Self-tolerance in collagen induced arthritis

Tove Eneljung

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Department of Rheumatology and Inflammation Research

Institute of Medicine

at Sahlgrenska Academy

University of Gothenburg

UNIVERSITY OF GOTHENBURG

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tove.eneljung@rheuma.gu.se

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ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune chronic disease that results in damage to tissues throughout the body due to the inability of the immune system in these patients to discriminate between self-tissues and foreign invaders. Currently available treatment strategies consist of immunosuppressive drugs, which are efficacious but are associated with side-effects, such as increased risk for infections. Re-establishment of the ability of the immune system to discriminate between self and non-self through the induction of self-tolerance is an attractive treatment strategy that might lead to a cure for RA. Another interesting treatment option for RA is the design of a disease-regulated therapy, which would only be activated during a flare of the disease.

The aims of this thesis are to: 1) investigate the induction of antigen-specific tolerance in an animal model of RA (i.e., collagen induced arthritis; CIA); and 2) investigate whether disease-regulated production of an anti-inflammatory cytokine can ameliorate CIA.

We used gene therapy to express collagen type II peptide (CII) on antigen-presenting cells, so as to induce antigen-specific tolerance in animals with CIA. Our results show that gene therapy that targets haematopoietic stem cells induces strong resistance to the development of arthritis, and that B cells play a major role in the induction of tolerance. This effect is accompanied by increases in the suppressive capacities of T-regulatory cells and decreased levels of autoantibodies. We also show that gene therapy administered after immunisation with CII reduces the severity of CIA by decreasing the levels of autoantibodies and enhancing the suppression caused by T-regulatory cells.

Disease-regulated therapy was investigated using lentiviral-mediated transcription of IL-10 regulated by an IL-1 enhancer and IL-6 promoter. Our results show that gene therapy with an inflammation-dependent IL-10 gene construct generates increased levels of IL-10 in the lymph nodes, decreased levels of IL-6 in the serum, decreased levels of CII antibodies, and decreased severity of CIA.

In conclusion, we have developed gene therapy modalities and model systems that are well suited to investigations of the immunological mechanisms of antigen-specific tolerance and disease-regulated therapies in animal models of RA.

Keywords: tolerance, autoimmune, antigen-specific, collagen type II, gene therapy, disease-regulated therapy, collagen induced arthritis, mice, rheumatoid arthritis

LIST OF PAPERS

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

I. Sara Tengvall*, Tove Eneljung*, Kajsa Wing, Pernilla Jirholt, Jan Kihlberg, Rikard Holmdahl, Anna Stern, Inga-Lill Mårtensson, Louise Henningsson, Kenth Gustafsson, Inger Gjertsson.

Gene therapy mediated antigen presentation by B cells establishes tolerance in collagen induced arthritis

Submitted

II. Tove Eneljung, Sara Tengvall, Pernilla Jirholt, Louise Henningsson, Rikard Holmdahl, Kenth Gustafsson, Inger Gjertsson.

Antigen specific gene therapy post immunisation reduces the severity of collagen induced arthritis.

Submitted

III. Louise Henningsson*, Tove Eneljung*, Pernilla Jirholt, Sara Tengvall, Ulf Lidberg, Wim B. van den Berg, Fons A. van de Loo, Inger Gjertsson.

Disease-dependent local IL-10 production ameliorates collagen induced arthritis in mice.

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ABBREVIATIONS

ACPA	Anti-citrullinated protein antibody
AICD	Activation-induced cell death
AIRE	Autoimmune regulator
APC	Antigen-presenting cell
BCR	B cell receptor
CCIA	Chronic collagen induced arthritis
cDNA	Complementary DNA
CFA	Complete Freund’s adjuvant
CIA	Collagen induced arthritis
CII	Collagen type II
CLIP	Class II–associated invariant chain peptide
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
cPPT	Central polypurine tract
CSR	Class switch recombination
cTEC	Cortical thymic epithelial cell
CTLA-4	Cytotoxic T-lymphocyte antigen 4
D	Diverse
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalitis
ELISA	Enzyme-linked immunosorbent assay

ER	Endoplasmic reticulum
Fab	Antigen-binding fragment
FACS	Fluorescence-activated cell sorting
Fc	Crystallizable fragment
FCS	Foetal calf serum
FMO	Fluorochrome minus one
FO	Follicular cell
FoxP3	Forkhead box P3
GC-B	Germinal center B cell
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced tumour necrosis-factor-receptor-related protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HC	Heavy chain
HLA	Human leukocyte antigen
HSC	Haematopoietic stem cell
ICOS	Inducible T cell co-stimulator
IDO	Indoleamine 2,3-dioxygenase
IFA	Incomplete Freund’s adjuvant
Ii	Invariant chain
Im-B	Immature B-cell
IPEX	Immune dysregulation polyendocrinopathy enteropathy X-linked
i.v.	Intravenous
LC	Light chain
LNT-CII	Lentivirus containing the SFFV promoter and CII peptide

LNT-Ctrl	Lentivirus containing the SFFV promoter and CLIP peptide
LNT-GFP	Lentivirus containing the SFFV promoter and GFP
LNT-Igκ-CII	Lentivirus containing the Igκ promoter and CII peptide
LNT-Igκ-Ctrl	Lentivirus containing the Igκ promoter and CLIP peptide
LPS	Lipopolysaccharide
LTR	Long term repeat
MCP-1	Monocyte chemoattractant protein-1
mDC	Myeloid dendritic cell
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
mTEC	Medullary thymic epithelial cell
MPP	Multipotent progenitor
MZ	Marginal zone
NK	Natural killer cell
NKT	Natural killer T cell
PBS	Phosphate-buffered saline
PC	Plasma cell
pDC	Plasmacytoid dendritic cell
PolyA	Polyadenylation tail
RA	Rheumatoid arthritis
RRE	Rev responsive element
RT-PCR	Reverse transcriptase polymerase chain reaction
SFFV	Spleen focus-forming virus

SHM	Somatic hypermutation
SOCS	Suppressor of cytokine signaling
T1	Transitional cell 1
TCR	T-cell receptor
Tfh	T follicular helper cell
Th	T helper
TLR	Toll-like receptor
Tr1	T-regulatory cell 1
TRA	Tissue restricted antigen
Treg	T-regulatory cell
TNF	Tumor necrosis factor
V	Variable
VSV-G	Vesicular stomatitis virus-G
WPRE	Woodchuck post-transcriptional regulatory element

1 INTRODUCTION

1.1 Self-tolerance in autoimmune diseases

The immune system protects the body against foreign invaders, such as bacteria, viruses, parasites or fungi, and helps to repair injuries to tissues and cells. The immune system is designed to differ between an invading microbe and self-tissues. If this ability is lost and the immune response is directed against self-tissues the outcome is autoimmune inflammation and eventually tissue destruction i.e. an autoimmune disease. Autoimmune diseases affect at least 3% of the US population and are one of the most common causes of death among young and middle-aged women in the United States [1, 2]. Existing treatment strategies comprise substitution therapies or immunosuppressive drugs that down-regulate the autoimmune response to self-tissues as well as the appropriate immune defence against microbes. An attractive approach to treating autoimmunity would be to re-establish self-tolerance to eliciting autoantigens in each specific disease. In this thesis, we explore the immunological mechanisms of self-tolerance in an animal model of rheumatoid arthritis (RA). As my research was performed with the model of collagen-induced arthritis (CIA) in mice, I will focus on the murine immune system.

1.2 The cells of the immune system

1.2.1 Haematopoietic stem cells

The pluripotent haematopoietic stem cells (HSCs) differentiate into the entire repertoire of the leukocyte compartment [3, 4] in a multistep fashion, as shown in Figure 1. Initially, specialised committed cells, called multipotent progenitor (MPP) cells, appear, followed by oligopotent progenitor cells, the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP) cells. The end-differentiation stage is represented by the mature effector cells, which include the platelets, erythrocytes, granulocytes, macrophages, dendritic cells (DCs), B cells, T cells, and natural killer (NK) cells. The granulocytes, macrophages, monocytes, NK cells, dendritic cells, and mast cells constitute the innate immune system, which reacts immediately to foreign invaders, while the B cells, T cells, DCs, and NK cells form the adaptive immune system, which generates a specific and longlasting but delayed response to specific antigens.

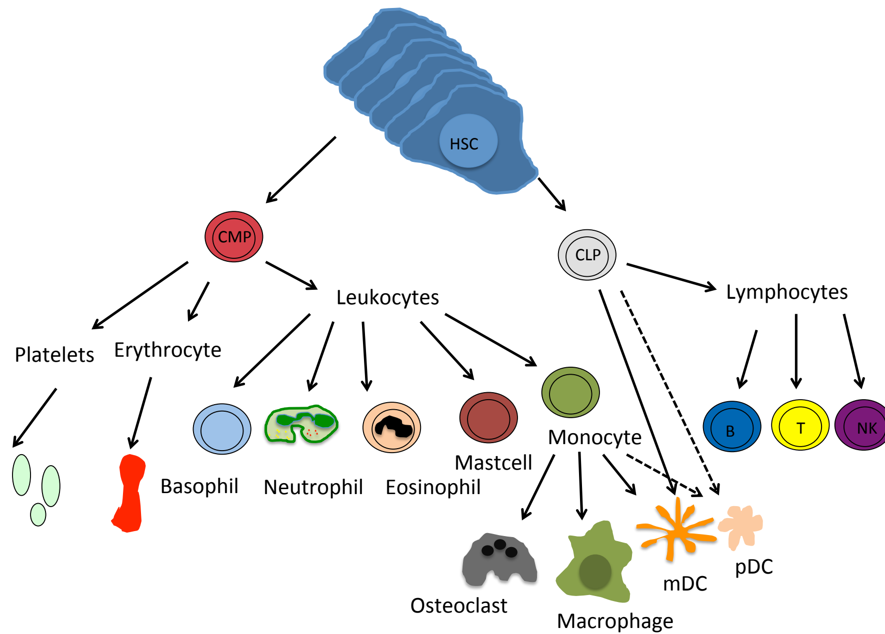


Figure 1. Haematopoietic stem cells (HSCs) give rise to the common myeloid progenitor cells (CMPs) and the common lymphoid progenitor cells (CLPs), which in turn differentiate into platelets, erythrocytes, basophils, neutrophils, eosinophils, mast cells, monocytes, osteoclasts, macrophages, myeloid dendritic cells (mDC), plasmacytoid DCs (pDC), B cells, T cells, and NK cells.

1.2.2 Monocytes

Monocytes circulate in the blood and the lymph before being recruited by inflammatory signals into peripheral tissues where, depending on the environment, they differentiate into macrophages and dendritic cells, which comprise the mononuclear phagocyte system, or osteoclasts [5-9]. An important recruiting signal for monocytes to migrate to inflammatory tissues is monocyte chemotactic protein 1 (MCP-1). Monocytes can be divided into two subsets based on surface expression of receptors for chemokines and adhesion molecules [10]:

1. *"Inflammatory monocytes":* Have the phenotype of F4/80+, CD11b+, CCR2+, CD62L+, CX3CR1^{low}, Ly6C+(Gr1+). These cells respond to the MCP-1, which recruits the monocytes to lymph nodes and sites of inflammation. These cells produce tumour necrosis factor (TNF) and interleukin 1 (IL-1), and they can differentiate into dendritic cells (DCs).

2. *"Resident monocytes":* Have the phenotype of CD11b+, CCR2-, CD62L-, CX3CR1^{hi}. These cells are present under non-inflammatory conditions and they differentiate into tissue macrophages and DCs.

1.2.3 Macrophages

Macrophages are present throughout the body in connective tissues, the basement membranes of blood vessels, the spleen, and lymph nodes. In addition, they form Kupffer cells in the liver, microglial cells in the central nervous system, Langerhans cells in the skin, alveolar macrophages in the lungs, osteoclasts in the bone, and mesangial cells in the kidneys. Macrophages are extremely important sentinels that respond to danger signals, phagocytose microbes, clear dead cells and toxic compounds, present antigens, produce cytokines such as TNF, IL-1, IL-6 and IL-10, and activate other cells of the immune system.

1.2.4 Dendritic cells

Dendritic cells (DCs) are important macrophage-like cells that act as a communication linkage between the innate and adaptive immune systems. A DC can be derived from a myeloid progenitor cell or a lymphoid progenitor cell. DCs are very potent antigen-presenting cells and deliver co-stimulatory or co-inhibitory signals to T cells. DCs reside in tissues, where they are activated as a part of the innate immune response when antigen is encountered and ingested. After activation, they migrate to the lymph nodes to present antigen bound to major histocompatibility molecule class II (MHC II) molecules and to present co-stimulatory or co-inhibitory molecules on their surfaces, so as to activate or inhibit antigen-specific T cells. The differentiation of DCs from monocytes *in vitro* requires the addition granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4. Dendritic cells are further subdivided into the myeloid (mDC) and plasmacytoid (pDC) subsets.

Myeloid or conventional DCs (mDC/cDC)

The most abundant type of DC is the mDC, which is a professional antigen-presenting cell (APC) that is very important for T-cell activation in lymph nodes. The mDCs express CD80, CD86, B7-H1 (PD-L1), and B7-H2 (ICOSL), the surface marker CD11c, as well as a variety of cytokines depending on the stimulus to which they are exposed. The maturation status of the mDC is governed by exposure to microbial and viral antigens and the cytokine milieu [11, 12].

Plasmacytoid DCs (pDCs)

The pDC is a rare cell subset that is important for defence against viruses. The pDC is activated by unmethylated viral DNA acting *via* Toll-like receptor (TLR)9 in endosomes. The pDC is responsible for IFN-α production and it expresses CD11c, B220, BST-2 (mPDCA), and Siglec-H, whereas it is negative for CD11b. pDCs can be differentiated *in vitro* from murine bone marrow cells by the addition of the Flt3 ligand.

1.2.5 B cells

B cells are important components of the adaptive immune defence in that they produce antibodies and cytokines and present antigens. B cells develop in the foetal liver and in the bone marrow of adults from the common lymphoid progenitor cell (CLP). They go through multiple maturation stages as pro-B cells, pre-B cells, immature B cells, mature B cells, and activated B cells to become memory B cells, plasma cells, and B-regulatory cells. The specificity of an individual B cell clone is achieved through gene recombination, and under certain circumstances, mutations, leading to a unique membrane-bound antibody that forms the B-cell receptor (BCR) (Figure 2). Each antibody is composed of a heavy chain (HC) encoded by gene segments termed variable (V), diverse (D), and joining (J), in addition to the exons that encode the constant (C) region. The light chain (LC) is encoded by the V, J, and C segments. The process that generates the heavy and light chains is called 'V(D)J recombination' and it is mediated by the RAG-1 and RAG-2 enzymes. The VDJ recombination process starts in the pro-B cell, and results in a complete heavy chain that assembles with a surrogate light chain, which forms the pre-B cell receptor (pre-BCR) in the pre-B cell. Thereafter, the LC loci undergo VJ recombination, express a LC that is assembled with

the HC and finally, a BCR in the immature B cells in the bone marrow. These cells emigrate to the spleen and are termed transitional 1 (T1) cells [13-15]. The T1 cell expresses a BCR of IgM and the T2 cell expresses BCRs of IgM and IgD on their surfaces. B-1 cells originate from the foetal liver and express CD5 and high levels of surface IgM [16, 17]. These cells are mainly localized to the peritoneal and pleural cavities. They produce low-affinity IgM natural antibodies. The presence of IL-10 is important for the proliferation of B-1 cells.

Antibodies

Antibodies are composed of two identical heavy chains and two identical light chains, which form two variable antigen-binding fragments (Fabs) and one constant fragment, named Fc (crystallizable fragment) (Figure 2). The constant region of the heavy chain determines the antibody class; the μ-chain forms IgM; the δ-chain forms IgD; the ε-chain forms IgE; the α-chain forms IgA; and the γ-chain forms IgG. There are two types of light chains, termed κ and λ. The Fab fragment is important for the specific recognition of antigens, while the Fc part is important for effector functions, such as complement activation, activation of innate immune cells, and transportation of antibodies, e.g., to the foetus, *via* the Fc receptors present on diverse types of cells. The B cell can be activated through antigen recognition and co-stimulation by an activated T-helper cell for antibody production or by a T-cell-independent antigen. The T-helper cell binds *via* its T-cell receptor (TCR) to the major histocompatibility complex class II (MHC II), which presents an antigen on the B-cell surface. This contact allows the CD40 ligand (CD40L) on the T cell to bind CD40 on the B-cell surface. The activated T-helper cell secretes cytokines, such as IL-4, leading to clonal expansion of the stimulated B cell. Various cytokines induce differentiation into IgM-, IgD-, IgG- (subclasses 1, 2a-c, 3), IgA- or IgE-producing plasma cells. To produce IgG, IgA, and IgE antibodies, the genome needs to undergo class switching, which means that the region encoding the antibody constant region such as IgM is excised from the genome through recombination.

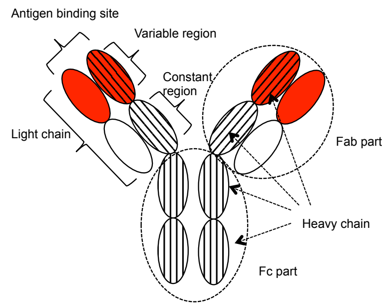


Figure 2. The B cell receptor (BCR) is a membrane-bound antibody that consists of two heavy chains and two light chains. Each chain consists of a variable region and a constant region.

The maturation of B cells

The B-2 cells or conventional B cells are the most common B-effector cells. They originate from the transitional T1 and T2 cells. The B-2 cells constitute the *marginal zone (MZ) B cells* ($IgM^{hi}IgD^{lo}$) and the *follicular (FO) B cells* ($IgM^{+}IgD^{hi}$) and can differentiate into plasma cells, memory B cells and possibly regulatory B cell subsets [18-21]. MZ B cells reside in the marginal zones of the spleen and are characterised by rapid antibody responses to circulating antigens, and are activated both by T-cell-dependent and independent antigens [22]. FO B cells differentiate into germinal center B cells after T-cell-dependent activation and subsequently into memory B-cells or plasma cells.

