General Introduction and Aims

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GENERAL INTRODUCTION

Dendritic cells: the sentinels of the immune system.

Our immune system consists of cells belonging to the innate immune system and the adaptive immune system. Dendritic cells (DCs) are a family of antigen presenting cells (APCs) that bridge these two systems[1]. They scan the internal milieu for self and foreign antigens and present these to T cells of the adaptive immune system through a process termed 'antigen presentation' (**Figure 1**). T cells express a T cell receptor (TCR) that can specifically recognize a particular antigen in the form of a short peptide bound to major histocompatibility complex (MHC) molecules on APCs. Whereas $CD8^+$ T cells recognize antigens bound to MHC class I molecules, MHC-II is used to present antigens to $CD4^+$ T cells (**Figure 1**). While exogenous antigens, in contrast to intracellular antigens, are normally presented to $CD4^+$ T cells, cross-presentation enables presentation of exogenous antigens onto MHC-I and thereby activation of $CD8^+$ T cells[2] (**Figure 2**).

Ready, set, go: 3 signals necessary for complete T cell activation.

Antigen presentation resulting in TCR stimulation, is a first signal, but it does not automatically result in activation of the receiving T cell. Three signals are necessary in total to achieve full T cell activation[5]. Based on the expressed co-stimulatory molecules or co-inhibitory molecules (**Figure 3**) on the surface of DCs, an activating or inhibiting response is mediated[6, 7], which is known as the second signal. Besides engagement of TCR, the signal via CD28 on T cells which binds CD86 on DCs is crucial for T cells, because without this interaction a T cell will become anergic[8]. Positive stimulation can further be induced by inducible costimulator (ICOS) that binds ICOS-ligand (ICOS-L) (in **Figure 3** as 'B7c')[9]. Inhibitory signals can be mediated via PD-1 and CTLA-4, which can bind PD-L1/PD-L2 or CD80/CD86, respectively. CTLA-4 binds CD80/CD86 with greater affinity and avidity than CD28, thus enabling it to outcompete CD28 for its ligands. Cytokines, either produced by DCs or already available in the milieu, are the third signal, and can further define the T cell response e.g. differentiating them into T helper cell subsets or unlocking their full potential.

DC subsets

Four types of DCs can be distinguished: Conventional type 1 or 2 DCs (cDC1s and cDC2s), plasmacytoid DCs (pDCs)[6] and inflammatory monocyte derived DCs (Mo-DCs)[11]. The functions of DCs are different during steady state or during inflammation. In steady state, cDC1s and cDC2s are primarily involved in peripheral tolerance as they can present tissue-associated self-antigens and effectively induce regulatory T cells (Tregs) and $CD4^+$ and $CD8^+$ T cell tolerance[2, 12-14]. During activation, however, primarily (but not exclusively) $CD8^+$ T cells are activated by cDC1s, and $CD4^+$ T cells by cDC2s[14-16]. pDCs can

also activate both T cell types, but in addition are known for their high type 1 interferon signature[17]. During inflammation, Mo-DCs arise from a monocyte precursor and their functions overlap with dendritic cells[11]. The most used markers to identify DC subsets are listed in **Table 1**.

