Unravelling the mechanisms for mucosal tolerance induction using the CTA1R7K-X-DD immunomodulating fusion protein

Effective treatment options for autoimmune diseases

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Gothenburg 2016

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Unravelling the mechanisms for mucosal tolerance-induction using the CTA1R7K-X-DD immunomodulating fusion protein: Effective therapeutic treatment of autoimmune disease

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“But the plans were on display…”
“On display? I eventually had to go down to the cellar to find them.”
“That’s the display department.”
“With a flashlight.”
“Ah, well, the lights had probably gone.”
“So had the stairs.”
“But look, you found the notice, didn’t you?”
“Yes”… “yes I did. It was on display in the bottom of a locked filing cabinet stuck in a disused lavatory with a sign on the door saying ‘Beware of the Leopard.”

Douglas Adams, The Hitchhiker’s Guide to the Galaxy

Unravelling the mechanisms for mucosal tolerance induction using the CTA1R7K-X-DD immunomodulating fusion protein:

Effective treatment options against autoimmune diseases

Charlotta Hansson
Department of Microbiology and Immunology, Institute of Biomedicine,
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Tolerance is a physiological mechanism that prevents attacks on our own cells and tissues whilst allowing immune responses directed against tumours and invading pathogens. Autoimmunity is an aggressive attack by the immune system on tissues and tissue functions that results from a loss of tolerance which can cause chronic and debilitating symptoms. Hence, reinstating tolerance to achieve physiological homeostasis is highly warranted.

In recent years significant progress has been made in our understanding of tolerance and how auto-aggression is actively controlled by multiple layers of immune regulatory mechanisms. This thesis describes a unique tolerance-inducing platform that may find clinical use in the treatment of several autoimmune diseases. The CTA1R7K-X-DD platform is a fusion protein that consists of three elements with their own respective properties; a targeting unit (DD), a disease-specific peptide (X), and an inactivated mutant of the immunomodulatory cholera toxin A1-subunit (CTA1R7K). I have exploited this platform using different peptide inserts (X) to investigate its mechanism of action and function in two experimental models of human autoimmune diseases, namely rheumatoid arthritis (RA) and multiple sclerosis (MS).

The tolerogenic CTA1R7K-COL-DD fusion protein is a therapeutically effective means to prevent collagen-induced arthritis (CIA) in mice and my aim was to understand the mechanisms by which the fusion protein reinstates tolerance. My research will contribute to a better understanding of immune regulation during autoimmunity, and provide a strategy to interfere with the progression of autoimmune diseases. By comparing the tolerogenic fusion protein with its immunoenhancing, enzymatically active, counterpart (CTA1-X-DD), I identified a yin-and-yang effect of the two fusion proteins on migratory dendritic cells (DCs), which were found to be the primary target cells *in vivo* after intranasal immunizations. While the enzymatically active CTA1-X-DD fusion protein induced the expression of co-stimulatory molecules on the DC, inactive CTA1R7K-X-DD instead promoted a set of genes associated with Tr1 induction and co-inhibition. Most importantly, the IL-27 cytokine was upregulated in both DCs and T cells, a signalling pathway known to be important in Tr1 induction and the regulation of effector responses. The differential effects observed on the DC populations were dependent on enzymatic activity and translated into differences in downstream T cell responses. I studied these two populations in detail to dissect the immunomodulating events that participated in the development of tolerance or immunity.

In the second half of my thesis work I have investigated the therapeutic effect of the fusion protein carrying disease-relevant peptides in the experimental autoimmune encephalitis (EAE) model. I used this model to further demonstrate the effects of our fusion protein on disease development, focusing on regulatory CD4 T cell subsets and the functional importance of the IL-27 pathway in tolerance induction. Finally, to meet the need for simple treatments in the clinic I have evaluated whether there would be a formulation that is cost-effective to produce, which may also secure good patient compliance. To this end, I have collaborated with a group that expressed the fusion protein in an edible plant. This allowed me to test whether oral administration of the fusion protein, when bioencapsulated, could be used to treat CIA. I found that treated mice overall exhibited significantly reduced symptoms and in fact, some mice showed no symptoms at all. Thus, this proof-of-principle study showed that a protein-based pharmaceutical administered in the form of a transgenic plant might be clinically feasible for tolerance induction.

My thesis identifies several molecular features in the early tolerization process in targeted DCs and the subsequent generation of CD4 T cells that help explain the tolerogenic functions of the CTA1R7K-X-DD fusion protein. My research also provides experimental evidence which indicate that the CTA1R7K-X-DD fusion protein could be a potential therapeutic agent for treatment of RA, MS and possibly other autoimmune diseases.

Keywords: immunological tolerance, dendritic cells, Tregs, autoimmunity, multiple sclerosis, experimental autoimmune encephalitis, rheumatoid arthritis, collagen-induced arthritis, plant vaccination
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ORIGINAL PAPERS

This thesis is based on the following studies, which are referred to in the text by their Roman numerals (I-III)

I. **Tolerance-induction by a mutant cholera toxin-derived fusion protein depends on migratory CD103+ DCs**
Hansson C, Schön K, Lycke NY
Manuscript

II. **IL-27R is critical for tolerance-induction by the CTA1R7K-MOG-DD fusion protein in experimental autoimmune encephalitis**
Hansson C, Verolin M, Schön K, Chandode R, Quintana F, Lycke NY
Manuscript

III. **Feeding plants that express a tolerogenic fusion protein effectively protects against arthritis**
Hansson C, Schön K, Kalbina I, Stridh Å, Andersson S, Bokarewa MI, Lycke NY
Plant biotechnol J. 2016 Apr 14(4): 1106-15. Epub 2015 Sep 25.

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ABBREVIATIONS

7AAD	7-aminoactinomycin D
ActB	Beta actin
ADP	Adenosine diphosphate
AI	Arthritic index
ANOVA	Analysis of variance
APC	Antigen presenting cell
AT	Adoptive transfer
Batf3	Basic leucine zipper and transcription factor ATF-like 3
BM	Bone marrow
BMDC	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD	Cluster of differentiation
cDC	Conventional dendritic cell
cDNA	Complementary DNA
CFA	Complete Freunds adjuvant
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CIA	Collagen-induced arthritis
CLN	Cervical lymph node
CNS	Central nervous system
cpm	Counts per minute
CT	Cholera toxin
CTA	Cholera toxin subunit A
CTB	Cholera toxin subunit B
CTLA-4	Cytotoxic T lymphocyte antigen-4
DC	Dendritic cell
DD	D fragment of *Staphylococcus aureus* protein A dimer
dLN	Draining lymph node
EAE	Experimental autoimmune encephalitis
ELISA	Enzyme-linked immunosorbent assay
Flt3L	FMS-like tyrosine kinase 3 ligand
FoxP3	Forkhead box P3
GM-CSF	Granulocyte monocyte-colony stimulating factor
$G_{s\alpha}$	Adenylate Cyclase stimulator
GVHD	Graft-versus-host disease
HPRT	Hypoxanthine guanine phosphoribosyltransferase
IDO	Indoleamine 2,3-dioxygenase
IFA	Incomplete Freunds adjuvant
IFN	Interferon
IL	Interleukin
IL-27Rα	Interleukin 27 receptor alpha subunit

ILN	Inguinal lymph node
in	Intranasal
ip	Intraperitoneal
ITAM	Immunoreceptor tyrosin-based activation motif
ITIM	Immunoreceptor tyrosin-based inhibition motif
iv	Intravenous
KO	Knock-out
LN	Lymph node
MALT	Mucosa-associated lymphoid tissue
medLN	Mediastinal lymph node
MHCII	Major histocompability complex class II
migDC	Migratory dendritic cell
MMP	Matrix metalloproteinase
MOG	Myelin oligodendrocyte glycoprotein
mRNA	Messenger RNA
MS	Multiple sclerosis
NF_KB	Nuclear factor kappa B
nTreg	Natural regulatory T cell
OVA	Ovalbumin
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
pLN	Popliteal lymph node
PLP	Myelin proteolipid protein
pMHC	peptide-MHC complex
PPARγ	Peroxisome proliferator-activated receptor gamma
PTx	Pertussis toxin
RA	Rheumatoid arthritis
resDC	Resident denritic cell
RT-qPCR	Real time quantitative PCR
sc	Subcutaneous
SIRPα	Signal-regulatory protein alpha
SP	Spleen
TCR	T cell receptor
t-DNA	Transfer-DNA
Tfh	T follicular helper cell
Tg	Transgenic
Th	T helper cell
TGFβ	Transforming growth factor beta
Treg	Regulatory T cell
Tr1	Type 1 regulatory T cell
wt	Wildtype

A BRIEF HISTORY OF TOLERANCE

Although it would take many experimental efforts to prove and define the concept of immune tolerance, its story simply begins with inquisitive observation. In 1945, Ray D. Owens found that dizygotic twin cattle, who shared blood systems before birth, were born tolerant to a mixture of red blood cells from each other; a chimerism that was retained throughout life. He never used the term “tolerance”, but his observations were the embryo from which an entire scientific field developed.

In 1960, Peter Medawar and Macfarlane Burnet received the Nobel Prize for their discovery of acquired immunological tolerance. In the Nobel lecture, the concept was defined as "a state of indifference or non-reactivity towards a substance that would normally be expected to excite an immune response”. This is a postulate that has not changed much since. According to Burnet himself, he had only a minor role in these findings and stated that “my part in the discovery… was the formulation of the hypothesis that called for experiment” [1]. This formed the basis for a series of experiments, where Medawar was first to demonstrate immunological tolerance in 1953 when he showed that early engraftment of allogeneic splenocytes in fetal mice conferred resistance against rejection of future grafts from the same, but not other, donor strains of mice [2].

Since then, great progress has been made on the subject. Some historical seminal findings include the discovery of the T-cell receptor genes which extended the field of tolerance to include T cells [3, 4]. Later, specific cell-death, or ***deletion***, became the first described mechanism of immune tolerance by demonstrating negative selection of B-cells [5, 6] and T-cells [7]. This was closely followed by a second mechanism in which tolerance was maintained in the periphery by the induction of cellular non-responsiveness, or ***anergy*** [8]. Finally, Le Douarin and colleagues demonstrated a dominant form of tolerance in several studies [9] that would eventually culminate in the discovery of ***regulatory T cells***.

In more recent years, the field has exploded in size and complexity and continues to expand as it involves several disease conditions where immune tolerance has either critical or detrimental roles, such as allergy, autoimmunity, tumour progression and transplant rejection. Though overall consensus remains to be attained as to which mechanisms are most important to break or reinstate tolerance, it is certainly clear that a better understanding of these concepts will have a critical clinical impact. Autoimmune disease is, broadly speaking, a result of failing check-points within the immune system and clinical symptoms vary depending on the tissues targeted. However, they often leave their victims with crippling morbidity, decreased quality of life or even death. In conclusion, harnessing the regulatory arm of the immune system is one way to address these issues.

In my thesis, I aimed to further characterize the interface at which either tolerance or immunity is determined in T cells by antigen presenting cells (APCs). The CTA1-DD adjuvant is a known immunostimulant whereas the inactive mutant, CTA1R7K-DD, promotes tolerance. We exploited this bimodular platform to investigate the different expression profiles that were induced in relevant cell types during the initiation of an immune response. For example, IL-27 was uniquely upregulated during tolerance and CD39, which has been shown to be induced by IL-27 signalling and implicated in tolerance, was upregulated downstream of this. In addition, my thesis provides proof-of-concept that our mutated protein vector can be used to ameliorate autoimmune reactions in the host. We successfully treated mice with experimental autoimmune encephalitis (EAE), a mouse model for human multiple sclerosis (MS). Furthermore, we used the fusion protein to demonstrate that protein expressed in an edible plant, without any subsequent processing, could be used to alleviate arthritis in mice upon feeding.

But first, the following sections will review key concepts and more recent findings and breakthroughs that have been highly relevant to the field and in moving this particular project forwards.

THEORETICAL BACKGROUND

The mucosa

Mucosal membranes cover all internal body surfaces exposed to the outside world and protects the underlying connective tissue from dehydration and microbial assault. Besides the powerful physical and chemical barriers put in place, the lamina propria harbours an exceptionally intricate network of innate and adaptive immune cells [10]. The mucosa is constantly bombarded with materials from the external environment, such as innocous particles, food-antigens and commensal bacteria, but also by invasive pathogenic micro-organisms and detrimental particulate substances. Furthermore, the host itself constists of self-antigens that have to be recognized as such. It is in this context, ridden with antigenic noise, that the mucosal immune system is bestowed with the difficult task of discriminating between self and non-self, between innocous and harmful - and mount a fitting response accordingly. Naturally, the evolutionary product is a system of tighly regulated processes in which the epithelium and cells from the innate and adaptive immune system act in concert to achieve clearance of harmful substances without excessive tissue damage.

Within the mucosa, there are immunological inductive sites and effector sites. Briefly, APCs continously sample antigens in their surroundings and migrate towards the organized secondary lymphoid organs, such as mucosa-associated lymphoid tissues (MALT) and draining lymph nodes (dLN). Here, they present their antigens to naïve T cells *en route*. If a T cell recognizes the specific antigen, it responds by expanding and aquiring effector functions so that it is able to 1) help B-cells mount a humoral response and/or 2) leave the lymphoid organ and enter the mucosal layer via the blood circulation where the inflammatory cue was first detected. Homing is regulated by the selective expression of molecules that instructs the lymphocytes where to go, but a leakiness in this process leads to, for example, intranasal immunization giving robust reproductive tract immunity [11].

THE RESPIRATORY TRACT

The respiratory tract consists of two functionally distinct compartments, namely the upper respiratory tract, which is covered by a mucosal layer populated by commensal bacteria, and the lower lung parenchyma which is specialized for gas exchange and hosts no bacteria. Bronchial mucus, secreted IgA and a ciliated epithelium cooperatively prevent large particles (>5µM) from reaching the alveoli, however smaller particles and pathogens still access these areas and consequently, well-orchestrated immunological defenses are necessary throughout the entire respiratory tract [12].

Respiratory M cells, which are specialized cells that provide rapid transfer of antigens and pathogens to the immune system, cover the follicle area in both the upper and lower respiratory tract [13] and dendritic cells (DCs) are strategically positioned within and beneath the epithelium for optimal antigen uptake. DC subsets will be described in greater detail in subsequent sections, but with regards to their anatomical location; conventional

CD103+ DCs intersperse the epithelium whereas CD11b+ DCs are positioned in the underlying connective tissues and the overall density of DCs gradually decreases upon descention of the respiratory tract [14]. A schematic overview of immune cells in the respiratory muosa is presented in figure 1.

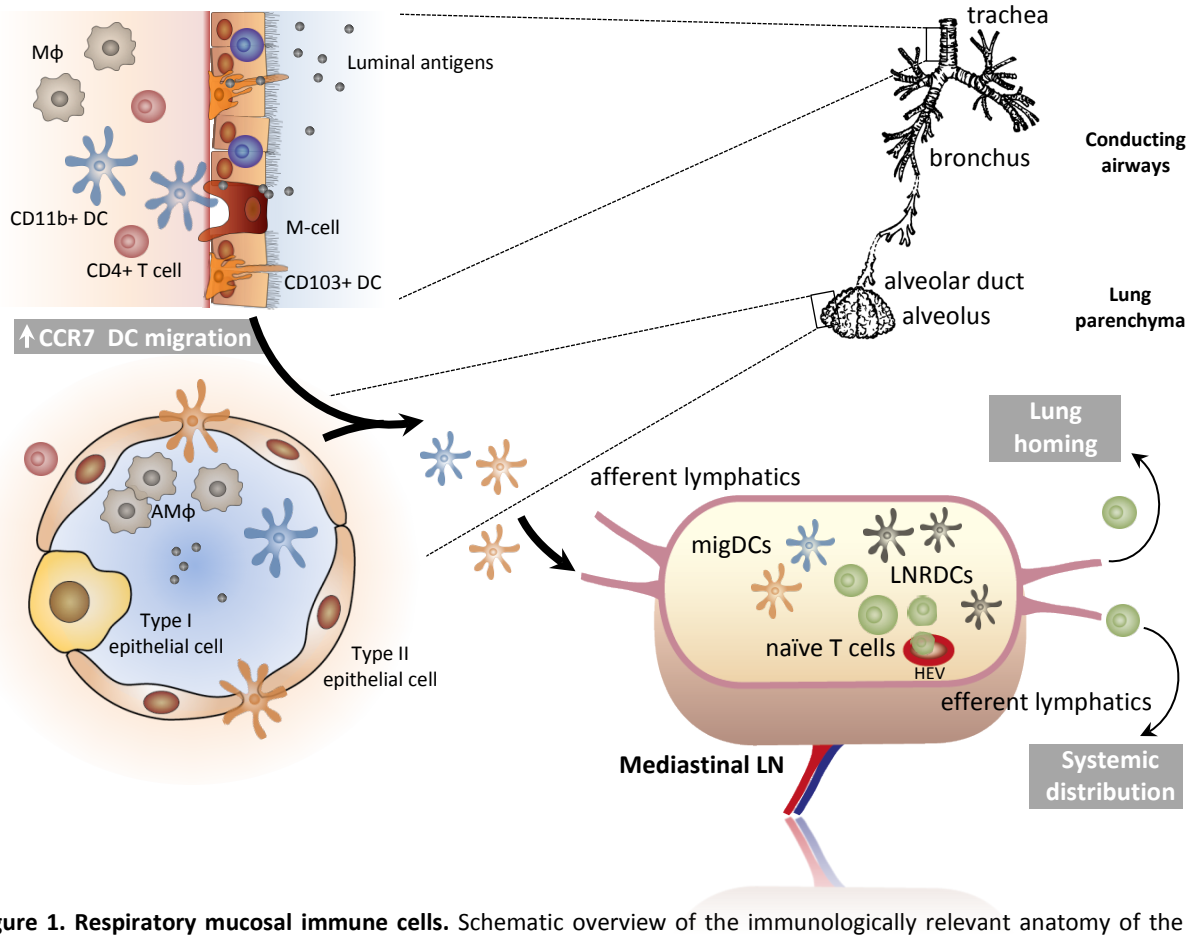


Figure 1. Respiratory mucosal immune cells. Schematic overview of the immunologically relevant anatomy of the respiratory tract, adapted from [14, 15]. Two subsets of conventional DCs (cDC) are present. Intra-epithelial CD103+ DCs (orange) and CD11b+DCs (blue) in the lamina propria. They are also present in the alveoli, which is otherwise mainly populated by resident alveolar macrophages. Upon uptake of airborne antigens, the cDCs upregulate CCR7 and migrate to the draining lymph node where they interact with naïve antigen-specific T cells. Mϕ: Macrophage, HEV: high endothelial venule, LNRDCs: lymph node resident dendritic cells, migDCs: migratory dendritic cells, AMϕ: alveolar macrophage.

MUCOSAL ADJUVANTS

Adjuvants are molecules or formulations that in some way improve the immunogenicity of vaccines and, as such, they are able to enhance, accelerate, prolong or modulate immune responses towards a co-administered antigen. They are usually classified as immuno-modulators or delivery systems, although some adjuvants may have both of these properties. Their mode of action is still not fully understood despite decades of adjuvant research. In fact, the use of oil emulsion adjuvants date as far back as 1916 [16] and was soon followed by the discovery of aluminum salts (alum) which is still the most commonly used adjuvant in human vaccines to date. However, immunogenic vaccine adjuvants lie outside the main scope of this thesis, and for a more comprehensive review on the matter the reader is referred elsewhere [17, 18]. Here, two experimental mucosal adjuvants relevant to the fusion protein used in this thesis work are described, CTA1-DD and cholera toxin (CT).

Cholera toxin (CT) is an enterotoxin produced by *Vibrio cholera* that causes acute diarrhoea, ultimately by phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel which causes massive release of water and electrolytes in the small intestine [19]. As a result, the severe dehydration that follows causes thousands of deaths every year in regions with poor sanitation and health care [20].

CT is an AB_5-toxin; a two-component toxin comprised of the enzymatically active A1-subunit (CTA1) coupled, via an A2 subunit, to a pentamer of B-subunits that facilitate binding (CTB). Other members of the AB_5-toxin family include heat-labile toxin (LT) from *Escherichia coli* and pertussis toxin (PT) from *Bordella pertussis.* The mode of action of CT is well characterized and depends on CTB binding to GM1 gangliosides, present on all nucleated cells [21-23]. Once bound, the entire complex is endocytosed and transported by retrograde vesicular transport through the golgi apparatus to the endoplasmatic reticulum (ER) where CTA1 is released from the A2- and CTB-subunits by reduction of a disulphide bond. The CTA1 protein subsequently unfolds and is delivered to the ER membrane where it is released into the cytosol by ER oxidoreductin 1 (Ero1) via the Sec61 channel [24, 25]. Avoiding ubiquitination and degradation, CTA1 refolds and interacts with cytosolic ADP-ribosylation factor 6 (Arf6) which enables the catalytic activity of the enzyme. Next, CTA1 facilitates the ADP-ribosylation of the α-subunit of the stimulatory G-protein (Gsα) using nicotinamide adenine dinucleotide (NAD) after which Gsα is rendered chronically active. In turn, Gsα activates adenylate cyclase which converts intracellular ATP to cAMP. Increased concentration of intracellular cAMP has many different effects within the cell, but CFTR activation in enterocytes leads to increased secretion of chloride ions and forced efflux of water due to osmotic pressure [26].

More importantly in the context of this thesis, CT is a highly potent experimental adjuvant. Its immunoenhancing abilities are not completely understood, but it has been shown to be mediated by CD11b+ DCs in a Gsα-dependent manner [27] which upregulates co-stimulatory molecules such as CD80, CD86, CD40 and MHCII [28]. However, it is not suitable for human use in its native form since oral ingestion results in severe dehydration. In addition, CT can cause inflammation in the central nervous system (CNS) after intranasal delivery via CTB-mediated binding of olfactory nerves [29]. **CTA1-DD** was developed as an alternative approach to avoid the toxicity of CT [30]. The issue of CTB promiscuity was addressed by replacing it with a dimer of the D-fragment from *Staphylococcus aureus* while retaining the Gsα-activating CTA1 subunit. The fusion protein was initially thought to bind exclusively to B-cells as the DD-domain targets Fc and Fab fragments of immunoglobulins, but has since been shown to also interact with DCs [31, 32]. The adjuvant has been tested in combination with numerous antigens and has proven highly effective at augmenting immune responses in addition to being completely non-toxic [31-34]. Moreover, the incorporation of specific peptides within the vector itself allows for specific co-targeting of adjuvant and antigen, which has proven highly effective in protection against influenza in mice immunized with the vector containing a universal flu vaccine candidate, the matrix protein 2 (M2e) [32].

The immunogenicity of CTA1-DD is dependent on the enzymatic activity of CTA1, as evidenced by the lack of adjuvant function in a mutant construct rendered inactive by a single-point mutation. Interestingly, the substitution of Arginine (R) into Lysine (K) in the resultant **CTA1R7K-DD** mutant construct promoted the induction of tolerance and was

shown to ameliorate collagen-induced arthritis (CIA) in mice upon intranasal (i.n) administration [35]. The observed effect resulted from a population of IL-10 producing FoxP3- T helper cells specific to the incorporated antigen. Because IL-10 knock-out mice failed to develop tolerance, it was concluded that tolerance most likely depended on the formation or suppressive Tr1 cells. Furthermore, Th17 and Th1 responses were specifically inhibited; the exact opposite outcome to that after treatment with the active CTA1-DD adjuvant [36].

As described, **Gsα** is the target protein of CTA1. Though it is currently incompletely understood whether the CTA1 subunit ends up in the cytoplasm in a non-degraded form when coupled with the DD moiety, it is clear that ADP ribosylation is a critical element in the adjuvanticity of CTA1-DD. Gα is one of three subunits (α, β and γ) which make up the heterotrimeric guanine nucleotide-binding proteins (G-proteins). They play a central role in G-protein-coupled receptor (GPCR)-mediated signal transduction, a family of receptors that have very diverse functions and ligands [37, 38]. Engagement of the stimulatory Gα protein (Gsα) leads to elevated intracellular cAMP concentrations, while activating the inhibitory α subunit (Giα) has the opposite outcome. In the immune system, elevated **cAMP** levels have generally been shown to inhibit T-cell function. For example, increased cAMP has been shown to mediate tolerance in neonatal Tregs [39] as well as in a model of HIV infection [40]. Furthermore, removing the gene encoding Gsα (*gnas*) in CD11c+ cells resulted in Th2-skewed responses and prominent allergic asthma whereas increased cAMP inhibited this phenotype [41]. However, these are complex concepts in light of other somewhat contradictory findings, since cAMP has also been shown to enhance Th17 cell differentiation and function after CT administration [42]. In addition, feedback responses to decomposition products of cAMP often have immuno-suppressive effects. For example, after cAMP efflux through a specific transportation system termed multidrug resistance proteins (MRP), it may be metabolized into adenosine by ectophosphodiesterases and ectonucleotidases. Adenosine interacts with four different adenosine receptors within the GPCR family; A_1, A_{2A}, A_{2B}, and A_3 that have either suppressive or exciting effects on the immune system as summarized in figure 2 [43, 44]. Thus, in this context, immunological outcome depends on the cell-specific expression of these receptors.