In a T-cell-dependent B-cell response the antigen peptide on MHC II is recognised by its specific T-helper cell which further activates the B-cell. The B-cell genes encoding the BCR can undergo somatic hypermutation (SHM) and/or class switch recombination (CSR). SHM is a process whereby random point mutations in the variable region of the immunoglobulin gene (VDJ) results in a variety of different Fab parts with different affinities for its antigen, which may result in *affinity maturation* of the BCR. CSR involves excision of the exons that encode the constant part of the IgM or IgD heavy chain, so as to allow the production of IgG, IgA or IgE class antibodies in response to cytokine stimulation.

The *plasma cells* are recognised by a large cytoplasm due to an expanded endoplasmic reticulum and Golgi apparatus for production of antibodies.

Short-lived plasma cells produce antibodies during a limited time period while long-lived plasma cells, often situated in the bone marrow, produce antibodies as a memory of an earlier activation by antigen.

Memory B-cell subsets comprise an immunological memory of previous immune responses which after antigen re-encounter provides rapid activation of antigen-specific B cells and production of antigen-specific antibodies [23].

Recently, an IL-10-producing B-cell subset that can down-regulate inflammation, e.g., as in collagen induced arthritis (CIA), was described [20, 24-27]. These cells, which have been named *'B-regulatory cells'*, can be induced from marginal zone, follicular or memory B cells through antigen-specific stimulation *via* the BCR and co-stimulation by T cells *via* CD40 binding to CD40L. TLR stimulation of B cells can also contribute to IL-10 production by B cells [28, 29]. B-regulatory cells can induce a regulatory T cell subset, namely the Tr1 cells that suppress inflammation *via* IL-10 production [21, 24, 30, 31]. Mice that lack the IL-10 gene only in their B cells develop a severe form of CIA that involves increased levels of Th1 and Th17 cells and decreased levels of IL-10-producing T-regulatory cells (Tr1 cells) [32]. B-cell depletion in experimental autoimmune encephalitis (EAE) decreases the numbers of FoxP3 positive T-regulatory cells (Tregs) and levels of IL-10, probably due to the deficiency of B-regulatory cells [33]. Although the origin and phenotype of B-regulatory cells remain uncertain, there are data that indicate that these cells can be sub-divided into: 1) *T2 MZP B-regulatory cells* (transitional 2 marginal zone precursors), which are characterised as $CD19^{+}CD21^{hi}CD23^{hi}CD1d^{hi}$ and produce IL-10 following CD40 stimulation; and 2) *B10 cells*, which are characterised as $IL\text{-}10^{+}CD19^{+}CD5^{+}CD1d^{hi}$ and produce IL-10 following TLR2 or TLR4 stimulation.

1.2.6 B-cell tolerance

Several processes act to limit the survival and the functionality of autoreactive B cells [34, 35] (Figure 3). These include:

Factors intrinsic to the B cell:

a) Self-reactive B cells are deleted.

b) Receptor editing: Self-reactive B cells undergo editing of the light chain variable region of the BCR, and only B cells with BCRs that are not directed against self are allowed to survive.

c) Clonal anergy or tuning: Self-reactive B cells are inactivated or are made less responsive to stimuli.

Factors extrinsic to the B cell:

a) Helplessness: Self-reactive B cells do not undergo co-activation by T-helper cells.

b) Active suppression: Self-reactive B cells are suppressed by Tregs.

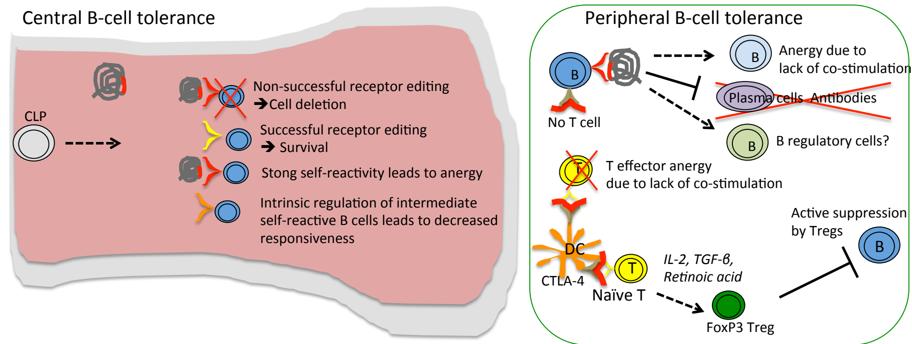


Figure 3. B-cell tolerance mediated by central processes in the bone marrow and peripheral processes in the secondary lymphoid organs, such as lymph nodes and spleen.

1.2.7 T cells

T cells are antigen-specific cells that differentiate into a variety of T-cell subsets. These T-cell subsets act to: kill other cells; enhance inflammatory responses; activate B cells; and suppress inflammatory responses. T cells develop from HSCs into CLPs in the bone marrow, and then migrate to the thymus for further development into antigen-specific CD4+ T cells or CD8+ T cells. The T-cell receptor (TCR) determines the antigen specificity of the T cell. A small subset of T cells have a γδ-chain TCR, while the αβ T cells constitute the majority of all T cells and are usually the type of cells that is referred to when one is talking about ”T cells”. In addition, natural killer T cells (NKTs) develop from αβ T cells. The NKT cell is a hybrid between a T cell and NK cell. The antigen-specificity of the αβ T cells is regulated stringently to prevent the generation of self-reactive T-effector cells, which could initiate a harmful immune response directed against self-tissues. This regulation is performed both centrally in the thymus and peripherally in the lymph nodes, spleen, and other local tissues (Figure 4) [36]. Natural Tregs

are also induced in the thymus, although the underlying mechanisms are not fully understood, as discussed later in this section.

1.2.8 Central tolerance in the thymus

The T cells that enter the thymus have a double-negative phenotype, which means that they do not express either CD4 or CD8. The selection process in the thymus is defined by two major steps (Figure 4).

First, *positive selection* or MHC restriction is performed in the cortex of the thymus [37]. The double-negative T cells develop into double-positive T cells that express both CD4 and CD8. Depending on whether the newly produced TCR binds to a MHC I or to a MHC II molecule on the cortical thymic epithelial cell (cTEC), the T cell is converted into a single-positive CD4+ MHC II-binding cell or a CD8+ MHC I-binding cell. The affinity of TCR binding to self-MHC determines the fate of the T cell. Only those T cells that express CD4 or CD8 and bind with intermediate affinity to self-MHC on the cTEC cell surface will survive and be able to migrate into the thymic medulla. The T cells with low affinity or lack of affinity for self-MHC molecules will be deleted through apoptosis.

Second, in a *negative selection process*, only those T cells with low or moderate avidity for MHC / antigen will survive. The medullary thymic epithelial cells (mTECs) express a transcription factor, called autoimmune regulator *(AIRE)*, which is essential for the presentation of tissue-restricted antigens (TRAs), i.e., antigens that are not normally localised to the thymus. The mTECs, macrophages, and DCs in the thymus present self-antigens on MHC, which results in the deletion of T cells that have strong binding to self-antigen [38, 39].

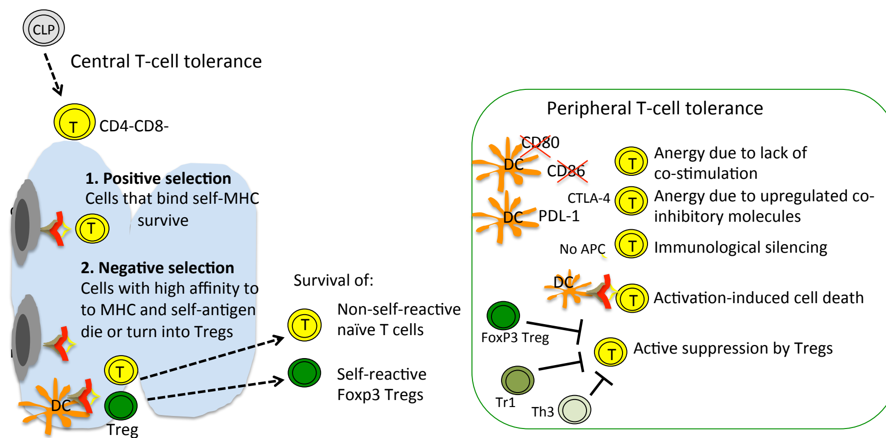


Figure 4. T-cell tolerance mediated by central mechanisms in the thymus and peripheral mechanisms in the secondary lymphoid organs, such as the spleen and lymph nodes or in local tissues.

1.2.9 Thymic induction of CD4+CD25+FoxP3+ natural Tregs

Although the processes of development and selection of CD4+CD25+FoxP3+ natural Tregs in the thymus are not fully known, they appear to differ from the processes of selection of other T-cell subsets [34, 40]. In this case, the high affinity of the TCR for the self-antigen presented on MHC II molecules leads to the survival of natural Tregs instead of negative selection. Experiments on transgenic mice that express a TCR with a variable affinity for self-antigens have shown that only high-affinity TCRs induce natural Tregs [41]. Van Santen *et al.* showed that CD4+CD25+ T cells are more resistant to negative selection when they are bound to self-antigen, as compared with CD4+CD25- T cells [42]. Other pivotal molecules for thymic Treg generation include the Treg transcription factor forkhead box P3 (FoxP3) and co-inhibitory molecules, such as CTLA-4 [43] (discussed in the section on "**Treg markers"** and the section on "**Co-inhibitory molecules"**).

Peripheral T-cell subsets

CD4+ or CD8+ T cells that survive thymic education migrate into secondary lymphoid organs, such as spleen and lymph node, or local tissues. I will only address the CD4+ T cells. Upon stimulation by cytokines, the T cells differentiate further into CD4+ T-helper cells [44, 45]. The T-helper cells are divided into multiple subsets, e.g., Th1, Th2, Th17, Th23, and Tregs (Figure 5). These subsets are not generated in a rigid irreversible fashion but are instead characterised by a pronounced plasticity [44, 46].

Th1 cells, which were described by Mosmann in 1986 [47], are considered to be primarily involved in combating intracellular bacteria and viruses. Th1 cells are differentiated from naive T cells after stimulation with IL-12, IFN-γ, IL-23, and IL-27, released mainly by DCs. Th1 cells activate macrophages *via* IFN-γ stimulation in combination with engagement of the CD40L on Th1 cells to the CD40 on macrophages. Th1 cells secrete IL-2, which activates cytotoxic CD8+ T cells that are stimulated by antigen bound to MHC I molecules on an APC. The released IFN-γ increases IL-12 production by dendritic cells in a positive feedback loop. Up-regulated CD40 on DCs binds to CD40L on T cells, thereby inducing additional IL-12 secretion. Th1 cells also release GM-CSF and IL-3, which stimulate further production of neutrophils and macrophages from haematopoietic progenitors.

Th2 cells secrete IL-4, IL-5 and IL-13, which stimulate B cells to proliferate, class-switch, and mature. Th2 cells are induced by IL-4 and IL-2, which is mainly produced by DCs and NK cells. IL-4 down-regulates the IL-12β_2 receptor subunit, thereby inducing IL-12 unresponsiveness, which leads to an activated Th2 response and an inhibited Th1 response. Th2 cells also secrete IL-3 and GM-CSF, which stimulate neutrophil and macrophage expansion.

Th17 cells produce IL-17A, IL-17F, IL-21 and IL-22, which enhance the inflammatory response through the stimulation of production of pro-inflammatory cytokines (by for instance, fibroblasts), recruitment of neutrophils, and activation of B cells, events that are important for both defence against extracellular pathogens (bacteria and fungi) and the pathogenesis of autoimmune diseases, such as RA [48-50]. In the presence of IL-6, TGF-β seems to contribute to Th17 generation [51, 52], while IL-21 and IL-23 stimulate Th17 expansion.

T-follicular helper (Tfh) cells represent a T-cell subset that is specialised in the activation of B cells into plasma cells or memory B cells in the germinal centres of the spleen or lymph node [53-55]. Tfh cells produce IL-21, which is important for both B cells and the Tfh cells themselves. Other factors that seem important for Tfh cell differentiation is IL-6 and the transcription factor

Bcl-6. The chemokine receptor CXCR5 and the co-stimulatory molecule ICOS are important for Tfh function.

T-memory cells represent a long-lasting, activated, antigen-specific $CD4^+$ or $CD8^+$ T-cell population that is located in the thymus, spleen, blood, lymph node and peripheral tissues [56, 57]. T-memory cells can be divided into two subsets; T-central-memory cells ($CCR7^+CD62L^+$) and T-effector-memory cells ($CCR7^-CD6^{lo}$) [58-60]. Rosenblum *et al.* have described a memory Treg subset that consists of antigen-activated, resident Tregs that can expand in response to a renewed encounter with the antigen [61].

Tregs are antigen-specific cells that have suppressive and anti-inflammatory capacities [62-64]. Tregs act in both a contact-dependent manner and a cytokine-mediated manner *via* IL-10 and TGF-β. The main function of Tregs is to prevent or reduce inflammation by regulating the activation of T-effector cells and APCs, and to inhibit the generation of plasma cells from mature B cells [65]. Currently, the Treg compartment is sub-divided into the subsets of natural and induced T-regulatory cells.

Natural or thymic-derived Tregs are antigen-specific CD4+CD25+FoxP3+ cells that are generated in the thymus and that serve to limit immune responses to foreign and self-antigens [66, 67]. In healthy mice, these cells constitute 5%-10% of the CD4+ T-cell compartment. A strong co-inhibitory signal, such as the binding of CTLA-4 on the natural Treg cell to CD80 or CD86 on the APC, is essential for Treg function [68]. Natural Tregs exhibit high and stable expression of CD25. CD25 is the α-chain of the IL-2 receptor and it is up-regulated on activated T-effector cells, as well as on Tregs. Transcription factor FoxP3 is essential for the Treg phenotype, as it suppresses differentiation into other T-cell subsets, such as Th1, Th2, and Th17. Although Tregs need IL-2 for survival, in contrast to other T-effector cells, they do not have the ability to produce it themselves.

Induced Tregs or adaptive Tregs are generated in peripheral tissues upon antigen encounter [69]. While the complete characteristics of these cells have not yet been reported, three different subgroups have been described:

Th3 cells are mainly found in the mucosa, produce IL-4, IL-10 and TGF-β, and are believed to be important for oral tolerance.

Tr1 cells are activated by their cognate antigen in the presence of IL-27. They produce IL-10 and TGF-β [70-72]. They can also be induced by tolerogenic dendritic cells [73].

Peripherally induced FoxP3+ Tregs are induced after antigen stimulation of the TCR in the presence of TGF-β and IL-2 [74]. Inducible FoxP3+ Tregs are enriched in gut-associated lymphoid tissues.

Other possible Treg subsets? CD8+ cells, CD4-CD8- and γ/δ-T cells have been discussed in the context of cells that have suppressive capacities [75].

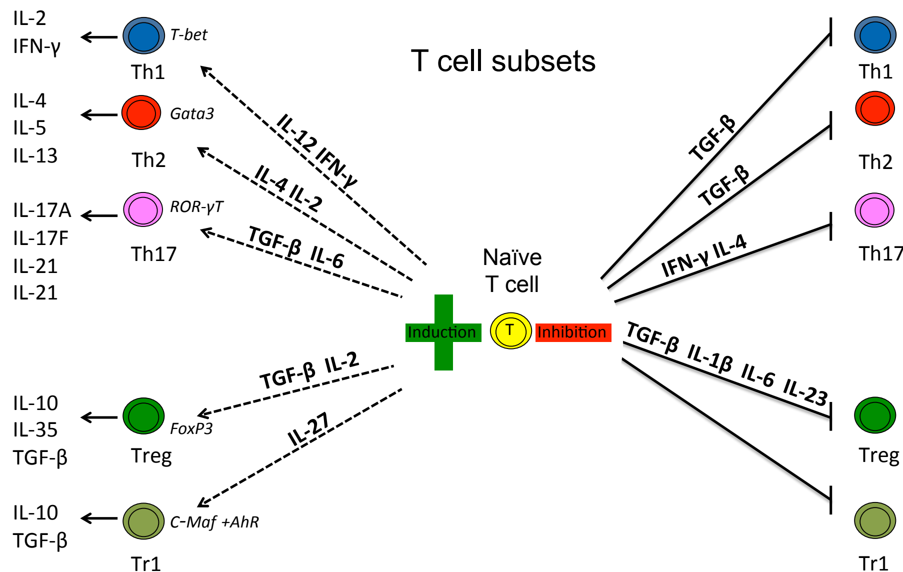


Figure 5. Cytokine production by the T-cell subsets of Th1, Th2, Th17, Treg, and Tr1 (left). Factors that induce T-cell differentiation into the respective T-cell subset (left side) and factors that inhibit differentiation (right side) are depicted. Transcription factors specific for each cell subset (*italicised*) include T bet in Th1 cells, Gata 3 in Th2 cells, ROR-γT in Th17 cells, FoxP3 in Tregs, and C-Maf and AhR in Tr1 cells.

Treg markers

Forkhead box P3 (FoxP3) is a transcription factor that stimulates the transcription of the genes for IL-2, CTLA-4, and GITR, whereas it represses the transcription of the genes for IL-2 and IFN-γ. FoxP3 is not required for the survival of Treg precursors but it is essential for Tregs to maintain suppressive function [76, 77]. FoxP3 seems to inhibit cytokine production and T-effector cells *via* interactions with transcription factors, such as NFAT [78] and NF-κB [79]. Depletion of FoxP3 leads to severe autoimmune diseases, such as scurfy in mice [80] and immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) in humans [81, 82].

Expression of FoxP3 has been described in response to different stimuli, such as the transcription factors NFAT or Smad and anti-inflammatory TGF-β, which are important for induced Tregs [83], and by TCR activation and IL-2 stimulation, which are important for natural thymic-derived Tregs [84], and by the transcription factor c-Rel, which is important for both induced and thymic-derived Tregs [85].

Helios, which is a transcription factor that is used as an intracellular marker for T cells, was discovered in 1998 [86, 87]. Initially, it was described by Thornton *et al.* [88] as a marker for Tregs of thymic origin. However, it was later found to be also expressed as an activation marker on peripherally induced Tregs, as well as on T-effector cells [89]. In mice, Helios has recently been shown to repress IL-2 secretion by Tregs *via* binding to the IL-2 promoter [90]. Helios deficiency does not impair the functions of Tregs in mice [91], whereas human Tregs seem to depend on Helios for their suppressive capacity [92, 93].

How does a Fox P3^{+} Treg suppress inflammation?

