TH17 CYTOKINES IN AUTOIMMUNITY

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Th17 Cytokines in Autoimmunity

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Introduction

IMMUNITY

The immune system

The human immune system is composed of several organs and many different cell types that protect the body against any particle that is not part of the body, the non-self. Mostly, these will be invading pathogens or cancer cells. The immune system can be divided into two main components: an innate component and an adaptive component (figure 1). These two components do not operate separately, but are in fact closely linked and depend upon each other.

When the body is invaded by pathogens, cells of the innate immune system are quickly activated to limit the infection. However, they are not specific for one type of antigen; rather they respond to epitopes present on many different pathogens, like peptidoglycans and lipopolysaccharides, which have been strongly conserved during evolution. Contrary to the innate immune system, cells of the adaptive immune system are highly specific for one particular epitope or antigen. Development of this specific response takes several days, but leads to a much stronger and long lasting immune response. Furthermore, the high specificity provides the possibility of developing memory cells that will be able to respond more quickly if a similar pathogen invades the body again.

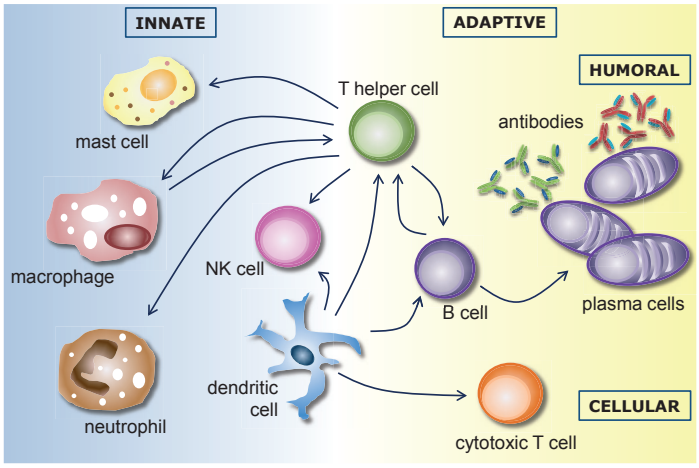

Figure 1. Overview of several cell types involved in the innate and adaptive immune system and some oftheir interactions. NK cell = natural killer cell.

The innate immune system

The first line of defence against invading pathogens is provided by the epithelial cell surface. These cells are tightly packed together to form a mechanical barrier. Some epithelial surfaces have small hairs, called cilia, which move mucus across the barrier to remove pathogens. In addition, these cell layers often produce fluids containing enzymes that kill pathogens.

If pathogens do cross the epithelial cell layer, they can be recognized by several cell types through pathogen recognition receptors (PRRs), toll-like receptors (TLRs) and many others. Macrophages will recognize and phagocytose pathogens, as well as produce signalling molecules, called cytokines, to attract other cell types to the site of inflammation. Phagocytosis can also be enhanced by activation of the complement system. The complement system has three pathways, the classical, the lectin and the alternative pathway. While the lectin and alternative pathways play a role in targeting pathogens for phagocytosis, the classical pathway serves to clear apoptotic debris or immune complexes (ICs, antibodies bound to their antigen) from the body.

Dendritic cells (DCs) will respond to an antigen by producing chemokines, cytokines that attract other cells like neutrophils and natural killer (NK) cells and allow them to cross the blood vessel wall to enter the site of inflammation. In addition, DCs will take up the antigen and present it to cells of the adaptive immune system through two different molecules, the major histocompatibility complex (MHC) class I or MHC class II.

The adaptive immune system

B and T lymphocytes are the primary cells of the adaptive immune system. These cells have highly specific antigen receptors that are assembled during lymphocyte development. All B and T cells express receptors with a single, unique specificity, of which they can express many on their membranes. This ensures that the cells only respond to one target and therefore that the adaptive immune response can be specifically guided.

The adaptive immune system can be divided into two parts, the cellular and the humoral immune response. Key players in the cellular immune response are cytotoxic T cells. These cells are characterized by expression of CD8 molecules on their membrane. The cellular immune response is required to eliminate infected or damaged cells from the body. When cells are infected by a pathogen, e.g. a virus, they will express proteins of this pathogen. Such proteins are brought to the surface of an infected cell by the MHC class I (MHC-I) molecule. In the context of MHC-I these proteins can be presented to cytotoxic T cells. Specifically those cytotoxic T cells that express a T cell receptor (TCR) that recognizes such an antigen expressed on the infected cell are activated. CD8 molecules expressed on the T cells act as co-receptors, binding MHC-I and enhancing the activation signal. Upon activation, the T cells kill the infected cell, removing the intracellular pathogen from the body. This process is facilitated by another T cell subtype, the T helper cell. T helper cells are so named because they are able to activate other cells of the

immune system. Through cytokine production, T helper cells can activate and stimulate cytotoxic T cells and other effector cells involved in cellular immunity.

The humoral immune response is involved in the clearance of extra-cellular pathogens. The two main players in the humoral immune response are T helper cells and B cells. T helper cells are characterized by CD4 expression on their membranes. B cells will take up an antigen through binding and subsequent internalization of their B cell receptor (BCR). They then present the antigen to the T helper cell on MHC class II molecules. The T helper cell recognizes the antigen with its T cell receptor and enhances the activation signal through binding of CD4 to MHC class II. B cells will then differentiate into plasma cells that produce large amounts of antibodies with the same specificity. In fact, antibodies are secreted forms of the B cell receptors (BCRs) secreted by plasma cells. These antibodies can bind the surface of pathogens, thereby blocking e.g. cell entry of a viral particle. Specific receptors for antibodies, called Fc receptors, are present on phagocytic cells and upon antibody binding the pathogens are subsequently destroyed. In addition, antibodies can be bound by complement factors, which facilitate phagocytosis by activating phagocytic cells, like macrophages.

AUTOIMMUNITY

Self-tolerance

Antigen receptors on lymphocytes are highly specific for their target. The development of this specificity is a tightly regulated process, involving complex gene rearrangement events at antigen receptor loci whereby the resulting specificity is completely random [1]. This ensures the highest possible diversity and therefore provides optimal protection against a wide range of pathogens. In adult humans, the estimated number of individual B cell receptors circulating through the body at any given time is 10^{11} with 10^{9} new B cells being produced each day.

Because all possible antigen specificities may arise during lymphocyte development, many cells will develop that are specific for a self-antigen. Through a number of selection stages present during lymphocyte development and maturation, many of these autoreactive cells will undergo programmed cell death, called apoptosis, or change their specificity. In early B cell development in the bone marrow, about 75% of B cell receptors are autoreactive, but in healthy individuals this is reduced to only 20 % of mature B cells in the circulation [2]. These remaining circulating autoreactive cells can subsequently be silenced through signals that render them unsusceptible to antigen stimulation, a state called anergy [3]. These mechanisms provide protection against immune reactions to a self-antigen, thereby ensuring self-tolerance.

Loss of self-tolerance may lead to the development of an auto-immune disease. In patients with auto-immune diseases, the number of circulating autoreactive cells is increased. For example, in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) patients, around 45% of circulating B cells is autoreactive [2]. Circulating B and T cells play an important role in

the maintenance of tolerance, as subsets of both cells have been described to have regulatory functions. For example, regulatory T cells can inhibit both B and T cells in the humoral immune response to limit the production of autoreactive antibodies [4].

B cell development

B cells arise from precursor cells in the bone marrow. B cells express a highly specific B cell receptor (BCR) that is formed during B cell development (figure 2). It is composed of an immunoglobulin heavy chain (IgH) and a light chain (IgL). In the pro-B cell stage, the variable region of the IgH gene locus undergoes a process called V(D)J recombination, whereby a random variety (V) segment, diversity (D) segment and joining (J) segment are joined [5]. The combination of V, D and J segments determine the specificity of the IgH chain. The newly formed IgH chain is then expressed on the membrane with a surrogate light chain (SLC), as the IgL has not yet been formed. Signaling through this pre-BCR is essential for survival and continuation of B cell development [6, 7]. Also, to ensure that only one specific BCR is expressed in the B cells, successful recombination of one IgH chain allele will downregulate recombination on the other allele, which is called allelic exclusion [7, 8]. Upon signaling, the pre-BCR is internalized and surrogate light chain expression down regulated. The IgL chain locus then undergoes recombination, enabling the formation of IgL chain protein, which together with the IgH chain forms the BCR.

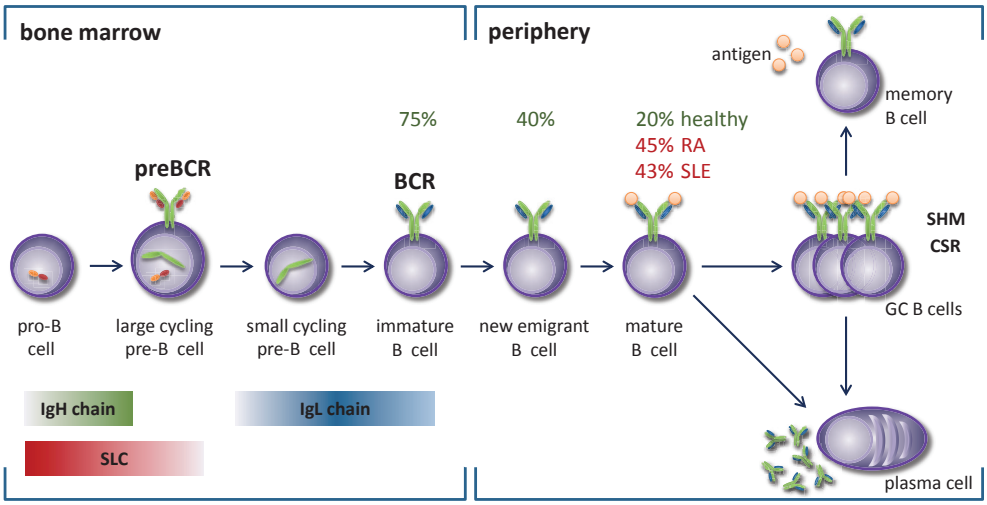

Figure 2. B cell development and differentiation. B cells develop in the bone marrow. IgH and IgL chain recombination and SLC expression are indicated. Immature B cells migrate to the periphery (mostly the spleen and lymph nodes), where they await antigen presentation. Upon stimulation by an antigen, the cells differentiate into GC B cells or plasma cells. Somatic hypermutation (SHM) and class switch recombination (CSR) take place in GCs. Percentages indicate the proportion of autoreactive B cells.

Because V(D)J recombination results in random BCR specificities, several checkpoints exist in B cell development to limit the number of autoreactive BCRs that reach the periphery. If in immature B cell present in the bone marrow reacts with a self-antigen, it can go into apoptosis. Alternatively, additional rearrangement of the IgL chain locus, termed receptor editing, can be induced to reduce auto-reactivity [9-11]. However, this can only occur several times, and failure to form a non-autoreactive BCR will lead to apoptosis of an immature B cell in the bone marrow. Finally, in mature, circulating B cells, binding of an antigen to the BCR in absence of co-stimulation by T cells induces an inhibitory signal rather than an activation signal, rendering them unsusceptible to antigen stimulation. This state is called anergy [3].

Defects in developmental checkpoints lead to increased numbers of autoreactive B cells and auto-immune disease [2]. In RA patients, mature B cells have been found in the circulation and synovium that still express the SLC next to the IgL chain [12]. These cells have an autoreactive (pre-)BCR repertoire [13, 14]. Furthermore, autoreactive BCRs can leak into the periphery through a process called allelic inclusion, whereby both alleles are recombined and therefore multiple BCRs are expressed on one cell [15]. Signaling by the autoreactive BCR is masked by the non-autoreactive BCR, allowing the B cell to migrate from the bone marrow.

B cell differentiation

Immature B cells with a fully formed BCR will migrate from the bone marrow to peripheral lymphoid organs such as the spleen and lymph nodes. In a process that is still not very well understood, B cells with particular specificities are selected into a long-lived pool of circulating mature, naïve B cells. If they are not selected into this pool (e.g. because they are autoreactive), they die within several days. Upon activation, naïve, mature B cells can differentiate into antibody producing plasma cells or become memory B cells. A rapid humoral immune response to a pathogen is accomplished by the formation of IgM plasma cells. However, IgM antibodies have a lower affinity for their antigen and have a shorter half-life than antibodies of other isotypes. The isotype of a BCR and antibody is determined by the IgH chain, the μ region (forming IgM) being the default. Other isotypes arise in germinal centers, highly specialized areas in lymphoid organs where B cells can undergo class switch recombination (CSR) to IgG, IgE or IgA [16, 17]. In addition, B cells can undergo a process called somatic hypermutation (SHM) in germinal centers. During SHM, numerous point mutations are introduced in the V(D)J joining regions that enhance the affinity of the antibody for an antigen [16, 18, 19]. Tfh cells play an important role in the germinal center reaction, enhancing survival of B cells and inducing antibody production [20].

Autoantibodies, immune complexes and complement

Autoantibodies are a hallmark of auto-immune diseases. Often, these autoantibodies deposit in small capillaries in target organs, like the kidneys in SLE patients [21]. There, they initiate an

immune response causing damage of the surrounding tissue. Antibodies deposit in the form of ICs, which are formed when antibodies bind to their antigen or to each other. When an antigen is bound by an antibody, this facilitates phagocytosis of the antigen by immune cells by activation through Fc receptors. In addition, certain antibody isotypes (IgG1 in humans, IgG2 in mice and IgM in both) can activate the complement system. Binding of complement factors to the antibodies further targets the pathogen for degradation.

The importance of the classical complement pathway in clearing ICs and apoptotic debris is underlined by the fact that more than 50% of SLE patients have reduced circulating C1q levels accompanied by enhanced apoptosis, either because of decreased production of C1q, or of suppression of C1q through anti-C1q auto-antibodies [22-24]. Also more than 90% of patients with lower C1q levels develop SLE [25, 26]. Decreased C1q leads to continued activation of immune cells through the ICs or the persistent availability of auto-antigens from apoptotic debris. Furthermore, C1q has an immune modulatory effect on DCs, stimulating production of IL-10 and inhibiting IL-12 and IL-23 production [27].

In contrast, the other complement pathways are involved in facilitating phagocytosis of pathogens and can be pathogenic in SLE, because they can induce local tissue damage. This is why a normally functioning complement response can be pathogenic in SLE, whereas specific defects in the classical pathway predispose to SLE.

T cell development

Precursor T cells develop from stem cells in the bone marrow and migrate to the thymus. These cells enter the thymus at the cortico-medullary junction and move through the cortex during development [28]. This is necessary to receive the required signals from stromal cells in the thymus that aid proper development. Like B cells, T cells express an antigen receptor that is highly specific for a single antigen. This (TCR) is formed in a similar way as the BCR, the different TCR loci also undergoing V(D)J recombination (figure 3). TCRs on CD4+ and CD8+ T cells have an α and a β subunit. A small but distinct T cell population expresses a γδTCR and remains CD4 and CD8 negative [29].

The earliest developmental stage that can be distinguished is the CD4-CD8- double negative 1 (DN1) stage (figure 3). These cells express CD44 but lack CD25 expression. The cells then upregulate CD25 (DN2) and commence rearrangement of the γ, δ and β loci. Cells will then either express the γδTCR, or a pre-TCR composed of a rearranged TCR β with a precursor TCRα (DN3). In this stage CD44 is downregulated. Signaling through the preTCR is essential for progression in development and downregulates CD25 (DN4). Subsequently, the TCRα locus is rearranged. The resulting αβTCR must be MHC restricted, meaning it must be able to bind an MHC molecule. Failure to do so will lead to additional rearrangement of the TCRα locus and ultimately cell death [30, 31].

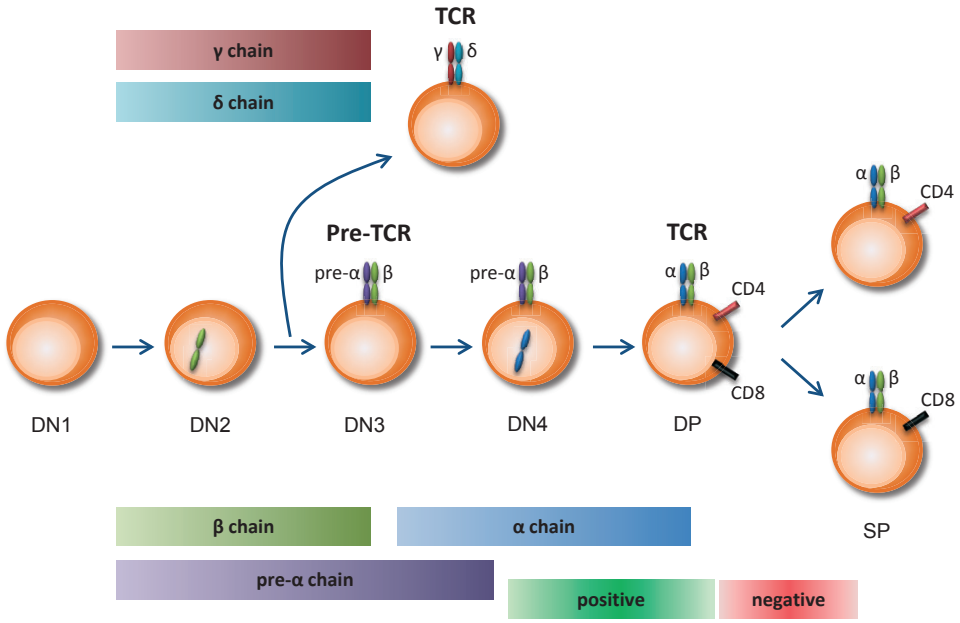

Figure 3. T cell development in the thymus. Expression of the pre-α chain, rearrangement of the β, γ, δ and α chains and positive and negative selection are indicated. DN double negative; DP double positive; SP single positive; TCR T cell receptor.

As in B cell development, several checkpoints exist in T cell development to ensure self-tolerance. During the double positive (DP) stage, both CD4 and CD8 are expressed on the T cells independent of antigens. In addition, low affinity selfpeptides are presented to the cells and if they bind the newly formed TCR, subsequent signaling will induce survival, positively selecting the T cell for further development [32]. Important transcription factors in this process are RORγ and TCF-1, which induce expression of the survival factor Bcl-X_L [33, 34]. However, if the signal is too weak or too strong, the cells will die through 'death by neglect' or apoptosis, respectively [32]. Affinity for an antigen presented on an MHC class I or MHC class II molecule determines whether the cell ultimately becomes a CD8+ or a CD4+ single positive (SP) T cell, respectively.

A second checkpoint involves negative selection, whereby cells that express TCRs with affinity for antigens present in the thymus (self-antigens) are clonally deleted [35]. Expression of autoimmune regulator (AIRE) by thymic epithelial cells (TEC) induces the expression of genes encoding tissue specific antigens, which are presented to the developing T cells [36]. This ensures deletion of self-reactive T cells before they enter the periphery. In contrast, CD4(+) Foxp3(+) regulatory T cells (Treg cells) are largely autoreactive yet escape clonal deletion in the thymus. The molecular mechanisms involved are largely unknown, although it was recently

demonstrated that CD27-CD70 co-stimulation involving TECs in dedicated niches in the thymus rescues developing Treg cells from apoptosis and thereby promotes Treg cell generation [37]. After selection, less than 5% of TCRs remain, indicating that T cell selection is far more stringent than B cell selection. In this context, it was recently shown that recent thymic emigrants have an increased potential to acquire a FoxP3+ Treg phenotype in the periphery [38].

T helper cell differentiation

T helper cells, characterized by CD4 expression, are so named because they assist in the activation of other cells. Several different subsets of T helper cells have been identified. Depending on the invading pathogen, DCs will direct the differentiation of naïve T helper cells towards the required subset. These subsets can be distinguishes based on their cytokine profile, chemokine receptor profile and the transcription factors they express.

In 1986, two T helper subsets were distinguished, the T helper 1 (Th1) and Th2 subsets [39]. Th1 cells are involved in the response against intracellular bacteria and protozoa, whereas Th2 cells play a role in the response against parasites. It was proposed that over-activation of Th1 cells could lead to autoimmune responses and over-activation of Th2 cells to allergies. In addition to these two subsets, recently other subsets have also been described, including Th17 cells, Th22 cells, follicular Th (Tfh) cells and T regulatory (Treg) cells. Th17 cells are involved in immune responses against extracellular bacteria and fungi, Tfh cells play a role in B cell immunity and Treg cells are required to limit the immune response.

Distinct signals from DCs lead to polarization of naïve T cells to different subsets (figure 4). IL-27 produced by DCs induces up-regulation of the transcription factor T-bet expression on naïve Th cells [40, 41], which induces expression of IL-12 receptor (IL-12R) [41-43]. Subsequent binding of IL-12, also produced by DCs, by these receptors initiates the expression of STAT4 [44], which promotes the production of IFNγ [45], the signature cytokine of Th1 cells. IFNγ predominantly activates macrophages to phagocytose intracellular bacteria [46]. Th1 cells can also be distinguished from other Th cells by their expression of CXCR3 [47, 48], a chemokine receptor that enables the cells to migrate to the site of inflammation. Th1 cells have been implicated in the pathogenesis of several autoimmune diseases, including psoriasis, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [49-51].

Th2 cells arise after production of IL-4 by DCs [52]. This induces the expression of transcription factors STAT6 and GATA3 [53, 54], leading to the expression of IL-4, IL-5 and IL-13. IL-4 stimulates mast cells [55] and is involved in class switch recombination [56], a process in B cells during plasma cell formation. IL-5 activates eosinophils to attack helminths [57] and enhances histamine release by basophils [58]. IL-13 is closely related to IL-4, inducing the production of IgE antibodies by plasma cells [59]. Th2 cells also express the chemokine receptor CCR4 [48]. Over-activation of Th2 cells can lead to hypersensitivity to non-pathogenic stimuli (allergies) or asthmatic disease

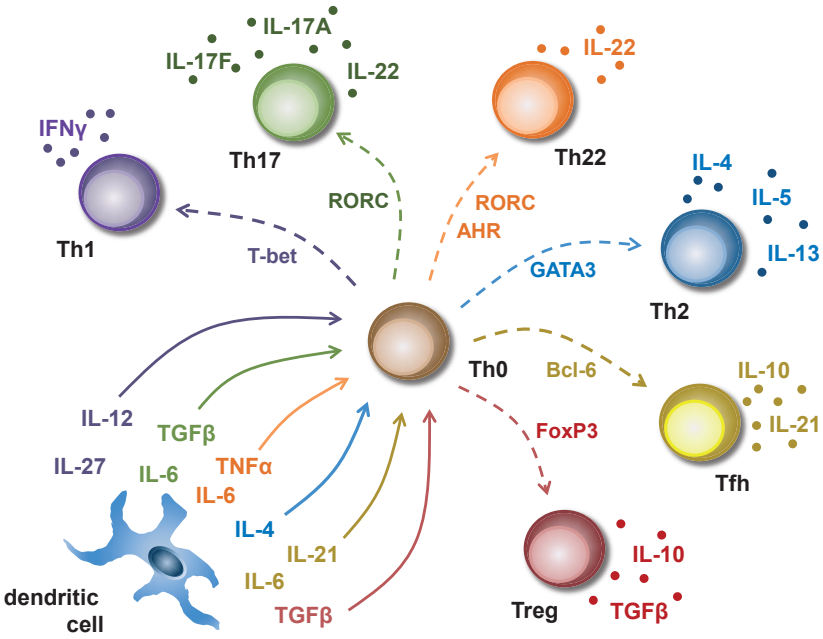

Figure 4. T helper cell differentiation. Dendritic cells produce cytokines that induce T helper cell differentiation (dashed arrows) from naïve T helper (Th0) cells. The cytokines produced by the dendritic cell determine the T helper subset (depicted by different colours) through activation of transcription factors (indicated next to dashed arrows).

[60, 61]. In contrast, Th2 cells have been shown to be protective in auto-immune diseases through their capacity to inhibit pathogenic T cell subsets [62-64].

In 2005, a new Th subset was described. These cells are characterized by IL-17A production and were therefore named Th17 cells [65]. In addition, they also produce IL-17F, IL21, IL-22 and IL-26 [66-68] and express the chemokine receptor CCR6 [69]. Naïve T cells can differentiate towards the Th17 lineage under the influence of IL-6 and TGFβ that induce expression of transcription factors STAT3 and RORC [70]. Stability and expansion of the population are directed by IL-21 and IL-23, respectively [71, 72]. IL-17A and IL-22 produced by Th17 cells are involved in the defence against extracellular bacteria [66]. IL-17F induces angiogenesis and production of several cytokines by epithelial cells and fibroblasts [73]. It has become clear that, next to Th1 cells, Th17 cells are also involved in the pathogenesis of many auto-immune diseases [74].

Tregs dampen the immune response by limiting the effector function of other cells, including T and B cells, but also by limiting activation of DCs [75]. Reduced numbers or dysfunction of

Tregs is associated with autoimmune disease or graft versus host disease [76-79]. Tregs arise under the influence of TGFβ and transcription factor FoxP3 expression [80]. Because TGFβ is also required for Th17 differentiation, it is thought that local IL-6 levels determine the balance between Tregs and Th17 cells. Tregs can be distinguished from other Th cells through high CD25 on their membrane [81]. Treg cells produce several cytokines that have immune-modulatory effects. IL-10 and IL-35 are required for dampening of the immune response at mucosal barriers [82, 83]. Furthermore, TGFβ inhibits Th1 and Th2 responses [84].

Th22 is a recently described Th subset. These cells express IL-22 and TNFα, but not IL-17 and are therefore distinct from Th17 cells [85, 86]. The cells also express CCR6, but they can be separated from Th17 cells based on the additional expression of CCR10, which is hardly expressed by Th17 cells [85, 86]. Th22 cells develop under the influence of TNFα and IL-6, and IL-22 expression depends on expression of transcription factor RORC and aryl hydrocarbon receptor AHR [86]. IL-22 targets non-hematopoietic cells as the IL-22R is expressed in stromal cells but not on cells of the immune system [87]. It is involved in tissue repair and maintaining mucosal barrier function in the defence against bacteria [88]. The role of IL-22 in auto-immune diseases is very heterogeneous, as both a pathogenic role for IL-22 as well as a protective role have been described in different diseases [89].

Although a role has been described for other Th subsets in B cell immunity, like IL-4 production by Th2 cells and IL-17A by Th17 cells, the key Th subset in the activation of B cells appears to be the Tfh cell. IL-6 and IL-27 production by DCs upregulates the expression of transcription factors Bcl-6 and c-Maf to induce Tfh effector differentiation [90-93]. Tfh cells produce IL-21 and IL-10, and express CD40 ligand (CD40L) in a STAT1 and 3 dependent fashion [90, 94]. Binding of CD40L with CD40 on B cells rescues B cells from apoptosis and IL-21 induces differentiation of B cells and production of antibodies [94, 95]. Furthermore, IL-21 further enhances Tfh function [96]. The interaction between Tfh cells and B cells takes place at the borders of B cell follicles and in germinal center light zones in lymphoid organs like the spleen or lymph nodes. The Tfh cell migrate to this location through expression of the chemokine receptor CXCR5 [97, 98] and downregulation of CCR7 [99].

AUTOIMMUNE DISEASES

Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease of the joint synovium, leading to joint destruction. In Western countries the prevalence of RA is around 1% [100]. Although treatment strategies and the outcome of disease have improved markedly over the past years, no cure for RA is currently available. This can be attributed to the fact that the cause of RA is still unknown.

The current understanding is that RA is a multifactorial disease with multiple possible events

triggering the onset. Many studies have shown that genetic factors contribute to the disease. HLA-DRB1 is a known haplotype associated with increased RA susceptibility [101]. Furthermore, many single-nucleotide polymorphisms (SNPs) in genes involved in immune regulation have been found in RA patients [101]. Several environmental factors, including mucosal barrier stress, caused by for example smoking, and gut microbiota, may contribute to the onset of disease [102]. In addition molecular mimicry has been proposed as a possible trigger [103]. This is a process whereby a cross-reaction occurs after or during an immune response to a pathogen epitope that is similar to a self-epitope. EBV and CMV infections have been suggested to lead to such a cross-reaction [104, 105]. However, the mechanism by which the auto-immune reaction is subsequently transferred to the joints is unknown.

The exact sequence of events of synovial inflammation leading to joint destruction is unclear. Many different inflammatory processes occur simultaneously in the synovium. During inflammation arginine residues in proteins can be converted to citrulline, sometimes significantly alters how the protein is folded. Loss of tolerance to citrullinated proteins induces the production of anti-citrullinated protein antibodies (ACPA) [106]. The role of ACPA in the pathogenesis of RA is not fully understood. ACPA can be found in the blood of some patients years before onset of disease [107], and it is clear that ACPA+ patients have a poorer disease outcome than ACPA- patients [108]. Eliminating B cells with anti-CD20 treatment is very effective in RA patients [109], and also decreases ACPA and rheumatoid factor (RF) (antibodies against the Fc portion of IgG antibodies) [110]. ACPA can activate synovial macrophages and fibroblasts to produce pro-inflammatory cytokines and chemokines that attract other cells to the synovium [111-113]. In addition, ACPA can induce osteoclastogenesis and bone loss [114]. Alternatively, macrophages can be activated through TLRs by endogenous damage-induced antigens or invading pathogens [115]. In addition, synovial fibroblasts and endothelial cells are activated, producing pro-inflammatory cytokines, chemokines and adhesins, thereby enabling inflammatory cells to migrate into the synovium [113, 116]. Amongst these are B and T cells, which can sometimes form tertiary lymphoid structures to further promote plasma cell formation [117]. They also produce pro-inflammatory cytokines like IL-6 and TNFα to further activate synovial cells.

Together the influx of cells, interaction of cells and production of pro-inflammatory cytokines leads to pro-inflammatory feedback loops and the ultimate destruction of the joint (figure 5). T helper cells can activate synovial macrophages and fibroblasts through the production of IL-17A [118]. This leads to production of IL-6, which in turn stimulates cytokine production by the T cells. The synovial cells also produce matrix metalloproteinases (MMPs), proteases that degrade extracellular matrix proteins, which in RA induce cartilage damage by breaking down collagen type II [119]. In addition, IL-1β and IL-17A reduce the number of chondrocytes that normally regulate cartilage regeneration [120]. Furthermore, IL-1β induces the production of GM-CSF, a potent growth factor for leucocytes. GM-CSF and RANK-L, a critical factor for normal bone resorption and homeostasis, produced by synovial cells enhance osteoclast activation, leading to bone erosion.

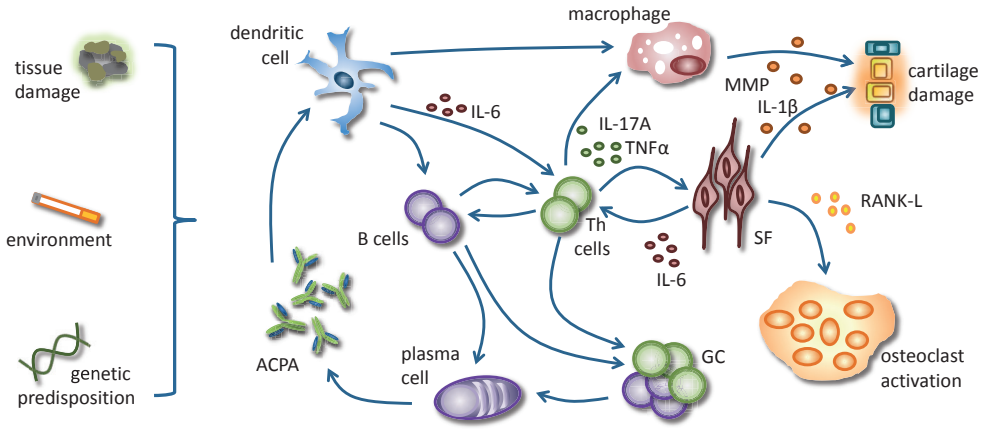

Figure 5. Pathogenesis of RA. Several cells and their interactions (arrows) as well as cytokines produced are depicted. SF synovial fibroblast.

Treatment of RA is now focussed on dampening the autoimmune response. Disease-modifying anti-rheumatic drugs (DMARDs), like methotrexate, are the cornerstone of RA treatment. However, these therapies limit the immune response in a non-specific way and have many side effects. The introduction of biologicals, antibodies directed against cytokines like TNFα or the IL-6 receptor, has greatly improved the outcome of disease [121, 122]. However, these treatments have many side effects, are very costly and do not cure the disease.

Role of Th cells in RA

Before the discovery of Th17 cells, Th1 cells were thought to be the dominant subset in the pathogenesis of autoimmune diseases. IFNα treatment, which induces Th1 differentiation, can lead to autoimmunity, including RA symptoms [123]. The number of IFNγ producing CD4+ T cells is increased in the synovium of RA patients [108, 118]. Furthermore, the number of IFNγ producing cells in the circulation of early RA patients correlates with disease severity [107]. IFNγ activates synovial macrophages, which are major inducers of osteoclast activation and differentiation [124-126]. Apart from IFNγ, Th1 cells also produce TNFα. This pro-inflammatory cytokine can activate synovial fibroblasts and chondrocytes [127, 128]. In addition, TNFα can limit the immune regulatory capacity of Tregs [106]. However, it has also been shown that IFNγ can limit osteoclastogenesis [129].

Despite these lines of evidence supporting a role for Th1 cells, to date a growing body of research shows that Th17 cells are the most pathogenic Th cells in RA. Upon entering the synovium, Th17 cells engage in a pro-inflammatory feedback loop with synovial fibroblasts [118].

IL-17A produced by the Th17 cells enhances survival and proliferation of synovial fibroblasts [130]. Furthermore, IL-17A induces IL-6 production by the fibroblasts, which in turn enhances IL-17 production by the T cells. IL-17A also synergizes with TNFα to induce fibroblast and chondrocyte activation [131, 132]. Furthermore, IL-17A, IL-6, IL-1 and TNFα induce activation of osteoclasts, leading to bone destruction [133-135]. In addition to Th17 cells, Th22 cells and IL-22 have also been implicated in RA. Increased numbers of IL-22 producing cells have been found in the circulation of RA patients [136, 137], and IL-22 serum levels correlate with disease severity [138, 139]. Several studies have shown a role for IL-22 in osteoclastogenesis and bone degradation [140, 141].

Tregs limit Th1 and Th17 responses by TGFβ production and inhibition of IL-6 production by DCs [142]. However, the inhibitory capacity of Tregs can be suppressed by IL-6, which can even induce IL-17A production by the Tregs, giving them a pro-inflammatory phenotype [143].

Apart from Tregs, Th2 cells also have a protective function in RA. RA patients who also have allergies, which is associated with Th2 activity, have lower disease severity [110]. A combination of IL-4 with IL-10 has been shown to reduce synovial infiltrates and cartilage damage in collagen induced arthritis, a mouse model for RA [109]. In addition, IL-4 reduces bone resorption by inhibiting osteoclasts [144].

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease affecting many different organs. Symptoms include butterfly rash, arthritis, sun sensitivity, Raynaud phenomenon, pleuritis and nephritis [145, 146]. Genetic predisposition plays an important role in the disease, which more commonly affects young Afro-American and Asian females [146]. The disease is characterized by the production of auto-antibodies mostly directed against nuclear structures like DNA. These auto-antibodies form immune complexes that can deposit in capillaries in organs such as the kidneys. There, they activate innate immune cells through Fc receptors and the complement system, leading to the production of pro-inflammatory cytokines and chemokines [145]. This induces influx of more immune cells, including IL-17 producing T cells, which have been found in the kidneys of patients with lupus nephritis [123].

Exactly which trigger initiates the production of auto-antibodies against nuclear structures in SLE is unknown. It is possible that reduced clearance of apoptotic cells in SLE patients provides an increased load of nuclear antigens to the immune system, but normally these antigens should not induce an immune response [147]. However, apoptosis induced modifications to chromatin, like acetylation or binding of HMGB1, may induce maturation of myeloid derived DCs (mDCs) through TLRs [148, 149]. Upon activation by nuclear antigens, the mDCs can induce T helper cell differentiation [150].

Recent studies also suggest a role for neutrophils. Upon activation, neutrophils undergo a

special form of cell death called NETosis, which is distinct from apoptosis and necrosis [151, 152]. Chromatin and granular proteins are excreted from the dying cells in the form of neutrophil extracellular traps (NETs) that facilitate the killing of bacteria [152]. However, these NETs can also activate plasmacytoid DCs (pDCs). These cells play an important role in the defense against viral infections by sensing virus derived nucleic acids [153, 154]. Upon apoptosis or NETosis, sensing of extracellular DNA through TLR 9 induces production of interferon (IFN) type I [155-157]. Production of IFN type I is regulated by interferon regulatory factors (IRFs) [158]. Antibodies directed against Ro52, a factor that induces the degradation of IRFs and thereby limits the IFN type I response [159], are often found in SLE patients. High IFN type I levels induce maturation of mDCs and mDC driven T helper cell differentiation [160]. In addition, mDCs produce BAFF, a cytokine that enhances B cell survival [161]. This leads to the production of more auto-antibodies, which can further induce IFN type I production by pDCs through binding of Fc receptors, thereby forming a pro-inflammatory loop (figure 6).

Treatment of SLE is limited to reducing the symptoms and tissue damage caused by inflammation. Patients are treated with corticosteroids and immune suppressive medication

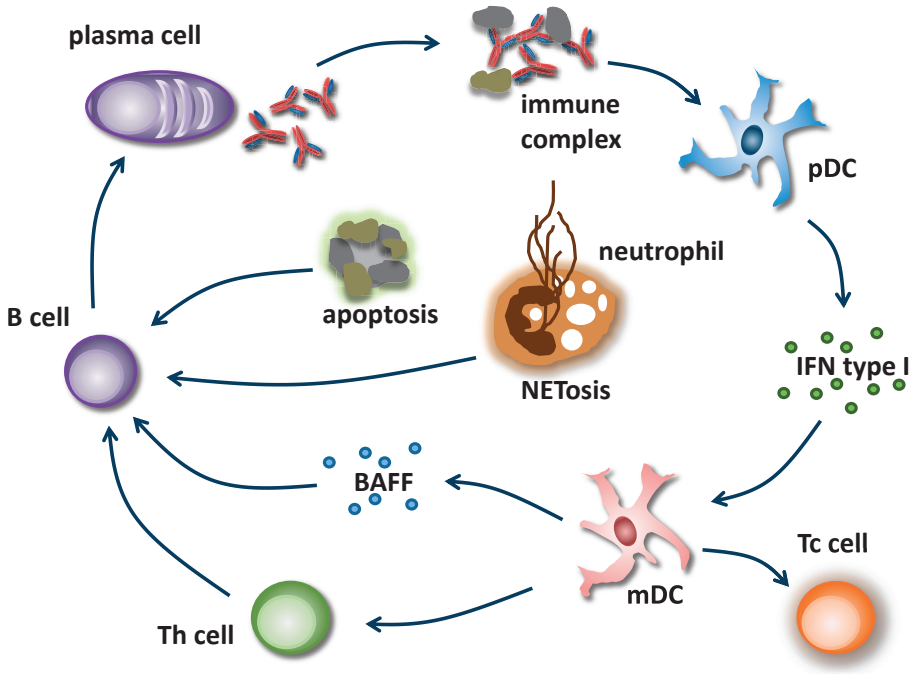

Figure 6. Pathogenesis of SLE. Several cell types and their interactions (arrows) as well as cytokines produced are depicted. pDC plasmacytoid DC; mDC myeloid derived DC, IC immune complex.

to limit inflammation, and additional medication is based on the clinical manifestation of the disease in the individual patient [146].

Role of Th cells in SLE

Many defects in Th cell signalling have been found in SLE patients. T cells have been found to be hyper-responsive to T cell receptor (TCR) signalling due to alterations in the TCR complex expressed on the membrane [162]. This complex recruits alternative downstream signalling molecules that enhance the signal strength >100 fold [163]. Th cells provide help to B cells through binding of CD40 ligand expressed on the T cell with CD40 expressed on the B cell. In SLE patients, CD40 expression on Th cells was found to be higher at baseline than healthy controls, and increased and prolonged on activated cells [164, 165].

Both Th1 and Th2 cytokines are implicated in the pathogenesis of SLE. IL-4 induces class switch recombination in B cells to isotypes that activate complement. In a mouse model of SLE, IFNγ is essential for lupus nephritis development [166]. Increased levels of IFNγ have also been found in the serum of SLE patients [167].

Recent studies have suggested a role for Th17 cells in SLE. IL-17A levels were increased in SLE patients and correlated with disease severity [168]. Also, the number of IL-17A producing PBMCs in SLE patients is higher compared to healthy controls [169, 170]. In mouse studies, lack of IL-23R signalling protected mice from SLE development, and blocking of IL-23 cytokine ameliorated the disease [171]. Furthermore, IL-17A producing cells have been found in infiltrates in the kidneys of lupus nephritis patients, although these are not CD4+ cells, but rather double negative T cells (expressing CD3, but not CD4 or CD8) [123].

The increased number of Th17 cells in SLE is accompanied by a decrease in Treg cell numbers [170]. This could be caused by an increased amount of IL-6, which shifts Th differentiation towards the Th17 lineage. These cells produce TNFα, which reduces the immune modulatory function of Tregs [172]. Also, IL-17A production by Tregs could be induced, making them pathogenic rather than protective. In addition, IL-10 can have a pathogenic role in SLE through its positive effects on B cell survival and activation [173], and IL-10 expression by PBMCs of SLE patients has been found to be increased [174].

A key cell type in the activation of B cells and autoantibody production are Tfh cells. In SLE patients, the numbers of circulating CD4+CXCR5+ T cells and IL-21+ T cells are increased and appear to correlate with autoantibody titres and disease severity [175-177]. Furthermore, IL-21 plasma levels are increased in SLE patients [178]. Although B cells in SLE patients have lower IL-21R expression than healthy controls [179], SNPs in the IL-21R gene are a risk factor for SLE [180]. Furthermore, amelioration of symptoms in SLE patients upon treatment with corticosteroids is accompanied by a reduction in the number of circulating Tfh cells [176].

AIMS AND OUTLINE OF THIS THESIS

Mouse models in autoimmunity

The pathogeneses of RA and SLE show many overlaps, but why the similar pathogeneses can lead to clinically different diseases remains unclear. The aim of this thesis is to unravel the differential roles of several Th17 related cytokines in the control of lymphocytes in autoimmunity, and more specifically in RA and SLE. Therefore we use mouse models, including several knock-out mice, collagen induced arthritis (a mouse model for RA) and the spontaneous B6.lpr lupus model, and human material from RA patients, SLE patients and healthy controls. The data presented in this thesis contribute to the understanding of the role of Th17 cytokines in the immune system and the pathogenesis of autoimmune diseases like RA and SLE.

To investigate the role of cytokines in the early stages of autoimmune disease, we make use of several mouse models to mimic these diseases. A well-established mouse model for RA is the collagen induced arthritis (CIA) model. In this model, mice are injected with chicken type II collagen (CII) emulsified in complete Freund's adjuvant (CFA). The model is based on molecular mimicry: the chicken CII is very similar to mouse CII and immune cells will therefore cross react with mouse CII upon activation. CFA is a strong activator of the immune system and will facilitate this process. The model is both T and B cell mediated. More specifically, Th17 cells are essential in this model as mice lacking IL-23 (and consequently having severely reduced Th17 numbers) are completely protected against CIA [181]. Auto-antibody production is also crucial for disease development, as mice lacking B cells are also protected against disease [182]. In addition, transfer of serum containing auto-antibodies from diseased mice induces the disease in naïve mice [183]. In the CIA protocol, mice are immunized on day 0 and again on day 21 to induce a memory response. Wild type mice will typically develop arthritis shortly before or after the second injection, with a total incidence of 30-50%. Disease severity is scored macroscopically and reaches its peak in wild type mice around day 35-40.

To investigate the role of cytokines in SLE, we employ the spontaneous lymph-proliferation (lpr) mouse model [184]. Mice with the lpr mutation are deficient in FAS, an important receptor that induces apoptosis. This receptor is normally expressed on B cells and is required for negative selection of autoreactive B cells during B cell development. As a result of the lpr mutation, autoreactive B cells survive and subsequently migrate to the periphery where they can activate T cells and produce auto-antibodies leading to SLE like symptoms. As this is a spontaneous model, the disease course is very heterogeneous, affecting mostly female mice. In mice with a C57BL/6 background, the disease develops relatively slowly with the first symptoms occurring around 16-20 weeks of age. The disease is characterized by proliferation of B cells and DN T cells, auto-antibody production and IC deposition in small capillaries of the kidneys and lungs.

To investigate the role of cytokines in RA and SLE, we have used several knock-out mouse lines that are deficient for a cytokine or cytokine receptor. These mice were immunized with CII/CFA to induce CIA or backcrossed onto the C57BL/6-lpr mouse line.

Aims of this thesis

Chapter 2 The role of IL-17A in RA has been studied extensively. IL-17 family members signal through different heterodimers of IL-17 receptor (IL-17R) family members, however, the IL-17RA subunit appears to be the common subunit for all IL-17 cytokines [185, 186]. In chapter 2 we investigate the effect of loss of IL-17RA signalling, and therefore loss of signalling of all IL-17 family cytokines, in the pathogenesis of CIA.

Chapter 3 In addition to IL-17A, several studies have suggested a pro-inflammatory role for IL-22 in joint inflammation [140]. However, other studies have shown a role for IL-22 in mucosal barrier homeostasis, and that IL-22 is protective in autoimmunity [187]. In chapter 3, we therefore investigate the differential effects of Th17 cells, which produce both IL-17A and IL-22, and Th22 cells, which only produce IL-22 but not IL-17A, in RA.

Chapter 4 In chapter 4 we explore the potential role of IL-22 in arthritis in the mouse, focussing particularly on the role of IL-22 in B cell immunity.

Chapter 5 B cells play an important role in RA and many RA patients are ACPA positive. A small population of B cells has been found in the periphery that expresses both the surrogate light chain (SLC) as well as the IgL chain and accumulates in RA patient synovium [12]. These cells display an autoreactive BCR repertoire [14, 117]. However, their contribution to auto-immune disease is not clear. In chapter 5, we investigate the effect of overexpression of the SLC in mature B cells in autoimmunity in mice.

Chapter 6 IL-27 reduces Th17 differentiation [188], and as a consequence in IL-27R KO mice, more T cells express IL-17A [189]. Also, follicular T helper cells, which are crucial for germinal center formation and function, depend on IL-27 [91]. The IL-27R is also expressed on B cells, but little is known about its direct effects on these cells. In chapter 6 we investigate the direct effect of IL-27R signaling on B cells, and the effect on B cell immunity.

Chapter 7 Both the IFN type I pathway and IL-17A producing cells have been implicated in SLE pathogenesis [168, 190]. Several studies show possible links between IFN type I and IL-17A producing cells [191, 192], but so far no studies have been performed to confirm the association of IFN type I with IL-17A in SLE patients. We therefore aimed to identify a possible link between these two pathways in SLE patients.

Chapter 8 A pathogenic role of Th17 cells in SLE has been suggested in both humans and mice. B6.lpr mice deficient for IL-23R expression are protected against development of SLE symptoms [171]. However, the role of the IL-17 family in this model remains to be investigated. In chapter 8 we investigate the role of IL-17RA signalling in the pathogenesis of SLE in mice.

REFERENCES

1. Bossen C, Mansson R, Murre C: **Chromatin topology and the regulation of antigen receptor assembly.** *Annu Rev Immunol* 2012, **30:**337-356.
2. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC: **Predominant autoantibody production by early human B cell precursors.** *Science* 2003, **301:**1374-1377.
3. Goodnow CC, Crosbie J, Adelstein S, Lavoie TB, Smith-Gill SJ, Brink RA, Pritchard-Briscoe H, Wotherspoon JS, Loblay RH, Raphael K, et al.: **Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice.** *Nature* 1988, **334:**676-682.
4. Fujio K, Okamura T, Sumitomo S, Yamamoto K: **Regulatory cell subsets in the control of autoantibody production related to systemic autoimmunity.** *Ann Rheum Dis* 2013, **72 Suppl 2:**ii85-89.
5. Schatz DG, Ji Y: **Recombination centres and the orchestration of V(D)J recombination.** *Nat Rev Immunol* 2011, **11:**251-263.
6. Hendriks RW, Middendorp S: **The pre-BCR checkpoint as a cell-autonomous proliferation switch.** *Trends Immunol* 2004, **25:**249-256.
7. Martensson IL, Rolink A, Melchers F, Mundt C, Licence S, Shimizu T: **The pre-B cell receptor and its role in proliferation and Ig heavy chain allelic exclusion.** *Semin Immunol* 2002, **14:**335-342.
8. Schlissel M: **Allelic exclusion of immunoglobulin gene rearrangement and expression: why and how?** *Semin Immunol* 2002, **14:**207-212; discussion 225-206.
9. Gay D, Saunders T, Camper S, Weigert M: **Receptor editing: an approach by autoreactive B cells to escape tolerance.** *J Exp Med* 1993, **177:**999-1008.
10. Radic MZ, Erikson J, Litwin S, Weigert M: **B lymphocytes may escape tolerance by revising their antigen receptors.** *J Exp Med* 1993, **177:**1165-1173.
11. Tiegs SL, Russell DM, Nemazee D: **Receptor editing in self-reactive bone marrow B cells.** *J Exp Med* 1993, **177:**1009-1020.
12. Meffre E, Davis E, Schiff C, Cunningham-Rundles C, Ivashkiv LB, Staudt LM, Young JW, Nussenzweig MC: **Circulating human B cells that express surrogate light chains and edited receptors.** *Nat Immunol* 2000, **1:**207-213.
13. Meffre E, Chiorazzi M, Nussenzweig MC: **Circulating human B cells that express surrogate light chains display a unique antibody repertoire.** *J Immunol* 2001, **167:**2151-2156.
14. Meffre E, Schaefer A, Wardemann H, Wilson P, Davis E, Nussenzweig MC: **Surrogate light chain expressing human peripheral B cells produce self-reactive antibodies.** *J Exp Med* 2004, **199:**145-150.
15. Liu S, Velez MG, Humann J, Rowland S, Conrad FJ, Halverson R, Torres RM, Pelanda R: **Receptor editing can lead to allelic inclusion and development of B cells that retain antibodies reacting with high avidity autoantigens.** *J Immunol* 2005, **175:**5067-5076.
16. Victora GD, Nussenzweig MC: **Germinal centers.** *Annu Rev Immunol* 2012, **30:**429-457.
17. Stavnezer J, Guikema JE, Schrader CE: **Mechanism and regulation of class switch recombination.** *Annu Rev Immunol* 2008, **26:**261-292.
18. Berek C, Berger A, Apel M: **Maturation of the immune response in germinal centers.** *Cell* 1991, **67:**1121-1129.
19. Jacob J, Kelsoe G, Rajewsky K, Weiss U: **Intraclonal generation of antibody mutants in germinal centres.** *Nature* 1991, **354:**389-392.
20. Ma CS, Deenick EK, Batten M, Tangye SG: **The origins, function, and regulation of T follicular helper cells.** *J Exp Med* 2012, **209:**1241-1253.
21. Oates JC, Gilkeson GS: **Mediators of injury in lupus nephritis.** *Curr Opin Rheumatol* 2002, **14:**498-503.
22. Siegert C, Daha M, Westedt ML, van der Voort E, Breedveld F: **IgG autoantibodies against C1q are correlated with nephritis, hypocomplementemia, and dsDNA antibodies in systemic lupus erythematosus.** *J Rheumatol* 1991, **18:**230-234.

23. Moosig F, Damm F, Knorr-Spahr A, Ritgen M, Zeuner RA, Kneba M, Ernst M, Schroder JO: **Reduced expression of C1q-mRNA in monocytes from patients with systemic lupus erythematosus.** *Clin Exp Immunol* 2006, **146:**409-416.

24. Antes U, Heinz HP, Loos M: **Evidence for the presence of autoantibodies to the collagen-like portion of C1q in systemic lupus erythematosus.** *Arthritis Rheum* 1988, **31:**457-464.

