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Modulation of dendritic cell function through delivery of effector proteins by genetically modified *Lactococcus lactis*

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Michaela

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LIST OF ABBREVIATIONS

Akt	*protein kinase B (PKB)*
AP-1	*activator protein 1*
APC(s)	*antigen-presenting cell(s)*
Bad	*Bcl-xL/Bcl-2 associated death promoter*
BAG	*BCL2-associated athanogene*
BM-DC(s)	*bone marrow-derived dendritic cell(s)*
CARD	*caspase recruitment domain*
CBA	*cytometric beads array*
CCL(2)	*chemokine (C-C motif) ligand (2)*
CCR6/7	*chemokine (C-C) receptor 6/7*
CD	*Crohn's disease*
C/EBPβ	*CAAT/enhancer-binding protein β*
CFU	*colony forming units*
(μ)Ci	*(μ)Curie, a unit of radioactivity*
CLP	*common lymphoid progenitors*
CMP	*common myeloid precursors*
COX	*cyclooxygenase*
cpm	*counts per minute*
CRIP	*cysteine rich intestinal peptide*
DC(s)	*dendritic cell(s)*
DC-SIGN	*dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3) grabbing nonintegrin*
DMEM	*Dulbecco's Modified Eagle's Medium*
dsRNA	*double stranded RNA*
DSS	*dextran sodium sulfate*
EDTA	*ethylene-diamine tetra-acetic acid*
EGF	*epidermal growth factor*
ERK(s)	*extracellular signal regulated kinase(s)*
FCS	*fetal calf's serum*
FHA	*filamentous hemagglutinin*
FITC	*fluorescein isothiocyanate*

Flt3	*FMS-like tyrosine kinase 3*
Foxp3	*forkhead box P3*
GALT	*gut associated lymphoid tissue*
GlyCAM-1	*glycosylation dependent cell adhesion molecule 1*
GM	*genetically modified*
GM-CSF	*granulocyte/macrophage–colony-stimulating factor*
GRAS	*Generally Regarded As Safe*
Groα	*growth regulated oncogene α*
h	*hours*
HBSS	*Hank's balanced salt solution*
HEPES	*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*
HNF-3	*hepatocyte nuclear factor 3*
HPLC	*high performance liquid chromatography*
HSC	*hematopoietic stem cell*
IBD	*inflammatory bowel disease*
ICAM-1	*intercellular adhesion molecule 1*
IEC	*intestinal epithelial cell lines*
IECs	*intestinal epithelial cells*
IFN	*interferon*
Ig(A/E/G)	*immunoglobulin (A/E/G)*
IL	*interleukin*
IL-(10)R	*interleukin (10) receptor*
ILT3/4	*immunoglobulin like transcript ¾*
iNOS	*inducible nitric oxide synthase*
IP10	*IFNγ-inducible protein 10*
kb	*kilo base pairs*
kDa	*kilo Dalton*
LAB	*lactic acid bacteria*
LFA-1	*leukocyte function-associated antigen 1*
LL-pTmIL10	*Lactococcus lactis producing murine interleukin-10*
LL-pTREX	*Lactococcus lactis transformed with empty vector*
LMP2/7	*low molecular mass polypeptide 2/7*
LPS	*lipopolysaccharide*

LRR	*leucine-rich repeats*
mAbs	*monoclonal antibodies*
MAdCAM	*mucosal addressin cell adhesion molecule-1*
MAPK(s)	*mitogen-activated protein kinase(s)*
M cells	*microfold cells*
MCP-(1,…)	*monocyte chemotactic protein (1,…)*
MDC	*macrophage derived chemokine*
MFI	*mean fluorescence intensity*
MHC	*major histocompatibility complex*
MIP-(1α/β…)	*macrophage inflammatory protein (1α/β,…)*
MLN	*mesenteric lymph node(s)*
mTFF3	*murine trefoil factor family 3*
mRNA	*messenger RNA*
MyD88	*myeloid differentiation primary response gene 88*
NF-κB	*nuclear factor-kappaB*
NO	*nitric oxide*
NOD(2)	*nuclear type binding oligomerization domain (2)*
OVA	*ovalbumin*
PAMP	*pathogen associated molecular pattern*
PBS	*phosphate-buffered saline*
PCR	*polymerase chain reaction*
PD-L1	*programmed death-1 ligand*
PE	*phyco-erythrin*
PG (E_2, I_2)	*prostaglandin (E_2, I_2)*
PI	*propidium iodide*
PI3K	*phosphoinositide-3 kinase*
PLC	*phospholipase C*
PKC	*protein kinase C*
PP	*Peyer's patche(s)*
PRR	*pattern recognition receptor*
PS	*penicillin/streptomycin*
RANTES	*regulated on activation, normal, T-cell expressed and secreted*
ROCK	*Rho associated kinase*

RPL13A	*ribosomal protein L13A*
SCID	*severe combined immunodeficiency*
SD	*standard deviation*
SED	*subepithelial dome*
SEM	*standard error of the mean*
SHP2	*Src homology 2-containing tyrosine phosphatase*
SLAM	*signaling lymphocytic activation molecule*
SOCS	*suppressor of cytokine signaling*
Sp(1,3)	*specificity protein (1,3)*
Src	*sarcoma (tyrosine kinase)*
STAT	*signal transducer and activator of transcription*
TAP1	*transporter associated with antigen processing*
TBP	*TATA box binding protein*
TCR	*T cell receptor*
TFF	*trefoil factor family*
TFIZ1	*trefoil factor interactions(z)*
TGFβ	*transforming growth factor β*
Th	*T helper*
TLR	*Toll-like receptor*
TNBS	*trinitrobenzene sulphonate*
TNF	*tumor necrosis factor (α)*
TRAIL	*tumor necrosis-related apoptosis-inducing ligand*
Treg cell	*regulatory T cell*
TTFC	*tetanus toxin fragment C*
TUNEL	*terminal uridine deoxynucleotidyl transferase dUTP nick end labeling*
TXA2-R	*thromboxane A2 receptor*
UC	*ulcerative colitis*
USF	*upstream stimulating factor*
Usp45	*unknown secreted protein of 45 kDa*
Vangl1	*Van Gogh like protein 1*
VCAM-1	*vascular cell adhesion molecule 1*

PART I: GENERAL INTRODUCTION

1 SHAPING AN IMMUNE RESPONSE

The immune system is composed of many interdependent cell types that collectively protect the body from bacterial, parasitic, fungal and viral infections. This large variety of danger signals presents different challenges to the immune system in terms of recognition and mounting appropriate immune responses. Many of the immune cell types involved have specialized functions to optimally defend the body against foreign pathogens.

To establish an infection, the pathogen must first overcome numerous surface barriers. Any organism that breaks through these first barriers leads immediately or within hours after antigen exposure to the induction of an innate immune response (Figure 1). Innate (natural) responses are general defense mechanisms that are antigen-nonspecific. Cells of the innate immune system are designed to recognize a few highly conserved structures, called 'pathogen-associated molecular patterns' (PAMPs) such as LPS, peptidoglycan, lipotechoic acids, mannose, bacterial DNA, double-stranded viral RNA and glycans. These PAMPs are common in many different micro-organisms and are recognized by 'pattern recognition receptors' (PRRs) present on most cells of the innate immune sytem. PAMPs can also be recognized by a series of proteins in the blood that initiate the complement pathways. The innate response uses phagocytic cells (neutrophils, monocytes and macrophages), cells that release inflammatory mediators (basophils, mast cells and eosinophils), and natural killer cells. The molecular components of the innate responses include complement, acute-phase proteins and cytokines such as interferons. However, innate immune cells alone cannot always eliminate infectious organisms. B and T lymphocytes, which are cells of the acquired (adaptive) immune system, have evolved to mount a stronger and more effective immune response against any pathogen. Acquired immune responses are antigen-specific and need some time to develop but are improved upon repeated exposure to a given pathogen, which is called immunological memory. (for review see [1,2])

Acquired immune responses are generated in the lymph nodes, spleen and mucosa-associated lymphoid tissues. Two types of acquired immune responses exist: cellular and humoral immune responses. $CD4^+$ T cells and $CD8^+$ T cells, which are part of the cellular immune response, have evolved to eliminate intracellular pathogens and to provide help to B cells, which are part of the humoral immune response. T cells can eradicate intracellular pathogens by activating macrophages and by killing virally infected cells. B cells secrete immunoglobulins, antigen-specific

antibodies responsible for eliminating extracellular micro-organisms. To be activated, T cells need to recognize antigens presented by specialized cells, called antigen-presenting cells (APCs), in the context of major histocompatibility complex (MHC) molecules through interaction with their T cell receptor (TCR). In addition, APCs provide co-stimulatory signals necessary to fully activate T cells. In the absence of co-stimulation T cell anergy is induced. However, in addition to eradicate harmful pathogens, the acquired immune response is also responsible for the induction of allergies and autoimmune responses. (Figure 1)

A key cellular component of innate immunity is the dendritic cell (DC), a professional APC. These cells constantly but quietly endocytose antigens in the periphery. When antigens are encountered, they are activated and migrate to the draining lymph node where they efficiently activate naive T cells and prime an immune response for which immunological memory has not been established. DCs also determine the character of the activated T cells that mediate the effect against a specific pathogen.

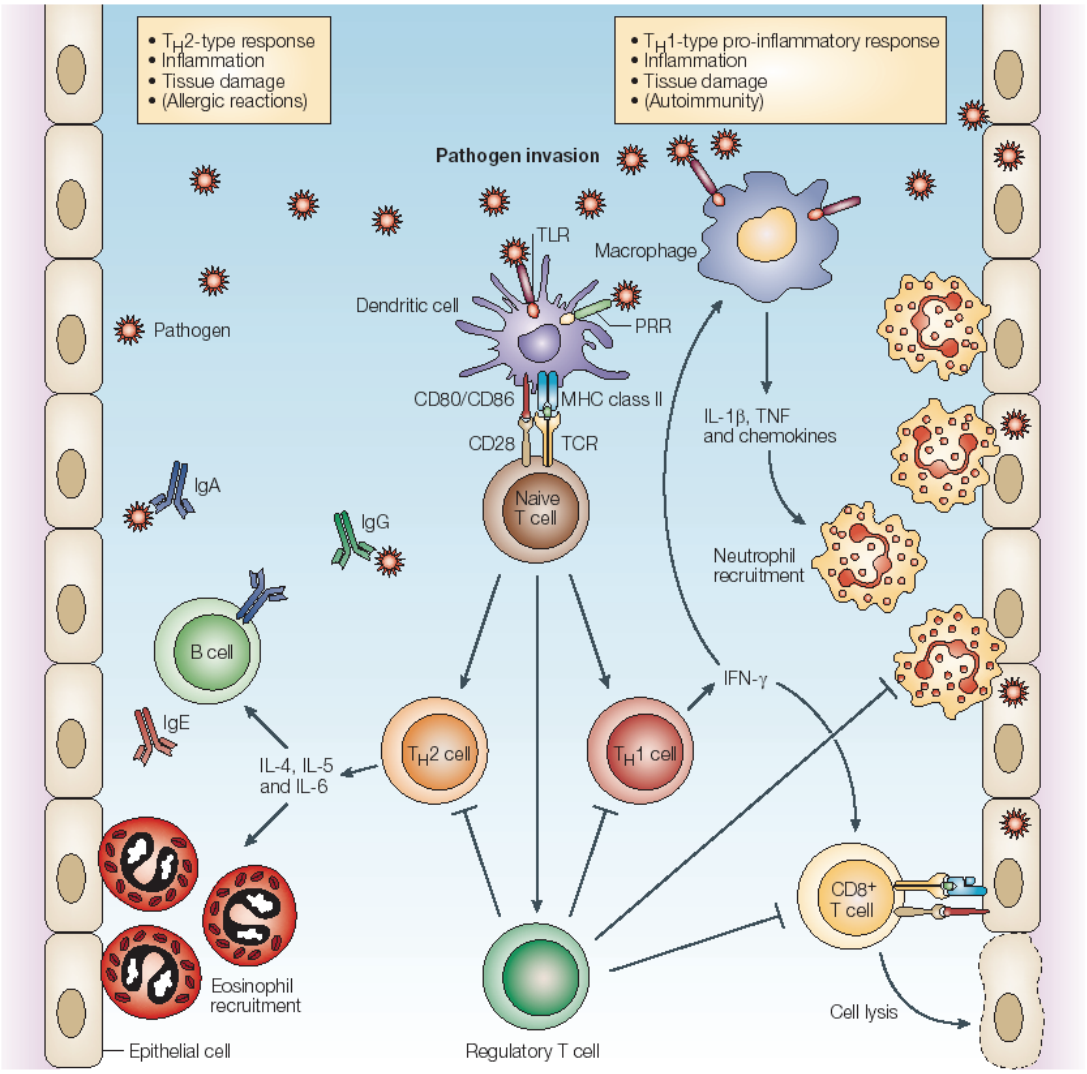

Figure 1 Immunity to infection.
Innate immune effector cells, including macrophages, dendritic cells (DCs), neutrophils and natural killer (NK) cells (not shown), together with various protein components of the complement system, provide the first line of defense against invading micro-organisms. Binding of conserved pathogen-derived molecules to pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), on the cell surface of macrophages and DCs activates the production of pro-inflammatory cytokines and chemokines, which help to attract other effector cells to the site of infection. Pathogen-activated DCs present pathogen-derived antigens to T cells and promote the differentiation of naive T cells to various subtypes of effector $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T helper 1 (Th1) cells secrete interferon-γ (IFN-γ), which activates the anti-microbial activity of macrophages and helps B cell production of IgG2a antibodies, whereas Th2 cells provide help for B cell production of IgG1, IgA and IgE. $CD8^+$ T cells lyse host cells infected with viruses, intracellular bacteria or parasites. In normal individuals, regulatory T cells (both natural regulatory T cells circulating in the periphery and those induced by infection) help to control these effector functions and the associated damage to host tissues. IL: interleukin; TCR: T cell receptor; TNF: tumor necrosis factor. Source: [3]

1.1 T cell subsets

The thymus is central to establish a functioning immune system. In the thymus, T cells mature from hematopoietic progenitors, driven by mutual interactions of stromal cells and the developing thymocytes. As a result, different types of T cells are generated, all of which have been carefully selected for their ability to mount an immune response to non-self without driving pathogenic self-reactive autoimmune responses.

As the emphasis of this work relates to the outcome of the DC-T cell interaction, I will further focus on $CD4^+$ T cells of which some different subsets have been identified over the last decades.

1.1.1 Effector T cells: Th1, Th2 and Th17

A major breakthrough was the classification of $CD4^+$ T cells into T helper 1 (Th1) and T helper 2 (Th2) subsets based on cytokine secretion *in vitro* [4,5].

The cytokine typical for Th1 responses is interferon γ (IFNγ). Besides increasing phagocytic activity, IFNγ also supports the development of cytotoxic $CD8^+$ T cells that are required for killing virus infected host cells. In contrast, Th2 cells produce mainly interleukin 4 (IL-4), IL-5 and IL-13, favoring humoral responses (Figure 2). Because Th2 cells secrete the anti-inflammatory cytokine IL-10 as well as IL-4, these cells might also have regulatory functions as well as effector functions. They are distinguished from the later described regulatory T cells (see 1.1.2) by the production of large amounts of IL-4 and smaller amounts of IL-10 as well as the lack of transforming growth factor β (TGFβ) production.

Appropriate responses against micro-organisms require selective forms of specific immunity mediated by these functionally polarized subsets of effector Th cells. For example, Th1 cells are necessary for immune responses against intracellular bacteria and viruses, promoting the production of opsonizing and complement-binding antibodies by plasma cells. They also activate macrophages and neutrophils. Immune responses against helminthes are dependent on Th2 cells, switching antibody production towards neutralizing immunoglobulin G (IgG) antibodies as well as IgE and lead to activation of eosinophils [5]. Conversely, deregulated Th responses promote several pathological conditions as in the case of allergy and asthma, which are characterized by excessive Th2 responses [6]. Th1-dominated responses may be involved in the pathogenesis of human diseases

characterized by granuloma formation, such as organ-specific autoimmune disorders, Crohn's disease and atherosclerosis [7,8].

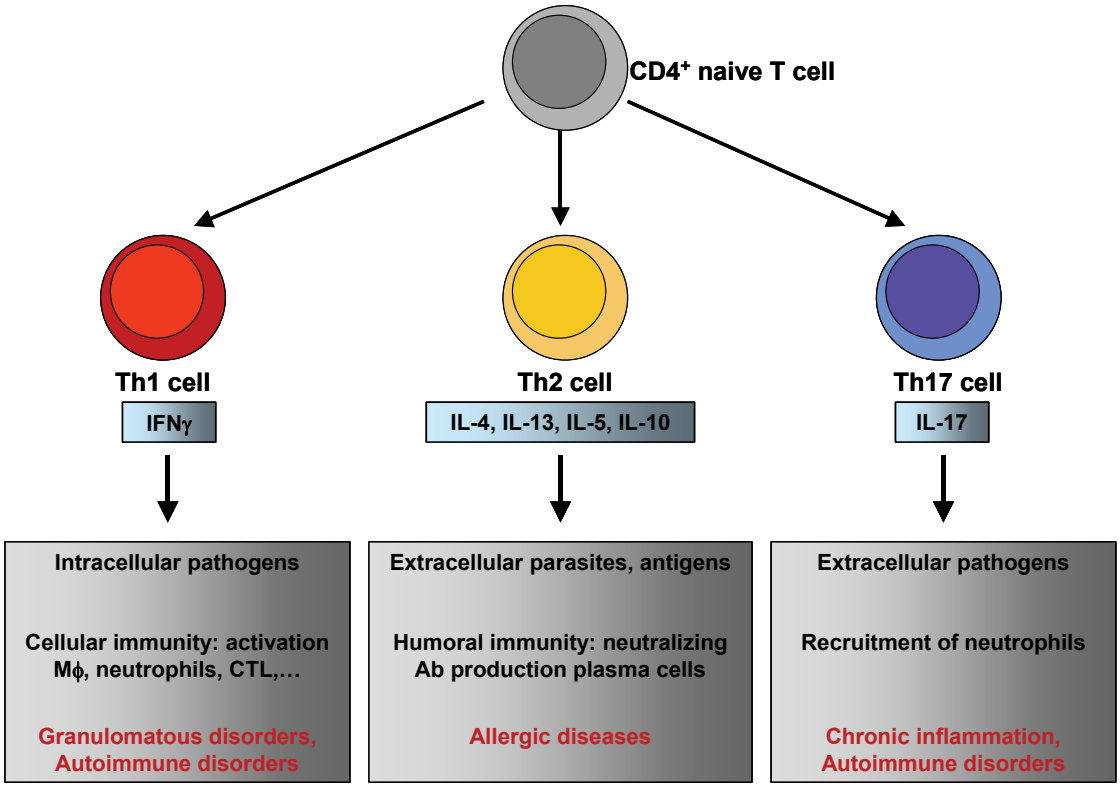

Figure 2 CD4$^+$ T helper subsets.
A CD4$^+$ naive T cell can develop into different effector cell subsets that can be recognized by their specific cytokine profile and are each responsible for a specific type of immune response. Next to pathogen elimination, deregulated T helper (Th) responses can also lead to several pathological conditions (in red). IFNγ: interferon γ; IL: interleukin; Mϕ: macrophages; CTL: cytotoxic T lymphocytes; Ab: antibodies.

Until recently the Th1 and Th2 subtypes were the only two CD4$^+$ effector cell types described since their discovery two decades ago. But a new type of CD4$^+$ effector cell has been added, the Th17 cells. These cells are characterized by their secretion of IL-17, IL-17F and IL-6, and have probably evolved to enhance host clearance of a range of pathogens distinct from those targeted by Th1 and Th2 (Figure 2). IL-17 is the founding member of a small family of cytokines that is generally thought to increase inflammation by recruiting other immune cells, like eosinophils, to peripheral tissues. The breakthrough leading to discovery of the Th17 lineage came from murine models of autoimmunity. The link with IL-12 and induction of Th1 responses in these models was questioned with the discovery that a new IL-12 family member, IL-23, shares with IL-12 the p40 subunit [9]. Given that key experimental data, linking

experimental autoimmune encephalitis and collagen induced arthritis to Th1 autoimmunity, were based on protection associated with manipulations that targeted the IL-12p40 subunit, the question arose whether protective effects might not involve inhibition of IL-23 instead of IL-12. After a range of studies it became indeed clear that IL-23 and not IL-12 is critically linked to autoimmunity in these models [10-12]. There is now plenty of evidence that IL-23, a cytokine produced by APCs, elicits production of IL-17 from a $CD4^+$ effector T cell that causes the harmful effects in autoimmune disorders [13-18].

1.1.2 Regulatory T cell subsets

Homeostasis in the immune system depends on a balance between the responses that control infection and the reciprocal responses that prevent inflammation and autoimmune diseases. It is now recognized that regulatory T cells (Treg cells) have a crucial role in suppressing immune responses to self-antigens and in preventing autoimmune diseases. Evidence is also emerging that Treg cells control immune responses to bacteria, viruses, parasites and fungi, preventing severe inflammation and collateral tissue damage.

The group of Treg cells can be further divided into naturally occurring (or constitutive) and inducible (or adaptive) Treg cells [3,19]. Both have complementary and overlapping functions in the control of immune responses. However the lineage relationship, if any, between these subsets remains to be defined.

The naturally occurring subset of $CD4^+$ Treg cells is characterized by its continuous expression of the α chain of the IL-2 receptor, CD25 [20]. They also express the transcription factor Foxp3 [21], the only phenotypic marker unique to this subset. Naturally occurring Treg cells represent 5-10% of the $CD4^+$ T cell pool in healthy adult humans and mice and are thought to be important for keeping auto-reactive T cells, which have escaped negative selection in the thymus, in check. Furthermore, they have been shown to induce tolerance to allo-antigens [22] and inhibit development of colitis in the T cell transfer SCID model [23,24].

A unique cytokine-production profile, rather than the expression of cell surface markers, has been used to define at least 2 populations of inducible Treg cells. These are not selected for in the thymus during T cell maturation, but are generated from naive T cells in the periphery during infection or can be induced by tolerance-inducing antigen administration regimens [3,25]. For experimental use, this cell population can also be derived from *in vitro* culture systems [26]. Both human

and mouse $CD4^+$ T cells, repeatedly stimulated in the presence of IL-10, differentiate into a new subset of $CD4^+$ T cells, termed Tr1 cells [26]. This subset is defined by its poor proliferative response, its ability to produce high levels of IL-10 and suppression of Th1 and Th2 responses *in vitro* and *in vivo* [26,27]. These cells may play a beneficial role in the tolerance to allo-antigens but also to allergens and self-antigens [28]. Weiner and co-workers showed that the induction of oral tolerance and the prevention of Th1-mediated autoimmune disease by feeding self-antigens were associated with the generation of TGFβ secreting T cells in the gut [29]. These T cells, which were distinct of Th2 cells in that they produce large amounts of TGFβ and varying amounts of IL-4 and IL-10, were named Th3. TGFβ promotes an isotype switch in B cells from IgM to IgA [30]. So Th3 cells help to explain the unique phenomena recognized in the gastro-intestinal tract; selective production of secretory IgA, suppression of IgG and IgM secretion and the induction of oral tolerance.

The exact mechanism by which natural and inducible T cells exert their suppressive effects is still subjected to speculations (Figure 3). Studies in animal models provide strong evidence for a role of cytokines in the effector function of Treg cells *in vivo*. Both IL-10 and TGFβ, produced by T cells, are important in providing protection from colitis in the SCID model [23,31]. Many studies have shown that using antibodies specific for IL-10 and TGFβ can reverse the suppression mediated by Tr1 and Th3 cells. Furthermore, mechanisms requiring cell-cell contact have been described for $CD4^+$ $CD25^+$ T cells [32], presumably via cytotoxic T lymphocyte associated antigen 4 (CTLA4) signaling [23], glucocorticoid-induced tumor necrosis factor receptor (GITR) [33] and/or membrane bound TGFβ [34,35]. Finally, it has been proposed that naturally occurring Treg cells might inhibit pathogenic effector T cells by competing for shared resources in the normal immune system [36]. So, although the mechanisms of suppression by Tr1 and Th3 cells seem to be mediated mainly by cytokines, $CD4^+CD25^+$ Treg cells might use many and as-yet-unidentified mechanisms to mediate suppression.

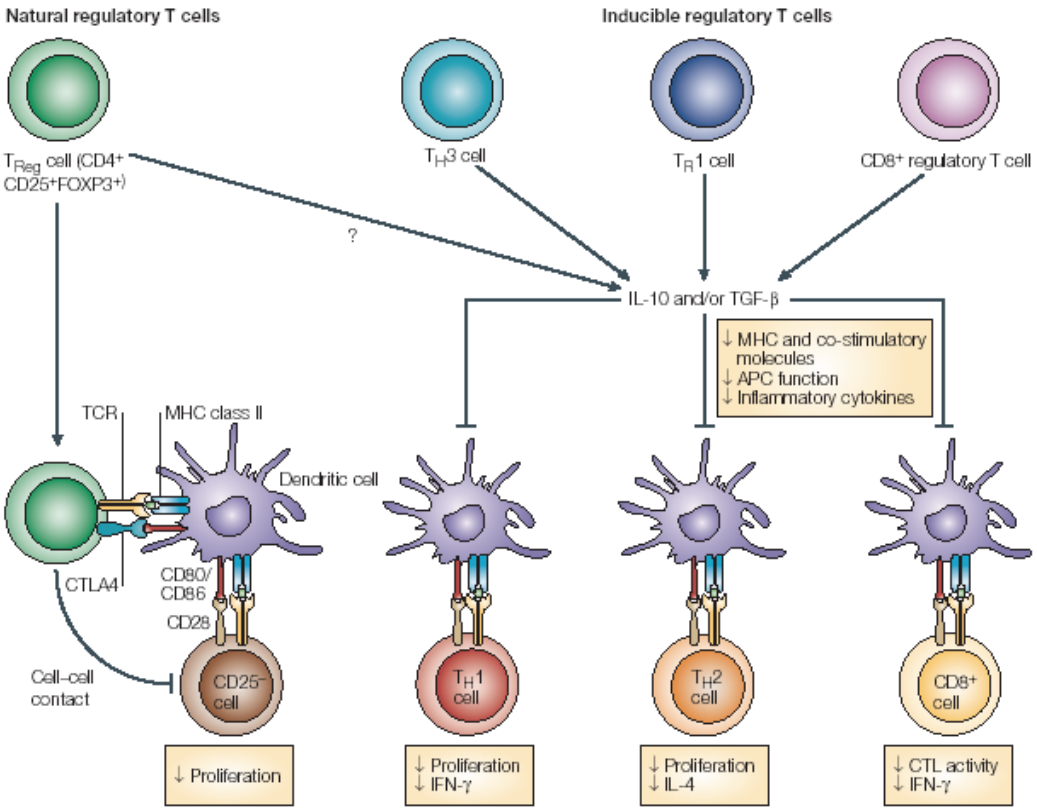

Figure 3 Targets of regulatory T cells and mechanisms of suppression.
$CD4^+$ $CD25^+$ $FOXP3^+$ (forkhead box P3) natural regulatory T cells (Treg cells) inhibit the proliferation of $CD25^-$ T cells. The mechanism of suppression seems to be multifactorial and includes cell–cell contact. $CD4^+$ $CD25^+$ Treg cells express cytotoxic T lymphocyte antigen 4 (CTLA4), which interacts with CD80 and/or CD86 on the surface of antigen-presenting cells (APCs) such as dendritic cells (DCs), and this interaction delivers a negative signal for T cell activation. There is also some evidence that secreted or cell surface TGF-β or secreted IL-10 might have a role in suppression mediated by natural Treg cells. Inducible populations of Treg cells, which include T regulatory 1 (Tr1) cells, T helper 3 (Th3) cells (and $CD8^+$ regulatory T cells), secrete IL-10 and/or TGF-β. These immunosuppressive cytokines inhibit the proliferation of and cytokine production by effector T cells, including Th1 cells, Th2 cells and $CD8^+$ cytotoxic T lymphocytes (CTLs), either directly or through their inhibitory influence on the maturation and activation of DCs or other APCs. The TGF-β producing Th3 cells are also important effector cells in mucosal immunity, inducing B cells to switch from immunoglobulin (Ig)M to an IgA isotype (not shown). TCR, T cell receptor. Source: [3]

1.2 Dendritic cells

DCs were first described in 1973 by Ralph Steinman [37] and are now recognized as crucial cells of the immune system, involved in T cell activation and differentiation. They are professional APCs that form a link between the adaptive and innate immune system. DCs originate from hematopoietic stem cells (HSCs) derived from the bone marrow. These precursors seed the blood and give rise to immature DCs,

which in the periphery act as sentinels, continuously sampling the environment for the presence of foreign antigens. 'Danger signals' induce activation of antigen-bearing DCs and migration into secondary lymphoid organs such as draining lymph nodes and the spleen. Mature DCs can initiate an immune response by efficiently presenting the sampled antigen to naive T cells. The interaction between the DC and a naive T cell will determine the outcome of T cell priming into either tolerance or immunity and the emergence of T cells carrying a Th1, Th2 or Th17 phenotype.

DCs were originally considered to be of myeloid origin and closely related to monocytes, macrophages and granulocytes. Recent studies, however, suggest that DCs can be generated along distinct developmental pathways and can originate from precursors of different hematopoietic lineages.

Furthermore, several DC types with different biological features have been identified in different tissues, including Langerhans cells in the epidermis, interstitial DCs in various tissues, thymic DCs and DC populations found in lymphoid organs. Differences in the tissue distribution, phenotype and function indicate the existence of a heterogeneous population of DCs. Another cell type, which belongs to the DC system, is the so-called plasmacytoid DC, originally identified as plasmacytoid T cells or plasmacytoid monocytes due to their morphological similarity to plasma cells and expression of certain T cell markers and MHC II molecules.

The development of DCs, their role in immunity and tolerance together with the existence of specific subsets and their functional differences will be described in this chapter, with specific emphasis on a population of DCs with special characteristics, namely the mucosal DCs.

1.2.1 Origin and differentiation of dendritic cells

DCs have been identified in lymphoid as well as non-lymphoid organs but their relationship and origin remain unclear and controversial. DCs were originally thought to be derived from myeloid precursors due to their functional, phenotypic and morphological similarities with macrophages and monocytes [38]. However, an initial study of DC reconstitution in bone marrow irradiated chimaeras showed that thymic DCs can be derived from $CD4^{low}$ early thymic precursors, which are devoid of myeloid reconstitution potential, and led to the concept that some DCs could be of lymphoid origin [39]. Furthermore, it has been shown that all DCs can develop from clonogenic bone marrow-derived common myeloid precursors (CMP) as well as from

clonogenic bone marrow-derived common lymphoid precursors (CLP) suggesting that all DCs can be generated along both myeloid and lymphoid pathways [40-42].

The fact that both CMP and CLP can generate all the DC populations suggests plasticity in developmental potentials of these early precursors [43]. It also suggests that the CMP and CLP that can give rise to DCs may share some common features. Moreover, it was demonstrated that most DC and plasmacytoid DC precursor activity was within the bone marrow hematopoietic precursors expressing FMS-like tyrosine kinase 3 (Flt3) [44,45]. The majority of mouse bone marrow CLP express high levels of Flt3 and these are the most efficient precursors of both DCs and plasmacytoid DCs [45]. In contrast, only a small proportion of the CMP express Flt3, yet the precursor activity for both DCs and plasmacytoid DCs resides in this minor $Flt3^+$ CMP fraction [45]. These findings demonstrate that the early precursors for all DC subtypes and for plasmacytoid DCs are within the bone marrow $Flt3^+$ precursor populations, regardless of their lymphoid or myeloid lineage orientation, and Flt3 signaling is required for the development of both DCs and plasmacytoid DCs.

However, it remains unclear whether DCs are generated from CMP and CLP within the bone marrow from where they migrate to the periphery, or alternatively, from circulating DC precursors that home to the peripheral lymphoid organs where they differentiate. Some recent reports support the concept that precursor DCs are recruited to the lymph nodes during infection where they differentiate into DCs [46-48].

A definition of DC-committed precursors has remained elusive both in humans and mice. Most articles on this subject either describe precursor populations with a differentiation potential not restricted to the DC lineage or describe immediate precursors of defined DC subsets such as plasmacytoid cells or monocytes, with limited DC differentiation potential. However, a $CD11c^+MHC\ II^-$ DC restricted precursor population which can fully reconstitute splenic $CD8\alpha^-$, $CD8\alpha^+$ and plasmacytoid $B220^+$ DC subpopulations and is devoid of lymphoid- or myeloid-differentiation potential, has recently been identified in mouse blood [46] (Figure 4). Whether this pre-DC is a common precursor for all DC subpopulations or an environmentally regulated DC precursor that is involved in the generation of only certain DC subpopulations, remains to be addressed.

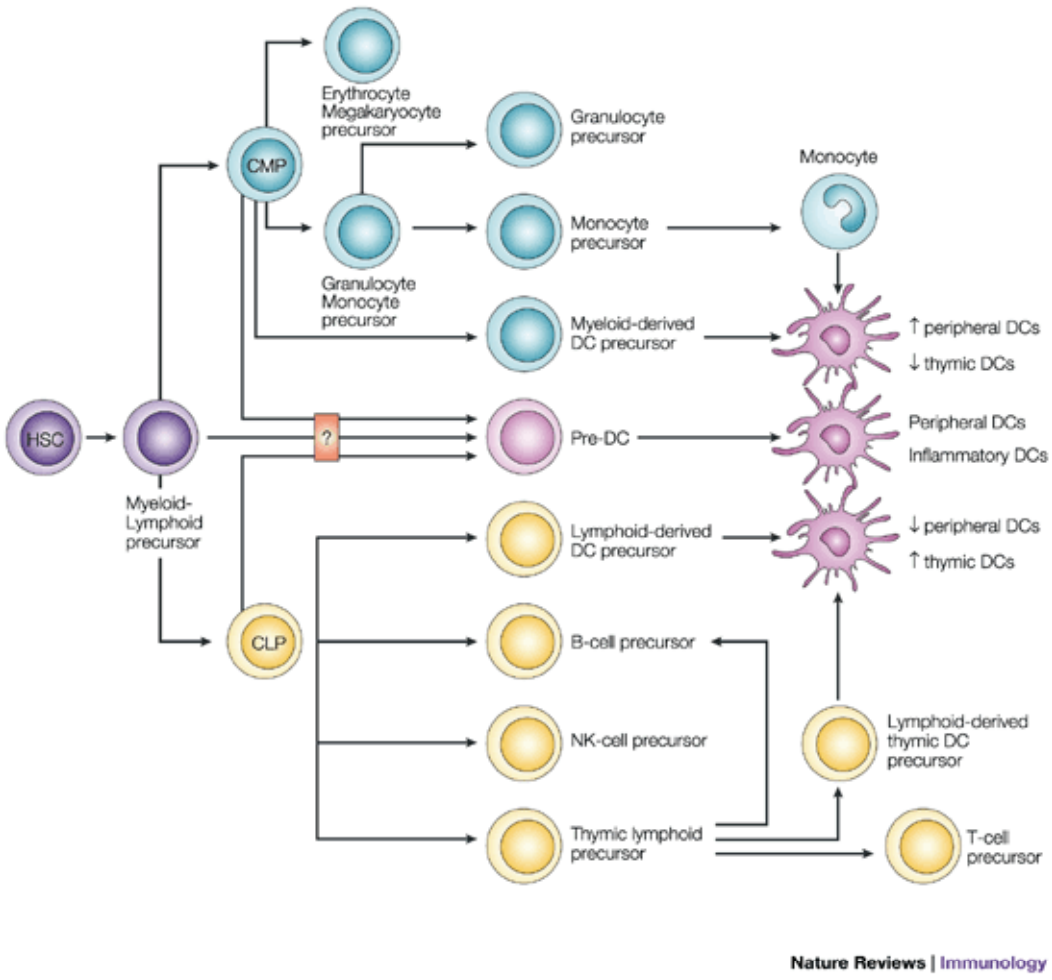

Figure 4 Theoretical model of the developmental origin of mouse dendritic cells.
The differentiation of dendritic cells (DCs) — including $CD8\alpha^-$, $CD8\alpha^+$, plasmacytoid $B220^+$ DCs and Langerhans cells — has been proposed to proceed directly through myeloid- and lymphoid-derived DC precursors [40], and through circulating common DC precursors (pre-DCs) [46]. On the basis of the relative DC differentiation potential and the absolute number of common lymphoid progenitors (CLP) and common myeloid progenitors (CMP), it has been established that thymic DC differentiation results from an equal contribution of both precursors, whereas peripheral DCs are derived mainly from CMP [41,42]. Myeloid-derived DC precursors have been shown to be derived from the fraction of CMP that express fms-related tyrosine kinase 3 ($Flt3^+$) [45]. Lymphoid-derived DC precursors could derive from CLP that are located in the bone marrow or from thymic lymphoid precursors, which give rise to T cells, thymic DCs and B cells. Pre-DCs and monocytes have been proposed to be involved in the generation of DCs after recruitment to reactive sites [46]. The origin of pre-DCs from myeloid and/or lymphoid progenitors, and their relative contribution to the generation of DCs in relation to CMP and CLP under steady state conditions have also to be determined. HSC: hematopoietic stem cell; NK: natural killer. Source: [49]

1.2.2 DC subsets

As mentioned before, a large variety of DC subsets have been described in lymphoid and non-lymphoid organs of humans as well as mouse. The distinction of these subsets has been made, depending on both functional and phenotypical differences but as explained before both the origin and development of these different subsets remain under investigation.

Several factors have made this field complex. First, considerable heterogeneity exist in the antibody panels used to characterize DC subsets, especially in the mouse where the ready availability of tissues and reagents has led to numerous studies. In humans, the lack of CD8α expression by any subset makes comparison with mouse DC subsets difficult [50]. Second, it is still a matter of debate whether the different subsets described represent different activation states of a single lineage or whether the distinguishable DC phenotypes are all products of separate developmental lineages. Reality probably is a mixture of these two models and furthermore a large degree of functional plasticity is a general feature of all DCs.

Murine subsets

Despite the use of different antibody panels, several common markers have been used in most studies so far, enabling some comparison between the described subsets and have lead to the identification of six distinct populations (Figure 5), some of which are only present in specific tissues.

Conventional DCs are characterized by the expression of high levels of CD11c. In lymphoid tissues they can be divided into CD8α^+ and CD8α^- subpopulations, which have been extensively studied over the past few years [51]. These subsets were previously known as lymphoid and myeloid DCs respectively. In the meantime it has been recognized that myeloid committed cells as well as lymphoid committed cells can give rise to both lymphoid (CD8α^+) and myeloid (CD8α^-) subsets. Moreover, until now it is still not clear whether CD8α expression on DCs indeed means an independent DC development pathway, or if it is merely a DC activation marker. Nevertheless, the terms myeloid and lymphoid DC subsets are still used in connection to their expression of myeloid versus lymphoid markers (CD11b versus CD8α) [49].

In the spleen, three conventional DC subsets can be found (Figure 5). A CD8α positive (lymphoid) subset is present in the T cell area with the following phenotype: CD8α^+ CD11b$^-$ CD4$^-$ CD205$^+$. This is also the dominant subtype of thymic DCs.

Furthermore two CD8α negative (myeloid) subsets can be defined, with differences in their CD4 expression: $CD8\alpha^- CD11b^+ CD4^+ CD205^-$ and $CD8\alpha^- CD11b^+ CD4^- CD205^-$. Both subsets are found in the marginal zones but migrate into T cell zones on stimulation with microbial products [52-54]. The functional relevance of the differential CD4 expression between those 2 subtypes remains controversial as it also is uncertain if $CD8\alpha^- CD4^+$ and $CD8\alpha^- CD4^-$ are two developmentally and functionally independent DC subsets or if the $CD8\alpha^- CD4^-$ subset might constitute a more activated or differentiated form of the $CD8\alpha^- CD4^+$ DC [51,55,56].

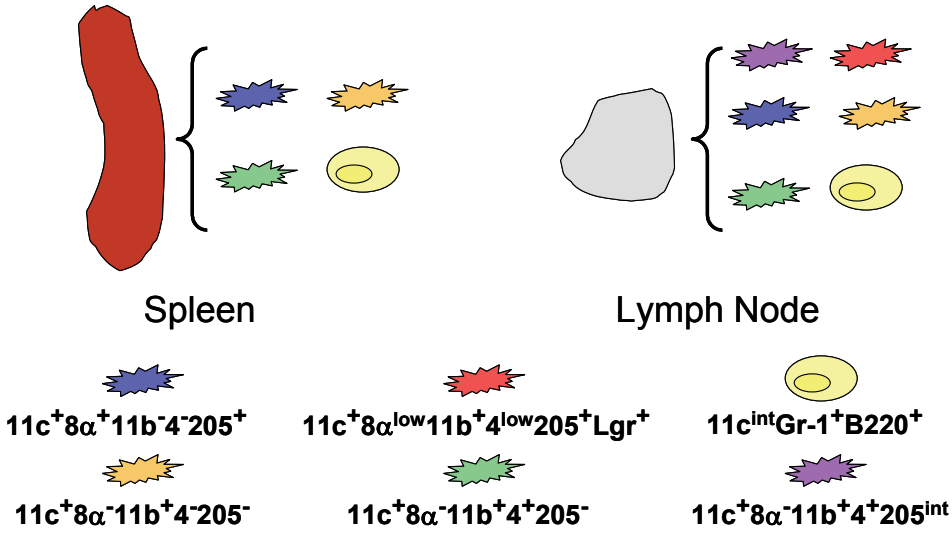

Figure 5 Murine dendritic cell subsets in spleen and lymph nodes.
Differentiation markers: (CD)11c; (CD)8α; (CD)11b; (CD)4; (CD)205; Lgr: langerin; Int: intermediate.

