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Immunomodulatory role of P28GST, a recombinant enzyme from the schistosome helminth parasite in the prevention of experimental colitis

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Abstract

Inflammatory bowel diseases are considered part of immune-mediated inflammatory disorders. Their pathogenesis was linked to an inappropriate exaggerated immune response to commensal bacteria normally present in the bowel, in genetically predisposed individuals. Increase of the level of hygiene and decrease exposure to helminthic infections was suggested as predisposing factors to IBD. Epidemiologic data have given a clue on the relation of prevalence of helminthic infections and the incidence of inflammatory bowel diseases in developing countries. The Th2 polarized T cell response driven by helminthic infection has been linked to the attenuation of Th1 driven inflammatory responses, preventing some Th1 mediated autoimmune diseases in their host, including Crohn's disease.

Our work focused on the immuno-modulatory effect of a Schistosome protein – P28GST (a Glutathion *S*-transferase). Its immuno-genetique, pro-Th2, characters have been previously demonstrated in experimental models as well as clinical trials. We showed that immunization with P28GST was able to significantly reduce experimentally induced colitis in two animal models.

Immunisation with this recombinant parasitic enzyme reduced clinical and histological scores of the TNBS induced colitis in both Sprague Dawley rats as well as in C57Bl/6 mice. This effect was associated with a decrease in the expression of inflammatory markers (Myeloperoxidase) as well as mRNA expression of pro-inflammatory cytokines (IL-1β, IL-17 and TNF) in the colon of sacrificed animals. We detected a shift of the immune response characterized with decrease of Th1 immune response assessed by the mRNA expression of IFNγ towards a less pathological Th2 immune response assessed by the mRNA expression of IL-4, IL-5 and IL-13. An increase in the ratio of mRNA expression of Arg1/iNOS2, as well as the immuno-histochemical detection of Arginase positive cells in the colon of the sacrificed animals suggested the presence of alternatively activated macrophages (AAMs) characterized by their anti-inflammatory effect and their association with the Th2 immune response. Similar results have been obtained in another animal model, the C57Bl/6 mice.

We have also compared the effect of a single recombinant Schistosome protein to two models of infection with living schistosome parasites, either with long standing infection (associated with a Th2-type response) or with a recent onset exposure (a Th1-type response). Our results showed that immunisation with a single Schistosome protein, the P28GST; give similar results to established infection in term of reduction of intestinal inflammation, whereas recently infected rats were not protected against colitis.

In conclusion, this study provides the first evidence that immunization with a recombinant protein from the Schistosome helminth parasite prevents hapten-induced colitis in two models of rodents.

Although further studies are needed to illustrate the exact mechanisms of action implicated in the immuno-modulatory effect, P28GST is a promising molecule exerting a potent anti-inflammatory role in the prevention of colitis. The potential effect of this helminthic enzyme is actually taken in consideration in the prevention of Crohn’s disease relapses in humans.

Résumé

Les maladies inflammatoires chroniques de l'intestin font partie des pathologies immunitaires. Leur pathogenèse est directement liée à une réponse immune exagérée dirigée contre des bactéries commensales normalement présentes dans l'intestin, chez des individus génétiquement prédisposés. Parmi les facteurs favorisants, on trouve l'amélioration du niveau d'hygiène ainsi qu'une diminution des infections parasitaires. Des études épidémiologiques ont suggéré une relation entre la prévalence des infections par les helminthes et l'incidence des maladies inflammatoires chroniques de l'intestin dans les pays en développement. Ces infections parasitaires induisant une réponse immune de type Th2, il est donc proposé qu'elles participent la régulation des maladies inflammatoires médiées par une réponse immune de type Th1, comme la maladie de Crohn.

Notre équipe s'est intéressée à l'effet immuno-modulateur d'une protéine du Schistosome, la P28GST (Glutathion *S*-transferase) dont les propriétés immunogénétiques pro-Th2 ont été démontrées précédemment dans des modèles expérimentaux et chez l'homme. Au cours de notre travail, nous avons montré que l'immunisation avec la P28GST était capable de diminuer de manière façon significative la colite expérimentale dans deux modèles animaux.

L'immunisation avec cette enzyme parasitaire, produite sous forme recombinante, a réduit les scores cliniques et histologiques obtenus après induction de colite expérimentale par injection de l'haptène TNBS chez des rats Sprague Dawley rats ainsi que chez des souris C57Bl/6. Cet effet est associé à une diminution des marqueurs de l'inflammation (Myéloperoxidase) et de lexpression de l'ARN messager codant pour des cytokines pro-inflammatoires (IL-1β, IL-17 et TNF) dans le colon des animaux. Nous détectons une modulation de la réponse immune caractérisée par une diminution du profil Th1 mesuré par la présence d'ARN messager codant pour l'IFNγ vers un profil de type Th2, associé à une augmentation de l'ARN messager codant pour l'IL-4, l'IL-5 et l'IL-13. L'augmentation du rapport ARN messager Arg1/iNOS2 ainsi que la détection de cellules Arginase positives par immuno-histo chimie dans le colon des animaux immunisés suggèrent la présence de macrophages alternatifs (AAM), dont on connait le rôle anti-inflammatoire et l'association à une réponse de type Th2. Des résultats similaires ont été obtenus dans un autre modèle expérimental, chez la souris.

Nous avons comparé l'effet de cette protéine du Schistosome avec l'effet de l'infection par des larves du parasite grâce à deux modèles d'infection : soit une infection au long cours (associé avec une réponse immune de type Th2), soit une infection récente (avec une réponse immune de type Th1). Nos résultats montrent que l'immunisation par une seule protéine de schistosome, la

P28GST, réduit l'inflammation intestinale aussi bien que l'infection au long cours, tandis que les animaux récemment infectés n'étaient pas protégés de la colite.

En conclusion, notre étude présente les premières évidences que l'immunisation avec une protéine recombinante de Schistosome pourrait réduire de manière préventive la colite expérimentale induite par l'injection d'une haptène dans deux modèles de rongeurs. Si les mécanismes d'action précis doivent encore être élucidés, nos travaux suggèrent que l'effet anti-inflammatoire de la P28 GST puisse avoir des applications dans la prévention de l'inflammation intestinale permettant d'envisager une utilisation chez l'homme, notamment dans la prévention des rechutes de la maladie de Crohn.

List of Abbreviations

- 5-ASA : 5 amino salicylic acid
- AAMs : Alternatively activated macrophages
- Ab : Antibody
- ADCC : Antibody dependent cell-mediated cytotoxicity
- Ag : Antigen
- AIDS : Acquired immunodeficiency syndrome
- APC : Antigen presenting cells
- Arg : Arginase
- ATG : Autophagy related protein
- B. fragilis : Bacteroides fragilis
- Bm : Brugia malayi
- C. jejuni : Campylobacter jejuni
- A. caninum : Ancylostoma caninum
- CARD : Caspase recruitment domain-containing protein
- CB4 : Coxsackie B4
- CCL : Chemokine ligand
- CCR : Chemokine receptor
- CD : Crohn's disease
- CDAI : Crohn’s disease activity index
- CDR : Complementarity determining regions
- DC : Dendritic cell
- DcR3 : Decoy receptor 3
- DLG : Drosophila discs large homologue
- DNBS : Dinitrobenzen sulphonic acid
- DR3 : Death-domain receptor 3
- DSS : Dextran sodium sulfate
- DTH : Delayed-type hypersensitivity
- E. coli : Escherichia coli
- Foxp3 : Forkhead box P3
- FUT : Fucosyltransferase
- GM-CSF : Granulocyte/macrophage colony-stimulating factor
- GSH : Glutathion

- GST : Glutathion *S*-transferase
- GWAS : Genome wide association studies
- H. diminuta : Hymenolepis diminuta
- H. polygyrus : Heligmosomoides polygyrus
- HD : Human defensin
- HLA : Human leukocyte antigen
- IBD : Inflammatory bowel disease
- IC : Immune complexes
- IEC : Intestinal epithelial cells
- Ig : Immunoglobulin
- IHC : Immune histo chemical
- IL : Interleukin
- IRGM : Immunity-related GTPase family M
- ISG : Interferon stimulated gene
- JAK : Janus kinase
- LP : Lamina propria
- LPMC : Lamina propria mononuclear cells
- LPS : Lipo-polysaccharide
- MAGUK : Membrane associated guanylate kinase
- MAPEG : Membrane-associated proteins involved in eicosanoid and glutathione metabolism
- MAPK : Mitogen-activated protein kinase
- M-CSF : Macrophage colony-stimulating factor;
- MDP : Muramyl dipeptide
- MHC : Major histocompatibility complex
- MLN : Mesenteric lymph node
- MNV : Murine norovirus strain
- MPO : Myelo-peroxidase
- MR : Mannose receptor
- MW : Molecular weight
- NF-Kb : Nuclear factor-[kappa]B
- NK : Natural killer
- NLR : Nod-like receptor
- NO : Nitric oxide

- NOD : Nucleotide-binding oligomerization domain
- NOS2 : Nitric oxide synthase 2
- OCTN : Organic cation transporter
- OxLDL : Oxidized low-density lipoprotein
- PAMP : Pathogen associated molecular pattern
- PG : Prostaglandin
- PRR : Pattern recognition receptor
- PSA : Polysaccharide A
- RANKL : Receptor activator of nuclear factor kappa-B ligand
- RANTES : Regulated and normal T cell expressed and secreted
- ROS : Reactive oxygen species
- RT-PCR : Real-time quantitative polymerase chain reaction
- S. enteric : Salmonella enteric
- S. flexneri : Shigella flexneri
- S. haematobium : Schistosoma haematobium
- S. mansoni : Schistosoma mansoni
- SD : Sprague Dawley
- SEA : Schistosme egg antigen
- SFB : Segmented filamentous bacterium
- ShGST : Schistosoma hematobium glutathion S-transferase
- SJapGST : Schistosoma japonicum glutathion S-transferase
- SMAD3 : Mothers against decapentaplegic homolog 3
- SNPs : Single nucleotide polymorphisms
- SR-A : Scavenger receptor-A
- STAT : Signal transducer and activator of transcription
- T. spiralis : Trichinella spiralis
- T. suis : Trichuris suis
- TAGAP : T-cell activation GTPase activating protein
- TCF : Transcription factor
- TcR : T cell receptor
- Teff : T effector cells
- TGFβ : Transforming growth factorβ
- Th: T helper
- Thr : T helper regulatory cell

- TIM : Triosephosphate isomerase
- TLR : Toll like receptor
- TNBS : Trinitrobenzene sulphonic acid
- TNF : Tumor necrosis factor
- TNSF15 : Tumor necrosis factor super family 15
- Tregs : Regulatory T lymphocytes
- TREM : Triggering receptor expressed on myeloid cells
- TSO : Trichuris suis ova
- TYK2 : Tyrosin kinase 2
- UC : Ulcerative colitis
- UCDAI : Ulcerative colitis disease activity index
- VAMP : Vesicle-associated membrane protein 3

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Introduction

Chapter I. Pathogenesis of Inflammatory Bowel Diseases

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Pathogenesis of Inflammatory Bowel Diseases

1.1. Spectrum and characterization of Inflammatory Bowel Diseases

Inflammatory Bowel Diseases involve both ulcerative colitis and Crohn's disease. These are chronic inflammatory disorders that result from a dysregulated immune response in genetically susceptible individuals. Usually relapsing diseases affecting young adults, characterized by chronic inflammation of the gastrointestinal tract.

The incidence of IBD has increased considerably in western countries since the second world war but is beginning to level off [1].

IBD affects approximately 1.4 million Americans. In the United States, the incidence of UC is 8-12 per hundred thousand population per year [2] while CD has an incidence of 5 per hundred thousand population per year [3]. In Europe the incidence of UC is 10-15 per hundred thousand population per year while CD incidence is 5-9 per hundred thousand population per year [4].

UC is characterized by continuous colonic mucosal inflammation involving the rectum. Patients classically present with abdominal pain and bloody diarrhea while CD is marked by focal, transmural inflammation involving both the upper and lower gastrointestinal tracts. This transmural inflammation can result in fibrotic and stenotic strictures or penetrating disease with fistula and abscess formation. Characteristic presentations include chronic bloody or non-bloody diarrhea, abdominal pain, anaemia and weight loss.

Data from European studies show that rates of UC and CD are highest in northern countries, and that the incidence of pediatric disease is increasing. Regional differences, however, are observed. The Registre des Maladies Inflammatoires Chroniques du Tube Digestif (EPIMAD) registry shows high rates in northern France. This population registry, started in 1988, covers regions in northern France and includes data on 5.7 million people, including 1.3 million children. The epidemiology of IBD in northern France is characterized by a higher incidence of CD than UC and large familial aggregations. Temporal trends show the incidence of CD in northern France is still rising due to pediatric CD. Data on 50 IBD multiplex families in northern France and Belgium were collected with evidence of clustering in space and time. Conjugal forms of IBD have also been observed, mostly CD [5].

There is longstanding evidence demonstrating that UC and CD result from an excessive and poorly controlled mucosal immune response to commensal flora in genetically susceptible hosts. The inherited susceptibility to developing IBD is complex and thought to be polygenic.

Previous studies in monozygotic twins show a higher concordance with CD compared to UC indicating a greater genetic contribution in the risk of developing CD [6].

1.2. Environmental factors affecting pathogenesis of IBD

Differences in incidence rates across age, time, and geographic areas suggest that environmental factors are involved in IBD, but only cigarette smoking and appendectomy have consistently been identified as risk factors [1].

The causes of either forms of inflammatory bowel disease, Crohn's disease or ulcerative colitis, remain unknown. Both epidemiological and molecular genetic studies offer persuasive evidence of genetic susceptibility, however, incomplete penetrance among monozygotic twins, the absence of familial aggregation in the majority of cases, the broad geographical variation or the rapid rise in the incidence of IBD over past decades cannot be explained by genetic predisposition alone, and reinforce the role of environmental factors in disease etiology [7, 8]. Increasing evidence supports that events early in life may have long term effects on health and disease. The current paradigm suggests that IBD has a complex pathogenesis, where genetic, environmental, and immunological factors interplay in unknown ways to induce bowel inflammation. Although much progress has recently been made in defining the genetic basis for susceptibility to the disease, the contribution of potential environmental risk factors remains poorly understood and several epidemiological studies have suggested an etiological role for perinatal and childhood events [9]. An appealing theory is that IBD is associated with an enhanced sanitary childhood environment. The “hygiene hypothesis” postulates that exposure to poor hygiene or an increased potential for infections during childhood can confer protection against the occurrence of these diseases later in life [10, 11]. However, there is no robust data indicating either a positive or negative role of hygiene in the development of IBD.

1.3. Genetics factors implicated in the pathogenesis of IBD

The field of IBD genetics is the fastest moving of all IBD research. Until recently geneticists struggled to advance the field beyond epidemiological studies pointing to a genetic contribution in IBD. Previously, only broad regions of the human genome likely to contain susceptibility genes were identified with the exception of *NOD2/CARD15*, the first susceptibility gene found in IBD.

Over the last 5 years, GWAS have revealed numerous IBD susceptibility loci, some of which overlap in CD and UC. GWAS involve genotyping numerous SNPs in patients with UC or

CD and comparing them with controls to identify regions of susceptibility through linkage disequilibrium. These regions are then more closely examined to further elucidate which genes are likely to contribute to disease susceptibility. The discovery of susceptibility genes has led to a greater understanding of how dysregulation in innate and adaptive immunity have contributed to the pathophysiology of IBD.

1.3.1. NOD2/CARD15 first identified susceptibility gene for CD

NOD2/CARD15 is one of the first discovered CD susceptibility genes. Homozygous mutations occur in 15% of patients with CD and contribute to the development of granulomatous small bowel disease [12, 13]. There are three main mutations resulting from amino acid substitutions in the receptor that lead to an inability to recognize its ligand [14]. NOD2 plays a multifaceted role in innate immunity and it is unclear if mutations in NOD2 result in a loss or gain of function [15].

The protein encoded by NOD2/CARD15 [nucleotide-binding oligomerization domain containing (NOD)2] functions as a cytosolic pattern recognition molecule for bacterial peptidoglycan. It specifically detects muramyl dipeptide, which is a major muropeptide that is released and recycled during bacterial growth. Binding of MDP to NOD2 activates transcription factors (including nuclear factor-[kappa]B) and kinases, thereby stimulating both innate and adaptive immunity [15]. The identification of Crohn's disease-associated mutations in NOD2/CARD15 created an immediate paradox, because it has been shown that these mutations result in a loss of function. Therefore, how can the decreased sensing of bacterial products due to mutation in NOD2/CARD15 be reconciled with the aberrant proinflammatory responses that are characteristic of Crohn's disease? For this paradox to be resolved, a change in the traditional paradigm for IBD is needed. Indeed, this indicates the possibility that, rather than representing an over-reactive immunologic condition, IBD may be a state of immunodeficiency. Possible defects may exist at different or even multiple cellular levels. First, there is defective NOD2 signaling in monocytes from patients carrying the Crohn's disease-associated mutations, leading to diminished MDP-mediated or Toll-like receptor-mediated secretion of IL-1β or IL-8 [16, 17]. This lack of response can lead to defective clearance of invading bacteria and perpetuate a chronic inflammatory response in the bowel wall, which could provide an explanation for the pathology of Crohn's disease.

Second, the major target of defective signaling may be the dendritic cell. This was demonstrated in a recent study that reported that dendritic cells from patients with Crohn's disease who harbored NOD2/CARD15 mutations cannot orchestrate an appropriate immune

response when stimulated with MDP, because of defective cytokine secretion [18]. Of specific interest, IL-10 secretion was significantly suppressed after stimulation with MDP. This in turn could lead to loss of regulatory elements, failure to contain the inflammatory response, and uncontrolled proinflammatory manifestations.

Third, it has been shown that NOD2 acts as an antibacterial factor in human epithelial cells [19]. It follows that defective NOD2 signaling may be associated with increased colonization/penetrance of the epithelial layer by bacteria and their products. The latter could subsequently function as a constant stimulus, initiating and perpetuating chronic inflammation.

Finally, it is possible that the critical dysregulation takes place at the level of Paneth cells. These cells reside at the epithelial crypts along the gastrointestinal tract and provide protection against intraluminal bacteria by secreting antibacterial substances, namely defensins [20, 21]. Indeed, Paneth cells are probably the major cellular source of NOD2. It was recently shown that patients with Crohn's disease, particularly those who carry mutations of the NOD2/CARD15 gene, exhibit decreased expression of human defensins 5 and 6 [22]. Once again, the proinflammatory response could be secondary to a primary inability to handle the bacterial burden from the intestinal lumen because of inadequate defensin secretion.

The forementioned studies have another important implication. It has now become clear that the primary defect in IBD most probably lies within the innate immune system. This is contrary to the long-held concept that IBD is a T cell mediated condition.

Nonetheless, these observations suggest that an impaired rather than an overly aggressive inflammatory response by a defective intestinal innate immune system may underlie the initial phase of IBD, most probably an inability to handle occasional invaders from the intestinal lumen. Activation of the acquired immune system would then be a subsequent phenomenon, mostly associated with the perpetuation of inflammation.

1.3.2. GWAS the era for the Human Genome Project

A number of advances beyond the field of IBD genetics facilitated the new era of genome-wide association studies (GWAS) including the Human Genome Project, the HapMap Project and the development of chip-based genotyping technology. These three factors not only resulted in researchers having a reference road map of the human genome, but also a significantly more efficient genotyping platform. This platform allows large scale studies examining the whole genome in a hypothesis free manner to be performed. Data from GWAS have identified approximately 70 genetic loci that confer susceptibility to CD and over 30 loci

that are associated with UC. These genetic loci guide the understanding of the molecular mechanisms of these gene products and reveal that alterations in the immune response underlie the pathophysiology of IBD [23].

GWAS examine tens to hundreds of thousands of single nucleotide polymorphisms across the human genome in cases and controls. Simply, the allele frequencies of these SNPs are statistically compared between cases and controls to determine any association between the SNP and the disease or condition in question. Independent confirmation of association is often performed for findings generated in a GWAS. Therefore, any confirmed SNP associations that are seen with a GWAS are likely to be in linkage disequilibrium with a true disease susceptibility allele. Several GWASs have been performed in IBD.

The first study to take advantage of these advances was performed by modern standards, in a modestly sized Japanese CD population in 2005. This study identified a strong association between CD and genetic variation in the tumor necrosis factor super family 15 gene, a finding reproduced in 2 cohorts of British Caucasian CD patients as well as in a British UC population [24]. The exact TNFSF15 variants that predispose to IBD remain to be identified and there are no obvious non-synonymous (amino acid changing) SNPs in this region identified to date [23].

The first GWAS in the Caucasian population was performed by the North American IBD genetics consortium in which variants in the IL-23 receptor and the autophagy related molecule immunity-related GTPase family M genes were identified [25, 26]. Autophagy was further implicated by another study in the Caucasian population that identified a non-synonymous SNP in ATG16L1 as a susceptibility variant [27]. Additional studies from the U.K. and a French-Belgian CD GWAS identified further variants associated with CD susceptibility including additional variants within the IL-23/Th17 pathway such as IL-12B and other genes related to novel pathogenic pathways [28-30].

In an effort to increase the power of the studies, the 3 Caucasian GWAS already mentioned were combined in a meta-analysis of over 3000 cases and approximately 5000 controls with an adequately powered independent replication cohort. This study resulted in the identification of over 30 loci (including the ones identified in earlier GWAS) although this explained only 20% of the total genetic contribution in this population. This study further emphasized the role of the IL-23/Th17 pathway in CD pathogenesis through the identification of STAT3, and JAK2 as susceptibility genes [31].

A further study demonstrated an association between Fucosyltransferase 2 non-secretors and CD [32] further implicating the importance of the mucus layer in host-microbial interactions.

The IBD international genetics consortium combined CD GWAS containing over 6000 CD cases and approximately 50,000 total individuals (cases and controls in both index and replication cohorts) resulting in the identification of a total of 71 confirmed loci for IBD [33]. A number of interesting genes were implicated in this study including genes further emphasizing the importance of the IL-23R/Th17 pathway (TYK2) as well as highlighting other processes including the TGFβ pathway (SMAD3), endoplasmic reticulum stress, T cell activation (TAGAP) and macrophage activation (VAMP3) among others. A number of other findings from this study included the calculation that these 71 loci only accounted for approximately 25% of the genetic variation in CD. This is likely to be a significant underestimate of the true contribution as the associations identified in this study are likely to have only partially tagged the true causative SNPs. The best example of this is the NOD2/CARD15 locus, tagged by a single SNP, but is known to have three common SNPs within the locus associated with CD [34].

Similar progress in UC disease identification has been slower in part because of the less heritable nature of the disease. Nevertheless, GWAS identified of a number of genes including IFNγ, IL-10, IL-23R, FcGR2a and the known HLA region that lead to UC susceptibility [35-38]. More recently a meta-analysis of 3 GWAS has implicated some novel genes associated with UC including CARD9 and orosomucoid1-like 3. In this study, association was seen with the 14 previously identified UC loci [39].

The identification of loci through genetic studies is only the first step in a multi-faceted pathway to understanding how variation in these genes lead to an increased risk of developing chronic mucosal inflammation. Fine-mapping genetic studies in large cohorts will be needed to elucidate the precise disease associated variants and these projects may require large-scale sequencing efforts. Given that IBD is a complex trait with both genetic and environmental aspects, researchers will need to assess host genetic risk and environmental interactions such as smoking and the microbiome and incorporate them into their analyses.

Remarkably, despite the discovery of new genetic susceptibility loci with GWAS, the identified IBD markers account for only 20% of the heritable risk [31]. Thus, a considerable amount of genetic research remains to be done to identify the risk and protective IBD genes to better elucidate IBD pathogenesis.

1.4. Role of microbiome in the pathogenesis of IBD

Of the 10-100 trillion organisms that reside within a human colon, the vast majority are bacteria but they also include viruses and members of Archaea. The relationship between the intestine and commensal organisms is one of symbiosis. The intestine provides nutrition to the

bacteria, which in return aid in digestive processes by breaking down non-absorbable complex sugars and xenobiotics and producing a variety of compounds such as short chain fatty acids and vitamins. In addition, these organisms help develop and modulate the immune response and minimize pathogenic invasion.

1.4.1. Impaired Clearance of Microbes in IBD

One of the etiologic theories on the pathogenesis of IBD is impaired clearance of foreign material leading to a sustained activation of monocytes and a compensatory induction of adaptive immune response [40]. The compensatory activation of adaptive immune response is temporally different from the initial acute inflammatory response and leads to perpetuation of the chronic inflammatory response contributing to IBD.

Recruitment of neutrophils to sites of trauma in the bowel is impaired in CD patients [40]. This finding led to the hypothesis that delayed neutrophil recruitment impairs the clearance of microbial organisms, which then leads to gut inflammation. This hypothesis was recently tested by injecting heat-killed *Escherichia coli* into the forearm of human CD, UC, and healthy control subjects. The investigators found that accumulation of neutrophils at the site of injection as well as the clearance of injected *E. coli* was markedly impaired in CD patients [41]. Together, these data support the notion that impaired clearance of antigenic material contributes to the pathogenesis of IBD.

1.4.2. Microbial characteristics in IBD

Advances in 16S ribosomal RNA gene and metagenomic sequencing provide extensive information regarding the prevalent species and their microbial gene functions that make up the intestinal microbiome. Metagenomic sequencing allows for the cataloging of microbial sequences from fecal samples, which provides insight into the species types and prevalent microbial genes that make up the intestinal flora. *Bacteroides* and *Firmicutes* comprise 90% of the gut microbiota [42]. Prominent clusters at the genus and family levels included bacteria of the Bacteroides and *Dorea/Eubacterium/Ruminococcus* groups, *bifidobacteria, proteobacteria* and *streptococci/lactobacilli* [43]. Individuals with IBD have a decrease in bacterial diversity evidenced by a fewer number of non-redundant bacterial genes compared to healthy controls. In addition, the bacterial profile not only differs between individuals with IBD and healthy controls but also between individuals with UC versus CD. There are a decreased number of commensal organisms including the *Lachnospiraceae* and *Bacteroides* and an increase in the number of *Proteobacteria* in individuals with IBD [44].

1.4.3. Host-microbial interactions

Host-microbe interactions are crucial in the development and modulation of the immune system and protection from pathogenic bacterial invasion. The host has evolved a variety of mechanisms to minimize the exposure of the intestinal epithelial surface to pathogenic organisms. Such factors include the compartmentalized mucus layer, the intestinal epithelial cell layer and the secretion of a variety of proteins and antimicrobial peptides [45].

Both commensal and pathogenic bacteria have evolved a number of mechanisms to aid in adherence to the epithelial cell layer, including the use of host cell surface molecules such as oligosaccharides. The ability of commensal organisms to adhere to the epithelial layer helps deeper invasion by displacing pathogenic bacteria. FUT2 is a gene located on chromosome 19 that encodes a type alpha fucosyltransferase [2, 3]. This enzyme regulates the secretion of the H1 antigen of the ABO antigens into the gastrointestinal mucosa by catalyzing the addition of a fucose molecule to oligosaccharides. Twenty percent of individuals are non-secretors and are unable to secrete the H1 antigen into body fluids such as saliva [46]. The FUT2 non-secretor status is associated with CD susceptibility [32]. The inability to secrete these ABO antigens into the gastrointestinal mucosa can affect how both commensal and pathogenic flora interact with the epithelial cell layer which may alter the mechanism by which the host minimizes pathogenic invasion. Specifically in IBD, the inability of commensal organisms to adhere to the epithelial layer and displace pathogens may result in increased susceptibility to infection, invasion and activation of the innate and adaptive immune response.

Commensal bacteria are involved in the development and modulation of the host immune system by promoting the development of certain lymphocyte subsets and suppression of others [45]. Sampling of the microbial antigens in the intestine occurs by a variety of mechanisms including via Fc receptors, microfold (M) cells and antigen presenting cells such as dendritic cells. This sampling allows the microbiota to modulate the intestinal immune response. DC sample intraluminal antigens by extending processes through the intestinal epithelial layer and present their displayed antigen to corresponding T lymphocytes.

Bacteroides fragilis normally resides within the large intestine and can act either as a commensal or pathogenic organism depending on the production of a metalloprotease toxin [47-49]. The capsule polysaccharide A of the commensal form of *B. fragilis* is thought to modulate the immune response to prevent the development of autoimmune-mediated diseases such as CD. PSA induces regulatory T lymphocytes that secrete IL-10, a negative regulator of mucosal inflammation [48].

Segmented filamentous bacterium is commensal, spore-forming, gram-positive organism of the genus *Arthromitus* that is found in the gut and is most closely related to bacteria from the genus *Clostridia* [50]. Observations that C57BL/6 (B6) mice from different commercial vendors exhibit different proportions of Th17 cells in the small intestinal lamina propria led to the discovery that mice expressing a higher number of Th17 cells are colonized with SFB [50]. Furthermore, mice lacking Th17 cells in the small intestinal LP under germ-free conditions develop increased numbers of Th17 cells when colonized with faeces from mice monocolonized with SFB and not other bacteria. SFB induces production of IL-22 and IL-17A but not IFNγ in CD4+ T cells. The ability of these commensal microbes to induce a Th17 response and to help protect against pathogenic invasion is further evidence that supports the role of the microbiota in modulation of the immune response. However, exuberant Th17 activation by such a commensal microbe can lead to the robust inflammatory response characteristic of CD. Thus, it is possible that manipulation of the number of SFB that colonize the gastrointestinal tract may alter the course of Th17 associated inflammatory diseases such as CD.

The intestinal microbiota is implicated in priming and enhancing the effects of the innate immune response. Pattern recognition receptors like Toll-like receptors and cytosolic Nod-like receptors play an important role in the detection and handling of bacteria.

1.5. Alteration in barrier function as a factor in the pathogenesis of IBD

The innate immune response is the host's first line of nonspecific defense and does not result in the development of long-term immunity. The gastrointestinal innate immune system is comprised of a mechanical barrier consisting of the mucus layer and epithelial cell barrier, phagocytes, natural killer cells, proteins and receptors involved in recognition of basic bacterial and viral motifs and autophagic mechanisms. Likewise, proteins such as defensins are secreted across this barrier by IECs and Paneth cells which aid in killing bacteria and other pathogenic organisms.

1.5.1. Mucus layer

Goblet cells secrete mucin, the principal component of the mucus layer, which further comprises two sub-layers - an inner layer adjacent to the IECs that is generally free of bacteria and an outer layer that is colonized with bacteria. The mucus layer forms a barrier that protects the epithelial cells from exposure to pathogens. To date, there have been 21 different

mucin genes identified [51]. MUC2 constitutes the majority of the mucin produced. In patients with IBD, both the production and composition of mucin are altered. Data suggest that in patients with UC the mucus layer may be thinner, and the amount of secreted MUC2 is reduced which may be related to the loss of goblet cells [52]. MUC2 knockout mice treated with dextran sodium sulfate develop a more severe colitis compared to wild-type controls [53]. Alterations in the composition or thickness of the mucus layer could contribute to the pathogenesis of IBD by facilitating exposure of the epithelial cell layer to pathogenic bacteria.

1.5.2. Alterations in the epithelial cell barrier

The IEC barrier consists of epithelial cells linked by tight junctions and forms a semi-permeable barrier, which allows nutrients to cross but prevents penetration by luminal bacteria. CD susceptibility genes, organic cation transporter and 2 types of membrane associated guanylate kinases relate to the integrity of the epithelial cell barrier.

Genes encoding the OCTN 1 and 2 transporters are located with the cytokine gene cluster known as the IBD5 locus on chromosome 5. These transporters are involved in transport of cationic proteins including amino acids and nutrients like carnitine. Two variant alleles that result from mutations within the OCTN1 and OCTN2 genes create proteins defective in cellular transport and lead to increased susceptibility to CD. The odds ratio for CD susceptibility is greatest in patients exhibiting mutations in genes encoding both OCTN and NOD2 [54]. This susceptibility is thought to result from impaired fatty acid oxidation, which in the setting of bacterial antigen exposure may cause colitis in experimental models [54-56].

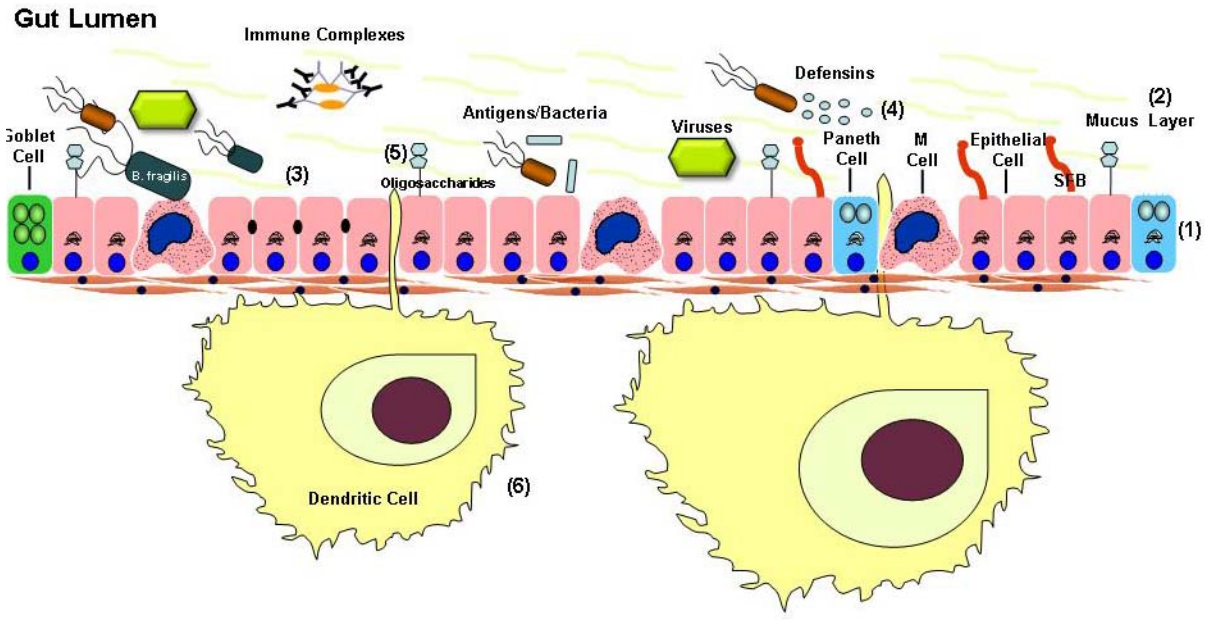

Figure 1: The intestinal immune system is protected from antigenic stimulation by the intestinal epithelial cell layer (1) and mucus layer (2). Defects in epithelial cell integrity may result from alterations in genes encoding OCTN1, OCTN2, DLG5, and MAGIK (3) which may result in free passage of microbes across the epithelial layer from the intestinal lumen. Paneth cells located at the base of the small intestinal crypts help minimize pathogenic invasion through secretion of defensins. Genes that relate to Paneth cell function include NOD2/CARD15, ATG16L1, IRGM, TNFSF15 and TCF-4. Microbial adherence to the epithelial layer may occur through host surface oligosaccharides (5). Alterations in FUT2 may affect the way that commensal and pathogenic organisms including norovirus adhere to the epithelium. Passage of pathogens across the epithelial layer results in activation of innate immune cells such as dendritic cells (6) that can then activate the adaptive immune response and production of inflammatory cytokines (Adapted from ***Vora P. et al, 2012*** [23]).