25. Leyva-Cobian F, Moneo I, Mampaso F, Sanchez-Bayle M, Ecija JL, Bootello A: **Familial C1q deficiency associated with renal and cutaneous disease.** *Clin Exp Immunol* 1981, **44:**173-180.

26. Marquart HV, Schejbel L, Sjoholm A, Martensson U, Nielsen S, Koch A, Svejgaard A, Garred P: **C1q deficiency in an Inuit family: identification of a new class of C1q disease-causing mutations.** *Clin Immunol* 2007, **124:**33-40.

27. Teh BK, Yeo JG, Chern LM, Lu J: **C1q regulation of dendritic cell development from monocytes with distinct cytokine production and T cell stimulation.** *Mol Immunol* 2011, **48:**1128-1138.

28. Lind EF, Prockop SE, Porritt HE, Petrie HT: **Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development.** *The Journal of experimental medicine* 2001, **194:**127-134.

29. MacDonald HR, Radtke F, Wilson A: **T cell fate specification and alphabeta/gammadelta lineage commitment.** *Current opinion in immunology* 2001, **13:**219-224.

30. Brandle D, Muller C, Rulicke T, Hengartner H, Pircher H: **Engagement of the T-cell receptor during positive selection in the thymus down-regulates RAG-1 expression.** *Proceedings of the National Academy of Sciences of the United States of America* 1992, **89:**9529-9533.

31. Borgulya P, Kishi H, Uematsu Y, von Boehmer H: **Exclusion and inclusion of alpha and beta T cell receptor alleles.** *Cell* 1992, **69:**529-537.

32. Starr TK, Jameson SC, Hogquist KA: **Positive and negative selection of T cells.** *Annual review of immunology* 2003, **21:**139-176.

33. Ioannidis V, Beermann F, Clevers H, Held W: **The beta-catenin--TCF-1 pathway ensures CD4(+)CD8(+) thymocyte survival.** *Nature immunology* 2001, **2:**691-697.

34. Sun Z, Unutmaz D, Zou YR, Sunshine MJ, Pierani A, Brenner-Morton S, Mebius RE, Littman DR: **Requirement for RORgamma in thymocyte survival and lymphoid organ development.** *Science* 2000, **288:**2369-2373.

35. Dzhagalov IL, Chen KG, Herzmark P, Robey EA: **Elimination of self-reactive T cells in the thymus: a timeline for negative selection.** *PLoS biology* 2013, **11:**e1001566.

36. Mathis D, Benoist C: **Aire.** *Annu Rev Immunol* 2009, **27:**287-312.

37. Coquet JM, Ribot JC, Babala N, Middendorp S, van der Horst G, Xiao Y, Neves JF, Fonseca-Pereira D, Jacobs H, Pennington DJ, et al: **Epithelial and dendritic cells in the thymic medulla promote CD4+Foxp3+ regulatory T cell development via the CD27-CD70 pathway.** *J Exp Med* 2013, **210:**715-728.

38. Paiva RS, Lino AC, Bergman ML, Caramalho I, Sousa AE, Zelenay S, Demengeot J: **Recent thymic emigrants are the preferential precursors of regulatory T cells differentiated in the periphery.** *Proc Natl Acad Sci U S A* 2013, **110:**6494-6499.

39. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL: **Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins.** *J Immunol* 1986, **136:**2348-2357.

40. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH: **A novel transcription factor, T-bet, directs Th1 lineage commitment.** *Cell* 2000, **100:**655-669.

41. Mullen AC, High FA, Hutchins AS, Lee HW, Villarino AV, Livingston DM, Kung AL, Cereb N, Yao TP, Yang SY, Reiner SL: **Role of T-bet in commitment of TH1 cells before IL-12-dependent selection.** *Science* 2001, **292:**1907-1910.

42. Takeda A, Hamano S, Yamanaka A, Hanada T, Ishibashi T, Mak TW, Yoshimura A, Yoshida H: **Cutting edge: role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment.** *J Immunol* 2003, **170:**4886-4890.

43. Lucas S, Ghilardi N, Li J, de Sauvage FJ: **IL-27 regulates IL-12 responsiveness of naive CD4+ T cells through Stat1-dependent and -independent mechanisms.** *Proc Natl Acad Sci U S A* 2003, **100:**15047-15052.

44. Jacobson NG, Szabo SJ, Weber-Nordt RM, Zhong Z, Schreiber RD, Darnell JE, Jr., Murphy KM: **Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4.** *J Exp Med* 1995, **181:**1755-1762.

45. Thierfelder WE, van Deursen JM, Yamamoto K, Tripp RA, Sarawar SR, Carson RT, Sangster MY, Vignali DA, Doherty PC, Grosveld GC, Ihle JN: **Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells.** *Nature* 1996, **382:**171-174.

46. Williams JG, Jurkovich GJ, Maier RV: **Interferon-gamma: a key immunoregulatory lymphokine.** *Journal of Surgical Research* 1993, **54:**79-93.

47. Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B, Mackay CR: **The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions.** *J Clin Invest* 1998, **101:**746-754.

48. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray PA, Mantovani A, Sinigaglia F: **Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s.** *J Exp Med* 1998, **187:**129-134.

49. Michalak-Stoma A, Pietrzak A, Szepietowski JC, Zalewska-Janowska A, Paszkowski T, Chodorowska G: **Cytokine network in psoriasis revisited.** *Eur Cytokine Netw* 2011, **22:**160-168.

50. Schulze-Koops H, Kalden JR: **The balance of Th1/Th2 cytokines in rheumatoid arthritis.** *Best Pract Res Clin Rheumatol* 2001, **15:**677-691.

51. Theofilopoulos AN, Koundouris S, Kono DH, Lawson BR: **The role of IFN-gamma in systemic lupus erythematosus: a challenge to the Th1/Th2 paradigm in autoimmunity.** *Arthritis Res* 2001, **3:**136-141.

52. Abehsira-Amar O, Gibert M, Joliy M, Theze J, Jankovic DL: **IL-4 plays a dominant role in the differential development of Tho into Th1 and Th2 cells.** *J Immunol* 1992, **148:**3820-3829.

53. Zheng W, Flavell RA: **The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells.** *Cell* 1997, **89:**587-596.

54. Kaplan MH, Schindler U, Smiley ST, Grusby MJ: **Stat6 is required for mediating responses to IL-4 and for development of Th2 cells.** *Immunity* 1996, **4:**313-319.

55. Mosmann TR, Bond MW, Coffman RL, Ohara J, Paul WE: **T-cell and mast cell lines respond to B-cell stimulatory factor 1.** *Proc Natl Acad Sci U S A* 1986, **83:**5654-5658.

56. Kuhn R, Rajewsky K, Muller W: **Generation and analysis of interleukin-4 deficient mice.** *Science* 1991, **254:**707-710.

57. Lopez AF, Sanderson CJ, Gamble JR, Campbell HD, Young IG, Vadas MA: **Recombinant human interleukin 5 is a selective activator of human eosinophil function.** *J Exp Med* 1988, **167:**219-224.

58. Miadonna A, Roncarolo MG, Lorini M, Tedeschi A: **Inducing and enhancing effects of IL-3, -5, and -6 and GM-CSF on histamine release from human basophils.** *Clin Immunol Immunopathol* 1993, **67:**210-215.

59. Punnonen J, Aversa G, Cocks BG, McKenzie AN, Menon S, Zurawski G, de Waal Malefyt R, de Vries JE: **Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells.** *Proc Natl Acad Sci U S A* 1993, **90:**3730-3734.

60. Ricci M, Rossi O, Bertoni M, Matucci A: **The importance of Th2-like cells in the pathogenesis of airway allergic inflammation.** *Clin Exp Allergy* 1993, **23:**360-369.

61. Steinke JW, Borish L: **Th2 cytokines and asthma. Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists.** *Respir Res* 2001, **2:**66-70.

62. Wurtz O, Bajenoff M, Guerder S: **IL-4-mediated inhibition of IFN-gamma production by CD4+ T cells proceeds by several developmentally regulated mechanisms.** *Int Immunol* 2004, **16:**501-508.

63. Powrie F, Menon S, Coffman RL: **Interleukin-4 and interleukin-10 synergize to inhibit cell-mediated immunity in vivo.** *Eur J Immunol* 1993, **23:**3043-3049.

64. Ouyang W, Ranganath SH, Weindel K, Bhattacharya D, Murphy TL, Sha WC, Murphy KM: **Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism.** *Immunity* 1998, **9:**745-755.

65. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT: **Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages.** *Nat Immunol* 2005, **6:**1123-1132.

66. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, Fouser LA: **Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides.** *J Exp Med* 2006, **203:**2271-2279.

67. Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, Basham B, Smith K, Chen T, Morel F, et al: **Development, cytokine profile and function of human interleukin 17-producing helper T cells.** *Nat Immunol* 2007, **8:**950-957.

68. Wei L, Laurence A, Elias KM, O'Shea JJ: **IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner.** *J Biol Chem* 2007, **282:**34605-34610.

69. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, Parente E, Fili L, Ferri S, Frosali F, et al: **Phenotypic and functional features of human Th17 cells.** *J Exp Med* 2007, **204:**1849-1861.

70. McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T, Cua DJ: **TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology.** *Nat Immunol* 2007, **8:**1390-1397.

71. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ: **IL-23 drives a pathogenic T cell population that induces autoimmune inflammation.** *J Exp Med* 2005, **201:**233-240.

72. Zhou L, Ivanov, II, Spolski R, Min R, Shenderov K, Egawa T, Levy DE, Leonard WJ, Littman DR: **IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways.** *Nat Immunol* 2007, **8:**967-974.

73. Starnes T, Robertson MJ, Sledge G, Kelich S, Nakshatri H, Broxmeyer HE, Hromas R: **Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production.** *J Immunol* 2001, **167:**4137-4140.

74. Maddur MS, Miossec P, Kaveri SV, Bayry J: **Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies.** *Am J Pathol* 2012, **181:**8-18.

75. Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T: **Regulatory T cells: how do they suppress immune responses?** *Int Immunol* 2009, **21:**1105-1111.

76. Valencia X, Yarboro C, Illei G, Lipsky PE: **Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus.** *J Immunol* 2007, **178:**2579-2588.

77. Chi LJ, Wang HB, Zhang Y, Wang WZ: **Abnormality of circulating CD4(+)CD25(+) regulatory T cell in patients with Guillain-Barre syndrome.** *J Neuroimmunol* 2007, **192:**206-214.

78. Behrens F, Himsel A, Rehart S, Stanczyk J, Beutel B, Zimmermann SY, Koehl U, Moller B, Gay S, Kaltwasser JP, et al: **Imbalance in distribution of functional autologous regulatory T cells in rheumatoid arthritis.** *Ann Rheum Dis* 2007, **66:**1151-1156.

79. Zorn E, Kim HT, Lee SJ, Floyd BH, Litsa D, Arumugarajah S, Bellucci R, Alyea EP, Antin JH, Soiffer RJ, Ritz J: **Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease.** *Blood* 2005, **106:**2903-2911.

80. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM: **Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.** *J Exp Med* 2003, **198:**1875-1886.

81. Suri-Payer E, Amar AZ, Thornton AM, Shevach EM: **CD4+CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells.** *J Immunol* 1998, **160:**1212-1218.

82. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, Treuting P, Siewe L, Roers A, Henderson WR, Jr., et al: **Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces.** *Immunity* 2008, **28:**546-558.

83. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DA: **The inhibitory cytokine IL-35 contributes to regulatory T-cell function.** *Nature* 2007, **450:**566-569.

84. Li MO, Wan YY, Flavell RA: **T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation.** *Immunity* 2007, **26:**579-591.

85. Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, Cianfarani F, Odorisio T, Traidl-Hoffmann C, Behrendt H, et al: **Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling.** *J Clin Invest* 2009, **119:**3573-3585.

86. Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H: **Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells.** *Nat Immunol* 2009, **10:**864-871.

87. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R: **IL-22 increases the innate immunity of tissues.** *Immunity* 2004, **21:**241-254.

88. Witte E, Witte K, Warszawska K, Sabat R, Wolk K: **Interleukin-22: a cytokine produced by T, NK and NKT cell subsets, with importance in the innate immune defense and tissue protection.** *Cytokine Growth Factor Rev* 2010, **21:**365-379.

89. Zenewicz LA, Flavell RA: **Recent advances in IL-22 biology.** *Int Immunol* 2011, **23:**159-163.

90. Choi YS, Eto D, Yang JA, Lao C, Crotty S: **Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation.** *J Immunol* 2013, **190:**3049-3053.

91. Batten M, Ramamoorthi N, Kljavin NM, Ma CS, Cox JH, Dengler HS, Danilenko DM, Caplazi P, Wong M, Fulcher DA, et al: **IL-27 supports germinal center function by enhancing IL-21 production and the function of T follicular helper cells.** *J Exp Med* 2010, **207:**2895-2906.

92. Cattoretti G, Chang CC, Cechova K, Zhang J, Ye BH, Falini B, Louie DC, Offit K, Chaganti RS, Dalla-Favera R: **BCL-6 protein is expressed in germinal-center B cells.** *Blood* 1995, **86:**45-53.

93. Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M, Ho IC, Sharpe AH, Kuchroo VK: **The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells.** *Nat Immunol* 2009, **10:**167-175.

94. Avery DT, Ma CS, Bryant VL, Santner-Nanan B, Nanan R, Wong M, Fulcher DA, Cook MC, Tangye SG: **STAT3 is required for IL-21-induced secretion of IgE from human naive B cells.** *Blood* 2008, **112:**1784-1793.

95. Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ: **Molecular mechanism and function of CD40/CD40L engagement in the immune system.** *Immunol Rev* 2009, **229:**152-172.

96. Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, King C: **A fundamental role for interleukin-21 in the generation of T follicular helper cells.** *Immunity* 2008, **29:**127-137.

97. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B: **CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function.** *J Exp Med* 2000, **192:**1553-1562.

98. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, Forster R: **Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production.** *J Exp Med* 2000, **192:**1545-1552.

99. Hardtke S, Ohl L, Forster R: **Balanced expression of CXCR5 and CCR7 on follicular T helper cells determines their transient positioning to lymph node follicles and is essential for efficient B-cell help.** *Blood* 2005, **106:**1924-1931.

100. Abdel-Nasser AM, Rasker JJ, Valkenburg HA: **Epidemiological and clinical aspects relating to the variability of rheumatoid arthritis.** *Semin Arthritis Rheum* 1997, **27:**123-140.

101. Kochi Y, Suzuki A, Yamada R, Yamamoto K: **Genetics of rheumatoid arthritis: underlying evidence of ethnic differences.** *J Autoimmun* 2009, **32:**158-162.

102. Arend WP, Firestein GS: **Pre-rheumatoid arthritis: predisposition and transition to clinical synovitis.** *Nat Rev Rheumatol* 2012, **8:**573-586.

103. Rashid T, Ebringer A: **Autoimmunity in Rheumatic Diseases Is Induced by Microbial Infections via Crossreactivity or Molecular Mimicry.** *Autoimmune Dis* 2012, **2012:**539282.

104. Toussirot E, Roudier J: **Epstein-Barr virus in autoimmune diseases.** *Best Pract Res Clin Rheumatol* 2008, **22:**883-896.

105. Pierer M, Rothe K, Quandt D, Schulz A, Rossol M, Scholz R, Baerwald C, Wagner U: **Association of anticytomegalovirus seropositivity with more severe joint destruction and more frequent joint surgery in rheumatoid arthritis.** *Arthritis Rheum* 2012, **64:**1740-1749.

106. Schellekens GA, de Jong BA, van den Hoogen FH, van de Putte LB, van Venrooij WJ: **Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies.** *J Clin Invest* 1998, **101:**273-281.

107. Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, Sundin U, van Venrooij WJ: **Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis.** *Arthritis Rheum* 2003, **48:**2741-2749.

108. De Rycke L, Peene I, Hoffman IE, Kruithof E, Union A, Meheus L, Lebeer K, Wyns B, Vincent C, Mielants H, et al: **Rheumatoid factor and anticitrullinated protein antibodies in rheumatoid arthritis: diagnostic value, associations with radiological progression rate, and extra-articular manifestations.** *Ann Rheum Dis* 2004, **63:**1587-1593.

109. Edwards JC, Szczepanski L, Szechinski J, Filipowicz-Sosnowska A, Emery P, Close DR, Stevens RM, Shaw T: **Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis.** *The New England journal of medicine* 2004, **350:**2572-2581.

110. Vancsa A, Szabo Z, Szamosi S, Bodnar N, Vegh E, Gergely L, Szucs G, Szanto S, Szekanecz Z: **Longterm effects of rituximab on B cell counts and autoantibody production in rheumatoid arthritis: use of high-sensitivity flow cytometry for more sensitive assessment of B cell depletion.** *J Rheumatol* 2013, **40:**565-571.

111. Clavel C, Nogueira L, Laurent L, Iobagiu C, Vincent C, Sebbag M, Serre G: **Induction of macrophage secretion of tumor necrosis factor alpha through Fcgamma receptor IIa engagement by rheumatoid arthritis-specific autoantibodies to citrullinated proteins complexed with fibrinogen.** *Arthritis Rheum* 2008, **58:**678-688.

112. Lu MC, Lai NS, Yu HC, Huang HB, Hsieh SC, Yu CL: **Anti-citrullinated protein antibodies bind surface-expressed citrullinated Grp78 on monocyte/macrophages and stimulate tumor necrosis factor alpha production.** *Arthritis Rheum* 2010, **62:**1213-1223.

113. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, Friday S, Li S, Patel RM, Subramanian V, et al: **NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis.** *Sci Transl Med* 2013, **5:**178ra140.

114. Harre U, Georgess D, Bang H, Bozec A, Axmann R, Ossipova E, Jakobsson PJ, Baum W, Nimmerjahn F, Szarka E, et al: **Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin.** *J Clin Invest* 2012, **122:**1791-1802.

115. Iwahashi M, Yamamura M, Aita T, Okamoto A, Ueno A, Ogawa N, Akashi S, Miyake K, Godowski PJ, Makino H: **Expression of Toll-like receptor 2 on CD16+ blood monocytes and synovial tissue macrophages in rheumatoid arthritis.** *Arthritis Rheum* 2004, **50:**1457-1467.

116. Pierer M, Rethage J, Seibl R, Lauener R, Brentano F, Wagner U, Hantzschel H, Michel BA, Gay RE, Gay S, Kyburz D: **Chemokine secretion of rheumatoid arthritis synovial fibroblasts stimulated by Toll-like receptor 2 ligands.** *J Immunol* 2004, **172:**1256-1265.

117. Humby F, Bombardieri M, Manzo A, Kelly S, Blades MC, Kirkham B, Spencer J, Pitzalis C: **Ectopic lymphoid structures support ongoing production of class-switched autoantibodies in rheumatoid synovium.** *PLoS Med* 2009, **6:**e1.

118. van Hamburg JP, Asmawidjaja PS, Davelaar N, Mus AM, Colin EM, Hazes JM, Dolhain RJ, Lubberts E: **Th17 cells, but not Th1 cells, from patients with early rheumatoid arthritis are potent inducers of matrix metalloproteinases and proinflammatory cytokines upon synovial fibroblast interaction, including autocrine interleukin-17A production.** *Arthritis Rheum* 2011, **63:**73-83.

119. Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, et al: **Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage.** *J Clin Invest* 1997, **99:**1534-1545.

120. Chabaud M, Aarvak T, Garnero P, Natvig JB, Miossec P: **Potential contribution of IL-17-producing Th(1)cells to defective repair activity in joint inflammation: partial correction with Th(2)-promoting conditions.** *Cytokine* 2001, **13:**113-118.

121. Mircic M, Kavanaugh A: **Inhibition of IL6 in rheumatoid arthritis and juvenile idiopathic arthritis.** *Exp Cell Res* 2011, **317:**1286-1292.

122. Taylor PC, Feldmann M: **Anti-TNF biologic agents: still the therapy of choice for rheumatoid arthritis.** *Nat Rev Rheumatol* 2009, **5:**578-582.

123. Crispin JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, Kyttaris VC, Juang YT, Tsokos GC: **Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys.** *J Immunol* 2008, **181:**8761-8766.

124. Zhang YH, Heulsmann A, Tondravi MM, Mukherjee A, Abu-Amer Y: **Tumor necrosis factor-alpha (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways.** *J Biol Chem* 2001, **276:**563-568.

125. Niki Y, Yamada H, Seki S, Kikuchi T, Takaishi H, Toyama Y, Fujikawa K, Tada N: **Macrophage- and neutrophil-dominant arthritis in human IL-1 alpha transgenic mice.** *J Clin Invest* 2001, **107:**1127-1135.

126. Okamoto H, Yamamura M, Morita Y, Harada S, Makino H, Ota Z: **The synovial expression and serum levels of interleukin-6, interleukin-11, leukemia inhibitory factor, and oncostatin M in rheumatoid arthritis.** *Arthritis Rheum* 1997, **40:**1096-1105.

127. Gitter BD, Labus JM, Lees SL, Scheetz ME: **Characteristics of human synovial fibroblast activation by IL-1 beta and TNF alpha.** *Immunology* 1989, **66:**196-200.

128. Bunning RA, Russell RG: **The effect of tumor necrosis factor alpha and gamma-interferon on the resorption of human articular cartilage and on the production of prostaglandin E and of caseinase activity by human articular chondrocytes.** *Arthritis Rheum* 1989, **32:**780-784.

129. Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, Takaoka A, Yokochi T, Oda H, Tanaka K, et al: **T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma.** *Nature* 2000, **408:**600-605.

130. Lee SY, Kwok SK, Son HJ, Ryu JG, Kim EK, Oh HJ, Cho ML, Ju JH, Park SH, Kim HY: **IL-17-mediated Bcl-2 expression regulates survival of fibroblast-like synoviocytes in rheumatoid arthritis through STAT3 activation.** *Arthritis research & therapy* 2013, **15:**R31.

131. Katz Y, Nadiv O, Beer Y: **Interleukin-17 enhances tumor necrosis factor alpha-induced synthesis of interleukins 1,6, and 8 in skin and synovial fibroblasts: a possible role as a "fine-tuning cytokine" in inflammation processes.** *Arthritis Rheum* 2001, **44:**2176-2184.

132. Koshy PJ, Henderson N, Logan C, Life PF, Cawston TE, Rowan AD: **Interleukin 17 induces cartilage collagen breakdown: novel synergistic effects in combination with proinflammatory cytokines.** *Ann Rheum Dis* 2002, **61:**704-713.

133. Wei S, Kitaura H, Zhou P, Ross FP, Teitelbaum SL: **IL-1 mediates TNF-induced osteoclastogenesis.** *J Clin Invest* 2005, **115:**282-290.

134. Kotake S, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, Saito S, Inoue K, Kamatani N, Gillespie MT, et al: **IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis.** *J Clin Invest* 1999, **103:**1345-1352.

135. Abu-Amer Y, Erdmann J, Alexopoulou L, Kollias G, Ross FP, Teitelbaum SL: **Tumor necrosis factor receptors types 1 and 2 differentially regulate osteoclastogenesis.** *J Biol Chem* 2000, **275:**27307-27310.

136. Zhang L, Li JM, Liu XG, Ma DX, Hu NW, Li YG, Li W, Hu Y, Yu S, Qu X, et al: **Elevated Th22 cells correlated with Th17 cells in patients with rheumatoid arthritis.** *J Clin Immunol* 2011, **31:**606-614.

137. Zhang L, Li YG, Li YH, Qi L, Liu XG, Yuan CZ, Hu NW, Ma DX, Li ZF, Yang Q, et al: **Increased frequencies of Th22 cells as well as Th17 cells in the peripheral blood of patients with ankylosing spondylitis and rheumatoid arthritis.** *PLoS One* 2012, **7:**e31000.

138. Leipe J, Schramm MA, Grunke M, Baeuerle M, Dechant C, Nigg AP, Witt MN, Vielhauer V, Reindl CS, Schulze-

Koops H, Skapenko A: **Interleukin 22 serum levels are associated with radiographic progression in rheumatoid arthritis.** *Ann Rheum Dis* 2011, **70:**1453-1457.

139. da Rocha LF, Jr., Duarte AL, Dantas AT, Mariz HA, Pitta Ida R, Galdino SL, Pitta MG: **Increased serum interleukin 22 in patients with rheumatoid arthritis and correlation with disease activity.** *J Rheumatol* 2012, **39:**1320-1325.
140. Kim KW, Kim HR, Park JY, Park JS, Oh HJ, Woo YJ, Park MK, Cho ML, Lee SH: **Interleukin-22 promotes osteoclastogenesis in rheumatoid arthritis through induction of RANKL in human synovial fibroblasts.** *Arthritis Rheum* 2012, **64:**1015-1023.
141. Marijnissen RJ, Koenders MI, Smeets RL, Stappers MH, Nickerson-Nutter C, Joosten LA, Boots AM, van den Berg WB: **Increased expression of interleukin-22 by synovial Th17 cells during late stages of murine experimental arthritis is controlled by interleukin-1 and enhances bone degradation.** *Arthritis Rheum* 2011, **63:**2939-2948.
142. McGovern JL, Nguyen DX, Notley CA, Mauri C, Isenberg DA, Ehrenstein MR: **Th17 cells are restrained by Treg cells via the inhibition of interleukin-6 in patients with rheumatoid arthritis responding to anti-tumor necrosis factor antibody therapy.** *Arthritis Rheum* 2012, **64:**3129-3138.
143. Xu L, Kitani A, Fuss I, Strober W: **Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta.** *J Immunol* 2007, **178:**6725-6729.
144. Miossec P, Chomarat P, Dechanet J, Moreau JF, Roux JP, Delmas P, Banchereau J: **Interleukin-4 inhibits bone resorption through an effect on osteoclasts and proinflammatory cytokines in an ex vivo model of bone resorption in rheumatoid arthritis.** *Arthritis Rheum* 1994, **37:**1715-1722.
145. Choi J, Kim ST, Craft J: **The pathogenesis of systemic lupus erythematosus-an update.** *Current Opinion in Immunology* 2012, **24:**651-657.
146. Frieri M: **Mechanisms of disease for the clinician: systemic lupus erythematosus.** *Annals of Allergy, Asthma, & Immunology* 2013, **110:**228-232.
147. Herrmann M, Voll RE, Zoller OM, Hagenhofer M, Ponner BB, Kalden JR: **Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus.** *Arthritis Rheum* 1998, **41:**1241-1250.
148. Dieker JW, Fransen JH, van Bavel CC, Briand JP, Jacobs CW, Muller S, Berden JH, van der Vlag J: **Apoptosis-induced acetylation of histones is pathogenic in systemic lupus erythematosus.** *Arthritis Rheum* 2007, **56:**1921-1933.
149. Urbonaviciute V, Furnrohr BG, Meister S, Munoz L, Heyder P, De Marchis F, Bianchi ME, Kirschning C, Wagner H, Manfredi AA, et al: **Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: implications for the pathogenesis of SLE.** *J Exp Med* 2008, **205:**3007-3018.
150. Fransen JH, Hilbrands LB, Ruben J, Stoffels M, Adema GJ, van der Vlag J, Berden JH: **Mouse dendritic cells matured by ingestion of apoptotic blebs induce T cells to produce interleukin-17.** *Arthritis Rheum* 2009, **60:**2304-2313.
151. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V, Zychlinsky A: **Novel cell death program leads to neutrophil extracellular traps.** *J Cell Biol* 2007, **176:**231-241.
152. Urban CF, Reichard U, Brinkmann V, Zychlinsky A: **Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms.** *Cell Microbiol* 2006, **8:**668-676.
153. Trinchieri G, Santoli D: **Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis.** *J Exp Med* 1978, **147:**1314-1333.
154. Trinchieri G, Santoli D, Dee RR, Knowles BB: **Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Identification of the anti-viral activity as interferon and characterization of the human effector lymphocyte subpopulation.** *J Exp Med* 1978, **147:**1299-1313.
155. Kerkmann M, Rothenfusser S, Hornung V, Towarowski A, Wagner M, Sarris A, Giese T, Endres S, Hartmann G: **Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells.** *J Immunol* 2003, **170:**4465-4474.

156. Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, Meller S, Chamilos G, Sebasigari R, Riccieri V, et al: **Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus.** *Sci Transl Med* 2011, **3:**73ra19.

157. Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, Xu Z, Punaro M, Baisch J, Guiducci C, Coffman RL, et al: **Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus.** *Sci Transl Med* 2011, **3:**73ra20.

158. Lazear HM, Lancaster A, Wilkins C, Suthar MS, Huang A, Vick SC, Clepper L, Thackray L, Brassil MM, Virgin HW, et al: **IRF-3, IRF-5, and IRF-7 coordinately regulate the type I IFN response in myeloid dendritic cells downstream of MAVS signaling.** *PLoS Pathog* 2013, **9:**e1003118.

159. Higgs R, Ni Gabhann J, Ben Larbi N, Breen EP, Fitzgerald KA, Jefferies CA: **The E3 ubiquitin ligase Ro52 negatively regulates IFN-beta production post-pathogen recognition by polyubiquitin-mediated degradation of IRF3.** *J Immunol* 2008, **181:**1780-1786.

160. Ronnblom L, Pascual V: **The innate immune system in SLE: type I interferons and dendritic cells.** *Lupus* 2008, **17:**394-399.

161. Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, Casali P, Cerutti A: **DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL.** *Nat Immunol* 2002, **3:**822-829.

162. Liossis SN, Ding XZ, Dennis GJ, Tsokos GC: **Altered pattern of TCR/CD3-mediated protein-tyrosyl phosphorylation in T cells from patients with systemic lupus erythematosus. Deficient expression of the T cell receptor zeta chain.** *J Clin Invest* 1998, **101:**1448-1457.

163. Nambiar MP, Fisher CU, Kumar A, Tsokos CG, Warke VG, Tsokos GC: **Forced expression of the Fc receptor gamma-chain renders human T cells hyperresponsive to TCR/CD3 stimulation.** *J Immunol* 2003, **170:**2871-2876.

164. Koshy M, Berger D, Crow MK: **Increased expression of CD40 ligand on systemic lupus erythematosus lymphocytes.** *J Clin Invest* 1996, **98:**826-837.

165. Desai-Mehta A, Lu L, Ramsey-Goldman R, Datta SK: **Hyperexpression of CD40 ligand by B and T cells in human lupus and its role in pathogenic autoantibody production.** *J Clin Invest* 1996, **97:**2063-2073.

166. Balomenos D, Rumold R, Theofilopoulos AN: **Interferon-gamma is required for lupus-like disease and lymphoaccumulation in MRL-lpr mice.** *J Clin Invest* 1998, **101:**364-371.

167. Kim T, Kanayama Y, Negoro N, Okamura M, Takeda T, Inoue T: **Serum levels of interferons in patients with systemic lupus erythematosus.** *Clin Exp Immunol* 1987, **70:**562-569.

168. Chen XQ, Yu YC, Deng HH, Sun JZ, Dai Z, Wu YW, Yang M: **Plasma IL-17A is increased in new-onset SLE patients and associated with disease activity.** *J Clin Immunol* 2010, **30:**221-225.

169. Yang J, Chu Y, Yang X, Gao D, Zhu L, Wan L, Li M: **Th17 and natural Treg cell population dynamics in systemic lupus erythematosus.** *Arthritis Rheum* 2009, **60:**1472-1483.

170. Shah K, Lee WW, Lee SH, Kim SH, Kang SW, Craft J, Kang I: **Dysregulated balance of Th17 and Th1 cells in systemic lupus erythematosus.** *Arthritis research & therapy* 2010, **12:**R53.

171. Kyttaris VC, Zhang Z, Kuchroo VK, Oukka M, Tsokos GC: **Cutting edge: IL-23 receptor deficiency prevents the development of lupus nephritis in C57BL/6-lpr/lpr mice.** *J Immunol* 2010, **184:**4605-4609.

172. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE: **TNF downmodulates the function of human CD4+CD25hi T-regulatory cells.** *Blood* 2006, **108:**253-261.

173. Tyrrell-Price J, Lydyard PM, Isenberg DA: **The effect of interleukin-10 and of interleukin-12 on the in vitro production of anti-double-stranded DNA antibodies from patients with systemic lupus erythematosus.** *Clin Exp Immunol* 2001, **124:**118-125.

174. Csiszar A, Nagy G, Gergely P, Pozsonyi T, Pocsik E: **Increased interferon-gamma (IFN-gamma), IL-10 and decreased IL-4 mRNA expression in peripheral blood mononuclear cells (PBMC) from patients with systemic lupus erythematosus (SLE).** *Clin Exp Immunol* 2000, **122:**464-470.

175. Terrier B, Costedoat-Chalumeau N, Garrido M, Geri G, Rosenzwajg M, Musset L, Klatzmann D, Saadoun D, Cacoub P: **Interleukin 21 correlates with T cell and B cell subset alterations in systemic lupus erythematosus.** *J Rheumatol* 2012, **39:**1819-1828.

176. Feng X, Wang D, Chen J, Lu L, Hua B, Li X, Tsao BP, Sun L: **Inhibition of aberrant circulating Tfh cell proportions by corticosteroids in patients with systemic lupus erythematosus.** *PLoS One* 2012, **7:**e51982.

177. Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, Manku H, Vyse TJ, Roncador G, Huttley GA, et al: **Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus.** *Arthritis Rheum* 2010, **62:**234-244.

178. Wong CK, Wong PT, Tam LS, Li EK, Chen DP, Lam CW: **Elevated production of B cell chemokine CXCL13 is correlated with systemic lupus erythematosus disease activity.** *J Clin Immunol* 2010, **30:**45-52.

179. Mitoma H, Horiuchi T, Kimoto Y, Tsukamoto H, Uchino A, Tamimoto Y, Miyagi Y, Harada M: **Decreased expression of interleukin-21 receptor on peripheral B lymphocytes in systemic lupus erythematosus.** *Int J Mol Med* 2005, **16:**609-615.

180. Webb R, Merrill JT, Kelly JA, Sestak A, Kaufman KM, Langefeld CD, Ziegler J, Kimberly RP, Edberg JC, Ramsey-Goldman R, et al: **A polymorphism within IL21R confers risk for systemic lupus erythematosus.** *Arthritis Rheum* 2009, **60:**2402-2407.

181. Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, Sedgwick JD, Cua DJ: **Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation.** *J Exp Med* 2003, **198:**1951-1957.

182. Svensson L, Jirholt J, Holmdahl R, Jansson L: **B cell-deficient mice do not develop type II collagen-induced arthritis (CIA).** *Clin Exp Immunol* 1998, **111:**521-526.

183. Holmdahl R, Jansson L, Larsson A, Jonsson R: **Arthritis in DBA/1 mice induced with passively transferred type II collagen immune serum. Immunohistopathology and serum levels of anti-type II collagen auto-antibodies.** *Scand J Immunol* 1990, **31:**147-157.

184. Cohen PL, Eisenberg RA: **Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease.** *Annu Rev Immunol* 1991, **9:**243-269.

185. Chang SH, Reynolds JM, Pappu BP, Chen G, Martinez GJ, Dong C: **Interleukin-17C promotes Th17 cell responses and autoimmune disease via interleukin-17 receptor E.** *Immunity* 2011, **35:**611-621.

186. Rickel EA, Siegel LA, Yoon BR, Rottman JB, Kugler DG, Swart DA, Anders PM, Tocker JE, Comeau MR, Budelsky AL: **Identification of functional roles for both IL-17RB and IL-17RA in mediating IL-25-induced activities.** *J Immunol* 2008, **181:**4299-4310.

187. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Stevens S, Flavell RA: **Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease.** *Immunity* 2008, **29:**947-957.

188. Diveu C, McGeachy MJ, Boniface K, Stumhofer JS, Sathe M, Joyce-Shaikh B, Chen Y, Tato CM, McClanahan TK, de Waal Malefyt R, et al: **IL-27 blocks RORc expression to inhibit lineage commitment of Th17 cells.** *J Immunol* 2009, **182:**5748-5756.

189. Batten M, Li J, Yi S, Kljavin NM, Danilenko DM, Lucas S, Lee J, de Sauvage FJ, Ghilardi N: **Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells.** *Nat Immunol* 2006, **7:**929-936.

190. Kirou KA, Lee C, George S, Louca K, Peterson MG, Crow MK: **Activation of the interferon-alpha pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease.** *Arthritis Rheum* 2005, **52:**1491-1503.

191. Axtell RC, de Jong BA, Boniface K, van der Voort LF, Bhat R, De Sarno P, Naves R, Han M, Zhong F, Castellanos JG, et al: **T helper type 1 and 17 cells determine efficacy of interferon-beta in multiple sclerosis and experimental encephalomyelitis.** *Nat Med* 2010, **16:**406-412.

192. Espinosa A, Dardalhon V, Brauner S, Ambrosi A, Higgs R, Quintana FJ, Sjostrand M, Eloranta ML, Ni Gabhann J, Winqvist O, et al: **Loss of the lupus autoantigen Ro52/Trim21 induces tissue inflammation and systemic autoimmunity by disregulating the IL-23-Th17 pathway.** *J Exp Med* 2009, **206:**1661-1671.


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Lack of IL-17 receptor A signaling prevents autoimmune inflammation of the joint and gives rise to a Th2-like phenotype in collagen-induced arthritis

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ABSTRACT

Introduction IL-17A signals through the IL-17 receptor (IL-17R) A and C heterodimer. The IL-17RA serves as a common receptor subunit for several IL-17 cytokine family members. Lack of IL-17RA signaling may therefore have additional effects to lack of IL-17A alone. We therefore aim to determine the role of IL-17RA signaling in auto-immune arthritis.

Methods Disease incidence and severity were scored in wild type, IL-17RA deficient and IL-23p19 deficient mice in collagen-induced arthritis (CIA). T helper cell profiles and humoral immune responses were analyzed at several time points. Pathogenicity of T cells and total splenocytes was determined in a functional assay in vitro and IL-17RA signaling was blocked in antigen induced arthritis (AIA) in vivo.

Results IL-17RA deficient mice were completely protected against CIA comparable to IL-23p19 deficient mice. However, IL-17RA deficient mice displayed an increased number of IL-4 producing CD4 T cells, distinct from IL-17A+ CD4 T cells. This was associated with fewer plasma cells and lower pathogenic IgG2a and increased IgG1 antibody production. Both isolated CD4+ T cells and total splenocytes from IL-17RA deficient mice were less capable of inducing IL-6 production by synovial fibroblasts in CIA in a functional in vitro assay. Furthermore, blocking IL-17RA signaling in AIA reduced synovial inflammation.

Conclusions These data show the critical role of IL-17RA signaling in the development of auto-immune inflammation of the joint. In addition, lack of IL-17RA gives rise to a Th2-like phenotype characterized by IL-4 production, suggesting active involvement of IL-17RA signaling in the regulation of IL-4 in CIA.

INTRODUCTION

The interleukin 17 (IL-17) cytokine family consists of IL-17A to IL-17F (1, 2). Of this family, the function of IL-17A has been most extensively studied. Its primary role is in immune responses against extracellular pathogens (3, 4). In addition, IL-17A is also implicated in several auto-immune diseases, including systemic lupus erythematosus (SLE), multiple sclerosis (MS), psoriasis and rheumatoid arthritis (RA) (5-11). Of the other family members, IL-17F is most similar to IL-17A, suggesting that they have similar functions (12, 13). IL-17A and IL-17F are both produced by memory T helper (Th) cells, in particular Th17 cells, but also by CD8+, γδ+ and NK T cells. Recent studies show innate sources of IL-17A and production by tissue specific cells, such as Paneth cells (14-17). Both cytokines can be excreted as homodimers or as IL-17A/F heterodimers (18, 19). It has been shown that the IL-17A/A homodimer is the most potent IL-17 family effector molecule in the induction of CXCL1, followed by the IL-17A/F heterodimer and finally the IL-17F/F homodimer (19). Interestingly, human CD4+ T cells express the IL-17A/F heterodimer and IL-17F homodimer, but almost no IL-17A homodimer (19). In contrast, mouse Th17 cells secrete all three isoforms (20).

IL-17A and IL-17F signal through a heterodimer of IL-17 receptor A (IL-17RA) and IL-17RC (21, 22). The affinity of IL-17A and IL17F for IL-17RA and IL-17RC is different between mice and humans (23). In mice, IL-17RA has a higher affinity for IL-17A, however, the murine IL-17RC binds primarily IL-17F, suggesting that some differential functions might exist between IL-17A and IL-17F (23). In humans, the IL-17RA binds preferentially to IL-17A, with a low affinity to IL-17F. The IL-17RC binds IL-17A and IL-17F with the same affinity (23). Whereas IL-17RA is ubiquitously expressed, the IL-17RC is expressed mostly on non-hematopoietic cells (21, 24, 25). The IL-17 cytokine / IL-17 receptor signaling axis is quite different in sequence and structure from other cytokine families. Signaling through the IL-17RA has been extensively studied revealing the importance of SEFIR (SEF/IL-17R), Act1 and TRAF6 leading to different downstream effector signaling pathways (26).

Observations in many models of autoimmune diseases strongly implicate IL-17A as an important mediator in many autoimmune diseases, including rheumatoid arthritis (27). Interestingly, IL-17A produced by Th17 cells is involved in a pro-inflammatory feedback loop with synovial fibroblasts in RA (28). Antibodies targeting IL-17A are being tested in numerous clinical trials, and early reports are promising showing efficacy in psoriasis, RA, and autoimmune uveitis (8, 29).

Recent studies also point to roles for other IL-17 family members in arthritis. Both IL-17B and IL-17C are pro-inflammatory in CIA (30). IL-17B signals through IL-17RB and IL-17C signals through a heterodimer of IL-17RA and IL-17RE (31, 32). IL-17E (also known as IL-25) signals through an IL-17RA/IL17RB heterodimer (33). Unlike the other IL-17 family members, expression of IL-17E promotes a Th2 immune response (34-36).

IL-17A deficient mice are not fully protected against collagen induced arthritis (CIA), a mouse model for RA, nor are IL-17A/IL-17F deficient mice (24, 37). This is in contrast to Act1 deficient mice, which are fully protected against CIA, indicating that there are differences between IL-17 ligand and IL-17R downstream signaling events in the development of autoimmune collagen arthritis (38). In addition, increasing data suggests that the IL-17RA subunit might act as a common receptor subunit for other IL-17 cytokine family members. However, the functionality of IL-17RA in the development of CIA and whether lack of IL-17RA has additional value compared to IL-17A deficiency in this autoimmune model of arthritis is still unknown.

Here we show that IL-17RA knock-out mice are completely protected against the development of autoimmune inflammation and joint destruction in CIA. Both plasma cell formation and collagen type II specific antibody production were decreased in the mice compared with controls. In addition, CD4+ T cells in IL-17RA-deficient mice developed a Th2-like phenotype with increased proportions of IL-4 producing cells. These data indicate that IL-17RA signaling clearly has a broader effect in the development of arthritis compared with IL-17A and suggests that IL-17RA could be an interesting therapeutic target in RA and potentially other IL-17 driven autoimmune diseases.

MATERIALS AND METHODS

Mice

IL-17 receptor A knock-out (IL-17RAKO) mice on a C57BL/6 background (39) were kindly provided by dr. J. Tocker, Amgen, Seattle, USA. IL-23p19 knock-out (IL-23KO) mice on a C57BL/6 background (40) were kindly provided by dr. N. Ghilardi, Genentech, Inc., South San Francisco, USA. Wild type C57BL/6 controls were purchased from Harlan Laboratories B.V. (Horst, the Netherlands). All mice were kept under specified pathogen free conditions and provided with food and water *ad libitum* at the Erasmus MC Experimental Animal Facility (EDC). Mice between 8 and 12 weeks old were used for all experiments. All experiments were approved by the Erasmus MC Animal Ethics Committee (DEC).

CIA

Mice were immunized with 100µg (2 mg/mL) chicken type II collagen (CII) (Chondrex, USA) emulsified in an equal complete Freund's adjuvant (CFA) volume containing 1 mg/ml heat-killed *Mycobacterium tuberculosis* (strain H37Ra; Difco Laboratories Inc., Detroit, USA) intra-dermally in the tail base at day 0 and day 21. Arthritis development was scored macroscopically, with a maximum score of 8 per mouse. Mice with a score >6 were sacrificed for ethical reasons. Mice that had not developed arthritis at day 50 were immunized a third time. Mice were sacrificed either at day 10, day 50 or at day 69.

AIA

For induction of AIA, mice were immunized as described previously (41). One hour before intra-articular induction of arthritis, mice were injected i.p. with murine IL-17R/human IgG1 Fc fusion protein (IL-17R:Fc, kindly provided by S. D. Lyman (Immunex, Seattle, WA) or OVA, as previously described (42). These injections were repeated on day 2, 4 and 6. Arthritis severity was scored 4, 7 and 14 days after immunization by measuring swelling of the knee joint.

Influenza

Wild type and IL-17RA KO mice were infected with 10^5 $TCID_{50}$ H3N2 influenza virus X-31 as previously described (43). Mice were sacrificed 10, 17 and 29 days after infection.

Flow cytometry

For intracellular cytokine staining, splenocytes were stimulated for 4 hours with PMA (0,05 µg/ml) and Ionomycin (0,5µg/ml) in the presence of Golgi stop (BD Biosciences). $2*10^6$ cells were stained per sample. Cells were stained for extracellular markers for 20 minutes at room temperature and subsequently fixed with 2% PFA and permeabilized in 0,5% saponin. Cells were then stained for 20 minutes at room temperature for intracellular markers. Anti-CD4, anti-IL-17A, anti-IL4 and anti-IFNγ antibodies were obtained from BD BioSciences (San Diego, CA, USA) and anti-IL-10 antibody was obtained from Biolegend (San Diego, CA, USA).

For B cell stainings, $2*10^6$ splenocytes were stained for extracelllar markers for 20 minutes at room temperature. For intracellular staining, cells were subsequently fixed and permeabilized using BD Cytofix/Cytoperm (BD BioSciences) and stained for intracellular markers at room temperature for 20 minutes. Anti-CD19, anti-B220 and anti-IgM were obtained from eBioscience (San Diego, CA, USA), anti-IgD, anti-CD95, anti-IgG1 and anti-IgG2ab from BD BioSciences and anti-PNA from Sigma-Aldrich (St Louis, USA).

Samples were acquired on a FACS Canto II HTS or a FACS LSR II flow cytometer (BD BioSciences). Analysis was performed using FlowJo v7.6 research software (Tree Star Inc. Ashland, OR).

Immuno-histo-chemistry/ histology

Spleen tissue samples were frozen in Tissue-Tec O.C.T. Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) and stored at -80^{o}C. 6µm thick samples were cut. Slides (Superfrost, Gerhard Menzel GmbH, Braunschweig, Germany) were fixed in acetone. Endogenous peroxidase was blocked using 30% H2O2 (Sigma-Aldrich). Slides were stained with anti-PNA (biotinylated, Sigma-Aldrich) followed by streptavidin (peroxidase labeled, Jackson ImmunoResearch, Westgrove, PA, USA) and rat-anti-IgD (eBioscience) followed by anti-rat (alkaline phosphatase labeled, Sigma-Aldrich). Pictures were made with a NanoZoomer

(Hamamatsu Photonics Deutchland GmbH, Herrsching am Ammersee, Germany).

For histological analysis of destruction of the knee joints, knees were were hematoxylin and eosin stained as previously described (41).

ELISA

For CII specific serum antibody level measurement, wells of microtiter plates were coated with 1µg/mL chicken or mouse CII (Chondrex) over night. Serum samples and reference sample were diluted serially and incubated in the plates for three hours at room temperature. Secondary antibodies goat-anti-mouse IgM, IgG1 and IgG2a (Southern Biotechnology Associated, Inc., Birmingham, AL, USA) were incubated for one hour at room temperature. Optical density at 450nm was measured.

Measurements of IL-6 levels in day 3 culture supernatant were performed using a mouse IL-6 ELISA Duoset (R&D Systems, Minneapolis MN). The ELISAs were performed according to the manufacturer's instructions.

Culture experiments

For T cell-fibroblast co-culture experiments, mice were immunized with 100µg CII/CFA emulsion intra-dermally and sacrificed 10 days after immunization. CD4+ CD62L- T cells were FACS sorted from the spleens. Purity of the obtained fraction was >98%. $2.5*10^4$ FACS sorted T cells were co-cultured with $2*10^4$ 3T3 fibroblasts for 96 hours in the presence of anti-CD3 and anti-CD28 (both 4µg/ml, BD BioSciences) in IMDM medium (Lonza, Verviers, Belgium), supplemented with 10% FCS (Invitrogen, Carlsbad, CA), 100 U/ml Penicillin/Streptomycin, 2 mM L-Glutamin (Lonza) and 50 mM b-mercapto-ethanol (Merck, Darmstadt, Germany). After 96 hours, IL-6 levels were measured in culture supernatant by ELISA.

For CII specific re-stimulation, CII was heat inactivated for 10 minutes at 80^{o}C. 10^5 total splenocytes were co-cultured with wild type mouse synovial fibroblasts obtained from naïve mice in IMDM medium (Lonza, Verviers, Belgium), supplemented with 10% FCS (Invitrogen, Carlsbad, CA), 100 U/ml Penicillin/Streptomycin, 2 mM L-Glutamin (Lonza) and 50 mM b-mercapto-ethanol (Merck, Darmstadt, Germany) alone, with addition of 100µg/ml CII or with addition of anti-CD3 and anti-CD28 (both 4µg/ml, BD BioSciences). After 96 hours, IL-6 levels in culture supernatant were measured by ELISA.

Statistical analysis

Data was analyzed using Prism software v5.04 (GraphPad Software Inc. La Jollla, CA). An unpaired two-sided T-test was performed, unless indicated otherwise. P-values <0,05 were considered significant.

RESULTS

IL-17RA KO mice are protected against CIA

To investigate the role of IL-17RA signaling in CIA, we immunized IL-17RA knock-out (IL-17RA KO) mice, IL-23p19 knock-out (IL-23 KO) mice and wild type mice. We chose the IL-23 KO mouse line as a control in this study. In IL-23 KO mice, development of the Th17 subset is abrogated, and all Th17 cytokines are expressed, but in significantly lower amounts (44). In IL17RA KO mice, signaling of the IL-17 family is lost, but signaling of other Th17 cytokines like IL-22 and GMCSF is intact. This is important to assess whether the observed effects are specific for signaling of the IL-17R. Mice were immunized on day 0 with CFA/chicken type II collagen (CII) and immunized again on day 21. Mice that had not developed arthritis by day 50 were immunized a third time. 73% of normal, C57Bl/6 wild type animals developed arthritis and, as expected, no IL-23 KO animals developed arthritis (figure 1A-C). Interestingly, IL-17RA KO mice were completely protected against CIA, even after a third immunization. This is in contrast to IL-17A knock-out mice, which are only partially protected against CIA (37). In addition, IL-17RA KO mice did not develop synovial inflammation of the knee joints (figure 1D) and histological slides showed no infiltrates or bone destruction (figure1E-G).

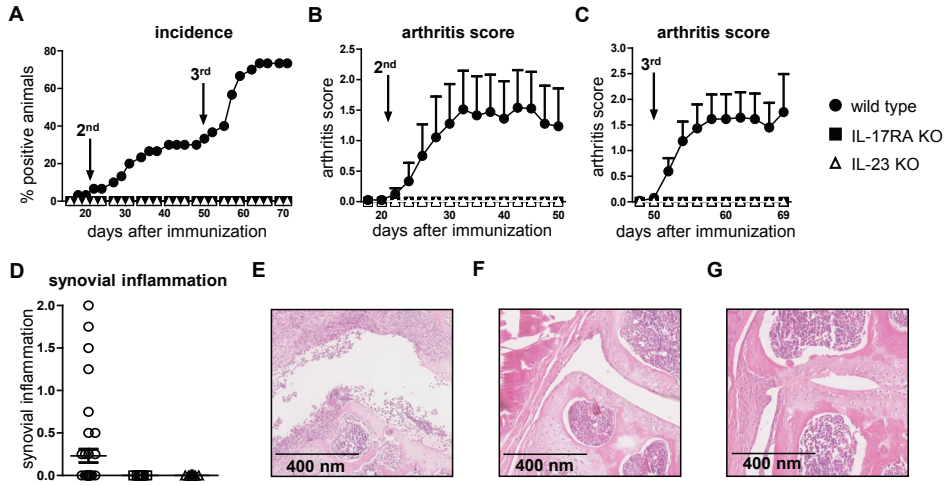


Figure 1. Arthritis incidence and severity in CIA in wild type, IL-17RA KO and IL-23 KO mice. Animals were immunized twice or three times with CFA/chicken CII i.d. [A] Arthritis incidence. [B] Arthritis score after two immunizations. [C] Arthritis score after three immunizations. [B-C] Arrows indicate immunizations. Maximum arthritis score per mouse is 8. Mean and SEM are shown for n = 7 to 40 animals per group. [D] Synovial inflammation score of the knee joints. Maximum score per knee is 2. Mean and SEM are shown for n = 12 to 40 knee joints per group. [E-G] HE stainings of knee joints of [E] positive control (FcγRIIB deficient mice on a C57Bl/6 background), [F] IL-17RA KO and [G] IL-23 KO mice. A representative image is shown for 12-40 knees per group. Figures are representative for 3 individual experiments.