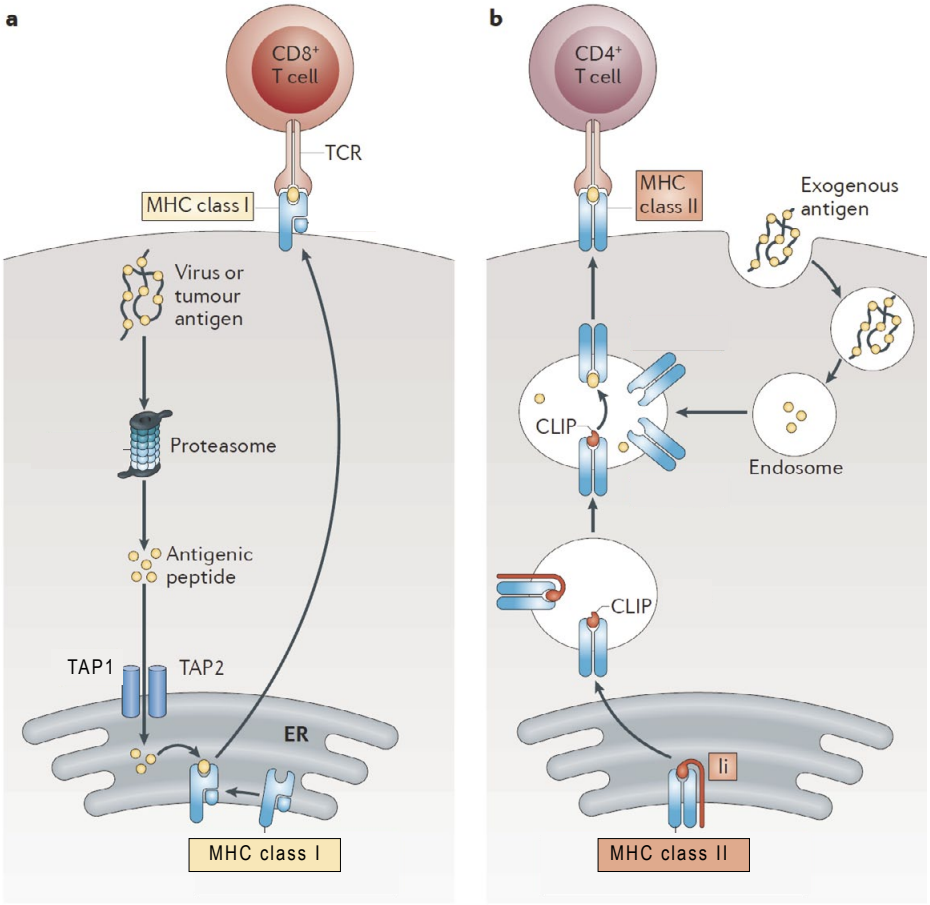

Figure 1: Antigen presentation to $CD8^+$ and $CD4^+$ T cells using MHC-I and MHC-II, respectively (**a**) Virus antigens or tumor antigens are primarily intracellular antigens and are commonly presented on major histocompatibility complex (MHC) class I complex molecules. Proteasomes are large proteins that degrade proteins, such as viral or tumor antigens into smaller peptides. Transporter associated with antigen processing (TAP) 1 and TAP2, carry antigen peptides into the endoplasmic reticulum (ER). Here, antigens are placed onto the presenting groove of MHC-I, which then leave the ER (and are transported via Golgi/secretory vesicles; not shown in figure) to the surface of the cell to present antigen to $CD8^+$ T cells on their T cell receptor (TCR). (**b**) Extracellular antigens, such as bacteria, are commonly presented on MHC-II molecules. The extracellular antigens are processed by endolysosomal enzymes into peptides. The presenting groove of MHC-II first contains Class II-associated invariant chain peptide (CLIP), which is derived from MHC class II-associated invariant chain (Ii). CLIP is displaced by the bacterial peptide and the MHC-II molecule is brought to the surface of the cell in order to present antigens to $CD4^+$ T cells. Adapted from Kobayashi et al[3].