Plasmacytoid $B220^+$ cells are a fourth subtype of DCs present in mouse spleen. Plasmacytoid DC precursors have been first described in humans as cells with a plasma cell-like morphology in T cell areas of lymphoid organs. They were recognized not to be classical plasma cells, but their true function remained unknown. As a consequence, they were called plasmacytoid monocytes or plasmacytoid T cells [57]. Recently, it has been discovered that plasmacytoid DC precursors produce massive amounts of type I IFN upon activation with viruses [58,59]. Moreover, once activated, these cells develop into genuine DCs that can potentially stimulate T cells [58]. Therefore they were renamed plasmacytoid DC

precursors. The precursor cell is characterized by the expression of low levels of CD11c and MHC II. In addition they stain positive for Ly-6G/C (Gr-1) and B220 [60].

Lymph nodes (LN) have been shown to harbor the same four DC subsets as the spleen does. Moreover, LN contain additional DC subtypes (Figure 5). The mesenteric LN, which belong to the intestine-draining LN, harbor one additional DC subset, whereas all skin draining LN harbor two additional DC subsets. The $CD8\alpha^-$ $CD11b^+$ $CD4^-$ subset with moderate expression of CD205, in contrast to the spleen $CD8\alpha^-$ DC, is found in all LN. These cells are believed to be the mature form of tissue interstitial DCs that gain access to the LN through the lymphatics. And, only present in skin draining LN, expressing high levels of langerin, is the mature form of Langerhans cells, $CD8\alpha^{low}$ $CD11b^+$ $CD4^{low}$ $CD205^+$ [61].

In addition to these main DC subpopulations, other DC subsets have been described in specific organs of the mouse, including the gut, lung, heart and kidney. The available functional and phenotypic data regarding these DCs have not yet allowed them to be ascribed to any of the DC subpopulations listed in Figure 5.

Human subsets

Relatively few studies have been performed on human DCs freshly isolated from tissues. The only readily available source of DCs is blood and although heterogenous in expression of markers, many of the differences in human blood DCs reflect other maturation or activation states rather than separate subsets. Therefore most of the data acquired are derived from several *in vitro* culture systems. As mentioned before, the lack of $CD8\alpha$ by human DCs makes comparison with mouse DC subsets difficult [50].

Four different mature subsets have been identified so far. Analogous to the murine homologue, Langerhans DCs have been described which express, in addition to CD11c, also langerin, E-cadherin and contain Birbeck granules in their cytoplasm. Interstitial, or 'dermal' DCs, are a second lineage which are $CD11c^+$, and can be further identified by CD68 and the coagulation factor XIIIa. Blood monocytes, termed pDC1, are the precursor cells for monocyte-derived DCs. After six days in the presence of granulocyte/macrophage-colony-stimulating factor (GM-CSF) and IL-4 they will generate DCs, called DC1 [62]. These cells are positive for CD11c and upon maturation induced by pro-inflammatory cytokines such as tumor necrosis factor (TNF) or microbial products (e.g. LPS), acquire a $CD14^-$ $CD83^+$ $CD86^+$ MHC II^{hi} phenotype. The final subset described in humans is the type I IFN producing plasmacytoid DC, termed pDC2. This subset of DCs was recognized well before their

murine counterpart, initially by their plasma-cell-like morphology and their unique surface phenotype. Typical for these cells are low levels of CD11c expression and the unique expression of IL-3 receptor α [63,64].

1.2.3 DC activation, migration and T cell activation

Immature DCs are spread throughout the periphery, especially at portals of pathogen entry such as the skin, airway and gastro-intestinal mucosa. In this immature state DCs are continuously sampling the microenvironment via three major mechanisms: receptor-mediated endocytosis, macropinocytosis and phagocytosis. Receptor-mediated uptake is accomplished via a myriad of receptors, including C-type lectin receptors (such as langerin, DC-SIGN, dectin, CD205), the mannose receptor for glycoproteins and Fc receptor for immune complexes [65,66].

Upon exposure to a pathogen, maturation of the immature DC is triggered. Maturation comprises a multi-step process that enables the DC to initiate both innate and adaptive effector cells (Figure 6). Initially DCs secrete cytokines and chemokines that recruit other innate effector cells, such as neutrophils and macrophages, to the site of infection. These phagocytic cells exert potent anti-microbial activities and control the pathogen. Toll-like receptor (TLR) mediated signaling leads to down-regulation of the chemokine receptor CCR6 and up-regulation of CCR7 [67] together with the enhancement of adhesion molecules, which allow the DC to enter the lymphatic system and govern the migration to the draining lymph nodes. During this transition phase DCs lose their endocytic capacity so that only antigens engulfed at the site of infection are processed and presented. In addition the DCs dramatically increase their levels of antigen-loaded MHC I and MHC II expression [68] together with their expression of co-stimulatory molecules, notably CD80 (B7.1) and CD86 (B7.2) but also CD40, OX40 ligand, 4-1BB ligand and SLAM.

In the lymphoid organs these mature DCs migrate to T cell areas and act as professional APCs by providing signal 1 (TCR crosslinking) and signal 2 (co-stimulation) to pathogen-specific, naive T cells, thereby inducing their activation and clonal expansion. Whereas signal 1 determines the antigen-specificity of the response, signal 2 is required to prevent T cell anergy (see also Figure 7). After expansion these activated T cells migrate back to the site of inflammation, clear the infection and give rise to memory. The character of the immune response is determined by the nature of the activated effector cells and the role of DCs herein will be discussed in the next section (1.3).

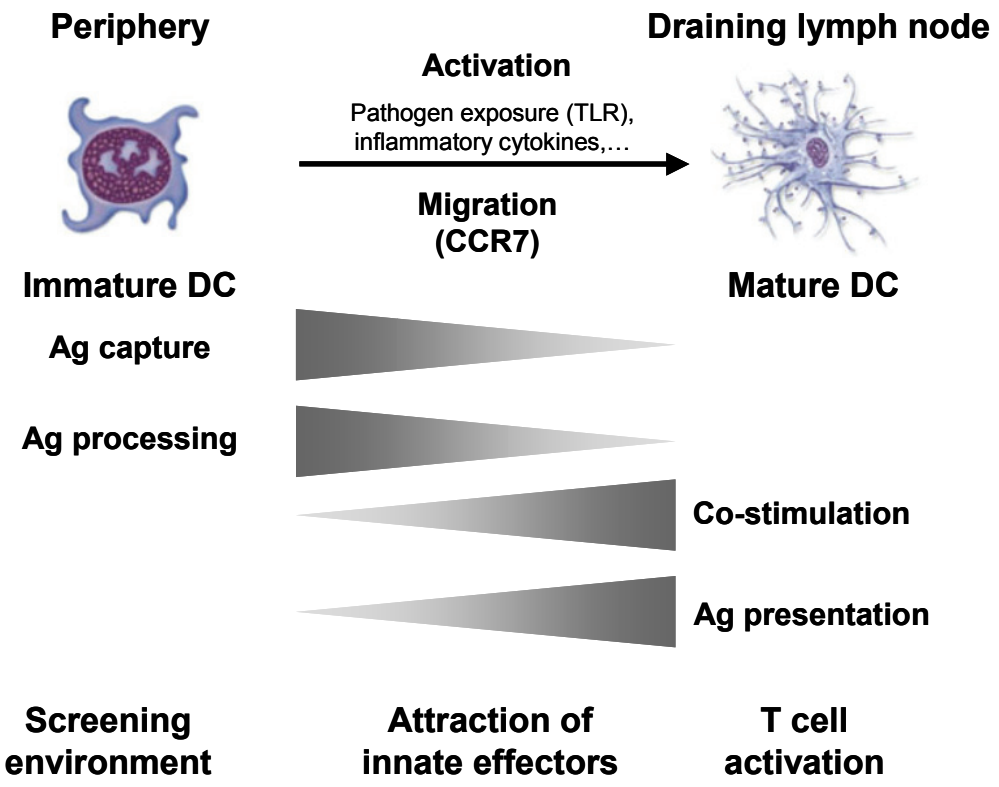

Figure 6 Pathogen triggered maturation of a peripheral dendritic cell (DC).
Upon exposure to a pathogen in the periphery, immature DCs are activated and migrate to the draining lymph nodes, thereby down-regulating their antigen (Ag) processing machinery but increasing their T cell activation capacities. Upon activation, the DC also secretes chemokines and cytokines to attract other cells of the innate immune system to the site of infection. TLR: Toll-like receptor, CCR7: chemokine C-C receptor 7

1.3 Polarization of the immune response

It has been well established that DCs have a pivotal role in the differentiation of naive $CD4^+$ T cells into Th1, Th2 or Th17 cells and evidence is accumulating that DCs are also able to induce T cell anergy or direct the development of Treg cells [69,70] (see 1.4). The final outcome of the differentiation process and thus the character of the immune response is determined by several factors, with the type of pathogen, the subset of DCs stimulated and the cytokine milieu at sites of inflammation being the major determinants.

1.3.1 DC subsets and polarization of the immune response

The existence of phenotypically distinct subsets of DCs that are located in distinct microenvironments raises the question whether they too, like distinct subsets of lymphocytes, may have evolved to serve distinct functions. Traditionally it was indeed

thought that the different human DC subsets had fixed instructive capacities, hence the DC1/DC2 nomenclature [71]. This view implies that myeloid DCs (DC1) induce IFNγ producing Th1 cells and thus pro-inflammatory responses whereas plasmacytoid DCs (DC2) mediate the more tolerogenic Th2 polarization. Studies on CD8α^+ (or formerly lymphoid-derived) and CD8α^- DCs (or formerly myeloid-derived) populations in mice also showed Th1 and Th2 priming capacities respectively [72,73]. In addition, it has been shown that CD8α^- DCs induce Th17 cells much more efficiently than other subsets in experimental autoimmune encephalomyelitis [74].

Nevertheless, in recent years it has become increasingly clear that DC subsets may not have an intrinsic capacity to direct either Th1 or Th2 cell development, but rather might be modified by environmental factors [75-77]. Colonna and co-workers showed that human blood plasmacytoid DCs stimulated with CD40 ligand or influenza virus matured and became potent stimulators of a Th1 response, driven by type I IFN and IL-12 production [77]. Another study showed that both mouse conventional and plasmacytoid DCs generated from bone marrow, as well as lymphoid tissue DCs can direct Th1 or Th2 responses depending on the dose of antigen presented [75].

All together the conclusion from these seemingly contradictory findings and theories might be that developmentally preprogrammed functions can be fine-tuned by environmental factors, leading to some degree of functional plasticity of the different DC subsets.

In spite of this plasticity in polarizing T cells, it is clear that, following stimulation, each DC subset secretes a lineage specific array of cytokines [78] and chemokines [79]. Stimulating human plasmacytoid DCs and myeloid DCs with a TLR7 ligand demonstrated the association of specific cytokine profiles with DC lineages. Although both subsets undergo similar phenotypic changes upon maturation, they show a different pattern of cytokine secretion. Specifically, myeloid DCs release IL-12 but no type I IFN and plasmacytoid DCs produce type I IFN but no IL-12. However, both subsets promoted the differentiation of T cells into Th1 effectors [78], indicating that the character of an adaptive immune response is ultimately determined by which PAMPs trigger immature DCs.

1.3.2 PAMPs, TLRs and polarization of the immune response

How do different DC subsets discriminate between pathogens when simultaneously present at the site of infection and ensure a proper type of immune response? The recognition of TLRs as main PRRs partially solved this question. The human myeloid

DC1 subset expresses all 10 TLRs with the exception of TLR9 and – according to some publications - TLR7 and therefore recognizes an abundant variety of PAMPs [80-82]. The plasmacytoid subset DC2 possesses a repertoire of TLRs (TLR1, TLR6, TLR7 and TLR9), which complements the TLRs expressed by DC1, enabling the two subsets of DCs to respond to distinct sets of PAMPs [80-82]. In mice the distribution pattern of TLR members shows more overlap between different subsets. While all subsets express TLR1, 2, 4, 6, 8 and 9, murine plasmacytoid DCs do not express TLR3 and CD8α^+ DCs lack TLR5 and TLR7 expression [83].

TLRs may be expressed as homodimers or heterodimers (TLR2 plus TLR1 or TLR6) and have broad specificity for conserved molecular structures of pathogens [84,85]. LPS from *Escherichia coli* signals through TLR4, whereas TLR2 appears to have several ligands including peptidoglycan from gram-positive bacteria, lipoproteins from *Mycobacterium tuberculosis* and zymosan from yeast as well as LPS from *Porphyromas gingivalis*. TLR3 recognizes dsRNA, TLR5 recognizes flagellin, TLR7 can be triggered by single stranded RNA and may thus be important for viral recognition; TLR9 recognizes certain types of CpG rich DNA found in some viruses and bacteria. TLR3, 7, 8 and 9 are located in the endosomes and require an acidic environment for activation. TLR1, 2, 4, 5 and 6 are expressed on the cell surface. The former TLRs are thus ideal for the detection of internalized viruses and bacteria whereas the latter recognize bacterial products.

A given DC population will only respond to the pathogen for which they have the appropriate TLRs. Transcriptional profiling by microarray analysis of human monocyte derived DCs treated with different classes of pathogens (bacteria, viruses and fungi) revealed the existence of common and pathogen specific genes [86]. Common genes respond to the presence of all pathogens and express proteins that are used in responses against all microbes, such as inflammatory cytokines and adhesion molecules necessary for the migration of maturing DCs to the lymphoid organs. In addition, distinct sets of genes are regulated in response to a specific pathogen, allowing for the generation of an adaptive immune response directed towards elimination of the eliciting pathogen. For example, activation of DCs with virus-derived dsRNA, a TLR3 ligand, but not with bacterial LPS, the prototypic TLR4 ligand, up-regulates the level of type I IFN, essential for anti-viral responses, as well as pro-apoptotic genes that may induce early cell death of infected cells.

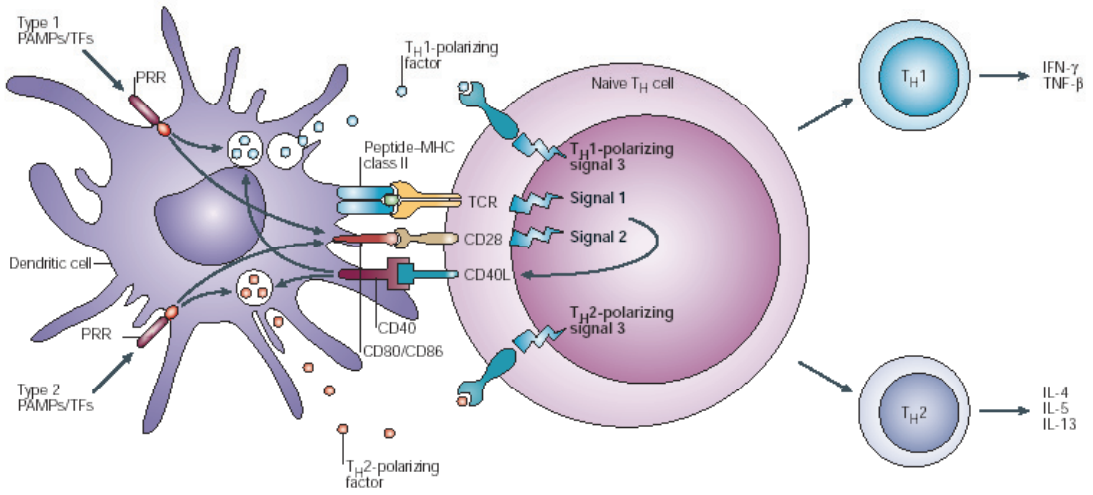

Figure 7 T cell stimulation and T helper 1 (Th1)/Th2-cell polarization
Signal 1 is the antigen-specific signal that is mediated through T cell receptor (TCR) triggering by MHC II-associated peptides processed from pathogens after internalization through specialized pattern recognition receptors (PRRs). Signal 2 is the co-stimulatory signal, mainly mediated by triggering of CD28 by CD80 and CD86 that are expressed by dendritic cells (DCs) after ligation of PRRs, such as Toll-like receptors (TLRs) that are specialized to sense infection through recognition of pathogen-associated molecular patterns (PAMPs). Signal 3 is the polarizing signal that is mediated by various soluble or membrane-bound factors (see 1.3.4), such as interleukin-12 (IL-12) and CC-chemokine ligand 2 (CCL2), that promote the development of Th1 or Th2 cells, respectively. The nature of signal 3 depends on the activation of particular PRRs by PAMPs but can also be influenced by the DC microenvironment (tissue factors: TFs) as discussed in 1.3.3. Type 1 and type 2 PAMPs can be defined as those that selectively prime DCs for the production of high levels of Th1 cell-polarizing or Th2 cell-polarizing factors. Whereas, the profile of T cell-polarizing factors is primed by recognition of PAMPs, optimal expression of this profile often requires feedback stimulation by CD40 ligand (CD40L) expressed by T cells after activation by signals 1 and 2. IFN-γ: interferon-γ; TNF-β: tumor necrosis factor-β. Source: [70]

These findings have been extended by several groups, showing that although PAMPs that stimulate different TLRs induce similar changes in surface phenotype, they often induce distinct patterns of cytokines, resulting in a Th1/Th2 polarization assumed to be the most appropriate attack towards the specific pathogen (signal 3 in Figure 7). For example LPS, CpG DNA, Poly(I:C) and TLR7 agonists induce IL-12p70 and/or IFNα and stimulate Th1 responses [55,78,87-90]. In contrast, other findings suggest that certain TLR ligands also mediate Th2 responses [88,91-97]. For instance, the TLR2 ligand zymosan promotes the release of IL-10 and the generation of Th2 effector cells in mouse splenic DCs, an effect also observed when using intact yeast [95,96]. *In vivo* studies also revealed that LPS from *P. gingivalis* causes DCs to prime a Th2 response [88,97], contrasting with the Th1 polarizing activities of DCs activated with *E. coli* LPS.

Not much is known concerning TLR ligands that mediate the induction of a Th17 response. Nevertheless it was shown in both human monocyte-derived and murine bone marrow-derived DCs that the TLR2 agonist peptidoglycan is a strong inducer of IL-23p19 mRNA [98,99]. IL-23 secreted by DCs is considered to be important in the induction of Th17 responses.

1.3.3 Conditioning of DCs in the peripheral microenvironment

The specific anatomical compartment where an immature DC resides and encounters a pathogen also profoundly impacts the character of the immune response generated by the DC after it has migrated to the lymph nodes. $CD11c^+$ DCs isolated from mouse mucosa such as Peyer's patches or respiratory tracts preferentially induce Th2 differentiation [100,101]. In contrast $CD11c^+$ DCs isolated from mouse spleen preferentially induce Th1 differentiation. DCs derived from bone marrow precursors or from liver precursors also displayed different effector functions in polarizing Th cells. While liver-derived DCs produced high IL-10 and induced allogeneic T cells to undergo Th2 differentiation, bone marrow-derived DCs produced low IL-10 and induced allogeneic T cells to undergo Th1 differentiation during primary mixed leukocyte reaction cultures or in allogeneic recipient mice after DC transfer [102].

A recent report showed that $CD8\alpha^+$ and $CD8\alpha^-$ subsets from the liver secrete similar amounts of IL-12 when stimulated with CD40 ligand, whereas in the spleen, only the $CD8\alpha^+$ subset secretes IL-12 [103]. As the two subsets are located in different areas of the spleen ($CD8\alpha^-$ DCs in the marginal zone and $CD8\alpha^+$ DCs in the T cell area [89]) it was hypothesized that these distinct microenvironments might differentially condition DCs to release IL-12 [103]. The functional differences between DCs originating from different tissues may thus result from differences in tissue cytokine microenvironments as well as in the lineage origin of different tissue DCs (Figure 8).

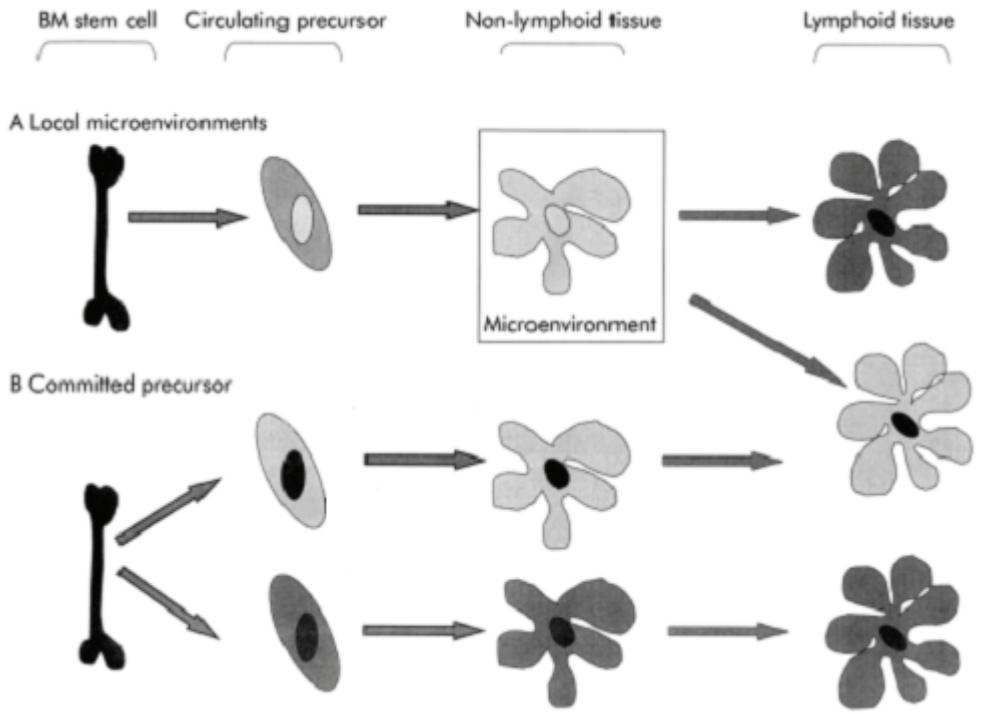

Figure 8 The origin of tissue specific dendritic cell functions.
DCs from mouse mucosal sites or liver (illustrated in light grey) and spleen (dark grey) have different T cell polarizing capacities after migration to the draining lymph node. These tissue specific functions of dendritic cells might result from the action of local microenvironmental factors on a common precursor (A) or from migration of distinct functionally committed precursors to the different tissues (B) or a combination of both mechanisms. BM: Bone marrow. Source: [104] with adaptations

1.3.4 DC-derived effector molecules polarizing the immune response

The conditions under which DCs are primed (microenvironment and pathogen derived compounds) thus strongly influence the Th balance. The T cell polarizing signal expressed by these primed DCs can be either cytokines or cell surface molecules. Some but not all of these DC-derived molecules have been identified.

One of the best documented is IL-12p70, a powerful inducer of effector Th1 cells [105]. Recently IL-23 and IL-27, which are closely related to IL-12, have also been implicated to drive Th1 differentiation [105,106]. IL-18 has also a Th1-promoting ability, amplifying the effects of IL-12, but not capable of Th1 differentiation [105]. Another group of important Th1-driving factors in humans, but not in mice, are type I IFNs (e.g IFNα/β). In both man and mice, type I IFN production is strongly associated with virus infection and may be crucial in the development of protective Th1 immunity

as they can induce IFNγ secretion from $CD4^+$ cells in humans [107]. Several cell surface molecules have also been shown to have a Th1-polarizing effect, among them intercellular adhesion molecule 1 (ICAM-1) and the Notch family ligand Delta [108,109].

A number of molecules implicated in Th2 differentiation have been identified up till now but no clear-cut picture has emerged yet. Some recently identified secreted mediators with Th2 inducing properties include the chemokine CCL2 (MCP-1), IL-25 and IL-6 [110,111]. IL-18 has been reported to be able to drive Th2 development in the absence of IL-12 [112]. Cell surface molecules with Th2-driving properties are OX40 ligand and Jagged family members [70,109].

Not much is known concerning DC-derived factors inducing the recently discovered Th17 lineage. The idea that IL-23 is the initiator of Th17 differentiation has been put to rest, although there is definitely a role for this cytokine in expanding and maintaining the Th17 population. TGFβ has recently been found to be a potent inducer of Th17 cells [113-115]. Analysis of DC-derived factors that act in concert with TGFβ led to the identification of IL-6, elicited by TLR-mediated signaling, as a critical cofactor. The combination of IL-6 in conjunction with TGFβ favors Th17 cells but inhibits the expression of Foxp3, a gene transcription factor essential for Treg cell differentiation [113,115]. Furthermore, both IL-1β and TNF were found to amplify the Th17 response but were not able to substitute for TGFβ or IL-6.

1.4 Dendritic cells and tolerance

The induction of antigen specific tolerance in the periphery is critical for the prevention of autoimmunity and maintenance of immune homeostasis. The role of DCs herein includes induction of T cell apoptosis, T cell anergy and generation of Treg cells.

In order to explain the role of DCs in the establishment of peripheral tolerance, the existence of “steady-state” migrating DCs has been suggested. These DCs migrate to the regional lymph nodes in the absence of infection and inflammation, loaded with tissue antigens. In this way self-antigens are transported to the lymph nodes and presented to autoreactive T cells for the induction of peripheral tolerance [116].

1.4.1 Induction of T cell apoptosis

DCs in the steady-state are capable of inducing apoptosis in T cells for the maintenance of immune homeostasis. This concept was derived from observations of

a Fas-ligand expressing DC subset, resident in murine lymph nodes. These DCs are able to mediate apoptosis in allogeneic $CD4^+$ T cells in a mixed leukocyte reaction [117]. *In vivo*, this seemed to be a mechanism to remove potentially self-reactive T cells from the periphery [118]. Treg cells appear to be rather resistant to Fas-ligand-mediated apoptosis [119] indicating that both DCs and Treg cells can act together to terminate a T cell response. Another apoptosis-inducing ligand, TRAIL, is expressed by human $CD11c^+$ blood DCs after stimulation with IFNγ or TNF. TRAIL confers the ability to kill TRAIL sensitive targets, including activated T cells [120].

1.4.2 Induction of T cell anergy

T cell anergy is yet another mechanism by which auto-reactive T cells are neutralized in the periphery. When T cells encounter antigen (self peptides) presented by resting APCs, not only do they fail to become optimally activated but they are also hyporesponsive upon rechallenge; they are anergic. The induction of T cell anergy by DCs might be due to incomplete maturation, blockade of the B7 family of co-stimulatory molecules or the influence of specific non-inflammatory molecules such as IL-10 and TGFβ.

The induction of T cell anergy by immunosuppressive mediators might be a mechanism to inhibit auto-reactive T cell responses by converting immature DCs into tolerance-inducing cells. It has been demonstrated that immature IL-10-treated human DCs release reduced amounts of inflammatory cytokines such as IL-1β, IL-6 and TNF and do not produce IL-12 [121-123]. As a result such DCs are not only less efficient at stimulating Th1 responses but can induce a state of antigen-specific tolerance due to the induction of T cell anergy [123,124]. The anergic T cells induced by IL-10-modulated DCs are characterized by inhibited antigen-specific proliferation, a reduced production of IL-2 and IFNγ, and a down-regulated expression of CD25 [123,125]. Induction requires direct cell–cell contact between T cells and DCs as well as soluble factors produced by the IL-10-treated DCs.

One way for DCs to promote and maintain anergy is through the presence of the cell surface receptor “Programmed Death Ligand 1” (PD-L1) or B7-H1 [126]. This inhibitory signaling molecule is overruled on mature DCs that express high levels of MHC II and co-stimulatory molecules. But when those signals are low, as is the case for IL-10-treated DCs, B7-H1 signaling may become critical. The finding that B7-H1 signaling might enhance IL-10 production in T cells, but inhibits IL-2 production, could explain how B7-H1 controls T cell anergy [126].

1.4.3 Induction of regulatory T cells

Finally, DCs may also control peripheral tolerance by inducing the differentiation of Treg cells (see 1.1.2). As discussed before, the activation status of the DC and local cytokine environment appear to play an important role in the outcome of T cell responses. This is also true for their function in Treg cell differentiation. Although T cells induced by immature DCs are anergic rather than regulatory, there is some evidence that immature DCs can selectively activate $CD4^+CD25^+$ Treg cells [127]. In contrast to the anergic T cells, the suppressive activity of these Treg cells seems to be antigen non-specific and can be partially inhibited by the addition of exogenous IL-2 *in vitro* [127]. Furthermore, it has been shown that DCs after treatment with vitamin D3 display an immature phenotype and are prone to induce $CD4^+CD25^+$ Treg cells, which are able to mediate transplantation tolerance [128].

DCs that direct the induction of IL-10 secreting Tr1 cells seem to have an intermediate phenotype, including increased expression of MHC II molecules and CD86 but low levels of expression of CD40 and ICAM-1 [129,130]. This is supported by studies showing that DCs lacking surface expression of CD40 can suppress a primed immune response and induce IL-10-secreting $CD4^+$ Treg cells [130]. Furthermore, the interaction between ICAM-1 and leukocyte function-associated antigen 1 (LFA-1) is thought to promote the induction of Th1 cells independently of IL-12 [108]. So, DCs in which CD40 and ICAM-1 expression is suppressed but CD80 and CD86 expression is increased might promote the induction of Tr1 cells but block the differentiation of Th1 cells.

Wakkach and colleagues [131] recently identified a subset of dendritic cells in the spleen and lymph node that appear to be a natural tolerizing dendritic cell subset. The cells have plasmacytoid morphology and remain immature even after *in vitro* activation with LPS or CpG, they have an unusual cell-surface phenotype ($CD11c^{low}CD45RB^{high}$), and they produce large amounts of IL-10 when stimulated. These cells are capable of directly generating Tr1 cells *in vitro* and *in vivo*, and may represent a naturally occurring dendritic cell subset involved in eliciting tolerance *in vivo*.

Th3 cells are a unique mucosal T cell subset with down-regulatory properties for Th1 and other immune cells [29]. In contrast to the other Th cell subsets, Th3 cells provide help for IgA production and primarily secrete TGFβ. The mechanism by which Th3 cells are induced remains unknown. It is also not known to what degree

the generation of these regulatory cells is related to unique APCs in the gut, the cytokine milieu, or other factors.

Many results support the concept that DCs are the inducers of Treg cells under certain circumstances. On the other hand, recent results imply that Treg cells also affect DC functions. For instance it has been shown that a subset of $CD8^+$ $CD28^-$ regulatory T cells induces up-regulation in DCs of the inhibitory receptors ILT3 and ILT4, which are responsible for DC-induced hyporesponsiveness of alloreactive $CD4^+$ T cells [132]. Similarly, anergic $CD4^+$ T cells have been shown to modify APCs that subsequently induce tolerance in naive T cells [133]. *In vitro*, human $CD4^+$ $CD25^+$ Treg cells also strongly suppressed TLR-triggered DC maturation, as judged by the blocking of co-stimulatory molecule up-regulation and the inhibition of pro-inflammatory cytokines secretion that resulted in poor antigen presentation capacity [134,135]. However, the exact mechanisms by which DCs induce Treg cells and vice versa remain to be defined.

Presently, microbial products that polarize DCs towards a Treg cell-inducing phenotype are beginning to be identified. Well-documented examples include: filamentous hemagglutinin (FHA) and adenylate cyclase toxin from *Bordetella pertussis*, *Schistosoma mansoni* lyophosphatidylserine, cholera toxin β-subunit, and hepatitis C virus glycoprotein NS4 (all reviewed in [3]). Through the generation of Treg cells, these pathogens are able to prevent the generation of protective Th1 or Th2 immune responses. In the case of FHA it has been shown *in vitro* that the induction of IL-10 and the inhibition of IL-12 production by DCs promotes the clonal expansion of IL-10 producing T cells. There is also evidence that some pathogens block DC maturation, leading to Treg cell development in order to protect themselves against detrimental effects of the immune response [70].

1.5 Dendritic cells in the gastro-intestinal immune system

The gastro-intestinal mucosa is the largest and most complex part of the immune system. It is in constant interaction with the luminal microenvironment, which contains an ever-changing commensal microflora as well as a variety of bacterial, viral and protozoan pathogens. Strong immune responses to these pathogens are required to prevent uncontrolled infections in the host. By contrast, active immunity against harmless antigens, such as food proteins or the commensal microflora, would not only be wasteful but more importantly would trigger hypersensitivity responses against these antigens and can lead to inflammatory disorders such as

Celiac disease and Crohn's disease, respectively. As a result, the usual response to harmless gut antigens is the induction of local and systemic immunological tolerance, known as oral tolerance. This physiological response is most often characterized by IgA production and the induction of Th2 responses, rather than Th1, next to other specific features. Specialized cells and organs that are involved in the uptake of antigens, distinctive subsets of APCs and several unusual populations of B and T cells might contribute to this tolerance-favoring environment.

The gut associated lymphoid tissues (GALT) can be divided into effector sites, which consist of lymphocytes scattered throughout the epithelium and lamina propria, and organized tissues responsible for the inductive phase of the immune response. These are the small intestinal Peyer's patches (PP) and mesenteric lymph nodes (MLN), as well as smaller, isolated lymphoid follicles, which have the appearance of microscopic PP and are distributed throughout the wall of small and large intestines.

As discussed in the previous sections, DCs play a pivotal role in decision between tolerance and immunity. Here we will discuss more specifically the role of mucosal DCs in the induction of oral tolerance. Diverse DC lineages with specific morphological characteristics have been recognized in the different GALT. Some of these subpopulations have unique functions when compared to DCs from non-mucosal sites, both with regard to the induction of Treg cells in the resting, or "steady-state", and in the sampling, processing and presentation of antigens following mucosal infection. It remains however unclear whether they work synergistically, as alternatives, or have distinct functions in the recognition of the intestinal microflora and the regulation of inflammation.

1.5.1 Antigen sampling

Fundamental to understanding how mucosal immune responses are induced and regulated is where different types of antigen are processed and presented by DCs to T and B cells. Primary sites for the induction of intestinal T and B cell responses are PP and MLN. Less clear are the mechanisms of antigen uptake and processing by intestinal DCs. Distinct dendritic cell subtypes may utilize diverse mechanisms to sample antigen in the intestine.

Antigenic material may interact directly with DCs in underlying tissue. This may occur in particular following intestinal epithelial barrier disruption, as occurs in inflammatory bowel disease (IBD). The population of DCs, identified within the lamina propria epithelium, may be the first APCs to come into contact with luminal antigens that cross the epithelial cell barrier.

Four other mechanisms have been described allowing for the uptake of antigens by mucosal DCs under normal physiological conditions, thereby remaining an intact epithelial cellbarrier (Figure 9). Firstly, luminal antigens are shuttled into the PP via the M cells, overlying the dome of the PP. Compared to absorptive epithelial cells, M cells have poorly developed brush borders, reduced enzymatic activity and a thin overlying glycocalyx. These features, possibly combined with a unique cytoskeleton and a pronounced capacity to form endocytic vessels, facilitate the ability of M cells to transport micro-organisms and macromolecules from the mucosal lumen to the subepithelial dome (SED) of the PP where DCs can take up the antigen. M cells are also scattered among the absorptive epithelium, where they could potentially transport antigens to the lamina propria [136].

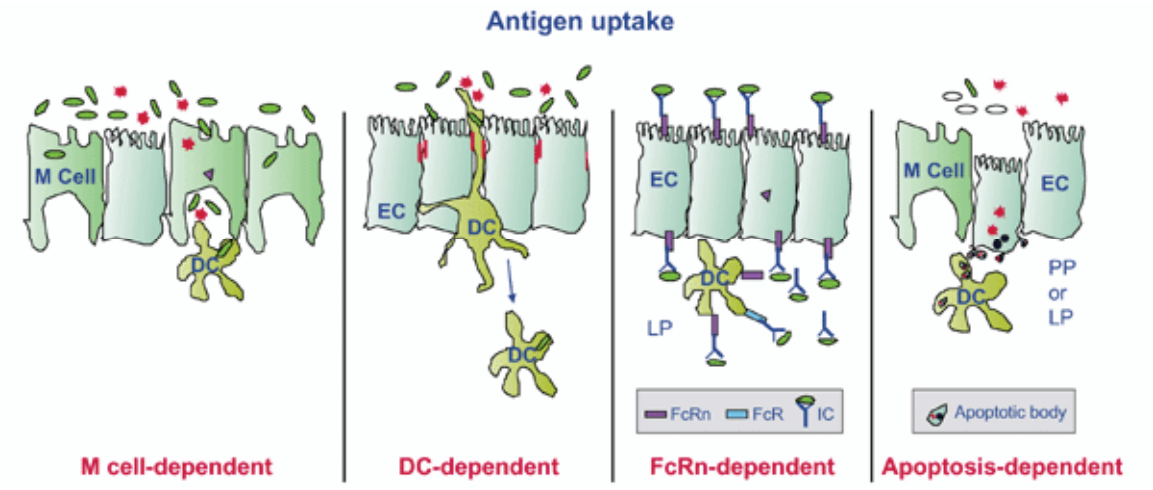

Figure 9 The uptake of antigens across mucosal surfaces.
M cells can transport antigens directly to underlying DCs. DCs can also extend dendrites between epithelial cells (EC) to directly sample antigens from the intestinal lumen. Neonatal Fc receptors (FcRn) mediate the bidirectional transport of IgG, resulting in transport into the lumen and trafficking back to the lamina propria (LP) of antigen-antibody complexes. Antigens associated with apoptotic epithelial cells can be taken up by DCs either in the steady state or after viral infection. PP, Peyer's patch; IC, immune complex. Source: [137]

Secondly, in the lamina propria, DCs have been described to sample bacteria from the gut lumen by extending their dendrites through the epithelial cell layer of the gut barrier [138,139]. The barrier integrity remains conserved by the expression and modulation of tight junction proteins, including occludin, claudin and zonula occludens [139]. This mechanism appears to require signals from the bacteria in the lumen. Recently, bacterial sampling was demonstrated in the steady-state

[140], indicating that DCs can sample commensal as well as pathogenic organisms from the lumen.

An alternative mechanism for antigen entry across a mucosal surface, that also targets DCs, was proposed [141]. It is mediated by neonatal Fc receptors expressed by adult human (but not mouse) intestinal epithelial cells that transport IgG across the intestinal epithelial barrier and, after binding with cognate antigen in the lumen, recycle the immune complexes back to the lamina propria [141] where this neonatal Fc receptor delivers the antigens in the form of immune complexes to DCs.

DCs in the SED of PP may take up antigen-bearing exosomes, shed from the basolateral surface of epithelial cells [142] or may internalize apoptotic epithelial cells both in the steady-state [116] and after reovirus infection [143], which constitutes a fourth mechanism that directly involves interaction of DCs with the epithelium.

1.5.2 Regulation of immune responses by intestinal DCs

Intestinal DCs have properties distinct from their non-mucosal counterparts, probably as a result of their association with the external environment. Based on the current information on intestinal DC subsets, a model was proposed of how mucosal DCs may play a role in the induction of oral tolerance [144] (Figure 10).

Under steady-state conditions, precursor DCs continuously enter the mucosal lamina propria, PP and colonic isolated lymphoid follicles, develop into immature DCs and localize to different regions due to the differential constitutive expression of specific chemokines, as there are CCL9, CCL19, CCL20 and CCL21. After uptake of antigens, via one of the described mechanisms, DCs migrate from the lamina propria to the MLN, or from the SED of PP to the interfollicular regions. This migration is accompanied by an up-regulation of chemokine receptors for T cell zone chemokines, such as CCR7, but does not affect the levels of co-stimulatory molecules and cytokines. Following migration of these antigen-loaded ‘quiescent’ DCs to T cell zones, the DCs stimulate T cells to differentiate into tolerizing T cells (Tr1 or Th3) or induce clonal deletion of T cells. These DCs could also interact with B cells and induce IgA responses to commensal bacteria [145].

The induction of regulatory T cells may involve one or more DC populations. $CD8\alpha^-$ $CD11b^+$ DCs are ideally located for antigen capture in the PP SED and lamina propria, produce IL-10 and induce T cells that produce IL-4, IL-10 and most likely also TGFβ [53,100,146]. In addition to $CD11b^+$ DCs, another candidate for Treg cell induction is the plasmacytoid DC. In particular, $CD8\alpha^+$ $B220^+$ plasmacytoid DCs from

the PP and MLN were shown *in vitro* to induce the differentiation of IL-10 producing Treg cells that could mediate suppression [147].