1.6. Alteration in the innate immune response

1.6.1. Pattern recognition receptors

The lack of specificity of the innate immune response requires the recognition of conserved molecular patterns of various antigens called pathogen associated molecular patterns. PAMPs are recognized by pattern recognition receptors such as toll-like receptors and cytosolic Nod-like receptors.

a) Toll-like receptors

In humans there are ten known TLRs, some of which are membrane-bound while others are located within the intracellular compartment. The ligands for TLRs are specific and are often components of various microbial and viral products. Signaling through a majority of TLRs results in the production of inflammatory cytokines such as TNFα and type I interferons. Production of type I interferons occurs independently of NF-KB and MyD88_ [57].

There is almost no aspect of the immune response that is not regulated by TLR. Initially described as drivers of the innate immune response to pathogens, it is now clear that the TLR family can also influence most aspects of adaptive immunity, as well as determine how tissue cells interact with microbes in their environment [58].

TLR are expressed by many different cell types in the intestine, including macrophages, DC, T lymphocytes and intestinal epithelial cells [59]. Although it is controversial exactly which TLR are expressed by each cell type, TLR function is likely to be central to protective immunity against intestinal pathogens by activating innate effector cells, stimulating the uptake of pathogens by M cells in Peyer's patches and inducing the production of cytokines

and anti-bacterial peptides [60]. Surprisingly, intestinal TLR also seem to be crucial for limiting inflammatory responses and maintaining tissue integrity, indicating a complex interplay between the pro-inflammatory and the homeostatic functions of these receptors [61]. As a result, it has become accepted that the balance between these processes is likely to be important for understanding the pathogenesis of inflammatory bowel diseases such as Crohn's disease and ulcerative colitis [62].

TLR2 is membrane-bound and is activated by a number of microbial components, one of which is PGN. Activation of TLR2 drives Th1 production of IL-12 and ultimately IFNγ and TNFα. TLR2 ligation has also been shown to induce retinoic acid production by DC, a property associated with gut-derived DC, which drive differentiation of $Foxp3^+$ Treg [60]. In addition, probiotic organisms can prevent acute colitis *in vivo* by inducing TLR2-dependent differentiation of IL-10-producing Treg [63]. In most cases, TLR2 simply plays an adjunct role in expanding or enhancing Treg activity, rather than being essential for their generation or function [58].

TLR3, TLR7, TLR8 and TLR9 are located within endosomes in the intracellular compartment and TLR7, TLR8 and TLR9 can activate or inhibit the inflammatory cascade [64-66]. The endosomal TLRs detect a number of different microbial components including single and double stranded RNA and CpG islands.

The gene encoding TLR8 lies on the X chromosome and is an IBD susceptibility gene. Haplotypes within the TLR8 gene confer protection (H1) or risk (H4) to the development of IBD [64]. TLR8 agonists can activate pro-inflammatory cytokines such as IFNγ, IL-12 and TNFα in peripheral blood mononuclear cells [64, 67]. TLR8 can also influence T regulatory lymphocytes, which suppress the immune response through production of factors like IL-10. Ligands that activate the TLR8 pathway, such as single stranded RNA, can reverse the normal function of these regulatory lymphocytes [68]. TLR8 agonists can also curb the inflammatory cascade through inhibition of TL1A expression induced by Fc gamma receptor signaling. The finding that TLR8 variants can protect against or predispose to the development of IBD coupled with its dual role in inducing and inhibiting critical inflammatory cytokines implicates its role as a modulator of inflammation in IBD.

TLR9 is another endosomal TLR that recognizes CpG islands in bacterial and viral genomes. Like TLR7, TLR9 uses adaptor proteins that allow it to move from the endoplasmic reticulum (ER) to endolysosomes. TLR9 can activate pro-inflammatory cytokines and type I interferons through two different pathways [69]. The production of pro-inflammatory cytokines such as IL-

12p40 is dependent on NF-KB activation whereas the production of type I interferons is dependent on the adaptor protein 3 complex.

b) Cytosolic Nod-like receptors

The NOD proteins are cytosolic pattern recognition receptors that recognize microbial components. NOD1 is an NLR that is expressed in non-myeloid cells and APC such as DC and macrophages [15]. NOD1 is encoded by the CARD4 gene, and various polymorphisms in CARD4 are associated with inflammatory disorders such as asthma and IBD. The ligand for NOD1 is a component of gram-negative bacteria. NOD2 is encoded by *CARD15* and detects muramyl dipeptide, a component of PGN that is present in both gram-negative and gram-positive bacteria. Stimulation of NOD1 and NOD2 results in the production of inflammatory cytokines through activation of the NF-KB or mitogen-activated protein kinase pathways [15]. NOD2, like the other pattern recognition receptors, plays an important role in the detection of intracellular invasion by pathogens. Alterations in these receptors impair the ability to sense organisms and may lead to defective microbial clearance and persistent antigenic stimulation of the immune response resulting in the mucosal inflammation and cytokine milieu typically seen in IBD.

Given that NOD2, an intracellular receptor, and TLR2, a membrane-bound receptor are both activated by PGN, it is possible that an interaction between the two modulate the immune response. MDP activation of NOD2 negatively regulates TLR2 activation by diminishing IL-12 production in APC [70].

A second role for NOD2 relates to its loss of function in IECs and Paneth cells. IECs exhibiting mutations in NOD2 have a decreased ability to control the overgrowth of *Salmonella enterica* (*S. enterica*) in the gastrointestinal tract [19]. This discovery is thought to be due to defective secretion of defensins by Paneth cells.

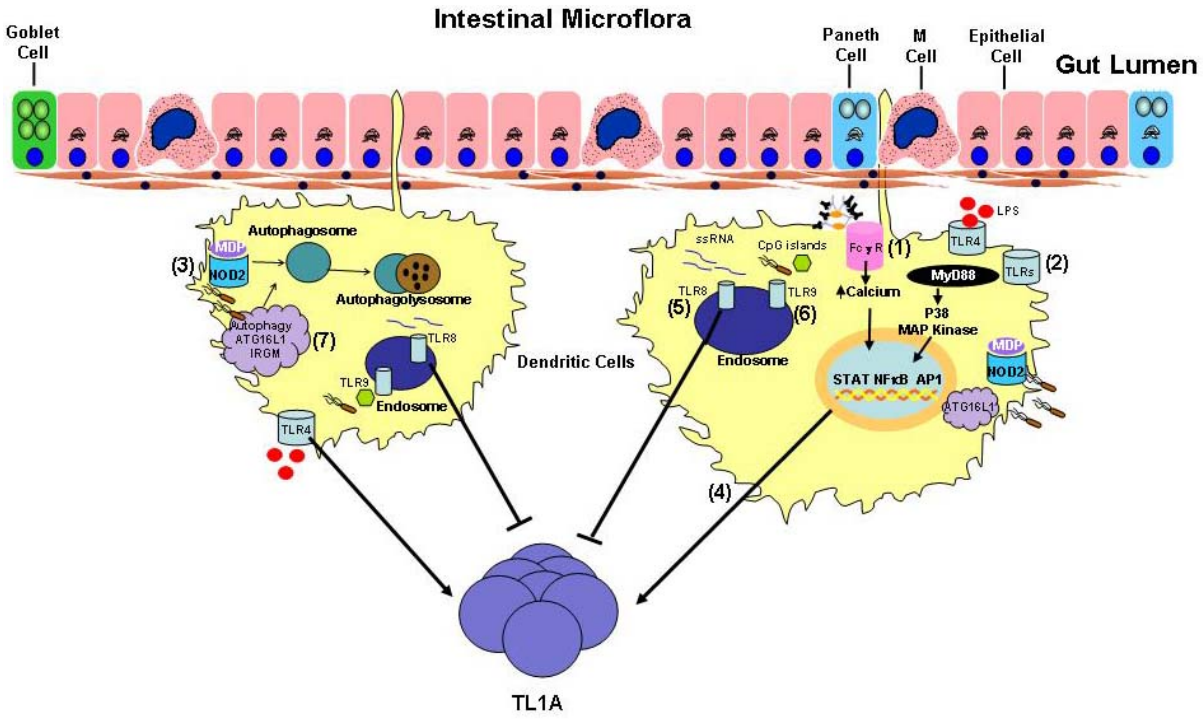

Figure 2: Activation of the innate immune response occurs when organisms breach the epithelial cell barrier. Microbial activation of innate immune cells such as dendritic cells can occur through different signaling pathways including Fc gamma receptors (1), Toll like receptors (2) and NOD2 (3). Signaling through Fc gamma receptors and certain TLRs results in induction of TL1A expression (4). Activation of TLR8 negatively regulates TL1A expression (5) which may make it a useful therapeutic target in the future. Impaired microbial clearance may result from alterations in pattern recognition receptors (2,3,5,6) or autophagy (7) leading to persistent activation of innate immune cells (Adapted from ***Vora P. et al, 2012*** [23]).

1.6.2. Paneth cells

Paneth cells are found mainly at the base of the crypts within the small intestine, although Paneth cell metaplasia can occur within the colon in setting of chronic inflammation. These cells are characterized by eosinophilic granules that contain alpha defensins. Defensins are antimicrobial proteins that bind to the cell membrane to create a hole and allow for leakage of nutrients and electrolytes. Paneth cells secrete a variety of alpha defensins in addition to lysozymes and secretory phospholipase A2 [71]. human eosinophils act as direct effectors in TLR2-mediated innate immunity with potent cytotoxic functions through defensin and eosinophil cationic protein production [72].

Reduced expression of HD-5 and HD-6 in patients with CD result from several alterations within Paneth cells including mutations in NOD2, decreased expression of transcription factors within the Wnt signaling pathway, and loss of normal autophagy function [71, 73, 74].

The Wnt signaling pathway is activated when the Wnt family protein binds to cell surface receptors. This receptor activation causes translocation of beta-catenin to the nucleus in order to form a complex with factors such as TCF-4. TCF-4 then acts as a transcription factor to control expression of various genes including those related to cell differentiation and production of defensins [74, 75]. Lower levels of TCF-4 expression are associated with lower alpha defensin levels in ileal CD, independent of NOD2 mutations and ileal inflammation and variants in the TCF-4 promoter are associated with small bowel CD [74]. There are several IBD susceptibility genes that relate to Paneth cell function such as NOD2/CARD15, ATG16L1, IRGM, TNFSF15 and TCF-4. Genetic alterations that result in defective secretion of defensins impairs microbial defense, which renders the host more susceptible to pathogenic invasion [75].

1.6.3. Autophagy

Autophagy is a biologic process in which autophagosomes engulf organelles and cytosolic macromolecules, followed by fusion of the autophagosome with a lysosome to form an autolysosome in which sequestered material is degraded. The degraded content can then be loaded onto human leukocyte antigen class II molecules or to compartments where recognition by TLR may occur. Autophagy is important for cellular homeostatic functions including structural remodeling, nutrient and energy generation, degradation of damaged or long-lived cytoplasmic components, and protection against invading microorganisms. Its protective role in infectious disease is an important part of the innate immune system and links to adaptive immunity by delivery of foreign antigens necessary for immune recognition [76]. The process of autophagy is not only involved in microbial defense but also in the degradation and elimination of normal intracellular components. The formation of the autophagosome relies on the generation of a complex of autophagy related proteins including ATG5, ATG12 and ATG16L1 at the cellular membrane .

Autophagic processes are involved in the innate immune response on multiple levels from interactions with TLRs to regulation of the pro-inflammatory cytokines [77]. Two autophagy genes, IRGM and ATG16L1 are implicated in the pathogenesis of IBD. The IRGM gene belongs to a family of interferon-inducible immunity-related GTPases (IRGs). It encodes a protein involved in multiple autophagocytic pathways including intracellular clearance of

pathogens that may occur through mitochondrial fission [78]. There are multiple IRGMs and they are involved in the elimination of intracellular pathogens such as mycobacteria, *Toxoplasma gondii* and *Chlamydia trachomatis* [79, 80]. Polymorphisms resulting in alteration of IRGM expression are associated with CD and UC [77, 81].

The ATG16L1 gene product is comprised of an N-terminal ATG16 domain thought to be essential for interaction with other autophagy proteins such as ATG5 and ATG12; which are essential for the formation of the autophagosome. ATG16L1 is broadly expressed by intestinal epithelial cells, APCs, $CD4^+$, $CD8^+$, and $CD19^+$ primary human T cells [82].

The autophagy protein ATG16L1 is involved in a number of autophagic pathways such as formation of the autophagosome and exocytosis of secretory granules in Paneth cells. Polymorphisms in the gene encoding ATG16L1 renders susceptibility to the development of CD. Decreased expression of ATG16L1 results in the induction of cytokines such as type I interferons, IL-1β, and IL-18 and defects in the formation and secretion of granules in Paneth cells [77, 83].

The ability to control and eliminate intracellular pathogens is a critical component of the innate immune response and autophagy clearly plays an important role in the clearance of these microbes.

Alterations in the autophagic machinery such as in ATG16L1 and IRGM may result in the ineffective elimination of pathogens and the persistent activation of the inflammatory cascade, which may contribute to the pathogenesis of IBD.

1.7. Eosinophils: differentiation and homing to the gut

Eosinophils originate from the bone marrow from pluripotent stem cells. Interleukin-3 (IL-3), IL-5, and granulocyte/macrophage colony-stimulating factor are the most important cytokines in eosinophil development, while IL-5 is the most specific cytokine for the selective differentiation of progenitors to the eosinophil lineage [84]. In addition to its relevance in differentiation, IL-5 is an important stimulus for the release of eosinophils from the bone marrow into the peripheral circulation [85]. Under physiological conditions the eosinophils migrate after approximately 8–12 h within the peripheral blood to their final tissue location, predominately the intestinal tract (with the exception of the oesophagus) [86].

Eosinophils in the gut are distinct, both phenotypically and in turnover kinetics, from those in other tissues [87]. Eosinophils are component of several forms of inflammation in the gut, including inflammatory bowel diseases.

The recruitment of eosinophils into the gastrointestinal tract is regulated by the constitutive expression of eotaxin-1, which mediates its action via the selectively expressed CC chemokine receptor [88]. Eotaxin-1 regulates homing of eosinophils to the gastrointestinal tract via the CCR-3 receptor [89]. Integrins expressed by eosinophils are involved in the adhesion processes necessary to induce eosinophil tissue accumulation. One of the more important integrins relevant for adhesion of gastrointestinal eosinophils is the $\alpha_4\beta_7$-integrin, which is expressed on eosinophils [90].

1.7.1. Role of Eosinophils in the pathogenesis of IBD

Eosinophils have been implicated in the pathogenesis of inflammatory bowel disease. Active inflammation in IBD is associated with an increase in eosinophils at sites of inflammation. Contrasting data have been presented concerning quiescent IBD [89]. Immuno-histopathological studies have revealed accumulation and activation of eosinophils in actively inflamed intestinal mucosa of Crohn's disease and ulcerative colitis patients. Elevated levels of chemokines relevant for eosinophil chemotaxis and mediator release from eosinophils can be detected in serum and faeces of patients with active IBD. Eosinophils in patients with active IBD are to a certain extent pre-activated in the circulation. Increased levels of eosinophil-activating mediators - eotaxin, which has been described for IBD patients, might explain the priming and activation of circulating eosinophils in IBD [91, 92].

Eosinophil granule cationic proteins are associated with tissue damage. However, at the same time they have been attributed to non-cytotoxic or at least more protective effects. Therefore the correlation of increased eosinophilia and their mediators with IBD cannot automatically be linked to pro-inflammatory effects. Potential anti-inflammatory effects have to be taken into account. Selective deletion of certain eosinophil-specific granule products results in attenuation of experimental intestinal inflammation [89].

Faecal levels of eosinophilic granule contents may be a helpful tool to monitor disease activity [89].

1.8. Alteration in adaptive immunity

The adaptive immune response consists of B and T lymphocytes, recognizes and generates specific responses to foreign antigens or infected cells and is responsible for immunologic memory. CD appears to result from Th1 production of IL-12, IFNγ, and TNFα and Th17 production of IL-17 and IL-23. UC however, is predominantly a Th2 and NK T cell disease and results in the production of IL-4, IL-13 and IL-5 [82]. Human studies and murine models of

IBD highlight the importance of CD4+ T cells in the production of inflammatory cytokines and development of mucosal inflammation. CD4+ T cells isolated from patients with CD produce more IFNγ and IL-17 compared to UC patients and controls [93]. The increased production of IFNγ occurs in a Th1-dependent fashion as there is increased RORgamma expression. Furthermore, DC isolated from CD patients stimulated with bacterial antigens produce more IL-23 compared to UC patients and controls [93]. Understanding the biological alterations underlying the dysregulated adaptive immune response in CD that drives production of IL-23, IL-17 and IL-12 may identify novel therapeutic targets in treatment of patients with IBD.

1.8.1. Th1/Th2/Th17 paradigm in IBD

A major dogma in the pathogenesis of IBD has been that Crohn's disease and ulcerative colitis express fully polarized, terminally differentiated immunophenotypes. Accordingly, Crohn's disease has traditionally been considered a prototypical Th1 condition mediated by the IL-12/IFNγ/TNFα cytokine axis. The successful recent application of anti-TNF and anti-IL-12 therapies to treat Crohn's disease has strengthened this hypothesis [94, 95]. On the other hand, it has been proposed that ulcerative colitis follows an atypical Th2 pattern of immunologic response, although this has never been supported by definitive evidence; rather it was a hypothesis of ‘exclusion’ that served to emphasize and account for its differences from Crohn's disease. This pathophysiological concept for IBD is rapidly changing as a result of recent advances in understanding of the pathogenesis of intestinal tissue injury.

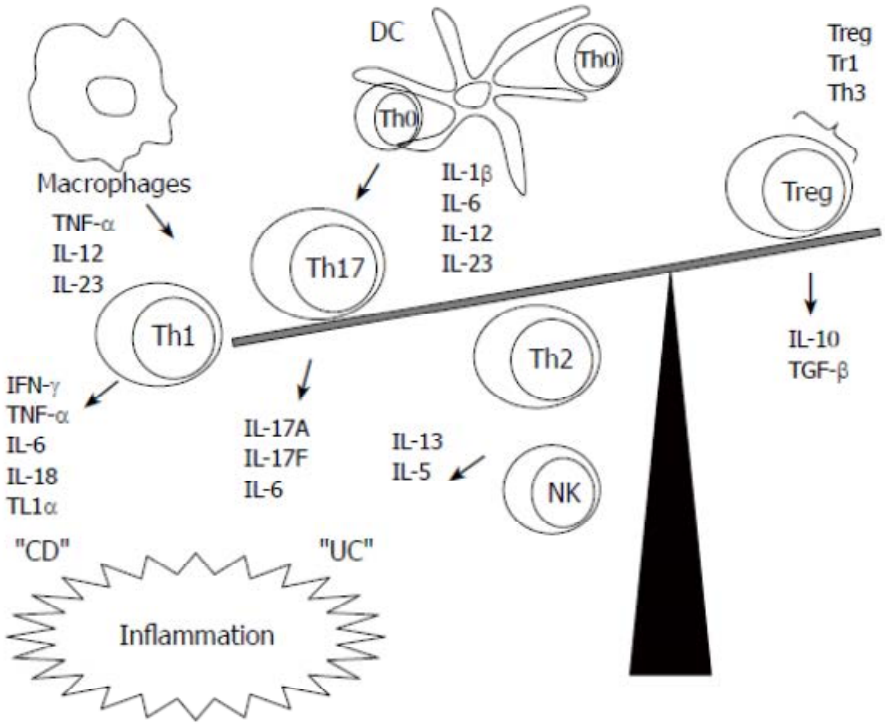

Figure 3: Cytokines imbalance between effector and T regulatory cells in IBD (Adapted from ***Sanchez-Munoz F. et al, 2008*** [96]).

A major breakthrough was the recent description of a third type of effector immunologic response, namely the IL-23/IL-17 pathway [97].

This pathway is induced by IL-23, a heterodimeric cytokine that shares the p40 subunit with IL-12, but couples it with the p19 instead of the p35 subunit [98]. IL-23 is produced by APC and induces expansion of Th17 cells but it is not required for their initial differentiation, as naïve T cells do not express the IL-23R [99-101]. IL-17 is a member of the IL-17 family of six cytokines along with IL-17F. It signals through the IL-17 receptor A and acts through activation of the NF-KB and MAPK pathways [102, 103]. The genes encoding IL-17 and IL-17F are located on chromosome 6, and these cytokines are produced by Th17 cells that proliferate in response to IL-23 [100]. Expression of IL-17 is associated with numerous inflammatory diseases including rheumatoid arthritis, asthma, CD and UC [104-106].

IL-23 drives a population of T lymphocytes that produce IL-17, IL-6 and TNFα (Th17 cells) [100]. These cells are involved in tissue damage in many diverse pathologic conditions.

Several recent publications have reported that the Th17 pathway may be of pivotal importance during chronic intestinal inflammation. It was shown that IL-23, but not IL-12, is essential for the development of intestinal inflammation in the IL-10 knockout model of colitis [107]. IL-23 induces activation of a proinflammatory memory T cell population that is capable of producing IL-17 and IL-6, which mediates tissue damage.

The downstream effects of the activation of the Th17 pathway are currently under intense investigation. In the trinitrobenzenesulfonic acid model of colitis, it appears that IL-17 affects the innate immune response by enhancing expression of macrophage inflammatory protein-2 and facilitating the sequestration of neutrophils within the inflamed tissue [108]. IL-17 producing $CD4^+$ cells may also interact with colonic subepithelial myofibroblasts, and modify the secretion of cytokines and chemokines from the latter, leading to an acceleration of proinflammatory responses and deterioration of tissue damage [109].

It is now clear that the Th17 pathway is critical for the development of inflammation in the majority of the animal models of colitis investigated thus far. In addition, levels of expression of both IL-23 and IL-17 are increased in inflammatory lesions in patients with Crohn's disease [106, 110]. These observations raise the possibility that the immunologic mechanisms elucidated in the mouse are equally important in the human condition. Interestingly, a recently reported genome-wide association study reported a highly significant association between Crohn's disease and a gene on chromosome 1p31 that encodes a subunit of the receptor for IL-23. Replication studies confirmed the association with the IL-23 receptor in independent cohorts of patients with Crohn's disease or ulcerative colitis, further supporting the potential pathogenetic role of IL-23 in IBD [25]. There is increased expression of IL-23 by LP macrophages isolated from patients with CD implicating IL-23 as an important cytokine in the development of colitis [111].

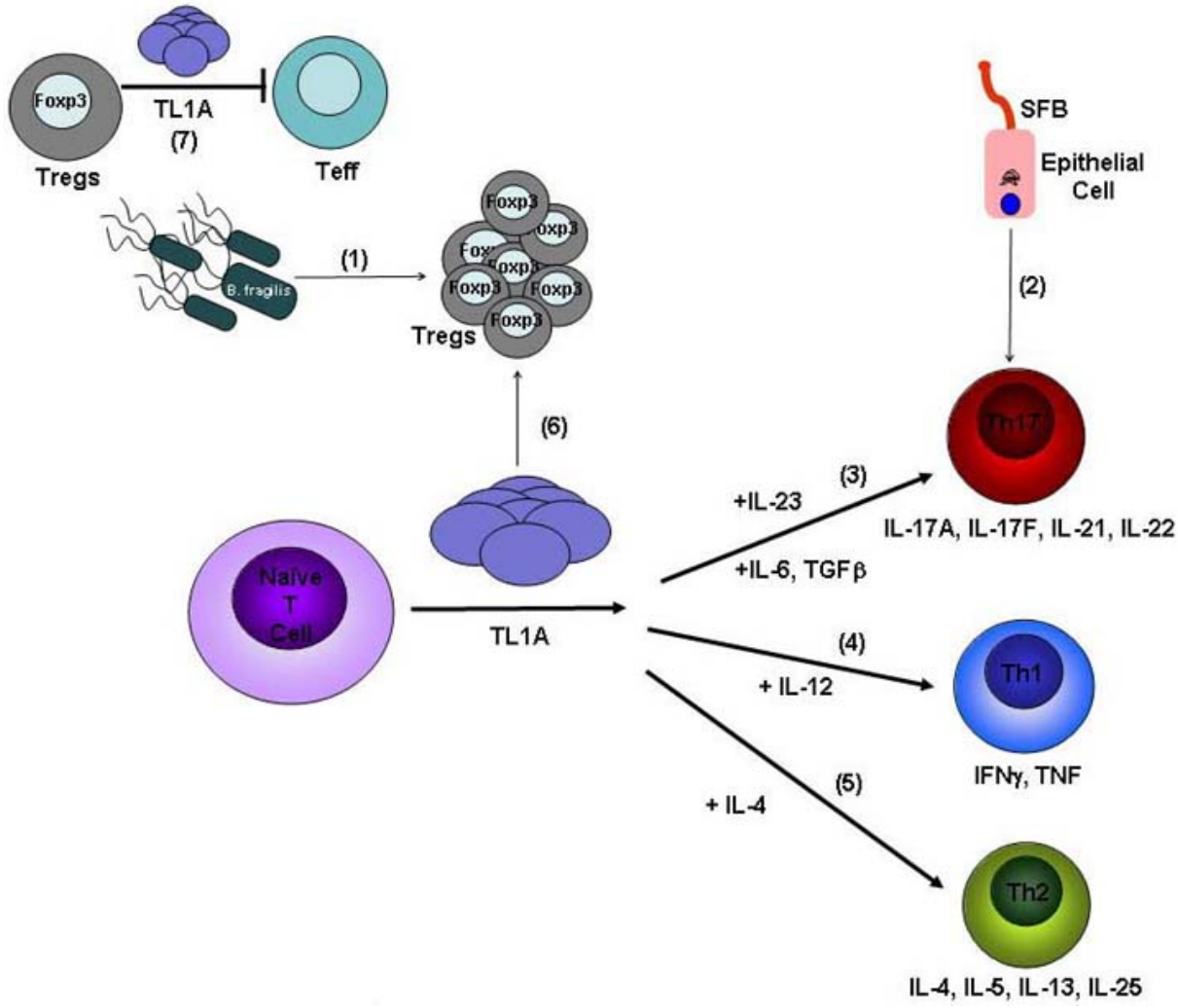

Figure 4: Modulation of the adaptive immune response occurs by the intestinal microflora such as *B. fragilis* (1) and SFB (2) through induction of T regulatory and Th17 lymphocytes respectively. TL1A in combination with IL-23, IL-6 and TGFβ induces generation of Th17 lymphocytes (3) while in combination with IL-12 and IL-4 enhances the development of Th1(4) and Th2 (4) lymphocytes respectively. Th1, Th2 and Th17 lymphocytes produce specific effector cytokines (indicated in the figure) that characterize the cytokine milieu seen in CD or UC. TL1A also appears to increase the number of Treg lymphocytes (6) but diminishes their suppressive ability on T effector cells (7). The ability of TL1A to expand Teff lymphocytes and suppress Treg function may make it an ideal therapeutic target in treatment of IBD (Adapted from ***Vora P. et al, 2012*** [23]).

Targeted treatments against IL-23 include an anti-p40 antibody (antibody against p40 subunits of IL-12 and IL-23). In patients with active CD, two trials using an anti-p40 antibody demonstrate higher response rates defined as a reduction in CDAI within the induction phase of treatment compared to placebo [95, 112].

1.8.2. Role of IL-12 in the pathogenesis of IBD

IL-12, a heterodimer composed of a p40 and p35 subunit, is secreted by activated myeloid cells [113]. It is responsible for Th1 maturation and secretion of IFNγ and TNFα both by NK cells and T cells [114]. Binding of IL-12 to its receptor initiates a cascade of events that lead to activation and nuclear translocation of STAT4 with promotion of Th1 responses [115]. Th1 cells are characterized by their transcription factor T-bet and play a role in chronic inflammatory conditions [116-118]. GWAS show that variants in the IL-12B gene are associated with CD [33]. Furthermore, increased expression of IL-12 is noted in the lamina propria mononuclear cells of patients with CD [119]. Elevated expression of IL-12 is seen in several models of colitis including DSS, TNBS [120, 121].

Initiation of adaptive immunity requires interactions between activated APC and antigen specific T cells. This contact between T cells and APC regulates the immunological synapse [122, 123]. Secretion of IL-12 by DC requires T cell polarity [124].

Although the IL-12 secreted by myeloid cells drives naïve T cells towards a Th1 phenotype, recent data suggest that IL-12 may be involved in the development of Th1/Th17 cells which exhibit features of both Th1 and Th17 cells and produce IFNγ and IL-17 [125]. These Th1/Th17 cells are thought to be generated from "unstable" Th17 cells in an intermediate differentiated state [126]. Th1/Th17 cells can be generated from Th17 cells by exposure to IFNγ and IL-12 [125].

1.9. Interference of innate and adaptive immune systems

Antigen presentation to T cells by professional antigen-presenting cells such as dendritic cells is critical for the activation of adaptive immune responses. There are three main pathways to transport luminal antigen to lamina propria. The first is mediated by microfold (M) cells through the capture of luminal antigens and their presentation to T cells. The second pathway, performed by LP DCs, involves dendritic cell extension to the intestinal lumen across epithelial cells, facilitating the capture of luminal antigens. The third pathway involves the neonatal Fc receptor (FcRn), which serves as the vehicle for transporting LP IgG across the colonic epithelial layer into the lumen where the IgG can bind enteric bacterial antigens to form immune complexes. The FcRn then recycles the antigens/IgG IC back across the colonic epithelial cells into the LP for processing by APCs such as DCs, which are capable of presenting the antigens to T cells [76]. One of the ways that host discerns foreign from self antigen is through pattern recognition receptor, which recognizes specific molecular patterns of pathogens.

One of the mechanisms by which sentinel APCs augment innate immune response is enhancement of the lytic potential of NK cells and their ability to produce interferon IFNγ [127]. Recently, a unique NK cell subset that specializes in the production of IL-22 and is responsive to IL-23 was identified [128]. Several murine studies in the past year point to a protective role for these IL-22–producing NK cells in host defense and IBD [127]. Lastly, genetic associations between autophagy genes and CD highlight the importance of intracellular processing of bacterial components in mucosal homeostasis.

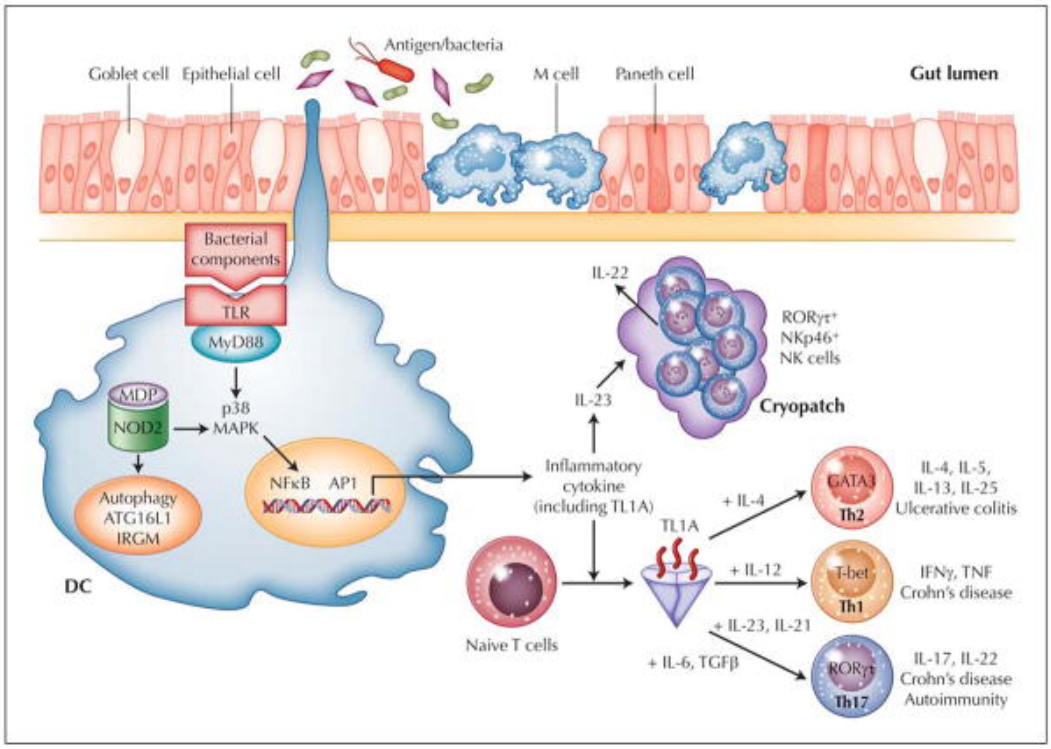

Figure 5: Different working hypotheses of pathogenesis of inflammatory bowel disease. Intestinal immune system is in close apposition to luminal antigen/bacteria, which are separated by a single layer of epithelial cells. Goblet cells contribute to the formation of the protective mucus layer, and M cells and dendritic cells sample intestinal luminal contents. Over response to antigens, either through Toll-like receptors, intracellular sensor NOD2, or antigen processing via autophagy, results in stimulated DC that recruit and generate T-cell (Th1, Th2, and Th17) and NK-cell subtypes. One subpopulation of RORγt^{+}NKp46^{+} NK cells that resides in gut cryptopatches is found to readily produce IL-22 in response to IL-23, likely released by DC. IL-22 may play an important role in epithelial homeostasis, bacteria

clearance, and tissue repair. TL1A appears to be a critical factor important in generating Th1, Th2, and Th17 cells. For each T-helper cell differentiation program, specific transcription factors and cytokine milieu are required (indicated in the figure). Terminally differentiated T-helper cells are characterized by a specific combination of effector cytokines (indicated in the figure) that orchestrate effector function of the adaptive immune system. Crohn's disease is a predominantly Th1- and Th17-mediated process, whereas ulcerative colitis appears to be mediated by Th2 and possibly Th17 (Adapted from ***Shih DQ. et al, 2009*** [76]).