To be able to suppress inflammation, the Treg needs to be activated by its specific antigen presented on an APC [94]. This ensures that the anti-inflammatory effect is not turned on randomly. The suppression exerted by Tregs is non-specific after the initial antigen-specific activation [94]. Tregs act *via* cytokines that are secreted (IL-10) or membrane-bound (TGF-β), *via* co-inhibitory molecules, such as CTLA-4 and LFA-1, and *via* cytolytic substances, such as perforins and granzyme (Figure 6). These suppressive pathways target T-effector cells (Th1, Th2, Th17, CD8+), Tregs, DCs, macrophages, B cells, NK cells, NK T cells, mast cells, and osteoblasts [95, 96].

Anti-inflammatory cytokines

FoxP3 Tregs also exert suppressive effects *via* membrane-bound TGF-β, secreted IL-10 and IL-35, which induce the expansion of FoxP3 Tregs, induce Tr1 cells, and down-regulate the inflammatory actions of APCs. However, the effects of these cytokines seem to be weaker than the contact-mediated suppressive mechanisms [97].

Cytotoxic effects

FoxP3^{+} Tregs secrete cytotoxic compounds, such as perforin and granzyme A or B, which act to kill effector T cells, B cells, DCs, and monocytes [98-100].

Contact-dependent suppressive mechanisms

Separation of the $FoxP3^+$ Tregs from the T-effector cells and APCs using a semi-permeable membrane results in inhibition of the suppressive effects [97], suggesting that cell-to-cell contact is of the uttermost importance for the functions of $FoxP3^+$ Tregs. The contact-dependent mechanism is suggested to act *via CTLA-4* on $FoxP3^+$ Tregs, which binds to CD80/86 on APCs to down-regulate the expression of co-stimulatory CD80 and CD86 molecules from the APCs [101] and this may also influence the activity of indoleamine 2,3-dioxygenase (IDO) in DCs [102, 103]. IDO is an enzyme that acts in the kynurenine pathway that leads to the depletion of tryptophan, which causes T-cell death. Depletion of CTLA-4 leads to increased numbers of Th17 cells, indicating that CTLA-4 inhibits Th17 differentiation [104]. In summary, CTLA-4 down-regulates the stimulatory effects of DCs on T cells and directly inhibits the effects on T-effector cell subsets.

CD39 is an enzyme produced in $FoxP3^+$ Tregs that hydrolyses ATP into ADP or AMP, and this results in the down-regulation of CD80 on DCs [105]. LAG3 on FoxP3 Tregs binds to MHC II on DCs and down-regulates the immunostimulatory properties of DCs *via* inhibitory intracellular signalling pathways [106].The glucocorticoid-induced tumour necrosis factor receptor-related (GITR) protein on FoxP3 Tregs binds to GITRL on DCs in the presence of IL-2, and this leads to expansion of the Treg compartment [107].

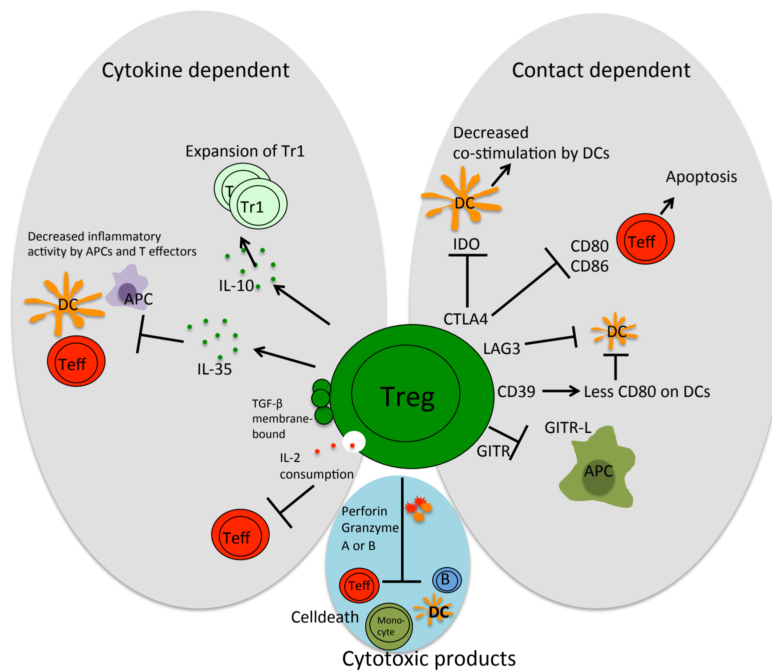


Figure 6. Mechanisms of suppression exerted by Tregs. The cytokine-dependent pathways (left) use IL-10, IL-35, TGF-β or IL-2. The contact-dependent pathways (right) act *via* CTLA-4 binding to IDO or CD80/CD86, LAG3 binding to MHCII on DC, CD39 acting on the DC to down-regulate CD80 or GITR binding to GITR-L. The cytotoxic products secreted by Tregs include perforin and granzymes A and B.

1.2.10 Peripheral T-cell tolerance

T cells that are released from the thymus are regulated peripherally to prevent self-reactivity. The phenomenon of peripheral tolerance is not fully understood but has been suggested to act through four different mechanisms (Figure 5) [34, 35, 108]: 1) *Anergy,* which involves the paralysis of the T cell due to a lack of co-stimulation by, for instance, CD80 or CD86 on the APC or by the up-regulation of co-inhibitory molecules, such as CTLA-4 on the T cell or PDL-1 on the APC; 2) *immunological silencing or ignorance*, whereby self-antigens are hidden from the T cells, for instance by localisation to a closed compartment, e.g., the lens of the eye, or when there are no APCs that are able to present the self-antigen; 3) *activation-induced cell death (AICD)*, in which self-reactive cells are induced to undergo apoptosis, e.g.,

via a Fas-Fas ligand interaction; and 4) *active suppression,* which is exerted by Tregs that are able to limit pro-inflammatory T-effector responses.

1.3 Antigen presentation

1.3.1 The MHC II molecule

MHC II molecules are present on several cell types, including professional APCs (i.e., macrophages, DCs, and B cells), rheumatoid synovial fibroblasts [109, 110], certain endothelial cells and specialised epithelial cells in the thymus, mTECs, and cTECs. The MHC II molecule consists of an α-chain and a β-chain, which form an antigen-binding cleft (Figure 7A). The MHC II molecules present extracellular antigens that are endocytosed, digested, and loaded into the antigen-binding cleft. Antigens presented on MHC II molecules activate CD4+ T cells. The invariant chain (Ii) is a protein that stabilises and transports MHC II molecules through the endoplasmic reticulum (ER) and trans-Golgi network and into the endosomes [111] (Figure 7B). The human MHC II molecules are designated as HLA-DR, HLA-DP, and HLA-DQ, and the corresponding murine MHC II molecules are termed H2-Q, H2-T, and H2-M.

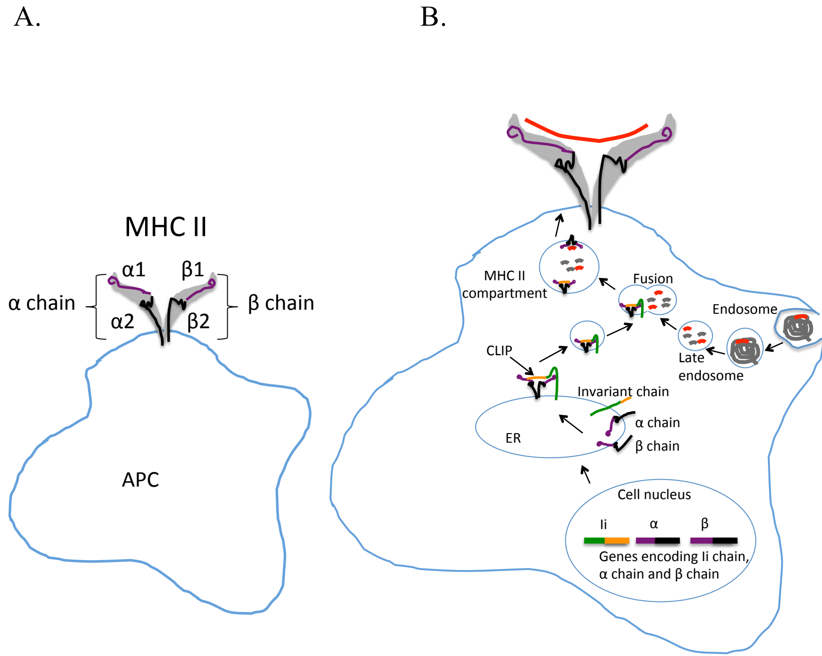


Figure 7A. The MHC II molecule consists of an α-chain and a β-chain. The α-chain is composed of α1 and α2 domains. The β-chain is composed of β1 and β2 domains. The α1 and the β1 domains form the antigen-binding cleft. **B.** Synthesis, transport and presentation of the invariant chain (Ii) and MHC II. The uptake of extracellular antigens and their degradation into peptides and presentation on MHC II are depicted.

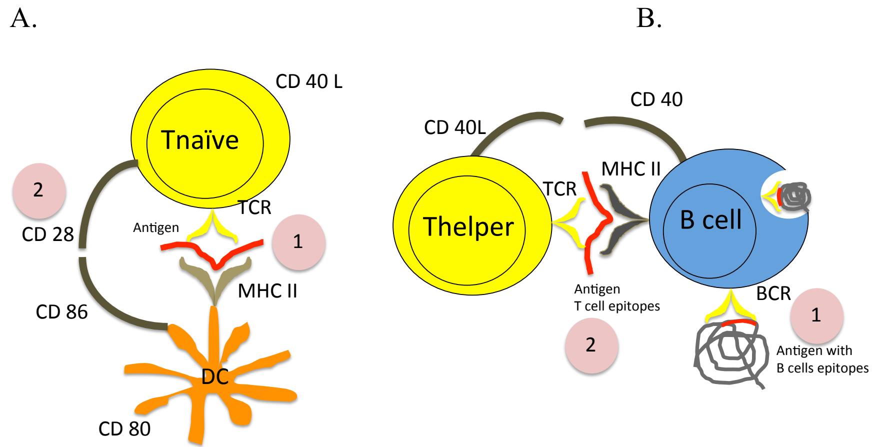


Figure 8A. T-cell activation by a DC. Signal 1: Binding of TCR to MHC II with antigen. Signal 2: CD28 on the T cell binds to co-stimulatory CD80 or CD86. **B**. B-cell activation by a T cell. Signal 1: Binding of BCR to a soluble antigen leads to endocytosis of the bound antigen. Signal 2: MHC II with antigen on the B cell binds to the TCR, and CD40 on the activated T cell binds to CD40L on the B cell.

1.3.2 Co-stimulatory molecules

The activation of a T cell involves several steps. First, for Signal 1, the TCR binds to its cognate antigen on MHC II (Figure 8A). Second, for Signal 2, co-stimulatory CD80 or CD86 on the APC binds to CD28 on the T cell (Figure 8A). Co-stimulatory signals are induced by infectious agents or by inflammation, e.g., through stimulation of TLRs on APCs. This T cell-APC interaction induces the expression of CD40L on the T cell and CD40 on the APC or B cell. This causes a positive feedback loop, which further enhances the expression of CD80 or CD86, thereby increasing the activation of T cells. Activation of a B cell is achieved by antigen binding to the BCR (Signal 1) and T cell binding *via* the TCR to MHC II/antigen on the B cell and co-stimulation by CD40 binding to CD40L (Signal 2) (Figure 8B). Furthermore, the inducible T-cell co-stimulator (ICOS) is expressed on activated T cells [112, 113]; through its binding to the ICOS ligand (ICOSL) on B cells it induces Bcl-6 expression, which leads to the development of Tfh cells [112].

1.3.3 Co-inhibitory molecules

The pro-inflammatory immune response is regulated not only by the Treg population, but also by co-inhibitory molecules, such as CTLA-4 and ICOS. CTLA-4 is a ligand that binds more strongly than CD28 to CD80 and CD86 and provides an inhibitory signal to the T cell. This inhibitory signal reduces the production of IL-2 and causes down-regulation of CD80 or CD86 on DCs. PD-L1 is a co-inhibitory molecule that is expressed, for instance, on APCs and binds to PD-1 on T cells, resulting in the expansion of Tregs or reduced activation of T-effector cells [114-116].

1.4 Cytokines

1.4.1 IL-1α and IL-1β

IL-1α is stored as an active precursor in the cytoplasm of platelets, endothelial, epithelial and mesenchymal-originating cells [117]. IL-1α acts both in the cell nucleus and on extracellular IL-1 receptors (IL-1R). It is rapidly released during cell injuries, such as ischemia, leading to the recruitment of neutrophils, which induce a sterile inflammation [118]. IL-1β is only produced upon stimulation, mainly by macrophages and DCs. It is

synthesised as an inactive precursor protein that has to be activated through cleavage by caspases. Active IL-1β acts on the same IL-1R as IL-1α. IL-1R binding leads to the up-regulation of adhesion molecules and the recruitment of macrophages, neutrophils, and lymphocytes to inflammatory sites. IL-1α and IL-β induce the differentiation of Th1, Th2, and Th17 cells and activate osteoclasts towards bone resorption [119, 120].

1.4.2 IL-2

IL-2 is produced by activated T cells and is an essential cytokine for the proliferation of T cells (reviewed by Malek [121]). Only T cells that are activated through antigen recognition produce IL-2 [122] and express the IL-2 receptor, which consists of non-covalently bound CD25, CD122, and CD132 components. T-cell activation as a result of antigen presentation by MHC I or MHC II leads to IL-2 production and IL-2 receptor up-regulation on the surfaces of T cells within hours, and this results in an autocrine feedback that down-regulates IL-2 secretion [121]. While FoxP3+ Tregs do not produce IL-2, they need IL-2 to be activated [123].

1.4.3 IL-6

IL-6, which is a cytokine that exerts multiple effects, is secreted by macrophages, endothelial cells, and fibroblasts. The pro-inflammatory effects of IL-6 include the stimulation of antibody production, stimulation of haematopoiesis in the bone marrow, and the synthesis of acute phase proteins in the liver [124]. IL-6 also plays important roles in the termination of the inflammatory response, in that it reduces the production levels of pro-inflammatory IL-1 and TNF-α by macrophages and initiates neutrophils to phagocytose dead cells. The phagocytosis of apoptotic cells leads to MCP-1 secretion by macrophages and endothelial cells. MCP-1 is responsible for the recruitment of monocytes and neutrophils. In contrast to other cytokines that act primarily in local tissues, IL-6 has distal effects on organs, such as the liver, and the bone marrow. Inflammatory diseases, such as RA, are associated with increased levels of IL-6 in the synovial fluid [125]. The humanised anti-interleukin-6 (IL-6) receptor antibody tocilizumab has shown efficacy in the treatment of RA through reduced IL-6 levels acting via expansion of Tregs, decreased levels of IL-21 and decreased levels of autoantibodies [126-128].

1.4.4 IL-10

IL-10 is a regulatory cytokine that possesses both anti-inflammatory and pro-inflammatory properties [129]. It is produced by monocytes, T cells (especially Th2 and Tr1 cells), B cells, and macrophages. The IL-10 receptor consists of the R1 part and R2 part, which form the two chains of the receptor. R1 is expressed on macrophages, monocytes, T cells, B cells, and NK cells and signals *via* the JAK1 pathway [129]. R2 is expressed on almost all cell types and is up-regulated by IFN-γ or TNF-α stimulation and signals *via* the Tyk1 pathway [129]. IL-10 binding to IL-10R leads to SOCS1 and 3 expression, which results in termination of the effects of IL-10 [130]. IL-10 regulates antigen presentation *via* the down-regulation of MHC II, co-stimulatory molecules (such as CD86), and adhesion molecules (such as CD54). IL-10 enhances phagocytosis and regulates cytokine production, e.g., the production levels of TNF-α, IL-1β, IL-6, IL-8, IL-12, IL-23, G-CSF, and GM-CSF are decreased, while the levels of IL-1R antagonist and TNF-α soluble receptor is increased.

Anti-inflammatory effects of IL-10 are:

- *decreased inflammatory activity in the innate immune system*
- *decreased Th1 activity and reduced IFN-γ and IL-2 production by Th1 cells*
- *inhibited IL-4 and IL-5 production by Th2 cells*
- *induction of Tr1 cells*
- *decreased induction of Th17 cells [131]*

Pro-inflammatory effects of IL-10 are:

- *activation of B cells by increased proliferation, increased MHC II expression and promotion of Ig class switching*
- *enhanced cytotoxicity of NK cells*
- *contribute to expansion and increased activity of cytotoxic T cells*

1.4.5 IL-17A

IL-17A is a pro-inflammatory cytokine that is mainly produced by Th17 cells and other T-cell subsets [49, 132]. It acts on fibroblasts and epithelial cells and increases the production of IL-6, IL-8, G-CSF, chemokines, and acute phase proteins [133]. The level of IL-17A is increased in the synovial fluids

of RA patients [48, 134] and it has, in combination with TNF-α, a bone-resorbing effect [135]. Antibodies to IL-17 or IL17 receptor have been used in clinical trials involving patients with psoriasis and RA, and showed impressing effects in psoriasis but more discrete effects in RA [136-138].

1.4.6 TGF-β

TGF-β is present at moderate levels during the normal non-inflammatory steady state. It prevents autoreactive T cells from being activated and inhibits the differentiation of naïve T cells into Th1, Th2 or cytotoxic T cells. TGF-β in combination with IL-1β, IL-6, and IL-23 stimulate Th17 generation and TGF-β in combination with IL-2 stimulate Treg differentiation (Figure 5) [139]. Depletion of TGF-β leads to decreased levels of Tregs and increases the T-effector cell responses that cause inflammatory diseases [139]. TGF-β is important for tolerance, embryogenesis, wound healing, and the resolution of inflammatory processes.

1.4.7 IFN-γ

IFN-γ is produced by Th1 cells, B cells, professional APCs, and NK cells. It binds to IFN-γR and its effects have traditionally been considered to be pro-inflammatory, in that it activates cells of the innate immunity system, such as macrophages and neutrophils, induces a Th1 response, inhibits Th2 or Th17 responses, and up-regulates adhesion molecules to increase leukocyte recruitment. However, several researchers have recently reported immunoregulatory activities for IFN-γ, which may be exerted through the inhibition of IL-17 [140, 141].

