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Tolerance and Immunity: Opposite Outcomes of Microbial Antigen Stimulation

Eroukhmanoff, Lena

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Lena Eroukhmanoff

Section for Immunology, Department of Experimental Medical Science,
Faculty of Medicine, Lund University

LUND UNIVERSITY

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Supervisor: Professor Fredrik Ivars
Faculty opponent: Associate professor Per-Arne Oldenborg,
Section for Histology and Cell Biology, Umeå University, Sweden

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ABBREVIATIONS

Ag	Antigen
APC	Antigen presenting cell
BM	Bone marrow
CD	Cluster of differentiation
DC	Dendritic cell
DN	Double negative
DP	Double positive
Foxp3	Forkhead box P3
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
KO	Knockout
LN	Lymph node
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MyD88	Myeloid differentiation factor 88
NO	Nitric oxide
OVA	Ovalbumine
PLN	Peripheral lymph node
SEB	Staphylococcal enterotoxin B
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TRIF	TIR-domain-containing adapter-inducing IFN-β
WT	Wild type

INTRODUCTION TO THE IMMUNE SYSTEM

The immune system is in many aspects a remarkable physiological network. Not only does it recognize and defend us from numerous infectious agents and foreign particles, it also manages to separate harmful from harmless and thereby avoids development of allergies. Finally, the immune system is intelligent enough to distinguish foreign from self. This is crucial to avoid destruction of the own body, which is what autoimmunity is all about. A functional immune system is taken for granted by most of us. We tend to ask ourselves why we develop infections, allergies, cancer and autoimmune diseases, rather than asking why we do not. However, the more one learns about the immune system, about tolerance and immunity, the more amazed one gets by its actions.

The immune system is divided into two branches, the innate and the adaptive. They both protect us from invading pathogens such as viruses, bacteria, fungi and parasites. The innate immune system provides the first line of defence and acts rapidly, within minutes or hours upon infection. It is comprised of barriers like skin, mucosal membranes and antibacterial enzymes, preventing microorganisms to enter the body. In addition, specialized immune cells such as macrophages, mast cells, neutrophils and dendritic cells (DCs) are included in the innate defence. These cells express different pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), that recognize and react to a limited set of evolutionary conserved microbial structures, termed pathogen-associated molecular patterns (PAMPs). Examples of such structures are components of the bacterial cell wall and viral RNA/DNA. Upon pathogen contact innate immune cells bind these patterns, become activated and thus initiate an inflammatory response in order to eliminate the pathogen. Additionally, DCs migrate to the secondary lymphoid organs (spleen and lymph nodes (LNs)) where, if necessary, an adaptive immune response is elicited.

In contrast to innate immune cells that only recognize a limited number of patterns and where different cells react to the same antigen, the adaptive immune system recognizes nearly an unlimited number of structures. Furthermore, unlike innate immunity which

acts rapidly, an adaptive immune response occurs only after several days. Lymphocytes; T and B cells, are the main effectors of adaptive immunity. They develop in the primary lymphoid organs (thymus and bone marrow (BM) respectively), each cell expressing a unique receptor with the ability to recognize a specific antigen. Lymphocytes circulate between blood, lymph, and secondary lymphoid organs. In the secondary lymphoid organs activated antigen presenting cells (APCs), mainly DCs, present antigens to naive T lymphocytes. When lymphocytes recognize their specific antigen, they become activated, begin to divide and differentiate into effector cells. These cells then promote an efficient immune response. Effector cells have a limited life-span and most of them undergo apoptosis once the antigen is eliminated. However, some cells persist and are termed memory cells. Upon a second encounter with a specific antigen, a more rapid and effective immune response is elicited. Memory therefore enables vaccination and prevents re-infection with pathogens that have already been defeated once by an adaptive immune response.

T CELLS AND TOLERANCE

T cell development

T cells originate from hematopoietic stem cells (HSCs) in the bone marrow (BM). HSCs have the capacity to self-renew, thus generating daughter-cells with identical properties, including the ability to generate all lineages of blood cells. T cell progenitors migrate from the BM to the thymus where T cell development is initiated. During this process the T cell receptor (TCR) is formed, consisting of either αβ chains or γδ chains. From here on, only αβ T cells will be discussed. To achieve great diversity in the TCR repertoire, gene segments coding for the variable domain of the TCR-α and TCR-β proteins of the receptor are randomly assembled in a great number of combinations. The different types of gene segments that constitute the TCR are called V (variable), D (diversity) and J (joining) and the process of combining these is called V(D)J recombination [1]. Cleavage of DNA is part of these gene rearrangements and the proteins recombination-activating gene (RAG)-1 and RAG-2 are essential catalysts for this reaction [2-4].

The earliest thymocyte population is termed CD4CD8 double negative (DN) since it does not express the characteristic T cell surface proteins CD4 and CD8. DN cells differentiate into a series of developmental stages based on their expression of CD44 and CD25; DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$) [5]. This process has been shown to be dependent on Notch signaling [6] and during these stages, the TCR is gradually formed. During the DN stages, the gene segments of the β-chain assemble and the β-chain protein is expressed, together with a pre-T α-chain (pTα), constituting the pre-TCR [7]. This receptor complex, together with CD3, signals for survival and progression to the following $CD4^+CD8^+$ double positive (DP) stage where the α-chain is rearranged [8]. Finally, the αβTCR is expressed and the next critical step for the DP T cell is to undergo the mechanisms of positive and negative selection.

Positive and negative selection

Thymocytes that have made it all the way to the $CD4^+CD8^+$ DP stage still have a long way to go. Only functional cells that do not react to self-antigens are selected to survive and eventually migrate to the periphery. The rest of the cells, comprising the large majority of all produced T cells, die by apoptosis in the thymus.

Positive selection is based on the ability of the TCR to react with major histocompatibility complex (MHC) molecules expressed by thymic epithelial cells [9-11]. If the TCR is able to bind the MHC it survives and continues its journey, whereas cells failing to recognize MHC are eliminated through apoptosis. The purpose of positive selection is to ensure that surviving T cells recognize self-MHC on APCs in the periphery, which is required for the T cells to react against foreign antigens. The ultimate outcome of positive selection is development of the $CD4^+CD8^+$ DP T cells into single positive (SP) ones. Cells that maintain the expression of CD8 will be able to react to antigens presented by MHC class I molecules in the periphery whereas CD4-expressing cells will recognize MHC class II-presented antigens [12]. Two models have been suggested to explain how it is determined which one of the CD4 or CD8 molecules is down-regulated. The stochastic model suggests random down-regulation whereas the instructive one implies that the cell is instructed to stop transcribing a certain gene, depending on the MHC-restriction of the T cell [13].

SP T cells that survive positive selection will further be submitted to negative selection. The purpose of this process is to eliminate self-reactive T cells to avoid auto-immunity in the periphery. This is referred to as central tolerance. During negative selection, self-antigens are presented to T cells by MHC molecules on APCs [14]. Recognition of these self-antigens induces apoptosis in the T cell whereas cells that are not self-reactive survive and migrate from the thymus to the periphery. However, the mechanism of negative selection is not perfect. One constraint is the requirement for self-antigens to be present in the thymus, at sufficient levels to induce T cell deletion. Tissue-specific antigens (TSAs) cause a particular problem since they often are present at high concentrations in the peripheral organs, in contrast to the

thymus where only trace amounts are detected. The transcription factor autoimmune regulator (Aire) partly solves this problem by inducing the expression of several TSAs in the thymus, enabling T cell tolerance to these. Mice and humans with defective Aire expression develop a multi-organ autoimmune syndrome [15]. However, despite the expression of a functional Aire, some self-reactive cells do survive and escape into the periphery.

T cell activation

When the mature, naive T cell leaves the thymus it recirculates between blood, lymph nodes and spleen [16]. To become activated it needs to encounter its specific antigen which will induce proliferation and differentiation of the T cell. Finally, some of these cells leave the lymphoid organs as effector cells, migrate to peripheral tissues and exert their effector functions at the site of infection, whereas others stay and act in the lymphoid organs.

DCs pick up antigens at sites of infection and thereby become activated. This leads to migration of the cells to draining lymph nodes where they act as mature, potent APCs [17]. Antigens are processed into peptides and subsequently presented by MHC molecules on the DC. Peptides derived from intracellular pathogens are presented by MHC class I molecules to $CD8^+$ T cells whereas peptides from extracellular pathogens are generally presented by MHC class II to $CD4^+$ T cells [17]. However, binding the MHC-peptide complex is not sufficient for T cell activation to occur, co-stimulatory signals delivered by the APC are required as well [18].

Naive T cells are attracted to APCs by chemokines [19, 20]. APCs and T cells interact transiently with each other in an antigen-independent manner [21], enabling T cells to sample a large number of MHC molecules on each APC, thus increasing the chances of recognizing the cognate antigen. Upon recognition of the specific antigen, strengthening of the transient adhesive interactions occurs, resulting in the formation of the immunological synapse [22]. This complex is formed by interactions of lymphocyte function-associated antigen 1 (LFA-1), LFA-2, and CD28 on the T cell,

with inter-cellular adhesion molecule 1 (ICAM-1), LFA-3 and CD80/CD86 respectively on the APC. Next, intracellular signalling cascades are activated through CD3, a complex associated with the TCR, ultimately resulting in activation of the transcription factors nuclear factor (NF)-κB, nuclear factor of activated T-cells (NF-AT) and activator protein 1 (AP-1). These together initiate the transcription of several important genes, such as interleukin (IL)-2 [23, 24]. Upon T cell activation, the inhibitory molecule cytotoxic T lymphocyte associated antigen (CTLA)-4 is up-regulated. It negatively regulates activation of T cells by binding CD80/CD86 with a much higher affinity than CD28 [25-27]. CTLA-4 expression is critical and protects mice from lymphoproliferative disease [28, 29].

Following activation, T cells start to divide extensively and differentiate into effector cells which express new gene patterns important for their effector functions. $CD4^+$ T cells differentiate into different kinds of T helper (Th) cells (Th1, Th2, Th17 or follicular T helper cells (Tfh)) or regulatory T cells (Tregs), whereas $CD8^+$ T cells differentiate into cytotoxic T cells (CTLs) [30, 31]. Activated cells are subsequently directed to their effector sites, in the lymphoid tissues or in the periphery.