The phenotype of PP, and lamina propria DCs that induce Treg cells may be influenced by local stromal factors, such as prostaglandin E_2 (PGE_2) and TGFβ [148-150]. Furthermore, it seems that epithelial cells may have a chief function in 'instructing' mucosal anti-inflammatory DCs. It was demonstrated in co-culture studies of epithelial cell monolayers, DCs and bacteria, that products of epithelial cells condition DCs to release IL-6, which drives the development of IgA producing plasma cells [151], and to prime Th2 responses in an allogeneic proliferation assay [152]. This process was mediated by a combination of thymic stromal lymphopoetin and other factors that are constitutively expressed by epithelial cells. Thus it is possible that the mucosal environment 'educates' DCs to mount non-inflammatory responses to preserve gut homeostasis.

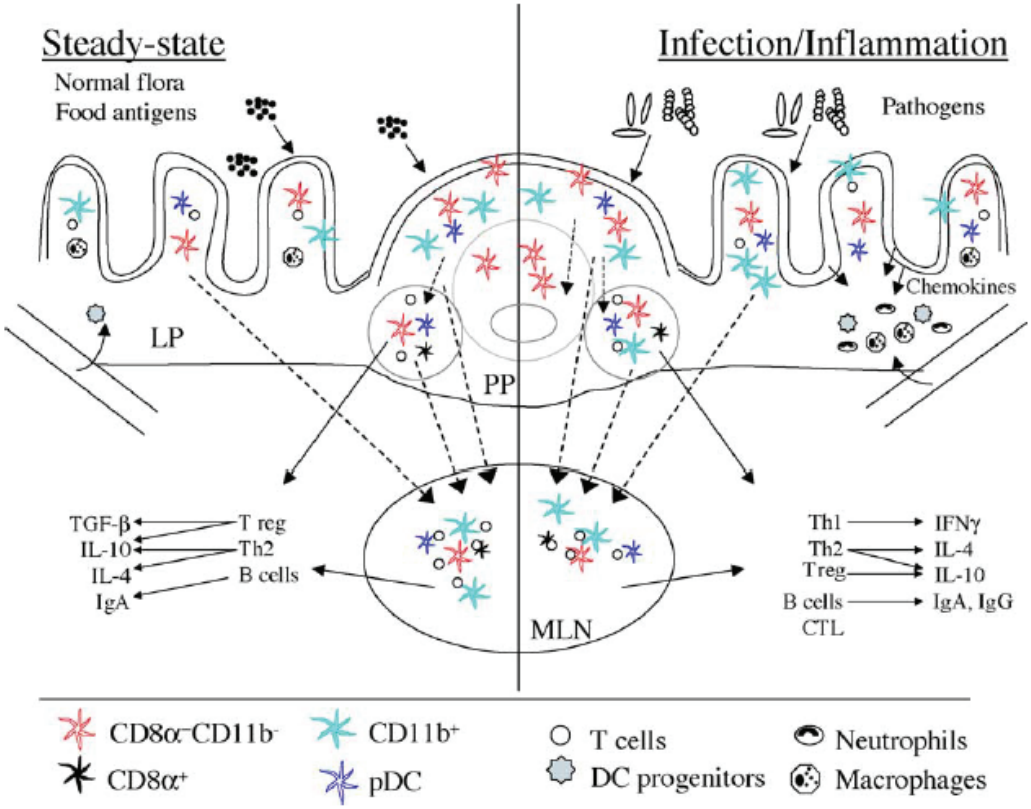

Figure 10 Mucosal dendritic cells (DCs) during steady-state and inflammatory conditions.
See text for details. CTL: Cytotoxic T-lymphocyte; Ig: Immunoglobulin; IL: interleukin; LP: lamina propria; MLN: mesenteric lymph nodes; pDC: plasmacytoid DC; PP: Peyer's Patches; TGFβ: Transforming Growth Factor β; Treg: regulatory T cell. Source: [144]

In contrast to tolerogenic responses to innocuous antigens, DCs become activated during infection or inflammation, migrate more rapidly and induce both innate and adaptive immune responses (Figure 10). In the mouse, $CD11b^+$ DCs or $CD8\alpha^-$ $CD11b^-$ DCs in the SED of PP or lamina propria are, due to their localization, most likely involved in the initial interaction and uptake of invading pathogens. As for peripheral DCs, the induction or expansion of specific T cell responses by mucosal DCs following infection will depend on the subset of DCs involved, the signals to which they are exposed during their activation and subsequent interaction with T cells and the combined effects of antigen dose and duration and/or frequency of T cell-DC contacts.

1.5.3 Probiotics and intestinal DCs

Probiotic bacteria, mainly belonging to the lactic acid group of bacteria, are well known for their health-promoting properties [153]. Regular intake of probiotic bacteria contributes to immune homeostasis by altering microbial balance or by interacting with the gut immune system, explaining their therapeutic effect in IBD [154-156] (see also 2.3). Although there is a considerable amount of information concerning the protective efficacy of probiotics, little is known about the precise mechanisms of action by which such bacteria may exert their beneficial effects. Evidence that intestinal DC populations can interact with luminal organisms is discussed above. As DCs are equipped with a myriad of receptors to recognize microbial structures (C-type lectins, TLRs…), it is likely that these DCs can be modulated by orally administered micro-organisms such as probiotics. Furthermore, the effect of probiotics may also result from soluble factors that mediate their effects on DCs.

Most studies on the probiotic effects on DCs were performed on murine *in vitro* generated bone marrow-derived DCs or on peripheral blood derived human DCs. Therefore one can only hypothesize as to the effects on gut-associated DCs. Oral administration of VSL#3, a probiotic formulation, has been described to ameliorate Th1-mediated murine colitis by the induction of Treg cells [157]. This mixture was also used with some success in clinical trials [156,158]. Incubation of bone marrow-derived DCs with VSL#3 induced some degree of maturation and increased levels of IL-10 production. Furthermore, this combination of strains also inhibits the generation of pro-inflammatory Th1 cells *in vitro* [159,160]. For single probiotic strains, different effects on DCs have been described. *Bifidobacteria* reduce the expression of co-stimulatory molecules CD40 and CD80 and up-regulate IL-10 production by human blood DCs [160]. This increase in IL-10 production may act

both by having direct anti-inflammatory effects and by enhancing the generation of Treg cells. On the other hand, some *Lactobacillus* strains appear to generate a semi-mature DC phenotype, characterized by increased co-stimulatory expression but low production of pro-inflammatory cytokines [160-162]. For specific *Lactobacillus* strains, the *in vitro* induction of Treg cells through the targeting of the C-type lectin DC-SIGN in human DCs has been shown [163]. Another study showed that the tolerization of DCs by probiotic lactic acid bacteria was dependent on MyD88, nuclear type binding oligomerization domain 2 (NOD2) and TLR2 signaling whereas the *in vivo* protective effect of these tolerized DCs in murine colitis required $CD4^+$ $CD25^+$ regulatory T cells in an IL-10 independent pathway [162]. All these studies show a pleiotropy of possible effects of probiotic strains on DCs. This capacity is however not restricted to lactic acid or even gram-positive bacteria, as it was observed that e.g. *Bordetella pertussis* and *Vibrio cholerae* compounds can selectively commit DCs to induce polarizing signals via different mechanisms [76] (and also discussed in 1.4.3). As most of the effects of probiotics were obtained from *in vitro* studies, the results should be interpreted with great caution. Indeed, not only are many potential players of the mucosal regulatory response lacking *in vitro* but, possibly more importantly, in an *in vivo* situation the DC receives signals from the patients entire microflora and thus any DC modulation by the probiotics will ultimately be dependent on variation of strains and concentrations present in the gut.

2 INFLAMMATORY BOWEL DISEASE

IBD is a group of chronic intestinal inflammatory diseases, of which the most common forms are ulcerative colitis (UC), an inflammation of the large intestine, and Crohn's disease (CD), which can affect any part of the gastro-intestinal tract. IBD affects 1-2 in every 1000 individuals in Western societies and is a significant public health problem mainly because of its poorly understood etiology. Clinically, IBD is characterized by chronic inflammation, resulting in diarrhea, abdominal pain, weight loss, rectal bleeding and nausea. If left untreated the disease can be fatal due to malnutrition, dehydratation and anemia. Although many patients are managed successfully with anti-inflammatory medication, some stay refractory to treatment, most will have recurrent activity and two-thirds will require surgery.

2.1 The pathogenesis of IBD

IBD is a multi-factorial disorder involving genetic and environmental elements. It is now widely accepted that the pathogenesis of IBD is due to a dysfunctional interaction between bacterial microflora of the gut and the mucosal immune system. In one version of this view, the disease defect lies within the mucosal immune system, reacting against a normal microflora. In this case, the normal state of immunological tolerance to microbial antigens in the gastro-intestinal tract is disturbed. The second major hypothesis regarding the pathogenesis of IBD views the disease as resulting from one or more causative agents present in the gut microflora, which induce a pathological response from a normal mucosal immune system. (for review see [164]).

Many data support the hypothesis that an aberrant immune response to intestinal bacteria seems to be crucial in the pathogenesis of IBD [165]. Important evidence comes from the identification of the first CD susceptibility gene. The caspase recruitment domain protein 15 gene (CARD15), which encodes NOD2, is located on chromosome 16, associated with the IBD1 locus [166-168]. NOD2 belongs to a family of intracellular proteins possessing a NOD linked at its carboxy terminus by a leucine-rich repeat domain (LRR). NOD2, expressed mainly by APCs, is intimately involved in intracellular bacterial sensing and generation of the innate immune response as its LRR domain recognizes muramyl dipeptide, derived from peptidoglycan, present in the cell wall of most bacteria. Through its CARD domain NOD2 activates NF-κB [169]. The 3 NOD2 polymorphisms associated with CD are located in and around of the LRR domain and are all loss-of-function mutations,

preventing binding of the muramyl dipeptide. How this loss of NF-κB activity can be linked to excessive inflammation is still a matter of debate. Additional studies have led to the hypothesis that signaling through NOD2 normally causes an overall down-regulation of the inflammatory response through dampening of NOD2-independent NF-κB activation e.g. the peptidoglycan-induced activation of the TLR2 [170]. In the absence of effective NOD2 signaling, this TLR2 dependent NF-κB activation can thus results in downstream activation of IL-12 and subsequent inflammation. Regardless of the mechanism, the association of CD with a defect in a microbial receptor defines a role for innate immunity and recognition of the microflora in disease pathogenesis.

Animal models of colitis have provided essential clues to the role of luminal flora in the pathogenesis of IBD. The list of genetic and acquired aberrations that lead to intestinal inflammation is strikingly long and diverse [171]. These models include animals with disturbed immune regulation such as the IL-2, IL-10 and TCR α knock-outs as well as models whit a disruption of the epithelial barrier function, such as the G2αi knockout mouse. In every model thus far explored, it has been shown that gnotobiotic conditions greatly diminish or prevent the onset of gut inflammation. With the introduction of non-pathogenic bacteria, each of these models then proceeds to maintain their typical phenotype of colonic inflammation.

Some studies, however, support the hypothesis suggesting that IBD is associated with pathological organisms that establish a low-grade infection of the mucosa and in doing so evoke the inflammatory response that characterizes the disease. One of the leading infectious candidates is *Mycobacterium avium paratuberculosis* [172], but also *Listeria monocytogenes* and *Helicobacter hepaticus* have been linked to IBD. However, so far there is no compelling evidence for an absolute requirement for any of the proposed causative agents in the onset of IBD.

Taken together these results suggest that the fundamental basis of IBD is the presence of one or more genetically determined defects that result in a mucosal immune system that overreacts to normal constituents of the intestinal microflora. These defects can be accompanied by genetically determined alterations in gut epithelial barrier functions that enhance exposure of the mucosal immune system to microflora components. For instance, recently it has been shown that CD patients exhibit decreased secretion of cationic peptides with antibacterial properties, known as defensins [173,174].

2.2 Intestinal dendritic cells in IBD

The fact that IBD is related to deregulated immune responses against intestinal flora, has elicited a closer examination of the role of the DC as a central mediator.

DCs from inflamed lamina propria of Crohn's patients show enhanced expression of TLR2 and TLR4, which may contribute to altered microbial recognition [175]. These DCs also express higher levels of CD40 than DCs from non-inflamed lamina propria or from healthy control tissue. Also an increased proportion of them release inflammatory cytokines. The up-regulation of CD40 on isolated cells is consistent with immunohistological studies showing increased numbers of DCs expressing CD80 and CD83 in mucosal tissue from CD and UC patients [176]. These altered DCs are likely to contribute to the initiation or perpetuation of intestinal inflammation, either as a local effector cell population active in innate immunity or by modifying the response of lymphocytes that the DCs activate as part of the immune response.

Other groups studying DC phenotype and expansion in murine colitis models have demonstrated expansion of colonic lamina propria DCs that exhibit up-regulated expression of co-stimulatory molecules, such as CD40, CD80 and CD86 [177]. Recent experiments suggest that DCs play an early and fundamental role in the disease. DC aggregates were identified under the basal crypt epithelium of immune deficient mice. When pathogenic T cells were transferred to these mice, they clustered and proliferated in these aggregates 5-10 days before colitis could be detected, suggesting that the DCs were involved in the initial activation or restimulation of pathogenic T cells [178]. Furthermore, in the SCID model, activated (OX40 ligand^{+}) DCs were increased in the MLN, after induction of colitis by the transfer of CD45RB^{hi} T cells [179]. Anti-OX40 ligand blocked the development of colitis, supporting an important role for activated DCs.

These studies suggest a role for activation of intestinal DCs in the initiation and possibly continuation of IBD, both in mouse and humans. Identifying a means to interrupt the activation of DCs *in vivo* may be a key to controlling the disease.

2.3 Current and potential therapies for IBD

Regardless of the particular defects, IBD finally results in a common immunopathological pathway comprised of either a Th1-mediated inflammation (CD) or a Th2-mediated inflammation (UC) [180]. This implies that, regardless of the nature of the fundamental defects, one could potentially treat IBD with therapies that

address an essential element of the final common pathway. Existing conventional treatments, such as corticosteroids, mesalamine and immunosuppressants are good examples as they aim to block downstream inflammatory events such as the secretion of cytokines and the expansion of immune cells and neutrophils regardless of the nature of the underlying T cell responses that generate these events. Despite their shortcomings and toxicities these agents have long been the main drugs for treatment of IBD.

Understanding the role of cytokines along with the development of monoclonal antibodies targeting specific disease related cytokines has led to a major advance in the development of IBD therapeutics. The greatest advance in this area has been the introduction of anti-TNF therapy. TNF is a pro-inflammatory cytokine, which is abundantly expressed in the gut of CD. As a result, this cytokine was considered to be an attractive target for the treatment of IBD and several anti-TNF reagents have thus been developed. These reagents include infliximab, CDP571, CDP870, etanercept, onercept and adalimumab (for a review see [181]). Infliximab is a mouse/human chimeric IgG1 monoclonal antibody against TNF, which was created in late 1980s, and it has been demonstrated to be effective in reducing intestinal inflammation in CD. Most of the other anti-TNF reagents are modified by a reduction of the mouse peptide sequence or are completely humanized in order to reduce the immunogenicity. Not all of these anti-TNF reagents have been proven to be as effective in the treatment of CD as infliximab. The efficacy seems to be dependent, not only on the ability to neutralize soluble TNF, but also on the capacity to bind to membrane-bound TNF, thereby mediating the apoptosis of the effector cells [182]. Since UC is characterized by a Th2-mediated inflammation, TNF was not initially thought to play an important role in the pathogenesis. However, next to the excess of Th2 cytokines IL-5 and IL-13, increased levels of TNF were found in the colonic mucosa, serum and stool of UC patients [183-185] and infliximab was shown to be extremely efficacious in two randomized placebo controlled clinical trials (for review see [186]). Although successful, the anti-TNF strategy has also been associated with significant complications [187-189].

Other cytokine-directed therapies are at earlier stages of development. Early studies with anti-IL-12p40 show some activity in active CD [190]. This antibody may also bind to the IL-12p40 subunit as a component of the heterodimeric pro-inflammatory cytokine IL-23. In support of this, polymorphisms in the IL-23 receptor gene appear to confer protection against the CD [191]. Furthermore, administration of a monoclonal antibody against the IL-23 specific subunit IL-23p19 was effective in both prevention

and treatment of active colitis in a mouse model [192]. This suggests that specific inhibition of IL-23, and not IL-12, may be an effective treatment of IBD. Also under investigation are anti-IFNγ [193] and targeting of IL-6 [194]. While the above-mentioned therapies aim at correcting the immune balance in IBD by interfering with the action of pro-inflammatory cytokines, IL-10 treatment tends to exploit the anti-inflammatory properties of the product itself. Intravenous administration of recombinant IL-10 initially resulted in reduced CD activity scores and increased remission [195]. However, larger trials could not confirm these positive effects [196]. Furthermore, many patients developed systemic side effects such as headache, fever and the induction of IFNγ [197]. It is possible that the pharmacodynamics of daily systemic IL-10 delivery do not lead to sufficient mucosal penetration while causing negative systemic effects. Therefore, alternative methods for the delivery of IL-10 might be a solution. Experiments in our department showed that topical delivery of IL-10 by means of intragastric administration of a recombinant *Lactococcus lactis* strain, secreting murine IL-10, prevented onset of colitis in IL-10 knockout mice and caused a 50% reduction of the inflammation in dextran sulfate sodium (DSS)-induced chronic colitis [198] (see also 4.2.1). Furthermore, in a phase I trial, the use of these genetically modified (GM) bacteria for mucosal delivery of IL-10 was shown to be a feasible strategy in human beings [199]. As this novel strategy avoids systemic side effects and is biologically contained [200], it is suitable for long-term treatments of chronic intestinal diseases. More details about *L. lactis* and its use as a live topical delivery system will be discussed in the chapter 4.

A second area where understanding of the pathogenesis has lead to novel therapies is the area of cellular adhesion and recruitment. The use of an anti-integrin antibody could prevent accumulation of leukocytes at areas of inflammation by inhibiting its binding to cellular adhesion molecules on the endothelial surface, such as VCAM, ICAM and MAdCAM. Although effective in clinical trials, the use of the humanized anti-α4 integrin has been associated with a fatal infection of the central nervous system [201].

A third area of interest for therapeutic intervention is the process of T cell activation. Both the antigen specific interaction between a T cell and the APC and co-stimulatory signaling offer opportunities for therapeutic intervention. Current therapies in progress are the administration of αCD3 antibodies for TCR targeting [202] or the inhibition of co-stimulation by binding of CD28 to B7 with Abatacept, an approved treatment for rheumatoid arthritis [203]. Unexploited as a therapy is the induction of

Treg cells to restore tolerance, as these can prevent and even cure colitis in various experimental models [24,204].

In parallel to the growing importance of the innate immune system came the first study of therapies intended to enhance the innate immune responses rather than to suppress adaptive immune responses. In this context, treatment with GM-CSF, for stimulation of neutrophils, shows promising results in CD [205].

Given the critical role for the luminal flora in IBD, it is not surprising that antibiotics seem useful as a treatment for CD with colonic localization or have a prophylactic effect for disease recurrence after ileal resection [206,207]. In addition, elemental diet has been demonstrated to be an effective therapy for CD [208]. This diet contains all essential nutrients but is free of proteins. Effects of elemental diet may be complex but are thought to include a favorable effect on the bowel flora composition and may minimize immunologic stimulation of the host immune response. Finally, the potential use of probiotics to restore immune regulation, enhance the epithelial barrier or change the composition of the microflora, is currently under investigation. The influence of certain probiotics on dendritic cells and thus on immune regulation has been described earlier (see 1.5.3). Some preparations seem to have beneficial effects in both UC and CD (for reviews see [154,155]).

3 INTERLEUKIN-10 AND THE TREFOIL FACTOR FAMILY

The focus of this work will be on the modulation of (mucosal) dendritic cell function by *L. lactis* delivered murine IL-10 or trefoil factor family 3 (TFF3). Both strains have healing effects in mouse models for chronic and/or acute colitis albeit with an entirely different functional basis. On the one hand we have IL-10, an anti-inflammatory cytokine with an important role in regulating the immune response. On the other hand, TFF peptides are mainly known for their epithelial repair function and only recently, attention is given on possible immune-modulating effects.

3.1 Interleukin-10

The immune system's inflammatory response is essential to protect the host from infection, injury and neoplasia. However, the immune response has to be of the appropriate amplitude and duration to prevent unnecessary destruction of healthy tissue. Excessive production of inflammatory factors can result in diseases such as rheumatoid arthritis, septic shock and, as described above, IBD. The immune system has developed multiple anti-inflammatory mediators to prevent such "out-of-control" inflammatory responses. One of the most potent of these anti-inflammatory factors is IL-10. IL-10 was originally described in 1989 as a cytokine synthesis inhibitor factor produced by Th2 cells that inhibited the production of cytokines such as IL-2, TNF, IFNγ and GM-CSF by Th1 cells, in response to antigens presented by APCs [209]. However its expression profile has now been widened and expression in various subsets of T cells, macrophages, monocytes, dendritic cells, mast cells, B cells, eosinophils, keratinocytes and epithelial cells has been shown [210]. Here, I will briefly review the IL-10 structure, its receptor, the signaling pathway and the main biological functions of IL-10 on cells of the immune system.

3.1.1 IL-10 protein, gene and expression

IL-10 belongs to the class II family of α-helical cytokines that is composed of the type I interferons, IFNγ and IL-10. The main structural feature is a left-handed anti-parallel four-helix bundle [211]. Recently five novel cytokines that display structural similarity to IL-10 have been identified from the human genome: IL-19, IL-20, IL-22, IL-24 and IL-26 [211,212], together comprising the cellular IL-10 subfamily. Similar to IL-10 these IL-10 related cytokines are α-helical proteins, whose amino acid sequences are 20-30% identical. These new IL-10 family members are strongly involved in immune regulation and inflammatory responses but further studies are needed to

provide a better understanding. The viral (v)IL-10 subfamily includes IL-10 gene homologues found in the Epstein-Barr virus, equine virus type 2, poxvirus Orf and human cytomegalovirus genomes [212]. Although the *in vivo* roles of vIL-10 in the viral life cycle remain to be defined, it is nevertheless sensible to speculate that these viral homologues play a key role in suppressing the host immune system, allowing the growth of virus induced tumors and survival of the viruses in the host. Members of this subfamily have sequence homologies with human (h)IL-10 ranging from 20%-80%.

The hIL-10 molecule consists of 160 amino acids, has a molecular weight of 18.5 kDa and shows 73% amino acid homology with murine (m)IL-10 that has 157 amino acids with a molecular weight of 18.5 kDa. Both are synthesized as monomers but form homodimers in biological fluids. Although an engineered human monomeric IL-10 molecule can bind to the IL-10 receptor, the homodimer that forms spontaneously has 60-fold greater affinity for the receptor and 10-fold higher biological activity [213]. hIL-10 and vIL-10 are not glycosylated, whereas mIL-10 appears to be heterogeneously N-glycosylated at a site near the N-terminus. Nevertheless, glycosylation of mIL-10 has no known influence on biological activity [210]. Furthermore, hIL-10 is active on both mouse and human cells, whereas mIL-10 is effective only on mouse cells.

Both mIL-10 and hIL-10 genes are encoded by 5 exons on the respective chromosomes 1. Activation of IL-10 gene expression results in $\pm$2 kb (hIL-10) and $\pm$1.4 kb (mIL-10) mRNAs. IL-10 can be expressed by a variety of cells, usually in response to an activation stimulus and its expression is regulated by different mechanisms in different cell types. In contrast to other cytokines, IL-10 transcription can be regulated by transcription factors Sp1 and Sp3, which are expressed constitutively by many different cell types [214]. Combined with control of mRNA stability at the post-transcriptional level [215], this suggests that the IL-10 gene is transcribed to some degree constitutively and subject to control by alteration of post-transcriptional RNA degradation mechanisms. This situation results in a more rapid induction of IL-10 expression than can be achieved by activation of transcription.

3.1.2 The IL-10 receptor: structure and expression

The IL-10 receptor (IL-10R) is a heterotetramer comprised of two molecules of the IL-10Rα (or IL-10R1) and two molecules of the IL-10Rβ (or IL-10R2) chain. (for reviews see [210,211])

IL-10R1 is the ligand binding subunit to which IL-10 binds with high affinity. Consistent with the observed species-specificity of IL-10, mIL-10R1 binds both mIL-10 and hIL-10, while hIL-10R1 does not bind mIL-10. Most hematopoietic cells express this R1 chain, although generally at very low levels. Upon activation of T cells its expression is down-regulated both at the mRNA and protein levels. In contrast, activation of monocytes is associated with up-regulation of IL-10R1 expression, consistent with the inhibitory function that IL-10 exerts on these cells. IL-10R1 expression has also been observed on non-hematopoietic cells, although it is more often induced rather than constitutive. Nevertheless constitutive IL-10R1 expression has been described in colonic epithelium.

The IL-10R utilizes an accessory subunit for signaling, IL-10R2. This chain was originally described as an orphan IFNR family member, located in the IFNR gene complex of both mouse and human. Several lines of evidence support IL-10R2's role in the IL-10R complex. First it can be crosslinked with IL-10/IL-10R1 in IL-10 responsive cells. Moreover, IL-10R2$^{-/-}$ mice, like IL-10$^{-/-}$ animals develop severe chronic enterocolitis and cells from these mice are unresponsive to IL-10. Finally, anti-hIL-10R2 monoclonal antibodies block IL-10 responses. IL-10R2 contributes little to IL-10 binding affinity and its principal function appears to be recruitment of a Janus kinase (Tyk2) to the signaling complex. Most cells and tissues examined constitutively express IL-10R2 and there is no evidence for significant activation-associated regulation of IL-10R2 expression in immune cells. Thus, any stimulus activating IL-10R1 expression should be sufficient to render most cells responsive to IL-10.

3.1.3 Signaling pathway

Binding of IL-10 to its receptor causes the activation of the receptor associated Janus tyrosine kinases, Jak1 and Tyk2 [216]. These kinases are responsible for the phosphorylation of tyrosine residues within the intracellular domain of IL-10R1 that serve as docking sites for STAT molecules [217]. IL-10 was initially shown to activate STAT1, STAT5 and STAT3 [218] but only STAT3 signaling seems to be required for the anti-inflammatory effects of IL-10 [219,220]. Homodimers of STAT3 form and translocate to the nucleus where they drive the transcription of STAT3 responsive genes. The obligate role of STAT3 in IL-10 signaling raised the issue of pathway redundancy and specificity as many receptors use STAT3. For example IL-6 signaling also activates the Jak1-STAT3 pathway yet is incapable of activating the anti-inflammatory response. An explanation involves the effects of suppressor of

cytokine signaling (SOCS)3 on the IL-6R. SOCS3 expression is strongly induced by both IL-10 and IL-6 stimulated cells and is required to regulate STAT3 signaling. It binds to specific phosphorylated tyrosine residues on cytokine receptors thereby rendering them sensitive for degradation. In contrast to the IL-6R, the IL-10R appears refractory to the effects of all members of the SOCS family [221]. Binding of IL-10 to the receptor thus rapidly activates STAT3 and it remains phosphorylated over a sustained period.

Perhaps the most complex aspect of IL-10 signaling involves the selective reduction of pro-inflammatory gene transcription in APCs. Microarray analysis revealed that expression of most LPS-induced genes was unaffected in the presence of IL-10. Only 15-20% of the genes were reduced by IL-10 and these genes predominantly encoded proteins crucial to inflammation, including cytokines, chemokines and cell surface receptors [222]. IL-10 therefore inhibits only subsets of genes activated by TLR stimulation. The exact mechanisms underlying this process are unknown but are dependent on *de novo* gene transcription induced by STAT3. These unknown STAT3 induced molecules then target the transcription of inflammatory genes [223].

3.1.4 Anti-inflammatory functions

The main biological functions of IL-10 seem to be to limit and terminate the inflammatory responses, block pro-inflammatory cytokine secretion and regulate the differentiation and proliferation of several immune cells such as T cells, B cells, natural killer cells and mast cells. The IL-10 gene homologues found in viral genomes are also thought to act as virulence factors to manipulate the immune system. Furthermore, many tumors also acquire an IL-10 secreting phenotype that may permit malignant cells to evade cell-mediated immune defenses [224,225]. The convergence of these evolutionary distinct mechanisms for immune evasion underscores the importance of IL-10 as a central immune modulator.

The major physiological function of IL-10 is to suppress the functions of APCs, such as monocytes/macrophages and DCs. Pathogens can stimulate these APCs to produce a variety of inflammatory mediators through activation of their PRRs (see 1.3.2 for DC). IL-10 modulates expression of cytokines, soluble mediators and cell surface molecules by these cells with important consequences for their ability to activate and sustain immune and inflammatory responses.

IL-10 potently reduces expression of TNF, IL-1, IL-12, IL-6 and GM-CSF by activated monocytes/macrophages [226-229]. The inhibitory effect of IL-10 on IL-1 and TNF are crucial to its anti-inflammatory activities because these cytokines often have

synergistic effects on inflammatory pathways and processes, and amplify these responses by inducing secondary mediators such as chemokines, prostaglandins and platelet activating factor. Furthermore IL-10 also inhibits the production by activated monocytes of the CC chemokines MCP-1, MCP-5, MIP-1α, MIP-1β, MIP-3α, MIP-3β, MDC and RANTES as well as the CXC chemokines IL-8, IP10, MIP-2 and Gro-α. These chemokines are implicated in the recruitment of monocytes, dendritic cells, neutrophils and T cells. Thus IL-10 inhibits expression of most inducible chemokines that are involved in inflammation [210]. Next to this IL-10 also reduces expression of inflammatory enzymes such as cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS), resulting in reduced prostaglandin and nitric oxide (NO) levels [210,230-232].

IL-10 not only inhibits production of effector molecules but also further augments its inhibitory activity by enhancing the release of natural antagonists such as soluble TNF receptor and the IL-1R antagonist [210]. Similarly, the potential destructive activities of matrix metalloproteinases (MMP) are limited by IL-10 as it not only inhibits the production of MMP-1 and MMP-9 through decreased PGE_2 expression but also induces the production of tissue inhibitor of MMP (TIMP) [233,234].

IL-10 also induces apoptosis of human plasmacytoid DCs and alters DC migration by modulating the expression of chemokine receptors [210]. In a recent study it was shown that both externally added and activation induced autocrine IL-10 production reduce the lifespan of mature myeloid DCs [235]. The mechanism behind this is likely associated with the down-regulation of anti-apoptotic Bcl-2 proteins.

Another key function of IL-10's immune suppressive capabilities is its effectiveness in disabling antigen presentation and subsequent T cell activation by inhibiting the expression of MHC II, CD80 and CD86 on macrophages and DCs thus effectively blocking antigen presentation to T cells [236-239].

Somewhat contradictory seems the up-regulation of activatory CD16 and CD64 FcγR expression by IL-10 on macrophages, which correlates with an enhanced capacity of macrophages/monocytes to phagocytose opsonized particles, bacteria or fungi [240,241]. However, as the increased uptake is accompanied with reduced antigen presentation and reduced co-stimulation, it rather serves to clear inflammatory stimuli from the site of infection. In fact, IL-10 induces the differentiation of monocytes into a type of macrophage prone to resolve inflammation by clearing bacteria without triggering the release of inflammatory mediators [242]. This phenotype is also seen

upon addition of IL-10 during GM-CSF/IL-4 driven differentiation of monocytes into DCs or during LPS-stimulated maturation of DCs [243-245].

Most of the inhibition of T cell activation by IL-10 seems to be caused indirectly via suppressing crucial APC functions. However, direct effects of IL-10 on T cells also have been described. IL-10 shows inhibitory activities towards Th1 and Th2 cells via the suppression of IL-2, IFNγ, IL-4 and IL-5 [246,247]. IL-10 can also inhibit expression of the chemokine receptor CXCR4, induced on activated T cells, and the chemotactic response to its ligand stromal-derived factor-1 [248].

3.1.5 Induction of tolerogenic DCs by IL-10

As mentioned above, the immunosuppressive properties of IL-10 on DCs result in a reduced expression of MHC II as well as co-stimulatory and adhesion molecules. Furthermore, DCs matured in the presence of IL-10 showed a reduced production of inflammatory cytokines and a lack of IL-12 synthesis [121-123]. The induction of T cell anergy by these IL-10-treated DCs has been described before (see 1.4.2). Briefly, IL-10 induced anergy is characterized by an antigen-specific inhibition of T cell proliferation and reduced production of IL-2 and IFNγ [125]. In contrast to Treg cells, anergic T cells induced by IL-10-treated DCs are characterized by a markedly reduced expression of the IL-2 receptor α-chain (CD25), and anergic T cells produce no immunomodulatory cytokines, such as IL-10 or TGF-β [123,125]. The presence of IL-10 during DC maturation thus favors the emergence of anergy-inducing APCs. However, the immunosuppressive activity of IL-10 is limited to IL-10 sensitive immature DCs. During terminal differentiation, DCs down-regulate the IL-10R. Therefore mature DCs are IL-10 resistant [210,242].

It has been shown *in vitro* that IL-10 plays an indirect role in the differentiation of Tr1 cells, through the induction of a specific tolerogenic DC subset [131]. DCs from the mucosal system, which are known to be more tolerogenic than systemic DCs derived from lymph nodes and spleen, also participate in the generation of Treg cells by expressing high levels of IL-10 upon stimulation and priming naive T cells to differentiate in Th2 and Tr1 directions and to secrete high levels of IL-4 and IL-10 [116]. This T cell derived IL-10 can then in turn further augment tolerance through converting DCs into a tolerogenic phenotype.

3.2 Trefoil factor family of peptides

The TFF is a family of peptides that plays an important role in mucosal defense and repair of the gastro-intestinal tract. This family consists of three members: the gastric peptides pS2, now called TFF1 and Spasmolytic Polypeptide or TFF2 together with the intestinal trefoil factor TFF3. They are small (7-12 kDa) protease-resistant proteins that are abundantly secreted onto the mucosal surface by mucus-secreting cells of the gastro-intestinal tract. Trefoil factor peptides have been shown both *in vivo* and *in vitro* to have major effects on such physiological processes as epithelial restitution, wound healing, apoptosis and cell motility. Here we will describe the region specific expression, structure, regulation and known biological effects of the TFF with some proposed mechanisms. As will become apparent, most mechanisms are still not completely understood.

3.2.1 TFF expression and gene regulation

Mammalian TFF peptides were originally detected as secretory products of the gut. Expression of the TFF in the gastro-intestinal tract is abundant and is second, in weight of protein, after the mucins. The trefoil peptides reside on a 50 kb section of the human chromosome 21q23 and on the murine chromosome 17 and likely share 5' regulatory sequences which suggests a common or connected regulation [249,250].

Under normal conditions, TFF1 and TFF2 show complementary cellular localization in the stomach: TFF1 is mostly restricted to the gastric pits and TFF2 to the mucous neck cells of the gastric gland. TFF3 has a quite different localization pattern being minimally expressed in the stomach, but massively produced by goblet cells throughout the intestine [251] where its local concentration on the gastro-intestinal surface is estimated at 1-5 mg/ml [252]. Nowadays a number of other human mucous epithelia have been identified as TFF secreting organs e.g. the conjunctiva, the salivary glands, the respiratory tract and the uterus [253-256]. Some reports also mention the expression of TFF peptides at less expected places than the mucosa-associated expression sites. First of all expression of the TFF has been shown in the brain [257,258], with again a differential localization for the different trefoil peptides. Furthermore, both TFF2 and monomeric TFF3 are expressed in rat lymphoid tissue [259]. The expression levels are much lower than in the stomach and intestine.

Next to the homeostatic expression, inflammation induces rapid and sustained, TFF expression with loss of the tight control over regional specificity [260]. Aberrant expression of TFF peptides was observed during various chronic inflammatory diseases; for example in ileal CD, in colon mucosa of patients with UC, in gastric glands during gastric ulcer disease and in various types of metaplasia [254,261-263]. The discovery that all three TFF peptides are typically secreted by a specific gland-like structure termed the ulcer-associated cell line was certainly a major hallmark [254,264,265]. This mucin-secreting glandular structure appears during a variety of chronic inflammatory conditions. It develops from stem cells, most commonly in the small intestine and is thought to represent a natural repair kit, which is activated after mucosal damage [265].

The differential expression pattern in health and inflammation can be explained by an epigenetic mechanism, DNA methylation of the TFF promoters. In tissues where the respective TFF is normally expressed, the CCGG sites in its promoter are not methylated. In other mucosal tissues, where expression might be induced upon damage or inflammation, the CCGG sites are partially demethylated. In contrast, in organs that do not express the TFF, the promoters of the three genes are methylated [250].

Current knowledge of basal activities largely derives from TFF3 promoter studies in transgenic animals. It has been found that high specific basal transcription of murine TFF3 is provided by 6.35 kb of the promoter and that goblet-cell specific transcription is partly conferred through a nine base pair ‘goblet cell responsive element’ present in the proximal promoter [266,267]. Adjacent positive and negative elements also contribute to this selective transcription [268]. Another element designated ‘goblet-cell silencer inhibitor’, located further upstream of the transcriptional start site, enables human and murine goblet cell-like cell lines to override the silencing effect of more proximal elements. Goblet cells but not non-goblet cells possess a nuclear protein that binds to the ‘goblet-cell silencer inhibitor’ regulatory element.

Analysis of human and murine TFF genes has also led to the identification of consensus binding sequences of known transcription factors: HNF-3, a member of the forkhead or winged helix transcription factors, is activated during the acute-phase response after injury. The consensus HNF-3’ binding motif lies within 100 base pairs of the transcriptional start site of all three human and murine TFF genes [250,269]. The GATA transcription factors are zinc finger proteins and constitute a family of six related members. Consensus binding sites for GATA transcription factors are present in TFF promoters and indeed GATA 6 expression resulted in activation of TFF1 and

TFF2, but not TFF3 [270]. Furthermore, the promoters of the three trefoil genes contain binding sites for the helix-loop-helix leucine zipper USF, in a region known as the E-box [270]. USF proteins are associated with growth inhibition, as are certain TFF peptides, suggesting that activation of USF by certain endogenous stimuli may contribute to inhibition of cell proliferation.

Furthermore, it has been shown that low concentrations of trefoils can stimulate their own release as well as that of the other family members by binding cis-regulatory elements of their respective promoters. This cross-induction is dependent on the ERKs/MAPKs 1 and 2 and requires epidermal growth factor (EGF) receptor phosphorylation [271].

Considering their up-regulation during inflammation, it is not surprising that TFF peptides can be regulated by pro-inflammatory cytokines. Both IL-1β and IL-6 are important regulators of trefoil gene expression. Dossinger *et al.* showed that these cytokines repress TFF promoter activity and gene expression synergistically via inhibition of NF-κB and C/EBPβ factors, respectively [272]. Like IL-1β and IL-6, TNF also negatively regulates TFF3 gene expression in a NF-κB dependent fashion [273]. On the other hand, *in vivo* data, using mice with a defective IL-6/IL-11 signaling receptor gp130, have demonstrated that TFF1 gene expression is positively regulated by IL-6 via SHP2/ERK/AP-1 activation [274]. Furthermore, IL-6 signaling via STAT3 strongly induces TFF3 [274]. These apparently conflicting data may simply reflect the complexity and timing of initiation of IL-6, IL-1β and TNF signaling cascades.

A role for the anti-inflammatory Th2 cytokines, IL-4 and IL-13, in TFF2 induction has first been shown in the lung. TFF2 is regulated by these cytokines in a STAT6 dependent fashion during the acute phase and by IL-4 in a STAT6 independent fashion during the chronic phase of experimental asthma [275]. In the gut, the goblet cells are an important target for these Th2 cytokines. Furthermore, the induction of both TFF3 and mucin 2 by IL-4 and IL-13 in a STAT6 dependent fashion was shown in colon cancer cell lines [276]. The TFF3 gene has a STAT6 binding site, which suggests direct regulation by IL-4 and IL-13 [276].

3.2.2 TFF structure

The trefoil factor family peptides all possess a characteristic three-loop structure (the trefoil domain). This trefoil domain is defined as a sequence of 38 or 39 amino acid residues wherein three disulfide linkages occur between 6 paired cysteine residues

to form the three loops. This structure is essential for the protease resistance of the TFF in the gut milieu [277].

In vivo, the predominant form of each TFF comprises two trefoil domains that are formed either through inter-chain disulfide bonding through a seventh cysteine residue, as occurs in TFF1 and TFF3, or by genomic duplication of the trefoil domain, as in TFF2. Recombinant TFF1 and TFF3 can thus be produced both as monomers or disulphide-linked dimers, whereas TFF2 consist of two trefoil domains in its monomeric form. Dimerization of TFF1 and TFF3 is necessary for some but probably not all TFF functions.