1.4.8 SOCS

Suppressors of cytokine signalling (SOCS) proteins are induced by cytokines and function to suppress intracellular signalling. The SOCS1 protein is induced by IL-10 and promotes the down-regulation of the type 1 IFN receptor and IFN-γ receptor [130, 142]. The SOCS3 protein is induced by LPS, IL-6, and IL-10 [143-145]. Both SOCS1 and 3 have been suggested to suppress IL-6 signalling *via* the inhibition of different STATs [146, 147].

1.5 Self-tolerance

Self-tolerance is a state of homeostasis in which potentially self-reactive immune responses are effectively regulated and no inflammatory responses are detectable. As previously described, if this balance is disturbed, self-tolerance is broken and a dysregulated immune response directed against self-tissues may lead to the development of autoimmune diseases. Self-tolerance is breached when autoreactive B cells are activated by soluble self-antigens or autoreactive T cells are activated by self-antigens presented on APCs. Human MHC molecules can present up to 30 million self-antigen peptides, which in theory could contribute to a self-reactive immune response [148]. However, multiple mechanisms prevent the breaching of self-tolerance. There are two main strategies for tolerance: 1) a recessive mechanism that diminishes the active effector response towards self-antigens; and 2) a dominant mechanism that actively suppresses inflammation *via* Tregs $_{\text{and}}$ B-regulatory cells [34].

The *recessive* mechanism involves:

1. Negative selection through clonal deletion of autoreactive T and B cells in the thymus or bone marrow;

2. Receptor editing of autoreactive BCRs [149];

3. Induction of anergy (unresponsiveness) after contact with self-antigen. Anergy refers to the inactivation, unresponsiveness or arrested development of a cell after recognition of an antigen [150]. Anergy, which can be permanent or reversible, is caused by incomplete T-cell activation, incomplete co-stimulatory binding or excessive binding of co-inhibitory factors. Infectious anergy is exerted by anergic T cells that surround a naïve T cell and prevent it from being activated by an APC, instead making the T cell anergic. Tregs (CD4+CD25+FoxP3+) are often anergic and the spread anergy in this fashion, probably through the down-regulation of MHC II, CD80, and CD86 on the APC. Anergic Tregs can also induce apoptosis directly in DCs or indirectly cause DCs to induce the apoptosis of T cells;

4. Negative signalling molecules or activation-induced cell death lead to insufficient stimulatory signals for the cell to survive [151].

The *dominant* mechanism involves:

1. Suppression of T-effector cells by Tregs;

2. Most likely, IL-10 and TGF-β secretion from B-regulatory cells to suppress T-effector cells [21, 24, 32, 152].

1.6 Collagen type II

The cartilage is made up of collagen of types II, VI, IX, X, and XI, as well as proteoglycans, water, and chondrocytes. The major component of cartilage is collagen type II (CII), which constitutes 90%-95% of cartilage [153]. The immunodominant T-cell epitope of CII comprises amino acid residues259–270. Compared with the rat and human CII sequences, the murine sequence differs by one amino acid, such that at position 266 the mouse has an aspartic acid while the rat and human have a glutamic acid [154]. In the mouse model of CIA, rat CII induces a more severe form of arthritis than murine CII [155].

1.7 Rheumatoid arthritis versus collagen induced arthritis

1.7.1 Rheumatoid arthritis

RA is an autoimmune inflammatory disease that affects 0.1%–1.0% of the population [156, 157]. Autoimmune diseases are caused by an immune response directed against self-tissues, such as cartilage, bone, blood vessels, lung alveoli, and nerves. RA is a heterogeneous disease that ranges from a mild arthritis to a severe inflammation that might affect the internal organs. The aetiology and pathogenesis of RA are not completely known. Studies on monozygotic twins have shown that the heritability of anti-citrullinated protein antibody (ACPA)-negative and ACPA-positive RA is about 66% [158]. The shared epitope hypothesis proposes that the β-chains of MHC II molecules HLA-DRB1 0401 and 0404, called HLA-DR4, exhibit similar activation of the T cell *via* presentation of an arthritogenic peptide [159, 160]. The shared epitope is not associated with RA development *per se* but by the development of ACPAs, which means that the shared epitope increases susceptibility to RA *via* ACPAs [158, 161, 162]. The shared epitope hypothesis argues that since the shared epitope on the HLA-DR4 molecule is strongly associated (at 11%–18%) with the development of RA, this antigenic epitope in combination with antigen presentation elicits RA [158, 163, 164].

What is known regarding the aetiology and pathogenesis in RA?

- There is a very strong correlation with the HLA-DR genotype;
- Accumulation of lymphocytes in synovium occurs during active disease [165, 166];
- Increased levels of several cytokines, e.g. IL-17 and TNF, are detected in synovial fluids;
- Increased levels of autoantibodies directed against citrullinated proteins, e.g., cyclic citrullinated peptide 2, fibrinogen, and α-enolase, are detectable even before the onset of disease [167, 168];
- Biological therapies, such as TNF blockers and CTLA-4 immunoglobulins that affect the T-cell compartment, have an effect on RA [169, 170];
- B-cell-depleting therapies are effective in rheumatoid factor-positive RA [171].

What is not completely known?

- The nature of the eliciting autoantigen;
- The time-point at which self-tolerance is lost;
- The location of self-tolerance loss.

1.7.2 Collagen type II-induced arthritis (CIA)

The experiments that we performed as part of the work of this thesis could not have been carried out in humans with RA, as we wanted to investigate the relative importance of the participating cells during different tolerogenic situations and the effect of a cytokine during the development of arthritis. Collagen induced arthritis (CIA) shares many similarities with RA, and it is the most commonly used animal model for studying RA [172-175]. The choice of mouse strain influences the frequency, severity, and chronicity of arthritis in this model [174]. We used inbred DBA/1 mice, which allow us to study a low number of individuals, all of which carry the HLA haplotype $H\text{-}2^q$. Murine CIA can be induced by immunisation with CII of bovine, deer, murine, rat, chicken or human origin [175-180]. Heterologous CII, e.g., rat CII, induces a more severe form CIA in mice than autologous mouse CII [155]. The similarities and differences between RA and CIA are listed in Table 1. A disadvantage of the CIA model is the observed variability in

severity of arthritis, which is attributed to CII quality, laboratory conditions, such as stress, and environmental agents.

Table 1. Similarities and differences between RA and CIA.

Similarity	Difference
Strong association with MHC II molecules	RA is a chronic disease, while CIA is an acute and self-limiting disease
Polyarticular peripheral arthritis	RA is a symmetric arthritis
Partly mediated by T and B cells	RA is not as dependent upon CII-specific IgG antibodies as CIA is
Presence of CII-reactive T cells	
Presence of CII-specific antibodies	
Synovial hyperplasia, inflammatory cell infiltration, pannus formation and erosion of cartilage and bone	

CIA pathogenesis

To induce CIA, the animals need to be immunised with both an adjuvant, e.g., Complete Freund's Adjuvant (CFA), and CII, and in most cases, they have to be boosted with a combination of Incomplete Freund's Adjuvant (IFA) and CII. When visible arthritis develops at 20–30 days post-immunisation, the synovial membranes are found to be infiltrated by B and T cells and macrophages and the synovial fluid contains high numbers of neutrophils [181]. The arthritis is probably initiated by the adjuvant, which starts an innate immune response in which neutrophils and macrophages are activated by danger signals [181], leading to inflammatory joint damage and an adaptive immune response directed against CII.

Post-translational modifications of CII in CIA and RA

Proteins are glycosylated by glycosidases and glycosyltransferases in the ER and Golgi apparatus before presentation on MHC II molecules [182]. Glycosylation stabilises the proteins, participates in their transportation to cell surface, positions the proteins correctly in the cell, and facilitates cell adhesion and cell communication. It has been shown that a glycosylated

lysine at position 264 in the immunodominant CII peptide is of major importance for the development of CIA and for tolerance in CIA [183-185].

1.8 Tolerance to CII in CIA and RA

Different protocols for the induction of CII-specific tolerance in CIA have been used with success. Below, I list some of the important studies on this topic in chronological order.

Already in 1986, Nagler-Anderson *et al.* [186] could show that 500 µg of bovine CII administered intragastrically eight times over a period of 2 weeks prior to CIA induction in DBA/1 mice reduced the incidence of arthritis, and that this was associated with lower levels of IgG2a CII antibodies in the non-arthritic mice. Denatured CII or CII at higher doses had no effect on the incidence of arthritis.

Matsumoto *et al.* [187] showed that when denatured or digested CII of bovine origin was administered intranasally 7 days prior to CIA induction there was a reduction in arthritis in DBA/1 mice. The levels of CII-specific IgG2a antibodies were decreased, as were the IFN-γ levels in the supernatants from CII-stimulated lymph node cultures, indicating suppression of the Th1 response.

Myers *et al. [188]* used a synthetic CII analogue peptide (amino acids 256–276) injected i.v. on days 11, 12, and 14 after CIA induction in HLA-DR1-transgenic mice. The incidence of arthritis was reduced, the levels of CII-specific antibodies were reduced, and the levels of the Th2 cell cytokine IL-4 were increased in these mice.

Dzhambazov *et al.* [189] used a galactosylated or naked CII (amino acids 259–273) in a complex with MHC II (A^q), which was administered i.v. or intranasally on days 21 and 34 after CIA induction in B10.Q mice. The incidence and severity of CIA were decreased when the galactosylated peptide, but not the naked peptide, and MHC II complex were administered i.v. to the mice. The levels of CII-specific IgG antibodies were decreased on days 35 and 70 during the course of CIA. Transfer of T cells on Day 5 prevented or delayed the development of CIA in naïve recipients.

Hasselberg *et al.* [190] used a CII peptide (amino acids 259–274) in a complex with MHC II and cholera toxin B, which was administered intranasally on days 26, 27, and 28 after CIA induction in DBA/1 mice. The

results showed a 60% reduction in the incidence of arthritis, lower levels of CII-specific IgG antibodies, decreased levels of IFN-γ, IL-6, and IL-17, and increased levels of IL-10 in these mice.

1.9 Disease-regulated therapies

Current treatment strategies for RA involve continuous administration of immunosuppressive drugs that limit the immune reactions directed against self-tissues. To reduce the side-effects associated with the immunosuppressive drugs, disease-regulated administration of the treatment only during active inflammatory flares would be preferable. Inflammation-dependent transcription of cytokine genes has previously shown success in the treatment of experimental autoimmune encephalitis (EAE) using IL-2-dependent transcription of IL-10 in T cells [191]. In addition, Adriaansen *et al.* showed that transcription of TNF-receptor-blocking immunoglobulins regulated by a nuclear factor kappa B (NF-κB)-responsive promoter could ameliorate CIA [192]. Garaulet *et al.* showed reduction in the severity of arthritis using E-selectin as a regulator of IL-10 transcription [193] and Geurts *et al.* showed that transcription of IL-4 using the IL-1enhancer/IL-6 promoter prevented cartilage erosivity in CIA [194].

2 AIM

To investigate the immunological mechanisms underlying tolerance induction and maintenance in collagen type II induced arthritis.

2.1 Objectives

1. To investigate the roles of participating antigen-presenting cells in antigen-specific tolerance in CIA;

2. To elucidate the time-points at which and the mechanisms by which antigen-specific tolerance in CIA can be induced in the inflammatory phase of the disease;

3. To determine whether inflammation-dependent release of the regulatory cytokine IL-10 can induce tolerance in CIA.

3 MATERIALS AND METHODS

3.1 Gene therapy

To induce antigen-specific tolerance through endogenous expression of an immunodominant CII epitope on all APCs (**Papers I** and **II**) and exclusively on B cells (**Paper I**), and to create inflammation-regulated increases in IL-10 expression (**Paper III**) we used lentivirus-based gene therapy. Lentivirus-based gene therapy ensures permanent integration of the delivered gene(s) into the genomes of transduced cells. Lentiviral particles can transduce both dividing and non-dividing cells [195]. Normally, the transcription of the lentivirus genome is driven by the promoter and enhancer in the 5′- and 3′-long term repeats (LTRs), but we used self-inactivated vectors in which the promoter and enhancer in the 3′-LTR were deleted, leading to reliance on an added incorporated promoter [196]. Lentiviral vectors represent an excellent tool for targeting protein expression to a specific cell population or cell compartment, so as to facilitate mechanistic studies.

Lentiviral particles

Second-generation lentiviral particles are produced by co-transfection of the human embryonic kidney cell line HEK 293FT with the following three vectors: 1) a transgene vector that contains the gene of interest driven by a chosen promoter; 2) a packaging plasmid (pCMVΔR8.74) that contains the *gag* gene (encodes the viral core proteins), the *pol* gene (encodes the reverse transcriptase), and the *rev* gene (responsible for the transport of viral mRNA); 3) a plasmid (pMD.2) that contains the genes for the vesicular stomatitis virus-G (VSV-G) envelope proteins. The transgene vector also contains all the sequences required for efficient viral replication, called *cis*-acting elements, which include the woodchuck post-transcriptional regulatory element (WPRE), the central polypurine tract (cPPT), the rev responsive element (RRE), and the polyadenylation signal (polyA) [197]. The HEK 293FT cells were grown, transfected with the three vectors, and after 72 h in culture, the medium was harvested and concentrated by ultracentrifugation at 90,000 × *g*. The pellets were resuspended in PBS that contained 2% foetal calf serum (FCS) and stored at -80ºC.

Lentiviral vector LNT-CII (LNT-Ii-CII)

The lentiviral construct LNT-CII contains CII amino acids 259–270 cloned into the MHC class II–associated invariant chain peptide (CLIP) position of the invariant chain (Ii), carefully conserving the anchor residues. The invariant chain is a protein that stabilises and transports MHC II molecules from the ER to the cell surface (Figure 9A-B). Transcription of Ii-CII was driven by the general spleen focus-forming virus (SFFV) promoter. The LNT-CII was named *LNT-Ii-CII* in **Paper II**.

Lentiviral control vector LNT-Ctrl (LNT-Ii-CLIP)

Under normal conditions, CLIP remains in the antigen-binding cleft when Ii is dissociated from MHC II, until an antigenic peptide with higher affinity for MHC II replaces CLIP. We used Ii with CLIP as a lentiviral control, which was named LNT-CLIP or LNT-Ctrl. Transcription of Ii-CLIP was driven by the SFFV promoter.

Lentiviral vector LNT-Igκ-CII (LNT-Igκ -Ii-CII) or LNT-Igκ-Ctrl (LNT-Igκ-CLIP)

To achieve expression of the CII peptide or the CLIP peptide exclusively in B cells, a lentiviral construct that contained the Igκ promoter was used [198].

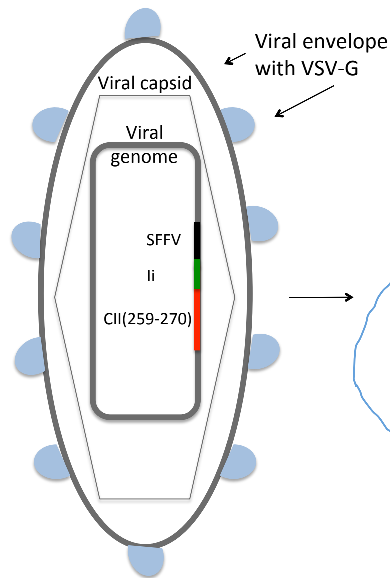
Lentiviral vector LNT-IL-10

To achieve endogenous expression of IL-10, a lentiviral construct that contained the IL-1 enhancer and IL-6 promoter was used [199].

Lentiviral vector LNT-GFP

As a lentiviral control for LNT-CII or LNT-IL-10, a lentiviral construct that contained green fluorescent protein, LNT-GFP, was used.

A. B.



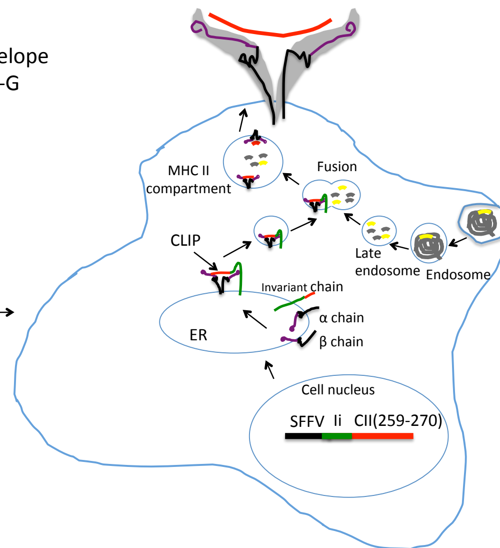


Figure 9. A. The lentiviral particle LNT-CII consists of an envelope with VSV-G, the capsid, and the viral genome containing the SFFV promoter, and invariant chain (Ii) fused to CII(259–270). **B.** Transduced cell with integrated SFFV, Ii and CII(259–270) in the murine genome. Ii stabilises the transport of MHC II through the ER, while CII binds to the antigen-binding cleft. In the MHC II compartment, the CII peptide and endocytosed antigens compete for binding to MHC II and for presentation by MHC II on the cell surface.

3.1.1 Administration of lentiviral particles

Transplantation of haematopoietic stem cells

Bone marrow was collected from naïve DBA/1 donors. The cells were selected using a HSC selection kit. The selected HSCs were transduced for 24 h with the lentiviral particles LNT-Ctrl, LNT-CII, LNT-Igκ-Ctrl, LNT-

Igκ-CII, LNT-IL-10 or LNT-GFP with a multiplicity of infection (MOI) of 75, i.e., 75 lentiviral particles/cell. The cells were then harvested, washed, and counted before injection into lethally irradiated (8.5 Gy) syngenic naïve recipient mice. The mice were allowed to reconstitute their bone marrow for a minimum of 10 weeks before CIA was induced.

Adoptive transfer of sorted T cells, B cells, and MHC II-positive cells

Mice that received LNT-Ctrl- or LNT-CII-transduced HSCs were sacrificed 18 weeks after bone marrow transplantation. The spleens were sorted by FACS into purified cell subsets based on CD3 for T cells, CD19 for B cells, and MHC II for non-B-cell APCs. The T and B cells and APCs were injected i.v. into naïve syngenic mice, and CIA was induced 2 days later (Figure 10).