Peripheral T cell tolerance

Self-reactive cells that escape thymic negative selection are controlled by peripheral tolerance in healthy individuals. The mechanisms of peripheral tolerance are deletion, anergy and active suppression.

Deletion of self-reactive T cells occurs via activation-induced cell death (AICD), primarily through the Fas-FasL (CD95/CD95L) cell death pathway, as an accumulation of activated cells in the periphery with increased rates of autoimmune disease has been observed in Fas deficient *lpr* mice or FasL deficient *gld* mice [32-34].

Clonal T cell anergy is a tolerance mechanism defining a state of non-responsiveness in T cells following antigen exposure. It affects IL-2 production and proliferation upon restimulation but the T cell remains alive in a hyporesponsive state for an extended period of time [35]. Anergy is a cell-intrinsic mechanism, although the induction might be initiated by other cells. It is important to separate *in vitro* clonal T cell anergy with the related *in vivo* adaptive tolerance state. *In vitro* clonal anergy can be induced by a strong TCR engagement with antigen or anti-CD3 antibodies (Abs) in the absence of costimulation, resulting in a defective proliferation [36]. Costimulation via CD28 is essential to prevent clonal anergy, as the old concept of anergy says: "signal 1 leads to tolerance and signal 1 plus 2 leads to activation". However, it is not known whether it is a direct effect of CD28 signaling or an indirect effect on IL-2 expression that is important [37]. *In vitro* clonal anergy does not require antigen persistence and is in many cases reversible upon the addition of exogenous IL-2 [38]. *In vivo* adaptive tolerance is usually induced after vigorous but transient proliferation caused by prolonged antigen stimulation of naive T cells. It requires persistence of the antigen and is not reversible by exogenous IL-2 [38].

Suppression, mediated by regulatory T cells, is a third important mechanism of peripheral T cell tolerance. A number of distinct subsets of regulatory T cells with different suppressive mechanisms have been identified; The $CD4^+$ type 1 regulatory T cells (Tr1) that secrete high levels of the suppressive cytokine IL-10 [39] and $CD4^+$ Th3 cells producing soluble transforming growth factor beta (TGF-β) [40]. These cells are induced in the periphery and are currently not thought to represent a distinct T cell lineage. Other inducible regulatory T cells that have been discovered are the forkhead box P3 (Foxp3) expressing cells derived from naive $CD4^+CD25^-$ T cells in the presence of TGF-β [41]. Even though $CD4^+$ regulatory T cells have been most extensively studied, $CD8^+$ suppressor cells have been identified as well, for example $CD8^+CD28^-$ cells that inhibit upregulation of costimulatory molecules on APCs [42]. Finally, the most studied regulatory T cells are the thymically derived $Foxp3^+CD4^+CD25^+$ natural regulatory T cells (Tregs). These cells are produced in the thymus and will be further discussed in the following section.

FOXP3$^+$ NATURAL REGULATORY T CELLS

Background

Foxp3$^+$CD4$^+$CD25$^+$ natural regulatory T cells (Tregs) play key roles in maintaining tolerance to self and thus in controlling autoimmunity. In 1995, Sakaguchi *et al.* demonstrated that a subset of CD4$^+$ T cells constitutively expressed the IL-2 receptor alpha chain (CD25) and that these cells were highly immunosuppressive [43]. Transfer of CD4$^+$CD25$^-$ T cells from normal mice into syngeneic T cell-deficient nude mice resulted in the development of various autoimmune diseases in the recipients, whereas disease could be prevented by co-transfer of CD4$^+$CD25$^+$ cells. This was a major breakthrough in the field of Tregs. However, CD25 could not serve as a specific marker for these cells, since it is highly expressed on both activated CD4$^+$ and CD8$^+$ T cells. Therefore, the discovery of the transcription factor Foxp3 was of great importance.

Foxp3 is an X chromosome-encoded gene recently discovered to be mutated in the autoimmune disorder "immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome" (IPEX) in humans, and in the analogous lymphoproliferative disease observed in the spontaneous mouse mutant "scurfy" [44-46]. Subsequent studies revealed that Tregs constitutively express Foxp3 and that this transcription factor is required for Treg differentiation and function [47-49]. Since then, Tregs have been extensively studied and although a lot of questions have been answered, many of them still remain. Foxp3 is a specific marker for Tregs in mice, since it is not expressed by conventional T cells [50, 51]. Other markers expressed by Tregs are glucocorticoid-induced tumor necrosis factor receptor (GITR), CTLA-4 and OX-40L [52].

Tregs comprise approximately 5-10% of the peripheral CD4$^+$ T cells in mice. They are anergic *in vitro* upon conventional T cell stimuli but the anergy may be broken by the addition of exogenous IL-2, which also results in abolished suppressive properties [53, 54]. Interestingly, Tregs do proliferate when exposed to antigen *in vivo* and yet they maintain their ability to suppress [55-57]. It has been demonstrated that continuous

Foxp3 expression in peripheral Tregs is required to actively maintain their suppressive function and phenotypic characteristics [58].

Treg differentiation

Tregs are derived in the thymus as a separate T cell lineage. Early studies using CD25 as a marker, demonstrated that Tregs are not found in the periphery until day 3 of life and that neonatal thymectomy at day 3 after birth results in autoimmunity in mice [59]. A more recent report using Foxp3-green fluorescent protein (GFP) transgenic mice enabled observation of Tregs in the thymus [60]. It was demonstrated that less than 0.1% of the SP $CD4^+$ thymocytes expressed Foxp3 12h after birth. However, between day 3 and 4 the population had increased to 2.1-4.5% of the SP $CD4^+$ cells and at the age of 3 weeks, the proportion had reached the adult level of 10%. These studies confirmed what was earlier proposed, that the development of Tregs is delayed relative to that of conventional T cells.

Foxp3 induction and thus Treg differentiation occurs relatively late during thymic differentiation. Several studies have reported that $Foxp3^+$ cells are detected, although at very low levels, among DP $CD4^+CD8^+$ thymocytes [50, 51, 60, 61]. Furthermore, Liston *et al.* proposed that the DP subset represented precursors to SP $Foxp3^+$ cells [61]. However, a recent study reports that the majority of DP $Foxp3^+$ cells detected by flow cytometry are doublets comprised of DP $Foxp3^-$ and SP $CD4^+$ cells [62]. Although it remains to be established whether Foxp3 is expressed in DP cells or not, these studies do agree on the fact that the majority of Foxp3 expressing cells in the thymus are SP $CD4^+$ cells.

How thymocytes are committed to the Treg lineage is still under investigation. It has been proposed that TCR specificity would be an important factor and that Tregs are self-reactive in contrast to conventional T cells [63]. However, others claim that Tregs are not particularly self-reactive [64]. In addition, Tregs and conventional T cells have been shown to have an overlapping TCR repertoire [65, 66].

Another important issue that has been addressed is whether Foxp3 is necessary and/or sufficient for commitment to the Treg lineage. Studies were performed with mice expressing a non-functional Foxp3 protein. Cell surface phenotype and gene-expression profile of these cells appeared similar as that of wild type (WT) Tregs but distinct from that of conventional T cells. Thus, lineage commitment does not require the expression of a functional Foxp3 protein [67, 68].

Recently, Lio *et al.* looked for the immediate precursors to Tregs within SP $CD4^+$ thymocytes. They found that the $CD4^+CD25^{hi}$ population was highly enriched in Treg cell precursors, and that the presence of IL-2 induced Foxp3 expression in these cells [69]. However, previous data have shown that IL-2 signaling is important, although not crucial, for Treg development, since mice lacking IL-2 or IL-2Rα show a 50% decrease in proportion and number of $Foxp3^+$ thymocytes [70]. Other molecules that have been shown to participate but not to be indispensable for Treg development are CD28, CD80 and CD86 [71, 72]. It is not clear whether TGF-β is important or not for thymic Treg development [73, 74].

Mechanisms of suppression

The mechanisms by which Tregs suppress their cellular targets are not well understood. *In vitro* studies have shown that activation and/or expansion of multiple cell types such as $CD4^+$ T cells, $CD8^+$ T cells, B cells, natural killer (NK) cells and NKT cells, might be suppressed by Tregs [75]. It has become clear that Tregs may exert a number of different suppression mechanisms that can be divided into four groups, further discussed below; (1) inhibitory cytokines, (2) cytolysis, (3) metabolic disruption, and (4) modulation of APC function. The growing number of mechanisms by which Tregs suppress suggests that their function is context dependent, and that the type and magnitude of the immune response might be of importance. Furthermore, different target cells might be susceptible or resistant to distinct Treg mechanisms.

Development of an *in vitro* model system [53, 54] to study Treg function was thought to represent their actions also *in vivo*. However, a number of molecules and processes contributing to Treg suppression *in vitro* have not been confirmed to be important *in vivo* [76]. *In vitro* studies strongly suggest that the suppressive capacity of Tregs requires activation through their TCR but that once activated, suppression is antigen independent [54]. Whether this is true also *in vivo* is not clear. It has been discussed whether Tregs act directly on responder cells or whether they mediate their suppression via APCs. Suppression assays *in vitro* have been performed in two distinct ways, in the presence or absence of APCs. Suppression was observed in both systems, meaning that Tregs are able to suppress responder cells directly. However, this does not necessarily occur when APCs are present, as a number of mechanisms of suppression involving APCs have been observed as well.

In vitro suppression has been shown to be dependent on cell-cell contact or at least vicinity between Tregs and responders, since abrogated suppression was observed when cells were separated by a permeable membrane [53, 54]. This does not rule out a potential role for cytokines or other secreted molecules in suppression, since they may require proximity between cells.

Inhibitory cytokines such as IL-10 and TGF-β have been shown to be important for Treg suppression *in vivo* [77-82]. However, neither of these cytokines are required for *in vitro* suppression of proliferation [54, 83]. More recently, IL-35 and galectin-1 (Gal-1) have been proposed to be implicated in Treg suppression *in vitro* [84, 85].

It has been demonstrated that activation of Tregs results in the upregulation of granzyme B expression, and one study claimed that Tregs kill responder cells by a granzyme B-dependent, perforin-independent mechanism [86]. However, this could not be confirmed by other *in vitro* studies [87], although an *in vivo* study demonstrated that a population of Tregs in a tumor microenvironment expressed granzyme B and could kill T cells in a granzyme B- and perforin-dependent manner [88].