CD4+ T cells in IL-17RA KO mice have a Th2 phenotype in CIA

To determine whether lack of IL-17RA signaling has an effect on T helper cell profile, we analyzed the CD4+ T cell compartment of IL-17RA KO, IL-23 KO and wild type controls in CIA. These groups of mice did not show differences in the total numbers of T cells, T cell subsets (CD4+, CD8+), not in naïve mice nor at any time point after immunization (data not shown). As determined by intracellular flow cytometry, the total numbers of cytokine expressing CD4+ T cells was similar in the three groups of naive mice. As expected, IL-23 KO mice had fewer IL-17A+ CD4+ T cells at the first two time-points, when most inflammatory cells are found in the spleen (figure 2A) (44). No significant differences were found in the number of IFNγ+ CD4+ T cells at any time point (figure 2B). Interestingly, at all three time-points after immunization, IL-17RA KO mice had a higher number of splenic IL-4+ CD4+ T cells, indicating that lack of IL-17RA signaling gives rise to a Th2-like phenotype (figure 2C). These cells are distinct from IL-17A producing CD4+ T cells, as no IL-17A and IL-4 double producing cells were found at any time point after immunization (data not shown and supplementary figure 1). Importantly, we did not find an increase in the number of IL-10 expressing CD4+ T cells in IL-17RA KO mice (figure 2D), nor in the number of FoxP3+ regulatory T cells between wild type and IL-17RA KO mice at any time point after immunization (data not shown). These findings indicate that the protection against CIA development in IL-

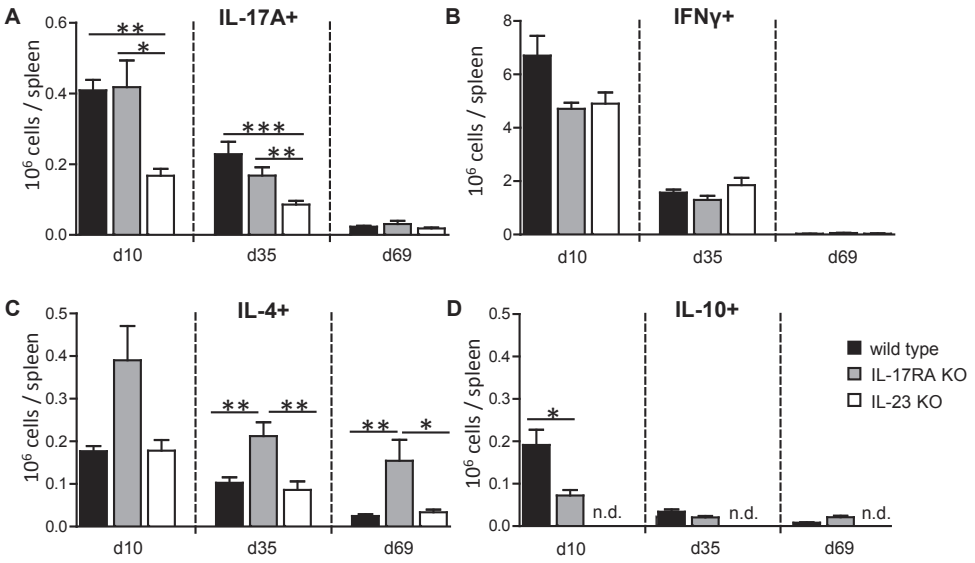


Figure 2. Cytokine expression by splenic CD4+ T cells. Total numbers of [A] IL-17A, [B] IFNγ, [C] IL-4 and [D] IL-10 expressing splenic CD4+ T cells at 10 days, 35 days and 69 days after immunization in CIA in wild type, IL-17RA KO and IL-23 KO mice. Mean and SEM are shown for n = 3 to 12 animals per group. Figures are representative for 3 individual experiments. * p<0,05; **p<0,1; *** p<0,001.

17RA-deficient mice is not caused by a higher number of regulatory T cells. Furthermore, we found no differences in the number of TNFα expressing CD4+ T cells (data not shown).

IL-4+ CD4+ T cells induce IgG1 class switching in B cells

Th1 and Th17 cells induce plasma cell formation and class switch recombination (CSR) to IgG2c producing plasma cells in C57BL/6 mice, which is essential for disease development in CIA (45). In contrast, Th2 cells induce CSR to IgG1 producing plasma cells. To investigate whether the observed shift towards a Th2 phenotype affected B cell immunity in IL-17RA KO mice, we investigated plasma cell formation and CSR in these mice. No differences were found in the total numbers of B cells, nor in specific B cell subsets (follicular, marginal zone) in the spleens of wild type and IL-17RA KO mice (data not shown), before immunization or at any time point after immunization. However, fewer plasma cells were formed in IL-17RA KO mice after three immunizations (figure 3A). Interestingly, anti-*chicken* CII IgG1 serum levels were increased in IL-17RA KO mice, compared with wild type controls at 35 days after immunization (figure 3B), indicating an increased Th2 response. No differences were found in anti-*chicken* CII IgG2c production. Additionally, we found

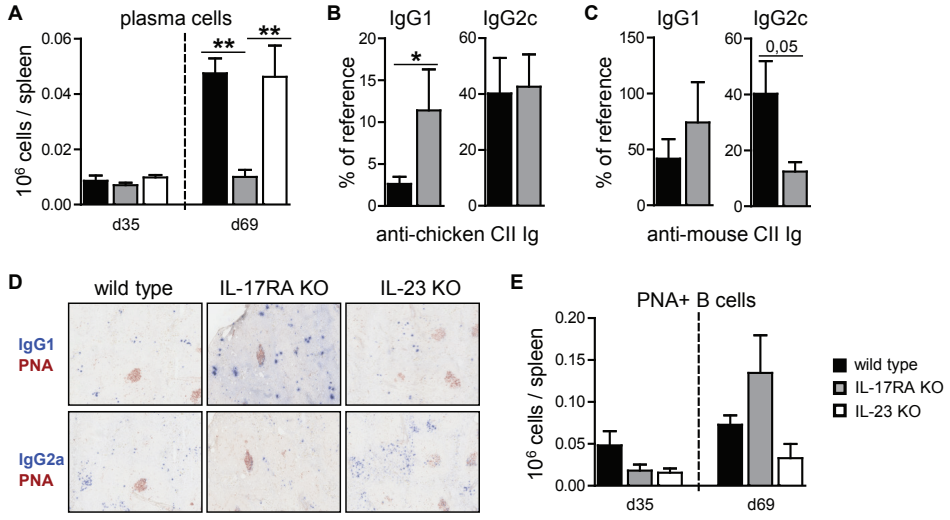


Figure 3. Plasma cells and antibody production in wild type, IL-17RA KO and IL-23KO mice in CIA. [A] Total number of splenic CD19-CD138+ plasma cells of total splenocytes after the third immunization (day 69). [B] Serum anti-*chicken* CII IgG1and IgG2c levels and [C] serum anti-*mouse* CII IgG1 and IgG2c levels in wild type and IL-17RA KO mice at 69 days after immunization. Mean and SEM are shown for n = 6 to 15 animals per group. [D] IHC stainings of splenic PNA+ GC B cells (brown) and IgG1+ or IgG2a+ plasma cells (blue) after the third immunization (day 69). A representative image is shown for n = 9-12 animals per group. [E] Total number of splenic PNA+ GC B cells in wild type and IL-17RA KO mice at 35 and 69 days after immunization. Mean and SEM are shown for n = 6 to 15 animals per group. * p<0,05; ** p<0,01.

that the induction of the pathogenic self-reactive IgG2c antibodies, directed against *mouse* CII, were not induced in IL-17RA KO mice (figure 3C), indicating that these mice were protected against development of autoimmunity. The shift towards IgG1 producing plasma cells was confirmed by immuno-histo-chemistry of the spleens after three immunizations (figure 3D). To exclude that the reduction in serum levels of IgG2c antibodies was caused by a defect in germinal center formation, we quantified the numbers of PNA+ germinal center B cells in the spleen. We found that these numbers were not affected by loss of IL-17RA signaling (figure 3E). To further exclude global defects in T cell activation in IL-17RA-deficient mice, we examined germinal center formation upon influenza virus infection. We found no differences in weight loss or cytokine production between wild type and IL-17RA KO mice, indicating that these mice are capable of mounting a normal immune response against influenza virus (supplementary figure 2A and B). In addition, we found equal proportions and total numbers PNA+ germinal center B cells in the mediastinal lymph nodes of the two groups of mice (supplementary figure 2C). Together, these data indicate that the shift towards a Th2 like phenotype in IL-17RA KO mice correlates with a less pathogenic B cell response in CIA.

IL4+ CD4+ T cells in IL-17RA KO mice in CIA are functionally protective

To investigate whether the Th2-like phenotype in IL-17RA KO mice was functionally protective in CIA, we isolated splenocytes from IL-17RA KO and wild type mice at ten days after immunization with CFA/CII. We then isolated CD4+ effector T cells by FACS sorting, defining them as CD3+CD4+CD62L-. Again, the proportion of IL-4 producing CD4+ T cells in the spleens of IL-17RA KO mice was significantly higher than in wild type mice (data not shown) and the proportions of IL-17A, IL-10 and IFNγ producing cells were comparable between wild type and IL-17RA KO mice (data not shown). The isolated T cells were then co-cultured with 3T3 fibroblasts. After three days the capacity of the T cells to induce a pro-inflammatory response by synovial fibroblasts was tested by measuring IL-6 production by the fibroblasts in the culture supernatant by ELISA. No IL-6 production was detectable in the supernatants of cultures of wild type or IL-17RA KO T cells that were cultured without fibroblasts (figure 4A). Remarkably, the change in phenotype of the T cells to a Th2-like phenotype in IL-17RA KO mice was accompanied by a lower induction of IL-6 production by fibroblasts after co-culture (figure 4A). To further determine the pathogenicity of T cells in the IL-17RA KO mice in CIA in vitro, we co-cultured total splenocytes from IL-17RA KO and wild type mice ten days after immunization with wild-type synovial fibroblasts from unimmunized mice, which do express the IL-17RA (46) in the presence of CII for three days. No IL-6 was detected in supernatant from cultures of wild-type or IL-17RA KO splenocytes cultured alone (figure 4B). Again, splenocytes from IL-17RA KO mice were less capable of inducing a pro-inflammatory response by fibroblasts (figure 4B). These data show a lower pathogenic capacity of the T

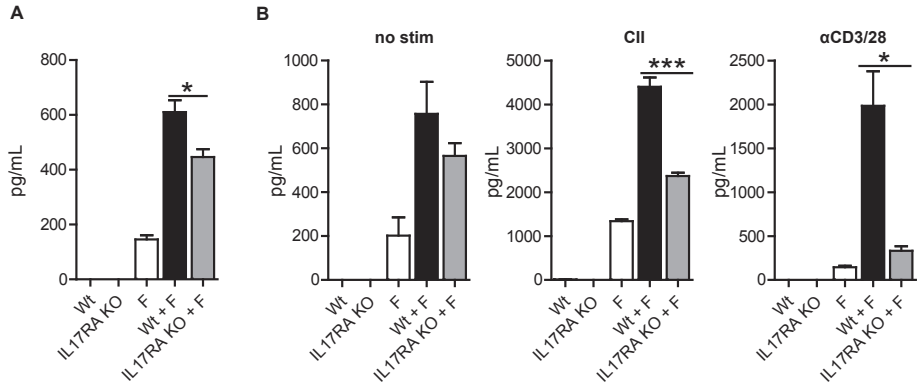


Figure 4. Pathogenicity of CD4+ T effector cells and splenocytes in wild type and IL-17RA KO mice *in vitro*. [A] CD3+ CD4+ CD62L- cells were FACS sorted from spleens of wild type and IL-17RA KO mice ten days after immunization with CII/CFA [A] $2,5*10^4$ sorted T cells were co-cultured with $2*10^4$ 3T3 fibroblasts. After three days IL-6 levels were measured in supernatant by ELISA. Mean and SEM are shown for n = 5 animals per group. [B] Splenocytes were isolated from wild type and IL-17RA KO mice ten days after immunization with CII/CFA. 10^5 total splenocytes were cocultured with $2*10^4$ synovial fibroblasts in IMDM alone or with CII or anti-CD3 and anti-CD28. After three days IL-6 levels were measured in supernatant by ELISA. Mean and SEM are shown for n = 6 animals per group. * $p<0,05$; *** $p<0,001$. Figures are representative of two individual experiments.

cells in IL-17RA KO mice and indicate that the change to a Th2-like phenotype in these mice is associated with protection against CIA.

Blocking IL-17RA signaling ameliorates arthritis development

To investigate at which stage of the T cell response IL-17RA signaling is involved, we blocked IL-17RA signaling in an antigen-induced arthritis experiment in wild-type mice. Mice were immunized with mBSA/CFA intra-dermally and boosted 21 days later. One week after the boost, arthritis was induced in right hind legs by intra-articular mBSA injection. Simultaneously, mice were injected with a soluble IL-17R protein (IL-17R:FC), which was repeated at day 2, 4 and 6 after mBSA injection. Synovial inflammation was markedly decreased in mice treated with the IL-17R:FC compared with untreated controls (figure 5), indicating that blocking IL-17RA signaling after onset of the disease is still effective. This is in line with previous work showing beneficial effects of blocking IL-17RA signaling in CIA (42). These data indicate that IL-17RA signaling has an effect mostly in the effector phase of the T cell response, after activation and priming of the T cells. Furthermore, they suggest that intervention of the IL-17RA signaling pathway could be beneficial in the treatment of arthritis.

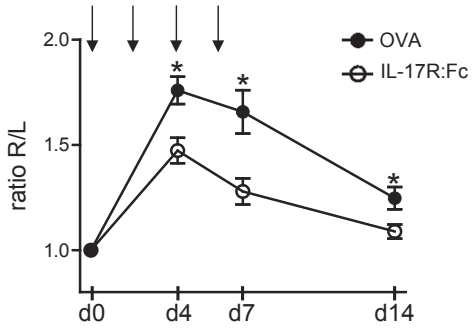
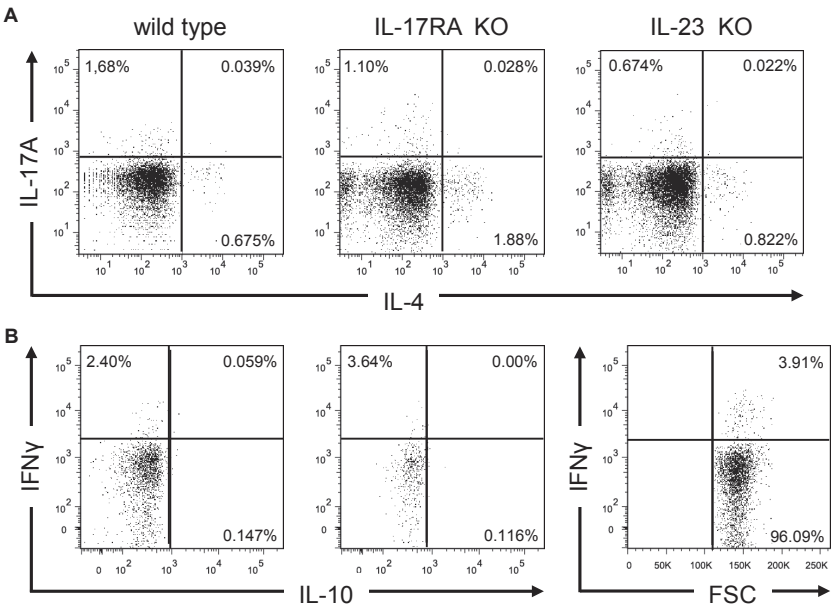
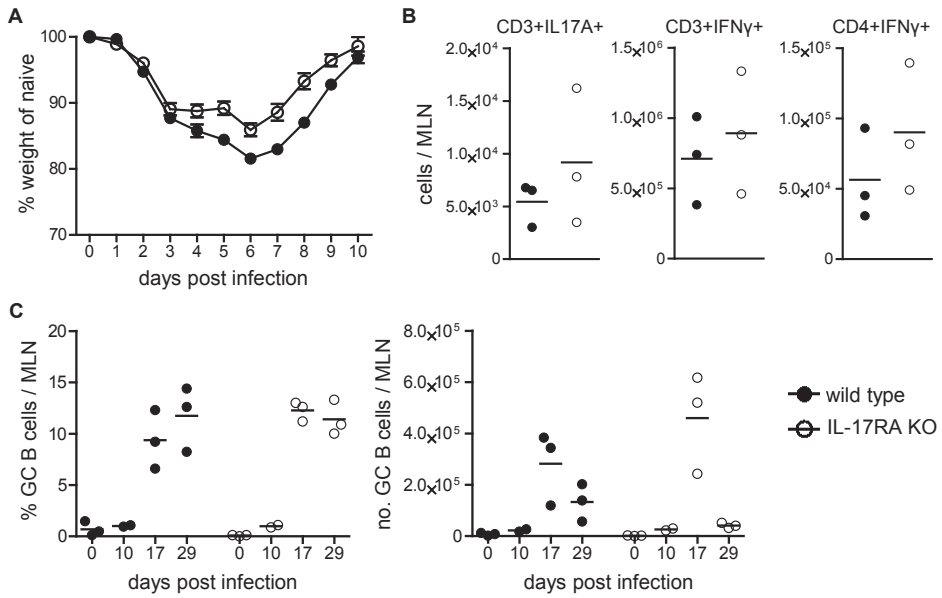


Figure 5. Blocking of IL-17RA signaling in AIA. Ratio of knee thickness of right hind knees (injected with mBSA) and left hind knees (not injected) of wild type mice injected with IL-17R:Fc or mice injected with OVA (control). Arrows indicate injections with IL-17R:Fc or OVA. Mean and SEM are shown for n = 6 or 7 mice per group. *p<0,05



Supplementary figure 1. Cytokine staining on splenic CD4+ T cells in CIA. Representative graphs of cytokine stainings of CD4+ T cells in wild type, IL-17RA KO and IL-23 KO mice at day 35 after immunization. Top panels depict IL-17A (y-axis) and IL-4 (x-axis), lower panels depict IFNγ (y-axis) and IL-10 (x-axis). Proportions indicated are proportions of CD4+ splenic T cells.



Supplementary figure 2. IL-17RA KO mice mount a normal anti-influenza immune response. [A] % weight loss in n = 16 mice per group after X31 influenza virus infection. Mean and SEM are shown. [B] Total numbers of IL-17A producing CD3+, IFNγ producing CD3+ and IFNγ producing CD4+ T cells in n = 3 mice per group in mediastinal lymph nodes 17 days after infection. [C] Proportions of total B220+ B cells with PNA+CD95+ germinal center phenotype (left) and total numbers of PNA+CD95+ germinal center B cells (right) in mediastinal lymph nodes in n = 2 or 3 mice per group. [B-C] Line indicates mean values.

DISCUSSION

Here we demonstrate that IL-17RA signaling is critical for CIA development. Although IL-17A knock-out mice were only partially protected against CIA (37), IL-17RA KO mice were fully protected, showing a complete absence of synovial inflammation and joint destruction, comparable to IL-23 KO mice. Interestingly, splenic T cells from IL-17RA KO mice showed a shift towards a Th2-like phenotype, with an increased number of IL-4 producing cells. In addition, IL-17RA KO mice had increased anti-*chicken* CII-specific IgG1 and reduced anti-*mouse* CII-specific IgG2a antibodies compared with WT mice. With a functional assay we showed that splenocytes from IL-17RA KO mice are indeed less pathogenic in CIA than wild type cells. Importantly, blocking of the IL-17RA had profound beneficial effects on arthritis development in AIA even after activation of T cells, indicating a role for IL-17RA signaling in the effector phase of the T cell response. These data provide new insight in the role of IL-17RA signaling in autoimmune arthritis and suggest active involvement of this pathway in the regulation of T cell IL-4 production in CIA.

The involvement of IL-17A as a pro-inflammatory cytokine in the induction and progression of CIA has been previously demonstrated (37, 42, 47). Using IL-17A deficient mice, Nakae et al clearly showed the involvement of IL-17A in the development of CIA (37). However, development of CIA was also observed in a small percentage (~20%) of mice lacking IL-17A, suggesting IL-17A-independent induction of autoimmune arthritis. Interestingly, these mice developed significantly less severe CIA compared with control mice (37). In the present study, we showed the essential role of the subunit IL-17RA of the IL-17 receptor in the development of CIA. In line with this, lack of Act1 which is a downstream signaling molecule in the IL-17 receptor signaling pathway, also completely prevented the development of collagen arthritis (38). These data indicate differences between IL-17 ligand, IL-17 receptor signaling / IL-17 receptor downstream signaling events in the development of autoimmune arthritis and suggest differences in efficacy using different therapeutic approaches such as neutralizing antibodies or inhibition of IL-17 receptor signaling molecules.

In a previous study, lack of IL-17F signaling had no additional effect to lack of IL-17A signaling in arthritis (24). However, IL-17B and IL-17C play an important role in CIA by inducing TNFα (30). Importantly, IL-17C binds the IL-17RE, but also requires IL-17RA/Act1 for signaling. Possibly, IL-17A and IL-17C have complementing effects in CIA, explaining the discrepancy between IL-17A KO and IL-17RA KO mice in CIA. In this context, IL-17C is also described as a factor involved in the development of Th17 cells, and the induction of IL-17A expression (32). Further studies are needed to unravel the involvement of other IL-17 family members in the IL-17RA-mediated prevention of CIA. In addition, cell-specific receptor knock-out mice should help to identify the target cells that are critical in the IL-17 mediated arthritis.

We have previously shown that by their IL-17A production Th17 cells initiate a pro-inflammatory feedback loop upon interaction with synovial fibroblasts (28). IL-17A induces IL-6 production by synovial fibroblasts, which in turn stimulates more IL-17A production by Th17 cells. In a functional assay, we showed in this report that CD4+ T cells from IL-17RA KO mice were less pathogenic in CIA in co-culture with normal synovial fibroblasts. This is remarkable, as the CD4+ cells from IL-17RA KO mice produced comparable IL-17A compared with wild type controls. Also, the synovial fibroblasts used in these cultures do express the IL-17RA (46). In addition, total splenocytes were also less pathogenic when co-cultured with synovial fibroblasts in the presence of CII, suggesting modulation through lack of IL-17RA directly on antigen specific T cell and indirectly on other cells. We found increased numbers of IL-4 producing CD4+ T cells in the spleens of IL-17RA KO mice in CIA. Although IL-4 is protective in the pro-inflammatory feedback loop, the effect of blocking IL-4 in CIA has been shown to be limited, unless IL-10 is also blocked (48). However, we found no increase in the numbers of IL-10 producing CD4+ T cells in the IL-17RA KO mice, nor in the number of IL-10 producing B cells (data not shown). It is therefore likely that more factors are involved in the loss of pathogenicity of CD4+ T cells in IL-17RA KO mice.

To our surprise, we found increased numbers of IL-4 producing CD4 T cells in IL-17RA KO mice in CIA. These cells do not co-express IL-17A, and we found hardly any IL-17A/IL-4 double positive cells. We therefore regard them as a distinct subset from Th17 cells in these mice. This means the shift towards a more Th2 like phenotype in these cells is established early in the differentiation of naïve CD4 T cells, and is not a consequence of plasticity of Th17 cells. In line with this, we found that lack of IL-17RA signaling resulted in lower auto-reactive IgG2a antibody levels and favored IgG1 antibody production. This decrease of autoreactie IgG2a was not due to impaired germinal center formation. The increase in IL-4 is remarkable, as we did not find a decrease in the numbers of IFNγ-producing cells. This suggests that signaling through the IL-17RA negatively regulates IL-4 production by CII specific CD4+ T cells in CIA. However, the mechanism involved in the shift in T cell phenotype needs to be identified.

Data from a phase II study using an IL-17RA neutralizing antibody (Brodalumab) in patients with psoriasis show significant improvement of plaques (49). In contrast, RA patients with an inadequate response to methotraxate did not show any clinical response (50). However, our data in the present study show that lack of IL-17RA signaling completely protects mice against CIA and gives rise to a Th2 like phenotype. In addition, we find that blocking IL-17RA signaling in mice decreases arthritis development in AIA even after T cells are activated. This is in line with an earlier study showing efficacy in CIA (42). The discrepancy between lack of clinical response by IL-17RA antibody treatment in patients with RA and full protection of CIA in IL-17RA-deficient mice and beneficial effects of blocking of IL-17RA signaling in AIA is not clear. Whether IL-4 was induced in this human study is not known. Differences in IL-17 receptor signaling between mouse and men are known and may influence the response. Furthermore, the stage of the disease might be critical for intervention of IL-17 receptor activity. However, data presented in the present study warrant further studies to examine specific modulation of IL-17RA signaling, possibly by direct deletion of IL-17RA activity or downstream by small molecule approaches.

REFERENCES

1. Gaffen SL. Recent advances in the IL-17 cytokine family. Curr Opin Immunol 2011;23(5):613-9.
2. Iwakura Y, Ishigame H, Saijo S, Nakae S. Functional specialization of interleukin-17 family members. Immunity 2011;34(2):149-62.
3. Dubin PJ, Kolls JK. Th17 cytokines and mucosal immunity. Immunol Rev 2008;226:160-71.
4. Curtis MM, Way SS. Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens. Immunology 2009;126(2):177-85.
5. Doreau A, Belot A, Bastid J, Riche B, Trescol-Biemont MC, Ranchin B, et al. Interleukin 17 acts in synergy with B cell-activating factor to influence B cell biology and the pathophysiology of systemic lupus erythematosus. Nat Immunol 2009;10(7):778-85.
6. Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, et al. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. Am J Pathol 2008;172(1):146-55.
7. Venken K, Hellings N, Hensen K, Rummens JL, Stinissen P. Memory CD4+CD127high T cells from patients with multiple sclerosis produce IL-17 in response to myelin antigens. J Neuroimmunol 2010;226(1-2):185-91.
8. Hueber W, Patel DD, Dryja T, Wright AM, Koroleva I, Bruin G, et al. Effects of AIN457, a fully human antibody to interleukin-17A, on psoriasis, rheumatoid arthritis, and uveitis. Sci Transl Med 2010;2(52):52ra72.
9. Chabaud M, Fossiez F, Taupin JL, Miossec P. Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. J Immunol 1998;161(1):409-14.
10. Leipe J, Grunke M, Dechant C, Reindl C, Kerzendorf U, Schulze-Koops H, et al. Role of Th17 cells in human autoimmune arthritis. Arthritis Rheum 2010;62(10):2876-85.
11. Pisitkun P, Ha HL, Wang H, Claudio E, Tivy CC, Zhou H, et al. Interleukin-17 cytokines are critical in development of fatal lupus glomerulonephritis. Immunity 2012;37(6):1104-15.
12. Starnes T, Robertson MJ, Sledge G, Kelich S, Nakshatri H, Broxmeyer HE, et al. Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. J Immunol 2001;167(8):4137-40.
13. Hymowitz SG, Filvaroff EH, Yin JP, Lee J, Cai L, Risser P, et al. IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. EMBO J 2001;20(19):5332-41.
14. Takatori H, Kanno Y, Watford WT, Tato CM, Weiss G, Ivanov, II, et al. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. J Exp Med 2009;206(1):35-41.
15. Passos ST, Silver JS, O'Hara AC, Sehy D, Stumhofer JS, Hunter CA. IL-6 promotes NK cell production of IL-17 during toxoplasmosis. J Immunol 2010;184(4):1776-83.
16. Michel ML, Keller AC, Paget C, Fujio M, Trottein F, Savage PB, et al. Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. J Exp Med 2007;204(5):995-1001.
17. Takahashi N, Vanlaere I, de Rycke R, Cauwels A, Joosten LA, Lubberts E, et al. IL-17 produced by Paneth cells drives TNF-induced shock. J Exp Med 2008;205(8):1755-61.
18. Liang SC, Long AJ, Bennett F, Whitters MJ, Karim R, Collins M, et al. An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment. J Immunol 2007;179(11):7791-9.
19. Wright JF, Guo Y, Quazi A, Luxenberg DP, Bennett F, Ross JF, et al. Identification of an interleukin 17F/17A heterodimer in activated human CD4+ T cells. J Biol Chem 2007;282(18):13447-55.
20. Chang SH, Dong C. A novel heterodimeric cytokine consisting of IL-17 and IL-17F regulates inflammatory responses. Cell Res 2007;17(5):435-40.
21. Kuestner RE, Taft DW, Haran A, Brandt CS, Brender T, Lum K, et al. Identification of the IL-17 receptor related molecule IL-17RC as the receptor for IL-17F. J Immunol 2007;179(8):5462-73.

22. Toy D, Kugler D, Wolfson M, Vanden Bos T, Gurgel J, Derry J, et al. Cutting edge: interleukin 17 signals through a heteromeric receptor complex. J Immunol 2006;177(1):36-9.

23. Ho AW, Gaffen SL. IL-17RC: a partner in IL-17 signaling and beyond. Semin Immunopathol 2010;32(1):33-42.

24. Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, et al. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. Immunity 2009;30(1):108-19.

25. Yao Z, Fanslow WC, Seldin MF, Rousseau AM, Painter SL, Comeau MR, et al. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. Immunity 1995;3(6):811-21.

26. Gaffen SL. Structure and signalling in the IL-17 receptor family. Nat Rev Immunol 2009;9(8):556-67.

27. Lubberts E. IL-17/Th17 targeting: on the road to prevent chronic destructive arthritis? Cytokine 2008;41(2):84-91.

28. van Hamburg JP, Asmawidjaja PS, Davelaar N, Mus AM, Colin EM, Hazes JM, et al. Th17 cells, but not Th1 cells, from patients with early rheumatoid arthritis are potent inducers of matrix metalloproteinases and proinflammatory cytokines upon synovial fibroblast interaction, including autocrine interleukin-17A production. Arthritis Rheum 2011;63(1):73-83.

29. Genovese MC, Van den Bosch F, Roberson SA, Bojin S, Biagini IM, Ryan P, et al. LY2439821, a humanized anti-interleukin-17 monoclonal antibody, in the treatment of patients with rheumatoid arthritis: A phase I randomized, double-blind, placebo-controlled, proof-of-concept study. Arthritis Rheum 2010;62(4):929-39.

30. Yamaguchi Y, Fujio K, Shoda H, Okamoto A, Tsuno NH, Takahashi K, et al. IL-17B and IL-17C are associated with TNF-alpha production and contribute to the exacerbation of inflammatory arthritis. J Immunol 2007;179(10):7128-36.

31. Shi Y, Ullrich SJ, Zhang J, Connolly K, Grzegorzewski KJ, Barber MC, et al. A novel cytokine receptor-ligand pair. Identification, molecular characterization, and in vivo immunomodulatory activity. J Biol Chem 2000;275(25):19167-76.

32. Chang SH, Reynolds JM, Pappu BP, Chen G, Martinez GJ, Dong C. Interleukin-17C promotes Th17 cell responses and autoimmune disease via interleukin-17 receptor E. Immunity 2011;35(4):611-21.

33. Rickel EA, Siegel LA, Yoon BR, Rottman JB, Kugler DG, Swart DA, et al. Identification of functional roles for both IL-17RB and IL-17RA in mediating IL-25-induced activities. J Immunol 2008;181(6):4299-310.

34. Kim MR, Manoukian R, Yeh R, Silbiger SM, Danilenko DM, Scully S, et al. Transgenic overexpression of human IL-17E results in eosinophilia, B-lymphocyte hyperplasia, and altered antibody production. Blood 2002;100(7):2330-40.

35. Pan G, French D, Mao W, Maruoka M, Risser P, Lee J, et al. Forced expression of murine IL-17E induces growth retardation, jaundice, a Th2-biased response, and multiorgan inflammation in mice. J Immunol 2001;167(11):6559-67.

36. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. Immunity 2001;15(6):985-95.

37. Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. J Immunol 2003;171(11):6173-7.

38. Pisitkun P, Claudio E, Ren N, Wang H, Siebenlist U. The adaptor protein CIKS/ACT1 is necessary for collagen-induced arthritis, and it contributes to the production of collagen-specific antibody. Arthritis Rheum 2010;62(11):3334-44.

39. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, et al. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. J Exp Med 2001;194(4):519-27.

40. Ghilardi N, Kljavin N, Chen Q, Lucas S, Gurney AL, De Sauvage FJ. Compromised humoral and delayed-type hypersensitivity responses in IL-23-deficient mice. J Immunol 2004;172(5):2827-33.

41. van Hamburg JP, Mus AM, de Bruijn MJ, de Vogel L, Boon L, Cornelissen F, et al. GATA-3 protects against severe joint inflammation and bone erosion and reduces differentiation of Th17 cells during experimental arthritis. Arthritis Rheum 2009;60(3):750-9.

42. Lubberts E, Joosten LA, Oppers B, van den Bersselaar L, Coenen-de Roo CJ, Kolls JK, et al. IL-1-independent role of IL-17 in synovial inflammation and joint destruction during collagen-induced arthritis. J Immunol 2001;167(2):1004-13.

43. GeurtsvanKessel CH, Willart MA, Bergen IM, van Rijt LS, Muskens F, Elewaut D, et al. Dendritic cells are crucial for maintenance of tertiary lymphoid structures in the lung of influenza virus-infected mice. J Exp Med 2009;206(11):2339-49.

44. Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, et al. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J Exp Med 2003;198(12):1951-7.

45. Svensson L, Jirholt J, Holmdahl R, Jansson L. B cell-deficient mice do not develop type II collagen-induced arthritis (CIA). Clin Exp Immunol 1998;111(3):521-6.

46. Lubberts E, Schwarzenberger P, Huang W, Schurr JR, Peschon JJ, van den Berg WB, et al. Requirement of IL-17 receptor signaling in radiation-resistant cells in the joint for full progression of destructive synovitis. J Immunol 2005;175(5):3360-8.

47. Joosten LA, Radstake TR, Lubberts E, van den Bersselaar LA, van Riel PL, van Lent PL, et al. Association of interleukin-18 expression with enhanced levels of both interleukin-1beta and tumor necrosis factor alpha in knee synovial tissue of patients with rheumatoid arthritis. Arthritis Rheum 2003;48(2):339-47.

48. Joosten LA, Lubberts E, Durez P, Helsen MM, Jacobs MJ, Goldman M, et al. Role of interleukin-4 and interleukin-10 in murine collagen-induced arthritis. Protective effect of interleukin-4 and interleukin-10 treatment on cartilage destruction. Arthritis Rheum 1997;40(2):249-60.

49. Papp KA, Leonardi C, Menter A, Ortonne JP, Krueger JG, Kricorian G, et al. Brodalumab, an anti-interleukin-17-receptor antibody for psoriasis. N Engl J Med 2012;366(13):1181-9.

50. Abstracts of the American College of Rheumatology & Association of Rheumatology Health Professionals, Annual Scientific Meeting. November 9-14, 2012. Washington, D.C., USA. Arthritis Rheum 2012;64(10 Suppl):S1-1216.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IL-17/Th17 mediated synovial inflammation is IL-22 independent

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ABSTRACT

Background IL-17A and Th17 cells are critically involved in T cell-mediated synovial inflammation. Besides IL-17A, Th17 cells produce IL-22. Recently, Th22 cells were discovered, which produce IL-22 in the absence of IL-17. However, it remains unclear whether IL-22 and Th22 cells contribute to T cell-mediated synovial inflammation. Therefore, we examined the potential of IL-22 and Th22 cells to induce synovial inflammation and whether IL-22 is required for T cell-mediated experimental arthritis.

Methods Peripheral and synovial Th17 and Th22 cells were identified and sorted from patients with rheumatoid arthritis (RA). Co-culture experiments of these primary T cell populations with RA synovial fibroblasts (RASF) were performed. The *in vivo* IL-22 contribution to synovial inflammation was investigated by inducing T cell-mediated arthritis in IL-22 deficient mice and wild-type mice.

Results Peripheral Th17 and Th22 cell populations were increased in patients with RA and present in RA synovial fluid. In T cell-RASF co-cultures, IL-22 in the presence of IL-17A had limited effects on IL-6, IL-8, MMP-1 and MMP-3 production. Furthermore, primary peripheral blood and synovial Th17 cells were more potent in the induction of these factors by RASF compared to Th22 cells. In line with this, similar synovial inflammation and disease severity was found between IL-22 deficient and wild-type mice in T cell-mediated experimental arthritis.

Conclusion These findings show that IL-17A/Th17 cell-mediated synovial inflammation is independent of IL-22 and Th22 cells. This implies that targeting IL-17A/Th17 cells, rather than IL-22/Th22 cells, should be the focus for treatment of T cell-mediated synovial inflammation.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by synovial inflammation and destruction of cartilage and bone [1, 2]. Pro-inflammatory cytokines, such as TNF-a and IL-17A, play a central role in RA [3, 4]. IL-17A producing T-helper-17 (Th17) cells are implicated in the pathogenesis of RA and other T cell-mediated diseases [5-10]. In patients with RA elevated proportions of peripheral and synovial Th17 cells were found [11-14] and the pathogenic role for IL-17A in arthritis has been shown in experimental mouse models [15, 16]. Furthermore, we have shown that Th17 cells from patients with RA, induce a pro-inflammatory loop upon interaction with RA synovial fibroblasts (RASF). This loop includes autocrine IL-17A production, which may explain the progression of an early inflammatory arthritis to a chronic destructive arthritis [14, 17].

Besides IL-17A, Th17 cells express the cytokine IL-22 [18, 19]. Among target cells of IL-22 are mainly tissue epithelial cells. IL-22 is involved in antimicrobial defense and tissue regeneration [20-22]. The IL-22 receptor is not expressed by immune cells and no effects of IL-22 have been identified in these cells. In addition to T cells, TCRgd, lymphoid tissue inducer (LTi) and NK cells also produce IL-22 [23, 24]. Recently, the novel human Th22 cell subset was identified producing high levels of IL-22 in the absence of IL-17A production. Th22 cells are implicated in skin homeostasis and pathology [25] and IL-22 production in T cells is dependent on the aryl hydrocarbon receptor (AHR) and the transcription factor ROR-C [26, 27]. Besides cytokine production, Th17 and Th22 cells can be characterized by a specific chemokine receptor expression pattern. Whereas both Th17 and Th22 cells are negative for CXCR3 expression and positive for CCR6 and CCR4 expression, only Th22 cells are positive for the expression of CCR10 [25, 27, 28].

In patients with RA increased IL-22 expression levels or elevated proportions of IL-22 producing T cells were found in peripheral blood and in the inflamed synovium [11, 29-31]. The pathological role of IL-22 in RA has been suggested by an association of IL-22 serum levels with bone erosions. Also elevated Th22 and Th17 cell proportions were found in patients with RA compared to healthy controls [32-34]. In addition, RASF have been shown to produce IL-22 and respond to IL-22 with increased proliferation and chemokine production [35]. Moreover, IL-22 deficient (IL-22$^{-/-}$) mice develop less severe collagen induced arthritis (CIA) compared to wild-type mice, indicating an involvement of IL-22 in the pathogenesis of CIA [36].

However, the involvement of IL-22 in autoimmune disease development appears to be context dependent. In skin inflammatory disorders, such as psoriasis, IL-22 acts as a key mediator of pathogenic psoriatic skin features [37]. Moreover, IL-22 produced by innate and adaptive immune cells is required for imiquimod induced skin inflammation in mice [38]. In contrast, IL-22 is not required for the development of experimental autoimmune encephalomyelitis [39] and IL-22 is even protective for experimental inflammatory bowel disease [40]. These discrepancies in the role of IL-22 in autoimmune disease development and the various IL-22 target cells and

sources of IL-22 production prompted us to investigate the direct effect of IL-22 and Th22 cells in T cell-mediated synovial inflammation. Using human *in vitro* T cell-RASF co-cultures and murine T cell-mediated experimental arthritis, we show that IL-22 and Th22 cells do not contribute to IL-17/Th17 mediated synovial inflammation.

METHODS

Subjects

For this report 10 healthy volunteers (8 women and 2 men, mean age ± SD; 47.4 ± 24.5), 10 treatment naive patients with early RA (6 women and 2 men, mean age ± SD; 49.7 ± 13.7) and 8 patients with established RA and active disease were studied. All patients fulfilled the American College of Rheumatology 1987 revised criteria for RA. Blood was obtained at the second visit after obtaining informed consent. Clinical and laboratory data of treatment naive patients with early RA are shown in supplementary table 1. This study was embedded in the Rotterdam Early Arthritis Cohort Study (REACH) and was approved by the Medical Ethics Committee of the Erasmus MC Rotterdam.

Supplementary Table 1. Clinical and laboratory data

ACR 1987 criteria	(No patients (%))*
Morning stiffness	5/8 (63)
Arthritis of 3 or more joint areas	6/8 (75)
Arthritis of hand joints	7/8 (88)
Symmetric arthritis	7/8 (88)
Rheumatoid nodules	0/8 (0)
Serum rheumatoid factor positive	5/8 (63)
Serum ACPA positive	7/8 (88)
Bone erosions	0/8 (0)
Clinical parameters	(mean ± S.E.M.; range)
DAS 28 4.3 ± 0.4 (2.7-5.8)	
Swollen joint count 3.6 ± 0.8 (0-7)	
Tender joint count 5.3 ± 1.5 (0-13)	
Disease duration (month)	4.3 ± 1.2 (1-11)

*All patients were classified as RA ≥ 4 criteria

Animals

IL-22$^{-/-}$ mice on the C57BL/6 background [21] were kindly provided by Dr. Wenjun Ouyang,

Genentech Inc., USA. Wild-type C57BL/6 mice were purchased from Harlan Laboratories B.V. (Horst, the Netherlands). Mice were kept under specific pathogen free conditions and provided with food and water *ad libitum*. Mice between 8-12 weeks of age were used for experiments. All experiments were approved by the Erasmus MC Animal Ethics Committee (DEC).

Flow cytometry and cell sorting

Monoclonal antibody preparations, intracellular cytokine detection, flow cytometry and cell sorting were described previously [14, 41]. The following human monoclonal antibodies (MoAb) were obtained from BD Biosciences (San Diego, CA): CD45RO, CCR6, CD4 and IFN-g. IL-22 and IL-17A MoAb were obtained from eBioscience (San Diego, CA). Murine CD4, CD8, IL-17A and IFN-g MoAb were obtained from BD Biosciences and TCRgd MoAb from Biolegend Inc. (San Diego, CA). Samples were acquired on a FACScantoII flow cytometer (BD Biosciences) and analyzed using FlowJo v7.6 research software (Tree Star Inc. Ashland, OR). T cell populations were sorted from peripheral blood mononuclear cells (PBMC) or synovial fluid mononuclear cells (SFMC) using a FACSAria cell sorter (BD Biosciences).

Cell cultures

RASF isolation and subsequent culture has been described [14]. 10.0 x 10^3 RASF were co-cultured with sorted allogeneic 25.0 x 10^3 peripheral blood total CCR6+ T, Th17 or Th22 cells or 3.0-10.0x10^3 synovial fluid Th17 or Th22 cells. Cells were cultured for 72 hours in Iscove's Modified Dulbecco's Media (IMDM, Lonza, Verviers, Belgium), supplemented with 10% FCS (Invitrogen, Carlsbad, CA), 100 U/ml Penicillin/Streptomycin, 2 mM L-Glutamin (Lonza) and 50 mM b-mercapto-ethanol (Merck, Darmstadt, Germany) and stimulated with soluble aCD3 and aCD28 (0.3 mg/ml and 0.4 mg/ml respectively, Sanquin, Amsterdam, The Netherlands). Cells were cultured in the absence or presence of 10 mg/ml neutralizing IL-22 Moab (R&D systems, Minneapolis, MN) or 2 mM FICZ (Enzo Life Sciences Inc., Farmingdale, NY)

Cytokine measurements

Human IL-6, IL-8 and IFN-g production was determined using ELISA (Invitrogen). Human, IL-17A, IL-22, TNF-a, MMP-1, MMP-3 and murine IL-17 expression was measured using Duoset ELISA kits (R&D systems, Minneapolis, MN). ELISA was performed according to the manufacturer's instructions.

T cell-mediated arthritis

8 mg/ml methylated bovine serum albumin (mBSA, Sigma-Aldrich, St. Louis, MO) was emulsified in an equal volume of complete freund's adjuvant (CFA), containing 1 mg/ml heat-killed

Mycobacterium tuberculosis (strain H37Ra; Difco Laboratories Inc., Detroit, Mi). At day –7, mice were immunized by intra-dermal injection of 100 ml mBSA/CFA emulsion into the tail base. On day 0, arthritis was induced by injecting mice intra-articularly (i.a.) with 60 mg mBSA in 6 ml 0.9% NaCl into both knee joints. The arthritis severity was scored macroscopically, 7 days after immunization, on a scale of 0 to 2. Rear limbs were removed and prepared for histology. Sections were hematoxylin and eosin stained as previously described [41]. The analysis of murine IL-17A cytokine expression in synovial washouts was performed as described previously [41]. To determine mBSA specific effector cell responses, draining lymph node cells were cultured in the presence or absence of 50 ug/ml mBSA for 3 days. IL-17A production was measured by ELISA. To measure DNA synthesis, cells were pulsed at day 2 with thymidine [^{3}H] for 16–20 h, harvested, and counted using standard methods.

Statistical analysis

Differences between experimental groups were tested with a two-sided paired t-test or stated otherwise, using Prism software v5.04 (GraphPad Software Inc. La Jollla, CA). *P*-values <0.05 were considered significant.

RESULTS

Both Th17 and Th22 cells are increased in peripheral blood of RA patients and present in the inflamed synovium

Recently, we have shown that the proportion of IL-17A and IL-22 producing CD4+CD45RO+ (memory) CCR6+ T cells was increased in patients with early RA [14, 29]. Through a flow cytometric approach combining specific chemokine and cell surface receptor expression it is now possible to distinguish in more detail Th17 and Th22 cells, without the need to perform intracellular cytokine stainings [25, 27, 28]. First, memory CD25-CCR6+ T cells were gated to exclude CD25+ regulatory T cells. Subsequently, Th17 and Th22 cells were defined within this CCR6+ gate as CXCR3-CCR4+ whereby Th22 cells, but not Th17 cells were CCR10+ (Figure 1A).

By following this gating strategy, significantly increased peripheral blood memory total CCR6+ T cell, Th17 cell and Th22 cell proportions were identified within the total memory T cell population of patients with early RA compared to age and sex matched healthy controls. The increase of Th17 and Th22 cell proportions (~2.5 and ~3.3 fold respectively) was even relatively larger than the total CCR6+ T cell population (~1.6 fold) (Figure 1B).

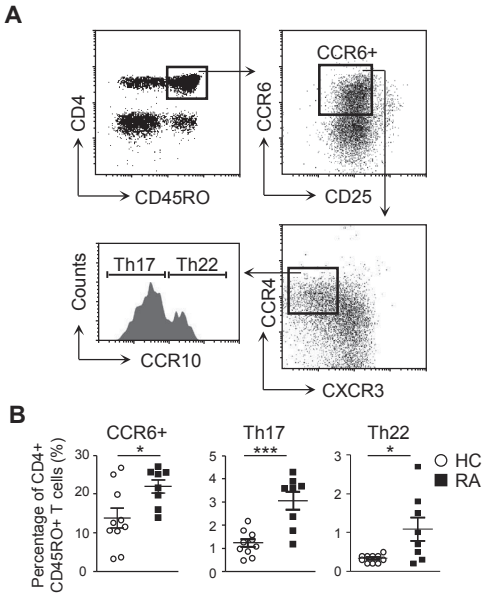


Figure 1. Th17 and Th22 cell populations are increased in peripheral blood of patients with early RA compared to age and sex matched healthy individuals. (A) Flow cytometric gating strategy for the identification of peripheral blood Th17 and Th22 populations. CCR6+ T cells are defined as CD4+CD45RO+CCR6+CD25- and within this CCR6+ T cell population, Th17 cells are defined as CXCR3-CCR4+CCR10- and Th22 cells as CXCR3-CCR4+CCR10+ (B) Flow cytometric analysis for total CCR6+ T, Th17 and Th22 cells in PBMC of patients with early RA (black squares) and age and sex matched healthy controls (HC, open circles). Mean and SEM are given in a scatter plot for 10 healthy controls (HC) and 8 RA patients. For statistical analysis a two-sided unpaired t-test was performed (* = $p < 0.05$, *** = $p < 0.001$).

In addition, total memory CCR6+ T cell, Th17 cell and Th22 cell populations were present in matched peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) from patients with established RA and active disease. Moreover, total memory CCR6+ T cell, Th17 cell and Th22 cell proportions were similar in SFMC compared to PBMC (Figure 2A). The presence of Th17 (IL-17A+IL-22+/-) and Th22 (IL-17A-IL-22+) cells in SFMC was also indicated by performing intracellular IL-17A, IL-22 and IFNg stainings (Figure 2B).

These data show that in addition to the total memory CCR6+ T cell population, Th17 and Th22 cells are increased in peripheral blood of patients with early RA and that both Th17 and Th22 cells are present in synovial fluid.

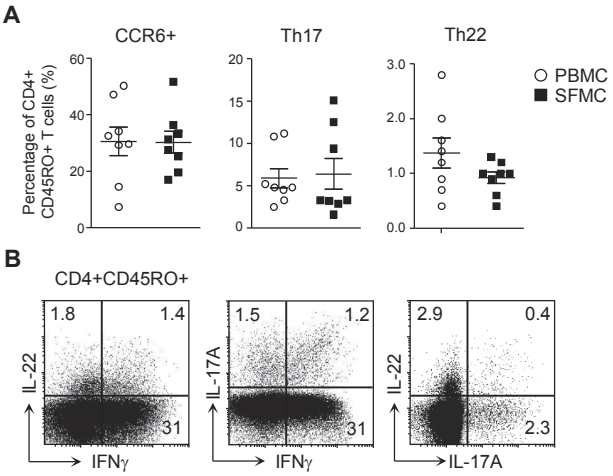


Figure 2 Identification of Th17 and Th22 cells in peripheral blood and synovial fluid of patients with established RA. (A) Flow cytometric analysis for total CCR6+ T cells, Th17 and Th22 cells in PBMC (open circles) and SFMC (black squares). Mean and SEM are given in a scatter plot for 8 patients with established RA and active disease per group. (B) Flow cytometric analysis for intracellular IL-17A, IL-22 and IFN-g cytokine expression in CD4+CD45RO+ synovial T cells in patients with established RA. Numbers in representative dot plots indicate the proportion of cytokine expressing cells per quadrant.

IL-17A, but not IL-22 is up-regulated in CCR6+ T cell-RASF cultures

The interaction of CCR6+ T cells and RASF induces a pro-inflammatory loop leading to increased autocrine IL-17A production [14]. To verify whether this interaction leads to increased IL-22 production as well, CCR6+ T cell cultures with or without RASF were analyzed for IL-17A, IL-22 and TNF-a production. Increased IL-17A producing CCR6+ T cell proportions and increased IL-17A protein levels were detected in CCR6+ T cell RASF co-cultures compared to CCR6+ T cell mono-cultures as shown before [14]. In contrast, no difference was found in the fraction of IL-22 producing CCR6+ T cells as well as IL-22 and TNF-a protein levels (Figure 3A and 3B). This clearly shows a specific induction of IL-17A, but not of IL-22 and TNF-a production by CCR6+ T cells upon RASF interaction.