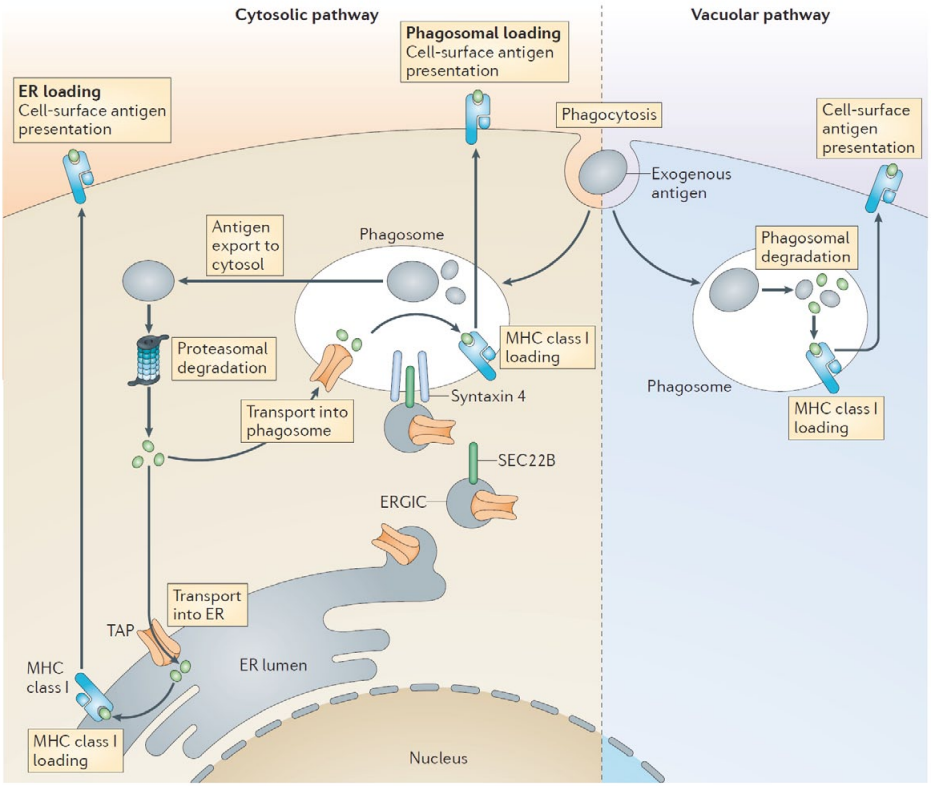

Figure 2: Processing exogenous antigens onto MHC-I molecules within antigen presenting cells in order to cross-present.
Exogenous antigens can be placed onto MHC-I molecules via 2 pathways: the cytosolic pathway or the vacuolar pathway. Within the cytosolic pathway (left), phagocytosed exogenous antigens are processed in an engulfed vesicle called phagosome, then released into cytosol where they can further be degraded by proteasomes. After this, they can be re-transported into the phagosomes, where they can be loaded on available MHC-I molecules and expressed on the cell surface. Alternatively, anitgens can be transported into the ER and loaded on MHC-I molecules there. The ER contains the transporter protein TAP, which is located to phagosomes with the help of other proteins such as the SNARE protein SEC22B and Syntaxin 4. ER-Golgi intermediate compartment (ERGIC) is where SEC22B interacts with Syntaxin 4. The vacuolar pathway (right) allows phagosome-degraded exogenous antigens to be directly loaded on MHC-I molecules and then go to the cell surface for antigen presentation. From Joffre et al[4]

Effector adaptive immune cells in autoimmunity

DCs are central orchestrators of the immune response: they activate T cells and - besides antigen presentation and co-stimulation - produce cytokines to direct naïve T cells into a particular differentiation pathway (**Figure 4**). T helper subsets produce different cytokines (**Figure 4**) and thus have different functions. For a long time, the T helper 1 cell (Th1 cell) and Th2 cell paradigm dominated our understanding of (auto)immune diseases versus allergic diseases, respectively[19, 20]. The Th1 subset is important in host defenses to

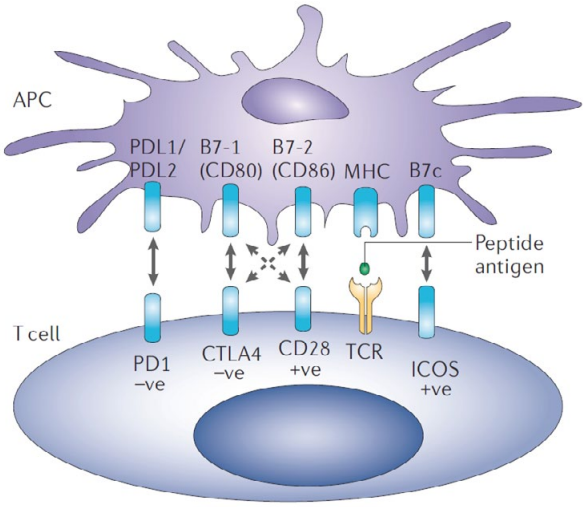

Figure 3: Co-stimulatory and co-inhibitory molecules set thresholds for T cell activation
A selection of common co-stimulatory and co-inhibitory molecules are depicted, that results in positive (+ve) or inhibitory (-ve) effects on T cell activation. The most important co-stimulation for activation is CD28 interaction on T cells with CD80 or CD86 on APCs. Classic interactions are bold arrows, while dashed arrows are weaker affinity connections that are known. From Gregersen et al[10].

