each cycle.

4) Data Expression

Quantitative PCR reactions were used to quantify gene expression of related DC cell markers, immunosuppressive and pro-inflammatory cytokines. The housekeeping genes: Glyceraldehyde-3-Phosphate DeHydrogenase (GAPDH), Hypoxanthine guanine PhosphoRibosyl Transferase (HPRT) and 18S RNA were used as controls. All primers were designed for real-time PCR (Table VIII) and purchased

from Sigma Aldrich (Sigma Aldrich, St. Louis, USA). Quantitative analysis was achieved based on the cycle threshold (Leading to arbitrary value of 1 for the reference group, results were expressed in $2^{-\Delta\Delta CT}$). The value of each well was calculated using MxPro software (Livak and Schmittgen, 2001).

Table VIII: Genes and corresponding primer sequence (Sense and antisense) used for RTqPCR.

Gene Name	HGNC Symbol	Sense primer	Antisense primer
18S	RNA18S	5'-TCAAGAACGAAAGTCGGAGG	5'-GGACATCTAAGGGCATCACA
CCR1	CCR1	5'-GAAGGTGAACGAGAGGGCCT	5'-CTGGTCATGTTTTTTAGCCTCTTGT
CCR5	CCR5	5'-GTCAAGTCCAATCTATGACATCAATTATT	5'-CGGGCTGCGATTTGCTT
CCR6	CCR6	5'-CCATTCTGGGCAGTGAGTCA	5'-TTTAGCAACTTGCACGTGGC
CD58	CD58	5'-CTGTATCCCAAGCAGCGGT	5'-ATTGGAGTTGGTTCTGTCTGG
CD80	CD80	5'-CCTCAATTTCTTTCAGCTCTTGGT	5'-AGGACAGCGTTGCCACTTCT
CD86	CD86	5'-GGGACTGAGTAACATTCTCTTTGTGA	5'-GGCTTTGGTTTTGAGAGTTTGC
GAPDH	GAPDH	5'-GCCAAGGTCATCCATGACAACTTTGG	5'-GCCTGCTTCACCACCTTCTTGATGTC
GILZ	TSC22D3	5'-CCGAAATGTATCAGACCCCCA	5'-AACGGAAACCACATCCCCTC
HLADR	CD74	5'-TGTTCTGCCTCACTCCCGAGC	5'-GAGCGCTCATCAGCACAGCTA
HPRT	HPRT1	5'-CCCTGGCGTCGTGATTAG	5'-ATGGCCTCCCATCTCCTT
ICOSL	ICOSLG	5'-GCTCTTCAGCAGCCTTCGA	5'-CTCACTGGTTTGCCAATATACGTAA
IDO1	IDO1	5'-GGAGCAGAGACTACAAGAATGGCA	5'-CCTGTGGATTTGGCAGAGCAA
IL-6	IL6	5'-ATGTAGCCGCCCCACACA	5'-CCAGTGCCTCTTTGCTGCTT
IL-10	IL10	5'-GAGAACCAAGACCCAGACATCAA	5'-CCACGGCCTTGCTCTTGTT
IL-12p70	IL12B	5'-CTTTCTAAGATGCGAGGCCAAG	5'-AGAGAGTGTAGCAGCTCCGCAC
ILT3	LILBR4	5'-CCCATGGGACATGAGTAGCC	5'-AGCACTTCTCTGCGATGACG
ILT4	LILRB2	5'-GATGCCCCACTCCGTCTAAG	5'-AGTTGAGTGAGCCGTAGCAC
TGFβ	TGFB1	5'-CGAGCCTGAGGCCGACTAC	5'-CGGAGCTCTGATGTGTTGAAGA
TLR4	TLR4	5'-CCCGACAACCTCCCCTTCT	5'-TGCCCCATCTTCAATTGTCTG
TNFa	TNF	5'-ATCTTCTCGAACCCCGAGTGA	5'-GGAGCTGCCCCTCAGCTT

S. Statistical analysis

Graphpad Prism 7 software was used for data treatment and statistical analysis. Ordinary one-way Analysis of Variance (ANOVA) statistical tests were carried out on all samples. Significance of p values are as following: $p>0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****), with $p<0.05$ being considered statistically significant and smaller p values highly significant.

Results

VII. Results

A. EV characterisation

To verify that isolated EVs are indeed exosomes, we first analysed them by TEM after PTA contrasting (Figure 23A and B). The samples of EVs isolated from either healthy donor plasma or tumour cell supernatant are homogenous in nature and shape. The mean diameter of HDexo and C15exo is 61nm and 80.7nm respectively. Thus, the samples correspond to the expected exosome morphology and size of 30-120nm in diameter.

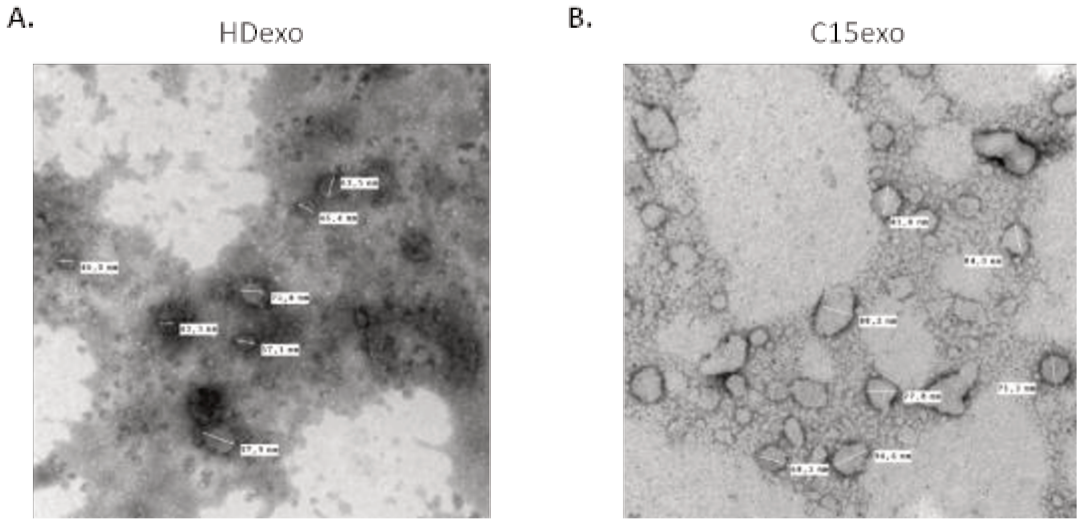


Figure 23: Morphological analysis of nasopharyngeal Carcinoma-derived exosomes (C15exo) and healthy donor exosomes (HDexo). A. TEM analysis of HDexo and B. C15exo suspension in PBS, contrasted with 2% PTA.

We also analysed HD and C15 samples using the Nanosight NS300. This method allows a precise count of EVs and gives the average size of detected particles which helps determine the purety and size of the samples. HD samples show an average size of 111nm with little number of bigger particles (Figure 24A). C15 samples show two peaks at 169nm and 214nm, which is slightly above the classical exosomes size range (Fig. 24B). It must be mentioned that Nanosight measurement of particle size can be subject to variability that depend on multiple factors including operator proficiency, cleanliness of the buffer and/or circuit, instalment of the circuit, duration of measurement, vibrations, etc. Although, Nanosight study has shown slightly larger EV size than with TEM measurements, the particles are still contained in the expected size of exosomes.

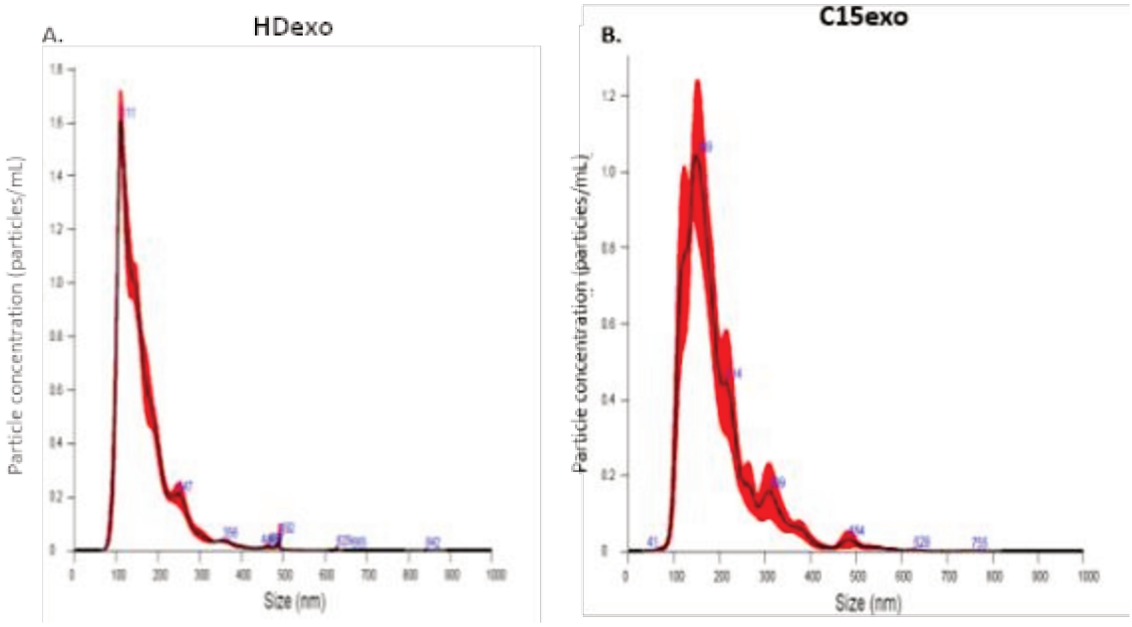


Figure 24: Phenotypical analysis of EV samples. A. HD samples and B. C15 samples were studied using the Nanosight NS300 in PBS buffer. Results are represented in a size (nm) per concentration (particles/mL) curve, showing the distribution of detected particles.

We then sought to verify that EVs express the appropriate exosome markers by western blot testing (Figure 25). Common exosomal markers CD63, HLADR were found on both samples, with HLA-DR found highly expressed on C15 samples. Moreover, the heat shock protein grp94 was absent, as expected for exosomes. We also wanted to validate the expression of NPC exosome marker LMP1, which is indeed present on C15 samples only. Altogether, the TEM, Nanosight and Western Blot analysis led us to confirm that the EVs isolated are indeed exosomes. From now on, we will refer to the isolated EVs as exosomes.

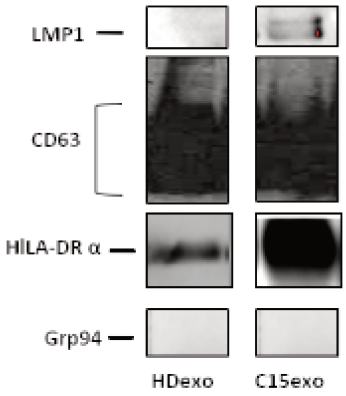


Figure 25: Western Blot study of HD and C15 samples. Western blots were carried out on exosome samples (HDexo and C15exo) to verify the presence of exosome markers (CD63, HLADR), the absence of grp94 and the presence of NPC exosome marker (LMP1).

Furthermore, it has been described that NPC exosomes suppress the proliferation of immune cells (Klibi et al., 2009a). To verify this property, human PBMCs were activated and co-cultured with C15exo for a proliferation assay. As expected, NPC exosomes significantly inhibit the proliferation of activated PBMCs by 2 folds (Figure 26).

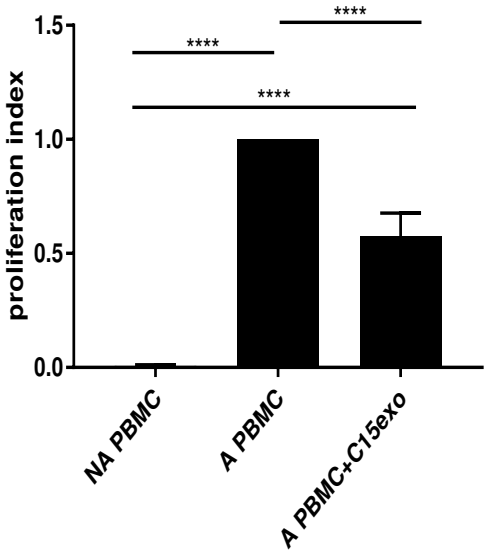


Figure 26: Functional assay of exosomes (C15exo) co-cultured with human PBMCs. The immunosuppressive function of C15exo were tested on activated PBMCs. Non-activated (NA) PBMCs were used as a negative control and activated (A) PBMCs as a positive control. Results were normalised to activated PBMCs and expressed as a proliferation index, n= 4, p<0.0001.

Thus, phenotypical and functional characterisation of isolated exosomes show that they are of the expected size, express the predicted markers and possess the immunosuppressive properties described in literature. A full characterisation is not possible for each lot of isolated exosomes. However, functional testing of exosomes is systematically carried out on at least three donors before the exosomes are used in experiments.

B. Morphological and phenotypical characterisation of MoDCs cultured with C15 exosomes

In order to determine if the tumour exosomes have an impact on MoDC maturation, a phenotypical study is firstly carried out. The cells' morphology was observed by photonic microscopy, the transcriptome was analysed by RTqPCR and expression of maturation markers was studied by flow cytometry.

1) Morphology study of MoDCs

After differentiation with GM-SCF and IL-4 (day 5), monocytes showed a clearly distinct morphology to DCs (Figure 27A), which comforts the idea that monocytes were effectively differentiated into iDCs. However, there was no distinguishable difference between the morphology of control iDCs, HDexoDCs and C15exoDCs (Figure 27B, C and D). All three conditions of DCs were loosely adherent to the plate and showed small dendrites.

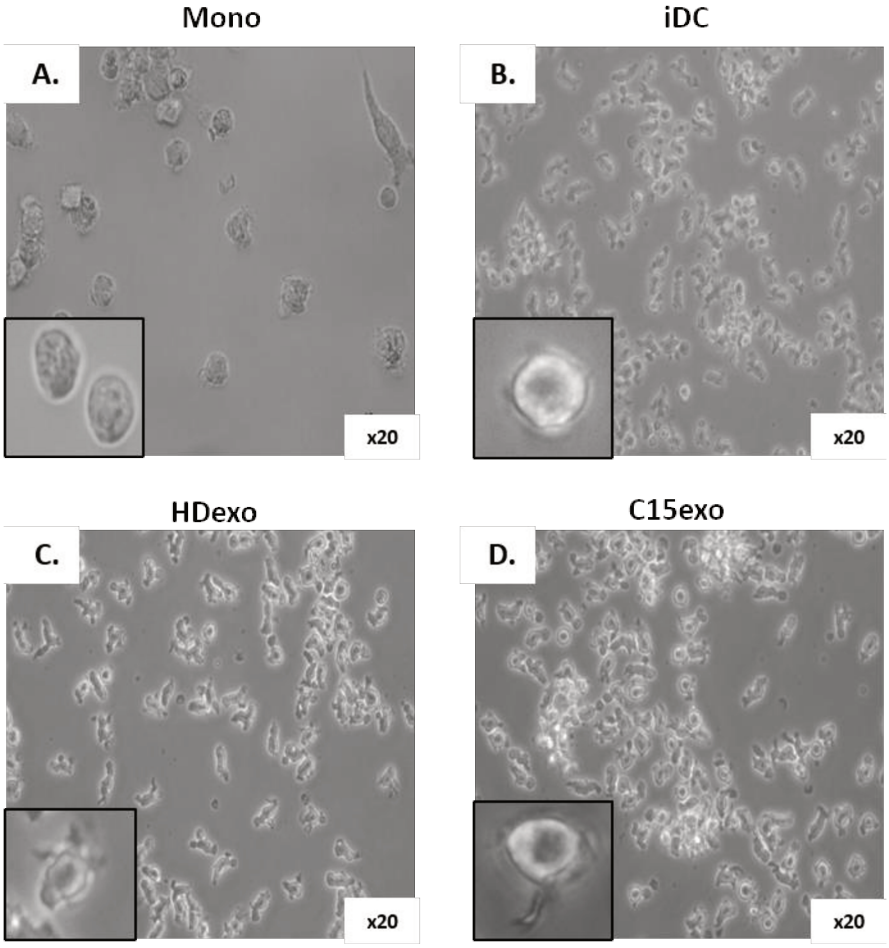


Figure 27: Morphological study of MoDCs after 5 days of culture in the presence or absence of exosomes. DCs were differentiated with cytokines GM-CSF and IL-4. A. Photonic microscopy image of mature monocytes, B. iDCs, C. MoDCs cultured with either HDexo D. or with C15exo. Magnification of each image is x20, a x40 image of a single cell is showed inset.

Nevertheless, after 2-day maturation with maturing agents IL-1β and TNFα (day 7), control mDCs show typical DC characteristics such as adherence to the culture plate, presence of dendrites and growth in clumps (Figure 28A). These aspects are also found in the cells cultured with the HDexo (Figure 28C). The control tDCs generated by adding dexamethasone and vitamin D3 show a drastically different morphology as the cells are either much longer and thinner or rounder with less dendrites (Fig. 28B).

This is also the case for cell pre-cultured with C15exo (Figure 28D). So, from a morphological view point, control tDCs are closer to C15exo DCs and HDexo DCs are more similar to control mDCs.

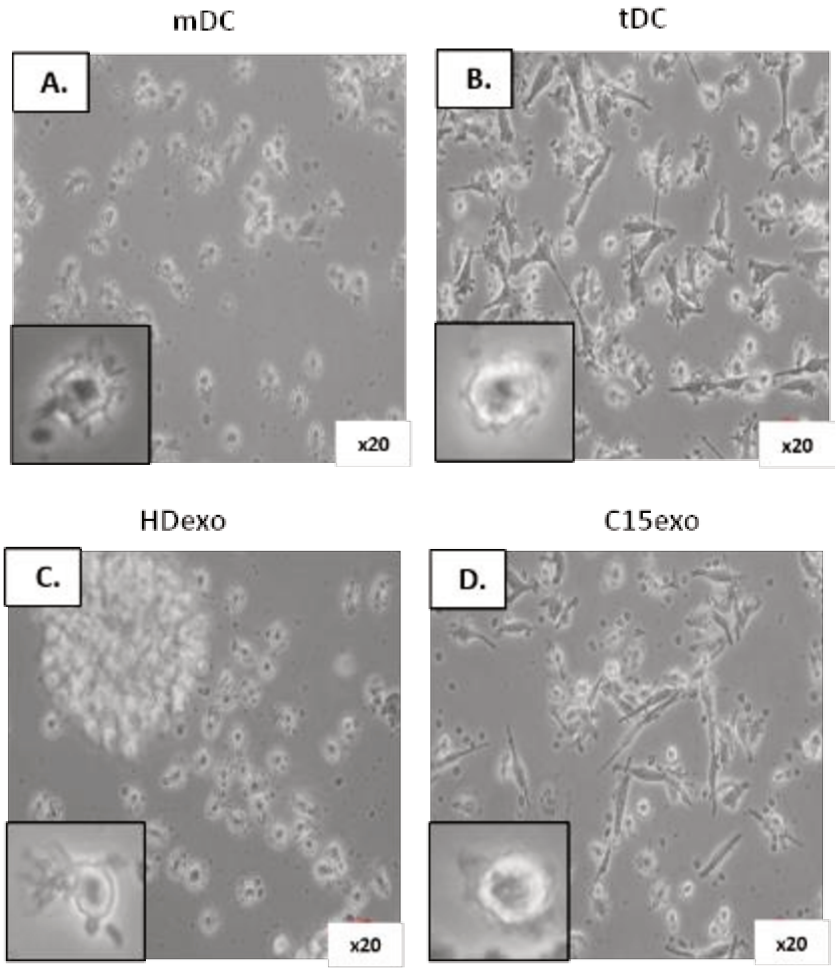


Figure 28: Morphological study of MoDCs after 7 days of culture in the presence or absence of various exosomes. DCs were matured with cytokines TNFα and IL-1β for the last 48 hours of culture. A. Photonic microscopy image of mature DCs B. tolerogenic DCs C. MoDCs cultured with either HDexo D. or with C15exo. Magnification of each image is x20, a x40 image of a single cell is showed inset.

2) Transcriptomic study of MoDCs

We then began the phenotypical study by analysing the transcriptome of MoDCs after maturation by RTqPCR (Figure 29). C15exoDCs seem to overexpress some maturation markers (HLADR, ICOSL and CD58) whereas other maturation markers were less expressed than control mDCs (CD80 and CD86). This is in line with literature, often describing tDCs as semi-mature DCs (Figure 29A). To go further, we looked at iDCs markers such as LPS receptor TLR4 and chemokine receptors (CCR1, CCR5 and CCR6) that contribute to the recruitment of iDCs to the site of inflammation (Figure 29B) (Lukacs-Kornek et

al., 2008). C15exoDCs expressed higher levels of these markers compared to mDCs. In fact, the chemokine receptors are more highly expressed than control tDCs. This indicates that although C15exoDCs express maturation markers they also express iDC markers, thus, verifying the expected semi-mature phenotype. We then looked at tDC markers: Immunglobulin-Like Transcript 3/4 (ILT3/4), GC-Induced Leucine Zipper (GILZ) and the immunosuppressive enzyme IDO1 (Figure 29C). IDO was highly expressed in HDexoDCs and completely downregulated in C15exoDCs.The expression of inhibitory receptors ILT3 and 4 are both highly expressed in C15exoDCs. Moreover, GILZ's expression is also upregulated in C15exoDCs compared to mDCs. GILZ favours immune suppression by inducing the secretion of IL-10 and TGF-β, thus inhibiting DC maturation. In fact, such immunosuppressive cytokines were also studied by RTqPCR and showed an increased level of transcription for IL-10 but TGF-β remained the same as control mDCs (Figure 29D). Finally, we determined the transcription levels of effector cytokines such as IL-12 and TNFα that were increased in C15exoDCs, but IL-6 was less expressed in C15exoDCs (Figure 29E). Thus, this first look at C15exoDC phenotype shows that their maturation seems incomplete with an increased expression of some iDCs and tDCs markers as well as more IL-10 transcription.

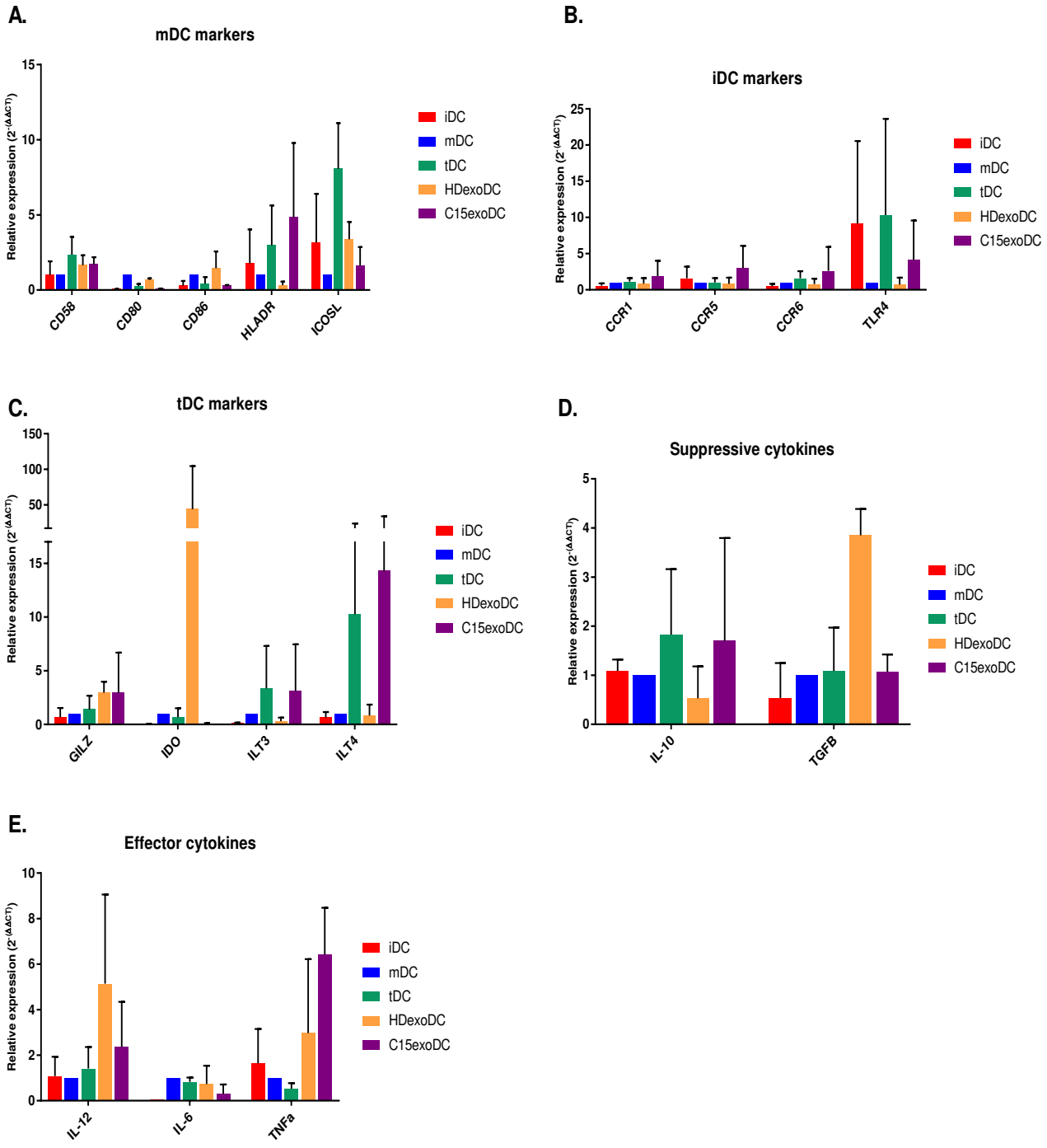


Figure 29: Transcriptomic analysis of MoDC phenotype after maturation. The transcription levels of various DC phenotype genes were studied by RTqPCR: A. mDC markers (CD58, CD80, CD86, HLADR and ICOSL), B. iDC markers (CCR1, CCR5, CCR6 and TLR4), C. tDC markers (GILZ, IDO1, ILT3 and ILT4), D. suppressive cytokines (IL-10 and TGFβ) and E. effector cytokines (IL-12, IL-6 and TNFα). Results were expressed in relative expression $2^{-(\Delta\Delta CT)}$, n=2.

3) Study of the membrane expression of maturation markers in MoDCs

A phenotypical study of DC maturation markers was then carried out by flow cytometry in order to determine the maturation status of the cells. After differentiation, there is no significant difference in the expression of CD86 and HLADR of C15exoDCs, iDCs and tDCs. C15exoDCs show slightly higher mfi than iDCs and tDCs for CD40, CD80, CD83 and DC-SIGN (table IX). Following maturation, the mfi of C15exoDCs are lower for all the markers tested when compared to control mDCs. But compared to control tDCs, their expressions are similar, except for HLADR and DC-SIGN which are higher than tDCs (table X).