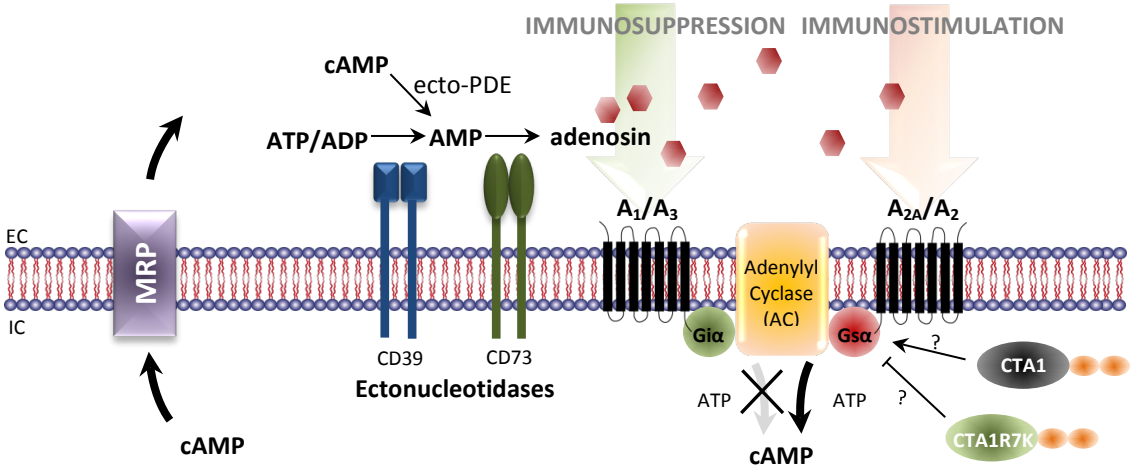


Figure 2. On the complexity of Gsα, cAMP and immune function. Schematic overview of the AC-mediated cAMP production, hijacked by the cholera toxin. Also, subsequent feedback pathways via adenosine receptors are shown, which also includes the metabolization of extracellular ATP via CD39. CTA1 has ADP-ribosylating abilites whereas the interaction between Gsα and the CTA1R7K mutant bindin to Gsα does not result in activation. MRP: multidrug resistance protein, ecto-PDE: ectophosphodiesterase. Adapted from [43].

Immunological tolerance

There are multiple layers of tolerance, a condition which is maintained through mechanisms such as clonal deletion, clonal diversion, receptor editing, anergy and the induction of suppressor cells. While **central tolerance** is broadly a consequence of the selection processes during B- and T-cell maturation in the primary lymphoid organs, **peripheral tolerance** refers to the negative regulatory pathways that instruct the immune system not to respond inappropriately to innocuous antigens in the periphery.

It is well established that antigens administered via the mucosal route typically induce tolerance to subsequent systemic challenge, and **mucosal tolerance** represents a specific type of peripheral tolerance. The phenomenon is best described in the gastrointestinal tract, termed oral tolerance, and scientific reports from as early as 1829 describes how chewing poison ivy prevented skin rashes upon future contact with the plant [45]. In 1911, Wells and Osborne showed that feeding OVA to guinea pigs made them resistant to anaphylactic reactions after systemic challenge with the same antigen [46, 47]. Since then, although many questions remain unanswered, our understanding of the various mechanisms that mediate immunological tolerance have increased considerably.

CENTRAL TOLERANCE

After immature T cells have left the bone marrow (BM), they undergo two rounds of selection in the thymus; positive selection followed by negative selection - or clonal deletion. T cells are CD4+CD8+ double-positive when they first enter the thymic cortex and immediately interact with cortical thymic epithelial cells (cTECs) from which they acquire survival signals given that they bind self-peptides on MHC (self-pMHC) with moderate affinity. The T cells that do not recognize the self-pMHC complex with sufficient affinity die by neglect [48, 49]. Surviving cells upregulate their expression of extracellular-signal-regulated kinase (ERK) and migrate to the thymic medulla in a CCR7-dependent manner where medullary epithelial cells (mTECs) and thymic DCs (tDCs) present a large repertoire of extra-thymic self-antigens that are normally only expressed in specific peripheral tissues (peripheral tissue antigens, PTAs). This process allows for the removal of T cells that are strongly autoreactive by apoptosis. The promiscuous expression of PTAs is mediated by the transcription factor autoimmune regulator (AIRE) through poorly defined mechanisms. However, its function is absolutely vital, as demonstrated by the fact that humans lacking Aire suffer from severe autoimmune syndrome poly-endocrinopathy-candidiasis-ectodermal-dystophy (APCED) [50]. Interestingly, Aire-mediated expression of PTAs is not restricted to the thymus, but gut-associated PTAs have also been shown to be expressed in lymph node stromal cells demonstrating a functional overlap between the thymus and periphery [51].

CD4+CD25^{hi}FoxP3+ natural regulatory T cells (nTregs) are also products of thymic selection and play an important role in the contraction of a normal immune response. Furthermore, they also prevent spontaneous auto-aggressive reactions, as evidenced by scurfy mice which lack a functional FoxP3 gene and succumb to severe autoimmune disease [52]. How nTregs, with their high-affinity TCR for self-peptide recognition, escapes the negative selection process is largely unknown, but may involve specific inhibition of apoptosis in Treg precursors via CD70 and CD27 interactions [53, 54].

PERIPHERAL TOLERANCE: DC-INDEPENDENT MECHANISMS

Although thymic selection is efficient in shaping the circulating T cell repertoire, it fails to eliminate all self-reactive lymphocytes that could induce autoimmunity within a conducive inflammatory context [55]. Moreover, immune responses directed towards certain exogenous antigens, e.g. food derived, would be detrimental to the host. Hence, several peripheral processes exist to rectify these problems.

Firstly, naïve T cells are physically separated from non-lymphoid tissues, a phenomenon referred to as **immunological ignorance**. They are confined to the blood circulation from which they enter secondary lymph nodes via high endothelial venules (HEV). If they do not encounter the antigen that they are specific to, they are returned to the circulation via efferent lymphatics, and thus are precluded from encountering potential cognate antigens concentrated in the parenchyma [56].

Secondly, swift **removal of apoptotic cells** by phagocytosis forms a barrier of ignorance between self-antigens and potential autoreactive T cells. Dying cells will attract macrophages by releasing "find-me" signals, such as fractalkine [57], and exposed phosphatidyl serine (PtdSer) on their cell membrane triggers subsequent engulfment. Furthermore, the clearing of apoptotic bodies actively induces the expression of IL-10 and TGFβ in macrophages, proactively impeding the release of potentially immunogenic content [58, 59]. Indeed, the importance of antigen ignorance is demonstrated by the observation that defects in these processes can lead to leakage of apoptotic bodies and release of pro-inflammatory intracellular content, which have implications for autoimmune disorders such as lupus and rheumatoid arthritis [60].

Lastly, inducing **anergy in T cells** is yet another mechanism of maintaining immunological unresponsiveness to innocuous or self-antigens. The classical model of the induction of a T cell response comprises three signals. *Signal 1* refers to the interaction between the T cell TCR and its specific peptide-MHC (pMHC) complex on the APC, whereas *Signal 2* involves the engagement of co-inhibitory or co-stimulatory molecules. *Signal 3* is a polarizing signal, mediated mainly by cytokines, which promotes the differentiation of specific T cell subsets [61-63]. TCR ligation without sufficient co-stimulation results in apoptosis or clonal anergy, and the T cell is consequently prohibited from differentiating into an effector cell. This typically occurs under homeostatic or sub-immunogenic conditions. In addition, the induction of actively regulatory CD4 T cells (Tregs) provides further suppression when ignorance or anergy is not sufficient to prevent autoimmunity. There are by now a vast number of co-signalling molecules described that are able to promote or inhibit both naïve and effector T cells during and/or after antigen recognition (see page 19).

Dendritic cells

In a series of seminal papers between 1973 and 1974, Steinman and Cohn identified a novel cell type with a distinct morphology in that they could "…assume a variety of branching forms, and constantly extend and retract many fine cellular processes" [64]. Because of their appearance, they were termed dendritic cells (DCs). It would take several years before the mechanisms of DC function were understood, but Steinman postulated early that they had an immune function, and was first to describe their ability to potently induce T cell proliferation *in vitro* [65]. More than a decade later, Charles Janeway introduced the concept

of co-stimulation and unified the innate and adaptive immune system by stressing the point that DCs express pattern-recognition receptors (PRRs) [66]. Today, DCs are regarded as the most potent of all APCs due to their broad spectrum of functions, encompassing the prompt sensing of microbial and self-derived danger-signals followed by efficient migration and subsequent priming of T cells in secondary lymphoid organs. Since then, numerous sub-classifications have added nuance and complexity to our perception of DC heterogeneity, phenotype and function. Currently, DCs are chiefly divided into four major groups; conventional DCs (cDCs), IFN-producing plasmacytoid DCs (pDCs), Langerhans cells and monocyte-derived DCs (moDCs). Precursors in the circulation originate from the BM and differentiate in the peripheral tissues and their respective development and maintenance in these compartments are dependent on FMS-like tyrosine kinase 3 ligand (Flt3L) [67, 68]. An overview of DC ontogeny is simplified in figure 3, and comprehensively reviewed elsewhere [69, 70]. Briefly, pDCs are sensors of viral infection and produce vast amounts of Type I IFNs [71] while circulating moDCs are rapidly recruited to sites of infection where they can produce chemoattractants, cytokines and also participate in tissue repair [72]. However, focus will next be placed primarily on cDCs.

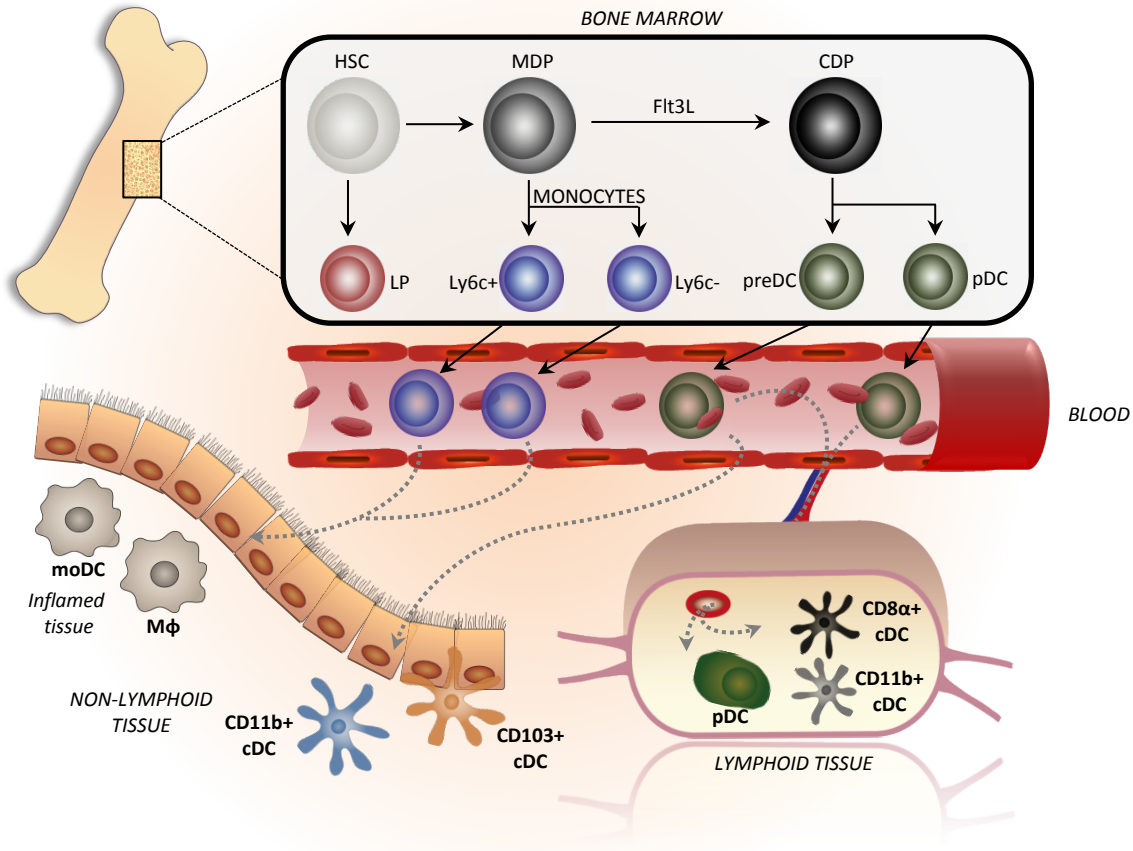


Figure 3. DC development from take-off to tissue. Ly6c+ "classical monocytes" have an inflammatory function whereas Ly6c- "non-conventional" or "patrolling" monocytes have an anti-inflammatory phenotype that is important in wound healing etc [73]. They normally circulate in the blood but are readily recrtuited to inflamed tissues where they differentiate. HSC: hematopoietic stem cell, LP: lymphoid progenitor, MDP: macrophage and DC precursor, CDP: common DC percursors, pDC: plasmacytoid DC, cDC: conventional DC, Mϕ: macrophage, moDC: monocyte-derived DC.

cDCs can be subdivided into two major groups; migratory (migDC) and lymphoid resident DCs (resDC). The canonical life cycle of a migDC revolves around patrolling the non-lymphoid tissues in an immature state, followed by rapid upregulation of genes important for APC-function after uptake of material recognized as detrimental to the host. Next, the migDC will traffic to the lymph node T cell area and pass along their information to potentially present antigen-specific T cells before dying. The comparative lifespan of DC subsets in inflammatory vs steady-state conditions is not extensively elucidated, but a study on splenic myeloid DCs showed that stimulation via CD40 cross-linking or TLR-agonists increased the expression of anti-apoptotic Bcl-xL [74, 75]. In contrast, IL-10 was shown to inhibit Bcl-2 and Bcl-xL, which shortened the life-span of the DCs [76]. Furthermore, the activation status of T cells affects DC survival, as naïve T cells were shown to promote prolonged survival of DCs in the lymph node [77]. Conversely, DCs underwent rapid cell death upon interaction with antigen-specific T cells *in vitro* [78]. In summary, though not fully explored, DC survival may be another potential strategy for either maintaining homeostasis or enhancing immunity.

Immature tissue-patrolling **migDCs** are highly phagocytic and express only low levels of MHCII and co-stimulatory molecules. They sense their environment by the abundant expression of PRRs (e.g. the TLR, RLR, NLR and CLR families) that recognize generic motifs common to many different microbes but not self, such as bacterial cell wall components, dsRNA and fungal polysaccharides, reviewed in [79]. In addition, damage-associated molecular patterns (DAMPs), or self-components associated with e.g. necrosis, results in non-infectious maturation of DCs. Upon maturation, MHCII and a wide range of co-stimulatory molecules, such as CD40, CD80, CD83 and CD86, are upregulated. In addition, the phagocytic activity of the DC halts and CCR7 is upregulated which enables migration towards a CCL19 and CCL21 gradient, produced in the T cell zone of secondary lymphoid tissues. Currently, migDCs are divided into two distinct lineages; CD11b+ DCs and Batf3-dependent CD103+ DCs, although a compensatory Batf3-independent pathway was recently described [80].

ResDC occupy the LN, SP or thymus where they present foreign and host-derived lymph-borne antigens. Compared to migDCs, they are less mature and more phagocytic, but can be activated following the same mechanisms. They are phenotypically classified into CD4+CD11b+CD8α-, CD4-CD11b-CD8α- or Batf3-dependent CD4-CD11b-CD8α+ DCs. Generally speaking, resident CD8α+ and migratory CD103+ DCs are better at uptake of apoptotic cells and cross-presentation of antigens to CD8+ T cells whereas CD11b+ DCs are better at priming CD4 T cells. However, there is also significant functional plasticity between these subsets.

ANTIGEN UPTAKE, PROCESSING AND PRESENTATION

Once the DC has taken up proteins from its surrounding, these must be processed and loaded onto vesicular MHCII molecules which subsequently traffic to the cellular membrane and becomes available for interaction with antigen-specific T cells. In addition, endogenous antigens are constitutively presented onto MHCI molecules, present on all nucleated cells. However exogenously derived peptides may also be cross-presented on MHCI via poorly characterized pathways, predominantly carried out by batf3-dependent DCs *in vivo* [81]. As CD8+ T cell priming is not reviewed in this thesis, the following section will only briefly

summarize the MHCI pathway and instead focus on antigen processing and presentation by MHCII. Briefly, **MHCI** antigen presentation involves ubiquitination of misfolded self-peptides or cytosolic peptides from intracellular bacteria and viruses, which are all subsequently degraded by the proteasome. APCs constitutively express an immuno-proteasome, specialized for production of peptides suitable for CD8+ T cell priming. Further trimming by cytosolic peptidases sometimes precede translocation to the ER by transporter associated with antigen presentation (TAP). After ER translocation, peptides are loaded onto MHCI, a process mediated by specific chaperones, and transported to the cell membrane.

MHCII processing is typically carried out by professional APCs and results in the presentation of endogenous and exogenous peptides by autophagic or classical pathways respectively. The uptake of extracellular antigens is carried out by receptor-mediated endocytosis, pinocytosis or phagocytosis, as summarized in figure 4. Principally, after uptake the antigen is processed and shuttled through progressively acidic endosomal vesicles and finally merge with MHCII-containing late endosomes. Here, the MHCII chaperone invariant chain (Ii) is cleaved into the shorter class II associated invariant chain (CLIP) and the chaperone H2-M mediates the substitution of CLIP to antigenic peptides prior to membrane

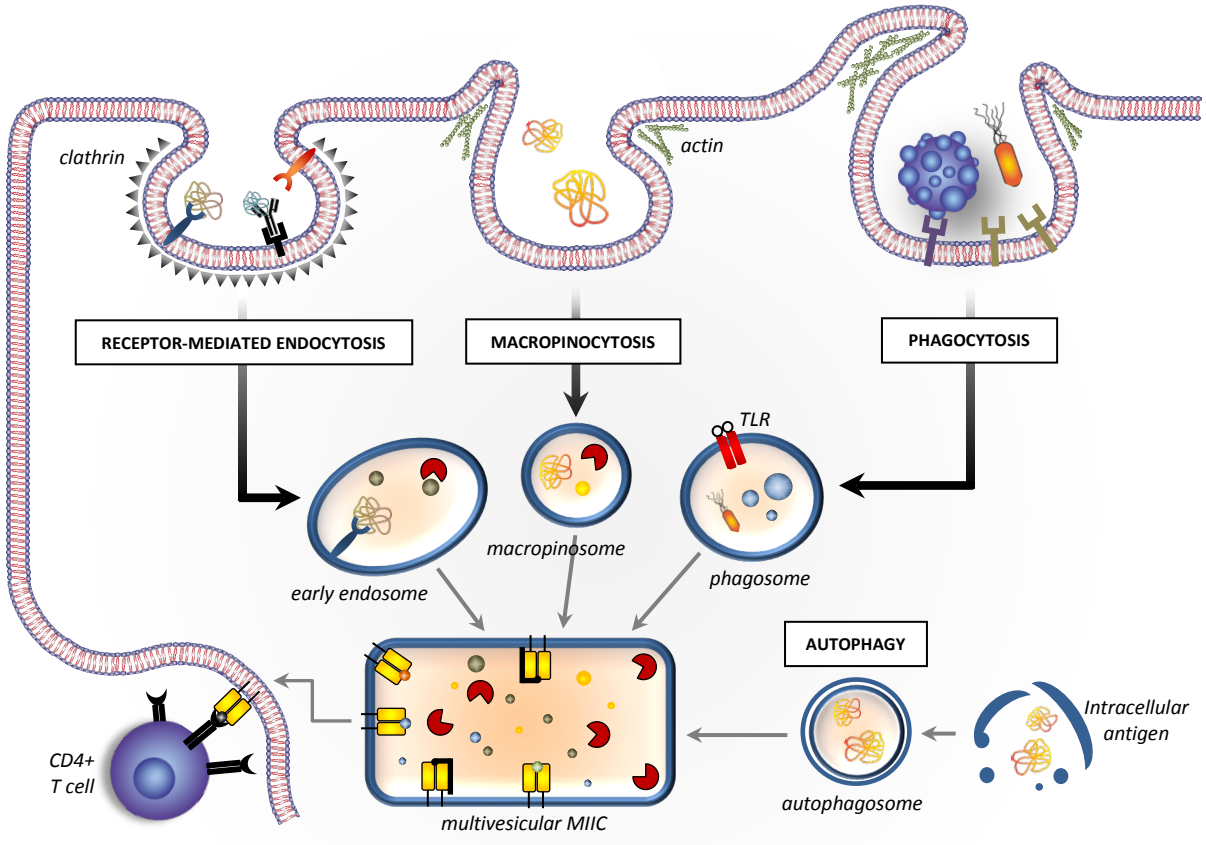


Figure 4. A simplified overview of antigen uptake and processing pathways, adapted from [82]**.** Endocytosis via clathrin-coated pits is mediated by several endocytic receptors on the APC, e.g. Fc-receptors, complement-receptors and lectin-receptors among others. Macropinocytosis is actin-dependent and leads to a non-specific uptake of exogenous soluble material, whereas opsonized particles, pathogens or dead cells are phagocytosed via scavenger-receptors or complement-dependent pathways etc. During autophagy, ER-derived membrane envelop intracellular constituents and the subsequent autophagosome merge with MHCII-containing vesicles.
MIIC: MHCII-loading compartment

transport via tubules. These processes involve several different proteases, named cathepsins. Their function considered, cathepsins are important editors of the MHCII surface epitope repertoire and their expression will significantly influence the induction of both immunity and autoimmunity. For example, inhibiting serine-protease cathepsin S (CatS) resulted in delayed loading of peptide onto MHCII which reduced overall antigen presentation and had beneficial effects in murine models for MS [83] and Sjögrens syndrome [84]. Furthermore, CatS is selectively upregulated in psoriasis patients [85] and CatS mRNA levels are elevated in atherosclerotic tissues [86]. *In vivo*, the activity of CatS is regulated during DC maturation via its inhibitor, cystatin C. These findings illustrate the fact that manipulating antigen presentation pathways is yet another mechanism by which immunity and tolerance may be regulated.

DECISION-MAKING PROCESSES IN TOLERANCE AND IMMUNITY

As previously stated, pMHCII complexes on the DC trigger antigen-specific TCR-signalling, but it is the co-stimulatory or co-inhibitory surface molecules that control T cell differentiation, effector function and survival. As such, the combined expression of these molecules is critical in fine-tuning the immune response to innocuous or detrimental antigens. Importantly, the co-receptor repertoire undergoes dynamic changes in a spatiotemporal fashion, thus adding to the complexity. Although relatively little is known about how these different co-signalling pathways integrate on a larger scale, much work has gone into understanding and characterizing individual pathways.

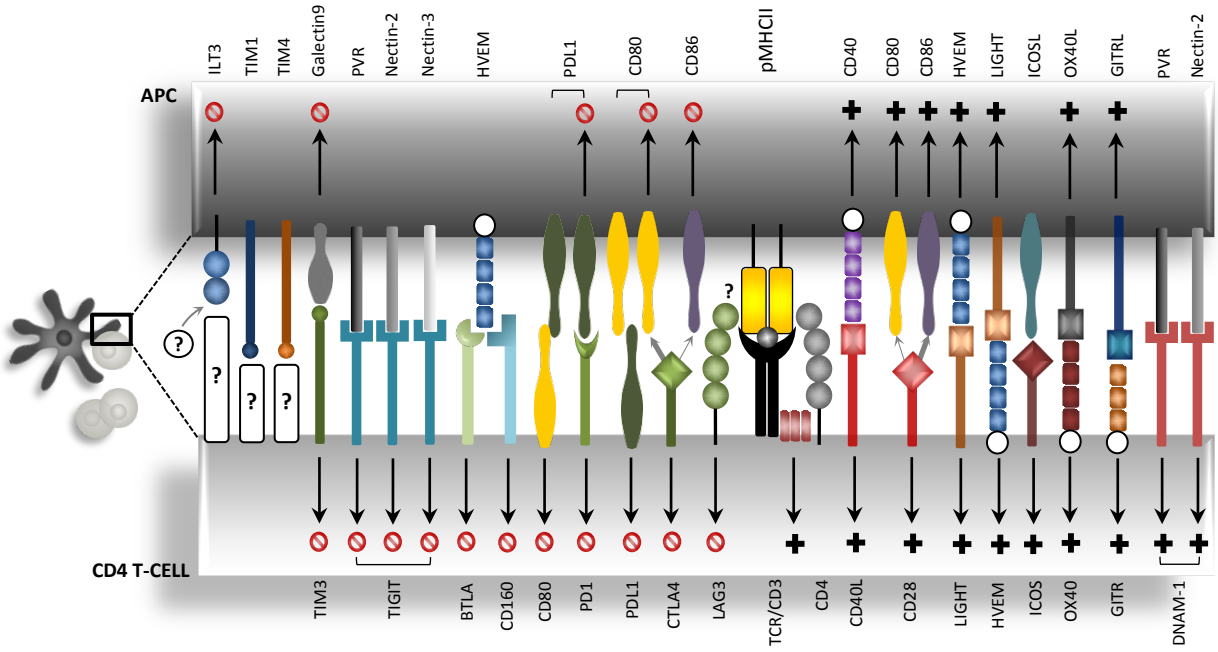


Figure 5. Selected co-signaling molecules on the APC and on the CD4 T cell, adapted from [63, 87-89]. Arrows indicate signaling direction and "+" translates to co-stimulation and co-inhibition is shown as a "stop"-sign. ILT: Immunoglobulin-like transcript, TIM: T cell immunoglobulin and mucin domain, PVR: Poliovirus receptor, TIGIT: T cell immunoreceptor with immunoglobulin and ITIM domains, BTLA: B and T lymphocyte attenuator, HVEM: Herpes virus entry mediator, PD: Programmed cell death protein, PDL: programmed death ligand, CTLA: Cytotoxic T-lymphocyte-associated protein, LAG: Lymphocyte activation gene, ICOS: Inducible T-cell co-stimulator, GITR: Glucocorticoid-induced TNFR-related protein.

Co-signalling molecules are generally members of two superfamilies, namely the immunoglobulin superfamily (IgSF) and the TNF receptor superfamily (TNFRSF). Some of these molecules, which are either relevant to this thesis or amongst the most well-characterized, are represented in figure 5. To briefly summarize; members of TNFRSF typically contain at least one extracellular cysteine-rich domain (CRD), and co-stimulatory HVEM, CD40, and OX40 are examples of such molecules. IgSF members, however, consist of immunoglobulin (Ig) domains, of which the CD28 and B7 families (e.g. CD80 and CD86) have been best described. They typically interact with each other, save for the co-inhibitory BTLA molecule which binds to HVEM. Other members of IgSF include TIMs, which have both stimulatory and inhibitory functions [90] but need to be further characterized and LAG3, a CD4-homologue that interacts with MHCII and possibly other unidentified proteins [91]. DNAM1 and TIGIT both interact with nectin-like ligands but have co-stimulatory and co-inhibitory functions, respectively. Lastly, the ligand of ILT3 is unidentified, but activation has been shown to suppress CD4 T cell responses [92] as well as NF_KB signalling in the DC itself [93-95].