1.9.1. IL-22–producing NK cells: protective role in host defense and IBD

The recent identification of IL-22 as a UC susceptibility gene implicates this cytokine in the pathogenesis of IBD [35]. IL-22 is a member of IL-10–related cytokines and plays a role in maintaining the integrity of epithelial cell barrier in the gut from pathogens [127]. Binding of IL-22 to its receptor initiates a STAT3 signaling pathway that induces the production of antimicrobial proteins including β-defensin-2, β-definsin-3, S100A7, S100A8, S100A9, RegIIIβ, RegIIIγ, and lipocalin-2, which protects the epithelial barrier against microbes [129]. Direct induction of antimicrobial proteins may be one of the pathways for IL-22 in regulating early defense mechanisms against extracellular bacterial infection caused by *Citrobacter rodentium* [130].

Several recent studies implicated a protective role for IL-22–producing NK cells in IBD. Adoptively transferring $CD4^{+}CD45RBhi$ T cells from wild-type or IL-22–deficient mice into $Rag1^{-/-}$ or $Rag1^{-/-}IL22^{-/-}$ mice showed that the absence of exogenous and endogenous non-T non-B cell sources of IL-22 led to worsened murine colitis than with lack of either exogenous T-cell or endogenous sources of IL-22. NK cells were identified as the likely population that produced IL-22, the absence of which led to worsened murine colitis [131]. These results implicate that $ROR\gamma t^{+}NKp46^{+}$ NK cells provide an innate source of IL-22 that has a protective role in host defense and mucosal inflammation.

1.9.2. TL1A bridges innate and adaptive immune response in IBD

Apart from the well described Th17 pathway, there are also indications that other cytokine-mediated mechanisms play important roles during mucosal inflammation. TL1A (TNF superfamily member 15) is a novel TNF-like cytokine that can bind to two different receptors with opposing functions: the functional receptor death-domain receptor 3 and decoy receptor 3, which acts in an inhibitory manner, competing with DR3 for TL1A binding [132]. TL1A, its

expression is increased in inflamed tissue of colon and small bowel of CD patients especially in chronic ileitis and colocalizes to macrophages and T cells.

TL1A is primarily expressed on mucosal dendritic cells, and acts preferentially on memory $CD4^+$ cells to provide co-stimulatory signals for proliferation and IFNγ secretion [133]. Interactions between TL1A expressed on antigen-presenting cells and DcR3 on activated lymphocytes may therefore be of particular importance for the pathogenesis of IBD, especially in light of the fact that both molecules are upregulated in patients with active Crohn's disease and ulcerative colitis [134]. Moreover, single nucleotide polymorphisms in the gene encoding TL1A (TNFSF15) confer susceptibility to Crohn's disease [135]. Interestingly, this association is independent of ethnic background, making TNFSF15 the first gene to be globally linked with predisposition to Crohn's disease. The role of TL1A in the pathology of mucosal inflammation was recently tested by using neutralizing antibodies to TL1A where inactivating the function of TL1A leads to attenuation of inflammation in two different murine colitis models [136].

the potential interaction of TL1A in microbial-host interaction was illustrated by a report showing microbial organisms can induce TL1A in APCs [137].

TL1A plays an important role in modulating adaptive immune response. In the Th1 effector arm, TL1A was found to augment IFNγ production by IL-12/IL-18–stimulated $CD4^+/CCR9^+$ T cells that are specifically enriched in the intestinal immune compartment [82]. Furthermore, in autologous monocyte–T-cell co-cultures, TL1A production by monocytes potentiated IFNγ production by $CD4^+$ T cells [137]. In addition to mediating Th1 response, the role of TL1A in Th2-mediated functions and disease pathology was demonstrated by signaling to exert Th2 effector function in Th2-polarized CD4 cells and co-stimulation of IL-13 production by activated NK T cells [138]. Furthermore, IFNγ and IL-17 production induced by IL-12 and IL-23 respectively, can be synergistically enhanced by TL1A [136]. Interestingly, a report using DcR3-deficient mice showed that TL1A/DcR3 signaling is dispensable for polarization of naïve $CD4^+$ T cells into Th1, Th2, or Th17 effector cell subtypes [139]. Instead, DcR3 expression is required on T cells for immunopathology, local T-cell accumulation, and cytokine production, suggesting that TL1A/DR3 signaling is important to co-stimulate antigen-induced expansion of primed T cells in the target organ of T cell–mediated autoimmune and inflammatory diseases. Together, TL1A/DcR3 signaling appears to have pleiotropic effects that include amplifying the innate immune response, modulating adaptive immunity by augmenting Th1, Th2, and Th17 effector cell function, and T-cell accumulation and immunopathology of inflamed tissue. The role TL1A plays in promoting a Th1 and Th17 response is complex as

recent data indicate that TL1A may act as a differential regulator in generation of Th17 cells from naïve T cells. The ability of TL1A to enhance effector T cell function through production of inflammatory cytokines and to inhibit regulatory T cell function makes it an ideal therapeutic target. The importance of TL1A as a potential therapeutic target is validated by the fact that TL1A antibodies attenuate gastrointestinal inflammation in multiple mouse models of acute and chronic colitis [23].

1.10. Molecular pathway interaction

The identification of IBD susceptibility genes is just the beginning in comprehending the pathophysiology of IBD. Given that IBD is a complex polygenic disease and each susceptibility gene only has modest effects on the development of disease, it is possible that interactions between genes affect the risk of developing disease. Determining the functionality of these susceptibility gene products and how they interact in molecular pathways to cause a dysregulated immune response is critical in understanding the inflammatory disease process and in developing novel therapeutic agents.

1.10.1. Gene - gene interactions

a) TLR8 and TL1A

Genes that encode TL1A and TLR8 are susceptibility genes in the development of CD and variants in each gene contain both risk and protective haplotypes [64, 140]. TL1A appears to be an important regulatory cytokine, and it may play a significant role in modulating the transition from the innate to adaptive immune response.

Activation of TLR8 by its ligand in human monocytes leads to production of the pro-inflammatory cytokines IL-6 and TNFα and the anti-inflammatory cytokine, IL-10, but inhibits Fc gamma induction of TL1A mRNA and protein expression. In addition, activation of TLR8 signaling prevents the production of IFNγ from CD4+ T cells [141]. The ability of TLR8 signaling to induce pro-inflammatory and anti-inflammatory cytokines hints at TLR8's role in the modulation of inflammation. This role is further supported by the fact that certain haplotypes within the TLR8 gene confer risk or protection. The ability of TLR8 signaling to inhibit TL1A expression may make TLR8 a potential therapeutic target in the treatment of CD.

b) NOD2 and ATG16L1

Interactions between NOD1, NOD2 and ATG16L1 play critical roles in the innate immune response. There is accumulating evidence suggesting that alterations in NOD2 and ATG16L1,

two different CD susceptibility genes, interact to affect microbial handling [142]. Transfection of HeLA cells with tagged NOD1, NOD2, ATG5 and ATG16L1 reveals that a fraction of NOD1, NOD2 and ATG16L1 is present at the plasma membrane with NOD1 and NOD2 co-localizing with ATG16L1 at the cell surface. Furthermore, interactions between NOD proteins and ATG16L1 are important for the sequestration of bacteria into autophagosomes as NOD1 or NOD2 co-localize with ATG16L1 at the site of entry of *Shigella flexneri* into the cell [23].

NOD2-mediated autophagy is also required in microbial handling and in the development of major histocompatibility complex class II CD4+ T cell responses. Exposure of DC exhibiting variant *NOD2* to GFP-labeled *S. enterica* or *E. coli* results in a decreased number of bacteria localized within lysosomes compared to controls. Antithetical findings exist with rapamycin (an activator of autophagy) treatment suggesting that *NOD2* variants affect the cells' ability to clear intracellular bacteria via autophagy [143].

These data highlight the importance of NLRs and autophagy in the clearance of intracellular microbes and provide further evidence to the theory that impaired microbial clearance leads to persistent activation of the innate and adaptive immune responses and the production of inflammatory mediators.

1.10.2. Environmental - gene interactions

Through GWAS there is a better understanding of the genetic susceptibility of IBD; however, little is known about the environmental triggers. Recent data show how infection with norovirus in ATG16L1 variant mice produces the development of features of CD. Persistent infection with a murine norovirus strain in mice with hypomorphic ATG16L1 expression and reduced autophagy leads to abnormalities in granule formation and exocytosis in Paneth cells [144]. These features are similar to those found in patients homozygous for the CD risk allele ATG16L1. Exposure of these mice to a mild concentration of DSS, in the setting of persistent infection with MNV-CR6, results in colonic ulcerations with associated mucosal inflammation, mesenteric stranding, and small intestinal villous atrophy. This inflammatory response depends on TNFα, IFNγ, and the commensal intestinal flora, given that blocking antibodies and broad spectrum antibiotics attenuate or prevent the inflammatory response [144].

These data highlight the concept that the development of IBD is a complex, multiple hit process that results from environmental triggers in genetically predisposed individuals.

Chapter II

Immuno-modulation induced by infectious agents

The development of some autoimmune diseases is increasing in the developed world faster than can be accounted for by genetic change. The development of these autoimmune diseases is known to be influenced by both genetic and environmental factors. Environmental factors which have been considered to play a role include infectious agents such as viruses or bacteria. In the developing world changes have occurred such that many chronic infections have been eliminated and this may have led to the emergence of autoimmune pathology.

In fact, infections are major players in the environmental factors which modulate the development of autoimmune diseases, both on the positive and negative sides. Underlying mechanisms are multiple and complex, probably different according to pathogens. It is extremely interesting to correlate these mechanisms and more generally the infections in question with the polymorphism of genes predisposing to or protecting against the various autoimmune diseases. At the therapeutic level, these concepts should open up new perspectives either based on treatment or prevention of infections or immune stimulation attempting to safely reproduce the immune stimulatory effect of infections.

2.1. Relation between infections and autoimmune diseases

Immunological studies performed in animal models of autoimmune diseases strongly suggest that infections represent the best candidates for the environmental factors triggering human autoimmune disease. The study of animal models has clearly shown that infections may trigger autoimmune diseases, as in the case *of Coxsackie B4* virus in type I diabetes and the *encephalomyocarditis* virus in autoimmune myositis, two models in which viruses are thought to act by increasing immunogenicity of auto-antigens secondary to local inflammation. The induction of a *Guillaine-Barré* syndrome in rabbits after immunisation with a peptide derived from *Campylobacter jejuni* is explained by mimicry between *C. jejuni* antigens and peripheral nerve axonal antigens. Other models involve chemical modification of auto-antigens, as in the case of iodine-induced autoimmune thyroiditis. These mechanisms have so far only limited clinical counterparts (rheumatic fever, *Guillaine-Barré* syndrome and drug-induced lupus or myasthenia gravis) but one may assume that unknown viruses may be at the origin of a number of chronic autoimmune diseases, such as type I diabetes and multiple sclerosis.

Autoimmune Diseases triggered by Infections

Autoreactive B and T cells are present in all healthy subjects. Their repertoire is defined by intrathymic negative selection which eliminates autoreactive T cells presenting high affinity

receptors for auto-antigens expressed in the thymus. Peripheral physiological autoreactive T cells recognize a wide spectrum of major auto-antigens distributed in all organs and known to be the target for a multitude of autoimmune diseases [145].

The question is posed of the mechanisms by which dormant autoreactive T cells are activated in patients with autoimmune diseases in whom the infectious agent protein is not an auto-antigen. Three mechanisms can be proposed to explain the modalities of the T cell activation:

1) Polyclonal lymphocyte activation

The first mechanism involves polyclonal B or T cell activation. The reality of the involvement of such mechanisms is elusive. It would imply, as far as autoreactive T cells are concerned, that one should find no or few somatic mutations in the autoantibody gene segment corresponding to complementarity determining regions since auto-antigen-driven selection is not a primary event in this setting. This is in fact rarely the case, with the exception of some forms of systemic lupus erythematosus [146]. It is possible, however, that the major B and T cell activation which is observed in some diseases, notably viral and parasitic diseases, may explain some autoimmune states.

2) Antigen mimicry

The second mechanism is antigen mimicry. It has been noted that the protein sequence of a number of bacterial or viral proteins present a homology with auto-antigen sequences. There is a significant homology between the *Coxsackie B4* virus protein and the glutamic acid decarboxlyase sequence and between the *hepatitis B* virus polymerase sequence and a segment of myelin-basic protein which has been incriminated in the pathogenesis of multiple sclerosis [147]. The list of such homologies is long. However, the consideration of such homologies often does not show definite evidence for a possible role for shared antigenic determinants between the infectious agent in question and the auto-antigen.

The bioinformatics-based search for homologies reveals the existence of a large number of medium-length homologies, the relevance of which is elusive. Such evidence has only been obtained in a very limited number of diseases [145].

3) Increased immunogenicity of organ auto-antigens secondary to infection-mediated inflammation

A number of infectious agents induce localized inflammation of the target organ. This is notably the case for a wide spectrum of viruses. This information may be at the origin of an

organ-specific autoimmune response which will enhance and perpetuate the inflammation. Two experimental models illustrate this mechanism. In *Theiler's* disease, the infection initially provokes a virus-specific encephalomyelitis associated with T cell reactivity to viral proteins [148]. However, within a few weeks, the virus-specific immune response is replaced by a bona fide autoimmune response, including myelin-basic protein and proteolipid protein - specific T cell reactivity. It is this autoimmune response which is at the origin of the chronicity of the disease. Similarly, infection of mice with the *Coxsackie B3* virus induces a long-term cardiomyositis which develops in two phases, the first viral, second autoimmune [149]. The role of inflammation in triggering autoimmune disease is also supported by data obtained using the *Coxsackie B4* virus in non-obese diabetic mice. CB4 has been incriminated in the etiology of human type 1 diabetes. It is an enterovirus with clear pancreatotropism. Infection with CB4 can induce diabetes in non-autoimmune-prone mouse strains and accelerate diabetes onset in diabetes-prone mice. It was suggested that the diabetogenic effect of the virus is mediated by inflammation, rather than by antigen-mimicry, which is very unlikely to be instrumental in mice showing a highly skewed T cell repertoire [150].

In these models, it is assumed that the initial virus-induced inflammation triggers over expression of molecules participating in auto-antigen recognition by T cells.

These molecules include MHC molecules (class one and class two), costimulatory and adhesion molecules.

It is tempting to believe that human counterparts of such experimental models explain some of the infection associated autoimmune diseases. One has to admit, however, that no direct demonstration of such a mechanism has been made in human autoimmune diseases, possibly due to the difficulties met in identifying etiological agent or due to the long lag time between the initial causal infection and onset of clinical disease [145].

Surprisingly, infections may also protect from autoimmune diseases. That was the core of the "Hygiene Hypothesis".

2.2. Protective effect of infections on autoimmune diseases

Accumulating evidence from various sources suggests that the increase in autoimmune diseases observed in western countries is partly caused by a decline in infectious diseases and progress in hygiene. This notion, which was first developed for allergic diseases, applies to most, but not necessarily all, autoimmune diseases.

There is a concomitant decline in infections and increase in autoimmune diseases in western countries. The incidence of most autoimmune diseases has been steadily increasing over the last three decades in North America and Europe [151].

This trend has been particularly spectacular in type 1 diabetes, inflammatory bowel disease, and multiple sclerosis, though for the last disease, it seems a plateau has been reached. In the case of type 1 diabetes, the increased incidence is associated with a worrying decrease in age of onset with frequent involvement of very young children (less than 2-3 years old) [152]. This ‘‘epidemic’’ is not observed in less developed countries. Within developed countries, it involves more northern than southern countries. Such a difference is not explained for the most part by genetic differences, since as shown for multiple sclerosis and type 1 diabetes, children from families having recently immigrated from low-incidence to high-incidence countries develop the disease with high incidence [153, 154].

At the same time, the incidence of major infectious diseases has decreased in developed countries, even though some serious infections have persisted and new ones have appeared, such as AIDS. Particular attention should be given in this context to gastrointestinal infections, which are very prominent in underdeveloped countries and relatively rare today in western countries. This trend is clearly explained by the dramatic improvement in the quality of drinking water and food (cold chain) in western countries, particularly in young children. The correlation of the decline in infections and the increase in autoimmune diseases is further suggested by the correlation which has been observed between socio-economic levels (including quality of sanitation), and the frequency of major autoimmune diseases, either when considering whole countries or individual patients [145].

It has been proven in several animal models of autoimmune diseases that reduced exposure to infections increases risk of disease. It should be mentioned, however, that the protective role of infections is not a general rule, since some specific pathogen-free animals may develop autoimmune diseases, in particular conditions (transgenic T cell receptors or depletion of regulatory cells) and that infections may be required for the development of some autoimmune diseases, such as arthritis in the SKG mouse model [155] and inflammatory bowel disease [156].

How infections protect against allergic and autoimmune diseases is unknown. The mechanisms of the protective effect of infections on autoimmune diseases are most likely multifactorial. Most data are presently derived from animal models. Although autoimmune diseases are essentially Th1 diseases, while allergic diseases are Th2 diseases, it appears that the protective effect of infections on both types of disease is similar in each case.

This is interesting, since it contradicts the notion initially put forth that infections exclusively protect against allergic diseases through stimulation of Th1 cells. The development of most autoimmune diseases depends on the cytokines IL-2 and IFNγ produced by type 1 helper T cells, whereas the development of allergic diseases requires IL-4 and IL-5, both of which are produced by type 2 helper T cells. The reciprocal down-regulation of Th1 cells by Th2 cytokines and of Th2 cells by Th1 cytokines raises the possibility that these cytokines are involved in the infection mediated protection against allergy or autoimmunity. Contrary to initial reports, there is a trend toward an association between allergic and autoimmune diseases in individual patients: the frequency of atopic diseases is increased in patients with diabetes and rheumatoid arthritis [157]. These observations would fit with the concept of common mechanisms underlying infection mediated protection against autoimmunity and allergy. In fact, there is a tendency toward increased incidence of concomitant occurrence of allergic and autoimmune diseases in single individuals [158].

2.3. Hygiene Hypothesis

The high percentage of disease-discordant pairs of monozygotic twins demonstrates the central role of environmental factors in the etiology of autoimmune diseases. More than 50 and sometimes 70 or 80% of monozygotic twins are discordant for major autoimmune diseases. Such discordance is particularly striking when considering the fact that twins share much the same environment, at least during childhood. Numerous investigations have been devoted to the search for environmental factors controlling the onset of autoimmune diseases.

Is has become progressively apparent that infection could protect against autoimmune diseases, according to the hygiene hypothesis initially formulated for allergic diseases [145], infectious agents can suppress allergic and autoimmune disorders. The main factor in the increased prevalence of these diseases in industrialized countries is the reduction in the incidence of infectious diseases in those countries over the past three decades. This concept is not new. In 1966, it was suggested that the risk of multiple sclerosis is increased among persons who spent their childhood in a home with a high level of sanitation [159]. About 20 years later, the “hygiene hypothesis” was proposed by D.P. Strachan in an article that claimed an inverse relationship between the occurrence of hay fever and numbers of siblings [160]. Strachan observed that the risk of allergic rhinitis was inversely linked to birth order and the size of the family. He proposed that infections within households in early childhood have a role in preventing allergic rhinitis [160].

The “Hygiene Hypothesis” as described by Strachan was directed at understanding why the incidence of allergic responses might be increasing in the population [160, 161]. It was suggested that reduced exposure to infectious agents in infancy might predispose to hay fever. It was assumed that exposure to some infectious diseases would probably play major roles historically in shaping our immune systems [162]. The effects of certain parasitic and bacterial infections are to “tune” and moderate the immune response to prevent an over exuberant response that can have a pathological outcome.

2.3.1. Epidemiological data indicating a direct link between the decreasing level of infectious burden and the rising incidence of immunological disorders

Western countries are being confronted with a disturbing increase in the incidence of most immune disorders, including autoimmune and allergic diseases. Epidemiologic data provide strong evidence of a steady rise in the incidence of allergic and autoimmune diseases in developed countries over the past three decades. The incidence of many diseases of these two general types has increased: asthma [163], rhinitis [164] and atopic dermatitis [165]; representing allergic diseases, and multiple sclerosis [166], insulin dependent diabetes mellitus (type 1 diabetes) — particularly in young children [167] — and Crohn’s disease [168]; representing autoimmune diseases. The incidence of Crohn’s disease more than tripled in northern Europe from the 1950s to the 1990s [168]. Concomitantly, there has been an obvious decrease in the incidence of many infectious diseases in developed countries as a result of antibiotics, vaccination, or more simply, improved hygiene and better socioeconomic conditions. Figure 1 shows the estimated incidence of tuberculosis, rheumatic fever, measles, and mumps in the United States and of hepatitis A in France over a 50 year period. Intestinal infections are notable, because their frequency has decreased in developed countries as compared with less developed countries, particularly among children. Moreover, the age at which colonization of the intestinal flora occurs differs among countries: intestinal colonization with gram-negative bacteria, for instance, occurs later in developed than in less-developed countries, both quantitatively and qualitatively [169, 170].

The high prevalence of parasitic infections, notably with *plasmodia* and Schistosoma in southern countries, contrasts with the absence of these diseases in developed countries. Furthermore, the frequency of infestation by minor parasites such as *Enterobius vermicularis* (pinworms) over the past decade has decreased in developed countries [171].

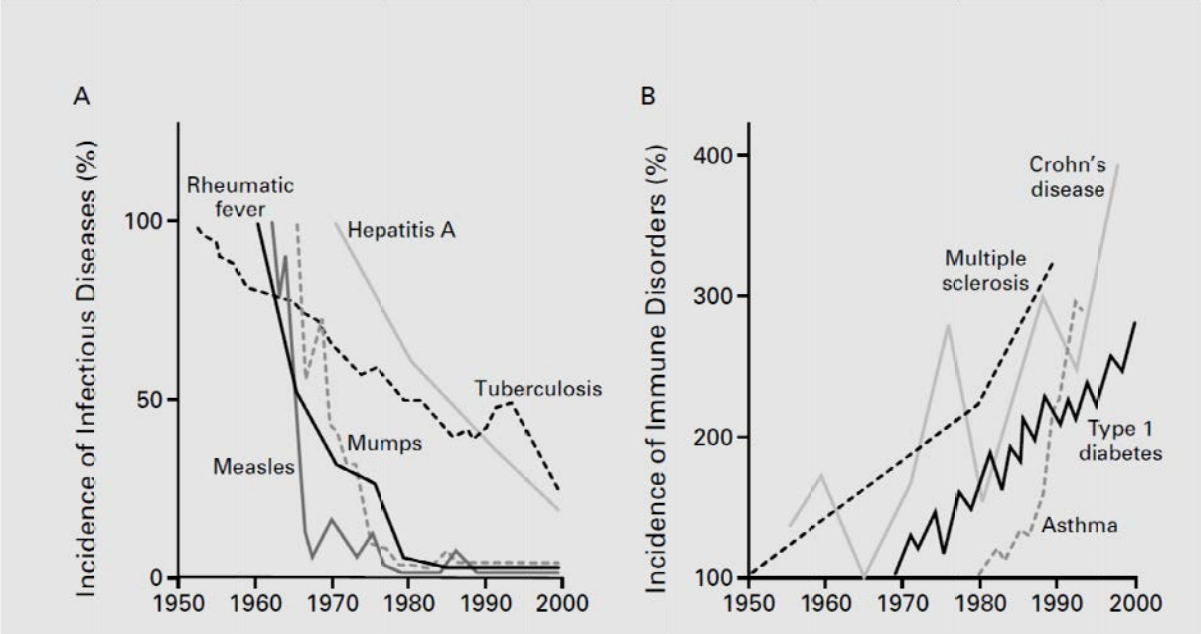

Figure 6: Inverse relation between the incidence of prototypical infectious diseases (Panel A) and the incidence of immune disorders (Panel B) from 1950 to 2000. In Panel A, data concerning infectious diseases are derived from reports of the Centers for Disease Control and Prevention [172]. In Panel B, data on immune disorders [168, 173-175]. (Adapted from ***Bach JF. 2002*** [151]).

The hygiene hypothesis does not exclude an etiological role for specific pathogens in a given immune disorder as might notably be the case in inflammatory bowel diseases.

2.3.2. Other environmental factors implicated in the modulation of the immune system

1) The North–South Gradient

Allergic and autoimmune diseases are not evenly distributed among continents, countries, well-circumscribed regions within a given country, or ethnic groups. An examination of the distribution reveals several important and probably interrelated phenomena. One is the north–south gradient: the incidence of disease decreases from north to south in the Northern Hemisphere (and reciprocally from south to north in the Southern Hemisphere).

2) Genetic Factors

There are several explanations for the gradient other than underdiagnosis. One is the role of genetic factors.

3) Interactions between Genetic and Environmental Factors

The degree to which genetic and environmental factors influence susceptibility to autoimmune and allergic diseases is still ill defined. The best hint derives from the concordance rates of such diseases in monozygotic twins. The rate is 25 percent in the case of multiple sclerosis [176], 40 percent in the case of type 1 diabetes [177] and 75 percent in the case of asthma [178].

One may assume that the concordance rate is directly related to penetrance of the disease, with the proviso that it is impossible to include in such analyses pairs of twins in which both twins have all the predisposing genes but are disease-free.

4) Socioeconomic Status

An obvious factor in the north–south gradient is socioeconomic differences. Several studies have found a lower frequency of immunologic diseases in populations with a low socioeconomic status.

5) Childhood Infection

When infection is an incriminating factor it often occurs early in childhood. Young children with older brothers and sisters at home and those who attend a day-care center during the first six months of life subsequently have a lower incidence of asthma and type 1 diabetes than children who do not attend a day-care center and who have no older siblings [179].

Another influential factor is the quality of medical care, which varies substantially from country to country with respect to the use of vaccines and antibiotics. The administration of antibiotics to children has been suspected to increase the risk of asthma and allergy.

6) Other Factors

Other factors that may not influence the rate of infection should not necessarily be dismissed. The climate (notably the extent of exposure to sunlight) and a number of culturally based differences in behavior, notably diet, may also be important. Air pollution may have a role in asthma. But even though air pollution can worsen the clinical status of patients with asthma, it does not appear to affect the incidence of asthma. Other risk factors may include organ damage by environmental toxins and the immunomodulatory effect of vitamin D deficiency [151].

2.3.3. Understanding the Hygiene Hypothesis

Independently of the need for confirmation by epidemiological prospective studies, the hygiene hypothesis still poses numerous questions concerning the nature of protective infectious agents, the timing of their involvement with regard to the natural history of immune diseases and, most importantly, the mechanisms of protection. Four orders of mechanisms are

being explored. Antigenic competition is the first hypothesis (immune responses against pathogens compete with autoimmune and allergic responses). This is probably an important mechanism but its modalities are still elusive in spite of considerable experimental data. Its discussion in the context of homeostatic regulation of lymphocyte pools has shed new light on this hypothesis with possible competition for self MHC peptide recognition and IL-17. Another hypothesis deals with immunoregulation. Infectious agents stimulate a large variety of regulatory cells (Th2, CD25C, Tr1, NKT,...) whose effects extend to other specificities than those which triggered their differentiation (bystander suppression). Infectious agents may also intervene through components which are not recognized as antigens but bind to specific receptors on cells of the immune system. Major attention has recently been drawn to Toll receptors (expressed on macrophages and possibly on regulatory T cells) and TIM proteins present on Th cells, which may express the function of the virus receptor (as in the case of the *Hepatitis A* virus and Tim-1) [145].

2.4. Parasitic infections modulate immune response

Not only residents of western countries but also immigrants from developing countries are at high risk of developing inflammatory bowel diseases and asthma [180]. In the case of type 1 diabetes, a similar geographical distribution to the diseases above and an inverse correlation to hygiene conditions are observed [181]. A population-based ecologic study in Canada showed that IBD, including ulcerative colitis and Crohn's disease, correlated with a high socioeconomic status, low rate of enteric infection, and high rate of Multiple sclerosis [182].

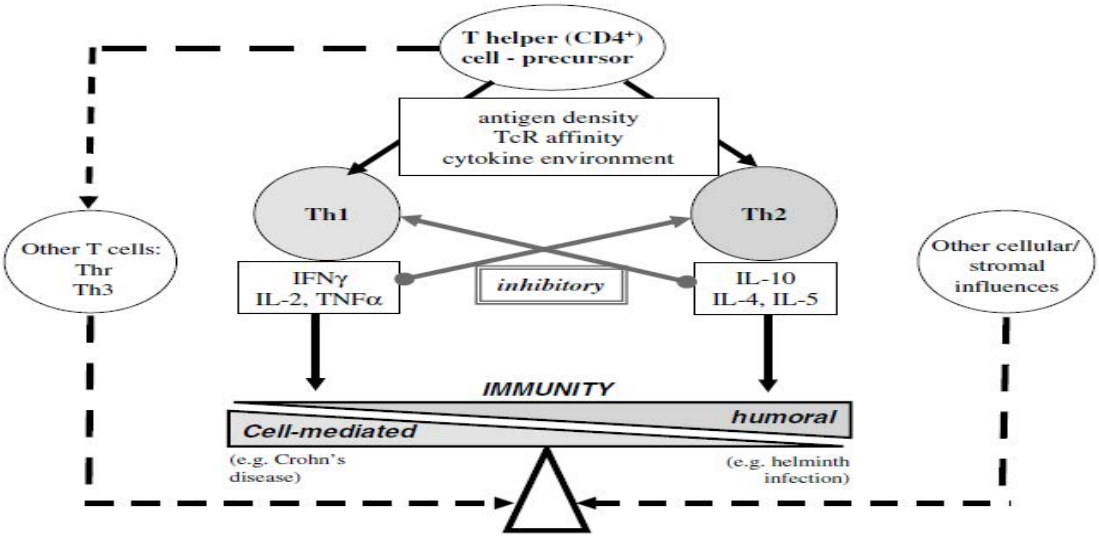

Figure 7: showing how T helper (Th) type 1 and 2 cells develop. The ability of their respective mediators to reciprocally down regulate the other cell type and how each

preferentially promotes either (i) antibody/humoral immune responses directed against extracellular stimuli or (ii) cell-mediated immunity (e.g. enhanced cytotoxic cell activity) to combat intracellular pathogens, viruses and abnormal self antigen expression. Note that in the balance of immunity, while humoral or cell-mediated events can predominate the other type of immune response can still be found (Adapted from ***Mosmann TR. et al, 1986*** [183]).

Infection with parasitic helminths reliably induces a Th2 immune response in the host characterized by high IgE synthesis and eosinophilia [184]. Helminths are master regulators of host immune responses utilizing complex mechanisms to dampen host protective Th2 type responses and favors long-term persistence. Such evasion mechanisms ensure mutual survival of both the parasite and the host. A characteristic feature of host immune response to parasite is the implication of both the innate and the adaptive immunity – a feature that is only shared with allergic reaction.

The key players in Th2 type immunity are CD4+ Th2 cells and involve the cytokines IL-4, IL-5, IL-9, IL-10, IL-13 and Ig E. CD4+ Th2 cells also express some of these cytokines as well as the chemokine ligand CCL11 and the chemokine receptor CCR3 [185-187].

Three main features are present in Th2 type immune response: inflammation, wound repair, and, most importantly, resistance to helminths [188].

Parasites are capable of secreting of a wide range of immunoregulatory molecules, which are able to target various host cells and alter them to induce a highly directed host response known as a “modified Th2 type response.” This response is designed to limit a possibly detrimental Th2 immune response, thus restraining the extreme symptoms that are often observed in allergy or in aspects of helminth diseases such as fibrosis in *Schistosoma mansoni* [189].

Several mechanisms are implicated in the immune regulatory pattern observed with Helminth infection; including both modulation of immune cells, induction of innate and adaptive regulatory cells, anti-inflammatory cytokines and specific inhibitory antibody isotypes [186] leading to alteration of the host immune response.

When considering the multitude of Helmith parasites agents that can induce protection from various immunological disorders, several theories have been studied – none of which is exclusive.

2.4.1. T helper Th1 –Th2 deviation

Th1–Th2 deviation was the first major candidate mechanism for explaining the protective influence of infectious agents from immunological disorders. Some authors suggested initially that in developed countries the lack of microbial burden in early childhood, which normally favours a strong Th1 biased immunity, redirects the immune response towards a Th2 phenotype and therefore predisposes the host to allergic disorders.