Metabolic disruption is another mechanism of suppression mediated by Tregs, and the modulation of cyclic adenosine monophosphate (cAMP) is one such example. Elevated levels of cAMP are associated with inhibition of proliferation, differentiation and repression of IL-2 and interferon (IFN)-γ gene expression in lymphocytes, by the blocking of NF-κB activity [89]. Tregs have been demonstrated to increase cAMP levels in target cells through direct transfer of cAMP from Tregs into activated T cells via gap junctions [90]. Furthermore, two other studies describe the importance of CD39 or CD73, both expressed on activated Tregs, for the generation of adenosine which was shown to suppress proliferation and cytokine production by effector T cells [91, 92]. This was dependent on the adenosine receptor A2A.

Several *in vitro* studies have demonstrated that Tregs inhibit mRNA-induction of IL-2 and other effector cytokines in responder Foxp3$^-$ T cells [53, 54, 93]. Another study reports that Tregs compete for IL-2 with Foxp3$^-$ cells, and that their large consumption due to constitutive expression of CD25 causes apoptosis of Foxp3$^-$ cells [94].

As previously mentioned, Tregs may also act via APCs and several mechanisms of suppression affecting APCs have been proposed. Tregs constitutively express CTLA-4 on their cell surface, and downregulation of the costimulatory molecules CD80 and CD86 on DCs, in a CTLA-4 dependent manner, has been reported [95]. This has recently been confirmed *in vivo* [96], and results in a limited capacity of APCs to stimulate T cells through CD28. Additional *in vivo* studies have been performed supporting the important role of DCs in Treg suppression. Using two-photon laser-scanning microscopy, Tang *et al*. demonstrated that no stable interactions were formed between Tregs and effector T cells, whereas Tregs formed long-lasting conjugates with antigen-loaded DCs [97]. Tadokoro *et al*. applied the same technique and showed that contacts between T cells and antigen-bearing DCs were of shorter duration in the presence of Tregs than in their absence [98].

Tregs have also been demonstrated to induce expression of the tryptophan degrading enzyme indoleamine 2,3-dioxygenase (IDO) in DCs, by interactions between CTLA-4

on Tregs and CD80/CD86 on DCs [99, 100]. However, it is not clear whether IDO is involved in Treg suppression. Another cell surface molecule that has been suggested to play a role in DC-mediated Treg suppression is lymphocyte activation gene 3 (LAG-3), a CD4 homolog binding MHC class II molecules on DCs, resulting in suppressed DC maturation and thus reduced immunostimulatory function [101]. Finally, two additional molecules proposed to be implicated in Treg suppression are fibrinogen-like protein 2 (FGL2) and neuropilin 1 (Nrp-1), both interacting with DCs [102, 103].

Foxp3 has been shown to physically interact with the transcription factors NF-κB and NFAT [104]. Further studies have demonstrated that the NFAT-Foxp3 complex is important for upregulation of the Treg markers CD25 and CTLA-4, and that it is required for the suppressive activity of Tregs [105].

In summary, Tregs are able to suppress by numerous different mechanisms and more studies have to be performed to better understand this area. Further questions to be answered are *where* Tregs exert their suppression, in the secondary lymphoid organs, in peripheral tissues or both, and whether the suppression mechanisms are dependent on the site where the Tregs act.

Migration and site of suppression

Where Tregs act and how they migrate *in vivo* is still under investigation. Tregs have been shown to affect the initiation of immune responses by suppressing activation and proliferation of naive T cells, and were therefore suggested to act in the secondary lymphoid organs [106]. However, other papers report that Tregs also migrate to and function in tumors, transplanted organs and inflammatory tissues [107]. Using a TCR-transgenic diabetic model, Chen *et al.* demonstrated that Tregs had no effect on the priming of T cells in the lymph nodes but rather inhibited the T cell effector functions at the inflammatory site [108]. Another study reported an accumulation of Tregs in the skin, where they dampened the immune response to an infectious agent [80]. Migration of Tregs to the skin was dependent on their expression of the integrin $\alpha_E\beta_7$ (CD103) [109].

CD103 has been used to distinguish Treg subsets [110], and the migration of T cells to different tissues is determined by the cell surface expression of adhesion molecules and chemokine receptors (CCRs) [111]. Naive Tregs are $CD103^-$, express L-selectin (CD62L) and CCR7, and recirculate through lymphoid tissues. In contrast, $CD103^+$ Tregs display an effector/memory phenotype, expressing high levels of E/P-selectin binding ligands, multiple adhesion molecules as well as receptors for inflammatory chemokines, allowing efficient migration into inflamed tissues [112]. CCR7 is crucial for recirculation of naive Tregs through the lymph nodes and the lack of CCR7 abolishes their ability to control the priming of an immune response [113]. In contrast, CCR7-deficient effector/memory Tregs accumulate at the site of inflammation and exert enhanced suppression compared to WT cells.

A more recent study confirms that Tregs play an important role both in the lymph nodes and at the site of inflammation. Using an allograft model, these authors particularly demonstrate that sequential migration of Tregs is required, from blood to inflamed tissues and then to the draining lymph node, for efficient inhibition of effector T cell responses [114].

Thus, Tregs may act in the lymph nodes, at the site of inflammation or both, however it is still uncertain where they primarily exert their suppression.

Role in infectious disease

To survive an infection, a controlled immune response is required where the invading pathogen is recognized at the same time as the collateral damage to self-tissues is limited. Several studies have demonstrated the importance of Tregs during parasitic, fungal, viral and bacterial infections. Common consequences of Tregs preserving homeostasis by controlling excessive immune responses are enhanced pathogen survival or even long-term persistence.

During cutaneous infection with *Leishmania major*, Tregs accumulate at the site of infection where they suppress the response to the parasite [80]. This prevents immunity against the pathogen and thus allows a persistent infection. Further studies demonstrated that Tregs were parasite-specific rather than self-reactive [115]. In this model, parasite persistence was necessary for maintaining immunity to re-infection. Other examples where Tregs control immunopathology are a mouse model of *Pneumocystis* pneumonia [116], a model in which mice were infected in the eye with herpes simplex virus (HSV) [117], and mice infected with *Schistosoma mansoni* [118], affecting the liver. However, in other cases where Treg control is excessive, the survival of the host is compromised. Malaria might be one such example, since depletion of Tregs in a mouse model of malaria protects from death caused by the lethal strain of *Plasmodium yoelii* [119]. A restored immune response against the parasite and control of the infection was observed in the absence of Tregs.

TOLL LIKE RECEPTORS

Background

Toll like receptors (TLRs) are important for innate immune responses since they recognize a variety of microbial products. The TLR family was first identified in *Drosophila*, where the Toll protein was shown to play an important role during embryonic development [120]. Almost 10 years later subsequent studies revealed the involvement of Toll in resistance to fungal pathogens in the adult fly [121] and shortly thereafter, in 1997, the first mammalian TLR-homologue was identified [122]. At present, 10 TLRs have been discovered in humans and 13 in mice [123]. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are all situated at the cell surface, predominantly recognizing bacterial components. In contrast, TLR3, TLR7, TLR8, and TLR9 are located in intracellular endosomal or lysosomal compartments where they recognize microbial RNA and DNA.

Expression and structure

TLRs are expressed by hematopoietic cells, including mast cells, DCs, macrophages, and B cells, but they have also been detected on nonimmune cells such as fibroblasts and epithelial cells [124]. TLRs are transmembrane glycoproteins with an extracellular domain characterized by leucine-rich repeats (LRRs) of different size and abundance, responsible for binding pathogen-associated molecular patterns (PAMPs). The conserved residues within each repeat provide a rigid structure whereas the variable residues are the ones interacting with ligands [125]. The intracellular part of the receptor is composed of Toll/Interleukin-1 receptor (TIR) domains important during TLR activation [126].

Activation

Each TLR recognizes specific microbial patterns and numerous TLR ligands have been identified, some of them presented in table 1.

Table 1. TLR ligands, modified from Mogensen *et al.* (2009) and Kumar *et al.* (2009) [127, 128].

TLR	**Microbial Ligands**
TLR1/2	Triacyl lipopeptides
TLR2	Lipoproteins, peptidoglycan (PGN), lipoarabinomannan (LAM), hemagglutinin, zymosan, viral envelope glycoproteins
TLR3	dsRNA
TLR4	Lipopolysaccharide (LPS), mannan, glycoinositolphospholipids, viral envelope glycoproteins, HSP70
TLR5	Flagellin
TLR6/2	Diacyl lipopeptides, lipoteichoic acid (LTA)
TLR7	ssRNA
TLR8	ssRNA
TLR9	CpG DNA
TLR10	Unknown
TLR11	Uropathogenic bacteria, profilin-like molecule
TLR12	Unknown
TLR13	Unknown

TLR1 forms heterodimers with TLR2 and recognizes triacyl lipopeptides such as the synthetic ligand Pam_3Cys. TLR2 also heterodimerizes with TLR6, and then recognizes diacyl lipopeptides, of which lipoteichoic acid (LTA) is one example. Other TLRs rather form homodimers. TLR3 is activated by dsRNA, such as the synthetic ligand polyinosinic-polycytidylic acid (pI:C). TLR4 is a receptor with the ability to recognize structurally and biochemically unrelated ligands. Lipopolysaccharide (LPS), viral envelope glycoproteins and heat shock proteins (HSPs) are some examples. Flagellin is a ligand of TLR5. TLR7 and TLR8 both recognize ssRNA, whereas TLR9 is activated by CpG DNA. The ligands for TLR10, TLR12 and TLR13 are still not known. TLR11 has been reported to recognize uropathogenic bacteria and a profilin-like molecule in mice.

Most TLRs directly bind their ligands whereas others require accessory proteins to facilitate recognition and binding. LPS is recognized by a receptor complex consisting of TLR4 and myeloid differentiation protein-2 (MD-2). In addition, two other proteins have been reported to be involved in LPS-induced activation of TLR4, namely LPS-binding protein (LBP) and CD14. The role of these accessory proteins is not yet completely understood. The former is thought to be a shuttle protein that catalyzes the transfer of LPS from the outer membrane of Gram-negative bacteria to CD14, which in turn binds to LPS and presents it to the TLR4/MD-2 receptor complex [129]. While MD-2 is indispensable for LPS-recognition [130], LBP and CD14 were reported to be crucial only for MyD88-independent LPS signaling of LPS [131-133]. Furthermore, CD14 was also shown to participate in TLR2/TLR6- and TLR2/TLR1 activation [134] and an additional accessory protein, CD36, was reported to be involved in the recognition of certain ligands by TLR2/TLR6 [134, 135].