Table IX: Cell surface expression of maturation and phenotypic markers of MoDCs after differentiation (Day 5). All median fluorescence intensity (mfi) values of marked samples were normalised with the corresponding isotypic control, n=4

	CD40	CD80	CD83	CD86	HLA-DR	DC-SIGN
Mono	4,3 ± 3,45	6,47 ± 3,22	2,4 ± 1,37	2,36 ± 1,38	21,76 ± 9,64	3,46 ± 2,17
iDC	55,98 ± 36,51	8,37 ± 4,92	2,45 ± 1,23	1,36 ± 0,66	15,11 ± 13,71	57,69 ± 21,69
tDC	53,01 ± 36,08	9,85 ± 1,78	1,82 ± 0,72	1,66 ± 0,3	21,67 ± 8,93	31,47 ± 18,6
HDexo	66,06 ± 37	9,92 ± 2,35	3,02 ± 1,32	1,71 ± 0,49	15,07 ± 6,21	66,28 ± 42,3
C15exo	62,12 ± 45,76	11,56 ± 2,67	3,35 ± 0,73	1,87 ± 0,78	19,3 ± 6,85	65,08 ± 71,03

Table X: Cell surface expression of maturation and phenotypic markers of MoDCs after maturation (Day 7). All median fluorescence intensity (mfi) values of marked samples were normalised with the corresponding isotypic control, n=2

	CD40	CD80	CD83	CD86	HLA-DR	DC-SIGN
iDC	110,06 ± 25,94	18,75 ± 5,64	3,33 ± 3,09	1,66 ± 0,43	19,57 ± 9,19	56,84 ± 6,04
mDC	225,3 ± 16,74	44,77 ± 16,49	11,58 ± 2,43	34,42 ± 27,55	114,55 ± 37,72	62,42 ± 23,71
tDC	92,07 ± 52,74	26,95 ± 3,71	5,58 ± 4,09	11,18 ± 13,51	44,01 ± 40,58	33,91 ± 2,67
HDexo	174,88 ± 10,07	31,08 ± 0,53	11,49 ± 0,6	20,71 ± 2,62	75,17 ± 5,71	51,47 ± 13,57
C15exo	123,44 ± 65,26	23,2 ± 8,14	8,48 ± 3,36	16,33 ± 2,68	73,2 ± 32,12	49,49 ± 48,99

Furthermore, overlays of a representative experiment are shown in Figure 30A. Day 5 overlays display no significant differences between the culture conditions. Nevertheless, after maturation, the

overlays clearly demonstrate that when the cells are pre-cultured with tumour exosomes the expression of some maturation markers are much lower (CD40, CD80, CD83). However, other maturation markers such as CD86 and HLADR show no major difference in intensity of expression when cells are cultured with C15exo. And the iDC marker DC-SIGN, shows that there are two populations, one that expresses DC-SIGN at a similar level to mDCs and HDexo, and the other that expressed DC-SIGN at a very low level (Figure 30B). Thus, the major differences discernible by flow cytometry are more observable after maturation and not before.

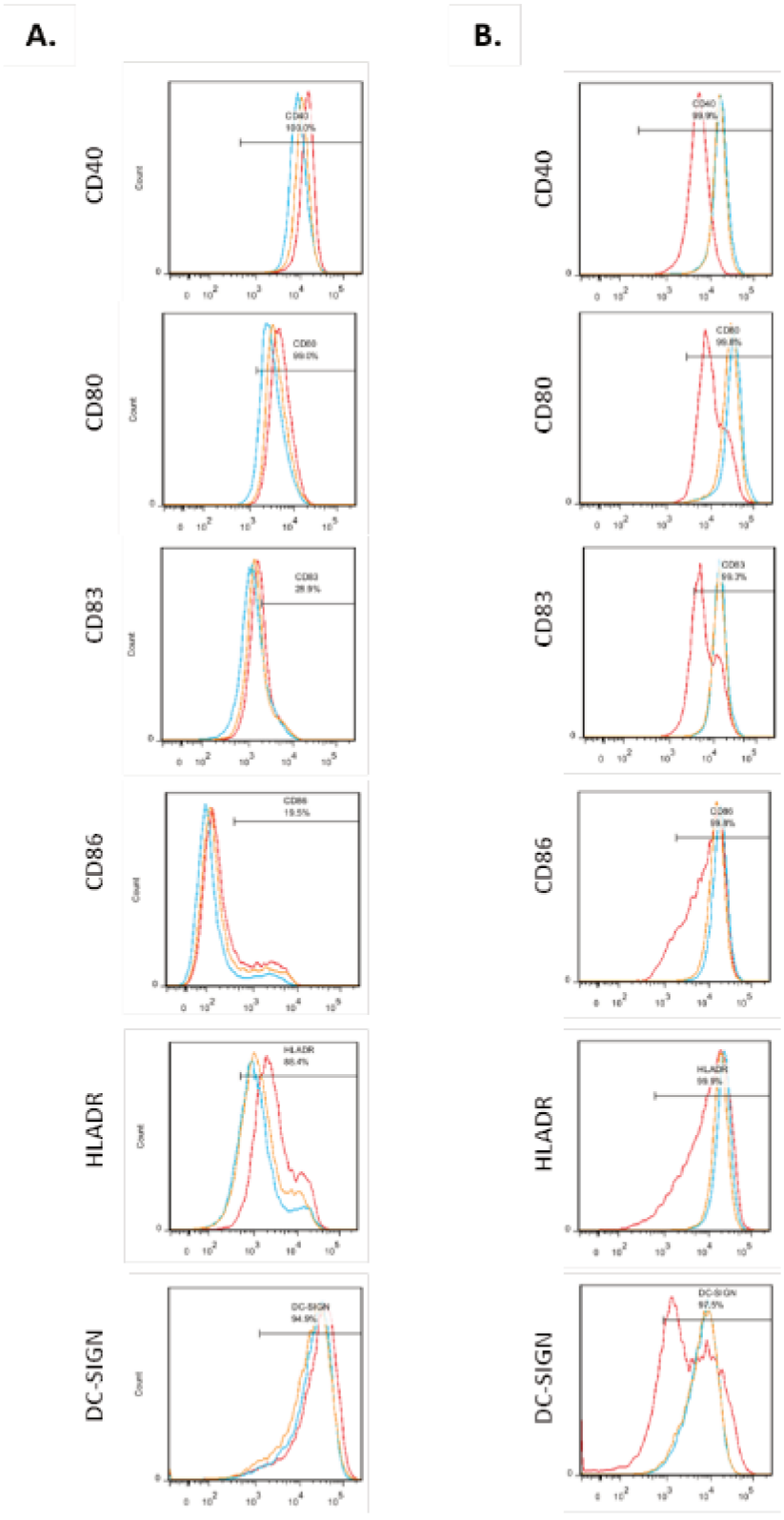


Figure 30: Cell surface expression of maturation markers on MoDCs. The expression of maturation markers was determined A. after differentiation with GM-CSF+IL-4: iDCs (red) ± HDexo (blue) or C15exo (orange) (Day 5) B. The maturation state was determined after a 2-day maturation with TNFα and IL-1β: mDCs (blue) ± HDexo (red) or C15exo (orange) (Day 7). Representative overlays of two independent experiments.

This first phenotypical study suggest that C15exo do indeed disrupt the maturation of MoDCs as shown by the morphological differences, the transcriptome study that translates a semi-mature phenotype and the incomplete membrane expression of maturation markers. Thus, C15exo seemingly inhibit maturation which is a major characteristic of tDCs. Nevertheless, functional studies still need to be carried out to confirm their tDC status.

C. MoDCs cultured with NPC exosomes promote a suppressive micro-environment through immunosuppressive cytokine secretion

The cytokine secretion of DCs was monitored after differentiation and maturation by ELISA dosage. Effector cytokines (IL-6, IL-12p70, TNFα and IFNγ) and regulatory cytokines (IL-10 and TGF-β) were studied. Although not significant, the trend of IL-10 secretion after differentiation shows an increase when the cells are cultured with C15exo compared to mDCs, tDCs and HDexoDCs (Figure 31A). No significant change in the levels of TGF-β secretion was discernible between the different cell conditions (Figure 31B). Moreover, the secretion of IL-6 does seem to be increased by the presence of C15exo, but monocytes display a higher secretion of IL-6 (Figure 31C). IFNγ release does not seem to be changed by C15exo nor HDexo. However, control tDCs appear to slightly decrease their secretion compared to control iDCs (Figure 31D). Thus, C15exo do not seem to significantly alter the cytokine secretion of MoDCs.

These tests need to be reiterated to confirm this last statement.

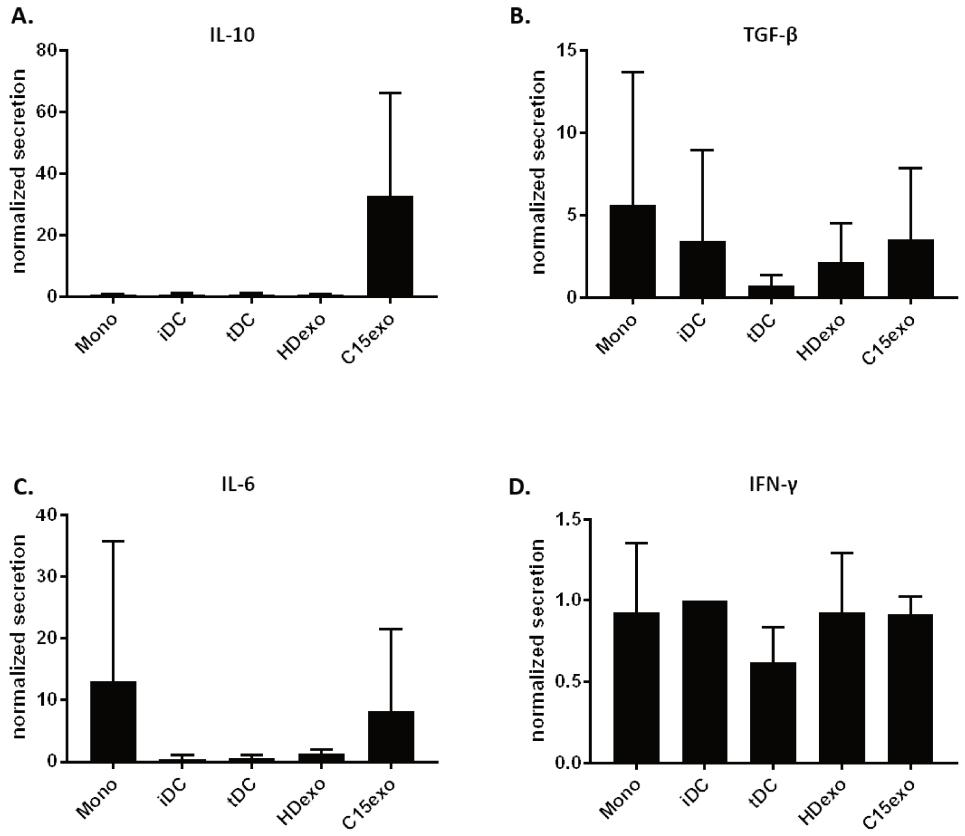


Figure 31: Dosage of cytokine secretion by MoDCs after differentiation. The secretion of various cytokines was dosed by ELISA in the culture supernatant of cells ± pre-treated with HDexo or C15exo, after differentiation. Secretion of suppressive cytokines A. IL-10 and B. TGF-β, as well as effector cytokines C. IL-6 and D. IFNγ were quantified. Results were normalised compared to control iDC condition, n=3 for monocytes and tDC, n=4 for iDC, HDExoDC and C15exoDC.

Furthermore, after maturation, the secretion of IL-10 strongly increases in control tDCs compared to control mDCs. This also seems to be the trend for C15exoDCs although not significant (Figure 32A). However, unlike IL-10, tDCs show the lowest levels of TGF-β secretion, while other DCs pre-treated with exosomes show no difference (Figure 32B). The dosage of the functional sub unit of IL-12, IL-12p70, displays a decrease in all cell conditions compared to mDCs, but most significantly for tDCs and C15exoDCs (Figure 32C). IL-6 secretion is null for iDCs, and comparatively to mDCs, all other conditions seem to secrete less IL-6 (Figure 32D). Significantly, effector cytokine TNFα is less secreted by iDCs and tDCs whereas HDexoDCs and C15exoDCs release more TNFα compared to mDCs (Figure 32E). Finally, IFNγ dosage shows no substantial difference although lower levels for iDCs and C15exoDCs seem to be the trend (Figure 32F). The analysis of the secretome allows to better understand the immune environment that the DCs promote. In this case, C15exoDCs increase the secretion of suppressive cytokine IL-10 and decrease the release of effector cytokines IL-6, IL-12 and IFNγ. This suggests that C15exoDCs promote an immunosuppressive micro-environment.

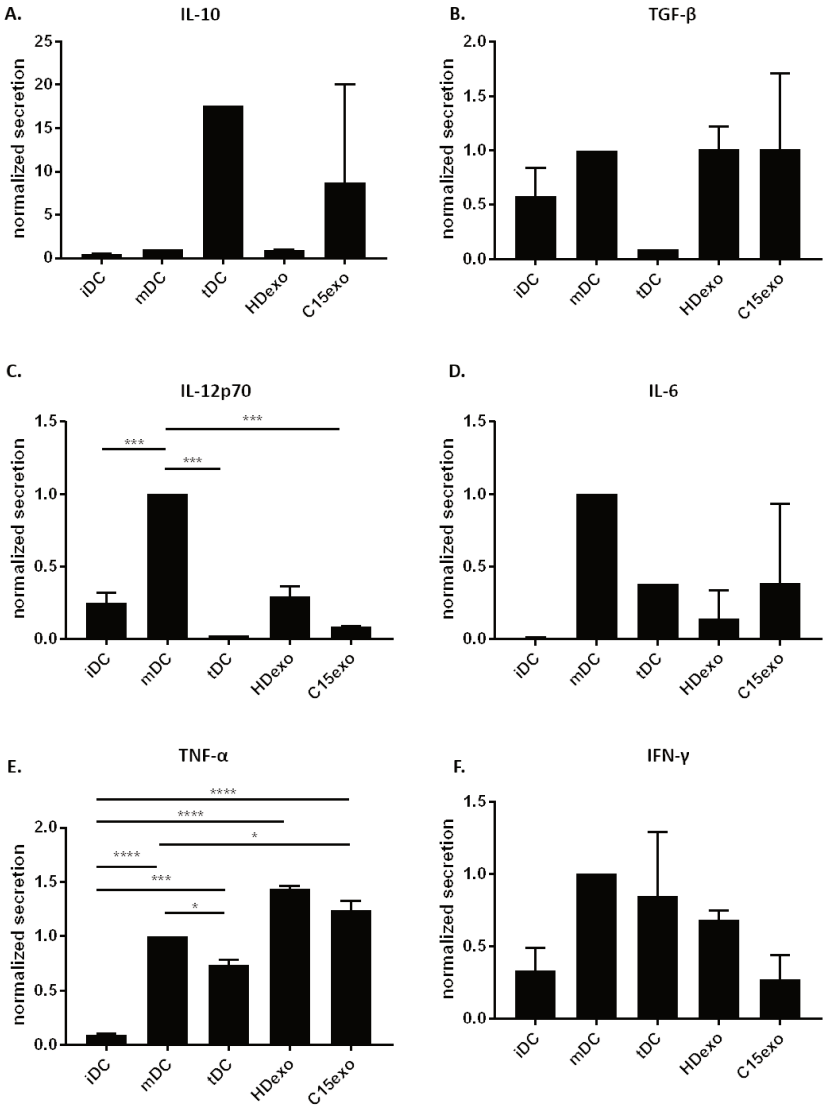


Figure 32: Dosage of cytokine secretion by MoDCs after maturation. The secretion of the following cytokines was dosed by ELISA in the culture supernatant after maturation: A. IL-10 secretion, B. TGF-β secretion, B. IL-12p70 secretion, D. IL-6 secretion, E. TNFα secretion and F. IFNγ secretion. The results are normalized with the control mDC condition. IL-10, TGF-β, IL-6, IL-12p70 n=2 ($p<0.001$), TNFα ($0.05<p<0.0001$), IFNγ and tDCs n=1.

D. NPC exosomes induced semi-mature DCs that modulate IDO expression and function

Another characteristic of tDCs is their expression of the immuno-regulatory enzyme IDO. Thus, we sought to determine if C15exoDCs express IDO and if so, is it biologically active? IDO expression was determined in cultured DCs by Western Blot, the biologic activity of IDO was assessed by HPLC dosage of its substrate and product (Trp and Kyn respectively) and IDO was dosed in the supernatant of DC/naïve T cell co-culture.

After the 5 first days of culture, the highest Kyn/Trp ratio is found in the supernatant of C15exoDCs, whereas all the other conditions exhibit similar levels of IDO activity (Figure 33A). This can be correlated to the Western Blot, where IDO expression levels are higher even than the control tDCs (Figure 33B). Thus, this first result shows that C15exosomes induce the expression of biologically functional IDO that contributes to immune suppression.

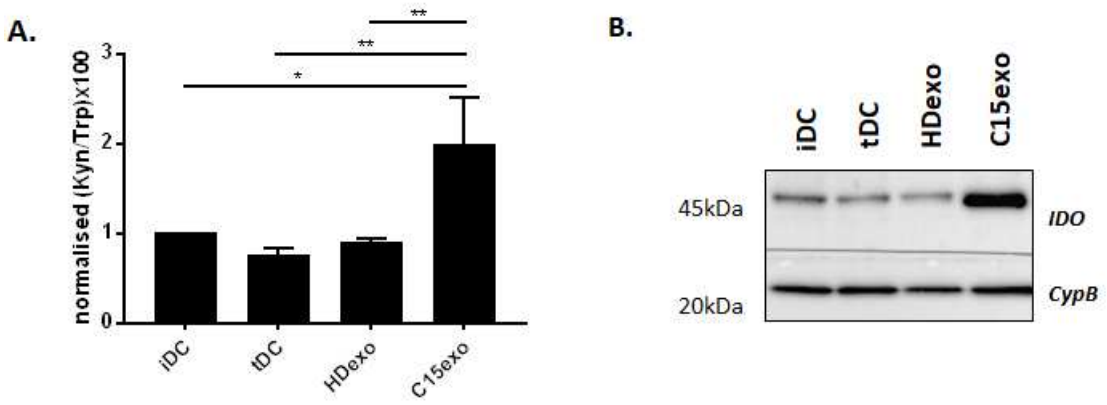


Figure 33: Study of IDO expression on DCs and biological activity after differentiation. A. The study IDO activity by HPLC dosage of Trp metabolites ((Trp/Kyn)x100) was carried out. B. IDO expression was determined by Western blotting, n=1 (0.05<p<0.01).

Furthermore, IDO activity after maturation is significantly lower for iDCs and C15exoDCs compared to control mDCs (Figure 34A). IDO expression shown by Western Blot can corroborate this finding, as there is no detectable signal in the iDC and C15exoDC lanes (Figure 34B). No measurable secretion of IDO was detected by ELISA after differentiation nor maturation of MoDCs (data not shown). Interestingly, these results are in contrast to the data obtained after differentiation. The sudden loss in C15exoDCs and surge in mDCs of IDO expression remains hard to completely explain although tDCs that do not express IDO have already been described in literature.

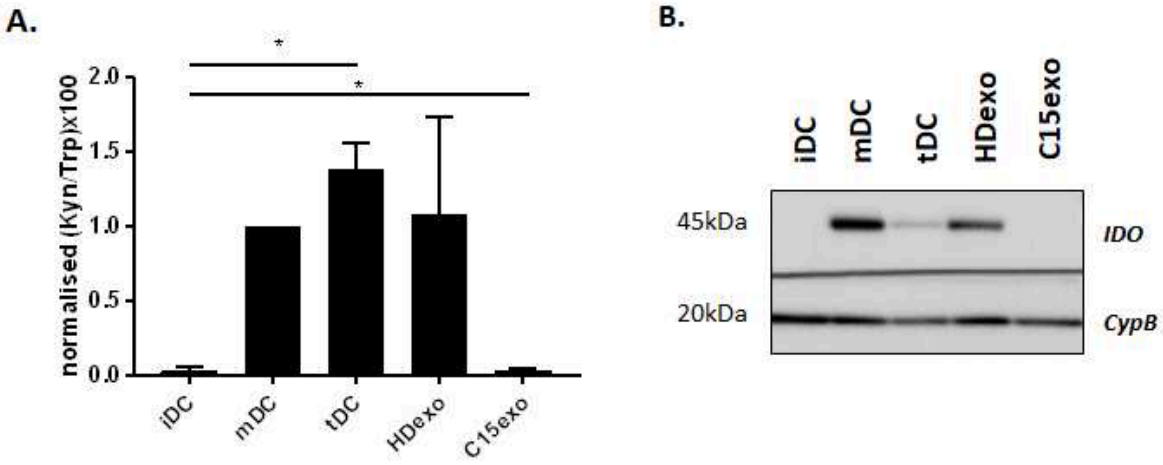


Figure 34: Study of IDO expression on DCs and biological activity after maturation. A. To study IDO activity, HPLC dosage of Trp metabolites ((Trp/Kyn)x100) were carried out. B. IDO expression was determined by Western blotting, n=2 (0.05<p<0.01).

Furthermore, after 5 days of DCs/naïve T cells co-culture, the Trp/Kyn ratio is over 20,000 folds higher for C15exoDC/LT comparatively to mDC/LT and HDexoDC/LT (Figure 35A). IDO secretion levels are three folds higher for C15exoDC/LT than mDC/LT (Figure 35B). In conclusion, these results show an increase in IDO levels and activity after differentiation of monocytes into iDCs. This peak is followed by a crash of IDO activity after maturation but picks up exponentially after DCs are cultured with naive T cells. Thus, these findings comfort the idea that C15exoDCs are indeed tolerogenic as they clearly favour an immunosuppressive micro-environment by secreting high amounts of IDO that starve the media in Trp, fill it with Kyn and leave effector T cells unable to induce and anti-tumoural response.

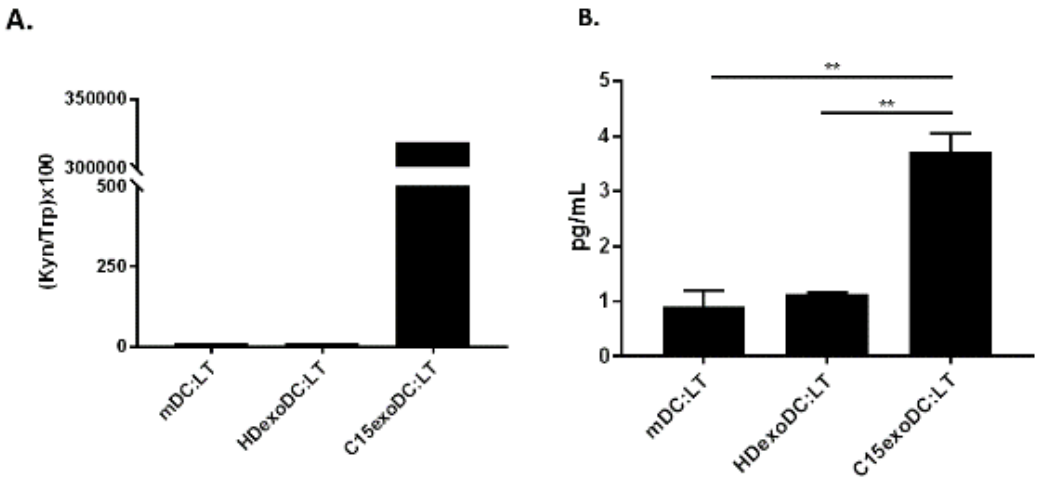


Figure 35: IDO expression on DCs and HPLC dosage of Trp metabolites after 5 days of co-cultivating DCs with naive T cells. A. HPLC dosage of Trp metabolites ((Trp/Kyn)x100) and B. an ELISA dosage of secreted IDO (pg/mL) were carried out, n=1 (0.05<p<0.01).

E. C15exoDCs inhibit the proliferation of effector T cells and favour an immunosuppressive microenvironment

To confirm the tolerogenic status of DCs pre-cultured with C15exo, their suppressive function is tested by a culture of the DCs with total autologous CD3+ T cells. This was achieved by a complex and lengthy experiment; the timeline of the experiment is detailed in the following figure (Figure 36).

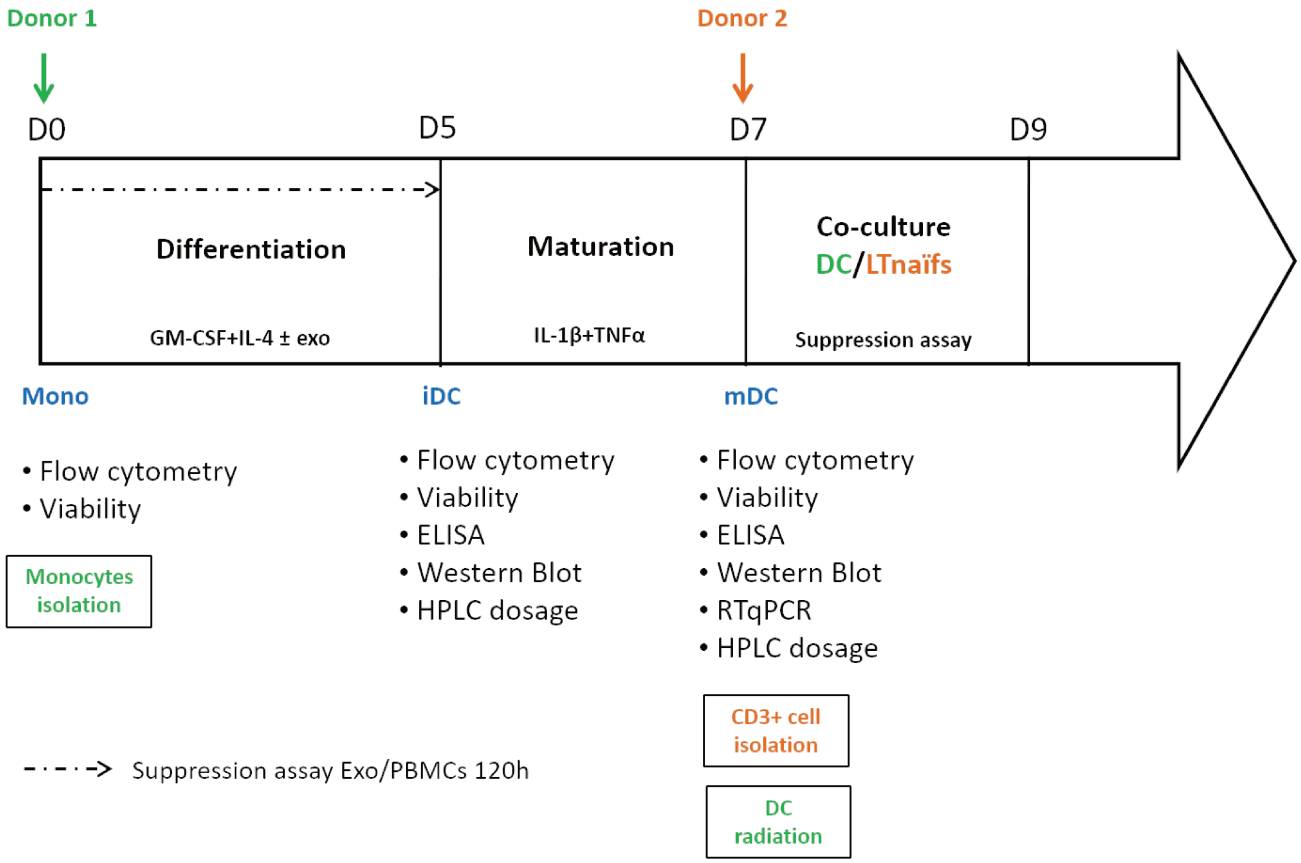


Figure 36: Experimental protocole for the co-culture of MoDCs with total CD3+ T cells. Monocytes from donor 1 are isolated, differentiated then matured into MoDCs (green). Then CD3+ T cells from a second donor are isolated and cultured with MoDCs for a suppression assay (orange). During the first 5 days of the experiment, the exosomes (HDexo and C15exo) used for the experiment are tested with the PBMCs from donor 1 to verify their function (dotted arrow). At each step, parts of the cells are collected to verify DC phenotype (Flow cytometry) and complementary tests are carried out (viability, RTqPCR, Western Blot, HPLC dosage of Trp metabolites and ELISA). DCs are irradiated before culture with CD3+ T cells, the co-culture ratio is 1:20 (DC:CD3+ T cells).