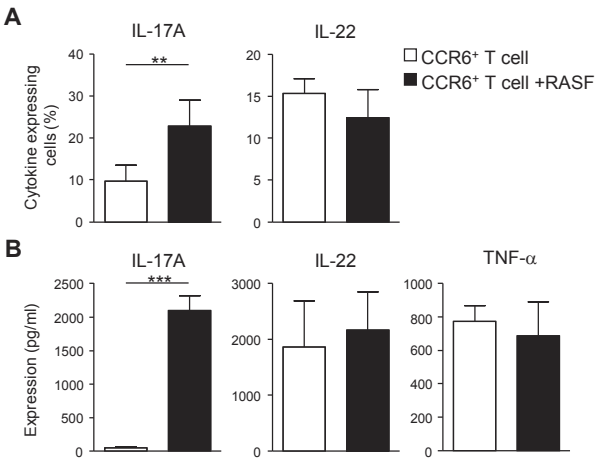


Figure 3 IL-22 production by CCR6+ T cells is not induced upon interaction with RASF. (A-B) Cultures of 25.0 x 10^3 sorted CCR6+ T cells from patients with early RA in the presence or absence of 10.0 x 10^3 RASF. Cells were cultured for 3 days in the presence of aCD3 and aCD28. (A) Flow cytometric analysis for intracellular IL-17Aand IL-22 expression in CCR6+ T cell cultures (white bars) and CCR6+ T cell RASF co-cultures (black bars). (B) IL-17A, IL-22 and TNF-a expression levels were determined by ELISA in supernatant of CCR6+ T cell cultures (white bars) and CCR6+ T cell RASF co-cultures (black bars). Mean and SEM are given for 5-7 treatment-naive early RA patients per group (** = $p < 0.01$, *** = $p < 0.001$).

IL-22 has limited effects on IL-6, IL-8, MMP-1 and MMP-3 production in CCR6+ T cell-RASF cultures

Upon interaction with CCR6+ T cells, RASF produce pro-inflammatory mediators such as IL-6, IL-8 and tissue destructive enzymes, such as matrix metalloproteinase-1 (MMP-1) and MMP-3. This production is largely dependent on IL-17A and TNF-a [14]. However, the contribution of IL-22 to IL-6, IL-8, MMP-1 and MMP-3 induction is unclear. Therefore, CCR6+ T cell-RASF co-cultures were performed wherein IL-22 signalling was neutralized by anti-IL-22 antibodies, or wherein IL-22 production was induced by an AHR agonist, 6-formylindolo[3,2-b]carbazole (FICZ) [27]. IL-22 neutralization had no effects on IL-17A, IL-6, IL-8, MMP-1 and MMP-3 production in CCR6+ T cell-RASF co-cultures (Figure 4). Treatment of CCR6+ T cell cultures with FICZ resulted in a ~4.7 fold induction of IL-22 expression. This had a slight, but not significant inhibitory effect on IL-17A production in CCR6+ T cell-RASF co-cultures. This effect on IL-17A was accompanied by a significant reduction of IL-6, but not of IL-8, MMP-1 and MMP-3 production. These findings show that IL-22 has limited effects on pro-inflammatory cytokine and MMP production in CCR6+ T cell-RASF co-cultures in the presence of IL-17A.

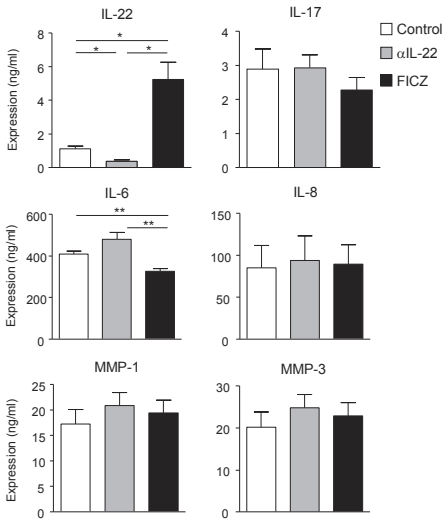


Figure 4 IL-22 has limited effects on pro-inflammatory cytokines and MMPs produced by RASF upon interaction with CCR6+ T cells. 10.0 x 10^3 RASF were co-cultured with 25.0 x 10^3 sorted CCR6+ T cells from patients with early RA. Cells were cultured for 3 days in the presence of aCD3 and aCD28 and in the absence or presence of aIL-22 (10 mg/ml) or FICZ (2 mM). Indicated cytokines and MMPs were determined in supernatant by ELISA. Mean and SEM are given for 5 treatment-naive early RA patients per group. Results are representative for at least 3 independent experiments (* = $p < 0.05$, ** = $p < 0.01$).

Th22 cells are less potent inducers of IL-6, IL-8 and MMP-1 production by RASF compared to Th17 cells

In addition, the effects of primary Th17 or Th22 cells from patients with early RA on RASF were investigated. Therefore, primary Th17 and Th22 cells were sorted according to the gating strategy as shown in figure 1A and cultured in the presence of RASF. To verify the phenotype of the sorted Th17 and Th22 cells, intracellular flow cytometric staining for IL-17A and IL-22 was performed. Th17 and Th22 cells both produced IL-17A and IL-22, but IL-17A production was higher in Th17 cells (4.4% vs. 1.3% in Th22) and IL-22 production was higher in Th22 cells (9.3% vs. 2.0% in Th17) (Figure 5A). These differences were reflected by protein expression levels in the culture supernatant. Th17 cells expressed higher levels of IL-17A compared to Th22 cells and IL-22 was expressed by both Th17 and Th22 cells. Furthermore, slightly higher levels of IFN-g and TNF-a were expressed by Th17 cells compared to Th22 cells (Figure 5B).

Importantly, compared to peripheral blood Th17 cells, Th22 cells were less efficient in the induction of IL-6 and MMP-1 production by RASF (Figure 5B). To verify whether this phenomenon is also true for synovial Th22 cells, synovial Th17 and Th22 cells were sorted and co-cultured

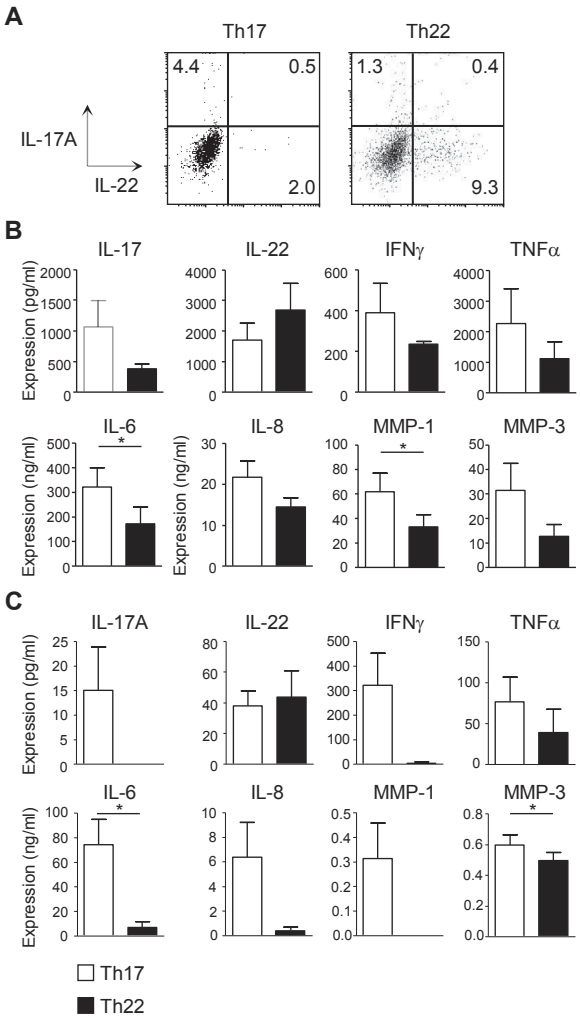


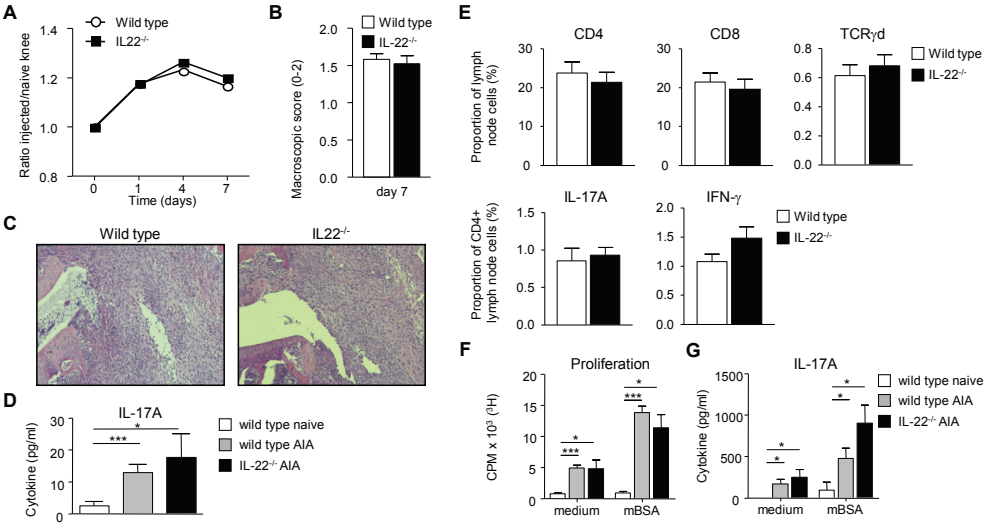
Figure 5 Effects of primary Th17 and Th22 cells of patients with early RA in RASF co-cultures. (A-B) 10.0 x 10^3 RASF were co-cultured with 25.0 x 10^3 sorted primary Th17 or Th22 cells from patients with early RA. Cells were cultured for 3 days in the presence of aCD3 and aCD28. (A) Flow cytometric analysis for intracellular IL-17A and IL-22 expression by primary Th17 or Th22 cells, co-cultured for 3 days with RASF. Numbers in representative dot plot represents proportion of cytokine expressing cells per quadrant. (B) Indicated cytokines and MMPs were determined in supernatant by ELISA. Mean and SEM are given for 5 treatment-naive early RA patients per group. (C) 10.0 x 10^3 RASF were co-cultured with 3.0 – 10.0 x 10^3 sorted primary synovial fluid Th17 or Th22 cells from patients with early RA. Indicated cytokines and MMPs were determined in supernatant by ELISA. Mean and SEM are given for 4 patients with established RA per group (* = $p < 0.05$, ** = $p < 0.01$).

with RASF. After co-culture, IL-17A and IFN-g expression was restricted to Th17-RASF co-cultures, whereas both Th17-RASF and Th22-RASF co-cultures expressed IL-22 and TNF-a. Compared to synovial Th17 cells, synovial Th22 cells are less potent in inducing IL-6 and MMP-3 production by RASF (Figure 5C).

When taken together, compared to peripheral and synovial Th17 cells, Th22 cells are less efficient in inducing pro-inflammatory cytokine and MMP production by RASF.

IL-22 does not contribute to IL-17A/Th17 mediated synovial inflammation in antigen induced arthritis

To investigate whether IL-22 directly contributes to T cell-mediated synovial inflammation *in vivo*, the murine mBSA antigen induced arthritis (AIA) model was used. This model is largely T cell-mediated and dependent on IL-17A. Furthermore, T cells in this model express IL-22 [41, 42]. AIA was induced in IL-22 deficient (IL-22$^{-/-}$) mice and wild-type mice. Knee swelling in time was measured and arthritis severity was macroscopically scored. No difference in knee swelling, macroscopic inflammation score, histology and IL-17A production between arthritic wild-type and IL-22$^{-/-}$ mice was detected, indicating that in the presence of IL-17A, IL-22 is not directly involved in T cell-mediated synovial inflammation in AIA (Figure 6A-D). This was further supported by similar CD4, CD8 and TCRgd T cell proportions, and similar proportions of IL-17A and IFN-g producing CD4 T cells in arthritic IL-22$^{-/-}$ and wild-type draining lymph nodes (Figure 6E . In addition, similar mBSA specific proliferation or cytokine production responses were observed between arthritic IL-22$^{-/-}$ or wild-type draining lymph node cells (Figure 6E and 6F).



Taken together, these findings show that lack of IL-22 has no *in vivo* contribution to IL-17A/Th17 mediated synovial inflammation in AIA.

DISCUSSION

By using cultures with primary human T cells of treatment naive patients with early RA and T cell-mediated arthritis in IL-22$^{-/-}$ mice, we showed that IL-17A/Th17 mediated synovial inflammation is independent of IL-22. IL-22 produced by CCR6+ T cells had limited effects on RASF produced pro-inflammatory cytokines IL-6, IL-8 and tissue destructive enzymes MMP-1 and MMP-3. In addition, primary human peripheral or synovial Th22 cells were markedly less potent than Th17 cells in inducing IL-6, MMP-1 and MMP-3 production by RASF. Moreover, deficiency of IL-22 *in vivo* has no significant effect on IL-17A/Th17 mediated synovial inflammation in AIA.

Despite, the limited observed effects of IL-22 and Th22 cells on IL-17A/Th17 mediated synovial inflammation, increased IL-22 levels or Th22 cell proportions are found in patients with early RA and are present in synovial fluid. Interestingly, Th22 cells correlate with elevated proportions of Th17 cells in patients with RA [32]. This and the shared developmental program and dependence on similar transcriptional regulators and cytokines, such as ROR-C and IL-23 [19], may imply that IL-22 expression is commonly accompanied with IL-17A mediated function. This may be highly relevant in local mucosal immune responses against microorganisms [19, 23, 24], whereas this is less relevant in non-mucosal environments, such as the joint synovium. We found an increase in IL-17A production, but not in IL-22 production, by CCR6+ T cells in

← **Figure 6 Effects of IL-22 deficiency in antigen induced arthritis (AIA).** Arthritis was induced in both IL-22$^{-/-}$ and wild type mBSA immunized mice by an intra-articular injection with 60 mg mBSA. (A) Synovial inflammation of wild-type (black squares) and IL-22$^{-/-}$ mice scored as the ratio between knee thickness before onset and 1, 4 and 7 days after onset of arthritis. Mean is given for 24 knee joints of 12 mice per group per time point. (B) Macroscopical scores of synovial inflammation of wild-type (white bars) and IL-22$^{-/-}$ (black bars) mice, 7 days after onset of arthritis. Mean and SEM are given for 24 knee joints of 12 mice per group. (C) Representative histological (H&E) stainings of knee joint sections obtained 7 days after immunization from wild-type and IL-22$^{-/-}$ mice. (D) Expression of IL-17A synovial washouts of naive wild type (white bars), arthritic wild type (grey bars) and arthritic IL-22$^{-/-}$ mice (black bars). Mean and SEM are given for 8 synovial washouts per group (E) Flow cytometric analysis for the population of CD4, CD8 and TCRgd T cells and intracellular IL-17A and IFN-g expression obtained from wild-type (white bars) and IL-22$^{-/-}$ mice (black bars), 7 days after the induction of arthritis. Data are obtained from at least 2 independent experiments. Mean and SEM are given for 6 mice per group. (F-G) mBSA specific responses of draining lymph node cells from naive wild type (white bars), arthritic wild type (grey bars) and arthritic IL-22$^{-/-}$ mice (black bars), cultured in the presence or absence of mBSA. (F) Proliferative response to mBSA stimulation, as determined by [^{3}H] thymidine incorporation. (G) IL-17A response to mBSA stimulation. Mean and SEM are given for 4-9 mice per group. For statistical analysis a two-sided unpaired t-test was performed. (* = $p < 0.05$, *** = $p < 0.001$).

the presence of RASF. However, in peripheral blood of RA patients, we found increased IL-22 levels and increased numbers of Th22 cells. This suggests that additional signaling events or cellular interactions are present, which induce IL-22 expression. Indeed, addition of LPS activated monocytes or IL-23 were able to induce the induction of IL-22 in our system (data not shown).

On the other hand, IL-22R is expressed by RASF and within synovial tissue IL-22 is produced by RASF [35], indicating a function for IL-22 in the inflamed synovium. Treatment of RASF with IL-22 has been shown to induce proliferation and expression of chemokines, such as CCL2 [35]. However, in our T cell-RASF co-cultures we were not able to identify an effect of IL-22 on the expression of CCL2 (data not shown). This lack of effect together with no inducing effects on the pro-inflammatory mediators IL-6 and IL-8 produced by RASF indicates that in comparison to IL-17A, which is a potent inducer of IL-6 and IL-8 [14], IL-22 has limited effects in T cell-mediated synovial inflammation.

Moreover, the effect of IL-22 is likely dependent on the stage of synovial inflammation. In IL-1Ra$^{-/-}$ mice, IL-17A is already expressed in the early stages of inflammation, whereas IL-22 is mainly expressed in highly inflamed synovia [43]. This may explain the mild effect on arthritis severity in IL-1Ra$^{-/-}$ mice after anti-IL-22 treatment compared to anti-IL-17A treatment [43]. The expression of IL-22 in later stages of arthritis and our finding that IL-22 deficiency has no effect in T cell-mediated arthritis, shows that IL-22 has a secondary effect on synovial inflammation rather than a direct role in the induction of synovial inflammation.

It might be that IL-22 has a role in the induction of bone erosions in later stages of RA. This would be in line with the finding that IL-22 expression levels correlate with bone erosions and the induction of osteoclastogenesis by RASF induced RANKL production [12, 34, 44].

The observation that the increase of IL-22 levels and Th22 cells in patients with RA correlates with elevated Th17 cells and progression of bone erosions [12], would argue for the use of IL-22 or Th22 cells as a biomarker in RA. For this purpose a combination of chemokine receptors and cytokines will be preferable to distinguish pure Th17 and Th22 cell populations (Figure 1)

From the findings that synovial inflammation was not affected in IL-22$^{-/-}$ mice it can be concluded that local IL-22 produced by adaptive or innate immune cells have no direct contribution to the induction of T cell-mediated synovial inflammation. However, this is in disagreement with findings obtained by collagen induced arthritis (CIA) in IL-22$^{-/-}$ mice, in which arthritis incidence and severity was lower compared to wild-type mice [36]. On the other hand, our data are in line with other experimental auto-immune disease models, such as EAE, in which IL-22 is not required for the induction of encephalomyelitis [39]. A possible explanation for these differences is the underlying pathological mechanism in these experimental autoimmune models. Whereas AIA and EAE are largely dependent on cellular immune responses, CIA is both dependent on humoral and cellular responses, suggesting a more important role of IL-22 in the humoral response and less in the cellular response [36, 41, 45]. After CIA induction high total IgG and collagen specific IgG levels were found in IL-22$^{-/-}$ mice [36], suggesting a role of IL-22 in

IgG production by B cells. In this context, a current study performed in our laboratory (Corneth et al., unpublished), confirmed the data as published by Geboes et al. [36], that IL-22 deficiency in the CIA model results in decreased arthritis incidence and severity. However, we observed impaired terminal B cell differentiation associated with a decreased number and size of germinal centers in IL-22$^{-/-}$ mice. As IL-22 is expressed in lymphoid organs the suppressive effects of IL-22 deficiency on arthritis severity may be caused by intrinsic defects in germinal center formation or maintenance and altered kinetics of collagen specific antibody production in IL-22$^{-/-}$ mice.

IL-22 serum levels in patients with RA correlated with serum titers of antibodies against citrullinated peptides [44]. It would be of interest to investigate whether these antibodies are of high affinity and whether the kinetics of antibody production is altered in IL-22$^{-/-}$ mice.

Studying the role of IL-22 is complicated, because (1) IL-22 is expressed by multiple cells of the immune system, such as T cells, NK cells and lymphoid tissue inducer cells and (2) IL-22 is expressed in different tissues, including the skin, gut and lymph nodes and (3) IL-22 has multiple context dependent functions, such as wound healing and microbial defense [46, 47]. The contrasting results of studies regarding IL-22, which show pro-inflammatory, protective or no effects of IL-22 in different diseases or disease models, may be inherent to these context dependent effects of IL-22. In psoriasis for example IL-22 can synergize with other pro-inflammatory cytokines to induce many of the pathogenic phenotypes from keratinocytes and exacerbate disease progression in psoriasis. In contrast, IL-22 plays a beneficial role in IBD by enhancing barrier integrity and epithelial innate immunity of the intestinal tract [21, 40, 46, 47]. Moreover, IL-22 has protective roles in airway inflammation and protects against liver pathology during malaria infection [48-50].When taken together, IL-22 does not contribute to the induction of IL-17A/Th17 mediated synovial inflammation. This implies that treatment of patients with early RA should focus on targeting IL-17A and Th17 cells, rather than on targeting IL-22.

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REFERENCES

1. **McInnes IB**, Schett G. The pathogenesis of rheumatoid arthritis. *The New England journal of medicine* 2011;**365**:2205-19.
2. **Isaacs JD**. The changing face of rheumatoid arthritis: sustained remission for all? *Nature reviews* 2010;**10**:605-11.
3. **McInnes IB**, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. *Nature reviews* 2007;7:429-42.
4. **Lubberts E**. Th17 cytokines and arthritis. *Semin Immunopathol* 2010;**32**:43-53.
5. **Lubberts E**. IL-17/Th17 targeting: on the road to prevent chronic destructive arthritis? *Cytokine* 2008;**41**:84-91.
6. **McGeachy MJ**, Cua DJ. Th17 cell differentiation: the long and winding road. *Immunity* 2008;**28**:445-53.
7. **Miossec P**, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. *The New England journal of medicine* 2009;**361**:888-98.
8. **Korn T**, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. *Annu Rev Immunol* 2009;**27**:485-517.
9. **Cornelissen F**, van Hamburg JP, Lubberts E. The IL-12/IL-23 axis and its role in Th17 cell development, pathology and plasticity in arthritis. *Curr Opin Investig Drugs* 2009;**10**:452-62.
10. **Annunziato F**, Cosmi L, Liotta F, Maggi E, Romagnani S. Type 17 T helper cells-origins, features and possible roles in rheumatic disease. *Nature reviews* 2009 ;**5**:325-31.
11. **Shen H**, Goodall JC, Hill Gaston JS. Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis. *Arthritis Rheum* 2009;**60**:1647-56.
12. **Leipe J**, Grunke M, Dechant C, Reindl C, Kerzendorf U, Schulze-Koops H, et al. Role of Th17 cells in human autoimmune arthritis. *Arthritis Rheum* 2010;**62**:2876-85.
13. **Nistala K**, Moncrieffe H, Newton KR, Varsani H, Hunter P, Wedderburn LR. Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. *Arthritis Rheum* 2008;**58**:875-87.
14. **van Hamburg JP**, Asmawidjaja PS, Davelaar N, Mus AM, Colin EM, Hazes JM, et al. Th17 cells, but not Th1 cells, from patients with early rheumatoid arthritis are potent inducers of matrix metalloproteinases and proinflammatory cytokines upon synovial fibroblast interaction, including autocrine interleukin-17A production. *Arthritis Rheum* 2011;**63**:73-83.
15. **Lubberts E**, Joosten LA, Oppers B, van den Bersselaar L, Coenen-de Roo CJ, Kolls JK, et al. IL-1-independent role of IL-17 in synovial inflammation and joint destruction during collagen-induced arthritis. *J Immunol* 2001;**167**:1004-13.
16. **Nakae S**, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. *J Immunol* 2003;**171**:6173-7.
17. **van Hamburg JP**, Asmawidjaja PS, Davelaar N, Mus AM, Cornelissen F, van Leeuwen JP, et al. TNF blockade requires 1,25(OH)2D3 to control human Th17-mediated synovial inflammation. *Annals of the rheumatic diseases*. 2012;**71**:606-12.
18. **Ouyang W**, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol* 2011;**29**:71-109.
19. **Eyerich S**, Eyerich K, Cavani A, Schmidt-Weber C. IL-17 and IL-22: siblings, not twins. *Trends Immunol* 2010;**31**:354-61.
20. **Sonnenberg GF**, Fouser LA, Artis D. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. *Nature immunology* 2011;**12**:383-90.
21. **Zheng Y**, Danilenko DM, Valdez P, Kasman I, Eastham-Anderson J, Wu J, et al. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* 2007;**445**:648-51.
22. **Akdis M**, Palomares O, van de Veen W, van Splunter M, Akdis CA. T(H)17 and T(H)22 cells: A confusion of antimicrobial response with tissue inflammation versus protection. *The Journal of allergy and clinical immunology* 2012;**129**:1438-49.

23. **Wolk K**, Witte E, Witte K, Warszawska K, Sabat R. Biology of interleukin-22. *Semin Immunopathol* 2010;**32**:17-31.
24. **Rubino SJ**, Geddes K, Girardin SE. Innate IL-17 and IL-22 responses to enteric bacterial pathogens. *Trends Immunol* 2012;**33**:112-8.
25. **Eyerich S**, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, et al. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *The Journal of clinical investigation* 2009;**119**:3573-85.
26. **Veldhoen M**, Hirota K, Westendorf AM, Buer J, Dumoutier L, Renauld JC, et al. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins *Nature* 2008;**453**:106-9.
27. **Trifari S**, Kaplan CD, Tran EH, Crellin NK, Spits H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nature immunology* 2009;**10**:864-71.
28. **Duhen T**, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nature immunology* 2009;**10**:857-63.
29. **Colin EM**, Asmawidjaja PS, van Hamburg JP, Mus AM, van Driel M, Hazes JM, et al. 1,25-dihydroxyvitamin D3 modulates Th17 polarization and interleukin-22 expression by memory T cells from patients with early rheumatoid arthritis. *Arthritis Rheum* 2010;**62**:132-42.
30. **Zhang L**, Li YG, Li YH, Qi L, Liu XG, Yuan CZ, et al. Increased Frequencies of Th22 Cells as well as Th17 Cells in the Peripheral Blood of Patients with Ankylosing Spondylitis and Rheumatoid Arthritis. *PLoS One* 2012;**7**:e31000.
31. **Mitra A**, Raychaudhuri SK, Raychaudhuri SP. Functional role of IL-22 in psoriatic arthritis. *Arthritis research & therapy* 2012;**14**:R65.
32. **Zhang L**, Li JM, Liu XG, Ma DX, Hu NW, Li YG, et al. Elevated Th22 cells correlated with Th17 cells in patients with rheumatoid arthritis. *J Clin Immunol* 2011;**31**:606-14.
33. **Leipe J**, Schramm MA, Grunke M, Baeuerle M, Dechant C, Nigg AP, et al. Interleukin 22 serum levels are associated with radiographic progression in rheumatoid arthritis. *Annals of the rheumatic diseases* 2011;**70**:1453-7.
34. **da Rocha LF**, Jr., Duarte AL, Dantas AT, Mariz HA, Pitta ID, Galdino SL, et al. Increased Serum Interleukin 22 in Patients with Rheumatoid Arthritis and Correlation with Disease Activity. *J Rheumatol* 2012;**39**:1320-5.
35. **Ikeuchi H**, Kuroiwa T, Hiramatsu N, Kaneko Y, Hiromura K, Ueki K, et al. Expression of interleukin-22 in rheumatoid arthritis: potential role as a proinflammatory cytokine. *Arthritis Rheum* 2005;**52**:1037-46.
36. **Geboes L**, Dumoutier L, Kelchtermans H, Schurgers E, Mitera T, Renauld JC, et al. Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice. *Arthritis Rheum* 2009;**60**:390-5.
37. **Ouyang W**. Distinct roles of IL-22 in human psoriasis and inflammatory bowel disease. *Cytokine Growth Factor Rev* 2010;**21**:435-41.
38. **Van Belle AB**, de Heusch M, Lemaire MM, Hendrickx E, Warnier G, Dunussi-Joannopoulos K, et al. IL-22 is required for imiquimod-induced psoriasiform skin inflammation in mice. *J Immunol* 2012;**188**:462-9.
39. **Kreymborg K**, Etzensperger R, Dumoutier L, Haak S, Rebollo A, Buch T, et al. IL-22 is expressed by Th17 cells in an IL-23-dependent fashion, but not required for the development of autoimmune encephalomyelitis. *J Immunol* 2007;**179**:8098-104.
40. **Zenewicz LA**, Yancopoulos GD, Valenzuela DM, Murphy AJ, Stevens S, Flavell RA. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity* 2008;**29**:947-57.
41. **van Hamburg JP**, Mus AM, de Bruijn MJ, de Vogel L, Boon L, Cornelissen F, et al. GATA-3 protects against severe joint inflammation and bone erosion and reduces differentiation of Th17 cells during experimental arthritis. *Arthritis Rheum* 2009;**60**:750-9.
42. **Cornelissen F**, Mus AM, Asmawidjaja PS, van Hamburg JP, Tocker J, Lubberts E. Interleukin-23 is critical for full-blown expression of a non-autoimmune destructivearthritis and regulates interleukin-17A and RORgammat in gammadelta T cells. *Arthritis research & therapy* 2009;**11**:R194.
43. **Marijnissen RJ**, Koenders MI, Smeets RL, Stappers MH, Nickerson-Nutter C, Joosten LA, et al. Increased expression of interleukin-22 by synovial Th17 cells during late stages of murine experimental arthritis is controlled by interleukin-1 and enhances bone degradation. *Arthritis Rheum* 2011;**63**:2939-48.

44. **Kim KW**, Kim HR, Park JY, Park JS, Oh HJ, Woo YJ, et al. Interleukin-22 promotes osteoclastogenesis in rheumatoid arthritis through induction of RANKL in human synovial fibroblasts. *Arthritis Rheum* 2012;**64**:1015-23.

45. **van Hamburg JP**, de Bruijn MJ, Ribeiro de Almeida C, van Zwam M, van Meurs M, de Haas E, et al. Enforced expression of GATA3 allows differentiation of IL-17-producing cells, but constrains Th17-mediated pathology. *European journal of immunology* 2008;**38**:2573-86.

[46] **Zenewicz LA**, Flavell RA. Recent advances in IL-22 biology. Int Immunol. Mar;23(3):159-63.

[47] **Ouyang W**. Distinct roles of IL-22 in human psoriasis and inflammatory bowel disease. *Cytokine Growth Factor Rev* 2010;**21**:435-41.

[48] **Mastelic B**, do Rosario AP, Veldhoen M, Renauld JC, Jarra W, Sponaas AM, et al. IL-22 Protects Against Liver Pathology and Lethality of an Experimental Blood-Stage Malaria Infection *Front Immunol* 2012;**3**:85.

[49] **Takahashi K**, Hirose K, Kawashima S, Niwa Y, Wakashin H, Iwata A, et al. IL-22 attenuates IL-25 production by lung epithelial cells and inhibits antigen-induced eosinophilic airway inflammation. *J Allergy Clin Immunol* 2011;**128**:1067-76.

[50] **Taube C**, Tertilt C, Gyulveszi G, Dehzad N, Kreymborg K, Schneeweiss K, et al. IL-22 is produced by innate lymphoid cells and limits inflammation in allergic airway disease. *PLoS One* 2011;**6**:e21799.


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IL-22 is essential for terminal B cell differentiation in collagen induced arthritis

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ABSTRACT

Objectives The Th17 cytokine IL-22 is thought to have a pro-inflammatory role in rheumatoid arthritis. It is associated with increased joint destruction in rheumatoid arthritis (RA) patients, suggesting IL-22 might be a useful therapeutic target in RA. Indeed, in collagen-induced arthritis (CIA), an animal model for RA, IL-22 knock-out mice are partially protected against pannus formation and bone destruction. However, the role of IL-22 on T and B cell immunity in the inflammatory phase of RA is not fully understood. This prompted us to further study the role of IL-22 in auto-immune arthritis.

Methods The role of IL-22 in T cell immune responses was investigated in vitro by co-culturing wild type (wt) and IL-22KO Th17 cells with synovial fibroblasts and in vivo by DTH in IL-22KO and wt mice. To investigate the role of IL-22 on B cells in auto-immune inflammation, CIA was induced in wt and IL-22KO mice.

Results T cell immune responses were normal in IL-22KO mice in vitro and in vivo compared to wt controls. However, terminal B cell immunity was impaired in these mice. Germinal center formation was decreased, with fewer and smaller germinal centers being formed in the spleen. This led to decreased plasma cell formation and lower auto-reactive anti-collagen specific IgG2a antibody production in the inflammatory phase of disease.

Conclusions These data show a critical role for IL-22 in functional germinal centers and terminal B cell differentiation, having implications for the use of IL-22 as a therapeutic target for RA and other auto-immune diseases.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting 1% of the Western population and is characterized by chronic inflammation of the synovium of the joints and subsequent destruction of cartilage and bone (1, 2). Interleukin 17 (IL-17), a T-helper-17 (Th17) cell cytokine, plays an important role in this disease (3-5). Th17 cells can be found in the inflamed joint synovium and IL-17 can be detected in the synovial fluid of patients with RA (6, 7). These Th17 cells, if not properly controlled, induce a pro-inflammatory feedback loop with synoviocytes (8, 9). In addition, numbers of circulatory Th17 cells and IL-17 levels in serum are higher in RA patients who do not respond to anti-TNF therapy (10). In collagen induced arthritis (CIA), an animal model for RA, IL-17 neutralizing antibodies protect mice against joint inflammation and destruction (11). In addition, CIA disease incidence and severity are reduced in IL-17A deficient mice (12).

Next to IL-17, Th17 cells also produce IL-22 (13). This IL-10 family member signals through a heterodimer of the IL-10 receptor 2 (IL-10R2) and the IL-22 specific IL-22R1 subunit, which is expressed on stromal cells, but not on immune cells (14). Therefore, IL-22 appears to be involved in the crosstalk between immune cells and their surrounding tissue. IL-22 is not only produced by Th17 cells, but also by CD8+ T cells, NK T cells and innate lymphoid cells (15-17). Recently, a new human T helper cell subset was described which produces IL-22, but not IL-17A (18-20). However, these Th22 cells have not been found in mice.

The role of IL-22 in immune responses is very heterogeneous. It plays a major role in the defense against microbes at mucosal barriers and it is involved in tissue repair (17, 21-23). Also, IL-22 is required to prevent systemic inflammation with commensal gut flora (24). In addition, IL-22 is also implicated in several autoimmune diseases (25). In RA patients, higher proportions of IL-22 expressing T cells are found in the circulation and in the inflamed syovium (7, 26-28). Furthermore, IL-22 induces osteoclastogenesis through fibroblast activation and serum IL-22 levels in RA patients correlate with radiographic progression of disease (29, 30). In patients with psoriasis, IL-22 producing cells are elevated in skin lesions and IL-22 is involved in the pathology of the autoimmune skin inflammation (15, 31) . IL-22 levels in serum of patients with psoriasis correlate with disease severity (32, 33). Furthermore, several mouse models of psoriasis depend on IL-22 (34, 35).

In contrast, IL-22 is not required for experimental autoimmune encephalomyelitis (EAE) (36). Even more so, although serum levels of IL-22 are increased in inflammatory bowel disease (IBD) patients and correlate with disease activity (37), IL-22 is protective in mouse models of IBD (38-40). In bleomycin induced airway inflammation, IL-22 can have both a protective and a pathological role, depending on the co-expression of IL-17A (41). These seemingly opposite roles of IL-22 in (auto)immune diseases prompted us to further study the role of IL-22 in autoimmune inflammation. Geboes et al. have shown that in CIA, IL-22 deficient mice are partially protected

against pannus formation and joint destruction (42). However, the role of IL-22 in the inflammatory phase of the disease, and especially the effect of IL-22 on B cell immunity, is not fully understood. Here we show that IL-22 is required for the formation or maintenance of functional germinal centers in CIA. In IL-22KO mice terminal B cell differentiation and pathogenic antibody formation are impaired. However, because lymphoid cells do not express the IL-22 receptor, this effect is indirect, pointing to a role for IL-22 in the formation of functional follicular structures. These data provide new insight in the role of IL-22 in B cell mediated autoimmune diseases and IL-22 as a target for the treatment of patients with RA.

METHODS

Mice

IL-22 knock-out mice on a C57Bl/6 background (22) were kindly provided by Dr. Wenjun Ouyang, Genentech Inc., USA. Wild type C57Bl/6 control mice were purchased from Harlan Laboratories B.V. (Horst, The Netherlands). Mice were kept under SPF conditions in the Erasmus Medical Center animal facility (EDC) and all experiments were approved by the Erasmus MC Animal Ethical Committee. Animals were provided with food *ad libitum*. All mice were 8-12 weeks of age at the start of the experiments.

Immunizations

For collagen induced arthritis (CIA), 2 mg/ml chicken collagen type II (CII) (Chondrex, USA) was emulsified in an equal freund's adjuvant (CFA) volume containing 1 mg/ml heat-killed *Mycobacterium tuberculosis* (strain H37Ra; Difco Laboratories, Inc., Detroit, Mi). Mice were immunized by intra-dermal (i.d.) injection with 100µg CII/CFA emulsion. 21 days later, mice were boosted subcutaneously (s.c.) with 100µg CII/CFA. Arthritis severity was scored macroscopically with a maximum score of eight per mouse.

For delayed type hypersensitivity reaction (DTH), 8 mg/ml methylated bovine serum albumin (mBSA, Sigma-Aldrich, St. Louis, MO) was emulsified in an equal complete freund's adjuvant (CFA) volume containing 1 mg/ml heat-killed *Mycobacterium tuberculosis* (strain H37Ra; Difco Laboratories). Mice were immunized with 100µg mBSA/CFA emulsion intra-dermally and were injected with 200µg mBSA in 10 ml 0.9% NaCl the footpads 7 days later. Swelling of the footpads was measured 24 and 48 hours after injections in the footpads.

Co-culture experiments

Th17 cells were sorted from spleens obtained ten days after CII/CFA immunization by FACS sorting CD4-high, CD62L-low, CD25-low, CCR6-high expressing cells. Antibodies were obtained

from BD BioSciences (San Diego, CA, USA) or Biolegend (San Diego, CA, USA) (anti-CCR6). Purity of obtained fraction was >98%. $15*10^3$ sorted cells were co-cultured with 10^4 synovial fibroblasts in IMDM medium (Lonza, Verviers, Belgium), supplemented with 10% FCS (Invitrogen, Carlsbad, CA), 100 U/ml Penicillin/Streptomycin, 2 mM L-Glutamin (Lonza) and 50 mM b-mercapto-ethanol (Merck, Darmstadt, Germany) and anti-CD3 and anti-CD28 (both 4µg/ml, BD BioSciences) for 96 hours. After 96 hours, IL-6 levels in supernatant were measured by ELISA and IL17A expressing cells were measured by flow cytometry.

Flow cytometry

For intracellular cytokine staining, $2*10^6$ splenocytes were stimulated with PMA (0,05 µg/ml) and Ionomycin (0,5µg/ml) in the presence of Golgi stop (BD Biosciences) for 4 hours. Cells were fixed with 2% PFA and permeabilized in 0,5% saponin. Anti-CD4 and anti-IL-17A antibodies were obtained from BD BioSciences.

For B cell stainings, $2*10^6$ splenocytes were stained with antibody mix for 20 minutes at room temperature. For intracellular staining, cells were subsequently fixed and permeabilized using BD Cytofix/Cytoperm (BD BioSciences) and stained for intracellular markers for 20 minutes at room temperature. Anti-CD19, anti-B220 and anti-IgM were obtained from eBioscience (San Diego, CA, USA), anti-IgD and anti-CD95 from BD BioSciences and anti-PNA from Sigma-Aldrich (St Louis, USA).

Samples were acquired on a FACS Canto II HTS or a FACS LSR II flow cytometer (BD BioSciences) and analyzed using FlowJo (Tree Star, Inc., Ashland, OR, USA) software.

ELISA

For CII specific serum antibody level measurement, wells of microtiter plates were coated with 1µg/mL CII (Chondrex). Samples and reference sample were diluted serially and incubated in the wells for three hours at room temperature. Secondary antibodies goat-anti-mouse IgM, IgG1 and IgG2a (Southern Biotechnology Associated, Inc., Birmingham, AL, USA) were incubated for one hour at room temperature. Optical density at 450nm was measured.

Measurements of IL-6 levels in culture supernatant were performed using a mouse IL-6 ELISA Duoset (R&D Systems, Minneapolis MN). ELISA was performed according to the manufacturer's instructions.

Immunohistochemistry

Spleen tissue was frozen in Tissue-Tec O.C.T. Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) and stored at -80^{o}C. 6µm thick samples were cut. Slides (Superfrost, Gerhard Menzel GmbH, Braunschweig, Germany) were fixed in acetone and endogenous

peroxidase was blocked using 30% H_2O_2 (Sigma-Aldrich). Slides were stained with anti-PNA (biotinylated, Sigma-Aldrich) and streptavidin (peroxidase labeled, Jackson ImmunoResearch, Westgrove, PA, USA) or rat-anti-IgD (eBioscience) and anti-rat (alkaline phosphatase labeled, Sigma-Aldrich). Pictures were made using a NanoZoomer (Hamamatsu Photonics Deutchland GmbH, Herrsching am Ammersee, Germany).

Statistical analysis

Data was analyzed using Prism software v5.04 (GraphPad Software Inc. La Jollla, CA). A two-sided students T-test was performed, unless indicated otherwise. P-values <0,05 were considered significant.

RESULTS

IL-22KO mice are protected against severe CIA

Geboes et al have suggested that IL-22 is involved in the development of CIA (42). To verify the effects of IL-22 deficiency on the induction and severity of arthritis, wild type and IL-22KO C57/Bl6 mice were immunized with chicken type 2 collagen (CII) in complete Freund's adjuvant (CFA) and boosted 21 days later. IL-22 has no significant effect on the incidence of arthritis, as the incidence of CIA at day 50 after immunization in wild type mice (~35%) was in the same range of the IL-22KO mice (~50%) (figure 1A). In contrast, IL-22 was critically involved in the severity of arthritis, which was significantly lower in IL-22KO mice compared with wild type controls (figure 1B). These findings show that although IL-22KO mice are susceptible to CIA, they are protected against severe arthritis.

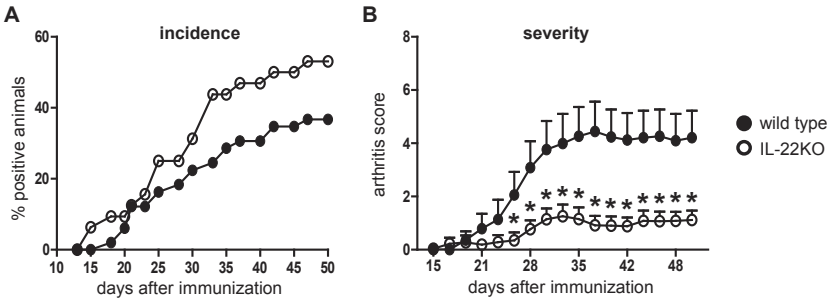

Figure 1. Incidence and severity of CIA in wild type and IL-22KO mice. [A] Incidence of CIA in wild type (black circles) and IL-22KO (clear circles) mice in CIA. Percentages of animals are shown. [B] Arthritis score of animals with disease. Maximum score is 8. Animals with a score >6 were sacrificed for ethical reasons. Mean and SEM are shown for n = 20 wild type and 21 IL-22KO animals, graph is representative for four individual experiments, * p<0,05.

Th17 cells from CII immunized IL-22KO mice are pathogenic in vitro

Next, we wondered which cell type could be involved in the apparent pathogenic role of IL-22 in CIA. Previous studies have shown that IL-17A and Th17 cells play a critical role in the incidence and progression of arthritis (11, 22, 43). Therefore, we analyzed whether IL-22 has an effect on the population size of IL-17A producing splenic CD4+ T cells in wild type and IL-22KO mice at different time points after CII-immunization. Flow-cytometric analysis revealed that the lack of IL-22 production had no inhibitory effect on the proportions of splenic CD4+ T cells that produced IL-17A in IL-22KO mice. Percentages of splenic Th17 cells were even higher in these mice ten days after immunization (figure 2A). As Th17 cells are crucial for the development of CIA, we next investigated whether Th17 cells that do not express IL-22 are equally pathogenic *in vitro* as Th17 cells that do produce IL-22. Wild type and IL-22KO mice were immunized with CII/CFA and sacrificed ten days after immunization. Fractions of CD4+CCR6+ effector T cells, which are enriched for Th17 effector cells (44), were FACS sorted from the spleens of these mice. The sorted cells were co-cultured with synovial fibroblasts to determine if they were equally capable of inducing a pro-inflammatory response in fibroblasts, which express the IL-22R (8, 45). After three days, IL-6 production by synovial fibroblasts in the culture supernatant was measured by ELISA. Interestingly, we found a higher production of IL-6 in IL-22KO T cell co-cultures, compared with wild-type T cell co-cultures, indicating an increased pathological capacity of CCR6+ T cells deficient for IL-22 (figure 2B). In line with this effect a significant increased proportion of IL-17A

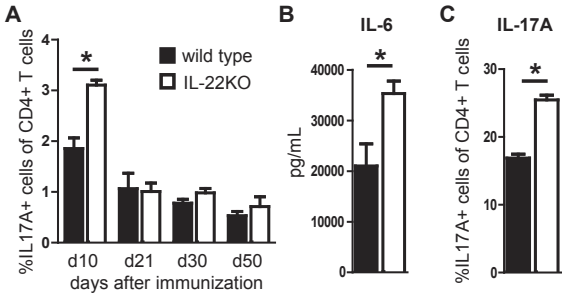

Figure 2. Pathogenicity of wild type and IL-22KO Th17 cells *in vitro*. [A] Percentage of IL-17A producing CD4+ T cells in wild type (black bars) and IL-22KO (clear bars) mice at several time points in CIA. Mean and SEM are shown for n = 6-10 animals per group. [B-C] Wild type and IL-22KO mice were immunized with CII/CFA and sacrificed ten days later. $15*10^3$ FACS sorted CD4+CCR6+ T cells from the spleens of these wild type and IL-22KO mice were co-cultured with 10^4 mouse synovial fibroblasts for three days. [B] Levels of IL-6 in co-culture supernatant after three days, as measured by ELISA. Mean and SEM are shown for n = 6 samples per group. [C] Percentage of IL-17A producing T cells after three days of co-culture with synovial fibroblasts. Graph is representative for two individual experiments, *p<0,05.

producing T cells was observed in the absence of IL-22 (figure 2C). These findings show that IL-17A producing T cells are present in the absence of IL-22 after CII immunization and that these cells retain pathogenic potential.

T cell responses in vivo in IL-22KO mice are normal

As the proportion of Th17 cells in the spleen during CIA and their pathologic potential appear not to be effected by a lack of IL-22 expression, we also tested whether the Th1/Th17 cell responses were normal in IL-22KO mice by measuring a DTH response *in vivo.* This model is Th1 mediated, but has been shown to be dependent upon IL-17A (46). Both wild type and IL-22 KO mice were immunized with mBSA in CFA. Seven days later, a secondary injection with mBSA in the footpads resulted in a comparable swelling of the footpads in IL-22KO and wild-type mice (figure 3). These findings show that Th1/Th17 cell responses in IL-22KO mice are normal. Therefore the partial protection against CIA development in IL-22KO mice is not likely to be caused by impaired T cell immunity.

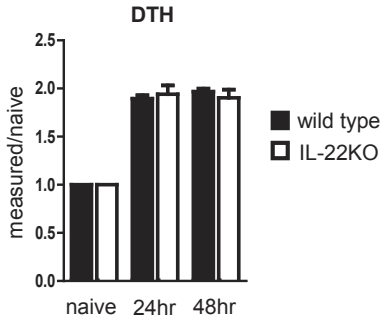

Figure 3. Pathogenicity of wild type and IL-22KO Th1/Th17 cells *in vivo*. Wild type and IL-22KO mice were immunized with mBSA/CFA. After seven days a trigger with mBSA was given directly in the footpads of these mice. Swelling of the footpad was measured to determine the inflammatory T cell response. Mean and SEM are shown for n = 5 animals per group.

Class switch recombination and production of CII specific antibodies depends on IL-22 in CIA

In addition to Th17 cells, CIA disease development depends highly on a humoral immune response with the production of pathogenic IgG2a antibodies, immune complex formation and complement activation (47). We therefore analyzed B cell immunity in wild type and IL-22KO mice in CIA. Proportions of splenic B cells were comparable between wild type and IL-22KO mice (figure 4A), as were total splenic B cell numbers (data not shown). Formation of intracellular IgM+

plasma cells in the spleen was comparable in wild type and IL-22KO mice, as was the production of CII specific IgM antibodies in serum (figure 4B and C, respectively). However, the formation of class-switched plasma cells was impaired in IL-22KO mice as shown by the significantly reduced proportion of intracellular IgG1+ and IgG2ab+ positive plasma cells in the spleen when compared with wild type controls (figure 4D and E). Importantly, the decrease in class switched-plasma cells was associated with a remarkable decrease in the concentration of pathogenic CII specific IgG2a antibodies in the serum of IL-22KO mice in CIA (figure 4F). From these data it becomes evident that IL-22 plays a crucial role in the induction of class-switch recombination and in the production of pathological collagen specific IgG2ab antibodies during CIA.

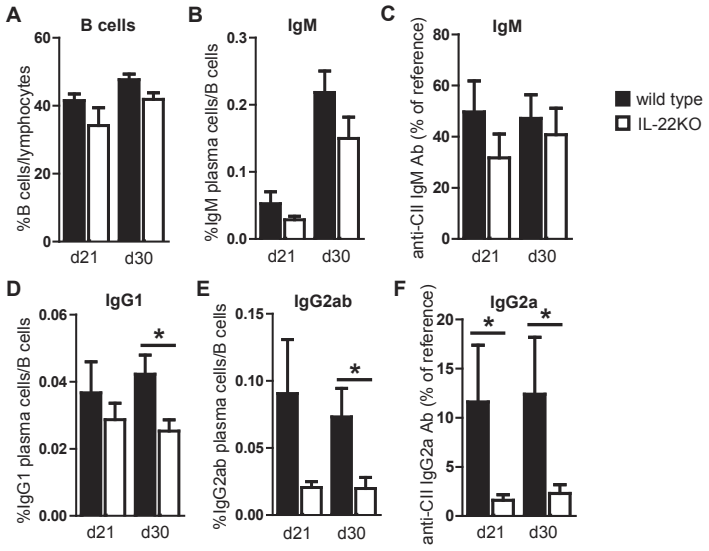

Figure 4. Plasma cell and auto-reactive antibody formation in wild type and IL-22KO mice in CIA. Proportions of splenic B cells [A] and splenic IgM [B], IgG1 [D] and IgG2ab [E] plasma cells in CIA, as measured by flow cytometry. Serum CII-specific IgM antibody [C] and IgG2a [F] levels in CIA, as measured by ELISA. Mean and SEM are shown for 10-20 animals per group, * p<0,05.

Impaired germinal center formation in the absence of IL-22 in CIA

Our finding that CII-specific IgM antibody levels were comparable between IL-22KO and wild type mice, but CII-specific IgG antibody levels were lower in IL-22 KO mice, suggests that in the absence of IL-22 class switch recombination is impaired. Proportions and total numbers of splenic B cells were comparable between wild type and IL-22KO mice at several time points in disease (figure 4A and data not shown). Interestingly however, at the peak of disease severity,

the proportions of PNA+ germinal center B cells were slightly decreased in IL-22KO mice (figure 5A, p=0,08). Immuno-histo-chemistry confirmed that only very few, smaller germinal centers were formed in spleens of IL-22KO mice when compared with wild type mice (figure 5B and 5C).

Together, these data show that lack of IL-22 production does not affect pathogenicity of Th17 cells in CIA. However, terminal B cell differentiation is severely impaired in IL-22KO mice in CIA, leading to reduced IgG plasma cell formation and reduced levels of pathogenic anti-CII IgG2a antibodies, which are crucial for severe disease.