A given pathogen may activate various different TLRs via alternative PAMPs and moreover, several structurally unrelated pathogens can activate the same TLR. Activation and homo- or heterodimerization of TLRs is believed to result in the recruitment of adaptor proteins that bind TIR domains of the receptors, and thereby initiate their signaling [136].

Signaling

Binding of microbial components to TLRs triggers the activation of signaling cascades, starting from the cytoplasmic TIR domains and ultimately resulting in the induction of genes important for antimicrobial responses. There are four TIR-domain containing adaptor molecules involved in TLR signaling, namely myeloid differentiation factor 88 (MyD88), TIR-associated protein (TIRAP)/MyD88 adaptor-like (MAL), TIR-domain containing adaptor protein-inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM) [127]. Distinct TLRs signal via different adaptors, which at least partly determine the specificity of the response.

The four adaptor molecules mentioned above do all have activating functions. However, a fifth TIR adaptor has been identified, sterile α- and armadillo-motif containing protein (SARM) [137]. SARM is a negative regulator of TLR-induced TRIF signaling, since its expression blocks the induction of genes downstream of TRIF but not of MyD88.

TLR signaling pathways are broadly divided into MyD88-dependent and MyD88-independent [138], since this adaptor molecule is the most widely used, recruited by all TLRs except TLR3. MAL is required for linking MyD88 together with TLR2 and TLR4 [139, 140], whereas other TLRs directly interact with MyD88. TLR3 and TLR4 are unique in their ability to signal via the MyD88-independent pathway, mediated by TRIF [141]. TLR3 directly binds to this adaptor whereas TRAM is required as a bridging molecule connecting TLR4 and TRIF [142, 143].

TLR signaling involves three major pathways responsible for mediating antimicrobial responses. These include NF-κB, mitogen-activated protein kinases (MAPKs), and IFN regulatory factors (IRFs), and result in transcription of proinflammatory genes and IFN production [127]. The MyD88-dependent pathway is mainly responsible for expression of proinflammatory cytokines such as IL-1, tumor necrosis factor (TNF)-α and IL-6 [144], induced by activated NF-κB and mitogen-activated protein kinases (MAPKs). An alternative MyD88-dependent pathway is triggered in plasmacytoid DCs (pDCs), where TLR7- and TLR9 activation results in the activation of IRFs and production of type I interferons (IFNs). A second alternative MyD88-dependent pathway was recently discovered in TLR2 activated cells, in which viral but not bacterial ligands resulted in IRF-dependent IFN production [145]. Finally, the MyD88-independent, TRIF dependent pathway is generally accepted as the main one for induction of type I IFN responses in non-pDCs, and it also contributes to the activation of NF-κB [127]. Figure 1 represents a simplified schematic overview of the different TLRs and their signaling pathways.

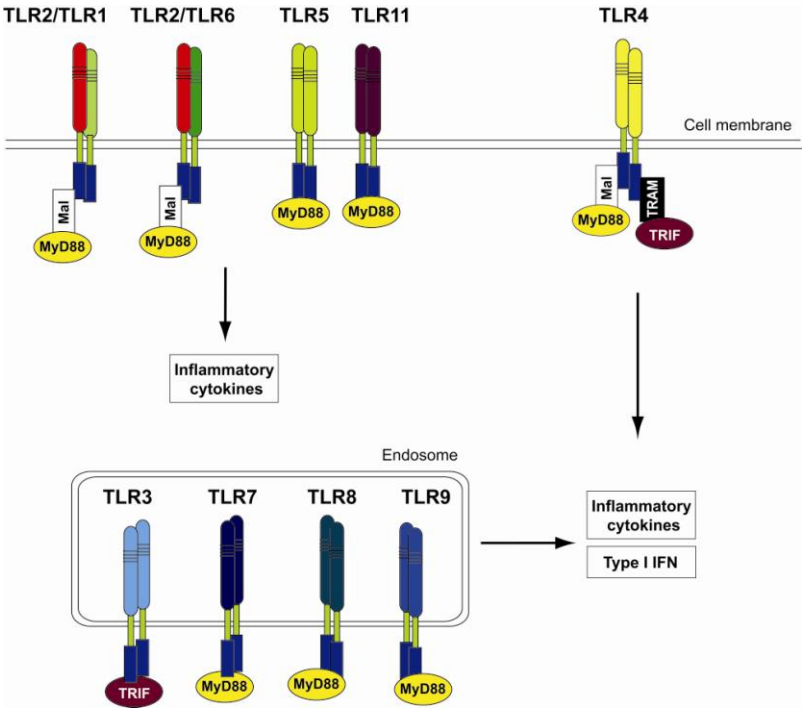

Figure 1. TLRs and their signaling pathways. Modified from Mogensen (2009) [127].

An interesting feature of TLR4 signaling was recently discovered by Kagan *et al*. This group demonstrated that TLR4 activates the MyD88-dependent and MyD88-independent pathways sequentially from different cellular compartments, the former from the plasma membrane and the latter from endosomes after endocytosis of the receptor complex [146]. Another study demonstrated that even TLR2-induced type I IFN production was dependent on receptor internalization [145].

Role in innate and adaptive immunity

TLRs play a key role in innate immunity where they initiate inflammatory responses. Rapidly after microbial invasion, TLRs on tissue-resident macrophages, DCs and mast cells are activated, leading to the production of proinflammatory cytokines and type I IFN [144]. This results in the expression of selectins, chemokines, and chemokine receptors important for recruitment of leukocytes to the site of inflammation [147]. Monocytes, neutrophils, basophils, eosinophils and NK cells are all part of the innate immune system and migrate from peripheral blood into the inflamed tissue where they participate in killing pathogens and infected cells.

TLRs are also involved in adaptive immune responses, which begin with the capture of antigens by DCs in peripheral tissues. Activation of TLRs on DCs is followed by phagocytosis of the pathogen, upregulation of MHC- and costimulatory molecules, a switch in chemokine receptor expression and migration to the draining lymph nodes where the processed antigens are presented by MHC molecules on the DCs to T cells [17]. In addition, DCs secrete cytokines and chemokines that are important for the differentiation of T cells. It is generally accepted that most TLR-mediated signals stimulate DCs to produce IL-12, which promotes development of Th1 cells [124]. However, TLR-mediated induction of IL-10 has been observed with certain ligands and this favors a Th2 response [148]. Furthermore, TLRs may also induce production of IL-6, TGF-β and IL-23, and thereby promote Th17 cells [149].

DCs activated by some TLRs have also been shown to affect the differentiation of $CD8^+$ T cells into cytotoxic T cells by inducing cross-presentation of extracellular antigens [150]. The role of TLRs in modulating antibody responses by B cells is not well understood [149]. However, it has been demonstrated that activation and differentiation of Th cells in mice is not sufficient for T-dependent B cell responses. In addition, TLR-mediated stimulation directly on B cells is required for the induction of a proper antibody response [151].

INTERFERON BETA

Background

Interferon (IFN)-β is part of the type I IFN family together with IFN-α and other less well characterized IFNs. Production of IFN-β is mainly controlled at the transcriptional level where multiple transcription factors are involved, including the IRFs [152]. In response to appropriate stimuli, several different cell types have been shown to express IFN-β, including monocytes, macrophages, neutrophils and DCs [153]. IFN-β was initially considered to display anti-viral effects only, however, today there are no doubts that this cytokine has multiple functions in addition to those inhibiting viral replication [154].

Receptor and signaling

IFN-β signaling is mediated by the type I IFN receptor [155, 156], which consists of two transmembrane subunits, IFN receptor 1 (IFNAR1) and IFNAR2, associated with the cytoplasmic tyrosine kinases Tyk2 and Jak1, respectively. Binding of IFN-β to the receptor induces rapid trans-phosphorylation of these kinases, followed by phosphorylation of critical residues of the intracellular domain of the receptor itself. These phosphorylated residues serve as recruitment sites for the transcription factors signal transducer and activator of transcription (Stat) 1 and Stat2, which are then phosphorylated by Tyk2 and Jak1. Once phosphorylated, these Stats form two distinct transcriptional activator complexes, namely, IFN-α-activated factor (AAF) and IFN-stimulated gene factor 3 (ISGF3). The former is a homodimer of Stat1 whereas the latter is a heterotrimer consisting of Stat1, Stat2, and IRF9. These complexes translocate from the cytoplasm into the nucleus where they bind specific DNA sequences, the IFN-γ-activated sequence (GAS) and the IFN-stimulated response element (ISRE), respectively [157]. This results in transcriptional induction of hundreds of target genes [158]. Figure 2 represents a schematic overview of the type I IFN receptor and its signaling.

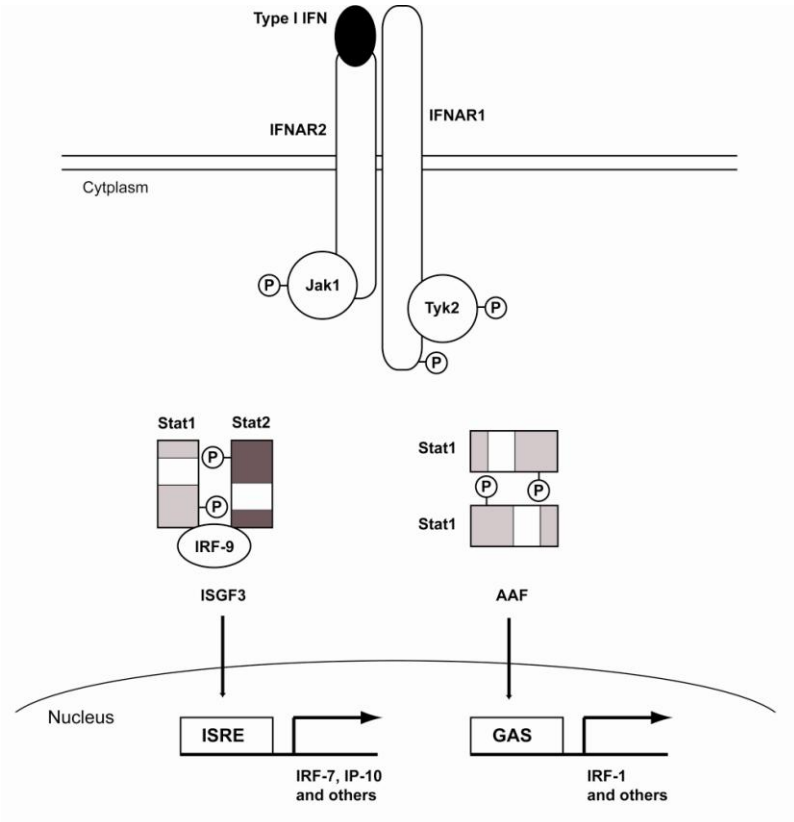

Figure 2. Type I IFN signaling. Modified from Taniguchi and Takaoka (2001) [159].