1) DC/CD3+ T cell suppression assay

The suppression assay shows that after 48 hours of co-culture, the C15exoDCs significantly decrease the proliferation of total T cells compared to mDCs. The C15exoDC/LTs' suppression rate is intermediate between the tolerogenic control and mature control. Thus, C15exo induce MoDCs capable of partly anergising T cells, which is a functional characteristic of tDCs.

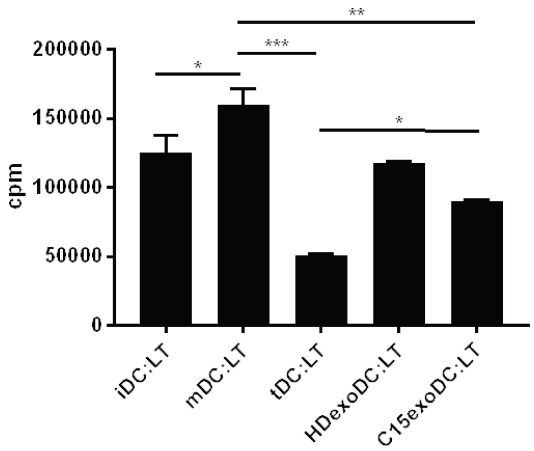


Figure 37: Suppressive function of DCs on CD3+ T cells. A suppression assay of CD3+ T cells co-cultured with irradiated DCs that were pre-cultured ± exosomes (HDexo or C15exo) was carried out after 48h. Results are shown in cpm, n=1, (0.05<p<0.001).

2) ELISA dosage of cytokine secretion after DC/CD3+ T cell co-culture

Thus, previous findings have shown that C15exoDCs can anergise T cells. This is generally mediated by cytokines, so we studied the cytokine secretion in the supernatant of this co-culture. Control tDCs/CD3+ LTs display no difference in IL-10 secretion when compared to mDC/LTs. Although not significant, IL-10 seems to be more secreted when the DCs were pre-treated with C15exo (Figure 38A). Likewise, TGF-β dosage showed similar results, where LTs cultured with C15exoDCs seem to secrete slightly more TGF-β than the controls mDC (Figure 38B). The immunostimulatory cytokines IL-12 was only secreted by C15exoDC/LTs and iDC/LTs (Figure 38C). Thus, the DCs pre-treated with C15 tumour exosomes seem to enhance the overall secretion of IL-10, TGF-β and IL-12 comparatively to mDCs which translates an overall immunosuppressive micro-environment.

In conclusion, our findings seem to suggest that C15exoDCs are able to block effector T cell proliferation and disrupt the cytokines balance by favouring an immunosuppressive micro-environment.

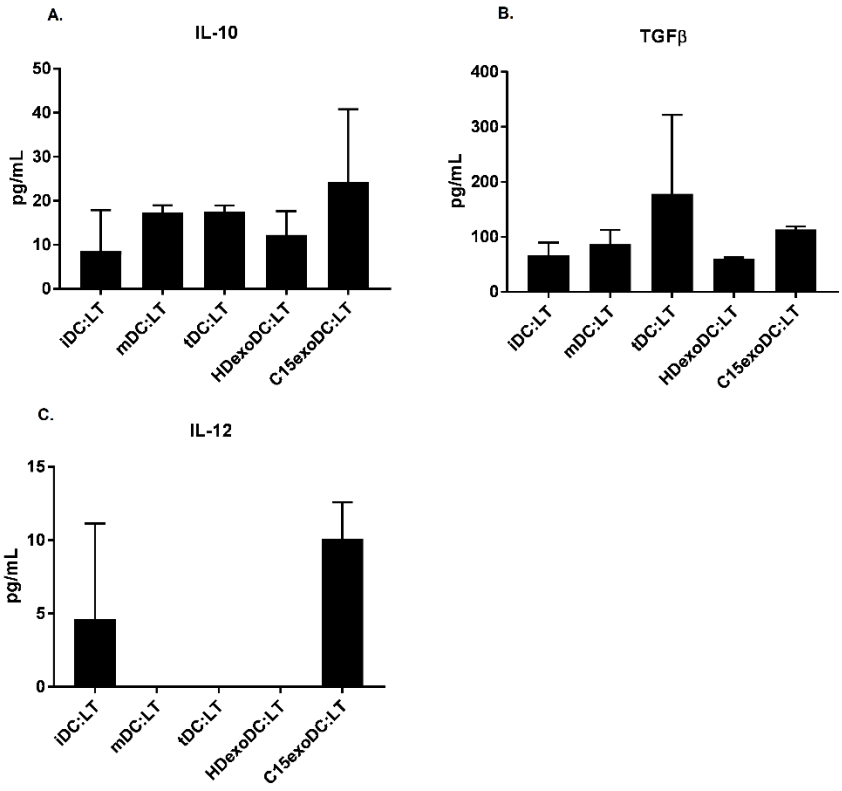
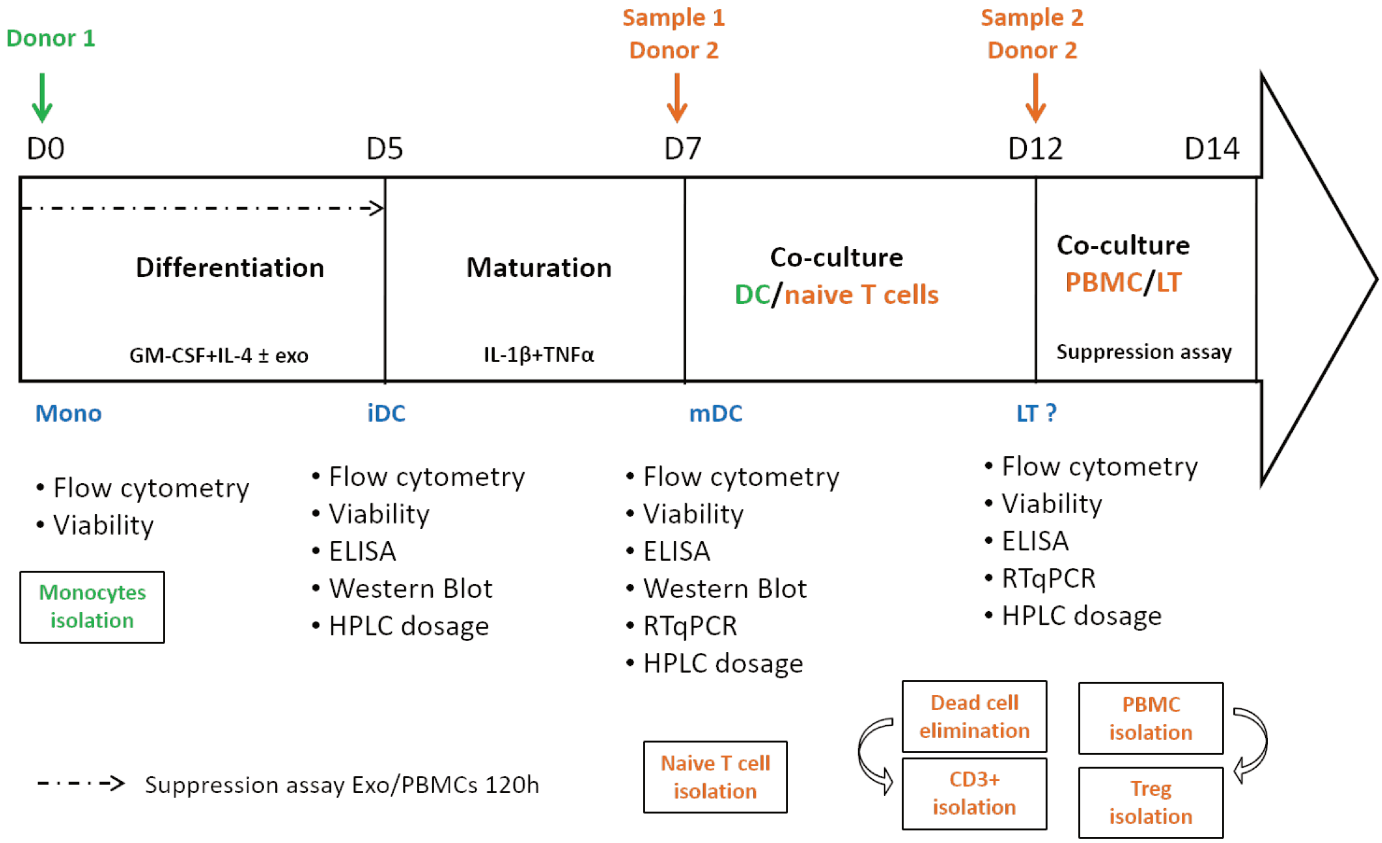


Figure 38: Cytokine secretion of irradiated DCs and CD3+ T cell co-culture after 48h. Cytokine secretion was quantified by ELISA in the co-culture supernatant of CD3+ T cells and irradiated DCs that were pre-cultured ± exosomes. The cytokines A. IL-10, B. TGF-β and C. IL-12 were dosed, results are presented in pg/mL, n=1.

F. MoDCs cultured with C15exo give rise to Tregs

The undeniable proof that C15 exosomes give rise to tDCs is to show that a co-culture of tDC with naive T cells generates regulatory T cells and anergise effector T cells. We have already found the latter, but the induction of Tregs by C15exoDCs still remains to be confirmed. To determine if C15exoDCs can induce Tregs, we have designed a long and fastidious experimental protocol (Figure 39).

1) Induced T cell phenotype and cytokine secretion



In order to determine if C15exoDCs induce Tregs we must characterise the conditioned T cells after co-culture with DCs. Firstly, a phenotypical study of T cells obtained after co-culture by flow cytometry aimed at determining if they expressed induced regulatory T cells (iTreg) markers. A higher frequency of a CD4+, CD25hi, CD127- FoxP3+ population of cells was observed after co-culture with C15exoDCs

Figure 39: Experimental protocole for the co-culture of MoDCs with naive T cells. Monocytes from donor 1 are isolated, differentiated then matured into MoDCs (green). Then nTL cells from a second donor are isolated (sample 1, orange) and cultured with MoDCs for 5 days. After, the conditioned T cells are collected from the co-culture by CD3+ isolation and subsequently cultured with autologous PBMCS for a suppression assay (sample 2 of donor 2, orange). Tregs are isolated the same day to act as a suppression control. During the first 5 days of the experiment, the lot of exosomes (HDexo and C15exo) used for the experiment are tested with the PBMCs from donor 1 to verify their function (dotted arrow). At each step, parts of the cells are collected to verify DC or T cell phenotype (Flow cytometry) and carry out complementary tests (viability, RTqPCR, Western Blot, HPLC dosage of Trp metabolites and ELISA).

(9,47%) comparatively to control mDCs and HDexoDCs (5.13% and 5.04% respectively) (Figure 40A). To further characterise obtained T cells, the secretion of immunoregulatory cytokines (IL-10 and TGF-β) and inflammatory cytokine (IL-6) of these cells was measured by ELISA. Indeed, in the supernatant of C15exoDCs/nTL co-culture, the secretion of IL-10 and TGF-β was higher comparatively to mDC/nTL (1.5 folds and 2.3 folds respectively) (Figure 40B and C). The release of IL-6 was significantly decreased by 5 folds in C15exoDC/nTL culture compared to mDC/nTL (Figure 40D). Thus, cells induced by C15exoDCs show a higher percentage of cells expressing iTreg markers, and secreted more suppressive cytokines (IL-10 and TGF-β) and less effector cytokines (IL-6).

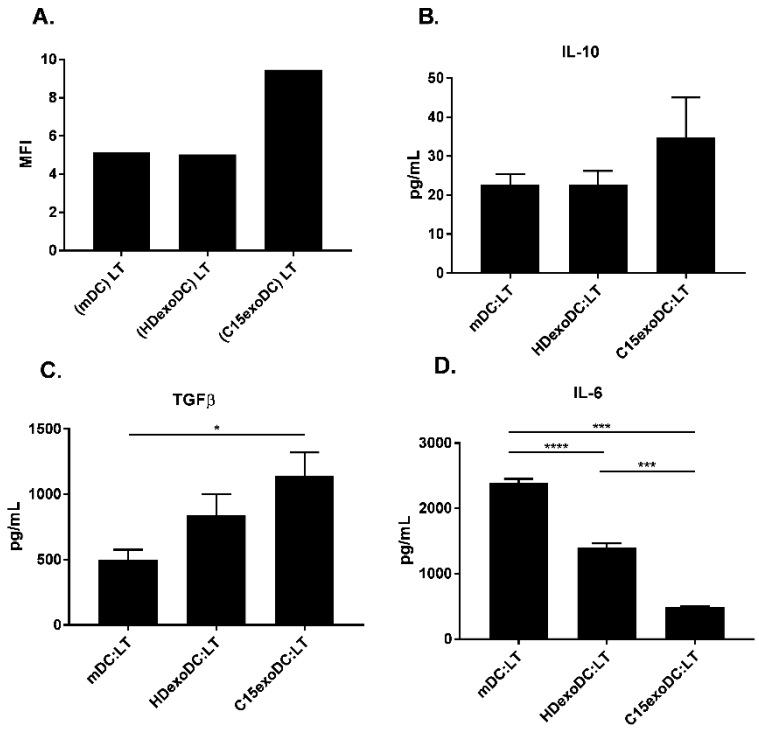


Figure 40: Characterisation of induced T cells following DC/nTL co-culture. After 5 days of co-culture of nTLs with conditioned DCs (± HDexo or C15exo), A. a flow cytometry analysis of Treg markers (CD4+ CD25hi CD127- FoxP3+) and dosage of B. IL-10, C. TGF-β and D. IL-6 secretion by ELISA were performed. Results are shown in pg/mL, n=1, (0.05<p<0.0001).

2) Induced T cell's immunosuppressive effect on autologous PBMCs

Finally, the suppressive function and anergy of the induced T cell is verified by suppression and proliferation assays. When the obtained T cells are cultured with activated human PBMCs, the T cells pre-cultured with C15exoDCs show an intermediate level of suppression between the Treg/PBMCs and mDC TL/PBMCs (Figure 41A). Significantly, this intermediate level was also observed when studying the proliferation of obtained T cells alone (Figure 41B). Hence, the T cells induced after culture with tumour exosomes-exposed DCs exhibit Treg markers, favours an immunosuppressive micro-

environment through modulation of cytokine secretion, seems to decrease PBMC proliferation and display a very low proliferation rate verging on anergy.

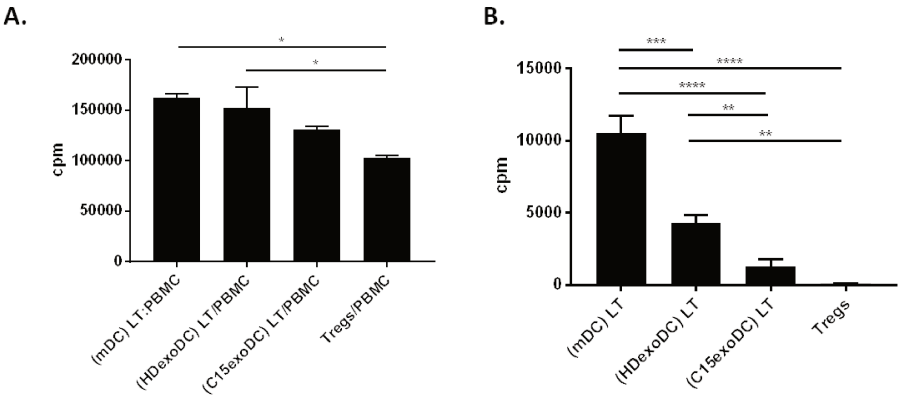


Figure 41: Characterisation of induced T cells following DC/nTL co-culture. After 5 days of co-culture of nTL with conditioned DCs (± HDexo or C15exo), A. the suppressive function of obtained T cells was determined by a co-culture of the cells with heterologous activated PBMCs for 72h. B. The proliferation of T cells alone was determined after 48 hours by radioactive-thymidine incorporation. Proliferation results are expressed in cpm, n=1, (0.05<p<0.0001).

G. Chemoattraction of DCs by C15exo

Previous work in the team has shown that NPC exosomes recruit T cells (effector T cells and Tregs) to the tumour site in a CCL20-dependant manner, which favours the tumour's immune evasion. Knowing this, we wondered whether C15exo could attract DCs, and if so, is it also CCL20-dependant? Chemoattraction assays were carried out in Boyden chambers at day 5 and 7 of culture. At day 5, three different DCs were cultured in the chamber, either control iDCs (black), control tDCs (grey) or C15exoDCs (light grey). Interestingly, adding C15exo to the lower chamber always significantly attracts higher numbers of immature or tolerogenic cells, with a 30 folds increase (Figure 42A). If the C15exosomes have been previously cultured with a CCL20 blocking antibody, the attraction seems partly abrogated for control iDCs and tDCs (both a 1.1folds decrease). However, the blocking antibody decreased the attraction of C15exoDCs by 2.3 folds. Furthermore, after maturation, mDCs, tDCs and C15exoDCs were tested for chemoattraction by C15exo. Remarkably, the C15exo in the lower chamber only seemed to attract control tDCs and C15exoDCs, but not control mDCs (migration index 6.67, 18.9 and 0.14 respectively). Moreover, once more, the attraction by C15exo is inhibited by the blocking of CCL20, most significantly for C15exoDCs (13.5 folds decrease). The same trend is observable for tDCs, with a 20.2 folds decrease (Figure 42B). Thus, C15exo only seem to attract immature, tolerogenic and C15exoDCs which once again, suggests that C15exoDCs are indeed tDCs. Moreover, this exosomal recruitment of cells can be disrupted by blocking CCL20 carried on C15 exosomes.

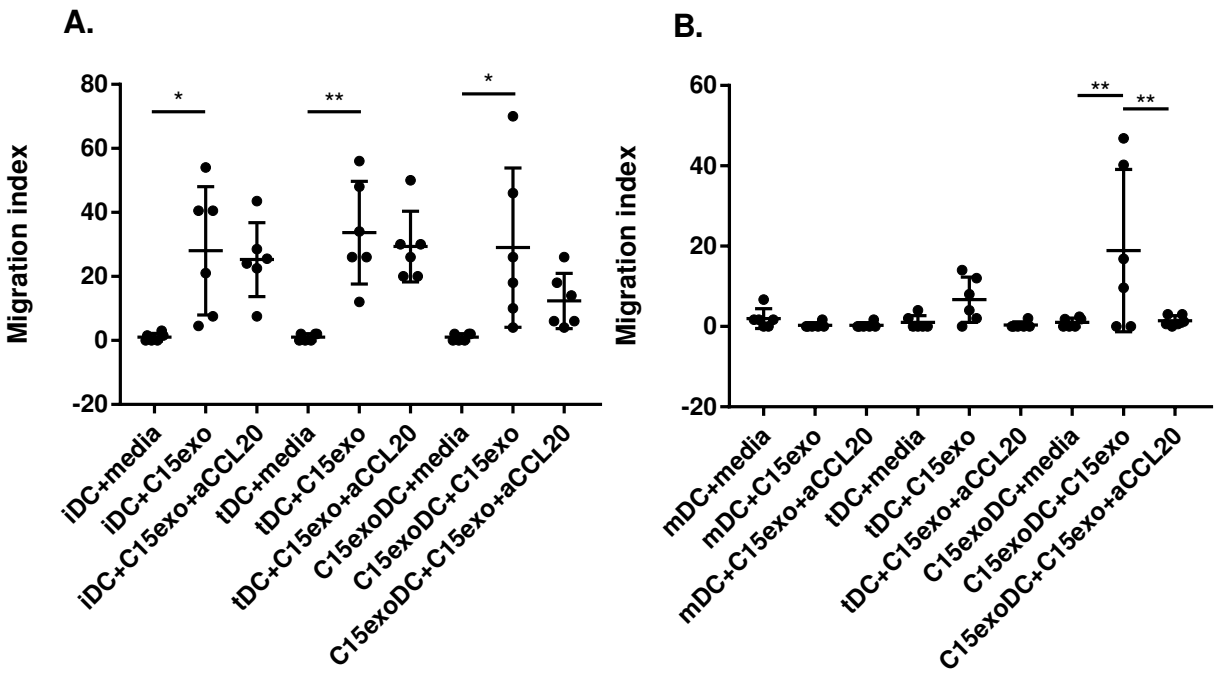


Figure 42: Chemoattraction of various treated DCs by C15exo. Chemoattraction assays were carried out in Boyden chambers. Cells were cultured with culture media only or with C15exo (5µg/mL) ± hu-αCCL20 blocking antibody (20µg/mL). A. Chemoattraction of iDCs, tDCs and C15exoDCs by C15exo was tested after differentiation. B. Chemoattraction of mDCs, tDCs and C15exoDCs by C15exo was tested after maturation. Results were obtained after three independent blind counts. Chemoattraction is expressed as a migration index using the cells cultured with culture media only as a reference condition, n=1 (0.05<p<0.01).

H. Effect of C15exo on the maturation of MoDCs matured with LPS

The main work of my thesis is focused on the effect of C15exo on the maturation of MoDCs matured with cytokines IL-1β and TNFα. This maturation was chosen early on, as it best resembles DC maturation in the physiopathological context of cancer. Nevertheless, another well-known and accepted maturing agent is *E. Colis* LPS. Thus, we aimed to determine if C15exo could also alter MoDC maturation when cells where matured using LPS.

As previously carried out, we did a phenotypical study by flow cytometry analysis of the expression of maturation markers. Table XI shows mfi of each marker after maturation with LPS. For maturation markers, C15exoDCs show consistently lower mfi values than control mDCs (CD40, CD80, CD83, CD86 and HLADR) and a higher expression for iDC marker DC-SIGN. Their expression for C15exoDC is more comparable to control tDCs and iDCs.

Table XI: Cell surface expression of maturation and phenotypic markers of MoDCs after maturation with LPS. All median fluorescence intensity (mfi) values of marked samples were normalised with the corresponding isotypic control, n=3

	CD40	CD80	CD83	CD86	HLA-DR	DC-SIGN
iDC	66,29 ± 12,95	13,87 ± 1,39	2,93 ± 1,14	1,68 ± 0,07	37,68 ± 31,22	44,86 ± 29,93
mDC	125,75 ± 32,97	36,49 ± 5,16	13,71 ± 2,66	25,06 ± 9,26	90,27 ± 28,18	40,48 ± 21,95
tDC	32,46 ± 4,02	25,93 ± 4,59	2,73 ± 0,92	4,55 ± 0,30	65,88 ± 17,90	19,16 ± 2,59
HDexoDC	150,78 ± 17,42	40,39 ± 4,49	14,63 ± 2,34	27,85 ± 6,89	95,09 ± 29,54	39,82 ± 19,33
C15exoDC	45,83 ± 29,75	12,88 ± 2,59	3,54 ± 0,48	2,39 ± 0,62	27,11 ± 12,71	47,44 ± 44,19

Overlay representation of the membrane expression of maturation markers illustrated well the differences notices in table XI. After differentiation, the C15exoDC expression of CD40 and CD83 is already noticably lower than control mDCs and iDCs (Figure 43A). After maturation with LPS, the difference in expression of maturation markers is clearly observable as the intensity of CD40, CD80, CD83 and CD86 are significantly lower than control mDCs (Figure 43B). The lower expression of HLADR is not so clear-cut but can still be considered lower than control mDCs. And finally, the expression of iDC marker DC-SIGN is the highest for C15exoDCs. Flow cytometry analysis has shown that C15exoDCs have an immature DC phenotype.

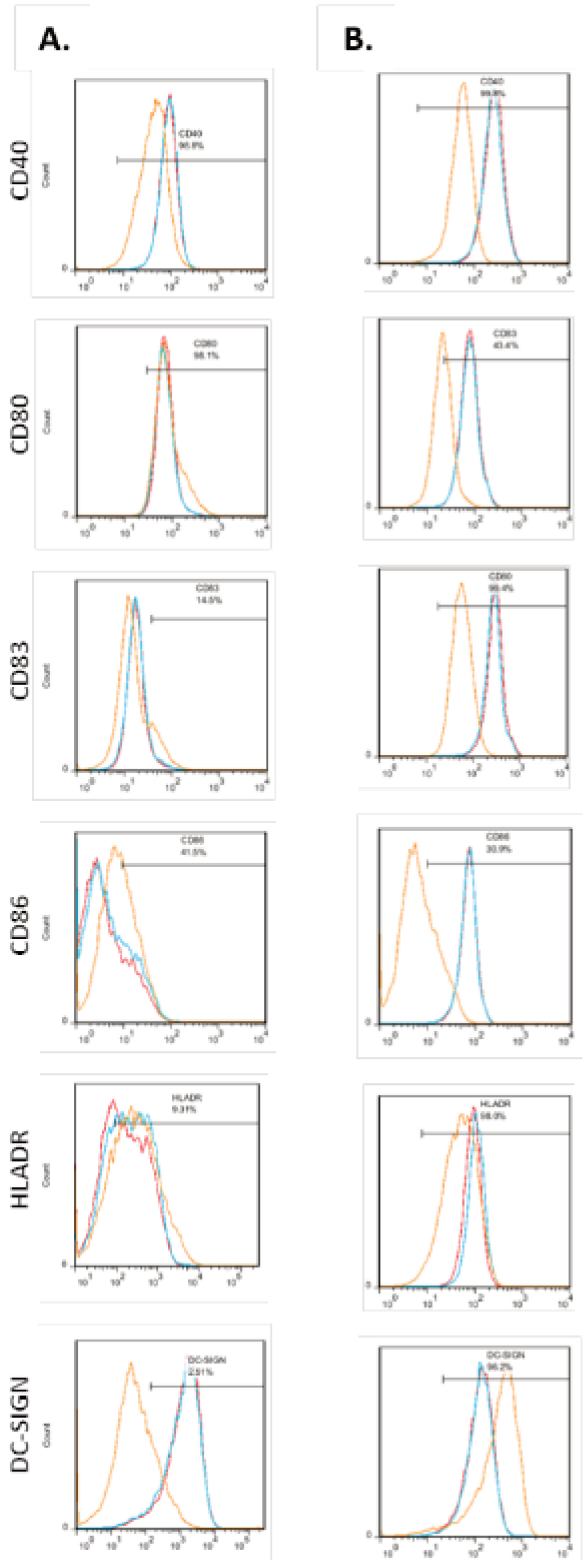


Figure 43: Cell surface expression of maturation markers on MoDCs. The expression of maturation markers was determined A. after differentiation with GM-CSF+IL-4: iDCs (red) ± HDexo (blue) or C15exo (orange) (Day 5) B. The maturation state was determined after a 2-day maturation with LPS: mDCs (blue) ± HDexo (red) or C15exo (orange) (Day 7). Representative overlays of two independent experiments.

I. Effect of C15exo on the secretion of MoDCs matured with LPS

Now that we know C15exo can alter the maturation of LPS-induced MoDCs, the next step is to determine if they also change their cytokine secretion. The secretion of suppressive cytokines IL-10 and TGF-β by C15exoDCs (Figure 44 A and B) seems to be lower than for control mDC, while HDexoDCs secrete similar levels to mDCs. Moreover, the secretion of effector cytokines IL-12, TNFα, IL-6 and IFNγ is significantly decreased when MoDCs are pre-treated with C15exo compared to control mDCs (Figure 44C, D, E and F). In contrast, HDexoDCs exude an immunostimulatory function as they secrete more IL-12 and TNFα as well as comparable levels of IL-6 and IFNγ than mDCs. Thus, ELISA assays have shown that C15exoDCs secrete less effector cytokines IL-12, TNFα, IL-6 and IFNγ to favour an increased secretion of immunosuppressive cytokines IL-10 and TGF-β, which enhances immune suppression.