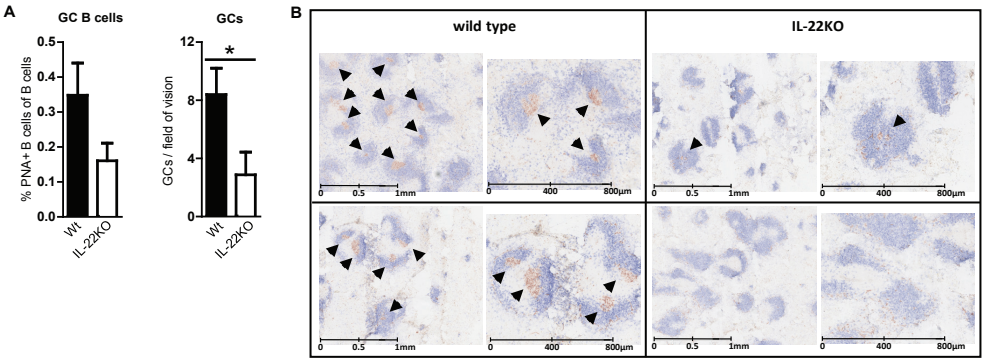

Figure 5. Impaired germinal centre formation in IL-22KO mice in CIA. [A] Proportions of splenic PNA+ germinal centre B cells in wild type and IL-22KO mice in CIA at the peak of disease severity (around day 35 after immunization). Mean and SEM are shown for n = 17 wild type and 16 IL-22KO mice. [B] Number of germinal centers in the spleen per field of vision at the peak of disease severity, analysed by immuno-histo-chemistry. Mean and SEM are shown for n = 10 wild type and 8 IL-22 KO animals; *p<0,05. [C] Immuno-histo-chemistry stainings of spleens of wild type and IL-22KO mice at the peak of disease severity, blue staining is IgD, brown staining is PNA. Two representative samples from n = 20 wild type and 21 IL-22KO mice are shown. Arrows indicate germinal centres.

DISCUSSION

Our data indicate that IL-22 is essential for B cell immunity in CIA. In line with a previous study (42), we found that IL-22KO mice are partially protected against CIA development. Mice had a significantly lower arthritis score compared to wild type controls. However, the role of IL-22 in T- and B-cell immunity during autoimmune arthritis has not been clearly identified. Here we show for the first time that IL-22 is essential for B cell, but not T cell immunity in CIA. By studying IL-22KO Th17 cells in CIA *in vitro* and in DTH *in vivo*, we found that these cells are equally pathogenic compared to wild type Th17 cells. In contrast, in CIA *in vivo*, fewer and smaller germinal centres were present in spleens of IL-22KO mice, leading to fewer class switched plasma cells and lower serum auto-reactive antibody levels, explaining the significantly lower CIA score in the IL-22KO compared to wild type mice.

We found a higher proportion of IL-17A producing T cells in the spleens of IL-22KO mice in CIA at ten days after immunization. At later time points, this difference was no longer present, perhaps because these pathogenic cells migrated away from the spleen to the joints. However, this increase in IL-17A expression might enhance susceptibility of IL-22KO mice to CIA. This would explain the slightly higher incidence of CIA in these mice, even though the severity of CIA is lower and associated with impaired B cell immunity. It also suggests a possible link between the regulation of IL-17A and IL-22 expression, possibly through competition for AHR or other regulatory factors.

The effect of IL-22 on B cell immunity in CIA is surprising, as neither IL-22 nor the IL-22 receptor is expressed by B cells (14). It is therefore unlikely that IL-22 has a direct effect on B cells. As splenic B cell numbers are normal in IL-22KO mice, IL-22 does not appear to have an effect on B cell development. In fact, it seems to play a role in the regulation of the formation or maintenance of functional germinal centres in the spleen, and possibly also in other lymphoid organs. This implies that the impaired formation of class switched plasma cells and the lower level of serum IgG antibodies in IL-22KO mice is not caused by a B cell intrinsic defect. This is further supported by the finding that the formation of IgM plasma cells and auto-reactive IgM antibody levels, which are independent of germinal centres, are normal in IL-22KO mice. In a previous study, IL-22KO mice were shown to have smaller B cell follicles in the lungs after infection with M. tuberculosis (48). Furthermore, lung fibroblasts produced CXCL13 upon exposure to IL-22, which is essential for B cell follicle and germinal centre formation (49). We therefore propose that the absence of fully functional germinal centres in IL-22KO mice in CIA could be caused by reduced expression of CXCL13 by splenic stromal cells in these mice.

The formation of class switched plasma cells, the production of auto-reactive IgG antibodies and the subsequent formation of immune complexes are essential in the development of CIA. For these factors, class switching of B cells is essential, and in CIA this depends on germinal centres. A disruption in the formation of germinal centres therefore explains the decreased severity of CIA in IL22KO mice. We therefore propose that, unlike IL-17A, IL-22 is not a major 'pro-inflammatory' cytokine in the inflammatory phase of CIA, but it has a vital role in the regulation of effective secondary follicular structures.

Previous studies have suggested the role for IL-22 in the destruction of the joints of mice in CIA and also of RA patients (13, 42). However, our data show that in addition, it has a more complex, indirect effect on the inflammatory phase of the disease. In a functional assay, we showed that Th17 cell pathogenicity is not affected by loss of IL-22 expression. Furthermore, another study by our lab indicates that in human T cell – synovial fibroblast (RASF) co-cultures, Th17 cells are much more potent inducers of IL-6, MMP-1 and MMP-3 production by RASF than Th22 cells (50). Indeed in the B cell independent antigen induced arthritis (AIA) mouse model, IL-22KO mice developed similar inflammation and destruction of the joints compared to wild type controls (50). These data suggest that, although IL-22 may contribute to a pro-inflammatory

response in the joints, in the presence of IL-17A it is not essential for the destruction of cartilage and bone.

Increased levels of IL-22 are present in the joints of RA patients (28). In addition, studies have shown a possible role for IL-22 in the induction of osteoclastogenesis (29). IL-22 has therefore been considered as a possible new therapeutic target in the treatment of RA. Importantly, our data indicate that IL-22 also has a significant effect on the formation of germinal centres and therefore on B cell immunity. Because this is a more regulatory effect on the organisational structure of lymphoid organs and not a direct effect on immune cells, blocking or neutralizing IL-22 in RA patients might not inhibit the inflammation of affected joints, as these processes will occur before the patient presents with symptoms of the disease. In addition, our data on the pathogenicity of human Th22 and Th17 cells in inducing IL-6, MMP-1 and MMP-3 production by RASF suggests that IL-17A might be a more promising target in the treatment of RA patients (50). Data from the present study provide new insight in the role of IL-22 in B cell mediated autoimmune diseases and IL-22 as a target for the treatment of patients with RA. This information may have significant implications for designing IL-22 neutralizing strategies for RA and other B cell mediated auto-immune diseases.

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REFERENCES

1. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med 2011;365(23):2205-19.
2. Isaacs JD. The changing face of rheumatoid arthritis: sustained remission for all? Nat Rev Immunol 2010;10(8):605-11.
3. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol 2007;7(6):429-42.
4. Lubberts E. Th17 cytokines and arthritis. Semin Immunopathol 2010;32(1):43-53.
5. Cornelissen F, van Hamburg JP, Lubberts E. The IL-12/IL-23 axis and its role in Th17 cell development, pathology and plasticity in arthritis. Curr Opin Investig Drugs 2009;10(5):452-62.
6. Chabaud M, Fossiez F, Taupin JL, Miossec P. Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. J Immunol 1998;161(1):409-14.
7. Shen H, Goodall JC, Hill Gaston JS. Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis. Arthritis Rheum 2009;60(6):1647-56.
8. van Hamburg JP, Asmawidjaja PS, Davelaar N, Mus AM, Colin EM, Hazes JM, et al. Th17 cells, but not Th1 cells, from patients with early rheumatoid arthritis are potent inducers of matrix metalloproteinases and proinflammatory cytokines upon synovial fibroblast interaction, including autocrine interleukin-17A production. Arthritis Rheum 2011;63(1):73-83.
9. van Hamburg JP, Asmawidjaja PS, Davelaar N, Mus AM, Cornelissen F, van Leeuwen JP, et al. TNF blockade requires 1,25(OH)2D3 to control human Th17-mediated synovial inflammation. Ann Rheum Dis 2012;71(4):606-12.
10. Chen DY, Chen YM, Chen HH, Hsieh CW, Lin CC, Lan JL. Increasing levels of circulating Th17 cells and interleukin-17 in rheumatoid arthritis patients with an inadequate response to anti-TNF-alpha therapy. Arthritis Res Ther 2011;13(4):R126.
11. Lubberts E, Koenders MI, Oppers-Walgreen B, van den Bersselaar L, Coenen-de Roo CJ, Joosten LA, et al. Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. Arthritis Rheum 2004;50(2):650-9.
12. Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. J Immunol 2003;171(11):6173-7.
13. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. J Exp Med 2006;203(10):2271-9.
14. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 increases the innate immunity of tissues. Immunity 2004;21(2):241-54.
15. Res PC, Piskin G, de Boer OJ, van der Loos CM, Teeling P, Bos JD, et al. Overrepresentation of IL-17A and IL-22 producing CD8 T cells in lesional skin suggests their involvement in the pathogenesis of psoriasis. PLoS One 2010;5(11):e14108.
16. Goto M, Murakawa M, Kadoshima-Yamaoka K, Tanaka Y, Nagahira K, Fukuda Y, et al. Murine NKT cells produce Th17 cytokine interleukin-22. Cell Immunol 2009;254(2):81-4.
17. Rubino SJ, Geddes K, Girardin SE. Innate IL-17 and IL-22 responses to enteric bacterial pathogens. Trends Immunol 2012;33(3):112-8.
18. Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, et al. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. J Clin Invest 2009;119(12):3573-85.
19. Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. Nat Immunol 2009;10(8):857-63.
20. Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. Nat Immunol 2009;10(8):864-71.

21. Sonnenberg GF, Fouser LA, Artis D. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. Nat Immunol 2011;12(5):383-90.

22. Zheng Y, Danilenko DM, Valdez P, Kasman I, Eastham-Anderson J, Wu J, et al. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. Nature 2007;445(7128):648-51.

23. Akdis M, Palomares O, van de Veen W, van Splunter M, Akdis CA. TH17 and TH22 cells: a confusion of antimicrobial response with tissue inflammation versus protection. J Allergy Clin Immunol 2012;129(6):1438-49; quiz1450-1.

24. Sonnenberg GF, Monticelli LA, Alenghat T, Fung TC, Hutnick NA, Kunisawa J, et al. Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. Science 2012;336(6086):1321-5.

25. Pan HF, Li XP, Zheng SG, Ye DQ. Emerging role of interleukin-22 in autoimmune diseases. Cytokine Growth Factor Rev 2012.

26. Colin EM, Asmawidjaja PS, van Hamburg JP, Mus AM, van Driel M, Hazes JM, et al. 1,25-dihydroxyvitamin D3 modulates Th17 polarization and interleukin-22 expression by memory T cells from patients with early rheumatoid arthritis. Arthritis Rheum 2010;62(1):132-42.

27. Zhang L, Li YG, Li YH, Qi L, Liu XG, Yuan CZ, et al. Increased frequencies of Th22 cells as well as Th17 cells in the peripheral blood of patients with ankylosing spondylitis and rheumatoid arthritis. PLoS One 2012;7(4):e31000.

28. Mitra A, Raychaudhuri SK, Raychaudhuri SP. Functional role of IL-22 in psoriatic arthritis. Arthritis Res Ther 2012;14(2):R65.

29. Kim KW, Kim HR, Park JY, Park JS, Oh HJ, Woo YJ, et al. Interleukin-22 promotes osteoclastogenesis in rheumatoid arthritis through induction of RANKL in human synovial fibroblasts. Arthritis Rheum 2012;64(4):1015-23.

30. Leipe J, Schramm MA, Grunke M, Baeuerle M, Dechant C, Nigg AP, et al. Interleukin 22 serum levels are associated with radiographic progression in rheumatoid arthritis. Ann Rheum Dis 2011;70(8):1453-7.

31. Ouyang W. Distinct roles of IL-22 in human psoriasis and inflammatory bowel disease. Cytokine Growth Factor Rev 2010;21(6):435-41.

32. Wolk K, Witte E, Wallace E, Docke WD, Kunz S, Asadullah K, et al. IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis. Eur J Immunol 2006;36(5):1309-23.

33. Lo YH, Torii K, Saito C, Furuhashi T, Maeda A, Morita A. Serum IL-22 correlates with psoriatic severity and serum IL-6 correlates with susceptibility to phototherapy. J Dermatol Sci 2010;58(3):225-7.

34. Ma HL, Liang S, Li J, Napierata L, Brown T, Benoit S, et al. IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation. J Clin Invest 2008;118(2):597-607.

35. Van Belle AB, de Heusch M, Lemaire MM, Hendrickx E, Warnier G, Dunussi-Joannopoulos K, et al. IL-22 is required for imiquimod-induced psoriasiform skin inflammation in mice. J Immunol 2012;188(1):462-9.

36. Kreymborg K, Etzensperger R, Dumoutier L, Haak S, Rebollo A, Buch T, et al. IL-22 is expressed by Th17 cells in an IL-23-dependent fashion, but not required for the development of autoimmune encephalomyelitis. J Immunol 2007;179(12):8098-104.

37. Schmechel S, Konrad A, Diegelmann J, Glas J, Wetzke M, Paschos E, et al. Linking genetic susceptibility to Crohn's disease with Th17 cell function: IL-22 serum levels are increased in Crohn's disease and correlate with disease activity and IL23R genotype status. Inflamm Bowel Dis 2008;14(2):204-12.

38. Sugimoto K, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, et al. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. J Clin Invest 2008;118(2):534-44.

39. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Stevens S, Flavell RA. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. Immunity 2008;29(6):947-57.

40. Wolk K, Witte E, Hoffmann U, Doecke WD, Endesfelder S, Asadullah K, et al. IL-22 induces lipopolysaccharide-binding protein in hepatocytes: a potential systemic role of IL-22 in Crohn's disease. J Immunol 2007;178(9):5973-81.

41. Sonnenberg GF, Nair MG, Kirn TJ, Zaph C, Fouser LA, Artis D. Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. J Exp Med 2010;207(6):1293-305.

42. Geboes L, Dumoutier L, Kelchtermans H, Schurgers E, Mitera T, Renauld JC, et al. Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice. Arthritis Rheum 2009;60(2):390-5.

43. Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, et al. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J Exp Med 2003;198(12):1951-7.

44. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nat Immunol 2007;8(6):639-46.

45. Ikeuchi H, Kuroiwa T, Hiramatsu N, Kaneko Y, Hiromura K, Ueki K, et al. Expression of interleukin-22 in rheumatoid arthritis: potential role as a proinflammatory cytokine. Arthritis Rheum 2005;52(4):1037-46.

46. Nakae S, Komiyama Y, Nambu A, Sudo K, Iwase M, Homma I, et al. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. Immunity 2002;17(3):375-87.

47. Svensson L, Jirholt J, Holmdahl R, Jansson L. B cell-deficient mice do not develop type II collagen-induced arthritis (CIA). Clin Exp Immunol 1998;111(3):521-6.

48. Khader SA, Guglani L, Rangel-Moreno J, Gopal R, Junecko BA, Fountain JJ, et al. IL-23 is required for long-term control of Mycobacterium tuberculosis and B cell follicle formation in the infected lung. J Immunol 2011;187(10):5402-7.

49. Ansel KM, Ngo VN, Hyman PL, Luther SA, Forster R, Sedgwick JD, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. Nature 2000;406(6793):309-14.

50. van Hamburg JP, Corneth OB, Paulissen SM, Davelaar N, Asmawidjaja PS, Mus AM, et al. IL-17/Th17 mediated synovial inflammation is IL-22 independent. Ann Rheum Dis 2013.


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Surrogate light chain expression beyond the pre-B cell stage promotes tolerance in a dose-dependent fashion

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ABSTRACT

While surrogate light chain (SLC) expression is normally terminated in differentiating pre-B cells, co-expression of SLC and conventional light chains has been reported in a small population of autoreactive peripheral human B cells that accumulate in arthritic joints. Despite this association with autoimmunity the contribution of SLC expressing mature B cells to disease development is still unknown. We studied the pathogenicity of SLC+ B cells in a panel of mice that transgenically express various levels of the SLC components VpreB and λ5 throughout B cell development. Here we report that although VpreB or λ5 expression mildly activates mature B cells, only moderate VpreB expression levels - in the absence of λ5 - can enhance IgG plasma cell formation. However, no autoantibody production was detectable in VpreB or λ5 transgenic mice and on susceptible genetic backgrounds VpreB expression could not accelerate autoimmunity. Instead, moderate VpreB expression partially protected mice from induced autoimmune arthritis. In support of a tolerogenic role of SLC-transgenic B cells, we observed that in a dose-dependent manner SLC expression beyond the pre-B cell stage enhances clonal deletion among immature and transitional B cells and renders mature B cells anergic. Collectively, these findings suggest that SLC expression in human B cells does not propagate autoimmunity but instead imposes tolerance.

INTRODUCTION

Over a decade ago in arthritic joints of RA patients a new candidate pathogenic B cell subset was identified of which ~68% expressed B cell receptors (BCRs) reactive to nuclear self-antigens (1, 2). These cells that constitute up to 1% of circulating B cells in peripheral blood of healthy individuals (1) remarkably co-express conventional Ig light chains (LC) and pre-B cell receptor (pre-BCR) components VpreB and lambda5 (λ5) at the cell surface (1, 3). Despite the pre-BCR expression these SLC^+LC^+ cells cannot be designated as immature bone marrow emigrants based on their low expression of CD10 and CD38 with concomitant upregulation of activation or memory markers, including CD5 and CD27 (1). The notion that SLC^+LC^+ B cells may represent a recently activated B cell population *in vivo* is further supported by the recapitulation of VpreB re-expression in a fraction of GL-7^+ murine B cells in mice subjected to T-dependent immunizations (3). Despite the recognition of their activated phenotype and predominant expression of autoreactive BCRs (1, 2), it is still unknown whether these SLC^+LC^+ B cells propel or modulate autoreactive B cell responses in rheumatic diseases.

Expression of the SLC at the pre-B cell stage is essential to verify successful rearrangement, stable expression, and possibly the IgL chain pairing capacities of the produced IgH chain (4). In addition, the SLC may also function as a counterselector for autoreactive IgH chains since SLC-deficient mice exhibit spontaneous autoantibody production (5). In large cycling pre-B cells with successfully recombined IgH chains SLC signaling induces a transient proliferative burst, ensures allelic exclusion through terminating gene expression that is required for Ig gene rearrangements, and silences its own transcription (reviewed in (6)). The SLC instantly provides such positive feedback signals to differentiating IgH expressing pre-B cells, since it is equipped with autonomous signaling capacity (7, 8). This capacity is not only conferred by the polyreactive nature of the non-immunoglobulin tail of λ5 that can bind multiple self-antigens such as heparin sulfate and galectin-1 (9, 10) but mainly by self-reactivity of the SLC to an N-glycosylation site of the IgH chain providing strong and instant pre-BCR signaling (11).

The importance of SLC signaling for pre-B cell development is evident from mice deficient for SLC components or downstream pre-BCR signaling molecules, such as Bruton's tyrosine kinase (Btk) or BLNK/Slp65 (6, 12). These mice exhibit defects in expression of pre-B cell maturation markers, Ig lambda (Igλ) LC rearrangements and an enhanced in vitro proliferative capacity in response to IL-7. Conversely, transgenic mice with continuous high-level SLC expression throughout B cell differentiation do not exhibit a delay in pre-B cell maturation or prolonged pre-B cell proliferation (13). Instead, constitutive BCR signaling induced by high-level transgenic SLC expression hampers normal B cell maturation but instead drives spontaneous differentiation of peripheral B cells into IgM plasma cells, thereby providing a first indication that constitutive pre-BCR signaling beyond the pre-B cell stage may jeopardize B cell tolerance. However, high-level SLC expression in mature B cells in these mice did not induce spontaneous IgG autoantibody production or systemic autoimmune

disease (13), leaving the possibility that lower doses of SLC signaling could still establish systemic autoimmunity. Importantly, in human SLC^+LC^+ B cells VpreB levels are lower than in pre-B cells, and VpreB levels in SLC^+LC^+ B cells markedly exceed those of λ5 (1).

To study whether pre-BCR signaling, as occurring in rare human SLC^+LC^+ B cells, could affect B cell tolerance, we studied autoimmunity in SLC transgenic (SLC-tg) mice with enforced expression of different doses of both or individual SLC components beyond the pre-B cell stage (13). Here we report that although enforced SLC expression promotes mild B cell activation *in vivo*, no spontaneous or enhanced autoimmunity could be observed on autoimmune-resistant or autoimmune-prone genetic backgrounds. In contrast, prolonged VpreB expression partially protects from experimentally induced arthritis by a dose-dependent induction of clonal deletion and anergy in developing and mature B cells, respectively. These findings imply that SLC re-expression in autoreactive SLC^+LC^+ B cells in humans is most likely not pathogenic but instead functions to silence these cells, mainly through tolerance mechanisms other than receptor editing.

MATERIALS & METHODS

Mice and genotyping

The CD19-VpreB Tg and CD19-λ5 Tg constructs and the generation of multiple independent mouse strains expressing different copy numbers of these constructs have been described (13). Three lines with increasing CD19-VpreB Tg expression ($Vpre^{low}$, $VpreB^{int}$ and $VpreB^{high}$) and two lines with increasing CD19-λ5 Tg expression ($λ5^{low}$ and $λ5^{high}$) were all crossed onto the c57bl/6 background (>10 generations) and were analyzed on this genetic background, or on c57bl/6 *FcγRIIB*-deficient (14) or c57bl/6 Eμ-Bcl2 (15) backgrounds, or upon crossing onto the dba/1 background for >10 generations. Mice were genotyped by genomic PCR on tail DNA according to standard protocols using the following primers for the CD19-VpreB Tg and CD19-λ5 Tg constructs: a forward primer aligning with the human genomic CD19-promoter fragment (5'-TGAGAAGGAGTCTATGTGCC-3'), a reverse primer specific for mouse genomic *vpreb1* (5'-GCCATAGGAGGAGCAAAGAA-3') and a reverse primer specific for mouse genomic *Igll1* (λ5; 5'- ACTCTGAGCTTCATTGACCC-3'). All mice were kept at specified pathogen free conditions at the Erasmus MC experimental animal care facility, and all experimental protocols were reviewed and approved by the Erasmus MC committee for animal experiments.

Collagen-induced arthritis (CIA) experiments

Mice on the c57bl/6 background were immunized intradermally at the tail base with 100 μg of chicken collagen type 2 (CII; Chondrex) emulsified in complete Freund's adjuvant (CFA), and a secondary immunization was performed at day 21 by injecting intradermally in dorsal skin 100

µg of chicken CII emulsified in CFA. Assessment of arthritis severity was performed as described in Lubberts et al. (16).

General flow cytometry procedures

Preparation of single-cell suspensions of lymphoid organs and fluorescent labeling of cell surface markers was performed according to standard procedures (17). Used monoclonal antibodies were specific for CD19 (ID3, eBioscience), CD23 (B3B4, eBioscience), CD25 (PC61.5, eBioscience), CD69 (H1.2F3, BD Biosciences), CD86 (GL1, eBioscience), CD93 (PB493/AA4.1, eBioscience), CD95 (Jo2, BD Biosciences), CD138 (281-2, BD Biosciences), B220 (RA3-6B2, eBioscience), IgM (II/41, eBioscience), IgD (11-26c.2a, eBioscience), IgG1 (A85-1, BD Biosciences), IgG2a/b (R19-5, BD Biosciences), IgG3 (R40-82, BD Biosciences), Igκ (187.1, BD Biosciences), Igλ (R26-46, BD Biosciences), VpreB/CD179a (R3/VpreB, BD Biosciences), and λ5/CD179b (LM34, BD Biosciences). Biotin-conjugated antibodies were subsequently stained with fluorochrome-coupled streptavidin (eBioscience), and staining of Gal-β(1-3)-GalNAc carbohydrates on B cells was performed with biotin-conjugated PNA (peanut agglutinin, Sigma-Aldrich). After completing cell surface marker labelling, intracellular stainings for Ig or SLC components were performed by fixing and permeabilizing/staining in Cytofix/Cytoperm™ and Perm/Wash™ buffers respectively (BD Biosciences) according to the manufacturer's instructions. All flow cytometric measurements were performed on a LSRII™ flow cytometer (BD Biosciences) and data were analysed using FlowJo software (Tree Star Inc.).

Flow cytometric measurement of Ca^{2+} influx

Splenic cell suspensions were prepared in loading buffer (HBSS/5% FCS/10mM HEPES) and splenocytes were loaded at a concentration of $10 \cdot 10^6$ cells/mL with 5 µM Fluo-3 (Molecular Probes) and 5 µM Fura Red™ (Molecular Probes) in loading buffer for 30 minutes at 37°C. Cells were washed and resuspended in flux buffer (HBSS/5% FCS/10mM HEPES/1mM $CaCl_2$) and during stimulation at 37°C with 20 µg/mL $F(ab'_2)$ goat anti-mouse-IgM (Jackson Immunoresearch) Fluo-3 and Fura Red™ fluorescence was measured in FITC (515-545 nm) and PerCP-Cy5.5 (675-715 nm) detection channels respectively on an LSRII™ flow cytometer (BD Biosciences). The ratio of Fluo-3/Fura Red™ fluorescence was calculated and normalized for maximum fluorescence upon stimulation with 2µg/mL ionomycin (Sigma-Aldrich).

Anti-nucleosome autoantibody ELISA

Measurements of anti-nucleosome autoantibodies in serum by ELISA was performed as described previously (17). Briefly, nucleosome-coated plates of an anti-nucleosome ELISA kit (Orgentec) were incubated with 100-fold diluted serum samples, and subsequently plate-bound antibodies

were detected using the protocol and reagents provided by the manufacturer. As secondary reagent polyclonal anti-mouse total IgG antibodies (Southern Biotech) were used.

B cell purification and *in vitro* stimulation

Magnetic-activated cell sorting (MACS) of naïve splenic B cells was performed by indirect labeling of non-B cells, activated B cells, B1 cells and plasma cells using biotinylated anti-CD5 (53-7.3), anti-CD11b (M1-70), anti-CD43 (S7), anti-CD95 (Jo2), anti-CD138 (281-2), anti-Gr-1 (RB6-8C5), and anti-TER-119 (PK136) antibodies (BD Biosciences) and streptavidin-conjugated magnetic beads (Miltenyi Biotec). After magnetic depletion of labeled cells the purity of naïve B2 cells typically exceeded 95% as verified by flow cytometric analysis. Purified naïve B2 cells were cultured for 24 hours and stimulated with F(ab'$_2$) goat anti-mouse-IgM (Jackson Immunoresearch) or LPS (own production) as described previously (17).

mRNA deep sequencing

mRNA was isolated using the RNeasy Mini Kit (Qiagen) from F(ab'$_2$) goat anti-mouse-IgM stimulated or unstimulated splenic naïve B cells. Sequencing of total mRNA was performed using a HiSeq 2000 sequencing system (Illumina). Reads were mapped to Ensembl transcripts base on the University of California at Santa Cruz (UCSC) mouse genome annotation (mm9). Fragments per kilobase of a transcript per million mapped reads (FPKMs) were calculated and assigned per transcript. Sequencing data analysis was performed with MultiExperiment Viewer (MeV) software v 4.8.1 (Dana-Farber Cancer Institute).

Statistical analysis

The Student's t-test was used for calculating levels of significance of differences between groups of continuous data. Levels of significance in the analysis of mRNA deep sequencing data were calculated using a one-way ANOVA with Bonferroni correction.

RESULTS

Expression of different doses of VpreB and λ5 in peripheral B cells

Since VpreB re-expression has been reported *in vivo* in germinal center B cells upon T-dependent immunization (3), we examined whether antigenic stimulation of murine mature B cells could induce SLC expression. We used total mRNA deep sequencing analysis to compare gene expression in MACS-purified naïve splenic B cells from wild-type mice that were either unstimulated or stimulated with F(ab')$_2$ anti-IgM for 12 hours. Whereas activation marker CD69 showed a robust

and significant upregulation upon anti-IgM stimulation, we could not detect a significant increase in transcription of *Vpreb1* or *Vpreb2* genes (Figure S1). Transcription of Vpreb2 was even reduced after BCR stimulation, but importantly *Vpreb3* transcription was strongly induced (Figure S1). Expression of *Igll* (λ5) was very variable. Since in human SLC^+LC^+ B cells expression of other pro- and pre-B cell genes as *Dntt* (TdT), *Rag1* and *Rag2* was reported (1), we examined whether anti-IgM stimulation could also instruct re-expression of these genes. No significant increase in *Rag1* or *Rag2* transcripts could be observed, while *Dntt* was minimally expressed (Figure S1).

From these findings it appears that antigenic stimulation of wild-type B cells differentially affects transcription of *Vpreb* and *Igll*. Therefore, we decided to employ mice that express individual VpreB or λ5 components or both of them in mature B cells to study the effects of SLC signaling on B cell tolerance. Since it is conceivable that potential effects of SLC expression on mature B cell tolerance are limited to a narrow range of lower SLC expression levels, we used multiple independent CD19-VpreB Tg and CD19-λ5 Tg mouse lines harboring different copy numbers of these transgenic constructs. Most of these CD19-VpreB and CD19-λ5 transgenic mouse lines (expressing high levels of VpreB and λ5, respectively throughout B cell development under the control of the CD19 promoter region) we have previously described on an mixed genetic background (13). After crossing these lines onto the relatively autoimmune-resistant c57bl/6 genetic background for >10 generations, we analyzed three independent CD19-VpreB transgenic mouse strains: Vpreb#1 ($VpreB^{high}$), Vpreb#3 ($VpreB^{int}$) and $VpreB^{low}$, as well as two independent CD19-λ5 transgenic mouse strains: λ5#2 ($λ5^{high}$) and λ5#1 ($λ5^{low}$). Flow cytometric analysis of splenic B cells revealed increasing, but very low membrane expression of VpreB and λ5 in mice with increasing transgene copy numbers (Figure 1A). In mice carrying both a VpreB and λ5 transgene membrane expression of individual SLC components was markedly increased (Figure 1A).

Previous studies have shown that in the absence of VpreB λ5 has very limited binding capacity to the IgH chain, and that the eighth β-strand in λ5 is aiding the pairing of VpreB to the IgH chain (18, 19). To confirm that the increase of VpreB and λ5 membrane expression in double-transgenic B cells was due to mutual VpreB and λ5 stabilization rather than altered transcriptional activity of the individual transgenes, we measured intracellular VpreB and λ5 expression in these cells. We noted that single versus double-transgenic B cells carrying the same transgenic constructs did not exhibit overtly different VpreB or λ5 cytoplasmic expression levels (Figure 1B). Furthermore, the discrepancy between membrane versus cytoplasmic VpreB or λ5 expression was larger in single-transgenic than in double-transgenic B cells, indicating that the low membrane expression of VpreB and λ5 in single-transgenic B cells may not only be resulting from enhanced BCR internalization, but partially from the mutual dependence of VpreB and λ5 for stable membrane expression.

Taken together, these analyses show that our panel of SLC transgenic mice express a range of doses of VpreB and λ5 in mature peripheral B cells that allows us to analyse whether individual or combined expression of SLC components at the mature B cell stage can induce autoimmunity.

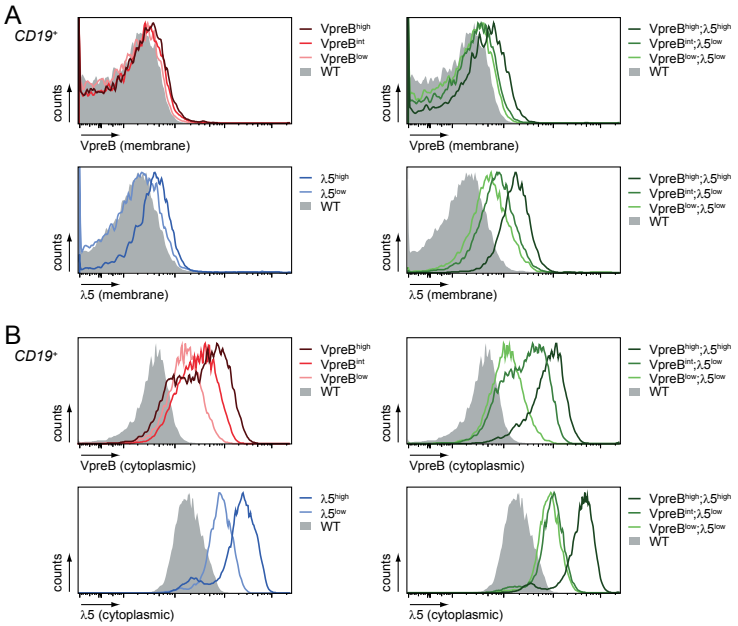


Figure 1. VpreB and λ5 expression in peripheral SLC transgenic B cells. Flow cytometric analysis of CD19^{+} gated wildtype (WT), VpreB and λ5 transgenic splenocytes for (A) membrane and (B) cytoplasmic expression of VpreB and λ5. Of each transgenic mouse strain representative histograms of >3 mice (10-14 weeks of age) are shown.

Moderate VpreB expression drives mild B cell activation and germinal center formation

Since the SLC has autonomous signaling capacity that may provide activating BCR signals to mature B cells independent of (allo-)antigens (11), we examined whether enforced SLC expression in mature B cells could induce their activation. To this end, we assessed membrane expression of various activation markers on transgenic splenic B cells. Flow cytometric analysis demonstrated a spontaneous mild upregulation of activation markers CD86 and to lesser extent CD69 on VpreB and/or λ5 transgenic B cells (Figure 2A), whereas CD25 was not differentially expressed. Upregulation of these B cell activation markers correlated with transgene expression levels in VpreB or λ5 transgenic B cells, while co-expression of VpreB and λ5 transgenes could only minimally increase CD86 and CD69 levels.

It has previously been published that high-level SLC expression in mature B cells could induce spontaneous differentiation of IgM plasma cells in vivo (13), but it is unclear whether lower levels of SLC expression may also drive activated B cells in SLC transgenic mice into T-cell dependent plasma cell differentiation. In all VpreB transgenic lines, but in particular in VpreB^{int} mice, we

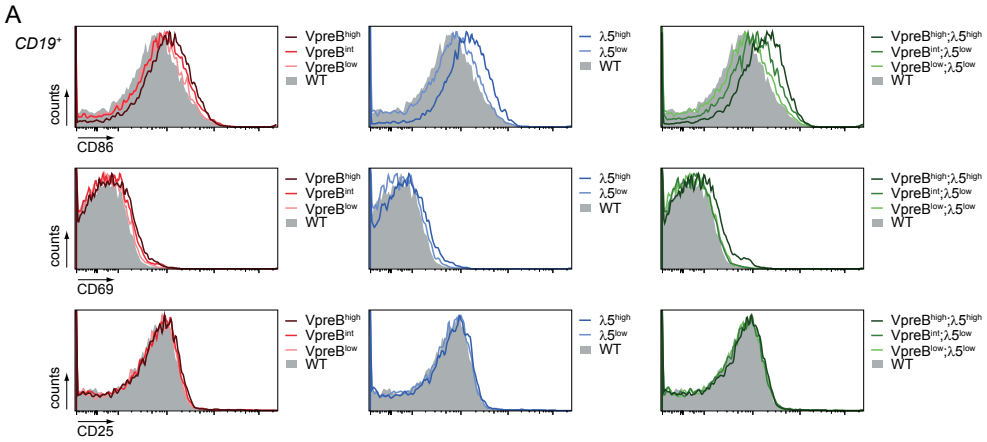
observed spontaneous germinal center (GC) formation in the spleen. This was reflected by a relative increase in $CD95^+PNA^+$ GC B cells among total $CD19^+$ gated cells (Figure 2B), whereas λ5 expression alone or in conjunction with VpreB could not induce this increase. Quantification by flow cytometry demonstrated however that the increase in GC B cell numbers was not significant between wild-type and VpreB transgenic mice (Figure 2C). Concomitantly we observed in $VpreB^{int}$ mice a near-significant (p=0.07) increase in splenic IgG plasma cell numbers, using flow cytometry (Figure 2D). These findings demonstrate that VpreB expression in mature B cells may facilitate GC formation and IgG plasma cell production, whereas λ5 expression can even preclude this spontaneous B cell differentiation.

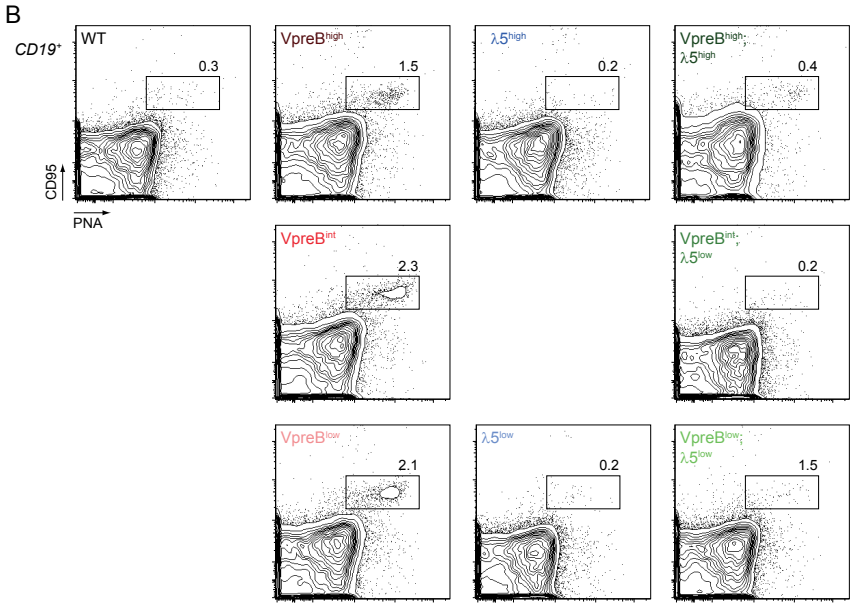
$VpreB^+$ B cells do not aggravate autoimmunity and ameliorate arthritis

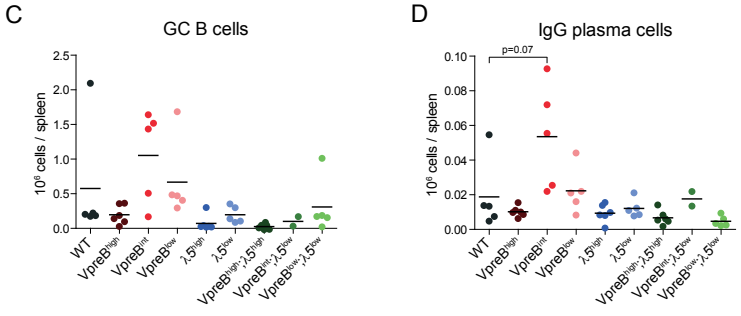
As $VpreB^{int}$ mice displayed a trend towards autonomous GC formation and enhanced IgG plasma cell formation in the absence of infection or immunization, we explored whether VpreB expression in mature B cells may facilitate the activation of self-reactive B cells. We examined the spontaneous development of autoimmunity in $VpreB^{int}$ transgenic mice on a pure c57bl/6 genetic background by screening serum from aging mice for auto-antibodies. Using ELISA we could not detect SLE-associated auto-antibodies, including IgG anti-nucleosome antibodies, in $VpreB^{int}$ transgenic mice nor in any other SLC transgenic strain (Figure 3A). Further screening for auto-antibody production by HEp2 autoreactivity assays did not reveal any anti-nuclear or anti-cytoplasmatic auto-antibody production in aging SLC transgenic mice (data not shown).

To investigate whether moderate VpreB expression in mature B cells could accelerate auto-immunity in SLE-prone mice, we bred $VpreB^{int}$ mice onto the *FcγRIIB*$^{-/-}$ background, which predisposes c57bl/6 mice to lethal SLE (20). In young (10 weeks) or aging (22 weeks) $VpreB^{int}$;*FcγRIIB*$^{-/-}$ versus *FcγRIIB*$^{-/-}$ mice we could not detect enhanced GC formation (Figure 3B) or IgG plasma cell formation (Figure 3C) using flow cytometry. Evaluation of IgG anti-nucleosome auto-antibody levels in serum showed a non-significant (p=0.18) trend towards higher auto-antibody levels in aging $VpreB^{int}$;*FcγRIIB*$^{-/-}$ versus *FcγRIIB*$^{-/-}$ mice (Figure 3D). Likewise, no significant increase in auto-antibody production could be observed in aging c57bl/6 Eμ-Bcl2 mice when a $VpreB^{int}$ or $VpreB^{low}$ transgene was introduced (Figure S2 and data not shown).

While SLC expression in mature B cells did not induce spontaneous arthritis in SLC transgenic strains, the enrichment of SLC^+LC^+ cells in inflamed joints of RA patients (1) prompted us to examine arthritis development in SLC transgenic mice. Since $VpreB^{int}$ mice exhibited a propensity to spontaneous GC and IgG plasma cell formation (Figure 2C and 2D) and a trend towards more auto-antibody production on the auto-immune prone *FcγRIIB*$^{-/-}$ background (Figure 3D), we provoked auto-immune arthritis in $VpreB^{int}$;*FcγRIIB*$^{-/-}$ versus *FcγRIIB*$^{-/-}$ mice using a collagen-induced arthritis (CIA) protocol (16). After the secondary immunization with chicken collagen type II (CII) on day 21, we observed a similar rise in arthritis incidence in both groups culminating


A
CD19+
counts
CD86
CD69
CD25
VpreBhigh
VpreBint
VpreBlow
WT
λ5high
λ5low
VpreBhigh;λ5high
VpreBint;λ5low
VpreBlow;λ5low


B
CD19+
CD95
PNA
WT
0.3
VpreBhigh
1.5
λ5high
0.2
VpreBhigh;λ5high
0.4
VpreBint
2.3
VpreBint;λ5low
0.2
VpreBlow
2.1
λ5low
0.2
VpreBlow;λ5low
1.5


C
GC B cells
10⁶ cells / spleen
2.5
2.0
1.5
1.0
0.5
0.0
WT
VpreBhigh
VpreBint
VpreBlow
λ5high
λ5low
VpreBhigh;λ5high
VpreBint;λ5low
VpreBlow;λ5low

D
IgG plasma cells
p=0.07
10⁶ cells / spleen
0.10
0.08
0.06
0.04
0.02
0.00
WT
VpreBhigh
VpreBint
VpreBlow
λ5high
λ5low
VpreBhigh;λ5high
VpreBint;λ5low
VpreBlow;λ5low

← **Figure 2. B cell activation and terminal B cell differentiation in SLC transgenic mice.** (A) Expression of activation markers CD86, CD69 and CD25 on splenic CD19^{+} cells in young (10-14 weeks old) VpreB and λ5 transgenic mice versus wild-type (WT) mice as determined by flow cytometry. Representative data of 3 independent experiments are shown. (B) Flow cytometric identification of CD95^{+}PNA^{+} germinal center B cells among gated CD19^{+} splenocytes in wildtype (WT), VpreB and λ5 transgenic mice (10-14 weeks of age). Representative data of at least 2 independent experiments are shown. (C and D) Flow cytometric quantification of germinal center (GC) B cells and IgG plasma cells in spleens of young SLC transgenic (10-14 weeks old). GC B cells were identified as CD19^{+}CD95^{+}PNA^{+} cells and IgG plasma cells as CD138^{high} cells with high cytoplasmic expression of either IgG1, IgG2 or IgG3. Collective data of more than 2 independent experiments are shown.

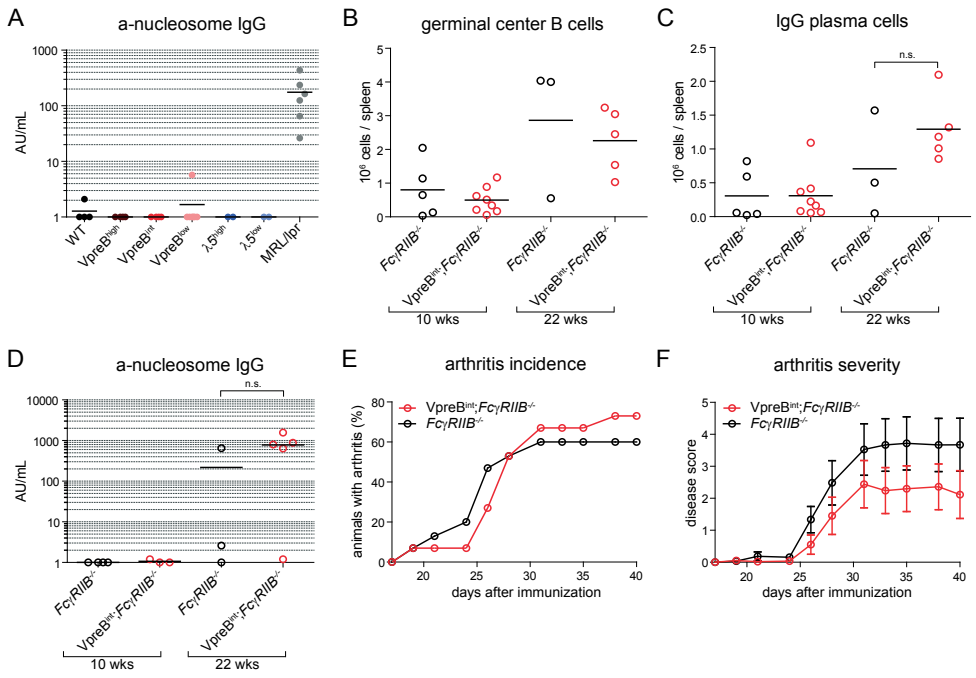


Figure 3. No spontaneous or enhanced autoimmunity in SLC transgenic mice. (A) Serum IgG anti-nucleosome levels were determined in wildtype (WT), SLC transgenic mice (all 22 weeks of age) and diseased MRL/lpr mice by ELISA. (B and C) Splenic germinal center B cells and IgG plasma cells in young (10 weeks old) and aging (22 weeks old) VpreB^{int} transgenic versus non-transgenic littermates on the *FcγRIIB*$^{-/-}$ background were quantified as described in Figure 2C and 2D. (D) ELISA measurement of IgG anti-nucleosome antibody levels in serum from young and aging VpreB^{int} transgenic versus non-transgenic littermates on the *FcγRIIB*$^{-/-}$ background. (E) Proportion of animals with signs of arthritis and (F) arthritis scores in young (10-14 weeks) VpreB^{int};*FcγRIIB*$^{-/-}$ and non-transgenic *FcγRIIB*$^{-/-}$ littermates monitored after immunization against chicken collagen type II (CII) on day0 and day 21. Data from 1 out of 2 independent experiments are shown; n=15 mice per group.

in ~73% of VpreB^{int};*FcγRIIB*$^{-/-}$ versus ~60% of *FcγRIIB*$^{-/-}$ mice with arthritis symptoms at day 40 (Figure 3E). Importantly however the severity of arthritis in VpreB^{int};*FcγRIIB*$^{-/-}$ mice mas markedly reduced compared to *FcγRIIB*$^{-/-}$ mice (Figure 3F). At day 40 the overall arthritis disease in groups of VpreB^{int};*FcγRIIB*$^{-/-}$ versus *FcγRIIB*$^{-/-}$ mice was 3.7 versus 2.1 respectively (p=0.17, Figure 3F) while the score of diseased VpreB^{int};*FcγRIIB*$^{-/-}$ versus *FcγRIIB*$^{-/-}$ mice was 6.2 versus 4.0 respectively (p=0.08).

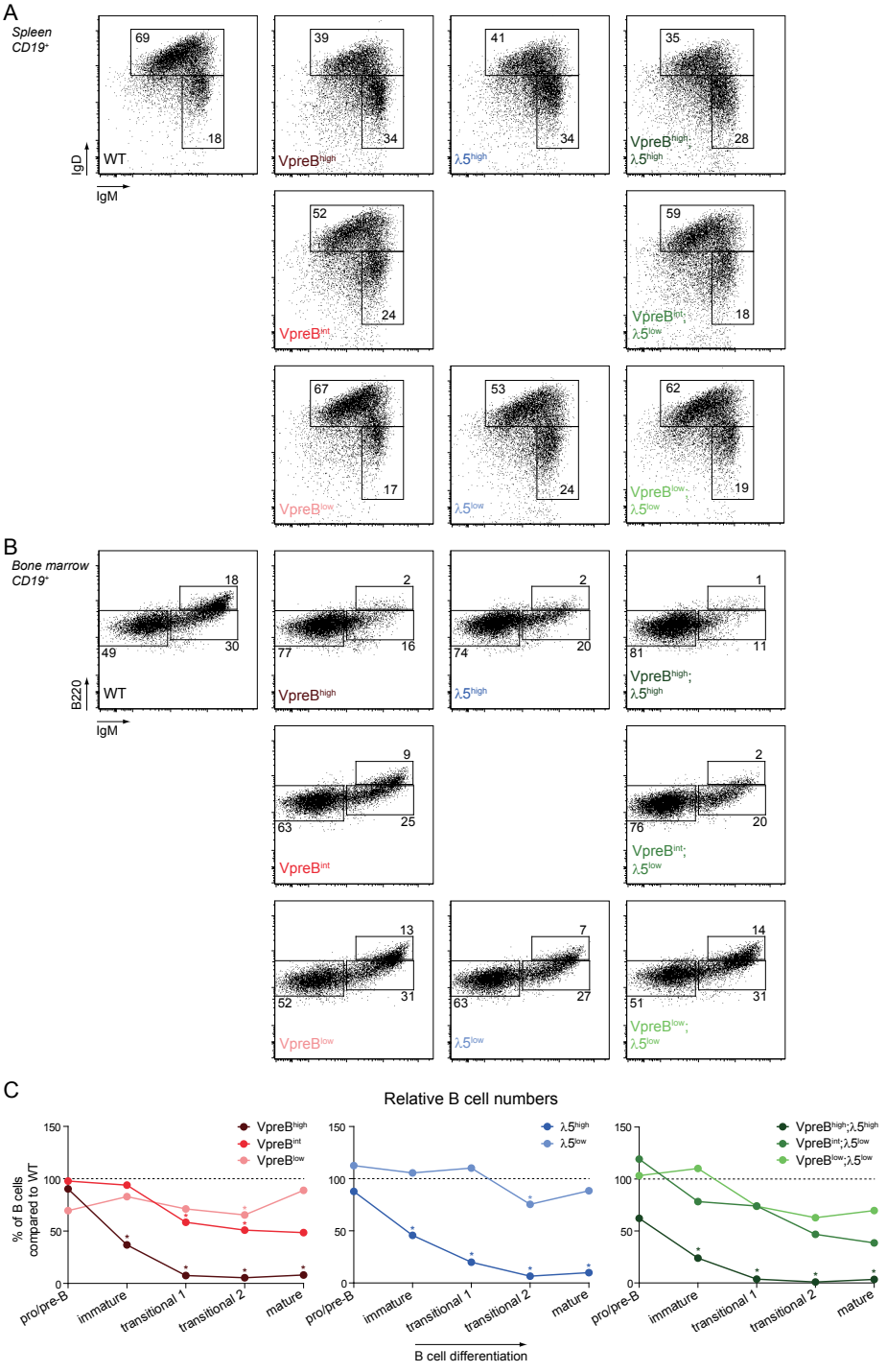
Taken together, these findings indicate that ectopic expression of SLC components in mature B cells cannot provoke or enhance systemic auto-immunity but rather alleviates autoimmune symptoms in auto-immune prone mice.

SLC expression induces clonal deletion in developing B cells

The SLC chain has the unique capacity to signal autonomously through the non-immunoglobulin tail of λ5 (7, 8, 11). It may therefore lower BCR membrane expression on mature B cells by inducing enhanced BCR internalization and thereby preclude the development of strong autoimmunity. We therefore examined IgD and IgM expression levels on the surface of SLC transgenic splenic B cells, but failed to detect a clear SLC-induced decrease in IgM or IgD expression (Figure 4A). However, correlating with increasing levels of transgenic VpreB and/or λ5 expression a relative decrease in IgD^{high} mature splenic B cells was observed in SLC transgenic mice (Figure 4A), suggestive of a loss of B cells during final B cell maturation stages.