Target genes

IFN-β induces transcription of numerous genes. Der *et al.* studied mRNA profiles of a human cell line and found that IFN-β stimulation resulted in a more than 2-fold increase of 268 genes, and a greater than 4-fold change in 56 genes [160]. Although the functions of many IFN-inducible genes remain to be elucidated, some are known and have been shown to have antiviral, antiproliferative, and immunomodulatory properties [161].

The important activity against viral infections is mediated by the expression of IFN-inducible genes that contribute to cellular resistance, inhibition of viral replication and spread. The best studied IFN-β-dependent antiviral proteins include the protein kinase R (PKR), the 2´,5´-oligo-adenyl synthetase (2´,5´-OAS) and the Mx proteins, involved in blocking *de novo* protein synthesis, preventing viral replication and promoting apoptosis of virally infected target cells, and blocking viral RNA transcription respectively. Other well-studied IFN-inducible genes are *STATs* and *interferon regulatory factors* (*IRFs)* [160].

IFN-β has been demonstrated to induce expression of ICAM-1 and CD47, which are important for the recruitment of lymphocytes to the infection site. Furthermore, expression of several molecules involved in MHC class I- and MHC class II presentation, as well as proapoptotic caspases and the important signaling molecules MAPKs, were also shown to be IFN-β dependent [161].

It is important to note that the genes transcribed, and thus the effects exerted by IFNs, depend on the type of microbial pathogen that initiates the immune response [153]. Further studies will have to be done to increase the understanding of the IFNs and their complex signaling.

QUINOLINE-3-CARBOXAMIDES

Background

Since the 1980´s, quinoline-3-carboxamides have been developed for treatment of human disease. Linomide, the first chemical compound in use, was tested in a series of Phase II- and III trials in multiple sclerosis (MS) and type I diabetes patients [162-164]. However, the development was interrupted due to unacceptable side effects, and a new set of quinoline-3-carboxamides was developed. One of them, laquinimod, showed efficacy in Phase II trials in MS patients [165, 166], and is now involved in Phase III studies of this disease. Laquinimod is also tested in a Phase II study in patients with Crohn´s disease. ABR-215757 (5757) is another quinoline-3-carboxamide which is currently in clinical development for treatment of the autoimmune rheumatic disorder systemic lupus erythematosus (SLE). The target molecule and mode of action of quinoline-3-carboxamides have remained unknown for years.

Target molecule and mode of action

The cytosolic calcium-binding protein S100A9, expressed by granulocytes and monocytes [167], was recently identified as a molecular target of 5757 and other quinoline-3-carboxamides [168]. The compounds were shown to inhibit interactions of S100A9 with the proinflammatory receptors TLR4 and receptor for advanced glycation end products (RAGE). Although binding of the quinoline-3-carboxamides to S100A9 correlated with their ability to inhibit autoimmune disease, it remains to find out whether their primary pharmacological activity is mediated by this blocking.

Quinoline-3-carboxamides have shown inhibiting effects in several models of T cell-mediated inflammatory autoimmune diseases and it would be reasonable to assume that these compounds affect a common mechanism involved in such disorders, for example T cell activation. One obvious target would be DCs, since they are involved in early stages of immune responses as professional APCs.

DENDRITIC CELLS

Background

Dendritic cells (DCs) are hematopoietic cells that are specialized in capturing, processing and presenting antigens to T cells. Depending on the type- and activation state of the DC, this may lead to tolerance or immunity. DCs are heterogeneous and can be divided into two major populations: (1a) nonlymphoid tissue migratory, (1b) lymphoid tissue-resident, and (2) plasmacytoid DCs (pDCs) [169]. The migratory DCs pick up antigens in peripheral tissues and then migrate through the lymph to the lymph nodes. Migration occurs also in the steady state, although at a lower rate [170]. The lymphoid tissue-resident DCs, in contrast, collect and present antigens in the lymphoid organs and thus do not migrate through the lymph. pDCs migrate from the blood into secondary lymphoid organs under steady-state conditions and their main function is to rapidly secrete massive amounts of type I IFN upon viral infection [171].

Lymphoid tissue-resident DCs and pDCs derive from the common macrophage/DC precursor (MDP) [172] that divides in the bone marrow and further differentiates into the common DC precursor (CDP) [173]. This is a precursor of pDCs and lymphoid tissue-resident DCs but it is unable to give rise to monocytes [174]. Furthermore, other precursors were reported to give rise to lymphoid tissue-resident DCs but not to pDCs [175-177].

Lymphoid tissue-resident DCs

Splenic lymphoid tissue-resident DCs constitutively express MHC class II and CD11c in mice. These cells are subdivided into three populations: $CD8\alpha^{+}CD4^{-}CD11b^{-}$ DCs, $CD4^{+}CD8\alpha^{-}CD11b^{+}$ DCs, and $CD4^{-}CD8^{-}CD11b^{+}$ double negative (DN) DCs [178, 179]. $CD8\alpha^{+}$ DCs are mainly localized in the T cell zone whereas $CD4^{+}$ DCs mainly reside in the marginal zone [180]. CD8α is not expressed on human DCs.

Lymph node DCs are more heterogeneous than those of the spleen and thymus, since they include migratory DCs entering via the afferent lymphatics, in addition to the spleen-equivalent populations $CD8\alpha^+$, $CD4^+$, and DN DCs. In steady state, approximately half the LN DCs seem to be lymphoid tissue-resident [181, 182], whereas the spleen and thymus include mostly tissue-resident cells [179]. One can distinguish between migratory- and lymphoid tissue-resident DCs by studying their phenotype; migratory DCs typically have a mature phenotype when they arrive in the lymph nodes, in contrast to lymphoid tissue-resident DCs that are immature [181].

Functional distinctions between $CD8\alpha^-$ and $CD8\alpha^+$ DCs have been demonstrated in mice. First, $CD8\alpha^-$ DCs are more efficient at presenting antigens via MHC class II to $CD4^+$ T cells while $CD8\alpha^+$ DCs efficiently present antigens to $CD8^+$ T cells via MHC class I [183]. Furthermore, $CD8\alpha^+$ but not $CD4^+$ DCs have been shown to cross-present cell-associated antigens [184]. $CD8\alpha^-$ and $CD8\alpha^+$ DCs have also been reported to differ in the production of IL-12, IFN-α, and IFN-γ [185].

PAPER I

T-cell tolerance induced by repeated antigen stimulation: Selective loss of Foxp3$^-$ conventional CD4 T cells and induction of CD4 T-cell anergy.

Background

Immunization of mice with bacterial superantigens (SAg) causes deletion of SAg-reactive CD4$^+$ T cells [186-188], while the remaining SAg-reactive cells become anergic and thus fail to respond to the immunizing SAg both *in vivo* and *in vitro* [189, 190]. Several reports have demonstrated that such anergic cell populations contain cells which are able to suppress immune responses to the antigen *in vivo* and *in vitro* [191-194]. These data suggest that Tregs might be involved in T cell anergy, stressing the difference between active suppression and inherent anergy, both possibly responsible for the unresponsiveness of T cells. During persistent infections, microbes are repeatedly in contact with immune cells of the host, and an increased frequency of Foxp3$^+$ cells has been observed in blood of infected individuals [195, 196]. It has been assumed that this increase was a result of *de novo* development of Tregs.

Our previous data show that both CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells from mice repeatedly immunized with SAg suppressed T cell proliferation and IL-2 production in cells stimulated with SAg *in vitro*. These results indicated a potential presence of Tregs in the CD4$^+$CD25$^-$ population and we addressed this hypothesis in paper I. We further investigated the role of Foxp3$^+$ cells in anergy caused by repeated antigen stimulation of mice, both *in vitro* and *in vivo*.

Summary

We report that repeated antigen stimulation results in an increased proportion of Foxp3$^+$ cells in the CD4$^+$ population of T cells in mice. This was caused by a decreased number of antigen-reactive conventional CD4$^+$ T cells rather than by *de novo* development of Foxp3$^+$ Tregs. Proliferation of naive transferred CD4$^+$ T cells was inhibited in repeatedly immunized mice and cells transferred from such mice into

naive recipients were shown to be anergic. Finally, we demonstrate that the *in vitro* anergy is partially dependent on $Foxp3^+$ cells whereas the *in vivo* anergy is not.

Results and discussion

We immunized DO11.10 TCR-transgenic (TCR-TG) mice three times with recombinant staphylococcal enterotoxin B (SEB) and observed, as expected, poor proliferation of spleen cells restimulated with SEB *in vitro*, confirming T cell anergy. Further, cells were analyzed by flow cytometry and we found that both $CD4^+CD25^+$ and $CD4^+CD25^-$ populations expressed Foxp3, in control- as well as in 3xSEB cells. However, the proportion of $Foxp3^+$ cells in the $CD4^+CD25^-$ population was significantly increased in 3xSEB- compared to control splenocytes, and by counting the total cell number, we concluded that this was due to selective reduction of $CD4^+CD25^-Foxp3^-$ cells. In support of this, Papiernik *et al*. have demonstrated that chronic stimulation with a viral SAg eliminated $CD4^+CD25^-$ T cells while the $CD4^+CD25^+$ cells were maintained [197]. Another study shows a selective deletion of $CD4^+CD25^-$ cells in septic shock patients [198].

Using an analogous immunization protocol, where OTII TCR-TG mice were injected with ovalbumine–peptide (OVAp) or OVA protein, we obtained similar results as with SEB in regards of T cell anergy and the increased frequency of $Foxp3^+$ cells. As expected from previous studies [199-202], these data demonstrate that the effects on $CD4^+$ T cells were not specifically caused by the SAg but were rather a result of repeated stimulation of these cells.