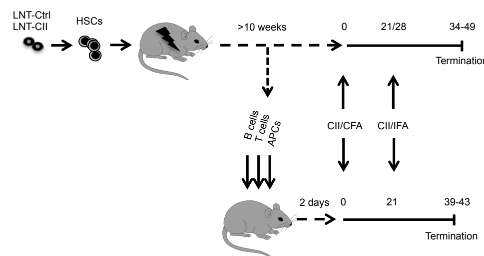


Figure 10. Lentiviral-transduced HSCs were injected i.v. into naïve mice at least 10 weeks prior to induction of CIA. CIA was induced by immunisation with CII and CFA on day 0 and boosted on day 21 with CII and IFA. Adoptive transfer of sorted B cells, T cells, and APCs from the LNT-Ctrl or LNT-CII mice (that previously received LNT-Ctrl- or LNT-CII- transduced HSCs) was performed by i.v. injection into naïve syngenic mice 2 days prior to CIA induction.

3.2 Mice

Male DBA/1 mice (6–8 weeks of age) were purchased from Taconic (Denmark). The DBA/1 strain contains the MHC II allele H2-A^q, which is associated with susceptibility to CIA and important for presentation of the CII epitope, resembling the way in which the MHC II allele HLA-DR1 in humans is associated with increased susceptibility to RA. Male mice develop a more severe form of CIA than female mice, which makes the male mice more suitable for investigations of anti-arthritic therapies.

3.3 Polymerase chain reaction

To ensure that the lentiviral constructs were integrated in the genomes of the mice (**Papers I** and **III**), DNA was purified from the bone marrow cells, splenocytes, lymph node cells, and thymus-derived cells. Quantitative polymerase chain reaction (qPCR) was performed on the DNA to quantify the integration of WPRE, i.e., the viral element in our constructs that does not exist normally in the murine genome. The levels of WPRE were compared to the levels of the normally occurring *titin* gene, to determine the number of viral copies integrated per cell using a primer/probe-based assay.

3.3.1 Reverse transcriptase-polymerase chain reaction

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed (**Paper III**) to compare the levels of mRNA for cytokines and suppressors of cytokine signalling (SOCS) with the levels of normally occurring *β-actin* mRNA. For this purpose, RNA was purified from lymph node cells and splenocytes using the RNeasy Mini Kit (Qiagen) prior to the synthesis of complementary DNA (cDNA) using a cDNA Reverse Transcription kit. PCR analysis was performed on the cDNA using primers for *IL-10*, *SOCS*, and *β-actin*. The reactions were prepared using the Power SYBR green PCR Master Mix and analysed on the Viia7 system. While the RT-PCR analysis estimates the quantities of the mRNA species in the lymph node cells and splenocytes, a major disadvantage is that the mRNA levels do not reflect the levels of secreted cytokine.

3.4 Detection of CII on MHC II

Unfortunately, there is no available antibody that recognizes the CII peptide in the MHC II grove. Instead, we used T-cell hybridomas that are T cells clones specific for only one post-translational modification of the peptide presented on the MHC II molecule. We used T-cell hybridomas specific for the naked CII peptide (HCQ4), hydroxylated CII peptide (HdBr1) or galactosylated CII peptide (HCQ3). After co-cultivation of the hybridomas and APCs from the LNT mice, the supernatants were analysed for IL-2 produced by the activated hybridomas.

3.5 Collagen induced arthritis (CIA)

We used rat CII emulsified in CFA to induce CIA, and CII in IFA for booster injections on day 21 or day 28 post-induction.

3.5.1 Assessment of arthritis

Clinical evidence of arthritis was assessed blindly every second to third day using a scoring scale that ranged from 0 to 3 in the evaluation of each paw for redness and swelling. Histological examinations were performed on the paws, knees, feet, elbows, and hands after fixation in formalin. Sections of the paws were cut, decalcified, and embedded in paraffin and stained with haematoxylin-eosin. The severity of synovitis and bone-cartilage erosions were assessed by two blinded observers, using a scoring scale that ranged from 0 to 3, based on synovial hypertrophy, pannus formation, and bone-cartilage erosions.

3.6 Antibody and cytokine analyses

The antibody levels were analysed by enzyme-linked immunosorbent assay (ELISA). Cytokine expression at the mRNA level was assessed by RT-PCR and at the protein level by ELISA or using FlowCytomix and measurement by FACS.

3.7 Flow cytometry (FACS)

Fluorescence-activated cell sorting (FACS) can be used to measure surface or intracellular expression of cell components. It is performed using fluorochrome-labelled antibodies against the molecule of interest. When intracellular markers are analysed, the cells are permeabilised and fixed to allow penetration of the antibodies into the cells. FACS uses lasers to detect the fluorochrome. The FlowCytomix assay (performed in **Paper III**) is based on fluorescent beads that are labelled with antibodies directed against the

cytokines of interest, with enables FACS analysis to determine the levels of cytokines in the analysed serum or supernatant.

Frequency of marker-positive cells

The FACS results are presented as the frequency of cells that express the analysed marker. Each fluorochrome has some degree of leakage into other laser channels and this leakage has be taken into account during analysis of FACS data. To set the cut-off, we used ”fluorochrome minus one” (FMO) gating, which means that for each fluorochrome, the cut-off was assigned based on the background or leakage from a sample that contained all the fluorochromes except the one for which the gating was set [200]. Thus, non-specific binding of the antibody could be subtracted to give the actual specific antibody staining.

Mean fluorescence intensity (MFI)

MFI represents the level of expression of the protein on each cell and is presented as the geometric mean.

Sorting of cell populations

To quantify the cell populations (**Papers I** and **II**), to perform the proliferation assays (**Paper I**), and to carry out the adoptive transfer (**Paper I**), cells were separated by FACS. After single-cell preparation, B cells, T cells, T-effector cells, Tregs, and MHC+ cells were stained for the expression of CD19, B220, CD3, CD4, CD25, FoxP3,and MHC and sorted in the Sy3200 flow cytometer.

3.8 Suppression assays

Suppression assays with Tregs were performed in the **Papers I** and **II**. In **Paper I**, T cells were sorted into the $CD4^{+}CD25^{-}$ cell T-effector cell population and the $CD4^{+}CD25^{+}$ Treg population, as described in the section above. APCs were sorted from spleen from naïve mice by negative selection using Mouse Dynabeads PanT kit (Life Technologies Ltd, Paisley, UK). To evaluate the suppressive capacity of the Tregs from LNT-CII mice, these cells were co-cultured with APCs and T-effector cells, labelled with tritiated thymidine and 12 h later the cells were harvested and analysed for proliferation in a Betacounter (1450 LSC & Luminescence Counter, MicroBeta TriLux; PerkinElmer Inc., Bath, UK). In **Paper II**, cell sorting

was performed using selection kits that are based on negative and positive selection after staining for surface markers, such as CD4 and CD25, which are labelled with magnetic particles. The T cells from the spleen were purified and sorted into $CD4^+$ T cells, $CD4^+CD25^+$ Tregs, and $CD4^+CD25^-$ T-effector cells using a $CD4^+CD25^+$ T-cell enrichment kit. To co-culture these T cells with APCs that presented CII, CD11c-positive cells were purified from the spleens of naïve mice using the EasySep kit (Stemcell Technologies). Purified $CD11c^+$ cells were pulsed with CII that was denatured at 56°C for 30 min, prior to incubation overnight. CII-pulsed $CD11c^+$ cells were co-cultured with T-effector cells and Tregs at different ratios. The supernatants were analysed for the levels of IFN-γ using an ELISA kit.

3.9 Statistical analyses

Mann Whitney U-test

To compare ordinal data between the groups, we used the Mann Whitney U-test. This test compares independent non-parametrically distributed groups. This test was used to compare the levels of antibodies and cytokines.

Student's t-test

To compare ordinal data between parametrically distributed groups, we used Student´s t-test. This was used to compare levels of cytokines produced by T-cell hybridomas.

Fisher´s exact test

When comparing nominal data, i.e., having arthritis or being healthy, Fisher´s exact test was used.

Linear regression

When the severity of arthritis during the time course was compared we used linear regression to compare differences between the groups.

Logistic regression

When the frequency of arthritis during the time course was compared we used logistic regression to compare differences between the groups.

Two-way ANOVA

Two-way ANOVA was used to compare differences between the two groups when the ratios of Treg to Teffector cells (**Paper I**) or the concentration of lentivirus (**Paper III**) was increased.

4 RESULTS

Paper I:

To investigate the roles of antigen-presenting cells during antigen-specific tolerance in CIA, transplantation of LNT-CII- or LNT-Ctrl-transduced HSCs was performed at least 10 weeks before CIA induction. Co-cultures of cells from the LNT-CII or LNT-Ctrl mice and T-cell hybridomas specific for the CII peptides showed that the LNT-CII mice had a significantly higher level of presentation of the naked CII peptide on MHC II than the LNT-Ctrl mice. Clinical and histopathological evaluations showed that the LNT-CII mice developed less severe CIA than the LNT-Ctrl mice (Figure 11A–C).

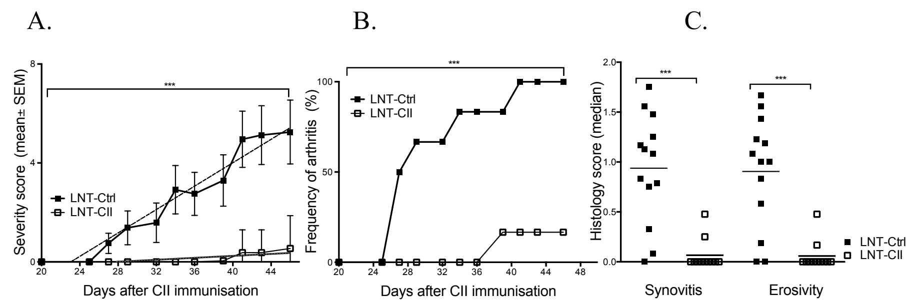


Figure 11. Development of CIA in LNT-CII and LNT-Ctrl mice. A. Severity of arthritis. B. Frequency of arthritis. C. Histopathological evaluations of synovitis and bone-cartilage destruction.

The levels of CII-specific IgG antibodies during CIA were lower in the LNT-CII mice than in the LNT-Ctrl mice (Figure 12). Subclasses of CII-specific IgG antibodies were analysed at the termination of the experiment, and the levels of CII-specific IgG1, IgG2a, and IgG2b antibodies were found to be lower in the LNT-CII mice than the control LNT-Ctrl mice. The capacity of the Tregs from the LNT-CII mice to suppress T-effector cells from arthritic mice was higher than that of the Tregs from the LNT-Ctrl mice.

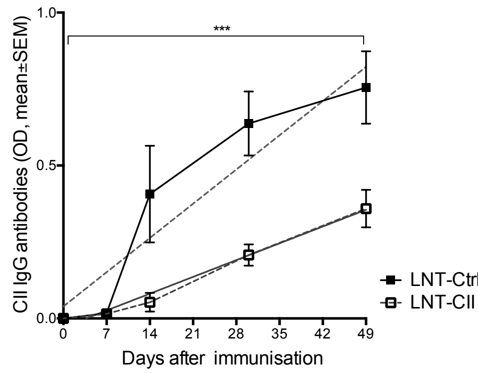


Figure 12. Levels of CII-specific IgG antibodies in the LNT-CII mice and LNT-Ctrl mice.

The adoptive transfer of B cells or T cells from the LNT-CII mice into naïve mice 2 days prior to CIA induction revealed that both the B and T cells were able to ameliorate the development of CIA.

To delineate the importance of B cells as APCs, a B-cell-specific promoter was employed to drive the CII epitope together with Ii (LNT-Igκ-CII) or CLIP (LNT-Igκ-Ctrl) expression. Transplantation of LNT-Igκ-CII-transduced HSCs followed by CIA induction showed that endogenous expression of the CII epitope on B cells significantly inhibited the development of CIA, as compared with transplantation of LNT-Igκ-Ctrl-transduced HSCs (Figure 13).

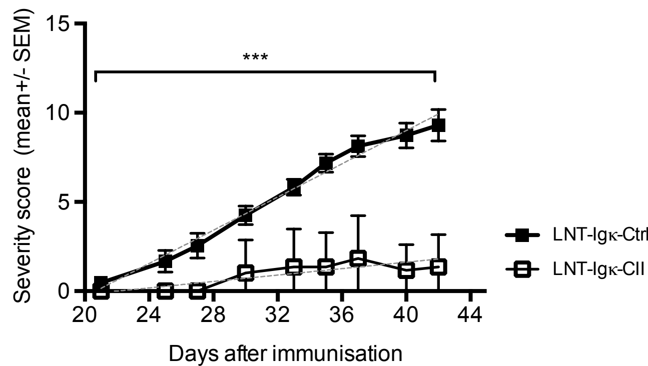


Figure 13. Severity of CIA in LNT-Igκ-CII or LNT-Igκ-Ctrl mice.

The levels of CII-specific IgG, IgG1, IG2a, and IgG2b antibodies were lower in the LNT-Igκ-CII mice than in the LNT-Igκ-Ctrl mice at the termination of experiment. The T-cell hybridoma assay showed presentation of the naked CII peptide on the MHC II on splenocytes and on cells from the peritoneal lavage from the LNT-Igκ-CII mice.

Paper II:

To investigate the induction of tolerance during the inflammatory phases of CIA, LNT-CII was administered i.v. on day 7 or day 26 after the induction of CIA. Evaluations of CIA showed arthritis of reduced severity in the LNT-CII mice, as compared with the LNT-GFP mice (Figure 14).

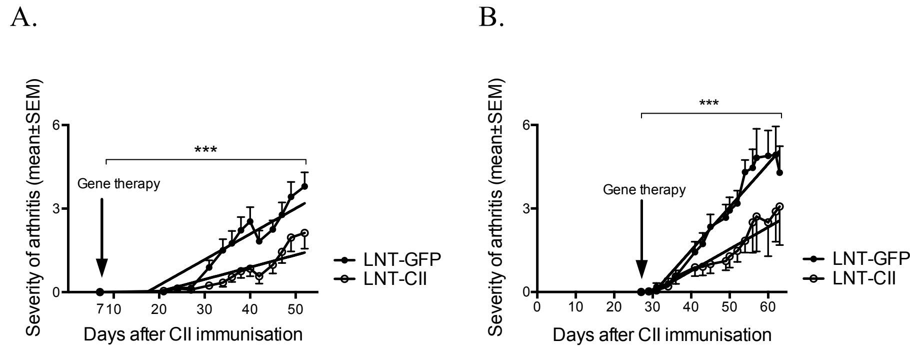


Figure 14. Severity of CIA in mice after administration of LNT-GFP or LNT-CII on day 7 (in A) or on day 26 (in B) after the induction of CIA.

The levels of CII-specific IgG antibodies were decreased at the termination of the experiments for treatment on day 7 and on day 26 post-immunisation of CIA. The suppressive capacity of the CII-specific Tregs on T-effector cells was higher in the LNT-CII mice than in the LNT-GFP mice after treatment on day 26 post-immunisation (Figure 15).

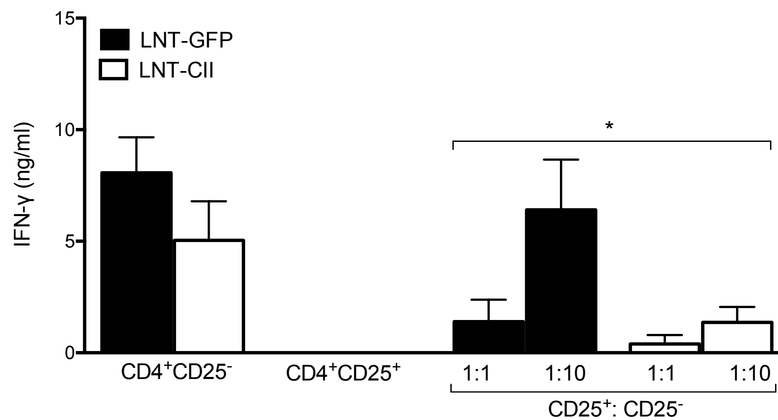


Figure 15. Sorted Tregs and T effector cells from LNT-GFP mice (black bars) or LNT-CII mice (white bars) were co-cultured alone or in ratios of 1:1 and 1:10 and the production of IFN-γ was used as a measurement of cell proliferation.

To investigate antigen-specific tolerance in manifest arthritis, LNT-Ctrl or LNT-CII was injected i.v. on day 31 post-immunisation. Evaluation of CIA showed a discrete anti-arthritic effect on CIA in the LNT-CII group. A decrease in the levels of CII-specific IgG antibodies accompanied by the inhibition of arthritis development in the LNT-CII mice, as compared with the control mice (Figure 16).

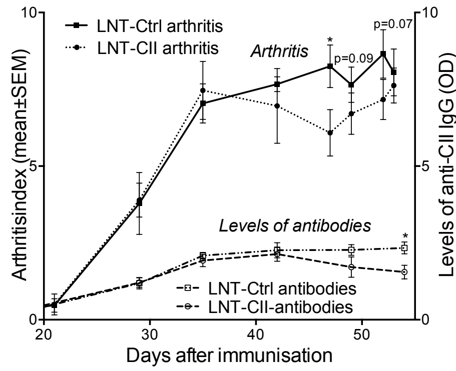


Figure 16. Severity of CIA in relation to levels of CII-specific IgG in sera of mice after administration of LNT-Ctrl or LNT-CII on day 31 post-immunisation with CII.

The levels of circulating Tregs in the LNT-CII and LNT-Ctrl mice were similar. Analysis of the proportions of Helios+ Foxp3+ Tregs in the blood showed a higher frequency of Helios-expressing Tregs in the LNT-CII group than in the control group (Figure 17).

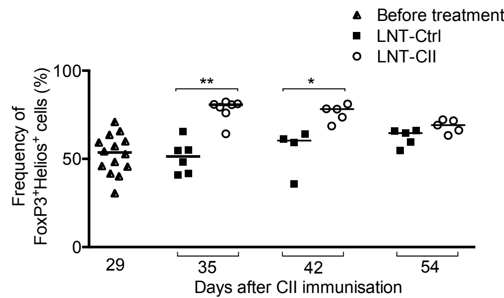


Figure 17. Frequencies of Helios+ Tregs in the blood samples of mice on days 29, 35, 42, and 54 post-immunisation after LNT-Ctrl or LNT-CII injection on day 31.