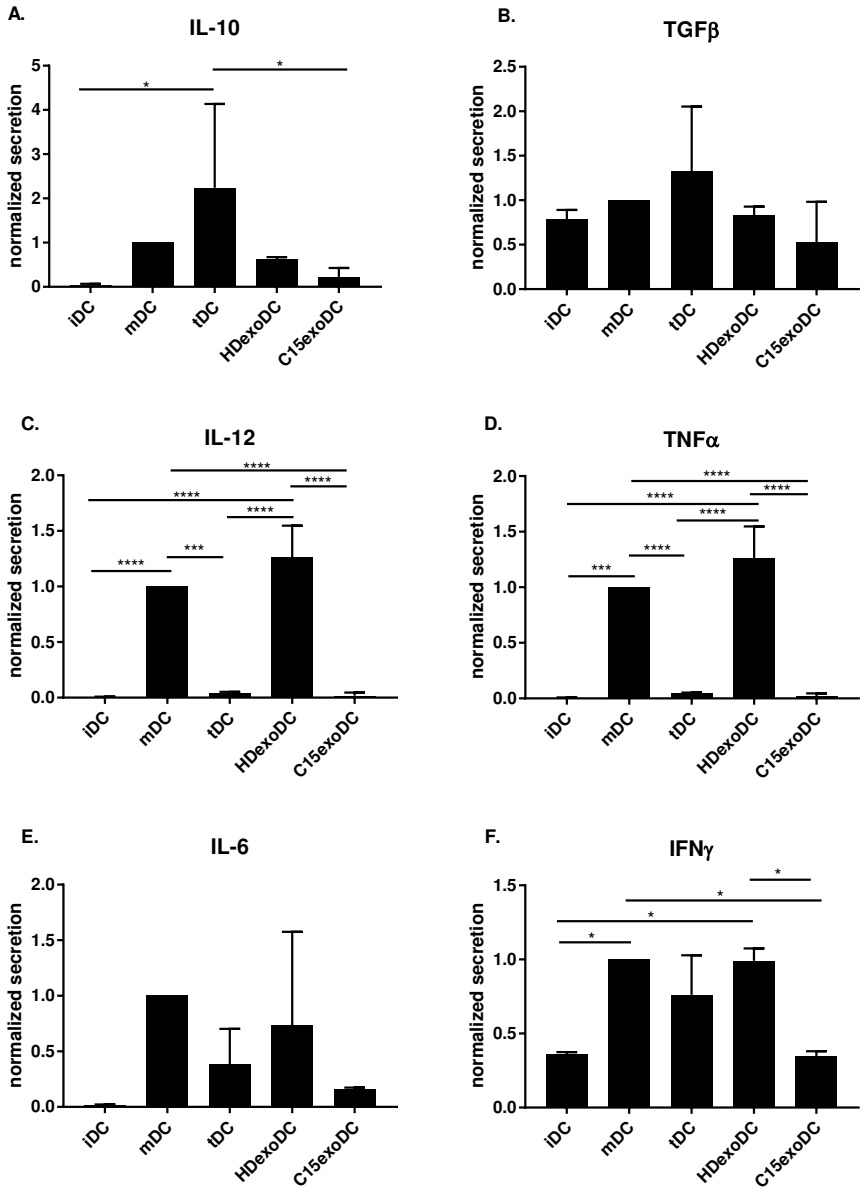


Figure 44: Dosage of cytokine secretion by MoDCs after maturation. The secretion of the following cytokines was dosed in the culture supernatant after maturation with LPS. A. IL-10, B. TGF-β, C. IL-12p70, D. TNFα, E. IL-6 and F. IFNγ secretion were measured by ELISA. The results are normalized with the control mDC condition. IL-10, IL-12, TGFβ and TNFα n=3, IL-6, IFNγ and tDC n=2 (0.05<p<0.0001).

In conclusion, the phenotypical study of C15exoDCs matured with LPS show that C15exo do indeed block maturation as we observe an immature phenotype. Moreover, the study of cytokine secretion shows the immunosuppressive functions of C15exoDCs induced by LPS. Thus, this mechanism of DC maturation blockade by C15exo seems to be true for both LPS and IL-1β and TNFα-matured MoDCs.

J. Effect of gal-9 on MoDC maturation

1) Phenotypical characterisation of MoDC cultured with galectin-9

It has been described that gal-9 is present on NPC tumours and our team has shown that gal-9 is greatly involved in NPC immune suppression (Mrizak et al., 2015). On this basis, we hypothesised that gal-9 could be the molecular mediator of MoDC maturation blockade observed in our experiments. To answer this question, we added recombinant gal-9 to human monocyte culture (day 0) and studied the impact on MoDC maturation by analysis of membrane maturation markers and dosage of cytokine secretion.

Firstly, we looked at the expression of membrane-bound maturation markers by flow cytometry. The mfi values for each marker, in each condition, is shown after differentiation (Table XII) and after maturation (Table XIII). On monocytes alone, gal-9 does not seem to have an effect whereas gal-9 added to monocytes that differentiate into iDCs exhibit a slight increase in their expression of CD40 (iDC 33,17 vs iDC+gal-9 50,10) and DC-SIGN (iDC 33,30 vs iDC+gal-9 43,18). After maturation, mDC alone show lower expression of maturation markers than mDCs pre-cultured with gal-9 (CD40, CD80 and CD83). Moreover, the presence of gal-9 seems to slightly decrease the expression of iDC marker DC-SIGN.

Table XII: Cell surface expression of maturation and phenotypic markers of MoDCs cultured ± Gal-9 after differentiation. All median fluorescence intensity (mfi) values of marked samples were normalised with the corresponding isotypic control, n=1

	CD40	CD80	CD83	CD86	HLADR	DC-SIGN
Mono	6,41	5,98	1,85	2,36	12,40	1,23
iDC	33,17	10,04	2,23	2,30	26,04	33,30
Mono+Gal9	8,29	6,10	1,88	2,33	13,67	1,32
iDC+Gal9	50,10	7,12	2,30	1,95	21,19	43,18

Table XIII: Cell surface expression of maturation and phenotypic markers of MoDCs cultured ± Gal-9 after maturation. All median fluorescence intensity (mfi) values of marked samples were normalised with the corresponding isotypic control, n=1

	CD40	CD80	CD83	CD86	HLADR	DC-SIGN
iDC	20,08	100,66	10,96	79,35	4,12	0,98
mDC	2,06	30,00	1,61	62,26	6,07	1,42
mDC+Gal9	20,16	101,91	10,47	67,74	4,06	0,98

Furthermore, overlay representation of the expression of maturation markers shows no major alteration after differentiation (Figure 45A) nor after maturation (Figure 45B) with gal-9. All together, these first phenotypical results suggest that gal-9 enhances MoDC maturation.

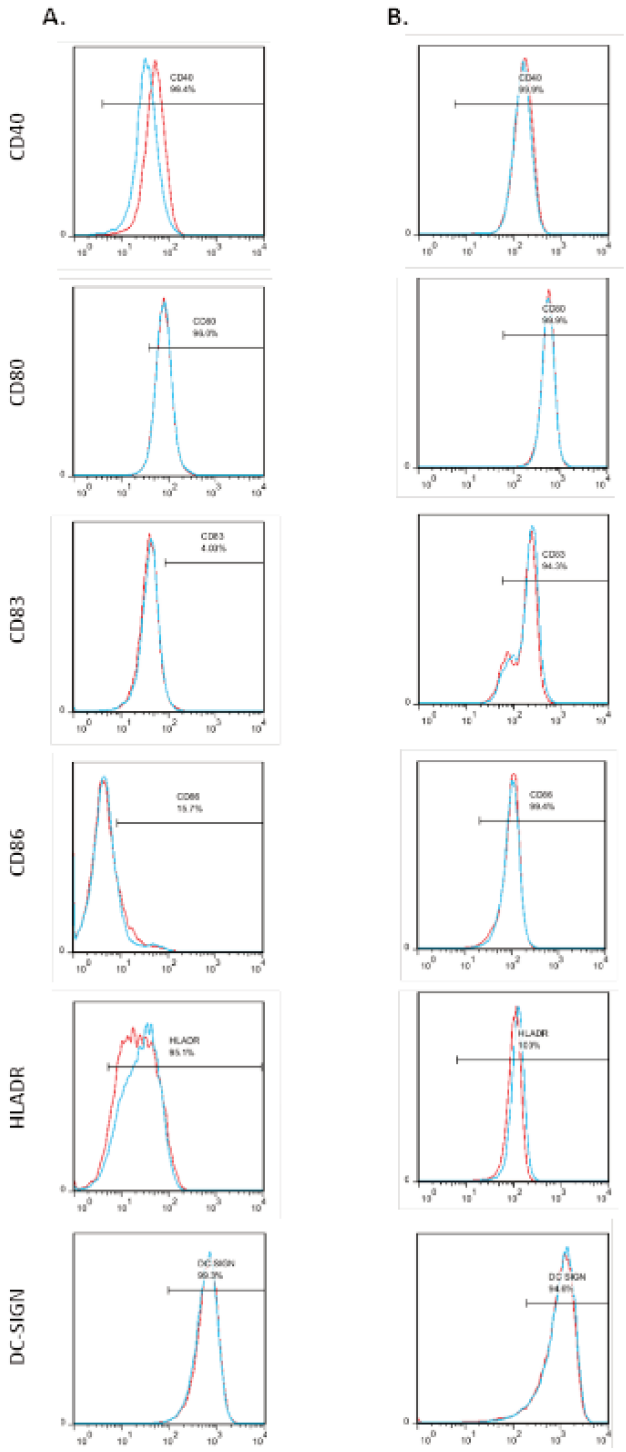


Figure 45: Cell surface expression of maturation markers on MoDCs cultured ± Gal-9. The expression of maturation markers was determined by flow cytometry A. after differentiation (iDC in blue) ± gal-9 (red), and B. after maturation: mDCs (blue) ± gal-9 (red), n=1.

2) Effect of gal-9 on the cytokine secretion of MoDCs

After phenotypically studying the impact of gal-9 on MoDC maturation by flow cytometry, we then sought to determine if cytokines secretion was altered. Quantification of cytokine secretion by ELISA is a preliminary functional study that allows us to see if the functions of MoDCs pre-treated with gal-9 are altered. After maturation, we analysed the secretion of immunosuppressive cytokines (IL-10 and TGF-β) (Figure 46A and B) as well as effector cytokines (IL-12 and TNFα) (Figure 46C and D). We have observed no major difference in the secretion of IL-10 (Figure 46A), TGF-β secretion is slightly decreases when MoDCs are pre-cultured with gal-9 (Figure 46B). The secretion of effector cytokine IL-12 is also lower (Figure 46C) whereas TNFα levels are identical in the compared conditions (Figure 46D).

Thus, our preliminary findings show that recombinant and exogenous gal-9 does not seems to significantly alter the maturation of MoDCs. If anything, it might even enhance the maturation as shown by mfi values of maturation marker expression.

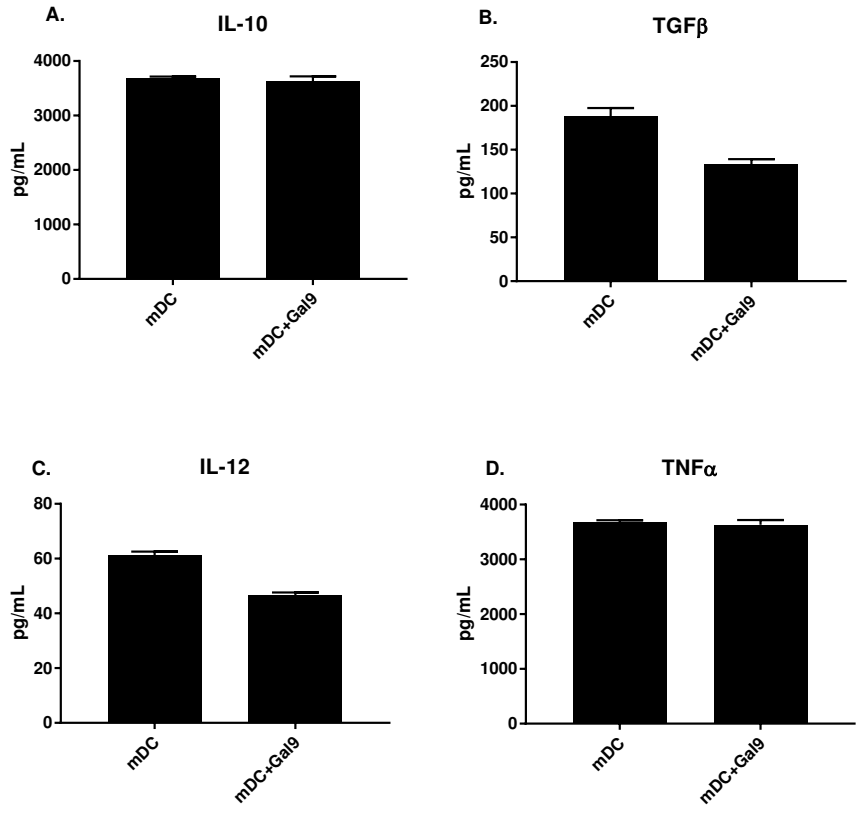


Figure 46: Dosage of cytokine secretion by MoDCs cultured ± Gal-9 after maturation. The secretion of the following cytokines was dosed by ELISA in the supernatant of DCs cultured ± Gal-9. A. IL-10 secretion, B. TGF-β secretion, C. IL-12p70 secretion or D. TNFα secretion, n=1.

K. NPC patient exosomes

With the collaboration of Dr. Hayet Rafa, we were able to collect plasma samples from NPC patients treated at the Mustapha-Bacha Hospital (Alger, Algeria). From these samples, total exosomes were isolated by the standard exosomes isolation method routinely carried out in the laboratory (cf. Material and Methods). Samples from a single patient are roughly 5-6mL of plasma which contain too few exosomes to be isolated individually. Thus, we pooled samples of patients based on their treatment, stage (TNM classification) and EBV status. Table XIV summarizes the pools and the clinical characteristics of the NPC patients.

Table XIV: Pools of NPC patients samples based on their stage (TNM classification), treatment and EBV status. Volume of plasma (mL) and quantity of exosome isolated (µg) are also shown.

	Stage	TNM Classification	Treatment(s)	EBV Status	Volume (mL)	Quantity of exosomes (µg)
Pool A	Stage II	T2aN0M0	TPx	EBV (+)	15	5,25
Pool B	Stage III	pT3bN0M0	TPx	EBV (+)	8	4,28
Pool C	Stage IV	pT4N2aM1 ; T4N0M0	TPx	EBV (+)	15	8,98
Pool D	Stage III	pT3N0M0 ; pT3N1M0	TPx	EBV (+)	10	5,72
Pool E	Stage IV C	pT4bN4M0 ; pT3N1M1 ; pT2N2aM1a	TPx	EBV (+)	10	4,75
Pool F	Stage II		TPX+Cetazima	EBV (+)	25	10,95
Pool G	Stage III	pT3NxM0	TPx	EBV (+)	9	4,53
Pool H	Stage III	pT3N0M0 III	No treatment	EBV (+)	24	25,92
Pool I	Stage IV		TPx	EBV (+)	16,5	6,43
Pool J	Stage II		TPx	EBV (+)	25	21,47
Pool K	Stage II		No treatment	EBV (-)	11	9,64

Perspectives

&

Discussion

VIII. Discussion- perspectives

NPC is an EBV-associated malignancy that exhibit a strong immunosuppressive microenvironment governed by tumour-derived exosomes and Tregs. These key players interact and help sustain the strong immunosuppression to favour tumour progression. Moreover, one well-known mechanism of Treg induction is *via* tDCs that are able to convert nTL into Tregs and to inhibit the proliferation of effector T cell. Thus, tDCs contribute to immune evasion by impairing the immune response. In this context, the aim of my thesis was to determine whether tumour exosomes could block the maturation of DCs to favour the emergence of tDCs. This ultimately leads to an increase in Treg prevalence and the worsening of cancer.

Summary of results

The results obtained during my thesis have shed light on new mechanism that enhances the NPC's immunosuppressive TME via exosomes. NPC secretes large amounts of exosomes that are known to directly interact with infiltrating T cells but also peripheral T cells. Indeed, they can inhibit the proliferation of effector T cells, convert conventional T cells into Tregs and enhance the suppressive functions of Tregs (Mrizak et al., 2015).

Based on this knowledge, our results allow us to see the bigger picture that includes indirect mechanisms involving dendritic cells (Figure 47). We have shown that NPC exosomes interact with monocytes and pushes them towards a tDC phenotype. Indeed, infiltrating monocytes and DCs have been found in NPC biopsies and are sometimes referred to as accessory cells (Zong et al., 1993).

We have also shown that the induced tDCs then block the anti-tumour response by angergising effector T cell and inducing Tregs. A blockade of effector T cells limits the immune response and Treg themselves inhibit and kill effector T cells.

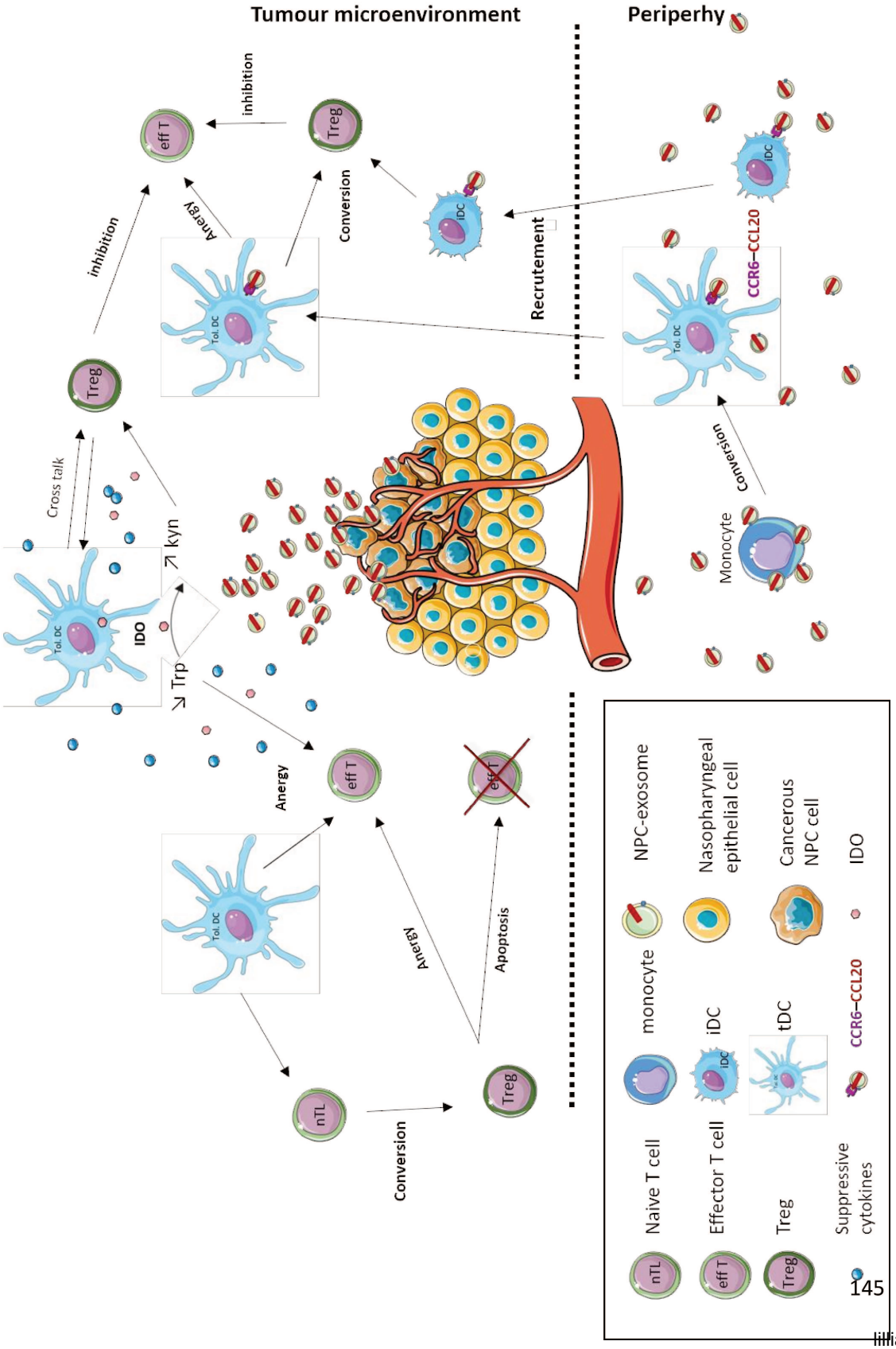
Moreover, tumour exosomes carry the CCL20 chemokine, this allows NPC exosomes to recruit effector T cells and Tregs to the tumour site, as previously discovered in our lab (Mrizak et al., 2015). We have also demonstrated that the exosomal-bound CCL20 attracts iDCs, tDCs and C15exoDCs in order to contribute to the TME immune suppression. This is likely to be mediated by the CCL20/CCR6 axis as the NPC-exosome-induced tDCs show increased transcriptomic expression of CCR6.

Finally, induced tDCs also set up an immunosuppressive microenvironment to disrupt the anti-tumour response and favour the emergence of regulatory immune cells. For this, tDCs secrete high levels of suppressive cytokines (IL-10 and TGF-β) and less effector cytokines (IL-12 and TNFα) which contributes to the rise of Tregs. Another important environmental factor is the enzyme IDO. Indeed, tDCs express IDO that depletes Trp from the surrounding media and inhibits effector T cell

proliferation. The production of kyn is toxic for effector T cells and kill them whilst Tregs thrive on kyn metabolites (Belladonna et al., 2009; Yan et al., 2010). Thus, this double hit effect suppresses effective T cell response and enhances Treg-mediated tolerance.

All of this strengthens immune suppression in the TME, which leads to tumour progression and overall worsening of cancer. This study has given new insight on immune evasion mechanisms, which are potential new targets for anticancer immunotherapies.

Figure 47: New proposed model of the NPC tumour microenvironment (Sarah Renaud)



General Conclusion

In conclusion, in this thesis, we defined a new indirect mechanism of Treg induction by NPC-derived exosomes via tDCs. We first sought to determine if tumour exosomes had an effect on the maturation of human MoDCs. For this, tests were carried out to determine the maturation status of MoDCs exposed to NPC exosomes. A complete phenotypical study led us to believe that NPC exosomes did indeed block MoDC maturation, as cells exhibited a semi-mature phenotype during transcriptomic and flow cytometry analysis of maturation markers. Then, we undertook a study of the function of C15exoDCs. This was done by examining cytokine secretion and the expression of regulatory enzyme IDO. And indeed, we found that functional assays correlate with phenotypical studies as C15exoDCs show tolerogenic potential by favouring an immunosuppressive environment. But the final nail in the coffin to verify that C15exoDCs are indeed tolerogenic DCs, is to verify their effect on T cells. For this, MoDCs exposed to NPC exosomes would have to block effector T cell proliferation and induce Tregs. Fastidious testing of T cells with C15exoDCs co-culture revealed that the latter showed both of the forementioned immunosuppressive properties. At this point, we had confirmed the tDC status of C15exoDCs, and in line with previous work carried out in the lab, we wanted to determine if NPC exosomes could attract DCs. Former studies revealed that NPC exosomes are able to recruit effector T cells and Tregs to the tumour site via the CCL20/CCR6 axis. As the C15exoDCs express high levels of CCR6, this seemed like a plausible hypothesis. Thus, chemoattraction assays showed that NPC exosomes preferentially attracted control iDCs, tDCs and C15exoDCs. Furthermore, it was also determined that this mechanism is dependent on the chemokine CCL20, as its blockade led to the disruption of DC recruitment.

Finally, with the aim of elucidating which molecular pathways are at play in exosomal tDC induction, we used recombinant gal-9 to see if it blocked MoDC maturation in the same way NPC exosomes do. Interestingly, results showed no major alteration in MoDC maturation. In fact, recombinant gal-9 seems to enhance MoDC maturation, so further tests need to be carried out using exosomal gal-9 blocking agents.

NPC exosomes not only directly block effector T cell response but also induce Tregs, which sustains immune suppression in the TME. This work has shed light on a new indirect mechanism that contributes to NPC's immune evasion. Indeed, NPC exosomes induce tDCs which themselves give rise to Tregs. Thus, in further elucidating tumour immune escape mechanisms, we are better equipped to develop new anticancer immunotherapy drugs. Although Tregs are the effectors of immune

suppression, tolerogenic DCs are the instigators. It is now clear that their role in immune evasion is crucial as they could be the next big trend in anticancer immunotherapy.

Perspectives and Questions regarding this project

How to better characterise exosomes

We also analysed our exosome samples by multiple methods including TEM and Nanosight. As briefly mentioned in the results, the size of exosomes measured by Nanosight are slightly bigger than when measured with TEM. It has been shown that Nanosight measures can vary depending on multiple factors including operator proficiency, cleanliness of the buffer and/or circuit, instalment of the circuit, duration of measurement, vibrations, etc. (Gardiner et al., 2013; Maas et al., 2015). Nonetheless, Nanosight still remains a valuable tool for the study of exosomes that require great dexterity.

Furthermore, the real confirmation of the exosome status is a phenotypical study of its maturation makers. Although there is not a single marker that allows their identification, the concomitant expression of specific exosomal markers, or lack of, is necessary. A few years ago, the International Society of Extracellular Vesicles (ISEV) published a paper describing which markers are necessary to define exosomes (Lötvall et al., 2014). The following table, summarizes the different categories of proteins expected to be present, or absent, on isolated exosomes. It should be noted that at least one protein from category 1, 2 and 3 should be quantified on isolated exosomes.

***Table XV: categories of exosomal markers and some exmaples (non-exclusive)**. * italics: official gene name, ** denotes the different possible family members. Adapted from Lötvall et al., 2014.*

1. Transmembrane or lipid-bound extracellular proteins	**2. Cytosolic proteins**	**3. Intracellular proteins**
Argues presence of a membrane in the isolate	With membrane- or receptor-binding capacity	Associated with compartments other than plasma membrane or endosomes
Present of enriched in EVs/exosomes	Present of enriched in EVs/exosomes	Absent or under-represented in EVs/exosomes, but present in other types of EVs

Examples: Tetraspanins (*CD9, CD63, CD81*) Intergins (*ITG***) or cell adhesion molecules (*CAM***) Growth factor receptors Heterotrimeric G proteins (*GNA***) Phosphatidylserine-binding *MFGE8*/lactadherin	Examples: Endosome or membrane-binding protein (TSG101, annexins=*ANXA**, Rabs=*RAB**) Signal transduction or scaffolding proteins (syntenin)	Examples: Endoplasmic Reticulum (grp94=HSP90B1, calnexin=*CANX*) Golgi (*GM130*) Mitochondria (cytochrome c=*CYC1*) Nucleus (Histones=*HIST*H**) Argonaute/RISC comple (*AGO**)

We demonstrated their expression by western blot but there are now other methods to do so. The Nanosight has developped methods to determine protein expression by fluorescent marking of exosomes. Moreover, flow cytometry analysis of exosomes is also becoming more and more common. Bearing in mind that the equipment needs to be sufficiently precise in focalisation to be able to analyse exosomes. ***We have currently started to develop protocols of exosomal analysis on the ATTUNE NxT flow cytometer from Thermofisher that is available at the BiCell plateform. This machine has an innovative acoustic focalisation which allows the visualisation of exosome samples. So far we have only managed to view exosomes, we now need to better define the machine configuration.***

Other NPC cell lines and patient-derived exosomes

The next step of this study would be to confirm these results on other NPC cell lines such as C17 and C666-1 to see if the tumour-derived exosomes also induce tDCs. Furthermore, it would add real clinical relevance to carry out the same tests on exosomes derived from plasma of NPC patients. This would allow us to measure the amplitude of NPC exosome's implication in tumour progression. Previous work carried out in our lab has revealed that all the circulating exosomes, which include healthy cell-derived exosome and tumour exosomes, still have an overall immunosuppressive quality (Mrizak et al., 2015). Thus, targeting tumour exosomes is a very promising lead for the treatment of NPC, and cancer in general.