A loss of B cells at the immature B cell stage has been previously described in high-level SLC-transgenic mice and may reflect aspecific toxicity effects of high-level SLC expression at this developmental stage (13). It is, however, conceivable that the loss of B cells could reflect a more stringent clonal deletion of developing auto- or polyreactive peripheral B cells. We therefore investigated whether such elimination of B cells would occur in a dose-dependent fashion at known autoreactivity checkpoints during B cell development. Flow cytometric analysis of bone marrow B cells confirmed a SLC dose-dependent relative reduction of immature (B220^{low}IgM^{pos})

Figure 4. SLC dose-dependent clonal deletion of immature and transitional B cells →
(A) B cell maturation in young (10-14 weeks) wildtype (WT) and SLC transenic mice was assessed by flow cytometric characterization of IgM and IgD expression on splenic CD19^{+} cells. Numbers indicate the percentage of B cells in quadrant gates. Results of 1 representative out of at least 2 independent experiments are shown. (B) Pro- and pre-B cells (B220^{low}IgM^{neg}), immature B cells (B220^{low}IgM^{pos}) and mature recirculating B cells (B220^{high}IgM^{high}) were identified by flow cytometry in bone marrow from young (10-14 weeks old) wildtype (WT) mice and SLC transgenic littermates. Numbers correspond to B cell percentages within the gates. Data from 1 out of at least 2 independent experiments are shown. (C) Relative numbers of pro/pre-B cells (bone marrow CD19^{+}B220^{low}IgM^{neg} cells), immature B cells (bone marrow CD19^{+}B220^{low}IgM^{pos} cells), transitional 1 (T1) B cells (splenic CD19^{+}CD93^{+}IgM^{high}CD23^{low} cells), transitional 2 (T2) B cells (splenic CD19^{+}CD93^{+}IgM^{high}CD23^{high} cells) and mature B cells (splenic CD19^{+}IgD^{high} cells) were quantified using flow cytometry in young (10-14 weeks old) SLC transgenic versus non-transgenic littermates. Asterisks indicate significant reductions (p<0.05) in B cell numbers, compared with wild-type mice. Collective data from 3 independent experiments are shown.


A
Spleen
CD19+
IgD
IgM
WT
69
18
VpreBhigh
39
34
λ5high
41
34
VpreBhigh;
λ5high
35
28
VpreBint
52
24
VpreBint;
λ5low
59
18
VpreBlow
67
17
λ5low
53
24
VpreBlow;
λ5low
62
19
B
Bone marrow
CD19+
B220
IgM
WT
18
49
30
VpreBhigh
2
77
16
λ5high
2
74
20
VpreBhigh;
λ5high
1
81
11
VpreBint
9
63
25
VpreBint;
λ5low
2
76
20
VpreBlow
13
52
31
λ5low
7
63
27
VpreBlow;
λ5low
14
51
31
C
Relative B cell numbers
% of B cells compared to WT
VpreBhigh
VpreBint
VpreBlow
λ5high
λ5low
VpreBhigh;λ5high
VpreBint;λ5low
VpreBlow;λ5low
150
100
50
0
pro/pre-B
immature
transitional 1
transitional 2
mature
B cell differentiation

and recirculating mature (B220^{high}IgM^{high}) B cells (Figure 4B). Quantification of B cell numbers throughout B cell differentiation in the SLC transgenic strains showed that no loss of B cells occurred before the immature B cell stage in the bone marrow. We noted a SLC dose-dependent reduction of absolute B cell numbers at the immature B cell stage in the bone marrow and at transitional B cell stages in the spleen (Figure 4C). While in transgenic mice expressing high levels of VpreB and/or λ5 a strong and significant reduction of B cell numbers already occurred at the immature B cell stage, this B cell loss was only noticeable during splenic transitional B cell stages in transgenic mice expressing intermediate or low VpreB and/or λ5 levels. In these analyses, the B cell reduction in most SLC transgenic strains was less prominent at the mature B cell stage compared to transitional B cell stages, possibly reflecting an compensatory enhanced survival of fully mature B cells to maintain peripheral B cell numbers.

To test whether next to clonal deletion SLC signaling in immature B cells could also enforce receptor editing in the bone marrow (21), we explored Igλ usage as a read-out of receptor editing. Using flow cytometry we could not detect a significant increase in Igλ usage of by peripheral B cells in any of the SLC transgenic lines (Figure S3A and S3B), arguing against enhanced receptor editing instructed by SLC signaling.

SLC expression in mature B cells instructs BCR unresponsiveness in a dose-dependent fashion

The deletion of auto- and polyreactive immature and transitional B cells strongly depends on activating BCR signals (22, 23). The gradual deletion of immature and transitional B cells in SLC transgenic mice prompted us to investigate the BCR signaling quality in SLC transgenic B cells. Using a Ca^{2+} mobilization assay we determined the BCR signaling strength of SLC transgenic B cells upon α-IgM stimulation. We noted a reduction in Ca^{2+} influx in SLC transgenic splenic B cells tightly correlating to the dose of VpreB and/or λ5 expression in these cells (Figure 5A). Although single-transgenic B cells exhibited an apparently normal initial Ca^{2+} influx upon BCR stimulation, the decay in cytoplasmic Ca^{2+} levels was faster than in wild-type B cells. High-level expression of both VpreB and λ5 invoked both a reduced initial Ca^{2+} influx and a quick loss of cytoplasmic Ca^{2+}.

This reduced Ca^{2+} mobilization in SLC transgenic B cells strongly resembles the BCR signaling characteristics of anergic B cells. First we tried using flow cytometry to identify anergic B cell populations in SLC transgenic mice *in vivo* by the expression of markers as CD93, CD23 and CD138, but we were unable to detect an increase in previously described anergic B cell fractions (data not shown) (24, 25). We next tested whether the expression of SLC on mature B cells renders them selectively unresponsive to BCR signals by comparing the activation of SLC transgenic B cells after BCR versus TLR stimulation. While enforced SLC expression could not induce spontaneous upregulation of activation marker CD25 on unstimulated B cells *in vitro*, CD25 upregulation was

significantly reduced on α-IgM stimulated SLC transgenic B cells compared to wildtype B cells (Figure5B and 5C). Again, VpreB and/or λ5 expression levels inversely correlated with B cell activation. Importantly, the reduced upregulation of CD25 in response to BCR stimulation was not resulting from a general unresponsiveness to B cell activating signals since B cells expressing low or intermediate levels of VpreB and/or λ5 showed normal CD25 upregulation (Figure 5B and 5C). A small but significant reduction in CD25 upregulation was observed in VpreB^{high} and λ5^{high} B cells upon LPS stimulation, but this reduction was not as large as observed in these cells upon α-IgM stimulation.

Taken together, these results demonstrate that continued SLC expression beyond the pre-B cell stage in a dose-dependent mode instructs deletion of developing immature and transitional B cells and anergy of surviving mature B cells.

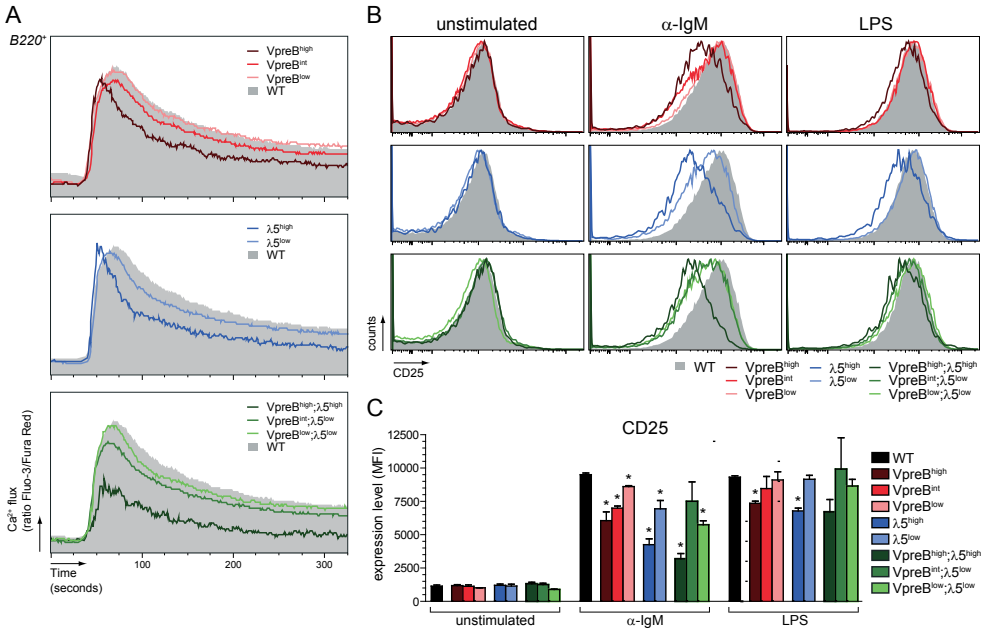


Figure 5. Dose-dependent induction of anergy by SLC expression. (A) Measurement of Ca^{2+} mobilization in B220^{+} gated splenocytes loaded with Fluo-3 and Fura Red ™ from wildtype (WT) versus SLC transgenic mice after stimulation with 20 µg/mL F(ab')$_2$ anti-IgM. Measurements were normalized for maximum Ca^{2+} influx upon ionomycin stimulation (2 µg/mL). (B) Flow cytometric evaluation of CD25 membrane expression after F(ab')$_2$ anti-IgM (10 µg/mL) or LPS (5 µg/mL) stimulation of MACS-purified splenic B cells from SLC transgenic mice and non-transgenic littermates. (C) Quantification by mean fluorescence intensity (MFI) of CD25 membrane expression levels as measured in the experiment of Figure 5B. Per group at least 3 mice were analyzed. Error bars represent standard deviations; asterisks indicate MFI levels significanty different from wildtype MFI levels ($p<0.05$).

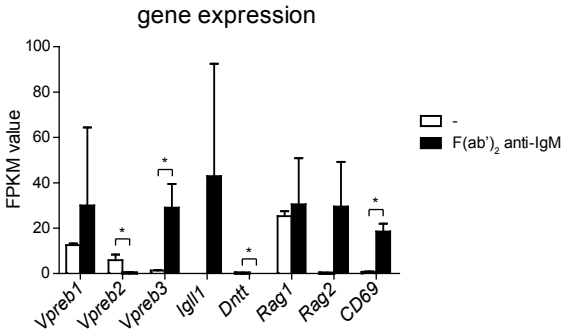


Figure S1. Expression of SLC components in BCR stimulated splenic B cells. MACS-purified naïve splenic B cells ($CD19^+CD43^-CD5^-CD138^-$) from 4 wild-type mice were cultured for 12 hours with 10 µg/mL $F(ab')_2$ anti-IgM (black bars) or left unstimulated (white bars). Transcript levels in these cultured cells are shown for the indicated genes as determined by total mRNA deep sequencing analysis; error bars represent standard errors of the mean. Asterisks indicate significant differences ($p<0.05$) in gene expression. FPKM: fragments per kilobase of a transcript per million mapped reads.

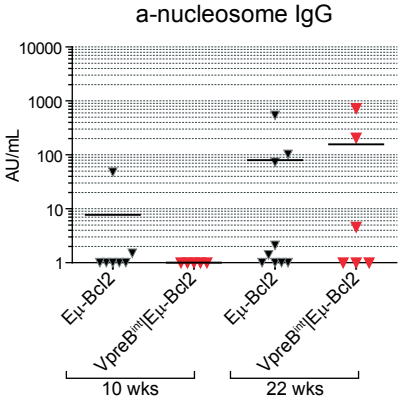


Figure S2. No enhanced autoantibody production in aging $VpreB^{int}$;Eμ-Bcl2 mice. Quantification by ELISA of serum IgG anti-nucleosome autoantibodies in young (10 weeks old) and aging (22 weeks old) $VpreB^{int}$ versus non-transgenic littermates on the Eμ-Bcl2 genetic background.

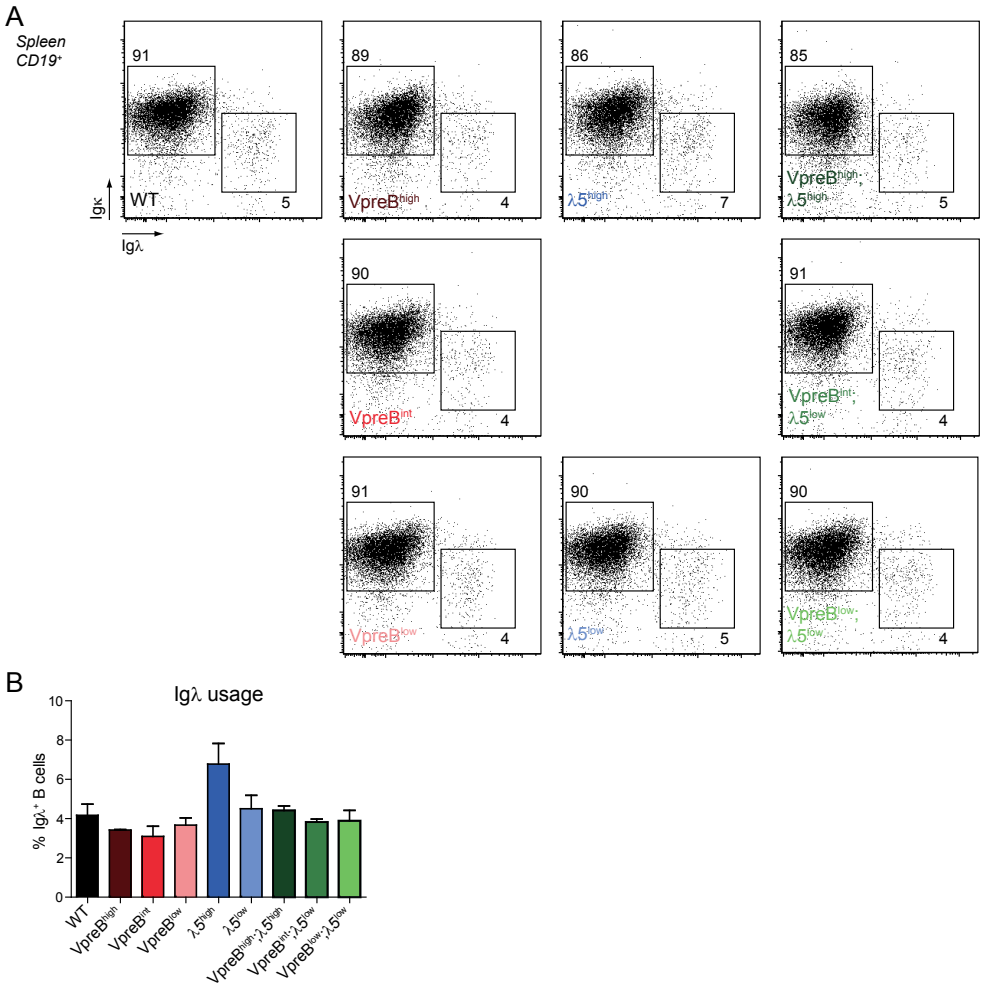


Figure S3. No increased Igλ usage by peripheral SLC transgenic B cells
(A) Relative frequencies of Igκ+ and Igλ+ cells among CD19+ gated splenic B cells as determined by flow cytometry. Numbers indicate the percentage of cells within the indicated gates. (B) Summarized data of the flow cytometric analysis in (A). Error bars indicate standard deviations; >3 mice per group were analyzed.

DISCUSSION

Over a decade ago, human SLC expressing mature B cells were described and readily associated with autoimmunity due to their abundance in arthritic joints and their frequent expression of autoreactive BCRs (1, 2). Since their discovery no studies have been performed that address whether SLC+ mature B cells may actively drive autoimmune disease. Here we report that in mice

transgenically expressing SLC in mature B cells no enhanced or spontaneous autoimmunity could be observed on multiple genetic backgrounds despite mild spontaneous activation of SLC^+ B cells. Instead, we found that SLC expression in mature B cells partially protects mice from autoimmune arthritis. This protective role of SLC re-expression can be attributed to a more stringent clonal deletion of immature and transitional B cells as well as induction of anergy in mature B cells.

Although SLC re-expression has been reported in splenic murine germinal center B cells upon their activation during a T-dependent antibody response (3), the role of SLC^+ mature B cells in autoimmunity has not been previously studied in murine models. This may be attributed to the very low frequencies of naturally occurring SLC^+ mature B cells. Despite our finding in mRNA deep sequencing experiments that *Vpreb3* transcription is upregulated upon BCR activation of splenic B cells *in vitro*, we were unable to detect considerable frequencies of $VpreB^+$ wildtype B cells in lymphoid organs that typically contain germinal centers, including Peyer's patches or mesenteric lymph nodes (data not shown). This discrepancy between our and previous studies (3) in the identification of SLC^+ mature B cells *in vivo* in wild-type mice may be explained by differences in (i) antigen dose and thus BCR stimulation, in (ii) the use of alum adjuvant, or (iii) in anatomical localization of antigen-activated B cells, leading to altered B cell engagement and therefore SLC re-expression in these different T-dependent responses.

Given the selective conditions that permit SLC re-expression in only a limited number of B cells in wildtype mice, we employed transgenic mice that express various doses of the SLC components VpreB and λ5 in B cells beyond the pre-B cell stage to study a possible contribution of the SLC to the development of autoimmunity. Our studies in SLC transgenic mice show enforced SLC expression on mature B cells induces mild B cell activation *in vivo* that in a dose-dependent fashion, whereas GC formation and IgG plasma cell differentiation and are not enhanced. Only in mice expressing moderate levels of VpreB in mature B cells ($VpreB^{int}$) we observed a discernible, but non-significant, increase in GC B cells and IgG plasma cells. The absence of spontaneous GCs in mice co-expressing VpreB and λ5 indicates that λ5 signaling prohibits a GC differentiation program in activated $VpreB^{int}$ B cells. Differences in B cell fate between activated mature B cells expressing either VpreB or λ5 may be explained by differences in the BCR signals provided to these cells, and indeed an extensive body of literature exists that links BCR signaling strength to B cell differentiation fate (26-29). However, in our models the absence of clear differences between VpreB or λ5 transgenic B cells in Ca^{2+} influx and activation marker upregulation in response to in vitro α-IgM stimulation contradicts a difference in BCR signaling quality as the primary cause of differences in GC formation.

To explain these differences in B cell fate between VpreB versus λ5 transgenic mice alternative causes must be considered, including the possibility that not λ5 but VpreB may alter terminal B cell differentiation by manipulating antigen-based B cell selection through allelic inclusion, a phenomenon that may rescue B cells with irrelevant BCR specificities. (30, 31). A prerequisite for allelic exclusion would be the stable membrane expression of either SLC component. Although

our studies show that neither VpreB nor λ5 can be individually expressed at the cell membrane at high levels, previous reports demonstrated that VpreB had a better IgH chain pairing capacity (19) and thus possibly a greater capacity to establish allelic inclusion. In naturally occurring dual light chain expressing B cells, the failing allelic exclusion of one of the two light chains is associated with very similar expression levels of the two light chains, thereby effectively reducing the signaling strength of either BCR by twofold.

If transgenic expression of SLC components would mimic allelic exclusion, one would expect allelic exclusion to be effective only if comparable membrane expression levels of SLC components and IgL are being achieved. This is not the case, based on the low membrane VpreB and λ5 levels on SLC transgenic cells (Figure 1A) while normal Igκ and Igλ membrane levels are being observed on these cells (Figure S3). However, the intrinsic autonomous signaling capacity of SLC components may compensate for low membrane expression levels, establishing a state of BCR signaling compatible with allelic inclusion while SLC components and LCs are unequally expressed.

Despite the spontaneous formation of germinal centers in VpreB^{int} mice in the absence of any challenge with exogenous antigen, we could not detect any spontaneous autoimmunity on the relatively autoimmune-resistant c57bl/6 background nor any aggravation of autoimmunity when moderate VpreB expression was introduced on autoimmune-susceptible genetic backgrounds (Figure 3A-D). In contrast, autoimmune arthritis proved to be less severe in VpreB^{int} transgenic *FcγRIIB*$^{-/-}$ mice compared with non-transgenic littermates, demonstrating a protective role of VpreB re-expression in autoimmune arthritis. It may be argued that this protection could be solely conferred by the approximate two-fold reduction in total IgD^{+} B cell numbers in VpreB^{int} mice. The deletion would then not have been random, but rather specifically affecting developing B cells with a polyreactive or self-reactive BCR in SLC transgenic mice, thereby establishing a more robustly purged mature B cell repertoire. The elimination of collagen type II-reactive B cells in VpreB^{int} mice is probably incomplete, since equal proportion of these mice and non-transgenic littermates develop arthritis symptoms. An additional mechanism that suppresses the pathogenicity of such cells is provided by our finding that SLC-component expressing B cells are anergic as evident from reduced Ca^{2+} mobilization and CD25 upregulation upon α-IgM but not LPS stimulation. Since anergy can only be induced by chronic BCR occupation (32), the anergic response of mature SLC transgenic B cells demonstrates the continuous signals provided by SLC components to these cells. Although our analyses on BCR-induced Ca^{2+} influx only include peripheral B cells, the SLC signals autonomously and therefore this chronic BCR signaling most likely is the driving force behind the enhanced deletion of immature and transitional B cells.

The finding that especially VpreB^{int} transgenic mice seem predisposed to spontaneous GC and IgG plasma cell formation is striking, since these cells most closely resemble human peripheral SLC^{+}LC^{+} B cells that are reported to express hardly any λ5 and VpreB levels that are lower than those in pro- and pre-B cells (1). In addition to moderate VpreB expression, human SLC^{+}LC^{+} B

cells also re-express early B cell genes as TdT and RAG at low levels, suggesting that SLC signaling may launch a transcriptional program to revise their (autoreactive) BCRs (33). In accordance with this view, in human the IgH and IgL chains of SLC^+LC^+ B cells frequently display characteristics of extended receptor editing, such as more distal J gene usage and D-D fusions (33). Such receptor revisions could abolish the pathogenic potential of autoreactive SLC^+LC^+ B cells, suggesting that SLC re-expression could be a mechanism to induce tolerance in mature B cells, either through the previously suggested induction of receptor editing, or by anergy and clonal deletion. Although our results in murine transgenic models may be difficult to directly extrapolate to the pathogenesis of human rheumatic diseases, our finding that moderate VpreB expression ameliorates rather than aggravates autoimmunity supports the idea that the largely autoreactive pool of SLC^+LC^+ B cells found in arthritic joints does not actively contribute to autoimmunity and therefore is not an eligible target in the treatment for autoimmune disease.

REFERENCES

1. Meffre, E., E. Davis, C. Schiff, C. Cunningham-Rundles, L. B. Ivashkiv, L. M. Staudt, J. W. Young, and M. C. Nussenzweig. 2000. Circulating human B cells that express surrogate light chains and edited receptors. *Nat Immunol* 1:207-213.
2. Meffre, E., A. Schaefer, H. Wardemann, P. Wilson, E. Davis, and M. C. Nussenzweig. 2004. Surrogate light chain expressing human peripheral B cells produce self-reactive antibodies. *J Exp Med* 199:145-150.
3. Meffre, E., F. Papavasiliou, P. Cohen, O. de Bouteiller, D. Bell, H. Karasuyama, C. Schiff, J. Banchereau, Y. J. Liu, and M. C. Nussenzweig. 1998. Antigen receptor engagement turns off the V(D)J recombination machinery in human tonsil B cells. *J Exp Med* 188:765-772.
4. Geier, J. K., and M. S. Schlissel. 2006. Pre-BCR signals and the control of Ig gene rearrangements. *Semin Immunol* 18:31-39.
5. Keenan, R. A., A. De Riva, B. Corleis, L. Hepburn, S. Licence, T. H. Winkler, and I. L. Martensson. 2008. Censoring of autoreactive B cell development by the pre-B cell receptor. *Science* 321:696-699.
6. Herzog, S., M. Reth, and H. Jumaa. 2009. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. *Nature reviews. Immunology* 9:195-205.
7. Ohnishi, K., and F. Melchers. 2003. The nonimmunoglobulin portion of lambda5 mediates cell-autonomous pre-B cell receptor signaling. *Nat Immunol* 4:849-856.
8. Kohler, F., E. Hug, C. Eschbach, S. Meixlsperger, E. Hobeika, J. Kofer, H. Wardemann, and H. Jumaa. 2008. Autoreactive B cell receptors mimic autonomous pre-B cell receptor signaling and induce proliferation of early B cells. *Immunity* 29:912-921.
9. Bradl, H., J. Wittmann, D. Milius, C. Vettermann, and H. M. Jack. 2003. Interaction of murine precursor B cell receptor with stroma cells is controlled by the unique tail of lambda 5 and stroma cell-associated heparan sulfate. *J Immunol* 171:2338-2348.
10. Gauthier, L., B. Rossi, F. Roux, E. Termine, and C. Schiff. 2002. Galectin-1 is a stromal cell ligand of the pre-B cell receptor (BCR) implicated in synapse formation between pre-B and stromal cells and in pre-BCR triggering. *Proc Natl Acad Sci U S A* 99:13014-13019.
11. Ubelhart, R., M. P. Bach, C. Eschbach, T. Wossning, M. Reth, and H. Jumaa. 2010. N-linked glycosylation selectively regulates autonomous precursor BCR function. *Nat Immunol* 11:759-765.
12. Hendriks, R. W., and R. Kersseboom. 2006. Involvement of SLP-65 and Btk in tumor suppression and malignant transformation of pre-B cells. *Semin Immunol* 18:67-76.
13. van Loo, P. F., G. M. Dingjan, A. Maas, and R. W. Hendriks. 2007. Surrogate-light-chain silencing is not critical for the limitation of pre-B cell expansion but is for the termination of constitutive signaling. *Immunity* 27:468-480.
14. Takai, T., M. Ono, M. Hikida, H. Ohmori, and J. V. Ravetch. 1996. Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice. *Nature* 379:346-349.
15. Strasser, A., S. Whittingham, D. L. Vaux, M. L. Bath, J. M. Adams, S. Cory, and A. W. Harris. 1991. Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. *Proc Natl Acad Sci U S A* 88:8661-8665.
16. Lubberts, E., M. I. Koenders, B. Oppers-Walgreen, L. van den Bersselaar, C. J. Coenen-de Roo, L. A. Joosten, and W. B. van den Berg. 2004. Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. *Arthritis Rheum* 50:650-659.
17. Kil, L. P., M. J. de Bruijn, M. van Nimwegen, O. B. Corneth, J. P. van Hamburg, G. M. Dingjan, F. Thaiss, G. F. Rimmelzwaan, D. Elewaut, D. Delsing, P. F. van Loo, and R. W. Hendriks. 2012. Btk levels set the threshold for B-cell activation and negative selection of autoreactive B cells in mice. *Blood* 119:3744-3756.
18. Minegishi, Y., L. M. Hendershot, and M. E. Conley. 1999. Novel mechanisms control the folding and assembly of lambda5/14.1 and VpreB to produce an intact surrogate light chain. *Proc Natl Acad Sci U S A* 96:3041-3046.

19. Seidl, T., A. Rolink, and F. Melchers. 2001. The VpreB protein of the surrogate light-chain can pair with some mu heavy-chains in the absence of the lambda 5 protein. *European journal of immunology* 31:1999-2006.

20. Bolland, S., and J. V. Ravetch. 2000. Spontaneous autoimmune disease in Fc(gamma)RIIB-deficient mice results from strain-specific epistasis. *Immunity* 13:277-285.

21. Nemazee, D. 2006. Receptor editing in lymphocyte development and central tolerance. *Nature reviews. Immunology* 6:728-740.

22. Goodnow, C. C. 2007. Multistep pathogenesis of autoimmune disease. *Cell* 130:25-35.

23. Shlomchik, M. J. 2008. Sites and stages of autoreactive B cell activation and regulation. *Immunity* 28:18-28.

24. Merrell, K. T., R. J. Benschop, S. B. Gauld, K. Aviszus, D. Decote-Ricardo, L. J. Wysocki, and J. C. Cambier. 2006. Identification of anergic B cells within a wild-type repertoire. *Immunity* 25:953-962.

25. Culton, D. A., B. P. O'Conner, K. L. Conway, R. Diz, J. Rutan, B. J. Vilen, and S. H. Clarke. 2006. Early preplasma cells define a tolerance checkpoint for autoreactive B cells. *J Immunol* 176:790-802.

26. Casola, S., K. L. Otipoby, M. Alimzhanov, S. Humme, N. Uyttersprot, J. L. Kutok, M. C. Carroll, and K. Rajewsky. 2004. B cell receptor signal strength determines B cell fate. *Nat Immunol* 5:317-327.

27. Gatto, D., D. Paus, A. Basten, C. R. Mackay, and R. Brink. 2009. Guidance of B cells by the orphan G protein-coupled receptor EBI2 shapes humoral immune responses. *Immunity* 31:259-269.

28. Phan, T. G., D. Paus, T. D. Chan, M. L. Turner, S. L. Nutt, A. Basten, and R. Brink. 2006. High affinity germinal center B cells are actively selected into the plasma cell compartment. *J Exp Med* 203:2419-2424.

29. Paus, D., T. G. Phan, T. D. Chan, S. Gardam, A. Basten, and R. Brink. 2006. Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. *J Exp Med* 203:1081-1091.

30. Li, Y., H. Li, and M. Weigert. 2002. Autoreactive B cells in the marginal zone that express dual receptors. *J Exp Med* 195:181-188.

31. Gerdes, T., and M. Wabl. 2004. Autoreactivity and allelic inclusion in a B cell nuclear transfer mouse. *Nat Immunol* 5:1282-1287.

32. Gauld, S. B., R. J. Benschop, K. T. Merrell, and J. C. Cambier. 2005. Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling. *Nat Immunol* 6:1160-1167.

33. Meffre, E., M. Chiorazzi, and M. C. Nussenzweig. 2001. Circulating human B cells that express surrogate light chains display a unique antibody repertoire. *J Immunol* 167:2151-2156.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IL-27 receptor signaling is essential for B cell immunity

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Manuscript in preparation

ABSTRACT

Introduction IL-27 receptor (IL-27R) signaling is involved in T helper cell (Th)1 differentiation and follicular Th cell function. However, its effects on B cell immunity are not fully understood.

Methods To study whether IL-27 directly affects B cell function, naïve wild-type splenic and peritoneal B cells were cultured in the absence or presence of recombinant IL-27 (rIL-27). In vivo effects of IL-27R signaling were investigated in wild-type and IL-27R deficient (WSX-1 deficient) mice in collagen induced arthritis (CIA) and the DNP-Ficoll immunization models.

Results We found that IL-27 had limited effects on splenic B cells in vitro and that IL-27R KO splenic B cells appeared normal. However, CIA development was decreased in IL-27R KO mice and was associated with impaired germinal center formation. Moreover, in the IL-27R KO mice the proportions of peritoneal B1 cells were increased, as well as the number of IgM plasma cells in the spleen and total IgM antibody levels in serum, suggesting enhanced B1 cell immunity. Although IL-27R-deficient B1 cells showed a normal response to LPS stimulation in vitro, T cell independent B cell responses in vivo were lacking in in IL-27R KO mice.

Conclusions Our findings show that IL-27 has limited direct effects on B cells, but is essential for B cell immunity in a non-B cell intrinsic fashion.

INTRODUCTION

Interleukin 27 (IL-27), a member of the IL-12 cytokine family, is a heterodimer consisting of an IL-27p28 and an Ebi3 subunit [1]. IL-27 is predominantly expressed by dendritic cells (DC) and binds to a heterodimeric receptor complex consisting of gp130 and WSX-1 [2, 3]. The effects of IL-27 on T cell development and function have been well studied [4], yet the IL-27 receptor (IL-27R) is expressed on many cell types, including B cells, NK cells, epithelial cells and mast cells [3]. The interpretation of data on IL-27 is further complicated because individual subunits of IL-27 can also bind to other factors to form different molecules. Ebi3 can bind to IL12p35 to form IL-35, which is expressed by regulatory T cells [5], and IL27p28 can bind to CLF-1 to form a heterodimer affecting NK and T cell cytokine production [6]. Furthermore, gp130 is a subunit also used for signaling by other cytokines, including IL-6, and IL27p28 alone has been shown to limit signaling of these cytokines by binding to gp130 [7].

IL-27 was initially described as an inducer of T helper 1 (Th1) cell differentiation. WSX-1 was shown to be essential for early commitment to the Th1 lineage [2, 8]. IL-27R signaling induces T-bet through STAT1 activation, leading to up regulation of IL-12β2 expression on naïve T cells, thereby enhancing susceptibility to Th1 differentiation [9-11]. Indeed, mice deficient for the IL-27R were more susceptible to infection with *Lysteria monocytogenes* [2] and *Leishmania major* [12] and developed more severe arthritis in an IFNγ-dependent proteoglycan induced arthritis model [13].

However, IL-27 is now regarded more as a regulatory cytokine in the immune system. IL-27 directly inhibits Th2 differentiation by limiting the expression of GATA-3 [14]. Mice lacking IL-27R showed exacerbated allergic asthma associated with an enhanced Th2 response [15]. IL-27 also limits Th17 differentiation by directly reducing the expression of RORγt [12, 16]. WSX-1 deficient mice showed enhanced numbers of Th17 cells in the central nervous system in experimental autoimmune encephalomyelitis [17] and wild-type mice that were administered IL-27 shortly before onset of disease in collagen induced arthritis (CIA) had attenuated arthritis [18]. IL-27 also affects effector T cells. IL-27 can induce IL-10 production by several T cell subsets through STAT3 [19-21]. IL-10 production was enhanced by ICOS, IL-21 and c-Maf, the expression of which were in turn enhanced by IL-27 [22].

IL-27 has an indirect effect on B cell immunity through follicular T helper T(FH) cells. T(FH) cells are vital in the regulation of germinal center (GC) reactions [23, 24]. GCs are specialized structures in B cell follicles where affinity maturation and high affinity B cell selection take place [25]. In the GCs T(FH) cells produce IL-21, which is a potent inducer of differentiation of B cells into plasma cells [26, 27]. IL-27 is critical for T(FH) effector function in GCs and lack of IL-27R signaling leads to impaired GC formation [28]. B cells express the IL-27R and several studies report an effect of IL-27 on human B cell activation and proliferation [29, 30], but it is unclear whether IL-27R signaling controls B cell function in a B cell-intrinsic fashion or whether IL-27R signaling in other cell types, particularly T cells, indirectly affects B cells.

In this study, we aim to investigate how IL-27R signaling controls B cell immunity. We show that whereas the direct effects of IL-27R signaling on B cells are limited, IL-27 is crucial for B cell immunity in vivo. In CIA, GC formation and auto-antibody formation was impaired in WSX-1 knock-out (IL-27R KO) mice, but we did not detects direct effects of IL-27 on follicular B cells. In addition, we examined the effect of IL-27R signaling on the in vivo B cell response to the T cell-independent antigen DNP-Ficoll and found that plasma cell and antibody formation were significantly decreased in IL-27R KO mice compared to wild-type controls. We therefore conclude that IL-27 is crucial for T cell dependent as well as T cell independent B cell immunity, whereby the molecular mechanisms involved are very different.

MATERIALS AND METHODS

Mice

WSX-1 knock-out (IL-27RKO) mice on a C57BL/6 background were kindly provided by dr. N. Ghilardi [2], Genentech, Inc., South San Francisco, USA. Wild type C57BL/6 controls were purchased from Harlan Laboratories B.V. (Horst, the Netherlands). All mice were provided with food and water *ad libitum* and were housed at the Erasmus MC Experimental Animal Facility (EDC) under specified pathogen free conditions. All mice were between 8 and 12 weeks old at the start of the experiments. All experiments were approved by the Erasmus MC Animal Ethics Committee (DEC).

Culture experiments

CD19+ CD62L+ naïve splenic B cells were isolated from wild type and IL-27RKO mice by FACS sorting. Anti-CD19 antibodies were obtained from eBioScience (San Diego, CA, USA) Purity of the acquired population was >98%. 10^5 purified cells were cultured for three days in IMDM medium (Lonza, Verviers, Belgium), supplemented with 10% FCS (**Invitrogen, Carlsbad, CA**), 100 U/ml Penicillin/Streptomycin, 2 mM L-Glutamin (Lonza) and 50 mM b-mercapto-ethanol (Merck, Darmstadt, Germany) with F(ab')2 anti-IgM (10µg/mL, Jackson Immunoresearch) and CD40L (20µg/mL, BD BioSciences) or LPS (5µg/mL, Sigma Chemical Co, St Louis, MO) in the presence or absence of recombinant IL-27 (20ng/mL, R&D systems, Minneapolis, MN). After 48 hours, proliferation in anti-IgM/CD40L conditions was measured by ^{3}H incorporation (3mCi/well, incubated for 16 hours). Expression of activation markers in anti-IgM/CD40L conditions was measured by extracellular flow cytometry after 48 hours. In LPS conditions, plasma cell formation was measured after 96 hours by intracellular flow cytometry.

Peritoneal B1 cells (CD19+B220lowCD43+CD5+) were isolated from peritoneal lavage by FACS sorting. Anti-CD19, anti-B220, anti-CD43 and anti-CD5 antibodies were obtained from eBioScience Purity of the acquired population was >98%

Cell cycle status of the peritoneal B cells cultured for 48 hours with LPS was determined after fixing in ice-cold ethanol and subsequent staining in PBS containing 0.02 mg/ml propidium iodide, 0.1% v/v Triton X-100, and 0.2 mg/ml RNase. Doublet cells were excluded by measuring peak area and width.

Collagen induced arthritis

Mice were immunized with 100µg (2mg/mL) chicken type II collagen (CII) (Chondrex, USA) emulsified in an equal complete Freund's adjuvant (CFA) volume containing 1 mg/ml heat-killed *Mycobacterium tuberculosis* (strain H37Ra; Difco Laboratories Inc., Detroit, USA) intra-dermally in the tail base on day 0 and day 21. Arthritis development was scored macroscopically; the maximum score was 8 per mouse. For ethical reasons, mice with a score >6 were sacrificed. Mice were sacrificed either at day 36 or at day 45.

DNP-Ficoll

Animals were immunized with 50µg DNP-Ficoll (Biosearch Technologies) in 0.9% NaCl intra-peritoneally (i.p.). Seven days later, the animals were sacrificed and splenic plasma cell numbers were determined by flow cytometry and serum DNP-specific antibody levels by ELISA [31, 32].

Flow cytometry

For B cell stainings, $2*10^6$ splenocytes were stained for extracellular markers for 20 minutes at room temperature. For intracellular staining, cells were subsequently fixed and permeabilized using BD Cytofix/Cytoperm (BD BioSciences) and stained for intracellular markers at room temperature for 20 minutes [32]. Anti-CD19, anti-B220, anti-IgM, anti-CD25 and anti-CD86 were obtained from eBioscience (San Diego, CA, USA); anti-CD69, anti-IgD, anti-CD95, anti-IgG1, anti-IgG2ab and anti-CD138 from BD BioSciences and anti-PNA from Sigma-Aldrich (St Louis, USA).

Samples were acquired on a FACS Canto II HTS or a FACS LSR II flow cytometer (BD BioSciences). Analysis was performed using FlowJo v7.6 research software (Tree Star Inc. Ashland, OR).

Histology and immunohistochemistry

For histological analysis of destruction of the knee joints, knees were hematoxylin and eosin stained as previously described [33].

Spleen tissue samples were frozen in Tissue-Tec O.C.T. Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) and stored at -80^{o}C. 6µm thick samples were cut and slides (Superfrost, Gerhard Menzel GmbH, Braunschweig, Germany) with samples were fixed in acetone. Endogenous peroxidase was blocked using 30% H_2O_2 (Sigma-Aldrich). Slides were stained with anti-PNA (biotinylated, Sigma-Aldrich) followed by streptavidin (peroxidase labeled,

Jackson ImmunoResearch, Westgrove, PA, USA) and rat-anti-IgD (eBioscience) followed by anti-rat (alkaline phosphatase labeled, Sigma-Aldrich). Pictures were made with a NanoZoomer (Hamamatsu Photonics Deutchland GmbH, Herrsching am Ammersee, Germany).

ELISA

For CII specific serum antibody level measurement, wells of microtiter plates were coated with 1µg/mL mouse CII (Chondrex) over night. Total IgM antibody and DNP-specific Ig subclass level measurements were performed by ELISA, as previously described [32].

Statistical analysis

Data was analyzed using Prism software v5.04 (GraphPad Software Inc. La Jollla, CA). For comparisons, a non-parametric Mann-Whitney U test was used. P-values <0.05 were considered significant.

RESULTS

CIA incidence and disease severity are decreased in IL-27RKO mice

CIA is strongly dependent on IL-17A production by Th17 cells, which is enhanced in IL-27R KO mice [17, 34]. However, collagen specific antibody production and immune complex formation are also critical for disease development [35]. We therefore determined the effect of loss of IL-27R signaling on CIA development. Wild-type and IL-27RKO mice were immunized with collagen type II (CII) and complete Freund's adjuvant (CFA) to induce arthritis. Both disease incidence and severity were significantly lower in IL-27RKO mice (figure 1A-B). In addition, synovial inflammation and destruction of the knee joints was also decreased in these mice (figure 1C-D), indicating that IL-27RKO mice are partially protected against CIA development.

We did not find an increase in the number of IL-17A-producing T cells in the spleens of the IL-27R KO mice in CIA compared with wild type controls (data not shown). The numbers of IgM and IgG2a plasma cells in the spleens of these mice were significantly decreased at 36 and 45 days after immunization (figure 1E). Although in the serum of IL-27R KO mice anti-CII antibodies were detectable, serum anti-CII IgG2a levels were decreased in IL-27R KO mice (figure 1F), when compared with wild-type mice.

Likewise, we saw an induction of GC B cells in the IL-27R KO mice at dat 36 and 45 after immunization, but their numbers were significantly reduced compared to immunized wild-type mice (figure 1G and H).

Taken together, these data show that the absence of IL-27R KO mice ameliorates arthritis and also reduces the formation of GC and auto-antibody production in CIA.

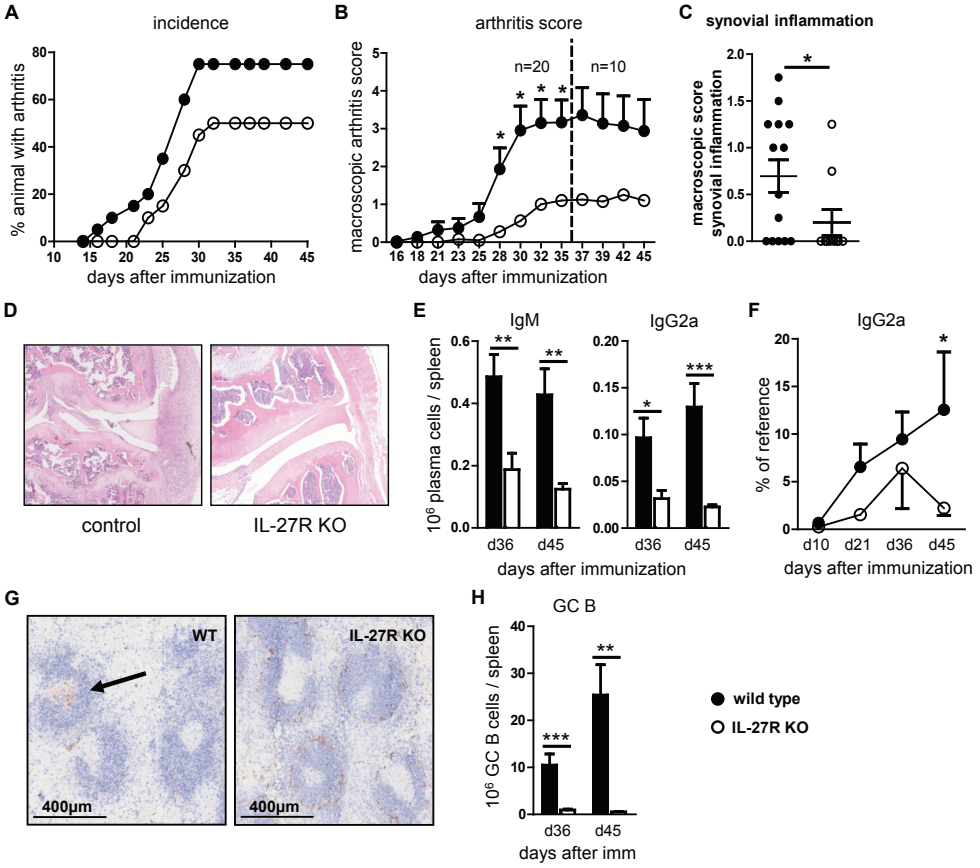


Figure 1. Impaired B cell immunity in IL-27R KO mice in CIA. [A-E] Mice were immunized with CII/CFA and boosted again 21 days later. [A] Incidence of CIA. [B] Arthritis score. Swelling and redness of paws were scored. The total score per mouse is 8; 0 meaning no arthritis. Mice with a score >6 were sacrificed for ethical reasons. [C] Synovial inflammation score of the knee joints. Maximum score per knee is 2; 0 meaning no inflammation. [D] HE stained sections of knee joints from FcγRIIB KO mice (control) and IL-27R KO mice 45 days after immunization. [E] IgM and IgG2a plasma cell numbers in spleens of mice in CIA. [F] Serum anti-mouse CII IgG2a antibody levels in CIA. [G] Anti-PNA (brown) and anti-IgD (blue) stained sections of spleens from wild type and IL-27R KO mice 36 days after immunization. [H] Numbers of splenic GC B cells in CIA. Mean and SEM are shown for n=10 or 20 mice per group; * p<0,05; ** p<0,01; *** p<0,001. Figures are representative of 3 individual experiments.

IL-27 has limited effects on follicular B cells

The finding that the lack of IL-27R signaling reduces B cell activation in our CIA models can be explained by impaired T cell activation or by an intrinsic B cell defect in IL-27R-deficient mice. To distinguish between these (not mutually exclusive) possibilities, we next investigated whether the lack of IL-27R signaling affected activation, proliferation and differentiation of naïve B cells. Expression of IL-27R was confirmed on naive splenic follicular B cells, marginal zone B cells and peritoneal B1 cells by RT-PCR (data not shown). Total naïve CD19+CD62L+ B cell fractions were FACS-sorted from the spleens of 8 week old naïve wild-type (and as a control also from IL-27R KO) mice. The cells were cultured with anti-IgM and CD40L for 48 hours with or without recombinant IL-27 (rIL-27). We observed that IL-27R+ and IL-27R- B cells proliferated equally, as measured by ^{3}H incorporation (figure 2A). In addition, rIL-27 had little effect on the expression of activation markers, except for upregulation of CD86 on wild type B cells with rIL-27 (figure 2B).

B cells were also cultured for 96 hours with LPS or LPS + IL-4 with or without rIL-27 to determine the effect on plasma cell formation. Upon LPS stimulation IgM and IgG3 plasma cell

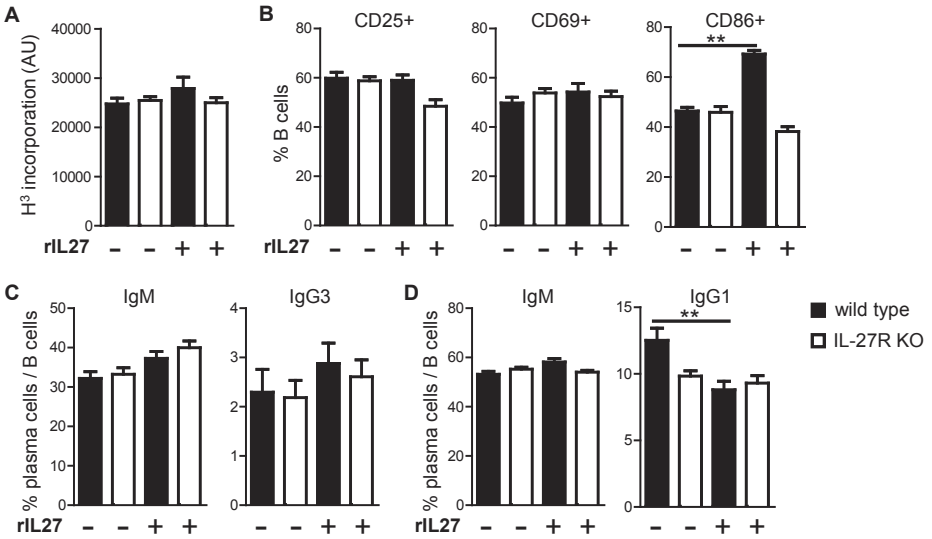


Figure 2. Naïve wild type and IL-27RKO B cells cultured in the presence or absence of rIL-27. [A-C] Naïve B cells (CD19+CD62L+) were FACS sorted from wild type and IL-27RKO mice. [A-B] 10^5 B cells were cultured for 48hrs with 10µg/mL anti-IgM and 20 µg/mL CD40 ligand in the presence or absence of 20ng/mL r-IL27. [A] Proliferation of cells was assessed by ^{3}H incorporation. [B] Activation of cells was assessed by measuring the proportion of CD86 expressing B cells by flow cytometry. [C-D] 10^5 B cells were culture for 96hrs with 5µg/mL LPS [C] or 5µg/mL LPS with 0,05µg/mL IL-4 [D] in the presence or absence of rIL-27. Plasma cell formation was measured by flow cytometry. Mean and SEM are shown for n = 6 samples per group, *** p<0,001

formation was similar in cells cultured with or without rIL-27 (figure 2C). This was also found for IgM and IgG1 plasma cell formation upon LPS + IL-4 stimulation, although IgG1 plasma cell formation was slightly decreased after addition of rIL-27 (figure 2D).

In summary, IL-27R KO B cells do not appear to have any defects in proliferation, activation or differentiation (figure 2A-D). We therefore conclude that intrinsic effects of IL-27R signaling on the activation, proliferation and differentiation of splenic follicular B cells are limited.

Proportion of B1 cells is increased in peritoneal lavage and spleen of IL-27R KO mice

To further examine B cell immunity in other compartments, we analysed the peritoneal lavage of these mice. Interestingly, the proportions of CD19+CD5+ B-1 cells in the peritoneum was increased in naïve IL-27R KO mice, compared to wild-type mice (figure 3A-B). In addition, serum IgM levels were increased in these mice at 9 weeks of age (figure 3C). As serum IgM is largely derived from B-1 B cells [36], this finding suggests that the absence of IL-27R enhanced B-1 cell function (figure 3C).

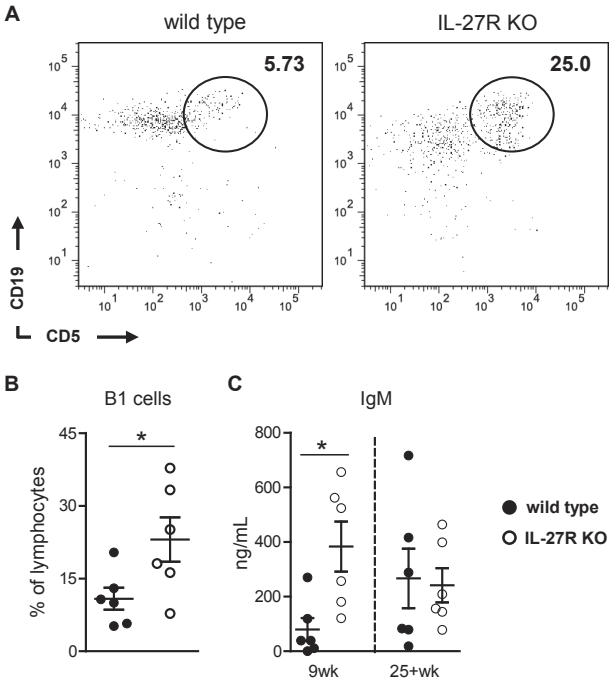


Figure 3. Increased B1 cells in peritoneum and serum IgM levels in IL-27R KO mice. [A] Flow cytometry analysis of B1 cells (CD19+CD5+) in peritoneal lavage of naïve mice. [B] Proportion of B1 cells in peritoneal lavage. [C] Serum IgM antibody levels at 9 and 25+ weeks of age. Mean and SEM are shown for n=6 mice per group; * p<0,05.