Paper III:

In **Paper III**, we investigated whether a lentiviral vector that contained the gene for the anti-inflammatory cytokine IL-10, regulated by an IL-6 promoter and with an IL-1 enhancer element, could suppress the development of arthritis. Transplantation of lentivirally transduced HSCs was performed at least 10 weeks prior to the induction of CIA in the mice. *In vitro* assays showed higher levels of IL-10 in the supernatants after LPS stimulation of bone marrow-derived macrophages from the LNT-IL-10 mice, as compared with the levels in the controls (Figure 18A). *Ex vivo* analysis of lymph node cells at the termination of the experiment showed higher levels of IL-10 mRNA than in the controls (Figure 18B) and higher levels of IL-10 protein in the APCs (Figure 18C) and B cells (data not shown), as compared with the controls. The IL-10 protein levels were not increased in the APCs or B cells from the spleens of the LNT-IL-10 mice, as compared with the controls. Taken together, these results show that IL-10 production is increased locally, mainly in the APCs and B cells in the lymph nodes of the LNT-IL-10 mice.

A. B. C.

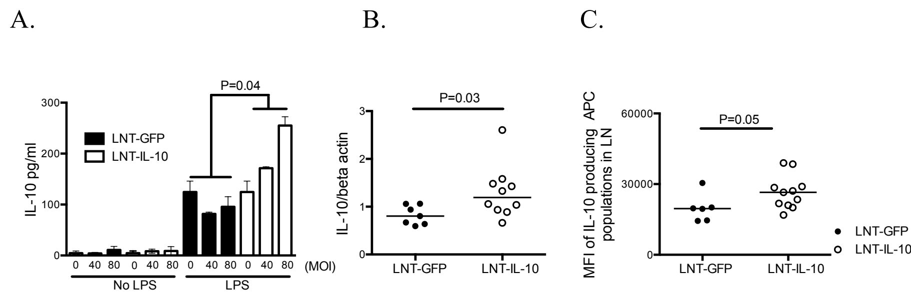


Figure 18. A. Levels of IL-10 in the supernatants 9 days after *in vitro* transduction of HSCs that were transduced with LNT-GFP or LNT-IL-10 at MOI of 0, 40 or 80, and with or without LPS stimulation. **B.** Levels of *IL-10* mRNA measured by PCR in the lymph node cells at the termination of the experiment. **C.** Mean fluorescence intensities (MFIs) of IL-10 protein in the APCs from the lymph nodes, as analysed by FACS.

Evaluation of the development of arthritis showed amelioration of CIA in the LNT-IL-10 mice, as compared with the control mice (Figure 19).

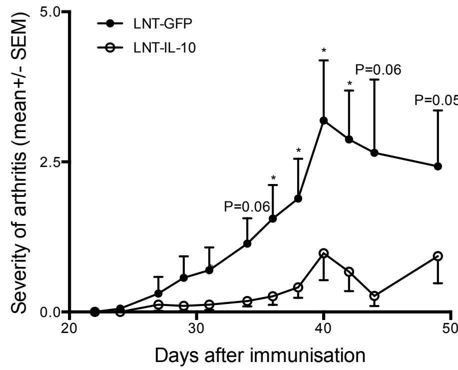


Figure 19. Severity of arthritis in the LNT-IL-10 mice and LNT-Ctrl mice.

The amelioration of CIA was accompanied by decreased serum levels of CII-specific IgG antibodies (Figure 20A) and IL-6 (Figure 20B) and increased levels of *SOCS1* mRNA in the lymph nodes (Figure 20C).

A. B. C.

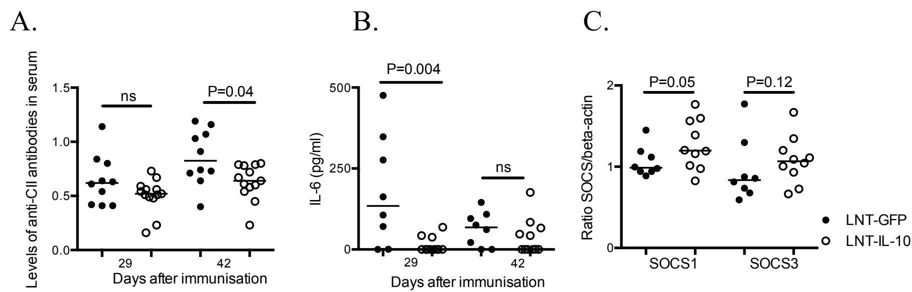
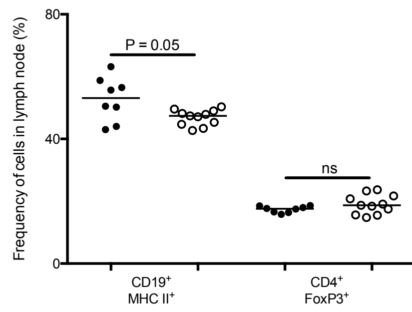


Figure 20. A. The serum levels of CII-specific antibodies are similar in the groups on day 29, and are lower in the LNT-IL-10 mice compared to the controls on day 42. **B**. The serum levels of IL-6 are lower in the LNT-IL-10 mice than in the control mice on day 29, whereas the IL-6 levels in the two groups are similar on day 42. **C.** The levels of *SOCS1* mRNA are lower in the LNT-IL-10 mice than in the control mice on day 42. The levels of *SOCS3* mRNA in the two groups on day 42 are similar.

The frequencies of B cells in the lymph nodes and spleens were lower in the LNT-IL-10 mice than in the control mice, while the frequencies of Tregs in the lymph nodes were higher in the LNT-IL-10 mice (Figure 21A-B).

A. B.



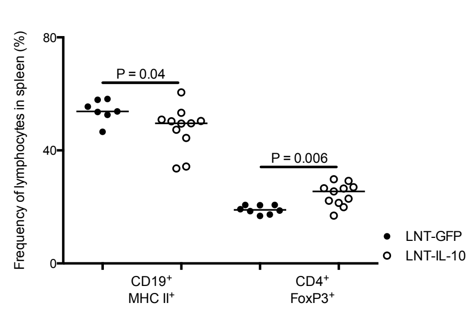


Figure 21. Cell populations in the lymph nodes and spleens. **A.** The frequencies of B cells in the lymph nodes and spleens are reduced in the LNT-IL-10 mice than in the control mice, while the frequencies of Tregs in the lymph nodes are higher in the LNT-IL-10 mice. **B.** The frequencies of splenic B cells are lower in the LNT-IL-10 mice than in the control mice, while the frequencies of splenic Tregs are similar in the two groups.

5 DISCUSSION

Re-establishment of self-tolerance is an attractive strategy to use in the treatment of autoimmune diseases, such as RA. To realise this therapeutic strategy, especially for application during ongoing inflammation, the basic immunological mechanisms need to be better defined. For example, in which compartment the crucial tolerogenic interaction takes place and which cells are involved in this interaction are currently unclear. To address these issues, we used gene therapy in an animal model of RA, CIA, to study the importance of endogenous expression of the tolerogen on MHC class II for different types of APCs. Our studies suggest that the B cell is crucial for tolerance induction, that antigen-specific tolerance can be attributed mainly to endogenous presentation of the self-antigen by B cells, and that disease-regulated IL-10 production, especially IL-10 produced by B cells, inhibits the development of arthritis. However, our studies also clearly show that the tolerogenic mechanisms are complex and involve several different cell populations acting in concert.

5.1 Paper I

5.1.1 Gene therapy – pros and cons

The advantages of the gene therapeutic approach for the induction of antigen specific tolerance are that: 1) it is a tool for manipulating the genome, thereby achieving permanent and endogenous expression of the desired cytokine or antigen; and 2) endogenous production of proteins is advantageous in terms of avoiding gastrointestinal degradation by enzymes and circumventing the need for repeated administrations of the antigen. Lentiviral transduction of HSCs results in the integration of the lentiviral vector into a large proportion of the target cells, which subsequently spread throughout the entire body. In addition, transgene expression driven by tissue-specific promoters allows studies of targeted cell populations. Several studies using gene therapy for the expression of self-antigens or cytokines have been performed with variable levels of success in CIA (reviewed by Adriaansen *et al.* [201]), as well as in diabetes and experimental autoimmune encephalitis [202-204].

The main disadvantage of the gene therapeutic approach is that the system cannot be readily transferred into clinical practice or the treatment of patients with RA due to the risks associated with performing such a serious intervention as the transplantation of HSCs, the risk of development of replication-competent viruses; and a minor risk of activation of proto-oncogenes. The loading of MHC II molecules with CII peptide might increase the risk for infections due to the reduced capacity of the MHC II to present foreign antigens with adequate efficiency. In addition, a drawback of this system is that it is a time-consuming model to implement, in that it requires the cloning of an appropriate lentiviral vector, synthesis of lentiviral particles, harvesting of bone marrow cells from donor mice, irradiation of the recipient mice, reconstitution of the bone marrow for a minimum of 10 weeks prior to the induction of CIA and finally, the development of CIA over a period of 4–8 weeks. Therefore, a single series of experiments using the lentiviral HSC strategy can take up to 6 months.

Despite these problems, gene therapy has a place in clinical practice. It provides a life-saving option for immunodeficient children when matched bone marrow donor is unavailable [205], it has been used in patients with thalassemia [206] and also in HIV-infected patients [207]. However, to date, gene therapy for autoimmune diseases, such as RA, has met with limited success (reviewed by Evans [208]).

In **Paper I**, we describe the use of a model in which the gene therapeutic approach almost completely abolishes CIA through endogenous presentation of the CII peptide on APCs. This system represents an excellent model to investigate the details of the immunological mechanisms operating during tolerance in CIA, which may provide future candidates for curative therapies.

5.1.2 Which antigen-presenting cell is the most important for tolerance induction?

The LNT-CII lentivirus was constructed to provide CII presentation on the MHC II molecules on all APCs. In **Paper I**, we used the SFFV promoter to achieve CII presentation on all bone marrow-derived professional APCs, i.e., the DCs, macrophages and B cells, while use of the Igκ promoter gave CII presentation on the B cells exclusively. Lentiviral transduction of HSCs led to integration of the transgene into both tissues that are important for peripheral tolerance, such as the synovium, draining lymph nodes, and spleen, and organs that are important for central tolerance, such as the bone marrow and thymus. All three types of professional APC possess the ability

to induce peripheral tolerance, while a recent study indicates that it is mainly the DCs that are essential for the induction of central tolerance [209].

DCs are important for antigen presentation both in peripheral tissues and in the thymus. They have the unique ability to collect antigens, migrate to lymph nodes, and present the antigens on their cell surface, which has a high density of MHC II molecules. DCs also express co-stimulatory surface-located molecules, such as CD80, CD86, and CD40, and co–inhibitory molecules, such as PDL-1, which regulates the T-cell response [210-212]. DCs are important for the negative selection of autoreactive T cells in the thymus [38], a process that is critical for the avoidance of lethal autoimmunity. In addition, a recent study by Guerri *et al.* [209] showed that thymic DCs are necessary for the induction of natural Tregs in the thymus. The DCs change their maturity status and their expression of surface molecules so as to activate or down-regulate T-cell responses [211, 213, 214]. This adaptation of the DCs to the surrounding milieu leads to immature DCs differentiating into semi-mature or mature DCs [215, 216]. The tolerogenic DCs, which are able to induce a tolerogenic T-cell response, mainly belong to the immature and semi-mature DC subsets, which are characterised by low levels of co-stimulatory molecules, increased levels of co-inhibitory molecules, and increased levels of IL-10 [216, 217]. However, mature DCs are also able to expand Tregs [218]. Tolerogenic DCs have been generated from murine resting DCs *in vitro* by treatment with IL-10, TNF or dexamethasone [219]. Human DCs can be made tolerogenic by exposure to e.g., vitamin D3 (VD3) or dexamethasone [220, 221]. In addition, DCs and the Tregs have been shown to influence each other during non-inflammatory conditions where expansion of DCs with FLT3L leads to the expansion of Tregs, which results in a more robust self-tolerance [222].

We explored if VD3 stimulated DCs either transduced with the LNT-CII or a lentiviral control construct, LNT-MOG can alter the outcome CIA induction in WT mice. VD3 stimulated DCs showed a decreased expression of CD40 and CD86 compared to control DCs and a decreased expression of MHC II on DCs compared to control DCs after LPS stimulation *in vitro*. Unexpectedly, injection of VD3-DC-CII i.v. 26 days after induction of CIA, caused a more severe arthritis than DC-CII controls (figure 22A), while, VD3-DC-MOG controls were significantly less arthritic than the VD3-DC-CII mice (figure 22A). When the experiment was repeated, we obtained the opposite result, where the VD3-DC-MOG controls developed a more severe arthritis than both VD3-DC-CII and the DC-CII controls (figure 22B), but the DC-CII mice had a more severe CIA compared to VD3-DC-CII (figure 22B).

However, in the second experiment none of the differences were significant. These experiments indicate that vitamin D3 treatment of the DC's has no major impact on the outcome of the CIA model.

In vitro experiments conducted by Anderton *et al.* with altered peptide ligands (APLs) showed that a specific APL gave decreased inflammation and prevented the development of EAE, whereas *in vivo* experiments showed that the same APL gave increased inflammation and worsened the disease [223].

A. B.

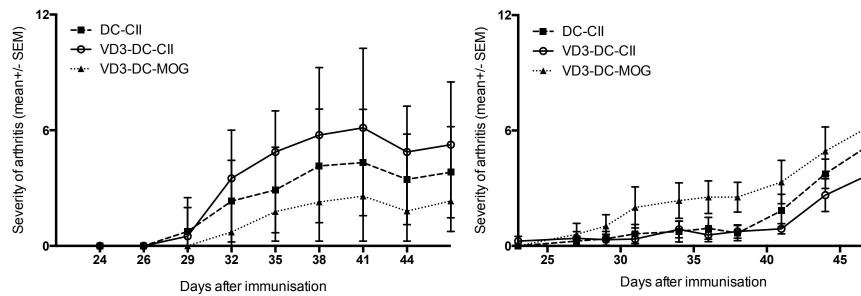


Figure 22. Severity of arthritis in mice injected with LNT-CII-transduced, VD3-stimulated DCs (VD3-DC-CII) or LNT-CII-transduced DCs (DC-CII; controls) or LNT-MOG-transduced, VD3-stimulated DCs (VD3-DC-MOG). **A.** The first *in vivo* experiment. **B.** The second *in vivo* experiment.

In summary, in our tolerance model where we use transduced HSCs, the DCs are probably the most important cell for the induction of central tolerance in the thymus, which effectively protects against CIA. However the transduced DCs are most likely also involved in the induction and maintenance of peripheral tolerance to CII.

Macrophages are present in most of the organs of the body, and can produce large quantities of cytokines in response to danger signals from microbial presence. Activated macrophages mainly present antigens from microbes that they have engulfed but also present self-antigens. Similar to DCs, macrophages can gain tolerogenic properties with low expression of MHC II, high secretion of IL-10, and capacity to expand the Treg compartment, e.g., after dexamethasone treatment [156]. We targeted the expression of CII to macrophages using a CD68 promoter [224], and found that even though the clinical severity of CIA was reduced, there were no significant differences regarding histopathological synovitis and erosivity between the groups (Figure 23A–B). Endogenous CII presentation by macrophages ameliorates

arthritis development but macrophages are not as effective as B cells in this regard.

A. B.

Figure 23. A. Severity of arthritis and **B.** histopathology after the transplantation of LNT-CD68-CII- or LNT-CD68-Ctrl-transduced HSCs into mice.

A regulatory macrophage subset that can be induced from monocytes by stimulation with M-CSF and IFN-γ has been identified in mice and this mechanism has been exploited in human RA trials [225]. **Taken together,** our results show that macrophages are able to reduce the severity of CIA by gene therapy-mediated endogenous CII presentation, albeit not as efficiently as CII presentation targeted to the B-cell compartment.

B cells differ from DCs and macrophages in that they are abundant in the lymph nodes and spleen and they are highly efficient at presenting selected antigens that are taken up by specific binding to the BCR for presentation on MHC II [226]. This unique presentation of antigens that are bound to the BCR makes B cells much more sensitive than other professional APCs to an antigen that is present at low concentration [227, 228]. The B cells can also possess tolerogenic properties. For instance, CD40-stimulated B cells have been shown to be superior to immature DCs with regard to the induction of Tregs [229], and antigen-stimulated B cells that are not stimulated with CD40 have the capacity to induce anergy in $CD4^+$ cells *via* CTLA-4 [230]. It has also been shown that long-term expression of self-antigen on B cells is decisive for the induction of tolerance [231].

The importance of B cells as APCs (Paper I) is evidenced by the reduction in arthritis both after adoptive transfer of B cells and when using the B-cell-specific Igκ promoter to present CII on B cells exclusively. Lentivirus-mediated presentation of CII peptide on B cells in a non-inflammatory

environment may lead to the induction of Tregs or anergic T cells, which in turn leads to lack of help to antigen-specific B cells resulting in decreased CII-specific antibody production.

To summarise, all professional APCs have the capacity to induce tolerance in various settings, and it remains to be determined which APC is the most important *in vivo* during establishment and maintenance of self-tolerance. In **Paper I**, we show that when all the APCs present the self-antigen on MHC II, tolerance is efficiently induced, and that this effect can be recapitulated when B cells only act as APCs. We detected thymic integration of the CII construct in our system, which indicates that thymic BM-derived APCs can participate in central tolerance induction in both the negative selection of T cells and the induction of CII-specific Tregs.

5.1.3 B cells in CIA and RA

In CIA, the B-cell compartment is important for the development of arthritis owing to its capacities for antigen presentation and antibody production, and its B-regulatory cells [31]. B-cell depletion in mice, achieved using an antibody against CD22, which is expressed on mature B cells but not on plasma cells, inhibited the development of CIA [232]. In the model of chronic CIA (CCIA) [233], CD40-stimulated, CII-specific B cells produced high levels of IL-10 and were able to prevent the development of arthritis [25]. Depletion of IL-10-producing B cells in CIA led to more severe disease, characterised by increased numbers of Th1 and Th17 cells and a decrease in the number of Tr1 cells [32].

In RA, B-cell depletion has shown good effects, mainly in seropositive patients [234-236]. B-cell depletion leads to decreased levels of autoantibodies without influencing the general immunoglobulin levels, and the treatment effect is not related to the reduction in autoantibody levels [237]. The most likely mechanisms for the effects of B-cell depletion are decreased antigen presentation, decreased expression of co-stimulatory molecules, and decreased generation of germinal centres in the synovia [237].