Nonetheless, it must be mentioned that each cancer type sheds different exosomes, that differ in both function and molecular composition. Thus, the challenge for this strategy is to successfully target and inhibit cancer exosomes without affecting surrounding healthy exosomes. Unfortunately, a unique tumour exosome marker has yet to be defined (Kosaka et al., 2014; R et al., 2016). Once light is shed on this matter, new targets for NPC immunotherapy could be developed. However, exosomes are currently used as biomarkers in cancer and other diseases (He et al., 2018; Kim et al., 2018a; Panagiotara et al., 2017; Soung et al., 2017). They are used to improve patient stratification, better determine treatment protocols and overall disease management.

By which molecular pathway do NPC exosomes induce tDCs?

After this, elucidating the molecular pathways involved in the induction of tDCs remains a question of great interest. A first lead would be to see how the exosomes interact with the monocytes, are they internalised or is it a kiss and run mechanism? If the exosomes are internalised, this could mean that the factors that affects monocytes are carried within the exosomes, such as miRNA for example. If it is a kiss and run mechanism, this would imply a receptor/ligand type interaction between the tumour exosomes and monocytes. Although it has been described that exosomes do interact with monocytes, the exact nature of the exchange remains unclear (Weiss et al., 2018).

EBV oncoprotein LMP1?

A first candidate is LMP1, which is specific to NPC tumour exosomes and has immunosuppressive properties (Keryer-Bibens et al., 2006). LMP1 is a viral oncogene involved in many major cellular pathways commonly disrupted in cancer, promotes NPC invasiveness (Aga et al., 2014) and inhibits T cell activation (Keryer-Bibens et al., 2006). LMP1 packaging into exosomes depends on CD63, its impairment leads to exosomes devoid of LMP1 which subsequently stops NF-KB and proto-oncogene bcl2 from being overexpressed (Verweij et al., 2011). Even though this makes LMP1 a very appealing candidate, we must bear in mind that LMP1 is a CD40 mimic. A study by Gupta et al. has shown that LMP1 enhances DC maturation when included in a HIV vaccine (Gupta et al., 2011). A later study by the same team demonstrated that human DCs showed enhanced activation, migration and IL-12 secretion when matured with LMP1 in a melanoma mouse model (Gupta et al., 2011). Thus, only the blockade of exosomal LMP1 will give us definitive answers on LMP1's involvement in the induction of tDCs by NPC exosomes. The challenging aspect of this study is the inhibition of LMP1. As mentioned in the introduction, LMP1 is a membrane-bound protein that has 6 transmembrane domains and both the Cter and Nter regions are intracellular. This makes blocking LMP1 with an antibody very difficult. Nevertheless, in a study by Verweij et al., the packaging of LMP1 into exosomes was blocked when cells were transfected with a "non-cleavable" mono-ubiquitylated (Ub)–LMP1 fusion protein (Verweij et al., 2011). This is an appealing alternative to regulate LMP1 expression in NPC exosomes and thus, determine if their induction of tDCs is LMP1-dependant. ***Recently, my colleague Dr. Alexandre Quilbe and I received a grant of 10.000€ from the Cancéropôle Nord-Ouest and Bristol Myers Squibb to test the effect of exosomal LMP1 blockade on the maturation of MoDCs.***

Galectin-9?

The second candidate is gal-9, which is also specific to NPC exosomes (Keryer-Bibens et al., 2006) and is involved in tissue homeostasis by blocking the immune response. Recent work in the lab has shown that gal-9 contributes to the suppressive functions of Tregs and a monoclonal humanised antibody is currently being developed for use in clinical trials (patent US20170283499A1). Nevertheless, the notion that gal-9 is an exclusive immunosuppressive factor is subject of debate. Most literature defines gal-9 as an actor of immune suppression. But in DC biology, gal-9 seems to promote anti-tumour immunity by increasing Tim3 expression (Nagahara et al., 2008). Indeed, work carried out during my thesis has shown that recombinant gal-9 enhances MoDC maturation, which is in line with a study by Dai (Dai et al., 2005). Further testing still needs to be carried out by blocking exosomal gal-9 to determine its involvement in exosome-mediated tDC induction.

Exosomal miRNA?

The suppressive effect of NPC exosomes on DCs could also be mediated by miRNA. Indeed, it has been shown that NPC exosomes carry miRNA that contribute to NPC development (Lung et al., 2018; Zhou et al., 2018). Ye et al. found that miR-24-3p, miR-891a, miR-106a-5p, miR-20a-5p, and miR-1908 promote the induction of Tregs (Ye et al., 2014). Thus, an analysis of miRNA contained in NPC exosomes could give us further insight on how they induce tDCs.

Which type Tregs are generated by NPC-exosome induced tDCs?

Another aspect of the study that needs to be further elucidated is the exact nature of the T cells obtained after nTL culture with C15exoDCs. Indeed, NPC exosomes favor T cells anergy, the generation of Tregs as well as potentiating Tregs suppressive function. However, the phenotype of the induced Tregs (iTregs) remains to be verified. Preliminary results show FoxP3+ iTregs that secrete high amounts of IL-10. Nevertheless, FoxP3+ iTregs generated *in vitro* are not very well described in the literature and little is known about them (Schmidt et al., 2016; Shevach and Thornton, 2014). To further confirm the phenotype of induced Tregs, other markers need to be studied. For example, it was found that other myeloid suppressive cells, such as regulatory macrophages, can be induce by a TIGIT+ FoxP3+ iTreg population. Furthermore, this population depends on IDO expression, IL-10 and TGF-β secretion which parallels our findings (Riquelme et al., 2018).

What about monocyte chemoattraction by NPC exosomes?

We established that NPC exosomes are able to specifically attract iDCs and tDCs to the tumour site via the chemokine CCL20. We also determined that NPC exosomes did not attract mDCs nor monocytes. This is in line with literature that links higher monocyte and mDC tumour infiltration with a better prognosis in NPC (Giannini et al., 1991). This would suggest that the monocytes present in the TME do not acquire a suppressive function after contact with tumour exosomes and maintain their immune functions. Thus, as monocytes are not attracted to the tumour site, maybe the exosome-led differentiation of monocytes into tDCs occurs in periphery. In addition, once monocytes have given rise to suppressive tDCs, they are recruited to the TME. However, it does seem unlikely that tumour-infiltrating monocytes remain unaffected by tumour exosomes whilst peripheral monocytes induce tDCs. Perhaps monocytes are indeed recruited to the tumour, but not via exosomes. Other factors such as MCP-1 or IFNγ and TNFα are shown to attract monocytes (Deshmane et al., 2009; Jehs et al., 2016). Further testing needs to be carried out to elucidate this mechanism.

A proof of concept in an in vivo mouse model?

A validation of these finding *in vivo* would evaluate the importance of this phenomenon in NPC carcinogenesis. In a SCID mouse model, xenografted with a NPC tumour, C15exoDCs would be injected into the mice. Then we would monitor the growth of the tumour and the immune infiltrate. Flow cytometry analysis of the infiltrate would allow us to determine if numbers of infiltrating tDCs and Tregs increase during the evolution of the tumour. Another method would be to follow the tumour growth in the same model but by injecting NPC exosomes and determining if the number of tDCs increases based on a CD11c+, CD80neg/low, CD86neg/low, DC-SIGN+, IDO+ and PD-L1+ phenotype. Furthermore, the study of Treg levels in correlation with tDCs would also of interest.

Metabolism evaluation as a tDC signature?

A challenging aspect of tDC biology is the lack of a universal tDC marker. Indeed, a signature of characteristics makes them identifiable but only a functional assay can confirm their tolerogenic status. A unique tDC marker would considerably aid in the study and understanding tDC biology as well as their involvement in autoimmune diseases, transplantation and cancer. Although this seems to be an increasingly utopic goal with tDC phenotype depending on multiple factors such as origin and generation conditions (Navarro-Barriuso et al., 2018). However, a new field in tDC research is the alternative metabolism identified in tDCs. Indeed, tDCs favour oxidative phosphorylation and fatty acid

oxidation whereas mDCs preferentially use glucose as a source of carbon (Sim et al., 2016). This new lead could possibly give us a new common marker of tDCs.

What is the real involvement of IDO?

Furthermore, a well-known mechanism of Treg induction by tDCs is via the immunosuppressive enzyme IDO. IDO catabolizes the vital amino acid Trp into Kyn and its downstream metabolites. A depletion of the media in Trp is a key immune regulation mechanism used by Tregs in order to kill effector T cell by starvation. Moreover, kyn and its metabolites are themselves toxic for effector T cells (Belladonna et al., 2009). Our study of IDO showed that after differentiation, C15exoDCs expressed high levels of functional IDO. However, IDO expression crashed after maturation leading us to believe that the C15exoDCs are IDO negative. A possible explanation could be linked to the maturing agent, *E.Coli* LPS. Studies have shown that LPS-matured DCs show an increase in IDO expression (Bubnoff et al., 2011). Thus, if LPS maturation is IDO-dependent, it would be in the interest of the tumour exosomes to block IDO and subsequently DC maturation. Moreover, Tregs thrives on the effects of IDO, Trp depletion and increased levels of kyn can enhance Treg suppressive function (Yan et al., 2010). Interestingly, tDCs and Tregs engage in cross talk, IDO expression can be induced by Tregs via factors such as IL-10, TGF-β and IFNγ (Fallarino et al., 2003; Janikashvili et al., 2011). Thus, the resurging levels of IDO after the DC/T cell co-culture could be induced by the T cells.

Although IDO is considered a hallmark of tDC phenotype, not all tDCs express IDO. Our findings can be correlated with other studies that use IDO-negative tDCs in clinical trials which are currently underway in kidney transplantation (Moreau et al., 2012). Therefore, IDO is not an exclusive tDC marker. Regardless, it is now clear that IDO plays a key role in the maintenance of tolerance. This is why IDO is now a sought-after immune checkpoint making it a target of choice for upcoming immunotherapy-based cancer treatments. Pre-clinical studies showed very encouraging results with a notable decrease of naive T cell conversion into Tregs when IDO1 was inhibited with what was later known as Epacadostat (INCB024360) (Liu et al., 2010). In a B16 melanoma model, tumour rejection was enhanced by coupling Epacadostat with anti-CTLA4, PD-1 or PD-L1 as the secretion of IL-2 and proliferation of CD8 T cells were restored (Spranger et al., 2014). However, recent data from the Epacadostat/Keytruda phase 3 clinical trial (Keynote-252/ECHO-301 - NCT02752074) has shown no major benefit of inhibiting IDO in melanoma, although the rational seemed to predict otherwise. Thus, IDO biology needs to be further elucidated to (i) understand why anti-IDO therapies failed and (ii) develop more efficient IDO-based treatments for cancer (Muller et al., 2018). Nevertheless, other enzymes that metabolise Trp such as IDO2 and Trp 2,3 dioxygenase (TDO) are also being evaluated as

possible alternatives. IDO2 shows high polymorphism and is only expressed in 50% of Caucasians. In light of the recent failing of IDO1 inhibitors, the 1-MT IDO1 inhibitors seems to better inhibit IDO2 (Metz et al., 2007). Furthermore, it has been described that TDO is highly expressed by cancer cells and so far TDO expression in human immune cells has not been observed (Pilotte et al., 2012). Further studies regarding these two alternatives are still needed. Nonetheless, anti-IDOs have sparked other interests as they seem to enhance the effects of chemotherapy (Muller et al., 2005). To date, this phenomenon is still difficult to explain.

MoDC vs MoMac differentiation: Do exosomes favour a polarisation?

Monocytes are the precursors of MoDCs but also of MoMac. The CD14hi CD16low monocytes give rise to MoDCs whereas CD14low CD16hi monocytes give MoMacs. In our work, we have considered this difference by preferentially isolating the CD14hi monocytes. Nevertheless, the question can be asked, do NPC exosomes favour a polarisation over another? Just as DCs, macrophages also have a regulatory counterpart named tumour-associated macrophages (TAMs). TAMs highly resemble M2 type macrophages, or alternatively activated macrophages, which are activated by Th2 cytokines like interleukin IL-4, IL-10 and IL-13. They have been found to infiltrate the tumour, interact with surrounding T cells and contribute to the progression of cancer (Yang and Zhang, 2017). Indeed, tumour cells are known to polarise macrophages into a regulatory phenotype (Wang et al., 2016a). This can be mediated by soluble factors like IL-10 and HIF-1α in a hypoxia context (Ambade et al., 2016). M2 macrophages are recruited to the hypoxic tumour regions via oncostatin M and Eotaxin (CCL11), which are secreted by tumour cells (Tripathi et al., 2014). But just as DCs, TAMs can derive from different origins and thus gives rise to different variants of TAMs that mostly depend on the expression of STAT (Van Overmeire et al., 2014). A culture of NPC exosomes with monocytes in different polarising conditions, followed by a transcriptomic and flow cytometry analysis of these cells, would give us answers to this question.

How NPC-derived exosomes and DC react to anti-tumour Photodynamic Therapy?

PDT is an innovative new treatment of cancer that specifically targets tumour cells that incorporate a photosensitising drug (Dupont et al., 2017; Korbelik and Dougherty, 1999). An increasing number of cancers are benefiting from PDT treatment; the key is to find the appropriate photosensitiser for each cancer type. Nevertheless, after illumination, tumour cells die by apoptosis

and release tumour antigens. PDT treatment has been associated with an increased anti-tumoural and anti-bacterial effect (Castano et al., 2006; Reginato et al., 2014). And thus, PDT not only targets and kills tumour cells but also seem to induce an anti-tumoural vaccination via the massive release of tumour antigens taken up by already present DCs (Zheng et al., 2016). Jung et al. confirmed just this as lysates of PDT-treated tumour cells were incubated with DCs and showed a greater decrease in tumour growth than PDT alone (Jung et al., 2012). Our team studies the effects of PDT on the immune system and it would be interesting to determine how DCs react to PDT. Also, to see if exosomes are changed by PDT and if they are still able to induce tDCs.

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Annexes

X. List of publications:

Published

- Radhia M'kacher, Corina Cuceu, Mustafa Al Jawhari, Luc Morat, Monika Frenzel, Grace Shim, Aude Lenain, William M. Hempel, Steffen Junker, Theodore Girinsky, Bruno Colicchio, Alain Deterlen, Leonhard Heidingsfelder, Olivier Moralès, **Sarah Renaud**, Zoé Van de Wyngaert, Eric Jeandidier, Nadira Delhem and Patrice Carde. *The transition between telomerase and ALT mechanisms in Hodgkin lymphoma and its predictive value in clinical outcomes*. *Cancers 2018 May 30;10(6). pii: E169. doi: 10.3390/cancers10060169.*

- Laurissa Ouaguia, Dhaffer Mrizak, **Sarah Renaud**, Olivier Moralès et Nadira Delhem. Control of the inflammatory response mechanisms mediated by natural and induced regulatory T-cells in HCV-, HTLV-1&, and EBV-associated cancers*. Mediators Inflamm. 2014 Nov 30;2014, 2014:e564296.*

Submitted

- **Sarah Renaud**, Alexandre Quilbe, Benjamin Hennart, Clémentine Deschutter, Chantal Havet, Anthony Lefebvre, Zachary Fitzpatrick, Joshua Mason, Delphine Allorge, Nadira Delhem* and Olivier Moralès* (* Equally contributing authors) Nasopharyngeal Carcinoma Exosomes Facilitate the Induction of Regulatory T Cells by generating Tolerogenic Dendritic Cells. [*Submitted to Oncoimmunology]*

- Dhafer Mrizak*, Rami Mustapha*, **Sarah Renaud**, Natahlie Martin, Hayet Rafa, Perdo Roman Puché, Fei Fei Liu, Toshiro Niki, Kwok-Wai Lo, Véronique Pancré, Yavn de Launoit, Pierre Busson, Olivier Moralès^{+} and Nadira Delhem^{+} (*,$^{+}$ Equally contributing authors). EBV-derived Peptides Provide Protection to Nasopharyngeal Carcinoma by Inducing Resistance against immunosuppressive exosomes. [*Submitted to Cancer Immunology Research*]

- Clara Milhem*, **Sarah Renaud***, Céline Ingelaere, Olivier Moralès, Alexandre Quilbé, David Pasquier, Xavier Mirabel and Nadira Delhem (* Equally contributed authors) Case Report: Immune Regulation after High dose Hypofractionated RadioTherapy leading to Complete Clinical Response in a Renal Adenocarcinoma Patient [*Submitted to Journal of Immunology Research*]

In preparation:

- **Sarah Renaud***, Romuald Bonnet*, Hayet Rafa, Hajer Jerraya, Eric Lartigo, Alexandre Quilbe, Olivier Moralès, Nadira Delhem (*Equally contributing authors). Effect of Triple Negative Breast cancer–derived exosomes on the surfacing of M2 type macrophage.

- Rami Mustapha*, Dhafer Mrizak*, **Sarah Renaud**, Clément Barjon, Chantal Samson, Toshiro Nikki, Fei Fei Liu, Olivier Dellys, Pierre Busson, Olivier Moralès^{+} and Nadira Delhem^{+} (*,$^{+}$ Equally contributing authors). Galectin-9 blocking: A Novel Cancer Immunotherapeutic Approach

Targeting Regulatory T Cells.

The transition between telomerase and ALT mechanisms in Hodgkin lymphoma and its predictive value in clinical outcomes

Radhia M'kacher, Corina Cuceu, Mustafa Al Jawhari, Luc Morat, Monika Frenzel, Grace Shim, Aude Lenain, William M. Hempel, Steffen Junker, Theodore Girinsky, Bruno Colicchio, Alain Deterlen, Leonhard Heidingsfelder, Olivier Moralès, **Sarah Renaud**, Zoé Van de Wyngaert, Eric Jeandidier, Nadira Delhem and Patrice Carde.

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MDPI

Article

The Transition between Telomerase and ALT Mechanisms in Hodgkin Lymphoma and Its Predictive Value in Clinical Outcomes

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Abstract: *Background*: We analyzed telomere maintenance mechanisms (TMMs) in lymph node samples from HL patients treated with standard therapy. The TMMs correlated with clinical outcomes of patients. *Materials and Methods*: Lymph node biopsies obtained from 38 HL patients and 24 patients with lymphadenitis were included in this study. Seven HL cell lines were used as in vitro models. Telomerase activity (TA) was assessed by TRAP assay and verified through hTERT immunofluorescence expression; alternative telomere lengthening (ALT) was also assessed, along with EBV status. *Results*: Both TA and ALT mechanisms were present in HL lymph nodes. Our findings were reproduced in HL cell lines. The highest levels of TA were expressed in CD30−/CD15− cells. Small cells were identified with ALT and TA. Hodgkin and Reed Sternberg cells contained high levels of PML bodies, but had very low hTERT expression. There was a significant correlation between overall survival ($p < 10^{-3}$), event-free survival ($p < 10^{-4}$), and freedom from progression ($p < 10^{-3}$) and the presence of an ALT profile in lymph nodes of EBV+ patients. *Conclusion*: The presence of both types of TMMs in HL lymph nodes and in HL cell lines has not previously been reported. TMMs correlate with the treatment outcome of EBV+ HL patients.

Keywords: Hodgkin Lymphoma; telomerase; alternative lengthening of telomeres; EBV

Control of the inflammatory response mechanisms mediated by natural and induced regulatory T-cells in HCV-, HTLV-1&, and EBV-associated cancers.

Laurissa Ouaguia, Dhaffer Mrizak, **Sarah Renaud**, Olivier Moralès et Nadira Delhem.

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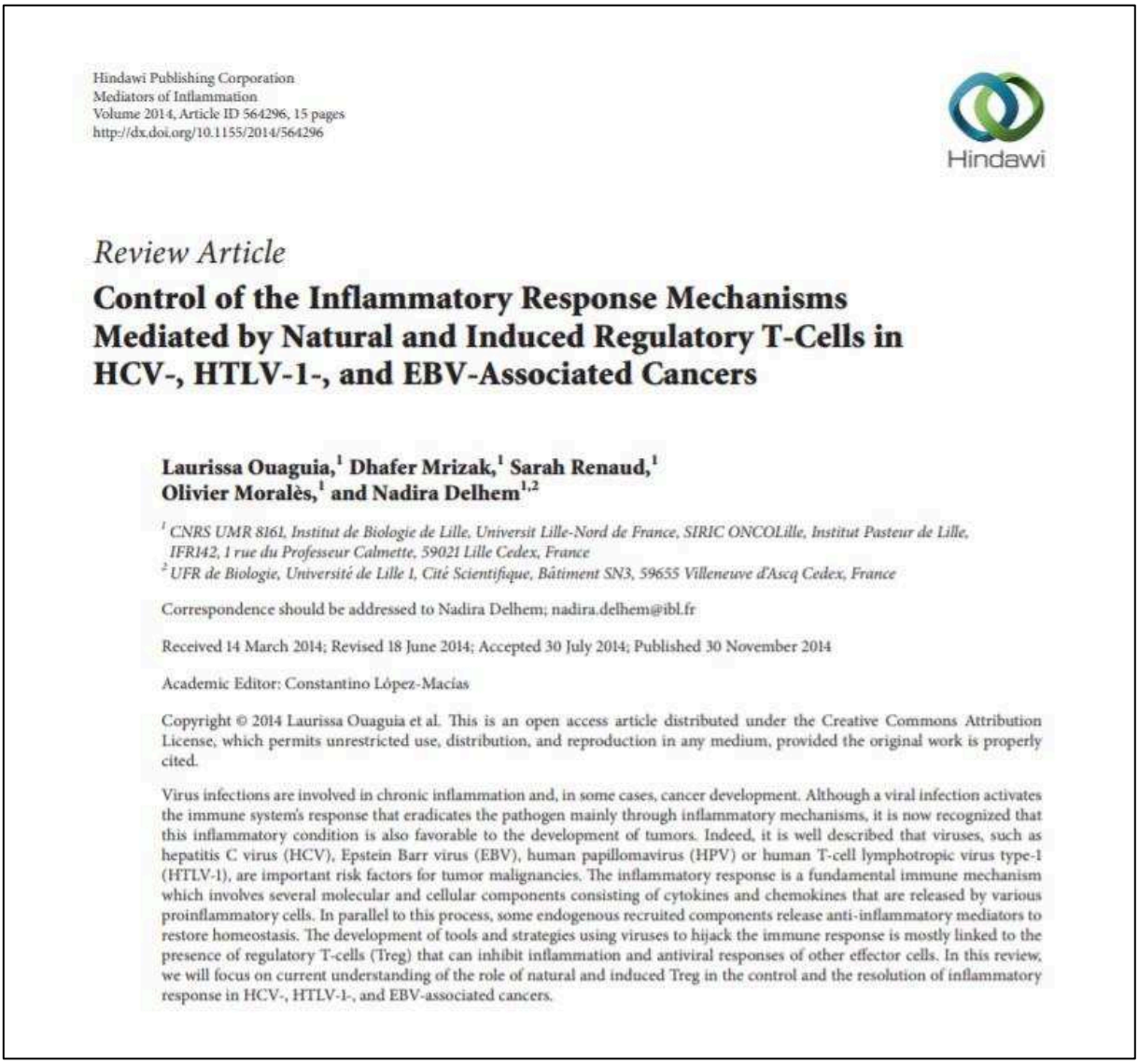

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Review Article

Control of the Inflammatory Response Mechanisms Mediated by Natural and Induced Regulatory T-Cells in HCV-, HTLV-1-, and EBV-Associated Cancers

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Virus infections are involved in chronic inflammation and, in some cases, cancer development. Although a viral infection activates the immune system's response that eradicates the pathogen mainly through inflammatory mechanisms, it is now recognized that this inflammatory condition is also favorable to the development of tumors. Indeed, it is well described that viruses, such as hepatitis C virus (HCV), Epstein Barr virus (EBV), human papillomavirus (HPV) or human T-cell lymphotropic virus type-1 (HTLV-1), are important risk factors for tumor malignancies. The inflammatory response is a fundamental immune mechanism which involves several molecular and cellular components consisting of cytokines and chemokines that are released by various proinflammatory cells. In parallel to this process, some endogenous recruited components release anti-inflammatory mediators to restore homeostasis. The development of tools and strategies using viruses to hijack the immune response is mostly linked to the presence of regulatory T-cells (Treg) that can inhibit inflammation and antiviral responses of other effector cells. In this review, we will focus on current understanding of the role of natural and induced Treg in the control and the resolution of inflammatory response in HCV-, HTLV-1-, and EBV-associated cancers.

Nasopharyngeal Carcinoma Exosomes Facilitate the Induction of Regulatory T Cells by generating Tolerogenic Dendritic Cells

Sarah Renaud, Alexandre Quilbe, Benjamin Hennart, Clémentine Deschutter, Chantal Havet, Anthony Lefebvre, Zachary Fitzpatrick, Joshua Mason, Delphine Allorge, Nadira Delhem* and Olivier Moralès*
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[Submitted to OncoImmunology]

Context and Objectives

Nasopharyngeal carcinoma (NPC) is characterised by an immunosuppressive microenvironment governed by regulatory T cells (Tregs) and tumour exosomes (TEXs). The aim of this study is to evaluate the impact of NPC exosomes on the generation of tolerogenic dendritic cells (tDCs) able to induce Tregs which contribute to the tumour's immune escape.

Methodology and results

First, we analysed the phenotype, cytokine secretion and expression of indoleamine 2,3-dioxygenase in exosomes-exposed DCs (exoDCs) to determine their semi-mature state. Then, a functional assay of exoDCs co-culture with either total CD3+ T cells or naive CD4+ T cells helped us show that exoDCs induce effector T cell anergy and convert naive T cells into Tregs. Finally, chemoattraction assays revealed that TEXs recruit immature DCs and tDCs via CCL20.

Conclusion

Taken together, our results strongly suggest that the presence of NPC exosomes favours the emergence and recruitment of semi-mature tDCs. Thus, these promising results should open new prospects for antitumor immunotherapies based on the inhibition of factors involved in the emergence of Tregs.

Proof of submission to OncoImmunology

OncoImmunology

Nasopharyngeal Carcinoma Exosomes induce Regulatory T Cells by producing Tolerogenic Dendritic Cells

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Abstract:	Nasopharyngeal carcinoma (NPC) is characterised by an immunosuppressive microenvironment governed by regulatory T cells (Tregs) and tumour exosomes (TEXs). This study is to evaluate the impact of NPC exosomes on the generation of tolerogenic dendritic cells (tDCs) able to induce Tregs which contribute to the tumour's immune escape. First, we analysed the phenotype, cytokine secretion and expression of indoleamine 2,3-dioxygenase in exosomes-exposed DCs (exoDCs) to determine the semi-mature state. Then, a functional assay of exoDCs co-culture with either total CD3+ T cells or naïve CD4+ T cells helped us show that exoDCs induce effector T cell anergy and convert naïve T cells into Tregs. Finally, chemoattraction assays revealed that TEXs recruit immature DCs and tDCs via CCL20. Taken together, our results strongly suggest that the presence of NPC exosomes favours the emergence and recruitment of semi-mature tDCs. Thus, these promising results should open new prospects for antitumor immunotherapies based on the inhibition of factors involved in the emergence of Tregs.
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Introduction

Nasopharyngeal carcinoma (NPC) is a malignant epithelial tumour of the upper aerodigestive tract (Wei et al., 2010). Foci of prevalence are essentially found in the Guandong region, South-East Asia and Northern Maghreb, indicating multidimensional etiologic factors such as genetic predispositions or environmental factors with the Epstein-Barr virus (EBV) as the main incriminating agent [1–4].