3.2.3 TFF receptor and signaling

Despite numerous reports on the signaling cascades triggered by the TFF, convincing evidence for a TFF-receptor or binding partner, unambiguously shown to take part in any of the known TFF functions, is still lacking. TFF3 has been shown to elicit a luminal chloride secretory event *in vitro* only when applied to the basolateral site of epithelial monolayers [278]. *In vivo* it has been demonstrated that intravenously infused radioactive labeled TFF2 binds to mucus producing cells of the rat stomach, a binding that could be displaced by unlabeled TFF2 in a dose-dependent matter [279]. Furthermore transcriptional regulation [280], cell motility [281,282] and other effects are all rapid events following TFF stimulation. These and many other reports strongly suggest that cellular responses to TFF peptides are likely mediated by a receptor-ligand interaction. Because TFF peptides are able to phosphorylate the EGF-receptor, it was considered that they are an alternative ligand for this receptor. However, no TFF-binding to the EGF-receptor could be demonstrated until now. Several TFF-binding proteins have been described, including the small-intestinal CRP-ductin (muclin), which binds TFF2 and the gastric proteins TFIZ1 (TFF1-binding) and blottin (TFF2-binding). But binding to these proteins does not lead to activation of signal transduction pathways or downstream target activation. Therefore, they cannot be considered as TFF-receptors.

Multiple signaling pathways appear to be linked to the biological actions of the TFF including the PI3K/Akt pathway, the Rho-ROCK cascade, COX-2/TXA2-R/Gαq signaling, PLC/PKC, MAPK and EGF-receptor signaling [283]. Nuclear magnetic resonance spectroscopy revealed that human trefoils have significant structural and electrostatic differences, which suggests that each trefoil peptide has a specific target or group of target molecules [284]. This finding is consistent with other data that suggest different functions for the structurally similar TFF peptides.

3.2.4 TFF functions

Although the integrity of the mucosa is dependent on the presence of a monolayer of epithelial cells joined by tight junctions, it is the mucus layer on the apical surface of this population that occupies the real interface between lumen and mucosa. This mucus layer is a viscous elastic coat, formed mainly by the large molecular weight mucin glycoproteins that are secreted by intestinal goblet cells or mucous cells in the stomach. TFF peptides are co-secreted with mucins and are intimate constituents of mucus gels. It is therefore widely accepted that the TFF plays a key role in maintenance of the surface integrity of mucosal epithelia.

There is some evidence to suggest that TFF peptides are involved in mucus polymerization. Some mucin regions are only sparsely glycosylated and these domains are shown to be responsible for oligomer formation and possibly for trefoil binding [285]. TFF peptide interaction with mucin probably influences the viscosity of the mucus. As each of the mucous epithelia secretes its own characteristic mucin/TFF combination, this could be a physiological mechanism to provide the optimal viscosity of the biopolymer on a specific epithelium. Furthermore, it has been suggested that the interaction between TFF2 and mucins inhibits, both *in vitro* and *in vivo*, proton permeation through the mucus layer [286] and a change in viscosity was observed when TFF2 was added to stomach mucin [287]. Another report shows enhanced protection of human T84 colon cancer cell monolayers from several insults when incubated with TFF3 or TFF2 in combination with human colonic mucin, compared to TFF3 or TFF2 alone [252].

The functional integrity of the gastro-intestinal mucosa is maintained through the constant renewal of the epithelium. In addition, the epithelium is constantly exposed to a wide spectrum of potentially injurious factors (pathogenic bacteria and their products, toxic dietary factors, etc.), which can induce mucosal damage. One of the first steps in mucosal wound healing is a shift in cell shape towards a specialized migratory phenotype, whereby cell-cell contacts are reduced, the cytoskeleton reorganizes and cells acquire a flattened appearance (cell spreading). The next key step is migration of the cells in order to cover the denuded basal lamina. In a final step, the monolayer of flattened cells re-establishes tight junction structure and cell polarity and thus barrier function is restored. This initial phase, which does not require cell proliferation is termed restitution and appears sufficient to reseal superficial injuries *in vivo.* Fast repair is required to restore epithelial continuity, limit fluid and electrolyte loss and prevent mucosal inflammation. When mucosal damage

extends deeper and requires regeneration, additional repair steps including angiogenesis and proliferation will take place within days. The final, often overlooked, stage is remodeling which can take months and is necessary to regain a normal-looking mucosa.

In contrast to other epithelial growth factors, TFF expression is rapidly up-regulated in response to mucosal injury [280,288]. All three TFF peptides have been shown in a variety of *in vitro* models to be involved in the different steps of restitution and regeneration particularly by modulating cell-cell contacts, cell migration, apoptosis and angiogenesis although the understanding of mechanisms through which the TFF peptides promote restitution is still incomplete. Consistent with all this, colonic restitution is absent in TFF3-null mice, resulting in lethality of even minor colonic injury [289]. Indeed, of the many proteins that have been described to augment and induce restitution, TFF3 is the only factor that has been shown to be essential [289].

Cell-cell adherens junctions are established and maintained by the adhesion molecule E-cadherin. The intracellular domain of E-cadherin binds directly to β-catenin and through association with α-catenin, linkage with the cytoskeleton occurs. TFF3 has been shown to induce phosphorylation of β-catenin [290], thereby disrupting adherens junctions. Furthermore, TFF3 also reduces E-cadherin, α- and β-catenin levels, leading to increased motility [291]. One report described Van Gogh like protein 1 (Vangl1) as a downstream factor required for TFF3-induced migratory response in intestinal epithelial cell lines (IEC) [292]. In these IEC, Vangl1 protein normally co-localizes with E-cadherin at the cell membrane and this association decreases by increased Vangl1 phosphorylation after TFF3 stimulation.

In contrast to other motogens, TFF2 and TFF3 are non-mitogenic when added to a variety of IEC [282]. However, in an *in vitro* wounding model, the addition of TFF2 or TFF3 resulted in a three- to six-fold increase in the rate of epithelial migration in the wound. Furthermore, the actions of TFF peptides as motogenic factors have been shown to be synergistic with EGF [281] and, in contrast to cytokine and growth factor mediated migration, independent of TGFβ [282]. TFF2 and 3 also rapidly activate intracellular signal transduction pathways and motogenic activity has been shown to depend on ERK 1 and ERK 2 activation [293,294]. The exact mechanisms downstream of ERK activation involved in migration still have to be elucidated.

As detachment of cells from cell-cell and cell-substratum contacts normally results in apoptosis (anoikis), there must be a mechanism that protects cells from dying during migration. Numerous studies on the anti-apoptotic effect of TFF peptides are in

agreement with their motogenic function during restitution [293,295-299]. TFF1 was found to protect cells from anoikis, chemical- or Bad-induced apoptosis by partially or completely blocking caspase-3, -6, -8 and -9 activities [295]. TFF2 reduces apoptosis induced by serum starvation and anoikis in the breast cancer cell line MCF-7 The anti-apoptotic effect of TFF3 has been reported to require intact TFF3 dimer, EGF-receptor activation and the PI3K pathway [293,299]. Another group described inhibition of anoikis by TFF3 via a PI3K/Akt/NF-κB pathway [297].

Restitution *in vivo* is dependent upon continuous mucosal blood flow and angiogenesis is a typical process observed when mucosal damage extends deeper than the superficial epithelium. Consequently, the pro-angiogenic activity of the TFF [300] perfectly supports restitution, particularly during the later stages of remodeling. Whereas all three TFF peptides act as pro-angiogenic factors through COX-2 and EGF-receptor dependent pathways, expression of vascular endothelial growth factor (VEGF) could also be induced by TFF3 via Src and activation of STAT3. Furthermore TFF3 is involved in NO formation through iNOS. NO is an endothelial growth factor important in inflammation and tumor vascularization [301].

3.2.5 TFF modulating the immune system

Trefoils are strongly induced after epithelial damage and facilitate repair processes by stimulating cell migration, inhibiting apoptosis and reducing antigen access to the healing epithelium by augmenting the barrier function of the mucus, as explained in the previous section. Since they are regulated by both pro-inflammatory and anti-inflammatory cytokine expression (see 3.2.1), an additional role for these peptides in regulation of immune responses can be postulated.

Both TFF2 and the monomeric form of TFF3 are found to be expressed in rat spleen and thymus and, to a lesser extend, in lymph nodes and bone marrow [259]. The expression levels are much lower than in the stomach and intestine, but LPS treatment could transiently induce higher TFF levels in the spleen. TFF1 mRNA was also detected in mouse spleen [302,303] and thymus [303]. At present, the function of trefoil peptides in lymphoid organs has not been fully elucidated. Since Cook *et al* co-localized TFF in spleen to cells with a plasma-like phenotype, they hypothesized that trefoils interact with immunoglobulins that are, like mucins, variably glycosylated. However, this interaction could not be confirmed [259]. A more recent study in $TFF2^{-/-}$ mice demonstrates that TFF2 deficiency is associated with increased cytokine secretion by macrophages and enhanced proliferative responses of splenic T cells in response to IL-1β [303].

Migration of various leukocyte subpopulations from the bloodstream into inflamed tissues or recirculation into lymphoid tissues is controlled by adhesion receptors and chemo-attractants, which direct them to the site of injury. The cell adhesion molecules and integrins are among the first adhesion molecules acting in this process. They aid in the docking of leukocytes to endothelial surfaces and participate in leukocyte rolling. GlyCAM-1, VCAM-1 and MAdCAM-1 are glycoproteins that may be classified as mucins on the base of their O-glycosylation pattern and proline-serine- and threonine-rich domains [304]. Whether or not the trefoils interact with these mucin-like glycoproteins to facilitate leukocyte adhesion and migration remains to be elucidated. However their induction by LPS in immune organs and the finding that both recombinant human TFF2 and TFF3 stimulate human monocyte migration at concentrations similar to those found in gastro-intestinal secretions [259], suggest a potential role for the trefoil peptides in the immunological response to tissue injury by regulating leukocyte recruitment. On the other hand, TFF treatment reduced the expression of the cell adhesion molecule VCAM-1 in colonic endothelium in an *in vivo* model of colitis [305]. Reduced VCAM-1 expression could thus lead to reduced infiltration of inflammatory cells at the site of infection, which would ameliorate inflammation.

Another important regulator of gut inflammatory responses is NO. Acute production of NO via the constitutively active nitric oxide synthase (cNOS) has beneficial effects on the mucosa by increasing gut blood flow and releasing local repair mediators [306,307]. Conversely, chronic production of NO, driven by iNOS and produced by infiltrating macrophages and neutrophils [307], leads to the production of reactive nitrogen species, including peroxynitrite and NO_2 [308]. These free radical species are highly damaging and induce further inflammation, leading to ongoing tissue injury [309]. Giraud *et al.* reported that the increased TFF2 concentrations induced in epithelial damage and inflammation might limit tissue damage in part by inhibiting this chronic NO production. *In vitro*, TFF2 indeed reduced LPS-induced NO and iNOS expression in monocytes [310]. This was verified *in vivo* in a model for experimental colitis, where TFF2-treated rats show less nitrated protein levels and a significantly reduced infiltration compared to control-treated mice. Thus, TFF2 can reduce inflammation by inhibiting inducible NO production, thereby likely down-regulating reactive nitrogen species and downstream pro-inflammatory targets.

TFF2 deficient mice show a mild phenotype but are more susceptible to nonsteroidal anti-inflammatory drug-induced colitis [311]. More evidence for an immunoregulatory role of TFF2 came from recent microarray analysis studies with these knockout mice

[312]. Out of 12,000 analyzed genes, 128 genes had significantly modulated expression when compared to wild-type mice, and the majority was implicated in immune regulation. The most prominent up-regulated genes belong to the cryptdin family (mouse orthologues of the human α-defensins), which have been shown to play a crucial role in innate immunity [313]. In addition to these antimicrobial peptides, another Paneth cell product was found to be up-regulated namely CRIP, which has a significant role in the regulation of cytokine balance and the immune response [314]. The other group of immune response-relevant genes whose expression was altered in the setting of TFF2 deficiency includes genes involved in MHC I presentation such as the immunoproteasomal subunit genes LMP2 and LMP7, BAG2, a member of the BAG family regulating chaperone activity, and the TAP1 transporter of peptides. Furthermore Cathepsin C, important for MHC II presentation was also up-regulated in intestine of $TFF2^{-/-}$ mice. In summary, TFF2 deficiency causes specific modulations of immune system relevant genes, demonstrating an indirect interplay of TFF2 with the immune response.

Indirect evidence for TFF3 in regulating immune responses also exists. First, TFF3 can induce the expression of decay accelerating factor in IEC [315]. This protein blocks complement activation and has also been described as a negative regulator of T cell immunity [316]. Also in IEC, it has been shown that TFF3 not only induces activation of NF-κB but also expression of TWIST, a negative regulator of NF-κB activity. Consequently, up-regulation in TFF3-treated IEC is only transient and probably results in an anti-inflammatory response since this NF-κB activation is not associated with IL-8 induction [317]. Furthermore TFF3 can induce COX-2, a key enzyme for the biosynthesis of prostaglandins both *in vitro,* in IEC, and *in vivo* in a mouse model of acute DSS-induced colitis [297,318,319]. COX-2 is normally expressed at a low or even undetectable level in unstimulated cells or tissues but is rapidly induced by cytokines, mitogens and other inflammatory stimuli. Moreover, the cytoprotective PGE_2 and PGI_2 are synthesized by COX-2 in the gastro-intestinal tract after inflammation. In IEC the TFF3 dependent up-regulation of COX-2 also leads to PGE_2 and PGI_2 production. The effects of these prostaglandins are pleiotropic. Next to the role of PGE_2 in the promotion of epithelial cell restitution and maintenance of the intestinal barrier function in the gastro-intestinal tract [320,321], the anti-inflammatory effects of this molecule on DCs have been extensively documented [322-325]. Furthermore PGE_2 is also able to inhibit NF-κB in macrophages thereby preventing iNOS protein expression [326]. All these data strongly suggest at least an indirect role for TFF3 in immune regulation.

4 *L. LACTIS* AS A DELIVERY TOOL IN THE GASTRO-INTESTINAL TRACT

Lactococcus lactis is a member of the Lactic Acid Bacteria (LAB), a taxonomically diverse group of gram-positive bacteria that share the property of converting fermentable carbohydrates primarily to lactic acid, thus acidifying the growth medium. Members of the LAB group are best known for their use in food industry, mainly for the preparation of fermented food and feed products such as preparation of dairy products and meat. One intrinsic advantage of food-grade LAB therefore lies in the knowledge that they have never been associated with any pathogenic effects. Even when given overt opportunity, as would be the case following LAB consumption during an ongoing intestinal disease, food-grade LAB display no health risk. Therefore, several LAB species have been granted a “Generally Regarded As Safe” (GRAS) status by the US Food and Drug Administration.

Many LAB species are members of the resident microflora of the gastro-intestinal tract of vertebrates. Over the past decades interest in the study of LAB has dramatically increased due to their application as so called “probiotics”, i.e. strains with particular health benefits to the host, next to their nutritional value [153]. The fact that selected probiotic strains, which may or may not be natural residents of the human gastro-intestinal tract, can influence the intestinal physiology through modulation of the endogenous flora or the immune system (see also 1.5.3) is presently well recognized. In particular members of the genera *Lactobacillus* and *Bifidobacterium* (this genus does not belong to the LAB group) have received much attention and their beneficial effect in reducing tissue damage in patients with IBD or in animal models for this disease is well documented [154-156].

L. lactis is a non-colonizing, non-invasive species that was originally isolated from cow’s udder. The species has been used in industry for many years in particular in the production of cheeses. Although some commercial formulations claim health-promoting properties, *L. lactis* is not generally considered a true probiotic strain. Mainly because it was the first LAB species to be characterized in great detail at the molecular and genetic level, *L. lactis* has enjoyed an increasing interest as a production host for heterologous proteins and in a later stage as *in situ* delivery vehicle of biologically active molecules.

4.1 Production and secretion of heterologous proteins

Many efforts have been made to better understand the molecular basis of LAB's technological properties and to obtain better control of industrial processes involving LAB. This knowledge has led to the investigation of their potential use for new applications, such as the production of heterologous proteins in bioreactors, in fermented food products or directly in the digestive tract of humans and other animals. *L. lactis*, as a model organism, can nowadays be genetically engineered quite efficiently. Many elaborate and highly effective genetic tools, i.e. well-characterized plasmids as well as an efficient transformation procedure, have become available over the last 15 years [327-329]. Moreover, the genomes of *L. lactis* ssp. *lactis* strains IL1403 and MG1363 are entirely sequenced [330,331]. Many expression- and targeting-systems have also been designed for *L. lactis* [332,333], allowing the intra- or extra-cellular production of a great variety of proteins of viral, bacterial or eukaryotic origins, and with molecular mass ranging from 9.8 kDa to 165 kDa (for review see [334]).

To produce a protein of interest in fermentors, secretion is generally preferred to cytoplasmic production because it allows continuous culture and simplifies purification. *L. lactis* has some characteristics that make it a very good candidate for heterologous protein secretion. First, relatively few homologous proteins are secreted by this bacterium, Usp45 (an unknown secreted protein of 45 kDa) being the most prominent one [335]. Second, laboratory strains do not produce any extracellular proteases and thus the secreted proteins are not prone to extracellular degradation, as would be the case in the natural industrial strains [336,337].

In general, the homologous Usp45 signal peptide has been most successfully used for high-level secretion of a variety of heterologous proteins. In a search for other homologous secretion signals a panel of signal peptides was evaluated for heterologous secretion, but none was found to be superior to the Usp45 signal peptide [338,339]. Notwithstanding this general observations, Lindholm *et al.* used a *Lactobacillus brevis* signal peptide for secretion of the *E. coli* FedF fimbrial adhesin by *L. lactis* and found it to be more efficient than the Usp45 secretion signal [340].

Not unexpectedly and in line with the observations in other bacterial species, high secretion efficiency most likely requires that (i) the composite precursor polypeptide assumes a configuration that allows efficient cleavage by the lactococcal signal peptidase, and (ii) the mature part of the heterologous protein is properly escorted by the secretion machinery. It is, therefore, not surprising that some heterologous

proteins are poorly, if at all, secreted [339,341]. Notably, charges at the N-terminal part of the mature moiety may greatly affect the translocation efficiency across the cytoplasmic membrane [342]. Le Loir and co-workers were first to show that the introduction of negative charges, trough the insertion of a nine-residue synthetic peptide in between the ultimate amino acid residue of the signal peptide and the first residue of the mature heterologous polypeptide, improved secretion efficiency and production yields of several heterologous proteins in *L. lactis* [343], an observation that was later confirmed by other workers for different proteins [344].

Our department was the first to obtain efficient synthesis and secretion of eukaryotic proteins in *L. lactis*. We used the Usp45 signal peptide to secrete bioactive cytokines, such as murine and human interleukins IL-2, IL-6 and IL-10, in quantities ranging from 50 µg (mIL-2) to 100 µg (mIL-6, mIL-10) per liter of culture [198,345-347]. The continuous secretion of these bioactive cytokines did not affect the growth rate of *L. lactis*. Cytokines in general display fairly simple structures. Their biological activity does not depend on complex glycosylation or other secondary modifications and in most cases, binding and activation of their receptors is straightforward. However, many of them critically depend on the correct formation of disulfide bridges in order to be biologically active. That lactococci are capable of correct disulfide bridge formation in the processing of heterologous proteins was proven by *in vitro* and *in vivo* bioactivity of recombinant mIL-2, mIL-6, mIL-10 and TFF peptides [198,318,346].

4.2 Local delivery of heterologous proteins

There exists a vast body of literature (over 2000 published papers) on the use of live bacteria, recombinant or not, as tools for vaccination via a non-parenteral route (recently reviewed in [348]). By far the most extensively explored are attenuated strains of pathogenic species belonging to the genera, *Salmonella, Shigella, Yersinia* and *Listeria*. Today, live vaccines based on attenuated *S. typhi* and *Vibrio cholerae* are available [349]. The development of bacterial vaccine vehicles carrying a heterologous gene is more problematic and none has yet reached the market.

Because of their GRAS status LAB are attractive for use as vaccine vehicles. However, LAB are non-invasive and the vaccine delivery to APCs may be less effective. Still, antigen specific immune responses have been obtained with several LAB (for reviews see [350,351]). Wells and co-workers were first to show that oral as well as intranasal inoculation with GM *L. lactis* that had accumulated intracellular

tetanus toxin fragment C (TTFC) led to the induction of high IgG serum titers and could protect mice against a lethal challenge with tetanus toxin [352-354]. A similar antibody titer was induced using dead or alive *Lactococcus* suggesting that *in situ* antigen synthesis is not essential [353]. In a recent study the efficacy of *L. lactis* as a vehicle for intestinal delivery of antigens for the induction of antigen specific peripheral tolerance was evaluated [355]. Therefore, OVA-immunized DO11.10 mice, which bear transgenic OVA-specific $CD4^+$ TCR, were fed with OVA-secreting *L. lactis*. The results suggest that this GM *L. lactis* strain induces OVA-specific tolerance through the induction of $CD4^+CD25^-$ Treg cells that exert their function in a TGFβ specific manner.

The first indication for the importance of active *in situ* delivery of a bioactive substance was obtained in a study, in which it was shown that intranasal administration of *L. lactis* that had accumulated intracellular TTFC and secreted either mIL-2 or mIL-6 produced significantly higher antibody titers than did the parental strain expressing TTFC alone [346]. The boosting effect was completely lost when the bacteria were killed prior to immunization, indicating that *in situ* secretion of the cytokines by viable bacteria is essential.

4.2.1 *Intestinal IL-10 delivery by genetically modified L. lactis*

As discussed before, IBD most likely occurs as a consequence of the breakdown of immune tolerance towards the intestinal microflora. IL-10 is a powerful anti-inflammatory cytokine and is a central factor in induction and maintenance of immune tolerance (see 3.1). Furthermore, the pivotal role played by IL-10 within the mucosal immune system is demonstrated by the chronic ileocolitis that develops in gene-targeted IL-10 knock-out mice [356]. This makes IL-10 a very attractive therapeutic for intestinal inflammation, which was confirmed in animal models of colitis [357-359]. The clinical results after systemic administration of recombinant IL-10 to IBD patients were however mostly disappointing with moderate side effects [196,360] and injection of high doses even lead to the systemic induction of pro-inflammatory IFNγ [197]. Therapeutic targeting at the site of inflammation, e.g. the intestine, could solve this problem but oral administration is hampered by the extreme acid sensitivity of IL-10 and, to date, no oral formulation of IL-10 is known.

Van Deventer and co-workers presented a novel method of IL-10 delivery to intestinal mucosal tissue by the use of *ex vivo* transduced $CD4^+$ T cells [361,362]. In these studies, the authors demonstrated that both human and mouse T lymphocytes could be engineered by retroviral transduction to express high levels of biologically

active IL-10 upon activation. In the classic $CD45RB^{high}$ model, these IL-10 transduced $CD4^+$ cells successfully prevented the development of colitis without interfering with systemic immune activation. Furthermore, the development of immune-suppressive Treg cells in the area of inflammation may be induced. This approach can thus be considered as a clinically viable approach to the treatment of CD.

Another alternative that requires fewer techniques and allows for oral treatment is the use of an *L. lactis* strain, constructed to secrete IL-10 [198]. The therapeutic effect of *L. lactis*-mediated delivery of IL-10 on inflammation of the colon was first evaluated in two murine models for IBD, (i) a model in which colitis is induced by chemical treatment, namely DSS and (ii) $IL\text{-}10^{-/-}$ mice in which colitis develops spontaneously [356]. Daily intragastric administration of these bacteria efficiently cured chronic DSS-induced colitis in mice (Figure 11A). The observed healing was comparable to systemic treatment with prominent anti-inflammatory drugs such as dexamethasone and anti-IL-12. When given systemically, recombinant IL-10 also decreases inflammation to the same level as IL-10 producing *L. lactis* but the amount of IL-10 required to achieve this effect is a 1,000-fold higher. This indicates that greatly improved IL-10 delivery has been achieved by using GM *L. lactis*. Daily treatment with IL-10 producing *L. lactis* also prevented spontaneous enterocolitis in $IL\text{-}10^{-/-}$ mice (Figure 11B). More recently the *L. lactis* IL-10 treatment was also validated in trinitrobenzene sulphonate (TNBS)-induced colitis [363].

Killing the IL-10 producing bacteria by UV-irradiation prior to inoculation abrogated their curative effects [356]. This shows that *in situ* production of IL-10 is an essential feature of the mechanism of action. Because of the localized production and since no IL-10 could be found in the systemic circulation one can speculate that side effects associated with systemic administration of IL-10 can be reduced or even avoided.

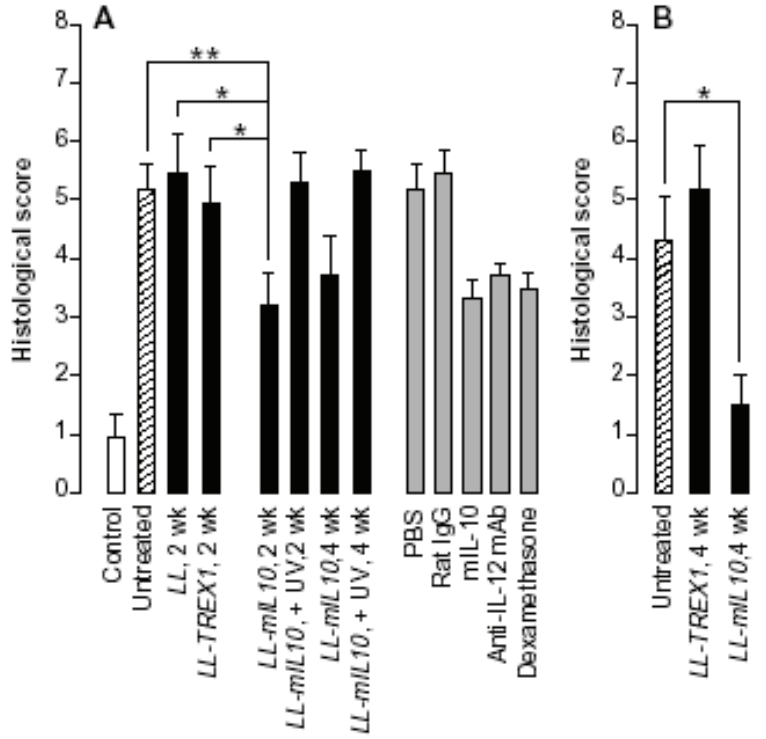

Figure 11 Treatment of murine colitis by *L. lactis* secreting IL-10 (*LL-mIL10*).
Bars represent the mean ± SEM. **P* < 0.025; ***P* = 0.0151. (**A**) Histological scores (sum of epithelial damage and lymphoid infiltrate, both ranging from 0 to 4) for the distal colon of groups (*n* = 10) of control female Balb/c mice (white bar) and of female Balb/c mice with DSS-induced colitis that were untreated (hatched bar), treated with the indicated *L. lactis* cultures (black bars), or treated with five daily intraperitoneal injections of the compounds indicated (gray bars) (mIL-10: 5 mg per mouse per day; anti–IL-12: 1 mg per mouse per day; dexamethasone: 5 mg per mouse per day; rat IgG: 5 mg per mouse per day). Mice treated daily for 2 or 4 weeks (wk) with 2×10^7 mIL-10 producing *LL-mIL10* showed significantly reduced inflammation when compared with untreated or control-treated (*LL* or *LL-TREX1*) mice. This effect was not observed when *LL-mIL10* cultures were UV-killed (+ UV). (**B**) Histological scores (sum of the degrees of inflammation in the proximal, middle, and distal colon, all ranging between 0 and 4) obtained after blinded interpretation of groups (*n* = 5) of 7-week-old untreated (hatched bar), *LL-TREX1*–treated, or *LL-mIL10*–treated female 129 Sv/Ev IL-$10^{-/-}$ mice (black bars). *LL-mIL10*–treated mice showed significantly less inflammation than untreated mice. Source: [198]

4.2.2 Intestinal delivery of TFF by genetically modified L. lactis

One of the characteristics that are similar between CD and UC is that they are relapsing diseases, characterized by silent episodes and acute active episodes. These active episodes of IBD are diagnosed as acute colitis that is mainly dominated by a prominent epithelial damage and an intense inflammation that is the result of the influx of luminal bacteria. In the case of first presentation of acute colitis in CD, the

inability to sense acute bacterial infection was shown to be a risk factor in the development of CD [167,168,364]. Therefore treatment of acute intestinal inflammation might be a means to prevent IBD, but the number of therapeutic strategies for acute colitis is rather limited.

TFF peptides, as epithelial healing factors (see 3.2), are promising tools for treatment of acute colitis that is characterized by extensive epithelial ruptures. There is strong *in vivo* evidence for protective and healing functions of various forms of mucosal injury by TFF peptides in the gastro-intestinal tract when delivered orally, subcutaneously or rectally [281,365-375]. Most studies indicate that systemic subcutaneous administration of TFF peptides reaching the basolateral site of the mucosa seems to be more effective than oral application, despite their extreme protease resistance. The main reason for this is that orally administered TFF peptides stick to the mucus and so become metabolically inert or are removed from the lumen at the caecum or small bowel [370]. Thus in the context of colitis, orally delivered TFF peptides do not reach the site of injury.

Topical and active delivery of TFF in the colon by localized synthesis from *L. lactis* provides an alternative approach to deliver trefoil peptides to the colonic mucosa and thus avoid the clinical disadvantages of rectal and intra-colonic administration [318]. Daily intragastric administration of the TFF-secreting strains, prior to or during disease induction, resulted in significant protection against DSS-induced acute colitis as observed by reduced mortality, reduced loss of body weight, substantial improvement of colon histology and the reduction of inflammatory infiltrate. The protective effect required *de novo* TFF synthesis by live *L. lactis*. Oral administration of high amounts of purified mTFF1 did not ameliorate acute colitis. Whereas rectal administration had some effect, it was much less effective than orally administered TFF-secreting *L. lactis*. Intimate basolateral contact between colon cells and *L. lactis*, as could occur following *L. lactis* transport by M cells or through ruptures in the epithelium, probably enable the TFF peptides to accumulate out of reach of complexing mucins and allow them to interact with the putative basolateral TFF receptors on enterocytes. Remarkably, *L. lactis*-mediated TFF treatment also ameliorated established chronic colitis in IL-10 knockout mice; a finding that broadens the potential therapeutic application of trefoil factors in IBD.

COX-2, a known target of TFF signaling [300,319], is strongly induced in the intestines of mice treated with TFF-secreting *L. lactis* (Figure 12). This finding proves that the bacteria produced TFF peptides that were biologically active *in situ* in the colon. Inhibition of COX-2 by meloxicam substantially abrogated the prophylactic

effect on acute DSS-induced colitis. This indicates that, although COX-2 is probably not the only TFF-induced factor that is involved, its up-regulation is important in prevention of colitis through *L. lactis*-mediated topical delivery of TFF [318].

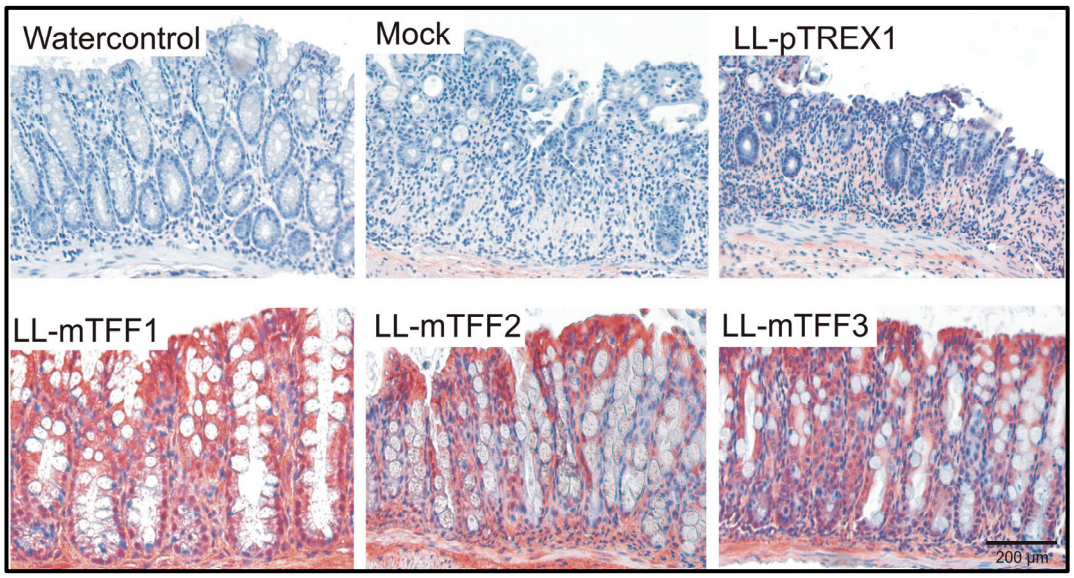

Figure 12 Induction of COX-2 expression by TFF-secreting *L. lactis*.
Representative immunohistochemical images for COX-2 expression in the distal colon of healthy control mice and mice with DSS colitis, either mock-treated or treated with the empty expression vector (LL-pTREX1) or the different TFF-secreting strains (LL-mTFF1, LL-mTFF2 and LL-mTFF3). Source: [318]

4.3 Biological containment of genetically modified *L. lactis*

When applicable in man, the technology of *L. lactis*-mediated topical delivery of therapeutic proteins may open a vast spectrum of new medical applications. The use of GM organisms in medicine, however, raises legitimate concerns on dissemination of antibiotic selection markers or transfer of the genetic modifications to other micro-organisms and on survival and propagation of GM organisms in the environment. There is therefore a need for an adequate containment system.

Biological containment systems have been designed to act in an active or a passive way. The former essentially provide control through the conditional production of a toxic compound whose expression is tightly controlled by an environmentally responsive element or suppressed by an immunity factor. Many examples of such systems, particularly in *E. coli*, have been described [376-379]. Although active

containment systems provide actual killing of the host, they have important drawbacks. Firstly these systems often involve the introduction of a large amount of foreign DNA. Secondly, most of these systems are plasmid borne and therefore prone to horizontal transfer to other micro-organisms.

Passive systems overcome these shortcomings. Here, growth is dependent on complementation of an auxotrophy or other gene defect, by supplementing either the intact gene or the essential metabolite. The *lacF* gene can be used as a selection marker in ∆*lacF L. lactis* [380]. A *supD* gene has been used as a selectable marker for plasmid maintenance to complement suppressible pyrimidine auxotrophs [381]. Passive systems, however, have the drawback that they are often bacteriostatic rather than bactericidal.

Thymidylate synthase is an essential enzyme in the synthesis of the DNA constituents thymidine and thymine. The choice of *thyA* as a target gene combines the advantages of both passive and active containment systems. “Thymine less death” was described as early as 1954 [382] and involves activation of the SOS repair system and rapid DNA fragmentation, thereby essentially constituting an indigenous suicide system. Thymine and thymidine growth dependence is intrinsically different from other auxotrophies in that lack of the essential component is bacteriocidal rather than bacteriostatic [383]. Genetic exchange of the chromosomal thymidylate synthase gene *thyA*, for a gene of interest, human IL-10, thus provides a robust means for inheritable growth control of engineered *L. lactis* [200]. The resulting GM *L. lactis* strain Thy12, developed in our department, is strictly dependent on the presence of thymidine or thymine for its growth. Thy12 provides a satisfactory solution to concerns about biosafety for several reasons. First, only the absolute minimal amount of foreign DNA - the gene of interest, i.e. hIL-10 - is present in the GM organism and no resistance marker is required to guarantee stable inheritance of the transgene. Second, accumulation of the GM organism in the environment is very unlikely, as rapid death occurs upon thymidine starvation. In addition, no acquisition of *thyA* from other micro-organisms could be observed [200]. Third, whenever the intact *thyA* is acquired by homologous recombination, the transgene would be removed. Fourth, the risk of disseminating the genetic modification through lateral gene transfer is minimized because the gene of interest is integrated in the *L. lactis* chromosome. Furthermore, a number of mechanisms for lateral gene transfer are disabled in the Thy12 parental strain *L. lactis* ssp. *cremoris* MG1363. The strain has been cured of all natural resident plasmids [384]. It lacks a host factor required for conjugative transposition and also phage replication is

severely impaired, thereby disabling phage-mediated transduction of host genetic material [385,386]. This approach thus provides a simple and robust system for biological containment.

Based on the above-described characteristics of *L. lactis* Thy12, the Medical Ethical Commission of the Academic Medical Center and the Dutch Administration of Public Health, Environment and Nature approved a limited clinical trial with this bacterium, in Crohn's patients under physical containment. This Phase I, open label clinical trial demonstrated that treatment of humans with viable *L. lactis* secreting hIL-10 is clinically safe and biologically contained, and gave indications of its clinical efficacy. In total 10 patients with moderate to severe CD were included in the study and a clinical benefit was observed in 8 of them: five patients went into complete clinical remission and three patients showed a clinical response [199].

AIM OF THE PROJECT

Aim of the project

DCs are bone-marrow derived professional antigen-presenting cells with immuno-regulatory functions. In the past years, it has become clear that DCs are likely to play a central role in the mucosal immune response to intestinal flora [139,140,144,152,387]. DCs are present in the intestine in the GALT and the lamina propria and lie in close proximity to the large and dynamic antigenic load in the gut lumen. Antigenic material may interact directly with DCs in underlying tissues. IBD is caused by a loss of tolerance against the normal enteric flora, due to a dysregulated mucosal immune response and/or a defective mucosal barrier function [164,388]. This has elicited a closer examination of the role of the DC as a central mediator in IBD and recent studies suggest a role for activation of intestinal DCs in the initiation and continuation of IBD [175-178]. Interrupting the activation of DCs *in vivo* may be a key to controlling the disease.

The potential of genetically engineered *L. lactis* for specific therapeutic applications through *in situ* delivery of immunomodulatory proteins at the intestinal mucosa has previously been described [198,318,346]. In this study we focus on *L. lactis*-delivered mIL-10 or mTFF3. Both strains have healing effects in mouse models for chronic and/or acute colitis but immunological processes of the *L. lactis*-delivered proteins mIL-10 and mTFF3 that regulate the therapeutic effects are only partially understood [198,318,363]. We suggest that intestinal DCs are attractive targets for the recombinant proteins delivered by the orally administered *L. lactis* bacteria.

We used an *in vitro* model to study modulation of DC function. For this, murine bone marrow cells were isolated and cultured with IL-4 and GM-CSF to generate DCs [389,390]. In some respects, DCs generated *in vitro* do not show the same behavior or capability as DCs isolated *ex vivo*. Nonetheless, they are often used for research, as they are still much more readily available than genuine DCs that constitute a heterogenous population, sparsely distributed throughout the body.

The TFF peptides are mainly known for their epithelial repair function [289,366,367]. Their possible immune-modulating properties were investigated only recently [259,303,391]. In a first part of our study, we therefore explored the effect of recombinant mTFF3 on DCs. We aimed to investigate whether mTFF3 could directly regulate DC function by determining its effect on LPS-induced maturation of bone marrow-derived DCs (BM-DCs). For this, immature BM-DCs pretreated with recombinant mTFF3 were stimulated with LPS and compared to non-treated LPS-stimulated BM-DCs. We studied phenotype, cytokine secretion pattern and

T cell-stimulating capacity of the BM-DCs. Because mTFF3 is strongly expressed in the intestinal tract, we also investigated whether mTFF3 preferentially regulates mucosal DC function. Mucosal DCs from PP and MLN were compared to peripheral DCs isolated from spleen.

The anti-inflammatory cytokine IL-10 is a well-known immune-modulator with inhibitory effects on maturation and cytokine production of immature DCs [210]. Furthermore, treatment of immature DCs with IL-10 leads to the induction of regulatory T cells, T cell tolerance or antigen specific T cell anergy [123-125,127]. In the second part of this work, we wanted to investigate the effect of *L. lactis* secreting murine IL-10 on DC function. Therefore, we determined the maturation phenotype, cytokine secretion profile and allogeneic T cell activating capacity of *in vitro* derived murine BM-DCs treated with the mIL-10 secreting *L. lactis* or treated with a *L. lactis* control strain. LPS-stimulated BM-DCs were used as control for maturation. Also, we were interested in the effect of this mIL-10 secreting strain on subsequent LPS-induced maturation of BM-DCs. Given the described effects of IL-10-treated DCs on the induction of regulatory or anergic T cells, we also aimed to study the induction of possible changes in T cell polarization by the BM-DCs treated with *L. lactis* secreting mIL-10.

PART II: EXPERIMENTS AND RESULTS

1 MURINE TREFOIL FACTOR 3 DOES NOT DIRECTLY MODULATE LPS-MEDIATED DENDRITIC CELL FUNCTION

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1.1 Abstract

Peptides of the trefoil factor family (TFF) are expressed along the gastro-intestinal tract. They protect mucous epithelia from damage and contribute to mucosal repair, which is essential for preventing inflammation. Moreover, it has been suggested that TFF2 and TFF3 in particular play a role in regulating immune responses. Depending on their activation status, dendritic cells (DCs) can initiate either tolerance or immunity. This study, by comparing LPS-induced maturation of mTFF3-treated DCs and non-treated DCs, aimed to investigate whether murine TFF3 directly regulates DC function. mTFF3-treated DCs and non-treated DCs did not differ phenotypically or functionally. Both populations expressed, both before and after LPS stimulation, similar levels of co-stimulatory molecules and cytokines, and were both efficient stimulators of T cells. Our results suggest that mTFF3 does not modulate immune responses on the level of DC function.