In our experiments using lentivirally induced tolerance in CIA, suppression of CIA was accompanied by decreased frequencies of plasma cells and reduced levels of CII-specific antibodies. Although the B-regulatory cell compartment was not investigated in our experiments, based on earlier studies in this area performed by Mauri *et al.* [25], we suspect that the continuous presentation of CII led to a tolerogenic response, most likely

characterised by the induction of B-regulatory cells that contributed to the reduction of arthritis.

5.1.4 CII-specific IgG antibodies in CIA and RA

In CIA, which is a disease elicited by the administration of exogenous CII together with adjuvant, the CII-specific antibodies are important but not sufficient for the development of arthritis. This was shown using B-cell depletion, which inhibited arthritis despite the development of CII-specific antibodies at levels that were as high as those in arthritic control mice [232]. In contrast, transfer of a combination of CII-specific antibodies induced arthritis in naïve mice, while a single antibody induced synovial proliferation without manifest arthritis [238-240]. In CIA, the levels of CII-specific antibodies are increased already on day 14 post-immunisation [241].

In RA, up to 70% of patients have anti-CII antibodies at diagnosis. The levels of these antibodies in the serum decline during worsening of arthritis, possibly due to the accumulation of immune complexes in the arthritic joints [242, 243]. Moreover, the levels of anti-CII antibodies are correlated with early bone erosivity and high inflammatory activity at the onset of disease [244]. The presence of antibodies to citrullinated CII has been noted several years before the onset of RA and the level of these antibodies increases after the onset of RA [168]. These antibodies do not appear to contribute to the aetiology of RA but rather can be attributed to epitope spreading and the creation of neo-epitopes during the pathogenesis of RA after self-tolerance has been broken [168]. Therefore, CII-specific antibodies are important in the pathogenesis of CIA, while their importance in RA remains to be proven. In RA, anti-CII antibodies may play a role in erosivity and may paradoxically correlate with a mild prognosis in a subgroup of patients. Antibodies to CII are part of the pathogenesis of CIA but probably not of RA. In our experiments, the LNT-CII mice had reduced levels of anti-CII antibodies already 2 weeks after the induction of CIA, before clinical arthritis was evident. This indicates that LNT-CII-induced expression of CII on APCs has a dampening effect on T-cell and B-cell activation, thereby inhibiting the production of anti-CII antibodies.

5.1.5 Is tolerance induction dependent upon post-translational CII peptide modifications?

The impact of the glycosylation status of the CII peptide was analysed using T-cell hybridomas that are reactive to various forms of the CII peptide presented on MHC II. We found that CII was mainly presented as a non-modified naked peptide on APCs from the peritoneal cavities and spleens of LNT-CII mice, both before and after CII immunisation. These findings suggest that antigen-specific tolerance can be induced by the non-modified, naked CII peptide. Is this naked CII peptide also important in CIA induction? Paradoxically, the rat CII that was injected subcutaneously for CIA induction has the glycosylated form and it is presented on MHC II as a glycosylated CII peptide [185]. Earlier studies showed that glycosylated CII also was more efficient at inducing tolerance in CIA [188, 189]. Healthy cartilage consists of glycosylated CII, while arthritic cartilage consists of both naked and glycosylated CII (as observed in both CIA and RA) [245]. This suggests that in healthy mice, glycosylated CII is tolerated as a self-antigen through processes of selection of autoreactive B and T cells, whereas naked CII is considered to be a foreign, non-self antigen that induces an inflammatory response. **Taken together,** these findings suggest that glycosylated CII is most efficient for the induction of CIA and for the induction of tolerance in CIA, despite the fact that the naked CII is associated with arthritic cartilage. In our experiments, the endogenous production and presentation of naked CII peptide (amino acids 259–270) induced powerful tolerance in CIA. It is possible that the glycosylation status of the peptide is less important for tolerance induction compared to the extensive and continuous presentation of the naked CII peptide on APCs both in the thymus and in the periphery.

5.1.6 Are CII-reactive T cells of importance in CIA and RA?

In CIA, glycosylated CII induces the disease through expansion of CII-reactive T-effector cells, mainly of the Th1 and Th17 subsets, and through the stimulation of B cells into class-switched and affinity-matured plasma cells that secrete high levels of CII-specific IgG antibodies [176, 246].

In RA, about 50% of the patients exhibit the presence of T cells that are reactive to CII (amino acids 255–274), as compared with <5% of patients with osteoarthritis or healthy controls [247, 248]. A low number of patients with RA have been examined for the presence of T cells that are reactive to glycosylated and naked CII, and the data show that T cells that are reactive to

both forms of CII are present in the blood and synovial fluids [184, 249, 250]. **Taken together,** these outcomes imply that CII-reactive T cells are important for the development of both CIA and RA. However, in CIA, CII-reactive T cells are known to be important both for aetiology and pathogenesis, while in RA, the sparse date indicate that these T cells could be involved in the pathogenesis of the disease, although a clear linkage remains to be established.

5.1.7 T-cell subsets in immunotolerant mice

The frequencies of T-helper cells and Tregs in the blood and spleen were similar in the LNT-CII mice and the controls over the course of arthritis. A possible explanation for this is that the increase in the total frequency of T-helper cells or Tregs was too small to be detectable; unfortunately we are unable to measure the numbers of CII-specific T cells. It is known that Tregs proliferate after antigen exposure in local lymph nodes [61], and it would be of great interest to measure only CII-specific T cells. However, the functional analysis of the T cells from the spleens showed that the Tregs from LNT-CII mice had increased suppressive capacities for T-effector cells, which is in line with the findings of Rosenblum *et al.* [61], who demonstrated that peripheral presentation of self-antigen leads to the expansion of thymus-derived Tregs with enhanced suppressive capacities. Peripheral effector memory T cells have also been shown to be important in antigen-specific immune responses upon re-challenge with antigen, mediating a rapid peripheral T-cell expansion [58]. There are several subsets of T-memory cells, and the peripheral effector memory T cells constitute a long-lived, antigen-specific T-cell subset that is characterised by surface expression of CD44, low expression of chemokine receptor CCR7 (responsible for lymph node homing), and low levels of the adhesion molecule CD62L [58]. In our studies, the expression of CD62L on T cells was examined in the spleen and blood on days 0, 7, 14, and 28, revealing similar levels for the different groups (data not shown). This can be explained by the fact that the memory T cells mainly reside in the synovia and draining lymph nodes. Taking all this information into account, we conclude that the Tregs have increased suppressive capacities, and we speculate that these cells originate from T-memory regulatory cells.

5.1.8 When is tolerance induced?

The time-point for tolerance induction, i.e., the point at which the CII-specific B and T cells are deleted or re-educated to tolerate CII, is not known. The selection processes of B and T cells in the mouse during early development are designed to delete cells with strong affinities for self-antigens, such as CII. B- and T-effector cells with low or intermediate affinities or Tregs with moderate affinity for CII are most likely present in the mouse. Our gene therapy allows the presentation of CII on APCs in the periphery (and probably also in the thymus) under non-inflammatory conditions at least 10 weeks prior to CIA induction. This presentation of CII may lead to the expansion of Tregs that are specific for CII [61]. When CIA is induced, the mice are immunised with rat CII in an emulsion with CFA, which leads to the presentation of CII on APCs in an inflammatory environment. This activates the CII-specific T and B cells, leading to T-cell co-stimulation of B cells, which results in affinity maturation and class switching of the B cells. An inflammatory response to endogenous CII is initiated that causes arthritis. In the control mice, this response leads to a continuous inflammation that progresses to severe synovitis and bone-cartilage erosions. Our hypothesis is that the mice that present higher levels of endogenous CII on APCs thanks to gene therapy have both increased frequencies of anergic CII-specific B and T cells and an expanded CII-specific Treg compartment that becomes activated by CII presentation; this decreases the inflammatory immune response towards the articular CII. An initial inflammatory burst after immunisation leads to the activation of T- and B-effectors cells, which within 1–2 weeks are suppressed by Tregs. This hypothesis is strengthened by data showing decreased levels of CII-specific IgG antibodies already on day 14 after CIA induction. The lower levels of CII-specific IgG antibodies are a consequence of decreased B-cell activation by activated T cells and weaker generation of class-switched plasma cells. Therefore, we speculate that tolerance to CII is ordained and induced already after the transplantation of HSCs followed by the endogenous presentation of CII on APCs. Tolerance can be maintained even after the induction of CIA through CII re-stimulation of an expanded CII-specific Treg compartment and the presence of anergic CII-specific T and B cells.

5.1.9 Can the effect on arthritis be explained by mechanisms other than antigen-specific tolerance?

Tolerance is defined as an absence of an inflammatory immune response directed against the antigen. As previously discussed, the antigen-specific

cells are constituted by B- and T-cells. We found decreased levels of CII-specific antibodies in the healthier LNT-CII mice, as well as an increased capacity of the Tregs from LNT-CII mice to suppress T-effector cells from control mice. Could these findings be explained by mechanisms other than tolerance? Indeed, treatments that are not antigen-specific, such as IL-10 therapy (**Paper III**), give decreased levels of CII-specific antibodies in healthy mice, as compared with arthritic controls. Natural Tregs require antigen-specific recognition of their antigen to be activated. However, after activation, they suppress in a non-specific manner, i.e., through bystander suppression. The suppression assay showed that the Tregs from LNT-CII mice were able to suppress T-effector cells from control mice. Could this be due to activation of Tregs by antigens other than CII? To me it seems unlikely that LNT-CII mice should have more extensive presentation of antigens other than CII. This notion is supported by our data that confirm that the CII peptide is presented on the APCs from both naïve and arthritic LNT-Ii-CII mice, but not on the APCs from control mice. To ascertain that the CLIP peptide did not have an immune-stimulatory or -inhibitory effect in itself, we used a lentiviral GFP control and detected similar development of arthritis. Taken together, our data suggest that the reduction in arthritis observed in LNT-CII mice is due to antigen-specific tolerance to CII.

5.2 Paper II

A key to the success of tolerance induction is that the self-antigen is presented in a non-inflammatory environment. Our group have earlier shown that prophylactic endogenous expression of self-antigen can ameliorate the development of CIA ([251] and **Paper I**). However, since we cannot predict which individuals will develop RA, the prophylactic induction of tolerance is not suitable for application in the clinic. Therefore, it is of major importance to investigate the possibility of inducing tolerance in active inflammatory disease. In **Paper II**, we address the effect of lentivirus-mediated expression of an immunodominant CII peptide on MHC II after the induction of CIA. Our results show that lentiviral gene therapy on days 7 and 26 post-immunisation reduces arthritis, while a discrete effect on arthritis is seen on day 31 post-immunisation. This means that the effect is stronger the earlier the therapy is administered.

5.2.1 Presentation of the CII epitope on APCs after i.v. injection

In this paper, we used the SFFV promoter to drive the expression of the CII peptide on all APCs. In addition to presentation on professional APCs, non-professional APCs, i.e., cells that non-constitutively express MHC II (such as certain epithelial cells, rheumatoid synovial fibroblasts, and endothelial cells), can also be induced to express MHC II and thus, to present antigens. The peripheral i.v. injection of LNT-CII (in **Paper II**) has the potential to influence presentation on all the MHC II-positive cells in the body [252]. Expression of co-stimulatory molecules on APCs is crucial for the induction of a T-cell response [212]. In line with this, it has been suggested that non-professional APCs, as a consequence of their reduced expression of co-stimulatory molecules, may limit T-effector cell responses [253, 254]. In **Paper II**, we showed that peripheral injection of lentiviral particles resulted in vector integration in the draining lymph nodes. However, as the extent of CII presentation by non-professional APCs has not been investigated to date, we can only hypothesise regarding the contribution of CII presentation on synovial fibroblasts and in the endothelial lymphatic vessels that drain the lymph nodes. Further experiments are needed to investigate the importance of fibroblasts and endothelial cells, and we can only speculate that these cells are important contributors as antigen presenters during active inflammation, acting to reinforce inflammation and contributing to a lesser extent to the reduced arthritis seen in healthier LNT-CII mice.

5.2.2 The timing of LNT-CII injections influences the tolerogenic effect

The fact that arthritis is reduced after the injection of LNT-CII on day 7 post-immunisation could be due to a resolved innate response at this time-point, leading to presentation of the CII peptide in a non-inflammatory environment. This scenario seems unlikely, as the immunisation with rat CII and CFA on day 0 triggers an inflammatory burst *via* an innate immune response that involves macrophages, neutrophils, and macrophages, as well as the release of inflammatory cytokines, such as IL-1, TNF-α, IL-6, IL-17, and IFN-γ [181]. A more likely explanation for the treatment effect seen on day 7 is that continuous presentation of the CII peptide is dominant and leads to a tolerogenic response, despite the inflammatory environment. We propose that the diminished effect of LNT-CII on manifest CIA (at day 31) is mainly due to the established inflammatory disease not only affecting the milieu of antigen presentation but more importantly, the creation of neo-epitopes, such

as citrullinated proteins, as a result of injuries caused to the cells in various tissues, such as the synovium, cartilage, and bone [255, 256]. Thus, patients with established RA have developed neo-epitopes as a consequence of damage to tissues, which produces a disease that is driven by multiple autoantigens. To be able to use tolerance as a treatment in this type of patient, a single effective antigen for tolerance induction will be difficult to identify. A common denominator, such as citrullinated antigens, might represent an elegant approach.

5.2.3 Could CII presentation lead to unexpected effects in CIA?

Our hypothesis was that CII presentation on APCs could induce antigen-specific tolerance due to continuous and endogenous presentation of the self-antigen. However, excessive presentation of CII in an inflammatory milieu could lead to an immunostimulatory response through the activation and expansion of CII-specific T-effector cells. Indeed, such a scenario has been described for patients with multiple sclerosis who were treated with disease-specific altered ligand peptides in a phase II trial; of the 142 patients who were treated, 13 developed hypersensitivity-type reactions [257]. It is known that thymic selection allows the survival of some T-effector cells with low self-reactivity, e.g., towards CII. If APCs that express CII on their MHC II are activated, e.g., by the adjuvant, and express co-stimulatory molecules, they may initiate a CII-specific T-cell-based inflammatory response. However, when we injected LNT-CII into arthritic mice on day 31 post-immunisation we could show also in a highly inflammatory environment that increased endogenous expression of CII on APCs decreased the severity of arthritis, albeit with less efficiency than in mice treated on day 26 and most evidently compared to mice treated on day 7. Importantly, there were no signs of an increased immnostimulatory response when the mice were treated on day 31. This indicates that lentivirus-mediated CII presentation on APCs is more prone to suppress T-effector cells or enhance Tregs than it is to activate T-effector cells.

5.2.4 Importance of Helios- and Foxp3-positive Tregs during tolerance

Investigation of the Foxp3$^+$ Treg compartment of the LNT-CII mice treated on day 31 showed that the numbers of Helios- and Foxp3-doublepositive Tregs in the blood were increased compared to the controls, and that this

increase was initiated rapidly (by day 35) after the injection of lentiviral particles. Helios was initially identified as a marker of thymus-derived $Foxp3^+$ Tregs [88, 92] but has later shown to be expressed also on peripheral $Foxp3^+$ Tregs [258]. Several groups have suggested that Helios expression on $Foxp3^+$ Tregs is associated with increased suppressive capacity and correlates with up-regulated expression of the homing receptor CD103 and glucocorticoid-induced TNF receptor (GITR) [93, 259]. However, it is also known that blocking Helios expression leaves the T-cell compartment, including the $Foxp3^+$ Tregs, intact [91]. Daley *et al.* showed that in self-reactive thymic Tregs, which co-express Helios and the pro-apoptotic molecule Bim, Helios could serve as a marker for strong self-reactivity [260]. Thus, while there have been different proposals regarding the implications of Helios expression for $Foxp3^+$ Tregs in the periphery and in the thymus, the precise roles of Helios remain to be clarified. We detected an increased proportion of Helios- and Foxp3- doublepositive T cells in the blood samples from the healthier LNT-CII mice. This finding is in line with the findings of Feuerer and Zabransky [93, 259], who proposed Helios as a marker of more suppressive $Foxp3^+$ Tregs.

5.2.5 Importance of CII-specific antibodies

The development of CII-specific antibodies was investigated after lentiviral injection on day 31 post-immunisation. At the termination of the experiment (day 54), there were significantly lower levels of these antibodies in the LNT-CII mice than in the controls. In this setting, on day 31 when LNT-CII was injected in the presence of manifest CIA, the levels of CII-specific antibodies were already increased due to active arthritis. We speculate that CII presentation on APCs increases the proportion of Tregs and decreases the proportion of T-effector cells, and that these changes decrease the T-cell activation of B cells and reduce the levels of CII-specific antibodies.

5.3 Paper III

For chronic autoimmune diseases, e.g., RA, the present treatment arsenal consists of agents for general or targeted immunosuppression. The accompanying and unwanted side-effects include increased risk of infections. A disease-regulated immunosuppression intervention during the

inflammatory flare would be a preferable option. To investigate this type of approach to therapy, we used an inflammation-dependent IL-1 enhancer/IL-6 promoter [194]. Since both IL-1 and IL-6 are cytokines that act early during inflammation, they appear to be ideal for initiating an anti-inflammatory response during a flare of CIA. Our data showed increased mRNA expression of IL-10 in the draining lymph nodes and increased levels of IL-10 in the B cells and other APCs in the draining lymph nodes. This was a satisfactory outcome, since IL-10 is a cytokine that exerts its effects locally and it is in the joints and local lymph nodes that the anti-inflammatory effect is needed.

5.3.1 Inflammation-dependent IL-10 production - an ideal regulator for therapeutic applications?

A disadvantage of using inflammation-dependent transcription of IL-10 is that the inflammatory response to a foreign invader can also trigger the production of IL-10 in a setting in which the body needs to have an intact inflammatory capacity. This risk could be minimised through local delivery of the gene therapy to the target tissue. A promoter that is activated by a molecule, the level of which is increased specifically during autoimmune inflammation and not during infections, would be a perfect regulator for the treatment system. However, to my knowledge, a good candidate molecule that behaves differently in autoimmune inflammation than during infections has not been identified to date. The T-cell integrin CD44, which mediates rolling to the endothelium *via* binding to hyaluronic acid, has been suggested as a marker of chronic inflammation [261]. However, the use of this molecule as a regulator will still be associated with susceptibility to chronic infections, although the risk of acute infections might be decreased. Thus, disease-regulated therapies using inflammatory molecules as regulators will continue to be associated with increased risk of infections until more-specific disease-associated molecules are uncovered.