One of the major features of NPC is the presence of an important lymphoid infiltrate within the primary tumour, including mostly T cells (LT), and a minority of B lymphocytes (LB), monocytes, dendritic cells (DCs) and eosinophils [5]. Nevertheless, the intratumoural infiltration of immune cells is not sufficient to prevent the development of cancer. It would therefore seem that the NPC cells resist their elimination and escape the immune response by subverting the immune system for their own benefit [6]. In this sense, an immunosuppressive tumour microenvironment (TME) has been described in NPC to be dominated by two major players. On the one hand, regulatory T cells (Tregs), which can represent up to 12% of tumour-infiltrating lymphocytes and in the periphery [7]. On the other hand, the tumour exosomes produced in large amount by NPC cells that also possess immunosuppressive properties [8].

Exosomes are nanovesicles of 30-120nm in diameter that arise from late endosomal compartments dubbed multivesicular bodies (MVBs). Exosomes are secreted into the extracellular media when the MVB membrane fuses with the cell plasma membrane. Exosomes are secreted by most cell types but especially immune cells, epithelial cells and tumour cells [9]. They can be found in various biological fluids such as plasma, urine, saliva or cerebrospinal fluids [10–12]. They play a role of intercellular mediators by transmitting the molecules they carry. Their composition and functions are directly linked to the nature of the cell from which they come from. The nature of their cargo can range from mRNAs or microRNAs [13], MHC I molecules, antigens etc. [14–16]. NPC-derived exosomes has been well described as major contributors of tumour progression [17]. They carry specific immunosuppressive markers such as galectin 9 and the EBV viral protein LMP1, which give NPC exosomes a suppressor role, favourable to the tumour's immune escape (Keryer-Bibens et al., 2006). In addition, we recently described in the team that NPC exosomes were able to recruit and activate Tregs, further promoting the immunosuppressive TME [18] .

Tregs are the major players in immune tolerance and are able to regulate the immune response by suppressing the proliferation of effector cells [19,20]. Thus, a large amount of Treg is systematically associated with tumour progression and a poor prognosis in most of cancer [21]. Several types of Treg are described, among them: (i) natural Tregs (nTregs) (5 to 10% of circulating CD4 +

lymphocytes) are educated within the thymus and (ii) induced Tregs (iTregs) (30% of all circulating Tregs) which are peripherally differentiate from naive CD4 Helper T cells [22,23]. One of the main cellular mechanism of Treg differentiation involves tolerogenic dendritic cells (tDCs), which also contribute to the immune escape of cancer [24].

DC have a pivotal role between the innate and adaptive immune system. At their immature state, they present the feature of a sentinel cells able to detect and phagocyte danger molecules. This leads to the uptake of the antigen and initiate their maturation process where they will become professional antigen presenting cell (APC), form an immunological synapse with lymphocytes within proximal draining lymph node and give rise to an appropriate immune response. Mature DCs (mDCs) increase the expression of their antigen-charged MHC II, co-stimulatory molecules (CD40, CD80, CD83, CD86), and C-C chemokine receptor type (CCR)- 7 allowing the migration to draining secondary lymphoid organs [25]. In the case of cancer, DCs give rise to anti-tumoral T-helper 1 cells and release cytokines such as IL-12, TNFα and IFNγ. Finally, the activation of T cells leads to an effective and potent immune response. However, there are always two sides to every story. Tolerogenic dendritic cells (tDCs) are the immunosuppressive counterpart of immunostimulatory mature DCs. tDCs regulate the immune response and stops us from developing autoimmune diseases by enabling immune peripheral tolerance. tDCs can be characterized by their semi-mature phenotype, their ability to block effector T cell proliferation whilst enhancing Treg function and differentiation [26].

We have previously demonstrated that exosomes produced by NPC tumour cells promote the recruitment and suppressive activity of Tregs, thus contributing to the escape of NPC to immune surveillance [18]. In this study, we aimed now to evaluate the impact of NPC tumour exosomes on the maturation of human monocyte-derived DCs (MoDCs). Our hypothesis being that in the particular context of NPC, tumour exosomes favour the emergence of tDCs that subsequently contribute to the worsening NPC by promoting tumour escape. In this study, we were able to complete the description of such direct and indirect effect of NPC-exosomes on the development of immunosuppressive cells. We showed here that NPC-exosomes are not only able to block the DC maturation process, but also that these semi-mature DCs are effective tDCs that induce a strong immunosuppressive environment by secreting IL-10 and TGF-β, and able to prime naive T cells to become effective regulatory T cells. NPC-exo are also able to attract iDCs and tDCs using the CCL-20/CCR-6 axis, thus reinforcing the immunosuppressive environment. Description of the interactions between NPC-exo and DCs is a new additional mechanism of how tumour-derived exosomes mediate immune evasion of NPC.

Materials and Methods

Mouse model and tumor exosomes generation

Patient-derived EBV-positive xenografted tumors (C15) were permanently propagated by subcutaneous passage in SCID mice as previously described [8]. 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 the IPL (Lille, France).

C15 tumor exosomes were isolated from in vitro conditioned culture media (C15exo). Control exosomes were isolated from plasma samples given by healthy donors (HDExo). Conditioned culture media were prepared by collagenase and DNAse dispersion of cells from the C15 xenografts and incubation of these cells for 48h in low serum clarified conditions (allowing collection of C15).

Exosome isolation

Isolation of exosomes from healthy donors, C15 xenografts or from NPC plasma was done by differential centrifugation and flotation on a D_20/sucrose cushion as previously reported [8]. Plasma and serum sample were initially diluted at a ratio of 1:50 and 1:2 respectively in phosphate buffered saline medium (PBS). Exosomes are stored at -80°C until further use.

Exosome protein dosage

Isolated exosomes were diluted 1:100 and total protein concentration was quantified according to the manufacturers' instructions (Biorad, USA) based on a Bradford dye-binding method and using Ascent™ Software. Exosomes were then added to cell culture at 5µg/mL.

Exosome characterization using Electron Microscopy

Around 2µg of Exosomes were diluted in PBS and placed on formvar-coated 200 mesh copper grids rinsed and contrasted with 2% phosphotungstic acid (PTA). Grids were rinsed with PBS once. Grids were then fixed with glutaraldehyde and contrasted with 2% PTA. Images were obtained with a Hitachi H7500 transmission electron microscope (TEM) equipped with a wide-field 1024x1024-pixel digital camera from AMT Advantage HR (Elexience, France).

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

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. PBMCs were isolated from peripheral blood samples by density gradient centrifugation

using Ficoll (GE healthcare, Uppsala, Sweden) and leucosep™ according to the manufacturer's instructions (Dutscher, France).

Immune cell isolation

Monocytes were isolated from PBMCs using a positive selection CD14+ isolation kit (Miltenyi Biotec, Berlin, Germany) according to the manufacturer's instructions with an average purity of 95%. The CD4+ naive T cells were also isolated by a negative selection using a CD4+ naive T cell isolation kit (Miltenyi Biotec, Berlin, Germany) with an average purity of 90%. After co-culture with DCs, T cells were isolated using a CD3+ selection kit (Miltenyi Biotec, Berlin, Germany) with an average purity of 75%.

Dendritic cell generation

$1x10^6$ monocytes were cultivated in complete RPMI 1640 medium (Life Technologies, UK) with 10% FCS (Life Technologies, UK) that was previously clarified, as well as 2mM L-glutamine and 50µg/mL of gentamycine.

Seeding of monocytes was done in 6-well plates (Nunc, Denmark) containing 10^6monocytes/mL in 4 conditions: only monocytes, with 25ng/ml GM-CSF and 10ng/ml IL-4 (PeproTech Inc, Rocky Hill, USA) for negative and positive controls, and 5µg/mL of exosomes from healthy donors or C15 for testing. On the 5th day of culture, the media was replaced with new medium containing ± IL-1β and TNFα as maturation controls. An alternate maturating agent also used at times, LPS (lipopolysaccharide of E. Coli, Sigma Aldrich, St.Louis, USA) at 100ng/mL. As a tolerogenic dendritic cell control Vitamin D3 (Life Technologies, UK) and Dexamethasone (Sigma Aldrich, St. Louis, USA) were added to the fresh media at day 5 of culture for 48 hours respectively at 39ng/mL and 393ng/mL. After 7 days of culture the maturation state of the DCs were evaluated. Cells were recovered and assessed by flow cytometry (BD FACS Canto II) for cell surface maturation markers. Supernatants were recovered at day 5 and 7 of the culture and stored at -80°C until further use.

Mixed Leukocyte Reaction (MLR) - DC/T cell co-culture

CD4+ CD45RA naive T cells were obtained from PBMCs exclusively from healthy donors by positive selection isolation kits (Miltenyi Biotec). Heterologous mature DCs were co-cultured with either naive T cells at a 1:5 ratio, at 10^6 cells/mL in complete RPMI for 5 days. At day 5 of the co-culture supernatants were firstly recovered and frozen at -80°C until further use. Then dead cells were eliminated using a Dead Cell Removal kit and afterwards T cells were purified with a positive

CD3 selection kit with an average purity of 95%. Both kits were used accordingly to the manufacturer's instructions (Miltenyi Biotec, Berlin, Germany).

DC phenotype was then assessed by flow cytometry (BD FACS Canto II) for cell surface intracellular markers. The state of maturation was determined using monoclonal mouse anti-human CD11c-BioBlue, -CD14-VioGreen, -CD40-PE, -CD80-APC, -CD83-PE-Cy7, -CD86-FITC, HLADR-PerCP and DC-SIGN-APC-Cy7 (Miltenyi Biotech, Germany).

The same co-culture procedure was also carried out using the conditioned DCs and total CD3+ T cells. CD3+ cells were isolated using a positive selection kit (Miltenyi Biotec, Berlin, Germany). Cells were co-cultured for 2-3 days with a ratio of 1:20 (DC:CD3+), at 10^6 cells/mL in complete RPMI. DCs were irradiated at 50Gy before the culture. T Cell proliferation was then tested using radioactive thymidine and supernatant was stored at -80°C until further use.

Mixed Leukocyte Reaction (MLR) - suppression assay

Suppressive activity of T cells after co-culture with DCs was measured by their ability to inhibit the proliferative response of autologuous PBMCs in a MLR. Assays were set up with a mixture of T cells: PBMCs (1:4; 1:2 and 1:1) in a round bottom 96-well plate (Corning Costar) and cultured for 48 and 72hours. Cells were activated with plate-bound anti-CD3 (10ng/mL) mAb, incubated at 37°C for 2 hours before the culture and soluble anti-CD28 (10ng/mL) mAb (Clinisciences, Montrouge, France) was added at the time of the culture. There are three types of T cells: $T_{IL1\beta TNF\alpha \text{ or } LPS}$, T_{HDexo} and T_{C15exo}. 5.10^4 irradiated (5000 cGy) autologous PBMCs were used as antigen-presenting cells. Culture with freshly isolated autologous Tregs, without irradiated PBMCs, activated PBMCs, T cells or activating agents were also done as proliferation controls.

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). Each proliferation assay was carried out in triplicate and estimated in count per minute (cpm) and results were normalized compared with the positive control.

Phenotypical analysis of T cells by flow cytometry

After DC-LT co-culture, the expression of iTreg surface antigens was tested using monoclonal mouse anti-human antibodies such as CD4-VioBlue, -CD18-FITC, -CD49b-PE-Cy7, -LAG3-PE and –CTLA4-APC. Monoclonal mouse anti-human CD4-BioBlue, -CD25-FITC, -CD127-PE-Cy7 (Miltenyi Biotech, Germany) were used for detection of cell surface antigens on pTregs. FoxP3-

APC intracellular staining was achieved with FoxP3 staining Buffer kit (Miltenyi Biotech, Berlin, Germany).

PBMC and exosomes co-culture

Exosomes' immunomodulatory properties were tested by a proliferation assay. They were set up with cultures of 10^5 PBMCs in contact with exosomes at 5µg/mL in a round bottom 96-well plate and cultured for 120 hours. The cells were activated with plate-bound anti-CD3 (1µg/mL) mAb, incubated at 37°C for 2 hours before the culture and soluble mouse anti-human CD28 mAb (100ng/mL) (Clinisciences, Montr uge, France) was added at the time of the culture. 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). Each proliferation assay was carried out in triplicate and estimated in count per minute (cpm).

Flow cytometry analysis

Cell immunophenotype was analysed by flow cytometry using BD FACSCanto II flow cytometer powered by FACS DIVA software. After their harvest, cells were washed with Phosphate-Buffered Saline (PBS) (GIBCO-Life technologies) and labeled with fluochrome-conjugated mAbs. For each assay, the appropriate isotypic control mAbs were used for positive signal setting. Finally, median fluorescence intensity (mfi) data were analysed with FlowJo software.

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 (14 000rpm, 30 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.

Briefly, proteins were separated by SDS-PAGE electrophoresis using gradient pre-casts gels (4-12% gradient, Bis-Tris, Invitrogen) in standard conditions except when planning detection of CD63, which require non-reducing conditions. Then proteins were transferred on PVDF membranes (Hybond™-C Extra, Amersham Biosciences, UK). The latter was blocked for 2 hours at room temperature in blocking buffer containing 2% casein, 0.1% Tween20 (Sigma-Aldrich) and PBS (1X), and then incubated

overnight at 4°C with primary antibodies directed against : rat anti-human Grp94 mAb 1:500 (ADI-SPA-850-F, Enzo Life Sciences), mouse anti-human HLA-Drα mAb 1:200 (sc-53449, Santa Cruz Biotechnology), mouse anti-human CD63 mAb 1:100 (ab59479, Abcam, UK), mouse anti-human LMP1 mAb 1:4 (S12) (Kerafast), mouse anti-human CD81 mAb, rabbit anti-IDO (Cell Signaling Technology, USA), Mouse anti-cyclophilin B mAB 1:400 (Provided by Dr. Fabrice Alain's team), mouse anti-human Galectin-9-CT-L1 mAb 1:200 (was 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 Western Lightning® Plus-ECL, Enhanced Chemiluminescence Substrate kit (PerkinElmer, Boston, MA, USA) and read in luminescent BioRAd ChemiDoc XRS+ machine using the ImageLab software.

High Performance Liquid Chromatography (HPLC)

The substrate and product i.e. L-Tryptophan (Trp) and Kynurenine (Kyn) of IDO were quantified in the supernatant of all the culture conditions at day 5 and 7. Supernatants were immediately frozen at -80°C for further analysis. Samples were tested by Professor Delphine Allorge's Laboratory of Toxicology at University Hospital of Lille, France. Concentrations of Trp and Kyn were assayed using an analytical procedure based on electrospray ionization liquid chromatography-tandem mass spectrometry (LC-ESI/MS/MS). This procedure was developed according to previously published methods, with slight modifications [27]. One hundred microliter of supernatants or culture medium were analyzed after the addition of 100µl acetonitrile containing tryptophane-D5 at 50 000 nM, as an internal standard. The samples were mixed and centrifuged and the supernatant (100µl) was added to deionized water (500µl). Fifteen microliters of this mixture were injected onto an UPLC-MS/MS system (Xevo TQ-S Detector, Waters, Milford, USA) equipped with an Acquity HSS C18 column (Waters, Milford, USA). Ions of each analysed compound were detected in a positive ion mode using multiple reaction monitoring. MassLinks software (Waters) was used for data acquisition and processing.

ELISA

In order to dose the cytokine secretion of the DCs and T cells at day 5, 7 and 12 of culture, the supernatants were tested for the secretion of the following cytokines: IL-10, TGF-β, IL-12p70, IL-6, TNFα and IFNγ (BD Pharmingen™, USA) and IDO (Uscn Life Science). The Enzyme-Linked ImmunoSorbent Assay (ELISA) method was carried out according to the manufacturer's recommendations (BD Pharmingem™). Results were expressed as the mean of normalized values for each well of a duplicate.

	Purified antibodies	Biotinylated antibodies
Anti-IL-10	IgG1 rat	IgG2a rat
Anti-IL-12p70	IgG1 mouse	IgG1 mouse
Anti-TGFβ	IgG2aRat	IgG2aRat
Anti-TNFα	IgG1 mouse	IgG1 mouse
Anti-IL-6	IgG1Rat	IgG2aRat
Anti-IFNγ	IgG1Mouse	IgG1Mouse

Briefly, purified primary antibodies were fixed in 96-well plates (MaxoSorb, NUNC, Denmark) overnight at 4°C for coating. After 4 washes in PBS 1X (Euromedex, France)-Tween (Sigma Aldrich, USA) 0.05%, plates were saturated with PBS-BSA 3% (Bovine Serum Albumin, Sigma-Aldrich®, USA) for 2 hour, at room temperature. Then, they were washed 3 times with PBS-Tween 0.05%. Culture supernatant were deposited in the plate and incubated overnight at 4°C. After 3 washes with PBS-Tween, anti-cytokine biotinylated detection secondary Ab (1µg/mL), was incubated for 90 minutes at room temperature. Followed by 3 washes, the reaction was amplified by adding streptavidin-peroxidase to 1/10000th (Interchim, UK) for 45 minutes at room temperature. After 4 washes, the plates were revealed by the addition of a solution of H_2O_2 (1/1000th) and Ortho-phenylenediamine Dihydrochloride (OPD) at 1mg/mL (Sigma-Aldrich®, USA) in development buffer. This reaction was stopped by addition of HCl (VWR, USA). The plates were then read at 492nm on the spectrophotometer (Multiskan EX, ThermoLabsystems, France) using Ascent™ Software.

Migration assay

The chemotaxis protocol was performed as previously described (23) using Boyden chambers (Neuroprobe) and 8µm pore polycarbonate filters (Nucleoprobe). The various populations of dendritic cells were harvested and suspended in RPMI 1640 at a concentration of 10^6cells/mL. RPMI only was used as a negative control. Tumor exosomes (5µg/mL) were harvested in the lower chamber either in the presence or absence of a blocking anti-CCL20 mAb (PeproTech, USA) at a concentration of 20µg/mL. For this, tumour exosomes were pre-incubated with the anti-

CCL20 for 2h at 37°C in 5% CO_2. The chemotaxis assay was performed in triplicates. The cells having migrated through the pores and into the lower well after 3h of incubations at 37°C in 5% CO_2, were counted three independent times.

Statistical analysis

Graphpad Prism 7 software was used for data treatment and statistical analysis. Ordinary one-way ANOVA statistical tests were carried out on all samples. Significance of p values are as following: $p>0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****), with $p<0.05$ being considered statistically significant and smaller p values highly significant.

Results

EV characterisation

To verify that isolated extracellular vesicles (EVs) are indeed exosomes, we analysed them by electron microscopy after PTA contrasting (Fig. 1A and B). The samples of EVs isolated from either healthy donor plasma or tumour cell supernatant are homogenous in nature, shape and correspond to the expected size of 30-120nm in diameter. The mean diameter of HDexo is 61nm and C15exo is 80.7nm.

EVs express the appropriate exosome markers by western blot testing (Fig 1C). Common exosomal marker CD63, HLADR were found on both samples, with HLA-DR found highly expressed on C15exo. Moreover, the heat shock protein grp94 was absent as expected for exosomes. Additionally, we sought to verify expression of NPC exosome marker LMP1, it was present on C15exo only.

Finally, as expected NPC exosomes suppress the proliferation of immune cells by 2 folds. Indeed, human PBMCs were activated and co-culture with C15 EVs for a proliferation assay (Fig. 1D). The proliferation of activated PBMCs is reduced in the presence of C15 EVs. All together, these analyses lead us to confirm that the EVs isolated are indeed exosomes. From now on, we will refer to the isolated EVs as exosomes.

Morphological and phenotypical characterization of MoDC cultured with C15 exosomes

In order to determine if the tumour exosomes have an impact on MoDC maturation, a phenotypical study is firstly carried out. The cells' morphology was analysed by photonic microscopy. After 2-day maturation with maturing agents IL-1β and TNFα (day 7), control mDCs show typical DC characteristics such as adherence to the culture plate, dendrites and growth packed together in clumps (Fig. 2A). These aspects are also found in the cells cultured with the HDexo (Fig. 2C). The control tDCs of dexamethasone and vitamin D3 show a drastically different morphology as the cells are either much longer and thinner or rounder with less dendrites (Fig. 2B). This is also the case for cell pre-cultured with C15exo (Fig. 2D). So, from a morphological view point, control tDCs are closer to C15exo DCs and control mDCs are more similar to HDexo DCs.

A phenotypical study of the cells was then carried out by a flow cytometry analysis of DC maturation markers in order to determine their maturation. After differentiation (day 5), there is no significant difference in the expression of CD86 and HLADR of C15exo-cultured cells and iDCs and tDCs. C15exoDCs show slightly higher mfi than iDCs and tDCs for CD40, CD80, CD83 and DC-SIGN (table 1). Following maturation (day 7), the mfi of C15exoDCs are lower for all the markers tested when compared to control mDCs. But compared to control tDCs, the expression of CD14are similar, except

for HLADR and DC-SIGN which are higher (table 2). Furthermore, overlays of a representative experiment are shown. Day 5 overlays show no significant differences between the culture conditions (Fig. 3A). Nevertheless, after maturation, the overlays clearly display that when the cells are pre-cultured with tumour exosomes the expression of some maturation markers are much lower (CD40, CD80, CD83). However, other maturation markers such as CD86 and HLADR show no major difference in intensity of expression when cells are cultured with C15exo. And the immature DC marker DC-SIGN, shows that there are two populations, one that expresses DC-SIGN at a similar level to mDCs and HDexo, and the other that expressed DC-SIGN at a very low level (Fig. 3B). Thus, the major differences discernible by flow cytometry are more observable after maturation and not before.

MoDCs cultured with NPC exosomes promote a suppressive micro-environnement through immunosuppressive cytokine secretion

The cytokine secretion of DCs was monitored after differentiation and maturation by ELISA dosage. Effector cytokines (IL-6, IL-12p70, TNFα and IFNγ) and regulatory cytokines (IL-10 and TGF-β) were studied. Although not significant, the trend of IL-10 secretion after differentiation shows an increase when the cells are cultured with C15exo compared to mDCs, tDCs and HDexoDCs (Fig. 4A). No significant difference in the levels of TGF-β secretion was discernible between the cell conditions (Fig. 4B). Moreover, the secretion of IL-6 does seem to be increased by the presence of C15exo, but monocytes display a higher secretion of IL-6 (Fig. 4C). IFNγ release does not seem to be changed by C15exo nor HDexo. However, control tDCs seem to slightly decrease their secretion compared to control iDCs (Fig. 4D.).

Furthermore, after maturation, the secretion of IL-10 strongly increases in control tDCs compared to control mDCs. This also seems to be the trend for C15exoDCs although not significant (Fig. 4bis E). However, unlike IL-10, tDCs show the lowest levels of TGF-β secretion, while other DCs pre-treated with exosomes show no difference (Fig. 4bis F). The dosage of the functional sub unit of IL-12, IL-12p70, displays a decrease in all cell conditions compared to mDCs, but most significantly for tDCs and C15exoDCs (Fig. 4bis G). IL-6 secretion is null for iDCs, and comparatively to mDCs, all other conditions seem to secrete less IL-6 (Fig.4bis H). Significantly, effector cytokine TNFα is less secreted by iDCs and tDCs whereas HDexoDCs and C15exoDCs release more TNFα compared to mDCs (Fig. 4bis I). Finally, IFNγ dosage shows no substantial difference although lower levels for iDCs and C15exoDCs seem to be the trend (Fig. 4bis J). The analysis of the secretome allows to better understand the immune environment that the DCs promote.

NPC exosomes induced semi-mature DCs that modulate IDO expression and function

Another characteristic of tDCs is their expression of the immuno-regulatory enzyme IDO. For this, IDO expression was determined in cultured DCs by Western Blot, the biologic activity of IDO was assessed by HPLC dosage of its substrate and product (Trp and Kyn respectively) and the enzyme was dosed in the supernatant of DC:naïve T cell co-culture.

After the 5 first days of culture, the highest levels of active IDO is C15exoDCs which can be correlated to the Western Blot. These levels are higher even than the control tDCs which are more similar to the iDCs or HDexoDC conditions (Fig.5A.1 and 2). Furthermore, IDO activity after maturation is significantly lower for iDCs and C15exoDCs (Fig.5B.1). IDO expression shown by Western Blot can corroborate this for iDCs and C15exo (Fig.5B.2). No measurable secretion of IDO was detected by ELISA after differentiation nor maturation (data not shown). Moreover, after 5 days of DCs/naïve T cells co-culture, the Trp/Kyn ratio is over 20,000 folds higher for C15exoDC/LT comparatively to mDC/LT and HDexoDC/LT (Fig.5C1). IDO secretion levels are three folds higher for C15exoDC/LT than mDC/LT (Fig.5C2). These results show an increase in IDO levels and activity after differentiation of monocytes into iDCs. This peak is followed by a crash of IDO activity after maturation but picks up exponentially after DCs are cultured with naive T cells.

C15exoDCs inhibit the proliferation of effector T cells and favour an immunosuppressive microenvironment

To confirm the tolerogenic status of DCs pre-cultured with C15exo, their suppressive function is tested by a culture of the DCs with total autologous CD3+ T cells (Fig. 6A). The suppression assay shows that after 48 hours of co-culture, the C15exoDCs significantly decrease the proliferation of T cells. Their suppression rate is intermediate between the tolerogenic control and mature control. Moreover, the tDC/LT control displays no difference in IL-10 secretion with the mDC/LTs. Although not significant, IL-10 seems to be more secreted when the DCs were pre-treated with C15exo. (Fig.6B). Likewise, TGF-β dosage showed similar results, where LTs cultured with C15exoDCs seem to secrete slightly more TGF-β than the controls mDC (Fig.6C). The immunostimulatory cytokines IL-12 was only secreted by C15exoDC/LTs and iDC/LTs (Fig. 6D). Thus, the DCs pre-treated with C15 tumour exosomes decrease CD3+ T cell proliferation and seem to enhance the overall secretion of IL-10, TGF-β and IL-12 comparatively to mDCs.

MoDCs cultured with C15exo give rise to iTregs

The undeniable proof that C15 exosomes give rise to tDCs is to show that a co-culture with naive T cells generates regulatory T cells and anergise effector T cells. A phenotypical study of T cells obtained after co-culture by flow cytometry aimed at determining if they expressed induced regulatory T cell (iTreg) markers. A higher frequency of a CD4+, CD25hi, CD127- FoxP3+ population of cells was observed after co-culture with C15exoDCs (9,47%) comparatively to control mDCs and HDexoDCs (5.13% 5.04% respectively) (Fig.7A). To further characterise obtained T cells, the secretion of immunoregulatory cytokines (IL-10 and TGF-β) and an inflammatory cytokine (IL-6) of these cells was measured by ELISA. Indeed, in the supernatant of C15exoDCs/nTL co-culture, the secretion of IL-10 and TGF-β was higher comparatively to mDC/nTL (1.5 folds and 2.3 folds respectively) (Fig.7B and C). The release of IL-6 was significantly decreased by 5 folds in C15exoDC/nTL culture compared to mDC/nTL (Fig.7D). Finally, the suppressive function and anergy of the induced T cell is verified by suppression and proliferation assays. When the obtained T cells are cultured with activated human PBMCs, the T cells pre-cultured with C15exoDCs show an intermediate level of suppression between the Treg/PBMCs and mDC TL/PBMCs (Fig.7E). Significantly, this intermediate level was also observed when studying the proliferation of obtained T cells alone (Fig.7F). Hence, the T cells induced after culture with tumour exosomes-exposed DCs exhibit Treg markers, secrete more immunosuppressive cytokines (IL-10 and TGF-β) and less inflammatory cytokines (IL-6), seem to decrease PBMC proliferation and display a very low proliferation rate verging on anergy.

