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Innate regulation of the adaptive immune system during autoimmunity

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Innate regulation of the adaptive immune system during autoimmunity

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“When you have exhausted all possibilities, remember this - you haven't”
- Thomas A. Edison

ABSTRACT

Immune activation comprises multiple biological checkpoints to ensure proper and regulated effector functions. Phagocytes such as macrophages, dendritic cells and neutrophils have important functions during inflammation, e.g. clearance of bacterial pathogens.

In this thesis, I have studied the regulatory properties of phagocytes and their crosstalk with adaptive immunity has been studied. Their role in the regulation of the adaptive immune system has been investigated at the site of inflammation and in the initiation of the immune response in the secondary lymphoid organs. Different animal models have been used to understand the regulatory properties of phagocytes in the context of autoimmunity and chronic inflammation.

We have shown that M2 macrophages can regulate and suppress autoimmunity in murine models of both type 1 diabetes and experimental autoimmune encephalomyelitis (EAE). The M2 macrophages were localized in the targeted organ and had the ability to suppress T cell activation and produce factors that promote wound-healing. Furthermore, we identified TGFβ as an important cytokine for the immunosuppressive properties of M2 macrophages, and also a crucial factor in the deactivation of inflammatory monocyte-derived cells during EAE remission.

We have also studied the role of neutrophils in the regulation of adaptive immunity in lymph nodes. We generated a neutropenic mouse model and studied how neutrophils interacted with T and B cells during adjuvant-induced inflammation. These studies revealed that neutrophils have an immense role in the activation of B cells and the generation of antibody-producing plasma cells.

LIST OF PUBLICATIONS

I. **Adoptive transfer of immunomodulatory M2 macrophages prevents type I diabetes in NOD mice.**
Roham Parsa, Pernilla Andresen, Alan Gillett, Sohel Mia, Xing-Mei Zhang, Sofia Mayans, Dan Holmberg, Robert A. Harris
Diabetes. 2012 Nov;61(11):2881-92

II. **Adoptive transfer of cytokine-induced immunomodulatory adult microglia attenuates experimental autoimmune encephalomyelitis in DBA/1 mice.**
Xing-Mei Zhang, Harald Lund, Sohel Mia, **Roham Parsa**, Robert A. Harris
Glia. 2014 May;62(5):804-17

III. **TGF-beta regulates persistent neuroinflammation by controlling T_H1 polarization and ROS production.**
Roham Parsa*, Harald Lund*, Ivana Tosevski, Xing-Mei Zhang, Ursula Malipiero, Jan Beckervordersandforth, Doron Merkler, Marco Prinz, Adriano Fontana, Tobias Suter, Robert A. Harris
Manuscript

IV. **Neutrophils regulate local T and B cell activation during adjuvant-induced emergency granulopoiesis.**
Roham Parsa, Harald Lund, Anna-Maria Georgoudaki, Xing-Mei Zhang, André Ortlieb Guerreiro-Cacais, Andreas Warnecke, Andrew Croxford, Maja Jagodic, Burkhard Becher, Mikael C.I. Karlsson, Robert A. Harris
Manuscript

*denotes equal contribution

PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. **Tumor-specific bacteriophages induce tumor destruction through activation of tumor-associated macrophages**
Fredrik Eriksson*, Panagiotis Tsagozis*, Kajsa Lundberg, **Roham Parsa**, Sara M Mangsbo, Mats AA Persson, Robert A Harris, Pavel Pisa
The Journal of Immunology, 2009 Mars;182(5):3105-11

II. **Toll-like receptor activation reveals developmental reorganization and unmasks responder subsets of microglia.**
Jörg Scheffel, Tommy Regen, Denise Van Rossum, Stefanie Seifert, Sandra Ribes, Roland Nau, **Roham Parsa**, Robert A. Harris, Hendrikus WGM Boddeke, Han-Ning Chuang, Tobias Pukrop, Johannes T Wessels, Tanja Jürgens, Doron Merkler, Wolfgang Brück, Mareike Schnaars, Mikael Simons, Helmut Kettenmann, Uwe-Karsten Hanisch
Glia. 2012 Dec;60(12):1930-43

III. **Strain influences on inflammatory pathway activation, cell infiltration and complement cascade after traumatic brain injury in the rat**
Faiez Al Nimer, Rickard Lindblom, Mikael Ström, André Ortlieb Guerreiro-Cacais, **Roham Parsa**, Shahin Aeinehband, Tiit Mathiesen, Olle Lidman, Fredrik Piehl
Brain, Behavior, and Immunity, 2013 Jan;27:109-22

IV. **A Silent Exonic SNP in Kdm3a Affects Nucleic Acids Structure but Does Not Regulate Experimental Autoimmune Encephalomyelitis**
Alan Gillett, Petra Bergman, **Roham Parsa**, Andreas Bremges, Robert Giegerich, Maja Jagodic
PloS One, 2013 Dec;8(12):e81912

V. **Genetic variability in the rat Aplec C-type lectin gene cluster regulates lymphocyte trafficking and motor neuron survival after traumatic nerve root injury**
RP Lindblom, Shahin Aeinehband, **Roham Parsa**, Mikael Ström, Faiez Al Nimer, Xing-Mei Zhang, Cecilia A Dominguez, Sevasti Flytzani, Margarita Diez, Fredrik Piehl
Journal of Neuroinflammation, 2013 May;10(60):2094-10

VI. **The multiple sclerosis risk gene IL22RA2 contributes to a more severe murine autoimmune neuroinflammation**
Hannes Laaksonen, André Ortlieb Guerreiro-Cacais, Milena Z. Adzemovic, **Roham Parsa**, Manuel Zeitelhofer, Maja Jagodic, Tomas Olsson
Genes and Immunity, 2014 Oct;15(7):457-465

*denotes equal contribution

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LIST OF ABBREVIATIONS

APC	Antigen-presenting cell
APRIL	A proliferation-inducing ligand
BAFF	B cell activating factor
BCR	B cell receptor
BM	Bone marrow
CD	Cluster of differentiation
cDC	Classical dendritic cell
CFA	Complete Freund's adjuvant
CLP	Common lymphocyte progenitor
CLR	C-type lectin receptor
CMP	Common myeloid progenitor
CNS	Central nervous system
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
Fc	Crystallizable fragment
FDC	Follicular dendritic cell
flt3L	FMS-like tyrosine kinase 3 ligand
G-CSF	Granulocyte-colony stimulating factor
GC	Germinal center
GM-CSF	Granulocyte macrophage-colony stimulating factor
GMP	Granulocyte-macrophage progenitor
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthases
LN	Lymph node
LPS	Lipopolysaccharide
M-CSF	Macrophage-colony stimulating factor
MHC	Major histocompatibility complex
moDC	Monocyte-derived dendritic cell
MOG	Myelin oligodendrocyte glycoprotein
MR	Mannose receptor
MS	Multiple sclerosis
MZ	Marginal zone
NOD	Non-obese diabetic mouse
NOX	nicotinamide adenine dinucleotide phosphate H oxidase
p.i.	Post-immunization
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PM	Peritoneal macrophages
PRR	Pattern recognition receptor
PV	Perivascular
ROS	Reactive oxygen species
SCS	Subcapsular sinus
SLE	Systemic lupus erthyematosus
T1D	Type 1 diabetes
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
WT	Wild type

1 Introduction

1.1 The immune system

The immune system has evolved to protect the host from invading pathogens such as bacteria and viruses through use of a large number of mediators such as recognition and activation receptors[1]. The immune system is also important for host homeostasis through removal of apoptotic cells[2,3], tissue remodeling and tissue repair[4,5]. One key aspect of the immune system is to discriminate between host molecules, also known as *self*, and molecules derived from potentially dangerous pathogens, defined as *non-self*[6]. Due to the immense cytotoxic and bactericidal properties of certain immune cells, the immune system has many built-in regulatory elements and checkpoints to control immune activation. Immune cells are broadly divided into two large functional groups, the *innate immune system* and the *adaptive immune system*[1]. The innate system, which is rapidly activated and has a broad but less specific capacity to recognize pathogen-derived molecules, is one of the first barriers that the pathogens will encounter. Conversely, the adaptive immune system is slower in its activation but is more specific and effective in the determination of pathogen-specific targets, and also has the unique capacity to develop immunological memory. The innate and adaptive immune systems work in a well-balanced symbiosis and communication between innate and adaptive immune cells is essential for proper immune function.

1.1.1 Innate immune system

The innate immune system is the first line of defense against invading pathogens. The first protection is the physical barrier built up by epithelium cells in our skin and mucosal surfaces[7]. Not only providing a barrier for the host, epithelium cells furthermore contribute to the innate immune system by producing anti-microbial peptides and molecules. Another passive part of innate immunity is a system of circulating plasma proteins termed the complement system[8]. These elements can directly bind to pathogens and alarm the immune system by recruiting local and blood-derived innate immune cells, facilitating phagocytosis or inducing direct lysis of the pathogen.

The earliest recruited immune cells are professional phagocytes, cells that actively engulfs extracellular content, including neutrophils, monocytes, macrophages, mast cells and dendritic cells (DC)[9]. Their primary role is to mediate uptake and lysis of the pathogens but also to produce inflammatory mediators such as cytokines and chemokines. The DCs have the ability to migrate to secondary lymphoid organs where they present antigens to the T cells of the adaptive immune system[10]. The recognition of pathogens, or of their pathogen-associated molecular patterns (PAMPs), by innate immune cells occurs through germline-encoded pattern recognition receptors (PRR) such as the Toll-like receptors (TLR) and the C-type lectin receptors (CLR)[11]. Innate cellular activation by PRRs is crucial for the later activation of the adaptive immune system.

1.1.1.1 Neutrophils

The neutrophilic granulocytes, commonly known as neutrophils, are the most abundant white blood cells in humans. Neutrophils are short-lived cells, spanning from hours to 5 days and are generated and fully differentiated from granulocyte-macrophage progenitors in the bone marrow (BM) before being released into the circulation[12-14]. The growth factor granulocyte-colony stimulating factor (G-CSF) is important for the activation and release of neutrophils from the BM but it is not crucial for neutrophil generation as G-CSF gene deleted mice are indeed neutropenic in the blood but still generate neutrophils in the BM[15]. During maturation in the BM neutrophils form intracellular granules (secretory granules) that contain proteins important for their anti-microbial functions[16]. Upon tissue infection endothelial cells, epithelial cells, monocytes and macrophages secrete agents such as G-CSF, KC (CXCL1) and MIP-2 (CXCL2) which are key chemokines for neutrophil attraction to the site of infection. Neutrophil activation by PRRs induces cytokine secretion and phagocytosis of the microorganism. The phagosome will fuse with the intracellular granules containing reactive oxygen species (ROS) and other noxious agents resulting in the elimination of the pathogen. In addition to intra-phagosomal killing, neutrophils have the ability to release

their nuclear content into the extracellular matrix and to prevent microbial growth, a mechanism termed neutrophil extracellular trap formation[17].

Neutrophils have historically been considered as suicidal killers with limited immune functions except for their antimicrobial abilities. It has always been challenging to study them *in vitro* due to their very limited survival. During the last decade our knowledge about neutrophils has increased dramatically due to the use of more sophisticated research tools. Several reports indicate their complex interactions with other innate cells such as macrophages[18] and NK cells[19], but also their ability to interact at the bridge between innate and adaptive immunity[20], by regulating the antigen-presenting capacity of DC and macrophages[21] or directly regulating T cell activation[22]. Neutrophils also interact with B cells and are an important source for B cell activating factor (BAFF)[23].

1.1.1.2 Monocytes

Monocytes are important innate cells that mostly reside in the blood but also have reservoir pools in the lungs and the spleen[24,25]. They represent about 5% and 10% of the nucleated cells in mouse and human blood, respectively. Monocytes are generated in the BM and the growth factor macrophage-colony stimulating factor (M-CSF) is crucial for their differentiation and survival, as mice deficient in M-CSF or its receptor M-CSFR demonstrate severe monocytopenia[26,27]. The release of monocytes from the BM is dependent on chemokine C-C motif ligand 2 (CCL2), but it is also important for the attraction of monocytes to the site of inflammation[28].

There are two types of monocyte subsets in mouse and human blood, named *inflammatory monocytes* and *resident monocytes* (Table 1). It is believed that resident monocytes are generated from inflammatory monocytes in the circulation and not from monocyte precursors in the BM[29]. As for their function, inflammatory monocytes are important precursors for tissue infiltrating inflammatory macrophages and monocyte-derived DCs (moDC) during infection or tissue trauma[30,31]. Recent reports have revealed interesting findings using intravital microscopy regarding resident monocytes; demonstrating that these cells crawl along the luminal side of the endothelium

wall and patrol for tissue injury in both mice and humans[32,33]. Taken together, monocytes are the source for inflammatory phagocytes during infections and tissue trauma, whereas tissue resident phagocytes are believed to be important for tissue homeostasis and repair[34].

Table 1 | **Monocyte subsets in mice and humans**

Mouse monocytes	**Human counterpart**	**Source**	**Function**
Inflammatory monocytes $CCR2^{+}Ly\text{-}6C^{hi}CX3CR1^{low}$	$CD14^{+}CD16^{-}$	Precursor in BM	Precursors for inflammatory macrophages and DCs
Resident monocytes $CCR2^{-}Ly\text{-}6C^{-}CX3CR1^{hi}$	$CD14^{+/mid}CD16^{+}$	$Ly\text{-}6C^{hi}$ monocytes	Patrolling the endothelial wall

1.1.1.3 Macrophages

Macrophages are tissue-resident phagocytes that can be found in almost every organ (Fig. 1). Macrophages not only act as sentinels surveying the local environment for unwanted microorganisms, but they are also important cells for tissue homeostasis by engulfing apoptotic cells and secreting growth factors[35]. For example, red pulp macrophages in the spleen are important for engulfing dying erythrocytes[36], or alveolar macrophages for assisting in the removal of allergens in the lungs[37]. Macrophage functions are adapted to their organ residency and are known to be plastic as the environment can drastically change. It has for a long time been believed that all tissue-resident macrophages are replenished by circulating monocytes throughout life, but this dogma has been challenged and rewritten by recent discoveries[38].

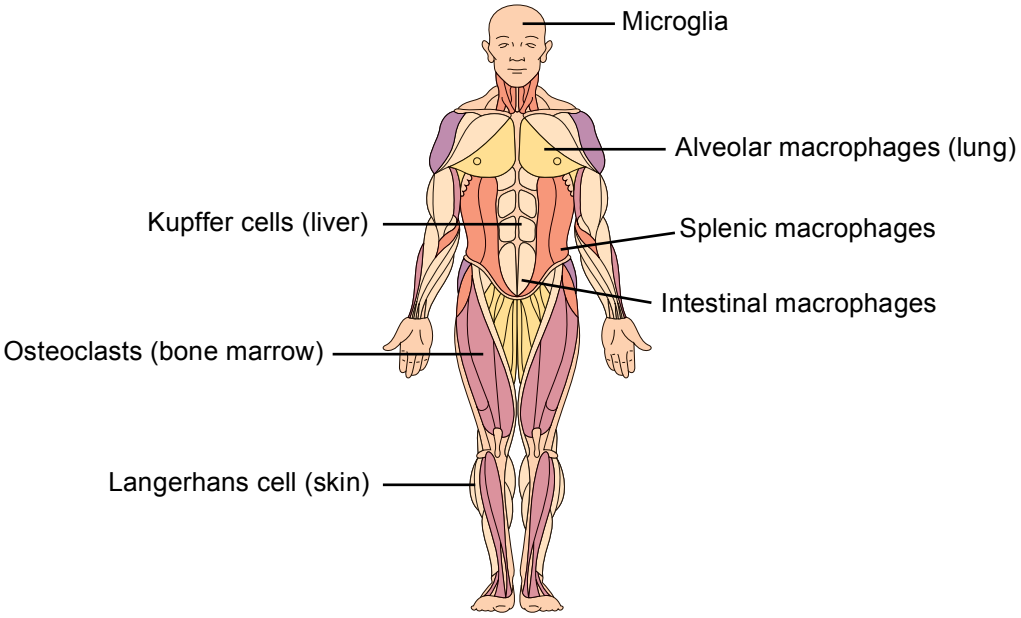

Figure 1 | **Macrophage distribution in the human body.** Macrophages are found in almost every organ throughout the body, where they have many functions such as nursing the tissue by secretion of growth factors but also immunosurveillance, including phagocytosis, secretion of anti-bacterial molecules and antigen presentation.

Tissue macrophages can arise prenatally, in adulthood or both depending on the population analyzed (Fig. 2). Macrophages can be derived from three different sources: the prenatal yolk sac, the prenatal fetal liver or from postnatal BM monocytes (Table 2). For example, the central nervous system (CNS) resident macrophages, microglia, are purely derived from the yolk sac, whereas red pulp macrophages are derived from the prenatal fetal liver. Macrophages in the adult liver, the Kupffer cells, originate from the yolk sac or fetal liver and are locally maintained independently from BM monocytes during adulthood[34,38,39]. Monocytes give rise to tissue macrophages during inflammation but are also important for the replenishment of macrophages in some specific organs such as the tissue-resident macrophages in the intestine.