1.2 Introduction

The trefoil factor family (TFF) encompasses three small (7-12 kDa) protease resistant proteins with a characteristic three-loop structure (the trefoil domain), formed by three conserved cysteine disulfide bonds. TFF1 and TFF3, formerly called pS2 and intestinal trefoil factor respectively, contain a single trefoil domain and occur as homodimers. TFF2, formerly called Spasmolytic Polypeptide, is a monomer containing two trefoil domains (for review, see [392]). Under normal circumstances,

TFF peptides are expressed in several tissues, but are most abundant in the gastro-intestinal tract, where they are secreted onto the mucosal surface in a tissue-specific manner [392].

The TFF plays an important protective role by promoting restitution and repair of epithelial cells [289,366,367]. In contrast to other epithelial growth factors, TFF expression is rapidly up-regulated in response to mucosal injury [280,288]. *In vivo* studies have suggested that TFF peptides play a major role in protecting the intestinal mucosa from various insults [318,373,374,393]. Mechanisms that mediate these responses are unique and only partially understood. Despite extensive studies no receptor has yet been identified [394].

Recent publications propose a role for TFF in regulating immune responses [259,391]. Both TFF2 and the monomeric form of TFF3 are expressed in rat lymphoid tissue [259]. The expression levels are much lower than in the stomach and intestine, but LPS treatment can transiently induce higher levels in the spleen. Also, both recombinant human TFF2 and TFF3 are chemotactic for human monocytes [259]. In addition, TFF2 has been shown to modulate the expression of several immunoregulatory genes, which are important for MHC I and MHC II presentation, such as cathepsin C, the immunoproteasomal subunit genes LMP2 and LMP7, and the TAP1 transporter [391]. Furthermore, in intestinal epithelial cells (IECs) TFF3 can induce the expression of decay accelerating factor, which blocks complement activation and can act as a negative regulator of T cell immunity [315,316]. In IECs it has also been shown that TFF3 not only induces activation of NF-κB but also expression of TWIST, a negative regulator of NF-κB activity. Consequently, NF-κB up-regulation in TFF3-treated IECs is only transient and probably results in an anti-inflammatory response [317]. Finally, we demonstrated a potential immune-regulatory function of TFF, in that intestinal delivery of TFF cures Th1-mediated colitis [318].

Dendritic cells (DCs) are bone marrow-derived professional antigen-presenting cells connecting innate and adaptive immunity [395,396]. Depending on the DC subset, its activation status or environment (mucosal versus peripheral [100,397]), the interaction between DC and naive T cell will lead to T cell priming towards either immunity or tolerance. This results in the emergence of T cells carrying a Th1 or Th2 phenotype, T cell anergy or regulatory T cells (Treg cells).

Although a role for TFF in modulating immune responses has been suggested, there is no conclusive data showing that TFF has a direct role in regulating cells of the

immune system. Therefore, we investigated whether murine TFF3 (mTFF3) can directly regulate DC function by determining its effect on the maturation of bone marrow-derived DCs (BM-DCs). Because BM-DCs are likely different from *in vivo* DCs, and mTFF3 is strongly expressed in the intestinal tract, we also investigated whether mTFF3 regulates mucosal DC function. Mucosal DCs from Peyer's patches (PP) and mesenteric lymph nodes (MLN) were compared to peripheral DCs isolated from spleen. Our results suggest that mTFF3 had no direct effect on LPS-induced maturation of BM-DCs or *ex vivo* isolated mucosal and peripheral DCs. Furthermore, mTFF3 did not alter the pattern of cytokines secreted by the different DC populations, or the DC's capacity to activate naive T cells.

1.3 Materials and Methods

1.3.1 Animals

Balb/c ($H2K^d$) and C57BL/6 (B6; $H2K^b$) female mice, 6-8 weeks old were purchased from Charles River, Italy. They were housed in a specific pathogen-free animal facility and treated according to the institutional guidelines of Ghent University.

1.3.2 Media, reagents and antibodies

Complete medium is RPMI-1640 medium supplemented with 10% heat inactivated FCS, L-glutamine, nonessential amino acids, sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol (all from Gibco BRL/Invitrogen, Gaithersburg, MD, USA).

The gene encoding murine TFF3 [398] was inserted into a yeast expression plasmid and the dimer form of TFF3 was expressed and purified essentially as previously described for human and rat TFF3 [399]. Purified murine TFF3 is routinely being analyzed by amino acid sequence analysis and mass spectrometry. The purity was determined by analytical HPLC and quantified by amino acid analysis. These analyses prove purity and presence of the dimer.

Purified LPS (*Escherichia coli* serotype O111b4) was obtained from Sigma-Aldrich, St. Louis, MO, USA. Recombinant murine IL-10 (mIL-10), mGM-CSF and mIL-4 were purchased from Peprotech, Rocky Hill, NJ, USA.

Monoclonal antibodies (mAbs) used for flow cytometry were hamster anti-mouse CD11c (clone HL3; FITC-conjugated), rat anti-mouse CD80 (clone 16-10A1; PE-conjugated), rat anti-mouse CD86 (clone GL1; PE-conjugated), rat

anti-mouse CD40 (clone 3/23, PE-conjugated), rat anti-mouse PD-L1 (B7-H1; CD274) (clone MIH5, PE-conjugated), and rat anti-mouse IA^d (clone AMS-32.1, PE-conjugated). All mAbs were from BD Pharmingen, San Diego, CA, USA. Isotype controls used were FITC-conjugated Armenian Hamster IgG1 (anti-TNP) and PE-conjugated rat IgG2a/IgG2b κ from BD Pharmingen. Before cell-staining, non-antigen-specific binding of IgG to the mouse Fcγ receptors was blocked with a rat anti-mouse CD16/CD32 mAb (Fc block; BD Pharmingen).

1.3.3 Bioassay for mTFF3

The murine cancer cell line with rectal epithelial phenotype CMT-93 was obtained from the American Type Culture Collection. Cells were maintained in DMEM supplemented with 10% FCS, 4 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin in 5% CO2 at 37°C.

CMT-93 cells were plated in 35 mm wells of a six-well culture plate and grown to confluence. Fresh medium with different concentrations of mTFF3 (0-10 μg/ml) was added to the cells. After 4 h cells were washed with PBS and total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Westburg BV, The Netherlands), with on column DNase treatment (Qiagen). One μg of total RNA was converted to single stranded complementary DNA by reverse transcription (Superscript, Gibco) with oligo dT priming. Subsequently, real time PCR for COX-2 was performed using the SYBR green kit (Eurogentec, Seraing, Belgium) and 300 nM of each primer. A two-step program was run on the iCycler (Biorad Laboratories, Hercules, CA, USA). Cycling conditions were 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Melting curve analysis confirmed primer specificities. All reactions were run in triplicate and normalized to ribosomal protein L13A (RPL13A) levels. RPL13A was chosen after checking the expression stability of a set of housekeeping genes in CMT-93 cells using the Genorm software [400]. The level of the normalized COX-2 mRNA is shown relative to the control sample without mTFF3. Data are expressed as mean ±SD. Primers were designed using the Beacon Designer software (PREMIER Biosoft International, Palo Alto, CA, USA). For murine COX-2 (reference sequence NM_011198) forward and reverse primers were 5'-AACAAAAGCTTCTACAAAGGAACTAA-3' and 5'-CCAGCACAAAACCAGGATCA-3' respectively. For murine RPL13A (reference sequence NM_009438) forward and reverse primers were 5'-CCTGCTGCTCTCAAGGTTGTT-3' and 5'-TGGTTGTCACTGCCTGGTACTT-3'.

1.3.4 Generation of bone marrow-derived DCs

A modified version of a published procedure was used to generate BM-DCs [389,390]. Briefly, bone marrow cells from femurs and tibias of Balb/c mice were depleted of red blood cells by lysis with ammonium chloride. Granulocytes, erythroid precursors, and B cells were killed by labeling with a cocktail of mAbs (anti-Gr-1, anti-TER-119/erythroid cells, and anti-CD45R/B220; all from BD Pharmingen), followed by incubation with low-toxicity rabbit complement (Cedarlane, Ontario, Canada). These cells were cultured in complete medium supplemented with 1000 U/ml mGM-CSF and 500 U/ml mIL-4. Medium was replaced on days 2 and 4. On day 6, nonadherent cells were removed, and fresh medium with cytokines was added. Two days later 60 to 90% of the new population of nonadherent cells were $CD11c^+$ BM-DCs. DCs were further purified to >90% using anti-CD11c magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified DCs were re-plated at 1×10^6 cells/ml and pre-incubated at 37°C for 6 h with different concentrations of mTFF3 ranging from zero to 250 µg/ml or with 10 ng/ml mIL-10 (positive control) in complete medium. For maturation, 10 ng/ml LPS was added after 6 h and cells were incubated overnight at 37°C. Supernatants were collected and stored at -70°C. Cells were harvested, washed, counted and added at the appropriate concentration for subsequent analysis.

1.3.5 Preparation of ex vivo DCs

DCs were prepared from spleen, MLN and PP of 20 naive Balb/c mice. To remove epithelial cells, PP were treated for 60 min at 37°C with media containing 145 µg/ml dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA), 25 mM HEPES (Sigma-Aldrich), 10% FCS (Gibco), 5 mM EDTA (Sigma-Aldrich) and 2.5 mM β-mercaptoethanol (Gibco) in HBSS (Sigma-Aldrich). Then they were washed extensively with HBSS. Spleens, PP and MLN were digested with collagenase (1 mg/ml, Sigma-Aldrich) and DNase (2 mg/ml, Boehringer Mannheim, Germany) for 45 min at 37°C and incubated in the presence of 5 mM EDTA at 37°C for 5 min. Spleen cells were depleted of red blood cells by treatment with ammonium chloride, and PP cells were purified on Lympholyte-M gradient (Cedarlane). Single cell suspensions of the three lymphoid organs were prepared and incubated with anti-mouse CD11c-coated magnetic beads (Miltenyi Biotech) and selected on MACS separation columns. After purification we obtain 10-15 x 10^6 $CD11c^+$ cells from MLN and PP of 20 naive Balb/c mice. For the spleen, only 10% of total cells were purified to obtain about 25 x 10^6 $CD11c^+$ cells. Purified DCs were then plated and treated as described above for BM-DCs.

1.3.6 Phenotypic analysis of DCs

For phenotypic analysis, DCs were double-stained with FITC-anti-CD11c and either PE-anti-CD80, -CD86, -CD40, -PD-L1 or -IA^d. The incidence of positive cells and mean fluorescence intensity (MFI) were determined by flow cytometry using a FACSCalibur flow cytometer (Beckton Dickinson) equipped with an argon laser (488 nm) and a helium neon laser (540 nm). The CellQuest software program (Becton Dickinson) was used for data acquisition and analysis. Propidium iodide (PI) (2 μg/ml) was added to the cells just before flow cytometry analysis. Gating was done on PI-negative cells to exclude dead cells.

1.3.7 Measuring cytokine concentrations with cytometric bead array

Supernatants were stored at –70°C. Cytokines were quantified using the Mouse Inflammation Cytometric Beads Array (CBA) kit (BD Pharmingen) according to the manufacturer's instructions. Briefly, six bead populations with distinct fluorescence intensities were coated with capture antibodies specific for IL-6, IL-10, MCP-1, IFNγ, TNF, and IL-12p70. The capture beads, sample lysates, and PE-conjugated detection reagent were incubated together to obtain sandwich complexes. The beads were washed and run through a flow cytometer. The MFI data were analyzed with Becton Dickinson CBA Analysis Software. Sample data were normalized with specific cytokine standards to quantify the proteins of interest.

1.3.8 Mixed lymphocyte reaction

C57BL/6 splenic CD4 positive T cells were purified by negative selection using magnetic bead separation (Miltenyi Biotec). They were stimulated with graded numbers of purified Balb/c DCs in round-bottomed 96-well plates (BD Falcon). Cultures were maintained at 37°C in a humidified incubator (5% CO_2). After three days, 1 μCi [3H]thymidine (Amersham GE Healthcare, Buckinghamshire, UK) was added. [3H]thymidine incorporation was determined after 18 h using a 96-well Topcount scintillation counter (Packard Instrument, Meriden, CT). The incorporated radioactivity is expressed as counts per minute (cpm) means ± SD.

1.3.9 Statistical analysis

SPSS was used to analyze MLR results. Statistical analysis was performed using the general linear model for repeated measurements and the Bonferroni post-hoc test. P-values < 0.05 were considered as significant.

1.4 Results

1.4.1 mTFF3 induces COX-2 mRNA expression in CMT-93 IECs

COX-2 has previously been described to mediate mTFF3 cytoprotective actions *in vitro* in IECs [319] and this was, more recently confirmed *in vivo* [318]. Biological activity of recombinant mTFF3 was confirmed by determining its ability to induce COX-2 expression in IECs. Murine CMT-93 cells were grown to confluence and treated for 4 h with different concentrations of mTFF3. COX-2 mRNA expression was quantified by real-time quantitative PCR (Figure 13). The cells that were treated with 0.1 μg/ml mTFF3 showed a 5-fold increase in COX-2 mRNA levels relative to the medium control (0 μg/ml mTFF3). A higher induction is reached with 1-10 μg/ml mTFF3. These results clearly show that mTFF3 used in these experiments is biological active.

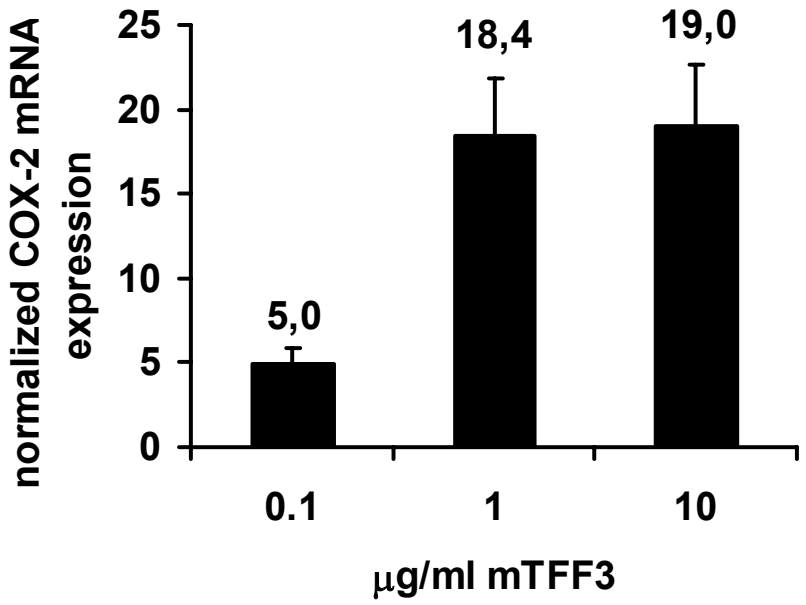

Figure 13 Induction of COX-2 mRNA expression in CMT-93 by mTFF3.
CMT-93 cells were grown to confluence and were then cultured for 4 h in the presence of 0, 0.1, 1 and 10 μg/ml mTFF3. Shown are the fold changes in RPL13A normalized COX-2 mRNA expression levels of every condition relative to the condition without mTFF3 addition. Bars represent the mean + SD.

1.4.2 mTFF3 does not inhibit LPS-induced phenotypic maturation of DCs.

In order to investigate whether mTFF3 can modulate DC maturation, we purified *in vitro* generated $CD11c^+$ BM-DCs and pretreated them for 6 h with mTFF3. They were then stimulated overnight in the presence of 10 ng/ml LPS. Because LPS-induced maturation of BM-DCs is inhibited by IL-10 [121,125,401], BM-DCs pretreated with mIL-10 were used as positive control for inhibition of maturation. BM-DCs cultured in medium were used as non-treated control cells. The phenotype of the different BM-DC groups was determined by flow cytometry. Mature DCs are known to express higher levels of MHC II, CD80, CD86 and CD40 than immature DCs. In the absence of LPS, non-treated (control) and mTFF3-treated BM-DCs expressed similar levels of co-stimulatory molecules (Figure 14A). mIL-10 treatment led only to reduced expression of MHC II. After LPS stimulation, mTFF3-treated BM-DCs up-regulated the expression of CD80, CD86, CD40 and MHC II to the levels expressed by control BM-DCs stimulated with LPS (Figure 14B). As expected, when 10 ng/ml of mIL-10 was added 6 h before LPS stimulation, the expression of CD80, CD86, CD40 and MHC II was inhibited compared to the other LPS-stimulated BM-DC groups. On the other hand, no difference in PD-L1 up-regulation after LPS stimulation was observed between non-treated, mTFF3-treated and mIL-10-treated BM-DCs (Figure 14B). These results indicate that mTFF3 treatment of BM-DCs does not alter their maturation in response to LPS. In contrast to mIL-10, mTFF3 cannot inhibit the expression of co-stimulatory molecules on BM-DCs. In addition, our data show that, unlike its effect on CD80, CD86, CD40 and MHC II, mIL-10 could not inhibit PD-L1 expression. This observation is consistent with the possibility that PD-L1 acts as a negative signal for T cell activation [126].

BM-DCs are useful for studying DCs *in vitro*, but given the complex biology of DCs *in vivo* and the postulated existence of different subsets, it is clear that *in vitro* results have to be evaluated carefully. In particular, emerging data show that mucosal DCs have unique functions that are not shared by DCs from other tissues [100,397]. Since TFF peptides are expressed mainly in the gastro-intestinal tract, it is possible that intestinal mucosal DCs are responsive to TFF whereas DCs from other origins are not. To test this hypothesis we isolated mucosal DCs from PP and MLN and compared their responses to LPS with or without mTFF3 pretreatment. As a control, we used peripheral DCs isolated from spleen. After LPS stimulation, mTFF3-treated DCs from the three different lymphoid organs up-regulated the expression of CD86, CD40, MHC II and PD-L1 to the same extent as non-treated DCs (data not shown). This result is in agreement with that obtained for BM-DCs.

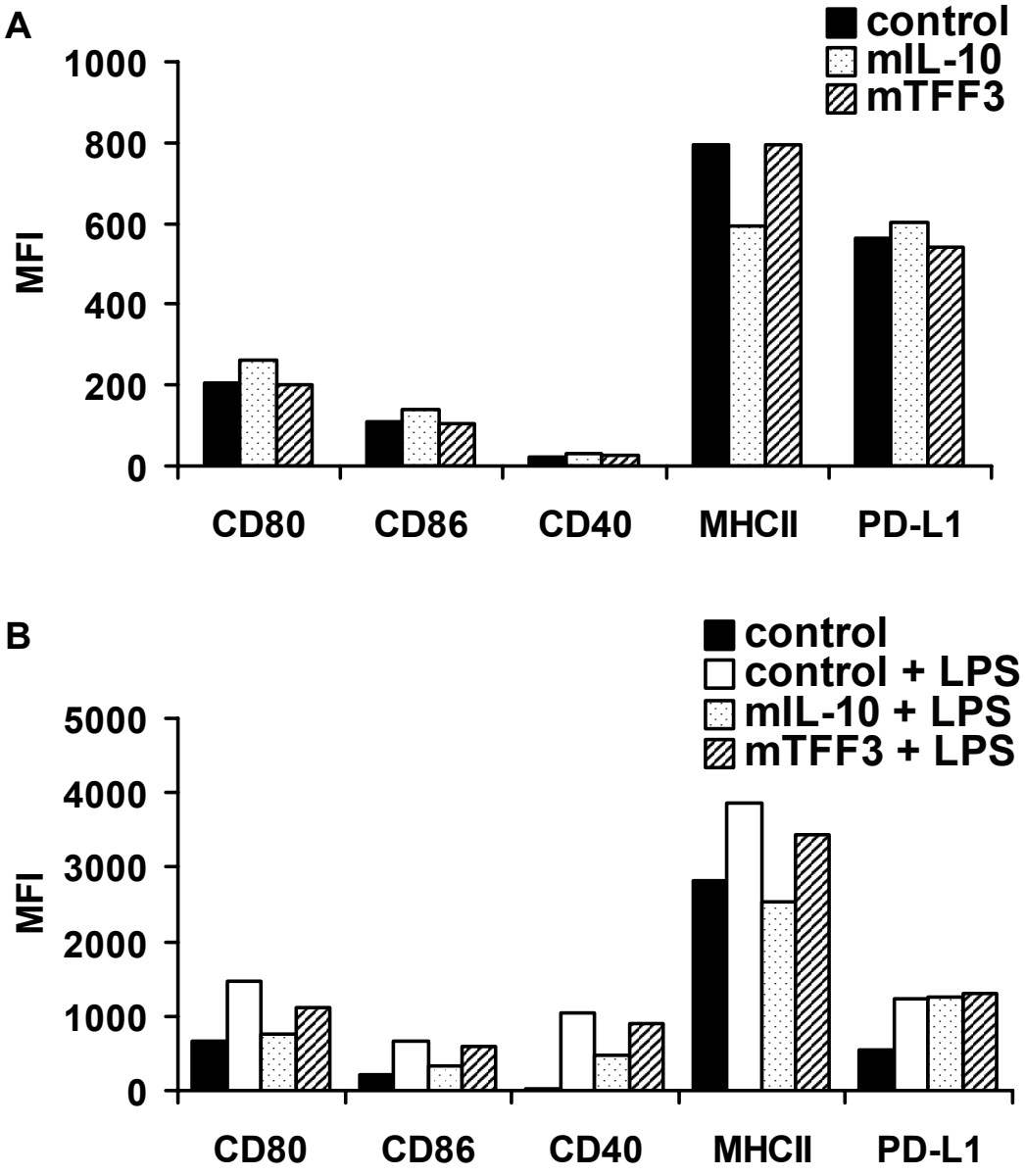

Figure 14 Phenotypic analysis of BM-DCs after LPS-induced maturation.
$CD11c^+$ BM-DCs (1 x 10^6 cells/ml) were cultured for 6 h in the presence of mIL-10 (10 ng/ml) or mTFF3 (10 μg/ml). After culturing the cells overnight without **(A)** or with LPS (10 ng/ml) **(B)**, they were harvested and analyzed by flow cytometry using anti-CD11c, anti-CD80, anti-CD86, anti-CD40, anti-MHC II and anti-PD-L1 mAbs. Gating was done on $CD11c^+$ cells. Bars represent MFI, the mean fluorescence intensity of the indicated mAb. Results are representative of three (A) and five (B) experiments.

1.4.3 mTFF3 does not alter LPS-induced cytokine secretion by DCs.

LPS is prone to induce a Th1 response [75], and cytokines secreted by DCs are important to determining whether the outcome of an immune reaction is Th1, Th2, or induction of Treg cells. To further evaluate the effect of mTFF3 on DCs, we determined the cytokine expression profile after LPS stimulation. As expected, LPS induced the secretion of pro-inflammatory cytokines and chemokines: IL-12p70, TNF, IL-6 and MCP-1, as well as of the anti-inflammatory cytokine IL-10 (Figure 15). Treatment of BM-DCs with mTFF3 did not influence any of the secreted proteins (Figure 15), whereas mIL-10 strongly inhibited the LPS-induced secretion of IL-12p70 and MCP-1, but only partially of IL-6 and TNF. These results indicate that, unlike mIL-10, mTFF3 cannot modulate the amount of cytokines secreted by BM-DCs.

The intrinsic difference between peripheral and mucosal DCs in their secretion of cytokines such as IL-10 is reported to be partially responsible for the different immune responses induced by mucosal and peripheral DCs [100,397]. To evaluate whether mTFF3 can differentially modulate cytokines expressed by mucosal DCs and peripheral DCs, we compared cytokines secreted by PP-, MLN- and spleen-DCs after LPS stimulation (data not shown). No significant difference was observed for secretion of IL-12p70, IL-10, IL-6, MCP-1 or TNF in response to LPS, for both mucosal and peripheral non-treated DCs compared to mTFF3-treated mucosal and peripheral DCs.

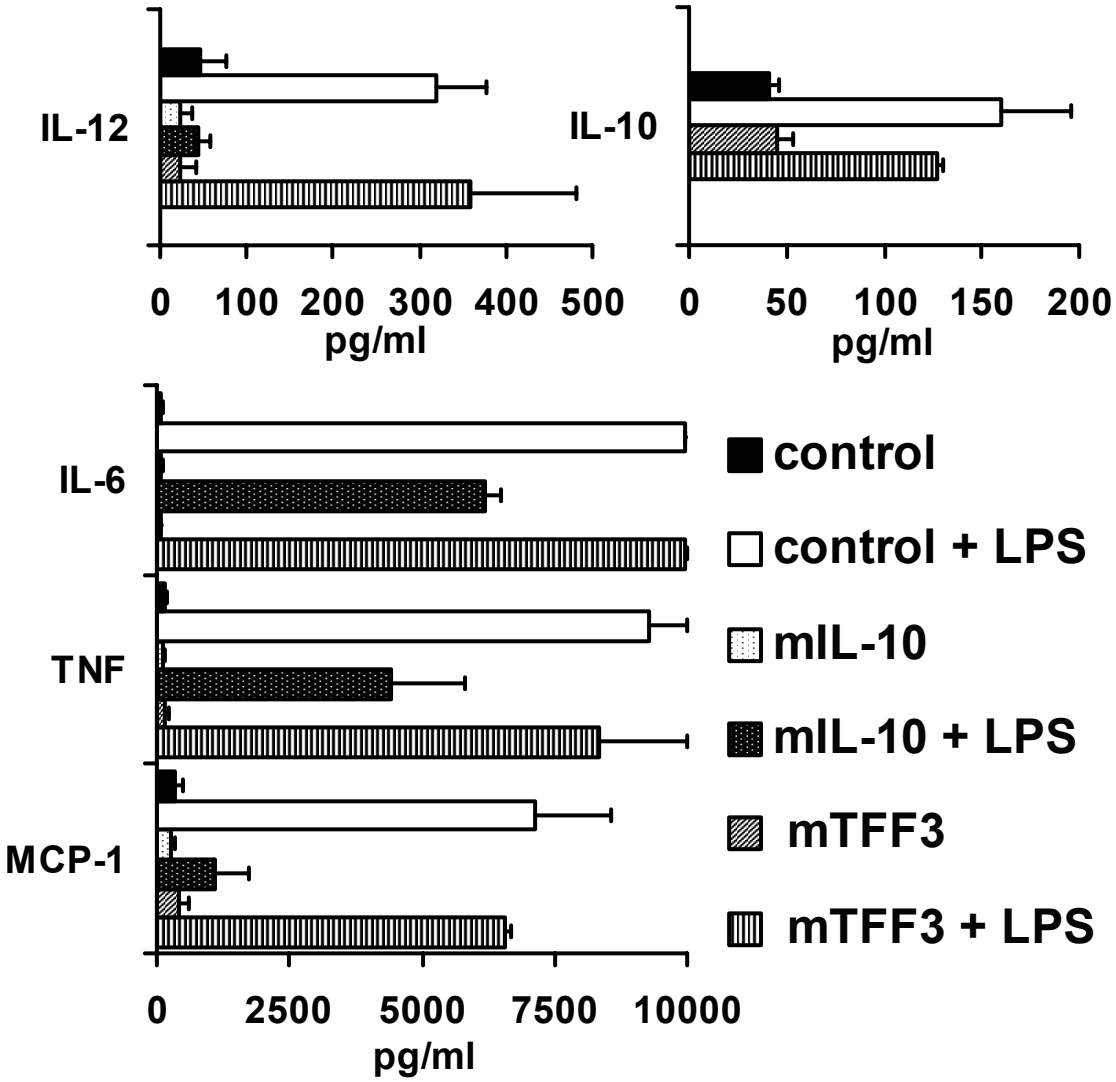

Figure 15 Cytokine production by BM-DCs after LPS-induced maturation.
$CD11c^+$ BM-DCs (1 x 10^6 cells/ml) were cultured for 6 h in the presence of mIL-10 (10 ng/ml) or mTFF3 (10 μg/ml). After overnight culture without or with LPS (10 ng/ml), supernatants were collected and cytokines were quantified with the Mouse Inflammation CBA kit. Results for IL-12p70, IL-10, MCP-1, TNF and IL-6 from 3 experiments are shown as mean pg/ml + SEM.

1.4.4 mTFF3 does not alter the capacity of DCs to stimulate allogeneic T cells.

To test whether the mature phenotype and inflammatory cytokine secretion of the BM-DCs after LPS treatment correlate with their capacity to stimulate T cells,

BM-DCs were co-cultured with allogeneic bead-purified $CD4^+$ T cells. mTFF3-treated BM-DCs stimulated with LPS were as effective at activating allogeneic $CD4^+$ T cells as non-treated control BM-DCs stimulated with LPS (Figure 16). Consistent with both cytokine secretion and phenotypic profile after LPS stimulation, mIL-10-treated BM-DCs stimulated with LPS were less effective in activating allogeneic $CD4^+$ T cells than non-treated LPS-stimulated BM-DCs (Figure 16). Similar results were obtained when BM-DCs were pretreated with up to 250 µg/ml of mTFF3 (data not shown).

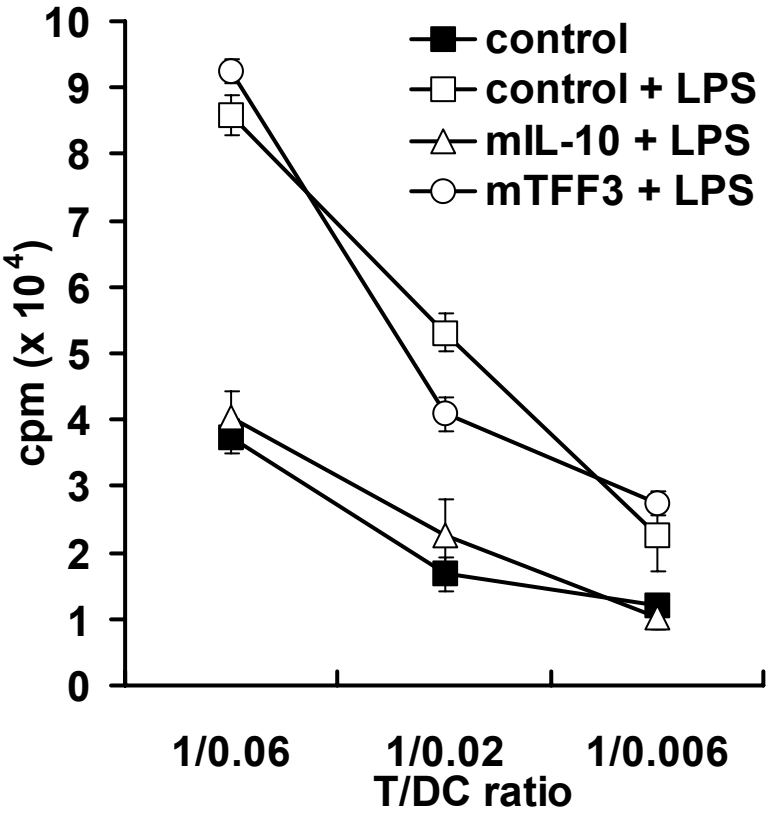

Figure 16 T cell-stimulating capacity of BM-DCs after LPS-induced maturation.
$CD11c^+$ BM-DCs (1 x 10^6 cells/ml) were cultured for 6 h in the presence of mIL-10 (10 ng/ml) or mTFF3 (10 µg/ml). After culturing the cells overnight with LPS (10 ng/ml), they were harvested. Graded numbers of LPS-stimulated BM-DCs were co-cultured with 150,000 naive allogeneic C57/BL6 splenic T cells. After three days of culture, [3H]thymidine was added. [3H]thymidine incorporation was measured after another 18 h. LPS-stimulated DCs, both mTFF3-treated and non-treated, show a significantly more potent allo-stimulatory activity than control DCs ($P<0.005$). LPS-stimulated DCs (pre)treated with mIL-10 do not stimulate T cell proliferation as compared to LPS matured DCs ($P<0.005$). Shown are mean values $\pm$ SD of triplicates from one experiment; data are representative for 3 experiments.

Analogous to BM-DCs, mTFF3-treated mucosal and splenic DCs stimulated with LPS were as efficient in activating allogeneic $CD4^+$ T cells as non-treated control DCs stimulated with LPS (data not shown).

In conclusion, our data show that mTFF3 does not inhibit LPS-induced activation of DCs, because mTFF3 treatment does not affect the capacity of LPS-stimulated DCs to activate naive allogeneic $CD4^+$ T cells. The absence of a difference between mTFF3-treated and non-treated cells is in agreement with the expression levels found for CD80, CD86, CD40, MHC II and PD-L1 and the secretion of pro-inflammatory cytokines. Furthermore, our experiments with *ex vivo* derived DCs confirm that regardless of whether DCs were retrieved from mucosal or peripheral sources, mTFF3 does not inhibit LPS-induced activation of DCs.

1.5 Discussion

Our results indicate that the presence of biologically active mTFF3 has no direct effect on LPS-induced maturation of *in vitro* generated BM-DCs. No difference was observed in expression of the co-stimulatory molecules CD40, CD86, CD80 and MHC II, and the negative stimulator of T cells, PD- L1 between mTFF3-treated and non-treated BM-DCs after LPS stimulation. Also for both groups we observed no difference in secretion of the pro-inflammatory cytokines IL-12p70, TNF, IL-6 and MCP-1, as well as the anti-inflammatory cytokine IL-10. Furthermore, they were equally effective in stimulating naive allogeneic $CD4^+$ T cells *in vitro*.

The maturation stimulus is a major factor that influences the pattern of cytokines released by DCs, and accordingly the Th balance. For stimulating DCs we used LPS, which is generally a Th1 inducing factor. As we could not determine any differences in DC profile, it is more than likely that the Th balance is not influenced by mTFF3-treatment. Furthermore, high IFNγ levels were measured during T cell proliferation when DCs, both mTFF3-treated and non-treated, were stimulated with LPS, suggesting the induction of a Th1 response (data not shown).

Although *in vitro* cultured BM-DCs are a useful tool, they may not be representative of all the different *in vivo* DC populations. As mTFF3 is secreted mainly in the small and large intestines, it may have evolved to preferentially modulate mucosal immune cells. However, our results suggest that mTFF3 does not specifically target mucosal DCs as no differences could be detected between mucosal and splenic DCs with respect to LPS-mediated maturation, cytokine secretion and capacity to stimulate T cells.

DCs determine the character of the immune response by secreting cytokines that drive the development of T cells into Th1, Th2, or T reg cells. Although Pathogen Associated Molecular Patterns that stimulate different Toll-Like Receptors induce similar changes in surface phenotype in DCs, they often induce distinct patterns of cytokines, resulting in a Th1/Th2 polarization that favors the pathogen [402]. Therefore it is plausible that mTFF3 can modulate DC functions if another maturation stimulus, such as αCD40, flagellin, zymosan or peptidoglycan, is used instead of LPS.

Alternatively, it is possible that mTFF3 showed no effect in our experimental setup due to the absence of the appropriate microenvironment or inflammatory mediators. After all, *in vivo* DC activation following infection is complex, and besides maturation stimulus and DC subset, inflammatory mediators at the site of infection also influence this process.

Since the TFF receptor is unknown [394], mTFF3 responsive cells have not been characterized. Nevertheless, as mTFF3 treatment did not alter the activation status, cytokine profile, and T cell-stimulating capacity of both *in vitro* cultured and *ex vivo* derived DC populations, we hypothesize that none of these DCs expresses the receptor for mTFF3. However, it is still possible that TFF peptides in the spleen or other lymphoid organs modulate immune functions by affecting other cells of the immune system and not through direct interaction with DCs. As suggested by Cook *et al*, plasma cells may secrete TFF peptides, which then interact with secreted immunoglobulins to modulate humoral immune responses [259].

Though we observed no direct modulation of DCs by purified recombinant mTFF3, it is possible that *in vivo* mTFF3 activates other cells to secrete molecules that can modulate DC function. Crosstalk between epithelial cells and DCs is considered to be very important to regulate intestinal mucosal immunity [152,403]. It has been shown that TFF induces COX-2 expression in epithelial cells [318,319], leading to the secretion of prostaglandin PGE_2 whose anti-inflammatory effects on DCs have been extensively documented [322-325]. In an *in vivo* model of colitis, TFF reduced VCAM-1 expression in colonic endothelium [305]. Reduced VCAM-1 expression could lead to reduced infiltration of inflammatory cells at the site of infection, which would ameliorate inflammation and affect DC stimulation. Furthermore, Giraud *et al* reported that TFF2 can reduce LPS-induced NO expression in monocytes [310], which may lead to inhibition of immune cell recruitment, thereby changing the microenvironment of DCs. As this effect on monocytes has been described only for

TFF2, it is possible that the effect of TFF2 on DCs is different from what we show for mTFF3.

In conclusion, our results demonstrate that recombinant mTFF3 does not influence LPS-induced DC maturation suggesting that mTFF3 does not affect immune responses on the level of DC functions directly. However, it remains possible that mTFF3 regulates DC maturation when other maturation stimuli or inflammatory mediators are present or that mTFF3 regulates DC function through modulation of other cells.

2 GENETICALLY ENGINEERED *LACTOCOCCUS LACTIS* SECRETING MURINE IL-10 MODULATES THE FUNCTION OF BONE MARROW-DERIVED DENDRITIC CELLS IN THE PRESENCE OF LPS

In preparation for submission to the European Journal of Immunology

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2.1 Abstract

Oral delivery of murine IL-10 by genetically modified *Lactococcus lactis* (*LL-pTmIL10*) has been shown to efficiently reduce intestinal inflammation in mice with chronic colitis but the mechanism(s) involved have not been elucidated. It has been suggested that IL-10 controls intestinal inflammation by inhibiting microbe-induced activation of dendritic cells (DCs). We therefore investigated whether *LL-pTmIL10* can modulate the functions of bone marrow-derived DCs (BM-DCs) responding to LPS. Incubation of these cells with *LL-pTmIL10* or with the control strain *LL-pTREX* reduced their ability to activate allogeneic T cell proliferation. These *L. lactis*-treated LPS-stimulated BM-DCs also significantly inhibited the capacity of fully matured BM-DCs to activate $CD4^+$ T cells. However, in contrast to *LL-pTREX*, *LL-pTmIL10* inhibited the LPS-stimulated secretion of MCP-1 by BM-DCs and reduced the synergistic up-regulation of IL-12/IL-23p40. In addition, *LL-pTmIL10* treatment of LPS-stimulated BM-DCs significantly inhibited their capacity to induce strong secretion of IL-17 by $CD4^+$ T cells. Our data suggest that the beneficial effects of *LL-pTmIL10* treatment during chronic colitis might involve inhibition of $CD4^+$ Th17 cells and a reduced accumulation of these cells as well as other immune cells at the site of inflammation.

2.2 Introduction

The mucosal immune system of the gastro-intestinal tract is responsible for inducing tolerance to commensal bacteria and food antigens and for providing protection against pathogenic micro-organisms. Ineffective immune regulation can result in dysregulated T cell responses to intestinal microflora and break-down of tolerance. Uncontrolled intestinal inflammation can ensue, as in chronic inflammatory diseases, such as Inflammatory Bowel Disease (IBD) [404,405]. IBD encompasses Crohn's Disease (CD) and ulcerative colitis, which are caused by a complex interaction between environmental, genetic and immuno-regulatory factors [168,388].

Steidler and co-workers [198] demonstrated that oral administration of recombinant *Lactococcus lactis* - a non-pathogenic, non-colonizing gram-positive bacteria - secreting murine IL-10 reduced inflammation in the chronic dextran sodium sulfate model by 50%, and prevented the onset of colitis in IL-$10^{-/-}$ mice. More recently this treatment was also validated in trinitrobenzene sulphonate induced colitis [363]. Furthermore, a biologically contained *L. lactis* strain secreting human IL-10 was constructed [200] and used in a phase I, open label clinical trial [199]. That trial demonstrated that treating CD patients with this strain is realistic and safe. However, the mechanisms mediating the therapeutic effects of *L. lactis*-mediated topical delivery of IL-10 in murine or human colitis remain unknown.

Studies have suggested that intestinal DCs could be involved in the initiation and possibly continuation of IBD in mouse and man [175-179]. It has been suggested that DCs, probably due to dysfunctional or exaggerated pattern recognition receptor responses, incorrectly recognize commensal bacteria and induce Th1 and possibly Th17 pro-inflammatory responses normally directed against pathogens [406]. Indeed, DCs are activated in IBD and up-regulate expression of TLR2 and TLR4, which might enhance responses to bacterial products and render $CD4^+$ T cells resistant to Treg cell-mediated suppression [407]. Furthermore, DCs in IBD produce increased amounts of pro-inflammatory cytokines, such as IL-12 and IL-6 [175]. Moreover, recent studies indicated that IL-23, which like IL-12 is produced by APCs such as DCs, has an important function in the local initiation of gut inflammation [408].