5.3.2 Activities and sources of IL-10 in arthritis

In this paper, the transplantation of LNT-IL-10-transduced HSCs increased the levels of IL-10 mRNA in the draining lymph nodes, and flow cytometry showed increased levels of IL-10 protein in the B cells and non-B APCs from the lymph nodes, while no increase in IL-10 protein levels was detected in the spleen. Mauri *et al.* [152] have shown that B- regulatory cells, as well as B-effector cells, are producers of IL-10, and it has also been shown that IL-10 from B cells efficiently suppresses CIA [32]. While the actions of IL-10 on B

cells are not completely understood, it seems that IL-10 stimulates B-cell survival and the expression of MHC II [32]. Other effects of IL-10 include the expansion of IL-10-producing Tr1 cells, suppression of the proliferation of Th1, Th2, and Th17 cells, inhibition of monocytes, DCs, and macrophages, and activation of NK cells [32, 129, 262]. In our system, HSC-derived cells, which are able to produce IL-1 and IL-6, contribute to IL-10 production. Apart from B cells, T cells, NK cells, DCs, macrophages, and osteoclasts can also produce IL-10. We did not find elevated levels of IL-10 in the T cells of the lymph nodes or spleen, possibly because the IL-10-producing T cells are localised to the synovia rather than to the lymph nodes. We found lower frequencies of B cells in the lymph nodes and spleens of the LNT-IL-10 mice than in those of the controls. Although further characterisation of B-cell subsets was not performed, we hypothesise that mainly the CII-specific B cells are deleted in the LNT-IL-10 mice. It is clear that the IL-10 production from the B cells and other APCs in the draining lymph node is sufficient to suppress arthritis.

5.3.3 Suppressors of cytokine signalling in arthritis

The suppressors of cytokine signalling (SOCS), SOCS1 and SOCS3 are negative regulators of cytokine signalling that are induced by, for example, IL-10. Depletion of SOCS1 in methylated BSA arthritis worsened the arthritis [263]. This is line with the results of the LNT-IL-10 experiment, in which increased IL-10 levels were associated with increased levels of SOCS1 and decreased levels of IL-6. SOCS3 seems to be important for suppression of the effects of IL-6 and enhancement of the anti-inflammatory effects of IL-10 [146]. This suggests that enhanced expression of SOCS1 and SOCS3 can ameliorate arthritis. In **Paper III**, higher levels of SOCS1 mRNA were found in the draining lymph nodes of the LNT-IL-10 mice, as compared with the controls. The levels of SOCS3 were increased, albeit not significantly (p=0.12). Based on this outcome, we speculate that the mechanisms underlying the LNT-IL-10 effect involve the transcription of IL-10 in the draining lymph nodes, thereby up-regulating SOCS1 and possibly SOCS3 expression, resulting in a decrease in the serum levels of IL-6, which ameliorates arthritis *via* effects on B cells, T cells, APCs, and NK cells (Figure 24).

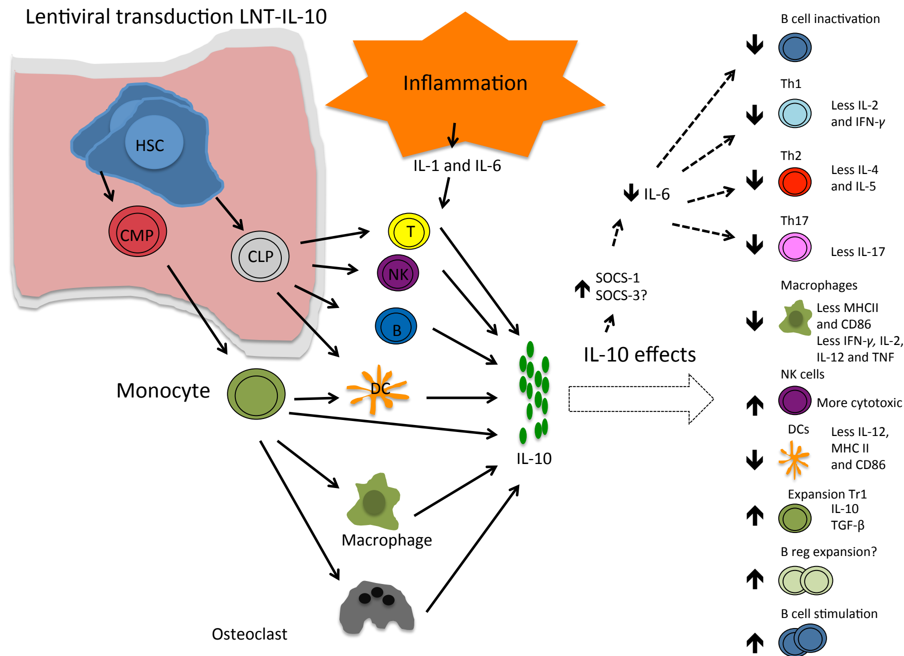


Figure 24. Summary of the suggested mechanisms described in **Paper III**. Bone marrow-derived HSCs are transduced with LNT-IL-10. Inflammation leads to the expression of IL-1 and IL-6, which activate IL-1 enhancer/IL-6 promoter-regulated transcription of IL-10. We show that IL-10 exerts effects on SOCS1 expression (and possibly SOCS3 expression), leading to a decrease in the serum levels of IL-6, which down-regulates CIA *via* the inhibition of B-effector, Th1, Th2, and Th17 cells, as well as a direct effect of IL-10 on macrophages, NK cells, and DCs, in addition to the expansion of Tr1, possibly B-regulatory cells and B cells.

5.4 General discussion

The lentivirus-mediated endogenous expression of CII on APCs provides a useful system to investigate the basic mechanisms that apply during antigen-specific tolerance in a mouse model of RA. Our findings suggest that the B cell is a major player in the establishment of tolerance, providing an interesting candidate for future therapies. We show that it is possible to induce tolerance after immunisation and after boosting in CIA when all the APCs express self-antigen on MHC II. At these time-points, investigation of the non-professional APCs, such as fibroblasts and endothelial cells, could suggest new possibilities for re-directing the immune response from

inflammation to tolerance. During the induction of tolerance in an inflammatory setting, the cytokine levels and expression co-stimulatory molecules on APCs are of importance. Disease-regulated transcription of IL-10 could facilitate such a tolerogenic immune response that re-educates the lymphocytic response to self-antigens.

5.4.1 Applicability to patients with RA?

Lentiviral transduction of HSCs as a tolerance-inducing treatment is presently not applicable as a therapy for patients with RA. These experiments were performed to gain knowledge regarding the mechanisms that are active during antigen-specific tolerance in an animal model of RA. **Paper I** shows that the B cells are of great importance as APCs during the induction of tolerance, and that FoxP3 Tregs have enhanced suppressive capacities. The antigen-specific antibodies remained at low levels in the tolerogenic mice, while the arthritic control mice had increased levels of antigen-specific antibodies from day 14 after the induction of CIA and throughout the course of arthritis. Further experiments on the relative importance of other APCs, such as DCs, macrophages, mTECs, synovial fibroblasts, and lymphatic endothelial cells, need to be performed to clarify the most potent APCs for tolerance induction. **Paper I** facilitates further studies through the establishment of a valuable model that is well suited to investigations of individual cell types. **In Paper II,** the lentiviral particles were injected i.v. after disease had been initiated by CII immunisation. This treatment strategy is not feasible in RA, as the eliciting autoantigen is unknown and the use of lentiviral vectors may be associated with risk. The experiments were performed to investigate the potential to induce antigen-specific RA after immunological or clinical onset of an autoimmune disease. The paper shows that it is possible to reduce the severity of arthritis when recombinant lentivirus injection is administered 7 or 26 days after the induction of CIA. The continuous presentation of endogenously synthesised CII peptide on APCs reduces the arthritis by enhancing the suppression mediated by Tregs and decreased levels of antigen-specific antibodies. It is remarkable that the presentation of CII on APCs can reduce inflammation despite an immunostimulatory injection of CII and CFA only 7 days earlier or the injection of a CII plus IFA booster only 5 days earlier. Further studies on the expression of co-stimulatory and co-inhibitory molecules on APCs on the day of lentiviral injection and 10 days thereafter might give important insights into the down-regulation of co-stimulatory molecules associated with tolerance. In **Paper III**, the gene therapeutic approach was found to be favourable because it allowed integration of the lentiviral vector in more cells

than could be achieved with a peripheral injection. The lentiviral integration into cells spread throughout the body and permitted us to investigate the effects of arthritis on cells in different tissues. Thus, gene therapy is a good system to investigate the importance of IL-10 and the possibilities for disease-regulated therapies that could be applied to the treatment of RA. **Taken together,** the insights gained from our studies on the importance of B cells during tolerance could be used in human RA to develop B-cell based approaches for tolerance induction and to derive novel therapies.

6 CONCLUSIONS

6.1 General conclusions

We have established a tolerogenic mouse model of human RA using gene therapy, and we demonstrate that endogenous expression of the tolerogen on the MHC II on all APCs, and on B cells in particular (**Paper I**), and B-cell-produced IL-10 (**Paper III**) induce tolerance in CIA. Furthermore, we show that it is possible to use this system to reduce CIA post-immunisation. The tolerogenic effect is mediated by enhancement of the functions or numbers of Tregs and decreased production of disease-specific IgG antibodies.

7 FUTURE PERSPECTIVES

To pursue our findings with respect to endogenous expression of the tolerogen on B cells, we would like to investigate whether these cells:

1) are the cells that generate the tolerogenic effect. For this purpose, we will use mice in which the DCs are deleted by treatment with diphtheria toxin (CD11c-DTR mice) to distinguish between the contributions of DCs and B cells as mediators of the tolerogenic effect. As our data indicate, B cells are key players in tolerance induction, we will investigate the mechanisms by analysing if these B cells:

2) can induce Tregs by analysing the suppressive capacity of Treg LNT-Igκ-CII mice *in vitro* and by adoptive transfer to naïve recipients. We will also study whether B cells that endogenously express the tolerogen can directly induce T-regulatory cells. Furthermore, we would like to study if B cell-induced tolerance:

3) alters the proportions of B-cell subpopulations by flow cytometric analyses of spleens, lymph nodes, and peripheral blood samples. These studies might have direct implications for future treatment strategies in RA, as it is possible to target a therapy to a specific cell population *in vivo* either by deletion or activation.

To pursue other aspects of my thesis we would like to investigate:

1) the role of non-professional APCs, such as synovial fibroblasts and lymphatic endothelial cells, using tissue-specific promoters;

2) if the continuous endogenous CII presentation on APCs influences the expression of co-stimulatory and co-inhibitory molecules, as detected by flow cytometry; and

3) if the presentation of CII is associated with increased risk of infections due to the binding of CII on MHC II that blocks presentation of foreign peptides, using i.v. inoculation of *Staphylococcus aureus*.

8 SAMMANFATTNING PÅ SVENSKA

8.1.1 Tolerans – hur funkar det egentligen?

Tolerans eller självtolerans betyder att tåla sig själv med en bevarad förmåga att reagera på allt främmande. Det låter så enkelt och självklart men det är oerhört svårt att förstå hur kroppen egentligen gör när den skapar tolerans. Vissa typer av vita blodkroppar, det vill säga T och B celler, är nycklarna till tolerans, tror vi i alla fall just nu.

I benmärgen bildas det hur många olika T och B celler som helst. Ganska slumpartat skapas miljontals varianter av cellerna, som ett oändligt memoryspel. På memorykortets bild, alltså på cellernas MHC (minneskort), står vilket antigen, vilket målkort, cellen har. Det skapas memorykort, MHC, riktade mot hud, hår, ben, brosk, bakterier, virus, hundar, katter, kor och i stort sett mot allt mellan himmel och jord. Redan innan B cellerna lämnar benmärgen gallras massvis med kort (celler), riktade mot kroppen själv, bort. De flesta T celler lämnar benmärgen för att ta sig till brässen (thymus). Där gallras de allra flesta celler bort eftersom de är riktade mot kroppen. En del självreaktiva celler överlever men blir förlamade (anergiska) eller också förvandlas de till T regulatoriska celler. T regulatoriska celler stoppar inflammation i kroppen.

När kroppen har valt vilka T och B celler som får överleva och vilka som omformats till T regulatoriska celler, så ska de sköta sitt jobb som vakter i kroppen och skydda oss mot bakterier, virus och dessutom hjälpa oss läka vävnadsskador som knivsår och brännsår, och döda baciller. En hel del självreaktiva vanliga T och B celler har ändå överlevt gallringen i benmärg och thymus och kommer att finnas på olika ställen i kroppen. Ibland kommer dessa celler leda till inflammation mot egna vävnader i form av en autoimmun sjukdom som ledgångsreumatism (reumatoid artrit, RA), eller andra celler som är riktade mot ofarliga saker som hundar och pollen vilket kan leda till allergier. Vissa celler som är självreaktiva kommer inte att utlösa någon sjukdom alls. Det beror bl. a. på vilka celler som presenterar antigenet och om det finns pågående inflammation i kroppen när antigenet presenteras.

8.1.2 Hur kan vi påverka de självreaktiva cellerna och skapa självtolerans?

Vi kan stoppa de självreaktiva celler genom att tillföra antigen hela tiden under icke-inflammatoriska förhållanden. Då förlamas eller dödas de självreaktiva cellerna eller så skapas många T regulatoriska celler som stänger av den autoimmuna inflammationen som de självreaktiva cellerna dragit igång. Detta har jag gjort i delarbete I och II.

Vi kan förändra den inflammatoriska miljön t ex genom att påverka inflammatoriska eller anti-inflammatoriska signaler, cytokiner. Detta har jag gjort i delarbete III.

8.1.3 Mina delarbeten:

I det första delarbetet använder jag genterapi mot stamceller i benmärgen. Genterapin går ut på att föra in en gen som är ansvarig för kollagenproduktion i musens celler. Genen förs in med hjälp av två olika lentivirus där det ena ger en kollagenproduktion i flera antigenpresenterande celler såsom dendritiska celler, makrofager och B celler medan det andra viruset bara ger kollagenproduktion i B celler. Stamceller från benmärgen behandlas med genterapi innan de injiceras in i en mus som strålats för att kunna ta emot cellerna som sin nya benmärg. Benmärgscellerna återhämtar sig under minst 10 veckor och därefter injiceras kollagen från råtta och en mykobakterieblandning för att ge musen en ledgångsreumatism-liknande sjukdom som kallas kollagen inducerad artrit. Det första arbetet visar att när antigenpresenterande celler med hjälp av genterapi producerar kollagen så leder detta till ett skydd mot utveckling av ledinflammation. Blodprover visar att de behandlade mössen har lägre nivåer av antikroppar riktade mot kollagen och att de T celler som bromsar inflammation, de T regulatoriska cellerna, har fått ökad förmåga att minska inflammationen. När jag använder det andra viruset som skapar en kollagenproduktion enbart i B celler, så visar det att även dessa möss får mindre ledinflammation och lägre antikroppsnivåer. Sammanfattningsvis så visar delarbete I att kontinuerlig kollagenproduktion i flera olika antigenpresenterande celler eller enbart i B celler skapar tolerans vid kollageninducerad artrit och att B cellerna är viktiga både som antikroppsproducenter och som antigenpresentatörer.

Det andra delarbetet handlar om att skapa tolerans under aktiv inflammation. Jag använder det första viruset som ger kollagenproduktion i alla antigenpresenterande celler. Viruset sprutas in i blodet i musen 7 dagar eller 26 dagar efter musen fått sin kollageninjektion som drar igång

sjukdomen. Resultaten visar att mössen inte får ett totalt skydd men ändå att ledinflammationen minskar. Ett tredje experiment där viruset sprutas in i möss som är riktigt sjuka, 31 dagar efter kollageninjektionen, visar att ledinflammationen minskar lite. De behandlade mössen får lägre antikroppsnivåer och vi ser högre andel av en viss sorts T regulatoriska celler.

Det tredje delarbetet handlar om genterapi för att skapa en skräddarsydd behandling som aktiveras när musen håller på att bli sjuk. Vid ledgångsreumatism tar man ständigt medicin för att dämpa immunsystemet och förhindra inflammation trots att sjukdomen förlöper i skov och att man dessemellan kan vara helt frisk. Den ständiga immundämpningen leder till att man blir känslig för infektioner och att vanliga förkylningar kan leda till svåra infektioner som kräver sjukhusvård och antibiotika behandling. Vår hypotes var att om man med hjälp av genterapi kunde låta patientens inflammation styra behovet av behandling så minskar risken för infektion. Vi använde ett virus som innehåller IL-10, ett antiinflammatorisk ämne som bromsar inflammation. IL-10 produktionen i viruset styrs av IL-1 och IL-6 som är ämnen som stiger vid inflammation. Målet var att när musens ledinflammation startade så skulle tillverkningen av IL-10 aktiveras och leda till att ledinflammationen minskade. Resultaten visar att kollageninducerad ledinflammation ledde till IL-10 tillverkning framförallt i i lymfkörtlar nära leden och att detta ledde till att ledinflammationen minskade.

8.1.4 Sammanfattning

Sammanfattningsvis visar min avhandling att vi med hjälp av genterapi mot stamceller kan framkalla en tolerans mot kollagen som finns i ledbrosket och på så sätt minska ledinflammationen. B cellerna är viktiga som antigen presentatörer för att skapa tolerans. De toleranta djuren har effektivare regulatoriska T celler, lägre nivåer av antikroppar mot kollagen och färre B celler. Vi visar också att även efter att sjukdomen initierats kan genterapi ha effekt. Genterapi gör det också möjligt att skapa en skräddarsydd behandling mot ledinflammation. Detta fungerar genom att en led som drabbas av ledinflammation aktiverar en gen som styr tillverkningen av ett anti-inflammatoriskt ämnen som i sin tur minskar ledinflammationen bl. a. genom att påverka vita blodkroppar. Våra experiment bidrar till förståelsen av tolerans och inflammationsreglerande behandlingar och gör att vi kommit en bit närmare att kunna behandla ledgångsreumatism hos människor med hjälp av dessa metoder.

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