Arguably, there are at least four different modalities by which co-signalling net effect is regulated. Firstly, the **actual expression of any given co-receptor** will naturally determine whether or not it will participate in the priming process. This would primarily be regulated at the transcriptional or post-transcriptional level, but there may be additional mechanisms that influence the amount of existing surface molecules. One such example is the trans-endocytosis of CD80 and CD86 by CTLA-4. Here, CTLA-4 expressed by the T cell physically rips the CD80 or CD86 molecules from the membrane of the APC and, in effect, downregulates their surface-available quantities [96]. Furthermore, a secondary level of co-signalling regulation is the **differential expression of interaction partners**. For example, HVEM is ubiquitously expressed on many different cell types, whereas expression of one of its ligands; LIGHT, is restricted to APCs and T cells [97]. Also, CD28 is constitutively expressed on naïve T cells but its ligands, CD80 and CD86, are only upregulated in the APC upon recognition of “danger-signals” in the periphery [98].

Next, as several co-signalling molecules are able to interact with more than one ligand, **multiple binding partners facilitates multiple outcomes**. There are numerous examples illustrating this concept. Firstly, both CD28 and CTLA-4 bind to CD80 or CD86, and net outcome depends on their relative expression; except for nTregs where expression is constitutive [99], CTLA-4 is typically upregulated on T cells at later stages of activation. Furthermore, CTLA-4 is a higher-affinity homologue of CD28 and thus readily outcompetes co-stimulatory signalling once expressed. Hence, the differential temporal expression pattern of CD28 and CTLA-4 constitute a natural negative feedback loop to preclude excessive immune responses. Furthermore, aside from CD80 interacting with CD28 and CTLA-4, PDL1 interactions have also been described [100, 101]. Therefore, CD80 expression confers both stimulatory and inhibitory immune signals in a context-dependent manner. Finally, the TIM receptors consist of an IgV domain and a mucin domain. Interestingly, using monoclonal antibodies (mAbs) which targets the IgV domain specifically leads to co-stimulation whereas antibodies directed towards the mucin domain leads to inhibited T cell responses. Thus, these motifs may be variably used to interact with different ligands [102], but whether these regulatory circuits reflect their *in vivo* function remains to be further examined.

Lastly, not only does CTLA-4 and CD80/86 interaction result in inhibitory signalling events within the T cell directly, but it also upregulates IDO expression in the APC which indirectly enforces T cell inhibition by tryptophan deprivation [103]. Thus, **bi-directional co-signalling** is another layer of regulation within these molecular networks. Interestingly, these pathways also transcend cellular networks, as CD80 on T cells can receive inhibitory signals after interaction with PDL1 on the APC, but PDL1 on T cells can also transduce inhibition via CD80 on other T cells [101]. However, whether PDL1 on T cells can induce co-inhibition via T cell-expressed PD-1 (fig. 5) is not yet known.

In conclusion, these examples collectively illustrate the many regulatory modalities that determine the final nature of the immunological synapse, through mechanisms of exclusion, competitive inhibition and potentially many others. How these pathways altogether control the differentiation of specific T cell subtypes is poorly understood, but it is likely to include many other secreted mediators that act both on the T cell and on the APC (signal 3). Thus, the intricacy of early decision-making processes in the immune system at large is a subject that we are only beginning to understand and requires further study using a meta-perspective.

PERIPHERAL TOLERANCE: DC-DEPENDENT MECHANISMS…

The contribution of DCs in maintaining peripheral tolerance is underscored by the break-down of CD4 T cell tolerance to self, which results in fatal autoimmunity, following their constitutive ablation [104]. However, this concept remains somewhat contentious, as Birnberg *et al.* found no such phenotype after DC removal, though not all DC subtypes were removed in that study [105]. Nevertheless, it seems most plausible that DCs are critical for immune tolerance as they instruct the differentiating CD4 T cells to acquire functional phenotypes, including FoxP3+ Tregs and Tr1 cells.

Upon infection or inflammation, all DC subsets have the ability to potently induce Th1, Th2 or Th17 effector responses but in the steady state they seem uniquely equipped to preclude excessive or autoreactive immune responses [89]. For example, pulmonary CD103+ DCs preferentially induce Tregs [106-108], whereas the CD103- DCs have similar function in the skin [109, 110]. In fact, much effort has gone into identifying a “tolerogenic DC” subtype but it is important to note that the tolerogenic properties observed in the steady state are also considerably plastic. The DCs are still subordinate to their microenvironment. It is by now well-established that CD103+ DCs in the intestine are crucial in promoting Treg formation and gut homing due to their unique capacity to metabolize vitamin A into retinoic acid (RA) [111-113]. Nevertheless, CD103+ DCs from colitic mice transferred to naïve recipients were impaired in their ability to induce Treg differentiation and instead favoured the development of IFNγ-producing Th1 cells [114]. Conversely, several studies demonstrate the tolerogenic conditioning of DCs *in vitro* using a variety of immunosuppressive agents such as vitamin D3, vitamin A, retinoids, IDO, IL-10, and TGFβ among others. These stimuli endow the DCs with the ability to induce T cell tolerance and suppress experimental autoimmune disease, such as type 1 diabetes and EAE [115-119]. Similarly, tumour-derived factors modulate DC function so that they promote the induction of Tregs that counteracts tumour clearance [120]. Lastly, PRR signalling typically induce DC maturation and immunogenicity; however in

some cases they instead promote quiescence. Thus, TLR2 signalling by yeast zymosan or ligation of the TAM receptor tyrosine kinase Mer (merTK) by ligands on apoptotic cells induces Tregs and inhibits DC maturation [89, 121, 122]. In summary, DCs confer T cell tolerance either by the ligation of tolerogenic receptors or by virtue of immaturity, resulting in the secretion of immunosuppressive cytokines or the lack of pro-inflammatory cytokines.

The transcriptional programs that govern DC tolerization are not completely understood, but NF_KB activation is crucial in the upregulation of MHCII and co-stimulatory molecules in DCs. Indeed, blocking this pathway subsequently inhibits DC maturation and reduces T cell responses [123-125]. Other key pathways include the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) proteins, which are phosphorylated downstream of several cytokine receptors and often act to enhance immune responses [126]. However, STAT3 deficiency restricted to CD11c+ cells results in spontaneous inflammation and mild autoimmunity [127], demonstrating that STATs have both immunogenic and tolerogenic functions. Furthermore, the suppressors of cytokine signalling (SOCS) proteins are important negative regulators of STATs by their competitive binding to JAKs. Indeed, upregulation of SOCS1 has been observed in tolerized DCs which correlated with decreased production of IL-12 and TNFα [128]. Finally, Galectins are S-type lectins that have also been shown to regulate DC function. For example, Galectin-1 promotes the expression of STAT3 and SOCS1 in DCs and knock-out mice are more susceptible to autoimmune disease [129, 130]. In addition, Galectin-9 interacts with TIM-3 and suppresses Th17 responses whilst promoting Treg differentiation [131].

…WITH FOCUS ON: THE IL-27 PATHWAY

The cytokines IL-10 and IL-27 are both linked to the development and function of regulatory T cells and receptor signalling for both cytokines is mediated by STAT3 phosphorylation [132-135]. More recently, DC modulation by IL-27 was shown to enhance the expression of the ectonucleotidase CD39 (fig. 2) in the DC itself, which conferred protection in the EAE model [136]. In addition, though the dominant source of IL-27 is thought to be myeloid cell populations [137], T cells were recently shown to produce IL-27 in a model of malaria parasite infection [138]. Thus, IL-10 and IL-27 have multiple sources, roles and targets during the induction and maintenance of peripheral tolerance, which will be further substantiated in the section that concerns T cell differentiation.

The IL-27 cytokine is a heterodimer composed of IL-27p28 and Epstein-Barr virus-induced gene 3 (EBI3), which upon engagement with the IL-27 receptor (IL-27R) activates JAK-STAT and mitogen activated protein kinase (MAPK) signalling. IL-27 belongs to the IL-12 family of cytokines, and can form various heterodimers with other subunits within this group. For example, the immunoregulatory cytokine IL-35 consists of EBI3 and IL-12p35 and have been linked to Treg activity [139]. Furthermore, administration of a recombinant heterodimer of IL-27p28 and IL-12p40 inhibited Th1 and Th17 responses while promoting the expansion of Tregs in a model of experimental autoimmune uveitis [140], though it is unclear whether this heterodimer forms naturally. Moreover, IL-27p28 has been shown to be secreted as a monomer in an EBI3-independent manner [138], and overexpression of this subunit alone resulted in attenuated anti-tumour responses and suppression of graft rejection [141].

IL-27 was initially thought to promote Th1 responses and, indeed, the observation that IL-27 signalling activated STAT1 and T-bet expression which increased IFNγ production justified this hypothesis [142, 143]. However, when challenging IL-27rα-/- mice with various pathogens [144-149], or using these mice in models of autoimmunity [150-153], IL-27 emerged as an important negative regulator in the duration and intensity of immune responses. It has since been shown to inhibit Th1, Th2, Th9 and Th17 responses in multiple studies [154]. With regards to Th17 development, IL-27 exerts its inhibiting effects through the activation of STAT1/STAT3 and T-bet expression, which counteracts expression of RORγt [150, 155]. Furthermore, a transcription analysis of CD4 T cells exposed to IL-27 revealed a specific upregulation of PD-L1 which inhibited Th17 responses through T-cell-T-cell interactions in the EAE model [156]. In fact, IL-27 mediated induction of PD-L1 has also been observed in human DCs [157]. In addition, the upregulation of CD39 on DCs reduces extracellular ATP (eATP), which otherwise downregulates IL-27p28 expression via purinergic receptors [158] and has pro-inflammatory properties in general [159].

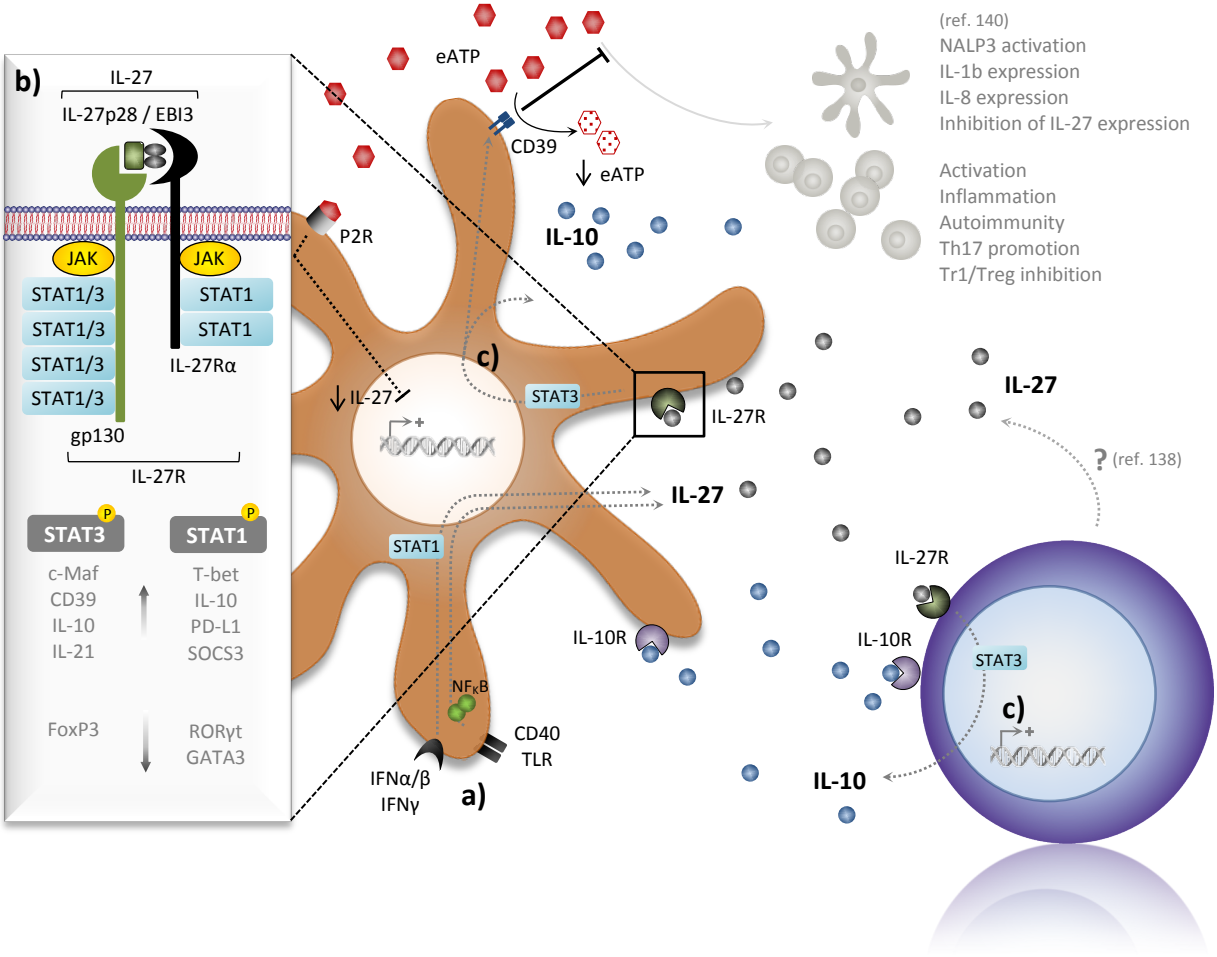


Figure 6. The 101 of IL-27 in peripheral tolerance, adapted from [158, 160-162]. **a)** IL-27 expression is induced after IFN signalling in a STAT1-dependent manner, or via e.g. TLR/CD40 ligation which triggers the $NF_{K}B$ pathway. **b)** IL-27 signals via the IL-27R that activates STAT1 and STAT3, and has several immunobiological functions. **c)** For example, immunosuppressive mediators CD39 and IL-10 are induced in DCs as well as CD4 T cells. Through these means and potentially many others, IL-27 produces a microenvironment that promotes tolerance. NALP3: NACHT, LRR and PYD domains-containing protein 3, eATP: extracellular ATP, P2R: purinergic receptors.

IL-27 has been implicated in the induction of IL-10 expression in that it upregulates the aryl hydrocarbon receptor (Ahr), which synergizes with c-Maf to drive IL-10 transcription. In addition, IL-27 promotes IL-21 production which, in turn, sustains IL-10 expression by further induction of c-Maf [163, 164]. However, their relationship is not an exclusive one, as IL-10-/- mice develop spontaneous colitis and have increased susceptibility to cancer whereas none of these phenotypes are found in mice devoid of IL-27 or IL-27rα. These discrepancies are thought to suggest that only IL-10 is critical in the preservation of homeostasis, whereas both cytokines are important during inflammation [158]. Indeed, multiple studies have established that IL-27 could promote IL-10 production in differentiated Th1, Th2, Th17, Treg and Tr1 cell subsets [158]. Interestingly, in the clinic IFNβ is used to treat multiple sclerosis (MS), and in the EAE model this pathway was shown to induce IL-27, which successfully prevented disease [165]. In addition, the amount of IL-27 that was produced in response to IFNβ treatment of MS patients correlated with therapy efficacy [166], further substantiating the link between IFNβ and IL-27.

In summary, though there are still some contradictions in the literature in need of reconciliation, our knowledge on IL-27 biology and function has profoundly increased since its discovery. In particular, many studies on the subject share the common theme that IL-27 antagonizes Th17 responses whilst augmenting IL-10 production in numerous cell types. Its additional abilities to promote the expression of PD-L1 and activating Tregs have led to the concept of IL-27 acting as a regulatory hub where several immunoregulatory pathways converge during antigenic challenge [158]. However, the context-dependent functions of IL-27 are still poorly characterized and its simultaneous impact on several immune populations needs further elucidation. Some of the pathways described in this section are summarized in **figure 6**.

Differentiation of T helper cells

A properly tailored CD4 T helper (Th) cell population is central to most adaptive immune responses where they act as positive or negative regulators, mainly by cytokine production, but also through cell-contact dependent interactions and metabolic alterations. They are present at the inductive site where they are essential during antibody class-switch and affinity maturation. Furthermore, they home to the effector site where they enhance the cellular functions required to either clear the host of a pathogen or protect it from autoreactive responses, excessive immunity and tissue damage. Naturally, such diverse scenarios require diverse strategies and functions. Thus, once CD4 T cells interact with their cognate antigen and become activated, they will differentiate into distinct lineages, a process largely dependent on the complex network of co-signalling molecules and the specific cytokine milieu [167]. Although T cell subsets were initially thought to only consist of the Th1 and Th2 lineages, described by Mosmann & Coffman in the 1980s [168], we now know that CD4 T cells are considerably more complex and heterogeneous than initially envisaged. The different subsets are defined by the activation of specific STATs, which in turn regulate the expression of specific master regulator transcription factors. Furthermore, aside from the different effector T cells described to date, there are also several subsets of regulatory T cells. They are broadly classified as thymus-derived nTregs and peripherally induced iTregs, of which the latter can be further divided into additional subsets. An overview of the differentiation of some T cell subsets is represented in **figure 7**.

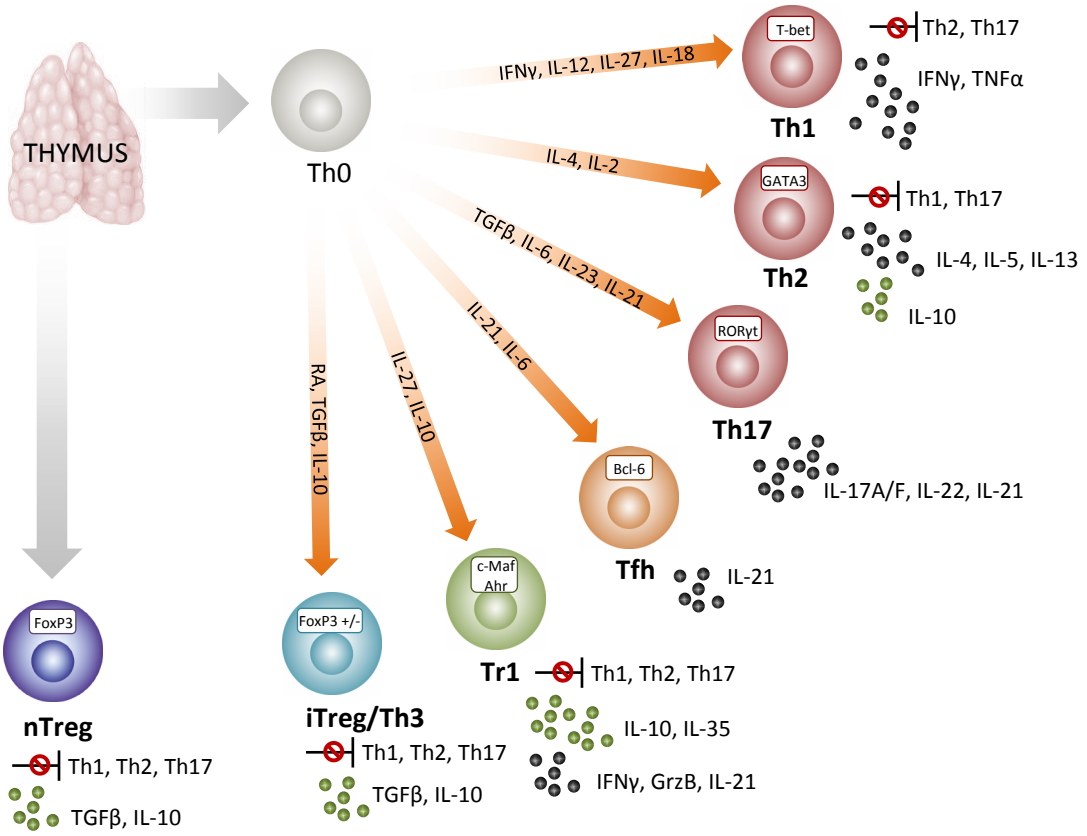


Figure 7. Schematic overview of the differentiation of some T helper cell subpopulations described in the literature. Other, less well established, subsets include Th9 and Th22, reviewed elsewhere [169]. Stop signs indicate the inhibition of differentiation of specific T cell subsets. FoxP3: Forkhead box P3, ROG: Repressor of GATA, Ahr: Aryl hydrocarbon receptor, Bcl-6: B-cell lymphoma 6, RORγt: RAR-related orphan receptor gamma, GATA3: GATA-binding protein 3, T-bet: T-box transcription factor.

Th1 cells primarily secrete pro-inflammatory cytokines IFNγ and TNFα and are important in the activation of macrophages and the expansion of cytolytic CD8+ T cells (CTLs). As such, they are particularly useful in the defence against viruses and intracellular bacteria. Their phenotype is governed by the master regulator T-bet, which promotes the expression of Th1-specific genes [170, 171] and suppresses genes specific to other lineages, such as GATA3 and IL-4 [172, 173], or RORγt [174]. Th1 differentiation is driven by several cytokines which act through their respective signalling pathways in a cooperative fashion. Briefly, the expression of T-bet is strongly dependent on STAT1 which is activated by IFNγ signalling [171, 175]. In turn, T-bet induces the expression of IFNγ as well as the IL-12β2 receptor subunit on the T-cell. IL-12 is secreted by APCs which activates STAT4 upon receptor binding and induces IFNγ expression in the T cell even further. Thus, the coordinated signalling by all these factors combined creates a positive feedback loop which amplifies the Th1 response. In addition, IL-18 and IL-27 signalling synergizes with IL-12 and enhance IFNγ and T-bet expression, respectively. Of note, the IL-18Rα is induced by the IL-12/STAT4 pathway and the contribution of both IL-18 and IL-27 in Th1 function is demonstrated by the impaired Th1 responses in mice lacking either of these cytokines [143, 176-178].

Th2 cells, on the other hand, produce their hallmark cytokines IL-4, IL-5 and IL-13. Th2 function involves the activation of mast cells and eosinophils and the promotion of IgG1 or IgE isotype class-switch. Therefore, they are typically generated in response to extracellular parasites, such as helminths and nematodes. However, dysregulated Th2 responses are also associated with aberrant IgE-mediated mast cell activation and release of histamines during allergic responses. IL-4 and IL-2 are critical in Th2 differentiation and expression of the master regulator, GATA3, is dependent on IL-4/STAT6 signalling. GATA3 has been postulated to enhance Th2 cytokine production, promote Th2-specific proliferation through growth factor independent-1 (Gf1) and to selectively inhibit Th1 differentiation by inhibition of STAT4 [179, 180]. In addition, IL-2 activates STAT5 which, together with GATA3, is required in IL-4 expression since the transcription factors bind to different sites of the IL-4 locus. The initial source of IL-4 remains unclear. However, GATA3 but not IL-4 has been shown to be indispensable for Th2 differentiation *in vivo*, suggesting that an IL-4 independent GATA3 induction pathway exists [167].

Th17 cells are important in the defence against extracellular pathogens, such as bacteria or fungi. The key cytokines in sequential Th17 differentiation include IL-6, TGFβ, IL-21, and IL-23, with RORγt as the master regulator that enforces the Th17 phenotype. Briefly, IL-6 and TGFβ initiate Th17 lineage commitment, IL-21 amplifies the response, and IL-23 stabilizes and maintains the Th17 population [167]. Interestingly, TGFβ on its own induces FoxP3 expression in iTregs while RORγt, IL-21 and IL-23R is preferentially induced if IL-6 is also present [181-184]. Thus, Th17 and iTreg responses seem antagonistically related in an IL-6 dependent manner. Unlike IL-6, IL-21 and IL-23; TGFβ does not signal directly via STAT3 but rather inhibits SOCS3, which otherwise negatively regulates STAT3 signalling [185]. STAT3 induces the expression of RORγt and binds to IL-17A and IL-17F promotors, which in conjunction with other factors induce the expression of these hallmark cytokines. IL-17 recruits monocytes and neutrophils by induction of numerous cytokines and chemokines, and indeed, dysregulated Th17 responses are implicated in several autoimmune diseases such as arthritis, multiple sclerosis and psoriasis [186]. Of note, recent studies have described a regulatory Th17 cell that appears to have anti-inflammatory functions when generated in the absence of IL-23. Their unique function was associated with an increased expression of the CD5L gene [187].

Tfh cells are critical in maintaining the germinal centre (GC) reaction, primarily by their secretion of IL-21 [188] but also via CD40:CD40L ligation [189], both of which promote B-cell survival. Bcl-6 is considered the master regulator as it induces and sustains the expression of IL-21 and CXCR5, while inhibiting the function of T-bet, GATA3 and RORγt [190]. IL-21 is important in Tfh and B-cell survival, whereas CXCR5 expression enables Tfh migration to the B-cell follicle in a CXCL13 dependent manner. Their differentiation is mainly determined by IL-6 and IL-21 signalling, which results in STAT3 activation and induction of Bcl-6. RORγt is not induced by the STAT3 pathway in the absence of TGFβ which it otherwise is during Th17 development. Regulatory **Tfr** cells were recently described, which are FoxP3+Blimp-1+CXCR5+ cells localized in the GC where they potently suppress both Tfh- and B-cells during the GC reaction [191-193]. They were initially thought to derive from nTreg precursors, suggesting that they would be specific to self-antigens and important in precluding the generation of auto-antibodies. This hypothesis was supported by the

observation that Tfr cells did not develop from TCR-transgenic T cells specific to an exogenous antigen [194, 195]. However, this idea has been challenged by more recent studies that suggest that they can also arise from naïve precursors [196]. Of note, the ratio of Tfh to Tfr cells is thought to be important in determining GC output, not least demonstrated by the defective humoral responses of aged mice due to a relative increase in functional Tfr cells [197]. Conversely, reduced Tfr numbers in relation to Tfh cells correlated with autoimmunity [198].

T-CELL MEDIATED SUPPRESSION…

Sakaguchi *et al.* first identified a population of CD4+CD25+ T cells that had suppressive properties and were subsequently termed regulatory T cells (Tregs) [199]. Almost a decade later, the discovery of their master regulatory transcription factor, FoxP3, represented a major breakthrough in that it allowed for Treg identification with unprecedented specificity [200]. As previously mentioned, their existence is essential since deletion or disruption of the FoxP3 gene results in fatal immunopathology [200, 201]. In addition, there are Tregs induced in the periphery that do not express FoxP3 but have important regulatory function; the IL-10 producing type-1 regulatory T cells (Tr1) and the TGFβ producing suppressive Th3 cells [202]. Principal mechanisms of suppression by Tregs include 1) cell-cell contact molecules, 2) secretion of inhibitory cytokines, and 3) alteration of metabolic pathways. For example, LAG3 is thought to have immunomodulatory functions on DCs via MHCII interactions and, in addition, some Tregs secrete Granzyme B which kills the recipient cell in a contact-dependent manner [203]. Furthermore, the secretion of TGFβ, IL-35 and IL-10 is closely related to immune suppression [139, 204, 205]. Finally, ectoenzymes CD39 and CD73 are expressed on the surface of Tregs, which are able to metabolize pro-inflammatory ATP into adenosine in the extracellular space, resulting in the metabolic disruption of neighbouring cells [206].