The extent of $CD4^+$ T cell deletion in mice immunized with SAg has been shown to be dose-dependent [189, 203]. Furthermore, the unresponsiveness of remaining T cells varies as well, depending on the immunization protocol [191, 204, 205]. In the present study, we have used two different immunization protocols. Initial experiments were performed with 5μg SEB i.v. on day 0, 4, and 8. Mice were sacrificed on day 10. For convenience, we changed the protocol and used i.p. injections instead. Here, mice were immunized with 20μg SEB on day 0, 2, and 4, and sacrificed on day 5.

Experiments with OVAp or OVA protein were all performed according to the latter protocol. We obtained similar results in 3xSEB mice with the different protocols.

It is well established that antigen exposure may induce conversion of peripheral naive $CD4^+$ T cells to $Foxp3^+$ Tregs [206, 207]. To determine whether our immunization protocol could convert $Foxp3^-$ cells into $Foxp3^+$ cells, we used *Rag*$^{-/-}$ mice, which do not express Foxp3 at steady state [49], but have been shown to induce expression of *Foxp3* upon tolerogenic antigen stimulation [206, 207]. By analyzing mRNA with quantitative RT-PCR and proteins with flow cytometry, we concluded that our protocol did not induce *Foxp3* expression. These data supported our early conclusions that the increased frequency of $Foxp3^+$ cells in repeatedly immunized mice was caused by selective reduction of $CD4^+CD25^-Foxp3^-$ cells.

Others have observed suppression in 3xSEB mice [192, 194]. To study this phenomenon in our models, we transferred naive CFSE-labeled $CD4^+$ T cells into repeatedly immunized mice and studied their proliferation in response to antigen. Thus, we could confirm the previously published data since SEB-induced proliferation of naive $CD4^+$ T cells was profoundly inhibited in 3xSEB mice, and we also observed corresponding results in 3xOVAp mice. Interestingly, inhibition was only transient, since proliferation was not significantly reduced when cells were transferred 1- or 2 weeks after the last antigen immunization. Although with a different protocol than ours, it has previously been demonstrated that OVAp could stimulate T cells for 5-10 days after injection [201]. This suggests that the absence of antigen might be a reason for the transient suppression in our experiments, since we transferred the cells 8- or 15 days after the last immunization. Interestingly, the increased frequency of $Foxp3^+$ cells remained in recipient mice, suggesting these cells did not play a major role in suppression. However, a possible role of $Foxp3^+$ cells in suppression could not be excluded, since these cells somehow gradually might have lost their capability to suppress. In a previous study, IL-10 was shown to be involved in suppression mediated by T cells from mice repeatedly immunized with SEB [192, 208]. To address whether this cytokine was involved also in our model, we used a neutralizing antibody

specific for IL-10. No difference in proliferation was observed in presence of this antibody, indicating that suppression was independent of IL-10. Another credible explanation for the suppression observed in our model would be competition, as antigen-primed cells in recipient mice can compete for antigen stimulation [209], which would result in reduced proliferation of transferred naive cells. Our preliminary data show that naive OT-I $CD8^+$ T cells are not suppressed when transferred into 3xOVAp mice. This is consistent with competition, since the MHC class II-restricted $CD4^+$ T cells of the repeatedly immunized recipient mice would not be expected to compete with the activation of MHC class I-restricted $CD8^+$ T cells.

To extend our studies of T cell anergy, we aimed to determine whether cells from repeatedly immunized mice were anergic also *in vivo*. CFSE-labeled cells from such mice were transferred into naive recipients and proliferation was measured upon antigen stimulation. The poor proliferation of cells transferred from immunized mice compared to that of controls, was a clear evidence for *in vivo* anergy. Similar to the suppression discussed in the previous section, anergy was also shown to be transient, despite a persistent increased frequency of $Foxp3^+$ cells in the repeatedly immunized mice. It is established that *in vivo* anergy is antigen-dependent [201, 210, 211], which suggests that the gradual loss of anergy in our study might be due to disappearance of the antigen.

In the final part of paper I, we studied the role of $Foxp3^+$ cells in T cell anergy. Based on our observation of an increased Foxp3-frequency, in combination with our previously published data where $CD4^+$ T cells from repeatedly immunized mice potently suppressed T cell proliferation *in vitro* [193], we thought that $Foxp3^+$ cells might contribute to this phenomenon as active suppressors. To address this possibility, we used flow cytometry to remove $Foxp3^+$ cells from the $CD4^+$ T cell population of control- and repeatedly immunized mice. The remaining cells were subsequently stimulated *in vitro*, in parallel with total $CD4^+$ T cells. Although $Foxp3^-$ cells from immunized mice did not proliferate to the same extent as those from control mice, their proliferation was slightly increased compared to total $CD4^+$ T cells from

repeatedly immunized mice. Thus, we concluded that the *in vitro* anergy of cells from repeatedly immunized mice was partially due to suppression by $Foxp3^+$ cells.

The observation that *in vivo* anergy was gradually lost despite of the remaining increased frequency of $Foxp3^+$ cells in repeatedly immunized mice, suggested that these cells might not be involved as suppressor cells during anergy *in vivo*. Total CFSE-labeled $CD4^+$ T cells or $CD4^+Foxp3^-$ cells from control- or repeatedly immunized mice were transferred into naive mice, and their proliferation in response to antigen was studied. Cells from immunized mice proliferated poorly compared to those from controls. Importantly, depletion of $Foxp3^+$ cells did not change the proliferative pattern of $CD4^+$ T cells from repeatedly immunized mice. Thus, we conclude that the anergy observed in our model *in vivo* is independent of $Foxp3^+$ T cells. We further performed *in vivo* anergy experiments with neutralizing antibodies specific for IL-10. Proliferation of transferred T cells was unaffected by these antibodies, suggesting that the *in vivo* anergy in our model is independent of IL-10. Instead, we propose it might be a T-cell intrinsic phenomenon, similar to previous observations in other models [212, 213]. Finally, *in vivo* T cell responsiveness to antigen varies in different studies and the relative contribution of T-cell intrinsic anergy and suppression seems to depend on the experimental model used [211, 212, 214, 215].

PAPER II

TLR2-induced expression of inducible nitric oxide synthase is interferon β-dependent.

Background

Toll like receptors (TLRs) play a central role in innate immune responses as they recognize various microbial products. Activation of TLRs triggers intracellular signaling cascades involving different adaptor molecules, ultimately resulting in the expression of genes important for an immune response. Distinct TLRs signal via different pathways and thus exert different immune responses. TLR signaling is generally divided into two major pathways, the MyD88-dependent and the TRIF-dependent [138]. TLR2 is known to signal only via the former, whereas TLR3 signals via the latter, and TLR4 uses both these pathways [216]. TLR3- and TLR4 activation via TRIF is known to induce the expression of IFN-β, a pleiotropic cytokine, and its downstream molecule inducible nitric oxide synthase (iNOS). Activation of TLR2 has also been reported to induce the expression of iNOS, and a recent study demonstrated that IFN-β expression was induced as well. However, this was only shown in presence of a viral ligand.

Summary

In this study we have used bone marrow-derived dendritic cells (BM-DCs) from IFN-$\beta^{-/-}$ mice to study the role of IFN-β in TLR2-mediated induction of iNOS. We demonstrate that the TLR2 ligands LTA and Pam_3Cys induce the expression of iNOS in an IFN-β dependent manner. Furthermore, iNOS activity induced by these ligands did not require receptor internalization or endosomal maturation, and was differentially dependent on the TRIF- and IRF3 molecules.

Results and discussion

We used BM-DCs from WT- and IFN-$\beta^{-/-}$ mice to study iNOS expression upon stimulation of TLR4 and TLR2. As expected, stimulation with the TLR4 agonist LPS

induced iNOS expression in an IFN-β-dependent manner. Interestingly, the iNOS response in cells stimulated with the TLR2 agonist LTA was also dramatically reduced in IFN-$\beta^{-/-}$ mice compared to WT, suggesting an important role of IFN-β in TLR2-mediated iNOS expression. We further showed that addition of rIFN-β or rIFN-γ to the cell cultures could compensate for the IFN-$\beta^{-/-}$ deficiency, and thus contribute to an increased iNOS response in the IFN-$\beta^{-/-}$ cells. In contrast to the iNOS response, TNF-α production was as expected independent of IFN-β.

Since our results regarding TLR2 were contradictory to previous reports [217, 218], we wanted to perform additional experiments to confirm our data. In addition to LTA and LPS, the TLR2- and TLR3 agonists Pam_3Cys and pI:C were used in these experiments. Firstly, we added a blocking antibody specific for the type I IFN receptor (IFNAR) to BM-DC cultures and observed a reduced iNOS response upon TLR2 stimulation, similar to that in TLR4-and TLR3-stimulated cells. Secondly, we investigated the possibility that IFN-γ, rather than IFN-β, would be the crucial factor for iNOS induction. It is well established that IFN-γ is able to induce iNOS expression in murine cells [219]. Furthermore, the expression of IFN-γ has been shown to be dependent on IFN-β [220]. We added an IFN-γ specific blocking antibody to the cell cultures without observing any effect on the iNOS response, suggesting that IFN-β rather than IFN-γ, is the essential factor for TLR2-mediated iNOS activity. Finally, we studied iNOS expression in cells from IFNAR$^{-/-}$ mice and observed a dramatically reduced iNOS response in TLR2-stimulated cells compared to WT. Together, these data support our finding that TLR2-induced iNOS expression is indeed dependent on IFN-β.

Expression of programmed cell-death ligand-1 (PD-L1) has also been suggested to be IFN-β-dependent [221]. We therefore used flow cytometry to study PD-L1 expression in TLR-stimulated BM-DCs, and observed increased levels of this protein in WT-compared to IFN-$\beta^{-/-}$ cells. This was true for cells stimulated with either LPS or LTA, and thus further reinforces the role of IFN-β in TLR2 signaling.