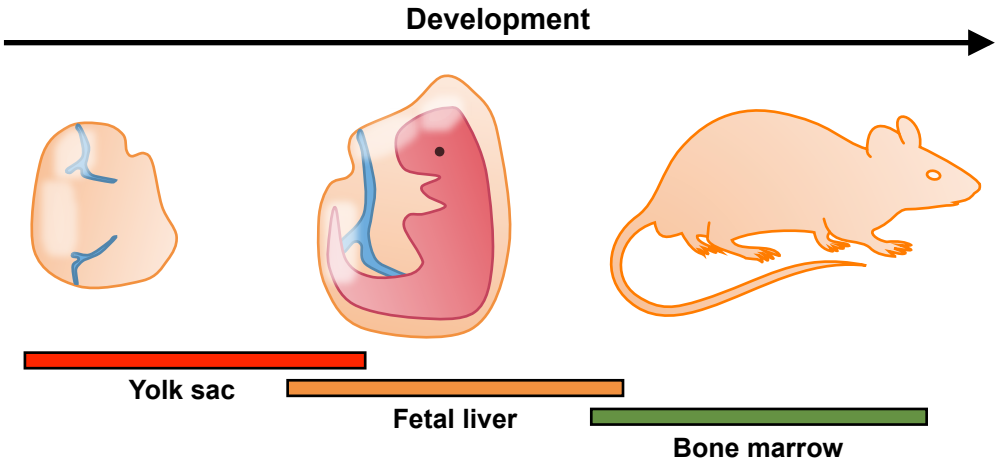

Figure 2 | **Origin of macrophage populations.** Macrophages can arise from the yolk sac, fetal liver or the adult bone marrow in mice. These macrophages will seed different organs during development. Some tissue macrophages will be dependent on mature monocytes from the postnatal bone marrow whereas other populations will be locally maintained in the tissue.

There are several important growth factors for generating and maintaining tissue-resident macrophages and monocyte-derived tissue macrophages. Studies in mice have revealed that M-CSF, GM-CSF and interleukin (IL)-34 are important growth factors for different types of tissue macrophages. For instance, M-CSF deficient mice are as mentioned earlier monocytopenic, but also lack kidney, peritoneal and intestine macrophages[40]. Interestingly, M-CSF receptor (M-CSFR) deficient mice demonstrate significant differences with the additional loss of skin Langerhans cells and CNS microglia. This phenomenon was later demonstrated to be dependent on the alternative ligand for M-CSFR, the cytokine IL-34[41,42]. Studies of irradiated chimeric mice and gene-deleted mice revealed the important function of granulocyte macrophage-colony stimulating factor (GM-CSF), as these mice lacked lung macrophages and developed alveolar proteinosis[43]. Collectively these studies demonstrate the heterogeneity and plasticity of macrophage generation and function.

All tissue resident macrophages have common functions in immune surveillance, detection and phagocytosis of pathogens and in alarming the tissue. Macrophages can detect microorganisms via the various types of PRRs they express, which leads to phagocytosis. Pathogens are trapped in the phagosome that will later be fused with lysosomes containing enzymes

and free radicals that will digest and eliminate the pathogen[35]. Activated macrophages will also produce mediators such as inflammatory cytokines and chemokines that will attract more immune cells[44]. Another capacity of some tissue macrophages is to locally present antigens from engulfed pathogens to recruited cells from the adaptive immune system, in particular to T cells[45]. There is an intimate cross-talk between macrophages and T cells involving both receptors and cytokines that will in turn affect cellular and functional effector programs of both cell types[46,47]. In addition to many functions of macrophages in innate immunity, they also conduct important roles at the bridge between innate and adaptive immunity and are a key effector cell during inflammatory responses.

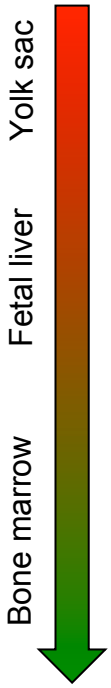

Table 2 | **Macrophage ontogeny**

Organ	Cell type	Origin
Brain	Microglia	Yolk sac
Skin	Langerhans cells Dermal macrophages	Yolk sac + fetal liver Bone marrow
Liver	Kupffer cells	Yolk sac + fetal liver
Heart	CCR2- macrophages CCR2+ macrophages	Yolk sac + fetal liver Bone marrow
Lung	Alveolar macrophages CD11b+ macrophages	Fetal liver Unknown
Kidney	Kidney macrophages	Fetal liver or bone marrow
Spleen	Red pulp macrophages Marginal zone macrophages	Fetal liver Unknown
Peritoneum	Peritoneal macrophages	Fetal liver
Intestine	Intestinal macrophages	Bone marrow
Blood	Monocytes	Bone marrow

1.1.1.4 Dendritic cells

The DCs have many similarities to macrophages and it has historically always been difficult to distinguish between these two cell types. But per definition DCs have three unique innate properties that make them different from macrophages: 1) they migrate to secondary lymphoid organs; 2) they are professional antigen-presenting cells (APC) with the ability to activate naive T cells via the major histocompatibility complex (MHC) class II complex; and 3) they have the ability to cross-present antigens via the MHC class I complex[10]. DCs can be subdivided into two major subsets, classical DCs (cDC) and non-classical DCs, or the more widely used name, moDCs. The cDCs can be further subdivided into different subclasses depending on anatomical location and specialized functions. In contrast to macrophages, cDCs are short-lived with an approximate half-life of 3-6 days. The seeding of cDCs is dependent on BM-derived progenitors that are strictly dependent on the growth factor fms-like tyrosine kinase 3 ligand (flt3L)[48]. Recruitment of monocytes to lymphoid and non-lymphoid organs is a consequence of inflammation that will differentiate moDCs. The moDCs are difficult to distinguish from cDCs as they

express similar surface markers, to some degree migrate to lymphoid organs and can activate naïve T cells *in vitro*, but in some circumstances they also express monocyte markers such as cluster of differentiation (CD)64 and F4/80 in mice[49]. Monocytes or BM cultured *in vitro* with GM-CSF generate moDCs[50]; however, GM-CSF deficient mice still develop moDCs, indicating that there are other ligands important for moDC generation[51]. The activation of DCs is very similar to macrophages as they express similar PRRs, which in turn activates an inflammatory program that includes phagocytosis, antigen loading of the MHC molecules, up-regulation of co-stimulatory receptors and cytokine secretion.

1.1.2 Adaptive immune system

To obtain an antigen-specific immune response the innate immune cells activate the adaptive immune system. T and B lymphocytes are key cells of the adaptive immune system. The generation of these cells occurs in the primary lymphoid organs such as the BM and the thymus, but their activation follows in the secondary lymphoid organs such as the lymph nodes (LN) and spleen. A unique characteristic of the cells of the adaptive immune system is their epitope-specific antigen receptors, the T cell receptor (TCR) and the B cell receptor (BCR)[52]. These receptors are generated by rearrangement of gene segments and mutations during maturation that induces high diversification and specificity. The TCR and BCR are both fundamental for T and B cell activation, respectively. These lymphocytes undergo positive and negative selection during their development and mature in the primary lymphoid organs. In simple terms, this is a process to ensure that lymphocytes accurately recognize foreign antigens[52,53]. The activation of T cells by the TCR is dependent on the MHC molecule and co-stimulatory ligands such as CD80 and CD86 expressed by APCs. B cell activation by the BCR is dependent on the tertiary structure of the antigen and cross-linking of the BCR. Another important attribute of T and B cells is their ability to induce immunological memory, which is the capability of the adaptive immune system to respond more rapidly and effectively to pathogens that have been previously encountered[54].

1.1.2.1 T cells

T cell development proceeds from early common lymphocyte progenitors (CLP) in the BM that later become recruited to the thymus for their maturation into naïve T lymphocytes. During thymic development T cells undergo gene rearrangement and clonal selection, and within the thymus the determination of T cell lineage takes place[55]. T cells can be divided into two subsets, $CD4^+$ T helper cells (T_H) and $CD8^+$ cytotoxic T cells (T_C). The antigen-specific activation of $CD4^+$ T_H cells in the periphery is primarily dependent on the presentation of peptides via the MHC class II molecule and co-stimulatory molecules that induce T cell proliferation. Furthermore, the cytokine milieu in the microenvironment, contributed by APCs and other stromal cells, is important for the differentiation of activated T cells and for their later effector functions such as cytokine profile and chemokine receptor expression[56]. Activated T_H cells have significant functions both locally in the secondary lymphoid organs, and most importantly peripherally at the sites of inflammation where they instruct and amplify innate immune cell functions. Within the secondary lymphoid organs T_H cells assist the activation of B cells through CD40-CD40L interaction which is essential for the development of antibody-producing plasma cells[57]. T_H cells are also involved in the 'licensing' of APCs, again dependent on CD40-CD40L, and these can then activate naïve $CD8^+$ T_C cells[58,59]. Similar to $CD4^+$ T_H cells, primary $CD8^+$ T_C cell activation is dependent on specific antigen recognition via the MHC class I molecule expressed by APCs. The primary role for activated T_C cells is to establish cell contact with a target cell, recognize the antigen and, if the antigen is recognized, to induce apoptosis of the targeted cell. Destruction of target cells by T_C cells is accomplished by perforin/granzyme-mediated apoptosis and FAS-FASL-mediated apoptosis[60].

1.1.2.2 B cells

B cells can be divided into three major subsets, the circulating follicular B cells, and the more innate-like B1 and marginal zone (MZ) B cells. Studies in mice, and to some degree in humans, have deduced that B1 cells are already generated in the fetus and undergo self-renewal in the periphery[61,62]. B1 cells

have less diversified and less specific BCRs that recognize highly conserved microbial elements[63]. The activation of B1 cells is T cell-independent and they produce circulating natural antibodies believed to be important for early defense prior to the development of postnatal follicular B cells. MZ B cells are generated in the spleen 2-3 weeks after birth in mice and after 1-2 years in humans[64,65]. Similar to B1 cells, MZ B cells also express less diversified BCRs and are T cell-independent for their activation. The generation and maturation of postnatal follicular B cells occurs in the BM and they are primarily derived from CLPs. These cells undergo many steps before becoming a mature circulating follicular B cell[66] (Fig. 3). B cells are activated by soluble or static antigen interactions with the BCR. Similar to T cells, B cells need further signals for proper activation and clonal expansion, this involves CD40-CD40L interactions and cytokines secreted by T_H cells and myeloid cells to instruct B cell functions and antibody isotype switch.

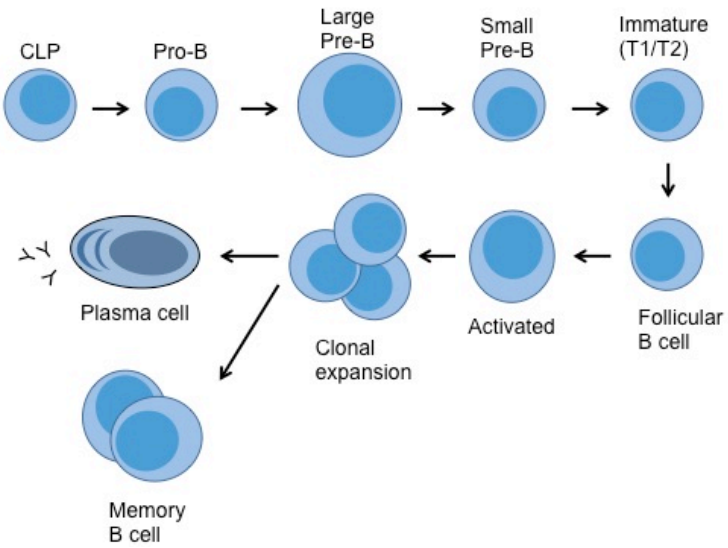

Figure 3 | **B cell development.** The figure illustrates the development of B cells and activation into becoming plasma cells or memory B cells.

1.2 Immune activation and regulation

The primary role of the immune system is to protect the host from unwanted microorganisms. The body tightly regulates the activation of the immune system, the balance between induction and resolution of inflammation being extremely important for survival of the host due to the potential cytotoxic properties of the immune cells. Hence the immune system has a very

sophisticated regulation with many checkpoints that control different aspects of innate and adaptive immune activation.

1.2.1 Inflammation

Inflammation is critical for protecting the host, but is also a key factor in the development of many complex states and disorders such as autoimmune diseases. Inflammation occurs in two manners: *acute inflammation*, which can be defined as a regulated activation of the immune system with defined initiation and resolution phases, and *chronic inflammation*, which can be defined as a dysregulated form of inflammation. Persistent injury, infection or prolonged exposure to toxins/antigens, can switch acute inflammation into chronic inflammation.

Acute inflammation is generated when innate cells such as mast cells, macrophages and endothelial cells are activated upon tissue trauma, either by infection or cellular damage, which activates a cascade of downstream effector molecules such as G-CSF, CXCL1, CXCL2, IL-1, IL-6 and tumor necrosis factor (TNF)[67]. This response enhances the local permeability of the blood vessels and recruits large numbers of neutrophils and monocytes to the site of inflammation[9]. The increased metabolic activity and enhanced cellular recruitment at the local site builds up the five core elements of inflammation, heat, redness, swelling, pain and loss of function[68] (Fig. 4). The recruited neutrophils possess a large arsenal of inflammatory mediators important for eliminating pathogens. They release granules containing proteases and antimicrobial polypeptides[69], produce and release ROS[70] and leukotrienes[71] but also secrete various cytokines, chemokines and growth factors[16]. Neutrophils are important for the later recruitment of inflammatory monocytes to the site of inflammation via the secretion of CCL2. The recruited monocytes is an important source of moDCs and inflammatory macrophages[72,73].

Neutrophils are normally short-lived cells but during inflammation they are exposed to growth factors that increase their life span. Factors such as G-CSF and GM-CSF secreted by tissue macrophages enhance neutrophil activity and postpone neutrophil apoptosis[74]. However, during prolonged infection or chronic inflammation neutrophils will undergo apoptosis that will result in neutrophil ‘consumption’. If the ongoing need for neutrophils is not

met, normal granulopoiesis is switched to emergency granulopoiesis[75]. This state is defined by enhanced serum levels of G-CSF, enhanced myeloid progenitor proliferation in the BM and the release of myeloid progenitors from the BM to the circulation. This adaptation of the immune system can in many cases be crucial for the survival of the host but also increases the risk of more tissue damage due to cytotoxic effects of innate cell-mediated inflammation.

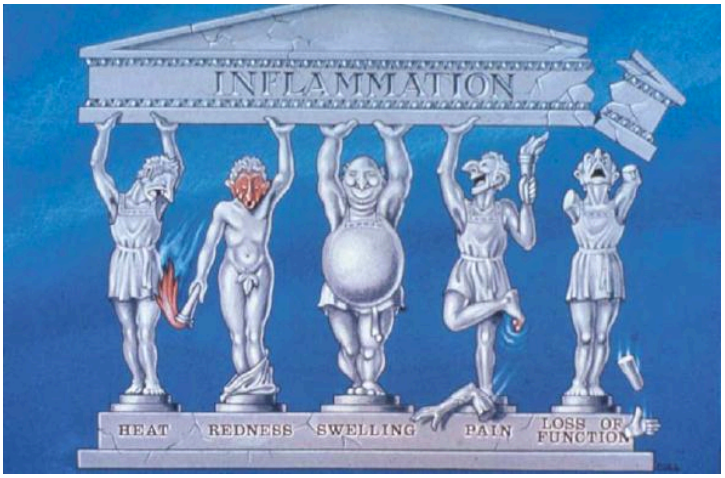

Figure 4 | **Definition of inflammation.** The figure illustrates the 5 different elements of inflammation. Heat, redness, swelling, pain and loss of function.

1.2.2 Innate cell migration and T cell activation

Tissue DCs and moDCs will engulf and digest pathogens at the site of inflammation. These activated DCs will express CCR7 and consequently migrate to the secondary lymphoid organs where they present antigens to the T cells via the MHC class II molecule and upregulate co-stimulatory receptors such as CD80 and CD86[76]. The surrounding tissue of the inflammation, the type of DC but also the type of PRRs that are stimulated will instruct the DCs to polarize the T cells differently[77]. The key cytokines secreted by activated APCs for T cell polarization are well established[78]. Bacterial, viral or protozoan infections stimulate APCs to produce and secrete IL-12 which promotes interferon (IFN)-γ-producing T_H1 cells[79]. Infections with helminth parasites or other macroparasites induce a different type of T cell response. How exactly these parasites interact with APCs and PRRs is not fully understood, but it has been reported that parasite antigens can interact directly with the mannose receptor (MR) and induce IL-4- and IL-13-producing

T_H2 cells[79,80]. IL-4 itself is a central cytokine for the induction of T_H2 cells, and granulocytes such as basophils are major producers of IL-4[81]. Fungal and bacterial infections can also induce another type of T_H phenotype that also has a pathogenic role in autoimmunity. Secretion of IL-6 and transforming growth factor (TGF)β drive IL-17 secretion by T cells and induces the T_H17 phenotype[82,83]. Additionally, it has also been demonstrated that APC production of IL-23 has a crucial role in the expansion and survival of T_H17 cells[84]. The early interaction with PAMPs and the PRRs on DCs at the site of inflammation are important for the later adaptive immune activation (Fig. 5).

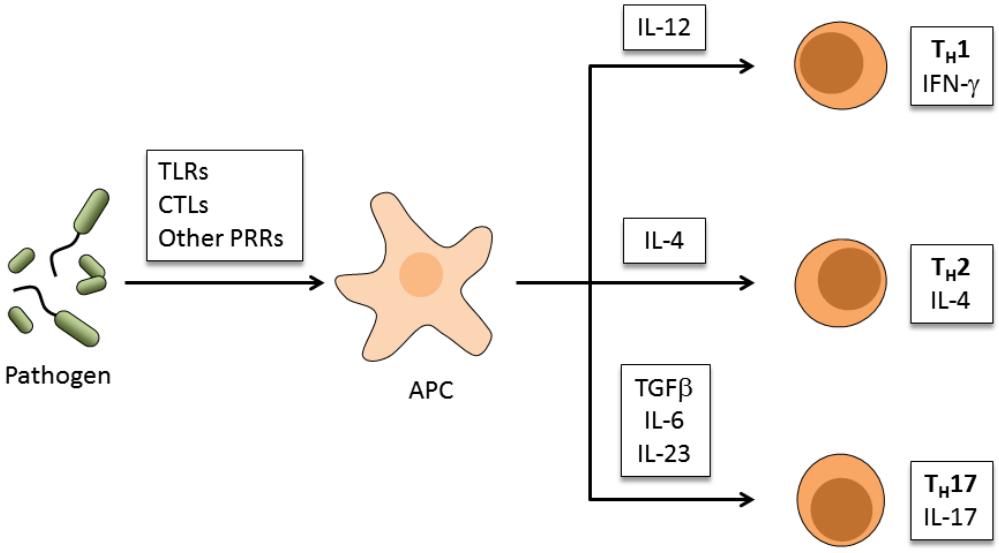

Figure 5 | **T cell polarization by APCs.** Pathogen or PRR-stimulated APCs produce cytokines that drive T cell polarization. IL-12 induces the T_H1 phenotype, IL-4 induces T_H2 cells and the combination of IL-6 and TGFβ induces T_H17 cells.