We then analysed the splenic B cell compartments of IL-27R KO mice. The numbers of total splenic B cells were comparable between naïve wild type and IL-27R KO mice. However, the numbers of marginal zone B cells were increased in IL-27R KO mice (figure 4A). They also appeared to contain higher numbers of B-1 cells, although differences with wild-type mice were not significant (Figure 4A). In addition, the numbers of IgM plasma cells in the spleens of IL-27R KO mice were increased (figure 4B).

Taken together, the increased proportions of B-1 cells in the peritoneal cavity, the increased numbers of IgM+ plasma cells in the spleen and increased levels of serum IgM in naïve IL-27R support enhanced activity of the B-1 B cell population.

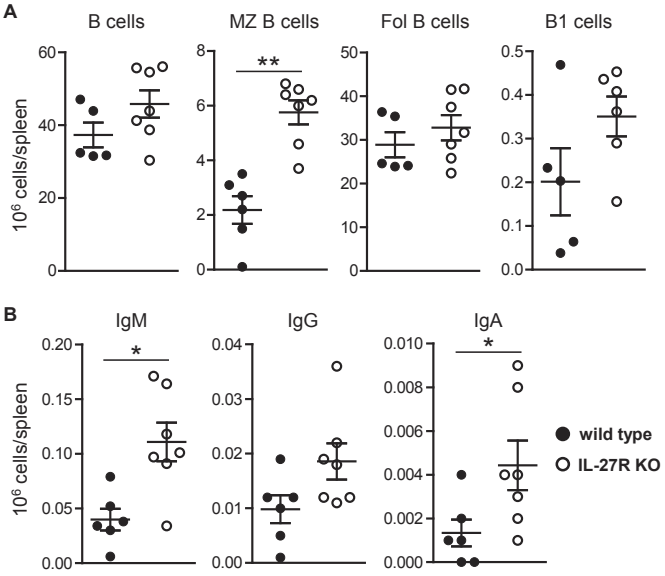


Figure 4. B cell compartment of spleens from naïve wild type and IL-27R KO mice. [A] Numbers of splenic B cells, marginal zone B cells, follicular B cells and B1 cells analysed by flow cytometry. [B] Numbers of splenic IgM, IgG and IgA plasma cells analysed by flow cytometry. Mean and SEM are shown for n = 5-7 animals per group; ** p<0,01. Figures are representative for 2 individual experiments.

IL-27R signaling has no direct effect on B1 cells

Next, we investigate whether the enhanced activity of the B-1 B cell population in IL-27R-deficient mice would reflect a B-1 cell intrinsic role for IL-27R signaling. To determine whether IL-27R KO B1 cells respond normally to TLR stimulation, we FACS-sorted CD19+CD5+CD43+B220^{low} B cells from peritoneal lavages of 8-week-old naïve wild type (and as controls IL-27R KO) mice. We performed

cell cycle analysis on these cells after culture for 48 hours with LPS. No differences were found between the proliferation of IL-27R KO and wild-type B-1 cells (figure 5A). In addition, we measured expression of surface activation markers, including CD25 and CD86, on cells cultured with or without LPS for 48 hours and found no evidence for impaired or increased activation of B-1 cells from IL-27R KO mice (figure 5B).

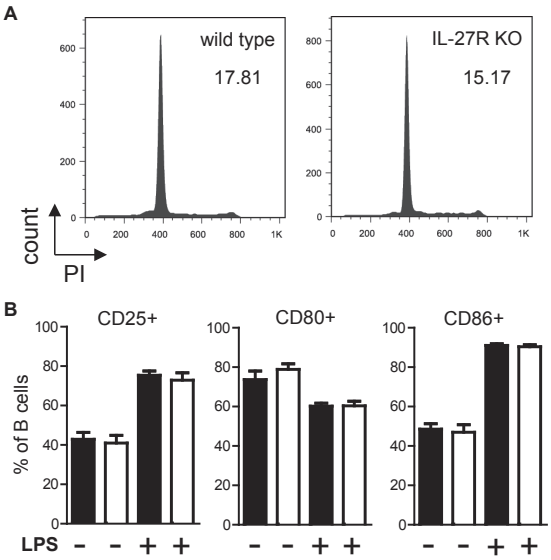


Figure 5. Wild type and IL-27R KO B1 cell responses in vitro. [A-B] B1 cells (CD19+B220^{low}CD5+) were FACS sorted from peritoneal lavage of naïve mice. 5*10^4 cells were cultured for 48 hours with or without 5µg/mL LPS. [A] Cell cycle status analysed with PI staining by flow cytometry. Numbers indicate % S/G2/M. [B] Expression of activation markers CD25, CD80 and CD86 analysed by flow cytometry. Mean and SEM are shown for n=3 animals per group.

B cell immunity is impaired in IL-27R KO mice after DNP-Ficoll immunization

Next, we investigated the effects of IL-27R signaling on T cell independent B cell responses in vivo. To this end, we made use of the T cell-independent type II model antigen DNP-Ficoll. Wild-type and IL-27RKO mice were immunized with DNP-Ficoll and sacrificed seven days later. Remarkably, the formation of IgM or IgG producing splenic plasma cells was significantly impaired in IL-27RKO mice, compared with wild type controls (figure 6A and supplementary figure 1A). In addition, serum DNP-specific antibody levels were significantly lower in IL-27RKO mice (figure 6B and supplementary figure 1B), showing that IL-27R signaling is essential for the generation of T-cell independent B cell responses in mice.

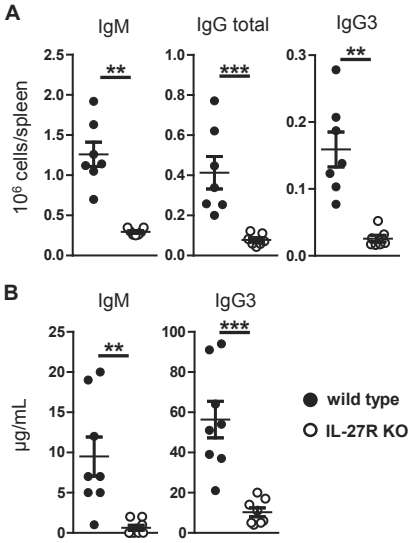
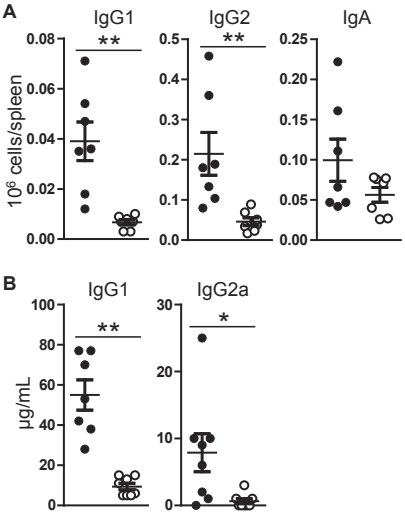


Figure 6. Plasma cell formation and DNP-specific antibody formation in wild type and IL-27RKO mice. [A-B] Mice were immunized i.p. with DNP-Ficoll and sacrificed seven days later. [A] Total numbers of splenic IgM, total IgG and IgG3 plasma cells measured by flow cytometry. [B] DNP-specific serum IgM and IgG3 antibody levels measured by ELISA. Mean and SEM are shown for n = 7-8 animals per group, ** p<0,01; *** p<0,001.



Supplementary figure 1. [A-B] Mice were immunized i.p. with DNP-Ficoll and sacrificed seven days later. [A] Total numbers of splenic IgG1, total IgG2 and IgA plasma cells measured by flow cytometry. [B] DNP-specific serum IgG1 and IgG2a antibody levels measured by ELISA. Mean and SEM are shown for n = 7-8 animals per group, *p<0,05; ** p<0,01.

DISCUSSION

Here we show that the IL-27 receptor is crucial for B cell immunity and that it mainly acts in a B cell non-intrinsic fashion in mice. IL-27R KO mice are partially protected against CIA and show impaired germinal center formation and anti-collagen antibodies. Nevertheless, IL-27R KO splenic B cells are intrinsically normal and respond normally to stimuli *in vitro.* Interestingly, the B-1 B cell population is also effected in IL-27R KO mice: the B cell response to the T-cell independent type II antigen DNP-Ficoll is impaired. Again, B1 cells from these mice respond normally to stimuli *in vitro.* Together these findings indicate an essential but not cell-intrinsic function of IL-27R in B cells, both in T cell-dependent and in T cell-independent B cell immunity.

Previously, it was reported that IL-27 induced proliferation and upregulation of CD86 expression on human B cells [29]. Although we do find upregulation of CD86 on mouse splenic B cells, we do not find any effect of IL-27 on proliferation in these cells upon CD40L and anti-IgM stimulation. In addition, enhanced G0/G1 to S transition by IL-27 has been reported in human B cells [30]. There was no reduction of proliferation in IL-27R KO B cells, nor any upregulation of proliferation in wild type or IL-27R KO B cells in the presence of rIL-27. Assuming that the differences between our findings and the published studies in human cannot be explained by the source of B cells (peripheral blood in human and spleen and peritoneum in mice), these data suggest differential effects of IL-27 on mouse and human B cells.

The limited effects of IL-27 on splenic B cells suggests that the impaired CIA development in IL-27R KO mice is due to indirect effects on B cell immunity. Indeed we found severely decreased germinal center formation in these mice in CIA, pointing to T(FH) involvement. However, Yoshimoto et al. have shown that IL-27 directly induces IgG2a class switching in mouse splenic B cells in vitro [37]. It is therefore possible that the decreased levels of auto-reactive IgG2a antibodies are due not only to impaired GC formation, but also impaired IgG2a class switching in vivo, thereby further protecting mice against CIA development. Interestingly, both overexpression of IL-27 and lack of IL-27R signaling ameliorate CIA through inhibiting effects of IL-27 on Th17 cells [18, 38] and its role in B cell immunity.

As with splenic B cells, we found no apparent defects in peritoneal IL-27R KO B cells. Cell cycle analysis revealed no differences with wild-type B-1 B cells and upregulation of expression markers was normal on these cells after LPS stimulation in vitro. However, T cell independent type II responses after i.p. DNP-Ficoll immunization were severely impaired in IL-27R KO mice. These data suggest that other cells present in the peritoneum and omentum are involved in B cell immunity in an IL-27 dependent fashion. Many cell types express the IL-27R, including monocytes, which are present in the peritoneum, and endothelial cells [21]. B-1 cells enter the peritoneum through milky spots in the omentum [39]. Although these structures lack FDC networks [40], stromal cells are present in milky spots, and these might be involved in homing of the cells.

We found increased levels of natural IgM antibodies in the serum of naïve IL-27R KO mice, the main producers of which are B-1 cells [36]. We also found increased numbers of IgM plasma cells and increased (although not significantly) B-1 cell numbers in the spleens of these mice. Also, the number of marginal zone B cells was increased. These cells can also produce natural IgM antibodies, possibly explaining the increased serum levels.

Additional experiments investigating the presence of B1 cells in milky spots in IL-27R KO mice and the migration pattern of IL-27R KO B1 cells upon DNP-Ficoll immunization should increase the understanding of this defect. In addition, the differential roles of IL-27 dependent B-1 and follicular B cell immunity could be e.g. better investigated with an influenza infection mouse model, since the activation and migration kinetics of the B-1 and follicular B cell population is known in extensive detail [36].

In this study we show that IL-27 has limited effects on B cells, but is crucial for B cell immunity. IL-27R deficient mice manifested defects both in T cell independent and in T cell dependent B cell immunity, but these defects were mainly non-intrinsic to B cells. Further studies will elucidate the cells involved in these IL-27-dependent pathways in B cell activation and provide a better understanding of the role of IL-27 in immune responses.

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REFERENCES

1. Pflanz S, Timans JC, Cheung J, Rosales R, Kanzler H, Gilbert J, Hibbert L, Churakova T, Travis M, Vaisberg E, et al: **IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells.** *Immunity* 2002, **16:**779-790.
2. Chen Q, Ghilardi N, Wang H, Baker T, Xie MH, Gurney A, Grewal IS, de Sauvage FJ: **Development of Th1-type immune responses requires the type I cytokine receptor TCCR.** *Nature* 2000, **407:**916-920.
3. Pflanz S, Hibbert L, Mattson J, Rosales R, Vaisberg E, Bazan JF, Phillips JH, McClanahan TK, de Waal Malefyt R, Kastelein RA: **WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27.** *J Immunol* 2004, **172:**2225-2231.
4. Hunter CA, Kastelein R: **Interleukin-27: balancing protective and pathological immunity.** *Immunity* 2012, **37:**960-969.
5. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DA: **The inhibitory cytokine IL-35 contributes to regulatory T-cell function.** *Nature* 2007, **450:**566-569.
6. Crabe S, Guay-Giroux A, Tormo AJ, Duluc D, Lissilaa R, Guilhot F, Mavoungou-Bigouagou U, Lefouili F, Cognet I, Ferlin W, et al: **The IL-27 p28 subunit binds cytokine-like factor 1 to form a cytokine regulating NK and T cell activities requiring IL-6R for signaling.** *J Immunol* 2009, **183:**7692-7702.
7. Stumhofer JS, Tait ED, Quinn WJ, 3rd, Hosken N, Spudy B, Goenka R, Fielding CA, O'Hara AC, Chen Y, Jones ML, et al: **A role for IL-27p28 as an antagonist of gp130-mediated signaling.** *Nat Immunol* 2010, **11:**1119-1126.
8. Yoshida H, Hamano S, Senaldi G, Covey T, Faggioni R, Mu S, Xia M, Wakeham AC, Nishina H, Potter J, et al: **WSX-1 is required for the initiation of Th1 responses and resistance to L. major infection.** *Immunity* 2001, **15:**569-578.
9. Kamiya S, Owaki T, Morishima N, Fukai F, Mizuguchi J, Yoshimoto T: **An indispensable role for STAT1 in IL-27-induced T-bet expression but not proliferation of naive CD4+ T cells.** *J Immunol* 2004, **173:**3871-3877.
10. Takeda A, Hamano S, Yamanaka A, Hanada T, Ishibashi T, Mak TW, Yoshimura A, Yoshida H: **Cutting edge: role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment.** *J Immunol* 2003, **170:**4886-4890.
11. Hibbert L, Pflanz S, De Waal Malefyt R, Kastelein RA: **IL-27 and IFN-alpha signal via Stat1 and Stat3 and induce T-Bet and IL-12Rbeta2 in naive T cells.** *J Interferon Cytokine Res* 2003, **23:**513-522.
12. Diveu C, McGeachy MJ, Boniface K, Stumhofer JS, Sathe M, Joyce-Shaikh B, Chen Y, Tato CM, McClanahan TK, de Waal Malefyt R, et al: **IL-27 blocks RORc expression to inhibit lineage commitment of Th17 cells.** *J Immunol* 2009, **182:**5748-5756.
13. Cao Y, Doodes PD, Glant TT, Finnegan A: **IL-27 induces a Th1 immune response and susceptibility to experimental arthritis.** *J Immunol* 2008, **180:**922-930.
14. Lucas S, Ghilardi N, Li J, de Sauvage FJ: **IL-27 regulates IL-12 responsiveness of naive CD4+ T cells through Stat1-dependent and -independent mechanisms.** *Proc Natl Acad Sci U S A* 2003, **100:**15047-15052.
15. Miyazaki Y, Inoue H, Matsumura M, Matsumoto K, Nakano T, Tsuda M, Hamano S, Yoshimura A, Yoshida H: **Exacerbation of experimental allergic asthma by augmented Th2 responses in WSX-1-deficient mice.** *J Immunol* 2005, **175:**2401-2407.
16. Yang J, Yang M, Htut TM, Ouyang X, Hanidu A, Li X, Sellati R, Jiang H, Zhang S, Li H, et al: **Epstein-Barr virus-induced gene 3 negatively regulates IL-17, IL-22 and RORgamma t.** *Eur J Immunol* 2008, **38:**1204-1214.
17. Batten M, Li J, Yi S, Kljavin NM, Danilenko DM, Lucas S, Lee J, de Sauvage FJ, Ghilardi N: **Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells.** *Nat Immunol* 2006, **7:**929-936.
18. Niedbala W, Cai B, Wei X, Patakas A, Leung BP, McInnes IB, Liew FY: **Interleukin 27 attenuates collagen-induced arthritis.** *Ann Rheum Dis* 2008, **67:**1474-1479.
19. Stumhofer JS, Silver JS, Laurence A, Porrett PM, Harris TH, Turka LA, Ernst M, Saris CJ, O'Shea JJ, Hunter CA:

Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol* 2007, **8:**1363-1371.

20. Fitzgerald DC, Zhang GX, El-Behi M, Fonseca-Kelly Z, Li H, Yu S, Saris CJ, Gran B, Ciric B, Rostami A: **Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells.** *Nat Immunol* 2007, **8:**1372-1379.
21. Awasthi A, Carrier Y, Peron JP, Bettelli E, Kamanaka M, Flavell RA, Kuchroo VK, Oukka M, Weiner HL: **A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells.** *Nat Immunol* 2007, **8:**1380-1389.
22. Pot C, Jin H, Awasthi A, Liu SM, Lai CY, Madan R, Sharpe AH, Karp CL, Miaw SC, Ho IC, Kuchroo VK: **Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells.** *J Immunol* 2009, **183:**797-801.
23. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, Forster R: **Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production.** *J Exp Med* 2000, **192:**1545-1552.
24. Tangye SG, Ma CS, Brink R, Deenick EK: **The good, the bad and the ugly - TFH cells in human health and disease.** *Nature reviews Immunology* 2013, **13:**412-426.
25. Victora GD, Nussenzweig MC: **Germinal centers.** *Annu Rev Immunol* 2012, **30:**429-457.
26. Bryant VL, Ma CS, Avery DT, Li Y, Good KL, Corcoran LM, de Waal Malefyt R, Tangye SG: **Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells.** *J Immunol* 2007, **179:**8180-8190.
27. Ozaki K, Spolski R, Ettinger R, Kim HP, Wang G, Qi CF, Hwu P, Shaffer DJ, Akilesh S, Roopenian DC, et al: **Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6.** *J Immunol* 2004, **173:**5361-5371.
28. Batten M, Ramamoorthi N, Kljavin NM, Ma CS, Cox JH, Dengler HS, Danilenko DM, Caplazi P, Wong M, Fulcher DA, et al: **IL-27 supports germinal center function by enhancing IL-21 production and the function of T follicular helper cells.** *J Exp Med* 2010, **207:**2895-2906.
29. Larousserie F, Charlot P, Bardel E, Froger J, Kastelein RA, Devergne O: **Differential effects of IL-27 on human B cell subsets.** *J Immunol* 2006, **176:**5890-5897.
30. Charlot-Rabiega P, Bardel E, Dietrich C, Kastelein R, Devergne O: **Signaling events involved in interleukin 27 (IL-27)-induced proliferation of human naive CD4+ T cells and B cells.** *J Biol Chem* 2011, **286:**27350-27362.
31. Maas A, Dingjan GM, Savelkoul HF, Kinnon C, Grosveld F, Hendriks RW: **The X-linked immunodeficiency defect in the mouse is corrected by expression of human Bruton's tyrosine kinase from a yeast artificial chromosome transgene.** *Eur J Immunol* 1997, **27:**2180-2187.
32. van Loo PF, Dingjan GM, Maas A, Hendriks RW: **Surrogate-light-chain silencing is not critical for the limitation of pre-B cell expansion but is for the termination of constitutive signaling.** *Immunity* 2007, **27:**468-480.
33. van Hamburg JP, Mus AM, de Bruijn MJ, de Vogel L, Boon L, Cornelissen F, Asmawidjaja P, Hendriks RW, Lubberts E: **GATA-3 protects against severe joint inflammation and bone erosion and reduces differentiation of Th17 cells during experimental arthritis.** *Arthritis Rheum* 2009, **60:**750-759.
34. Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, Sedgwick JD, Cua DJ: **Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation.** *J Exp Med* 2003, **198:**1951-1957.
35. Svensson L, Jirholt J, Holmdahl R, Jansson L: **B cell-deficient mice do not develop type II collagen-induced arthritis (CIA).** *Clin Exp Immunol* 1998, **111:**521-526.
36. Baumgarth N: **The double life of a B-1 cell: self-reactivity selects for protective effector functions.** *Nature reviews Immunology* 2011, **11:**34-46.
37. Yoshimoto T, Okada K, Morishima N, Kamiya S, Owaki T, Asakawa M, Iwakura Y, Fukai F, Mizuguchi J: **Induction of IgG2a class switching in B cells by IL-27.** *J Immunol* 2004, **173:**2479-2485.

38. Pickens SR, Chamberlain ND, Volin MV, Mandelin AM, 2nd, Agrawal H, Matsui M, Yoshimoto T, Shahrara S: **Local expression of interleukin-27 ameliorates collagen-induced arthritis.** *Arthritis Rheum* 2011, **63:**2289-2298.

39. Ansel KM, Harris RB, Cyster JG: **CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity.** *Immunity* 2002, **16:**67-76.

40. Rangel-Moreno J, Moyron-Quiroz JE, Carragher DM, Kusser K, Hartson L, Moquin A, Randall TD: **Omental milky spots develop in the absence of lymphoid tissue-inducer cells and support B and T cell responses to peritoneal antigens.** *Immunity* 2009, **30:**731-743.


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Th17 cytokines and Interferon type I: partners in crime in SLE?

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* ** authors contributed equally

Submitted for publication

ABSTRACT

Introduction A hallmark of systemic autoimmune diseases like systemic lupus erythematosus (SLE) is the increased expression of interferon (IFN) type I inducible genes, so-called IFN type I signature. Recently, T helper 17 subset (Th17 cells), which produces IL-17A, IL-17F, IL-21 and IL-22, has been implicated in SLE. As CCR6 enriches for Th17 cells, we used this approach to investigate whether CCR6+ memory T helper cells producing IL-17A, IL-17F, IL-21 and/or IL-22 are increased in SLE patients and whether this increase is related to the presence of IFN type I signature.

Methods 25 SLE patients and 15 healthy controls (HC) were included. SLE patients were divided into IFN type I signature positive (IFN+) (n=16) and negative (IFN-) (n=9) patients as assessed by mRNA expression of IFN inducible genes (IFIGs) in monocytes. Expression of IL-17A, IL-17F, IL-21 and IL-22 by CD4+CD45RO+CCR6+ T cells (CCR6+ cells) was measured by flow cytometry and compared between IFN+, IFN- patients and HC.

Results Increased percentages of IL-17A and IL-17A/IL-17F double producing CCR6+ cells were observed in IFN+ patients compared with IFN- patients and HC. IL-17A and IL-17F expression within CCR6+ cells correlated significantly with IFIG expression. In addition, we found significant correlation between BAFF – a factor strongly correlating with IFN type I – and IL-21 producing CCR6+ cells.

Conclusions We show for the first time higher percentages of IL-17A and IL-17A/IL-17F double producing CCR6+ memory T helper cells in IFN+ SLE patients, supporting the hypothesis that IFN type I co-acts with Th17 cytokines in SLE pathogenesis.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a debilitating systemic autoimmune disease characterized by the production of auto-reactive antibodies and multi-organ inflammation [1]. A hallmark of systemic autoimmune diseases is the increased expression of Interferon (IFN) type I in both blood and disease-affected tissues [2]. About half of the SLE patients exhibit an IFN type I signature or upregulation of IFN type I induced genes (IFIGs) which have been found to correlate with disease activity and severity [3-5].

Another key factor in the pathogenesis of SLE apart from IFN type I, is interleukin-17A (IL-17A). IL-17A is produced by several immune cell types, including CD4+ T cells (Th17 cells), CD8+ T cells, CD4-CD8-CD3+ (double negative, DN) T cells, NK cells, γδ-T cells and mast cells. Naïve CD4+ T cells differentiate to Th17 cells under the influence of IL-6 and TGFβ [6]. The expansion and stability of the Th17 population is regulated by IL-21 and IL-23, respectively [7, 8]. C57BL/6-lpr/lpr mice that lack IL-23 receptor signalling are protected for SLE development [9]. In SLE patients increased plasma levels of IL-17A correlated with disease severity (SLEDAI) [10]. In addition, in peripheral blood of SLE patients an increased number of IL-17 producing cells was observed. These cells correlated with disease activity and decreased upon treatment [11, 12]. IL-17-producing cells have also been found in several affected organs of SLE patients [11, 13].

Co-activity between IFN type I and IL-17/Th17 cells has been suggested in autoimmune diseases [14, 15]. In EAE, a mouse model for MS, IFN type I treatment caused exacerbation if the disease was Th17 driven but was effective if the disease was Th1 driven [15]. In the same study, MS patients that did not respond to IFN type I therapy had higher serum levels of IL-17A before therapy onset [15]. These two observations suggest additional effects of the IFN type I and Th17 system co-acting in the pathogenesis of autoimmune diseases.

Co-activity of IFN type I and Th17 pathways has also been suggested for SLE by the Ro52/TRIM21-/- mouse model. Ro52/TRIM21 is involved in the ubiquitination of interferon regulatory factors (IRFs), a process which limits the IFN type I response [16]. After ear tagging Ro52/TRIM21-/- mice develop an SLE like phenotype [17]. Interestingly, when these mice are crossed on an IL-23p19-/- mouse line, they do not develop SLE, indicating that the development of a SLE phenotype through enhanced IFN type I production in these mice is dependent on the IL-17/Th17 pathway.

Yet another important factor involved in the pathogenesis of SLE is B cell activating factor of the tumor necrosis factor family (BAFF). BAFF transgenic mice develop lupus-like disease [18] and increased expression of BAFF protein has been found in SLE patients, correlating with increased disease activity [19-21]. We previously described a strong correlation between BAFF mRNA expression in monocytes and the IFN type I signature in primary Sjögren's syndrome (pSS) patients [22]. Interestingly, IL-21, a cytokine produced by Th17 cells, in combination with BAFF has been reported to synergistically induce the differentiation of human memory B cells into antibody-producing plasma cells in absence of further co-stimulation [23]. BAFF is known to be

involved in germinal center formation [24], a process in which IL-17 is also involved [25].

The above mentioned literature suggests an association between the pathogenic IFN type I and Th17 pathway. So far, no studies have been performed on the co-occurrence of these pathogenic pathways in SLE patients. In this study, we report for the first time a higher percentage of IL-17A and IL-17A/F producing CCR6+ T memory cells in IFN type I positive SLE patients. Moreover, BAFF gene expression in monocytes correlates significantly with IL-21 expression in these CCR6+ cells, supporting the concept of co-activity of IFN type I, Th17 and BAFF in the pathogenesis of SLE.

PATIENTS AND METHODS

Patients

25 patients fulfilling the American College of Rheumatology revised criteria for SLE [26] were recruited at the outpatient clinic of the Immunology department and the Rheumatology department of the Erasmus Medical Center Rotterdam. The level of disease activity was assessed using the SLEDAI [27]. 15 healthy controls (HC) neither suffering from autoimmune diseases nor using corticosteroids were included. Characteristics of patients and controls are summarized in Table 1. Medical Ethical Review Committee of the Erasmus MC approved the study and written informed consent was obtained.

Table 1. Demographics and clinical characteristics of participants

Variable	SLE patients (n=25)		Healthy controls (n=15)
	IFN type I negative (n=9)	IFN type I positive (n=16)	
Demographics			
N females (%)	9/9 (100%)	14/16 (88%)	15/15 (100%)
Age (years)	41.3±17.5	39.8±15.7	41.0±14.0
Disease duration (years)	12.1±8.0	14.4±11.3	-
SLEDAI	4.0 (1.0,17.0)	4.50 (2.0,10.0)	-
			-
Treatment			
Hydroxychloroquine	7/9 (78%)	11/16 (69%)	-
Corticosteroids	5/9 (56%)	10/16 (63 %)	-
Mycophenolatemofetil	0/9 (0%)	4/16 (25%)	-
Azathioprine	2/9 (22%)	3/16 (19%)	-
Cyclophosphamide	0/9 (0%)	1/16 (6%)	-

Values are the mean ± SD, median (25% quartile,75% quartile) or number (%) of patients, depending on whether the data are continuous or dichotomous, and whether the data are normally distributed or not.

Blood collection and isolation of monocytes

Blood was collected in clotting tubes for serum preparation (stored at -80°C) and in sodium-heparin tubes for peripheral blood mononuclear cell (PBMC) preparation as described previously [28]. CD14 positive monocytes were isolated as described [28].

RQ-PCR

Total RNA was isolated from purified monocytes followed by cDNA preparation and RQ-PCR analysis using predesigned primer/probe sets (Applied Biosystems) [28]. For calculation of relative expression, all samples were normalized against expression of the household gene Abl[29]. Fold change values were determined from normalized CT values using 2ΔΔCT method (User Bulletin, Applied Biosystems, Foster City, California).

Flow cytometry

PBMCs were restimulated, stained and measured by flow cytometry as previously described [30]. For extracellular staining, CD4, CD45RO and CCR6 monoclonal antibodies were obtained from BD Biosciences (San Diego, CA), and CD25 antibodies from Biolegend Inc. (San Diego, CA). For intracellular staining, FoxP3, IL-17A, IL-17F and IL-22 monoclonal antibodies were obtained from eBioscience (San Diego, CA), and IL-17A monoclonal antibodies from Biolegend Inc. Samples were measured on a FACScantoII flow cytometer (BD Biosciences). Analysis was performed using FlowJo v7.6 research software (Tree Star Inc. Ashland, OR).

Factor analysis

Expression levels of 11 IFN type I inducible genes were submitted to a principal component analysis to identify correlated groups of genes to reduce data complexity. Kaiser-Meyer-Olkin measure of sampling adequacy was 0.839 with significant Bartlett's test of sphericity ($P< 0.001$). Eigenvalues were derived to assess the amount of variance explained by each component factor.

Statistical analyses

Statistical analyses were performed using SPSS 20.0 package. When data were not normally distributed, values were expressed as medians with interquartile ranges (IQRs) and comparisons were made using the non-parametric Mann-Whitney U test. In case of more than two samples the non-parametric Kruskal-Wallis test was performed. Correlations were assessed either using Pearson correlation test for normally distributed data or Spearman's rho when data were not normally distributed. Differences were considered statistically significant if $p<0.05$.

RESULTS

Prevalence of the IFN type I signature in SLE patients

In monocytes of 25 SLE patients and 15 HCs we assessed the expression levels of 11 IFIGs previously assessed in monocytes from patients with primary Sjögren's syndrome (pSS) (IFI27, IFI44L, IFIT3, IFITM1, SERPING1, IFIT1, IFIT2, LY6E, IFI44, XAF1 and MXA) [22]. To reduce data complexity, expression levels of the 11 genes were submitted to a principal component analysis to identify correlated groups of genes. The results of the principal component analysis identified a subset of 4 genes (IFI44L, IFITM1, SERPING1 and LY6E) to explain 95% of the total variance of the 11 IFN type I inducible genes within the SLE cohort. Given that the expression of these 4 IFN type I inducible genes was not normally distributed, log transformations of expression values were performed and IFN scores were calculated as described for pSS [22]. $Mean_{HC}$ and SD_{HC} of each gene in the HC-group were used to standardize expression levels. IFN scores per subject represent the sum of these standardized scores. When we set the threshold for a positive IFN type I signature at IFN score of 8 [22], 64% of SLE patients displayed an IFN type I signature and one of the 15 HC subjects (7%) (Figure 1A and 1B).

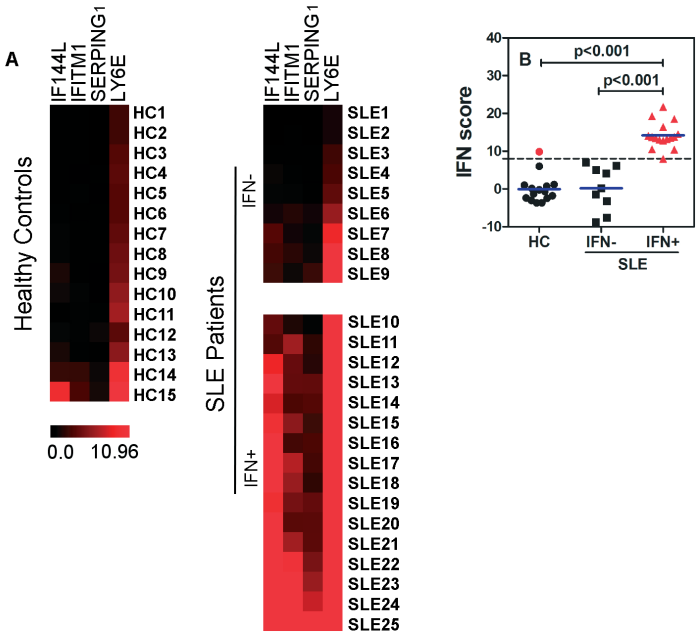

Figure 1. Prevalence of IFN type I signature in SLE patients. A, Heatmap showing gene expression of 4 IFN type I inducible genes in monocytes of SLE patients (n=25) and HC (n=15). On the left the HC are depicted and on the right the SLE patients are depicted and subdivided into IFN type I signature positive and negative patients. Red colour indicates high gene expression and cases are depicted according to ascending IFN scores. **B**, Distribution of IFN scores in IFN type I signature positive and negative patients and HC. In red IFN type I positive cases are depicted. Blue lines represent medians.

SLE patients with IFN type I signature show higher percentages of IL-17A and IL-17A/IL-17F producing CCR6+ cells

Because CCR6 enriches for Th17 cells [31-33], CCR6+ cells were selected after gating on lymphocytes and memory Th cells (CD4+CD45RO+ cells) within PBMCs and after CD25high cells were excluded. To investigate whether the IFN type I signature is associated with an increase in Th17 cytokines expressed by memory CCR6+ T cells, we measured the percentages of IL-17A, IL-17F, IL-22 and IL-21 producing CCR6+ T memory cells in SLE patients positive for the IFN type I signature (IFN+) and patients negative for the signature (IFN-) and HC. Interestingly, the percentages of CCR6+IL-17A+ cells were significantly increased in IFN+ patients as compared with IFN- patients (p=0.03) and a higher trend was observed compared with HC (p=0.07) (Figure 2A, D, E). The percentages of CCR6+IL-17F+ and in particular the

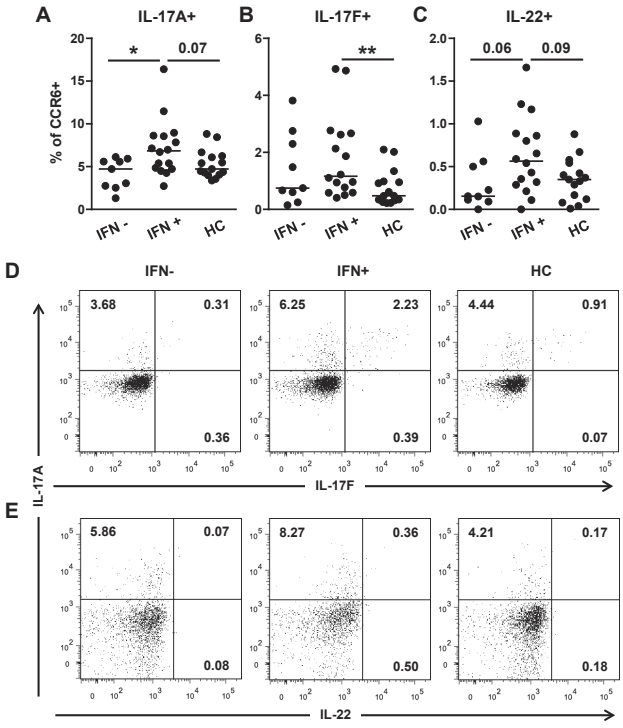

Figure 2. SLE patients with IFN type I signature show higher percentages of IL-17A and IL-17A/IL-17F producing CCR6+ cells. A-C, Proportions of IL-17A [A], IL-17F [B] and IL-22 [C] expressing cells within CCR6+ memory T cell population in PBMCs of IFN- (n=9) and IFN+ (n=16) patients and HC (n=15). All proportions were measured by intracellular flow cytometry. Horizontal line indicates median. *p<0,05; **p<0,01; to compare means Kruskal-Wallis test was used followed by Mann-Whitney U test. **B**, Representative graphs of proportions of IL-17A and IL-17F expressing cells within CCR6+ memory T cell population (defined as CD4+CD45RO+CD25-CCR6+) in PBMCs of IFN negative (IFN-) and IFN positive (IFN+) patients and healthy controls (HC). C, Representative graphs of proportions of IL-17A and IL-22 expressing cells within CCR6+ memory T cell population in PBMCs of IFN- and IFN+ patients and HC.

IL-17A/IL-17F double producers were significantly increased in the IFN+ group compared with HC (p=0.009) (Figure 2B and D). The percentages of CCR6+IL-22+ cells showed a higher trend for IFN+ vs. IFN- patients (p=0.06) and IFN+ patients vs. HC (p=0.09) (Figure 2C and E). These data suggest an association between the presence of the IFN type I signature and the expression of Th17 cytokines IL-17A and IL-17F in SLE. In addition, we investigated whether the Th17 cytokine production is associated with disease activity as assessed by SLEDAI scores. No significant correlations were observed between the SLEDAI scores and IL-17A and/or IL-17F expression (data not shown).

BAFF mRNA expression is correlated with IL-21 expression within CCR6+ memory T cells

Correlating the expression of IFIGs (as reflected by the total IFN score) with other parameters assessed in this study, we observed a significant positive correlation between the expression of IL-17A and IL-17F within CCR6+ cells and IFIG expression (Figure 3A and B). Also in this SLE cohort, IFIG expression correlated strongly with the BAFF mRNA expression in monocytes (r=0.527, p<0.0001) (Figure 3C). No correlation was observed between BAFF and IL-17A and/

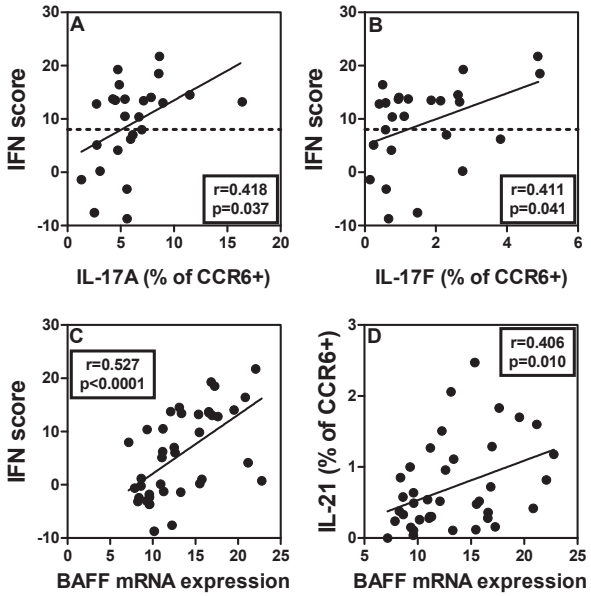

Figure 3. BAFF mRNA expression is correlated with IL-21 expression within CCR6+ memory T cells
A, Correlation between IFN score and IL-17A expression within CCR6+ memory T cells in SLE patients (n=25). **B**, Correlation between IFN score and IL-17F expression within CCR6+ memory T cells in SLE patients (n=25). **C,** Correlation between monocyte BAFF mRNA expression and IFN score in SLE patients and HC (n=40). **D,** Correlation between monocyte BAFF mRNA expression and IL-21 expression within CCR6+ cells in SLE patients and HC (n=40). The correlation coefficients (r) and *P* values are shown. For correlations Spearman's rho correlation test was used in A, B and D and Pearson correlation test was used in C.

or IL-17F expression. However we did find a significant correlation between BAFF mRNA and the percentages of IL-21 producing CCR6+ cells (r=0.406, p=0.010) (Figure 3D). Both BAFF and IL-21 are involved in the selection and activation of B cells, which is crucial in the pathogenesis of SLE, indicating that downstream factors of the IFN type I and Th17 pathways might also be associated.

DISCUSSION

Here we show for the first time a co-occurrence of increased IFN type I activity and increased IL-17/Th17 system in SLE patients. We found increased percentages of IL-17A and IL-17F producing CCR6+ T memory cells in IFN type I positive SLE patients. This finding further strengthens the hypothesis that IFN type I and Th17 cells by co-acting contribute to the pathogenesis of SLE. Further research to understand the link between these two pathways is warranted.

A possible mechanism explaining the co-occurrence of IFN type I and IL-17/Th17 immune pathway in SLE could be that both IFN type I and production of IL-6 and IL-23 by DCs are regulated through IRF-5 [34, 35]. Activation of TLR signalling on DCs will then lead to simultaneous enhancement of both pathways. Indeed there is evidence that TLR7 activation of plasmacytoid DCs, the main producers of IFN type I, promotes and modifies Th17 cell differentiation and function [36]. IFN type I itself is also able to promote Th17 differentiation and IL-17 production through induction of STAT-3 in T cells and IL-6 in DCs [37, 38]. In addition, IFN type I conditioned monocytes differentiate into DCs driving the development of Th17 cells from autologous naive CD4+ T cells [39].

In addition to the direct effect of IFN type I on Th17 cells, IFN type I may also act indirectly through the production of BAFF. BAFF is reported to be involved in DC maturation and DC driven Th17 cell differentiation *in vitro* [40]. BAFF gene silencing ameliorated joint pathology and inhibited the generation of Th17 cells in the joints of a collagen induced arthritis (CIA) mouse model [40]. In turn IL-17A can induce the formation of neutrophil extracellular traps (NETs) [41], which could potentially provide new auto-antigens to active TLRs on DCs, thereby forming a pro-inflammatory loop.

We find a correlation between BAFF, an IFN type I inducible factor, and the Th17 produced cytokine IL-21. Ettinger *et al.* showed that IL-21 together with BAFF promotes B cell responses by bypassing the need for T cell help or TLR signalling [23]. As these downstream factors are both involved in activation and selection of B cells, these findings again support the concept that IFN type I and the Th17 pathway act together in driving the disease process of SLE.

In contrast to others we did not find a correlation between SLEDAI and Th17 cytokines [10]. This might be due to the relatively low patient number, which is a limitation of our study.

Although we don't show a functional link between IFN type I and the Th17 pathway, our findings provide the first support for co-occurrence of increased IFN type activity and increased

IL-17/Th17 system in SLE. The Th17-IFN type I interaction found in this study might have implications for future treatment of SLE and other systemic autoimmune diseases where IFN type I plays a role. Preliminary results from a phase IIa trial with human IgG1κ anti-IFNα antibody in 87 SLE patients, showed so far a 40% reduction in IFN type I induced gene expression but no clinical effect compared to placebo (abstract Merrill J *et al.**). Our data indicate that IFN type I might act in concert with Th17 cytokines, paving the way for combination therapies possibly resulting in more significant clinical effects in the future.

REFERENCES

1. Choi J, Kim ST, Craft J: **The pathogenesis of systemic lupus erythematosus-an update.** *Current opinion in immunology* 2012, **24:**651-657.
2. Higgs BW, Liu Z, White B, Zhu W, White WI, Morehouse C, Brohawn P, Kiener PA, Richman L, Fiorentino D, et al: **Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway.** *Ann Rheum Dis* 2011, **70:**2029-2036.
3. Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ, Shark KB, Grande WJ, Hughes KM, Kapur V, et al: **Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus.** *Proceedings of the National Academy of Sciences of the United States of America* 2003, **100:**2610-2615.
4. Bennett L, Palucka AK, Arce E, Cantrell V, Borvak J, Banchereau J, Pascual V: **Interferon and granulopoiesis signatures in systemic lupus erythematosus blood.** *The Journal of experimental medicine* 2003, **197:**711-723.
5. Kirou KA, Lee C, George S, Louca K, Peterson MG, Crow MK: **Activation of the interferon-alpha pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease.** *Arthritis and rheumatism* 2005, **52:**1491-1503.
6. McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T, Cua DJ: **TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology.** *Nature immunology* 2007, **8:**1390-1397.
7. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ: **IL-23 drives a pathogenic T cell population that induces autoimmune inflammation.** *The Journal of experimental medicine* 2005, **201:**233-240.
8. Zhou L, Ivanov, II, Spolski R, Min R, Shenderov K, Egawa T, Levy DE, Leonard WJ, Littman DR: **IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways.** *Nature immunology* 2007, **8:**967-974.
9. Kyttaris VC, Zhang Z, Kuchroo VK, Oukka M, Tsokos GC: **Cutting edge: IL-23 receptor deficiency prevents the development of lupus nephritis in C57BL/6-lpr/lpr mice.** *J Immunol* 2010, **184:**4605-4609.
10. Chen XQ, Yu YC, Deng HH, Sun JZ, Dai Z, Wu YW, Yang M: **Plasma IL-17A is increased in new-onset SLE patients and associated with disease activity.** *Journal of clinical immunology* 2010, **30:**221-225.
11. Yang J, Chu Y, Yang X, Gao D, Zhu L, Wan L, Li M: **Th17 and natural Treg cell population dynamics in systemic lupus erythematosus.** *Arthritis and rheumatism* 2009, **60:**1472-1483.
12. Shah K, Lee WW, Lee SH, Kim SH, Kang SW, Craft J, Kang I: **Dysregulated balance of Th17 and Th1 cells in systemic lupus erythematosus.** *Arthritis research & therapy* 2010, **12:**R53.
13. Wang Y, Ito S, Chino Y, Goto D, Matsumoto I, Murata H, Tsutsumi A, Hayashi T, Uchida K, Usui J, et al: **Laser microdissection-based analysis of cytokine balance in the kidneys of patients with lupus nephritis.** *Clinical and experimental immunology* 2010, **159:**1-10.
14. Ambrosi A, Espinosa A, Wahren-Herlenius M: **IL-17: a new actor in IFN-driven systemic autoimmune diseases.** *European journal of immunology* 2012, **42:**2274-2284.
15. Axtell RC, de Jong BA, Boniface K, van der Voort LF, Bhat R, De Sarno P, Naves R, Han M, Zhong F, Castellanos JG, et al: **T helper type 1 and 17 cells determine efficacy of interferon-beta in multiple sclerosis and experimental encephalomyelitis.** *Nature medicine* 2010, **16:**406-412.
16. Higgs R, Ni Gabhann J, Ben Larbi N, Breen EP, Fitzgerald KA, Jefferies CA: **The E3 ubiquitin ligase Ro52 negatively regulates IFN-beta production post-pathogen recognition by polyubiquitin-mediated degradation of IRF3.** *J Immunol* 2008, **181:**1780-1786.
17. Espinosa A, Dardalhon V, Brauner S, Ambrosi A, Higgs R, Quintana FJ, Sjostrand M, Eloranta ML, Ni Gabhann J, Winqvist O, et al: **Loss of the lupus autoantigen Ro52/Trim21 induces tissue inflammation and systemic autoimmunity by disregulating the IL-23-Th17 pathway.** *The Journal of experimental medicine* 2009, **206:**1661-1671.

18. Mackay F, Woodcock SA, Lawton P, Ambrose C, Baetscher M, Schneider P, Tschopp J, Browning JL: **Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations.** *J Exp Med* 1999, **190:**1697-1710.

19. Becker-Merok A, Nikolaisen C, Nossent HC: **B-lymphocyte activating factor in systemic lupus erythematosus and rheumatoid arthritis in relation to autoantibody levels, disease measures and time.** *Lupus* 2006, **15:**570-576.

20. Petri M, Stohl W, Chatham W, McCune WJ, Chevrier M, Ryel J, Recta V, Zhong J, Freimuth W: **Association of plasma B lymphocyte stimulator levels and disease activity in systemic lupus erythematosus.** *Arthritis Rheum* 2008, **58:**2453-2459.

21. Stohl W, Metyas S, Tan SM, Cheema GS, Oamar B, Xu D, Roschke V, Wu Y, Baker KP, Hilbert DM: **B lymphocyte stimulator overexpression in patients with systemic lupus erythematosus: longitudinal observations.** *Arthritis Rheum* 2003, **48:**3475-3486.

22. Brkic Z, Maria NI, van Helden-Meeuwsen CG, van de Merwe JP, van Daele PL, Dalm VA, Wildenberg ME, Beumer W, Drexhage HA, Versnel MA: **Prevalence of interferon type I signature in CD14 monocytes of patients with Sjogren's syndrome and association with disease activity and BAFF gene expression.** *Ann Rheum Dis* 2013, **72:**728-735.

23. Ettinger R, Sims GP, Robbins R, Withers D, Fischer RT, Grammer AC, Kuchen S, Lipsky PE: **IL-21 and BAFF/BLyS synergize in stimulating plasma cell differentiation from a unique population of human splenic memory B cells.** *J Immunol* 2007, **178:**2872-2882.

24. Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, Corcoran LM, Godfrey DI, Toellner KM, Smyth MJ, et al: **IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism.** *The Journal of experimental medicine* 2010, **207:**365-378.

25. Hsu HC, Yang P, Wang J, Wu Q, Myers R, Chen J, Yi J, Guentert T, Tousson A, Stanus AL, et al: **Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice.** *Nature immunology* 2008, **9:**166-175.

26. Hochberg MC: **Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus.** *Arthritis Rheum* 1997, **40:**1725.

27. Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH: **Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE.** *Arthritis Rheum* 1992, **35:**630-640.

28. Drexhage RC, van der Heul-Nieuwenhuijsen L, Padmos RC, van Beveren N, Cohen D, Versnel MA, Nolen WA, Drexhage HA: **Inflammatory gene expression in monocytes of patients with schizophrenia: overlap and difference with bipolar disorder. A study in naturalistically treated patients.** *Int J Neuropsychopharmacol* 2010, **13:**1369-1381.

29. Beillard E, Pallisgaard N, van der Velden VH, Bi W, Dee R, van der Schoot E, Delabesse E, Macintyre E, Gottardi E, Saglio G, et al: **Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - a Europe against cancer program.** *Leukemia* 2003, **17:**2474-2486.

30. van Hamburg JP, Asmawidjaja PS, Davelaar N, Mus AM, Colin EM, Hazes JM, Dolhain RJ, Lubberts E: **Th17 cells, but not Th1 cells, from patients with early rheumatoid arthritis are potent inducers of matrix metalloproteinases and proinflammatory cytokines upon synovial fibroblast interaction, including autocrine interleukin-17A production.** *Arthritis Rheum* 2011, **63:**73-83.

31. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, Sallusto F, Napolitani G: **Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells.** *Nat Immunol* 2007, **8:**639-646.

32. Annunziato F, Cosmi L, Liotta F, Maggi E, Romagnani S: **The phenotype of human Th17 cells and their precursors, the cytokines that mediate their differentiation and the role of Th17 cells in inflammation.** *Int Immunol* 2008, **20:**1361-1368.

33. Ivanov, II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR: **The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells.** *Cell* 2006, **126:**1121-1133.

34. Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, Kano S, Honda K, Ohba Y, Mak TW, Taniguchi T: **Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors.** *Nature* 2005, **434:**243-249.

35. Lazear HM, Lancaster A, Wilkins C, Suthar MS, Huang A, Vick SC, Clepper L, Thackray L, Brassil MM, Virgin HW, et al: **IRF-3, IRF-5, and IRF-7 coordinately regulate the type I IFN response in myeloid dendritic cells downstream of MAVS signaling.** *PLoS pathogens* 2013, **9:**e1003118.

36. Yu CF, Peng WM, Oldenburg J, Hoch J, Bieber T, Limmer A, Hartmann G, Barchet W, Eis-Hubinger AM, Novak N: **Human plasmacytoid dendritic cells support Th17 cell effector function in response to TLR7 ligation.** *J Immunol* 2010, **184:**1159-1167.

37. Matikainen S, Sareneva T, Ronni T, Lehtonen A, Koskinen PJ, Julkunen I: **Interferon-alpha activates multiple STAT proteins and upregulates proliferation-associated IL-2Ralpha, c-myc, and pim-1 genes in human T cells.** *Blood* 1999, **93:**1980-1991.

38. Yasuda K, Richez C, Maciaszek JW, Agrawal N, Akira S, Marshak-Rothstein A, Rifkin IR: **Murine dendritic cell type I IFN production induced by human IgG-RNA immune complexes is IFN regulatory factor (IRF)5 and IRF7 dependent and is required for IL-6 production.** *J Immunol* 2007, **178:**6876-6885.