We next wanted to investigate what intracellular signaling molecules were involved in the TLR2-mediated iNOS expression. BM-DCs from MyD88$^{-/-}$, TRIF$^{-/-}$, and IRF3$^{-/-}$ mice were stimulated with TLR2-, TLR3- and TLR4 agonists and the iNOS response was measured. We confirmed that the TLR3- and TLR4-induced iNOS response was dependent on TRIF and IRF3 [141, 222, 223], but we also observed a reduced iNOS response upon TLR3- and TLR4 stimulation of MyD88$^{-/-}$ cells. This was rather unexpected, however these cells responded poorly to stimulation overall, even upon addition of exogenous rIFN-β. TLR2-induced iNOS expression was reduced in MyD88$^{-/-}$ cells, which was expected since TLR2 is known to signal via this adaptor [224]. However, the absence of TRIF and IRF3 also resulted in a decreased iNOS response in TLR2 activated cells, even though the impact of these molecules was different depending on the TLR2 agonist used. This is supported by previously published data where the inflammatory response induced by LTA was shown to differ from that of Pam_3Cys [225]. However, no firm conclusions may be drawn from these data concerning signaling molecules involved in TLR2-induced iNOS expression, as different results were obtained with the various ligands. Further experiments will therefore have to be done to elucidate this.

In previously mentioned experiments, NO concentration was determined in cell culture supernatants, as a measure of iNOS protein expression. To study mRNA expression of iNOS in BM-DCs stimulated with LPS or LTA, we performed a qRT-PCR. iNOS mRNA expression was markedly reduced in IFN-β$^{-/-}$ cells compared to WT, both upon LPS and LTA stimulation, and thus further shows the importance of IFN-β in TLR-2 induced iNOS expression. Similarly, reduced mRNA levels were observed in MyD88$^{-/-}$, TRIF$^{-/-}$, and IRF3$^{-/-}$ cells, indicating that MyD88, TRIF, and IRF3 are all involved in iNOS expression mediated by LPS or LTA.

We were further interested in studying the role of IFN-β in the TLR2-mediated iNOS expression *in vivo*. WT- and IFN-β$^{-/-}$ mice were immunized intra peritoneally with LPS or LTA, and peritoneal cells were isolated 2h thereafter. CD11b^{+} cells were sorted out and cultured for 48h *ex vivo,* without the addition of agonists. NO concentration was

then measured in the supernatants, and consistent with the *in vitro* data, it was dramatically reduced in the IFN-$\beta^{-/-}$ cells compared to WT. These results suggest that IFN-β plays an important role for the TLR2-induced iNOS activity also *in vivo*.

Previous studies have reported that LPS might contaminate commercial bacterially derived TLR agonists, which then results in incorrect conclusions regarding TLR activation and signaling [226, 227]. To investigate whether our results concerning the TLR2-mediated IFN-β-dependent iNOS response might be due to such a contamination, we studied BM-DCs from TLR4$^{-/-}$ mice. A reduced but not an abolished iNOS response was observed in cells stimulated with Pam_3Cys. Unexpectedly, iNOS was not expressed at all in LTA-stimulated cells. We do not have an explanation for this; however, it is consistent with previously published data [228]. We further used a highly purified LTA, specifically deprived of LPS, and studied iNOS expression in IFN-$\beta^{-/-}$ cells. Since we observed a dramatically decreased iNOS activity in these cells, we concluded that the IFN-β-dependent iNOS response observed upon TLR2 stimulation was not a result of LPS contamination.

TLR4-induced IFN-β expression has been demonstrated to require receptor internalization [146]. Furthermore, a viral agonist was recently shown to induce IFN-β downstream of TLR2, and this was dependent on endosomal maturation [145]. To determine whether the IFN-β-dependent iNOS expression induced by the bacterial TLR2 ligands used in our experiments involves a similar pathway, we used chloroquine and cytochalasin D, inhibitors of endosomal maturation and receptor internalization respectively. BM-DCs were preincubated with these inhibitors and subsequently stimulated with different TLR agonists. TLR3- and TLR4-induced iNOS expression was abrogated when cultured with chloroquine, which was expected since both these receptors signal in a vesicular compartment. Cytochalasin D did also have an inhibitory effect on TLR3- and TLR4 signaling but the iNOS response was only moderately reduced with this inhibitor. The effect of chloroquine and cytochalasin D appeared to be different in cells stimulated by TLR2 agonists. Low concentrations of the inhibitors increased the iNOS response in these cells, while it was reduced at the

highest concentration. However, the overall reduction was only minor, suggesting that the iNOS activity induced by LTA and Pam_3Cys does not require receptor internalization or endosomal maturation. Finally, TNF-α expression was hardly affected by the inhibitors, excluding the possibility that the reduced iNOS response in TLR3- and TLR4 activated cells would be due to toxicity.

Several reports have demonstrated that TLR2 stimulation induces iNOS expression in various cell types [229-233]. However, TLR2 signaling was reported not to involve the expression of IFN-β [217, 218], which is crucial for TLR4- and TLR3-mediated iNOS expression. Recently, Barbalat *et al*. demonstrated that TLR2 activation induced IFN-β in the presence of viral ligands only [145], which is contradictory to our results. We believe that the discrepancies between our results and those previously published might be due to different kinetics. Toshchakov *et al.* detect IFN-β mRNA in cells stimulated for 1h, while the expression is absent in cells stimulated for 6h [218]. Their Southern blot actually does reveal a certain IFN-β expression in cells stimulated for 1h with Pam_3Cys, although it is markedly reduced compared to that of LPS-stimulated cells. Han *et al.* did not observe any IFN-β mRNA expression in LTA-stimulated cells, but they only show expression at one time point [217]. Barbalat *et al.* also report the absence of IFN-β mRNA in cells stimulated by Pam_3Cys; however, they analyzed the cells after 12h of stimulation. Furthermore, Han *et al.* did observe phosphorylation of Stat1 in cells stimulated with LTA for 2h, while Toshchakov *et al.* claim they did not observe phosphorylated Stat1 in Pam_3Cys-stimulated cells.

To clarify the contradictions between our data and those previously published, it would be of relevance to study IFN-β expression *per se* at several time points, in parallel with the iNOS response.

PAPER III

Selective depletion of splenic CD4 dendritic cells in mice treated with immunomodulatory quinoline-3-carboxamide ABR-215757.

Background

Quinoline-3-carboxamides have been developed for treatment of human disease since the 1980´s. One such compound, ABR-215757 (5757), is currently in clinical development for treatment of the autoimmune rheumatic disorder systemic lupus erythematosus (SLE). We recently identified a molecular target of 5757, the S100A9 protein, and we further showed that 5757 blocks the interaction of this protein with the receptors TLR4 and receptor for advanced glycation end products (RAGE) [168]. Dendritic cells (DCs) are important for T cell activation and could therefore be a possible target for 5757 and other quinoline-3-carboxamides. 5757 has shown efficacy in mouse models of the autoimmune diseases multiple sclerosis (MS) and arthritis (unpublished data), but whether 5757-treatment has an effect during steady state conditions is not known.

Summary

We have investigated the impact of *in vivo* 5757-treatment on cells in lymph nodes and spleen of steady state mice. We show that the cell number of a specific DC-subset, $CD4^{+}CD8\alpha^{-}$, is selectively reduced in the spleen of 5757-treated mice. The reduction was reversible and was not caused by decreased cell division or increased apoptosis. Finally, the overall structure of the marginal zone (MZ), where $CD4^{+}CD8\alpha^{-}$ DCs normally reside, remained intact in mice treated with 5757, ruling out the hypothesis that these DCs would emigrate from the spleen because of a disrupted MZ.

Results and discussion

Mice were treated for 10 days with ABR-215757 (5757) dissolved in the drinking water and subsequently, spleen, mesenteric lymph nodes (MLNs), and peripheral lymph nodes (PLNs) were isolated, counted, and analyzed by flow cytometry. We did

not detect any significant changes in the number or frequency of $CD4^+$ T cells, $CD8^+$ T cells or B cells. In contrast, a selective reduced frequency of $CD4^+CD8\alpha^-$ ($CD4^+$) DCs was observed in the spleen but not in the lymph nodes of treated mice, whereas the frequency of two other DC populations, $CD4^-CD8\alpha^+$ ($CD8^+$) and $CD4^-CD8\alpha^-$ (double negative, (DN)), remained unchanged.

We next decided to study the kinetics of the 5757-effects on $CD4^+$ spleen DCs, and observed a continuous decrease in cell number of these cells during the 9 days of treatment. The number of DN- and $CD8\alpha^+$ DCs remained constant, confirming that the loss of $CD4^+$ DCs was not due to downregulation of CD4. We further aimed to determine whether the reduction of splenic $CD4^+$ DCs was reversible. Mice were sacrificed at different time points after the 10-day treatment with 5757. Already three days after terminated treatment, an increased number of $CD4^+$ DCs was detected in treated mice and normal $CD4^+$ DC levels were reached about nine days thereafter. These data are consistent with the previously determined half-life (about three days) for splenic $CD4^+$ DCs [234], and suggest that 5757-treatment might influence the replacement of $CD4^+$ DCs in the spleen.

Selective cell death could be one possible explanation for the reduced number of splenic $CD4^+$ DCs in 5757-treated mice. However, we did not observe a different frequency of apoptotic cells in treated- compared to control mice, which indicates that the reduction of $CD4^+$ DCs is not caused by direct toxicity of 5757.

$CD4^+$-, DN-, and $CD8\alpha^+$ splenic DCs are all generated from common precursors [176, 177]. Therefore, if 5757 would affect these cells, a general reduction of all three DC populations would be expected. It is well established that precursors of splenic DCs divide in the spleen [234, 235]. To investigate whether 5757 had an impact on the proliferation of $CD4^+$ DCs, mice were treated with 5757 for 5 days and during the last 12h of treatment, the thymidine analogue bromodeoxyuridine (BrdU) was injected. The frequency of $BrdU^+$ (dividing) cells was not decreased, but rather slightly increased in the $CD4^+$ DC population. These data excluded the hypothesis that the

reduction of $CD4^+$ DCs would be caused by a decreased proliferation of these cells. Proliferation of DN- and $CD8^+$ DCs was not influenced by the 5757 treatment.

A similar selective reduction of splenic $CD4^+$ DCs as in 5757-treated mice has previously been reported in $CD47^{-/-}$ mice [236, 237], raising the question whether 5757 somehow interferes with this receptor. In $CD47^{-/-}$ mice, migration of DCs from inflamed skin to local draining lymph nodes was shown to be reduced. To determine whether 5757-treatment had similar effects, DC migration experiments were performed. Inflammation was induced by painting abdominal skin or ear epidermis with fluorescein isothiocyanate (FITC) dissolved in irritant. After 24h, FITC-labeled DCs were counted in the draining inguinal- or auricular lymph nodes. There was no difference in frequency of FITC-labeled $CD11c^+$ DCs in lymph nodes from control- or 5757-treated mice. Thus, 5757-treatment does not seem to have the same functional consequences as the loss of CD47, indicating that the reduction of $CD4^+$ DCs in 5757-treated mice might be caused by a different mechanism than that in $CD47^{-/-}$ mice.