For instance, TLR4-stimulated DCs readily activate and polarize T cells towards T_H1 and T_H17 subtypes[85], whereas Dectin-1 and Dectin-2 stimulation specifically drive T cell activation towards the T_H17 phenotype[86]. Conversely, TLR2 stimulation of DCs drives IL-4 production by T_H2 cells[87]. A complete understanding of PRRs in T cell polarization by DCs is still lacking[77]. Other

myeloid cells such as neutrophils also migrate from the site of inflammation to the draining LN and regulate T cell activity[88,89]. Neutrophils are already evident just hours after tissue trauma in the draining LN cortex, residing close to follicular B cells and $CD169^+$ subcapsular sinus (SCS) macrophages[21,88]. The exact functions of these neutrophils are not known but it has been reported that neutrophils can eliminate parasite-infected SCS macrophages and that they regulate the antigen presentation capacity of macrophages and DCs through myeloperoxidase[90]. The regulation of antigen presentation by neutrophils thus limits the ensuing T cell activation by the APCs.

1.2.3 The role of innate cells in B cell activation

Activated $CD4^+$ T cells in the LNs will emigrate to the circulation via the inflammatory chemokine gradient, but a significant number of T cells are also important for local follicular B cell activation. These T cells, which are named follicular B helper T (T_{FH}) cells, migrate to the B cell follicles and assist in the formation of germinal centers (GC)[91,92]. T_{FH} cells secrete IL-21, which in turn provides proliferative signals to the activated B cells[93].

There are three fundamental steps for the activation of B cells: I) the recognition of unprocessed antigen by the BCR; II) interaction with the TCR and co-stimulatory receptors such as CD40L on T cells; and III) cytokine stimulation such as IL-6, IL-21, BAFF and a proliferation-inducing ligand (APRIL) for proliferation and survival of activated B cells[94]. The role of innate cells in B cell activation is already apparent during the early steps of B cell activation. SCS macrophages have the ability to trap draining antigens from the lymphatic vessels and to display them to the BCR expressed on follicular B cells[95]. Follicular dendritic cells (FDC) also present native antigens on their surfaces to B cells. These antigens have usually diffused into the LN and been retained on the FDCs via crystallizable fragment (Fc) receptors or complement[96]. FDCs also support B cell survival and proliferation through the production of BAFF[97].

Another important source of BAFF are neutrophils, studies in both mice and humans have shown that G-CSF-stimulated neutrophils from blood and spleen have the capacity to produce large amounts of BAFF following CXCL2

or lipopolysaccharide (LPS) stimulation[98,99]. These splenic neutrophils have been termed 'B helper' neutrophils for their dedicated role in B cell activation[23]. Importantly, B helper neutrophils only activate the innate-like marginal zone B cells in the spleen. Furthermore, it is evident that a large number of neutrophils infiltrate the LN several days later than the initial neutrophil infiltration wave post-immunization[22]. The reported function of these late wave neutrophils is to regulate the magnitude of T cell activation, but it was also noted that these neutrophils localize close to activated B cells and plasma cells.

Monocytes and DCs also play a crucial role in proliferation and survival of activated follicular B cells. Activated $CD11c^{+}CD8\alpha^{-}$ DCs in the lymph node paracortex secrete high amounts of IL-6, whereas $F4/80^{+}Ly6C^{hi}$ inflammatory monocytes entering the medulla from the circulation secrete APRIL which is important for plasma cell survival[100]. These different innate immune cells build up a gradient of stimulations for the activated follicular B cells as they leave the GC for the medulla (Fig. 6). The differentiation from an activated B cell towards an antibody-secreting plasma cell occurs during this journey.

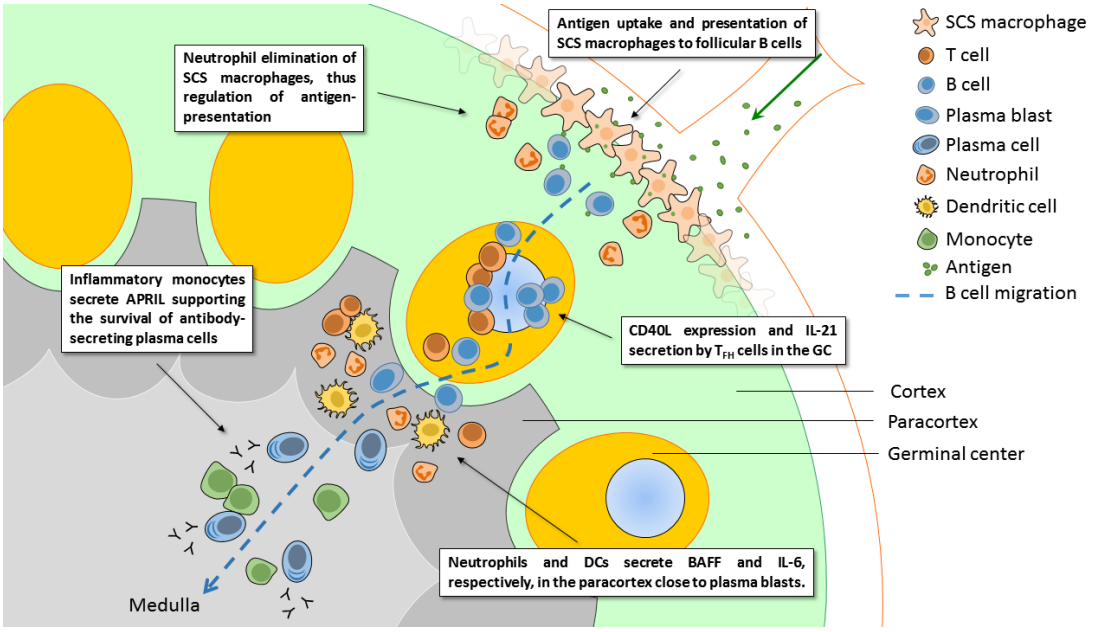

Figure 6 | **Innate immune cells regulating of B cell activation in the LNs.** Innate immune cells regulate B cell activation in many different aspects. SCS macrophages have the ability to capture antigens from the lymphatic vessels and display them to the naïve follicular B cells in the cortex. Neutrophils migrate quickly to the cortex of the LN during inflammation and it has been reported that neutrophils have the ability to eliminate SCS macrophages. One could speculate whether this phenomenon can also regulate antigen presentation of these macrophages. BCR-stimulated B cells form GC with the help of T_{FH} cells that express CD40L and IL-21. Activated B cells will migrate from the GC towards the medulla where they will pass a gradient of BAFF and IL-6 secreted from neutrophils and DCs, respectively. In the medulla, monocytes provide the cytokine APRIL to plasma cells that enhance their survivability and antibody production.

1.2.4 Macrophage activation

Macrophages are important in the initiation, effector and resolution phases of inflammation. They have the ability to communicate with a multitude of cells and have remarkable plasticity that allows them to effectively respond to different environmental signals. Historically, macrophages were believed to be activated by a combination of two cytokines secreted by activated T cells or NK cells, IFN-γ and TNF[101]. Such activated macrophages secrete high levels of pro-inflammatory cytokines such as IL-1, IL-6 and IL-12, and have enhanced microbicidal properties due to the production of radicals from the enzyme inducible nitric oxide synthases (iNOS) or nicotinamide adenine dinucleotide phosphate H oxidase 2 (NOX2)[102]. These macrophages were

named *classically activated macrophages* or M1 macrophages as they work in synergy with IFN-γ producing T_H1 cells. It was later shown that M1 macrophages also have the ability to support the differentiation of T_H17 cells as they can produce IL-23[103]. It is also known today that several PRRs steadily activate the M1 phenotype of macrophages by inducing TNF and IFNβ secretion, which in an autocrine fashion further stimulates the macrophages[104]. M1 macrophages are important effector cells during inflammation and have the ability to reactivate antigen-specific T cells at the site of inflammation. They also have a pathogenic role in disease states such as autoimmunity[35].

In contrast to M1 macrophages, *alternatively activated macrophages* or M2/M2a macrophages are induced by IL-4 and/or IL-13[105,106]. The name is derived from the specific upregulation of the mannose receptor, CD206, but also via the link to the T_H2 cytokine IL-4. These M2 macrophages are associated with wound-healing as they have high arginase expression which allows them to convert arginine to ornithine, a precursor for collagen and polyamines which are important building blocks for the extracellular matrix[4,107]. M2 macrophages also secrete chemokines such as CCL17 and CCL22[108], which attract IL-4-producing T_H2 cells that will enhance the M2 phenotype. They also secrete growth factors that enhance stromal cell and endothelial angiogenesis. These properties have made cancer cells evolve to induce macrophages towards the M2 phenotype which in turn support tumor growth and survival[46,109]. M2 macrophages are associated with parasite immunity, such as nematode and helminth infections, but the mechanisms of parasite elimination are still not very well understood[110]. Another described M2-like phenotype of macrophages is regulatory macrophages, also known as M2c or M2r macrophages. They are also termed deactivated macrophages (dM) in some circumstances due to their inability to produce pro-inflammatory cytokines such as IL-6 and IL-12, as well as having a lower expression of co-stimulatory molecules such as CD80 and CD86[101,111,112]. Regulatory macrophages can be induced by IL-10, TGFβ or glucocorticoids secreted by stromal cells or regulatory T cells (Treg)[113]. Interestingly, it has also been shown that regulatory macrophages can induce Tregs by secreting IL-10 and

TGFβ themselves[114]. Regulatory macrophages have the ability to suppress both CD4 and CD8 T cell proliferation in an IL-10- and TGFβ-dependent manner[114-116]. Furthermore, regulatory macrophages and M2 macrophages can also downregulate CD80 and CD86 expression on M1 macrophages (Fig. 7).

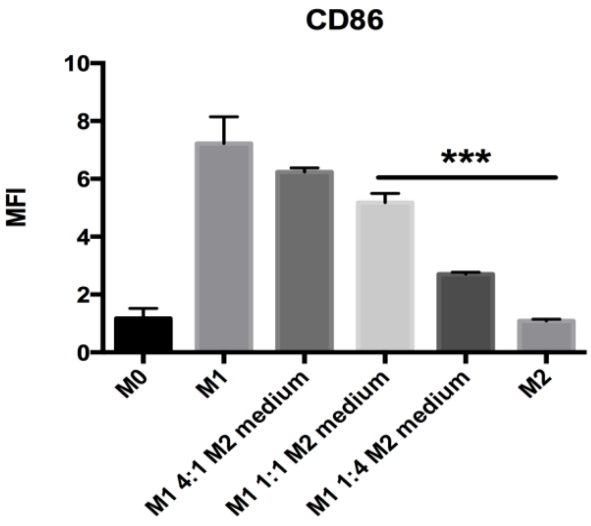

Figure 7 | **M2 macrophages can suppress M1 macrophage activation.** Downregulation of CD86 on M1 macrophages when they are co-cultured with M2-conditioned medium.

Macrophages need two signals before they obtain a full regulatory phenotype. The first signal, such as the anti-inflammatory cytokine IL-10 has low stimulatory function by itself, but combined with a secondary signal such as a TLR stimulation, induces a prominent regulatory phenotype indicated by high IL-10 secretion[117]. In addition, pro-inflammatory cytokine production such as IL-12 and IL-23 is downregulated. Many of these findings are based on data collected from *in vitro* experiments based on murine BM- and peritoneal-derived macrophages. These exemplified activation phenotypes are extremes in a large spectrum of macrophage activation, more recent data have revealed that macrophages in tissues have the ability to express both M1 and M2 markers at the same time, and unorthodox stimulations inducing novel activation pathways, indicating broad macrophage plasticity[101,118].

Table 3 | **Macrophage activation phenotypes.**

	M1 macrophages	M2 macrophages	dM/M2c/M2r macrophages
Context	Bacteria, autoimmunity anti-tumor	Parasites, allergy, repair, pro-tumor	Anti-inflammatory, pro-tumor
Ligands	PAMPs/DAMPs, IFNγ, GM-CSF	IL-4, IL-13, M-CSF	IL-10, TGFβ, glucocorticoids
Induction	Th1 and Th17 response Neutrophils	Th2 response Eosinophils, basophils	Tregs
Markers	MHCII, CD86, iNOS, PDL1 IL-12	CD206, PDL2, Arg1	TGFβ, IL-10, IDO
< Activation states >			

1.2.5 TGFβ – an important cytokine for the resolution of inflammation

Resolution of inflammation is a crucial phase for limiting tissue damage and initiating the healing process. One key process is limiting leukocyte infiltration[119], changes in the composition of lipid mediators and chemokines have an important role in limiting neutrophil recruitment and infiltration[120,121]. Neutrophils in the tissue will undergo apoptosis, this process involves the presentation of 'eat me' signals, such as phosphatidylserine, on the surface of the apoptotic neutrophils. Tissue macrophages recognize this signal, which will duly promote phagocytosis and induce an M2 or regulatory macrophage phenotype[122-124]. These macrophages will not produce pro-inflammatory cytokines such as TNF, IL-12 or IL-23, but instead produce and secrete anti-inflammatory cytokines such as IL-10 and TGFβ. TGFβ has many functions during resolution, enhancing the repair functions of fibroblasts and endothelial cells but also having an overall anti-inflammatory action on many leukocytes[125]. The important role of TGFβ in immunosuppression was discovered when mice deficient in TGFβ were generated. These mice developed multiorgan inflammation and died at 3-4 weeks of age[126].

There are three isoforms of TGFβ in mammals, TGFβ1, TGFβ2 and TGFβ3. TGFβ1 is the predominant isoform in the immune system and it will be denoted TGFβ from now on in this thesis. TGFβ signaling occurs via the TGFβ receptor complex[125,127] which is composed of two receptors, TGFβR1 and TGFβR2. TGFβ binds to TGFβR2 which then forms a copmlex with and

phosphorylates the cytoplasmic tail of TGFβR1. TGFβ is a potent suppressor of both T_H1 and T_H2 differentiation, it inhibits the production of their respective transcription factors, T-bet and GATA2[128,129]. TGFβ is also a key cytokine for Treg induction by activating the transcription factor FoxP3. There are two major populations of Tregs, the inducible Tregs which are generated in the periphery, and the naturally occurring Tregs which develop in the thymus early in life[130]. However, TGFβ also has an important role in the differentiation of pro-inflammatory T_H17 effector T cells. How exactly TGFβ can induce such functionally diverse effector T cells is not very well understood. It has been reported that high concentrations of TGFβ favor Treg induction by blocking IL-23 receptor expression and enhancing FoxP3 expression, whereas low concentrations of TGFβ in combination with IL-6 upregulate the IL-23 receptor and the T_H17 transcription factor RORγt[131].

TGFβ also has important immunomodulatory effects on innate immune cells, inhibiting the maturation of DCs by limiting the expression of MHC class II, CD80 and CD86[132]. In addition, TGFβ deactivates macrophage production of TNF, and in combination with IL-10 less nitric oxide (NO) is produced[114,133]. It has also been reported that TGFβ has a potent inhibitory effect on IL-12 production in macrophages and DCs *in vitro*[134]. TGFβ is thus a pleiotropic cytokine with different functions on immune cells. The immunomodulatory functions of TGFβ are both dependent on time, location and cell type during inflammation, which makes TGFβ biology complex and challenging to study.

1.3 Autoimmunity

A fundamental feature of the immune system is to not recognize and react to self-antigens. Autoimmunity is the failure of the immune system to control and regulate self antigen-induced inflammation. There are more than 70 distinct autoimmune diseases affecting 3-5% of the total global population[135]. Autoimmunity can be organ-specific, for example the pancreas is attacked in Type 1 Diabetes (T1D), and the central nervous system (CNS) is affected in Multiple Sclerosis (MS). Autoimmunity can also be systemic such as in Systemic Lupus Erthyematosus (SLE) in which many different organs such as

the skin, joints, kidney, lungs and other tissues are attacked. Why some people develop autoimmunity is still not well understood, but a combination of environmental and genetic factors has an important role in many autoimmune diseases[135]. Most autoimmune reactions are antigen-specific and can involve many self-antigens for one specific disease. However, healthy people also have self-specific B and T cells but most people never develop autoimmune disease[136,137]. This phenomenon first and foremost indicates that central immune tolerance is not fail-safe and that self-reactive lymphocytes can escape negative selection in the thymus and BM. It also signifies that peripheral tolerance have an important role as a reserve system for regulating and controlling self-reactive lymphocytes in healthy people[138].

There are three postulated models for peripheral tolerance, (I) induction of anergy, (II) clonal deletion and (III) immune suppression. If a self-reactive lymphocyte recognizes its specific antigen in the absence of co-stimulatory signals, for instance from an immature DC or a deactivated macrophage, the lymphocyte will become anergic or in a state of unresponsiveness[139,140]. In some circumstances, in the absence of both co-stimulatory signals and survival factors, the self-reactive lymphocyte can undergo activation-induced cell death or clonal deletion[141,142]. A third way of ensuring peripheral tolerance is through immunosuppression by Tregs and other regulatory innate immune cells via cellular interactions and cytokine secretion[143,144].

1.3.1 Type 1 Diabetes

T1D is an autoimmune disease in which the insulin-producing β-cells in the pancreas are attacked and eliminated by the immune system[145]. Insulin has a key function in the regulation of the blood glucose levels and deficiency in insulin leads to hyperglycemia. Symptoms of untreated T1D patients include polyuria, polydipsia, fatigue, and in severe conditions ketoacidosis and coma. Since β-cells do not regenerate, patients with T1D require a life-long daily treatment of exogenous insulin.