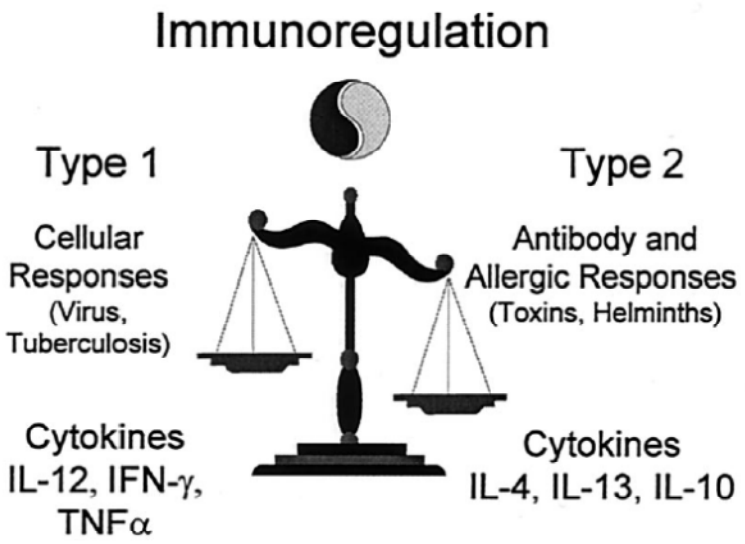

Figure 8: Infestation with helminths down modulates the Th1 immune response to unrelated parasitic, bacterial, and viral infections (Adapted from ***Weinstock JV. et al, 2002*** [190]).

The problem with such an explanation is that autoimmune diseases, which in most cases are Th1 cell mediated, are protected by infections leading to a Th1 response and that atopy may be protected, as seen above, by parasites which induce a Th2 response. These observations fit with the concept of a common mechanism underlying infection-mediated protection against allergy and autoimmunity [191].

2.4.2. Antigenic competition /homeostasis

It has been known for several decades that two immune responses elicited by distinct antigens occurring simultaneously tend to inhibit each other. Numerous mechanisms were evoked to explain antigenic competition that might be pertinent to the hygiene hypothesis. The development of strong immune responses against antigens from infectious agents could inhibit responses to ‘weak’ antigens such as auto-antigens and allergens [191].

We have long been aware of antigenic competition: immune responses to single antigens are usually stronger than the response to the antigen administered concomitantly with other antigens. Several mechanisms have been described to explain antigenic competition, including the pre-emption effect on macrophages, competition for cytokines or growth factors, and competition for peptide binding to MHC molecules. These mechanisms have been revisited in the context of the new concepts on lymphocyte homeostasis. It is now apparent that lymphocyte proliferation and survival depend on a number of homeostatic signals, including cytokines such as IL-7 and self-peptide MHC recognition. One may postulate that the strong immune responses that are elicited by infectious agents compete with immune responses against weaker antigens, such as autoantigens and allergens, for homeostatic signals [192].

2.4.3. Immunoregulation

It has been shown in various models of immunoregulation that the suppressive effect induced by a defined antigen may extend to immune responses specific to other antigens (bystander suppression).

This mechanism involves regulatory T cells which can suppress immune responses distinct from reponses against the antigen in question, here antigens expressed by infectious agents. The problem is complicated by the multiplicity of regulatory lymphocytes involving diverse cytokines that mediate their differentiation or their regulatory effects [193-196].

It is thus conceivable that regulatory cells stimulated by infectious agents dampen autoimmune responses. This mechanism may of course include Th2 cells, although there is only limited data supporting it. Studies performed where NOD mice are protected from diabetes after administration of a Gram-positive bacterial extract have shown that TGFβ played an important role in conditions in which Th2 cytokines were not involved [197].

2.4.4. Non-antigenic ligands

All the mechanisms mentioned previously are based on the notion that the hygiene effect is due to the decrease of immunological responses elicited against infectious agents.

A number of experiments indicate that infectious agents can promote protection from allergic diseases through mechanisms independent of their constitutive antigens, leading to stimulation of non-antigen specific receptors. This concept is well illustrated by the example of TLRs. Knowing the capacity of TLRs to stimulate cytokine production and immune responses, it might be predicted that TLR stimulation by infectious ligands should trigger or

exacerbate allergic and autoimmune responses. Surprisingly, and paradoxically, it has also been observed that TLR stimulation could prevent the onset of spontaneous autoimmune diseases such as Type 1 Diabetes in non obese mice, an observation made for TLR-2, -3, -4, -7 and -9 [198]. Similar data have been observed in an ovalbumin-induced model of asthma [199]. Concerning *Hepatitis A* virus, it was shown initially that atopic diseases were less common in subjects that have been exposed to the virus [200].

2.4.5. Gene – environment interactions

An interesting approach to identify mechanisms underlying allergic and autoimmune diseases consists in searching for associations between these diseases and polymorphisms of various genes, notably those coding for molecules involved in immune responses. It is interesting to note that such an association has been found for genes implicated in the control of infection. Among them, polymorphism in genes of the innate immune response such as CD14, TLR2, TLR4, TLR6 or TLR10, and intracellular receptors such as NOD1 and NOD 2, appears to be important [201]. Mouse studies have shown that these gene – environment interactions explain a proportion of the phenotypic variance [202].

Many studies have demonstrated that helminth infections lower the risk of autoimmunity or allergy. Experimental studies have also shown protective effects of helminth infections in animal models of autoimmunity (e.g., colitis, arthritis, and diabetes) and allergy (e.g., airway hypersensitivity) [203, 204]. **(Table 1)**

Animal models	**Helminths**	**Treatment**	**Proposed suppressive mechanisms**	**Refs**
Collagen-induced arthritis (CIA)	*Ascaris suum*	Worm Ag		[205]
Collagen-induced arthritis (CIA)	*Acanthocheilonema viteae*	Purified Ag (ES-62)	IFNγ↓, TNFα↓, IL-6↓, Anti-collagen IgG↓	[206]
Experimental autoimmune encephalomyelitis (EAE)	*Fasciola hepatica*	Infection	IFNγ↓, IL-17↓, Dependent on TGFβ	[207]
Experimental	*Trichinella spiralis*	Infection		[208]

autoimmune encephalomyelitis (EAE)				
Type 1 diabetes in NOD mice	*Litomosoides sigmodontis*	Infection, Worm Ag	IL-4↑, IL-5↑, Treg	[209]
Type 1 diabetes in NOD mice	*Heligmosomoides polygyrus*	Infection	Independent of IL-10 and Treg	[210]
Streptozotocin-induced diabetes	*Taenia crassiceps*	Infection	AAMs	[211]
TNBS/DNBS-induced colitis	*Hymenolepis diminuta*	Infection	IL-10↑	[212]
Piroxicam-induced colitis	*Heligmosomoides polygyrus*	Infection	IL-17↓, Independent of IL-10	[213]
Asthma/Airway hypersensitivity or inflammation	*Heligmosomoides polygyrus*	Infection	Treg	[214]
Asthma/Airway hypersensitivity or inflammation	*Ascaris suum*	Purified Ag (PAS-1)	IL-4↓, IL-5↓, Eotaxin↓, RANTES ↓, IL-10↑	[215]
Asthma/Airway hypersensitivity or inflammation	*Ascaris suum*	Worm Ag	IL-4↓, IL-5↓, Eotaxin↓, IgE↓	[216]
Asthma/Airway hypersensitivity or inflammation	*Litomosoides sigmodontis*	Infection	TGFβ↑, Treg	[217]

Table 1 a: Suppression of experimental immunological disorders by helminths.

Collagen-induced arthritis (CIA)	*Schistosoma mansoni*	Infection	IL-17 ↓, TNFα↓, IL-6 ↓, RANKL ↓, Anti-collagen IgG ↓	[218]
Experimental autoimmune encephalomyelitis (EAE)	*Schistosoma mansoni*	Infection	IL-12p40↓, IFNγ↓, TNFα↓, IL-4↑	[219]
Experimental autoimmune encephalomyelitis (EAE)	*Schistosoma mansoni*	Egg	IFNγ↓, IL-4↑, TGFβ↑, IL-10↑	[220]
Experimental autoimmune encephalomyelitis (EAE)	*Schistosoma japonicum*	Egg Ag	IFNγ↓, IL-4↑	[221]
Type 1 diabetes in NOD mice	*Schistosoma mansoni*	Infection, Eggs	Inhibition of Ab class switch (Anti-insulin IgG↓)	[222]
Type 1 diabetes in NOD mice	*Schistosoma mansoni*	Egg Ag	Treg	[223]
TNBS/DNBS-induced colitis	*Schistosoma mansoni*	Infection	IL-2 ↑, IL-4 ↑	[224]
TNBS/DNBS-induced colitis	*Schistosoma mansoni*	Eggs	IFNγ↓, IL-4↑	[225]
TNBS/DNBS-induced colitis	*Schistosoma mansoni*	Worm Ag	IFNγ↓, IL-17↓, TGFβ↑, IL-10↑	[226]
TNBS/DNBS-induced colitis	*Schistosoma japonicum*	Egg Ag	IFNγ↓, IL-4↑, IL-10↑, Treg	[227]
DSS-induced colitis	*Schistosoma mansoni*	Infection (male worm)	Macrophage infiltration	[228]
Systemic anaphylaxis	*Schistosoma mansoni*	Infection	IL-10-producing Bcell	[229]
Asthma/Airway hypersensitivity or inflammation	*Schistosoma mansoni*	Infection (male worm)	IL-5 ↓, IL-10 ↑	[230]
Asthma/Airway hypersensitivity or inflammation	*Schistosoma japonicum*	Egg Ag, Eggs	Treg	[231]

Table 1 b: Suppression of experimental immunological disorders by helminths (Schistosomes).

Autoimmune disorders had been considered as a Th1- mediated diseases for a long time. As the Th2 biasing ability of parasitic helminths and consequent downregulation of Th1 responses have been well known, anti-autoimmune effects of such parasites have been attributed to the downregulation of Th1 responses in infected animals. However, some major autoimmune diseases are now considered to be dependent on Th17, a newly found pathogenic T-cell subset that mainly produces IL-17 [232]. With this finding, the anti-autoimmune

properties of helminths have been revisited. In recent years, down-modulation of Th17 responses by parasitic helminths has been reported [207, 213, 218, 226]. If the suppressive activity on both Th1 and Th17 is common to parasitic helminths, helminths may become ideal sources of drug screening for treatment of autoimmune disorders [233].

2.5. Schistosoma infection "Master of immune regulation"

Schistosomes are parasitic worms that are a prime example of a complex multicellular pathogen that flourishes in the human host despite the development of a pronounced immune response. Understanding how the immune system deals with such pathogens is a daunting challenge [234].

2.5.1. Life cycle

Schistosoma infection causes a chronic disease, called Schistosomiasis, which is associated with periportal fibrosis, hepatosplenomegaly and ascites. Despite considerable therapeutic efforts and prevention measures, Schistosomiasis still affects about 200 million people in developing countries with more than 300,000 deaths per year in sub-Saharan Africa alone [235]. It is a part of the great neglected tropical diseases.

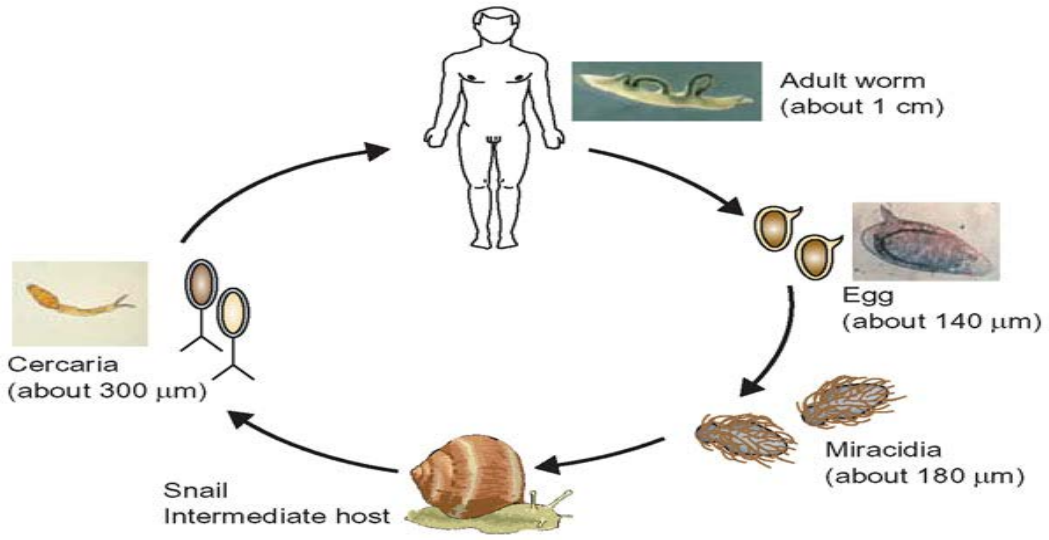

Figure 9: The Schistosome life cycle. Schistosomes reproduce asexually in freshwater snails; a larval form, the cercaria, is released from the snail and can burrow into the skin of the definitive host, man. In humans, Schistosomes migrate to the bloodstream where they mature

into adult worms. Eggs produced by the female worm are released into the environment where they hatch into a second larval form, the miracidia, which can infect the snail (Adapted from ***Hoffmann KF. et al, 2003*** [236]).

Schistosomes are members of the class Trematoda, a large, diverse and medically important group of parasitic worms within the phylum Platyhelminthes (flat worms).

Schistosomiasis (or bilharziasis) has been a scourge of man for tens of thousands of years. Schistosome eggs have been found in ancient mummies in both Egypt and China. In 1851, working in Egypt, *Theodore Bilharz* first reported the presence of adult Schistosome worms in human blood vessels [236].

Adult Schistosomes, in contrast to other parasites, live as couples with the female laying in the ventral groove of the male. The final habitat of adult S. mansoni is the mesenterial vasculature of the host, where they produce about 300 eggs per couple per day. About half of the eggs cross the vascular endothelium at the site of deposition, the intervening tissues and the mucosal epithelium to reach the gut lumen, from where they are finally released with the feces. If the feces get into fresh water, both the drop in salt concentration and exposition to sun light trigger hatching of the miracidia from the eggs. The miracidia are ciliated larvae, which actively approach and invade the intermediate host, a water snail. Within 4-6 weeks each miracidium multiplies via two sporocyst generations into a few thousand fork-tailed larvae (cercariae). These are released from the snail into the water ready to enter their definitive host, i.e. the human. Upon penetrating the skin of the host, cercariae lose their tails and transform into so-called Schistosomula. The Schistosomula then travel with the blood stream through several organs including the lung and, after reaching the liver, mature into adult worms. Following mating, the adult couples crawl against the blood flow into the portal veins to start egg production [184].

For the host immune system, infection with *S. mansoni* means that it has not only to deal with a multi-cellular pathogen, but is, moreover, confronted with several different developmental stages of this invader: cercariae, Schistosomula, adult worms and eggs. Interestingly, adult worms live in the blood for 7–10 years nearly unrecognized, whereas Schistosome eggs are a very prominent target of the host's immune response. When passing the endothelial barrier at the site of deposition, the eggs are immediately attacked by recruited immune cells resulting in the formation of Th2 type granulomas consisting mainly of $CD4^+$ T cells, eosinophils, and macrophages [234]. Moreover, as known from immune-compromised mice, an intact T cell response as a precondition for granuloma formation seems to be important for the eggs to

migrate through endothelium and gut wall, although the mechanism by which the eggs move through the tissues has yet to be elucidated [237]. On the other hand, about half of the eggs are washed away with the blood stream mainly into the liver, where they become trapped in the liver sinusoids again inducing granuloma formation. In the liver, granuloma formation seems to be host-protective by keeping hepatotoxic egg products away from hepatocytes [238]. However later-on, this has also its price: fibrotic processes during granuloma involution and reorganization may lead to liver fibrosis, hepatosplenomegaly and ascites namely in severe chronic disease with high egg loads.

In the first weeks of murine Schistosoma infection, while the host is exposed to Schistosomula and semi-mature Schistosomes, a more Th1-like immune response is observed. After 5-6 weeks with the onset of egg deposition, however, a pronounced Th2 immune response comes up being characterized by high production of IL-4 and IL-13, IgE synthesis as well as eosinophilia and mastocytosis. By week 12, when infection becomes chronic and egg production continues, a general down-modulation of immune reactivity sets in leading to a diminished Th2 response together with a smaller size of newly formed granulomas and reduced numbers of granuloma $CD4^+$ T cells [234]. The fact that the Th2 response develops with onset of egg production suggests that the egg stage is responsible for the Th2 shift, although a Th2 biased response has been reported to infective larvae penetrating the host skin [239] and to be induced also in the pre-patent stage [240]. The importance of the egg stage for Th2 induction, however, is highlighted by the finding that during infection with irradiated or single-sex cercariae, where no egg laying takes place, both, induction of a Th2 response and down-regulation of the immune response do not occur [234]. Moreover, the capacity for Th2 induction is not restricted to a full-blown infection, but is likewise seen when live Schistosome eggs, water-soluble *S. mansoni* egg antigens (SEA) or excretory/secretory egg products, respectively, are injected into mice [241]. This indicates that *S. mansoni* eggs contain and release components, which can trigger a powerful Th2 response. Since secretory products are released from live *S. mansoni* eggs, they are the first egg molecules that come into contact with the host's immune cells and are, thus, likely to convey the immunomodulatory properties of Schistosome eggs. In contrast, structural egg proteins become accessible to the immune cells only later, when the eggs are dying and disintegrating. Secretory egg products represent a much less complex mixture of egg molecules than whole SEA, and recent proteomic studies revealed two glycoproteins, omega-1 and IPSE/alpha-1, to be the most abundant products secreted [242, 243].

2.5.2. Immune response to Schistosoma infection

Cells targeted by parasitic immuno-modulation are now becoming a focus for immunological studies. This is more dependent on live parasites as shown by the recovery of cellular hypo responsiveness in patients treated with anti helminthic chemotherapy [244]; restoration of immune responses to a bystander vaccine targeting human immunodeficiency virus-1, which were greatly diminished in the presence of the parasite [245]; and elimination of intestinal helminths in patients vaccinated with bacille Calmette-Guerin that resulted in greater protective responses to the vaccine compared with patients who did not receive anti helminthic chemotherapy [246].

These studies showed that the presence of live worms or active infection ensured the secretion of immune modulatory molecules which activates and recruits immune regulatory cells. Despite the different anatomical locations target similar host cells employing comparable regulatory mechanisms.

Murine Schistosomiasis is characterized by a gradual switch from a predominant Th1 cytokine response (before the egg laying) to a Th2-dominated response, an event that favors the formation of granulomas around viable eggs in the liver [247, 248]. Egg-derived glycoconjugates play a key role in skewing the immune response towards a Th2 phenotype. Among the candidates responsible for this effect, complex-type N-glycans containing the core α 3-fucose and core β 2-xylose determinants, two glycan epitopes found in some invertebrate- and plant-derived allergens, may be important [249].

Previous reports have demonstrated the effect of Schistosoma infection on the down regulation of inflammation through inhibition of Th1 function [248]. It was proven in other animal models of inflammation - bronchial asthma or collagen induced arthritis - alternatively-activated macrophages together with $CD4^+CD25^+$ cells both can participate in the down-modulation of inflammatory allergic response in *S. mansoni*-infected animals by systemic and local suppression of pro-inflammatory mediators [218, 250].

a) Dendritic cells

DCs are the most important cells of the innate immune system in terms of antigen presentation and polarization of naïve T helper cells [251]. Therefore, DCs represent an important link between the innate and the adaptive immune system. As “sentinel cells” DCs carry pathogen recognition receptors on their surface to screen their environment for pathogen-derived signals, so-called pathogen-associated molecular patterns [252]. The nature of the pathogen and signals (cytokines, chemokines) released from a variety of recruited

inflammatory cells evoke functional changes in the DCs such as loss of phagocytic activity, up-regulation of co-stimulatory molecules (e.g. CD80, CD86) and cytokine production (e.g. IL-12, IL-10). DCs conditioned this way then migrate to the lymphatic organs and instruct naïve T helper cells to become effector Th cells [253]. The mechanism of the Th2 polarization is less well understood. A major role in driving a Th2 response has been assigned to the cytokine IL-4. As DCs do not produce IL-4 by themselves, this cytokine has to be provided to the DCs by another cellular source [254] or alternative mechanisms must come into play. The latter possibility is supported by the more recent findings that DCs do not need IL-4 to promote Th2 polarization and that Th2 induction can occur independently of IL-4, IL-4Rα- and STAT6 mediated signaling [255, 256].

As DCs are the main messenger cells to communicate with T cells and initiate an immune response, interference with their functions represents a key mechanism for helminths to induce an environment conducive to their survival.

i. DC activation during S. mansoni infection

Stimulation of dendritic cells (DCs) by the egg stage of the helminth parasite *Schistosoma mansoni* activates a signaling pathway resulting in type I interferon and IFN-stimulated gene (ISG) expression [257]. It was shown - for the first time from a non viral pathogen - that Schistosome eggs dsRNA may act as an inducer of the innate immune system through TLR3 [257].

SEA-pulsed DC proved to be extremely potent activators of naïve T cells, inducing a definitive SEA-specific Th2 response after transfer into naïve recipient mice [258].

Murine DCs stimulated with lipids from *S. mansoni* eggs matured to induce specifically Tregs that produced IL-10, reducing Th1 responses whilst producing a modulated Th2 response [259].

DCs are capable of downregulating costimulatory molecules, leading to induction of a Th2 response [260]. They can suppress both Th1 and Th2 responses.

Nevertheless, DCs are clearly essential for efficient priming of the Th2 response, as depletion of CD11c+ cells during development of the adaptive CD4+ T cell response in *S. mansoni* infection drastically impaired the Th2 response [261].

ii. Role of DCs in Th2 induction by Schistosoma

SEA is a complex mix of diverse components that is crudely analogous to the metabolic secretions of live eggs. Notably, each of SEA, live or dead *S. mansoni* eggs provoke a striking Th2 response when injected directly into naïve recipient mice, without the need for additional

adjuvant [258]. Indeed SEA, like some other helminth products or extracts, could itself be described as an adjuvant, as it is able to promote Th2 development to co-administered model antigens[262]. Further, constituents of SEA have been identified that interact with and activate a variety of innate cells, including mast cells and eosinophils [263].

In the case of *S. mansoni* infection, live eggs, egg extracts and egg-secreted products have been shown to condition human as well as murine DCs to promote the differentiation of naïve T helper cells towards the Th2 phenotype both in vitro and in vivo [264, 265]. Schistosome eggs or products seem to affect the activation status of the DCs, their expression of co-stimulatory molecules and their mode of interaction with T cells. Several studies have shown that DCs conditioned with SEA, in contrast to DCs conditioned with LPS or Th1 inducing agents, do not mature in the conventional way. They do not secrete any IL-12 or IL-10 and do not up-regulate the co-stimulatory molecules CD80 and CD86 on their surface [266]. Low expression of co-stimulatory molecules, however, is thought to go along with impaired DC T cell interaction and suboptimal T cell activation. Correspondingly, T cell cycling is transiently delayed, when $CD4^+$ T cells are co-cultured with SEA-conditioned DCs [267]. Thus, while SEA reduces CD80 and CD86 expression of DCs, other co-stimulatory molecules must be present on DCs at normal or increased rates for induction of Th2 cells.

In the early stages of infection, Schistosome larvae in the skin site of exposure induce an inflammatory reaction and cellular influx that includes DC. There is also evidence for the activation and maturation of DC in the skin following cercarial penetration, as determined by up-regulation of MHC II and CD86 expression, albeit coinciding with delayed migration [268]. It appears that phenotypic activation of DC by *S. mansoni* in vivo is promoted by CD40:CD154 interaction and inhibited by IL-10. It is currently unclear how other types of DC (including liver, gut and plasmacytoid DC) respond to *S. mansoni* exposure, either in vivo during infection or in vitro.

Taken together, Schistosome eggs or products thereof condition DCs to drive a Th2 response. Mechanisms involved are decreased expression of the co-stimulatory molecules CD80 and CD86 on the DCs, impaired DC T cell interaction and reduced DC IL-12 production.

The host's response to soluble secretions from mature eggs is characterized by an intense delayed-type hypersensitivity reaction that is principally mediated by egg Ag-specific MHC class II-restricted CD4+ Th cells. Analysis of the immune response in *Schistosoma mansoni*-infected mice shows that the Th0/Th1 cytokine response shifts toward a strong Ag-specific as well as nonspecific Th2 response with the maturation of the granulomas [247].Ag presentation of

parasite glycoconjugates to CD1d-restricted T cells may be important in the early events leading to the induction of Th2 responses and to egg-induced pathology during murine Schistosomiasis [258].

b) Regulatory T cells

CD4+ Tregs are particularly implicated in controlling innate and adaptive immunity. In a murine model of Schistosomiasis CD4+CD25+ T cells expressing high quantities of IL-10 were shown to play a significant role in reducing immunopathology, especially in the chronic stage of infection [269]. In *S. mansoni*-infected mice CD4+CD25+Foxp3+ cells produced significant levels of IL-10 that were required to prevent DC derived IL-12, thereby suppressing Th1 responses [270]. Isolated Schistosome eggs were demonstrated to be capable of modulating Th2 responses, without the need for the former life cycle stages that develop during infection. Schistosome eggs injected into mice were shown to induce a population of Foxp3+ cells that expanded to control the CD4+ T cell response, preventing inflammatory Th1 responses while modulating Th2 responses to prevent Th2 mediated immunopathology [271]. Both natural and adaptive Tregs have been described in filarial-infected persons, with the adaptive Treg population producing high levels of IL-10 [272]. During parasitic infection, Tregs may therefore be seen as important effector cells required to prevent or reduce pathology in the host by modulating the ensuing Th2 response, thereby simultaneously allowing establishment of chronic infection.

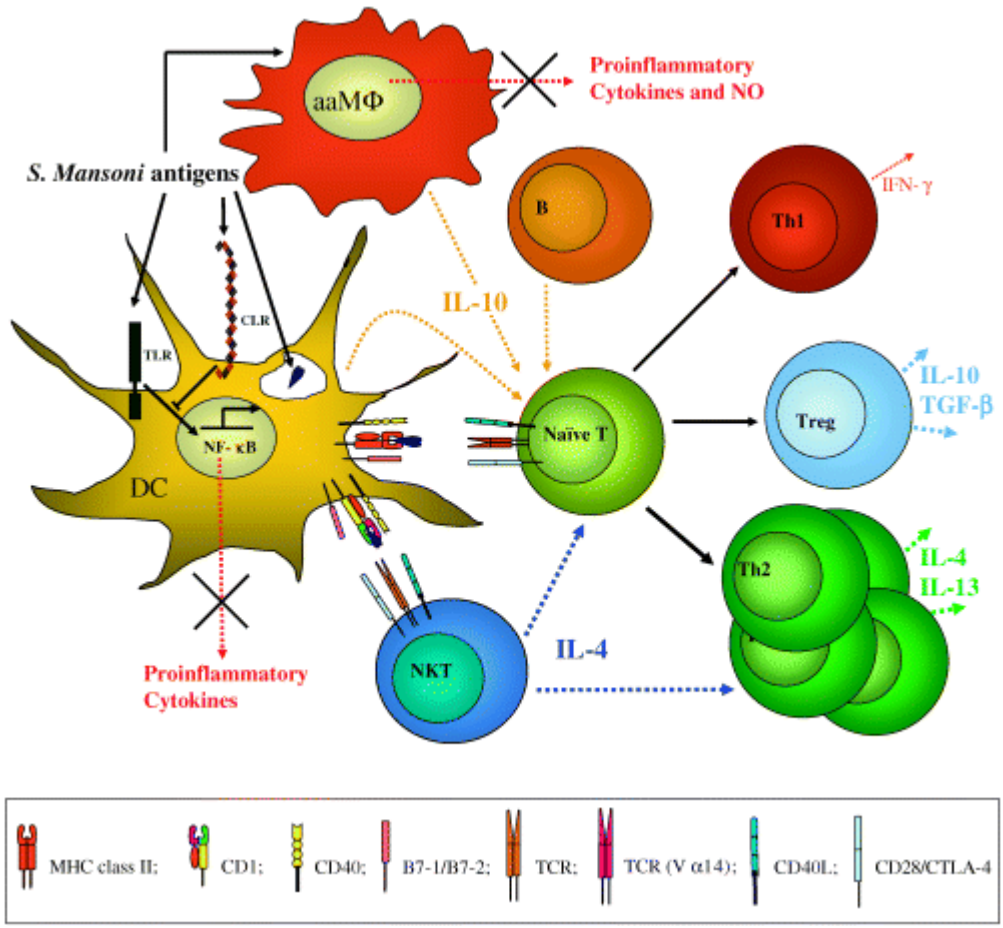

Figure 10: *S. mansoni* modulation of the immune response. *S. mansoni* live helminth and antigens modify cells of the innate immune system through interaction with TLRs and CLRs arresting the production inflammatory mediators and eliciting instead, the release of immunoregulatory cytokines such as IL-10. This results in the generation of suppressive Treg and a bias towards a Th2 response (Adapted from ***Zaccone P. et al, 2006*** [181]).

c) Regulatory B cells

Host protection as well as regulation by antibodies and B cells is being recognised as an essential component in Th2 responses in helminth infections [273]. In *S. mansoni*-infected mice, where the dominant isotypes are IgG1 and IgE, blockade of B cell production resulted in high levels of the proinflammatory cytokines IFNγ and IL-12 but low levels of the Th2 cytokines IL-4 and IL-10 in acute infection [274]. Antibody isotypes are demonstrated to have an important role in determining the outcome of helminth infection in the host. IgG4 correlates with high levels of IL-10 and the presence of adult worms in hyporesponsive persons. The development of specific antibody isotypes, including induction of IgG4 accompanied by a decrease in IgE, as well as IL-4 and IL-5, while IL-10 levels from different regulatory cell sources increase [275].

IgE is known to activate degranulation of mast cells, basophils and eosinophils and induce antibody-dependent cell-mediated cytoxicity [273]. IgG1 in mice and IgG4 in humans compete with IgE for binding sites. Thus, inhibitory IgG4 may prevent immunopathological responses in helminth asymptomatically infected individuals and can simultaneously provide an indication of the clinical outcome in infected persons [188].

Helminth infections can induce specific B cell phenotypes with regulatory properties as shown in infection with *S. mansoni* and *H. polygyrus* [276]. In *S. mansoni* infection, a particular subset of B cells has been described that are CD1dhigh and express high levels of IL-10 (defined as CD19 + IL10 + CD1d high CD5 + CD21 high CD23 + IgD + IgM high cells)[277].

d) Alternatively activated macrophages

Macrophages play crucial roles in the immune response, as they can initiate, modulate and also be final effector cells during immune responses to infections. Macrophages are derived from myeloid precursor cells in bone marrow and are widely distributed in every tissue of the body.

The concept of an alternative pathway of macrophage activation induced by the T helper 2 cytokines IL-4 and IL-13, distinct from IFNγ-mediated classical activation, has gained considerable ground over the past decade. Macrophages that are activated by the Th2-type cytokines IL-4 and IL-13 develop an alternatively activated phenotype and have a well described role in helminth infections. Alternatively activated macrophages (AAMs) are recruited in large numbers to the sites of helminth infection where they can proliferate.

IL-4 and IL-13 are the prototypical direct inducers of AAMs, but other cytokines such as IL-33 and IL-25 amplify AAM induction indirectly, through Th2 cells [278].

i. Inflammasome activation

Little is known about inflammasome activation and IL-1β release by AAMs, compared with CAMs. Decrease of caspase 1 expression and pro-IL-1β processing has been described for human monocytes stimulated with IL-13 [279]. A similar downregulation is observed in some human macrophage models. In fact, IL-13 is critical for worm expulsion and also plays an important role in Schistosome egg granuloma formation and fibrosis.

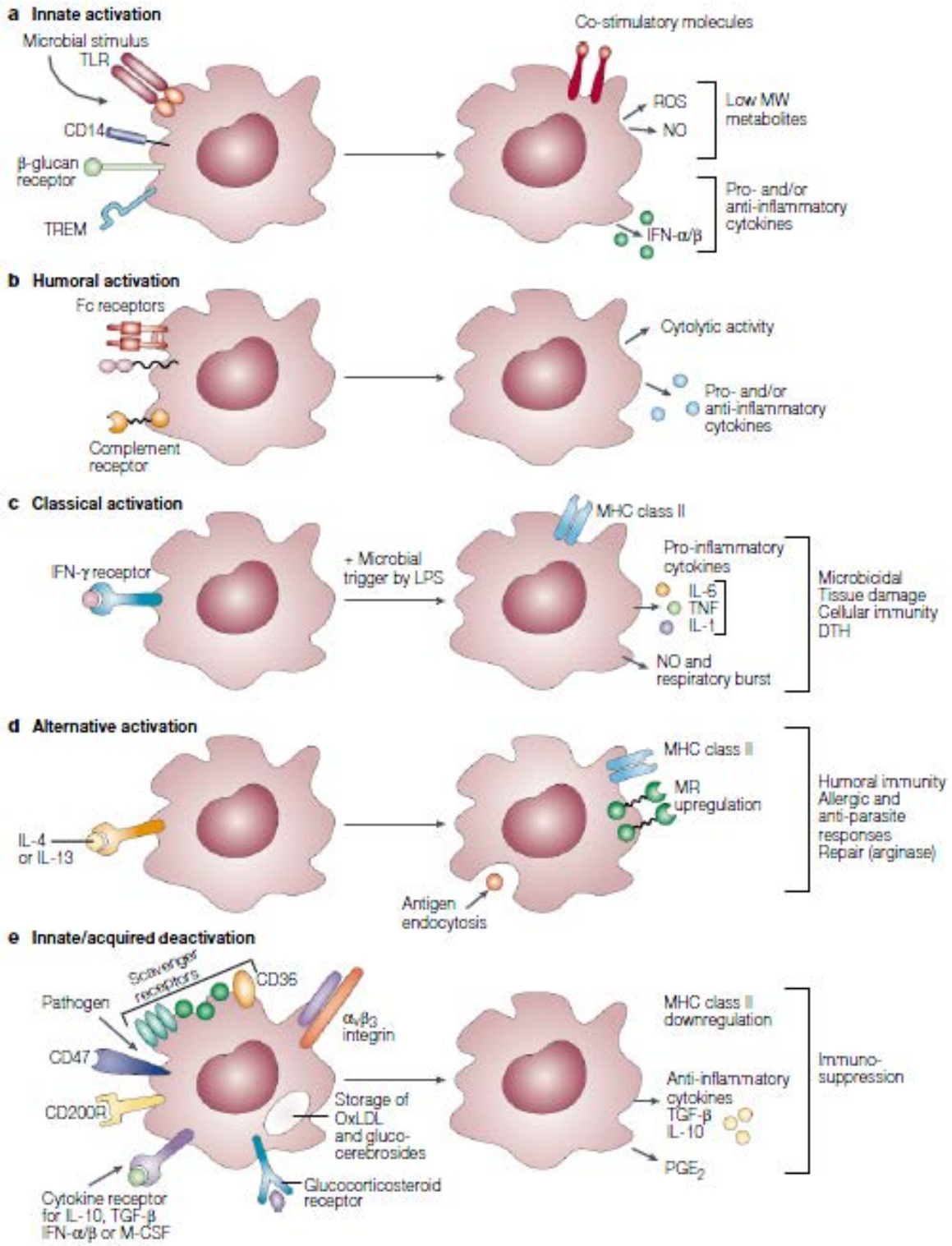

Figure 11: Innate and acquired immune activation of macrophages. a | Microbial stimuli are recognized by pattern-recognition receptors, such as Toll-like receptors, CD14/lipopolysaccharide-binding protein and a range of non-opsonic receptors. These stimuli induce the production of pro-inflammatory cytokines, such as IFN α/β), and reactive oxygen species and nitric oxide, followed by a regulated anti-inflammatory response. Enhanced expression of co-stimulatory surface molecules favours antigen presentation. Scavenger

receptor-A and mannose receptor promote the phagocytosis and endocytosis of host, as well as exogenous, ligands. b | Humoral activation and phagocytosis are mediated by some Fc and complement receptors, whereas other receptors downregulate responses. c | Classical activation is mediated by the priming stimulus IFNγ, followed by a microbial trigger LPS. d | Alternative activation is mediated by IL-4 and IL-13, acting through a common receptor chain (IL-4Rα). e | Deactivation can be innate or acquired in origin. The uptake of apoptotic cells or lysosomal storage of host molecules generates anti-inflammatory responses. Cellular activity is modulated by the interactions of macrophages with T cells, fibroblasts and matrix, through a range of receptors. Cytokines and glucocorticosteroids are potent modulators of activation. Pathogens can deactivate macrophages by various mechanisms (Adapted from ***Gordon S. 2003*** [280]).