The expression of FoxP3 is evidently critical for Treg function, but in fact, the induction of several surface markers associated with Tregs is not dependent on this transcription factor. Importantly, FoxP3 is located on the X chromosome. Thus, in female mice, all cells express only one of the two inherited alleles through a stochastic process termed X chromosome inactivation. This phenomenon was exploited in an elegant study, in which female mice were heterozygous for a non-functional FoxP3 gene that also expressed enhanced Green flourescent protein (EGFP). Here, EGFP+ (FoxP3-) cells were shown to express several Treg-associated markers such as CD25, CTLA-4 and GITR [207]. Thus, expression of these factors was FoxP3-independent. Interestingly though, their suppressive capacity was lost in the absence of a functional FoxP3 gene. Further studies on the transcriptome of Tregs isolated from different anatomical sites revealed distinct co-regulated gene clusters specific to their localization, indicating that the Treg population is indeed comprised of several subsets and that their suppressive function is regulated by the microenvironment [208, 209]. No major differences in the transcriptional signature of nTregs and iTregs were observed. However, their TCR repertoire is very different in that iTregs recognize primarily foreign antigens and nTregs are selected on the basis of high-affinity binding to self-antigens [210]. Thus, iTregs are thought to be of particular importance at mucosal surfaces where they balance or preclude immune responses to non-self antigens whereas nTregs are more important in non-mucosal tissues such as CNS and pancreas.

nTregs are difficult to phenotypically distinguish from peripherally induced FoxP3+ iTregs as they both express many of the canonical Treg markers, such as CD25, CTLA-4 and PD-1 (see page 22-23). Therefore, it remains a challenge to dissect whether nTregs have different or overlapping functions compared to iTregs, or both [211]. Helios was first described as a transcription factor specifically important for nTreg function, as inhibited expression resulted in downregulated FoxP3 and attenuated suppressive function in these cells. Furthermore, the marker was not expressed in antigen-specific iTregs [212, 213]. However, the use of Helios to distinguish between nTregs and iTregs is controversial, as it has since been found to be transiently expressed during T cell activation and expansion, in particular during Th2 and Tfh responses [214]. More recently, neuropilin 1 (Nrp-1) has been proposed as a promising new discriminative marker as it is selectively expressed on nTregs in the steady state [215, 216]. Indeed, though conditional deletion of Nrp-1 in T cells had no effect on the overall subset repertoire [217], EAE disease severity was exacerbated in mice with this genetic defect [218]. Moreover, Nrp-1 is a high affinity receptor for TGFβ1, and receptor-ligand interaction results in increased Treg cell activity [219, 220]. These data collectively demonstrate that Nrp-1 has functional relevance for the maintenance of immunological homeostasis. However, under inflammatory conditions such as during chronic EAE, expression of Nrp-1 was not restricted to nTregs but also included iTregs, both in the CNS and in the spleen [215]. Therefore, though there are still questions with regards to Nrp-1 expression, function and distribution during inflammatory challenge, it may serve as a marker for nTregs under steady-state conditions [211, 221].

iTregs that express FoxP3 can be formed *in vitro* from CD4+FoxP3- precursors in the presence of IL-2 and TGFβ [222]. In fact, when naïve CD4 T cells specific to a non-self antigen are transferred to lymphopenic mice that express this protein systemically, an autoimmune disease profile is transiently manifested, similar to that of graft-versus-host disease (GVHD). Subsequent spontaneous disease recovery is associated with the formation of FoxP3+ iTregs. However, in the absence of IL-2, disease recovery does not occur but rather continues to progress [223]. Thus, IL-2 is critical for iTreg generation, and their suppressive function has been proven essential in several inflammatory models [206]. However, the mechanistic details of IL-2 in iTreg development *in vivo* remains opaque. IL-2 signaling was shown to limit Th17 polarization in a STAT5-dependent manner [224] which may help to explain the IL-2 dependency in FoxP3+ iTregs. Many other conditions and signalling pathways are also important in iTreg formation. Firstly, low antigen doses promotes iTreg development due to decreased net TCR signaling [225] and similarly, strong CD28 signaling or CTLA-4 inhibition results in reduced iTreg induction [206]. TCR-signaling activates the PI3K-AKT-mTOR signaling pathway which ultimately results in increased proliferation and impaired Treg formation. In fact, this pathway is normally repressed in FoxP3 positive Tregs, and overexpression of AKT in mice results in reduced Treg induction and autoimmunity [226]. Conversley, inhibitors of mTOR such as Rapamycin®, are used in the clinic to prevent organ transplant rejection [227]. Finally, TGFβ signaling both induces and maintains FoxP3 expression by histone remodelling of an enhancer region within the FoxP3 gene, mediated by NFAT and Smad3 transcription factors [228].

Of note, **Th3** cells represent an alternative iTreg subset that is associated mainly with TGFβ production during oral tolerance [229]. The lack of specific markers to this subset precludes in-depth study, but they are thought to arise from naïve precursors after low-dose antigen

exposure when TGFβ and retinoic acid (RA) is present. Whether they express FoxP3 or not remain unclear and indeed, whether they actually constitute a distinct subpopulation also [230, 231].

…*WITH FOCUS ON: Tr1 CELLS*

Historically, Tr1 cells were identified by their lack of FoxP3 expression and their unique cytokine signature, which is typically dominated by IL-10, but also includes TGFβ, IFNγ, IL-5, low to no levels of IL-2, and a distinct lack of IL-4 [232]. More recently, Roncarolo *et al* suggested that the co-expression of surface molecules LAG3 and CD49b was specific to both human and mouse Tr1 cells and could be used in combination to identify these cells [233]. Membrane-bound LAG3 has inhibitory effects on TCR-mediated signal transduction whereas CD49b is an integrin and receptor to many extracellular matrix molecules [234] and is also expressed by NK cells and a subset of memory CD4 T cells that produce TNFα [235]. In another recent study, CD4 T cells that were generated *in vivo* by several, successively escalating, doses of antigen exhibited a gradual increase in markers associated with inhibitory or regulatory function. Thus, with each consecutive dose, the frequency of cells expressing IL-10, c-Maf, NFIL3, LAG3, TIGIT, PD-1 and TIM-3 increased, but not FoxP3 [236]. Importantly, although it has been clearly demonstrated that Tr1 cells are important in mediating tolerance in several T cell-mediated diseases [237], firmly establishing these cells as a unique lineage and finding specific markers is still under intense investigation [238]. Similar to Th3 cells, they may or may not represent an altered state of differentiation rather than a distinct T cell lineage.

Early studies showed that IL-10 signalling was essential for the induction of Tr1 cells [239, 240] and more recent studies have added that Tr1 generation also depends on IL-27 [241]. Interestingly, DCs co-cultured with FoxP3+ Tregs secrete higher levels of IL-27, as well as IL-10 and TGFβ, suggesting that the presence of FoxP3+ Tregs promote Tr1 differentiation [242]. Furthermore, IL-27 activates STAT1 and STAT3 (see fig. 6) in T cells, which are subsequently recruited to the IL-10 promoter [243]. In addition, IL-27 mediated activation of STAT3 results in Blimp-1 upregulation, which also enhances IL-10 expression in an early growth response protein-2 (Egr-2) dependent manner [244, 245]. Thus, the IL-27 and IL-10 signalling pathways are intimately linked and are both important for Tr1 development and function. To date, there is no known master regulator that uniquely determines Tr1 differentiation, although many transcription factors have been identified that cooperatively control their maintenance and function. These include the repressor of GATA-3 (ROG), the cellular homologue of the avian virus oncogene musculoaponeurotic fibrosarcoma (c-Maf), the aryl hydrocarbon receptor (Ahr), interferon regulatory factor (IRF4) and early growth response protein 2 (Egr-2) [232, 246-249]. *In vitro*, IL-6 signalling was shown to induce the expression of c-Maf, Ahr and IRF4 which are all crucial transcription factors for IL-10 expression. Moreover, IL-6 associated upregulation of IL-10 mRNA was dependent on IL-2 and IL-21, and IL-6 blockade *in vivo* was shown to exacerbate inflammation after polyclonal T-cell activation. This connection appears counterintuitive, as IL-6 and TGFβ are known to induce RORγt and Th17 responses. However, whereas IL-6 is indeed associated with exacerbated disease in EAE and arthritis [250-252], it was in fact shown to confer protection in murine type 1 diabetes (T1D) [253] and DSS-induced colitis [254]. In summary, the role of IL-6 mediated Tr1 generation is incompletely understood, but its effects are dependent on context or disease.

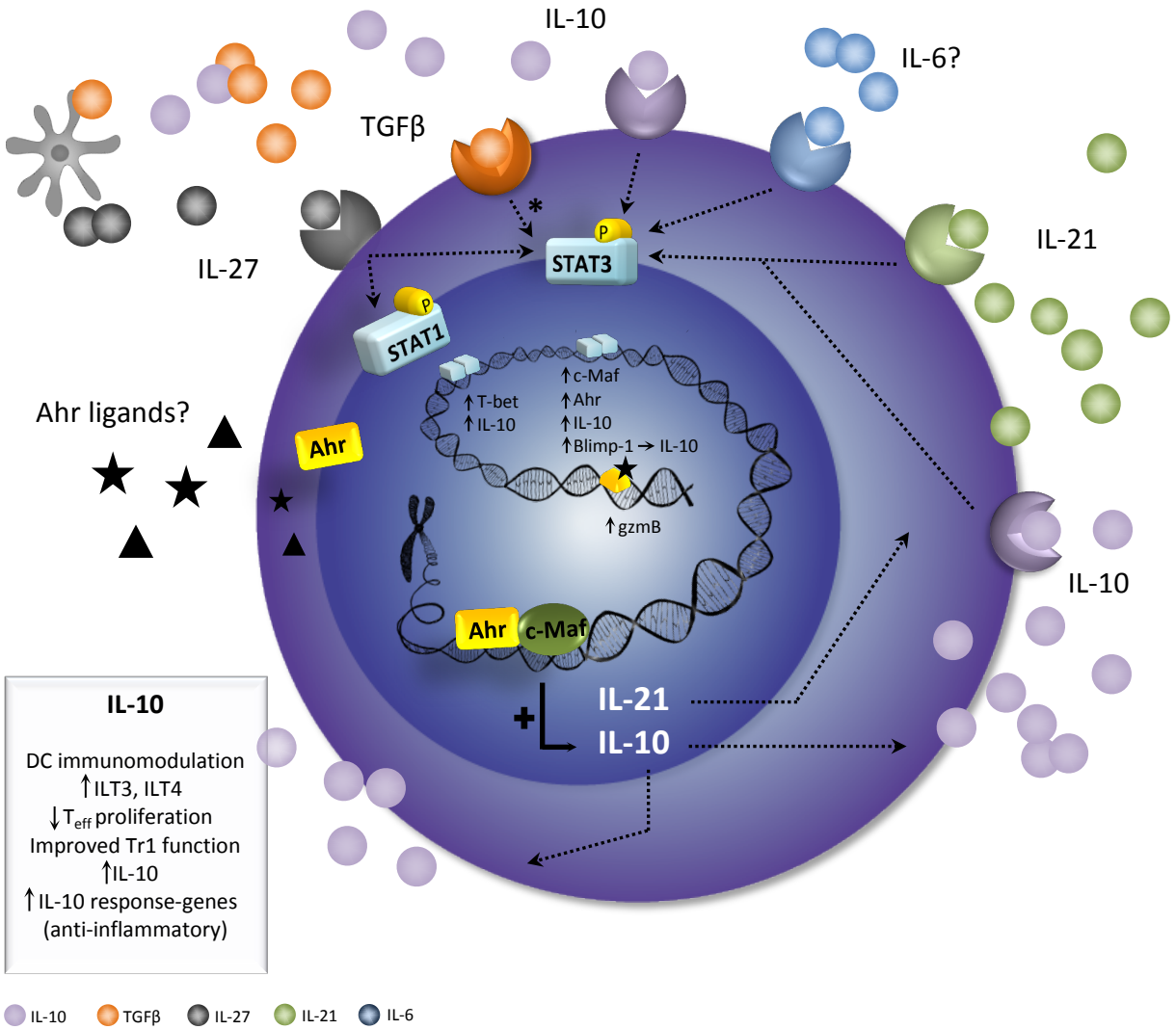


Figure 8. Simplified overview of Tr1-associated signalling pathways. Expression of Ahr and c-Maf is induced after STAT3 and Ahr activation. Next, Ahr physically associates with c-Maf and drives the expression of IL-10 and IL-21; cytokines crucial for the survival and function of Tr1 cells. *Note that TGFβ indirectly activates STAT3 via inhibiton of SOCS3, which is not depicted in this illustration. GzmB: Granzyme B

With regards to the mode of action of Tr1 cells; at least four principal modalities have been proposed thus far. First and foremost, the secretion of **IL-10** suppresses APCs as well as other T cells in the vicinity. The importance of this particular pathway is underscored by the fact that Tr1 functionality is completely compromised by blocking IL-10 using mAbs. This suppressive approach is an antigen-specific mechanism in that Tr1 cells need activation via TCR signalling to exert their suppressive function, although IL-10 itself targets any nearby cells that express the IL-10R. For example, IL-10 signalling in DCs upregulates their expression of ILT3 and ILT4 (see fig. 5) and subsequently inhibits DC maturation and promotes tolerance [95, 255]. In addition, Tr1 cells can kill myeloid APCs by their release of **Granzyme B and perforin**, which was found to occur upon engagement of CD2/CD58 and DNAM-1/TIGIT in human cells [256]. Moreover, expression of **co-signalling molecules** on Tr1 cells such as CTLA-4, PD-1, ICOS and LAG3 have contact-dependent immunomodulatory effects on the APC similar to that of FoxP3+ Tregs [233, 257]. For example, downregulation of MHCII on the APCs indirectly impaired the generation of antigen-specific effector responses. Finally, expression of **CD39** removes pro-inflammatory eATP and generates immunosuppressive adenosine in the extracellular environment [159, 258, 259].

Th CELL PLASTICITY

The differentiation of naïve T cells into unique subsets was once thought to be irreversible, however many studies have since demonstrated a considerable functional elasticity in most T cell types [260, 261]. Primed CD4 T cells acquire a distinct phenotype to combat a specific pathogen with unique characteristics, often located in a particular tissue. However, they retain their capacity to change their function upon re-activation in a microenvironment different from that within which they were originally generated. Indeed, elegant fate-mapping experiments, where cells that express or ever expressed FoxP3 can be traced, have shown that "ex-FoxP3" T cells lost their regulatory function and instead promoted autoimmune diabetes [262]. Conversely, inflammatory cells could also be reprogrammed to acquire regulatory function [263]. This is perhaps not too surprising, as TGFβ is central in the differentiation of both Tregs and Th17 cells and auxiliary cytokines promote the preferential expression of either FoxP3 or RORγt (see page 25). In addition, IL-12 induces T-bet and IFNγ in FoxP3+ Tregs [264, 265] and IL-27 can both induce IL-10 expression in various effector subsets, as well as generating Tr1 cells *de novo* [133, 249]. The intricate connections of T cell subsets and cytokines are visualized in figure 9. However, these processes are not only mediated by **cytokines** and **transcription factors**, but there are also other regulatory elements that we are only beginning to dissect.

The epigenetic landscape determines how stable the expression of subset-specific genes is and reorganization of **chromatin structure** creates access or blockade of key differentiation loci within the genome. This process requires a discrete number of cellular divisions [266-269] and is regulated by DNA methylation and various factors such as STATs, histone acetyltransferases (HATs), and histone deacetylases (HDACs). For example, recruitment of HATs CBP and p300 is indispensable for maintaining an open FoxP3 locus, and Tregs lose their FoxP3 expression and instead produce IL-17 upon loss of these factors [270].

The PI3K-AKT-mTOR **kinase signalling** pathway (see page 27) regulates the activation of pro-inflammatory T cell programs. In Tregs, this pathway is inhibited by their expression of phosphatase and tensin homologue (PTEN) which inactivates AKT, and PTEN deficiency leads to Treg conversion into Th1 and Th17 cells [271, 272].

Importantly, the use of distinct metabolic programs directly influences T cell function. For example, glucose catabolism by oxidative phosphorylation generates high amounts of ATP whereas glycolysis yields less ATP and favours production of amino-acid/nucleotide/lipid precursors. CD28 signalling controls the **metabolic switch** to glycolysis, a process dependent on PI3K-AKT-mTOR signalling. As Tregs negatively regulate the mTOR pathway, they do not use glycolysis but rather rely on the oxidation of fatty-acids. Thus, commensal bacteria in the gut may actively support Treg polarization by providing a localized abundance of short-chain fatty acids [273]. Hypoxia-inducible factor 1α (HIF1α) is another factor dependent on the PI3-AKT-mTOR pathway, and induces the expression of genes important for glycolysis as well as RORγt. Consequently, HIF1α activation results in Th17 polarization [274], effects which are actively suppressed in Tregs. Interestingly, HIF1α also controls early metabolic programming of Tr1 cells, but is later degraded in an Ahr-dependent manner [135].

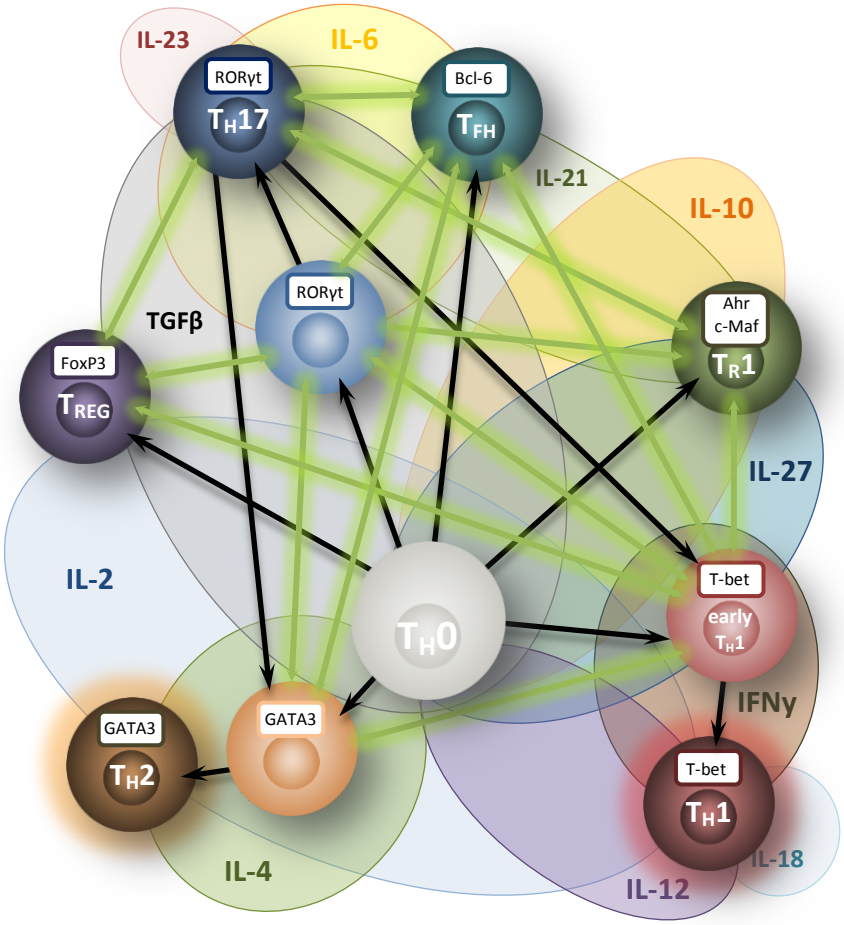


Figure 9. T cell plasticity, adapted from [260, 275]. Green arrows signify the possibility for bilateral differentiation whereas black arrows show irreversible changes. Generally, all T cell subsets can be manipulated into changing their cytokine secretion, but Th1 and Th2 cells that have undergone several rounds of division are highly stable. In contrast, Tregs, Th17- and other subsets are more plastic even at later stages of cellular division. Polarized Tfh cells from mice can be induced to make Th1, Th2 or Th17 cytokines if cultured with IL-12, IL-4 or IL-6 and TGFβ, respectively. Conversely, Th1, Th2 and Th17 can express IL-21, CXCR5 and PD-1 by culturing them in the presence of IL-21 and IL-6. The role of some key cytokines and their over-lapping functions in this process are visualized by a Venn(esque) diagram in this illustration, with each cytokine sphere positioned under the relevant CD4 T cell subsets they affect.

In summary; it appears as if T cell subsets are both stabilized and allowed to remain plastic through intimately linked pathways that include cytokines, transcription factors, metabolic programs and kinase signalling, which in turn regulate chromatin structure. The processes detailed here yet again demonstrate the multi-layered control that the microenvironment exerts over our immune system.

Immune-mediated diseases

The central role that Tregs have in controlling homeostasis and tolerance has made them an interesting therapeutic target for immune-mediated diseases. For example, manipulation of Tregs not only has beneficial effects in autoimmune diseases, but also prevents rejection of transplanted tissues. In fact, results from the clinic also indicate that adoptive transfer of Tregs in human patients is safe and effective in preventing transplant rejection [276]. Importantly, the function of Tregs represents a double-edged sword in that they are also capable of masking tumours from immune surveillance. Thus, by broadly manipulating the regulatory arm of the immune system during autoimmunity, we inevitably also introduce an

element of risk for tumour generation. However, it also means that there are multiple contexts from which we can learn about the fundamental mechanisms involved in immunological tolerance - knowledge that could potentially be applied in all of the above clinical conditions. For example, the suppressive function of PD-1 was first documented in knock-out mice which developed spontaneous lupus-like autoimmune disease, in the late 1990s [277]. Today, two antibodies directed against PD-1; Nivolumab® and Pembrolizumab®, are in clinical use for treatment of lung cancer and several other "immune check-point inhibitors" are also in clinical development [278].

In the present thesis, autoimmunity has been used as the model system of choice to understand how tolerance induction, specifically by the CTA1R7K-X-DD fusion protein, is achieved. The ultimate goal is to develop a therapeutic treatment for autoimmune diseases that can be used clinically. Autoimmunity is a term to describe aberrant immune responses directed towards healthy self-components, such as cells and tissues. There are at least 80-100 described conditions that stem from autoimmune reactions, many of which have similar and hard-to-diagnose symptoms. Some of the most common diseases include celiac disease, type 1 diabetes (T1D), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and multiple sclerosis (MS). For many of these diseases there is a clear hereditary component and women are typically more prone to develop disease. In some cases, as much as 75% of all patients are women. Currently, treatments are not curative but rather alleviate symptoms of disease, and novel treatment strategies with fewer side-effects are highly warranted [279]. In this thesis work we have focused on two diseases of particular interest, MS and RA, by exploring the therapeutic effects of our specific fusion protein in their corresponding experimental models, EAE and CIA.

MULTIPLE SCLEROSIS

MS is a disease of the central nervous system (CNS), where the insulating myelin cover of the axons of nerve cells in the brain and spinal cord is destroyed. Axon demyelination results in compromised communication within the affected neuronal networks and manifests as a wide range of symptoms, including debilitating motor and sensory dysfunction, fatigue, and sometimes cognitive issues [280]. In 2013 it was estimated by the MS international federation (MSIF) and WHO that MS affects approximately 2.3 million people worldwide, which was an increase compared to figures from 2008 (2.1 million) [281]. Risk factors are genetic as well as environmental and geographically, the incidence of MS is higher in areas farther from the equator, which is thought to correlate with fewer hours of sunlight and low vitamin D levels [282-284].

MS is a chronic disease and behaves according to two main clinical patterns; either as a relapsing-remitting condition with sudden flares that lasts days or months with intermittent periods of recovery, or as a successively deteriorating disease without remission. Alternatively, different combinations of these forms exist but are less common. Treatment options in the clinic are focused on improving functions between flares and delaying their onset using broadly immunosuppressive agents. First-line disease-modifying treatments include interferons and glatiramer acetate, but they have modest efficacy in relapsing-remitting MS and are associated with severe side-effects [285]. Thus, there are some key aspects that should be addressed for the successful development of novel MS therapies. Firstly, insights in the molecular pathways that are uniquely involved in the specific MS

disease patterns will pave the way for personalized medicine. In addition, the development of antigen specific immunomodulation, which reinstates tolerance to the disease-causing agent only, will reduce the adverse effects associated with currently available treatments. Encouraging results from two clinical phase I studies showed that intradermal injection of disease-related peptides ATX-MS-1467 had positive effects on MS.

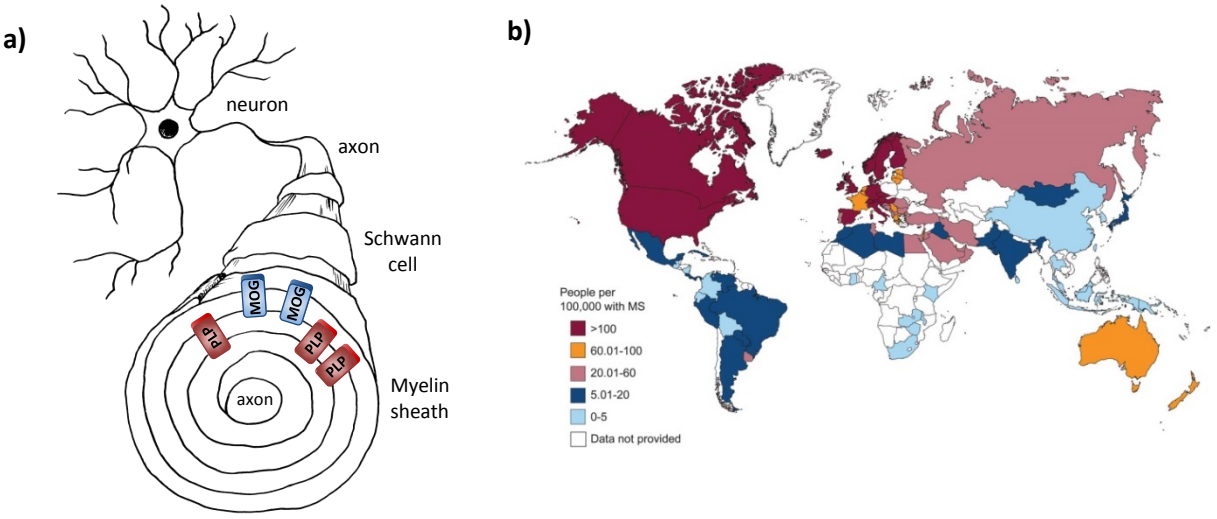


Figure 10. The anatomy and epidemiology of Multiple Sclerosis, adapted from [281, 286]. **a)** Schematic overview of a neuron with a myelinated axon. The protruding axons are wrapped by sheaths of myelin which is produced by oligodendrocytes (Schwann cells). Some protein constituents of the myelin sheaths relevant to this thesis are also depicted. MOG: myelin oligodendrocyte protein, PLP: proteolipid protein. **b)** Map of global distribution of MS 2013, reprinted with permission from MSIF.