1.3.1.1 The non-obese diabetic mouse and pathogenesis of T1D

It has always been difficult to study the immunopathogensis of human T1D due to the relative inaccessibility of the human pancreas. Animal models have thus been crucial in studying and understanding the pathogenesis of T1D. The non-obese diabetic (NOD) mouse strain was originally generated more than 30 years ago and has been fundamental to this end[146]. NOD mice are one of the most used animal models for T1D, as it clinically resembles human T1D in many ways. NOD mice display hyperglycemia and can be treated with insulin. They also have circulating autoantibodies prior to clinical manifestation[147]. However, there are other aspects of the NOD mouse model that do not resemble the human counterpart. First of all, these mice are highly inbred so they must be viewed as a single case study in humans. The onset of diabetes in NOD mice is around 12-15 weeks of age, an age when the mice can reproduce, whereas in humans most T1D cases are diagnosed during childhood or puberty[148].

The progression of T1D in NOD mice initiates with the accumulation of $CD4^+$ and $CD8^+$ at the pancreatic islets around 3-4 weeks of age, a process termed *insulitis*. Over the next 4-8 weeks the insulitis progressively increases with heightened T cell activity but also the recruitment of other leukocytes. Interestingly, the β-cells are not attacked during this time period[149]. The factors which trigger the immune cells to attack the β-cells are not very well understood, but events that may underlie this transition could be the acquisition of new immune effector functions, loss of sensitivity to negative signals, new self-antigens or the recruitment of accessory cell types[150]. However, it is well established that development of T1D in NOD mice is dependent on both $CD4^+$ and $CD8^+$ T cells[151]. $CD4^+$ T cells produce inflammatory cytokines such as IFN-γ that upregulate the FAS receptor on β-cells and also activate innate immune cells such as macrophages. Autoreactive $CD8^+$ T cells can eliminate β-cells by MHC class I-mediated cytotoxicity via perforin- or the FAS-dependent pathway. T cells also have a role in preventing onset of T1D. NOD mice lacking Tregs develop an accelerated disease onset and humans with IPEX (immunodysregulation,

polyendocrinopathy, enteropathy, X-linked) syndrome, with mutations in the FoxP3 gene, can spontaneously develop T1D[152,153].

1.3.1.2 The role of macrophages in T1D

Many studies in NOD mice and humans have highlighted the role of pathogenic T cells for the development of T1D. However, early studies have also shown the important role of M1 macrophages in pathogenesis of the disease. Inhibition of macrophage influx into the pancreas inhibits development of T1D[154]. Remarkably, M1 macrophages have been detected in the pancreas before lymphocyte infiltration and also in NOD/*scid* (severe combined immunodeficiency) that lack both T and B cells[155]. It has also been demonstrated that M1 macrophages have a key role in the activation of cytotoxic $CD8^+$ T cells[156]. M1 macrophages can produce pro-inflammatory cytokines such as IL-1β and TNF that can stress and induce apoptosis in β-cells[155,157]. Macrophages from NOD mice and T1D patients are also more prone to being M1 activated than are macrophages from healthy controls. NOD macrophages have an abnormal ratio of TNF:IL-10 production in relation to other mouse strains[158], and macrophages from T1D patients are hypersensitive to LPS stimulation[159]. It has also been speculated that there are defects in the transition from M1 to M2 macrophages in the pancreata of NOD mice. Interestingly, there is an approximate 80% incidence of T1D in female NOD mice and it was recently reported that transgenic NOD mice that did not spontaneously develop T1D expressed M2–associated genes in the pancreas[160]. These protected NOD mice that do not develop T1D possess regulatory macrophages with a phagocytic/immunosuppressive phenotype[161].

1.3.2 Multiple Sclerosis

MS is an autoimmune disease of the CNS and was already described in the mid 19^{th} century[162]. The immune system attacks the proteins of the myelin sheath and causes demyelination in the CNS. The myelin sheath isolates the axons of neurons and damage to this isolation results in weakening of the axonal signal transmission. MS patients display oligoclonal bands in the

cerebrospinal fluid (CSF), lesions on magnetic resonance imaging (MRI) and bouts of neurological symptoms[163]. MS patients develop symptoms such as disturbances in vision, sensation, motor function or autonomic problems, which all depend on the location of demyelinated plaques in the CNS[164]. The disease can also be classified into sub-types: relapsing-remitting MS (RRMS), primary progressive MS (PPMS), secondary progressive MS (SPMS) and progressive relapsing MS. The majority of the patients acquire the RRMS form of the disease but later progress into the SPMS sub-type. The effective treatments today target important immune functions and modulate immune mediators[165]. However, there is still today no treatment for the progressive forms of MS.

1.3.2.1 Experimental Autoimmune Encephalomyelitis

The animal model for MS is termed Experimental Autoimmune Encephalomyelitis (EAE) and can be induced in a number of species such as mice, rats, rabbits and monkeys. The animal model has been used for more than 80 years and it has become clear that EAE can reproduce many of the conserved immunological aspects of MS[166]. However, one should appreciate that EAE is a model of induced neuroinflammation in inbred animals and not a phenocopy of the heterogeneous human disease. EAE in mice can be actively induced by the injection of a myelin protein together with adjuvant. The adjuvant frequently used is complete Freund's adjuvant (CFA), which is mineral oil together with heat-killed *Mycobacterium tuberculosis*[167]. The use of pertussis toxin is also necessary for several mouse models. The adjuvant will stimulate PPRs on APCs, these innate cells will be activated and migrate to the draining LNs and present myelin antigen to the T cells. These activated T cells will then leave the LN to the circulation and migrate to the CNS[168].

It was established in the 1980s that T cells have a fundamental role in the induction of EAE. At that time the technique to obtain and culture myelin-specific T cells was developed, and Ben-Nun *et al* adoptively transferred myelin basic protein (MBP)-specific T cells into naïve rats which later developed EAE[169]. Initial findings of IFN-γ expression in the CNS led to the hypothesis that T_H1 cells were the driving force underlying inflammation in the

CNS. Paradoxically, mice deficient in the genes for either IFN-γ or IL-12 were not resistant to EAE[170-172]. This led to the finding of the important role of IL-23 and T_H17 cells and their pathogenic role in EAE[84,173]. Additionally, GM-CSF was recently reported to be a key pathogenic cytokine produced by T_H17 cells in EAE[174]. It has also been reported that autoreactive T cells derived from the inflamed CNS co-express IFN-γ and IL-17[175]. However, MS patients who received IFN-γ therapeutically experienced exacerbations during this treatment[176]. Furthermore, conflicting data have been reported regarding the role of T-bet, the major transcription factor for T_H1 cells in EAE, based on mice deficient for this gene[177,178].

1.3.2.2 The role of innate cells in EAE

Myelin-specific $CD4^+$ T cells are one of the first leukocytes to enter the CNS during adjuvant-induced EAE. These autoreactive T cells are reactivated by CNS-resident APCs. Microglia have been considered as poor APCs due to their low levels of MHC class II expression, but their importance for EAE is still significant as they can produce inflammatory cytokines and chemokines [179,180]. It has also been reported that CD40 expression on activated microglia is important for their interaction with T cells[181]. Perivascular (PV) macrophages express MHC class II and other co-stimulatory receptors such as CD80, CD86 and CD40[182]. These receptors are highly upregulated on PV macrophages during EAE and have been suggested to be important for the initial reactivation of autoreactive T cells[183]. These reactivated T cells will secrete factors important for opening of the blood brain barrier[184], and the production of IFN-γ and TNF activates PV macrophages and microglia towards an M1-like phenotype[182]. Activated PV macrophages, microglia and T cells will secrete chemokines important for leukocyte recruitment and importantly monocytes via CCL2[185]. Infiltrating monocytes are an important source of M1 macrophages and moDCs. EAE severity correlates with the number of infiltrating monocytes and inhibition of this infiltration protects mice from EAE[186,187]. It has also been postulated that M1 macrophages are the key effector cells in demyelination whereas microglia are important for scavenging cellular debris during EAE[188]. The exact mechanisms of demyelination are not

known but M1 macrophages in the CNS produce ROS that could lead to destruction of myelin[189,190].

2 Aims of the thesis

The aim of this thesis was to understand how modulation of innate immune cells could regulate adaptive immune responses during inflammation and autoimmunity.

The specific scientific goals for each study were:

Study I: to assess if regulatory M2 macrophages can be induced in NOD mice and to test the hypothesis that regulatory macrophages can suppress type 1 diabetes.

Study II: to investigate if microglia and macrophages can obtain a regulatory M2 phenotype and suppress EAE.

Study III: to understand the role of TGFβ in phagocytes and the subsequent TGFβ-dependent effects on adaptive immunity during EAE.

Study IV: to examine the role of neutrophils in regulating lymph node T and B cells after adjuvant-induced chronic inflammation.

3 Methods

I have used a variety of materials and methods in this thesis, all of which are described in the included studies. I would like to take the opportunity to review some of the specific methodologies in this section.

3.1 Animal models

We have been using mice in **study I**, **II**, **III** and **IV** for many reasons. First of all, there is a better availability for molecular tools such as antibodies, cytokines and growth factors for mice than there is for other murine strains such as rats and guinea pigs. Many standard operating procedures for assays and inflammatory models have been developed and described for mice. Additionally, numerous inbred and congenic strains are available for different purposes and a variety of genetically modified mice have been developed to specially study certain genes and cells.

We have been using two different mouse strains for EAE, C57BL/6 (B6) mice in **study III** (B6) and DBA/1 mice in **study II**. Both are dependent on CFA as adjuvant, while pertussis toxin is only necessary for EAE in B6 mice but not needed in DBA/1 mice. The majority of the gene-modified mice is on the B6-background or backcrossed onto the B6 background.

We have also used the Cre-Lox recombination technology to specifically delete or insert genes in specific cell types[191]. The technique is derived from bacteriophages and is based on the enzyme Cre recombinase, which can recombine a target sequence that is flanked by *LoxP* sequences. We have used mice expressing Cre under the *Lyz2* (LyzM)-promoter in **study III** and **IV**. In **study III** we deleted exon 3 in the TGFβ receptor (*Tgfbr2*) specifically in LysM-expressing phagocytes. In **study IV** we introduced the toxin DTA in LysM-expressing cells by activating Rosa26-driven expression of DTA in phagocytes. The Rosa26 cassette has a stop element that is flanked by *LoxP*. The Cre recombinase will remove the stop element which will lead to DTA expression under the constitutively expressed Rosa26 promoter, but only in LysM-expressing cells.

3.2 Bone marrow-derived macrophages and DCs

Analysis of macrophages and DCs was performed in **study I**, **II** and **III**. BM-derived macrophages were generated by the dissection of the femurs and the culture of RBC-lysed BM with either 20 ng/ml of mouse M-CSF or with 20% L929-conditioned complete medium. The conditioned medium was exchanged 2 times with 2-3 days interval. Harvesting of macrophages was performed using 0.5% Trypsin for 10 minutes at 37°C or with 0.5mM ethylenediaminetetraacetic acid (EDTA) for 30-45 minutes at 37°C.
BM-derived DCs were generated in a similar fashion to BM-derived macrophages, but instead 20 ng/ml GM-CSF was applied.

3.3 Suppression assay

To address the immunosuppressive capacity of M2 macrophages in **study I** and **II** we developed a co-culture assay in which we measured regulation of T cell proliferation by macrophages. We coated 96-well plates with 0.3 or 0.5 μg/ml of anti-CD3 antibody for 1-2 hours at 37°C. In some circumstance 0.5 μg/ml of anti-CD28 antibody was added to the splenocyte solution but not coated. Macrophages was later harvested in the antibody-coated wells and stimulated for 24 hours. Macrophages were thoroughly washed with pre-heated PBS and splenocytes or lymphocytes were subsequently added to the macrophages and cultured for 72 hours. Optimization experiments indicated that a macrophage:splenocyte ratio of 1:16 was optimal for sufficient T cell survival.

3.4 *In vivo* cell tracking

We wanted to track the migration of macrophages in **study I** and thereby used the *IVIS spectrum in vivo imaging system*. This system takes advantage of bioluminescent or fluorescent reporters in the blue or near-infrared wavelengths. The system is also built up of different filters that assist in the reduction of autofluorescence from skin and other organs. We used the lipid membrane-dye DiR (1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide) to stain our cells which emits light in the near-infrared spectrum upon

excitation. The staining of macrophages was performed with 3.5 μg/ml of DiR at 37°C for 5 minutes. The cells were carefully washed with an excess of ice-cold PBS. The fluorescently labeled macrophages were injected i.p before imaging. Mice were anesthetized and the ventral fur was shaved to minimize light interference. The anesthetized mice were imaged in the IVIS spectrum system with excitation and emission filters set at 710 and 760, respectively.

4 Results and Discussion

The studies in this Ph.D thesis were developed in order to understand the role of innate cells in the regulation of adaptive immune responses. I wanted to understand how innate immune cells such as macrophages, DCs and neutrophils communicate and interact with T and B cells in the context of inflammation and autoimmunity.

4.1 Study I: M2 macrophages in T1D.

Background

T1D is an autoimmune disease in which leukocytes progressively eliminate the insulin-producing β-cells in the pancreas, leading to the loss of glucose regulation.

Hypothesis

The destruction of β-cells in the pancreas is due to the pro-inflammatory environment induced by self-reactive T cells and M1 macrophages. We hypothesized that regulatory M2 macrophages should be able to regulate and reduce the activity of these pro-inflammatory leukocytes and thus reduce the loss of β-cells and T1D development in NOD mice.

Methods

A protocol for induction of anti-inflammatory M2 BM-derived macrophages was devised involving surface and functional immunophenotyping. The stability of these M2 macrophages was assessed by sequential stimulations. Stable M2 macrophages were adoptively transferred into NOD mice prior to clinical onset and the effect of therapy on the clinical course based on glucose levels was assessed. The fate of transferred cells was analyzed by live *in vivo* imaging and by immunohistochemistry. The effects on the cells in the pancreas were assessed by optical projection tomography (OPT) and immunological analysis of local T cell populations.

Results

IL-4/IL-10/TGFβ-treated BM-derived macrophages (M2r) were induced into a relatively stable regulatory phenotype based on their ability to produce IL-10 and TGFβ, to suppress T cell activation and their inability to produce pro-inflammatory cytokines. A single adoptive transfer of the M2r macrophages significantly protected >80% of the NOD mice from developing T1D and they remained protected for 3 months post-transfer. OPT and immunohistochemical analyses revealed increased numbers of β-cell islands. *In vivo* imaging revealed that the transferred M2r macrophages homed to the pancreas within 24 hours.

4.1.1 The source of macrophages

BM-derived macrophages have been a popular source to obtain large amounts of macrophages for *in vitro* studies. The BM contains both monocytes and GMPs that will respond to M-CSF stimulation and differentiate into macrophages. One can either use purified recombinant mouse M-CSF or M-CSF-conditioned media from the fibroblastic L929 cell line which produces large amounts of M-CSF[192,193]. The use of conditioned-media from L929 generates a large amount of macrophages without any obvious artifacts, but one could still consider if these macrophages should be termed 'L929-differentiated' macrophages as L929 cells produce many other factors. Another source of macrophages is the peritoneum. Peritoneal macrophages (PM) have been widely used since the 1960s, maybe due to the easy and quick access to mature macrophages. However, there is a big difference between BM-derived macrophages and mature PMs. PMs are larger than BM-derived macrophages and PMs also have higher expression of MHC class II and programmed death-ligand (PD-L)1 during steady-state, as well as higher IL-12 and iNOS expression after activation, indicating a more mature and specified phenotype in PMs relative to BM-derived macrophages[194]. The macrophage yield is also different, you can retrieve approximately 3-5 million macrophages from the peritoneum in contrast with 10-15 million macrophages from two mouse femurs. We used the BM as our source of macrophages as we wanted large numbers of macrophages that were as naïve as possible for different manipulations we systemically wanted to address.

4.1.2 The induction and stability of M2r macrophages

To determine the optimal M2 activation protocol we needed to evaluate different stimuli. We chose IL-4, IL-10, IL-13, TGFβ, dexamethasone and vitamin D for M2 induction based on previous knowledge[46]. We also combined IL-4, IL-10 and TGFβ to determine if there were any synergistic effects of these cytokines. IL-10, TGFβ and dexamethasone had a very potent effect in the downregulation of IL-6 secretion in M1 macrophages, whereas

TGFβ alone significantly upregulated IL-10 and TGFβ secretion (Fig. 8A). IL-4 enhanced the phagocytic capacity of macrophages and the expression of PD-L2. Interestingly, PD-L2 expression was further increased when IL-4 and IL-10 was combined. We also detected synergistic effects with IL-10 and TGFβ in the suppression of NO secretion and the expression of the anti-inflammatory B7-H4 receptor (Fig. 8B). These results indicate that a combination of IL-4, IL-10 and TGFβ could induce a macrophage phenotype (M2r) that included regulatory and wound-healing properties based on secretion of anti-inflammatory cytokines, the expression of specific receptors and their higher phagocytic capacity. We believed that these two distinct but combined phenotypes would be optimal for the environment in the pancreas to increase β-cell survival and suppress inflammatory responses.