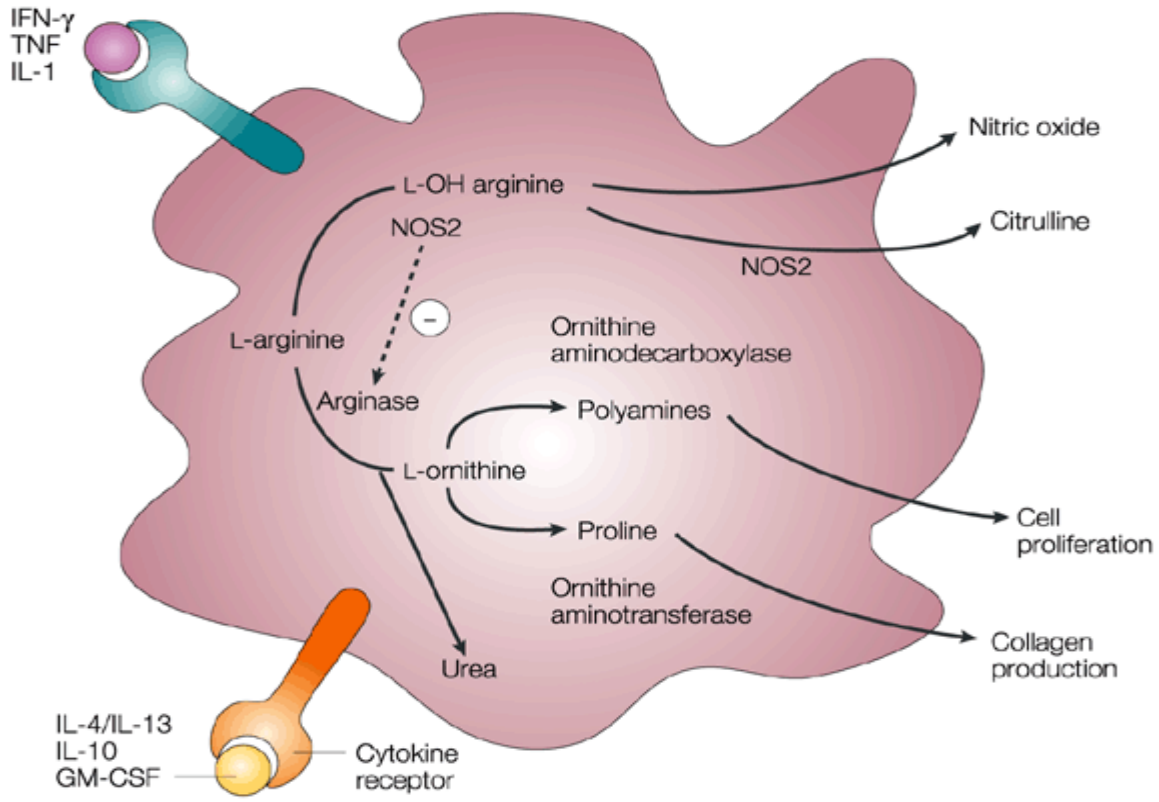

Figure 12: IL-4 and IL-13 promote arginase-dependent formation of L-ornithine and, ultimately, fibroblast proliferation and collagen production. IFNγ enhances the activity of nitric oxide synthase 2 to generate nitric oxide, and inhibits arginase (Adapted from ***Gordon S. 2003*** [280]).

ii. Role of AAMs in Infection, fibrosis and immunopathology

The generation and role of AAMs has been studied extensively in parasitic disease models providing the best evidence for a regulatory, protective role of AAMs in these Th2 cell-dominated infections. Murine markers of AAMs have provided a remarkably conserved signature of gene expression, considering the diversity of organisms studied. Parasite-specific features are associated with particular sites of migration during complex life cycles, e.g., in skin, lung, gut, and liver, illustrating the importance of tissue microenvironment in tuning the Th2 cell response. AAMs respond to Th2 cytokines and in turn regulate T helper cell functions. Delayed healing or excessive fibrosis occurs in mice with dysfunctional AAMs [281].

Chemokines produced by AAMs promote recruitment and activation of eosinophils, mast cells, and basophils, themselves sources of Th2 cytokines, as well as neutrophils [278].

Interactions of AAMs with antibody subclasses promoted by Th2 cytokines have received less attention, but include upregulation of FcR epsilon for IgE.

Parasitic infection induces many characteristics of AAMs, by unknown sensing receptors within the phagocytic vacuole and, on escape into the cytoplasm, by additional cytosolic sensors. The macrophages are induced to produce IL-4 and IL-13, which amplify the AAM phenotype through autocrine and paracrine mechanisms. The canonical signature markers are induced, depending on the IL-4 and IL-13 common alpha chain receptor and STAT6, incapacitating the cell's antimicrobial responses [282]. AAMs express enhanced Dectin-1 as well as mannose receptor [283]. Dectin-1 contains a essential for signaling, through Syk kinase and CARD9, thus inducing proinflammatory cytokines and activating a respiratory burst. The receptor contributes to host resistance to *Candida* and *Pneumocystis*. However, it is not clear whether AAMs are beneficial or deleterious to the host, because other innate (complement, neutrophils) and adaptive mechanisms, including classical activation by IFNγ and Th17 cell activation, come into play.

Studies with mouse macrophages in vitro and with cotton rats in vivo show that AAMs play a role in the response to infection. The macrophages are induced to express the marker signature of AAMs, which depends on the IL-4 and IL-13 receptor common alpha chain and STAT6, as well as TLR-4 and IFNβ. Infection in humans may also involve Th2 cytokines and AAMs. Although markers are insufficient to establish their role, other cell types, including innate and adaptive immune and epithelial cells, also modulate the host response [278].

Since the earliest reports on AAMs, it has been assumed that these cells promote repair of host tissues after inflammation. Induction of Arg1 by IL-4 and IL-13 has been implicated in

collagen deposition and degradation [284]. Imbalance in production and catabolism, associated with prolonged IL-13 effects on macrophages, promotes excessive fibrosis.

Although sterile foreign body reactions can depend on IL-4, IL-4Rα1 chain, and STAT6, repair of surgical wounds was independent of this pathway [285]. The IL-13-specific receptor was not investigated although it has been shown to be essential for fibrosis in a parasitic model of *S. mansoni* granuloma formation [286]. As noted, Arg1 can be induced by a variety of different pathways, and cells other than macrophages also express receptors for IL-13. The reduction of fibrosis in egg granulomata in macrophage-selective ablation of the IL-13Rα1, in which induction of AAM is unaffected, confirms the existence of AAM-independent pathways of fibrogenesis, and also independent of TGFβ or MMP9, which are enhanced in this model.

Macrophages isolated from liver granulomas of *S. mansoni*-infected mice were able to anergize T cell responses to specific and polyclonal stimuli [287].Granulomas in murine Schistosomiasis peak 7 or 8 weeks after infection, a time period in which hosts have developed already dominant Th2-type responses [234].

Macrophage-specific ablation of Arg1 enhances *S. mansoni* egg-induced fibrosis, an unexpected result. In wild-type mice, granulomata appear in liver after 7–10 weeks and diminish subsequently by Arg1-dependent downregulation, which is absent in Arg1-deficient mice. Macrophages express several transporters such as Cat2 as well as enzymes involved in L-arginine uptake and metabolism, but not a complete urea cycle. Ornithine decarboxylase, hydroxy-proline, and polyamines have been implicated in additional properties of AAMs.

It is not clear to what extent Th2 cytokines, AAMs, or fibroblasts contribute to noninfectious causes of fibrosis or whether combined targeting of IL-13 and TGFβ might prevent or reverse fibrosis.

e) Eosinophils

Eosinophils are innate Th2 related granulocytes that are elevated in asthma, allergy, and helminth infection. Eosinophils are evolutionarily conserved cells whose function has remained elusive, present in high levels in many naturally parasitized human populations. Eosinophils participate in integrating metabolic and immune signals to maintain homeostasis during chronic parasitism. Eosinophils are associated with helminth immunity and allergy, often in conjunction with alternatively activated macrophages (AAMs). Adipose tissue AAMs are induced by the cytokine IL-4. Eosinophils migrate into adipose by an integrin-dependent process and reconstitute AAMs through an IL-4/IL-13-dependent process [288].

Eosinophils are frequently associated with alternatively activated macrophages. Whereas adipose classically activated macrophages are associated with obesity and type-2 diabetes, AAM help maintain 'lean physiology'. Adipose AAM can be induced by the cytokine IL-4, although a definitive cellular source of IL-4 within adipose tissue is not known.

Although differences exist between mouse and human AAMs, including in the expression of arginase-1, there is overlapping profiles of IL-4/IL-13-conditioned human monocyte/macrophages that have also allowed recognition of human AAMs [289].
Eosinophil IL-4 production may contribute to sustaining AAMs; however, additional contributions from eosinophils are likely important, possibly through production of cytokines, chemokines or other mediators [288]. Furthermore, innate helper 2 cells, are implicated as an important source of IL-5 and IL-13, are present in adipose [290].

Despite their appearance in allergy and states of parasitism, particularly in response to intestinal helminthes, the biologic role of eosinophils remains incompletely defined. Although sparse in blood of persons in developed countries, eosinophils are often elevated in individuals in rural developing countries where intestinal parasitism is prevalent [291]. It was thought that eosinophils may have evolved to optimize metabolic homeostasis during chronic infections by ubiquitous intestinal parasites [292].

f) Role of Natural Killer T Lymphocytes

The CD1d-restricted NKT cells represent a heterogeneous population of unconventional, glycolipid-reactive, T lymphocytes that express NK cell markers (such as NK1.1) and comprise different categories of T cells [293, 294]. This cell population (termed invariant (i)NKT cells) recognizes a limited number of synthetic and naturally occurring α- and, to a lesser extent, β-anomeric glycosphingolipids in association with the MHC class I-like molecule CD1d on APCs, such as dendritic cells [295].
Mouse CD1d-restricted NKT cells, including invariant (i)NKT cells, are innate cells activated by glycolipid Ags and play important roles in the initiation and regulation of immune responses. Through their ability to promptly produce large amounts of Th1 and/or Th2 cytokines upon TCR engagement, iNKT cells exert crucial functions in the immune/inflammatory system during bacterial, protozoan, fungal, and viral infections. During the course of murine Schistosomiasis, iNKT cells exhibit an activated phenotype and following Schistosome egg encounter in the liver, hepatic iNKT cells produce both IFN-γ and

IL-4 in vivo. Schistosome egg-sensitized dendritic cells activate, in a CD1d-dependent manner, iNKT cells to secrete IFNγ and IL-4 in vitro. Interestingly, transfer of egg-sensitized DCs promotes a strong Th2 response in recipient wild-type mice, but not in mice that lack iNKT cells. Engagement of TLRs in DCs is not necessary for iNKT cell stimulation in response to egg-sensitized DCs, suggesting an alternative pathway of activation. Finally, self, rather than parasite-derived, CD1d-restricted ligands are implicated in iNKT cell stimulation [296]. CD1d plays an important role in the induction of Th2 responses during murine Schistosomiasis [258].

Helminths can activate iNKT cells to produce immunoregulatory cytokines in vivo, enabling them to influence the adaptive immune response. Both iNKT and non-iNKT cells do not have a major impact on the immune response during the early phase (1 and 4 weeks) of Schistosoma infection in murine animal models, they exert important, although opposite, effects on the immune response during the acute phase of the disease (7 and 12 weeks), after Schistosome egg production. Indeed, iNKT cells contribute to Th1 cell differentiation whereas non-iNKT cells might be mostly implicated in Th2 cell differentiation in response to parasite Ag. Schistosomes activate both iNKT and non-iNKT cells in vivo, enabling them to differentially influence the Th1/Th2 balance of the immune response [297].
CD1d-restricted cells are involved in the early immunological events leading to the generation of the Th2 response during Schistosomiasis. Activation of iNKT cells, in response to egg-sensitized DCs, does not require TLR-2 and TLR-3 expression on DCs, two TLR members recently described to be involved in DC maturation in response to parasite eggs. Activation of iNKT cell in response to Schistosome eggs is dependent on the presentation of self, rather than parasite-derived, CD1d-restricted ligands by DCs [296].

2.5.3. Regulation of the Th2 response in Schistosomiasis

Strikingly, all helminths – even free-living species when experimentally injected into mice – elicit a Th2 response in the host [298]. This provokes the question, what a Th2 response is for, and whether it helps the worm, the host or both? The uniformity of the immune reaction of mammalian hosts towards the phylogenetically distinct helminths indicates that the Th2 response is a profound and universal type of reaction of the host to get rid of these undesired guests. In case of gut-dwelling worms, the Th2 response very efficiently controls infection,. For helminths dwelling in tissues or blood, such as Schistosomes, however, the protective effect of a Th2 immune response is not that obvious, since it does not lead to clearance of the

parasites. Nevertheless, also the Th2 response against *S. mansoni* seems to be essential for survival of the host. IL-4 deficient mice [299] and IL-4Rα$^{-/-}$ mice [300] have impaired Th2 responses and die early due to massive intestinal inflammation, clearly indicating that IL-4 contributes to limiting inflammation and, thus, is beneficial to the host.

On the other side, strong Th2 responses can also have adverse effects such as allergy and asthma. Furthermore, the cytokine IL-13 was found to be responsible for liver fibrosis, a principal cause of pathology and even death in Schistosomiasis [301]. That means, the Th2 response is a two-sided sword: on one side, it helps eliminating or at least containing the parasite and dampens excessive inflammation, on the other side, it may have its price by causing organ damage. Thus, to minimize adverse consequences, the Th2 response during Schistosome infection has to be strictly regulated, which is usually the case in the chronic phase of infection. It was demonstrated that experimental allergic airway inflammation is enhanced when mice were sensitized with ovalbumin during the acute phase of Schistosome infection (8 weeks), i.e. when the Th2 response is peaking, while airway inflammation is suppressed upon sensitization in the chronic infection phase (16 weeks) [302]. It would have been also interesting to investigate this effect during the Th1 phase of infection (1week), which has not been tested in this study.

Major factors involved in regulating the Th2 response are elevated production of IL-10 and regulatory T cells [298]. IL-10 has a variety of anti-inflammatory functions including B cell immunoglobulin class switching from IgE to IgG4 [303]. Elevated IL10 production along with the switch from an IgE to an IgG4 response is a hallmark in chronic worm infections [304]. Notably, IgG4, in contrast to other immunoglobulin isotypes, has anti-inflammatory activity, a property that has been ascribed to its functional monovalency and poor ability to trigger complement and cell activation [305]. Beyond that, the anti-inflammatory effect of IL10 in *S. mansoni* infection is highlighted by the fact that IL-4/IL-10 as well as IL-12/IL-10 double-deficient mice rapidly succumb to lethal granulomatous inflammation, indicating that IL10 regulates both excessive Th2 and Th1 responses [306].

Tregs exert their regulatory function by producing the anti-inflammatory cytokines IL-10 and TGFβ [269] and/or by cytokine-independent mechanisms. A lipid contained in *S. mansoni* eggs and worms, lyso-phosphatidylserine, has been found to condition human monocyte-derived DCs via interaction with TLR-2 to promote the development of IL-10 producing Tregs in vitro [259]. Concerning the cytokine-independent mechanisms of the regulatory function of Tregs, an important way seems to be inhibition of T effector cell proliferation by growth factor deprivation. Natural Tregs are characterized by high expression of the IL-2 receptor

CD25 and – due to their high metabolic activity – by consumption of large amounts of IL-2 [307]. Effector T cells, on the other hand, have a lower CD25 expression, so they can hardly compete with Tregs for IL-2. In line with this, an enhanced apoptosis rate of $CD4^+$ T cells was observed in Schistosome egg granulomas, which could be reversed by addition of rIL2 [308, 309]. Therefore, limitation of this important growth factor results in inhibition of T effector cell proliferation as well as T cell hypo-responsiveness and anergy.

In addition to Tregs and IL10, alternatively activated macrophages were shown to contribute to the regulation of the Th2 response [310]. While the Th1 cytokine IFNγ leads to classical activation of macrophages, an alternative activation of macrophages is observed in a Th2 environment with high levels of IL-4 and IL-13 [289]. A key feature of AAMs is their expression of arginase-1, which is involved in the conversion of the substrate l-arginine into proline, an amino acid essential for collagen synthesis. Therefore, it was thought that AAMs promote fibrosis in chronic Schistosome infection. However recently, macrophage-specific arginase-1 was demonstrated to function as an inhibitor of egg-induced inflammation, fibrosis and Th2 responses in *S. mansoni*-infected mice, conversely, macrophage-specific depletion of arginase-1 led to increased production of Th2 cytokines and increased fibrosis [310]. Moreover, AAMs inhibited $CD4^+$ T cell proliferation in vitro, which was restored by addition of exogenous l-arginine, suggesting that the protective effect of AAMs results from arginase-dependent depletion of l-arginine, which is required for T cell proliferation and sustained T cell responses.

Taken together, although the Th2 response against *S. mansoni* is primarily elicited to contain the parasite and protect the host, it can also cause damage during chronic infection. Based on the endeavor of the worm to down-regulate immunity of the host without killing him (not to lose its habitat), and the endeavor of the host, to eliminate the undesired guest without causing "collateral damage", during the co-evolution of host and parasite anti-inflammatory mechanisms had been developed. These include the anti-inflammatory cytokines IL-10 and TGFβ, Tregs and AAMs.

To prevent damage to the host, in the chronic phase of Schistosome infection the Th2 response is controlled by anti-inflammatory measures including IL10, Tregs and AAMs. The identification of molecules used by Schistosomes to dampen the immune response and a detailed understanding of their function should help to develop new strategies for treatment and prophylaxis of chronic inflammatory states such as allergy, asthma and autoimmune disorders.

Chapter III

Prevention and treatment of Inflammatory Bowel Diseases by Helminth parasites

3.1. Mechanism of action

The core of parasitology is to understand a heterospecific relationship, in which the parasite seeks nutrients and shelter from the host at some detriment to the host species. Both species have coevolved in an arms race in which the host attempts to recognize and destroy/eliminate the intruder, while the parasite evolves to better counter or hide from the immune response mounted by the host. Thus, immuno-modulation of the host immune response is a goal of the successful parasite. Moreover, the notion of 'harmonious parasites', in which an individual with a specific parasitic infection is protected from other disease conditions is not new.

Helminth parasites had the benefit of immuno-modulation which could be of interest in diseases characterized with mounted immune response to normal human antigens as it is the case in inflammatory bowel diseases. Several theories had been studied but the main effector mechanism is not yet revealed. Here we summarize the proposed mechanisms of immuno-modulation secondary to parasitic colonization or infection in immune mediated colitis.

3.1.1. Changes in the gut flora homeostasis

- Increases in enteric goblet and mast cell numbers are hallmarks of intestinal helminth infection. The goblet cell response results in increased mucus production, and activated mast cells release a variety of mediators that lead to increased vascular and epithelial permeability *(i.e. the leak hypothesis)*, easing the movement of phagocytic cells, antibodies and complement into the gut lumen. In addition, mast cell mediators will evoke active epithelial ion transport and consequently water efflux into the lumen. The appearance of increased water, immune factors and mucus in the gut lumen would be expected to result in reduced contact with any lumen-derived pro-colitic agent [311].
- Altered muscle function and increased peristalsis would again limit contact time between the luminal contents and the epithelium.
- Helminth infection can affect the composition of the gut flora, and in theory this could be part of the anticolitic effect of helminths [311].

3.1.2. Modulation of the immune response

Firstly, helminth infection is associated with a strong Th2 response, which opposes the Th1 response associated with autoimmune diseases and CD.

Secondly, chronic infection with these organisms may generate a network of regulatory T (Treg) cells that secrete transforming growth factor TGFβ and IL-10 [312]. These cytokines may not only regulate aggressive Th1 responses but also control aberrant heightened Th2 responses that contribute to chronic allergic diseases such as asthma and food allergy. Interactions between parasites and their hosts are complex and multifaceted.

In their model of experimental colitis, Smith & et al proposed infection with *S. mansoni* results in an induction of Th2 responses (IL-4, IL-5 and IL-13 release), induction of regulatory cytokines (IL-10 , TGFβ), regulatory cells (CD 4 +; CD 25 +) with a role of a particular colon-infiltrating macrophage population [228].

During helminth infection the host evokes a strong Th2 immune response to provide protection against worm colonization. This response is characterized by eosinophil recruitment, mucosal expulsion mechanisms, and the secretion of IL-4, IL-5, and IL-13.

The cytokines produced by Th1 and Th2 cells cross-regulate each other's development and activity. Furthermore, Th1 and Th2 cytokines can inhibit Th17 development, while IL-17 does not seem to influence Th1 and Th2 effector cells [313]. In this way, helminths can evoke an immune response that might be able to attenuate the Th1/Th17 response found in CD. As previously discussed in Chapter II, the discovery of the new IL23/IL-17 pathway was a major breakthrough in the immunopathogenesis of IBD.

Furthermore, Treg cells have immunosuppressive properties and are characterized by the secretion of IL-10 and TGFβ. The Th2 and Treg cells that are upregulated in response to helminth infection are in turn capable of suppressing Th1 effector cells, the cells responsible for maintenance of inflammation in CD patients [314].

It was showed that IBD is associated with defective Tr1 cell activation, which may lead to an uncontrolled inflammatory reaction. In contrast, helminths induce the proliferation of Tr1 cells through a dendritic cell-mediated mechanism. Tr1 cells are characterized by the secretion of the immunomodulatory cytokines IL-10 and TGFβ. It is possible that helminths compensate for the defective Tr1 cells in IBD. This may explain why the experimental treatment with parasite eggs has beneficial effects in both patients with Crohn's disease and in those with ulcerative colitis [315].

3.1.3. Modulation of the neuroendocrine response

There is an evidence of neurone–immune cell juxtaposition, neuroimmune bidirectional communication; an association that is increased in the intestine of helminth-infected rodents. Involvement of the neuroendocrine system in mediating the beneficial effect helminth infection has not been fully examined [311].

3.2. Beneficial effect of infection with helminth parasites in experimental models of colitis

Several experiments have been conducted to show the beneficial effect of parasitic infection in relevant animal models of experimental colitis. They include infection with cestodes (*H. diminuta*), nematodes *(T. spiralis, H. polygyrus)* or trematodes (Schistosomes).

TNBS animal model of colitis is well established to test for mucosal immune mediated GIT changes that occur concurrently with inflammation. TNBS colitis which is characterized by densely packed transmural lesions differs greatly from the other type of hapten-induced colitis (oxalazone induced colitis). A second difference between trinitrobenzene sulfonic acid colitis and oxazolone colitis also emerged from the studies of the cytokine secretion patterns in these inflammations. These studies revealed that CD4+ T cells present in oxazolone-colitis lesions produce initially a large amounts of Th2 type cytokines (IL-4 and IL-5) in contrast to the increased amount of Th1 cytokines (interferon) found in trinitrobenzene sulfonic acid colitis [316].

3.2.1. Hymenolepis diminuta

The first full publication of helminth modulation in a murine model of colitis showed that *Hymenolepis diminuta* infection, either prophylactic or therapeutic, caused a significant amelioration of DSS-induced irregularities in stimulated ion transport to electrical nerve stimulation, the cholinergic agonist carbachol, and the adenylate cyclase activator forskolin compared to controls. In contrast, the histopathology (i.e., mixed immune cell infiltrate, oedema, and ulcerative damage) and elevated MPO levels that accompany DSS colitis were unaffected by concomitant *H. diminuta* infection. Similarly, there were no significant differences in levels of IFNγ, IL-12 or IL-10 in serum or tissue from any of the infected groups at the time of autopsy [317].

Further reasons to proceed carefully with helminth therapy for colitis related disorders were presented. The ability of *H. diminuta* to affect the course of oxazolone-induced colitis (a Th2 model) in the rat was examined [318]. In the study, disease severity was assessed by gross and

microscopic anatomy, myeloperoxidase and eosinophil peroxidase activity, and cytokine synthesis. They found that infection with *H. diminuta* caused a significant exacerbation of oxazolone-induced colitis.

3.2.2. Trichinella spiralis

It was subsequently showed that infection with the nematode, *Trichinella spiralis*, protected mice from colitis induced by intrarectal challenge with dinitrobenzene sulphate. Mice were infected with the intestinal nematode *Trichinella spiralis* and allowed to recover before induction of colitis. Prior nematode infection reduced the severity of colitis both macroscopically and histologically together with a decreased mortality and was correlated with a downregulation of MPO activity, Th1 type cytokine expression in colonic tissue, and emergence of a Th2 type immune response [319].

3.2.3. Heligmosomoides polygyrus

It was shown that colonization of piroxicam-treated colitic IL-10–/– mice with *Heligmosomoides polygyrus* (an intestinal helminth) suppressed established inflammation and inhibited mucosal IL-12 and IFNγ production. *H. polygyrus* augmented mucosal IL-13, but not IL- 4 or IL-5 production. Transfer of mesenteric lymph node T cells from IL-10–/– animals harbouring *H. polygyrus* into colitic IL-10–/– recipients inhibited colitis. (MLN) T cells from worm free mice did not. Foxp3 (scurfin) drives regulatory T cell function. *H. polygyrus* enhanced Foxp3 mRNA expression in (MLN) T cells leading to regulatory activity. This suggests that *H. polygyrus* inhibits ongoing IL-10–/– colitis in part through blocking mucosal Th1 cytokine production. Resolution of inflammation is associated with increased IL-13 production and can be adoptively transferred by MLN T cells [320].

Another study showed that mice infected with *H. polygyrus* were resistant to trinitrobenzenesulfonic acid (TNBS)-induced colitis, a Th1 cytokine-dependent inflammation. *Heligmosomoides polygyrus* did not change the normal microscopic appearance of the terminal ileum and colon and minimally affected lamina propria mononuclear cells composition. However, colonization altered (LPMC) cytokine profiles, blocking IFNγ and IL-12 p40 release but promoting IL-4, IL-5, IL-13, and IL-10 secretion. IL-10 blockade in vivo worsened TNBS colitis in *H. polygyrus*-colonized mice. *Heligmosomoides polygyrus* colonization inhibits Th1 and promotes Th2 and regulatory cytokine production in distant intestinal regions without changing histology or LPMC composition [321].

In a different, it was shown that prior *H. polygyrus* infection prevented TNBS-induced colonic damage and inflammation. TNBS induced upregulation of Th1 cytokines and normalized secretory responses to specific agonists with an absence of granuloma formation and submucosal oedema. The protective effect of prior *H. polygyrus* infection on colitis was associated with a decrease in mRNA expression of Th1 cytokines. TNBS-induced colitis did not alter *H. polygyrus*-induced mast cell infiltration or upregulation of Th2 cytokine expression. The results indicate that the protective mechanism of enteric nematode infection against TNBS induced colitis involves prevention of Th1 cytokine expression and improved colonic function by a mechanism that may involve mast cell-mediated protection of neural control of secretory function. Similar response patterns could account for the clinical improvement seen in inflammatory bowel disease with anti-helminthic therapy [322].

3.2.4. Schistosoma mansoni

It was demonstrated that Schistosome eggs had a protective effect on TNBS-induced colitis in mice. Schistosome egg exposure attenuated TNBS colitis and protected mice from lethal inflammation. Schistosome egg exposure diminished IFNγ and enhanced IL-4 production from CD3-stimulated spleen and mesenteric lymph node cells of TNBS-treated mice. Schistosoma egg exposure decreased colonic IFNγ but increased IL-10 mRNA expression in TNBS-treated mice. It was concluded that exposure to *S. mansoni* eggs can decrease murine colonic inflammation [225].

It was also demonstrated a protective effect of infection with *Schistosoma mansoni*, on trinitrobenzene sulfonic acid-induced colitis in rats. Concurrent infection with *S. mansoni* significantly reduced the duration of TNBS induced colitis to two weeks instead of four, as shown by macroscopic and microscopic damage scores and by a faster decrease in colonic MPO activity. TNBS increased colonic IL-2 production whereas S mansoni increased splenic IL-4 and IL-2 levels. In conclusion, concurrent infection with *S. mansoni* normalised longitudinal muscle contractility after one week whereas circular muscle contractility remained inhibited. Concluding that concurrent infection with *S. mansoni* significantly attenuates TNBS-induced colitis in the rats. However inflammation induced disturbances in contractility of longitudinal and circular colonic muscle strips may outlast the inflammatory reaction [224].

Mice infected with Schistosomes were refractory to DSS-induced colitis. Egg-laying Schistosome infections or injection of eggs did not render mice resistant to colitis induced by DSS. Schistosome worm infections prevent colitis by a novel mechanism dependent on

macrophages, and not by simple modulation of Th2 responses, or via induction of regulatory CD4+ or CD25+ cells, IL-10, or TGFβ. Infected mice had marked infiltration of macrophages (F4/80+) into the colon lamina propria and protection from DSS-induced colitis was shown to be macrophage dependent. Transfer of colon lamina propria F4/80+ macrophages isolated from worm-infected mice induced significant protection from colitis in recipient mice treated with DSS. Therefore, another mechanism whereby a parasitic worm suppresses DSS induced colitis via a colon-infiltrating macrophage population has been proposed [228].

All these experience has shown a promising role of helminth infections in the modulation of the immune response to different experimental models of colitis. The exact mechanism through which the parasites exert their immun-modulatory effect and the immune cell populations implicated in this beneficial role are not totally elicited.

3.3. Role of helminthic extracts in treatment of Inflammatory Bowel Diseases

Helminths secrete immunomodulatory substances that might reproduce similar effects to the parasite itself. Isolating and identifying such molecules from helminths could be a major focus of future research. Exploring how helminths modulate their host's immune response could yield greater understanding of the pathogenesis of IBD. If helminth parasites enhance IBD therapy, they might have a prophylactic role in preventing disease development in people at high risk of IBD.

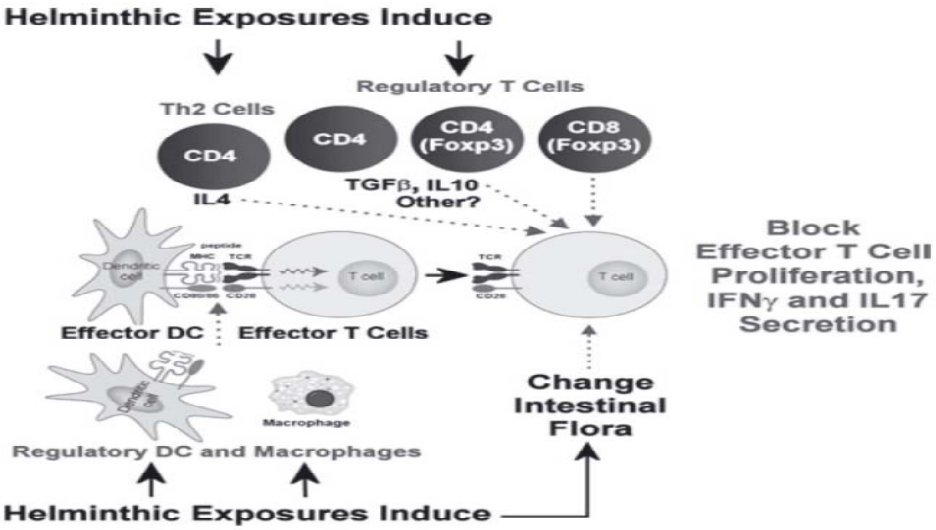

Figure 13: Helminth-induced regulatory circuits that limit inflammation (Adapted from ***Elliott DE. et al, 2012*** [323]).

Identification and characterization of helminth-derived immunomodulatory molecules that contribute to the anti-colitis effect could lead to new therapeutic approaches in IBD without the need for helminth infection. Using parasite extracts or synthetic drugs designed to mimic the immuno-modulating effect of helminth molecules also allows greater flexibility in dosing routes and therapeutic applications.