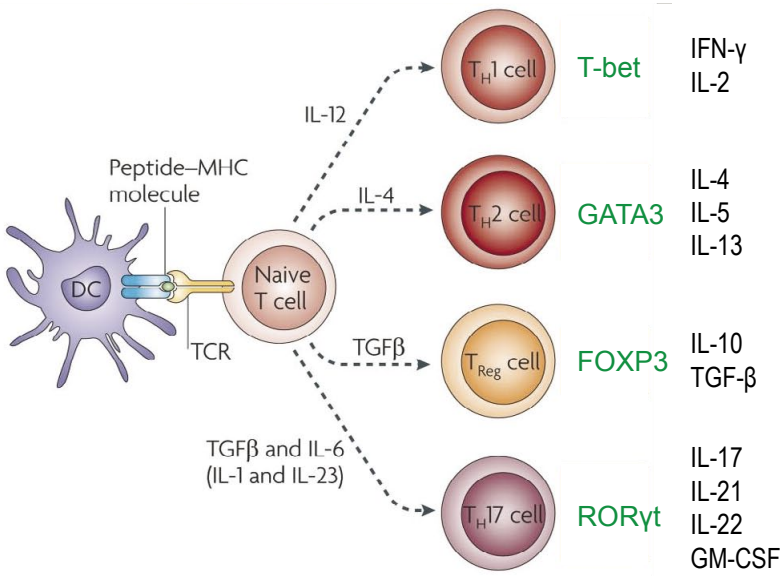

Figure 4: Differentiation of helper T cell (Th) subsets.. Naïve T cells can differentiate into various T helper cell (Th) subsets under influence of differentiating cytokines, that are shown adjacent to the dashed arrows. Responsible key transcription factors (green) and primary cytokines are listed per Th cell subset on the right side. Adapted from Zou et al Nat Rev Immunol 2010[36]

Table 1. Overview of cell surface markers to identify DC subsets in mice.

	Markers *)
cDC1	CD8α / CD103 Clec9a (DNRG1) CD207 (Langerin) XCR1
cDC2	CD4 / CD11b CD172a (SIRPα)
pDCs	B220 Ly6C PDCA.1 Siglec-H
moDCs	FcγRI (CD64) CD14 FcεRI CD11b CD172 (SIRPα) CD206

***) Based on markers listed by Gardner et al[18]**

intracellular bacteria and is involved in autoimmune diseases such as multiple sclerosis and diabetes mellitus. Their development is dependent on transcription factor T-bet, and can be induced by cytokine IL-12[21]. The Th2 subset, developmentally dependent on transcription factor GATA binding protein 3 (GATA3) and induced by cytokine IL-4, mediates protection against helminth infection[21], but also coordinates the characteristic eosinophilic, basophilic and mast cell response in diseases such as allergic asthma[22].

This Th1/Th2 model was challenged when in the early 2000's the Th17 cell was discovered[23]. The Th17 cell is involved in various autoimmune disorders, ranging from psoriasis, inflammatory bowel disease (IBD), rheumatoid arthritis (RA), multiple sclerosis (MS) to systemic lupus erythematosus (SLE)[24, 25]. In order of importance, the cytokines IL-6, IL-1β and TGF-β are relevant for differentiation of naïve T cells into Th17 cells[26], and rely on transcription factor RAR-related orphan receptor gamma (RORγt) for their development[27]. Expansion and survival of Th17 cells are maintained by IL-23[28].

Suppression of excessive immune responses and autoimmune reactions are performed by Tregs, that can be induced from naïve T cells by TGF-β and rely on transcription factor Forkhead box P3 (FoxP3) for their development[29]. Initially, it was thought that only the Th2 subset was important for humoral immunity, until the follicular T helper (Tfh) cell was discovered. It is primarily the Tfh cell that support germinal center B cell responses and plasma cell differentiation[30]. During certain conditions, such as helminth infections, Tfh cells can derive from Th2 cells[31], or the other way around, as has been reported in HDM-driven allergic airway inflammation[32]. This might also be cellular plasticity, which is known to occur for multiple Th-cell subsets[33, 34]. Plasma cells can produce autoreactive antibodies that play a key role in humoral autoimmune diseases such as SLE[35].