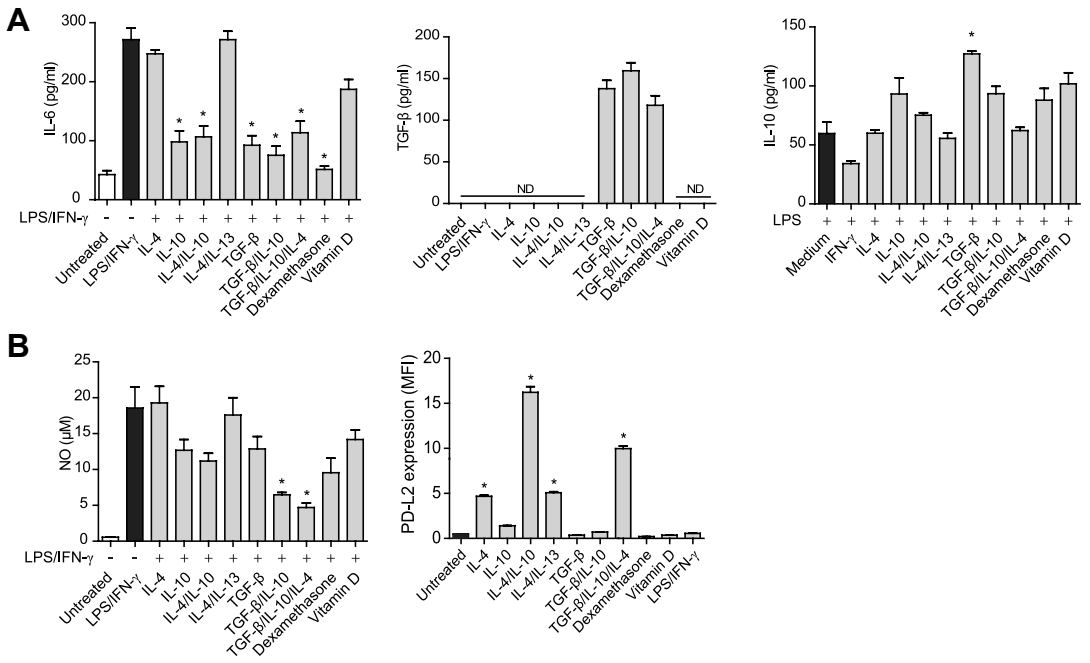

Figure 8 | **IL-4, IL-10 and TGFβ induces a unique M2 phenotype.** (**A**) Macrophages were stimulated with the respective stimuli and different cytokines were measured. (**B**) NO secretion and PD-L2 expression induced by different stimuli.

It is also known that macrophages are very plastic and can quickly adapt and change their phenotype. We therefore addressed the question if these M2r macrophage would retain their properties upon secondary pro-inflammatory stimuli. We thus first skewed naïve macrophages into M2r

macrophages and subsequently stimulated them with LPS/IFN-γ. The M2r macrophages did not obtain a strong M1 phenotype (based on their limited TNF secretion) in contrast to M1 stimulated macrophages, indicating a relatively stable M2r phenotype. Furthermore, we analyzed M1 and M2-associated genes by PCR to obtain a more detailed overview of their phenotype. We detected upregulation of M1-associated genes such as PD-L1, Nos2 and Cd86, yet the M2-associated genes such as Arg1, Tgfb1 and PD-L2 were still expressed. These results indicate that macrophages do respond to new stimuli and adapt to the new environment, but they do 'remember' their earlier phenotype. Importantly, the results were also confirmed *in vivo* using fluorescently-labeled M2r macrophages that had been transferred into NOD mice and then recovered from the pancreas.

4.1.3 M2r macrophages suppress T cell proliferation and induce Tregs

We wanted to evaluate the potential regulation of T cells by M2r macrophages as the pathogenesis of T1D is driven by self-reactive $CD4^+$ and $CD8^+$ T cells in the pancreas. To address this we developed an *in vitro* suppression assay by co-culturing M2 macrophages and anti-CD3-stimulated splenic T cells. The co-cultures revealed that TGFβ stimulation is crucial for the ability of M2 macrophages to suppress T cell proliferation. The suppression of T cells occurred via secreted factors or via a cell contact-dependent mechanism based on a transwell system and fixation of the macrophages, respectively. Furthermore, IL-10 and TGFβ had a synergistic effect on macrophages in the differentiation of inducible Tregs based on FoxP3 expression.

It has been speculated whether M-CSF-dependent resident tissue-macrophages have intrinsic immunosuppressive and M2-like properties as a homeostatic function in order to protect the local tissue[195,196]. For instance, M-CSF-generated macrophages produce and secrete more IL-10 in contrast to GM-CSF-generated macrophages. M-CSF has the ability to enhance the expression of M2-assoaciated genes both in mice and Man. Additionally, it has been reported that M-CSF signaling in BM myeloid precursors from NOD mice is dysregulated[197].

4.1.4 M2r macrophages prevent mice from developing T1D

The accumulated data for M2r macrophages indicates that these cells have enhanced phagocytic capacity, inability to produce pro-inflammatory cytokines but instead secrete anti-inflammatory cytokines, and have the capacity to suppress T cell proliferation and induce Tregs. We therefore wanted to understand the role of these M2 macrophages in T1D, but also to investigate if M2r macrophages could be used therapeutically for treating T1D in NOD mice. Adoptive cell transfer has been utilized before in NOD mice with the use of Tregs or tolerogenic DCs[198,199]. The main issue with Tregs is their antigen-specificity - in mice this is a controllable parameter whereas in humans it is much more difficulty to predict the active antigen(s)[200]. DCs with a tolerogenic phenotype indicated by low expression of co-stimulatory molecules such as CD80 and CD86 are usually induced by applying dexamethasone or IL-10 and have also been employed in adoptive transfer settings. The obvious risk with DCs is that they are good inducers of immunity as they have the capacity to express high amounts of MHC class II, CD80 and CD86. The half-life of DCs is also relatively short, which could make them less effective in their ability to suppress chronic inflammation.

We decided to transfer M2r macrophages at the later stage of the disease when the insulitis activity was at the highest but mice are still without any clinical manifestations. We transferred 2.5 million M2r macrophages i.p into 16 week- old female pre-diabetic NOD mice. We also injected vehicle (PBS) and untreated macrophages (M0) as controls for our therapeutic intervention. The first mice started to develop T1D just 1 week after the transfer, and following the mice for 3 months revealed that M2r macrophages protected more than 80% of the mice from developing T1D, in contrast to the two control groups which both developed normal frequencies of T1D onset in NOD mice (Fig. 9A). Immunohistochemistry of pancreas from 24-week-old M2r- and M0-treated mice visualized a significant increase of insulin^{+} islands in the pancreata of M2r-treated mice (Fig. 9B).

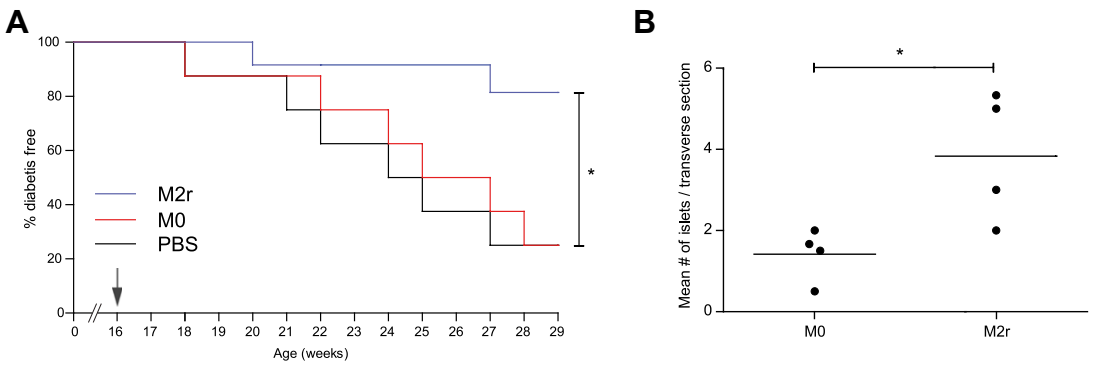

Figure 9 | **M2r macrophages prevent NOD mice from developing T1D.** (**A**) 2.5 x 10^6 M2r, M0 (untreated) or vehicle (PBS) was injected i.p into 16-week old pre-diabetic NOD mice. (**B**) Insulin$^+$ islets were counted in 24-week-old NOD mice after M0 or M2r macrophage transfer.

4.1.5 *In vivo* tracking of transferred M2r macrophages

To determine if the M2r macrophages migrated to the pancreas we took the advantage of using *in vivo* imaging to track the cells directly in living mice. We stained both M2r and M0 macrophages with the lipophilic fluorochrome DiR that will make the cells light up in the near-infrared range. Macrophage migration was already evident 2 hours post-injection and 24 hours later we could detect a strong signal around the area of the pancreas. Interestingly, we did not detect any different pattern in migration between M2r and M0 macrophages. Dissection of the liver, kidneys, spleen, pancreas and pancreatic LN (pLN) confirmed that the macrophages predominantly migrated to the pancreas but also to the pLN. It also indicates that a core macrophage chemokine receptor profile is involved in their migration to these tissues. Only a few chemokine receptors were differentially expressed in M2r compared to M0 macrophages based on genome-wide expression analysis (Table 4).

Table 4 | **Chemokine receptors on M2r relative to M0 macrophages**

Gene	P-value	Fold
Cxcr2	6,81E-07	3,05
Cxcr7	0,000730423	1,89
Cxcr4	3,12E-05	-1,64
Cxcr3	3,29E-05	-2,04
Ccr2	8,32E-05	-4,45
Cx3cr1	4,25E-06	-8,99

We next sought the answer for what these M2r macrophages were doing to protect the β-cells. We speculated two possible mechanisms: either that M2r macrophages regulated the activation or infiltration of self-reactive T cells, or that M2r macrophages nursed and supported β-cell survival through soluble factors. Immunohistochemical analysis and OPT imaging indicated no differences in T cell numbers. To confirm this we injected M2r or M0 macrophages into NOD mice and analyzed the T cell compartment in the pancreas and the draining pLN 1 week post-transfer. Our analysis of these organs confirmed that there were no differences in the $CD4^+$ and $CD8^+$ T cell pools, nor did we detect any increase in $FoxP3^+$ Tregs. These data indicate that M2r macrophages do not limit lymphocyte infiltration into the pancreas or pLNs. To address if the T cell activation or proliferation states were manipulated by M2r macrophages we used TCR transgenic BDC2.5 NOD mice in which the majority of the $CD4^+$ T cells are specific for a β-cell antigen. We injected either M0 or M2r macrophages into these mice and dissected the pLN 1 week post-injection. The lymphocytes from the pLN were then restimulated with the BDC2.5 mimotope for 72 hours in order to induce proliferation. Interestingly, we detected a significant reduction in T cell proliferation from mice that received M2r macrophages. Additionally, T cells in M2r-treated pLN displayed a trend to be less activated based on CD62L and CD44 expression.

These data indicate that M2r macrophages limit the proliferative capacity of T cells in the pLN and maybe also limit their activation status. However, we did not detect any differences in the numbers of Tregs in either the pancreas or the pLN. One could consider if the suppressive capacity of these Tregs is enhanced by M2r macrophages. Furthermore, a recent study has reported that M2 macrophages support β-cell proliferation in a TGFβ- and epidermal growth factor (EGF)-dependent manner. M2r macrophages secrete TGFβ, which could explain another possible therapeutic effect of M2r macrophages in T1D[201].

4.2 Study II: M2 macrophages and microglia in EAE

Background

MS is an autoimmune disease in which macrophages attack and destroy the myelin sheath surrounding the neuron axons, leading to neurological disability.

Hypothesis

Adoptive transfer of pre-activated anti-inflammatory macrophages or microglia will efficiently down-regulate experimental MS in mice.

Methods

A protocol for the cell culture of adult microglia was established. Induction of anti-inflammatory M2 macrophages and microglia was conducted based on a previously developed procedure. Immunophenotyping was performed on M2 macrophages and microglia, and the properties of these cells were also compared. Anti-inflammatory M2 macrophages and microglia were adoptively transferred into DBA/1 mice at different timepoints during EAE. The fate of transferred cells was followed by immunohistochemical analysis. Immunological analysis of CNS T cell populations in treated mice was investigated in order to determine the effect of macrophages/microglia on adaptive immunity.

Results

Stimulation with IL-4/IL-10/TGFβ induced M2 microglia with an immunosuppressive phenotype. These M2 microglia had decreased expression of CD86, PD-L1, NO and IL-6 and increased expression of PD-L2 and IL-10. M2 microglia suppressed T cell proliferation and induced regulatory T cells. A single transfer of M2 microglia attenuated the severity of established EAE, which was most obvious when the cells were transferred at 15 days post-immunization. M2 microglia-treated mice had reduced inflammation and less demyelination in the CNS.

4.2.1 Macrophages and microglia in the CNS

Microglia are considered to be the resident tissue macrophages in the CNS. These cells are originally derived from the yolk-sac progenitors during embryonic development[202]. In a healthy brain, microglia express low levels of immunomodulatory receptors such as MHC class II, CD80 and CD86. However, this quiescent state is still very dynamic as microglia survey the tissue continuously, which is important for tissue remodeling and wound healing[203]. It has been shown that acute and chronic inflammation in the CNS induces an M1 microglia phenotype with increased MHC class II expression and enhanced secretion of pro-inflammatory cytokines[204]. It has been debated if the M1 phenotype is specifically associated to microglia or if it is linked to monocyte-derived macrophages that resemble microglia during inflammation. Nonetheless, recent findings have described different roles for these two cell types, monocyte-derived cells being responsible for the inflammatory responses during EAE whereas microglia being more involved in the phagocytosis of cellular debris[188,205]. Another resident phagocyte in the perivascular space of the CNS is the PV macrophage. The PMs are believed to be derived from monocytes or monocyte progenitors during adulthood[206]. These cells have high expression of MHC class II and co-stimulatory molecules, and are considered to be responsible for the reactivation of infiltrating MOG-specific T cells in the perivascular space during EAE[207,208].

We used both macrophages and microglia in this study to understand their therapeutic role in EAE. The source of macrophages was from the BM, similar to **study I**. However, microglia have traditionally been cultured and expanded from neonatal CNS. We have reported that neonatal and adult microglia have different gene expression profiles and thus could act differently upon stimulation[209]. We therefore decided to use adult microglia as our EAE experiments were performed in adult mice.

4.2.2 Induction of M2 microglia

To determine if microglia can obtain an immunosuppressive M2 phenotype we took advantage of our findings in **study I**. We stimulated microglia with IL-4/IL-10/TGFβ and measured receptor expression and cytokine secretion.

Similar to macrophages, distinct profiles of microglia activation were observed. M2-stimulated microglia had increased expression of PD-L2 and IL-10 and secreted little or no pro-inflammatory cytokines. In contrast, M1-stimulated microglia had increased expression of CD86, PD-L1 and pro-inflammatory mediators such as IL-6 and NO (Fig. 10A). Interestingly, microglia that had been stimulated with LPS sequentially had decreased expression of pro-inflammatory markers, this phenomenon is termed 'endotoxin tolerance' and has been described as a 'memory' function of myeloid cells to limit tissue damage and enhance bacterial elimination[210]. To determine if microglia could retain their M2 phenotype we first pre-stimulated the cells with IL-4/IL-10/TGFβ for 24h. We then washed the cells to remove the M2-inducing cytokines and restimulated the cells with LPS for another 24h. Analysis of the supernatants 48h later revealed that M2 microglia indeed secreted pro-inflammatory mediators, but significantly less than M1-activated microglia (Fig. 10B). These data suggest that microglia can obtain an M2 phenotype similar to the M2r macrophages generated in **study I**.

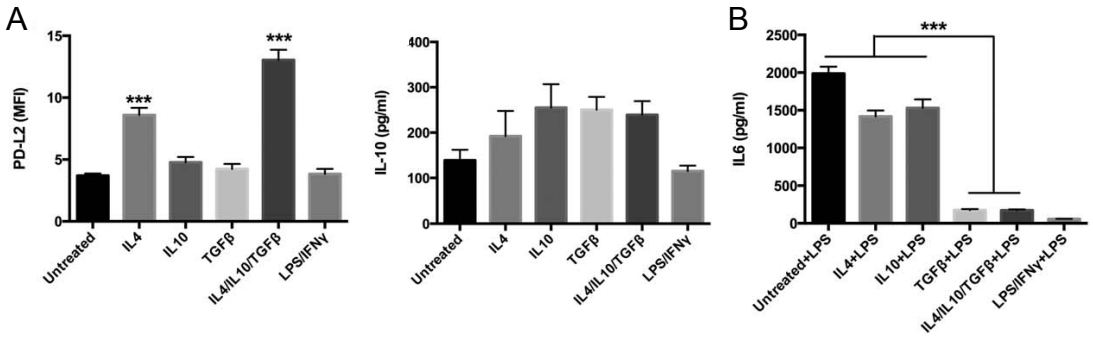

Figure 10 | **Induction of M2 microglia.** (**A**) Expression and secretion of PD-L2 and IL-10, respectively. (**B**) IL-6 secretion in M2-microglia after secondary stimulation of LPS.

4.2.3 Adoptive transfer of M2 macrophages and microglia in EAE

We next wanted to address the question if macrophages or microglia in an M2 state could reduce inflammation in the CNS and attenuate EAE in DBA/1 mice. The first question we had was the route for adoptively transferring M2 macrophages and microglia. We injected the cells i.p in **study I** as that was anatomically the closest route for macrophages to the pancreas. However, it was not likely that macrophages or microglia would travel from the peritoneal

cavity to the CNS. It has been described that peritoneal macrophages injected intravenously (i.v) are captured in the lungs and our own experiments indeed confirmed that BM-derived macrophages transferred by the i.v route did not enter the CNS[211]. We therefore decided to investigate if intranasal (i.n) administration could enhance migration of microglia to the CNS. The i.n. route has been widely used for the delivery of cells and molecules to the olfactory bulb (OB) in mice[212,213]. Using immunohistochemistry we could confirm that fluorescently labeled M2 microglia did migrate to the OB after i.n injection (Fig. 11A).