The immunopathology of MS is characterized by the CNS-infiltrating encephalitogenic CD4 T cells that produce either IL-17 or IFNγ. The inflammatory process that ensues perpetuates the disruption of the blood-brain-barrier, causing swelling, aggravated inflammation and recruitment and activation of immune cells. In patients with progressive MS, serum levels of IL-12 and IL-18 are increased, both of which promotes IFNγ production, which is known to exacerbate disease in humans [287, 288]. Of note, this is not the case for EAE in mice, where pathology depends on IL-23 and Th17 cells rather than Th1 and IFNγ responses [289, 290]. Th17 responses are also important in humans, where IL-17 activates pro-inflammatory responses in astrocytes and promote leukocyte recruitment to the CNS [291, 292]. The murine **EAE** model is currently the most well-established method to study human MS, and injection of myelin antigens in susceptible mouse strains results in CD4 T cell-mediated autoimmune encephalitis. Depending on the protein used for disease induction as well as the mouse strain, the clinical progression of EAE will vary. For example, injection of myelin oligodendrocyte protein (MOG) emulsified in CFA in C57/BL6 mice results in an acute monophasic disease, whereas proteolipid protein (PLP) in SJL mice leads to a slower relapsing-remitting model [293]. Another experimental research tool involves transgenic T cells that recognize disease-relevant epitopes, which have significantly contributed to our understanding on the specific role of CD4 T cells in CNS autoimmunity [294].

Th17 responses are crucial in murine EAE disease development, not least demonstrated by the finding that IL-23 deficient mice do not develop EAE at all [289]. Interestingly, in IL-17 knock-out mice, symptoms of EAE are certainly reduced, but not completely abolished [295, 296]. However, mice lacking Th17 cytokine GM-CSF are resistant to EAE [297], and CD4 T cells from untreated MS patients also secrete higher amounts of GM-CSF than treated patients [298]. Thus, IL-23 promotes Th17-mediated EAE by IL-17 dependent and independent pathways. Conversely, the development of Treg responses is important in preventing or attenuating disease. In the EAE model, transfer of nTregs prior to disease induction is associated with reduced disease progression [299, 300]. nTregs have also been shown to induce transient Tr1-like phenotypes in myelin basic protein (MBP)-specific T cells during their activation [301]. Finally, IL-10 producing Tr1 cells also have a protective role in EAE and IL-27 was shown to be important in promoting the generation of such responses as well as suppressing Th17 responses [152, 155, 242, 249, 302, 303].

RHEUMATOID ARHRTITIS

RA is one of the most common autoimmune diseases and is estimated to affect up to 1% of the adult population in developed countries. It is characterized by chronic inflammation and progressive cartilage destruction, and symptoms include joint swelling, pain, and stiffness which progresses to joint deformities due to bone erosion. Similar to MS, RA often manifests as periods of relatively mild disease disrupted by intermittent flares, but symptoms can also be constant and gradually progress in severity. In short, RA is a multifactorial disease where environmental cues can trigger autoimmunity under conditions of immune dysregulation in genetically predisposed individuals. For example, smoking and stress are thought to promote and worsen disease and human MHCII alleles HLA-DR4 or HLA-DR1 have been shown to correlate with a higher risk for disease. With regards to treatment options, three classes of drugs are available; non-steroidal anti-inflammatory agents (**NSAIDs**) and **corticosteroids** quickly alleviates pain and general inflammation, whereas disease modifying anti-rheumatic drugs (**DMARDs**), such as anti-TNF antibodies [304], are important in decelerating disease progression. However, DMARDs are also associated with numerous severe side-effects due to their influence on general immune function.

Overall, RA disease pathogenesis is complex and not fully elucidated, but involves CD4 T cells, pro-inflammatory cytokine networks as well as immune complexes and autoantibodies [305]. Autoantibodies directed towards the Fc-portion of IgG, termed rheumatoid factor (RF), form immunocomplexes that contribute to disease and in combination with clinical signs and symptoms, is often used to diagnose RA. Antibodies directed towards citrinullated proteins or peptides (ACPAs) are also highly prevalent in patient sera. Indeed, the role of B-cells in driving disease progression is further underscored by the successful introduction of Rituximab®, a monoclonal antibody which targets CD20 and is used in the clinic to deplete B-cells in RA patients [306]. Also, perhaps a somewhat overlooked function of TNFα is that of follicular dendritic cells (FDC) survival, which is crucial in GC formation [307-310]. Thus, another function of the DMARD anti-TNF antibody could be the disruption of B-cell responses [311].

Of note, B cells have also been shown to have regulatory functions by secretion of immunosuppressive cytokines IL-10 and IL-35 [312-314]. Furthermore, forced endogenous expression of a collagen type II (CII) peptide specifically in B cells was shown to prevent

severe arthritis in the experimental model collagen-induced arthritis (CIA). This correlated with increased Treg responses that negatively regulated disease upon transfer to wt recipients [315]. **CIA** is the most commonly used experimental rodent model to study RA, and is typically induced in susceptible animals by injecting CII emulsified in CFA. In fact, many findings from this model have been successfully translated to human RA [316], not least in the development of anti-TNF antibodies [317]. As in RA, joint destruction in CIA depends on the formation of autoantibodies, but they are specific to collagen in contrast to RFs and ACPAs in humans. Also, neutrophils are abundantly present in CIA as opposed to RA where macrophages are predominant [318]. In CIA, disease progression has been shown to critically depend on the presentation of CII peptides on a limited repertoire of MHCII alleles, such as $CII_{259\text{-}270}$ on $I\text{-}A^{q}$ in susceptible DBA/1 mice [319]. In the initial phases of disease, Th1 responses are apparent in the dLNs, while IL-1, TNFα and IL-6 dominate the cytokine network at the time of clinical onset [318]. The role of IFNγ appears biphasic, since early blockade of this pathway reduced arthritis severity but instead aggravated disease if blocked at later time points [320]. Furthermore, DBA/1 mice that lack either IFNγ or its receptor were highly susceptible to CIA [319] and Th17-associated cytokines IL-17, IL-21 and IL-23 have all been shown to exacerbate disease [321]. In summary, Th17- and B-cells are certainly important in driving disease, but whether Th1 cells are involved remains somewhat contentious.

Similar to EAE and MS, the concept of antigen-specific tolerance represents an attractive alternative to currently available RA treatments, and has been focused on in several preclinical studies. For example, forced expression of CII in APCs [315] or B-cells [322] by genetic manipulation resulted in decreased disease severity, and *in vitro* generation of CII-presenting tolerogenic DCs [323, 324] were protective during CIA upon transfer to recipient mice. Finally, Hasselberg *et al* showed that i.n administration of the CTA1R7K-COL-DD fusion protein successfully prevented disease or significantly attenuated symptoms of CIA [35]. In this study, the therapeutic effect was ascribed to IL-10 producing Tr1 cells which suppressed IFNγ, IL-6 and IL-17 responses.

Plant-based vaccination

"Let food be thy medicine and medicine thy food"
Hippocrates

The use of peptides or proteins for therapeutic purposes is part of an industry that, since the FDA approval of recombinant insulin some thirty years ago, has grown into a multibillion dollar business [325]. Protein drugs, such as hormones, enzymes and antibodies regulate many different cellular functions. In contrast to conventional small molecule drugs, they are typically highly specific to their target which ultimately manifests as reduced side-effects and toxicity [326]. On the other hand, protein expression, extraction and purification are costly procedures and in addition, the processed protein drugs have a very limited shelf-life that requires cold chain storage [327, 328]. Furthermore, they are generally administered by injection which requires health care personnel. Despite their prohibitively expensive production costs, the number of approved biopharmaceuticals are continually increasing for treatments of e.g. cancer, metabolic and immunological disorders and infectious diseases

[329]. Thus, optimization of the protein expression platforms available is critical for biopharmaceuticals to be economically feasible. Currently, protein drugs are most frequently manufactured using mammalian, yeast or bacterial cell cultures, which all have their unique merits. However, bacteria lacks many cellular post-translational processes vital for proteins of eukaryotic origin whereas setting up mammalian production units is incredibly expensive, even without the added costs of protein extraction, purification, cold storage and health care personnel [329]. As a result, transgenic (TG) plants have emerged as a highly cost-effective protein production platform.

There are several advantages in using TG plants as an expression system. Firstly, plants normally do not harbour human pathogens. Secondly, as a eukaryotic system they are able to perform important post-translational modifications (PTMs) such as disulphide bonds, protein folding and glycosylation [330-333]. Our glycobiome affects key proteins involved in antigen recognition and downstream effector functions [334, 335] and is therefore important in e.g. antibody conformation, stability and target recognition as well as the T cell recognition of epitopes derived from self-antigens [334-337]. For example, a galactosylated $COL_{259-273}$ peptide was superior to non-modified peptides in protecting DBA/1 mice against CIA [338, 339]. Naturally, these functions are of particular importance for the expression of immunorelevant protein drugs, such as monoclonal antibodies or T cell peptides. Lastly, although the expression capacity of plant cells is similar to that of mammalian cells, their protein can be stored at ambient temperature in lyophilized plant cells for several years without loss of structure or function [340].

Most importantly, the oral ingestion of edible TG plants without prior protein extraction or purification will substantially reduce manufacturing costs [331]. Normally, proteins are readily broken down in the acidic environment of the stomach by digestive enzymes. This has likely hampered the success of orally administered protein drugs and, as previously mentioned, they are currently delivered mainly by injection. However, the abundance of lignin and cellulose in the plant cell wall protects the protein drug from degradation, as human enzymes are incapable of breaking down their glycosidic bonds. It is not until the ingested plant material reaches the intestinal lumen, where commensal bacteria will break down the cell wall, that the drug will be released [341].

Subsequent to its release, the protein drug must somehow cross the gut epithelium for it to become available to the immune system. Of note, specific properties of proteins may differentially influence the bioavailability and tissue distribution of the protein drug. For example, in a recent study, the uptake of green fluorescent protein (GFP) fused with either CTB (see page 11) or a peptide that targets DCs (DCpep), was compared. CTB-GFP efficiently traversed both the gut epithelium as well as M-cells and quickly reached the circulation. DCpep-GFP, on the other hand, was not detected in epithelial cells, but rather used M-cells as their sole route of entry [342]. Of note, DCpep fusion proteins can also be taken up directly from the gut lumen by the extended dendrites of CX3CR1+ APCs [343]. The gut-associated lymphatic tissue (GALT) has the largest surface area for antigen entry and typically provides a microenvironment that is tolerogenic by default. Thus, a concentrated antigen uptake to this site may be particularly advantageous for tolerance treatments.

In fact, oral tolerance represents an effective and simple means to treat autoimmune disease, and it is well described in animal models that repeated feeding of antigen can inhibit future detrimental systemic immune responses to that same antigen [344]. However, human clinical trials have largely had poor clinical outcomes [345-349] which is likely due to the need for large quantities of purified protein. Tolerance induction by bioencapsulated antigens have indeed been established in several animal models of autoimmunity, such as diabetes [350-352], experimental autoimmune uveitis [353], and was also shown to confer tolerance to clotting factor VIII or IX in mouse models for haemophilia A and B [340, 354, 355]. Therefore, edible TG plants may be an attractive approach to efficiently treat autoimmune disease; by oral tolerization, using biopharmaceuticals that are produced at a very low cost, and is easily administered to the patient.

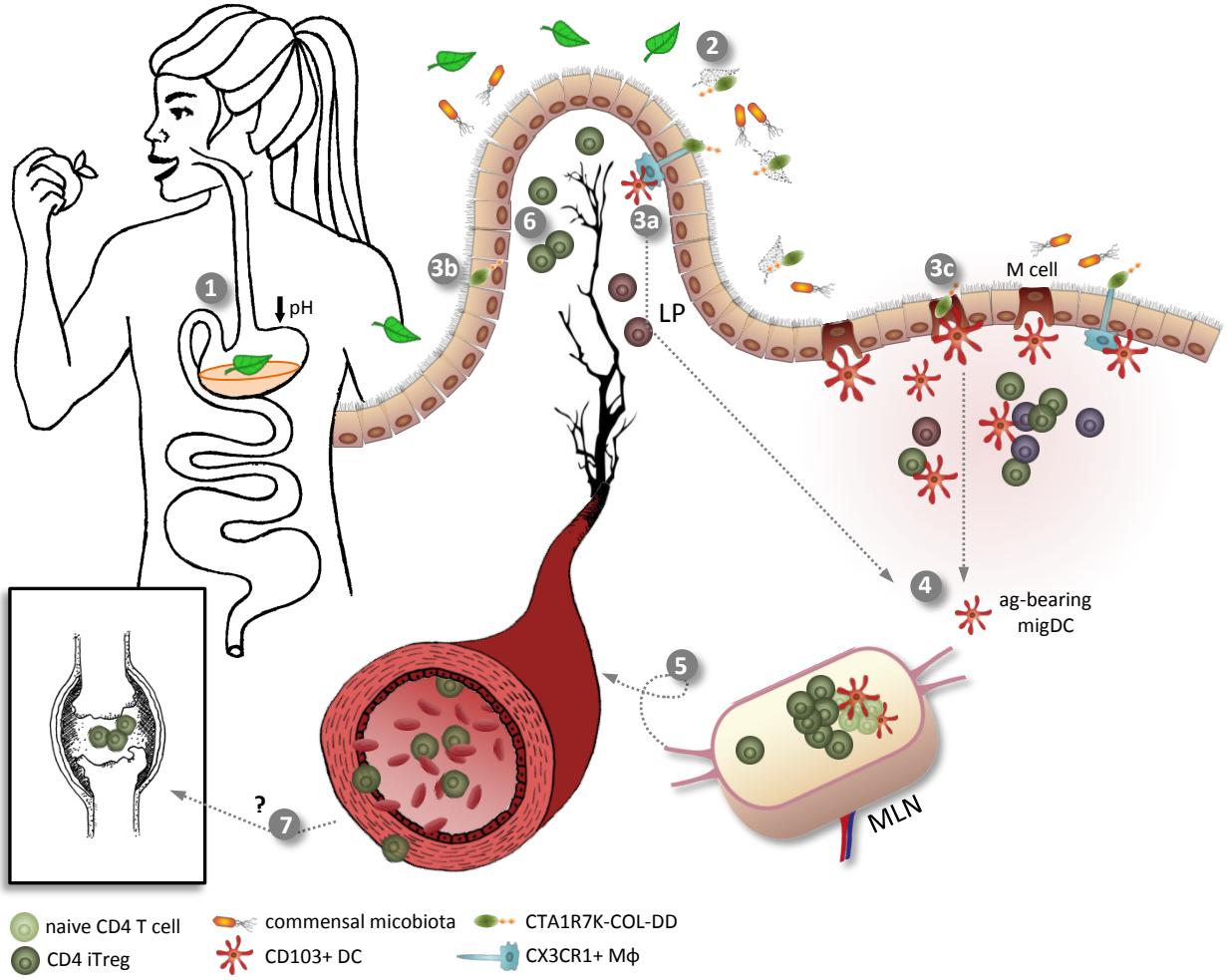


Figure 11. Oral ingestion of plant-expressed bioencapsulated biologicals and systemic tolerance, adapted from [341, 343, 356]. A multistep model of oral tolerance to food protein, presented by Pabst & Mowat, is summarized here [356]. **1)** The cellulose-rich plant cell wall protects the protein from degradation in the stomach. **2)** The plant cells are digested by the commensal microbiota and the protein is released into the gut lumen. **3)** Several routes of entry for protein uptake are possible; a) CX3CR1+ macrophages have dendrites that extend into the lumen, and captured antigens are tranferred to CD103+ migratory DCs. b) Proteins can to cross the epithelium directly, or pass through inbetween. c) Proteins are taken up by specialized M cells that cover the follicle area where DC readily take up antigens. **4)** Antigen-loaded DCs migrate to the MLN and induce iTregs in an RA-dependent manner. **5)** iTregs enter the circulation and home back to the LP. **6)** A subsequent putative mechanism is that iTregs home to LP, expand and may re-enter the circulation where they disseminate throughout the immune system to promote systemic tolerance. MLN: mesenteric lymph node, RA: Retinoic acid, LP: lamina propria

Summary

As is becoming increasingly evident, DCs play a critical role in preventing autoimmunity; mediated by their expression of co-signalling surface molecules as well as their secretion of tolerogenic cytokines they can promote Treg/Tr1 differentiation or induce deletion or anergy in pathogenic T cell clones. In the introduction to this thesis, I have specifically focused on cDCs and CD4 T cells and their bilateral interactions because of their particular relevance to my research.

To summarize the entire introduction in a few sentences; DCs exist as several distinct subpopulations **(p.15-17)**, and yet exhibit a remarkable degree of plasticity and functional overlap upon changes in the microenvironment **(p.21)**. These changes are registered by a limited set of well-conserved innate receptors that recognize evolutionary ancient molecular patterns. The sum of these patterns triggers specific gene sets, which remain to be fully characterized, within the DC that translates to the production of secreted and membrane-bound molecules **(p.19-24)** and serves as a goal-oriented communiqué to the adaptive immune system. CD4 T cells receives this information as a collection of dynamic, and often contrarious, signals **(fig. 5)** and, mainly by the aggregated activation of different JAK-STAT pathways, undergo fundamental changes in their transcription. The differential pathways activated in the CD4 T cell will trigger the specific expression of effector proteins, e.g. cytokines, capable of dealing with the specific challenge that triggered the immune response in the first place **(p.25-30)**. However, changes in the microenvironment can introduce differences in the net transcription of these effector proteins, challenging the original concept of stable and distinct CD4 T cell subsets **(p.31-32)**. This ensures flexibility within the adaptive immune system, but also introduces the risk of dysregulated CD4 T cell responses which, in turn, has potential implications for several other immune cells such as CD8 T cells, B cells and myeloid cells, in ways we do not fully comprehend yet. But the consequences of these dysregulated immune responses become evident in several autoimmune diseases, such as RA or MS **(p.32-36)**.

In a 2011 review by Pulendran *et al.* [89], multiple parameters important in DC-mediated tolerance were presented and sorted according to their hierarchical positioning in this process. It was a conceptual model that elegantly recapitulated some principal aspects of immunity, tolerance and homeostasis and will serve as a concluding summary to this thesis introduction. In this model, the DC constitutes ”ground zero” and information of successively higher resolution is obtained by studying innate receptors (hierarchy level -1) and their signalling pathways and transcription factors (hierarchy level -2). In contrast, studying the cellular interactions between relevant cell types, such as DCs and Tregs (hierarchy level +1) and characterizing the influence of the microenvironment within different immunological compartments (hierarchy level +2) yields a more global overview. Importantly, insights from each of these hierarchical levels alone offer only a limited view on the networks that orchestrate immunological tolerance. To fully appreciate these dynamic processes - and figuring out how to manipulate them - the knowledge from all the above modalities should be combined into a comprehensive model.

AIMS

The general aim of this thesis was to investigate whether the immunomodulatory fusion protein CTA1R7K-X-DD could induce tolerance that mediates protection against autoimmune disease. And if so, through which means.

The specific aims were:

- To explore the priming and potential feedback pathways that characterize the initial and recall interactions between a dendritic cell and a CD4 T cell during an immunogenic and tolerogenic response respectively.

- To investigate if the CTA1R7K-MOG/PLP-DD molecule could be used to protect against experimental autoimmune encephalitis (EAE).

- To better characterize the regulatory CD4 T cells that are induced after treatment of EAE using the CTA1R7K-MOG-DD molecule.

- To investigate if the tolerogenic CTA1R7K-COL-DD molecule could be used to treat collagen induced arthritis after bioencapsulation by expression in an edible plant.

EXPERIMENTAL PROCEDURES

This section briefly describes the key methodologies and the rationale behind choosing some of the particular methods used in this thesis. A more detailed description can be found in each individual paper.

MICE & IMMUNIZATIONS

Several different mouse strains were used in this thesis in order to address different questions. Knockout and transgenic mice were kept in a specific pathogen-free environment and bred at the experimental biomedicine (EBM) facility at the University of Gothenburg and wild-type mice were purchased from the distributors specified in each paper. Mice were 8-14 weeks old and age/sex-matched for all experiments.

TABLE 1. Mouse strains used in this thesis

BACKGROUND STRAIN	SPECIFIC STRAIN	PHENOTYPE
C57BL/6	wt	*Inbred mouse strain, H-2^b MHC allele (I-E null)*
	batf3-/-	*KO mice that lack CD103+ Dendritic cells*
	IL-27rα-/-	*KO mice that lack IL-27R α-subunit*
	Teα	*TG mice, CD4 T cell TCR recognizes Eα$_{52-68}$ peptide*
	2D2	*TG mice, CD4 T cell TCR recognizes MOG$_{35-55}$ peptide*
Balb/c	wt	*Inbred mouse strain, H-2^d MHC allele*
	DO11.10	*TG mice, CD4 T cell TCR recognizes OVA$_{323-339}$ peptide*
	JHD	*KO mice that lack B cells.*
SJL/J	wt	*Inbred mouse strain susceptible to encephalitis induction*
DBA/1	wt	*Inbred mouse strain susceptible to arthritis induction*

Several different fusion proteins were used with different incorporated peptides appropriate for each respective model. Unless stated otherwise, 5µg of the CTA1-X-DD/CTA1R7K-X-DD fusion protein was administered via the intranasal route according to the model-specific protocols. For *in vivo* antigen challenges in paper I, an oil-in-water emulsion (Sigma Adjuvant System) containing the relevant antigen (200ug of OVA protein or 50 µg of Eα$_{52-68}$ peptide) was injected intraperitoneally 8 days after tolerization or immunization. In paper III, mice were fed transgenic plants expressing the CTA1R7K-COL-DD fusion protein *ad libitum*.

RECOMBINANT PROTEIN PREPARATIONS

CTA1-DD and CTA1R7K-DD containing one copy of either of the peptides, listed in Table 2, were expressed and purified by transforming *E.coli* TG-1 cells with the different expression vectors. After o/n incubation in 2xYT medium with 50 mg/ml Kanamycin at 37^{o}C, inclusion bodies were collected and denatured using 8M Urea. Proteins were subsequently refolded by slow dilution in H_2O and purified by ion-exchange and size-exclusion chromatography. Endotoxin levels were low for all constructs at <100 units/mg.

TABLE 2. Fusion proteins used in this thesis

PEPTIDE	CONSTRUCT	AMINO ACID SEQUENCE
$OVA_{323-339}$	CTA1-OVA-DD	ISQAVHAAHAEINEAGR
	CTA1R7K-OVA-DD	
$E\alpha_{52-68}$	CTA1-Ea-DD	ASFEAQGALANIAVDKA
	CTA1R7K-Ea-DD	
MOG_{35-55}	CTA1R7K-MOG-DD	MEVGWYRSPFSRVVHLYRNGK
$PLP_{139-151}$	CTA1R7K-PLP(139)-DD	HCLGKWLGHPDKF
$PLP_{178-191}$	CTA1R7K-PLP(178)-DD	NTWTTCQSIAFPSK
COL $II_{259-274}$	CTA1R7K-COL-DD	GIAGFKGEQGPKGEPG

TRANSGENIC *ARABIDOPSIS THALIANA*

All plants were produced and grown by our collaborators at Örebro University. Transgenic *Arabidopsis thaliana* plants were engineered using a simplified *Agrobacterium*-mediated floral dip method. Briefly, the CTA1R7K-COL-DD or control constructs (R7K-DD and empty vector) were inserted into the pGreen plasmid containing a multiple-cloning-site flanked by t-DNA sequences. The assembled vector was used to transform *Agrobacterium tumefaciens* by electroporation. After culturing on selective medium (50 µg/mL Kanamycin and 5 µg/mL Tetracycline), positive clones were verified by PCR and subsequently used for transformation of the nuclear genome of 4-week-old *Arabidopsis thaliana* plants. Seeds were then harvested from these plants and grown on selective medium (10 µg/mL of herbicide BASTA and 400 µg/mL cephotaxime) after which resistant seedlings were grown for analysis, self-pollination and seed production. After successful transformation had been verified by PCR and protein expression analysed by Western blot, these plants were used for the feeding experiments in paper III.