IL-10 has profound anti-inflammatory effects on the immune system, including inhibition of cytokine production by DCs, T cells and macrophages, and inhibition of maturation and antigen-presentation by DCs [210]. The spontaneous development of IBD in IL-$10^{-/-}$ mice highlights the physiological importance of IL-10. IL-10 also appears to control intestinal inflammation by inhibiting TLR-mediated

pro-inflammatory responses, and DCs obtained from CD patients with NOD2 mutations showed impaired IL-10 production [409-411]. Kobayashi and co-workers showed that TLR-induced myeloid IL-12/IL-23 production is an important target of IL-10-mediated anti-inflammatory effects [409]. Based on these data, a model for the role of IL-10 and LPS in IBD has been proposed. This model hypothesizes that LPS induces APCs to produce IL-12/IL-23, which drive Th1/Th17 type immune responses. Normally, inflammation is controlled by APC- or lymphocyte-derived IL-10, which blocks further production of IL-12/IL-23 and other pro-inflammatory cytokines. This suppressive IL-10 signal might be important in the gut, were immune cells are continuously in close contact with LPS and other microbial products.

We hypothesized that IL-10 secreted by genetically modified *L. lactis* can modulate DC responses to TLR ligands such as LPS, partially explaining the anti-inflammatory effect in murine models for IBD. We show that *L. lactis,* independently of mIL-10 secretion, was less effective than LPS in inducing bone marrow-derived DC (BM-DC) maturation, demonstrated by their reduced capacity to activate naive T cells. Furthermore, we show that in the presence of LPS, mIL-10 secreted by *L. lactis* could specifically inhibit secretion of MCP-1 and IL-12p70. We also suggest that IL-10 secreted by *L. lactis* could also lead to a reduced expression of IL-23 by mature BM-DCs, which could explain the differential Th17-polarizing capacity of *L. lactis* secreting mIL-10 compared to the *L. lactis* control strain in the presence of LPS.

2.3 Materials and Methods

2.3.1 Animals.

Balb/c ($H2K^d$) and C57BL/6 (B6; $H2K^b$) female mice, 6-8 weeks old, were purchased from Charles River, Italy. They were housed in a specific pathogen-free animal facility and treated according to the institutional guidelines of Ghent University.

2.3.2 Media, reagents and antibodies.

The designation, RPMI-complete refers to RPMI-1640 medium supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.4 M sodium pyruvate, 10 IU/ml penicillin, 10 μg/ml streptomycin and 50 μM β-mercaptoethanol (all from Gibco BRL/Invitrogen, Gaithersburg, MD, USA). RPMI-complete PS^- is the above medium without antibiotics.

Purified LPS (*Escherichia coli* serotype O111b4) was obtained from Sigma-Aldrich, St. Louis, MO, USA. Recombinant murine IL-10 (rmIL-10), rmGM-CSF, rmIL-4 and rmIL-2 were purchased from Peprotech, Rocky Hill, NJ, USA.

Monoclonal antibodies (mAbs) for flow cytometry were hamster anti-mouse CD11c (clone HL3; FITC-conjugated), rat anti-mouse CD80 (clone 16-10A1; PE-conjugated), rat anti-mouse CD86 (clone GL1; PE-conjugated), rat anti-mouse CD40 (clone 3/23, PE-conjugated), rat anti-mouse PD-L1 (B7-H1; CD274) (clone MIH5, PE-conjugated), and rat anti-mouse IA^d (clone AMS-32.1, PE-conjugated). All mAbs were from BD Pharmingen, San Diego, CA, USA. Isotype controls were FITC-conjugated Armenian hamster IgG1 (anti-TNP) and PE-conjugated rat IgG2a/IgG2b κ from BD Pharmingen. Before cell-staining, non-antigen-specific binding of IgG to the mouse Fcγ receptors was blocked with a rat anti-mouse CD16/CD32 mAb (Fc block; BD Pharmingen).

2.3.3 Bacterial strains.

L. lactis subsp. *cremoris* strain MG1363 [384] was used throughout this study. Bacteria were cultured in GM17E medium, i.e. M17 (Difco Laboratories, Detroit, MI, USA) supplemented with 0.5% glucose and 5 µg/ml erythromycin. Stock solutions were stored at -20˚C in 50% glycerol in GM17E. Stock suspensions were diluted 1000-fold in fresh GM17E and incubated as standing cultures at 30˚C for 16 h. They reached a density of 2 x 10^9 colony forming units (CFU) per ml.

Plasmid pT1mIL10 is a derivative of pTREX1 [412] in which the coding region of mature murine IL-10 was fused to the lactococcal usp45 secretion leader [413], preceded by the coliphage T7 gene 10 ribosome binding site and the lactococcal P1 promoter [414]. Further in the text, *L. lactis* MG1363 transformed with plasmid pT1mIL10 is designated *LL-pTmIL10*. MG1363 harboring the empty control vector pTREX1 is designated *LL-pTREX.*

Before addition to the DCs, bacteria were diluted 200-fold in RPMI-complete PS^-, supplemented with 5 µg/ml erythromycin, and grown for 3 h at 37°C. The bacterial count reached ~1 x 10^8 CFU/ml. During co-culture the DC:bacterial CFU ratio was 1:1.

2.3.4 Generation of bone marrow derived DCs.

A modified version of a published procedure was used to generate bone marrow derived DCs [389,390]. Briefly, bone marrow cells from femurs and tibias of Balb/c

mice were depleted of red blood cells by lysis with ammonium chloride. Granulocytes, erythroid precursors, and B cells were labeled with a cocktail of mAbs (anti-Gr-1, anti-TER-119/erythroid cells, and anti-CD45R/B220; all from BD Pharmingen), and killed by incubation with low-toxicity rabbit complement (Cedarlane, Ontario, Canada). These cells were cultured in RPMI-complete supplemented with 1000 U/ml rmGM-CSF and 500 U/ml rmIL-4. Medium was replaced on days 2 and 4. On day 6, nonadherent cells were removed, and fresh medium with cytokines was added. Two days later 60 to 90% of the new population of nonadherent cells were $CD11c^+$ BM-DCs. DCs were further purified to >90% using anti-CD11c magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified DCs were re-plated at 1×10^6 cells/ml and pre-incubated at 37°C with approximately 1×10^6 CFU/ml *LL-pTmIL10* or *LL-pTREX*, or with 10 ng/ml rmIL-10 (positive control) in RPMI-complete PS^-, supplemented with 5 μg/ml erythromycin. To prevent bacterial overgrowth, 75 μg/ml gentamycin was added after 5 h. For maturation, 10 ng/ml LPS was then added and the cells were incubated overnight at 37°C. Supernatants were collected and stored at -70°C. Cells were harvested, washed and counted before use.

2.3.5 Phenotypic analysis of DCs.

For phenotypic analysis, DCs were double-stained with FITC-anti-CD11c and either PE-anti-CD80, -CD86, -CD40, -PD-L1 or $-IA^d$. The incidence of positive cells and MFI were determined by flow cytometry using a FACSCalibur flow cytometer (Beckton Dickinson) equipped with an argon laser (488 nm) and a helium neon laser (540 nm). The CellQuest software program (Becton Dickinson) was used for data acquisition and analysis. PI (2 μg/ml) was added to the cells just before flow cytometry analysis. Gating was done on PI-negative cells to exclude dead cells.

2.3.6 Mixed lymphocyte reaction.

C57BL/6 splenic CD4 positive T cells were purified by negative selection using magnetic bead separation (Miltenyi Biotec). They were stimulated with graded numbers of purified Balb/c DCs in round-bottomed 96-well plates (BD Falcon) in a total volume of 200 μl RPMI-complete. Cultures were maintained at 37°C in a humidified incubator (5% CO_2). After three days, 1 μCi/well [3H]thymidine (Amersham GE Healthcare, Buckinghamshire, UK) was added. [3H]thymidine incorporation was determined after 18 h using a 96-well Topcount scintillation

counter (Packard Instrument, Meriden, CT). The incorporated radioactivity is expressed as cpm means ± SD.

2.3.7 Measuring cytokine concentrations with cytometric bead array.

Supernatants were stored at –70°C. Cytokines were quantified according to the manufacturer's instructions, using the Mouse Inflammation Cytometric Beads Array kit (CBA; BD Pharmingen). Briefly, for every cytokine a bead population with distinct fluorescence intensity was coated with capture antibodies specific for this cytokine. The capture beads for every cytokine to be measured, sample lysates, and PE-conjugated detection reagent were incubated together to obtain sandwich complexes. For the Mouse inflammation kit, IL-12p70, IL-10, IL-6, TNF, IFNγ and MCP-1 were analyzed in one sample. The beads were washed and run through a flow cytometer. The MFI data were analyzed with Becton Dickinson CBA Analysis Software. Sample data were normalized with specific cytokine standards to quantify the proteins of interest.

2.3.8 Quantitative analysis of mRNA expression in DCs.

Purified $CD11c^+$ DCs were re-plated at 1×10^6 cells/ml and pre-incubated for 5 h at 37°C with *LL-pTmIL10* or *LL-pTREX* at 1×10^6 CFU/ml or with 10 ng/ml purified rmIL-10. Gentamycin (75 μg/ml) and LPS (10 ng/ml) were added after 5 h. After an additional 5 h, cells were washed with PBS and total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Westburg BV, The Netherlands), with on-column DNase treatment (Qiagen). One μg of total RNA was converted to single stranded complementary DNA by reverse transcription (Superscript, Gibco) with oligo dT priming. Subsequently, real-time PCR for murine IL-12p35, IL-12p40 and IL-23p19 was performed using the SYBR green kit (Eurogentec, Seraing, Belgium) and 300 nM of each primer. A two-step program was run on the iCycler (Biorad Laboratories, Hercules, CA, USA). Cycling conditions were: 10 min incubation at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Melting curve analysis confirmed primer specificities. All reactions were run in triplicate and normalized to TATA box binding protein (TBP) levels. TBP was chosen after checking the expression stability of a set of housekeeping genes in BM-DCs cells using the Genorm software [400]. The level of the normalized mRNA is shown relative to the control sample incubated with medium alone. Data are expressed as mean ± SD. Primers were designed using the Beacon Designer software (PREMIER Biosoft International, Palo Alto, CA, USA) (see table).

Gene symbol	*Reference sequence*	*Forward primer*	*Reverse primer*
mIL-12p35	NM_008351	ACTCTGCGCCAGAAACCTC	CACCCTGTTGATGGTCACGAC
mIL-12p40	NM_008352	ACCTGTGACACGCCTGAAGAAG	TGTGGAGCAGCAGATGTGAGTG
mIL-23p19	NM_031252	AGCAACTTCACACCTCCCTAC	ACTGCTGACTAGAACTCAGGC
mTBP	NM_013684	TCTACCGTGAATCTTGGCTGTAAA	TTCTCATGATGACTGCAGCAAA

2.3.9 Re-stimulation of T cells for the detection of IL-17 secretion.

Activated C57BL/6 splenic CD4 positive T cells were purified by negative selection using magnetic bead separation (Miltenyi Biotec). They were stimulated with purified Balb/c DCs at a ratio of 10:1 in round-bottom 96-well plates (BD Falcon). Cultures were maintained at 37°C in a humidified incubator (5% CO_2). After five days, T cells were recovered by density gradient centrifugation using Lympholyte-M (Cedarlane), to remove dead cells. Recovered cells were re-plated at 1×10^6 cells/ml in flat-bottom 96-well plates at 37°C in RPMI-complete with 5 U/ml rmIL-2 for 72 h. These cells (150,000) were then re-stimulated with 1.5×10^5 Balb/c splenocytes treated with mitomycin C (50 μg/ml; Sigma-Aldrich). After 72 h, the concentration of IL-17 was assayed using a murine IL-17 ELISA (Bender Medsystems, Burlingame, CA, USA) with a detection limit of 1.6 pg/ml. ELISA was performed according to the manufacturer's instructions.

2.3.10 Statistical analysis.

SPSS was used to analyze MLR results. Statistical analysis was performed using the general linear model for repeated measurements and the Bonferroni post-hoc test. For statistical analysis of DC regulatory activity and cytokine concentrations, we used a one-way ANOVA and the Bonferroni post-hoc test. P-values < 0.05 were considered as significant.

2.4 Results

2.4.1 L. lactis treatment induces a semi-mature BM-DC phenotype.

To determine whether *LL-pTmIL10* can modulate DC maturation, $CD11c^+$ BM-DCs generated *in vitro* and purified on immuno-beads were pretreated with *LL-pTmIL10* or *LL-pTREX* at a DC:bacterial CFU ratio of 1:1. Co-cultures of BM-DCs and *L. lactis* were initially maintained in antibiotic-free medium to allow bacterial activity and thus mIL-10 secretion by *LL-pTmIL10*. After 5 h, gentamycin was added to inhibit bacterial protein synthesis and growth. BM-DCs stimulated in the presence of LPS were used as a positive control for maturation. BM-DCs cultured in medium alone were used as an untreated control for spontaneous maturation, and BM-DCs cultured in the presence of rmIL-10 were used as a control for inhibition of spontaneous maturation. After overnight culture, the BM-DCs were harvested and analyzed by flow cytometry for the expression of co-stimulatory molecules. Both *LL-pTREX* and *LL-pTmIL10* induced lower levels of MHC II and co-stimulatory molecules CD40, CD86 and CD80, than LPS-treated BM-DCs, but they expressed higher levels than rmIL-10-treated and untreated control BM-DCs (Figure 17). In contrast, expression levels of the T cell inhibitory molecule Programmed Death-1 ligand (B7-H1, PD-L1) in BM-DCs stimulated or with *L. lactis* or LPS were comparable.

To test whether *LL-pTREX* or *LL-pTmIL10* could modulate LPS-induced maturation, BM-DCs were pretreated for 5 h with *L. lactis* and then stimulated overnight with LPS. Pretreatment with *LL-pTmIL10* could not prevent the LPS-induced up-regulation of maturation markers on BM-DCs. Similar results were obtained for *LL-pTREX*- and rmIL-10-pretreated BM-DCs, although the up-regulation of maturation markers was less pronounced for BM-DCs conditioned with rmIL-10. Furthermore, no difference in PD-L1 up-regulation after LPS stimulation was observed between non-treated, *L. lactis*-treated and rmIL-10-treated BM-DCs (Figure 17).

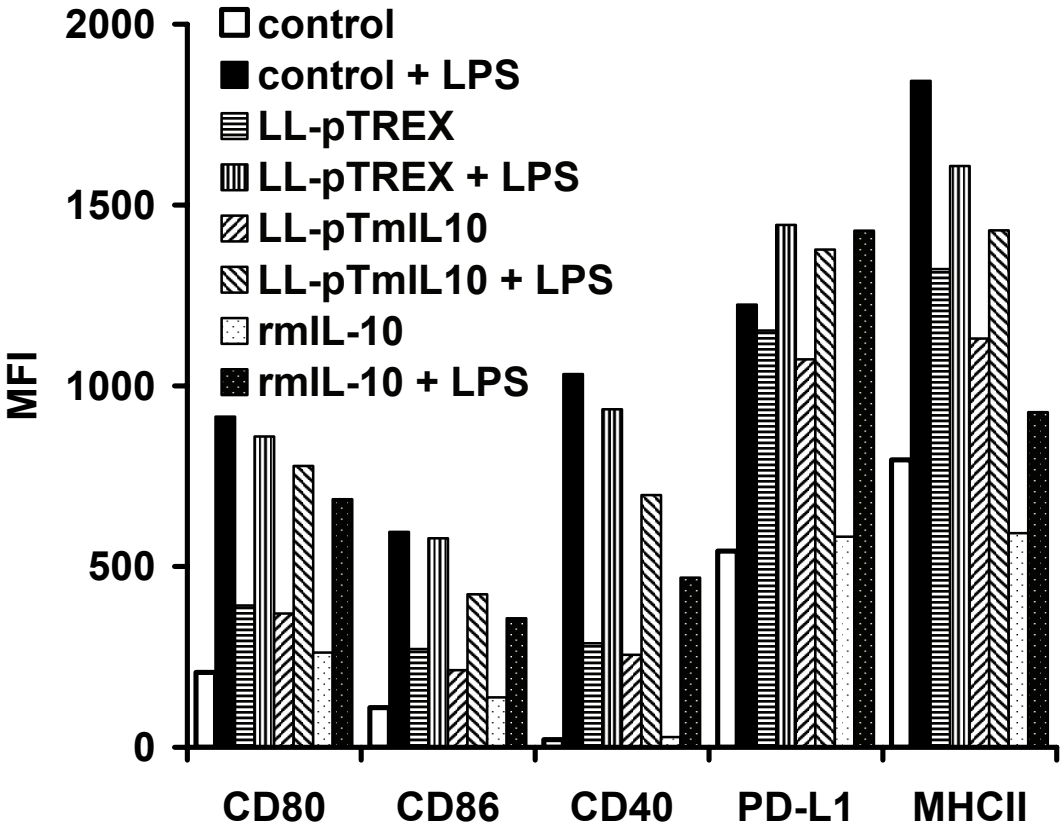


Figure 17 Phenotypic analysis of BM-DCs before and after LPS-induced maturation. $CD11c^+$ BM-DCs were treated as described in the Materials and Methods section. After culturing overnight without or with LPS (10 ng/ml), they were harvested and analyzed by flow cytometry using anti-CD11c, anti-CD80, anti-CD86, anti-CD40, anti-MHC II and anti-PD-L1 mAbs. Gating was done on $CD11c^+$ cells. Bars represent MFI, the mean fluorescence intensity of the indicated mAb. Results are representative of four experiments.

2.4.2 L. lactis treatment modulates the ability of BM-DCs to activate allogeneic T cells.

To determine whether the maturation phenotype induced in BM-DCs by *LL-pTREX* or *LL-pTmIL10* correlated with their functional maturation, BM-DCs were tested for their ability to stimulate allogeneic bead-purified naive $CD4^+$ T cells. As expected, LPS-activated BM-DCs were effective in stimulating allogeneic $CD4^+$ T cells, whereas *LL-pTREX*- or *LL-pTmIL10*-treated BM-DCs induced only weak T cell proliferation ($P<0.001$) (Figure 18). Although LPS stimulation of BM-DCs treated with *LL-pTmIL10* or with *LL-pTREX* increased their ability to stimulate allogeneic T cells, it was still

significantly less (P<0.05) than the ability of LPS-stimulated BM-DC controls. However, *LL-pTmIL10* or *LL-pTREX* treatment was less effective in inhibiting the ability of LPS-stimulated BM-DCs to activate allogeneic $CD4^+$ T cells compared to treatment of BM-DCs with rmIL-10 (P<0.001).

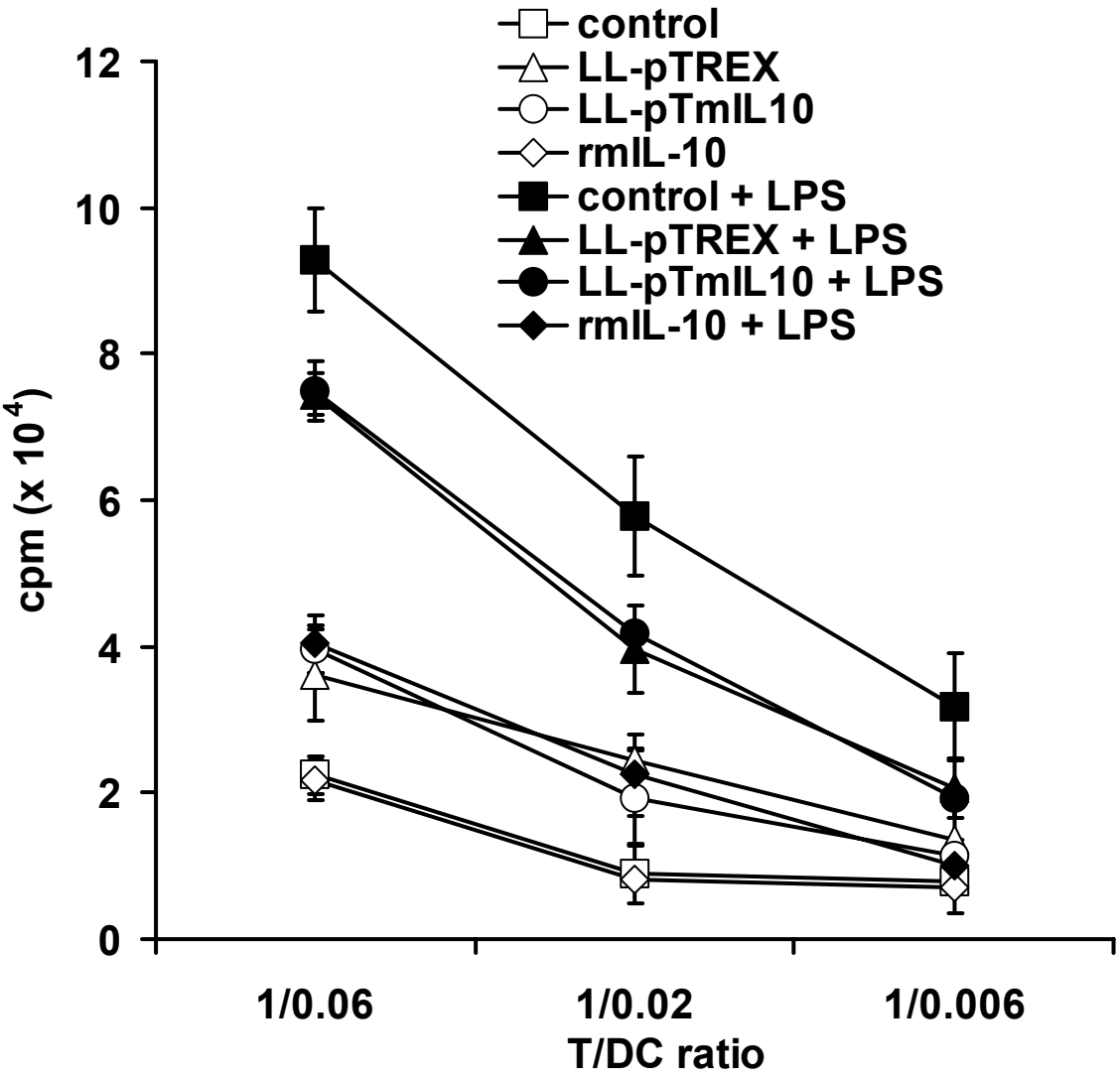


Figure 18 T cell-stimulating capacity of activated BM-DCs.
$CD11c^+$ BM-DCs were treated, harvested and used in a mixed lymphocyte reaction (see Materials and Methods). LPS-stimulated DCs (pre)treated with rmIL-10 do not stimulate T cell proliferation as compared to LPS-matured DCs (P<0.001). LPS-stimulated DCs also show a significantly more potent allo-stimulatory activity than the *L. lactis*-treated groups (P<0.001) and the *L. lactis*-treated, LPS-stimulated groups (P<0.05). *LL-pTmIL10* or *LL-pTREX* groups are not significantly different from each other. Shown are mean values ± SD of triplicates from one experiment; data are representative of four experiments.

Our results show that, consistent with their maturation phenotype, *LL-pTmIL10*- or *LL-pTREX*-treated BM-DCs were less effective in stimulating naive allogeneic T cells than LPS-stimulated BM-DCs, and no difference was found in the T cell proliferating capacity of *LL-pTmIL10*- or *LL-pTREX*-treated BM-DCs. In contrast to treatment with rmIL-10, *L. lactis*-treated BM-DCs only partially diminished the capacity of LPS-induced BM-DCs to activate naive T cells.

2.4.3 BM-DCs treated with L. lactis and stimulated with LPS inhibit the activation of allogeneic T cells by mature BM-DCs.

We previously showed that BM-DCs treated with *LL-pTREX* or *LL-pTmIL10* and stimulated with LPS were phenotypically as mature as LPS-stimulated control BM-DCs but less effective at stimulating naive T cells. Those results indicate that *L. lactis* alters the properties of BM-DCs to stimulate T cells independently of the expression of co-stimulatory molecules. To determine whether these LPS-stimulated BM-DCs treated with *L. lactis* can act as regulators, we investigated their ability to inhibit the capacity of control LPS-matured BM-DCs to activate naive T cells. Therefore, LPS-stimulated BM-DCs and naive T cells were co-cultured in the presence of graded numbers of LPS-stimulated BM-DCs pretreated with *LL-pTREX* or *LL-pTmIL10*. Untreated LPS-stimulated BM-DCs were used as negative controls. LPS-stimulated BM-DCs pretreated with *LL-pTREX* or *LL-pTmIL10* significantly inhibited the capacity of mature BM-DCs to activate naive allogeneic T cells (Figure 19). At a regulator:stimulator ratio of 1:10 the inhibitory capacity was higher when *LL-pTmIL10*-treated BM-DCs were used as regulators compared to *LL-pTREX*-treated BM-DCs.

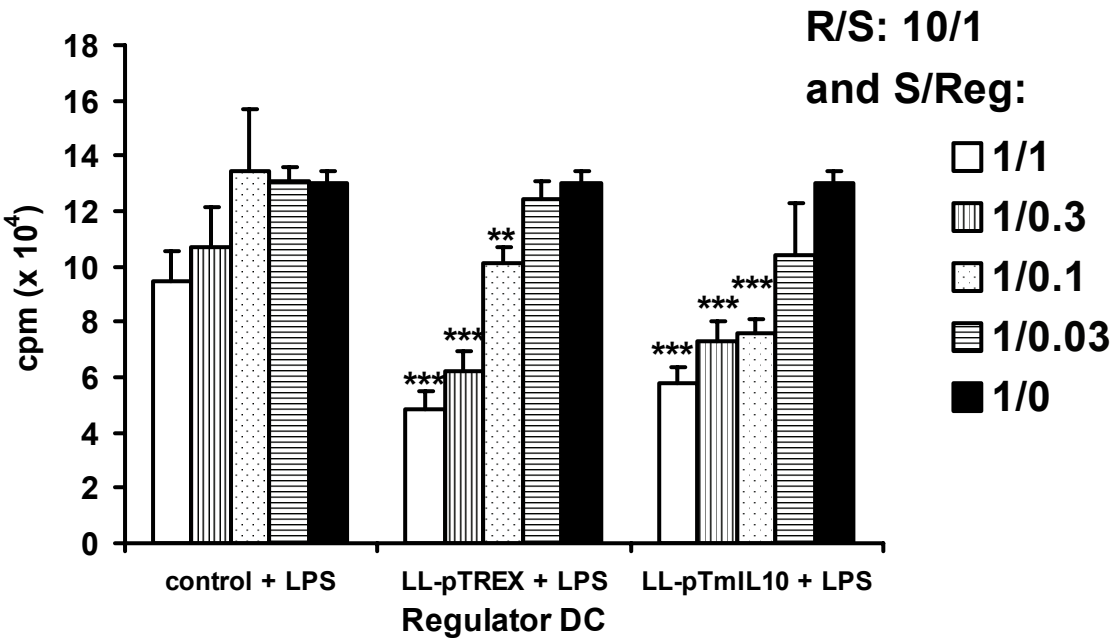


Figure 19 Modulation of T cell proliferation by the addition of regulator BM-DCs.
$CD11c^+$ BM-DCs were treated as described in the Materials and Methods section. After culturing the cells overnight with LPS (10 ng/ml), they were harvested. 150,000 naive allogeneic C57/BL6 splenic T cells (**R**esponders) were stimulated with tenfold fewer LPS-stimulated BM-DCs (**S**timulators), and to this mixed lymphocyte reaction graded numbers of the different pretreated and LPS-stimulated DCs were added to assess their regulatory capacity (**Reg**ulators). After three days of culture, [3H]thymidine was added. [3H]thymidine incorporation was measured after another 18 h. The black bar represents proliferation of the responders in the absence of regulators. *** and ** represent statistical significant differences in comparison with this condition of $P < 0.001$ and $P < 0.01$ respectively. Shown are mean values + SD of triplicates from one experiment; data are representative of three experiments.

2.4.4 BM-DCs treated with LL-pTmIL10 or LL-pTREX differ in their ability to secrete MCP-1 and IL-12p70 in response to LPS.

Since cytokines expressed by DCs can influence the outcome of an immune response, we investigated whether mIL-10 secreted by *LL-pTmIL10* could specifically modulate BM-DC function by modulating cytokine expression. As expected, LPS stimulation of BM-DCs induced strong expression of pro-inflammatory cytokines and chemokines, such as IL-12p70, MCP-1, TNF and IL-6, compared to the untreated and rmIL-10-treated BM-DCs (Figure 20 and data not shown). Furthermore, the expression levels for all cytokines examined after *L. lactis* treatment were increased compared to the non-treated and rmIL-10-treated control BM-DCs.

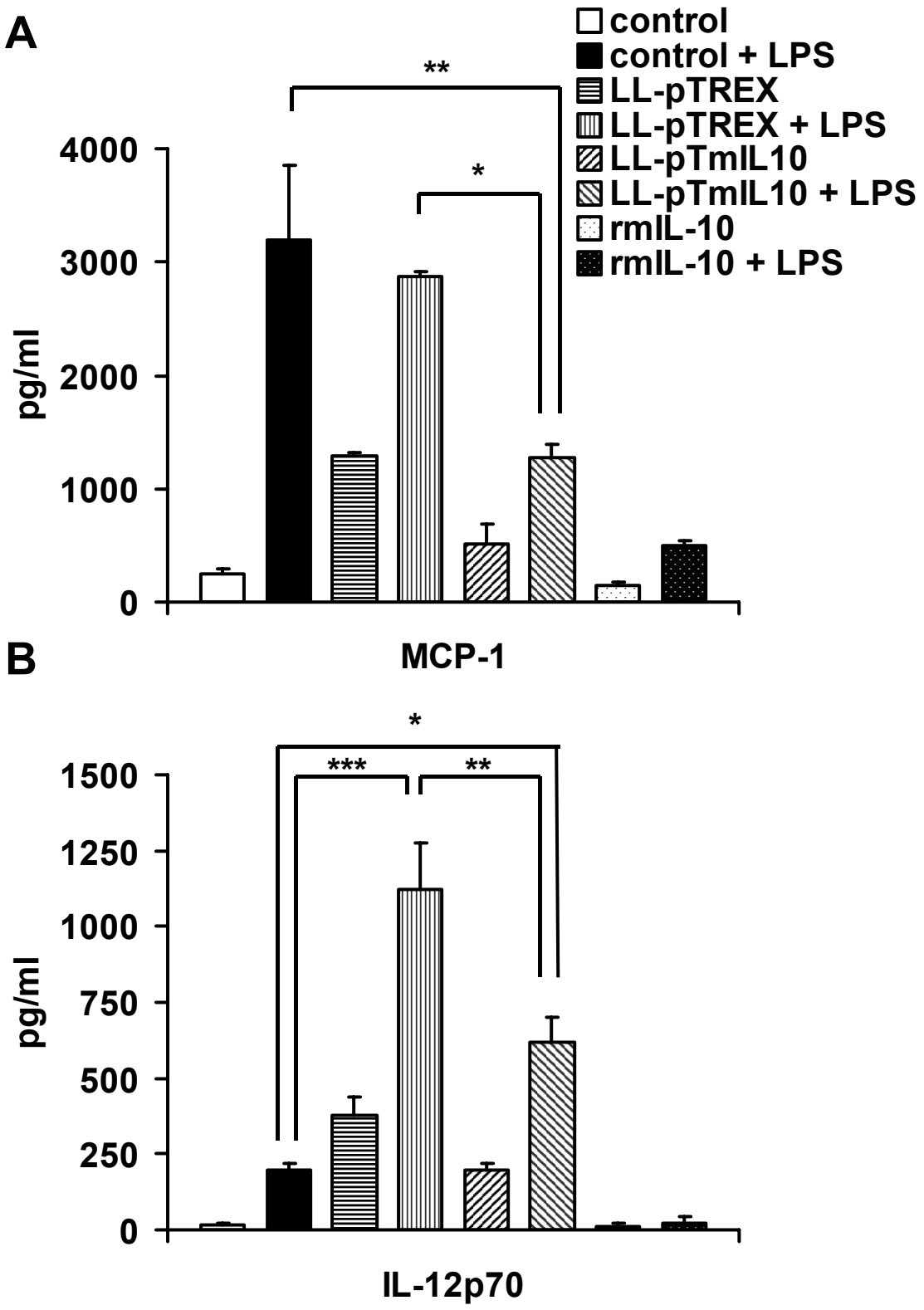


Figure 20 Cytokine production by activated BM-DCs.
$CD11c^+$ BM-DCs were treated as described in the Materials and Methods section. After overnight culture with or without LPS (10 ng/ml), supernatants were collected and cytokines were quantified with the mouse inflammation CBA kit. Results for **(A)** MCP-1 and **(B)** IL-12p70 from three experiments are shown as mean pg/ml + SEM. ***, ** and * denote significance of differences at $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively.

Treatment of BM-DCs with *LL-pTREX* or *LL-pTmIL10* induced less secretion of MCP-1 than treatment with LPS (Figure 20A, *LL-pTREX* = P<0.01 or *LL-pTmIL10* = P<0.001), whereas no difference was found for IL-12p70 (Figure 20B), TNF and IL-6 secretion (data not shown).

Pretreatment of BM-DCs with *LL-pTmIL10* (but not with *LL-pTREX*) significantly reduced LPS-induced MCP-1 up-regulation (P<0.01), but less effectively than treatment with rmIL-10 (P<0.001) (Figure 20A). Furthermore, we found that BM-DCs pretreated with *L. lactis* and stimulated with LPS expressed IL-12p70 more strongly than LPS-stimulated controls. *LL-pTmIL10* pretreatment significantly inhibited this synergistically up-regulated expression of IL-12p70 (P<0.01) (Figure 20B). In some of the experiments performed we observed a similar pattern of *LL-pTmIL10*-induced inhibition of TNF (data not shown). No inhibition was observed for IL-6 secretion after LPS stimulation of BM-DCs pretreated with *LL-pTmIL10* or *LL-pTREX*, whereas rmIL-10 treatment could partially inhibit LPS-induced expression of both IL-6 and TNF (data not shown).

2.4.5 LPS-stimulated BM-DCs pretreated with LL-pTmIL10 express less IL-12/IL-23p40 mRNA than LL-pTREX-pretreated cells.

IL-12 is a heterodimer formed by the subunits IL-12p35 and IL-12p40. The latter is also part of the pro-inflammatory cytokine IL-23 heterodimer produced by DCs. To test whether the difference in IL-12p70 expression by BM-DCs treated with *LL-pTmIL10* could also lead to a difference in IL-23 expression, we investigated if *LL-pTmIL10* treatment alters the expression of IL-12/IL-23p40, IL-12p35 or IL-23p19 after LPS stimulation (Figure 21). LPS-stimulated *LL-pTREX*- or *LL-pTmIL10*-treated BM-DCs were harvested and gene-specific mRNA expression was quantified by real-time quantitative PCR. *LL-pTREX*- and *LL-pTmIL10*-pretreated BM-DCs stimulated with LPS showed similar levels of IL-12p35 mRNA expression, whereas *LL-pTREX* treatment induced a threefold higher expression of IL-12/IL-23p40 mRNA. These data suggest that the reduced protein secretion of IL-12p70 by LPS-stimulated BM-DCs treated with *LL-pTmIL10*, compared to *LL-pTREX*-treated BM-DCs, is reflected by a difference in IL-12/IL-23p40 induction. In contrast, the inhibition of IL-12p70 secretion in LPS-stimulated BM-DCs treated with rmIL-10 is accomplished by affecting transcription of both IL-12/IL-23p40 and IL-12p35, correlating with an efficient inhibition of LPS-induced IL-12 secretion (Figure 20B). *LL-pTmIL10*- and *LL-pTREX*-treated BM-DCs did not differ in IL-23p19 mRNA levels but given their differential expression of IL-12/IL-23p40 mRNA, which leads to a reduced expression

of IL-12p70 protein, it is likely that BM-DCs matured with LPS in the presence of *LL-pTmIL10* secrete less IL-23 compared to their *LL-pTREX*-treated counterparts.

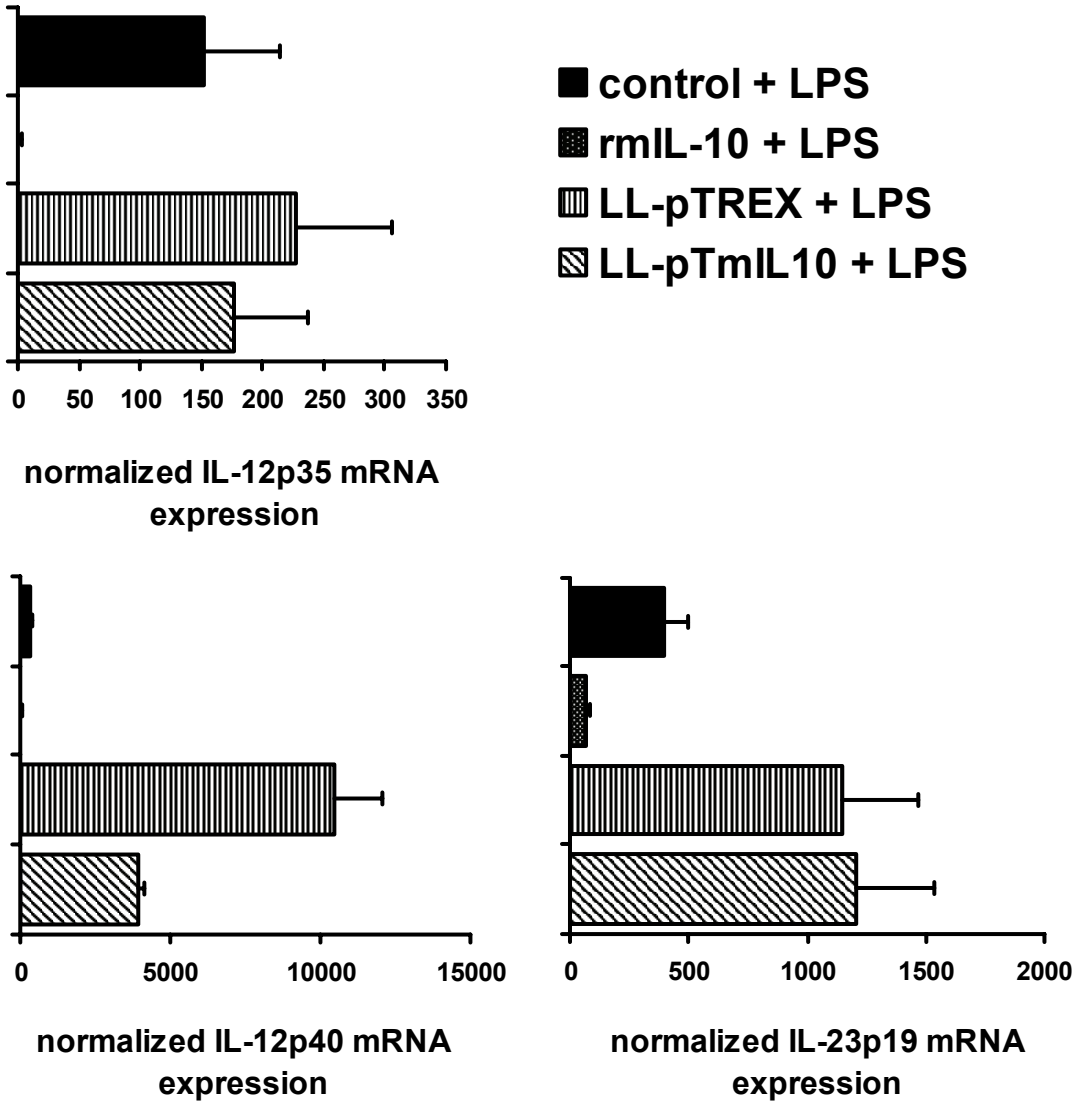


Figure 21 IL-12p35, IL-12p40 and IL-23p19 mRNA expression in activated BM-DCs. $CD11c^+$ BM-DCs were treated and harvested for mRNA isolation as described in the Materials and Methods section. Shown are the fold changes in TBP normalized mRNA expression levels of every condition relative to the control condition without stimulation. Bars represent the mean + SD. Results are representative of three experiments.

2.4.6 Allogeneic T cells stimulated by LL-pTREX- or LL-pTmIL10-treated BM-DCs differ in the ability to secrete IL-17.

Expression of IL-23 by DCs participates in expanding and maintaining the Th17 population *in vivo*. These recently discovered effector cells are identified by their secretion of IL-17 [13] and are probably involved in clearance of pathogens other than those targeted by Th1 and Th2 [415-417]. To further investigate immune-modulation by *L. lactis* and define the functional effect of *LL-pTmIL10*-specific modulation of IL-12/IL-23p40 expression by BM-DCs, we determined IL-17 expression by different activated T cells. Therefore, allogeneic bead-purified $CD4^+$ T cells were isolated after co-culture with the different BM-DCs and subsequently re-stimulated with cytostatic Balb/c splenocytes. After 72 h the presence of IL-17 in the supernatants was assessed (Figure 22). Corresponding to the high induction of the IL-12/IL-23p40 subunit, T cells activated by LPS-stimulated, *LL-pTREX*-treated BM-DCs induced significantly stronger expression of IL-17 ($P<0.001$) than LPS-stimulated, *LL-pTmIL10*-treated BM-DCs. Since DCs in the gut are confronted by both gram-negative and gram-positive bacteria, these data suggest that IL-10 secreted by *LL-pTmIL10* could modulate DC cell function by regulating IL-23 expression during inflammation, thereby inhibiting Th17 development.