$CD4^+$ DCs are mainly localized in the marginal zone (MZ) of the spleen [238, 239] and we hypothesized that a change of this structure might have caused the loss of these DCs in 5757-treated mice. To address this question, we stained spleen tissue sections with antibodies and studied them in a fluorescence microscope. As expected, a reduction in MZ DCs was observed in spleen from 5757-treated mice compared to controls. However, no change in the overall structure of the MZ was observed, as staining of marginal metallophilic macrophages (MMMs) was similar in spleen from control- and 5757-treated mice. In addition, we injected the fluorescently labeled polysaccharide FITC-dextran, previously demonstrated to rapidly be taken up by marginal zone macrophages (MZMs) [240], and observed a similar distribution pattern of these cells in spleen from control- and from mice treated with 5757. Taken together, these data indicated that the loss of $CD4^+$ splenic DCs in 5757-treated mice was not due to a disrupted MZ structure.

To conclude, 5757-treatment of steady-state mice causes a selective reduction of splenic $CD4^+$ DCs. It is not known whether these cells are the primary target of 5757, or whether the effects observed are rather a secondary phenomenon. Finally, DCs do not express S100A9, the only molecular target of 5757 known so far.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Immunförsvaret består av flera olika sorters celler vars uppgift är att skydda oss från sjukdomsframkallande mikroorganismer, såsom bakterier och virus. Immuncellerna har proteiner på sin yta som känner igen olika delar av dessa främmande inkräktare och därmed bidrar till att de kan bekämpas. Förutom förmågan att kunna utveckla immunitet har immunförsvaret en annan viktig uppgift, nämligen att skapa tolerans. Dels mot goda bakterier, men även mot främmande födoämnen och inte minst mot den egna kroppen. Om detta misslyckas kan allergi eller autoimmuna sjukdomar uppstå, där immunförsvaret alltså felaktigt reagerar mot ofarliga ämnen eller kroppsegna strukturer. Exempel på autoimmuna sjukdomar är ledgångsreumatism, MS, typ 1 diabetes och psoriasis.

Artikel I

I det första projektet har vi studerat fenomenet tolerans, närmare bestämt T-cells tolerans, vilket utförs av en viss sorts immunceller, så kallade T-celler. Vi använde oss av en toleransmodell där T-celler i möss upprepade gånger utsattes för ett främmande ämne. Detta förlopp skulle kunna liknas vid en kronisk inflammation. Som förväntat enligt teorin utvecklade mössen tolerans mot ämnet i fråga och vi kunde därmed studera de bakomliggande mekanismerna. Man vet nämligen att flera olika sorters celler och molekyler är involverade i T-cells tolerans men många frågor kvarstår. Regulatoriska T-celler har visat sig vara en av de viktigaste faktorerna för att upprätthålla tolerans och vi ville därför ta reda på vilken roll dessa celler spelade i vår modell. Regulatoriska T-celler, vilket hörs på namnet, reglerar immunsvar som utförs av andra vanliga T-celler.

Vi kunde ”mäta” tolerans genom att titta på hur mycket T-cellerna i mössen delade sig; toleranta celler är vilande och delar sig inte, medan aktiva celler som är redo för försvar delar sig för att bli många. Tolerans i mössen visade sig vara ett övergående fenomen, det vill säga den avtog med tiden. Vi upptäckte en ökad andel regulatoriska T-celler i möss som utvecklat tolerans, jämfört med obehandlade kontrollmöss som inte hade några toleranta T-celler. Detta antydde att de regulatoriska

T-cellerna var inblandade i toleransmekanismen. Men, vidare resultat var lite motsägelsefulla; vid en tidpunkt då toleransen nästan avtagit helt kvarstod fortfarande den ökade andelen regulatoriska T-celler i mössen. Man skulle därmed kunna tro att dessa celler inte var ansvariga för toleransen, eftersom den då borde bibehållits. För att utreda om regulatoriska T-celler spelade någon roll för T-cells tolerans i vår modell sorterade vi ut dessa celler från möss och kunde därmed mäta tolerans i frånvaro av regulatoriska T-celler. Vi observerade liknande tolerans i möss som blivit av med sina regulatoriska T -celler och de som hade dem kvar, vilket fick oss att dra slutsatsen att regulatoriska T-celler inte var involverade i T-cells tolerans i vår modell. Däremot visade det sig att de regulatoriska T-cellerna delvis var inblandade i den tolerans T-cellerna utvecklade när de plockades ut från mössen och odlades i cellkultur.

Slutsatsen är alltså att regulatoriska T-celler inte är ansvariga för den T-cells tolerans som uppstod *in vivo* (i den levande kroppen) medan de är inblandade i den tolerans som observerades *in vitro* (i glaset).

Artikel II

Vår andra studie handlar om motsatsen till tolerans, nämligen immunitet. Immunförsvarets celler har speciella proteiner på sin yta, receptorer. Det finns flera olika sorters receptorer med olika funktion och vi har fokuserat på en viss sort som kallas Toll-lika receptorer (TLRs). TLRs har till uppgift att känna igen och binda till specifika strukturer som finns hos främmande mikroorganismer. När detta sker skickar receptorn en signal in i cellen, som talar om för den att ett immunsvar behövs. Cellen blir då aktiv och börjar uttrycka många olika gener som kodar för flertalet proteiner som behövs för att bekämpa den invaderande mikroorganismen.

Det finns flera olika TLRs och de känner alla igen olika strukturer. Vissa binder till exempel virus medan andra endast binder bakteriella delar. Även signaleringen hos de olika receptorerna varierar, vilket gör att olika gener aktiveras och därmed också olika proteiner bildas. TLR-signalering är ett välstuderat ämne men det är mycket komplext. Oklarheter kvarstår bland annat angående en TLR, den så kallade TLR2, vilken vi har studerat närmare.

Tidigare resultat från andra forskargrupper har visat att signalering via TLR2 leder till bildandet av ett visst protein, iNOS. Andra TLRs, såsom TLR3 och TLR4, är också kända för att signalera och få cellen att producera iNOS när de stimuleras av sina respektive mikrobiella strukturer. Man vet också att TLR3 och TLR4 endast kan instruera cellen att producera iNOS i närvaro av ett annat protein, IFN-β, som alltså först måste bildas i cellen. Man säger att iNOS produktionen är IFN-β-beroende. TLR2 har däremot i tidigare studier visats inkapabel att instruera cellen att producera IFN-β, vilket innebär att produktionen av iNOS i dessa celler, till skillnad från dem som aktiverats via TLR3 eller TLR4, skulle vara oberoende av IFN-β. Nyligen publicerades en motsägelsefull studie där man visade att TLR2-stimulering kunde leda till produktion av IFN-β. Om det inträffade eller inte berodde dock på vilken mikrobiell struktur som användes för att stimulera receptorn; en bakterieliknande struktur ledde inte till IFN-β-produktion medan en viral struktur gjorde det. Vi har använt immunceller framtagna från benmärg i möss, för att studera förhållandet mellan IFN-β och iNOS i dessa när de aktiverats via TLR2 med olika bakteriella strukturer.

Vi använde oss av möss vars celler är inkapabla att bilda IFN-β överhuvudtaget, eftersom genen som kodar för detta protein förstörts (en så kallad knockout mus (KO)). Cellerna odlades i cellkultur (*in vitro*) och stimulerades via TLR2, varpå bildandet av iNOS studerades. Endast mycket lite iNOS visade sig produceras i celler från IFN-β KO möss jämfört med celler från normala möss, vilket tydde på att iNOS-produktion i celler stimulerade via TLR2 är beroende av IFN-β. Om vi tillsatte rent IFN-β till våra KO celler ökade produktionen av iNOS markant. Vi fortsatte sedan att undersöka signaleringsvägen från TLR2-stimulering till iNOS-produktion genom att använda KO möss för andra proteiner, men vi kunde inte direkt dra några tydliga slutsatser från dessa försök. Slutligen stimulerade vi TLR2 på celler direkt i mössen (*in vivo*), och fick liknande resultat som i *in vitro* experimenten, det vill säga att produktionen av iNOS var beroende av IFN-β.

I denna studie har vi alltså visat att stimulering av TLR2 leder till produktion av iNOS och att detta är beroende av IFN-β.

Artikel III

I det tredje projektet har vi studerat ett potentiellt blivande läkemedel vid namn 5757. Denna molekyl ingår just nu i kliniska prövningar på patienter med den autoimmuna sjukdomen systemic lupus erythematosus (SLE), där den har visat sig dämpa sjukdomsförloppet. Dock känner man ännu inte till hur 5757 verkar och vår uppgift var att studera dess effekter på immunceller i friska möss för att få ökad kunskap om denna molekyl.

Vi började med att undersöka om antalet av olika sorters immunceller i de lymfoida organen mjälte och lymfkörtlar förändrades i möss som behandlades med 5757. Sammansättningen T och B celler påverkades inte av drogen i något av de organ vi studerade. Däremot upptäckte vi att antalet av en annan sorts immunceller, så kallade dendritiska celler (DCs), minskade. Detta skedde endast i mjälten och det var bara en viss typ av DCs som påverkades. Vi såg att antalet av dessa celler minskade mer ju längre tid mössen behandlats med 5757 och att antalet återställdes ca 10 dagar efter att behandlingen avslutats.

En möjlig förklaring till att DCs minskade i antal skulle kunna vara att 5757 fick dem att genomgå programmerad celldöd, eller att drogen helt enkelt hindrade cellerna från att dela sig. Vi undersökte detta men såg ingen effekt av 5757, varken på celldöd eller på celldelning. Antalet minskade DCs skulle också kunna bero på att 5757 påverkar den specifika struktur i mjälten där just dessa DCs finns. Vi studerade därför mjältens struktur i mikroskop men såg inga märkbara förändringar i mjälte från 5757-behandlade möss jämfört med obehandlade.

Slutsatsen av denna studie är alltså att 5757 leder till ett minskat antal av en viss sorts DCs i mjälte i friska möss. Om drogen har en direkt effekt på dessa celler eller om det är en sekundär effekt vet vi ännu inte. Fler experiment måste göras för att ta reda på hur 5757 verkar.

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