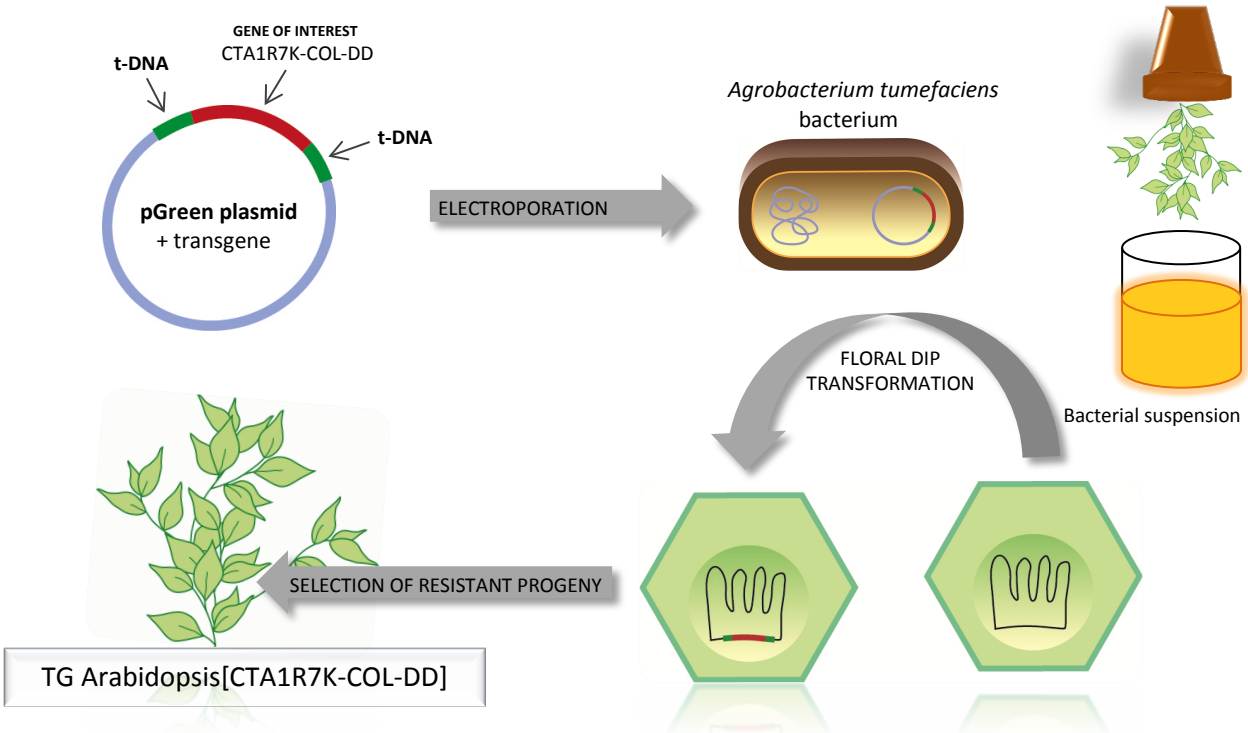


Figure 12. Schematic overview of the floral dip method used in this thesis.

EXPERIMENTAL MODELS OF AUTOIMMUNE DISEASE

There are inherent limitations when modelling a human chronic inflammatory disease in murine short-term experimental settings. Nevertheless, some scientific inquiry doesn't lend itself to investigation in humans. Thus, experimental tools such as gene knock-out or transgenic mice and the adoptive transfer of specific cells have been immensely helpful in identifying, understanding and dissecting different properties of the immune system. Furthermore, *in vivo* animal models recapitulate the anatomical aspect and the complex microenvironment of immune responses as opposed to *in vitro* cultures of cells and tissue, which are not sophisticated enough to emulate all these parameters. In this thesis, two T-cell mediated models of autoimmune disease were used, namely:

Experimental Autoimmune Encephalitis (EAE): To induce acute EAE, 100 µg of MOG_{35-55} peptide was emulsified at a 1:1 ratio in CFA (4mg/ml *Mycobacterium tuberculosis*) and injected s.c at the base of the tail of 8-9 weeks old C57B/6 mice. Furthermore, on day 0 and 2, 200ng of Pertussis toxin was given i.p and treatment was administered i.n as indicated in paper II. The procedure was similar for relapse-remitting EAE except for that instead, $PLP_{139-151}$ peptide was emulsified in CFA (7mg/ml *Mycobacterium tuberculosis*) and 8-week old SJL/J mice were used. Mice were examined for symptoms of EAE according to a 0-5 standard scoring protocol (1: limp tail, 2: limp tail and attenuated movement, 3: partial hind limb paralysis or severely affected movement, 4: complete hind limb paralysis and 5: moribund). To avoid any bias, group names on the cages were not accessible until finalizing the experiment and spot-check scoring was also occasionally carried out by other investigators in a blinded fashion.

Collagen-induced Arthritis (CIA): Arthritis was induced in 8-12 weeks old male DBA/1 mice by a s.c injection of 100 µg collagen type II (CII) emulsified in CFA (4mg/ml *Mycobacterium tuberculosis*) in a 1:1 ratio. 21 days later, a s.c booster dose of 100 µg CII emulsified in IFA was given and disease progression was regularly monitored hereafter, where each limb was scored 0-3, where 0.5: finger or toe swelling, 1: mild swelling or redness, 2: swelling or redness and 3: marked swelling, redness and/or ankylosis. To preclude unconscious bias, group names on the cages were covered after all treatments. Also, for histopathological assessment, tissue sections stained with haematoxylin and eosin were examined by an experienced pathologist in a blinded fashion.

BONE MARROW CHIMERAS

When generating bone marrow chimeras, one essentially transfers a genotype of interest to the hematopoietic compartment of the recipient mice. Thus, these mice represent a powerful tool which we used to investigate the role of IL-27R signalling during tolerance induction in the context of EAE. To this end, we transferred the bone marrow i.v from either *il27rα-/-* or wt mice into wt C57BL/6 recipients that had been lethally irradiated 24 hours prior to the transfer. Mice were given antibiotics (Baytril®) in their drinking water for two weeks and allowed to rest for an additional six weeks before starting the EAE induction protocol.

FLOW CYTOMETRY & CELL SORTING

Flow cytometry was a central methodology in this thesis, and in particular in paper I and II. In principle, antibodies differentially labelled with fluorescent compounds are used to target specific markers on/in cells of interest within a diverse bulk population. In addition, relative size and granularity is determined by the unique laser light scatter, forward and side, of each cell. As a single cell pass through a series of laser beams, the antibodies will emit their specific fluorescence which is filtered and detected by wavelength-specific sensors (photo multiplying tubes; PMTs). Next, the detected light signals are converted into electronic signals that specialized software display as a numerical value for each cell on an analysis plot. By comparing the specific fluorescent signals for each cell, one is able to distinguish specific cell subsets, assess their phenotype and measure cell division. In my thesis, I used this technology to identify FoxP3+ Tregs in **paper III**. I also identified T cell populations unique to our treatment that I was then able to track during disease progression in **paper II**. This technique was also heavily used in **paper I** to determine DC and T cell phenotype during immunity or tolerance induction, respectively. Finally, it is possible to sort cells of a specified fluorescent profile into separate tubes which enables further analysis of specific cells of interest. Thus, I was able to sort multiple cells from the same environment and analyse their transcriptional profiles subsequent to immunization as well as antigen challenge.

RNA EXTRACTION & qRT-PCR

Cells from the medLN were sorted at different time-points subsequent to immunization and challenge. Resident DCs were Ly6c^{neg}CD11c^{pos}MHCII^{int} (**resDC**) whereas migratory DCs were either Ly6c^{neg}CD11c^{pos}MHCII^{hi}CD103^{pos} (**migCD103**) or Ly6c^{neg}CD11c^{pos}MHCII^{hi}CD11b^{pos} (**migCD11b**). Finally, TEα T cells were identified as CD4^{pos}Vα2^{pos}Vβ6^{pos} (**TEα**). Purity from a test-sort was >98% (but was not specifically assessed in samples that were sorted directly into RLT lysis buffer).

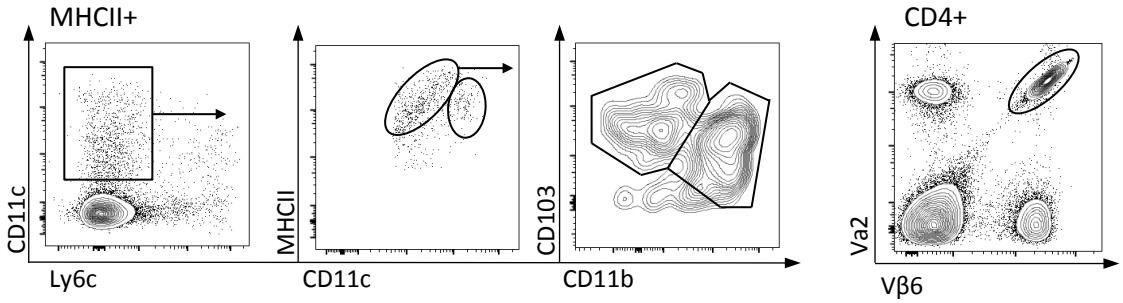


Figure 13. FACS gating strategy of sorted cell populations within the dLN.

Cells to be analysed were sorted directly into RLT lysis buffer (Qiagen) and stored in -80^{o}C until use. After thawing, samples were vortexed in order to disrupt the cellular membrane and the RNeasy micro kit (Qiagen) was subsequently used to extract total RNA according to manual instructions. The RT^2 first strand kit (Qiagen), optimized for our specific downstream application, was used to generate cDNA. Next, pre-optimized custom designed PCR gene array plates (Qiagen) were used to assess the mRNA transcriptional profile of some selected key genes in the different cell populations, using SYBR green for double-stranded DNA detection and relative comparisons of transcripts. 27 genes were used to analyse the DCs,

selected based on their previously described involvement in CD4 T cell tolerance. For T-cells, 43 genes were chosen to reflect subset differentiation, activation and co-inhibitory molecule expression. All genes are summarized in **table 3**.

TABLE 3. Gene arrays used in paper I

DC GENE ARRAY			
IL-27p28	*il27*	**IFNb**	*ifnb1*
CD80	*cd80*	**IDO1**	*IDO1*
CD86	*cd86*	**IDO2**	*IDO2*
CD40	*cd40*	**ILT3**	*lilrb4a*
CCR7	*ccr7*	**Arg1**	*tinagl1*
PD-L1	*cd274*	**IL-12p35**	*il12a*
ICOSL	*icosl*	**IL-12p40**	*il12b*
CD276	*cd276*	**BTLA**	*btla*
CD39	*entpd1*	**HVEM**	*tnfrsf14*
IL-1b	*il1b*	**IL-15**	*il15*
IL-6	*il6*	**MHCIIβ**	*h2-ab1*
IL-10	*il10*	**Galectin-9**	*lgals9*
IL-10R	*il10ra*	**TIM-4**	*tim4*
TGFb1	*tgfb1*		

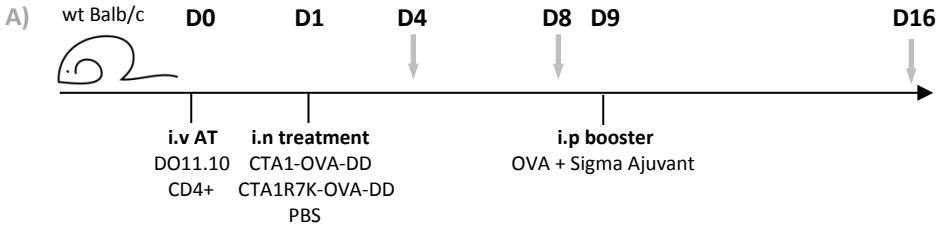
CD4 T-CELL GENE ARRAY					
IL-27p28	*il27*	**c-maf**	*maf*	**mTOR**	*mTOR*
FoxP3	*foxp3*	**CTLA-4**	*ctla4*	**CD80**	*cd80*
LAG3	*lag3*	**TIM3**	*havcr2*	**PDL1**	*cd274*
IFNy	*ifng*	**TGFBR1**	*tgfbr1*	**CD160**	*cd160*
IL-10	*il10*	**TIGIT**	*tigit*	**LIGHT**	*tnfsf14*
IL-17A	*il17a*	**ICOS**	*icos*	**CD69**	*cd69*
TGFb1	*tgfb1*	**ROG**	*zbtb32*	**TGFb3**	*tgfb3*
EBI3	*ebi3*	**Ahr**	*ahr*	**S1PR1**	*s1pr1*
IL-12p35	*il12a*	**Tbet**	*tbx21*	**NFkB**	*NFKB1*
GITR	*tnfrsf18*	**Roryt**	*rorc*	**bcl-6**	*bcl6*
CD40L	*cd40lg*	**GATA3**	*gata3*	**PPARγ**	*pparg*
CD39	*entpd1*	**HIF-1a**	*hif1a*	**CD25**	*il2ra*
CD49b	*itga2*	**PD-1**	*pdcd1*	**IL-2**	*il2*
KLRG-1	*klrg1*	**BTLA**	*btla*	**IL-10R**	*il10ra*
IL-15R	*il15ra*				

For analysis of the qRT-PCR data, all samples were normalized to HPRT and GAPDH house-keeping genes. The gene expression of cells from immunized (CTA1-Eα-DD) and tolerized (CTA1R7K-Eα-DD) mice were then compared to the corresponding cells from naive control mice. Finally, any up- or downregulated genes (fold change >2 was considered biologically relevant) between the treatment groups were also compared.

RESULTS & DISCUSSION

The following chapter aims to summarize and discuss key results from paper I-III upon which this thesis is based. During my time as a PhD-student, I have asked myself two principal questions; can the CTA1R7K-X-DD antigen vector be used to protect an individual against autoimmune disease and what is the nature of the induced state of tolerance?

In **paper I: Tolerance by a mutant cholera toxin-derived fusion protein depends on migratory CD103+ DCs** we dissected early events in the induction of either mucosal tolerance or immunity by the CTA1/R7K-X-DD fusion protein. We used two well-established model epitopes, the $OVA_{323-339}$ peptide and the $E\alpha_{52-68}$ peptide, incorporated in either the CTA1-DD or the enzymatically inactive CTA1R7K-DD construct. Furthermore, transgenic DO11.10 (balb/c) and TEα (C57BL/6) mice were used to follow antigen-specific CD4 T cell responses. In addition, the Yae antibody, which recognizes the $E\alpha_{52-68}$ peptide when loaded onto MHCII ($I\text{-}A^b$), was used to define the relevant APC and allowed us to compare antigen presentation efficiency and longevity of the different constructs in the draining lymph node. Finally, we used qRT-PCR to characterize the differential modulatory effects that the enzymatically active or inactive fusion proteins had on the gene expression profiles of the APC and the CD4 T cell in the draining lymph node at early time points.



B)

	DAY 4				DAY 8				DAY 16			
	proliferation	%DO11.10	IL-10	IFNγ/IL-17	proliferation	%DO11.10	IL-10	IFNγ/IL-17	proliferation	%DO11.10	IL-10	IFNγ/IL-17
CTA1-OVA-DD	+++	+++	+	+++	+++	+	-	+++	+++	+	-	+++
CTA1R7K-OVA-DD	+++	+++	+++	-	-	+	+++	+	-	+	+++	+
PBS	-	-	-	-	-	-	-	-	++	+	-	++

Figure 14. A: Overview of the model protocol used in this experiment. Grey arrows indicate time points of analysis. B: Enzymatically active CTA1 gave rise to robust effector T cell responses over time with upregulated pro-inflammatory cytokine levels. In contrast, although CTA1R7K induced similar early T cell expansion, the resulting responses were regulatory in that the T cells had reduced proliferative capacity *in vitro.* This was concomitant with increased IL-10 production and furthermore, the T cells were able to retain their regulatory behaviour after a systemic pro-inflammatory challenge (D16).

ADP-ribosylation controls the outcome of tolerance or immunity

In order to determine what role ADP-ribosylation had in early priming of antigen-specific CD4 T cells as well as downstream differentiation of effector responses, we transferred $OVA_{323-339}$ specific DO11.10 CD4 T cells to balb/c recipients. We subsequently administered CTA1-OVA-DD, CTA1R7K-OVA-DD or PBS i.n and analysed splenic responses at the indicated time points (**fig. 14A**). We found that early expansion of CD4 T cells occurred irrespective of enzymatic activity, but that *in vitro* proliferative responses to recall antigen were impaired at later time points when the inactive CTA1R7K-OVA-DD fusion protein was used (**fig. 14B**). Furthermore, CTA1-OVA-DD treatment resulted in markedly upregulated pro-inflammatory cytokine responses whereas CTA1R7K-OVA-DD induced IL-10. It was clear that the expansion of antigen-specific CD4 T cells primarily occurred in the lung-draining mediastinal lymph node (medLN) because, although CD4 T cell frequencies increased in the nasal-draining cervical lymph node (CLN) to some extent, the effect was 10-20-fold higher in the medLN. Furthermore, *in vitro* proliferative responses were absent in CLN samples. Similar cytokine patterns after restimulation of lymphocytes was observed in two different mouse strains, both for OVA-specific DO11.10 T cells (balb/c) (**fig 14**; spleen) as well as Eα-specific TEα cells (C57BL/6) (**fig 15**; medLN). In summary, the CTA1R7K-X-DD fusion protein promoted the induction of a tolerogenic T cell population in the medLN which could inhibit downstream systemic effector responses.

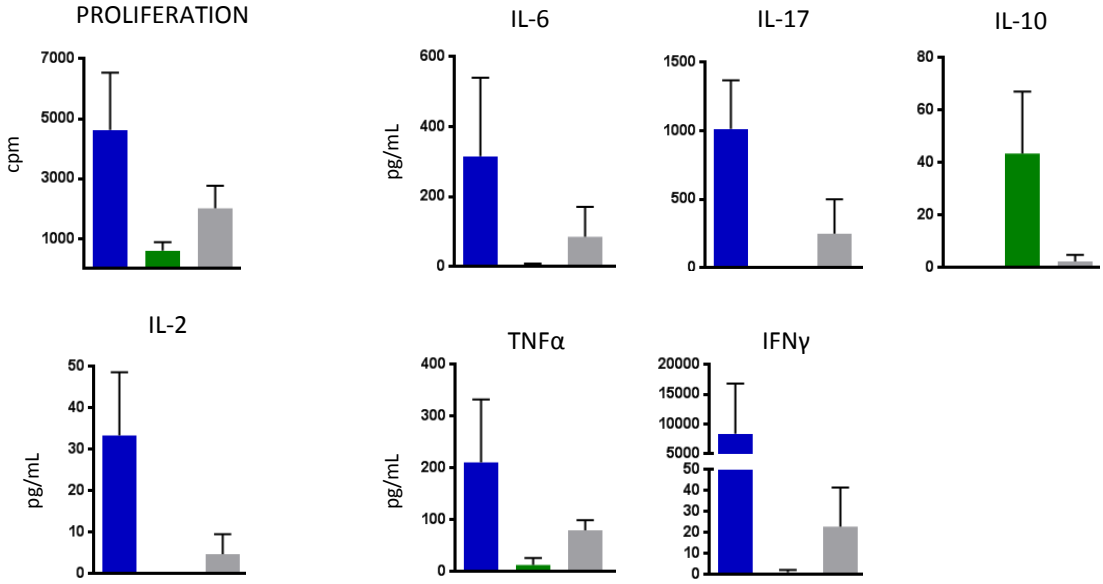


Figure 15. Proliferation and cytokine secretion after $Eα_{52-68}$ peptide restimulation of medLNs. Lymph nodes were harvested from mice on day 16 after AT of TEα CD4 T cells. Mice were also immunized with the indicated constructs and challenged i.p by Sigma Adjuvant and Eα peptide prior to restimulation. Responses in spleen were similar (not shown).

Regulatory CD4 T cells affect the outcome of naïve responses upon challenge

Clearly, antigen-specific effector responses in mice treated with the CTA1R7K-X-DD tolerogen were impaired, an effect which was concomitant with production of IL-10. We asked whether the residual T cell population after tolerization could exert any regulatory force on naïve T cells upon a pro-inflammatory challenge *in vivo*. To address this question,

we transferred antigen-specific DO11.10 CD4 T cells to recipient mice which were subsequently immunized (CTA1-X-DD) or tolerized (CTA1R7K-X-DD) i.n. After 8 days, all mice received new naïve CFSE-labelled DO11.10 CD4 T cells and were subsequently subjected to an i.p challenge immunization with OVA protein and Sigma Adjuvant®. Thus, we were able to simultaneously analyse the $CFSE^{neg}$ pre-primed and tolerized CD4 T cell population and the naive $CFSE^{pos}$ CD4 T cells during a pro-inflammatory response. Indeed, similar to our *in vitro* findings, naïve CD4 T cells proliferated poorly in pre-tolerized mice. In fact, not only was the proliferation of naïve CD4 T cells impaired, but their differentiation was also altered in that a greater proportion of the CD4 T cells were positive for FoxP3, a marker for Tregs. Taken together, it appeared as if treatment with the CTA1R7K-X-DD construct results in a population of actively regulatory T cells that produces IL-10 (fig. 14-15) and are possibly also FoxP3+ Tregs (fig. 16).

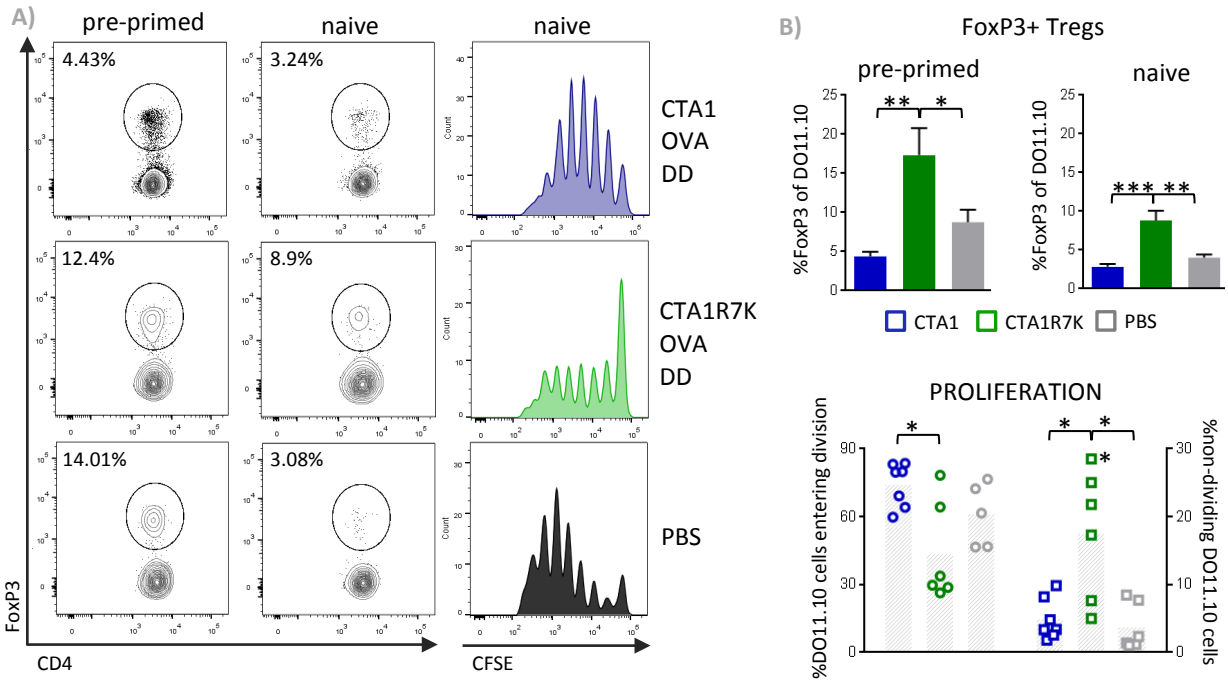


Figure 16. **A:** Representative FACS dot plots of one individual mouse from each treatment group depicting FoxP3+ antigen-specific CD4 T cells in the pre-primed population and in the naïve population that were subjected only to a systemic pro-inflammatory challenge. The CFSE profiles of these cells are also shown. **B:** Summary and statistics of non-dividing cells (first CFSE-peak) and dividing cells (all other CFSE-peaks) as well as FoxP3+ cells within the pre-primed and the naïve population.

Migratory Dendritic cells present the fusion protein irrespective of enzymatic activity but with different kinetics

The CTA1-DD construct was originally developed to be a B-cell targeting adjuvant, since the DD-fragment efficiently binds to immunoglobulins *ex vivo* [357]. However, the following observations suggested that this was probably not the case *in vivo*; firstly, DO11.10 CD4 T cell responses were unimpaired in B-cell deficient JHD mice. Secondly, DCs pulsed *ex vivo* with CTA1R7K-OVA-DD conferred tolerance after i.v transfer into balb/c mice, as demonstrated by reduced antibody responses to a pro-inflammatory antigen challenge. However, this effect was not mimicked by transferred B-cells (paper I supplementary data).

In the continuing studies, we aimed to characterize the relevant APC population *in vivo* in greater detail by using an antibody that detects the presentation of the $E\alpha_{52\text{-}68}$ peptide on MHCII I-A^b specifically. We immunized mice i.n with either CTA1-Eα-DD or CTA1R7K-Eα-DD and investigated antigen presentation of the peptide insert in the medLN at various time points. Of note, because of the detection limit of the Yae antibody, we had to administer a much higher dose of our fusion protein than normal protocols to reliably measure antigen presentation (100µg). Although this introduced the risk of saturating the model system, we were indeed able to discern differences in the density and kinetics of $E\alpha_{52\text{-}68}$ presentation. Importantly, the primary aim of this *in vivo* experiment was merely to give us an indication as to what APC subtype to focus on for further study. Whatever functional implications that these data may or may not have had would have to be confirmed by other experiments, in which a physiological dose of the fusion protein was used (fig.17B-19).

In summary, we found that it was primarily the CD103+ and, to a lesser extent, CD11b+ migratory DCs that processed and presented both CTA1-Eα-DD and CTA1R7K-Eα-DD, and that the DCs positive for antigen presentation appeared after approximately 24 hours (**fig. 17A**). We did not detect a noticeable signal of antigen presentation on monocyte-derived DCs, B-cells, or macrophages for either of the constructs. However, the fusion proteins differed in that CTA1-Eα-DD was presented at detectable levels both earlier and later in time compared to CTA1R7K-Eα-DD (not shown). Whether these construct-dependent differences in antigen presentation arose because of increased protein processing or an induced MHCII expression by the CTA1-Eα-DD construct remains to be investigated, but was most likely a combination between the two. In either case, it is possible that tolerance is mediated by the CTA1R7K-Eα-DD in part by the suboptimal presentation of the antigen, as lower pMHCII density on DCs has previously been shown to favour the development of Tregs rather than CD4 effector T cells [225, 358].

Since antigen presentation seemed to preferentially occur on migratory CD103+ DCs, we explored if their removal would have any consequences to our treatment. To this end, we utilized Batf3-/- mice that lack Batf3-dependent DCs, most strikingly migratory CD103+ DCs in the medLN. Treatment with our tolerogenic vector CTA1R7K-MOG-DD normally reduces disease severity of experimental autoimmune encephalitis (EAE) in mice (paper II, fig. 19). However, when treating Batf3-/- mice, only a partial treatment effect was observed indicating that the CD103+ DCs are not essential, but certainly important, during tolerance induction with the fusion protein (**fig. 17B**).