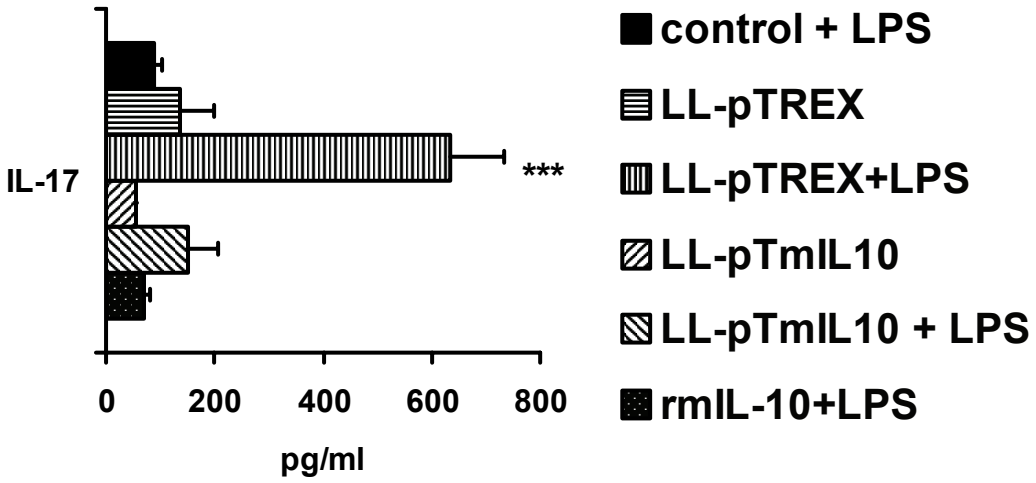


Figure 22 IL-17 production of activated T cells.
Re-stimulation of T cells was performed as described in the Materials and Methods section. Supernatants were collected after 72 h for IL-17 detection with a commercial IL-17 ELISA. Shown are mean pg/ml values + SD of triplicates from one experiment; results are representative of four experiments. *** denotes a significance of difference at $P < 0.001$.

2.5 Discussion

Here we report on the ability of mIL-10 secreted by a genetically modified *L. lactis* strain to modulate BM-DC functions in response to LPS. We show that *L. lactis*, independently of mIL-10 secretion, was less effective than LPS in inducing BM-DC maturation, demonstrated by their reduced capacity to activate naive T cells. In addition, *L. lactis*-conditioned, LPS-stimulated DCs had a reduced ability to stimulate naive allogeneic T cell proliferation compared to untreated LPS-stimulated controls and could suppress the capacity of LPS-stimulated BM-DCs to activate naive T cells. Furthermore, mIL-10 secreted by *L. lactis* specifically inhibited LPS-induced MCP-1 secretion as well as the synergistic effect of LPS and *L. lactis* on secretion of IL-12p70. Compared to *LL-pTREX*, mIL-10 secreted by *L. lactis* inhibited the strong, synergistic LPS-induced expression of IL-12/IL-23p40 mRNA, which correlated with the reduced expression of IL-12p70. This inhibition of IL-12/IL-23p40 could also lead to reduced expression of IL-23 by mature BM-DCs, which would explain the different Th17 cell polarizing effect of *LL-pTmIL10* and *LL-pTREX* in the presence of LPS.

Previous studies have shown that both viable and killed probiotic LAB species display strain-specific effects on the phenotype of human and murine DCs [159-161] and on polarizing T helper responses via modulation of DC function [163,418,419]. Although some commercial formulations claim health-promoting properties, *L. lactis* is not generally considered a true probiotic strain and little is known about its effects on DCs. Comparison of the immunomodulatory effects of different LAB on human peripheral blood monocytes showed that *L. lactis* MG1363 has a pro-inflammatory profile with a very low IL-10/IL-12 ratio, compared to other LAB and a commensal *E. coli* strain [420]. Furthermore, this observation was validated in murine BM-DCs, in which *L. lactis* also strongly up-regulated co-stimulatory molecules [162]. The discrepancy between previous results and ours might be due to the different experimental setups. We used viable bacteria at a DC:bacterial CFU ratio of 1:1 to avoid acidification of the growth medium by lactic acid production. Foligne and co-workers used a ratio of 1:10. Furthermore, we added gentamycin after 5 h of bacterial activity whereas in the other study gentamycin was present at the start of the co-culture. It has also been reported that exposure of cord blood-derived DCs to *L. lactis* strain W58 lead to moderate up-regulation of co-stimulatory molecules but did not induce full maturation compared to LPS [421]. This resembles our results suggesting that *L. lactis* MG1363 induces a semi-mature phenotype compared to

LPS. In addition, no difference was found between *LL-pTREX* and *LL-pTmIL10*. This indicates that the effect on BM-DC maturation, in the absence of LPS, is independent of the secretion of mIL-10 by *L. lactis*; it is an intrinsic property of the bacterial strain.

L. lactis treatment of BM-DCs induced lower levels of MHC II and co-stimulatory molecules, compared to treatment with LPS, but induction of the inhibitory molecule PD-L1 was similar. This might explain the difference between T cell activation capacities, as it has been shown that expression of alternative or inhibitory co-stimulatory molecules on DCs, such as PD-L1, can inhibit T cell activation when co-stimulation is sub-optimal [126]. This indicates that *L. lactis* induces an anti-inflammatory phenotype rather than the fully mature phenotype obtained with LPS.

Unexpectedly, LPS-stimulated BM-DCs pretreated with *LL-pTmIL10* or *LL-pTREX* were weaker stimulators of allogeneic T cells compared to LPS-stimulated BM-DCs. This could not be predicted from their maturation phenotype, which resembles that of untreated LPS-stimulated BM-DCs. One possible explanation might be the presence of large amounts of endogenous or exogenous mIL-10 during BM-DC maturation. Chang and co-workers showed that exposure of DCs to exogenous IL-10 upon maturation with LPS severely impaired their capacity to promote naive $CD4^+$ T cell proliferation. Besides the effects of exogenous IL-10 on DC apoptosis, they also found that endogenous IL-10 acted as a suicidal factor for DCs in an autocrine fashion [235]. Because our findings showed that treatment with 10 ng/ml rmIL-10 completely reduced the ability of LPS-stimulated BM-DCs to activate naive allogeneic $CD4^+$ T cells, the induction of apoptosis in mature DCs by the presence of high concentrations of IL-10 might also explain the impaired capacity of *LL-pTmIL10*- or *LL-pTREX*-pretreated LPS-stimulated BM-DCs to promote T cell proliferation. We indeed showed for this study that *LL-pTREX* treatment significantly up-regulated the expression of endogenous mIL-10 in LPS-stimulated BM-DCs compared to untreated LPS-stimulated BM-DCs, whereas high concentrations of exogenous mIL-10 (2-5 ng/ml) secreted by *LL-pTmIL10* were present in the BM-DC cultures pretreated with *LL-pTmIL10* (data not shown). In these cultures, endogenous mIL-10 secretion could not be determined. In contrast to rmIL-10 treatment, *L. lactis*-treated BM-DCs only partially inhibited the T cell activating capacity. This might be due to different concentrations of mIL-10 present during LPS-maturation; rmIL-10 at 10 ng/ml, *LL-pTmIL-10* secreted 2-5 ng/ml whereas with *LL-pTREX* $\pm$1 ng/ml was present. However, although different amounts of mIL-10 were detected in the cultures of *LL-pTmIL10*- and *LL-pTREX*-treated LPS-stimulated BM-DCs, there was no

difference in their capacities to stimulate naive T cells. Therefore, another explanation might be that the presence of *L. lactis* partially inhibits the potential apoptosis inducing effects of IL-10 on BM-DCs. Alternatively, these *L. lactis*-treated BM-DCs could lead to increased induction of allogeneic T cell apoptosis. The latter explanation might also explain why *LL-pTmIL10*- and *LL-pTREX*-treated BM-DCs were equally effective in inhibiting the proliferation of naive allogeneic T cells in the presence of fully potent, mature stimulator DCs. This cannot be explained by the apoptosis-inducing effects of endogenous IL-10 from the regulator BM-DCs, as it has been reported that mature DCs down-regulate their expression of the IL-10 receptor and are thus unresponsive to IL-10 [210,242].

Recently, commensal LAB were combined with a gram-negative strain or simply LPS for the stimulation of human monocyte-derived DCs [422]. This resulted for all tested strains in a strong synergistic induction of IL-12 and TNF, and at least an additive effect on the up-regulation of co-stimulatory markers. It has also been shown that weak IL-12- and TNF-inducing LAB possess the capability to inhibit the IL-12 and TNF responses induced by otherwise strong IL-12- and TNF-inducing strains, as well as DC maturation [422]. Our work shows no synergistic effect of *L. lactis*-treatment on the expression of co-stimulatory molecules of LPS-stimulated BM-DCs. However, synergism was shown for IL-12p70 secretion when the BM-DCs were treated with the empty vector control, *LL-pTREX*, and LPS, and secretion of mIL-10 by *L. lactis* reduced this synergism. A similar effect on TNF was seen in some but not all experiments. This is a unique characteristic of *LL-pTmIL10* as none of the commensal LAB tested by Zeuthen and co-workers showed this property in the presence of LPS [422]. Furthermore, the synergistic effect of LPS and *L. lactis* on IL-12p70 secretion is reflected by up-regulated IL-12/IL-23p40 mRNA expression, which was indeed reduced in the presence of *LL-pTmIL10* confirming its inhibitory effect on IL-12p70 secretion. In contrast, the IL-12p35 mRNA expression levels were similar in non-treated, *LL-pTREX*-treated and *LL-pTmIL10*-treated BM-DCs stimulated with LPS. This is consistent with the finding in human DCs that IL-12p35 mRNA is not induced by the gram-positive TLR2 agonist, peptidoglycan [99]. It has been proposed that mucosal inflammation occurs as the consequence of aberrant IL-12 responses to constituents of the commensal microbial flora, which in turn induces a pathological Th1 response [409,423]. We suggest that *in vivo* administration of *LL-pTmIL10* during inflammation might inhibit the stimulating effects of gut microflora on IL-12 secretion of DCs, which can lead to a reduced Th1-mediated inflammation.

However, it became clear with the discovery of IL-23 [9] that this cytokine rather than IL-12 drives chronic intestinal inflammation. Furthermore, in contrast to IL-12, IL-23 activates $CD4^+$ Th17 cells characterized by the production of IL-17, which have recently been shown to play an important role in colitis and several other autoimmune diseases [11,424,425]. Our study strongly indicates that BM-DCs stimulated with LPS in the presence of *L. lactis* indeed drive Th17 polarization through the induction of IL-23, which was clearly inhibited by the secretion of mIL-10 by *L. lactis*. Expression of IL-23p19 mRNA transcripts was strongly induced when BM-DCs were stimulated with LPS in the presence of the gram-positive *L. lactis* bacteria, compared to LPS alone. This could be explained by previous indications that peptidoglycan is more potent than LPS at inducting IL-23p19 mRNA transcripts in DCs [98,99]. Interestingly, *LL-pTmIL10*-pretreated, LPS-stimulated BM-DCs showed a reduced induction of IL-12/IL-23p40 mRNA transcripts compared to *LL-pTREX*-pretreated LPS-stimulated BM-DCs. This result indicates that p40 might be a limiting factor in the formation of the IL-23 complex, which might lead to a reduced secretion of the cytokine. Reduced secretion of IL-23 would be in agreement with the differential capacity of *LL-pTREX*- or *LL-pTmIL10*-pretreated, LPS-stimulated BM-DCs to induce IL-17 within the $CD4^+$ T cell population.

During inflammation, LPS and stimuli from LAB probably influence maturation of intestinal DCs. This may lead to a synergistic induction of IL-23 and subsequent sustained Th17 polarization, comparable to our *in vitro* data showing synergistic effects of *LL-pTREX* and LPS on BM-DCs. We hypothesize that IL-10 secreted by *LL-pTmIL10* can regulate this synergistic effect by reducing IL-23 secretion during intestinal inflammation, resulting in inhibition of otherwise sustained activation of Th17 cells.

Despite its ability to induce Th17 cells *in vitro*, IL-23 does not seem to be required for initiation of Th17 differentiation *in vivo*, although it definitely plays a role in expanding and maintaining the Th17 population [13,113-115]. In addition to its effect on T cell responses, IL-23 also has potent effects on cells of the innate immune system, inducing the production of inflammatory cytokines, such as IL-1, IL-6 and TNF, by monocytes and macrophages [417,426]. Furthermore, IL-23 also induces secretion of IL-17 by non-T cells in an inflammatory environment [427]. Therefore it has been proposed that, independently of its effect on Th17 cell activation, IL-23 production in response to intestinal bacteria triggers a pro-inflammatory cytokine cascade that, if left unchecked, can lead to the development of chronic intestinal inflammation [427].

Consequently, the proposed regulation of IL-23 by *LL-pTmIL10* might lead to reduction of the innate pathology induced by this cytokine *in vivo*.

During chronic inflammation, such as IBD, chemokines play a key role in the pathogenic infiltration of immune cells and the establishment of tissue destructive processes [428]. MCP-1, of the C-C chemokine family, plays a role in recruitment of monocytes to sites of injury and infection and also attracts a range of other cells, such as T cells, NK cells, basophils and neutrophils. The important role of MCP-1 during intestinal inflammation has been demonstrated by the marked increase in its tissue levels in IBD patients [429-432]. Other reports also demonstrated increased MCP-1 mRNA or protein levels in mouse models of (chronic) colitis [433-436]. Our data showed that *LL-pTmIL10*-pretreatment of BM-DCs strongly inhibited LPS-induced MCP-1 secretion. As previous data showed that *LL-pTmIL10* treatment of dextran sodium sulfate-induced murine colitis reduced both infiltration and epithelial damage, we suggest that the ability to reduce inflammation-induced MCP-1 expression might be part of the therapeutic mechanism of *LL-pTmIL10* [198,363]. *In vivo*, mIL-10 secreted by *LL-pTmIL10* may also target other MCP-1-secreting cell types in the gastro-intestinal tract. Although monocytes and macrophages are thought to be the main source of MCP-1, secretion by non-immune cells has also been demonstrated in IBD [429,430,432]. In fact, a role for IL-10 in the reduction of MCP-1 secretion by IL-1β-stimulated intestinal epithelial cells has been demonstrated in an *in vitro* study [437].

Of interest is that CCR2, the receptor for MCP-1, can be expressed on $CD4^+$ T cells, which produce much larger amounts of IL-17 than $CCR2^-$ cells, indicating the preferential expression of CCR2 on Th17 cells [438]. These $CCR2^+$ $CD4^+$ T cells are considerably increased in the ileum of CD patients [439]. Thus, MCP-1 reduction during oral treatment of murine colitis with *LL-pTmIL10* may reduce the accumulation of activated pathogenic $CCR2^+$ $CD4^+$ Th17 cells in the inflamed intestine, thereby contributing to the healing effect of *LL-pTmIL10.*

To conclude, we demonstrate that *LL-pTmIL10* can modulate functional properties of activated BM-DCs in the presence of LPS. We show that *LL-pTmIL10* in the presence of LPS inhibited MCP-1 and IL-12p70 secretion by BM-DCs and also their ability to induce $CD4^+$ Th17 cells. Furthermore our data indicate that *LL-pTmIL10* in the presence of LPS could reduce the secretion of IL-23 by BM-DCs. Our combined data suggest that *LL-pTmIL10* treatment during chronic colitis might lead to a

diminished promotion of $CD4^+$ Th17 cells and a reduced accumulation of these pathogenic Th17 cells as well as other immune cells at the site of inflammation, which together contribute to the beneficial effects of *LL-pTmIL10* administration.

PART III: SUMMARY AND DISCUSSION

Summary and Discussion

DCs are a heterogeneous population of professional APCs. They originate from bone marrow-derived hematopoietic stem cells. These precursors seed the blood and give rise to immature DCs that form a network of sentinels in peripheral tissues, especially at sites of pathogen entry. Immature DCs screen the environment for antigen. They are specialized in antigen uptake and processing. After interaction with microbial products or other maturation stimuli such as inflammatory cytokines, immature DCs change their pattern of chemokine receptors and migrate to the draining lymphoid tissue. During this process, DCs down-regulate their antigen-acquisition machinery and up-regulate the cell surface expression of antigen-loaded MHC molecules and co-stimulatory molecules such as CD40, CD86 and CD80. Once in the lymphoid organs, the phenotypically mature DCs have the ability to initiate immune responses by presenting antigen to naive T cells. Cytokines or cell surface molecules expressed by the activating DCs influence the outcome of T cell priming into either tolerance or immunity and the emergence of T cells carrying a Th1, Th2 or Th17 phenotype or Treg cells. Factors that influence the expression of these DC-derived effector molecules and thus the outcome of an immune response are the DC subset, its activation status, the antigen encountered and the environmental conditions under which DCs are primed.

The gastro-intestinal immune system has the important task to develop an immune response to invading pathogens while at the same time inducing tolerance against the normal microflora and food antigens. Different subsets of DCs are present in the intestinal mucosa, both at sites of antigen uptake and within inductive lymphoid tissue [440]. These intestinal DCs have unique functions when compared to DCs from non-mucosal sites and are thought to be critical in maintaining gut homeostasis [53,100,144,146,152]. IBD is a group of intestinal inflammatory diseases that can be subdivided in ulcerative colitis and Crohn's disease based on clinical manifestations. Increasing evidence suggests that IBD is caused by a loss of tolerance against the normal microflora [164]. Given the important role of DCs in the maintenance of mucosal tolerance, DCs may play a role in IBD as well. Recent studies indeed suggest that activated DCs are central mediators in the initiation and possibly also perpetuation of the disease [175-178]. Therefore, manipulation of DCs may be an effective way to treat IBD.

Previous studies demonstrated the therapeutic efficacy of *L. lactis*-delivered mIL-10 and TFF in experimental murine models for IBD [198,318]. So far, hardly any data

exist on the precise mechanisms of these therapies . We hypothesized that intestinal DCs are attractive targets for *L. lactis* delivered effector proteins. The *in vivo* study of DC modulation however is complex due to the relative infrequency of DCs and the existence of different subsets. Alternatively, DCs can be generated *in vitro* from murine bone marrow cultures supplemented with GM-CSF and IL-4 [389,390]. These BM-DCs are often used to study DC functions as they can be reproducibly obtained at high purity and in large numbers from a small number of donors.

In a **first part** of this work we investigated whether recombinant mTFF3 can directly regulate DC function by determining its effect on the maturation of BM-DCs. We purified *in vitro* generated $CD11c^+$ BM-DCs and pretreated them with mTFF3. They were then stimulated overnight in the presence of LPS. Data were compared to control BM-DCs cultured in medium. The phenotype of the different BM-DC groups was determined by flow cytometry. In the absence of LPS, non-treated and mTFF3-treated BM-DCs expressed similar levels of co-stimulatory molecules. After LPS stimulation, mTFF3-treated BM-DCs up-regulated the expression of CD80, CD86, CD40 and MHC II, and the negative stimulator of T cells, PD-L1 to the levels expressed by control BM-DCs stimulated with LPS. These results indicate that mTFF3 treatment of BM-DCs does not alter their mature phenotype in response to LPS. Furthermore, as expected, LPS induced the secretion of pro-inflammatory cytokines and chemokines: IL-12p70, TNF, IL-6 and MCP-1, as well as of the anti-inflammatory cytokine IL-10. Pretreatment of BM-DCs with mTFF3 did not influence any of the secreted proteins. In addition, our data show that mTFF3 does not affect the capacity of LPS-stimulated DCs to activate allogeneic bead-purified $CD4^+$ T cells.

The biology of DCs *in vivo* is complex and different subtypes exist. Therefore, results from BM-DCs have to be evaluated carefully as it is unclear how closely this *in vitro* obtained population resembles its *in vivo* counterpart. Since mTFF3 is secreted mainly in the small and large intestines, it is possible that intestinal mucosal DCs are responsive to this peptide whereas DCs from other origins are not. To test this hypothesis we isolated mucosal DCs from PP and MLN and compared their responses to LPS with or without mTFF3 pretreatment. As a control, we used peripheral DCs isolated from spleen. We found that mTFF3 does not specifically target mucosal DCs. Following treatment with mTFF3 no differences could be detected between mucosal and splenic DCs with respect to LPS-mediated maturation, cytokine secretion and capacity to stimulate T cells.

TFF peptides were demonstrated to be present in the spleen and other lymphoid organs [259]. Our results suggest that these TFF peptides do not modulate immune functions through direct interaction with DCs but rather affect other cells of the immune system. A recent study compared immune cells isolated from wild-type and TFF2-deficient mice [303]. They demonstrated that after stimulation, $TFF2^{-/-}$ splenic T cells exhibited increased IL-2 and IL-4 secretion and an enhanced proliferative response compared to wild-type T cells. Thymocytes, on the other hand were unaffected by TFF2 deficiency. Furthermore, TFF2 deficiency did not affect splenic B cell proliferation. Thus mature T cells may be the major targets of TFF2 regulation in the spleen. However, each trefoil factor has its own properties so a possible effect of mTFF3 on T cells requires further investigation. Furthermore, Giraud *et al* reported that TFF2 can reduce LPS-induced NO expression in monocytes [310], which may lead to inhibition of immune cell recruitment. As this effect on monocytes has also been described only for TFF2, it is possible that the effect of TFF2 on DCs is different from what we show for mTFF3.

Though we observed no direct modulation of DCs by purified recombinant mTFF3, it remains possible that *in vivo* mTFF3 regulates DC function through modulation of other cells. Crosstalk between epithelial cells and DCs is considered to be very important to regulate intestinal mucosal immunity [152,403]. It has been shown that TFF induces COX-2 expression in epithelial cells [318,319], leading to the secretion of prostaglandin PGE_2 whose anti-inflammatory effects on DCs have been extensively documented [322-325] Thus, TFF peptides might serve as key players in the epithelial-immune signaling system.

We found that recombinant mTFF3 does not influence LPS-induced DC maturation. However, it remains possible that mTFF3 can modulate DC maturation when the maturation stimulus is other than LPS. DCs determine the character of the immune response by secreting cytokines that drive the development of T cells into Th1, Th2, Th17 or Treg cells. PAMPs that stimulate different TLRs often induce distinct patterns of cytokines, thereby influencing T cell polarization [402]. Therefore it is plausible that mTFF3 can modulate the functions of DCs if these are stimulated by another PAMP (e.g. flagellin, zymosan, peptidoglycan). Furthermore, inflammatory mediators at the site of infection can also influence DC activation. Consequently, it is also possible that mTFF3 showed no effect in our experimental setup due to the absence of an appropriate microenvironment. Recent data from $TFF2^{-/-}$ stress the importance of the stimulus [303]. $TFF2^{-/-}$ macrophages secreted much higher levels of IL-6 than wild-type macrophages in response to IL-1R stimulation. On the other hand, wild-type

and TFF2$^{-/-}$ mice had very similar responses to LPS, suggesting that the hyperresponsiveness of TFF2 deficient mice was specific for IL-1β/IL-1R signaling pathways despite the fact that the IL-1R pathway shares many common adapters with the LPS/TLR4 pathway.

In conclusion, our results in this first part demonstrate that recombinant mTFF3 does not influence LPS-induced DC maturation suggesting that mTFF3 does not affect immune responses on the level of DC functions directly. However, it remains possible that mTFF3 regulates DC maturation when other maturation stimuli or inflammatory mediators are present or that mTFF3 regulates immune function through modulation of other cells.

In a **second part** of this work we focused on possible modulation of the immune system by genetically engineered *L. lactis* secreting mIL-10 (*LL-pTmIL10*). We hypothesized that IL-10 delivered via oral administration of recombinant *L. lactis* can modulate DC functions in response to microbial products, such as LPS, explaining partially the anti-inflammatory effect in murine models for IBD. We therefore studied the effect of *LL-pTmIL10* on LPS-induced BM-DC function. *In vitro* derived CD11c^{+} BM-DCs were treated with *LL-pTmIL10* or control *L. lactis* transformed with an empty vector (*LL-pTREX*). LPS was added to these BM-DCs after 5 hours. After overnight culturing the BM-DCs were analyzed and compared to untreated, rmIL-10-treated and LPS-stimulated BM-DCs. Figure 23 is an overview of the most important results.

In the absence of LPS, both *LL-pTmIL10* and *LL-pTREX* induced lower levels of MHC II and co-stimulatory molecules CD40, CD80 and CD86 compared to LPS-stimulated BM-DCs but expressed higher levels compared to rmIL-10 and non-treated control cells. This is in agreement with a recent study that demonstrated a generally lower induction of DC maturation by commensal LAB compared to LPS [422]. In addition, in our study, the level of the T cell inhibitory molecule PD-L1 induced by *LL-pTmIL10* or *LL-pTREX* was not different from the LPS-induced level. It has been shown that the expression of PD-L1 can inhibit T cell activation when co-stimulation is sub-optimal [126], suggesting that *L. lactis* induces an anti-inflammatory phenotype rather than a full mature phenotype as seen with LPS. Consistent with their phenotype, *L. lactis*-treated BM-DCs induced only weak T cell proliferation. As for the phenotype, no difference was found between *LL-pTREX*- and *LL-pTmIL10*-treated BM-DCs in their T cell proliferating capacity.

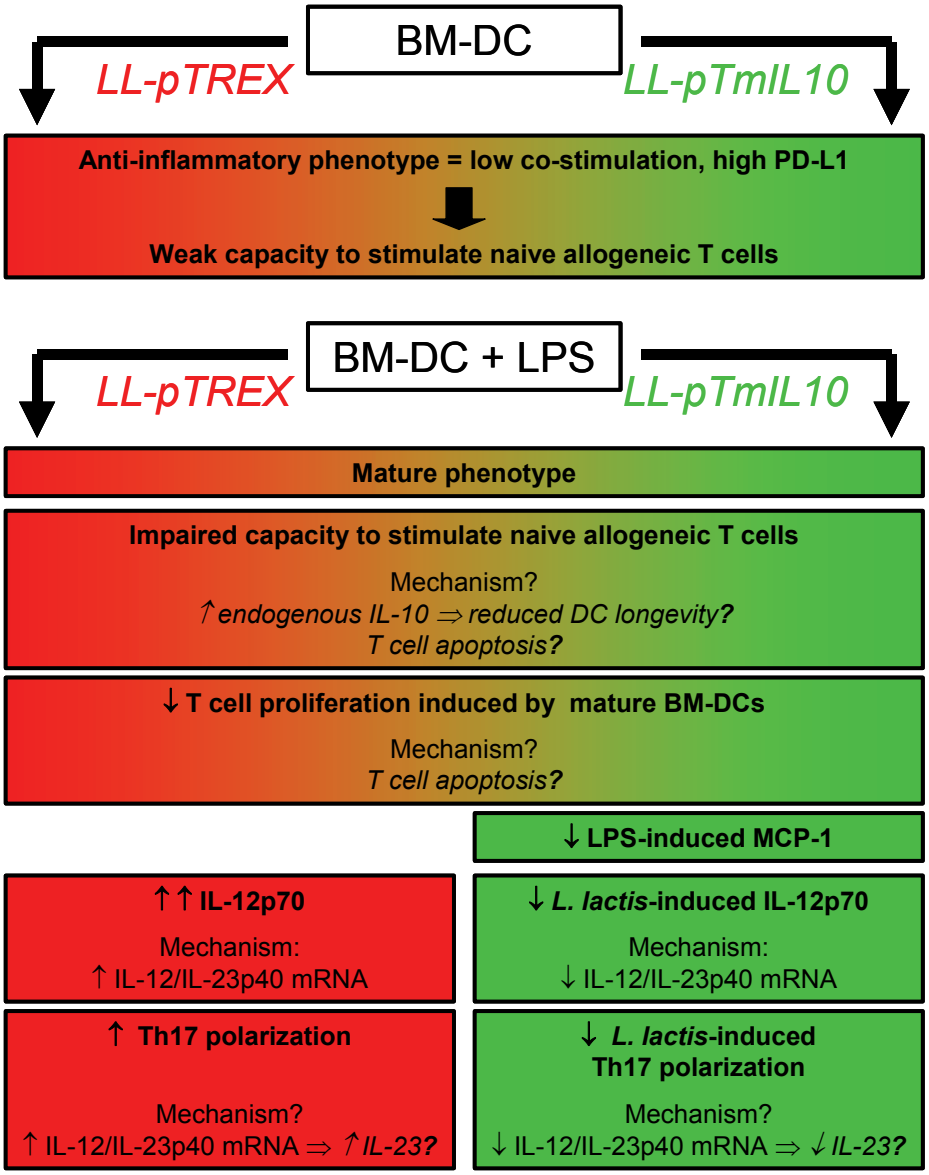


Figure 23 Summary of the effects in BM-DCs treated with *LL-pTREX* or *LL-pTmIL10*. Suggested mechanisms are indicated in *italics*

Rather unexpected was the finding that LPS-stimulated BM-DCs pretreated with *LL-pTmIL10* or *LL-pTREX* were reduced in their capacity to stimulate allogeneic T cells compared to LPS-stimulated BM-DCs. Chang and co-workers showed previously that exposure to exogenous IL-10 upon maturation with LPS severely impaired the capacity of DCs to promote naive $CD4^+$ T cell proliferation [235]. This effect has been ascribed to a reduced DC lifespan caused by the IL-10 specific suppression of anti-apoptotic proteins that are induced during DC maturation. In addition, they also found endogenous IL-10 to act as a suicidal factor for DCs in an autocrine fashion [235]. In agreement with this study, our findings showed that

treatment with 10 ng/ml rmIL-10 completely abolished the ability of LPS-stimulated BM-DCs to activate naive allogeneic $CD4^+$ T cells. We propose that the induction of apoptosis in mature DCs due to the presence of high concentrations of IL-10 might also explain the impaired capacity of *LL-pTmIL10*- or *LL-pTREX*-treated LPS-stimulated BM-DCs to promote T cell proliferation. Indeed, *LL-pTREX* treatment significantly up-regulated the expression of endogenous IL-10 in LPS-stimulated BM-DCs compared to untreated LPS-stimulated BM-DCs, whereas high concentrations of mIL-10 (2-5 ng/ml) were secreted by *LL-pTmIL10* in the BM-DC cultures pretreated with this strain. However, in contrast to rmIL-10, *L. lactis* treatment of BM-DCs only partially prevented the LPS-induced T cell activating capacity and despite different concentrations of IL-10 in the *LL-pTmIL10*- and *LL-pTREX*-treated LPS-stimulated BM-DC cultures, we found no difference in their respective capacities to stimulate naive T cells. An explanation might be that the presence of *L. lactis* partially inhibits the apoptosis inducing effects of IL-10 on BM-DCs.

Alternatively, *L. lactis* treatment of LPS-stimulated BM-DCs might reduce allogeneic T cell proliferation by the induction of T cell apoptosis. This might also explain our observation that *LL-pTmIL10*- and *LL-pTREX*-treated LPS-stimulated BM-DCs can act as regulators, effectively inhibiting the proliferation of naive allogeneic T cells in the presence of fully potent, mature stimulator DCs. This cannot be explained by induction of apoptosis in the mature DCs through secretion of endogenous IL-10 by the regulator BM-DCs, as it has been reported that mature DCs down-regulate their expression of the IL-10 receptor and are thus unresponsive to IL-10 [210,242].

Clearly, additional experiments need to be performed to confirm or reject any of the previous theories. To investigate the possibility that IL-10 induced DC apoptosis causes the reduced T cell activating capacity of LPS-stimulated BM-DCs after *L. lactis* treatment, we propose the addition of anti-IL-10 to the co-cultures of *LL-pTREX* or *LL-pTmIL10* with BM-DCs. In addition, any inhibiting influence of *L. lactis* on the apoptosis inducing effects of IL-10 can be investigated by adding exogenous rmIL-10 during *LL-pTREX* treatment of BM-DCs. Furthermore, various tools exist to determine the induction of apoptosis in either BM-DC or T cell populations.

Our data show a synergistic effect on IL-12p70 secretion when the BM-DCs were treated with *LL-pTREX* and LPS. This is in agreement with the study of Zeuthen *et al* that demonstrated a synergistic up-regulation of IL-12 and TNF in monocyte derived DCs when co-exposed to LAB and LPS [422]. However, the secretion of mIL-10 by

LL-pTmIL10 significantly reduced this synergistic induction of IL-12p70 by LPS and *L. lactis*. A similar effect on TNF was seen in some but not all experiments. This is a unique characteristic of *LL-pTmIL10* as none of the commensal LAB tested by Zeuthen and co-workers showed this ability in the presence of LPS.

IL-12 is a heterodimer formed by the subunits IL-12p35 and IL-12p40. The synergistic effect of LPS and *L. lactis* on IL-12p70 secretion is reflected by up-regulated IL-12p40 mRNA expression, which was indeed inhibited in the presence of *LL-pTmIL10*, confirming its inhibitory effect on IL-12p70 secretion. In contrast, the IL-12p35 mRNA expression levels of non-treated, *LL-pTREX*-treated or *LL-pTmIL10*-treated BM-DCs stimulated with LPS are similar. However, this is consistent with the finding in human DCs that IL-12p35 mRNA is not induced by the gram-positive TLR2 agonist, peptidoglycan [99]. The p40 subunit is also known to be part of the pro-inflammatory cytokine IL-23, which is produced by DCs upon microbial stimulation. Expression levels for IL-23p19 mRNA transcripts were highly induced when BM-DCs were stimulated with LPS in the presence of the gram-positive *L. lactis* bacteria, compared to LPS alone. This could be explained by previous results indicating that peptidoglycan is more potent than LPS at the induction of IL-23p19 mRNA transcripts in DCs [98,99]. However, the reduced induction of IL-12/IL-23p40 mRNA transcripts in *LL-pTmIL10*-pretreated LPS-stimulated BM-DCs compared to *LL-pTREX*-pretreated LPS-stimulated BM-DCs, suggests that p40 might be a limiting factor in the formation of the IL-23 complex, which might lead to a reduced secretion of this cytokine.

A recent study showed that IL-23 supports the proliferation of a new subset of IL-17 producing T helper cells called Th17 [441] and several reports suggest that IL-23 driven IL-17 is important in the pathogenesis of IBD [427,442-448]. Our results strongly suggest that BM-DCs stimulated with LPS in the presence of *L. lactis* drive Th17 polarization, whereas the secretion of mIL-10 by *L. lactis* clearly inhibits this effect. Reduced secretion of IL-23 would be in agreement with this differential capacity of *LL-pTREX*- or *LL-pTmIL10*-pretreated LPS-stimulated BM-DCs to induce IL-17 within the $CD4^+$ T cell population.

During chronic inflammation, invading bacteria and microbial components like LPS will activate mucosal DCs, causing aberrant responses to constituents of the commensal microbial flora [409,423]. Our results suggest that administration of *LL-pTmIL10* during inflammation might inhibit the synergistic effects of the gut microflora on IL-12p70 secretion by DCs, which might in turn lead to a reduced Th1-mediated inflammation. However with the discovery of IL-23, it became clear

that this cytokine rather than IL-12 drives chronic intestinal inflammation by inducing pathogenic Th17 responses. Our results allow us to hypothesize that IL-10 secreted by *LL-pTmIL10* might also be able to down-regulate the synergistic effect of LPS and commensal LAB on IL-23 secretion during intestinal inflammation resulting in the inhibition of otherwise sustained Th17 activation.

In addition to its effect on T cell responses, IL-23 has also potent effects on cells of the innate immune system, inducing the production of inflammatory cytokines, such as IL-1, IL-6 and TNF, by monocytes and macrophages [417,426]. Furthermore, an *in vivo* study by Hue and co-workers indicates that IL-23, produced in response to intestinal bacteria, also induces the secretion of IL-17 by non-T cells, including granulocytes and monocytes, in an inflammatory environment [427]. The down-regulation of microbial-induced IL-23 by *LL-pTmIL10* may thus inhibit both innate and adaptive pro-inflammatory immune responses *in vivo*.

However, despite its capacity to induce Th17 cells *in vitro*, IL-23 does not seem to be required for the initiation of Th17 differentiation *in vivo*, although there is definitely a role for this cytokine in expanding and maintaining the Th17 population [13,113-115]. Furthermore, as mucosal DCs have functional properties different from peripheral DCs, our *in vitro* demonstrated effects have to be carefully interpreted. However, a recent publication demonstrated the existence of an intestinal $CD11b^+$ DC population that is proposed to be responsible for the differentiation of Th17 cells *in vivo* [449]. These DCs are suggested to produce pro-inflammatory cytokines (IL-6, IL-23…) in response to bacterial flora, which, in combination with TGFβ promote Th17 responses.

Another important *LL-pTmIL10* specific effect we could demonstrate is the strong inhibition of LPS-induced secretion of the chemokine MCP-1 in BM-DCs. The important role for MCP-1 during intestinal inflammation has been demonstrated in several studies in which markedly increased tissue levels were found in IBD patients [429-432] or in animal models of chronic colitis [433-436]. The effective suppression of MCP-1 release from inflammatory cells might be a valuable means to decrease infiltration and ameliorate IBD. Previously obtained data showed that *LL-pTmIL10* treatment of DSS-induced murine colitis resulted in a reduction of both infiltration and epithelial damage [198]. The ability to reduce inflammation-induced MCP-1 chemokine expression might thus be part of the mechanism that leads to the efficient healing of murine colitis by *LL-pTmIL10*. Although monocytes and macrophages are suggested to be the main source of MCP-1, secretion by epithelial cells [429,432], smooth muscle cells and endothelial cells has also been demonstrated in IBD

mucosa [430]. Furthermore, a role for IL-10 in the reduction of MCP-1 secretion by IL1β-stimulated IECs has been demonstrated in an *in vitro* study [437]. Consequently, mIL-10 secreted by *LL-pTmIL10* may also target other MCP-1 secreting cell types in the gastro-intestinal tract.

Very interesting is the observation that CCR2, the receptor for MCP-1 is preferentially expressed on IL-17 producing T cells [438]. These $CCR2^+$ $CD4^+$ T cells are significantly increased in the ileum of Crohn's disease patients [439]. Thus, MCP-1 reduction during oral treatment of murine colitis with *LL-pTmIL10* may lead to a reduced accumulation of the activated pathogenic $CCR2^+$ $CD4^+$ Th17 cells within the inflamed intestine, additionally contributing to the healing effect of *LL-pTmIL10*.

To verify if our *in vitro* results can indeed be translated to the proposed *in vivo* effects that help explain the mechanism of *LL-pTmIL10* treatment in murine models of colitis, additional experiments are required. Figure 24 summarizes possible future experiments that can be performed to confirm the influence of *LL-pTmIL10* on DC modulation *in vivo* in an experimental model of chronic colitis.

To conclude, our results in the second part of this study demonstrate that *LL-pTmIL10* can modulate functional properties of activated BM-DCs in the presence of LPS. We show that *LL-pTmIL10* in the presence of LPS inhibited MCP-1 secretion by BM-DCs and also their ability to induce $CD4^+$ Th17 cells. In addition, we have data suggesting that *LL-pTmIL10* in the presence of LPS could reduce the secretion of IL-23 by BM-DCs. Together our data suggest that *LL-pTmIL10* treatment during chronic colitis might lead to a diminished promotion of $CD4^+$ Th17 cells and a reduced accumulation of these pathogenic Th17 cells as well as other immune cells at the site of inflammation all contributing to the beneficial effects of *LL-pTmIL10* administration.

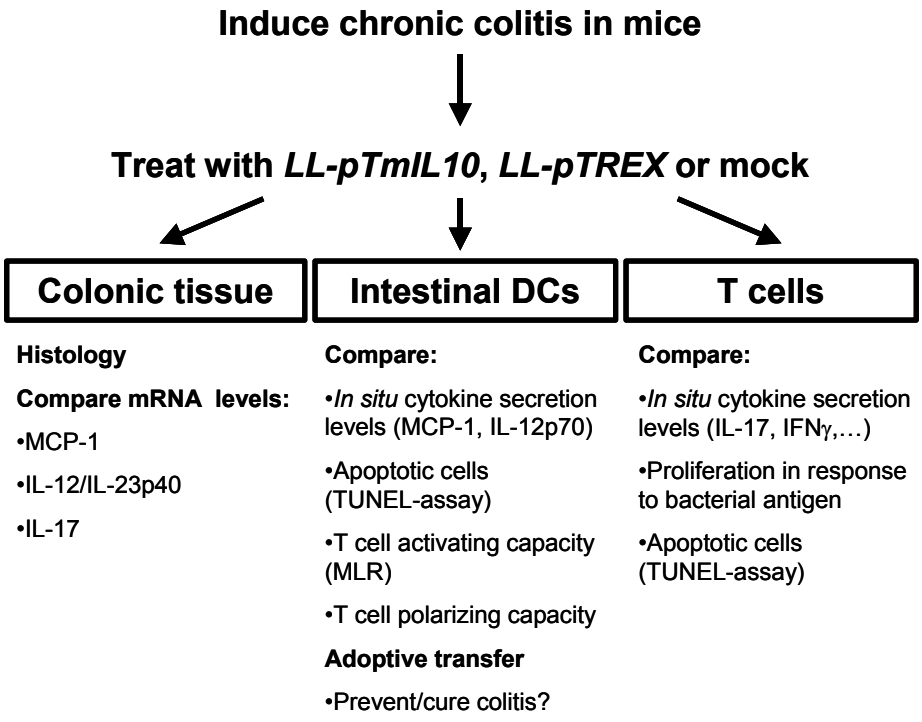


Figure 24 Overview of *in vivo* experiments.
First, mRNA expression levels of MCP-1, IL-12/IL-23p40 and IL-17 could be determined in colonic tissue of mock-treated, *LL-pTREX*-treated and *LL-pTmIL10*-treated mice. In addition, intestinal dendritic cells from these 3 groups could be isolated from the lamina propria and MLN. The cytokine secretion profile after *ex vivo* stimulation of these intestinal DCs could be compared to detect differences in MCP-1 and IL-12p70 levels between *LL-pTmIL10*-treated mice and the control groups. Furthermore, both T cell activating capacity of the intestinal DCs and the cytokine secretion profile of the activated T cells could be assessed and compared. The *in vivo* influence of intestinal DCs on T cells could also be assessed by directly studying the T cells isolated from MLN and spleen. We could determine the proliferative responses and the level of Th1, Th2 and Th17 cytokines after stimulating T cells derived from *LL-pTmIL10*-, *LL-pTREX*- and mock-treated mice with bacterial antigen. To define the importance of potential *LL-pTmIL10*-mediated effects on DCs, we could adoptively transfer intestinal DCs from *LL-pTmIL10*-treated mice into untreated mice with chronic colitis.