Control of the immune system

Homeostasis of the immune system is of crucial importance. During times of foreign pathogens, rapid activation of the immune system is necessary for the extermination of antigens. However, immune cell activation is strictly kept in check, as exaggerated innate and adaptive immune responses can tip the immune system towards autoimmune diseases. The nuclear factor-κB (NF-κB) signaling pathway is a well-studied molecular pathway that promotes cellular activation. The end products of the NF-κB pathway result in a pro-inflammatory milieu, differentiation and cell survival[37]. It also results in transcription of a key regulatory zinc finger (de)ubiquitinating enzyme A20/tumor necrosis factor α-induced protein 3 (TNFAIP3), that is known to contribute largely to the inhibition of NF-κB signaling[38], thereby bringing a balanced halt to inflammation.

A20/TNFAIP3: ubiquitin-modifying enzyme

A20/Tnfaip3 is a ring finger ubiquitin-modifying enzyme, with a dual function being ubiquitination and de-ubiquitination[38]. In the TNFα signaling pathway both functions are utilized to inhibit NF-kB signaling. Briefly, A20/TNFAIP3 removes an activating type of polyubiquitin, (K63–polyubiquitin) from accessory proteins such as RIP1 and NEMO, thereby inhibiting signals to downstream proteins[38]. Furthermore, A20/TNFAIP3 adds an inhibitory type of polyubiquitin (K48-polyubiquitin), to these accessory proteins including RIP1, thereby targeting them for degradation by proteasomes[38].

A20/TNFAIP3 in humans

TNFAIP3 is one of the few genes that has been linked by genome-wide association studies (GWAS) to multiple immune diseases[39, 40]. Single nucleotide polymorphisms (SNPs) in the vicinity of the *TNFAIP3* gene are associated to characteristic autoimmune diseases such as SLE, RA and psoriasis[40]. Over the years, the list of *TNFAIP3* gene SNP-associated clinical diseases keeps expanding, with recent additions of autoimmune hepatitis (AIH), Primary Biliary Cirrhosis (PBC) and colitis ulcerosa (CU)[41-43].

A20/Tnfaip3 in mouse models

The identification of various associations of the *A20/Tnfaip3* gene with diseases has spiked interest of immunologists to study the importance of this gene in cell lines and mouse models. *A20/Tnfaip3* $^{-/-}$ mice develop severe multiorgan inflammation and cachexia, resulting in early death[44]. Cre-LoxP recombination bioengineering made it possible to study cell-specific effects of *A20/Tnfaip3* gene deletion[45]. As an example, *A20/Tnfaip3* deletion in myeloid cells, using LysM-cre, resulted in a spontaneous autoinflammatory disease, characterized by paw inflammation[46]. Furthermore, *A20/Tnfaip3* deletion in B cells using a CD19-cre resulted in a model that resembles SLE[47].

Another key publication in the field was by Kool et al, who utilized a DC-specific cre (CD11c-cre) to ablate *A20/Tnfaip3*[48]. Aged *Tnfaip3*$^{CD11c\text{-}KO}$ mice had activated DCs and developed systemic T and B cell activation. In addition, germinal center B cells, plasma cells and autoantibodies were produced, resulting in an autoimmune phenotype resembling human SLE. Another laboratory used a similar DC-specific *A20/Tnfaip3* deletion strategy, but this resulted in an IBD resembling phenotype[49], indicating that most likely the local microbiome may influence the phenotype.

AIMS AND OUTLINE OF THIS THESIS

The paper by Kool et al.[48] has been the starting point for several chapters in this thesis, which we will touch upon later in our aims. In **chapter 2** we summarize the latest knowledge on A20 as a protein, its molecular function, SNPs associated to human disease, as well as all immune cell-specific conditional deletions of *A20/Tnfaip3* known to date.