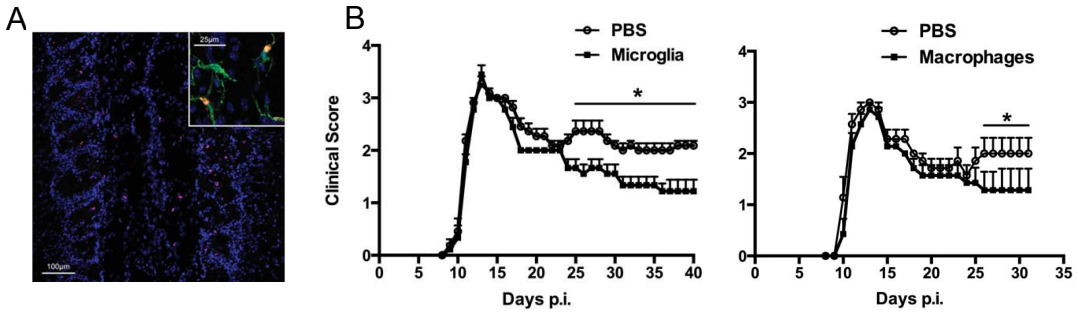

Figure 11 | **Adoptive transfer of M2 microglia.** (**A**) I.n. injection of fluorescently labeled M2-microglia (red). Analysis of the OB 24h post-transfer and staining of Iba1 (green) (**B**) I.n injection of 3 x 10^5 M2 microglia (left) or M2r macrophages (right) at day 15 post-immunization.

We transferred M2 microglia i.n at different time points to determine the effect of these cells on EAE. Transfer of M2 microglia at day 0 post-immunization (p.i) did not affect EAE. However, transfer on day 5, 12 and 15 p.i had a significant reduction of EAE severity at the late persistent stage of the disease (Fig. 11B). Similar effects were also observed when we transferred BM-derived M2r macrophages at day 15 post-injection. These data suggest that M2 microglia have an important role during the later phase of EAE, a period when remyelination and tissue healing is crucial. A recent study has reported a switch from M1 microglia into M2 microglia in a model of lysolecithin-induced demyelination[214]. These M2 microglia enhanced remyelination by promoting oligodendrocyte differentiation, and depletion of M2 microglia reduced remyelination and oligodendrocyte differentiation. Furthermore, depletion of M2 microglia enhanced the activity and numbers of

M1 microglia, indicating an important crosstalk between M1 and M2 microglia *in vivo*. We also observed this crosstalk *in vitro* when M1 macrophages and M2 microglia were co-cultured in different ratios and M1 markers were measured.

4.2.4 Immunomodulation of M2 microglia in the CNS during EAE

We next wanted to explore if M2 microglia could regulate the inflammatory response in the CNS during the persistent phase of EAE. Immunohistochemical analysis indicated a reduced infiltration of leukocytes and less demyelination in M2-treated mice in contrast to control mice. Qualitative analysis of the inflamed CNS by flow cytometry confirmed these findings and revealed a significant reduction of $CD11b^+$ myeloid cells and $CD3^+$ T cells. The effector phenotype of the T cells was altered, with reduced activation status as defined by CD62L and CD44, but also reduced IL-17 production by the T cells following M2 microglia treatment. These findings were confirmed when we co-cultured M2 microglia with anti-CD3 activated T cells *in vitro* and recorded reduced T cell proliferation. Similar to M2r macrophages in **study I**, M2 microglia also induced Tregs *in vitro*.

Taken together, these findings demonstrate that M2 microglia have the ability to reduce inflammation in the CNS either by limiting leukocyte infiltration or by modulating their effector functions. However, we also detect M2 microglia in the draining LNs, which could regulate lymphocyte emigration and activation. The transferred microglia were not pulsed with any antigen and still had an immunomodulatory effect on T cells. M2 microglia have enhanced phagocytosis that could lead to local uptake of debris and antigens, including of self-antigens.

4.3 Study III: The role of TGFβ in moDCs during EAE

Background

MS is an autoimmune disease characterized by immune-mediated demyelination and axonal loss. TGFβ is a pleotropic cytokine with many functions in both homeostasis and during inflammation. The role of TGFβ-signaling in phagocytes during autoimmunity has been poorly defined.

Hypothesis

TGFβ signaling in phagocytes within the CNS is critical for resolution of experimental neuroinflammatory disease.

Methods

We crossed LysM-CRE mice with TGFbrII$^{flox/flox}$ mice in order to generate a mouse specifically lacking the expression of TGFβRII on myeloid cells. Extensive immunophenotyping of both phagocytes and lymphocytes was performed in order to assess the role of TGFβ in monocyte-derived cells during EAE. Chimeric mice were generated to determine the role of TGFβ on BM-derived or CNS-resident cells during EAE.

Results

TGFbr2-deficiency in myeloid cells resulted in a more severe disease during the persistent phase of EAE, without clinical remission. TGFbr2-deficient BM-derived macrophages and DCs had increased IL-12 production that skewed T cells to produce more IFN-γ. This IFN-γ in turn enhanced the activation of CNS-infiltrating moDCs by increasing the release of ROS. The enhanced ROS production promoted demyelination and persistent disease severity in mice deficient of TGFbr2 on moDCs.

4.3.1 TGFβ signaling in phagocytes is important for EAE remission

TGFβ has multiple functions during both homeostasis and inflammation. The activity of TGFβ is dramatically increased in the CNS during acute EAE[215]. TGFβ and its receptors have been identified in CNS lesions of human MS patients[216]. However, studies of the function of TGFβ in the CNS during EAE have yielded conflicting results. TGFβ can have both pro- and anti-inflammatory functions, administration of TGFβ reduces the symptoms of EAE and suppress T cell proliferation[217,218], similar to the *in vitro* findings in **study I** and **II**. However, in those studies TGFβ was injected systemically (s.c and i.v) and not directly at the site of inflammation. Paradoxically, neutralization of TGFβ by antibodies exacerbated EAE and pharmacological inhibition of TGFβ signaling resulted in earlier onset of EAE[215,219]. Another study reported that overproduction of TGFβ in the CNS worsens EAE[220]. Furthermore, TGFβ has an important role in the differentiation of both pathogenic T_H17 cells and protective Tregs. These conflicting data could be explained by the complex biology of TGFβ, the net effect of this cytokine being dependent on the tissue, phase of inflammation and the cell that is targeted[125].

We wanted to address the effect of TGFβ on myeloid cells during EAE, and therefore crossed LysM-Cre with TGFβRII$^{flox/flox}$ mice to generate TGFβRII-deficiency specifically in the myeloid compartment (LysM-TGFbR2). LysM is expressed in phagocytes such as macrophages, monocytes and neutrophils and has been widely used for phagocyte gene-targeting[221]. We immunized s.c the LysM-TGFbR2 mice and littermate controls (WT) with myelin oligodendrocyte glycoprotein (MOG) in CFA and determined the clinical outcome of EAE. There was no difference in the onset or the acute phase of EAE between LysM-TGFbr2 and WT mice. However, WT mice entered the remission phase around day 17 with reduced disease severity, whereas the disease severity of LysM-TGFbr2 mice was not attenuated and did not convert into remission throughout the period of observation (Fig. 12A). Similar findings have been reported when the promoter of CD11c was used to drive the expression of a dominant negative form of TGFβRII in the cDC subset[222]. Analysis of the persistent phase of EAE (day 24-28) by flow

cytometery and immunohistochemistry indicated enhanced leukocyte infiltration in the LysM-TGFbr2 CNS, primarily comprising activated moDCs and $CD3^+$ T cells (Fig. 12B). Furthermore, LysM-TGFbr2 mice had considerably increased demyelination compared to WT controls at the persistent phase of EAE. This could either indicate enhanced inflammatory responses or dysfunctional remyelination.

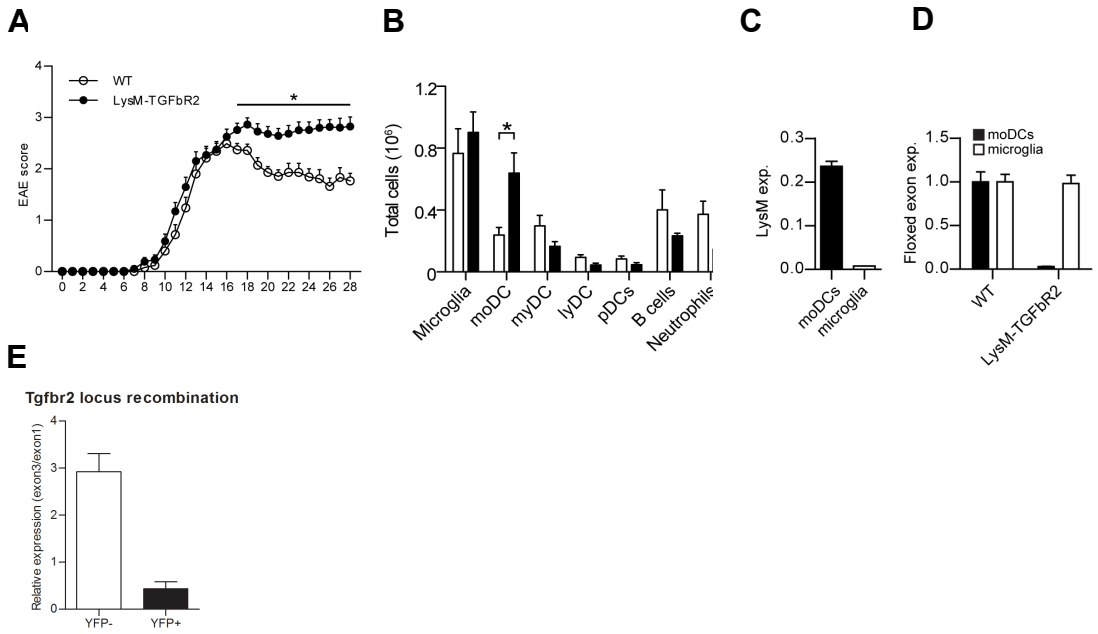

Figure 12 | **LysM-TGFbr2 mice have severe EAE without remission with increased moDC infiltration to the CNS.** (**A**) Clinical score of EAE on LysM-TGFbr2 and WT mice (**B**) Infiltrating cell types in the CNS at day 24. White bars: WT, black bars: LysM-TGFbr2 (**C**) LysM gene expression in sorted moDCs and microglia (**D**) Recombination of exon 4 in the *Tgfbr2* locus in sorted moDCs and microglia (**E**) Recombination of exon 3 in the *Tgfbr2* locus in sorted YFP^+ monocytes from LysM-TGFbr2 / Rosa26-EYFP mice.

To determine if the loss of TGFβ signaling was in blood-derived myeloid cells or in tissue-resident cells we first analyzed LysM expression in moDCs and microglia. We only detected LysM gene expression in moDCs and not in microglia (Fig. 12C). Furthermore, we only detected high recombination of the *Tgfbr2* locus in moDCs (Fig. 12D). Additionally, we crossed LysM-TGFbr2 mice with Rosa26-stopflox-EYFP mice to be able to sort out monocytes and analyze recombination of the *Tgfbr2* locus in those cells. Splenic YFP^+ monocytes, similar to moDCs in the CNS, had recombination of the *Tgfbr2*

locus, indicating that TGFβRII-deficiency already appears at the monocyte stage (Fig. 12E).

Analysis of the draining inguinal LNs (iLN) at day 7 and the CNS at the acute phase (day 16) p.i did not reveal any differences in lymphocyte or myeloid numbers nor their activation. We also detected no differences in the differentiation of T_H1 or T_H17 T cells, arguing that TGFβRII signaling in myeloid cells during immune priming is expendable. We generated chimeric mice to further confirm that moDCs had a crucial role in determining EAE severity. Reconstitution of LysM-TGFbr2 BM into irradiated LysM-TGFbr2 or WT mice phenocopied the EAE severity during the persistent phase. In contrast, when WT BM was transferred to irradiated LysM-TGFbr2 mice they displayed similar clinical EAE as WT BM transferred into irradiated WT mice. These data suggest that the observed increased EAE severity in LysM-TGFbr2 mice is dependent on TGFβr2-deficency in circulating monocytes and specifically in moDCs.

4.3.2 TGFβRII-deficiency in moDCs enhances T_H1-responses

Our analysis of the brain during the persistent phase indicated that there were higher frequencies of moDCs and T cells in LysM-TGFbr2 mice. Further analysis of the T cell compartment revealed significantly increased numbers of IFN-γ-producing $CD4^+$ T cells in the CNS with a decrease of IL-17-producing T cells. However, we did not detect any differences in IFN-γ^+IL-17^+ double-positive T cells, nor any alterations in the numbers of Tregs, indicating that T_H1 cells had an important role in the persistent phase of EAE in LysM-TGFbr2 mice.

An important factor for the differentiation of T_H1 cells is IL-12. We thus addressed if TGFβ regulated IL-12 secretion from macrophages and DCs in LysM-TGRbr2 mice. We generated BM-derived macrophages and DCs by using M-CSF or GM-CSF, respectively. Stimulation of macrophages and DCs with LPS induced IL-12 secretion from both LysM-TGFbr2 and WT cells. However, TGFβ was able to attenuate the LPS-induces IL-12 secretion in WT macrophages and DCs, but not in cells from LysM-TGFbr2. We next

developed an *in vitro* APC-T cell reactivation assay to further investigate if TGFβ had an important role in the regulation of T_H1 cells by limiting the production of IL-12 from DCs (Fig. 13A). We co-cultured sorted $CD4^+$ T cells from day 7 iLNs of MOG-immunized WT mice with BM-derived DCs from WT or LysM-TGFbr2 mice. The DCs were pre-stimulated with LPS in the presence or absence of TGFβ and then later co-cultured with iLN-derived $CD4^+$ T cells and MOG_{35-55} peptide for 3 days. Analysis of the $CD4^+$ T cells after 3 days revealed that TGFβ-signaling in moDCs indeed regulated the differentiation of T_H1 cells. We found that IFN-γ^+ T cell frequencies were significantly reduced when T cells were co-cultured with LPS-TGFβ-stimulated WT DCs, but not when co-cultured with stimulated DCs from LysM-TGFbr2 mice (Fig. 13B and C).

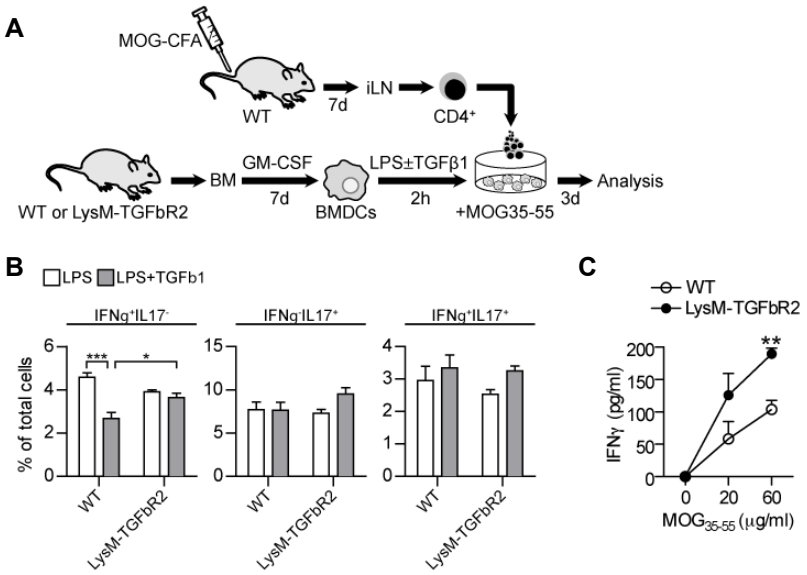

Figure 13 | **Loss of TGFβ signaling in APCs polarizes T cells to produce IFN-γ.** (**A**) Experimental design and setup for the re-activation assay. (**B**) Intracellular staining of CD4+ T cells from day 7 MOG-immunized iLNs co-cultured with stimulated BM-derived DCs for 3 days. (**C**) Soluble IFN-γ was measured from the co-cultures

4.3.3 IFN-γ regulates ROS production in moDCs during EAE

The mechanisms underlying myelin and axonal damage induced by the immune cells in MS and EAE are still not very well understood and many models have been proposed. It is considered that radicals from the ROS

pathway might have an important role in inducing tissue damage during inflammation. However, it is important to remember that physiological concentrations of ROS are important for many functions during homeostasis and immune defense, such as cellular respiration and elimination of pathogens. These processes are also regulated, exposure of electrophiles or ROS activating nuclear factor E2 related factor 2 (Nrf2) that in turn activates antioxidant enzyme gene transcriptions such as superoxide dismutase, glutathione peroxidases, peroxiredoxins, catalase and NAD(P)H: quinone oxidoreductase 1[223-227]. The proposed model is that high amounts of ROS during EAE and MS that overpower the antioxidant capacity will result in tissue damage such as modifications of lipoproteins and proteins in the cell membranes. High amounts of ROS can also induce oxidative stress in the tissue cells that will result in mitochondrial injury and DNA damage[228]. High levels of radicals have been demonstrated in cerebrospinal fluid from patients with MS and oxidative damage to cellular membrane lipids have been detected in MS lesions[229,230]. Inflammatory macrophages and moDCs are important sources of ROS during CNS inflammation. NOX-derived oxidative burst from phagocytes is also associated with tissue damage in MS and EAE[228,231-233].

We investigated if TGFβ signaling in moDCs was important for the regulation of ROS during EAE since we detected increased numbers of activated moDCs and severe demyelination in LysM-TGFbr2 mice. Analysis of NOX subunits and iNOS gene expression in CNS on day 28 of EAE indicated that the expression of the Nox2 gene was upregulated in LysM-TGFbr2 mice. Sorting of different myeloid cell populations revealed that the highest expression of Nox2 was indeed in moDCs and not in resident microglia or infiltrating neutrophils, which we later confirmed by immunohistochemistry. We also detected increased ROS activity in LysM-TGFbr2 mice during the persistent phase of EAE by employing DCFDA staining of the spinal cord at day 28 post-immunization. To further establish the importance of ROS synthesis from moDCs in LysM-TGFbr2 mice we measured Nox2 mRNA expression in BM-derived DCs after they had been stimulated with LPS in the presence or absence of TGFβ. We determined that TGFβ regulated the

expression of Nox2 mRNA in BM-derived DCs. However, TGFβ did not decrease ROS production from LPS-stimulated BM-derived DCs measured by fluorescent dihydrorohadmine-123 (DHR123). TGFβ only decreased ROS production from WT BM-derived DCs when IFN-γ was included as a co-stimulus (Fig. 14). These results thus indicate that TGFβ can regulate LPS-IFN-γ-induced ROS production by DCs in WT mice, but not in LysM-TGFbr2 mice.