PART IV: SAMENVATTING EN DISCUSSIE

Samenvatting en Discussie

Dendritische cellen (DC's) vormen een heterogene populatie van zogenaamde professioneel antigeenpresenterende cellen (APC's). Ze ontwikkelen zich vanuit de hematopoietische stamcellen van het beenmerg. Deze precursoren verspreiden zich via de bloedbaan naar de perifere weefsels waar ze zich ontwikkelen tot immature DC's. In het bijzonder op plaatsen waar de gastheer in contact kan komen met ziektekiemen, zoals de huid, de slijmvliezen en de mucosa van de darm, vormen deze immature DC's ware netwerken die de omgeving aftasten naar lichaamsvreemde antigenen. Ze maken dan ook deel uit van de eerste lijn van verdediging tegen binnendringende pathogenen. Nadat een immature DC in contact is gekomen met microbiële producten of andere 'gevaarsignalen, zoals inflammatoire cytokines, ondergaat hij verschillende wijzigingen die leiden tot zijn maturatie. De expressie van chemokine receptoren wijzigt waardoor de DC zal migreren naar de drainerende lymfeknopen of de milt. Tegelijkertijd vermindert de DC zijn antigeenopname en verhoogt hij de expressie van MHC-antigeen complexen en costimulatorische moleculen zoals CD40, CD80 en CD86 aan het celoppervlak. Deze fenotypisch mature DC bezit nu de capaciteit om een immuunantwoord op te wekken door antigeen te presenteren aan naïeve T cellen. De interactie tussen de DC en een T cel kan resulteren in immuniteit, onder de vorm van Th1, Th2 of Th17 responsen, of tolerantie door het opwekken van T cel anergie, apoptose of de inductie van Treg cellen. Het resultaat wordt in sterke mate beïnvloed door de expressie van costimulatorische moleculen of andere oppervlakte eiwitten en cytokines door de DC. Factoren die de expressie van deze effectormoleculen beïnvloeden zijn het subtype en activatiestatus van de DC, het betrokken antigeen en ook de aanwezige signalen in de omgeving van de DC tijdens zijn maturatie.

De gastro-intestinale mucosa vormt het grootste en meteen ook meest complexe onderdeel van het immuunstelsel. Door zijn continue interactie met de inhoud van het lumen heeft het de belangrijke taak om een onderscheid te maken tussen invaderende pathogene micro-organismen enerzijds en de steeds aanwezige normale microflora en voedselantigenen anderzijds. Terwijl tegen de eerstgenoemden een efficiënte immuunrespons vereist is om verdere verspreiding in de gastheer te voorkomen is het van groot belang dat tolerantie wordt opgewekt tegen de laatstgenoemde, onschadelijke groep antigenen. Hiervoor beschikt het intestinale immuunsysteem over gespecialiseerde immuuncellen en -organen [440]. Ook de intestinale DC's hebben unieke functies in vergelijking met DC's uit andere

perifere weefsels en vormen een belangrijke schakel in het onderhouden van de homeostase in de darm [53,100,144,146,152].

Inflammatoir darmlijden (IBD) is de algemene term voor een groep chronische ontstekingsziekten van de darm die aan de hand van klinische verschijnselen kunnen worden onderverdeeld in ulceratieve colitis en de ziekte van Crohn. Hoewel de precieze etiologie niet gekend is, vermoedt men dat naast genetische aanleg en omgevingsfactoren, een abnormale immuunreactie tegen de normale darmflora een belangrijke rol speelt in de oorzaak van IBD [164]. Zoals gezegd spelen DC's een belangrijke rol in mucosale tolerantie en het is dan ook niet verwonderlijk dat een aantal recente studies suggereren dat geactiveerde DC's cruciaal zijn bij het ontstaan en mogelijk ook het aandrijven van de inflammatie in IBD [175-178]. Hieruit kan besloten worden dat methodes om de activatie van DC's te onderdrukken zinvol kunnen zijn in de behandeling van IBD.

Lactococcus lactis is een niet-pathogene, niet-invasieve, niet-koloniserende gram-positieve melkzuurbacterie die voornamelijk gebruikt wordt voor de productie van gefermenteerde voeding. Steidler en medewerkers zijn de haalbaarheid nagegaan om via orale toediening van genetisch gewijzigde *L. lactis*, biologisch actieve, immuunmodulerende eiwitten af te leveren aan de mucosa [198,318,346]. Hierbij resulteerde intragastrische toediening van muis interleukine 10 (mIL-10) secreterende *L. lactis* in 50% reductie van inflammatie in natrium dextraan sulfaat (DSS) geïnduceerde chronische colitis. Bovendien verhinderde deze stam het ontstaan van spontane colitis in IL-$10^{-/-}$ muizen [198]. Daarnaast resulteerde dagelijkse intragastrische toediening van trefoil factor secreterende *L. lactis* in een beschermend en therapeutisch effect tegen DSS geïnduceerde acute colitis en in een verbetering van chronische geïnstalleerde colitis in IL-$10^{-/-}$ muizen [318]. Deze studies tonen, naast de mogelijkheid om *L. lactis* te gebruiken als leverancier van biologisch actieve eiwitten in de darm, ook de therapeutische efficiëntie van *L. lactis* aan in muismodellen voor IBD.

Ondanks de aangetoonde efficiëntie van de door *L. lactis* afgeleverde effectormoleculen mIL-10 en trefoil peptiden, zijn er nauwelijks data over de precieze werking van deze therapieën. Door hun aanwezigheid in de darm en hun immuunmodulerende rol vormen intestinale DC's een aantrekkelijk doelwit voor de door *L. lactis* aangeleverde therapeutische eiwitten. De *in vivo* studie van DC's wordt echter bemoeilijkt door de heterogeniteit van deze cellen en hun relatief lage aantallen. Daarom hebben wij in deze studie gebruik gemaakt van *in vitro*

gegenereerde beenmerg DC's (BM-DC's). Door toevoegen van GM-CSF en IL-4 kunnen op reproduceerbare wijze relatief grote aantallen DC's gegenereerd worden uit beenmergculturen [389,390]. Bovendien kunnen deze cellen op eenvoudige wijze sterk opgezuiverd worden. BM-DC's vormen dus een waardig en veel gebruikt alternatief voor de studie van DC's en hun functies.

De trefoil factor familie (TFF) is een familie van protease resistente peptiden die abundant gesecreteerd worden door mucus producerende cellen van het gastro-intestinale stelsel. Deze TFF, bestaande uit het gastrische TFF1, TFF2 en intestinale TFF3, dragen bij tot de bescherming en het herstel van gastro-intestinaal epitheel bij schade [289,366,367]. De signaalwegen die bij deze processen betrokken zijn, zijn nog maar gedeeltelijk ontrafeld [450]. Er is ook reeds bewijs geleverd dat het toedienen van trefoil peptiden een helend effect heeft in proefdiermodellen voor intestinale inflammatie [318,373,374,393] maar er is nog geen diepgaand onderzoek verricht naar het mechanisme waarmee deze peptiden de inflammatoire processen in de darm onderdrukken. De succesvolle behandeling van Th1 gemedieerde colitis in IL-$10^{-/-}$ muizen met TFF secreterende *L. lactis* duidt op immuunmodulerende eigenschappen van de TFF naast hun functie in epitheliale restitutie [318]. Sommige recente publicaties wijzen ook sterk in de richting van een rol voor de TFF in immuunregulatie maar er werd nog geen directe invloed aangetoond [259,303,310,315,317,391].

In **een eerste deel** van dit werk wilden we een mogelijke directe invloed van recombinant muis TFF3 (mTFF3) op de functionele capaciteiten van DC's onderzoeken. Daarom hebben we het effect van mTFF3 op de door LPS geïnduceerde maturatie van BM-DC's bepaald. Hiervoor werden $CD11c^{+}$ BM-DC's voorbehandeld met mTFF3 alvorens ze overnacht te stimuleren met LPS. Deze cellen werden vergeleken met controle BM-DC's die niet werden voorbehandeld. Het fenotype van de BM-DC's werd bepaald door middel van flow cytometrie. In de afwezigheid van LPS expresseerden onbehandelde en mTFF3 behandelde BM-DC's gelijkaardig lage hoeveelheden aan costimulatorische moleculen. Na LPS stimulatie zagen we een sterk opgereguleerde expressie van CD80, CD86, CD40 en MHC II en van de negatieve stimulator van T cellen, PD-L1 op de mTFF3 behandelde BM-DC's. De expressieniveaus verschilden bovendien niet van de LPS gestimuleerde controle BM-DC's. Uit deze data konden we dus besluiten dat mTFF3 behandeling van BM-DC's geen invloed heeft op de inductie van een matuur fenotype ten gevolge van LPS stimulatie. Zoals verwacht induceerde LPS ook de secretie van de pro-inflammatoire cytokines IL-12p70, TNF en IL-6, het chemokine MCP-1 evenals

van het anti-inflammatoire IL-10. Voorbehandeling van de BM-DC's met mTFF3 had geen enkele invloed op de secretie van deze eiwitten. In overeenstemming met deze data bleek mTFF3 ook niet in staat om de T cel stimulerende capaciteit van LPS gestimuleerde BM-DC's te wijzigen.

Resultaten bekomen met BM-DC's dienen steeds kritisch geëvalueerd te worden. Het is immers onduidelijk in welke mate deze *in vitro* populatie overeenstemt met de *in vivo* populatie die bestaat uit verschillende subtypes. Aangezien mTFF3 voornamelijk in de dunne en dikke darm gesecreteerd wordt, leek het ons mogelijk dat intestinale DC's responsief zijn voor dit peptide in tegenstelling tot DC's van andere weefsels. Om die hypothese te testen isoleerden we mucosale DC's uit de Peyerse platen (PP) en mesenteriale lymfeknopen (MLN) en vergeleken hun gevoeligheid aan LPS met en zonder mTFF3 voorbehandeling. Ons onderzoek toonde aan dat mTFF3 niet in staat is om specifiek mucosale DC's te moduleren. Na stimulatie met LPS konden immers geen verschillen waargenomen worden tussen mTFF3 behandelde en onbehandelde mucosale DC's op gebied van maturatie, cytokine secretie en capaciteit om allogene T cellen te stimuleren. Hetzelfde gold voor DC's uit de milt die als bron van niet-mucosale DC's werden gebruikt.

Cook en medewerkers hebben de aanwezigheid van de TFF in de milt en andere lymfoide organen aangetoond [259]. Uit onze resultaten kunnen we concluderen dat TFF peptiden het immuunsysteem niet kunnen moduleren via rechtstreekse interactie met DC's en dat hun aanwezigheid en functie in de immunologische organen dus op een andere manier verklaard moet worden. In een recente studie heeft men de immuuncellen van TFF2 deficiënte muizen vergeleken met deze van wild-type muizen [303]. Hierin werd aangetoond dat $TFF2^{-/-}$ T cellen uit de milt na stimulatie sterker prolifereerden en meer IL-2 en IL-4 secreteerden dan wild-type T cellen. Anderzijds kon geen verschil worden waargenomen tussen de thymocyten van beide muizen. Ook de proliferatie van B cellen uit de milt was niet aangetast in TFF2 deficiënte muizen. Hieruit kan worden besloten dat mature T cellen het voornaamste doelwit vormen voor TFF2 regulatie in de milt. Elk TFF peptide heeft echter zijn eigen karakteristieken en individuele functies en het effect van mTFF3 op T cellen dient nog onderzocht te worden. In een andere studie toonden Giraud en medewerkers aan dat TFF2 in staat is om LPS geïnduceerde NO productie in monocyten te onderdrukken [310] wat kan leiden tot een verminderde aantrekking van immuuncellen. Ook dit effect op monocyten werd enkel voor TFF2 waargenomen waardoor het aannemelijk lijkt dat de effecten van TFF2 op het immuunsysteem, en DC's in het bijzonder, verschillend zijn van wat we hier besluiten voor mTFF3.

Hoewel wij geen directe modulatie van DC's konden aantonen met gezuiverd recombinant mTFF3 blijft het nog steeds mogelijk dat mTFF3 onrechtstreeks in staat is om de activatie van DC's te reguleren door een modulerende invloed op andere cellen uit te oefenen. Zo wordt communicatie van het epitheel naar de onderliggende DC's beschouwd als een belangrijk onderdeel van mucosale immuniteit [152,403]. Wij konden, net als in eerdere studies, TFF geïnduceerde COX-2 expressie aantonen in intestinale epitheelcellen [318,319,451]. Dit leidt o.a. tot de secretie van PGE_2 waarvan de anti-inflammatoire effecten op DC's reeds uitvoerig werden beschreven [322-325]. TFF peptiden zouden langs deze weg dus een belangrijke rol kunnen spelen in de signalisatie van het epitheel naar het immuunsysteem.

We hebben aangetoond dat recombinant mTFF3 niet in staat is om LPS geïnduceerde maturatie van DC's te beïnvloeden. Het is dus wel nog mogelijk dat mTFF3 de maturatie van DC's onder invloed van een andere stimulus dan LPS kan onderdrukken. Zoals eerder aangehaald beïnvloeden de DC's het type immuunrespons o.a. door polariserende cytokines te secreteren die de ontwikkeling van naïeve T cellen naar Th1, Th2, Th17 of Treg cellen sturen. Het cytokinepatroon van de DC's wordt onder andere bepaald door de pathogeen geassocieerde moleculaire patronen (PAMP's) die via 'Toll-like' receptoren (TLR's) de DC stimuleren [402]. Het is dus goed mogelijk dat mTFF3 in staat is om de functies van DC's te moduleren indien deze worden aangedreven door een ander PAMP (bvb. zymosan, peptidoglycaan of flagelline). Bovendien kunnen DC's tijdens een infectie additioneel beïnvloed worden door de aanwezige inflammatoire moleculen. Het is dus ook mogelijk dat wij geen mTFF3 gemedieerde effecten waargenomen hebben door de afwezigheid van een gepaste micro-omgeving. Het belang van de juiste stimulus werd nog eens onderstreept in de recente studie van $TFF2^{-/-}$ muizen [303]. TFF2 deficiënte macrofagen bleken na stimulatie van de IL-1 receptor (IL-1R) significant hogere hoeveelheden IL-6 te produceren dan wild-type macrofagen. Anderzijds vertoonden deze macrofagen wel gelijkaardige reacties na LPS stimulatie wat wijst op een IL-1β/IL-1R specifieke hyperresponsiviteit in TFF2 deficiënte muizen en dit ondanks het feit dat de IL-1R signaalweg veel adaptor-moleculen deelt met de LPS/TLR4 signaalweg.

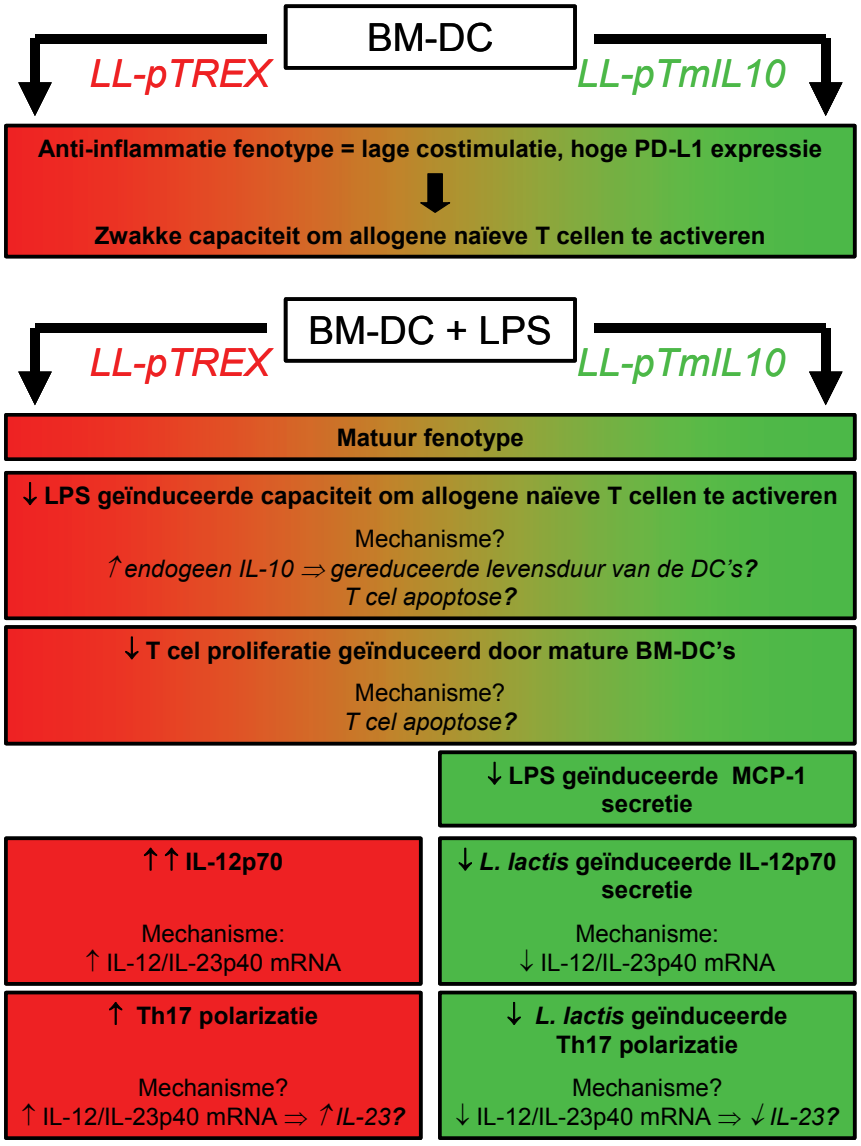
Uit dit alles hebben wij besloten dat recombinant mTFF3 niet in staat is om de LPS geïnduceerde maturatie van DC's te onderdrukken, wat suggereert dat mTFF3 zijn invloed op het immuunsysteem niet uitoefent via directe modulatie van de DC's. Niettegenstaande bestaat de mogelijkheid dat mTFF3 de maturatie van DC's reguleert in de aanwezigheid van een andere maturatiestimulus of van inflammatoire

moleculen of dat mTFF3 immuunantwoorden reguleert door het beïnvloeden van andere cellen.

In het **tweede deel** van dit werk wilden we de mogelijke invloed van de mIL-10 secreterende genetisch gewijzigde *L. lactis* stam (*LL-pTmIL10*) op het immuunsysteem nagaan. Een belangrijke fysiologische functie van IL-10 is het onderdrukken van de expressie van cytokines en andere gesecreteerde moleculen of oppervlakte-eiwitten door DC's. Dit heeft een verminderde capaciteit van deze DC's om het immuunsysteem te activeren tot gevolg, waardoor aan IL-10 een ontstekingsremmende werking kan worden toegeschreven [121,122,125,235,245,401]. Wij hebben de hypothese gesteld dat lokale aflevering van IL-10 in de darm door recombinant *L. lactis* een regulerende invloed kan uitoefenen op de activatie van intestinale DC's door microbiële producten, zoals LPS, wat een gedeeltelijke verklaring zou kunnen zijn voor de vastgestelde onderdrukte inflammatie in de muismodellen voor IBD. Om deze mogelijkheid te onderzoeken zijn we het effect van *LL-pTmIL10* op de functie van LPS gestimuleerde BM-DC's nagegaan. *In vitro* gegenereerde $CD11c^+$ BM-DC's werden gedurende 5 uur voorbehandeld met *LL-pTmIL10* of de controlestam *LL-pTREX* - dit is *L. lactis* getransformeerd met de uitgangsvector - en vervolgens al dan niet gestimuleerd met LPS. Na overnacht cultuur werden deze BM-DC's geoogst en vergeleken met onbehandelde, recombinant mIL-10 (rmIL-10) behandelde en LPS gestimuleerde BM-DC's. Een overzicht van de belangrijkste resultaten is weergegeven in Schema 1.

Zowel *LL-pTmIL10* als *LL-pTREX* induceerden lagere niveaus van MHC II en costimulatorische moleculen CD40, CD80 en CD86 in BM-DC's vergeleken met LPS gestimuleerde BM-DC's maar expresseerden meer van deze oppervlaktemoleculen dan onbehandelde of rmIL-10 behandelde BM-DC's. Deze resultaten zijn in overeenstemming met een recente studie die voor verschillende commensale melkzuurbacteriën een lagere inductie van DC maturatie aantoonde in vergelijking met LPS [422]. Wij stelden verder ook vast dat het door *LL-pTREX* of *LL-pTmIL10* geïnduceerde expressieniveau van PD-L1 niet verschilde van het door LPS geïnduceerde niveau. Expressie van deze T cel inhiberende molecule door DC's in combinatie met suboptimale expressie van costimulatorische moleculen, kan activatie van T cellen verhinderen [126]. Onze resultaten suggereren bijgevolg dat *L. lactis* een anti-inflammatoir fenotype induceert in vergelijking met het mature fenotype van met LPS geactiveerde BM-DC's. In overeenstemming hiermee induceerden de met *L. lactis* behandelde BM-DC's slechts een zwakke T cel

proliferatie. Net als voor het fenotype was er hierbij geen verschil tussen de T cel inducerende capaciteit van *LL-pTREX* en *LL-pTmIL10* behandelde BM-DC's.



Schema 1 Overzicht van *LL-pTREX* en *LL-pTmIL10* geïnduceerde effecten in BM-DC's. Hypothetisch mogelijke mechanismen staan *schuingedrukt.*

Eerder onverwacht was de vaststelling dat de met LPS gestimuleerde BM-DC's die voorbehandeld waren met *LL-pTREX* of *LL-pTmIL10* een gereduceerde capaciteit vertoonden om allogene T cellen te stimuleren ten opzichte van onbehandelde met LPS gestimuleerde BM-DC's. Dit kon niet worden afgeleid van hun fenotype, dat gelijkaardig was aan dat van de onbehandelde met LPS gestimuleerde BM-DC's. Chang en medewerkers hebben eerder aangetoond dat DC's die tijdens de maturatie

met LPS aan exogeen IL-10 werden blootgesteld een sterk gereduceerde capaciteit vertoonden om naïeve $CD4^+$ T cel proliferatie te induceren zonder evenwel de door LPS geïnduceerde fenotypische maturatie te onderdrukken [235]. Dit effect werd toegeschreven aan de IL-10 gemedieerde suppressie van anti-apoptotische eiwitten waardoor de mature DC's sneller in apoptose treden. In dezelfde studie werd ook aangetoond dat de aanwezigheid van endogeen IL-10 op autocriene wijze een gelijkaardige invloed uitoefent op maturerende DC's. In overeenstemming met deze studie konden ook wij aantonen dat LPS gestimuleerde BM-DC's behandeld met 10 ng/ml rmIL-10 een sterk gereduceerde capaciteit vertoonden om naïeve allogene $CD4^+$ T cellen te stimuleren. De inductie van apoptose in mature DC's door de aanwezigheid van IL-10 zou ook een verklaring kunnen zijn voor de waarneming dat LPS gestimuleerde BM-DC's voorbehandeld met *LL-pTREX* of *LL-pTmIL10* een de verminderde T cel activerende capaciteit vertoonden. *LL-pTREX* behandeling zorgde immers voor een significante verhoging van de endogene IL-10 expressie in LPS gestimuleerde BM-DC's vergeleken met onbehandelde door LPS gestimuleerde BM-DC's. Anderzijds zorgde voorbehandeling van de BM-DC's met *LL-pTmIL10* voor de aanwezigheid van hoge concentraties exogeen IL-10 (2-5 ng/ml) tijdens LPS stimulatie. In deze co-cultuur kon de hoeveelheid endogeen IL-10 bijgevolg niet exact bepaald worden. In tegenstelling tot rmIL-10 reduceerde de voorbehandeling met *LL-pTREX of LL-pTmIL10* slechts gedeeltelijk de T cel activerende capaciteit van LPS gestimuleerde BM-DC's. Dit zou te maken kunnen hebben met het verschil aan IL-10 concentraties aanwezig tijdens LPS maturatie: rmIL-10 was aanwezig aan 10 ng/ml, *LL-pTmIL10* secreteerde 2-5 ng/ml en in de aanwezigheid van *LL-pTREX* kon $\pm$ 1 ng/ml endogeen IL-10 worden gedetecteerd. Ondanks de verschillende hoeveelheden IL-10 werd echter geen verschil waargenomen in T cel stimulerende capaciteit van *LL-pTREX* of *LL-pTmIL10* behandelde LPS gestimuleerde BM-DC's. Een alternatieve verklaring zou daarom kunnen zijn dat de aanwezigheid van *L. lactis* het apoptose inducerend effect van IL-10 op DC's gedeeltelijk onderdrukt.

Een andere mogelijke verklaring voor de gereduceerde T cel activerende capaciteit van de met *L. lactis* behandelde, LPS gestimuleerde BM-DC's is dat deze BM-DC's in staat zijn om apoptose te induceren in de T cel populatie. Dit zou bovendien ook de efficiëntie verklaren waarmee zowel *LL-pTREX* als *LL-pTmIL10* behandelde, LPS gestimuleerde BM-DC's optreden als regulators en de proliferatie van naïeve allogene T cellen in aanwezigheid van mature, potente stimulator DC's, kunnen onderdrukken. Dit fenomeen kan immers niet uitgelegd worden aan de hand van apoptose inductie in de stimulator DC's door endogene IL-10 productie van de

regulator populatie, aangezien beschreven werd dat in mature DC's de expressie van de IL-10 receptor onderdrukt wordt waardoor deze ongevoelig zijn voor IL-10 [210,242].

Bovenstaande theorieën vragen uiteraard bijkomend onderzoek. Om de rol van endogeen en exogeen IL-10 te onderzoeken, kan een blokkerend anti-IL-10 antilichaam worden toegevoegd aan de co-culturen van BM-DC's met *LL-pTREX* of *LL-pTmIL10*. De eventuele inhiberende invloed van *L. lactis* op de apoptose inducerende werking van IL-10 kan onderzocht worden door exogeen rmIL-10 toe te voegen tijdens *LL-pTREX* behandeling van BM-DC's. Tenslotte bestaan er verscheidene mogelijkheden om de inductie van apoptose rechtstreeks aan te tonen in de BM-DC's of in de T cel populatie.

Zeuthen en medewerkers hebben recent aangetoond dat er een groot verschil is in de capaciteit van verschillende melkzuurbacteriën om TNF en IL-12 secretie te induceren in humane, uit monocyten ontwikkelde DC's. Niettegenstaande deze species-specifieke verschillen stelden zij vast dat beide cytokines steeds synergistisch werden opgereguleerd wanneer additioneel LPS werd toegevoegd [422]. In overeenstemming hiermee stelden ook wij een synergisme vast in de secretie van IL-12p70 wanneer de BM-DC's werden gestimuleerd met LPS in de aanwezigheid van *LL-pTREX*. Secretie van mIL-10 door *LL-pTmIL10* bleek evenwel in staat om deze synergistische inductie van IL-12p70 te reduceren. Een algemeen beschreven oorzaak van mucosale inflammatie is een aberrante respons gericht tegen de commensale microflora en IL-12 productie door APC's is daarbij een belangrijke factor door het induceren van pathologische Th1 responsen [409,423]. We suggereren aan de hand van onze data dat orale toediening van *LL-pTmIL10* tijdens inflammatie het stimulerend effect van de microflora op IL-12 secretie kan reduceren wat op zijn beurt zou kunnen leiden tot een verminderde Th1 gemedieerde inflammatie.

IL-12p70 is een heterodimeer cytokine, bestaande uit de subunits IL-12p35 en IL-12p40. De p40 subunit van IL-12 maakt ook deel uit van een ander pro-inflammatoir cytokine, het heterodimere IL-23 [9]. Dit cytokine wordt eveneens door DC's tot expressie gebracht na contact met microbiële producten [9,452]. Om na te gaan of de gereduceerde expressie van IL-12p70 in *LL-pTmIL10* behandelde BM-DC's ook een verschil in IL-23 expressie impliceert, zijn we het effect van *LL-pTmIL10* op de mRNA expressie van de subunits IL-12p35, IL-12/IL-23p40 en IL-23p19 nagegaan na LPS stimulatie. Zowel het synergistische effect van LPS en *L. lactis* op IL-12p70 secretie als de inhibitie hiervan in aanwezigheid van

LL-pTmIL10 werden weerspiegeld in de mRNA expressieniveaus van IL-12/IL-23p40. Daarentegen waren de IL-12p35 mRNA expressieniveaus van onbehandelde, *LL-pTREX* behandelde en *LL-pTmIL10* behandelde BM-DC's gestimuleerd met LPS, gelijkaardig. Dit is in overeenstemming met een eerdere studie in humane DC's waarin, in tegenstelling tot LPS, het gram-positieve TLR2 ligand peptidoglycaan niet in staat bleek om IL-12p35 mRNA expressie te induceren [99]. In vergelijking met LPS inductie bleek het mRNA expressieniveau van de IL-23p19 subunit sterk geïnduceerd te worden door de gram-positieve *L. lactis* bacterie in de LPS gestimuleerde BM-DC's. Dit is ook in overeenstemming met eerdere studies in DC's die aantoonden dat peptidoglycaan een sterkere capaciteit heeft dan LPS om IL-23p19 mRNA te induceren [98,99]. Zoals reeds gezegd resulteert *LL-pTmIL10* voorbehandeling van LPS gestimuleerde BM-DC's wel in een sterk verminderde inductie van IL-12/IL-23p40 mRNA transcripten in vergelijking met *LL-pTREX* voorbehandeling. Dit suggereert dat in de *LL-pTmIL10* behandelde BM-DC's de p40 subunit een limiterende factor vormt in de vorming van het IL-23 complex, wat dus een gereduceerde secretie van dit cytokine zou kunnen veroorzaken.

IL-23 ondersteunt de proliferatie van een nieuwe subset IL-17 producerende T helper cellen, de Th17 cellen [441] en verscheidene studies toonden reeds het belang aan van IL-23/IL-17 gemedieerde inflammatie in de pathogenese van IBD [427,442-444,446,448]. Uit onze resultaten bleek dat BM-DC's gestimuleerd met LPS in aanwezigheid van *L. lactis*, T cellen aanzetten tot de productie van IL-17. Secretie van mIL-10 door *LL-pTmIL10* bleek evenwel een onderdrukkend effect te hebben op deze Th17 polariserende capaciteit. Verminderde secretie van IL-23 door deze *LL-pTmIL10* behandelde BM-DC's vergeleken met de *LL-pTREX* behandelde BM-DC's zou een verklaring kunnen zijn voor dit fenomeen.

Een drijvende factor tijdens inflammatie is de activatie van intestinale DC's door zowel gram-negatieve LPS als gram-positieve microbiële stimuli. Vermoedelijk, en vergelijkbaar met onze *in vitro* data waarbij we synergistische effecten van *LL-pTREX* en LPS op BM-DC's waarnamen, leidt dit o.a. tot een synergistische inductie van IL-23, die op zijn beurt kan zorgen voor sterke Th17 polarisatie. Uit onze data menen wij te kunnen veronderstellen dat *LL-pTmIL10* in staat is om deze synergistische inductie van IL-23 te onderdrukken, resulterend in een verminderde Th17 gemedieerde pathologie.

IL-23 heeft naast zijn effect op T cellen ook sterke effecten op cellen van het aangeboren immuunsysteem. Zo induceert het de productie van pro-inflammatoire cytokines zoals IL-1, IL-6 en TNF in macrofagen en monocyten [417,426]. De *in vivo*

studie van Hue en medewerkers toonde bovendien aan dat tijdens inflammatie het door intestinale bacteriën geïnduceerde IL-23 onder andere ook granulocyten en monocyten aanzet tot de productie van IL-17. De onderdrukking van microbieel geïnduceerd IL-23 door *LL-pTmIL10* zou dus naast de T cel gemedieerde ook aangeboren pro-inflammatoire immuunresponsen kunnen inhiberen.

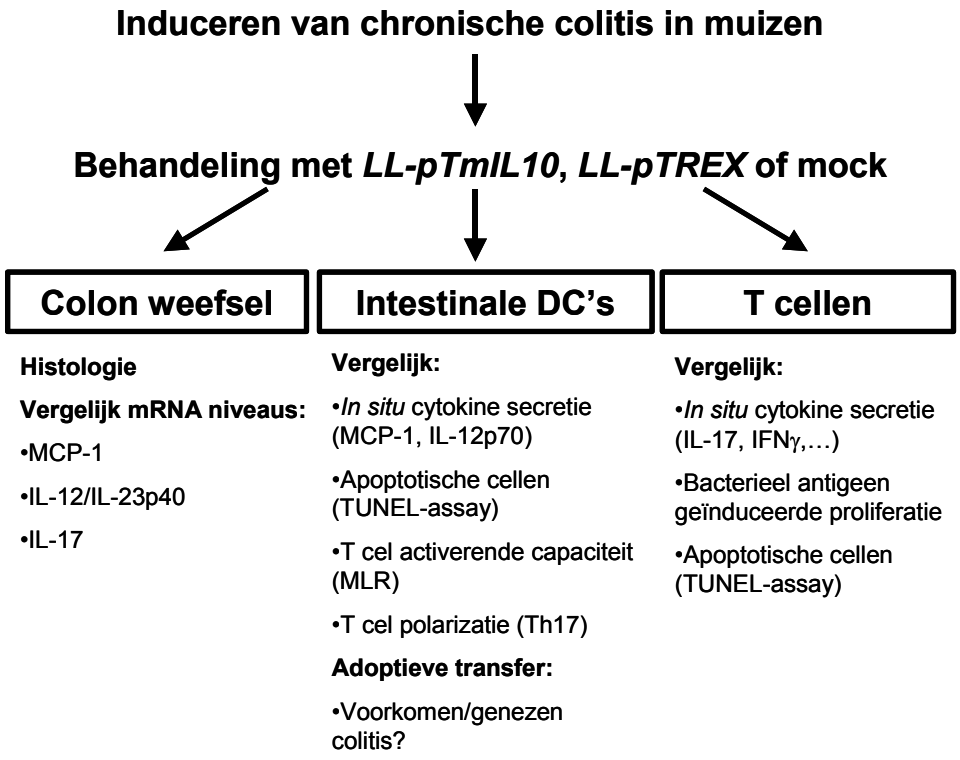
Uit onze data konden we verder ook besluiten dat *LL-pTmIL10* in staat is om de LPS geïnduceerde secretie van het chemokine MCP-1 in BM-DC's te onderdrukken. IBD wordt gekarakteriseerd door een continue infiltratie van de ontstoken weefsels door inflammatoire cellen uit de circulatie en chemokines spelen in dit proces een belangrijke rol [428]. Verscheidene studies beschrijven een verhoogde expressie van MCP-1 in geïnflammeerd weefsel van IBD patiënten [429-432] en in proefdiermodellen van chronische colitis [433-436], wat erop wijst dat de onderdrukking van MCP-1 secretie door inflammatoire cellen een efficiënte manier zou kunnen zijn om infiltratie te verminderen en IBD te verbeteren. De behandeling van chronische colitis met *LL-pTmIL10* resulteerde naast een vermindering van de epitheliale schade ook in een verminderde infiltratie van inflammatoire cellen [198]. De eigenschap van *LL-pTmIL10* om inflammatie geïnduceerde secretie van MCP-1 te onderdrukken zou dus deel kunnen uitmaken van het mechanisme dat leidt tot de genezing van colitis. Monocyten en macrofagen vormen de belangrijkste bron van MCP-1. Daarnaast werd echter ook expressie van dit chemokine door epitheelcellen, gladde spiercellen en endotheelcellen in IBD mucosa aangetoond [429,430,432]. In een *in vitro* studie in intestinale epitheelcellen demonstreerde men bovendien een rol voor IL-10 in de onderdrukking van IL-1β geïnduceerde MCP-1 secretie [437]. Naast DC's zouden bijgevolg ook andere MCP-1 secretende cellen in de darm het doelwit kunnen zijn van het door *LL-pTmIL10* gesecreteerde mIL-10.

Een interessante waarneming is de preferentiële expressie van CCR2, de receptor voor MCP-1, op IL-17 producerende T cellen [438]. In het ileum van Crohn patiënten werd bovendien een significante toename van deze $CCR2^+$ $CD4^+$ T cellen vastgesteld [439]. Bijgevolg zou een daling van MCP-1 tijdens de orale behandeling met *LL-pTmIL10* ook kunnen leiden tot een verminderde accumulatie van geactiveerde pathogene $CCR2^+$ $CD4^+$ Th17 cellen in de geïnflammeerde darm wat additioneel zou kunnen bijdragen aan het genezend effect van deze stam.

We moeten ons wel bewust blijven van het feit dat onze *in vitro* bekomen data niet noodzakelijk overeenstemmen met de *in vivo* situatie. Zo werd onlangs aangetoond dat IL-23, ondanks zijn *in vitro* capaciteit om Th17 cellen te induceren, niet cruciaal is

voor de *in vivo* differentiatie van deze populatie [113-115]. Ook moeten we rekening houden met het bestaan van gespecialiseerde mucosale DC's wiens functionele capaciteiten kunnen verschillen van perifere en dus ook van *in vitro* gegenereerde DC's. Om na te gaan of *LL-pTmIL10* ook *in vivo* in staat is om de functies van DC's te moduleren en of dit bijdraagt tot de therapeutische effecten van deze stam in muismodellen voor chronische colitis, zijn bijkomende experimenten noodzakelijk. Schema 2 is een overzicht van mogelijke *in vivo* experimenten die hierover uitsluitsel kunnen geven.

Samenvattend hebben wij in het tweede deel van dit werk aangetoond dat *LL-pTmIL10* een modulerend effect kan uitoefenen op de functionele karakteristieken van LPS geactiveerde BM-DC's. In de aanwezigheid van LPS inhibeert *LL-pTmIL10* de secretie van MCP-1 door BM-DC's en hun capaciteit om $CD4^+$ Th17 cellen te induceren. In een bredere zin suggereren onze data bijgevolg dat *LL-pTmIL10* behandeling tijdens chronische colitis zou kunnen leiden tot een verminderde ontwikkeling van IL-17 producerende $CD4^+$ T cellen en een verminderde accumulatie van deze pathogene Th17 cellen en andere immuuncellen in het geïnflammeerde weefsel wat collectief zou kunnen bijdragen tot de gunstige effecten van *LL-pTmIL10* therapie.



Schema 2 Mogelijke *in vivo* experimenten.
In een muismodel voor chronische colitis kunnen colonweefsels geïsoleerd worden van mock, *LL-pTREX* of *LL-pTmIL10* behandelde muizen. De mRNA expressieniveaus van MCP-1 maar ook van IL-12/IL-23p40 en IL-17 kunnen dan worden vergeleken tussen de verschillende groepen. Daarnaast kunnen we van deze 3 groepen ook de DC's uit de lamina propria en mesenteriale lymfeknopen isoleren voor verdere studie. Het cytokine profiel na *ex vivo* stimulatie zou kunnen worden vergeleken om verschillen te detecteren in MCP-1 en IL-12p70 secretie tussen de DC's van *LL-pTmIL10* behandelde muizen en de 2 controle groepen. Daarnaast kunnen deze DC's ook getest worden op hun T cel activerende capaciteit en kan worden gekeken naar cytokine secretie van de geactiveerde T cellen. Een indicatie voor de invloed van de intestinale DC's op T cellen *in vivo* kan eventueel ook worden nagegaan door het analyseren van T cellen geïsoleerd uit de verschillend behandelde muizen. We denken hierbij aan een vergelijking van de proliferatieve respons en de secretie van Th1, Th2 of Th17 cytokines na stimuleren met bacterieel antigeen. Om het belang van mogelijke *LL-pTmIL10* gemedieerde effecten op DC's te bepalen, kan tenslotte overgegaan worden tot een adoptieve transfer van intestinale DC's uit *LL-pTmIL10* behandelde muizen naar muizen met chronische colitis.

PART V: REFERENCES

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