DCs and T cells undergo time-dependent transcriptional changes during priming

We aimed to advance our understanding of the observation that tolerization of batf3-/- mice was partially impaired. We hypothesized that differential modulation of DC subsets *in vivo* was the reason for the bifurcation observed in downstream T cell responses. Hence, we sorted antigen-specific CD4 T cells and migratory CD11b+ and CD103+ DC's subsequent to either immunization or tolerization and analysed their expression of relevant genes that could potentially be involved in immunity or tolerance (table 3).

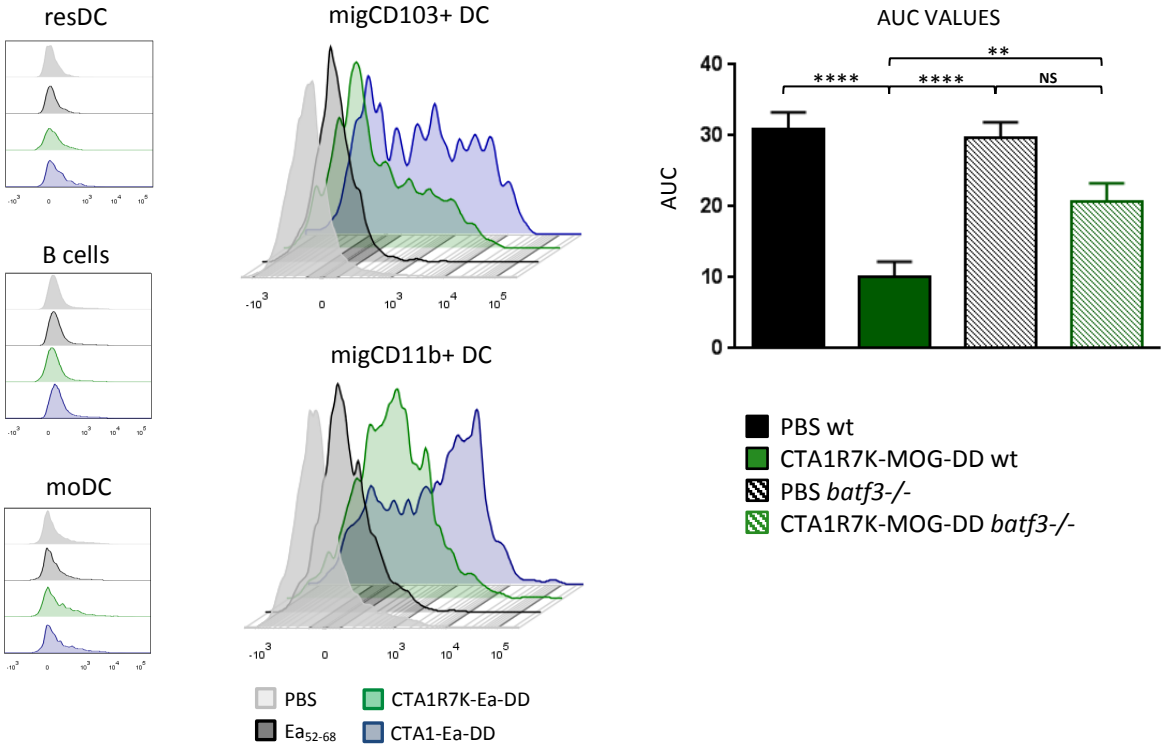
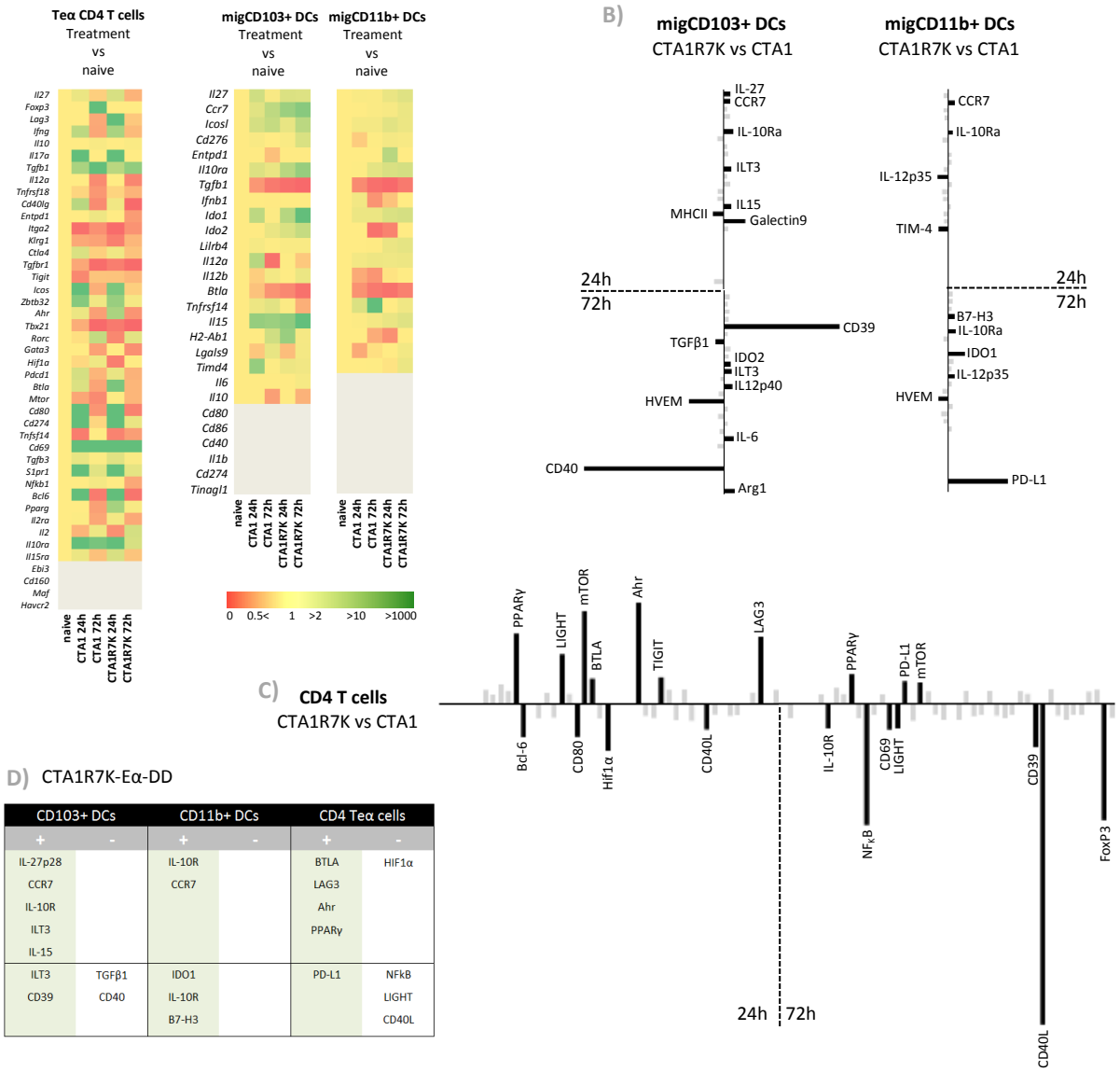


Figure 17. Representative histograms of antigen presentation at 24 hours using the Yae antibody ($\alpha Ea_{52\text{-}68}$ on I-A^b). **Left:** There was no notable increase in antigen presentation in resident DC, B cells or monocyte-derived DCs at 24 hours. **Right:** Representative histograms of antigen presentation in CD103+ and CD11b migratory DCs after treatment with the different fusion proteins compared to peptide alone or PBS. The peak of antigen presentation occurred at 24 hours, as shown here, and primarily in migratory DC subsets. Both CTA1R7K and CTA1 constructs facilitated efficient presentation of the incorporated antigen, but CTA1 displayed a higher density of antigen per APC.

Rather than being transcriptionally unaffected, tolerized DCs exhibited a unique upregulation of tolerogenic molecules compared to steady-state DCs. This phenotype was seen primarily in CD103+ DCs after 24 hours, but was extended to involve both CD103+ and CD11b+ DCs 72 hours after tolerization. Among the specifically upregulated genes in tolerized migCD103+ DCs, we found factors that influence migration (CCR7), T cell survival (IL-15), Tr1 differentiation (IL-27p28), autocrine tolerance maintenance (IL-10R) and negative T cell regulation (ILT3) to name a few (fig. 18). The expression of these genes was maintained at 72 hours post-tolerization, at which point the expression of CD39 was upregulated. Tolerized migCD11b+ DCs expressed similar early genes, such as IL-15, IL-27p28, CCR7 and IL-10R but none were unique to CTA1R7K-Eα-DD with the exception of CCR7. In contrast, 72 hours after tolerization, migCD11b+ DCs acquired a more pronounced tolerogenic phenotype with specifically increased expression of IL-27p28, IL-10R as well as genes involved in negative CD4 T cell regulation (B7-H3) and tryptophan metabolism (IDO1, IDO2).



D) CTA1R7K-Eα-DD

CD103+ DCs		CD11b+ DCs		CD4 Teα cells	
+	-	+	-	+	-
IL-27p28		IL-10R		BTLA	HIF1α
CCR7		CCR7		LAG3	
IL-10R				Ahr	
ILT3				PPARγ	
IL-15					
ILT3	TGFβ1	IDO1		PD-L1	NFkB
CD39	CD40	IL-10R			LIGHT
		B7-H3			CD40L

FIGURE 18. Overview of differences in the transcriptional profiles of immunized and tolerized DC subsets and antigen-specific TEα CD4+ T cells. A: Gene expression of FACS sorted cells from immunized (CTA1) and tolerized (CTA1R7K) compared to naïve control samples (fold change). B: Fold change differences between DCS from CTA1- and CTAR7K-treated mice post-immunization. C: Fold change differences between antigen-specific CD4 T cells from CTA1- and CTAR7K-treated mice post-immunization. D: Genes specifically regulated by the CTA1R7K-Eα-DD fusion protein (compared to both steady-state and CTA1-Eα-DD)

Most striking was the early upregulation and maintained expression of ILT3, IL-10R, IL-15 and IL-27p28 compared to both steady-state and immunized CD103+ DCs. Though the ligand for ILT3 is currently unidentified, it has been shown to suppress CD4 T cell responses [92]. It also contains a cytoplasmic ITAM motif which inhibits NF_KB and the transcription of several co-stimulatory molecules in the DC itself [93-95]. IL-27p28 has dual functions in that it promotes Tr1 differentiation, but also enable a tolerogenic phenotype in DC's by inducing expression of CD39 [136]. Indeed, we found that CD39 was upregulated in the CD103+ DCs 72 hours after tolerization, but not immunization. Of note, IL-15 was upregulated in CD11b+ DCs, but also specifically in tolerized CD103+ DCs. Furthermore, IL-15Rα was transiently upregulated in tolerized T cells. While it has been described that IL-15 promotes survival of

antigen-specific CD8+ T cells, the anti-apoptotic effects of IL-15Rα signalling in CD4 T cells during primary immune responses is not well characterized. Paradoxically, other published work has demonstrated that the removal of cells expressing IL-15Rα signalling has beneficial effects on autoimmunity and transplant rejection [359, 360]. Thus, further investigation of this pathway and how it translates to tolerance induction in our model is needed.

Finally, gene expression in CD4 T cells was assessed in parallel and while both tolerization and immunization resulted in upregulation of factors involved in both positive and negative regulation, uniquely upregulated genes in tolerized T cells involved IL-27p28, LAG3, Ahr, PPARγ and IL-15R. Pro-inflammatory genes such as NFkB, LIGHT and CD40L were profoundly downregulated in tolerized T cells but, unexpectedly, only increased PD-L1 expression was specific to the tolerogen.

In summary, in this study we aimed to shed light on the early pathways that determine the outcome of immunity or tolerance using our CTA1/CTA1R7K-DD platform. We identified the specific APC that is targeted by our fusion protein *in vivo* and examined CD4 T cell functions after administering the fusion proteins, which were either skewed towards Th1- and Th17-like responses (CTA1-DD), or dominated by Tregs (CTA1R7K-DD). In conclusion, this study describes an antigen delivery system that can be used to study APC function. But more importantly, we present a tolerogenic adjuvant that should be further explored for treatment of autoimmune disorders.

In **paper II: IL-27R is critical for tolerance induction by the CTA1R7K-MOG-DD fusion protein in experimental autoimmune encephalitis** we explored the functional impact of our tolerogenic vector by using models of experimental autoimmune encephalitis (EAE). In the present study, we used both an acute and a relapsing-remitting EAE model to mimic different aspects of human MS disease. Furthermore, *IL-27rα-/-* mice were used to generate bone marrow chimeras in the acute EAE model to dissect the role of the IL-27 pathway in tolerance induction after CTA1R7K-MOG-DD treatment.

CTA1R7K-MOG-DD treatment protects against acute encephalitis

To test whether the CTA1R7K-X-DD fusion protein would be effective as a treatment of autoimmune encephalitis, we generated fusion proteins in which disease-specific peptides were incorporated. The induction of EAE in C57BL/6 mice, using a peptide from myelin oligodendrocyte protein (MOG_{35-55}), results in rapidly progressing acute encephalitis. We incorporated the same peptide in our fusion protein and followed EAE disease progression in mice treated with the **CTA1R7K-MOG-DD** tolerogen (fig. 19A-B). A marked reduction of clinical symptoms was observed, which also correlated with reduced proliferative responses to recall MOG peptide in both the spleen and the inguinal lymph node (ILN) (fig. 19B). Of note, *in vitro* restimulation of lymphocytes from CLN, medLN or lung had no effect on proliferation or cytokine production. In SP and ILN, pro-inflammatory cytokines in the supernatant of restimulated lymphocytes from treated mice were significantly reduced (not shown). However, contrary to previous findings in other mouse models, reduced effector responses did not correlate with increased secretion of IL-10.

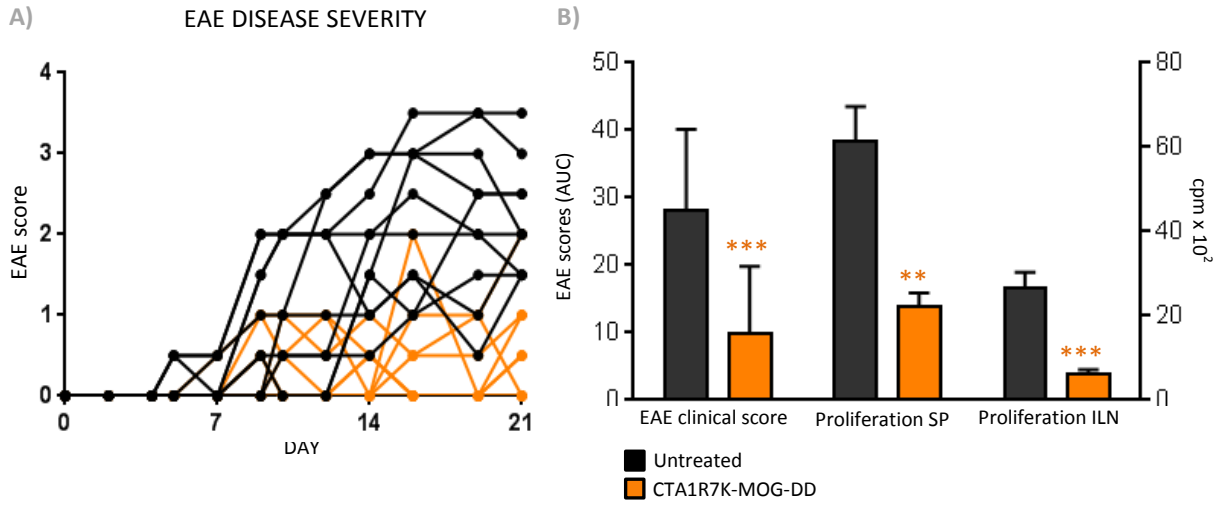


Figure 19. A: Protocol of EAE disease induction and treatment schedule. B: Clinical scores of each individual mouse over time in the MOG-induced EAE-model during treatment with PBS or CTA1R7K-MOG-DD. C: **Far left:** Mean clinical scores of encephalitis in the acute EAE-model, shown as AUC values over the whole period of disease (D0-D21) after i.n treatment with the CTA1R7K-MOG-DD fusion protein compared to untreated control mice. **Right:** Impaired proliferation of *in vitro* restimulated lymphocytes in the treated group on D21 in both spleen and inguinal lymph node is shown in the right y-axis.

To our surprise, the protective effect that the CTA1R7K-MOG-DD construct clearly had on disease outcome did not seem to coincide with an existing population of IL-10 producing Tregs. However, effector responses were clearly lacking, as indicated by reduced proliferative responses *in vitro* (**fig. 19B**), but also by significantly fewer infiltrating CD4 T cells into CNS. In fact, at no time point measured did CD4 T cells infiltrate the CNS of treated mice (**fig. 20A**). These observations prompted us to investigate effector responses at earlier time points during EAE progression rather than at the final stages of EAE disease. We hypothesized that CTA1R7K-MOG-DD treatment either a) directly impaired the generation of effector T cells during the induction phase, or b) indeed did promote the formation of regulatory T cells, but which already had exerted their function by the time we analysed these mice.

Treatment of EAE correlates with the expansion of Tr1 cells in the medLN

We found that T cells readily underwent initial expansion at the site of induction, and yet did not induce severe clinical manifestations in mice that were treated (**fig. 20B**). This observation suggested that regulation most likely occurred somewhere in between the stage of priming and blood-brain barrier extravasation, but was not instructive as to whether this suppression occurred at the site of disease induction (ILN), site of tolerization (medLN) or in the effector tissues (CNS). Therefore, we followed the expansion of LAG3+CD49b+ Tr1 cells as well as FoxP3+ Tregs at different time points in the medLN, ILN, SP, CNS and dCLN. A dramatic increase of Tr1 frequencies during the progressive phase of disease (day 11), was observed (**fig. 20C**), which was back to baseline at day 21. The expanded Tr1 cell population seemed unique to the medLN, because we did not find a similar increase in any of the other organs analysed. Because LAG3 and CD49b are membrane-expressed surface proteins, we were able to sort the Tr1 cells to test whether they had suppressive functions *ex vivo*. Thus, sorted LAG3+CD49b+ cells from the medLNs of treated mice were co-cultured at different ratios with splenic effector T cells (CD4+CD44+LAG3-) from untreated mice, naïve CD11c+ APC's and MOG_{35-55} peptide. Indeed, proliferation was impaired when adding increasing numbers of Tr1 cells, which correlated with an increased IL-10 secretion in the culture supernatants. Hence, the Tr1 cells could be actively suppressive during disease progression by producing IL-10. In fact, we have previously shown that tolerance induction with the fusion protein could not be achieved in IL-10 deficient mice.

There were no significant differences in the proportion of FoxP3+ Tregs in either of the groups or when compared to naïve mice (not shown). In this regard, these results differ from our findings in other models (paper I and paper III). In these studies, we observed higher frequencies of FoxP3+ Tregs that correlated with tolerance induction, although we did not investigate their functionality (paper I/III) or antigen specificity (paper III). These discrepancies could either be model-dependent or a consequence of the different methodologies used and time points analysed. For example, in paper III we observed an increased frequency of FoxP3+ T cells in the circulation of treated mice in which arthritis was induced. However, we did not investigate whether these Tregs were responsive to collagen. Furthermore, the treatment was administered via the oral route in the form of a bioencapsulated plant-expressed protein and the final, bioavailable dose reaching the GALT was not possible to assess. In paper I, we measured the frequency and absolute numbers of FoxP3+ CD4 T cells within a distinct population of antigen specific DO11.10 T cells, which was

significantly higher for mice treated with the CTA1R7K-X-DD tolerogen. However, overall FoxP3+ frequencies of the total CD4 T cell population, which includes both OVA-specific and endogenous T cells, was not different between groups. This clearly illustrates the importance of high-resolution gating of the cell to be studied, and using the transgenic MOG-specific 2D2 CD4 T cells in future experiments will address these technical issues. It may well be that FoxP3+ Tregs and Tr1 cells cooperatively establish tolerance during CTA1R7K-X-DD treatment, by mechanisms involving IL-27 signalling.

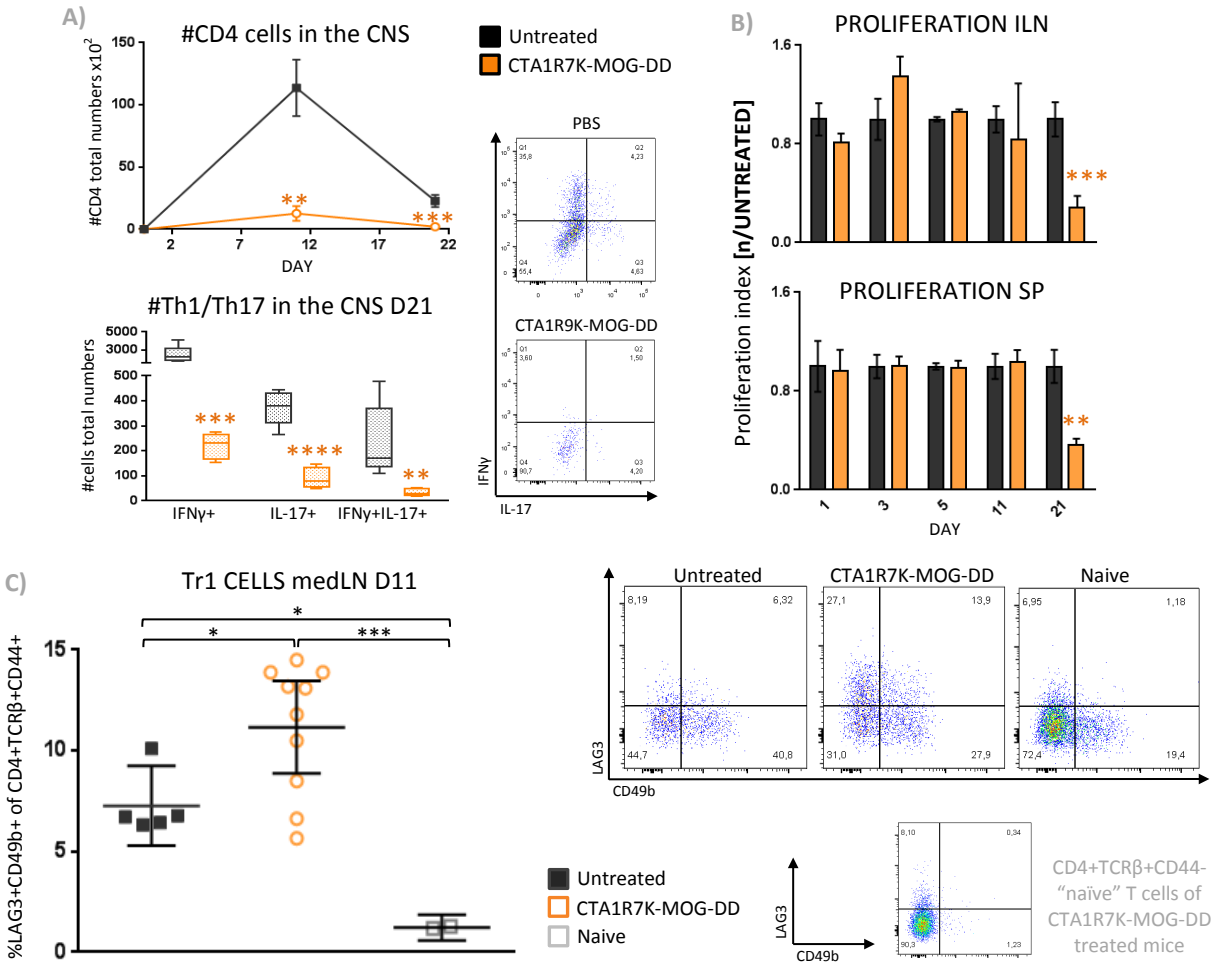


Figure 20. A: #CD4 and Th1/Th17 infiltration in the CNS of treated and untreated mice. B: Relative proliferation; cpm treated/$\overline{cpm}$ untreated (orange) vs cpm untreated/$\overline{cpm}$ untreated (black). C-D: CD4+TCRβ+CD44+CD49b+LAG3+ Tr1-like cells in medLN, 11 days after EAE induction.

The finding that Tr1 cells specifically and uniquely expand in the medLN is striking but somewhat puzzling, as the connection to the effector CD4 T cells induced in the distant ILN is not obvious. Interestingly, Odoardi *et al* showed in rats that before encephalitogenic CD4 T cells infiltrate the CNS, they are sequestered in the lung in which they undergo a shift in their transcriptional program and subsequently become licensed to migrate to the effector site,

i.e. the CNS. They leave the lung parenchyma via the lymph and recirculate through the medLN before reaching CNS. Furthermore, memory cells were reactivated and caused paralytic disease upon intra-tracheal antigen challenge [361]. Whether the medLN constitute an interface in which our administered fusion protein affects the effector CD4 T cells on their way to the CNS remains to be investigated, but it is certainly a plausible theory in light of these findings. This hypothesis consequently raises the question as to whether, in our model, the Tr1 expansion observed in the medLN arises from naïve precursors or is a result from a phenotypic shift of previously primed effector CD4 T cells that renders them unable to traverse the BBB and enter the CNS.

IL-27R signalling is critical for tolerance induction

Previous findings from other models of autoimmunity indicated that IL-27 production could be specific to CTA1R7K-X-DD treatment (T1D, unpublished data). Therefore, we aimed to further investigate the involvement of the IL-27 pathway in the context of EAE. Firstly, a polarisation assay was set up in which freshly isolated CD11c+ APCs were pulsed with different fusion proteins overnight and subsequently washed. Next, 2D2 T cells were co-cultured with these APCs for 6 days and restimulated via polyclonal activation, after which culture supernatants were analysed for cytokine content. Cells stimulated with CTA1R7K-MOG-DD produced lower amounts of pro-inflammatory cytokines compared to positive controls but secreted more IL-10. Also, we confirmed that in line with previous observations, IL-27 was significantly upregulated in restimulated co-cultures (**fig. 21A**). Whether this IL-27 was produced by DCs or the 2D2 cells in this experimental setting has not yet been evaluated.