Chemoattraction of DCs by C15exo

Chemoattraction assays were carried out in Boyden chambers at day 5 and 7 of culture. At day 5, three different DCs were cultured in the chamber: control iDCs (black), control tDCs (grey) and C15exoDCs (light grey). Interestingly, adding C15exo to the lower chamber always significantly attracts higher numbers of immature or tolerogenic cells, with a 30 folds increase (Fig. 8A). If the C15exosomes have been previously cultured with a CCL20 blocking antibody, the attraction seems partly abrogated for control iDCs and tDCs (both a 1.1 folds decrease). However, the blocking antibody decreased the attraction of C15exoDCs by 2.3 folds. Furthermore, after maturation, mDCs, tDCs an C15exoDCs were tested for chemoattraction by C15exo. Remarkably, the C15exo in the lower chamber only seem to attract the control tDCs and the C15exoDCs, but not the control mDCs (migration index 6.67, 18.9 and 0.14 respectively). And once more, the attraction by C15exo is inhibited by the blocking of CCL20, most significantly for C15exoDCs (13.5 folds decrease). The same trend is observable for tDCs, with a 20.2 folds decrease (Fig. 8B). Thus, C15exo only seem to

attract immature, tolerogenic and C15exoDCs and this effect can be disrupted by blocking CCL20 found on the C15 exosomes.

Discussion

Dendritic cells are most commonly known for their role in the promotion of the adaptive immune response through T cell activation. However, tDCs regulate our immune response, stopping us from developing autoimmune diseases and enabling immune peripheral tolerance. tDCs can be characterized by their semi-mature phenotype and their ability to block effector T cell response whilst promoting Treg function and differentiation. The semi-mature tDC phenotype is notably characterized by lower expression levels of some co-stimulatory markers such as CD40, CD80, CD83 and CD86 and higher DC-SIGN, a c-type lectin receptor, which serves as a maker of iDCs. Indeed, our results showed that the C15exoDCs has a similar morphology to control tDCs, and unlike mDCs. This first indication was correlate with flow cytometry testing of the membrane co-stimulatory markers expression. C15exoDCs exhibited a lower expression of CD40 and CD83 but similar levels of CD86 and HLADR. These observations show that our C15exoDCs have a semi-mature phenotype, which is in line with the literature [28]. However, a general tDC phenotype still needs to be better defined. A unique tDC marker would considerably aid in the study and understanding tDC biology as well as their involvement in autoimmune diseases, transplantation and cancer. Although this seems to be an increasingly utopic goal with tDC phenotype depending on multiple factors such as origin and generation conditions [29]. Additionally, an increased secretion of anti-inflammatory cytokines as well as lower secretion of pro-inflammatory cytokines are tDCs characteristics and contribute to the development of Tregs and anergy of effector T cells. The dosage of cytokines secretion by C15exoDCs confirms this premise with higher levels of IL-10 secretion, but not TGF-β. These findings correlate with many studies that show tDCs secrete more IL-10, a cytokine that favours tolerance, impairs effector T cells [30,31] and maintains Tregs suppressive functions [32–35]. However, TGF-β has a more pleiotropic role in both inflammation and tolerance. It is important for Treg biology, but in this work TGF-β secretion does not seem to be altered by C15exo [36]. Moreover, C15exoDCs released lower levels of effector cytokines IL-6, IL-12 and IFNγ.

Furthermore, a well-known mechanism of Treg induction by tDCs is via the immunosuppressive enzyme IDO1. IDO catabolizes the vital amino acid Tryptophan into Kyn and its downstream metabolites. A depletion of the media in Trp is a key immune regulation mechanism used by Tregs in order to kill effector T cell by starvation. Moreover, Kyn and its metabolites are themselves

toxic for effector T cells [37]. Our study of IDO showed that after differentiation, C15exoDCs expressed high levels of functional IDO. But IDO expression crashed after maturation leading us to believe that the C15exoDCs are IDO$^-$ after maturation. A possible explanation could be linked to the maturing agent, *E.Coli* Lipopolysaccharide (LPS). Studies have shown that LPS-matured DCs show an increase in IDO expression [38]. Thus, if LPS maturation is IDO-dependent, it would be in the interest of the tumour exosomes to block IDO and subsequently DC maturation. Moreover, Tregs thrives on the effects of IDO, Tryptophan depletion and increased levels of kyn, which can enhance Treg suppressive function [39]. Interestingly, tDCs and Tregs engage in cross talk, IDO expression can be induced by Tregs via factors such as IL-10, TGF-β and IFNγ [40,41]. Thus, the resurging levels of IDO after the DC/T cell co-culture could be induced by the T cells. This also comforts the idea that the naive T cells have indeed become Tregs after exposure to C15exoDCs. Although IDO is considered a hallmark of tDC phenotype, not all tDCs express IDO. Our findings can be correlated with other studies that use IDO-negative tDCs in clinical trials which are currently underway in kidney transplantation [42]. Therefore, IDO is not an exclusive tDC marker. Regardless, it is now clear that IDO plays a key role in the maintenance of tolerance. This is why IDO is now a sought-after immune checkpoint making it a target of choice for upcoming immunotherapy-based cancer treatments. However, recent data from the Epacadostat/Keytruda phase 3 clinical trial (Keynote-252/ECHO-301 - NCT02752074) has shown no major benefit of inhibiting IDO in melanoma, although the rational seemed to predict otherwise. Thus, IDO biology needs to be further elucidated to (i) understand why anti-IDO therapies failed and (ii) develop more efficient IDO-based treatments for cancer [43].

To go further, another aspect of the NPCexo-treated DCs that could be studied is their metabolism. Indeed, a characteristic change in metabolism can help to identify tDCs as they favour oxidative phosphorylation and fatty acid oxidation whereas mDCs preferentially use glucose as a source of carbon [44]. Testing and characterising the metabolism of C15exoDCs could further validate their tDC status.

It has long been described that tDCs maintain immune tolerance through induction of T cell anergy and the generation of Tregs as they are themselves highly potent immune regulators. The only way to be sure the C15exoDCs are indeed tolerogenic was to verify such functions. MLR co-culture of C15exoDCs with either naive T cells or total CD3+ T cells shows that they do indeed give rise to immunosuppressive T cells and induce T cell anergy. Given that co-cultures were carried out in a heterologous system, one could expect that the mismatch should above all induce a non-self-immune response. Nevertheless, the induction of Tregs and inhibition of effector T cells proliferation is a testament of the tolerogenic potency of NPC-exo treated DCs.

Knowing this, it is not surprising that many types of cancers subvert Tregs and tDCs to favour immune evasion. Indeed, high levels of circulating Tregs are associated with a poor prognosis in most cancers [24,45–48]. Thus, in cancers such as nasopharyngeal carcinoma (NPC) tDCs as well as Tregs all contribute to the establishment of an immunosuppressive tumor microenvironment (TME) [49,50]. In addition to this, our team has recently shown that tumor-derived exosomes also favour immune evasion [18]. Indeed, NPC exosomes favor T cells anergy, the generation of Tregs as well as potentiating Tregs suppressive function. However, the exact nature of the induced Tregs (iTregs) remains to be verified. Preliminary results show FoxP3+ iTregs that secrete high amounts of IL-10. Nevertheless, FoxP3+ iTregs generated *in vitro* are not very well described in the literature and little is known about them [51,52]. To further confirm the phenotype of induced Tregs, other markers need to be studied. For example, it was found that other myeloid suppressive cells, regulatory macrophages, can be induce by a TIGIT+ FoxP3+ iTreg population. Furthermore, this population depends on IDO expression, IL-10 and TGF-β secretion which corresponds well to our findings [53].

Furthermore, previous work in our team demonstrated that NPCexo can recruit Tregs at the tumour site in a CCL20-dependant manner [54]. Therefore, to determine if the C15exo could also recruit iDCs or tDCs to the tumour site in the same way it recruits Tregs, we carried out Boyden chamber assays. And indeed, chemoattraction tests found that C15exo preferentially recruit iDCs, tDCs and C15exoDCs but not control mDCs. Moreover, the use of an α-CCL20 blocking antibody partly or completely abrogated the chemoattraction, meaning that C15exo's attraction of iDCs and tDCs is also CCL20-dependant. These results corroborate with the literature that CCR6, the CCL20 receptor, is highly expressed on iDCs and is key their localisation [54]. The CCR6/CCL20 axis allows iDCs recruitment across tissues [55] and our unpublished data indicates that C15exoDCs express high levels of CCR6 (data not shown). Interestingly, it was shown that NPC patients show high levels of CCL20, secreted by the tumour and found on NPC exosomes. A meta-analysis carried out on 2429 NPC patients concluded that high levels of CCL20 and CCR6 was associated to a poor prognosis and could be novel attractive new drug candidates for the treatment of NPC [56].

Given that our study has shown that C15exo favour tolerance via DCs, the next step would be to determine by which pathway this disruption occurs. A possible candidate could be galectine-9 which is also specifically found on NPC exosomes and is involved in Treg suppressive function and would thus, promote the progression and immune escape of NPC [8,57]. However, preliminary results from our team has shown that recombinant human Gal-9 does not inhibit DC maturation but in fact enhances it (unpublished data). This observation correlates with work from another team that described the same phenomenon [58]. Nevertheless, this needs further investigations and it would be more rational to inhibit Gal-9 found on NPC-exo. Another possible target is LMP1, the viral oncoprotein that is

specifically found on NPC exosomes. Indeed, LMP1 is dysregulated in many cancers as it is involved in many major cell pathways such as proliferation and survival [59]. Interestingly, it has also been shown that LMP1 favours immune suppression [60]. To test this lead, blocking LMP1 on NPC-exo would determine if it is involved in NPC-exo mediated tDC induction.

This study has shown for the first time that tumour derived exosomes contribute to NPC immune evasion via induction of tolerogenic dendritic cells. This indirect mechanism enhances the emergence and functions of regulatory T cells, and thus contribute to the immunosuppressive tumour microenvironment. Yet again, tumour exosomes seem to be a major player in tumorigenesis and are of great interest as therapeutic targets. We are now one step closer to deciphering the immune evasion mechanisms put in place by the tumour although there are many others still to discover. At this time of great promise for anticancer immunotherapy, better understanding tumour immune evasion helps us better understand immunotherapy and develop new drugs.

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Figures

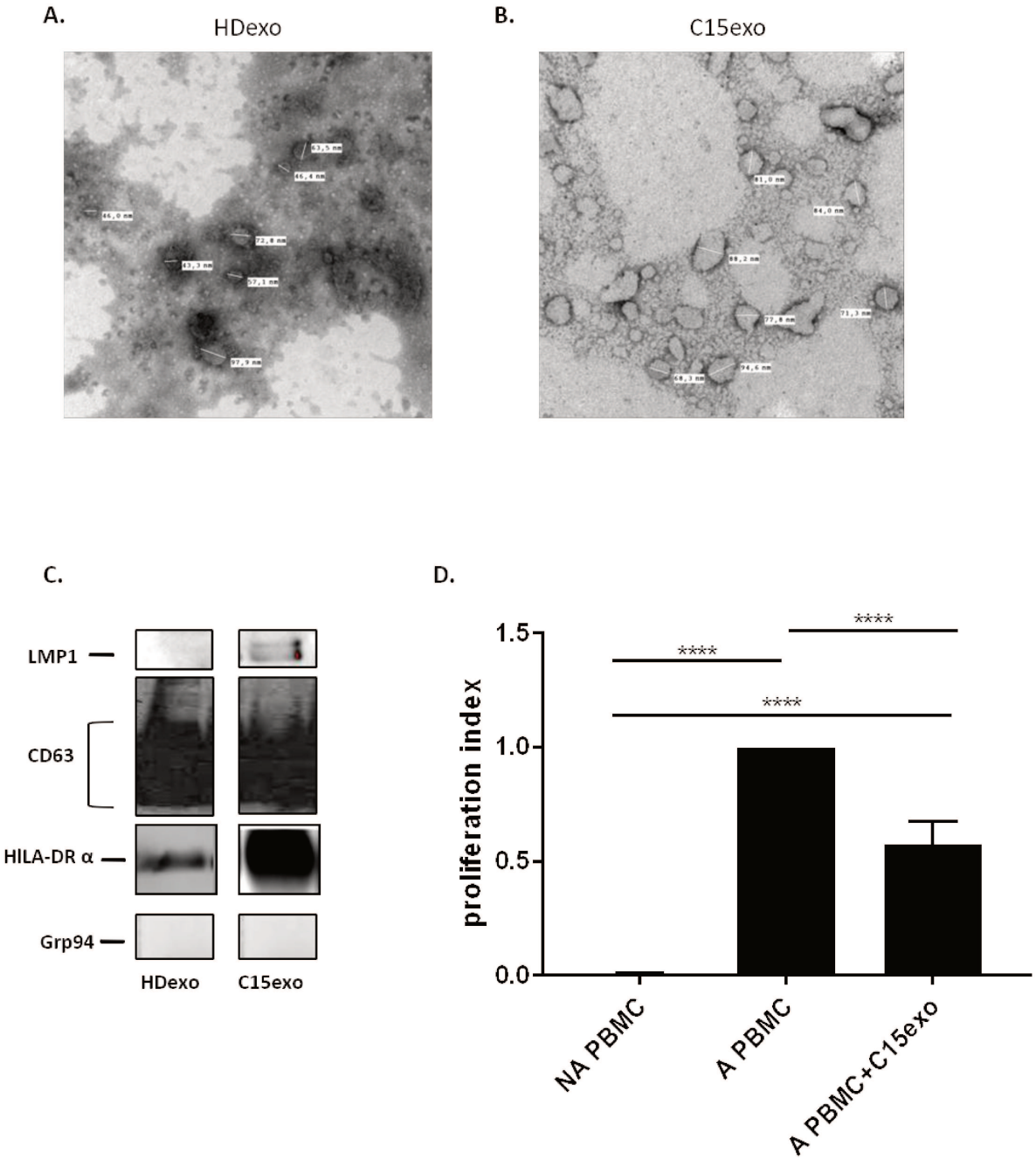
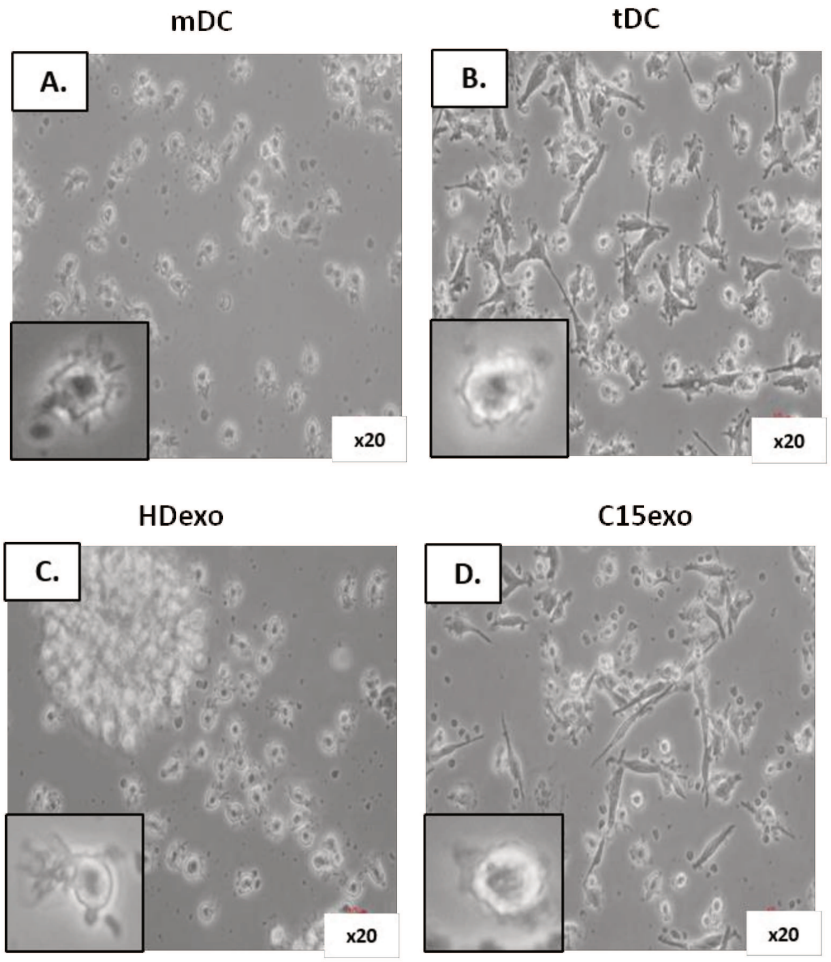


Figure 1



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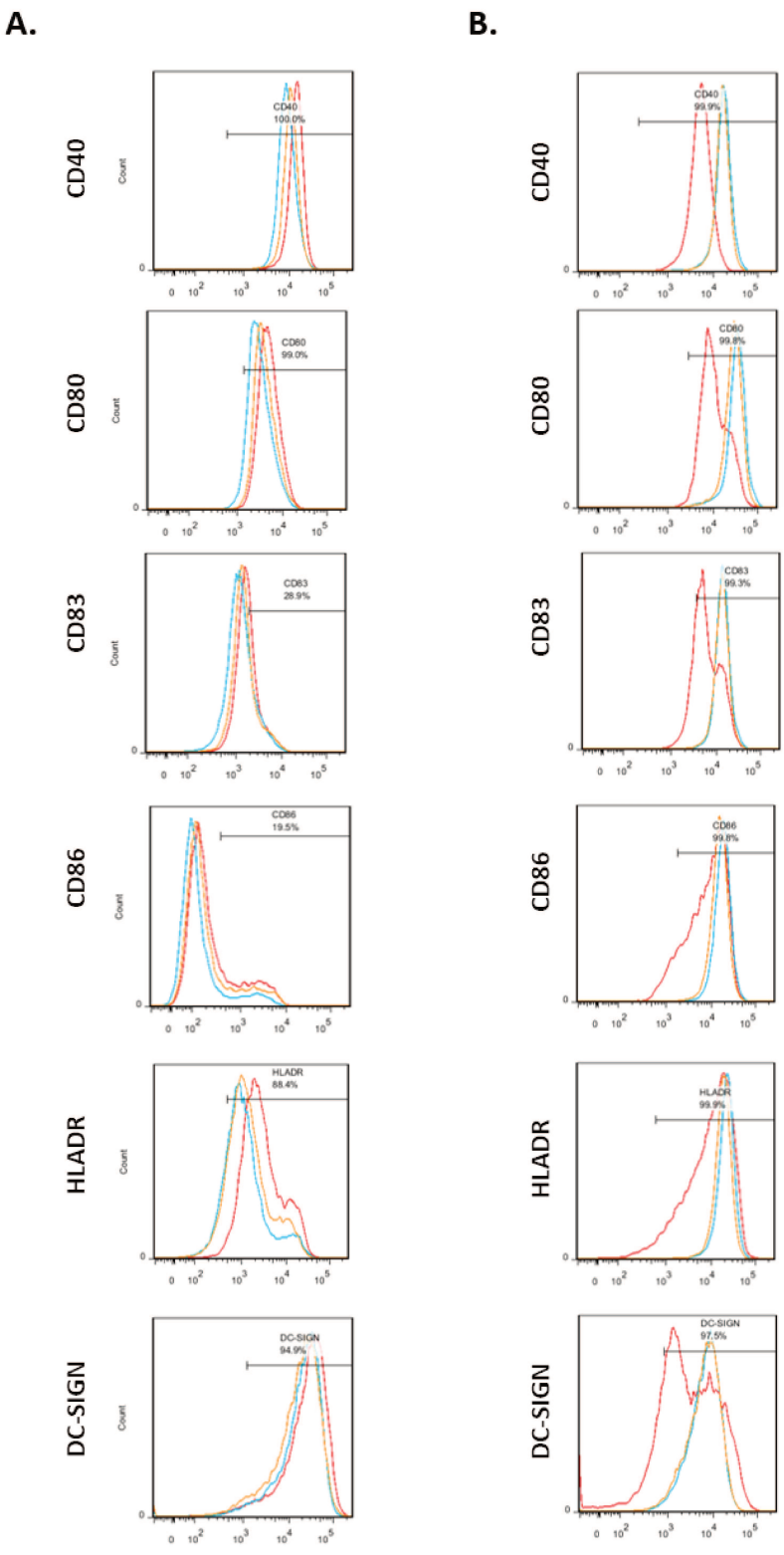

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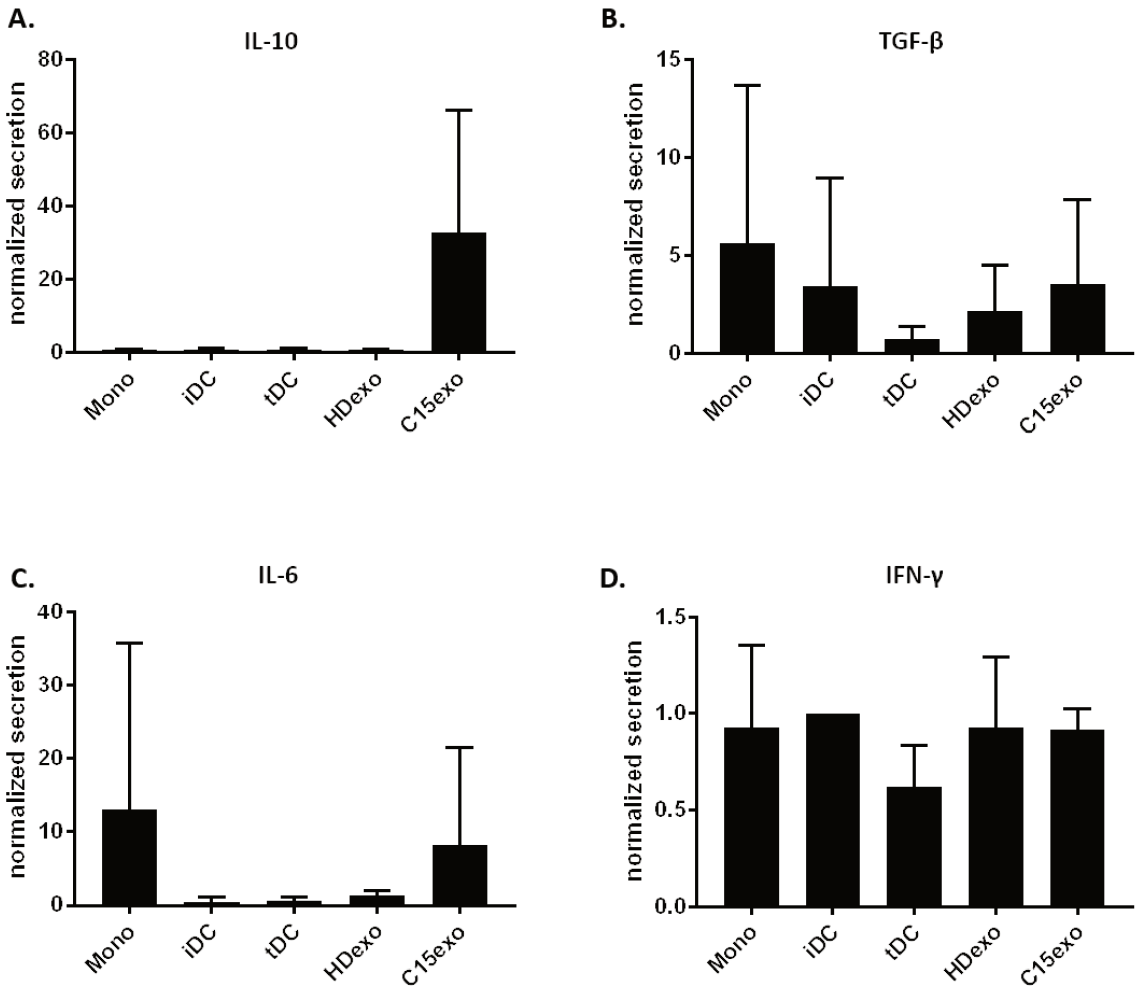
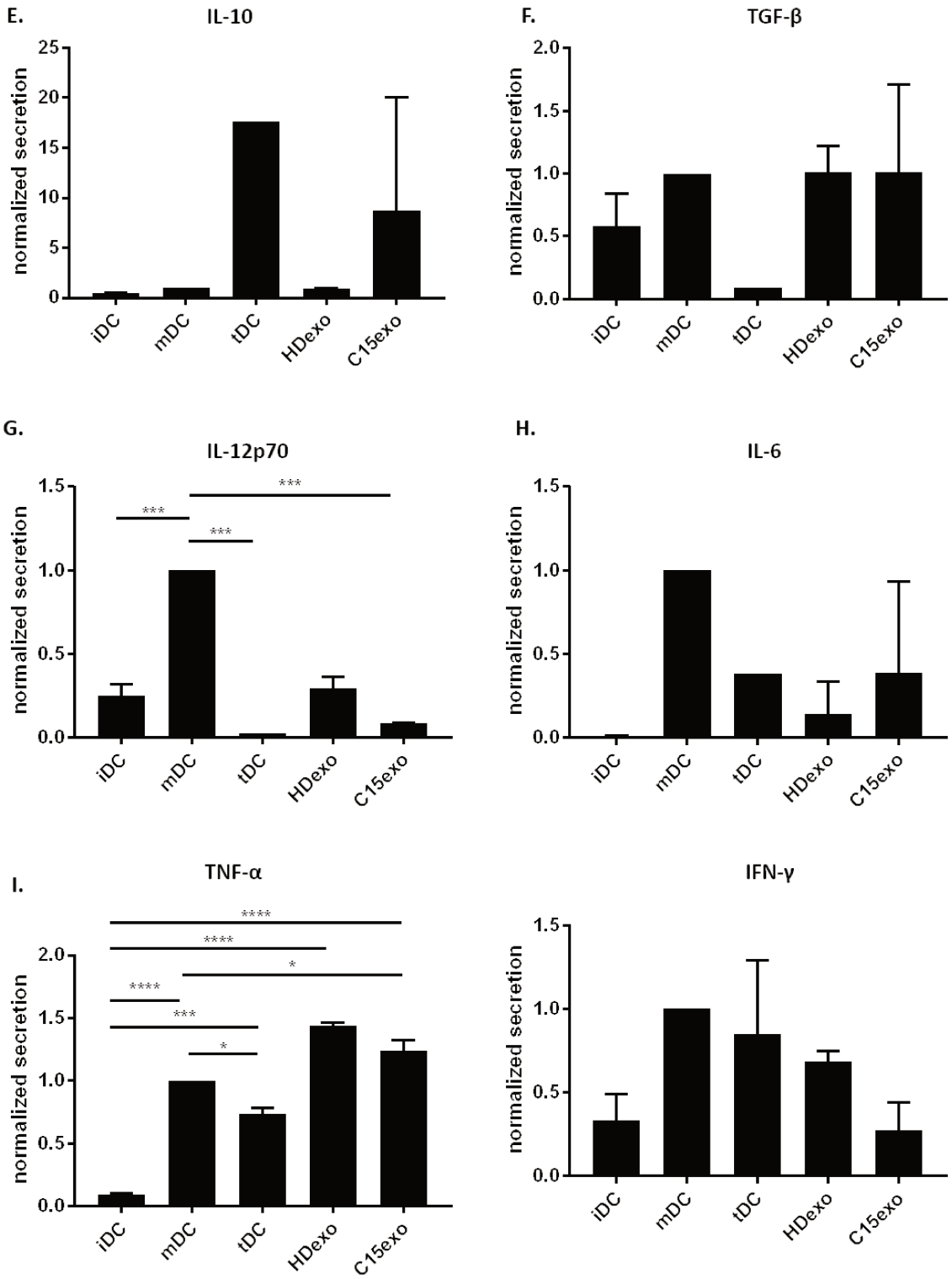