Helminths or their products might be useful in managing other immune-mediated disorders such as asthma, other allergic disorders and multiple sclerosis.

a) For example, the filarial nematode *Brugia malayi* secrete a cysteine protease inhibitors which interfere with antigen presentation and increase IL-10 secretion from macrophages [324].

It produces also homologues of the mammalian cytokine TGF*β*. Bm-tgh-2 is secreted by adult worms and binds to mammalian TGF*β* receptors thus performing an immunomodulatory function in the host [189].

b) In another work, treatment with proteins of *Schistosoma mansoni* and *Ancylostoma caninum* ameliorated TNBS-induced colitis in mice. *S. mansoni* proteins increased mRNA expression of regulatory cytokines while suppressing expression of proinflammatory cytokines.

Treatment of mice with colitis with *S. mansoni* or *A. caninum* proteins decreased the macroscopic inflammation score, extent of inflammation, and MPO activity. Immunologically, induction of colitis significantly increased expression of IFNα mRNA in the inflamed colon. Treatment with *S. mansoni* proteins caused a decrease of proinflammatory cytokines (IFNα, IL-17) in colon and MLN, whereas the production of regulatory cytokines (IL-10, TGFβ) increased significantly in colon tissue [226].

c) The effect of rectal submucosal administration of helminth antigens on subsequent experimental colitis was also examined. Mice treated with Trichinella spiralis antigens prior to the induction of DNBS-induced colitis were killed 3 days post-DNBS to assess colonic damage macroscopically, histologically and by MPO activity, inducible nitric oxide synthase (iNOS) and cytokine levels [325]. Previous treatment with T. spiralis antigens reduced the severity of colitis significantly, as assessed macroscopically and histologically, and reduced the mortality rate. This benefit was correlated with a down-regulation of MPO activity, interleukin IL-1b production and iNOS expression and an up-regulation of IL-13 and transforming growth factor-b production in colon.

Taken together, these results clearly show a beneficial role of local treatment with helminth antigens for experimental colitis and prompt consideration of helminth antigen-based therapy for IBD instead of infection with live parasites.

However, these experimental results have been obtained with either crude extracts, “excretory–secretory” products or poorly characterized parasite proteins, which are far to be ready to a large scale production at a clinical grade.

Even if the use of these unpurified parasite proteins may provide a more socially acceptable form of therapy for patients with IBD, as opposed to using living worms, these results support the urgency of further isolation and recombinant expression of the active products responsible for the beneficial effects on colitis.

3.4. Use of live parasites for treatment of Inflammatory Bowel Diseases in humans

The most provocative data in support of pursuing helminth infection as a therapeutic option for human inflammatory bowel disease have been provided by Weinstock and colleagues. They postulated that eradicating helminthic colonization would increase the risk of developing IBD by eliminating a protective parasitic influence, and that reintroducing helminths into people with active disease would inhibit immune-mediated mucosal injury through their ability to produce immunoregulatory factors [326].

They also put forward the hypothesis that using animal parasites, unable to fully develop in humans, will prevent or at least minimize the risk of active inflammation.

A proof of benefit was demonstrated in four patients with active CD and three with ulcerative colitis. In an initial treatment and observation period, a single dose of 2500 live eggs from the porcine nematode *Trichuris suis* was given orally, and patients were followed every 2 weeks for 12 week. Baseline medications were continued at the same dose throughout the study. Safety was monitored by following the patients' clinical status and laboratory studies at regular intervals. Patients also were monitored regularly using the Crohn's Disease Activity Index, Simple Clinical Colitis Activity Index and the Inflammatory Bowel Disease Quality of Life Index. To assess safety and efficacy with repetitive doses, two patients with CD and two with UC were given 2500 ova at 3-week intervals as maintenance treatment using the same evaluation parameters. During the treatment and observation period, all patients improved clinically without any adverse clinical events or laboratory abnormalities. Three of the four patients with CD entered remission according to the Crohn's Disease Activity Index; the fourth patient experienced a clinical response but did not achieve remission. Patients with UC experienced a reduction of the Clinical Colitis Activity Index to 57% of baseline. According to the IBD Quality of Life Index, six of seven patients (86%) achieved remission. The benefit derived from the initial dose was temporary. In the maintenance period, multiple doses again caused no adverse effects and sustained clinical improvement in all patients treated every 3

weeks for >28 week. This open trial demonstrated that it is safe to administer eggs from the porcine whipworm, *Trichuris suis*, to patients with CD and UC. It also demonstrated improvement in the common clinical indices used to describe disease activity. The benefit was temporary in some patients with a single dose, but it could be prolonged with maintenance therapy every 3 weeks. The study suggested that it was possible to downregulate aberrant intestinal inflammation in humans with helminths [327].

The safety of *T. suis,* was based on the following arguments: there is no systemic phase, it does not multiply in the host, it is not a human parasite; its eggs are capable of colonizing a human host for several weeks and are eliminated thereafter without any specific therapy, and is not directly transmittable to contacts.

The same group performed a double-blind controlled trial in patients with active ulcerative colitis treated with 2500 helminth ova every 2 weeks for 12 weeks. According to an intention-to-treat analysis, more patients treated with *T. suis* ova had a decrease in the ulcerative colitis disease activity index ≥4 points than those treated with placebo. Improvement according to the intent-to-treat principle occurred in 13 of 30 patients (43.3%) with ova treatment compared with 4 of 24 patients (16.7%) given placebo [328].

The trial included a second 12-week double-blind crossover phase. Patients that initially received placebo changed to *T. suis* ova and patients that initially received *T. suis* ova changed to placebo. At the end of the second phase, 56.3% of patients given *T. suis* ova responded, whereas only 13.3% improved on placebo. Combining data from both 12-week periods indicated response rates of 47.8% with ova and 15.4% with placebo. There were no side effects or complications attributable to *T. suis* in either phase.

Twenty nine patients with active CD, defined by a CD activity index (CDAI) ≥220, were enrolled in an open label study in which all patients ingested 2,500 live *T. suis* eggs prepared from pathogen-free animals, every 3 weeks for 24 weeks, other medications were maintained. Disease activity was monitored by CDAI. Remission was defined as a decrease in CDAI to less than 150, whereas a response was defined as a decrease in CDAI of greater than 100. Four patients withdrew at or before week 12 because of disease activity, and one withdrew between weeks 12 and 24 because of pregnancy. At week 12, 22 (75.9%) patients responded (a decrease in CDAI >100 points) and 19 (65.5%) of 29 were in remission (final CDAI <150 points). At week 24, 23 (79.3%) patients responded to therapy and 21 (72.4%) were in remission. These results were much better than any expected placebo response. No adverse clinical effects were detected as a result of therapy [329].

These two studies provided evidence that *T. suis* ova therapy is effective in both active ulcerative colitis and Crohn's disease. Therapy was beneficial in many patients whose disease was longstanding and refractory to conventional medications, and it was effective alone or in conjunction with other IBD drugs. Most patients were successfully maintained on treatment for a year, and some for more than 3 years. Thus, *T. suis* ova seem to be effective not only in active disease, but also in maintaining remission [330].

Summers et al stated that in their experience providing doses of TSO *(Trichuris suis ova)* to more than 120 patients (some of whom received treatment for more than 4 years), adverse events associated with these treatments were rare. Initially, all subjects in these studies had actively inflamed gut mucosa and many were on prednisone, azathioprine, 6-mercaptopurine or other immune suppressants during their course of treatment with TSO suggesting relative safety even in immune compromised hosts [331].

Another proof of concept study showed clinical efficacy of experimental infection with the human hookworm *Necator americanus* on Crohn's disease [332].

Five CD subjects with longstanding but mostly inactive disease and three reservoir donors each received a carefully measured inoculum (Infective Larvae cultured from faeces provided by a reservoir donors). Subsequently, four additional CD subjects with chronic and mostly active disease were inoculated with the same inoculum, and they were reinoculated from week 27 to week 30.

Neither respiratory symptoms nor detectable aberrant migration occurred. In the CD cohort, blood eosinophilia developed from week 5 compared to week 1 in RDs. CD activity index remained unchanged until week 17. After 20 weeks, the IBD questionnaire was improved.

Reinoculation of the five CD subjects first exposed caused no apparent adverse effect. Disease reactivation, as defined by a CDAI >150, occurred in two after the doses of long term immune suppressive drugs had been reduced. The other five CD subjects first inoculated were in remission at week 45.

This pilot study has established a potential for the hookworm *Necator americanus* as a candidate parasite to inoculate those with autoimmune disease. The natural advantages are lifecycle and migration predictability, ability to control the size of and eliminate a colony, and the parasite's longevity. Inoculation proved safe, even in immune suppressed patients.

Drawbacks:

Although helminth infections appear to be effective against IBD, treatment of patients with living helminths may envision drawbacks. Persistent infection and/or invasion of the parasite

(particularly zoonotic ones) to other tissues in the human host, where they might cause pathology, should be considered. The possibility of persistent active infection in man should be raised [333].

Many human helminth parasites could not be used because there are no available sources other than a human carrier. Eggs from such a source would risk inadvertent transmission of pathogenic microbial agents.

Infection with certain nematodes may induce enhanced intestinal propulsive activity, goblet cell hyperplasia, and increased mucus secretion. As a consequence, intestinal helminths may alter gastrointestinal motility, possibly resulting in intestinal symptoms like diarrhoea and abdominal cramps. Moreover, the idea of being infected with a living parasite could be psychologically hard to accept for some patients.

Finally, maintenance of parasite life cycles under strict conditions for large-scale therapeutic use is not feasible.

Reported a case of biopsy-proven iatrogenic infection by the pig whipworm *Trichuris suis* in a patient with Crohn's disease in whom the deliberate therapeutic ingestion of *T suis* ova has been adopted as an experimental approach to the treatment of Crohn disease [334].

Other dangers exist;

i) ''histotropic'' migration, a potential for the larvae to find their way into lymphatics or venules. The idea that they are non-invasive is false and all the more troublesome when one recognizes that there is mucosal damage with increased vascularity and often disruption of the muscularis mucosae in inflammatory bowel disease, potentially facilitating deep penetration of larvae into the bowel wall.

ii) Larval parasites in unnatural hosts travel peculiar and often unpredictable paths. There are numerous examples of aberrant migrations of parasites that find themselves in unfamiliar hosts. There is no predicting where *T. suis* larvae will go in humans, the abnormal host in this controversy. It may only be a matter of time and numbers of larvae before retinal or CNS disease occurs in a patient ''treated'' with *T. suis* [333].

These pilot studies revealed the interest of using helminth parasites in the prevention and treatment of Crohn's disease resistant or not responding to conventional therapy. However, the idea of using living organism in the treatment of these fragile patients is not acceptable or ethical.

The ongoing research projects to identify recombinant parasitic proteins capable of inducing or simulating the effect of real infection with a safe human usage profile are of interest.

Chapter IV

Glutathion *S*-transferase

4.1. Glutathion *S*-transferase enzymatic family

The glutathione *S*-transferase (GST, previously known as ligandins) family of enzymes are composed of many cytoplasmic, mitochondrial, or membrane-associated proteins involved in eicosanoid and glutathione metabolism [335]. GSTs are present in eukaryotes and in prokaryotes, where they catalyze a variety of reactions using endogenous and xenobiotic substrates. Members of the GST super-family are extremely diverse in amino acid sequence, and a large fraction of the sequences deposited in public databases are of unknown function [336-339]. The MAPEGs separate into two families: trimeric integral membrane proteins and those more structurally related to the cytosolic enzymes, membrane bound by a single N-terminal membrane anchor [340, 341].

GSTs can constitute up to 10% of cytosolic protein in some mammalian organs. GSTs catalyze the conjugation of reduced glutathione — via a sulf-hydryl group — to electrophilic centers on a wide variety of substrates. This activity detoxifies endogenous compounds such as peroxidised lipids, as well as breakdown of xenobiotics. GSTs may also bind toxins and function as transport proteins, which gave rise to the early term for GSTs of “ligandins”. The mammalian GST super-family consists of cytosolic dimeric isoenzymes of 45–55 kDa size [342-344]. They can be grouped into seven classes, based on sequence identity. Members within a class share >40% identity but they share less than 25% between different classes. The classes include alpha (GSTA), mu (GSTM), pi (GSTP), sigma (GSTS), theta (GSTT), omega (GSTO), and zeta (GSTZ), whereas the mitochondrial form belongs to a separate kappa (GSTK) class. This classification scheme can also accommodate GSTs from other organisms, although organism-specific classes are necessary to accommodate novel GSTs [338, 345].

The cytosolic GSTs are hetero or homodimeric proteins with subunit molecular masses ranging between 23 and 28 kDa. Each monomer has two domains: an N-terminal domain, with an α, β thioredoxin-like fold fused to an all α-helical C-terminal domain analogous to the bacterial enzyme, glutaredoxin-2 [346].

Most mammalian isoenzymes have affinity for the substrate 1-chloro-2,4-dinitrobenzene, and spectrophotometric assays utilizing this substrate are commonly used to report GST activity. However, some endogenous compounds, e.g., Bilirubin, can inhibit the activity of GSTs. In

mammals, GST isoforms have cell specific distributions (e.g., alpha GST in hepatocytes and pi GST in the biliary tract of the human liver) [347].

The sigma class is widely distributed in nature, with isoforms found in both vertebrates and invertebrates. It contains examples of proteins that have evolved specialized functions, such as the mammalian hematopoietic prostaglandin D2 synthase and the helminth 28-kDa antigen.

4.2. Biological activity of Parasites sigma Glutathion *S*-transferases

a) Host parasite interaction

PGD_2 generated by the mammalian sigma-class GST has roles in inflammation that depend on the etiology of the response. Some parasitic nematodes may adopt this mechanism as a means to influence their survival in the host. During its residence in the skin, *S. mansoni* larvae activate Langerhans cells and inhibit their migration from the epidermis to skin-draining lymph nodes. Parasite larvae synthesize and secrete PGD_2 that can block TNF-α−stimulated Langerhans cell migration, by signaling through the DP1 receptor [348]. The secreted *F. hepatica* sigma-class GST is also involved in host immune response modulation, although through a different mechanism, stimulating dendritic cells via the TLR4 receptor and altering a subsequent T-cell response [349].

b) Clearance of reactive oxidation products

It was suggested that the GST may serve a protective role in highly aerobic tissues or tissues sensitive to oxidative damage [350], a hypothesis supported by its protective effect in a *Drosophila* model of the human neurodegenerative disease, spinocerebellar ataxia type 1 [351].

4.3. Characterization of Schistosoma Glutathion *S*-transferase

Schistosoma haematobium 28-kDa glutathione *S*-transferase is a multifunctional enzyme involved in host-parasite interactions. The structure of this GST exhibits a unique feature, absent in previous GST structures, concerning the crucial and invariant Tyr10 side chain which occupies two alternative positions. The presence of two conformers of Tyr10 provides a clue about clarifying the multiple catalytic functions of Sh28GST. The relatively tight binding of GSH by Sh28GST explains the residually bound GSH in the crystal and supports a possible role of GSH as a tightly bound cofactor involved in the catalytic mechanism for prostaglandin D2 synthase activity [352].

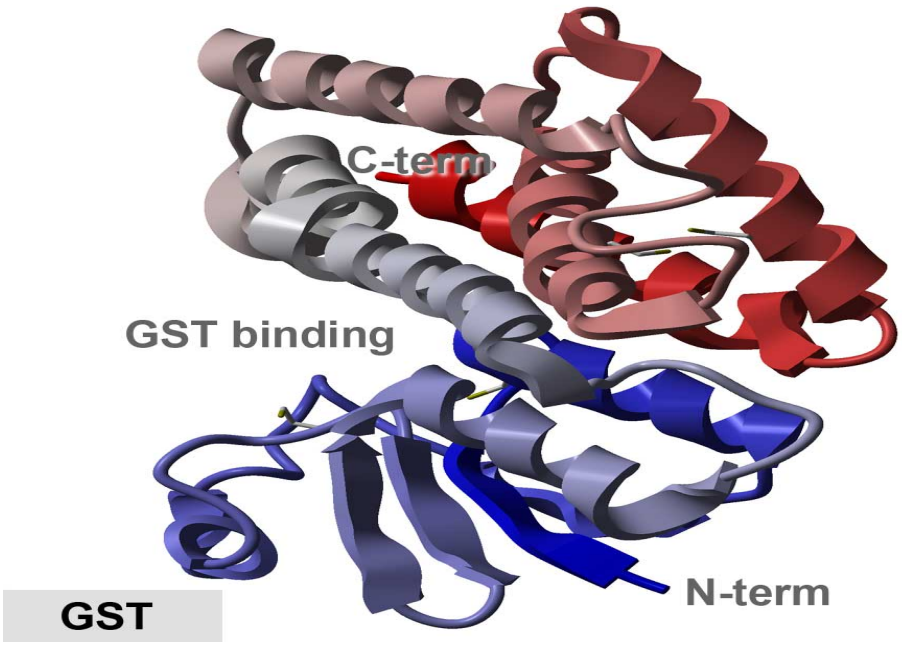

Figure 14: Three dimensional structure of non mammalian GST. It shows the C-terminal and the N-terminal as well as the GST binding site (Adapted from ***Udomsinprasert R. et al, 2005*** [336]).

The antigenic and phylogenetic variations between the 28kDa GSTs from 4 species of Schistosomes have been studied through cloning and sequencing of the 28kDa GSTs from *Schistosoma haematobium* (Sh28GST) and *Schistosoma bovis* (Sb28GST). Nomenclature is usually preceded by the type of Schistosoma species concerned. There are major common antigenic properties between the different species GSTs however Sb28GST and Sh28GST are more similar to each other (97%) than to Sm28GST (90%) and less to the 28kDa GST from *Schistosoma japonicum* (Sj28GST, 77%). Antisera directed against the major Sm28GST epitopes revealed differences in the recognition of the 28kDa GSTs from the other Schistosome species suggesting that these regions have been subjected to evolutionary pressure [353].

4.4. Preclinical trials of the P28GST

It was at the late eighties that a formulation of a vaccine against Schistosomiasis was identified by the team of André Capron, based on the principle that the worm itself is not immune vulnerable and it is the entrappement of the eggs in the walls of the different viscera which is involved in pathology. The identification and molecular cloning of a target antigen, a

glutathione *S*-transferase, has made it possible to demonstrate its vaccine potential in several animal species (rodents, cattle, primates) and to establish consistently the capacity of vaccination to reduce female worm fecundity and egg viability through the production of neutralizing antibodies (IgA and IgG) [354-356].

A number of lessons have been derived from series of preclinical trials reported in international publications [357-361]. From multiple experiments performed in rodents, cattle and primates, clear evidence has been gained that:

1- The major effect of immunisation with Schistosome GSTs is to significantly reduce female worm fecundity and egg viability.
2- Structure function studies and 3D structure analysis have allowed the identification of the major epitopes implied in the catalytic site of the enzyme and the binding site of the glutathione.
3- The dramatic reduction of female worm fecundity observed in primates (up to 85%) or in cattle (94%) is associated with a strong immune response leading to significant reduction of infectious pathology and its consequences on the animal health status (urinary bladder inflammation, weight loss).

These results led to the conclusion that P28 GST could be considered as an anti-pathology vaccine associated with anti-inflammatory properties.

4.5. Vaccine strategy against Schistosomiasis based on Schistosomes GST

For the development of vaccine strategies to generate efficient protection against chronic Schistosomiasis, controlling pathology could be more relevant than controlling the infection itself. In such strategy, focus is directed to parasite molecules involved in fecundity.

One of the interesting features of the Glutathion *S*-transferase is its expression at different parasitic stages, including cercariae, worms and eggs, as well as its strong inductory role of a Th2 type immune response [362]. The relevance of the induction of humoral immune response against specific domains of the 28GST was pointed out by the study of the immune status of infected human populations.

Clinical studies in *Schistosoma mansoni* infected individuals revealed a parallelism between the age-dependent evolution of IgA antibody levels to Sm28GST and the acquisition of resistance to reinfection. Functional analysis revealed that IgA antibodies to Sm28GST displayed a potent neutralizing effect and markedly impaired Schistosome fecundity, by limiting both the egg laying of mature worms and the hatching capacity of Schistosome eggs into viable miracidia [363].

Thus the core of the vaccine strategy is to limit the fecundity of the female worm by initiation of a strong immune response that leads to inhibition of this a crucial enzyme - the 28 kD glutathion *S*-transferase.

4.6. Immune response in immunised individuals to infection with Schistosomes: clinical trials of the use of recombinant Sh28GST in human

The recombinant P28 GST protein was produced by EUROGENTEC (Be/UK) under GMP conditions. After completion of all requested security control tests, five phases of clinical trials with the preparation named Bilhvax and only dedicated to safety have been so far completed (phases 1a and 1b, Phases 2a, 2b, 2d).

Clinical trials have been undertaken using the recombinant form of *Schistosoma haematobium* GST, P28GST, with alum, an adjuvant compatible with human use and this vaccine preparation was named *Bilhvax*.

The endpoint of these trials was the evaluation of the immune response resulting to the administration of the P28 GST *(Bilhvax)*. After two subcutaneous injections of 100 µg of P28GST, an immune response was elicited in all immunised individuals. No production of Th1 cytokines was observed in phase Ia and phase Ib studies. In contrast, there was a prominent production of IL-5 and IL-13 strongly boosted after the third injection in all vaccinated individuals. Identical cytokine profiles were observed in vaccinated children during phase Ib and the production of IL-5 and IL-13 was higher and significant production of IL-2 and IL-10 was observed. High titers of neutralizing antibodies were produced (IgG3 and IgA) together with Th2 cytokines [364, 365].

In a recent trial in healthy adult volunteers tested for the safety, tolerability and immunogenicity of P28GST in alum as a first vaccine candidate against Schistosomiasis.
A secondary objective of this study was to evaluate the immunogenicity of rSh28GST in humans, particularly in terms of the antibody response [366].

The overall conclusion of the study was that no severe side effects were detected and that only very few minor, clinically insignificant adverse effects were caused by the vaccination. Some

mild (grade 1) adverse effects, restricted to local swelling and erythema around the injection site, were reported in some subjects receiving P28GST [366].
The P28GST (Sh28GST) is the first candidate to reach actually phase III clinical trials [367].

4.7. Rationale of using Schistosoma Glutathion S-transferase to prevent against experimentally induced colitis in animal models

As we have discussed in the previous chapters that Schistosomes are considered "masters of regulation" and extensive studies have led to the in-depth characterization of one Schistosome parasite enzyme – P28GST. On the other hand, infection with living helminths, including Schistosomes, has been shown to prevent or reduce experimentally induced colitis.
The proposed hypothesis was that the Schistosome P28 GST, combining immune-regulatory and free radical scavenger properties may represent one of the major Schistosome molecule implicated in the down-regulation of intestinal inflammation. In addition, this molecule, produced in recombinant form, has been already successfully used in clinical trials for safety and immunogenicity studies, including in children, representing therefore a highly promising therapeutic anti- inflammatory agent, particularly valuable in inflammatory diseases such as IBD.

Indeed, it is well established that increased oxidative stress and/or impaired anti-oxidant defenses are associated with IBD and animal models of colitis [368].
The importance of anti-oxidants in the pathogenesis of intestinal inflammation is evident from experimental studies showing that antioxidant therapy improves colitis [369].

All together, these results indicate that the Schistosome P28 GST, combining immunoregulatory and anti-oxidant properties might represent one of the major factors implied in the down-regulation of intestinal inflammation.

Objectives

The actual treatment of inflammatory bowel diseases consists in the moderate to severe forms of corticosteroids in addition of immunosuppressive therapy. In cases who do not respond to this therapy, the addition of anti TNF therapy should be considered [370].

Concerns about immunosuppressive therapy on the long term use as well as their side effects (increased risk of opportunistic infections and malignancies specially lymphomas) and their limitations of use in special conditions (pregnancy, hypersensitivity, active infections, demyelinating disorders, severe congestive heart failure and malignancy) in addition to their cost present a continuous challenge to test for new molecules capable of preventing relapses and decrease the need for this group of drugs, with a special attention in children.

The interest of using helminth proteins has been demonstrated for prevention of intestinal inflammation both in animals as well as in humans. The protective effect is explained essentially by immuno- modulation from an immune profile of Th1 to Th2 through secretion of immune regulatory interleukins and stimulation of regulatory cells; however it is evident that this simple paradigm can't explain all the preventive features. The mechanism of action is not fully elicited and further studies are needed.

The main objective of our work was to evaluate the anti-inflammatory effect of the Sh28GST, previously tested in humans for another application, in the prevention of colitis in an experimental model - highly relevant to Crohn's disease. That molecule - that is easily reproduced through recombinant techniques and with a previously tested human safety profile - could represent a new therapeutic approach for the prevention and the control of Crohn's disease relapses.

In our research project, we aimed to show whether a Schistosome molecule, whose efficacy and safety have been proven in animal models and in humans can prevent hapten- induced colitis in rodents.

Immunisation with the *Schistosoma haematobium* Sh28GST has the potential to induce a strong Th2 response, and the production of regulatory markers.

The aim of our thesis was to further explore the beneficial role of Schistosomes on intestinal inflammation, by using the unique properties of a well-characterized Schistosome molecule, the Sh28GST, in 2 experimental models of chemically induced colitis classically admitted as relevant models for human Crohn's Disease.

The objectives of our project are:

- To show whether immunisation with Sh28GST can prevent intestinal inflammation experimentally induced by hapten sensitization of rodents.
- To compare with the immune response induced by Schistosoma infection and its evolution according to the time.
- To compare the effect induced by that molecule to the beneficial effect of a drug of reference in the treatment of inflammatory bowel diseases – 5 Amino Salicylic acid.
- To investigate the mechanism involved by measurement of inflammatory and regulatory cytokines in the colon of sensitised animals.
- To compare the efficacy of Sh28GST with anther Schistosoma GST.
- To show that even in high doses the Sh28GST has no harmful effect on the colon and that it safe to use as a prophylaxis against relapses of IBD.

Material & Methods

Animals:- Rats: Male Sprague-Dawley (SD) rats, 6-8 weeks of age (200-250 g) and - Mice: Male C57BL/6 mice, 6-8 weeks of age (18 - 20 g), were purchased from Janvier Europe SAS ®. They were maintained in pathogen free animal holding facilities. Animal use adhered to National Institutes of Health Laboratory Animal Care Guidelines. Animals were used for infection, immunisation and colitis induction.

Induction of TNBS Colitis: Induction of TNBS colitis in rats and mice has previously been described in detail [371-373]. Animals were put to fasting with free water access for 18 hours before the induction of colitis. Their abdomen was massaged to evacuate any residual faeces to allow better contact of TNBS with the mucosa.

Rats were anesthetized with intraperitoneal injection of pentobarbital at a dose of 40 mg/kg. Injected volume was 250µl de TNBS diluted in 40% of ethanol (2,4,6 Trinitrobenzene sulfonic acid, from Sigma-Aldrich ©) at a dose of 100 mg/Kg. Negative control group was injected with ethanol 40%

Mice were anesthetized with Xylasine (2% Rompun ®, from Bayer ©) – Ketamine (Imalgene ® 1000, from Rhone Meriux ©) at a dose of 50 mg/Kg each through a subcutaneous injection allowing an anaesthesia time of one and half hour. Injected volume was 150µl de TNBS diluted in 50% of ethanol (2,4,6 Trinitrobenzene sulfonic acid, Ref 92823, Sigma-Aldrich ©, 1M) at a dose of 150 mg/Kg. Negative control group was injected with ethanol 50%.

TNBS colitis was induced by once administration of TNBS/ethanol per rectum via a 3.5F catheter equipped with a 1-mL syringe; the catheter was advanced into the rectum until the tip was 8 cm (rats) proximal to the anal verge 4 cm (mice) and, at which time the TNBS was installed. To ensure distribution of TNBS within the entire colon and caecum, animals were held in a vertical position for 30 seconds after the intrarectal injection.

All animals were sacrificed at 96 hours (rats) after the induction of the colitis (48 hours for mice). The macroscopic score was assessed on whole colonic tissues; samples were harvested for histology, immunohistochemistry, myeloperoxidase activity and mRNA expression of cytokines, transcription factors and iNOS:Arginase.

Infection Protocol: approved by Institute Pasteur of Lille (MA-02-01a).

Animals had received anaesthesia according to their weight in the form of Valium (Diazepam ®, from Roche ©) at a dose of 100µl/250g in one leg and Ketamine at a dose of 100µl/200g in the other leg. After the induction of anaesthesia, the skin over their abdomen was shaved

and cleaned with cotton and natural water. Rats were fixed on their back with their arms and legs scotched to the plates and metal rings were scotched on their abdomen. Schistosoma cercariae (2000 cercariae diluted in 2 ml physiological saline) were diffused in the metal ring and then the ring was covered by opaque scotch and lights of the room were turned off to insure darkness. After 30 minutes, scotch was removed and the skin was cleaned.

Two protocols of infections were used: 1- Established infection model: in which rats were infected with Schistosoma cercariae 4 weeks before the induction of the TNBS colitis. 2- Recent infection model: in which animal were exposed to the infection only 1 week before colitis induction.

Reagents and drugs:

Sh28GST: Immunisation with Sh28GST with alum: has been performed following the human recommended schedule: 2 subcutaneous injections 50 µg/Kg with 4 weeks interval.

We also tested for the effect of the immunisation with the Sh28GST diluted with physiological serum without alum.

The used batches of rSh28GST were produced and purified from recombinant *Saccharomyces cerevisiae* culture (TGY73.4 - pTG8889 strain) under Good Manufacturing Practice (GMP) conditions by Eurogentec S.A. (Belgium). The rSh28GST clinical batch (# B98H11) was conserved lyophilized (124 µg per vial for the administrated dose of 100 µg; 352 µg per vial for the administered dose of 300 µg) by Sterilyo (France) under GMP conditions. The lyophilized preparation was re-suspended extemporaneously using 0.6 ml of apyrogenic and sterile aluminium hydroxide solution 0,2% (Al_2O_3 0.2%; $Al(OH)_3$ 3%; NaCl 9 g/L; ammonium carbonate buffer 10 mM, pH7.8) (Alum from Superfos, Denmark ®) and administered in a volume of 0.5 ml.

SJapGST: Glutathione S-transferase (GST) is a 26 kDa enzyme originally identified in *Schistosoma japonicum*, presently isolated from a recombinant E. coli source was purchased from Sigma-Aldrich ®. It was used in the same scheme as for ShGST.

We also tested for the effect of the immunisation with the SJapGST diluted with physiological serum without alum.

Aluminium: Alum hydroxide wet gel suspension (Alhydrogel ® 2% from InvivoGen ©). It was used as GST adjuvant and was tested alone in the same conditions.

5-ASA: in the form of Mesalazine ® 500 milligram sachets (from Ferring ©) was used to form pellets with mice food pouder at a dose of 4 gm/Kg of pouder. The mice had free access one week before the induction of the colitis till the day of sacrifice.

Tissue preparation: Animals from respective groups were sacrificed. The distal colon (4 cm for the rat model and 1 cm for the mice model) was removed then the following distal segment (1 cm) of the colonic segment was used for histology. The remaining segment was opened and rinsed. After scoring the gross morphological damage, the most distal 1 cm was used for the myeloperoxidase assay. The middle 2 cm was used to determine the mucosal content of different cytokines [224].

Macroscopic damage score: Macroscopically visible damage of the opened colonic segment was blindly scored on a 0–10 scale using the scoring system for TNBS induced colitis in rats described by Wallace and Keenan[373]. A score of 0 represents no visible damage whereas overall colitis has a maximal score of 10.

Macroscopic score of Wallace

Score	Macroscopic findings
0	No damage.
1	Hyperemia. No ulcers.
2	Hyperemia and thickening of the bowel. No ulcers.
3	One ulcer without thickening of the bowel wall.
4	Two or more sites of ulceration or inflammation.
5	Two or more sites of ulceration or inflammation or one site of ulceration/inflammation extending >1 cm along the lenght of the colon.
6	If damage covered >2 cm along the length of the colon. Then score will be increase by a scale of 1 for each cm in addition.
7	>3 cm
8	>4 cm
9	>5 cm
10	>6 cm

Table 3: showing the macroscopic damage score according to the presence or absence of mucosal hyperaemia, the number and the size of superficial mucosal ulcers in TNBS model of colitis, as described by Wallace and Keenan[373].

Histopathological Assessment: After sacrifice, the colon was separated and fixed in 4% buffered formalin and paraffin embedded. The sections (5 mm) were stained with Haematoxylin & Eosin for light microscopic examination. Microscopic damage scores were assessed on histological samples of colons by trained pathologists blinded to treatments. The extent of colonic inflammatory damage was assessed using the scoring system described by Ameho and colleagues[374]. A score of 0 represents no histological damage whereas extensive colitis with necrosis into the muscularis propria involving 50% of the specimen has a maximal score of 6.

Histologic score of Ameho

Score	Microscopic findings
0	No lesion.
1	Mild mucosal or submucosal inflammatory infiltrate with edema. Punctuate mucosal erosions. Muscularis mucosae intact.
2	Score 1 changes involving 50% of the specimen.
3	Prominent inflammatory infiltrate with deeper areas of ulceration extending through the muscularis mucosae into the submucosa.
4	Score 3 changes involving 50% of the specimen.
5	Extensive ulceration with coagulative necrosis extending deeply into the muscularis mucosae.
6	Score 5 changes involving 50% of the specimen.

Table 2: showing the histological scoring system according to the extent of colonic inflammatory damage in TNBS model of colitis, as described by Ameho and colleagues[374].

Elisa for MPO activity assay: Tissue MPO activity, which is directly related to the number and activity of myeloid cell infiltrate in inflamed tissue, was assayed to monitor the degree of inflammation. MPO activity was measured in the colon using special kit according to the manufacturer's instructions (Hycult Biotech ©). Colonic MPO activity is expressed in units per gram of tissue. Versamax ® unable microplate reader (from molecular devices ©) was used to assess the activity. Softmax ® pro v 4.7.1 was used to analyse the obtained data.