These data together indicate that TGFβ regulates the production of IL-12 from moDCs that is important for the differentiation of T_H1 cells during the persistent phase of EAE. Furthermore, IFN-γ secretion from these T_H1 cells in combination with TLR stimulation further activates moDCs to produce and release ROS, a process that also was regulated by TGFβ. ROS was associated with enhanced demyelination during the persistent phase of EAE in LysM-TGFbr2 mice.

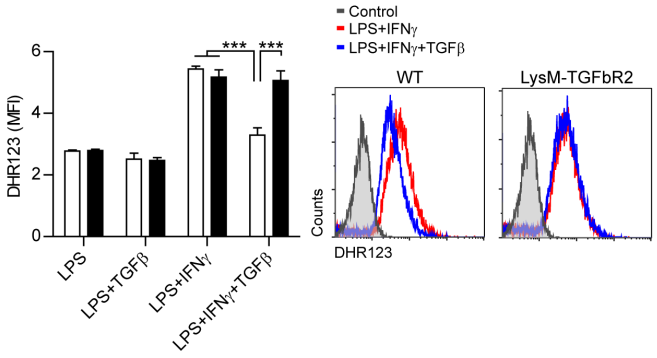

Figure 14 | **IFN-γ and TGFβ regulates ROS-production in moDCs during EAE.**
White bars: WT, black bars: LysM-TGFbr2

4.4 Study IV: The role of neutrophils in the regulation of adaptive immunity during emergency granulopoiesis.

Background

Neutrophils are important innate immune effector cells during acute and chronic inflammation. Deregulation of neutrophil cell counts during inflammation can induce a state of emergency granulopoiesis to maintain sufficient peripheral neutrophil numbers. Extensive research has been performed to understand the role of neutrophils in innate immunity during inflammation and emergency granulopoiesis. However, the role of neutrophils in the regulation of adaptive immunity during emergency granulopoiesis is not very well studied.

Hypothesis

Neutrophils regulate lymphocyte activation during immune priming in the draining lymph nodes.

Methods

We generated mice in which diphtheria toxin unit A was selectively expressed in $LysM^+$ cells (LysM-DTA). Analysis of cellular composition and immune function were conducted in both steady state and following adjuvant-induced pro-inflammatory activation. The mechanism of action of the observed effect was dissected through a combination of use of additional gene-deleted mouse strains, antibody-mediated cell depletion and antibody-mediated protein neutralization. The clinical significance of our model was examined using an experimental model of systemic lupus erythematosus (SLE).

Results

LysM-DTA was specifically neutropenic in steady state, but exhibited emergency granulopoiesis and extensive neutrophilia in draining lymph nodes following adjuvant-induced inflammation. Lymph node recruited neutrophils produced BAFF in a G-CSF-dependent manner, which triggered plasma cell formation and elevated antibody production. This mobilization was facilitated by increased CXCL1/CXCL2 expression and overlapped with increased numbers of IL-17-producing $CD4^+$ T cells. G-CSF neutralization and neutrophil starvation in turn significantly diminished plasma cell formation. In the context of B cell-driven autoimmunity, induced neutropenia in SLE mice increased plasma cell numbers and serum anti-DNA antibodies.

4.4.1 Induced neutropenia in LysM-DTA mice

Neutrophils have primarily been regarded as the first responding innate effector cells during inflammation. Their effector functions include the production and release of pro-inflammatory cytokines and chemokines, degranulation and secretion of anti-microbial peptides[16,234-236]. Neutrophils recruit inflammatory monocytes to the site of inflammation and they also have an important role during the resolution of inflammation. Apoptotic neutrophils release 'eat-me' signals to the tissue macrophages that upon phagocytosis will develop an M2-like phenotype that is associated with the release of TGFβ and IL-10.

Investigators have been using genetic tools such as gene-deficient mice, or molecular tools such as antibodies that neutralize neutrophil-released proteins, in order to understand and study the functions of neutrophils *in vivo*. Furthermore, neutrophil generation and blood counts also regulate their functions. It has been reported that neutrophil blood counts follow the circadian rhythm and that modulation of neutrophil circulating numbers has an impact on acute inflammation[237,238]. Live infections or adjuvant immunization can also modulate generation and release of neutrophils from the BM, a state defined as emergency granulopoiesis[75,239]. One way to study neutrophils is to induce neutropenia. It has been reported that neutrophils migrate to the LNs minutes after adjuvant provocation and depletion of these early neutrophils, by anti-Ly6G antibodies or by using G-CSFR deficient-mice, enhanced antigen presentation by APCs and increased T cell activation[21,22]. Another study used an N-ethyl N-nitrosourea (ENU) mutagenesis strategy and generated a neutropenic mouse strain[19]. They identified that mature neutrophils were required both in the BM and in the periphery for NK cell development. However, none of these studies had any signs of emergency granulopoiesis in their respective models.

We generated a neutropenic model in order to first investigate the role of neutrophils in the regulation of T and B cells. To achieve this we crossed LysM-Cre mice with Rosa26-stopflox-DTA mice and obtained a novel mouse strain called LysM-DTA. Analysis of the blood, spleen and BM in LysM-DTA mice clearly indicated neutropenia with around 85% reduction of neutrophils

(Fig. 15). However, as mentioned earlier in **study III**, LysM is also expressed in monocytes and macrophages. Interestingly, we did not see any differences in monocyte or any major macrophage populations in LysM-DTA mice compared to in WT mice. Analysis by flow cytometery and immunohistochemistry revealed that splenic red pulp macrophages, marginal zone macrophages and metallophilic macrophages were all intact in LysM-DTA mice. Further analysis of other myeloid cells such as DCs and eosinophils but also different macrophage populations in the brain, peritoneum, liver, skin and gut did not reveal any differences between LysM-DTA and WT mice.

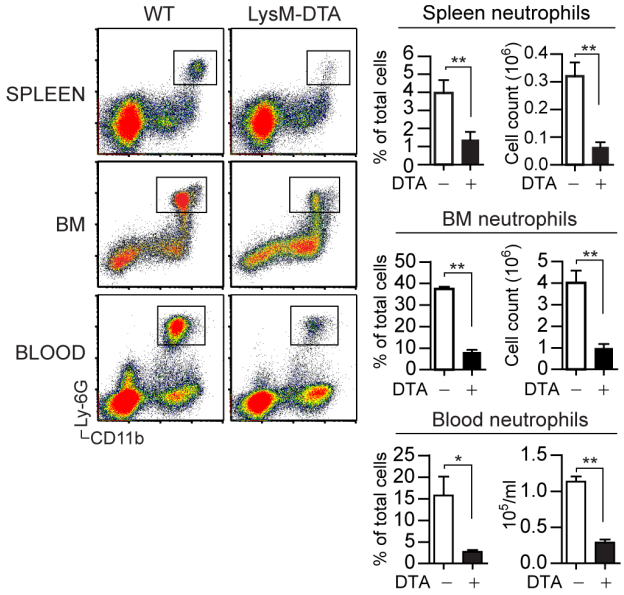

Figure 15 | **Drastic reduction of neutrophils in LysM-DTA mice.** Neutrophils ($CD11b^{+}Ly\text{-}6G^{+}$) were analyzed in spleen, BM and blood.

One explanation for this could be the fact that Cre recombination is never 100% efficient. For instance, recombination in peritoneal macrophages is around 80-90% based on YFP expression in LysM-CRE x Rosa26-stopflox-EYFP mice. The surviving non-recombined 10-20% macrophages are enough for the organ to repopulate itself during development and a similar mechanism can be applied for monocytes. However, neutrophils do not have peripheral progenitors, cannot proliferate at a mature stage and thus are dependent on BM progenitors for their generation. Furthermore, LysM is expressed relatively late during neutrophil maturation and the recombination rate of Cre in

neutrophils is around 90-95%, indicating high expression of LysM. This severely limits the generation of neutrophils in LysM-DTA mice and could be the reason for neutropenia but normal homeostasis in other myeloid populations such as monocytes and macrophages.

4.4.2 Emergency granulopoiesis and neutrophil recruitment

We next wanted to investigate the immunological consequences of neutropenia in LysM-DTA mice during induced inflammation. We thus immunized the mice s.c with CFA and analyzed innate and adaptive immune functions. We first noticed splenomegaly in LysM-DTA mice that was already evident at day 7 post-immunization (p.i.) (Fig. 16A). We also detected signs of lymphadenopathy at this timepoint, but the size of the draining iLNs was larger at day 14. We speculated that this could be due to emergency granulopoiesis, a triggered response due to neutropenia. Analysis of the serum displayed drastically enhanced levels of G-CSF in LysM-DTA compared to in WT mice (Fig. 16B). Another feature of emergency granulopoiesis is the accumulation and proliferation of myeloid progenitor cells in the spleen. Analysis of the spleen in LysM-DTA mice revealed a 2-fold increase in CMPs and a 4-8-fold increase in GMPs in comparison to WT spleens. The majority of the cells in the spleen were $CD11b^+$ but lacked markers for CMP/GMP progenitors such as C-kit or the neutrophil marker Ly-6G, indicating that these cells were caught in a stage between GMPs and mature neutrophils (Fig. 16C). Nonetheless, adjuvant-immunized LysM-DTA mice were still neutropenic in blood and BM throughout the 14 days of analysis despite the clear signs of emergency granulopoiesis.

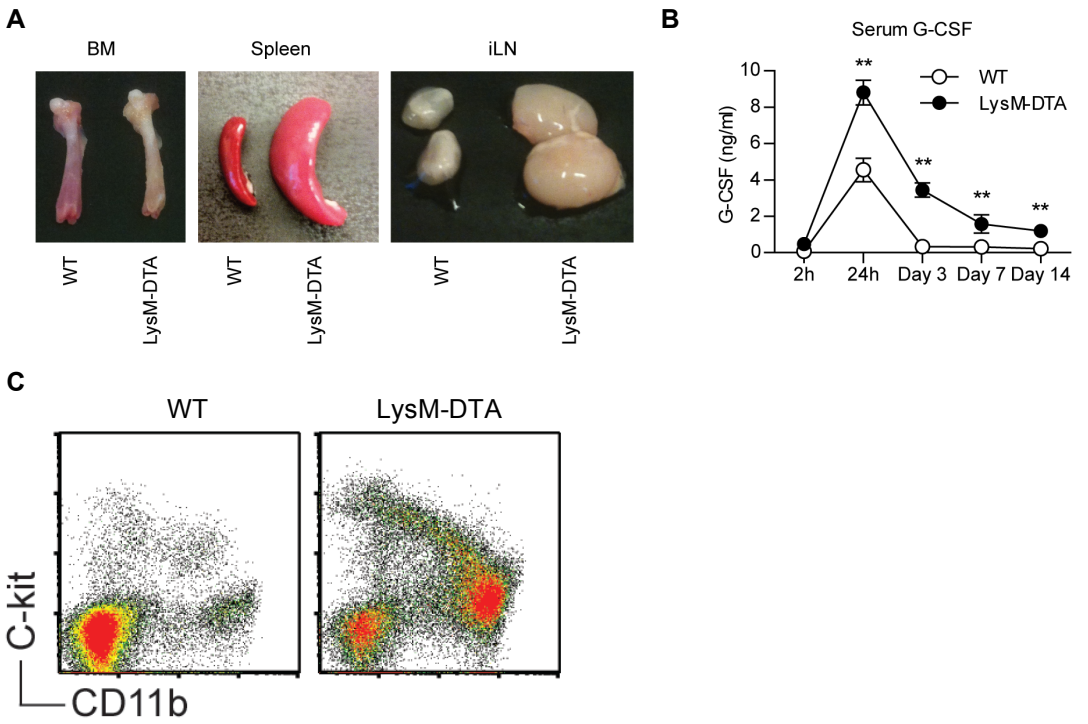

Figure 16 | **Emergency granulopoiesis is induced in LysM-DTA mice after CFA provocation.** (**A**) BM, spleen and iLNs 7 days p.i. (B) Serum G-CSF levels in LysM-DTA and WT mice after CFA immunization. (C) C-kit^{+} myeloid progenitors in the spleen of WT and LysM-DTA mice 7 days after CFA immunization.

It has been reported that neutrophils migrate to inflamed LNs and that this recruitment is divided into two phases[22]. The first neutrophil wave in WT mice was evident at 2 hours p.i. and localized in the LN cortex. The second wave of neutrophils was evident between day 7 and 14 but were confined instead to the LN paracortex. LysM-DTA mice lacked the first neutrophil wave but remarkably displayed a drastic 20-fold higher neutrophil influx into the LN paracortex during the second wave compared to in WT mice (Fig. 17). We hypothesized that it was a specific recruitment of neutrophils to the iLNs regulated by neutrophil-attracting chemokines. We observed a drastic increase of CXCL1 and CXCL2 mRNA in iLN cells in LysM-DTA compared to in WT mice. A key regulator for the induction of CXCL1, CXCL2 and G-CSF secretion by endothelial cells is IL-17. This cytokine is produced by T_H17 cells locally in the LNs and can thus recruit neutrophils to the tissue[89]. However, a recent study has reported that the induction of G-CSF is independent of IL-17 but is dependent on TLR4 signaling at the site of inflammation[240].

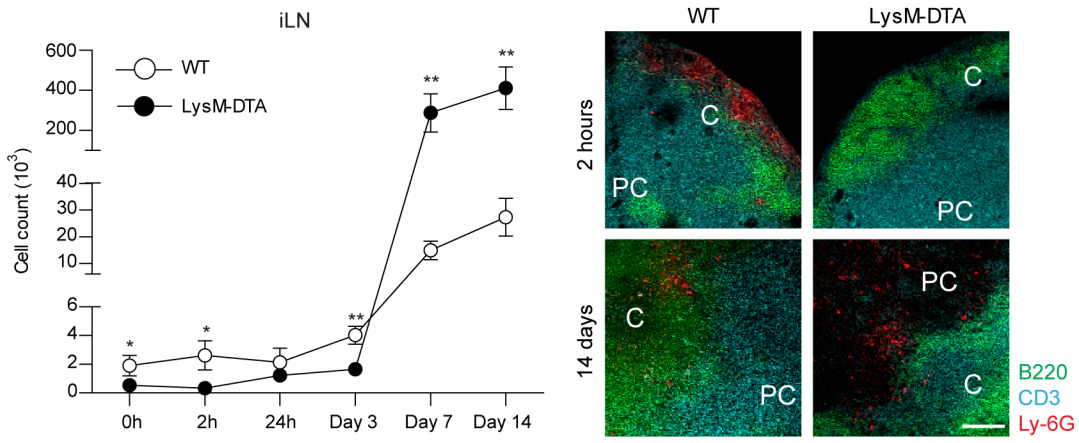

Figure 17 | **Neutrophils are recruited to the iLNs after CFA immunization.** Left: neutrophil recruitment to the iLN over time. Right: immunohistochemistry of iLNs (C: Cortex, PC: paracortex).

4.4.3 Neutrophil regulation of T cell differentiation and B cell activation

We next wanted to address if this deregulation in neutrophil LN recruitment affected T cell differentiation and B cell activation. There is already prominent evidence that neutrophils regulate T cell activation in the LNs, but if neutrophils regulate the differentiation program in T cells has not been previously reported. Analysis of iLNs in LysM-DTA mice at day 7 p.i. revealed increased numbers of IL-17-producing T cells compared to in WT mice. This increase of T_H17 cells was associated with the specific recruitment of neutrophils to the iLNs at day 7 (Fig. 18A). As mentioned earlier, some of the important gene targets of IL-17 are CXCL1, CXCL2 and G-CSF, which could explain the neutrophil recruitment phenomenon[241,242]. We could also detect increased production of IFN-γ in $CD4^+$ T cells at day 14 p.i. in LysM-DTA compared to in WT mice.

How do the T cells sense the loss of or the need for neutrophils? Earlier studies have demonstrated that neutrophils have the ability to regulate the antigen presentation capacity of APCs[21], and neutrophils can also specifically eliminate APCs in the LNs[88]. However, this does not explain the specific T cell differentiation into T_H17. The answer could be in the tissue where the initial inflammatory trigger occurs, at a time point where there are no or low numbers of circulating neutrophils in LysM-DTA mice. The s.c-

injected adjuvant will activate tissue macrophages and DCs to produce neutrophil-attracting chemokines and pro-inflammatory cytokines such as IL-23. The ingestion of apoptotic neutrophils by tissue APCs will in turn suppress the production of IL-23 and thereby reduce the stimulus for T_H17 differentiation[243]. However, this suppression of APCs does not occur in LysM-DTA mice due to the neutropenic state during the early phase of inflammation.