Figure 4



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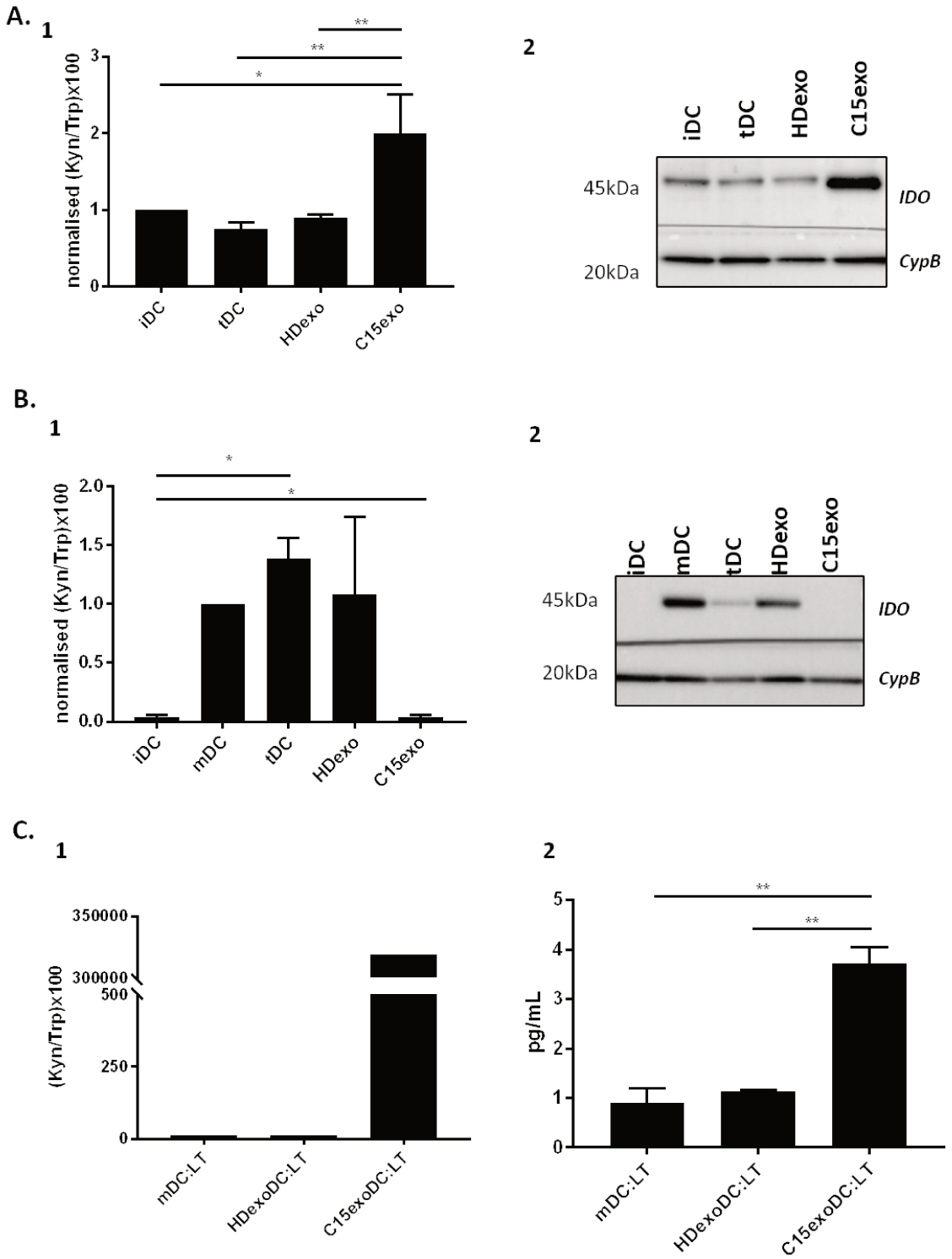

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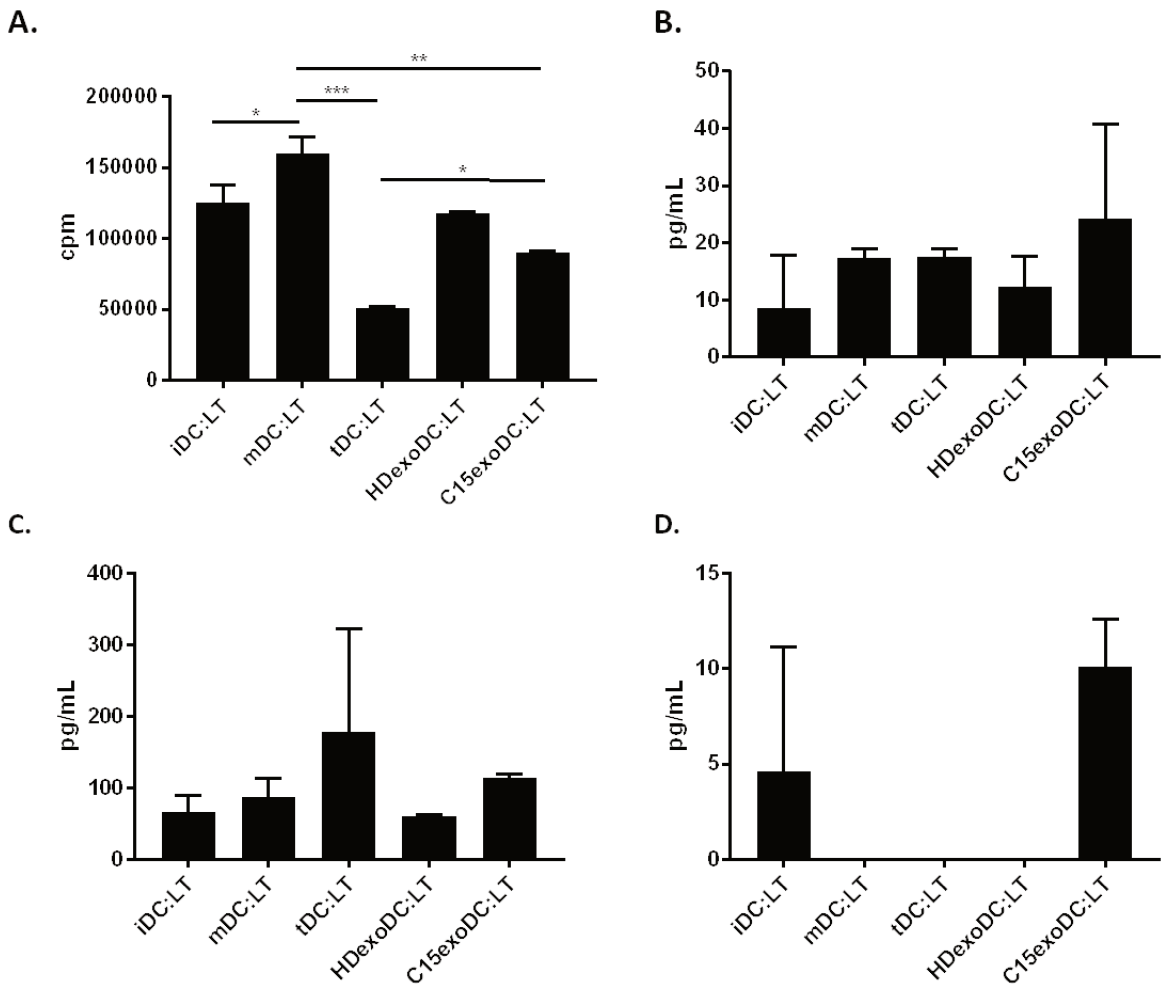

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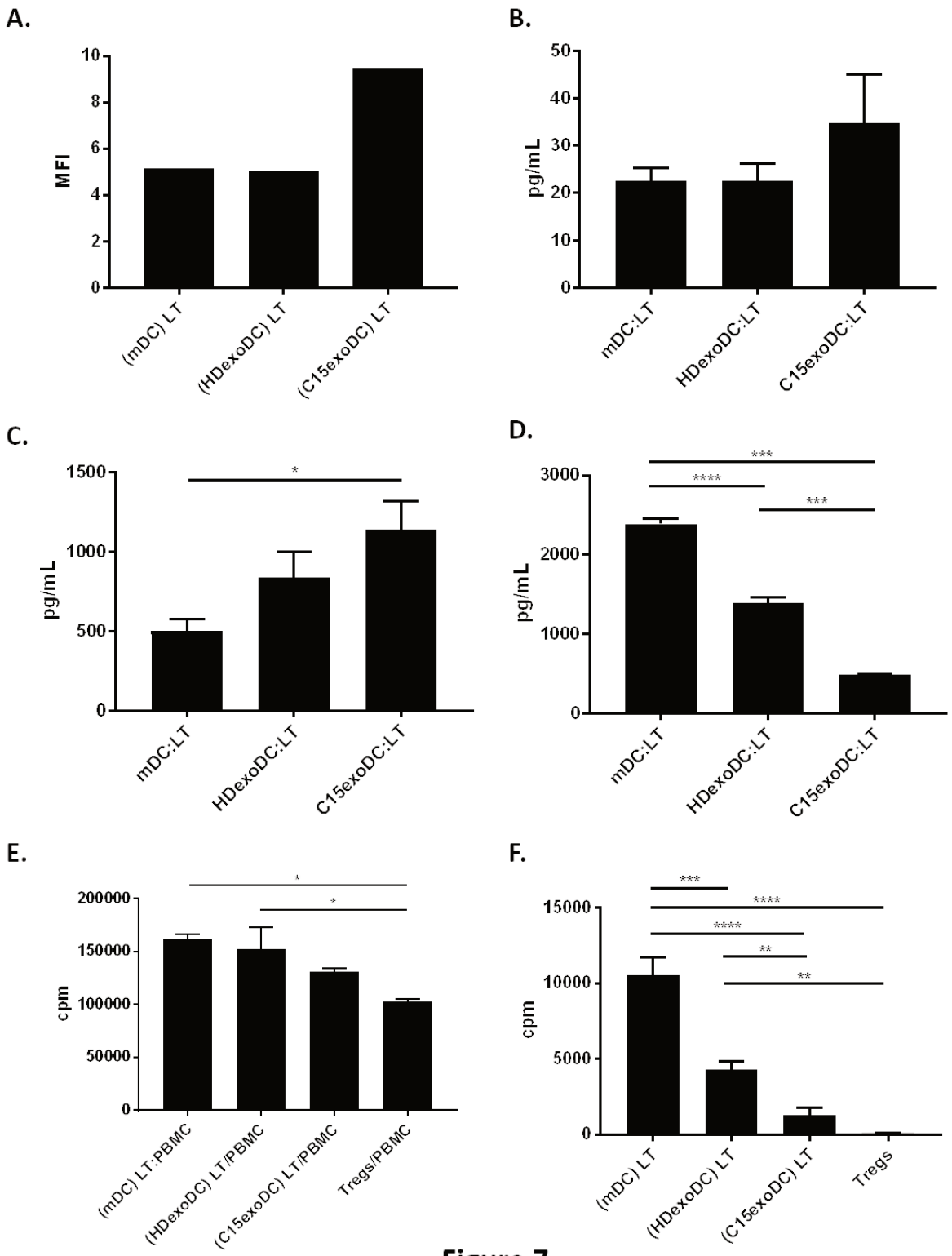
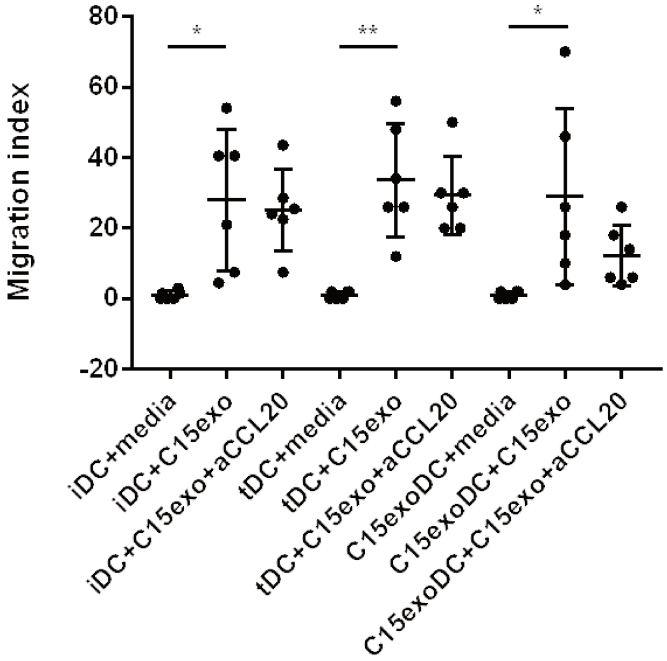


Figure 7

A.



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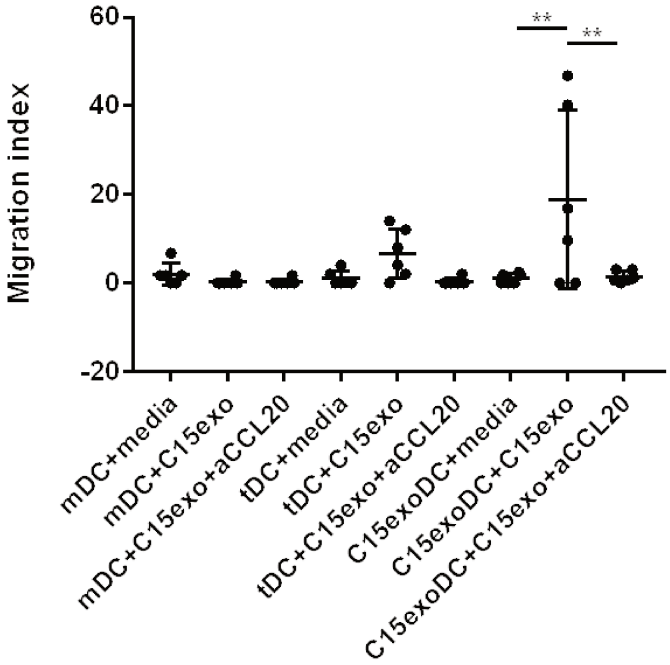

Figure 8

Legends of figures

Figure 1. Phenotypical and functional suppressive analyses of nasopharyngeal Carcinoma-derived exosomes (C15exo) and healthy donor exosomes (HDexo). **A.** Electron microscopy analysis of HDexo and **B.** C15exo suspension contrasted with 2% Phosphotungstic acid (PTA). **C.** Western Blot study of HDexo and C15exo. **D.** Functional assay of exosomes (HDexo or C15exo) co-cultured with human PBMCs. The immunosuppressive function of C15exo were tested, results expressed in cpm, n= 4, $p<0.0001$.

Figure 2. Morphological study of MoDCs after 7 days of culture in the presence or absence of various exosomes. DCs were matured with cytokines TNFα and IL-1β for the last 48hours of culture. **A**. Photonic image of mature DCs **B**. tolerogenic DCs **C.** MoDCs cultured with either HDexo **D.** or with C15exo.

Figure 3. Cell surface expression of maturation markers on MoDCs. The expression of maturation markers was determined **A.** after differentiation with GM-CSF+IL-4: iDCs (red) ± HDexo (blue) or C15exo (orange) (Day 5) **B.** The maturation state was determined after a 2-day maturation with TNFα and IL-1β: mDCs (blue) ± HDexo (red) or C15exo (orange) (Day 7). Representative overlays of two independent experiments.

Figure 4. Dosage of cytokine secretion by MoDCs after differentiation. The secretion of various cytokines was dosed by ELISA in the culture supernatant after differentiation. Secretion of suppressive cytokines **A.** IL-10 and **B.** TGF-β, as well as effector cytokines **C.** IL-6 and **D.** IFNγ were quantified.

Figure 4bis. Dosage of cytokine secretion by MoDCs after maturation. The secretion of the following cytokines were dosed by ELISA in the culture supernatant after maturation. **E.** IL-10 secretion, **F.** TGF-β secretion, **G.** IL-12p70 secretion, **H.** IL-6 secretion, **I.** TNFα secretion and **J.** IFNγ secretion. The results are normalized with the control condition, iDC for day 5, and mDC for day 7. IL-10, TGF-β, IL-6, IL-12p70 n=2 ($p<0.001$), TNFα ($0.05<p<0.0001$) and IFNγ n=1, tDCs n=1.

Figure 5. IDO expression on DCs and HPLC dosage of Tryptophane metabolites. These tests were carried out **A.** after differentiation, **B.** after maturation (n=2) or **C.** after 5 days of co-cultivating DCs with naive T cells (n=1). For this, **(1)** HPLC dosage of Tryptophane metabolites (Trp/Kyn)x100), **(A.2 and B.2)** testing of IDO expression by Western blotting and **(C.2)** an ELISA dosage of secreted IDO (pg/mL) were carried out, n=1 ($0.05<p<0.01$).

Figure 6. Suppressive function of irradiated DCs pre-cultured with exosomes. A. A suppression assay of CD3+ T cells co-cultured for 48h with DCs that were pre-cultured ± exosomes (HDexo or C15exo), results shown in cpm, n=1. Cytokine secretion was quantified in the co-culture supernatant by ELISA, **B.** IL-10, **C.** IL-12 and **D.** TGF-β were dosed in pg/mL, n=1 ($0.05<p<0.001$).

Figure 7. Characterisation of induced T cells following DC/naïve T cell co-culture. After 5 days of co-culture of naive T cells with conditioned DCs (± HDexo or C15exo), **A.** a flow cytometry analysis of Treg markers (CD4+ CD25hi CD127- FoxP3+) and dosage of **B.** IL-10, **C.** TGF-β and **D.** IL-6 secretion by ELISA were performed. **E.** The suppressive function of obtained T cells was determined by a co-culture of the cells with heterologous activated PBMCs for 72h. **F.** The proliferation of the T cells alone was determined after 48 hours by radioactive-thymidine incorporation. Proliferation results are expressed in cpm, n=1, ($0.05<p<0.0001$).

Figure 8. Chemoattraction of various treated DCs by C15exo. Chemoattraction assays were carried out in Boyden chambers. Cells were cultured with culture media only or with C15exo (5µg/mL) ± hu-αCCL20 blocking antibody (20µg/mL). **A.** Chemoattraction of iDCs, tDCs and C15exoDCs by C15exo was tested after differentiation. **B.** Chemoattraction of mDCs, tDCs and C15exoDCs by C15exo was tested after maturation. Results were obtained after three independent blind counts. Chemoattraction is expressed as a migration index using the cells cultured with culture media only as a reference condition, n=1 ($0.05<p<0.01$).

Tables

Table 1. Cell surface expression of maturation and phenotypic markers of MoDCs after differentiation (Day 5). All median fluorescence intensity (mfi) values were normalised with the corresponding isotypic control, n=4.

	CD40	CD80	CD83	CD86	HLA-DR	DC-SIGN
Mono	4,3 ± 3,45	6,47 ± 3,22	2,4 ± 1,37	2,36 ± 1,38	21,76 ± 9,64	3,46 ± 2,17
iDC	55,98 ± 36,51	8,37 ± 4,92	2,45 ± 1,23	1,36 ± 0,66	15,11 ± 13,71	57,69 ± 21,69
tDC	53,01 ± 36,08	9,85 ± 1,78	1,82 ± 0,72	1,66 ± 0,3	21,67 ± 8,93	31,47 ± 18,6
HDexo	66,06 ± 37	9,92 ± 2,35	3,02 ± 1,32	1,71 ± 0,49	15,07 ± 6,21	66,28 ± 42,3
C15exo	62,12 ± 45,76	11,56 ± 2,67	3,35 ± 0,73	1,87 ± 0,78	19,3 ± 6,85	65,08 ± 71,03

Table 2. Cell surface expression of maturation and phenotypic markers of MoDCs after maturation (Day 7). All median fluorescence intensity (mfi) values were normalised with the corresponding isotypic control, n=2.

	CD40	CD80	CD83	CD86	HLA-DR	DC-SIGN
iDC	110,06 ± 25,94	18,75 ± 5,64	3,33 ± 3,09	1,66 ± 0,43	19,57 ± 9,19	56,84 ± 6,04
mDC	225,3 ± 16,74	44,77 ± 16,49	11,58 ± 2,43	34,42 ± 27,55	114,55 ± 37,72	62,42 ± 23,71
tDC	92,07 ± 52,74	26,95 ± 3,71	5,58 ± 4,09	11,18 ± 13,51	44,01 ± 40,58	33,91 ± 2,67
HDexo	174,88 ± 10,07	31,08 ± 0,53	11,49 ± 0,6	20,71 ± 2,62	75,17 ± 5,71	51,47 ± 13,57
C15exo	123,44 ± 65,26	23,2 ± 8,14	8,48 ± 3,36	16,33 ± 2,68	73,2 ± 32,12	49,49 ± 48,99

Immune Regulation after High dose Hypofractionated RadioTherapy leading to Complete Clinical Response in a Renal Adenocarcinoma Patient

Clara Milhem[*], **Sarah Renaud**[*], Céline Ingelaere, Olivier Moralès, Alexandre Quilbé, David Pasquier, Xavier Mirabel and Nadira Delhem ([*] Equally contributed authors)

Case Report: [*Submitted to Journal of Immunology Research*]

Context and Objectives

Hypofractionated stereotactic radiotherapy (H-SRT) is a protocol of ionizing radiation delivery. It is now very important to characterize biological markers that could be used to determine the early efficiency of this type of treatment. The case reported here is a 53-year-old French male with renal adenocarcinoma treated by H-SRT for 3 sessions of 12 gray and who presents a complete remission with no progressive lesions after one year of follow up.

Methodology and results

Immunomonitoring of immune populations was carried out during the treatment. Immune cell frequencies, activation status and dosage of cytokine secretion in the patient's serum were all determined in the study. The findings suggest that several biomarkers evaluated could be useful as an early predictor of prognosis after H-SRT.

Conclusion

This study is a first indication of the effects of H-SRT on the immune system and describes a case of complete remission of renal adenocarcinoma after only 3 sessions of H-SRT.

EBV-derived Peptides Provide Protection to Nasopharyngeal Carcinoma by Inducing Resistance against immunosuppressive exosomes.

Dhafer Mrizak*, Rami Mustapha*, **Sarah Renaud**, Natahlie Martin, Hayet Rafa, Perdo Roman Puché, Fei Fei Liu, Toshiro Niki, Kwok-Wai Lo, Véronique Pancré, Yavn de Launoit, Pierre Busson, Olivier Moralès+ and Nadira Delhem+ (*,+ Equally contributing authors).

[*Submitted to Cancer Immunology Research*]

Context and Objectives

Nasopharyngeal carcinoma (NPC) is constantly associated with Epstein-Barr virus (EBV) but NPC cells are known to secrete large amounts of immunosuppressive exosomes. In this study, we aimed to develop a peptide-based approach to stimulate an EBV $CD4^+$ T cell response able to resist to NPC exosomes.

Methodology and results

HLA II promiscuous peptides derived from the 3 EBV latency II antigens were selected. These peptides, are well recognized by human PBMCs, after pre-incubation with NPC cell lines, inducing specific proliferation and IFN-γ release. Furthermore, generated peptide-specific $CD4^+$ T cell lines cytotoxic potential was assessed by the efficient and significant lysis of 3 NPC cell lines, even in the presence of autologous exosomes. Surprisingly, EBV specific $CD4^+$ T cell lines showed an unambiguous ability to overpass and resist to immunosuppressive effects of NPC exosomes. The ability of the cocktail to restrain tumor growth was then evaluated in an original NPC xenotransplanted humanised SCID mice model. We finally demonstrate that the EBV peptide cocktail was still recognized by PBMCs from a cohort of NPC patients regardless of the disease status and treatment.

Conclusion

Our data suggest that EBV latency II-derived peptides could be useful for immunotherapeutic adjuvant approach of NPC, after classical treatments.

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

Rami Mustapha, Dhafer Mrizak, **Sarah Renaud**, Clément Barjeon, Yvan de Launoit, Véronique Pancré, Pierre Busson, Olivier Moralès and Nadira Delhem

[In preparation]

Context and Objectives

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. Several studies have confirmed the presence of elevated levels of Tregs in cancer patients and have correlated this with a poor prognosis. Interestingly, it has been shown that *in vivo* treatment with Gal-9* leads to an increase of Treg cells, that Gal-9 is secreted by many types of tumors and correlated with a poor prognosis for cancer patients. Knowing this, the aim of our study was to determine if Gal-9 is a viable target for cancer immunotherapy.

Methodology and results

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 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-exosomes on TCR activated PBMC and Tconv proliferation. Then we moved to an NPC humanised 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.

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 that we are capable of reversing the immunosuppressed state observed in cancer by directly targeting Tregs. Since Gal-9 is over expressed only in pathological inflammatory conditions, this suggests that the monoclonal antibodies would have minimal side effects on the patient.

Abstract

Nasopharyngeal carcinoma (NPC) is a cancer of the upper aerodigestive tract that is associated in almost 100% of cases with an infection of the Epstein Barr virus (EBV). The microenvironment within NPC is characterized by a high prevalence of regulatory T cells (Tregs) and the presence of numerous tumour exosomes with immunosuppressive properties. Our team has recently shown that these exosomes promote the recruitment and suppressive activity of Tregs, which would contribute to the immune escape of the tumour (Mrizak et al., JNCI, 2015). In this context, the aim of the thesis is to evaluate the ability of NPC exosomes to promote the emergence of semi-mature tolerogenic dendritic cells (tDCs), known to promote tumour tolerance, notably by inducing Tregs and anergising effector T cells.

The results suggest that the NPC tumour exosomes favour the emergence of DCs with a semi-mature phenotype. Indeed, it has been found that DCs pre-treated with tumour exosomes are morphologically different from mature DCs (mDCs) and similar to controls tDCs. In addition, a transcriptomic RTqPCR study reveals that exosomes induce a downregulation of pro-inflammatory maturation markers and cytokines, whereas immature DCs (iDCs) markers and anti-inflammatory cytokines are overexpressed. In addition, flow cytometry analysis of the membrane expression of co-stimulation markers on DCs cultured with NPC exosomes shows they are only partly expressed, thus conferring a semi-mature phenotype, which is a well described characteristic of tDCs (Yoo and Ha, 2016). In addition to the phenotype, functional studies have shown that the generated DCs are immunosuppressive. In fact, the study of the immunosuppressive enzyme indolamine 2,3-dioxygenase 1 (IDO1) by western blot, ELISA and HPLC shows that this enzyme is overexpressed during differentiation, decreases during maturation and soars during co-culturing with T lymphocytes (TL). In addition, secretory analysis of DCs throughout the culture shows that tumour exosomes influence DC's cytokines secretion. DCs pre-treated with tumour exosomes have been shown to promote the secretion of immunosuppressive cytokines (IL-10 and TGF-β) and a decrease in effector cytokines (IL-6, IL-12 and TNFα) secretion after maturation. In addition, when studying the balance of anti- and pro-inflammatory cytokines in the culture supernatant of DC/TL co-culture, it reveals an overall immunosuppressive microenvironment. Furthermore, MLR tests demonstrate that NPC exosomes promote the emergence of DCs that inhibit the proliferation of TL effectors and induce Tregs from naive CD4 TLs. Finally, chemoattraction assays revealed that NPC exosomes can recruit iDCs and tDCs via exosome-bound CCL20. All these findings show that DCs pre-treated with tumour exosomes show tolerogenic potential and seem to favour an immunosuppressive microenvironment that would ultimately lead to the immune escape of the tumour.

To date, no other study has evaluated the impact of NPC exosomes on the maturation of human dendritic cells, even though tDCs play a major role in immune evasion of cancer. Thus, these promising results could open up new prospects of anti-tumour immunotherapies based on the inhibition of actors favouring the emergence of Tregs.

Key words: Tolerogenic Dendritic cells, Exosomes, Nasopharyngeal carcinoma, Epstein Barr Virus, Regulatory T cells