RNA Extraction, cDNA Synthesis, and Real-Time Quantitative Polymerase Chain Reaction

Total RNA was prepared from full-thickness sections of colon. Total RNA was extracted from colon sample tissue with NucleoSpin ® RNA II extraction kit (from Macherey-Nagel ©) following the manufacturer instructions.

RNA integrity, quantity, and genomic DNA contamination were assessed using the Nanodrop ® 1000 spectrophotometer (from Thrermo Scientific ©) and results were analysed with Nanodrop ® 1000 software v 3.7.1. Only those RNA samples with no DNA contamination were studied further.

RNA samples (20 µg) were reverse-transcribed to cDNA using the First Strand cDNA Synthase using the High Capacity cDNA Reverse Transcription Kits (from Applied Biosystems ©) with random hexamer primer.

Real-time quantitative polymerase chain reaction (PCR) was performed on a separate individual samples. Primer sequences were designed by using (Premier3 ® v0.4.0 and confirmed through standard nucleotide blast software. Primers were purchased from Eurogentec ®. Primer sequences are described in table ().

RT-PCR: Quantitative real-time RT-PCR was performed as described[375]. Real-time PCR was carried out with the SYBR ® Green detection reagent (from Applied Biosystems ©). Real-time RT-PCR was performed on an ABI Prism ® 7000 Sequence Detection System from Applied Biosystems ©). Results were analysed through ABI Prism ® 7000 SDS software version 1.1. Cycle threshold values for genes evaluated were determined, and fold induction was compared with GAPDH (for rats) and Bactine (for mice). The relative changes in gene expression was analyzed using the 2-ΔΔCt method [376]. The fold changes in mRNA expressions for targeted genes were relative to the respective control groups of rats or mice after normalization to the reference gene.

We calculated the ratio of colonic mRNA expression of Arginase1 and iNOS2 in both rats and mice and we tried to correlate that ratio to the polarisation of macrophages activation from M1 pathway to a M2 pathway.

Immuno-histo Chemistry: Immunohistochemistry was performed on sections embedded in paraffin (5 µm) of colon tissue. Sections were stained with the following antibodies: ED1as a marker for tissue macrophages and Arginase1 as a marker for AAMs

After dewaxing in xylene and rehydrating in a gradient concentration of ethanol, the slides were pre-treated with an antigen retrieval method by heating the slides in an autoclave: in a 10 mM sodium citrate buffer, pH 6 for 20 minutes for both antigens.

Sections were incubated first with a blocking buffer (5% goat serum diluted in PBS for 15 minutes) then incubated with the primary antibody diluted in the blocking buffer (1/100) at room temperature overnight. After wash with phosphate buffer saline, fluorescent conjugated secondary antibodies was diluted (1/200) in the same blocking buffer and incubated for 1 hour at room temperature. Finally, the secondary antibody was washed with PBS and slides were fixed with 1 µl Hachst bis-benzimide (from Sigma-Aldrich ©) in 1 ml Dakocytomation fluorescent mounting medium (from Dako ©).
A Leica ® CTR 5500 microscopy (from Leica Microsystems ©) was used and a Leica ® Application Suite software version 3.4.1 was used to interpretate the images.

Statistics: The nonparametric Mann–Whitney *U* test was used for assessing pathology scores. Data were expressed as mean ± standard error of the mean. Levels of significance were determined by Student *t* test. *P* values of <.05 were considered to be statistically significant.

Results

First question: Is Sh28GST effective in the prevention of TNBS induced colitis in a Sprague Dawley rat model?

In order to evaluate the effect of Sh28GST in a rat model of TNBS-induced colitis, we compared either immunisation using 2 subcutaneous injections of Sh28GST or infection with Schistosoma larvae. We used 2 models of infections by comparing established model of infection (of 4 weeks duration) with a recent model of infection (of 1 week duration).

Five groups of rats were studied and were either immunised with Sh28GST injected via two subcutaneous injections or exposed to transcutaneous infection with live Schistosoma cercariae.

At Day 35, induction of TNBS Colitis was performed at a dose (80 mg/kg) except for the negative control which received ethanol solution at (40% dilution – the vehicle of the TNBS).

All animals were sacrificed at Day 39.

	Day 0	Day 28	Day 35	Day 39
TNBS colitis (Positive control)			TNBS Colitis	Sacrifice
Sh28GST + TNBS colitis (Immunised group)	1st injection of Sh28GST	2nd injection of Sh28GST	TNBS Colitis	Sacrifice
Established infection + TNBS colitis	Infection with 2000 Schistosoma cercariae		TNBS Colitis	Sacrifice
Recent infection + TNBS colitis		Infection with 2000 Schistosoma cercariae	TNBS Colitis	Sacrifice
Ethanol (Negative control)			Rectal infusion of diluted Ethanol	Sacrifice

Table 3: Showing the experimental design of the Sprague Dawley rats experiments.

Animals were dissected and a blood sample, peritoneal lavage, colon dissection was done.

This experiment was repeated twice to confirm results and study data variability.

I) After gross macroscopic evaluation, we found that prior injection of Sh28GST decreased TNBS colitis in a rat model.

To assess the effect of immunisation of the Sprague Dawley rats with Sh28GST, we analysed the results of macroscopic assessment of markers of inflammation in the colon of animals in the different groups.

As expected, animals exposed to TNBS colitis only (positive controls) showed an important colitis as evidence by an increase in their mean of clinical damage score which was estimated at 8,1 (according to score of Wallace ranging from 0 – 10). **Figure 15 (B)** shows sample photo of the dissected colon from a rat in the positive control group. It shows thickening of the wall of the colon with extensive hyperaemia and a large mucosal ulceration that measured more than 3 cm in length.

We evaluated the gross macroscopic lesions in the colon of animals immunised with Sh28GST. Interestingly, we found that those animals were protected from the TNBS colitis by the Sh28GST that demonstrated a damping effect of the clinical damage score which was estimated at 5,35. There was a significant reduction compared to positive control group (with a 34% reduction in the macroscopic score of colitis according to the score of Wallace). **Figure 15 (B)** shows a sample photo of the dissected colon from a rat in the Sh28GST immunised group. It shows a decrease in the wall thickness of the colon with less hyperaemia. The size of the mucosal ulceration induced by the TNBS injection was less than 2 cm compared to 4 in the positive control group.

Sprague Dawley rats infection with Schistosome larva induced a protective effect against the TNBS colitis. However this anti-colitis effect was restricted to animals that were exposed to the infection 4 weeks before TNBS intra-rectal injection. They had a significant reduction in the mean of the macroscopic damage score which was evaluated at 5,9 (according to the score of Wallace).

The interesting finding was the comparison of both groups of animals immunised by Sh28GST and those from the established infection group as we found no significant difference in their anti-colitis effect as regard the macroscopic damage score (as evident in the sample photos).

Finally, animals exposed to Schistosome larvae only 1 week before the induction of the TNBS colitis had a gross accentuation of the colonic inflammation (macroscopic damage score estimated at 8,7 by the score of Wallace). This is clear in the sample photo in **Figure 15 (B)**, in which there is an increase in the thickness of the colonic wall and extensive mucosal ulcerations measured at more than 4 cm – with multiple ulcers in some animals.

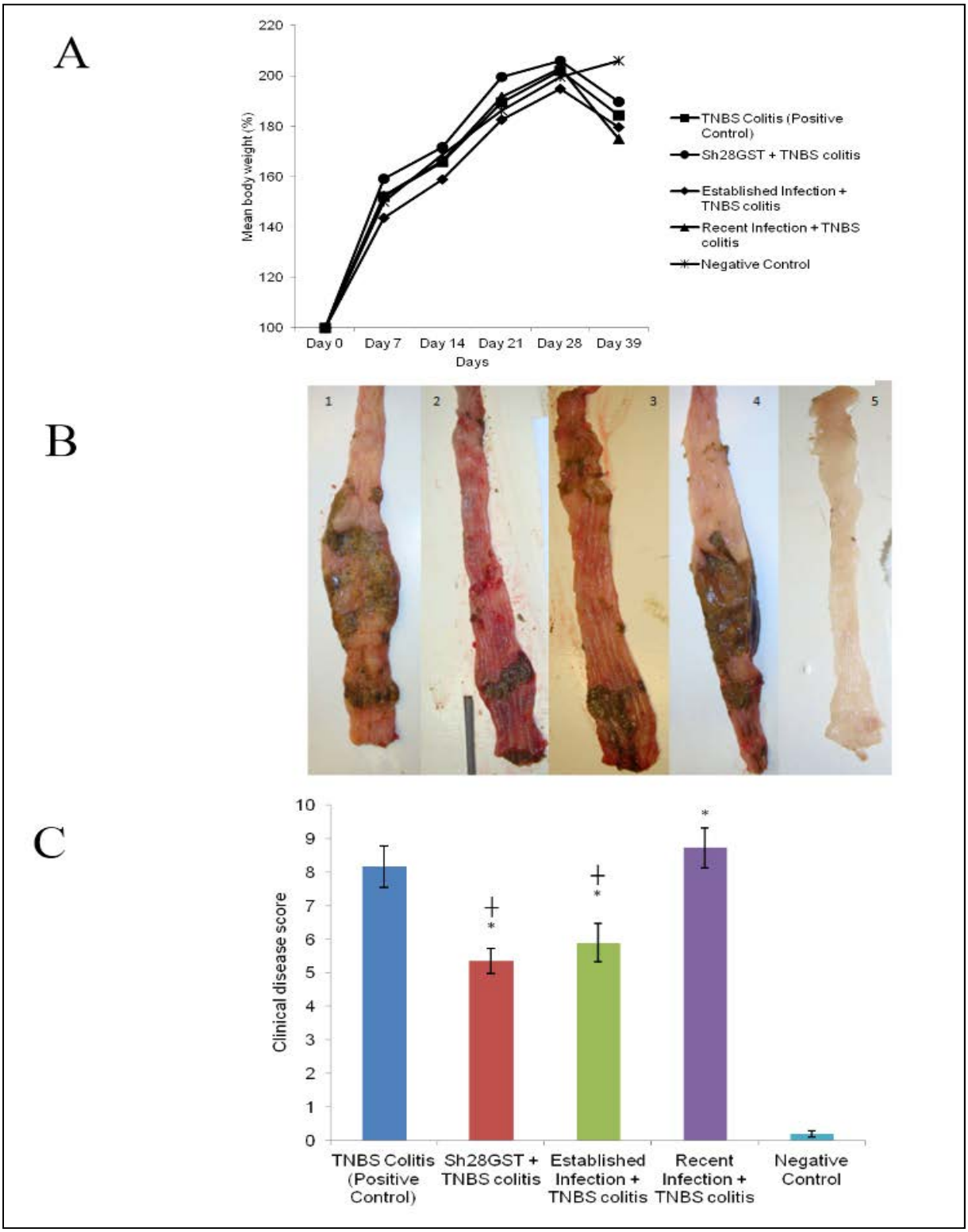

Figure 15 (A): mean body weight variation of Sprague Dawley rats from different groups over time of the experiments. **(B):** showing representive photos of the dissected colon of a rat

from the different groups 1) TNBS colitis group showing extensive mucosal ulceration with thickening of the wall of the colon.2) Sh28GST group showing a decrease in the colon thickness with less mucosal ulceration than previously shown in the group TNBS colitis. 3) Established infection group showing a decrease in the colon thickness with less mucosal ulceration than previously shown in the group TNBS colitis. 4) Recent infection group showing extensive mucosal ulceration with thickening of the wall of the colon. 5) Negative control group showing normal pattern of the mucosa covering the wall of the colon. **(C):** clinical disease score of the colon of the sacrificed Sprague Dawley rats from different groups according to the score described by Wallace and Keenan[373]. The results represent the pool of 2 independent experiments (mean ± SEM; n: 20/group); * p <0.05 compared with TNBS group (positive control); ┼ p <0.05 compared with recent infection group.

We concluded that Sh28GST was able to dampen macroscopic damage induced by TNBS in a Sprague Daley rats. This anti-colitis effect was as effective as the one observed in animals from established infection group.

II) Histologic findings as well as MPO activity confirmed previously obtained results that Sh28GST has an effective anti-colitis effect.

Further examination of the dissected colon from animals immunised with Sh28GST revealed a significant decrease in the histological damage score which was estimated at 1,2 compared to 3,4 of the animals of the positive control group (according to the score of Ameho which ranges from 0 – 6) as shown in **Figure 16 (A)**. There was a decrease of 65% of the degree of histological damage.

This was evident in the sample photos illustrated in **Figure 16 (B)**; as we show in the negative control group, all layers of the dissected colon could be identified with a 20 x zoom with no cellular infiltration or mucosal ulceration.

Animals immunised with the recombinant molecule were protected from the severe colitis observed in animals from the positive control group. There was less mucosal erosion with less cellular infiltration of polymorphs nuclear cells.

In animals with TNBS colitis, the colonic wall was markedly thickened to the extent that using the same zoom (20 x) was not sufficient to show all layers. The PMNs cellular infiltrate was so obvious with easy identification of neutrophils and eosinophils.

The decrease of the cellular infiltration of the submucosal layer of the dissected colon of Sh28GST immunised rats explained the significant decrease of the MPO activity in sample tissue analysed by ELISA technique, **Figure 16 (C)**. The value was evaluated at 4820 ng/mg protein compared to 12540 ng/mg protein in animals from positive control group (a percent reduction of 62%).

Histological findings were concordant with previous results obtained from gross evaluation of colon dissected from animals exposed to real Schistosoma infection. Again, animals infected 4 weeks prior to TNBS colitis were protected and had a mean of histological damage score of 0,9 (according to the score of Ameho). This effect was no different from that observed in Sh28GST immunised animals. Even the sample photos we show in **Figure 16 (B)**, reveals no difference in the epithelial erosion or cellular infiltrate between these animals.

The decrease in the MPO activity in the group immunised with Sh28GST was even more significant than that observed in the group with established infection (p = 0,009 in the Sh28GST group compared to p = 0.02465 in the established infection group).

Finally, the group with recent infection had an evidence of increase TNBS activity as estimated by histological assessment and MPO activity measurement.

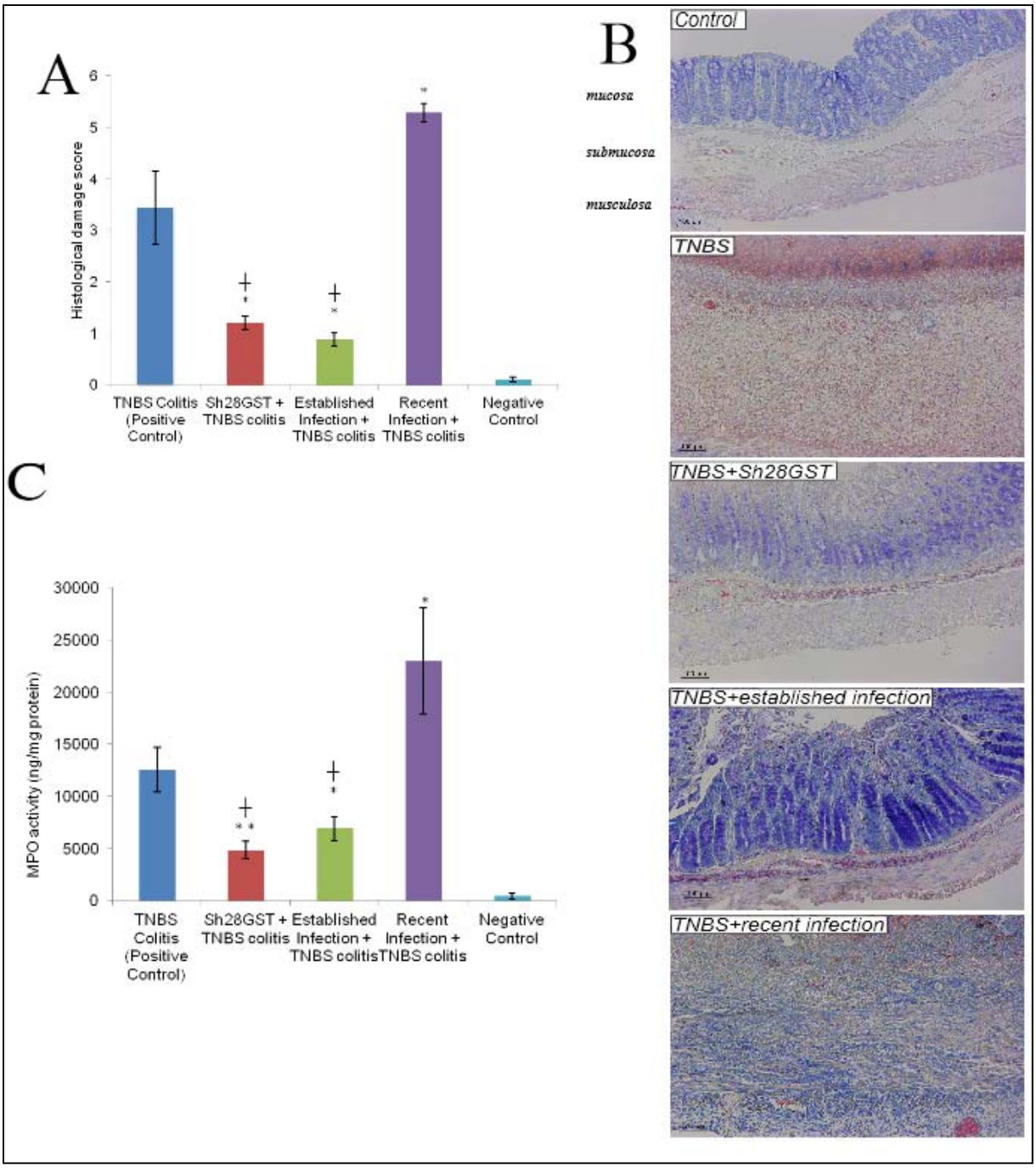

Figure 16 (A): showing histological damage score of the colon of the sacrificed Sprague Dawley rats from different groups according to the score described by Ameho and colleagues[374]. The results represent the pool of 2 independent experiments (mean ± SEM; n: 20/group); * p <0.05 compared with TNBS group (positive control); ┼ p <0.05 compared with recent infection group. **(B):** showing representive images of the H&E staining of a section of 5mm thickness the colon of a rat from different groups. In the negative control, there is normal thickness of the colonic wall with no ulceration, erosion or infiltration of the lamina propria (Zoom20X). In TNBS Colitis (positive control), there is an increase of the thickness of the colonic wall with ulceration and infiltration of the lamina propria with

polymorph neutrophils (Zoom20X). In the group immunised with Sh28GST, we show a decrease in the infiltration of the lamina propria with polymorph neutrophils than the TNBS colitis group (Zoom 40X). In the group of the established infection, there is a decrease in the infiltration of the lamina propria with polymorph neutrophils than the TNBS colitis group but more Eosinophils could be identified (Zoom 40X). In the group of recent infection, there is a severe ulceration with loss and thinning of the mucosal layer and appearance of ghost cells. Diffuse infiltration of the lamina propria with polymorph neutrophils more than the TNBS colitis group with appearance of ghost cells (Zoom 20X). **(C):** mean MPO expression in the colon tissue of the different rat groups. The results represent the pool of 2 independent experiments (mean ± SEM; n: 20/group); * p <0.05 compared with TNBS group (positive control); ** p <0.01 compared with TNBS group (positive control); ┼ p <0.05 compared with recent infection group.

To confirm previously presented results, we have showed with an objective parameter that prior injection of Sh28GST decreases TNBS colitis in a rat model as evidence by histological examination and MPO activity measurement.

From these results we concluded that prior immunisation with Sh28GST had an effect on decreasing the severity of the TNBS colitis in Sprague Dawley rat which is assessed by several marker of activity: clinical disease score, histological damage as well as the MPO activity in the colon tissue. This effect was no different from the protective effect of the long standing Schistosoma infection. In term of groups infected with Schistosoma cercariae, only the group that was infected 4 weeks before the induction of the TNBS colitis (established infection group) had a significant decrease of the inflammatory parameters compared to positive control group.

We tried to elicit the different molecular and cellular mechanisms implicated in the anti-colitis effect observed after immunisation of Sprague Dawley rats with Sh28GST.

III) Rat immunisation with Sh28GST induced immuno-regulatory changes at the level of different cytokines expression in the colon.

We studied the mRNA expression of different cytokines to illustrate the immuno-modulatory changes that occurs in the colon after immunisation of Sprague Dawley rats with Sh28GST upon exposure to TNBS colitis.

As shown in **Figure 17**, a significant decrease of mRNA expression of inflammatory cytokines was observed in the colon of animals immunised with Sh28GST compared with animals from TNBS colitis group.

As for the IL-1β there was a decrease of mRNA expression of 66% in the group of animals immunised with Sh28GST compared to TNBS colitis group. This percentage of reduction was estimated at 80% for IL-17 and at 45% for TNF. This decrease of the mRNA expression of pro-inflammatory cytokines in the colon of immunised rats can demonstrate some of the molecular changes that are implicated in the immuno-modulatory effect of Sh28GST which exerted anti-colitis effect.

This interesting finding has to be completed with the absence of difference in the mRNA expression of pro-inflammatory cytokines in both groups of animals immunised with Sh28GST and established infection with Schistosoma larva. We show in our work that long standing infection with Schistosomes induced a significant decrease of mRNA expression of pro-inflammatory cytokines (IL-1β, IL-17 and TNFα). Our results showed that immunisation with Sh28GST induced a similar reduction – which was even better for both IL-17 and TNF.

We didn't find in our work any statistically significant difference between the different groups in term of mRNA expression of the same previously tested cytokines in the serum of the sacrificed animals.

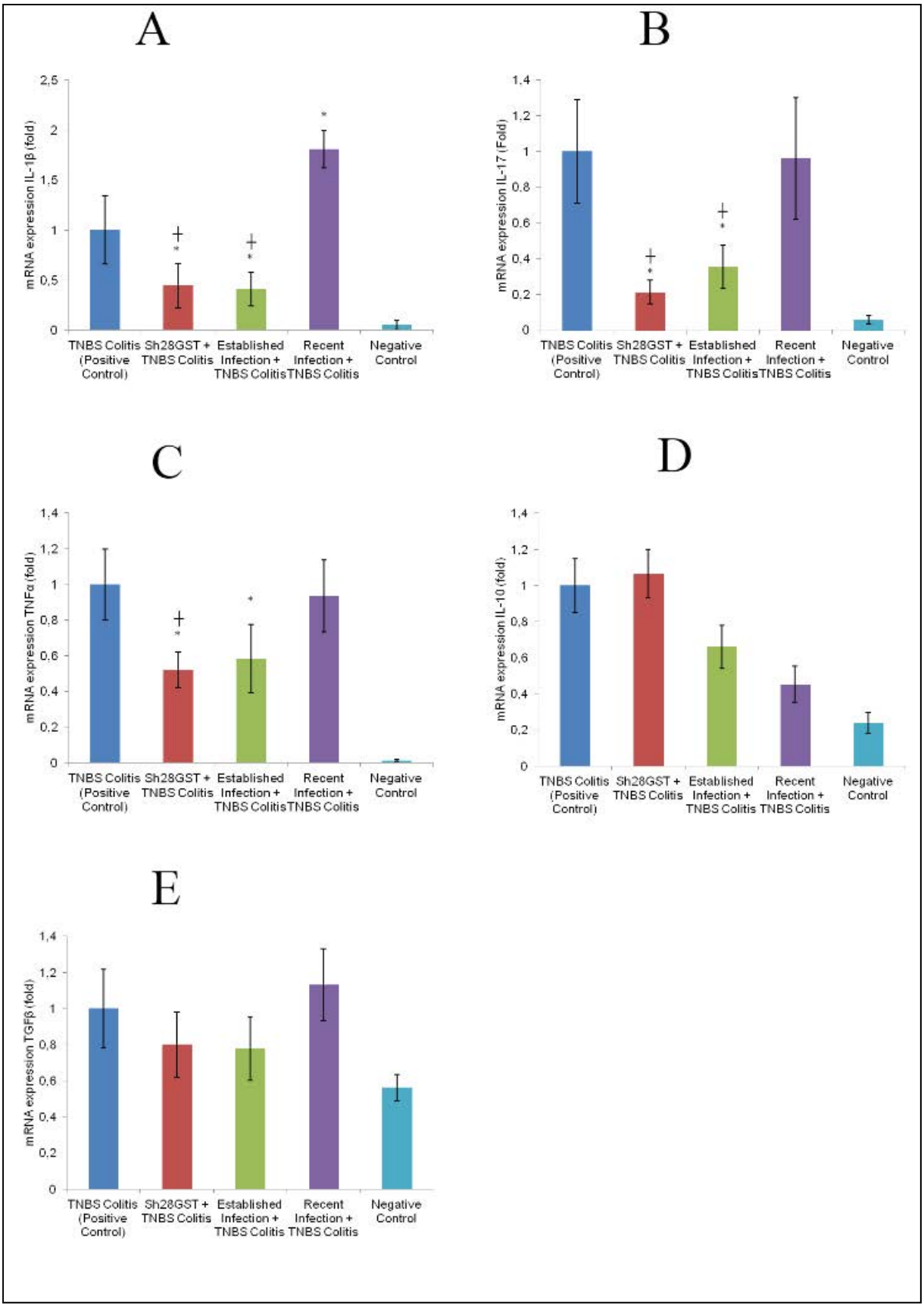

Figure 17 (A): showing changes in the mean mRNA expression of IL-1β in the colon tissue of the different groups. **(B):** showing changes in the mean mRNA expression of IL-17 in the

colon tissue of the different groups. **(C)**: showing changes in the mean mRNA expression of TNFα in the colon tissue of the different groups. **(D)**: changes in the mean mRNA expression of IL-10 in the colon tissue of the different groups. **(E)**: changes in the mean mRNA expression of TGFβ in the colon tissue of the different groups. Results represent the pool of 2 independent experiments (mean ± SEM; n: 20/group); * p <0.05 compared with TNBS group (positive control); ┼ p <0.05 compared with recent infection group.

From these results we concluded that Sh28GST had an anti-colitis effect mediated through a significant decrease of mRNA colonic expression of pro-inflammatory cytokines which was not different from the anti-colitis effect induced by the long standing Schistosoma infection.

In order to demonstrate the immune profile changes induced by the immunisation of Sh28GST, we studied mRNA expression in the colon of sacrificed animals from the different groups and we tried to show the changes in the immune profile balance.
We found a significant decrease of mRNA expression of cytokines of Th1 immune profile represented by IFNγ; which showed a significant reduction up to 40% in the group of animals immunised by Sh28GST compared to the positive control group as shown in **Figure 18 (A)**. Again, this decrease of IFNγ expression was no different from the group that was infection with Schistosoma 4 weeks before the induction of TNBS colitis.

Schistosoma infections are recognised for stimulation of an Th2 immune response after a long standing exposure to the parasite. An interesting finding in our result was the similarity of the level of mRNA expression Th2 cytokines in the colon of animals from both groups immunised with Sh28GST and those with established infection group. All Th2 cytokines mRNA expression (IL-4, IL-5 and IL-13) were no different from the group **Figure 18 (B, C and D)**.

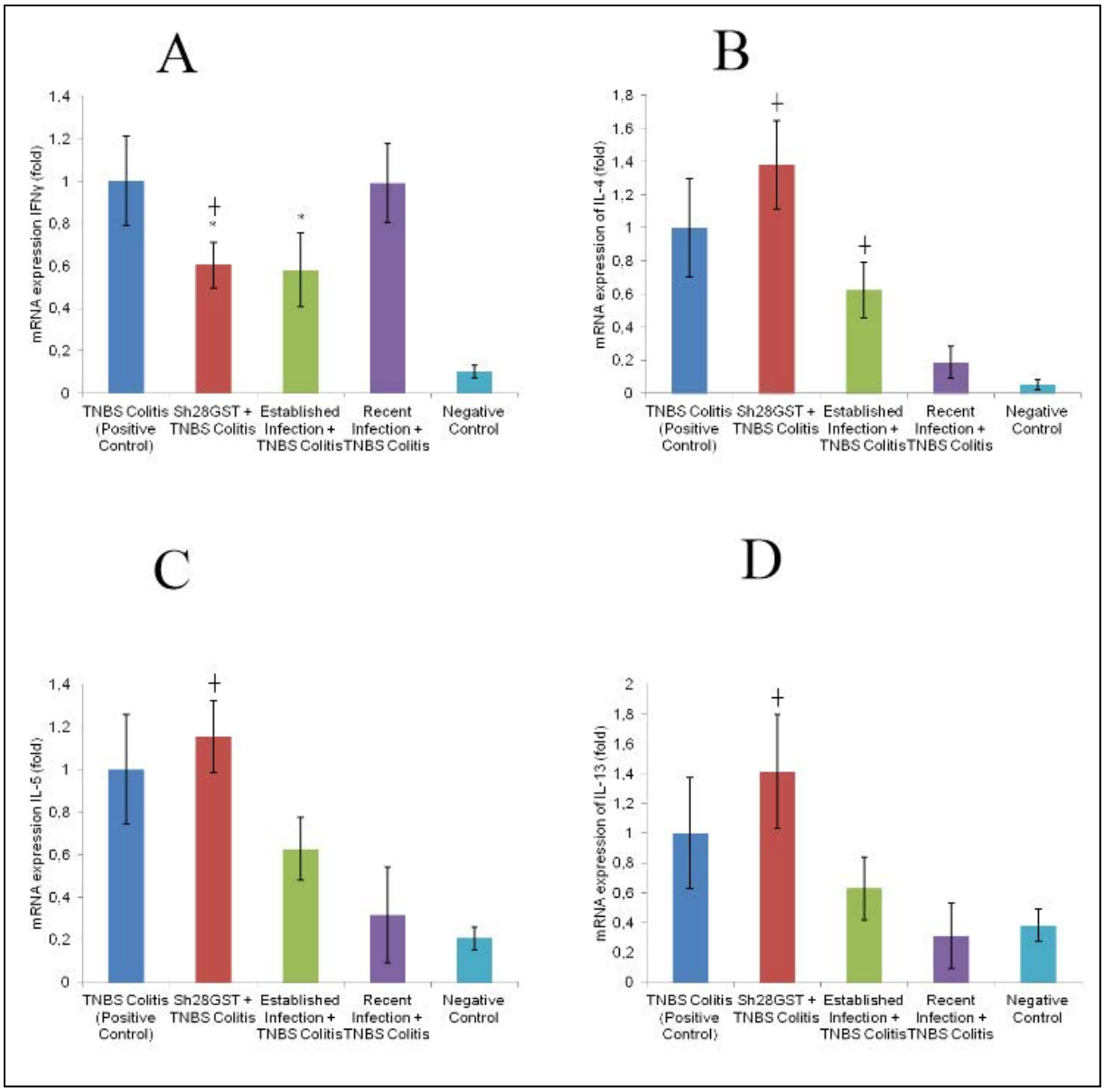

Figure 18 (A): showing changes in mean mRNA expression of IFNγ in the colon tissue of the different groups. **(B):** showing changes in mean mRNA expression of IL-4 in the colon tissue of the different groups. **(C):** showing changes in mean mRNA expression of IL-5 in the colon tissue of the different groups. **(D):** showing changes in mean mRNA expression of IL-13 in the colon tissue of the different groups. Results represent the pool of 2 independent experiments (mean ± SEM; n: 20/group); * p <0.05 compared with TNBS group (positive control); ┼ p <0.05 compared with recent infection group.

From these results we concluded that Sh28GST was capable of modulating the immune response of immunised animals through the changes induced in the Th1/Th2 immune profile balance.

IV) Immunisation with Sh28GST induces activation of different population of immune cells capable of modulation the immune response.

Eosinophils are polymorph nuclear cells with a very divergent role in the regulation of the immune response. Further analysis of histological examination of H&E stained sections from the dissected colon of the sacrificed animals from different groups revealed an infiltration with eosinophils in the colon of animals immunised with Sh28GST. This finding was no different to that of the infiltrate of the colon of animals exposed to infection 4 weeks before the induction of TNBS colitis, **Figure 19 (A)**. In fact, it has been proposed that eosinophils were capable of stimulating different populations of immune regulatory cells one of which is the alternatively activated macrophages (AAM) – a population of macrophages activated through a different pathway other than classically activated macrophages and playing a major role in the regulation and damping of the immune response through competition with CAM for the Arginin.

This was confirmed by an objective criterion, the ratio of mRNA expression in the colon of animals immunised with Sh28GST, which showed a increase of the ratio of Arginase/iNOS (up to twice increase compared to positive control group and up to 10 times compared to group with recent infection) **Figure 19(B)**. This ratio was at its maximal level in animals exposed to long standing infection with Schistosome larva. In **Figure 19 (C),** we show infiltration of the lamina propria of animals immunised with Sh28GST with AAMs as identified by immune histo-chemical staining.