Deletion of the *A20/Tnfaip3* gene in all DCs resulted in an SLE[48] or IBD phenotype[49], but it remained unknown which of the DC subsets is mainly responsible for the autoimmune phenotype. To address this issue, we investigated the phenotypic outcome of targeted deletion of A20/*Tnfaip3* in more specific DC subsets. Study of DC ontogeny has revealed several specific markers, such as DNGR1/Clec9a, which are primarily present on cDC1s[50]. In **chapter 3**, we studied the effect of loss of *A20/Tnfaip3* on the activation status of cDCs and moDCs in a *Tnfaip3*$^{DNGR1\text{-}KO}$ mouse model, in which the *Tnfaip3* was mainly deleted in cDC1s. We further analyzed the systemic effects on T cells and B cells that were associated with the activation of these cDCs/moDCs, and explored their immunohistopathology at the age of 31 weeks. Interestingly, while cDC1s were primarily targeted by *A20/Tnfaip3* deletion, cytotoxic $CD8^+$ T cell activation was hardly altered and, unexpectedly, mostly $CD4^+$ T cells and B cells were activated.

The myeloid cell-specific, LysM-cre-driven model of *A20/Tnfaip3* deletion is known to result in an autoinflammatory phenotype with paw inflammation in aged mice[46, 51]. In **chapter 4** we took a step outside of the field of autoimmunity and studied a house-dust mite (HDM)-driven airway inflammation model in *Tnfaip3*$^{LysM\text{-}KO}$ mice. These mice are known to develop a Th17-associated neutrophilic airway inflammation, rather than a Th2-associated eosinophilic airway inflammation[52]. Since neutrophilic recruitment is largely induced by IL-17[53], we wondered whether IL-17RA-signaling was essential for neutrophilic airway inflammation. To determine this, we crossed *Tnfaip3*$^{LysM\text{-}KO}$ mice onto an IL-17RA-signaling knockout background and stimulated the airways with HDM and assessed the responses.

It occurred to us during the study of aged *Tnfaip3*$^{CD11c\text{-}KO}$ mice, that develop an SLE phenotype, that the numbers of B cells were vastly reduced in spleens, pointing to

the possibility that a B cell lineage defect occurred in the bone marrow, resulting in disrupted generation of immature and mature B cell populations. In **chapter 5**, we therefore examined B cell development in the bone marrow of both young 6-week-old mice and 24-week-old *Tnfaip3*$^{CD11c\text{-}KO}$ mice. We further aimed to investigate whether in *Tnfaip3*$^{CD11c\text{-}KO}$ mice mature B cells that were released into the periphery were more responsive and thus more prone to B cell activation or immunoglobulin production than those in wild-type control mice.

B cell activation that is associated with germinal center reactions and plasma cell formation is dependent on help from T cells. In the case of autoimmunity, activated DCs might overly activate T cells, which can positively select autoreactive B cells in pathogenic germinal center reactions[54]. Activated T cells express CD40L which binds CD40 on B cells and thereby provides proliferation and survival signals besides those initiated by B cell receptor (BCR) engagement[55]. Apart from this T cell-dependent response, a T cell-independent activation of B cells by APCs is also known[56, 57]. A20/Tnfaip3-deficient DCs were known to activate B cells independently of T cell help *in vitro*[48]**.** In **chapter 6** we addressed the question whether A20/Tnfaip3-deficient DCs also had the capacity to directly activate B cells *in vivo* and thereby engaged these cells in autoimmune pathology. To this end, we crossed *Tnfaip3*$^{CD11c\text{-}KO}$ mice onto a *CD40lg* deficient background, thereby abrogating T-B cell communication. We also examined whether autoreactive immunoglobulins and kidney remodeling were altered.

It has been demonstrated that stimulated A20/Tnfaip3-deficient DCs *in vitro* have the capacity of highly inducing IL-17 in T cell co-cultures, likely due to elevated production of IL-6 and IL-23[48], both of which are beneficial for their survival. Given the importance of Th17 cells in autoimmune diseases including SLE[58], in **chapter 7** we examined whether IL-23 was dispensable for Th17 homeostasis *in vivo* and cytokine production in the *Tnfaip3*$^{CD11c\text{-}KO}$ SLE mouse model. To achieve this, we crossed *Tnfaip3*$^{CD11c\text{-}KO}$ mice to mice lacking IL-23. Since Th17 cells can stimulate B cells towards class-switched immunoglobulin-producing plasma cells[59], we further examined autoantibody production and kidney remodeling at the age of 24-weeks.

Finally, implications of our work and potential future directions in the field of A20/Tnfaip3 and (auto)immunity are described in **chapter 8.**

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