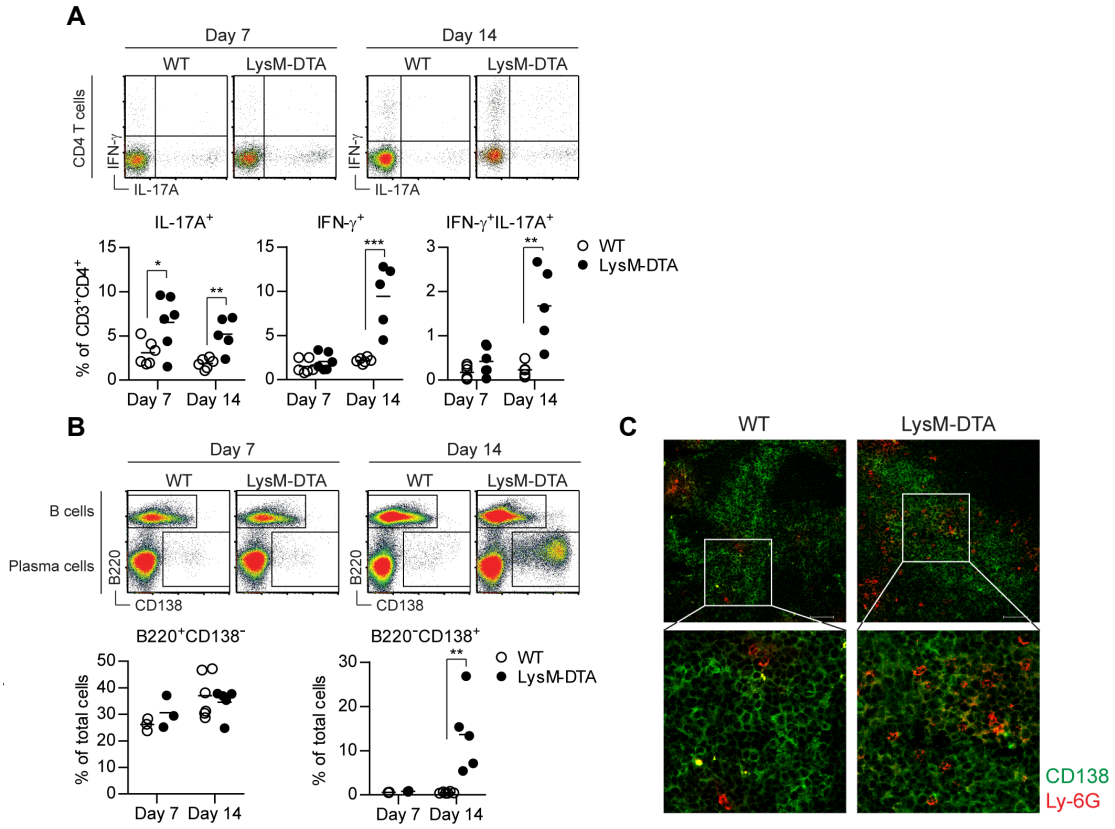

Figure 18 | **Enhanced numbers of IL-17-producing T cells and plasma cells in LysM-DTA iLNs.** (**A**) IL-17 and IFN-γ production by $CD3^+CD4^+$ T cells in the iLNs. (**B**) Analysis of $B220^+CD138^-$ B cells and $B220^-CD138^+$ plasma cells in the iLNs. (**C**) Immunofluorescent staining of iLN at day 14 p.i. visualizing plasma cells (green, CD138) and neutrophils (red, Ly-6G).

Moreover, we observed interesting alterations in the B cell compartment in the iLNs. We detected a 4-fold increase in numbers of B cells and a striking 25-fold increase in numbers of plasma cells in LysM-DTA mice at day 14 p.i. compared to in WT mice (Fig. 18B). Examination by immunofluorescent staining revealed that the recruited neutrophils at day 14 p.i. resided close to plasma cells in the paracortex and medulla of the iLNs (Fig. 18C). We also

confirmed that the $CD138^+$ cells were indeed *bona fide* plasma cells through their staining of intracellular IgG.

We thus speculated that recruited neutrophils had a role in the massive generation of plasma cells in the iLNs of LysM-DTA mice. A recent report has identified 'B cell helper' neutrophils in mice and humans that promote T cell-independent innate B cell activation in the marginal zone of the spleen[23]. However, our model is based on T cell-dependent follicular B cell activation during emergency granulopoiesis in the draining LNs. To further confirm that our model was not an intrinsic phenomenon only occurring in LysM-DTA mice we took advantage of antibody-mediated depletion of neutrophils that also induces emergency granulopoiesis[244]. In agreement with our findings in LysM-DTA mice, transient anti-Ly6G-mediated neutrophil depletion during 14 days p.i. induced increased numbers of B cells, plasma cells, T_H17 cells and neutrophilia in the iLNs of anti-Ly6G treated WT mice in comparison to isotype-treated WT mice.

Taken together, these data indicate that early neutropenia induces emergency granulopoiesis and specific differentiation of T cells into the T_H17 phenotype, which could be due to enhanced IL-23 production by emigrated tissue APCs. Enhanced CXCL1 and CXCL2 expression in LysM-DTA recruits increased numbers of neutrophils into the iLNs that reside close to B cells and plasma cells in the paracortex and medulla.

4.4.4 Neutrophil-mediated B cell activation is regulated by G-CSF

Since LysM-DTA mice had increased numbers of plasma cells in the iLNs we next wanted to investigate if neutrophils in LysM-DTA mice directly regulated B cell activation and plasma cell formation. A key factor for B cell activation is the cytokine BAFF and it has been reported that human neutrophils can produce this cytokine *in vitro*[98]. We therefore sorted neutrophils from the iLNs at day 14 from both LysM-DTA and WT mice p.i. We detected a significantly higher expression of BAFF mRNA in sorted LysM-DTA neutrophils compared to sorted WT neutrophils. Moreover, the average expression of BAFF was lower in the neutrophil-depleted fraction, indicating that neutrophils are an important source of BAFF in the iLNs. G-CSF has been suggested to be

important for the intracellular synthesis of BAFF in neutrophils and a secondary pro-inflammatory stimulation triggers the release of BAFF by neutrophils[99]. We sorted WT BM neutrophils and stimulated them with G-CSF together with either GM-CSF, IL-17, IFN-γ, LPS, IL-18 or CXCL2 for 24 hours. Analysis of the supernatants revealed that G-CSF alone did not induce the release of BAFF from neutrophils. However, BAFF was detected when neutrophils had been stimulated with G-CSF combined with either GM-CSF, IFN-γ, LPS or CXCL2, all factors that are present in the microenvironment of the inflamed LN (Fig. 19A).

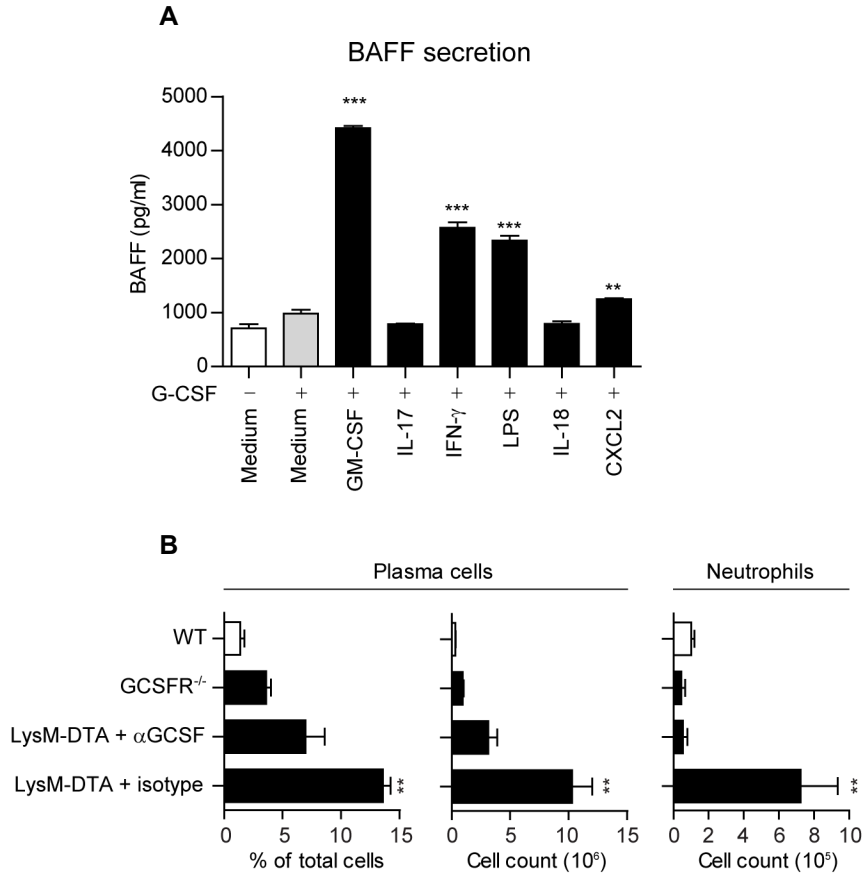

Figure 19 | **The release of BAFF from neutrophils is G-CSF-dependent** (**A**) BM-sorted neutrophils were stimulated for 24 hours with the respective stimuli (**B**) Analysis of plasma cells and neutrophils in iLNs at 14 days p.i. in WT, LysM-DTA and GCSFR$^{-/-}$ mice treated with anti-GCSF or isotype control.

Furthermore, we treated CFA-immunized LysM-DTA mice with G-CSF neutralizing antibody every second day for 14 days to limit the 'B cell helper'

functions of neutrophils *in vivo*, which indeed limited plasma cell formation and neutrophil influx in the iLNs in anti-GCSF treated mice in comparison to isotype-treated controls (Fig. 19B). These results were also reproduced in CFA-immunized G-CSFR deficient mice ($GCSFR^{-/-}$). Taken together, this uncovers the potential and importance of emergency granulopoiesis in increasing G-CSF systemically for the survival and effector functions of neutrophils.

The clinical outcome of LysM-DTA mice following adjuvant provocation has many similarities to Felty's syndrome (FS) in humans[245]. FS is defined by the coexistence of rheumatoid arthritis, lymphadenopathy, splenomegaly and neutropenia. FS has also been associated with other autoimmune diseases such as systemic lupus erythematosus (SLE). Among various indications of SLE, about half of the patients demonstrate some degree of neutropenia[246]. Increased B cell activity is also evident in these patients, with autoantibodies against neutrophil antigens and G-CSF having been reported[247,248]. Interestingly, irrespective of the presence or absence of autoantibodies against G-CSF, neutropenic FS and SLE patients have increased G-CSF levels. FS patients also have hyperplastic and active germinal centers in the enlarged spleen and LNs with neutrophil and macrophage infiltrates[249,250]. The association of neutropenia, exaggerated serum levels of G-CSF and increased B cell activation with autoimmunity led us to address whether induced neutropenia could enhance or accelerate SLE. We took advantage of hybrids between the New Zealand White and New Zealand Black strains (NZB/WF1) that represent a spontaneous SLE mouse model with pathological anti-DNA antibodies. The NZB/WF1 model has many lupus-like symptoms such as splenomegaly, lymphadenopathy, elevated serum autoantibodies and immune complex-mediated glomerulonephritis[251]. The spontaneous inflammation initiates when the mice are around 5-6 weeks of age.

We injected anti-Ly-6G or rat isotype control three times weekly for a month in 5 week old female NZB/WF1. Analysis of the spleen revealed increased numbers of both B cells and plasma cells in anti-Ly-6G treated mice in comparison to in isotype-treated mice. We also detected increased production of both IFN-γ and IL-17 by $CD4^+$ T cells. Furthermore,

measurement of serum antibodies revealed increased anti-DNA titers in neutrophil-depleted NZB/WF1 mice. These observations confirm that deregulation of neutrophils can enhance B cell-dependent autoimmunity as observed in SLE.

5 Concluding remarks and future perspectives

In this thesis the role of macrophages, DCs and neutrophils in the regulation of adaptive immunity during inflammation and autoimmunity has been studied. I have evaluated their regulatory properties both in the secondary lymphoid organs and in the targeted organs. The direct effects of innate myeloid cells in the activation of the adaptive immune system are well studied. However, the regulatory capacity of the myeloid compartment during inflammatory responses is still today not very well understood.

I have investigated different activation pathways in macrophages in **study I and II**. We generated BM-derived macrophages or extracted adult microglia and stimulated these cells with several activation factors to explore their immunomodulatory, wound-healing and homeostatic properties. Various M2-stimulations such as IL-4, IL-10 or TGFβ had the ability to deactivate macrophages, induce a state of immunosuppression or change their phagocytic activity. We reported for the first time that M2 macrophages could protect NOD mice from developing T1D in **study I**. However, we and others have shown that macrophages have a remarkable ability to quickly adapt to environmental signals and thus adapt to new situations. We reported that recovered M2r macrophages that had been transferred to NOD mice retained their M2 markers such as PD-L2, but also upregulated the M1 marker CD86. This indicates that M2r macrophages are relatively stable but still have the ability to adapt to the inflamed pancreas. We could also describe a TGFβ-dependent immunosuppression by the transferred M2 macrophages by evaluating the T cell proliferative potential *in vitro* and *ex vivo*. However, the unaltered T cell infiltration to the pancreas visualized by OPT and the T cell activation data did not fully explain the protective role of M2r macrophages in T1D pathogenesis. We speculated that the wound-healing and homeostatic properties of M2r macrophages could enhance β-cell survival and proliferation. Other research groups have partly confirmed these speculations but further investigation is needed to fully understand the mechanism of action. Similar to the findings in **study I**, we found in **study II** that M2 microglia and M2 macrophages had the ability to significantly attenuate

disease severity during the late phase of EAE. We could also detect lower T cell activity in the CNS and signs of lower degree of demyelination. We believe that M2 macrophages or microglia could have beneficial properties for wound-healing and remyelination, as the effects were important during the resolution phase.

Our findings in **study I and II** define the role of M2 macrophages in immunomodulation during inflammatory diseases such as autoimmune T1D and EAE. However, it should be further investigated whether M2 macrophages could be used as a cell therapy in autoimmunity. My colleague Dr. Mia dedicated a part of his Ph.D thesis to confirm that human monocyte-derived macrophages could obtain similar M2 properties to the murine counterparts, even those recovered from MS patients[252]. It would be interesting to investigate if epigenetic changes of M2 macrophages could enhance their phenotypical stability and hence 'lock-in' the M2 phenotype. Furthermore, I think it would be more beneficial, based on safety and cost efficiency, to develop and discover small molecules that can target macrophages *in vivo* and modulate their activation status into the M2 phenotype in settings of autoimmune disease.

During our investigation in **study I and II**, we discovered TGFβ-dependent immunosuppressive properties of macrophages. The regulatory functions of TGFβ-stimulated macrophages have been reported to be mediated by IL-10 and TGFβ *in vitro*. However, the TGFβ-induced immunomodulation by macrophages needed to be further investigated. We therefore decided in **study III** to generate mice in which the TGFβ receptor, TGFβRII, was specifically deleted in monocytes and macrophages in order to determine the role of TGFβ in these cells *in vivo* during autoimmunity. We took the advantage of mice expressing Cre recombinase under the LysM promoter, a gene that is expressed in phagocytes such as neutrophils, monocytes and macrophages. These mice were then crossed to TGFbr2$^{flox/flox}$ mice and we obtained LysM-TGFbr2 mice. Induction of autoimmune neuroinflammation in LysM-TGFbr2 mice revealed a more severe disease course during the persistent phase of EAE in comparison to littermate control mice. This

indicated that TGFβ had an important role in the initiation of the resolution phase and not during priming of the immune response. Furthermore, the EAE severity was associated with increased accumulation of T cells and moDCs. Interestingly, microglia still had functional expression of the TGFβR2 based on mRNA expression, only monocytes and moDCs were deficient for the receptor. I personally believe that moDCs could also be defined as inflammatory monocyte-derived macrophages as there is no specific maker or function that can separate them from DCs. Nevertheless, TGFβ had a strong inhibitory effect on the IL-12 production by macrophages and moDCs as LysM-TGFbr2-derived macrophages and moDCs produced more IL-12 in contrast to their WT counterparts. This was indeed also reflected in the CNS with increased numbers of IFN-γ producing $CD4^+$ T cells. The increased IFN-γ secretion by T cells further activated the phagocytes in the CNS that enhanced their ROS activity, which is associated with severe demyelination, a phenomenon we detected in the inflamed LysM-TGFbr2 CNS. The role of T_H1 cells has been somewhat neglected after the finding of pathogenic T_H17 cells and their effector functions in EAE. This study brings new light into the function of T_H1 cells and their ability to increase ROS activity in macrophages and other phagocytes during the persistent phase of EAE. Further investigations should be initiated to understand the role of IFN-γ and T_H1 cells during the later phases of EAE. We have demonstrated in **study I and II** that TGFβ-stimulated macrophages can induce Tregs *in vitro*. However, we did not detect any significant decrease of Tregs in the CNS of LysM-TGFbr2 mice, although we never investigated if the immunosuppressive capacity of the Tregs was affected. Another interesting question from **study III** is to define the cell type(s) that are responsible for the secretion of TGFβ at the peak of disease. These cells would be interesting drug targets for modulating and enhancing TGFβ secretion.

Finally, we addressed the role of phagocytes during the development of adaptive immune activation in the draining lymph nodes in **study IV**. To achieve this we crossed LysM-Cre mice with Rosa26-stopflox-DTA mice and generated LysM-DTA mice. LysM-DTA mice were neutropenic during steady-

state without any changes in other phagocyte populations such as macrophages and DCs. However, LysM-DTA mice generate emergency granulopoiesis with enhanced T, B and plasma cell responses and neutrophilia in the draining LNs post CFA-immunization. Increased CXCL1 and CXCL2 expression in the LN was perceived as a possible factor for the recruitment of neutrophils to the draining LNs. It would be interesting to investigate if IL-17 is an important upstream inducer of CXCL1 or CXCL2 in the LN. Another aspect of IL-17 is the induction of T_H17 cells in LysM-DTA mice. It has been reported that early antibody-induced neutropenia increases IL-23 production by local tissue macrophages and DCs. One could speculate if migratory APCs from the site of injection have amplified IL-23 production in LysM-DTA mice. We also showed that G-CSF is crucial for neutrophils in the activation of B cells. G-CSF-stimulated neutrophils synthesize BAFF and release it upon a secondary stimulation such as IFN-γ, GM-CSF or CXCL2, all factors present in the draining LN. Our data indicates that enhanced T cell responses due to neutropenia in combination with emergency granulopoiesis increase the systemic G-CSF level that amplifies B cell activation and the generation of plasma cells. It would be interesting to investigate if this model is active during prolonged bacterial infections and if manipulation of the neutrophil compartment could enhance both the cellular and humoral immunity against the pathogen during vaccination.

The studies in this thesis reveal the potential of M2 macrophages in regulation of inflammatory responses. I have also elucidated a novel mechanism in the regulation of B cell activation by neutrophils. Further investigations should be performed to develop possible drug-targets to exploit these different pathways.

6 Acknowledgements

During my years as a Ph.D. student (6 years if you want to know), I have learned that one needs a lot of inspiration and motivation to perform research. For me it comes from a lot of wonderful people who have surrounded me for many years and I want to take the opportunity to thank:

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