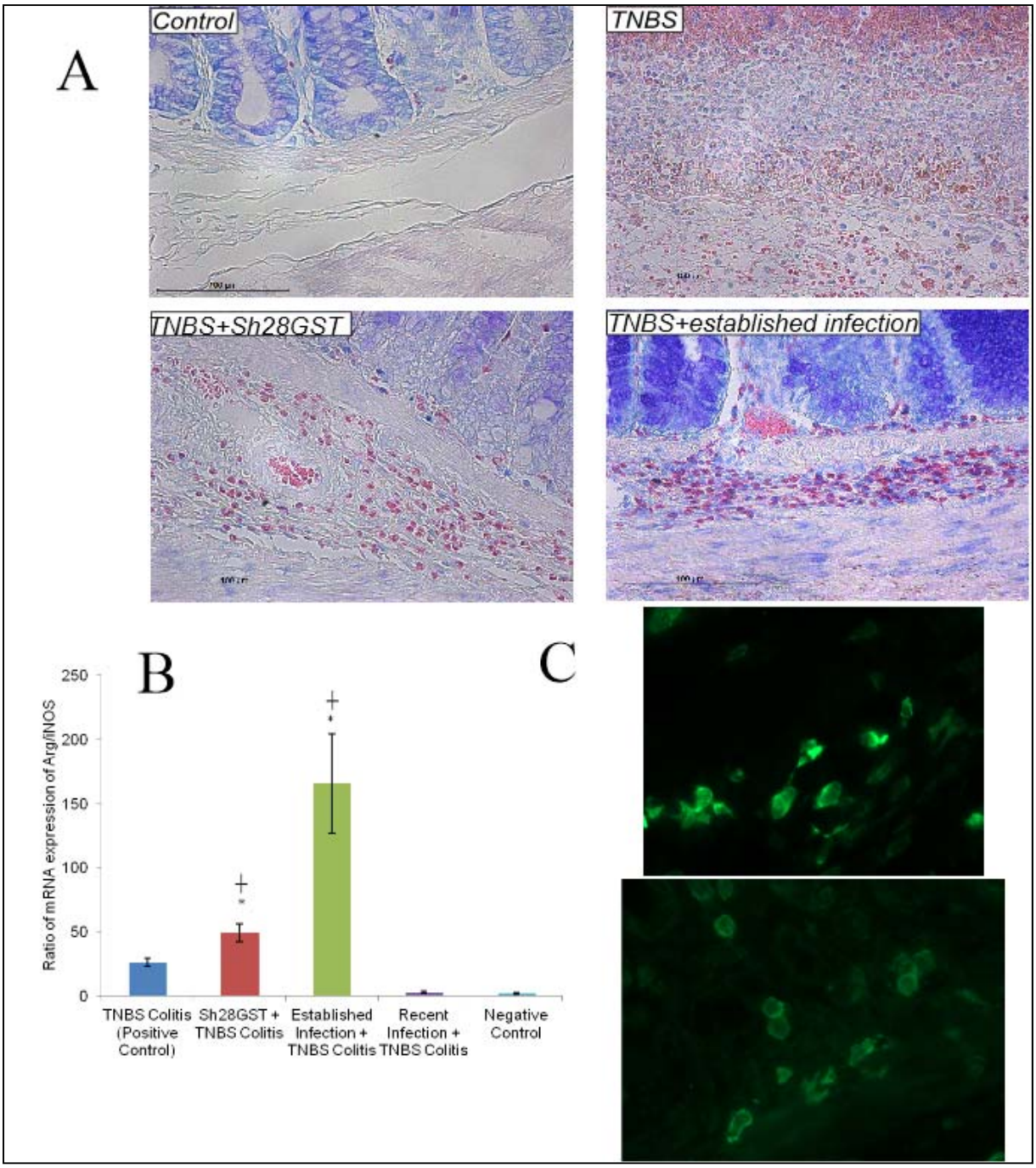
A
Control
TNBS
TNBS+Sh28GST
TNBS+established infection
B
C
Ratio of mRNA expression of Arg/iNOS
250
200
150
100
50
0
TNBS Colitis (Positive Control)
Sh28GST + TNBS Colitis
Established Infection + TNBS Colitis
Recent Infection + TNBS Colitis
Negative Control

Figure 19 (A): showing a representative sample of the H&E staining of a section of 5mm thickness the colon of a rat from different groups. In the negative control, there is normal no infiltration of the lamina propria (Zoom20X). In TNBS Colitis (positive control), there is an increase of the thickness of the colonic wall with ulceration and infiltration of the lamina propria with polymorph neutrophils mainly neutrophils (Zoom20X). In the group immunised with Sh28GST, we showed an infiltration of the lamina propria with polymorph nuclear cells mainly eosinophils (Zoom 20X). In the group of the established infection, there is also infiltration of the lamina propria with polymorph nuclear cells mainly eosinophils (Zoom 20X). In the group of recent infection, there is a severe ulceration with loss and thinning of the mucosal layer and appearance of ghost cells. Diffuse infiltration of the lamina propria with polymorph nuclear cells mainly neutrophils more than the TNBS colitis group (Zoom 20X). **(B):** showing ratio of mean mRNA expression of Arg/iNOS in the colon tissue of the different groups of rats. Results represent the pool of 2 independent experiments (mean ± SEM; n: 20/group); * p <0.05 compared with TNBS group (positive control); ┼ p <0.05 compared with recent infection group. **(C):** showing 2 representative photos showing immuno- histo chemical staining of the AAMs in the colon of Sh28GST immunised rats. Focus on the AAMs detected by anti Arginase 1 Ab in the lamina propria of this group (zoom 100x).

Sh28GST induces eosinophilic infiltration of the lamina propria of immunised rats. Cellular mechanisms implicated in the anti-colitis effect of Sh28GST include activation of AAMs population.

Second question: Is Sh28GST effective in the prevention of TNBS induced colitis in C57Bl/6 mice?

The aim of this experiment was to confirm the results previously obtained about the immune modulatory effect of Sh28GST in another animal model - the C57 Bl/6 mice.

Mice were immunised with the studied molecule Sh28GST by two subcutaneous injections in a dose and time schedule corresponding to those previously used for vaccination of children to prevent against recurrence of Schistosoma infection. Also we have used another GST from *Schistosoma japonicum* to study the specificity of the effect of Sh28GST. To evaluate the effect of the alum adjuvant, we have immunised 2 groups with the 2 GST without the adjuvant and also we have injected the adjuvant in a group of mice using the same dose and immunisation protocol as for the other groups.

We also compared the effect of Sh28GST immunisation to treatment with 5 ASA, the standard treatment for inflammatory bowel disease.

At Day 35, induction of TNBS Colitis was performed at a dose (150 mg/kg) except for the negative control which received ethanol solution at (40% dilution – the vehicle of the TNBS).

This experiment was repeated three times to confirm results and study data variability.

	Day 0	Day 28	Day 35	Day 37
TNBS colitis (Positive control)			TNBS Colitis	Sacrifice
Sh28GST + TNBS colitis	1st injection of Sh28GST	2nd injection of Sh28GST	TNBS Colitis	Sacrifice
Sh28GST without alum + TNBS colitis	1st injection Sh28GST without alum adjuvant	2nd injection Sh28GST without alum adjuvant	TNBS Colitis	Sacrifice
SJapGST + TNBS colitis	1st injection of SJapGST	2nd injection of SJapGST	TNBS Colitis	Sacrifice
SJapGST without alum + TNBS colitis	1st injection of SJapGST without alum adjuvant	2nd injection of SJapGST without alum adjuvant	TNBS Colitis	Sacrifice
Alum adj + TNBS colitis	1st injection of alum adjuvant	2nd injection of alum adjuvant	TNBS Colitis	Sacrifice
5-ASA + TNBS colitis		5-ASA	TNBS Colitis	
Ethanol injection (Negative Control)			Rectal infusion of diluted Ethanol	Sacrifice

Table 5: Showing the experimental design of the C57Bl/6 mice experiments.

I) Prior immunisation with Sh28GST decreases TNBS induced colitis in mice.

In order to confirm the protective effect of Sh28GST in another animal model using the same TNBS model of colitis, we immunised C57Bl/6 mice. We added other groups to compare the specificity of the Sh28GST in comparison with another GST - the SJapGST. We also used a group only injected with alum adjuvant and we used the 5-ASA in the human therapeutic dose adjusted to the weight of the mice.

The recombinant protein Sh28GST was capable of reproducing the previously described anti-colitis effect in a different animal model - C57Bl/6 mice. We show in our results a reduction which was statistically significant - of all markers of inflammation. Animals immunised with Sh28GST had a reduction of clinical disease score of 40% compared to animals from the positive control group (mean of the clinical disease score = 5,5 for animals of the positive control group compared to 3,3 to animals from the Sh28GST immunised group). The same finding was demonstrated in the histological damage score were the immunised group showed a reduction of 35% (mean of the histological damage score = 5,9 for animals of the positive control group compared to 3,5 to animals from the Sh28GST immunised group). Finally to confirm this fact; we calculated the reduction of the MPO activity in the colon of sacrificed animals from the different groups. There was a statistically significant reduction of the MPO activity in the group immunised with Sh28GST (mean of MPO activity in animals from positive control group was 2330 vs 1740 to animals immunised with Sh28GST). There was a 26% reduction in the mean of the MPO activity in the immunised group.

Only animals immunised with the SJapGST had a significant reduction in the markers of inflammation as noted with decrease of clinical disease score, histological damage score and MPO activity.

The alum adjuvant was essential for inducing a proper modulation of immune response and for the anti-colitis effect to be efficient. Both groups of animals immunised with the GSTs with no adjuvant were no protected from the marked inflammation induced by the injection of the TNBS as evident by absence of any significant reduction of any marker of inflammation. However, alum adjuvant alone had no effect and did not protect immunised mice with the same dose from the marked inflammatory process induced by TNBS injection.

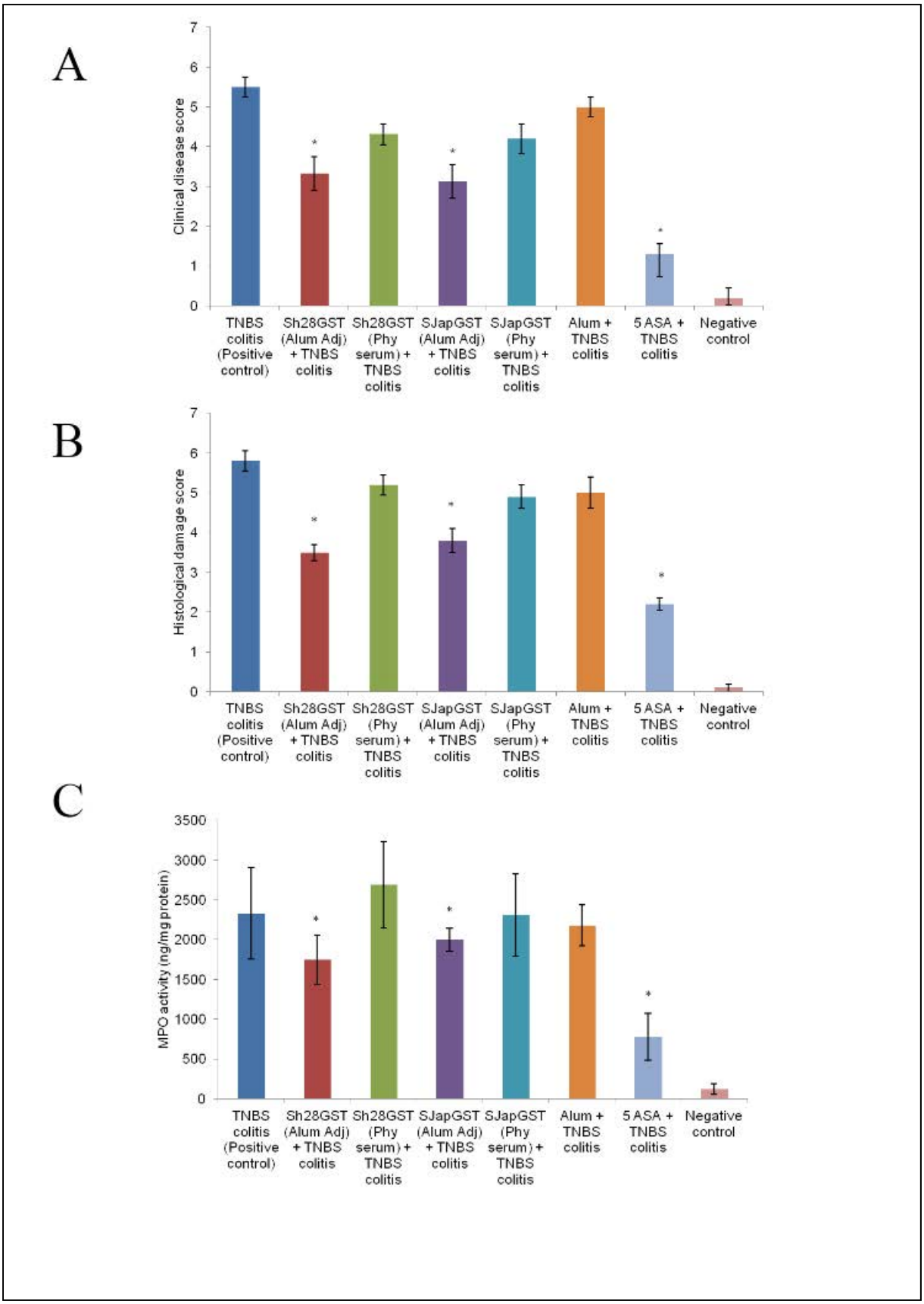

Figure 20 (A): showing the mean clinical disease score in the colon tissue of the different groups of mice according to the score described by Wallace and Keenan[373]. **(B):** showing

mean histological damage score in the colon tissue of the different groups of mice according to the score described by Ameho and colleagues[374]. **(C):** mean MPO expression in the colon tissue of the different mice groups. The results represent the pool of 3 independent experiments (mean ± SEM; n: 30/group). * p <0.05 compared with TNBS group (positive control).

We concluded that prior injection of both Sh28GST and SJapGST in a mice model of TNBS colitis, decreases the inflammation as evidence by decrease of the different parameters of TNBS colitis compared to positive control group.

The alum adjuvant – used together with the enzymatic molecule to form a gel in the subcutaneous tissue that increases immunological response to the molecule – was necessary to achieve the needed protection.

From these results, we confirmed the results previously obtained in the Sprague Dawley rats in C57Bl/6 mice. The Sh28GST was again effective in decreasing the TNBS colitis inflammatory markers. The SJapGST – not previously tested in humans – had a similar anti inflammatory effect as the Sh28GST.

II) Only prior immunisation with Sh28GST was capable of inducing immuno-modulatory changes with decrease of proinflammatory and Th1 cytokines expression in the colon of sacrificed mice compared to positive control group.

Only mice immunised with Sh28GST showed a significant decrease in the colonic mRNA expression of IL-1β and TNFα compared to positive controls (60% and 40% respectively). This confirmed the results previously described in the Sprague Dawley rats using the same protocol of immunisation and induction of TNBS colitis adapted the C57Bl/6 mice.

To elucidate the cells implicated and activated following the immunisation with recombinant molecule, we investigated for the alternatively activated macrophages known to have an anti-fibrotic as well as an anti-inflammatory role in the parasitic infection.

We analysed the mRNA expression of cytokines of both the classically activated macrophages (iNOS) in relation to those of the alternatively activated macrophages (Arginase) in the colon of sacrificed rats from different groups. We calculated a ratio of mRNA expression of Arg/iNOS to show the activation of different population in the colon of different groups and we found that the previously immunised group with Sh28GST had the highest level of expression of mRNA of Arginase in relation to iNOS.

As for the group of animals that received the 5ASA, they showed a decrease in the colonic mRNA expression of IL-1β as well as TNF compared to the positive control.

The ratio Arg/iNOS was at its higher value in animals that received 5-ASA as well as the animals immunised with Sh28GST.

These findings were not found neither in the group immunised with SJapGST. The group that was injected with the alum did not show any decrease in the colonic mRNA expression of IL-1β or the TNFα.

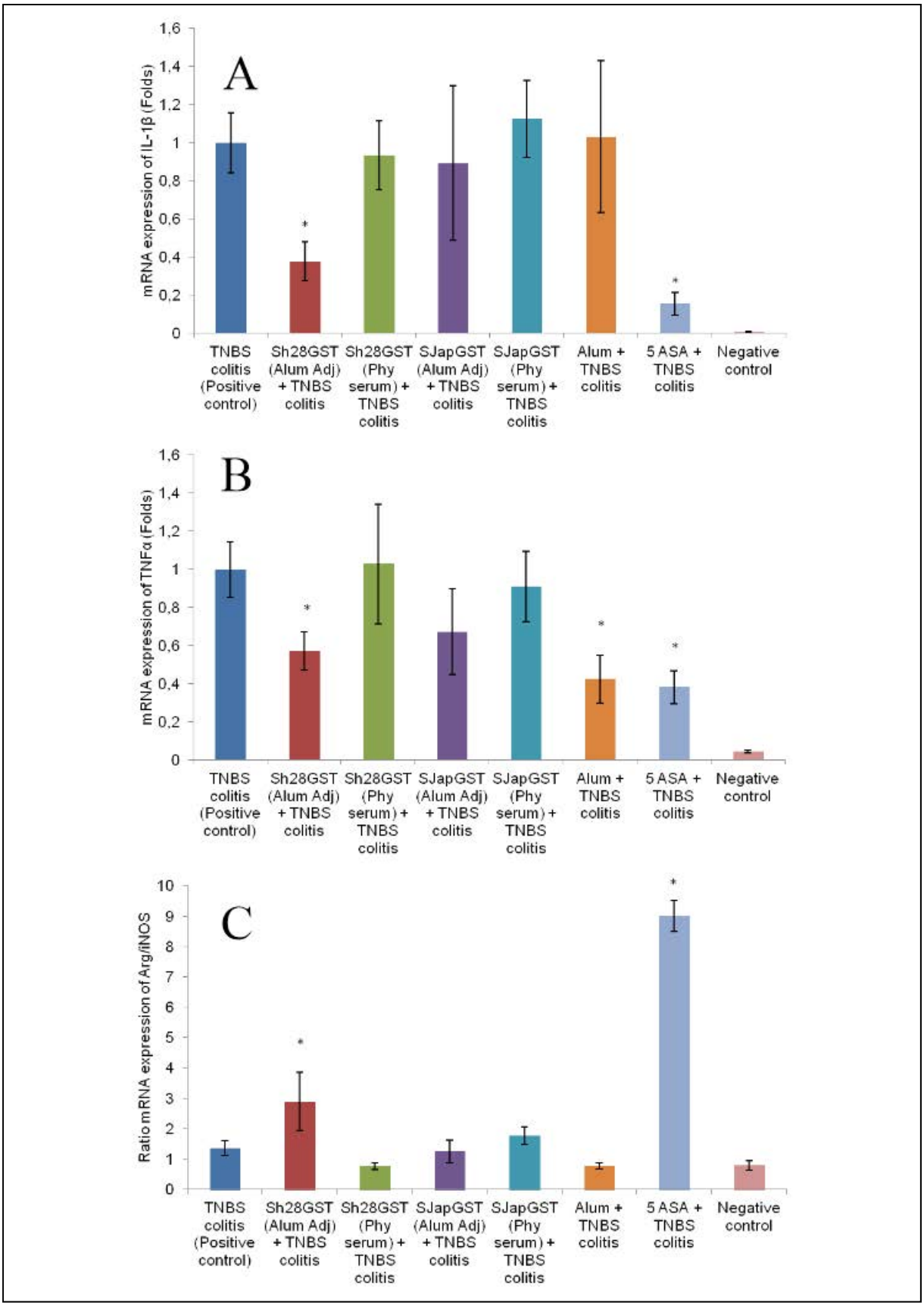
A
mRNA expression of IL-1β (Folds)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
TNBS colitis (Positive control)
Sh28GST (Alum Adj) + TNBS colitis
Sh28GST (Phy serum) + TNBS colitis
SJapGST (Alum Adj) + TNBS colitis
SJapGST (Phy serum) + TNBS colitis
Alum + TNBS colitis
5 ASA + TNBS colitis
Negative control
B
mRNA expression of TNFα (Folds)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
TNBS colitis (Positive control)
Sh28GST (Alum Adj) + TNBS colitis
Sh28GST (Phy serum) + TNBS colitis
SJapGST (Alum Adj) + TNBS colitis
SJapGST (Phy serum) + TNBS colitis
Alum + TNBS colitis
5 ASA + TNBS colitis
Negative control
C
Ratio mRNA expression of Arg/iNOS
10
9
8
7
6
5
4
3
2
1
0
TNBS colitis (Positive control)
Sh28GST (Alum Adj) + TNBS colitis
Sh28GST (Phy serum) + TNBS colitis
SJapGST (Alum Adj) + TNBS colitis
SJapGST (Phy serum) + TNBS colitis
Alum + TNBS colitis
5 ASA + TNBS colitis
Negative control

Figure 21 (A): showing mean mRNA expression of IL-1β in the colon tissue of the different groups of mice experiments. **(B):** showing mean mRNA expression of TNFα in the colon tissue of the different groups of mice experiments. **(C):** showing ratio of mean mRNA expression of Arg/iNOS in the colon tissue of the different groups of mice experiments. Results represent the pool of 3 independent experiments (mean ± SEM; n: 30/group); * p <0.05 compared with TNBS group (positive control).

From these results we concluded that only Sh28GST was capable of inducing immuno-modulatory changes at the level of the mRNA expression of pro-inflammatory and Th1 cytokines in the colon of the sacrificed animals. Again, the recombinant Sh28GST was capable of activating the alternatively activated macrophages in the lamina propria of the colon of immunised mice.

Even if the SJapGST had an anti-inflammatory effect against TNBS colitis, only the Sh28GST had an immuno-modulatory effect decreasing the expression of mRNA of inflammatory Th1 cytokines in the colons of immunised mice.

Discussion

Inflammatory bowel diseases are considered as part of immune mediated inflammatory disorders. Their pathogenesis was linked to an inappropriate exaggerated immune response to commensal bacteria normally present in the bowel, in genetically predisposed individuals. This has been linked to excess hygiene and decrease exposure to helminths [226, 327].

Epidemiologic data have given a clue on the relation of prevalence of helminthic infections and the incidence of inflammatory bowel diseases in developing countries [377]. Helminth parasites are the classic inducers of Th2 responses. The Th2 polarized T cell response driven by helminthic infection has been linked to the attenuation of some damaging Th1 driven inflammatory responses, preventing some Th1 mediated autoimmune diseases in the host, including experimentally induced colitis. The porcine whipworm *Trichuris suis* – although this is a non acceptable potentially iatrogenic infection - has been tested for treating IBD patients, resulting in clinical amelioration of the disease [327, 329]. Review papers and clinical trials have proposed the concept of gastrointestinal parasites as a potential therapy for refractory Crohn's disease [315]. Among all parasites, Schistosomes are considered as "Masters of Regulation" [189].

Our work focused on the immune modulatory effect of a recombinant parasitic Glutathion *S*-transferase – previously tested in young adult to prevent against relapses of Schistosoma infection. The human safety profile and potential side effects of this molecule were properly evaluated.
We investigated whether, this molecule, the "Sh28GST" could prevent or at least reduce colitis in 2 different animal models of hapten induced colitis and to study the possible mechanisms implicated in this immune-regulatory action.

In our first experiments performed in Sprague Dawley rats, we have shown that immunisation with Sh28GST decreased the inflammation induced by TNBS direct infusion in the colon. Interestingly, this effect was comparable to a decrease in the group of rats with established infection (4 weeks before TNBS injection) but not in the group of recently infected rats (just 1 week before TNBS).

These results confirm previous data suggesting a beneficial role of parasites in the prevention of hapten- induced colitis as well as other models of immune mediated inflammation. In the present work, we investigated the effect of immunisation with a single Schistosome protein the Sh28GST. and we showed that this recombinant enzyme was able to prevent inflammation induced by rectal instillation of TNBS. Concerning the potential mechanisms of action, we have shown that Sh28GST immunised animals had an increase in mRNA coding for Th2 cytokines (IL-4, IL-5 and IL-13) and a decrease in pro-inflammatory cytokines mRNA such as IFNγ and TNFα. This Th2-type cytokines play a major role to shift the host immune response from a Th1 profile to a less pathological Th2 profile. We also noted a significant decrease of IL1β as well as IL-17 expression after TNBS challenge of Sh28GST immunised animals. Both are pro-inflammatory cytokines with IL-17 secretion driven by IL-23 and shared by TNF.

Different populations of immune cells were studied in our work to show their role in the preventive role of the Sh28GST: the eosinophilic infiltrate was increased in the colons of the previously immunised rats (as shown in the sample photos of histological scoring of the colon of the sacrificed rats). Though the TNBS model of colitis is characterized by the diffuse infiltration of Polymorph neutrophils in the lamina propria of the exposed animals, in our experience there was a decrease of this infiltration with more eosinophil infiltration. We have also observed a significant increase in the IL-5 expression in the colon of the immunised rats which play a role in the differentiation of different Polymorph subpopulations. Is there a different activated population of eosinophils that may play an immune-modulatory role in the prevention of the TNBS colitis effect of the Sh28GST? Such question needs further characterization and identification of the different cells activated after immunisation with the Sh28GST?

The capacity of Sh28GST to activate and recruit alternatively activated macrophages is an innovative mechanism of action that we suggest by showing a significant increase in the ratio of mRNA expression of Arginase 1 in relation to iNOS 2 in the colonic mucosa of the immunised rats compared to controls. This was illustrated by immunohistochemistry showing positively cells with Arginase 1 a marker of AAM, in the lamina propria of the colon of immunised rats. The role of AAMs is crucial in the immuno-modulation induced by Schistosome infection. The AAMs are potent anti-inflammatory cells and have a major anti-fibrotic and healing capacity following the passage of the eggs of Schistosome parasites

through the wall of infected individuals. They help in deposition of collagen and induce healing of superficial mucosal damage. The possibility of activating this population of macrophages is a part of the mechanism by which Sh28GST exhibits its anti-inflammatory role.

Schistosomes exerts potent anti-inflammatory effects by directly regulating the ability of DC to respond to TLR ligands [378]. Regulatory T cells represent one of the host's mechanisms to prevent immune pathology during chronic immune stimulation. Enhancement of regulatory T-cell activity may be useful to control autoreactive T-cell responses and inhibit harmful inflammatory diseases such as asthma and IBD [379]. Tregs showed an ability to steer monocytes differentiation toward alternatively activated macrophages (AAM) [380].

Here in our experiments we studied the immune response to Schistosoma infection and the kinetics, the timing before the induction of colitis which is crucial for immuno-modulation. Our results raised the interest of comparing the immune-modulatory effect of a single recombinant protein to that of a parasitic infection.

In term of Schistosoma infection, our results were concordant with the previous reports showing that rats develop predominantly Th2 type response during a primary infection which may be involved in the immune reaction against Schistosome [381]. However in our work, the protective effect of a previous Schistosoma infection was restricted to the group with long standing infection rather than the group with a recent onset exposure to the parasite.

IL-4 plays a major role in inducing Th2-type CD4+ cells in the gut [382]. Our results showed that IL-4 as well as IL-5 mRNA was similarly increased in the colon of animals immunised with Sh28GST or infected for 4 weeks, when compared to recently infected rats. This confirms the association between a Th2 type response and the protective anti-inflammatory effect.

Natural killer cells seem to be the main source for IL-13 in the colon of animals challenged with TNBS [383]. However the anti-inflammatory effect of the secretion of IL-13 may be related to the stimulation of AAMs. Both IL-4 and IL-13 show an effect on alternative activation of macrophages that includes weak proliferative (possibly mediated by autocrine M-CSF), the fusion of cells, induction of expression of MHC class II molecules and enhancement of levels

of macrophage mannose receptor activity. Peritoneal macrophages show alternative activation pattern following exposure to recombinant IL-13, a pattern identical to that induced by IL-4 [280].

In our study, we tested the levels of various pro and anti-inflammatory cytokines in the serum of immunized animals. No changes in the pro or anti inflammatory cytokines levels were detected in the serum of animals immunised with Sh28GST following TNBS challenge. We thus suggest that no systemic changes of cytokine expression was implied, in contrast to the possibility of activation of local reticulo-endothelial system and local mesenteric lymph nodes leading to local activation of alternatively activated macrophages. We propose further investigation of the local reticulo-endothelial system following the Sh28GST immunisation in order to detect the mechanisms by which the AAMs are recruited and migrated to the colonic mucosa.

Previous experiences performed in the mice showed that Schistosome egg exposure attenuated TNBS colitis and protected mice from lethal inflammation. Schistosome egg exposure diminished IFNγ and enhanced IL-4 production from CD3-stimulated spleen and mesenteric lymph node cells of TNBS-treated mice[225].

In our work, we o confirmed the results obtained in rats by using C57Bl/6 mice and the same scheme of immunisation by Sh28GST and of TNBS colitis-induction – adapted to mice The results obtained in the Sprague Dawley rats were confirmed in C57Bl/6 mice. The Sh28GST was again effective in decreasing the TNBS colitis inflammatory markers. It reproduced the protective effect and induced most of the immune-modulatory changes observed in the rat experiment with decrease in the inflammatory Th1 cytokines expression in the colonic mucosa.

In our experience, the ratio of expression of mRNA of Arginase1 to iNOS2 in the colon of the immunised mice was at a higher value compared to other control groups.
The alum adjuvant – used together with the enzyme to form a gel in the subcutaneous tissue that increases immunological response to the molecule – was necessary to achieve the needed protection. However testing the alum alone had no effect on the TNBS colitis and did not reduce any inflammatory marker denoting the absence of any direct effect of the used adjuvant.

In conclusion, this study provides the first evidence that immunisation with a recombinant protein from the Schistosome helminth parasite prevents experimental colitis induced by hapten in two models of rodents.

We also concluded that Sh28GST is a promising recombinant molecule with a protective anti-inflammatory role in the prevention of TNBS colitis in both rats and mice. However, further experiments are needed to clearly understand the mode of action of Sh28GST.

The potential effect of this helminthic enzyme in the prevention of Crohn's disease relapses in human is actually taken in consideration in case-control clinical trials.

What is the role of eosinophils infiltration in the modulation of the immune response secondary to Sh28GST immunisation?

We have noted in our work an increase in the cellular infiltrate of essentially eosinophils in the lamina propria of the colon of the immunised rats with our recombinant molecule. This is concording with the increase in colonic IL-5 mRNA expression by RT-PCR. It has also to be mentioned that immunization of humans with Sh28GST induced increased IL-5 levels [366]. In fact IL-5 has been shown to be involved in protective immunity against several helminth infections in mice as suggested by IL-5 depletion studies [384]. Investigating the role of eosinophils in the anti-inflammatory effect of Sh28GST is crucial and we will use IL-5 knockout mice to test this implication.

Interleukin-5, a hematopoietic cytokine, plays a key role in the differentiation, maturation, and survival of eosinophils derived from bone marrow precursors. The link between IL-5 and eosinophils is highly conserved in mammals, which suggests a selective pressure for maintenance of this function in the immune repertoire [385, 386]. Two genetically modified IL-5 mice models could be used in further studies: IL-5 knockout (KO) and IL-5 transgenic (TG) mice.

Further questions:

- What is the role of alternatively activated macrophages infiltration in the modulation of the immune response secondary to Sh28GST immunisation?

We have brought some evidence favoring the activation of alternatively activated macrophages, secondary to immunisation with Sh28GST: by showing a significant increase

in mRNA ratio of Arg/iNOS in the colon of immunised rats by RT-PCR, confirmed by immuno histochemical techniques on the sections of the colon.
These results suggest that immunization with Sh28GST induced specific infiltration with AAM, well known to exert anti-inflammatory properties.
However, the exact role as well as the kinetics and mechanisms, of activation of this population in the modulation of the immune response are not well defined. For this, we propose to further investigate the protective effect of AAM either by specific depletion with arginase inhibitor or by passive transfer of purified AAM.

- What are the possible intercellular interactions affecting the immune response in Sh28GST immunised animals?

In fact the relation between eosinophils and macrophages is well established with mutual activation. Stimulated macrophages of mice with chronic schistosomiasis have a capacity to induce peripheral proliferation of eosinophil granulocytes. This capacity is independent of the quality of the intraperitoneal environment. It can be expressed after transferring macrophages elicited in schistosome infected mice into normal mice [387].
Aged apoptotic eosinophils are recognized and ingested as intact cells by macrophages. This action is modulated by IL-5. Apoptosis and ingestion by macrophages may represent a mechanism whereby the tissue longevity and removal of eosinophils is controlled [388].

For the study of correlation of both eosinophils and macrophages, we propose a model of extra-medullar proliferation of eosinophil granulocytes that can be induced by intraperitoneal implants of glass cover slips. Immature eosinophils are located in discrete foci on glass implants; they are not correlated with the eosinophil population of the peritoneal cavity, where only mature eosinophils can be observed. The same induction of eosinophil proliferation can be obtained by the transfer of macrophages elicited by glass implants in mice. This induction could not be done with cells mobilized in normal mice, either after transplant into normal mice or into schistosome infected ones [387].

- What is the possible role of different regulatory and anti-inflammatory cytokines in the anti-colitis effect of Sh28GST?

In our experience we have demonstrated that Sh28GST have induced changes in the level of mRNA expression of pro-inflammatory and Th1 cytokines. We propose to further investigate the mechanisms implicated in the modulation of the immune response with special interest on

the Treg cells and the regulatory cytokines (IL-10 and TGFβ). Multiple gene-knockout mice such as IL-2, IL-10 or T-cell receptor chains, develop bowel inflammation spontaneously, and are therefore possible models for IBD. Of these models the IL-10 knockout mice develop a disease closest to CD while the other resemble more closely ulcerative colitis [389].

- What is the possible role of alum in the immune response following animal immunisation with Sh28GST?

Alum-based adjuvant facilitates vaccine-driven humoral immunity, but their mechanism of action remains poorly understood. Alum administration enhances antigen presentation and upregulates expression of costimulatory molecules on APCs [390]. Recent reports suggest that Type II NKT cells may act as part of the Alum-sensing apparatus and facilitate Th2-driven humoral immunity [391].

In our experience, we could not show any direct effect of immunization with alum adjuvant alone, which did not induce any inflammatory reaction, in the conditions of immunization. However, alum was essential for the induction of the immuno-modulatory effect in mice immunised with Sh28GST, which concords with the helpful adjuvant effects of alum recommended in vaccines.

Conclusion

In this work, our aim was to demonstrate a new application of a previously tested recombinant parasitic protein in the prevention of experimental colitis. The Sh28GST is a new candidate to add in the arsenal of potential drugs to prevent from hapten-induced colitis. This study provides the first evidence that immunization with this recombinant protein from the Schistosome helminth parasite prevents hapten-induced colitis in two models of rodents. The safety profile – vigorously studied - in fragile population (children and young adults) makes its use in the treatment of Crohn's disease a nearby dream.

Although further studies are needed to illustrate the exact mechanisms of action implicated in the immuno-modulatory effect, P28GST is a promising molecule exerting a potent anti-inflammatory role in the prevention of colitis. Further experimental studies are needed to elicit, in details, the mechanisms that are implicated in its immune regulatory action.

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