Most importantly, to explore whether this finding had any functional implications, we generated bone marrow chimeras by transfer of either C57BL/6 wt or *IL-27rα-/-* bone marrow into wt recipient mice and induced EAE. Strikingly, we found that treatment in *IL-27rα-/-* chimeras showed no reduction of symptoms whatsoever (**fig. 21B**). These observations indicated that IL-27 signalling is fundamental for the induction of tolerance by the CTA1R7K-MOG-DD fusion protein. Of particular note, IL-27 has since its discovery emerged as a potent suppressor of Th1, Th2 and Th17 responses, which efficiently limits CNS inflammation in several animal models [362]. Furthermore, it has been convincingly shown that *IL-27rα-/-* mice develop a more EAE severe disease than wt controls and exhibit exacerbated Th17 responses [152]. Thus, IL-27 signalling has a central role in controlling CD4 effector T cell responses and in particular Th17 cells in the EAE model. Therefore, there is a risk that the lack of tolerance induction that we observed *in IL-27rα-/-* mice was more specific to the model that we used here, than to the intrinsic properties of our fusion protein. However, our data from paper I corroborate our findings from the EAE model in that migratory CD103+ DCs, and to a lesser extent CD11b+ migDCs, upregulated their gene expression of the IL-27p28 subunit after tolerization with the CTA1R7K-Eα-DD fusion protein during steady state conditions. Therefore, taken together our results indicate that CTA1R7K-X-DD specifically upregulates IL-27 which induces Tr1 differentiation, and that lack of IL-27R signalling abolishes the tolerogenic effect of the fusion protein during EAE disease progression.

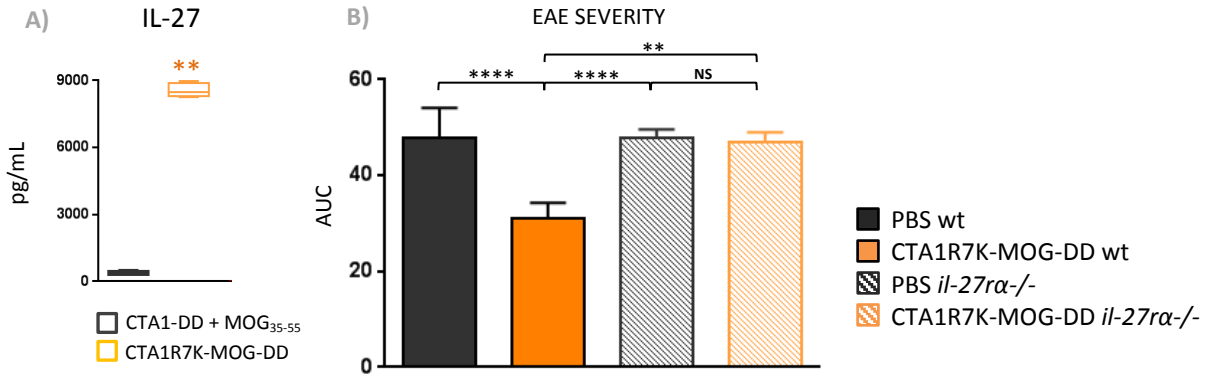


Figure 21. A: Polarized transgenic 2D2 T cells that recognize MOG_{35-55} specifically, co-cultured with naïve CD11c+ APCs and the indicated stimuli for 6 days prior to restimulation with αCD3 and αCD28 co-stimulatory factors. B: AUC values of clinical scores during the entire EAE disease course in treated and untreated wt and IL-27Rα-/- chimeric mice.

CTA1R7K-PLP-DD treatment protects against relapse-remitting encephalitis

Finally, we used the PLP-induced EAE model in SJL mice, which more closely resembles the typical clinical course of human MS [363]. We examined whether our fusion protein was able to mediate protection in a relapsing-remitting disease, where relapses are dominated by epitopes different from the incorporated $PLP_{139-151}$ peptide used in our treatment. While early administration of CTA1R7K-$PLP_{139-151}$-DD reduced symptoms throughout the entire disease course, therapeutic treatment using the same construct was unsuccessful (not shown). In contrast, treatment with a fusion protein cocktail containing $PLP_{139-151}$ and $PLP_{178-191}$ peptide inserts - of which $PLP_{178-191}$ constitutes the immunodominant epitope during the second disease peak [364] - was able to prevent all downstream relapses (fig. 23). Similar to the MOG-induced EAE model, treated mice had fewer infiltrating CD4 T cells in the CNS at the time of experiment termination, which typically occurred during the peak of the second relapse. In addition, CNS fewer infiltrates did not correlate with an increase in infiltrating FoxP3+ Tregs of LAG3+CD49b+ Tr1 cells.

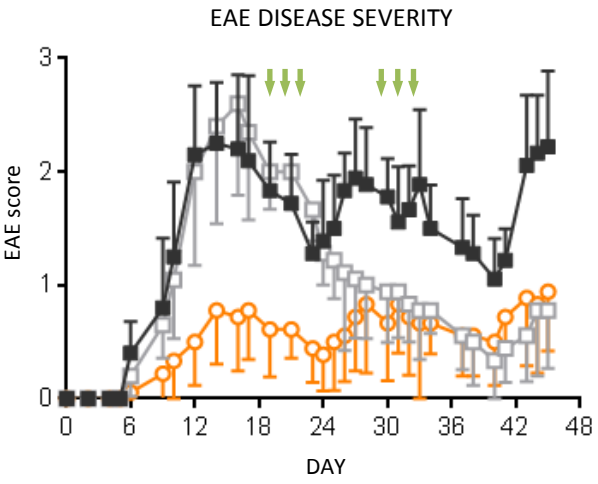


Figure 23. A: Polarized transgenic 2D2 T cells that recognize MOG_{35-55} specifically, co-cultured with naïve CD11c+ APCs and the indicated stimuli for 6 days prior to restimulation with αCD3 and αCD28 co-stimulatory factors. B: AUC values of clinical scores during the entire EAE disease course in treated and untreated wt and IL-27Rα-/- chimeric mice.

Untreated
CTA1R7K-PLP-DD (normal)
CTA1R7K-PLP-DD (therapeutic)

HLA polymorphism and heterogeneity in the epitope repertoire within the human population are issues that need to be taken into consideration if antigen-specific tolerance treatments are to be clinically feasible. Further investigations on the use of treatment cocktails, using combinations of the CTA1R7K-DD fusion protein with different peptide inserts, will probably be the way forward to successfully translate our experimental findings into the clinical setting.

In summary, in paper II we provide evidence that the tolerogenic adjuvant CTA1R7K-X-DD mediates tolerance to incorporated peptides in an inflammatory context and was therapeutically effective in a relapsing-remitting EAE model. Our data suggests that these effects are dependent on IL-27 signalling and is, possibly, also mediated by Tr1 induction in the medLN.

In **paper III: Feeding transgenic plants that express a tolerogenic fusion protein effectively protects against arthritis** we used the collagen-induced arthritis (CIA) model in which we had previously demonstrated effective therapeutic effect after intranasal administration of the CTA1R7K-COL-DD fusion protein [35]. Here, we used the CIA model for a proof-of-principle study in which the treatment strategy consisted of a transgenic edible plant as a carrier of our tolerogenic fusion protein for oral administration.

Feeding transgenic plants expressing CTA1R7K-COL-DD to mice protects them against severe arthritis

As it had been shown previously that we were able to prevent or alleviate arthritis after i.n administration of CTA1R7K-COL-DD [35], we decided to exploit this model to investigate whether we were able to achieve the same effect on disease outcome when administering the treatment orally as an edible plant vaccine. While edible transgenic plants have been used to successfully induce tolerance in some models of autoimmunity [350-352], few studies have explored the possibility of using collagen administered by dietary plants to treat CIA and have thus far only produced inconclusive results [365].

We successfully expressed CTA1R7K-COL-DD in *Arabidopsis thaliana* plants which were subsequently fed *ad libitum* to mice in which CIA was induced and disease progression was monitored. *Arabidopsis* was specifically chosen as opposed to, for example, carrots or other more intuitively palatable options, as the expression organism of choice. From an experimental perspective, *Arabidopsis* constitutes a model organism where the genome has been fully sequenced, which is useful when evaluating the impact of the insert location after transformation. In addition, because it is such a well-established plant research model, there is a wealth of knowledge available on how to engineer the optimal expression of recombinant proteins in these plants. Finally, *Arabidopsis* is small-sized and has a rapid development time and high seed yield, which makes it an affordable and efficient plant expression system [366].

The CIA model is frequently used to estimate the therapeutic effect of anti-inflammatory drug candidates for RA [319] and was also used in this study to assess the efficacy of our transgenic plants in reducing arthritis. Indeed, we were able to show that oral feeding of transgenic plant significantly reduced CIA disease whereas all control mice developed severe disease and tissue destruction (**fig.24A-B**). The effect was dependent on the peptide-antigen because oral feeding of a fusion protein without the incorporated COL peptide (CTA1R7K-DD) did not have any impact on disease outcome (not shown).

Protein expression of CTA1R7K-COL-DD was estimated to amount to 2.5% of total soluble protein (TSP), which roughly translates into 30µg of the fusion protein per gram of ingested plant material. For relevant comparisons to other studies; every gram of ingested plant contained approximately 1.5µg of the COL peptide and approximately 70g of *Arabidopsis* was given on each feeding occasion to groups of ten mice. Because they were fed *ad libitum*, we cannot determine the exact dose given to each individual mouse. In fact, our approach emulates a recognized practical problem with edible vaccines; that of batch-to-

batch variability in protein expression levels and dosage [367]. However, at the population level, the average available dose of 7g of plant, or 10µg of COL-peptide, was sufficient to significantly reduce disease (fig. 24-25) and, in fact, had we controlled for dosage we may have achieved an even better clinical outcome in the CIA model.

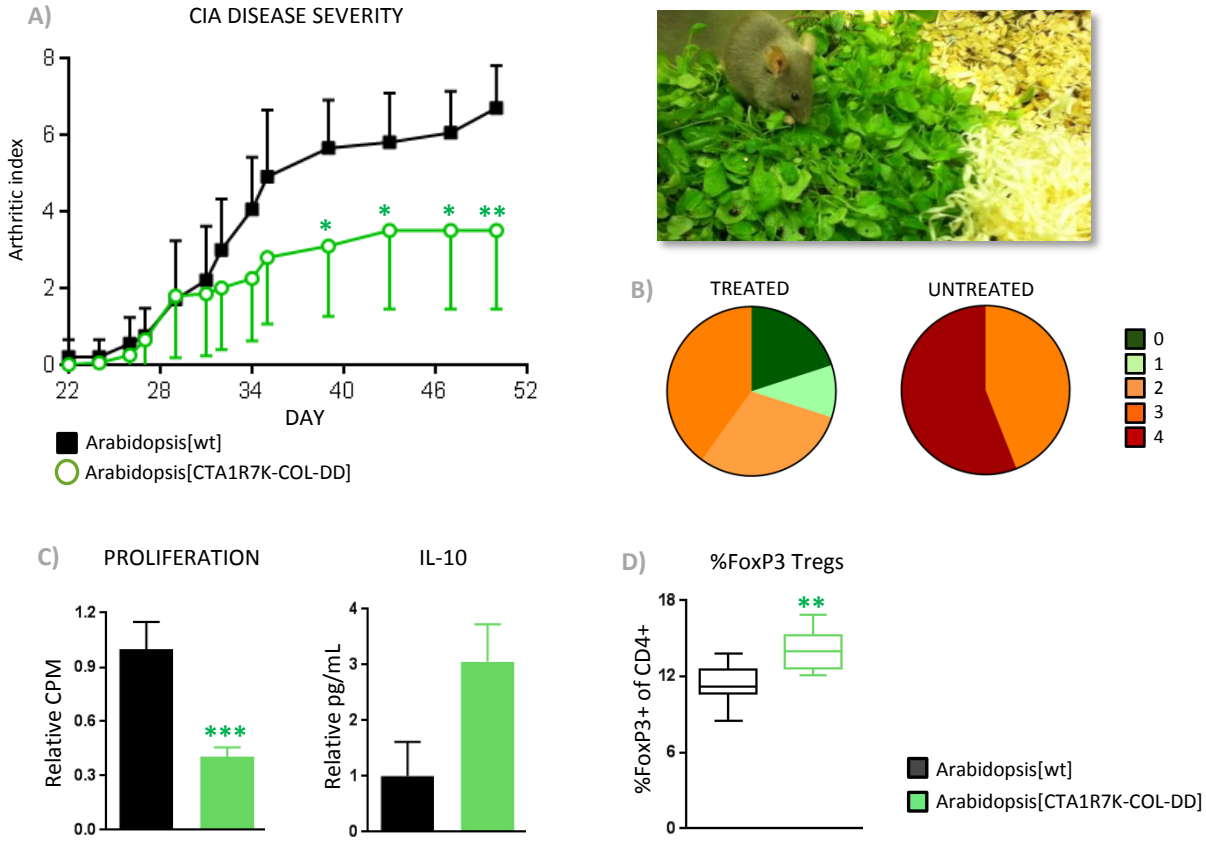


Figure 24. A: Mean clinical scores of arthritis in the CIA-model after feeding with either wt or transgenic *Arabidopsis thaliana.* B: Distribution of mild to severe disease, where 0 equals healthy mice and 4 the most severe arthritis in groups of 10 mice in total. C: Proliferation and IL-10 production of *in vitro* restimulated splenocytes D: FoxP3+ frequencies of CD4 T cells in peripheral blood.

To investigate the immunological properties of the observed therapeutic effect, humoral and cellular responses were analysed. Treated mice had significantly decreased levels of COL-specific IgG1 and IgG2a/b antibodies. This finding suggested that both Th1 and Th2 effector subsets were reduced, and we excluded the possibility of Th2 skewing being the underlying mechanism of our treatment. This was further substantiated by the fact that pro-inflammatory cytokine responses from *in vitro* restimulated splenocytes of treated mice were decreased compared to untreated controls; for IFNγ (Th1), Th17 (Th17) and IL-13 (Th2) alike. Similar to our previously published data on i.n. treatment of CIA using recombinant CTA1R7K-COL-DD, IL-10 production was increased in treated mice (fig. 24C). In addition, we observed higher frequencies of FoxP3+ CD4 T cells in the peripheral blood of treated mice (fig. 24D). However, FoxP3+ Tregs were not investigated in the previous study [36]. It should once again be emphasized that although the increase of FoxP3+ cell frequency in treated

mice was modest (approximately 3%), we did analyse the entire endogenous CD4 T cell population and were potentially diluting the difference between groups because of that. Thus, the biological relevance of this small, but statistically significant, increase should be further studied; both in terms of antigen specificity as well as suppressive capacity.

To validate our clinical observations of reduced CIA severity in treated mice, we measured the serum concentration of two biomarkers; the Matrix Metalloproteinase-3 (MMP-3) has been shown to correlate with RA disease intensity and joint destruction [368, 369], while IL-6 is a general marker of inflammation and indicative of a dysregulated Treg/Th17 axis during autoimmunity [370]. Serum levels of both MMP-3 and IL-6 were significantly reduced in treated mice, confirming the efficacy of our treatment. Furthermore, concentrations of these biomarkers were positively correlated to the individual clinical scores, as presented in figure 25A (r^2=0.6 for IL-6 and r^2=0.68 for MMP-3). Finally, a blinded histological examination of the joints revealed that treated mice had fewer infiltrates of inflammatory cells in the synovium which correlated with reduced overall tissue destruction and bone erosion (fig 25B).

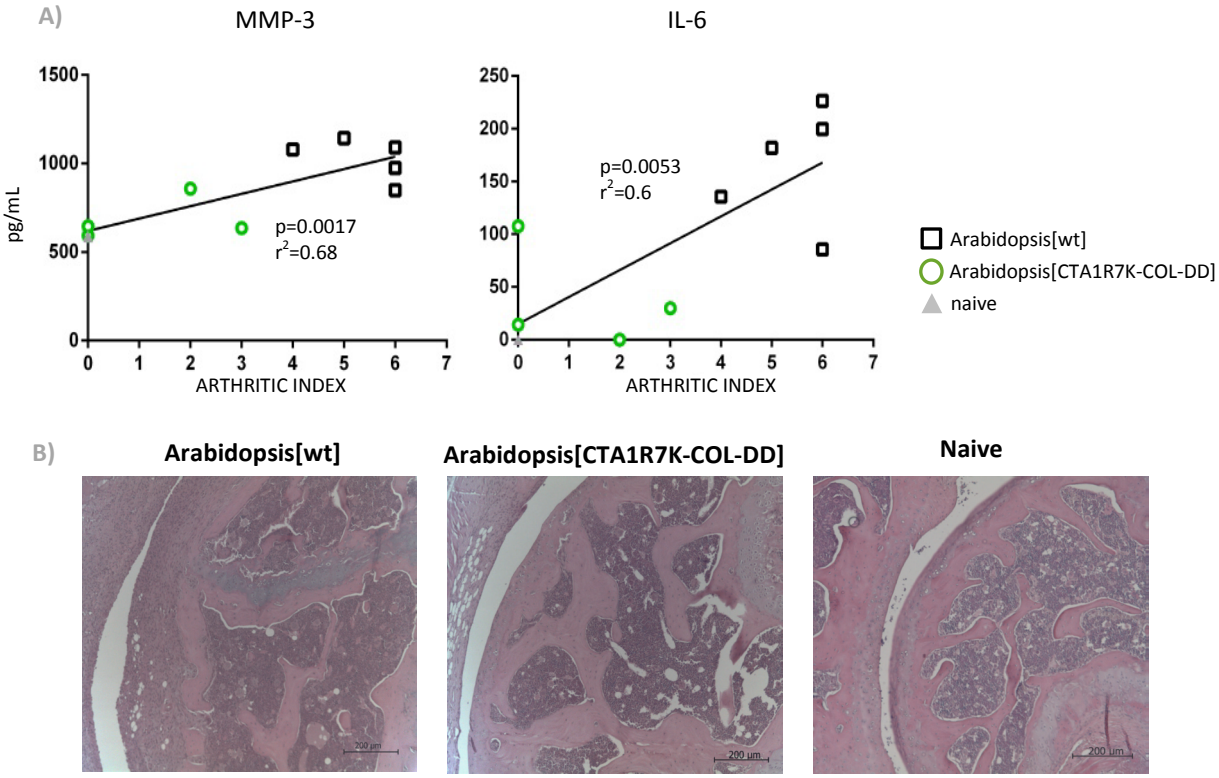


Figure 25. A: Serum biomarkers MMP-3 and IL-6 in collagen-induced arthritis. At the end of the experiment, serum was collected from treated, untreated and naive controls and IL-6 and MMP-3 was measured by ELISA. There was a positive correlation between disease severity and IL-6 or MMP-3, as well as between IL-6 and MMP-3 themselves. B: Histological images, reprinted from [371] with permission, were brightened 20% for better visualization.

Of note, our previous studies have shown that an equimolar dose of peptide alone, or whole OVA protein, has no effect on tolerance induction after i.n treatment compared to our fusion protein (paper II, unpublished observations and [36]). Although we did not address this

question in paper III for oral tolerance, Hashizume *et al*. [365] generated transgenic rice that expressed the COL peptide, and daily feedings corresponding to 25µg of peptide to mice for two weeks resulted in only a moderate decrease of IgG2a antibody responses [365]. This suggested that the peptide was indeed taken up in the gut and recognized by the immune system, but not at sufficient amounts or under conditions favourable enough to interfere with ongoing immune responses. Conjugating the disease-relevant antigen to a targeting moiety may be a viable approach to increase the efficiency of peptide-based treatment strategies of autoimmune disease. In support of this idea, Ruhlmann *et al.* demonstrated that a CTB-proinsulin fusion protein expressed in tobacco leaves could reduce insulitis in the T1D NOD mouse model. Indeed, our own data in paper III suggested that the CTA1R7K-COL-DD targeting properties relevant for tolerance induction, because we were able to achieve disease suppression at considerably lower doses than previous studies using peptides only (40µg vs 350µg of COL peptide in total).

In summary, paper III was a proof-of-principle study in which we investigated whether we could successfully reduce CIA after feeding of edible plants expressing our tolerogenic CTA1R7K-COL-DD fusion protein. Indeed, treated mice exhibited significantly milder symptoms of disease compared to untreated mice. We believe that our results argues in favour of using transgenic plants for antigen delivery and should be considered a realistic alternative to current administration strategies of biopharmaceuticals.

CONCLUDING REMARKS & FUTURE DIRECTIONS

Both RA and MS are debilitating autoimmune diseases that affect a significant minority of the human population. Current treatments are broadly immunosuppressive, and are only able to alleviate symptoms of disease. Therefore, the development of drugs that reinstate antigen specific tolerance has gained much attention, in the anticipation that they will be effective at reducing, or even reversing, autoimmunity without the severe side-effects that we associate with current treatment strategies.

The central topic of this thesis is to elucidate the mechanisms by which the CTA1R7K-X-DD fusion protein induces tolerance during auto-immunity. In my project, I have used several experimental models to **1)** characterize the modulatory effects that the fusion protein has on the DC, **2)** characterize the regulatory CD4 T cell population that is induced by our tolerogen and **3)** characterize the effects that this population has on effector CD4 T cell functions. Our aim has also been to describe the robustness of the patented CTA1R7K-X-DD platform technology by using several models of autoimmunity (paper II/III, [35] and unpublished data on T1D and EAMG). My data represents a considerable addition to our current knowledge on the early spatiotemporal regulation of mucosal immune responses after CTA1R7K-X-DD treatments. The main conclusions from the papers in this thesis could be summarized as follows:

I. CTA1R7K-X-DD induces tolerance in several models and mouse strains, and supports the notion that it could be be used in future treatments of human MS and RA diseases.

II. CTA1R7K-X-DD is preferentially presented by migratory CD103+ DCs upon nasal administration, and they play an important role in tolerance induction.

III. DCs uptake of CTA1R7K-X-DD is associated with the induced expression of several co-inhibitory molecules. In particular, IL-27 is upregulated for at least 72 hours in migratory CD103+ DCs, and IL-27R signalling appears to be crucial for effective tolerance induction in the EAE model.

IV. CD4 T cells aquire a Tr1-like phenotype upon treatment with CTA1R7K-X-DD, which subsequently suppress the priming of naïve neighboring cells.

V. The Tregs induced by CTA1R7K-X-DD during EAE progression is mainly detected in the lung-draining medLN, and yet pathogenic CD4 T cells generated at distant sites are prevented from entering the CNS and cause tissue destruction and progression of disease.

VI. Oral treatment with CTA1R7K-X-DD was successful when expressed by a transgenic plant and administered as an edible treatment in a mouse model for rheumatoid arthritis.

Although the results from this thesis has shed some light on the regulatory elements that promote tolerance during CTA1R7K-X-DD treatment, some of our existing data will need further investigation to confirm our hypotheses on the mechanism of action. Also, as with all new information, our results invariably open up as many new questions as they answer.

For example, future studies would have to address whether the CTA1R7K-X-DD fusion protein acts solely and directly on the migratory DCs or if there are additional innate elements *in vivo* that we have overlooked, but which are important in the tolerance-inducing effect.

Furthermore, the microarray in paper I demonstrated that the CD103+ migratory DCs were indeed affected by CTA1R7K-X-DD treatment. These data also showed that a single time point of analysis will not be adequately informative, because we observed that alterations in gene expression patterns changed over time and was highly dynamic.Therefore, we have already planned to extend our scope to include recall *in vivo* responses in order to better understand the transcriptional changes that the regulatory CD4 T cells undergo at different stages to different immune stimuli (tolerance induction vs pro-inflammatory challenge). Also, the proposed experimental design will answer whether the regulatory CD4 T cells may have secondary modulatory effects on newly arriving DCs, even when the fusion protein is no longer present. However, rather than using the limited microarray we have used so far, a more comprehensive NGS analysis on these cell populations will be undertaken. Such an analysis would have the potential to reveal novel genes or gene sets that may be regulated by the CTA1R7K-X-DD fusion protein.

Importantly, we observed a correlation (but not causality) between the transient expansion of a LAG3+CD49b+ Tr1-like cell population in the medLN and reduced infiltration of CD4 effector T cells in the CNS. Further phenotypic characterization and detailed study of this population could help us better understand their potential involvement in disease reduction.

It is my hope that the information that resulted from this thesis work will aid the future development of vaccines or biologics that induce antigen specific protection to autoimmune diseases. By reinstating tolerance, patients can revert to a healthier life without the adverse effects associated with treatments of today.

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- And that, as they say, was that –

THE PALE BLUE DOT

“Look again at that dot. That's here. That's home. That's us.
On it everyone you love,
everyone you know, everyone you ever heard of, every human being who ever was,
lived out their lives.
The aggregate of our joy and suffering, thousands of confident religions,
ideologies, and economic doctrines, every hunter and forager, every hero and coward,
every creator and destroyer of civilization, every king and peasant, every young couple in love,
every mother and father, hopeful child, inventor and explorer, every teacher of morals,
every corrupt politician, every "superstar,"every "supreme leader,"
every saint and sinner in the history of our species lived there
- on a mote of dust suspended in a sunbeam.

The Earth is a very small stage in a vast cosmic arena. Think of the endless cruelties
visited by the inhabitants of one corner of this pixel on the scarcely distinguishable inhabitants
of some other corner,
how frequent their misunderstandings, how eager they are to kill one another,
how fervent their hatreds.
Think of the rivers of blood spilled by all those generals and emperors so that,
in glory and triumph, they could become the momentary masters
of a fraction of a dot.

Our posturings, our imagined self-importance, the delusion
that we have some privileged position in the Universe,
are challenged by this point of pale light.
Our planet is a lonely speck in the great enveloping cosmic dark.
In our obscurity, in all this vastness,
there is no hint that help will come from elsewhere to save us from ourselves.

The Earth is the only world known so far to harbor life.
There is nowhere else, at least in the near future, to which our species could migrate.
Visit, yes. Settle, not yet.
Like it or not, for the moment the Earth is where we make our stand...

...There is perhaps no better demonstration of the folly of human conceits
than this distant image of our tiny world.
To me, it underscores our responsibility to deal more kindly with one another,
and to preserve and cherish the pale blue dot,
the only home we've ever known.”

Carl Sagan, Cosmos

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