39. Santini SM, Lapenta C, Donati S, Spadaro F, Belardelli F, Ferrantini M: **Interferon-alpha-conditioned human monocytes combine a Th1-orienting attitude with the induction of autologous Th17 responses: role of IL-23 and IL-12.** *PloS one* 2011, **6:**e17364.

40. Lai Kwan Lam Q, King Hung Ko O, Zheng BJ, Lu L: **Local BAFF gene silencing suppresses Th17-cell generation and ameliorates autoimmune arthritis.** *Proc Natl Acad Sci U S A* 2008, **105:**14993-14998.

41. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, Friday S, Li S, Patel RM, Subramanian V, et al: **NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis.** *Science translational medicine* 2013, **5:**178ra140.

* J. Merrill, V. Chindalore, J. Box, N. Rothfield, J. Fiechtner, J. Sun, D. Ethgen. **Results of a randomized placebo-controlled, phase 2a study of sifalimumab, an anti-interferon-alpha monoclonal antibody, administered subcutaneously in subjects with systemic lupus erythematosus [abstract].** [2011] [THU0411]


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Protective role of IL-17 receptor signaling in SLE pathology in B6.lpr mice

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ABSTRACT

Introduction A pro-inflammatory role for interleukin 17A (IL-17A) has been suggested in the pathogenesis of SLE. IL-17A plasma levels correlate with disease severity in SLE patients and IL-23R deficient lupus prone mice are protected against disease development. In addition HMGB1, a protein involved in DNA bending that can be excreted as an alarmin or during cell death, is correlated with disease severity in SLE patients. However, it is unclear whether IL-17 family members affect HMGB1 levels and how this contributes to SLE pathology.

Methods We crossed IL-17RA deficient mice with B6.lpr lupus prone mice. We followed the IL-17RA KO.lpr mice over time and analysed disease development. We measured lymphocyte proliferation and cytokine expression by flow cytometry. Serum HMGB1 and anti-HMGB1 levels were measured by Western blot and ELISA, respectively and kidney damage by histology.

Results Remarkably, we found an enhanced SLE phenotype in IL-17RA KO.lpr mice compared with B6.lpr mice, characterized by increased lymphocyte proliferation, expansion of double negative T cells and enhanced plasma cell formation. Also, kidney pathology in IL-17RA KO.lpr mice was more severe than in B6.lpr mice. Although total anti-nuclear antibody (ANA) titers were not increased in IL-17RA KO.lpr mice compared with B6.lpr mice, we did find increased presence of the DNA binding protein HMGB1 and anti-HMGB1 autoantibodies in the serum of these mice.

Conclusion These data indicate that IL-17RA signaling reduces pathology in lupus prone B6.lpr mice and suggest that IL-17RA signaling may be involved in the clearance of apoptotic debris.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease leading to multi-organ failure [1]. The incidence of SLE is highest in Afro-American and Asian females [2]. The cause of the disease is unknown, but both genetic and environmental factor play a role. Many single nucleotide polymorphisms (SNPs) in immune regulatory genes have been associated with increased SLE susceptibility [3]. In addition, infection with cytomegalovirus (CMV) or Epstein Barr virus (EBV) can predispose the disease and have been proposed as triggers that induce SLE in genetically predisposed patients [4, 5].

SLE is characterized by the presence of auto-antibodies directed against nuclear structures (ANAs) [1]. ANAs and immune complexes can induce T cell activation and the production of interferon (IFN) type I by plasmacytoid DCs (pDCs) [6, 7]. IFN type I expression is increased in around half of SLE patients [8, 9]. Monocyte derived DCs (mDCs) are activated by IFN type I and also induce T helper cell differentiation, and enhanced B cell activation and survival through BAFF production [10-12]. This leads to the production of auto-reactive antibodies, mostly directed against nuclear structures, which form immune complexes and deposit in small capillaries in for example the kidneys, inducing inflammation and tissue damage [13].

As nuclear structures including DNA are not usually accessible to immune cells, it is thought that aberrant apoptosis could be a trigger for ANA formation [14]. DNA normally does not induce an immune response, however, modifications to DNA associated with apoptosis can activate DCs [15, 16]. High mobility group box 1 (HMGB1) is a nuclear protein that binds DNA and is involved in DNA bending [17]. When excreted, it can serve as an alarmin or damage-associated molecular pattern (DAMP) to enhance clearance of damaged, apoptotic or necrotic cells [18]. However, during apoptosis, the presence of DNA together with this pro-inflammatory cytokine can lead to induction of ANA formation [19]. Recently it was shown that HMGB1 levels are increased in serum and urine of SLE patients and are related to disease activity [20, 21].

Recent studies have suggested a role for interleukin 17A (IL-17A) in the pathogenesis of SLE [22]. IL-17A is produced by many immune cells, including several T cell subsets like T helper 17 (Th17) cells, double negative T (DNT) cells, which do not express CD4 or CD8, and γδ T cells [22-24]. Stability of the Th17 cell population is dependent on IL-21 and IL-23 [25, 26]. IL-17A is a member of the IL-17 cytokine family and signals through an heterodimeric receptor complex composed of the IL-17 receptor A (IL-17RA) and IL-17RC subunits [27, 28]. The IL-17RA subunit appears to be the common receptor subunit for all IL-17 cytokine members [29, 30].

IL-17A plasma levels and numbers of IL-17A producing peripheral blood mononuclear cells (PBMCs) are increased in SLE patients and correlate with disease severity [31-33]. Furthermore, IL-17A producing DNT cells have been found in the kidneys of lupus nephritis patients [22]. In the BXD2 SLE mouse model, IL-17A was shown to be involved in germinal center formation, which is crucial for pathogenic auto-antibody production [34]. Furthermore, TRIM21/Ro52 knock-

out mice, which have increased IFN type I levels, develop an SLE phenotype after ear tagging. However, when they were crossed onto an IL-23p19 deficient background, which is characterized by reduced expression of RORγt and IL-17A, they were completely protected against SLE, indicating that the effect of IFN type I is mediated by the IL-23/IL-17 pathway in this model [35].

C57BL/6-lpr/lpr (B6.lpr) mice spontaneously develop an SLE phenotype due to a mutation in the Fas receptor (CD95) [36]. This mutation limits negative selection of auto-reactive lymphocytes and leads to systemic autoimmune symptoms, including ANA production and complement activation, forming immune complexes which infiltrate the kidneys. It was recently shown that B6.lpr mice lacking IL-23 receptor (IL-23R) signaling are completely protected against SLE development [37]. These mice had decreased numbers of IL-17A producing cells in the lymph nodes and decreased anti-DNA antibody production, suggesting a crucial role for the IL-23/IL-17A axis in SLE pathogenesis in this model [37]. However, these studies did not reveal whether IL-23 and IL-17A play a role in SLE pathogenesis independent of each other. Therefore, we used IL-17RA deficient mice to examine the role of IL-17 signaling in the presence of IL-23 in SLE development in B6.lpr mice.

Here we show that IL-17RA deficient B6-lpr mice have an enhanced SLE phenotype, characterized by increased lymphocyte proliferation and increased plasma cell formation and ANA production. Interestingly, serum levels of HMGB1 were enhanced in the IL-17RA KO mice, as well as levels of anti-HMGB1 antibodies. These data suggest a pathogenic role for IL-17RA signaling in the SLE phenotype of B6.lpr mice and suggest a possible role for IL-17RA signaling in clearance of apoptotic debris.

METHODS

Mice

C57BL/6-lpr/lpr (B6.lpr) mice were purchased from The Jackson Laboratory, USA, and IL-17 receptor A knock-out (IL-17RA KO) mice on a C57BL/6 background [38] were kindly provided by dr. J. Tocker, Amgen, Seattle, USA. For genotyping of the IL-17RA construct, 5'-CTTGTGTAGCGCCAAGTG, 5'-AGCTGCTGTTAGCACTTTGC and 5'-CGTACGCACACACTCTCGA primers were used. For genotyping of the lpr construct, 5'-GTAAATAATTGTGCTTCGTCAG, 5'-TAGAAAGGTGCACGGGTGTG and 5'- CAAATCTAGGCATTAACAGTG were used. The mouse lines were crossed to generate IL-17RA KO B6.lpr mice. Mice were housed under SPF conditions in the Erasmus Medical Center Animal Facility (EDC) and provided with food and water *ad libitum*. All experiments were approved by the Erasmus MC Animal Ethical Committee (DEC). Mice were sacrificed at the age of 8, 12, 20 or 26 weeks. Blood was drawn to isolate serum and the spleens, kidneys and cervical lymph nodes were harvested.

Flow cytometry

For B cell staining, $2*10^6$ splenocytes were stained for 20 minutes at room temperature. For intracellular Ig staining, cells were subsequently fixed and permeabilized using BD Cytofix/ Cytoperm (BD BioSciences) and stained at room temperature for 20 minutes. Anti-CD19, anti-B220 and anti-IgM antibodies were obtained from eBioscience (San Diego, CA, USA), anti-IgD, anti-CD95, anti-CD138, anti-IgG1 and anti-IgG2ab antibodies from BD BioSciences and anti-peanut agglutinin (PNA) antibodies from Sigma-Aldrich (St Louis, USA).

For T cells staining, splenocytes were stimulated for 4 hours with PMA (0.05µg/ml) and Ionomycin (0.5µg/ml) in the presence of Golgi stop (BD Biosciences). $2*10^6$ cells were stained per sample with antibody mix for 20 minutes at room temperature and subsequently fixed with 2% PFA and permeabilized in 0.5% saponin. Cells were then stained at room temperature for 20 minutes for intracellular markers. Anti-CD3, anti-CD4, anti-CD8, anti-IL-17A, anti-IL4 and anti-IFNγ antibodies were obtained from BD BioSciences (San Diego, CA, USA) and anti-IL-10 antibody was obtained from Biolegend (San Diego, CA, USA).

Samples were measured on a FACS Canto II HTS or a FACS LSR II flow cytometer (BD BioSciences) and analysis was performed using FlowJo v7.6 research software (Tree Star Inc. Ashland, OR).

Histology and immunohistochemistry

Kidney tissue samples from 26 week-old female IL-17RA KO lpr and B6.lpr mice were frozen in Tissue-Tec O.C.T. Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) and stored at -80 ^{o}C. Sections of 6µm thick were cut and used for stainings.

For Periodic acid Schiffs (PAS) staining slides were fixed with 4% formalin for 10 minutes. The slides were incubated with 0.5% periodic acid for 5 minutes and Schiff's reagent for 10 minutes. Mayer's Hematoxylin was used as counterstain and slides were covered with pertex.

For immunohistochemistry, slides were fixed with ethanol for 10 minutes. The slides were incubated with anti-$C3^{FITC}$ (1:50) or anti-IgG^{FITC} (1:80) (MP Biomedicals, Santa Ana, California, USA) for 45 minutes. DAPI was added shortly before the slides were covered with fluorglycerine.

Serum measurements

ANA titers were measured by immunofluorescence on HEp-2000 coated glass slides (Biomedical Diagnostics, Eindhoven, the Netherlands), using different dilutions of mouse serum in PBS, and rabbit anti-mouse IgG- FITC (Dako, Glostrup, Denmark) for detection.

Levels of HMGB1 were measured by Western Blotting. In short, 3 µl of mouse serum was added to 1X SDS (sodium dodecyl sulfate) loading buffer, samples were resolved on 12.5% SDS polyacrylamide gel electrophoresis (Criterion, BioRad, Veenendaal, the Netherlands) and blotted

on polyvinylidene fluoride membrane (Millipore, Amsterdam, the Netherlands). Detection was performed with polyclonal anti-HMGB1-biotin (Thermoscientific,Etten-Leur, the Netherlands), and streptavidin-IRDye800 (LI-COR Biotechnology, Lincoln, NE, USA). Blots were scanned with an Odyssey infrared Imaging System (LI-COR Biotechnology) and analyzed with Odyssey software. In each blot a cell lysate made of Jurkat cells was run as a standard. HMGB1 levels were presented as the fluorescence intensity against the standard.

Level of anti-HMGB1 were measured by ELISA. Costar polystyrene plates were coated with 1 µg/ml recombinant HMGB1 (Sigma, St. Louis, MO, USA) and after blocking mouse sera were added in dilutions of 20 and 80 times. Detection of antibodies was done with rabbit anti-mouse IgG-HRP (Dako, Glostrup, Denmark) and TMB colour reaction. Levels of anti-HMGB1 were calculated against a standard curve of a monoclonal anti-HMGB1 (R&D systems, Abingdon, United Kingdom)

Statistical analysis

Data was analyzed using Prism software v5.04 (GraphPad Software Inc. La Jollla, CA). For comparisons, a non-parametric Mann-Whitney U test was used. P-values <0.05 were considered significant.

RESULTS

Increased spleen size in IL-17RA KO.lpr mice compared to B6.lpr mice

To investigate the effect of IL-17RA signaling on the spontaneous SLE phenotype in C57BL/6 mice with the lpr mutation (B6.lpr), we crossed B6.lpr mice with IL-17RA deficient (IL-17RA KO) mice. We followed these IL-17RA KO.lpr mice over time to analyse the phenotype and sacrificed mice at 8, 12, 20 and 26 weeks of age. At 26 weeks of age, the spleens and lymph nodes from IL-17RA KO.lpr mice were greatly enlarged compared with spleens and lymph nodes from B6.lpr mice (figure 1A). The total number of splenocytes was comparable between wild-type and IL-17RA KO mice without the lpr mutation at 8 weeks of age. This number did not markedly increase over time in B6.lpr mice. However, the number of splenocytes in IL-17RA KO.lpr mice increased significantly over time to about threefold compared with B6.lpr mice at 26 weeks of age (figure 1B). This increase could be attributed to a significant increase in total numbers of B cells and T cells in IL-17RA KO mice (figure 1C-D).

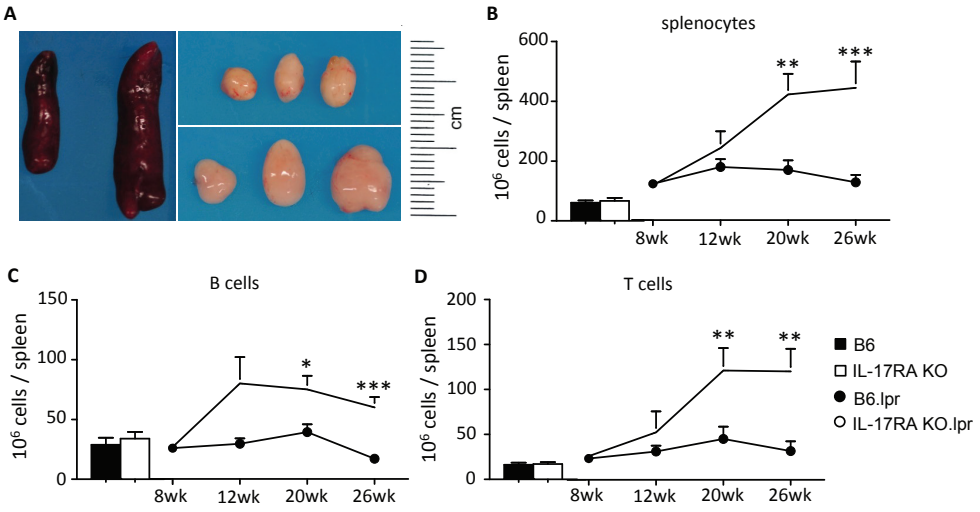


Figure 1. Increased spleen and lymph node size in IL-17RA KO.lpr mice. [A] Spleens and cervical lymph nodes from 26 week old B6.lpr (left and top) and IL-17RA KO.lpr (right and bottom). [B-D] Total number of splenocytes (all live cells in spleen) [B], B cells (CD19+) [C] and T cells (CD3+) [D] at different ages. Bars depict wild type B6 (black) and IL-17RA KO (white) mice at 8 weeks of age. Mean and SEM are shown for n = 3 – 21 mice per group; *p<0.05; **p<0.01; ***p<0.001.

HMGB1 and anti-HMGB1 are increased in the serum of IL-17RA KO.lpr mice

SLE is characterized by presence of ANA in the serum. We therefore measured ANA titers in the serum of B6.lpr and IL-17RA KO.lpr mice at several ages. Serum ANA titers increased with age in both groups (figure 2A). Surprisingly, serum ANA titers were not higher in IL-17RA KO.lpr mice than in B6.lpr mice at any age. ANA production is thought to be induced by insufficient clearance of apoptotic or necrotic debris containing DNA and HMGB1 [39, 40]. To determine whether higher serum ANA levels in IL-17RA KO.lpr and B6.lpr mice were associated with increased presence of HMGB1 in serum, we determined HMGB1 levels by Western blot. Interestingly, already at 8 weeks of age, HMGB1 levels were increased in IL-17RA KO.lpr mice compared to B6.lpr mice (figure 2B). Furthermore, anti-HMGB1 antibody levels increased in IL-17RA KO.lpr mice with age and reached a much higher level than in B6.lpr mice (figure 2C). In serum of control C57BL6 mice, we did not detect HMGB1 protein or antibodies to HMGB1 (data not shown). As HMGB1 is only released from late apoptotic or necrotic bodies [41], these data suggest a role for IL-17RA signaling in clearance of apoptotic bodies.

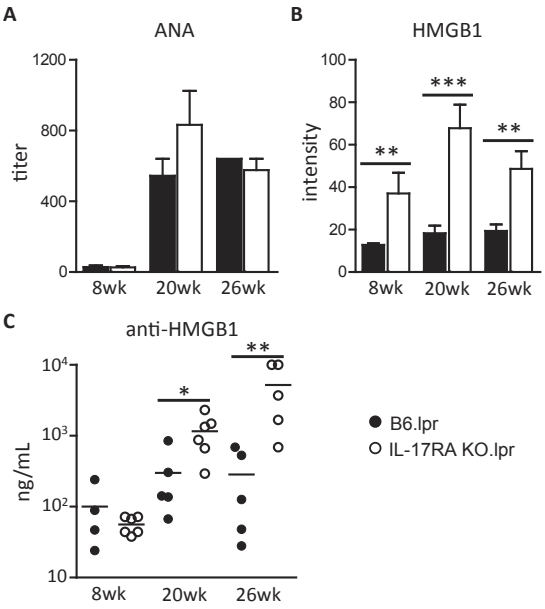


Figure 2. ANA, HMGB1 and anti-HMGB1 levels in B6.lpr and IL-17RA KO.lpr mice. [A] Serum ANA titers by immunofluorescence. [B] Serum HMGB1 levels measured by Western Blot. Mean and SEM are shown for n = 5 – 13 animals per group. [C] Serum anti-HMGB1 levels measured by ELISA. Bar indicates median. *p<0.05; **p<0.01; ***p<0.001.

Increased numbers of cytokine producing DNT and CD4+ T cells in IL-17RA KO.lpr mice

Both DNT and CD4+ T cells have been shown to play a role in the pathogenesis of SLE in patients [42]. To determine whether these subpopulations were increased in IL-17RA KO.lpr mice, we analysed the splenic T cell compartment of these mice by flow cytometry. The increased number of T cells in IL-17RA KO.lpr mice, when compared with B6.lpr mice, was due to an increase in the total numbers of DNT cells, and to a lesser extent of CD4+ and CD8+ T cells (figure 3A-C).

We found that specifically the proportions of DNT cells were increased in IL-17RA KO.lpr mice (supplementary figure 1). Total numbers of cytokine producing DNT cells were increased at 20 and 26 weeks of age in IL-17RA KO.lpr mice compared with B6.lpr mice (supplementary figure 2). However, proportions of IFNγ, IL-4, IL-17A and IL-10 producing DNT cells were in the same range in IL-17RA KO.lpr and B6.lpr mice (figure 4A-D), suggesting that there is no skewing within the DNT population. This was also true for CD4+ cytokine producing T cells (figure 4E-H), although only total numbers of IL-17A producing CD4+ T cells were increased (supplementary figure 3). Together, these data indicate that total numbers of DNT cells, CD4+ and CD8+ T cells in IL-17RA KO.lpr mice are enhanced and specifically skewed towards DNT cells, but not towards a specific cytokine profile within this subset.

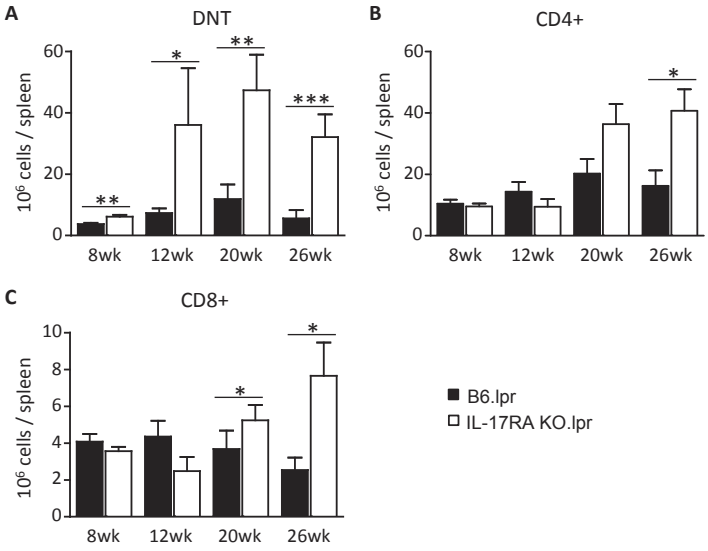


Figure 3. Expansion of T cell populations in IL-17RA KO.lpr mice. [A-C] Total numbers of splenic DNT [A], CD4+ [B] and CD8+ [C] T cells in B6.lpr and IL-17RA KO.lpr mice at different ages. Mean is shown for n = 9-21 animals per group; *p<0.05; **p<0.01; ***p<0.001.

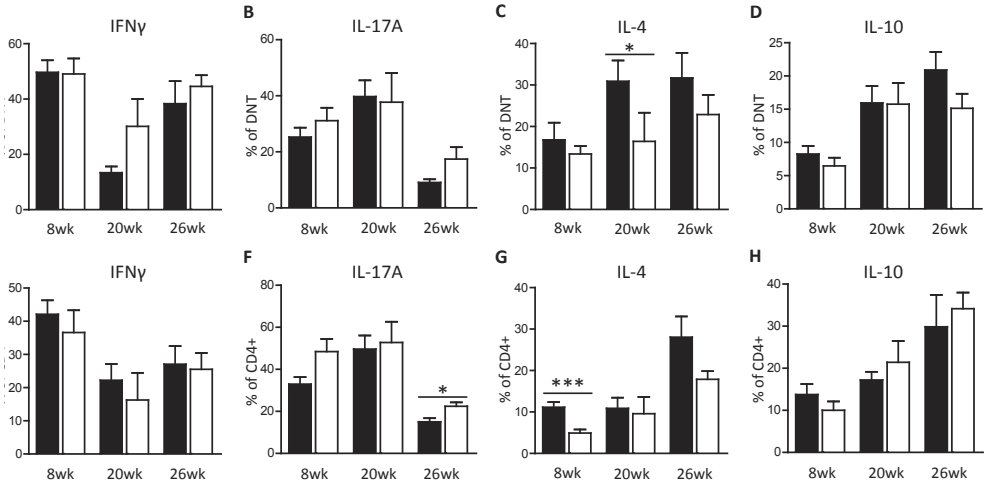


Figure 4. Proportions of cytokine producing DNT and CD4+ T cells. [A-D] Proportions of splenic IFNγ+ [A], IL-4+ [B], IL-17A+ [C] and IL-10+ [D] DNT cells in B6.lpr and IL-17RA KO.lpr mice at different ages. [E-H] Proportions of splenic IFNγ+ [E], IL-4+ [F], IL-17A+ [G] and IL-10+ [H] CD4+ T cells in B6.lpr and IL-17RA KO.lpr mice at different ages. Mean is shown for n = 9-21 animals per group; *p<0.05; ***p<0.001.

Plasma cell formation is enhanced in IL-17RA KO.lpr mice

As we found an increase in the number of B cells in IL-17RA KO.lpr mice compared to B6.lpr mice, we next analysed the splenic B cell compartment of these mice. Using flow cytometry with CD21 and CD23 [43], we found that the increase in the number of B cells can be attributed mostly to an increase in the number of follicular B cells, although the number of marginal zone and transitional B cells also increased in IL-17RA KO.lpr mice (figure 5A-C).

Next, we tested whether the increase in follicular B cells led to enhanced germinal center formation. Although after an initial rise at 20 weeks of age the number of germinal center B cells (PNA+CD95+CD19+) decreased in B6.lpr mice at 26 weeks of age, it was not significantly lower than in IL-17RA KO.lpr mice (figure 5D). However, intracellular flow cytometry analysis for Ig subclasses showed that the number of IgM, IgG1 and IgG2 plasma cells formed at 26 weeks of age was significantly larger in IL-17RA KO.lpr mice compared with B6.lpr mice, in line with the increased cell number in these mice and suggesting enhanced pathology (figure 5E-G).

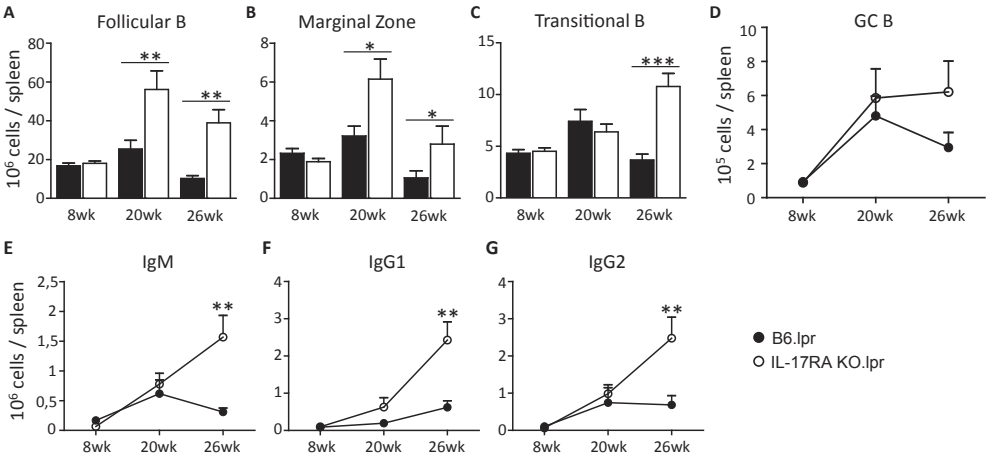


Figure 5. Expansion of B cell populations in IL-17RA KO.lpr mice. [A-C] Total numbers of splenic follicular (CD19+B220+CD21-CD23+) [A], marginal zone (CD19+B220+CD21+CD23-) [B] and transitional (CD19+B220+CD21-CD23-) B cells [C] in B6.lpr and IL-17RA KO.lpr mice at different ages. [D] Total numbers of germinal center B cells (CD19+B220+PNA+CD95+) at different ages. [E-G] Total numbers of IgM [E], IgG1 [F] and IgG2 [G] producing CD138+ plasma cells at different ages. Mean and SEM are shown for n = 9-21 animals per group; *p<0.05; **p<0.01; ***p<0.001.

Enhanced B cell immunity leads to increased kidney pathology in IL-17RA KO.lpr mice

To investigate whether the increase in cytokine producing T cells and plasma cells in the spleen was associated with enhanced pathology in IL-17RA KO.lpr mice, we performed PAS staining on kidney sections from B6.lpr and IL-17RA KO.lpr mice. Indeed the slides showed thickening of the

basal membrane of glomeruli in IL-17RA KO.lpr mice, indicating damage of the kidneys (figure 6A). In addition we found enhanced C3 complement deposition and enhanced IgG antibody deposition in kidneys of IL-17RA KO.lpr mice (figure 6B and 6C, respectively). Together, these data show that lack of IL-17RA in B6.lpr mice leads to an enhanced SLE phenotype with enhanced kidney pathology.

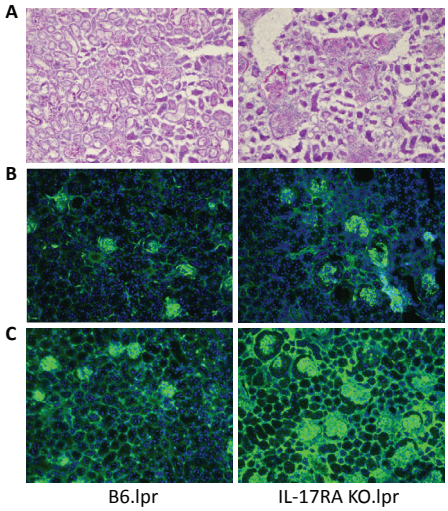
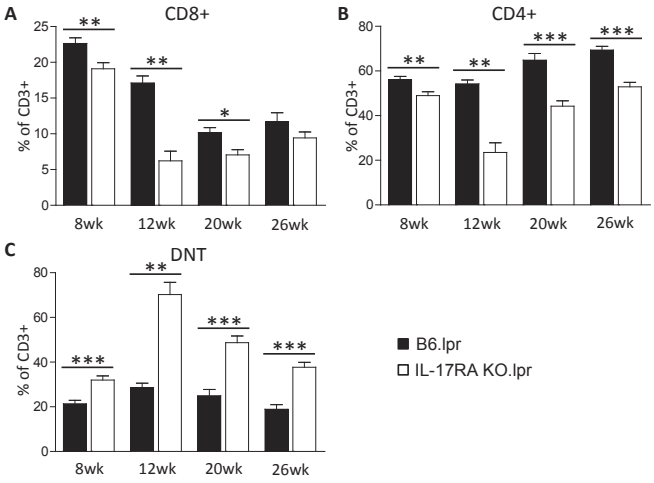
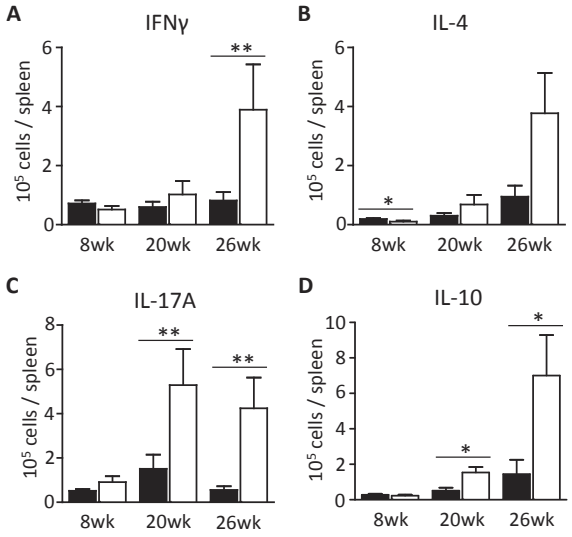


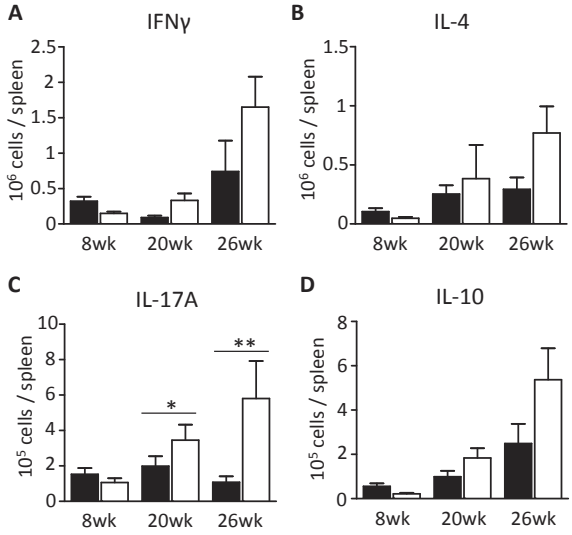
Figure 6. Kidney pathology in B6.lpr and IL-17RA KO.lpr mice. [A] PAS staining of kidney sections of 26 week old B6.lpr and IL-17R KO.lpr. [B-C] C3 deposition [B] and IgG deposition [C] in kidneys of 26 week old B6.lpr and IL-17R KO.lpr. Representative pictures are shown for n = 6 animals per group.



Supplementary figure 1. [A-C] Proportions of CD8+ [A], CD4+ [B] and DNT [C] CD3+ T cells in B6.lpr and IL-17RA KO.lpr mice at different ages. Mean and SEM are shown for n = 3 – 21 animals per group; *p<0.05; **p<0.01; ***p<0.001.



Supplementary figure 2. [A-D] Total numbers of IFNγ expressing [A], IL-4 expressing [B], IL-17A expressing [C] and IL-10 expressing [D] DNT T cells in B6.lpr and IL-17RA KO.lpr mice. Mean and SEM are shown for n = 8 – 21 mice per group; *p<0.05; **p<0.01.



Supplementary figure 3. [A-D] Total numbers of IFNγ expressing [A], IL-4 expressing [B], IL-17A expressing [C] and IL-10 expressing [D] CD4+ T cells in B6.lpr and IL-17RA KO.lpr mice. Mean and SEM are shown for n = 8 – 21 mice per group; *p<0.05; **p<0.01.

DISCUSSION

Here we show that lack of IL-17RA signaling enhances the SLE phenotype in B6.lpr mice. Spleen size is greatly enhanced in these mice, characterized by expansion of CD4+, CD8+ T and DNT cells and B cells and increased plasma cell formation. In addition, although total ANA titers were not different between the groups, serum HMGB1 and anti-HMGB1 antibody levels were elevated in IL-17RA KO.lpr mice compared to B6.lpr mice. This was accompanied with enhanced antibody deposition in the kidneys and kidney damage. These data suggest that loss of IL-17RA signaling enhances the development of autoimmunity in B6.lpr mice.

Interestingly, enhanced T cell proliferation was most prominent in CD4+ and DNT cells, although cytokine production was not skewed towards a specific cytokine producing subset as the distribution of cytokine production was similar in IL-17RA KO.lpr and B6.lpr mice. This shows that loss of IL-17RA signaling does not affect differentiation of the T cell subsets in the spleen, but suggests that IL-17RA signaling can limit proliferation or survival of the cells. In IL-17RA KO mice, loss of this regulatory function can apparently be rescued by other factors, however, additional loss of FAS-FASL signaling induces expansion of cells. Whether the IL-17RA and FAS-FASL are involved in the same pathway is unclear.

A role for IL-17RA signaling has been shown in disease progression in lupus prone BXD2 mice [34, 44]. When crossed with IL-17RA KO mice, BXD2 mice are partially protected against disease development. This can be attributed to impaired germinal center reactions in these mice. We do not find a decrease in the number of PNA+ germinal center B cells in the spleens of IL-17RA KO.lpr mice at any age investigated. Instead, the number of germinal center B cells appears to be slightly increased, although not significantly. In addition, auto-reactive antibody levels are not decreased in our model. As BXD2 mice have a mix of C57BL/6 and DBA genes, it is possible that background genes can explain these discrepancies, as we also find normal germinal center formation in IL-17RA KO mice on a C57BL/6 background in collagen induced arthritis and after influenza infection (Corneth et al, Arthritis and Rheumatism).

Recently, a new form of cell death by neutrophils has been described, called NETosis [45]. During this process, chromatin is reorganized in the neutrophils to form a net-like structure. This net-like structure is excreted from the cell as a neutrophil extracellular trap (NET) which engulfs extracellular pathogens and thereby facilitates phagocytosis by macrophages. It is vital that the NET is cleared sufficiently, as the extracellular presence of DNA/chromatin can lead to activation of immune cells and autoimmunity. IL-17A was recently reported to play an important role in the induction of a unique subset of macrophages (M2) that phagocytoses early apoptotic neutrophils, preventing exposure of immune cells to DNA and other nuclear antigens [46]. On the contrary, IFNγ and IL-4 induced apoptosis of pro-inflammatory macrophages, leading to an accumulation of apoptotic neutrophils and macrophages. Macrophages in kidneys of lupus resistant mice quickly shift to an M2 ‘protective’ phenotype upon damage induction, whereas

M1 macrophages are increased in the kidneys of MRL.lpr mice and are associated with enhanced renal damage [47]. It is possible that failure to induce the M2 macrophages through lack of IL-17RA signaling in B6.lpr mice leads to decreased clearance of apoptotic structures and NETs, increasing the auto-antigen load in already auto-immune prone mice. In addition, the increase in IFNγ and IL-4 producing Th cells in these mice can add to this pro-inflammatory feedback loop.

The role of IL-17A in clearance of apoptotic debris can explain the increased levels of HMGB1 in the IL-17RA KO.lpr mice. Even though disease symptoms (lymphoproliferation, ANA) are not visible in young IL-17RA KO.lpr mice, HMGB1 serum levels in these mice already significantly exceed the levels in B6.lpr mice at 8 weeks of age, indicating that a rise in HMGB1 levels precedes disease development. In MRL.lpr mice, HMGB1 levels were suggested to correlate with disease progression [48]. In line with this, our data indicate that high HMGB1 levels, as well as anti-HMGB1 levels, are associated with more severe disease in lupus prone mice.

Our data appears contradictory to studies that show a role for IL-23 in the SLE phenotype of B6.lpr and MRL.lpr mice [37, 49]. In IL-23R KO.lpr mice, which do not develop lupus, the total number of DNT cells is decreased as well as the total number of IL-17A producing DNT and CD4+ T (Th17) cells. This was accompanied with lower serum total IgG and ANA levels, decreased Ig and complement deposition in the kidneys and absence of kidney damage [37]. In contrast, IL-23 signaling in the IL-17RA KO.lpr mice is not deficient, and mice have normal or even increased numbers of IL-17A expressing DNT or Th17 cells, although IL-17A cannot signal. In addition, other cytokines produced by these populations, like IL-22, are normally expressed. It is therefore possible that these cytokines also play a role in the SLE phenotype in this mouse model. Interestingly, our preliminary data shows increased levels of HMGB1 in IL-22KO.lpr mice, although these levels do not rise as early as in IL-17RA KO.lpr mice (Corneth et al, unpublished data). Additional studies could shed light on a role for IL-22 and other pro-inflammatory cytokines produced by DNT or Th17 cells in experimental SLE.

REFERENCES

1. Choi J, Kim ST, Craft J: **The pathogenesis of systemic lupus erythematosus-an update.** *Curr Opin Immunol* 2012, **24:**651-657.
2. Hopkinson ND, Doherty M, Powell RJ: **Clinical features and race-specific incidence/prevalence rates of systemic lupus erythematosus in a geographically complete cohort of patients.** *Ann Rheum Dis* 1994, **53:**675-680.
3. Byrne JC, Ni Gabhann J, Lazzari E, Mahony R, Smith S, Stacey K, Wynne C, Jefferies CA: **Genetics of SLE: functional relevance for monocytes/macrophages in disease.** *Clin Dev Immunol* 2012, **2012:**582352.
4. James JA, Neas BR, Moser KL, Hall T, Bruner GR, Sestak AL, Harley JB: **Systemic lupus erythematosus in adults is associated with previous Epstein-Barr virus exposure.** *Arthritis Rheum* 2001, **44:**1122-1126.
5. Barzilai O, Sherer Y, Ram M, Izhaky D, Anaya JM, Shoenfeld Y: **Epstein-Barr virus and cytomegalovirus in autoimmune diseases: are they truly notorious? A preliminary report.** *Ann N Y Acad Sci* 2007, **1108:**567-577.
6. Bjorck P, Beilhack A, Herman EI, Negrin RS, Engleman EG: **Plasmacytoid dendritic cells take up opsonized antigen leading to CD4+ and CD8+ T cell activation in vivo.** *J Immunol* 2008, **181:**3811-3817.
7. Means TK, Latz E, Hayashi F, Murali MR, Golenbock DT, Luster AD: **Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9.** *J Clin Invest* 2005, **115:**407-417.
8. Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ, Shark KB, Grande WJ, Hughes KM, Kapur V, et al: **Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus.** *Proc Natl Acad Sci U S A* 2003, **100:**2610-2615.
9. Bennett L, Palucka AK, Arce E, Cantrell V, Borvak J, Banchereau J, Pascual V: **Interferon and granulopoiesis signatures in systemic lupus erythematosus blood.** *J Exp Med* 2003, **197:**711-723.
10. Yu CF, Peng WM, Oldenburg J, Hoch J, Bieber T, Limmer A, Hartmann G, Barchet W, Eis-Hubinger AM, Novak N: **Human plasmacytoid dendritic cells support Th17 cell effector function in response to TLR7 ligation.** *J Immunol* 2010, **184:**1159-1167.
11. Ronnblom L, Pascual V: **The innate immune system in SLE: type I interferons and dendritic cells.** *Lupus* 2008, **17:**394-399.
12. Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, Casali P, Cerutti A: **DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL.** *Nat Immunol* 2002, **3:**822-829.
13. Yung S, Chan TM: **Anti-DNA antibodies in the pathogenesis of lupus nephritis--the emerging mechanisms.** *Autoimmun Rev* 2008, **7:**317-321.
14. Herrmann M, Voll RE, Zoller OM, Hagenhofer M, Ponner BB, Kalden JR: **Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus.** *Arthritis Rheum* 1998, **41:**1241-1250.
15. Casciola-Rosen L, Andrade F, Ulanet D, Wong WB, Rosen A: **Cleavage by granzyme B is strongly predictive of autoantigen status: implications for initiation of autoimmunity.** *J Exp Med* 1999, **190:**815-826.
16. Fransen JH, Hilbrands LB, Jacobs CW, Adema GJ, Berden JH, Van der Vlag J: **Both early and late apoptotic blebs are taken up by DC and induce IL-6 production.** *Autoimmunity* 2009, **42:**325-327.
17. Bonaldi T, Langst G, Strohner R, Becker PB, Bianchi ME: **The DNA chaperone HMGB1 facilitates ACF/CHRAC-dependent nucleosome sliding.** *EMBO J* 2002, **21:**6865-6873.
18. Yang D, Chen Q, Yang H, Tracey KJ, Bustin M, Oppenheim JJ: **High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin.** *J Leukoc Biol* 2007, **81:**59-66.
19. Urbonaviciute V, Furnrohr BG, Meister S, Munoz L, Heyder P, De Marchis F, Bianchi ME, Kirschning C, Wagner H, Manfredi AA, et al: **Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: implications for the pathogenesis of SLE.** *J Exp Med* 2008, **205:**3007-3018.
20. Abdulahad DA, Westra J, Bijzet J, Limburg PC, Kallenberg CG, Bijl M: **High mobility group box 1 (HMGB1) and anti-HMGB1 antibodies and their relation to disease characteristics in systemic lupus erythematosus.** *Arthritis Res Ther* 2011, **13:**R71.

21. Abdulahad DA, Westra J, Bijzet J, Dolff S, van Dijk MC, Limburg PC, Kallenberg CG, Bijl M: **Urine levels of HMGB1 in Systemic Lupus Erythematosus patients with and without renal manifestations.** *Arthritis Res Ther* 2012, **14:**R184.

22. Crispin JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, Kyttaris VC, Juang YT, Tsokos GC: **Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys.** *J Immunol* 2008, **181:**8761-8766.

23. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT: **Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages.** *Nat Immunol* 2005, **6:**1123-1132.

24. Lockhart E, Green AM, Flynn JL: **IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection.** *J Immunol* 2006, **177:**4662-4669.

25. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ: **IL-23 drives a pathogenic T cell population that induces autoimmune inflammation.** *J Exp Med* 2005, **201:**233-240.

26. Zhou L, Ivanov, II, Spolski R, Min R, Shenderov K, Egawa T, Levy DE, Leonard WJ, Littman DR: **IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways.** *Nat Immunol* 2007, **8:**967-974.

27. Toy D, Kugler D, Wolfson M, Vanden Bos T, Gurgel J, Derry J, Tocker J, Peschon J: **Cutting edge: interleukin 17 signals through a heteromeric receptor complex.** *J Immunol* 2006, **177:**36-39.

28. Gaffen SL: **Structure and signaling in the IL-17 receptor family.** *Nat Rev Immunol* 2009, **9:**556-567.

29. Chang SH, Reynolds JM, Pappu BP, Chen G, Martinez GJ, Dong C: **Interleukin-17C promotes Th17 cell responses and autoimmune disease via interleukin-17 receptor E.** *Immunity* 2011, **35:**611-621.

30. Rickel EA, Siegel LA, Yoon BR, Rottman JB, Kugler DG, Swart DA, Anders PM, Tocker JE, Comeau MR, Budelsky AL: **Identification of functional roles for both IL-17RB and IL-17RA in mediating IL-25-induced activities.** *J Immunol* 2008, **181:**4299-4310.

31. Chen XQ, Yu YC, Deng HH, Sun JZ, Dai Z, Wu YW, Yang M: **Plasma IL-17A is increased in new-onset SLE patients and associated with disease activity.** *J Clin Immunol* 2010, **30:**221-225.

32. Yang J, Chu Y, Yang X, Gao D, Zhu L, Yang X, Wan L, Li M: **Th17 and natural Treg cell population dynamics in systemic lupus erythematosus.** *Arthritis Rheum* 2009, **60:**1472-1483.

33. Shah K, Lee WW, Lee SH, Kim SH, Kang SW, Craft J, Kang I: **Dysregulated balance of Th17 and Th1 cells in systemic lupus erythematosus.** *Arthritis Res Ther* 2010, **12:**R53.

34. Hsu HC, Yang P, Wang J, Wu Q, Myers R, Chen J, Yi J, Guentert T, Tousson A, Stanus AL, et al: **Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice.** *Nat Immunol* 2008, **9:**166-175.

35. Espinosa A, Dardalhon V, Brauner S, Ambrosi A, Higgs R, Quintana FJ, Sjostrand M, Eloranta ML, Ni Gabhann J, Winqvist O, et al: **Loss of the lupus autoantigen Ro52/Trim21 induces tissue inflammation and systemic autoimmunity by disregulating the IL-23-Th17 pathway.** *J Exp Med* 2009, **206:**1661-1671.

36. Lartigue A, Courville P, Auquit I, Francois A, Arnoult C, Tron F, Gilbert D, Musette P: **Role of TLR9 in anti-nucleosome and anti-DNA antibody production in lpr mutation-induced murine lupus.** *J Immunol* 2006, **177:**1349-1354.

37. Kyttaris VC, Zhang Z, Kuchroo VK, Oukka M, Tsokos GC: **Cutting edge: IL-23 receptor deficiency prevents the development of lupus nephritis in C57BL/6-lpr/lpr mice.** *J Immunol* 2010, **184:**4605-4609.

38. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, Oliver P, Huang W, Zhang P, Zhang J, et al: **Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense.** *The Journal of experimental medicine* 2001, **194:**519-527.

39. Sanford AN, Dietzmann K, Sullivan KE: **Apoptotic cells, autoantibodies, and the role of HMGB1 in the subcellular localization of an autoantigen.** *Journal of autoimmunity* 2005, **25:**264-271.

40. Kruse K, Janko C, Urbonaviciute V, Mierke CT, Winkler TH, Voll RE, Schett G, Munoz LE, Herrmann M: **Inefficient clearance of dying cells in patients with SLE: anti-dsDNA autoantibodies, MFG-E8, HMGB-1 and other players.** *Apoptosis : an international journal on programmed cell death* 2010, **15:**1098-1113.

41. Scaffidi P, Misteli T, Bianchi ME: **Release of chromatin protein HMGB1 by necrotic cells triggers inflammation.** *Nature* 2002, **418:**191-195.

42. Shivakumar S, Tsokos GC, Datta SK: **T cell receptor alpha/beta expressing double-negative (CD4-/CD8-) and CD4+ T helper cells in humans augment the production of pathogenic anti-DNA autoantibodies associated with lupus nephritis.** *J Immunol* 1989, **143:**103-112.

43. Oliver AM, Martin F, Gartland GL, Carter RH, Kearney JF: **Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses.** *Eur J Immunol* 1997, **27:**2366-2374.

44. Ding Y, Li J, Wu Q, Yang P, Luo B, Xie S, Druey KM, Zajac AJ, Hsu HC, Mountz JD: **IL-17RA Is Essential for Optimal Localization of Follicular Th Cells in the Germinal Center Light Zone To Promote Autoantibody-Producing B Cells.** *J Immunol* 2013.

45. Kaplan MJ, Radic M: **Neutrophil extracellular traps: double-edged swords of innate immunity.** *J Immunol* 2012, **189:**2689-2695.

46. Zizzo G, Cohen PL: **IL-17 stimulates differentiation of human anti-inflammatory macrophages and phagocytosis of apoptotic neutrophils in response to IL-10 and glucocorticoids.** *J Immunol* 2013, **190:**5237-5246.

47. Iwata Y, Bostrom EA, Menke J, Rabacal WA, Morel L, Wada T, Kelley VR: **Aberrant macrophages mediate defective kidney repair that triggers nephritis in lupus-susceptible mice.** *J Immunol* 2012, **188:**4568-4580.

48. Jiang W, Pisetsky DS: **Expression of high mobility group protein 1 in the sera of patients and mice with systemic lupus erythematosus.** *Ann Rheum Dis* 2008, **67:**727-728.

49. Kyttaris VC, Kampagianni O, Tsokos GC: **Treatment with anti-interleukin 23 antibody ameliorates disease in lupus-prone mice.** *Biomed Res Int* 2013, **2013:**861028.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9

General discussion

GENERAL DISCUSSION

RA and SLE affect millions of people worldwide. Although therapeutic strategies have significantly improved over the last two decades, at present there is no cure available. A better understanding of the pathogenesis of these diseases will help improve current therapies. In 2005, a new T helper subset was described that appears to play an important role in the development of auto-immune diseases [1]. These Th17 cells are characterized by the production of IL-17A, IL-17F, TNFα, IL-21 and IL-22. Extensive research in RA has shown that IL-17A can act as a pathogenic agent, activating cells in the joint, thereby contributing to tissue damage and joint destruction [2]. Emerging data now also suggest a pathogenic role for IL-17A in SLE [3]. However, the contribution of other Th17 cytokines to the pathogenesis of RA and SLE is not fully understood.

In this thesis we have further investigated the role of several Th17 related cytokines in the activation and differentiation of lymphocytes in the pathogenesis of RA and SLE. For these studies we have made use of mouse models of these diseases and materials obtained from RA and SLE patients. Our results shed light on the contribution of the studied cytokines in different stages of disease development and the potential of these cytokines as (novel) therapeutic targets.

MAIN FINDINGS OF THIS THESIS

1. Lack of IL-17RA signaling protects mice against CIA and gives rise to a Th2 like phenotype. Blocking IL-17RA signaling even after T cell priming has beneficial therapeutic effects. (**chapter 2**)

2. IL-17A mediated joint pathology in RA patients is IL-22 independent. Th17 cells, not Th22 cells contribute to joint inflammation. (**chapter 3**)

3. IL-22 deficient mice are partially protected against CIA through impaired B cell immunity, characterized by fewer and smaller germinal centers and lower pathogenic autoantibody levels. (**chapter 4**)

4. SLC expression on peripheral B cells protects mice against auto-immunity by inducing clonal deletion and anergy. (**chapter 5**)

5. In mice, IL-27 has no direct effect on B cells, but is essential for B cell immunity in both T cell dependent and T cell independent responses through effects on other cell types. (**chapter 6**)

6. IL-17A expression by CCR6+ memory T cells in SLE patients is correlated with IFN type I expression. In addition, IL-21 expression by these cells is correlated with increased expression of BAFF. (**chapter 7**)

7. Lack of IL-17RA signaling in lupus prone B6.lpr mice leads to an enhanced SLE phenotype characterized by increased lymphocyte proliferation and increased levels of HMGB1 and titers of anti-HMGB1. (**chapter 8**)

IL-17RA signaling in the regulation of lymphocytes

IL-17A has many pro-inflammatory functions in RA pathogenesis [2]. Other IL-17 family members have also been shown to play a role in CIA [4] and have been found in the inflamed synovium of RA patients [5]. The IL17 receptor A (IL-17RA) subunit is the common subunit of the receptors for several IL-17 family members [6-9]. We therefore investigated the effects of lack of IL-17RA signaling, and thereby loss of IL-17 family signaling in CIA. In **chapter 2** we show that IL-17RA-deficient mice are completely protected against CIA development. Intriguingly, we found that splenic T cells in these mice display an enhanced Th2 like phenotype, characterized by increased expression of IL-4. How deregulated IL-17 family signaling can lead to this shift in phenotype is unclear.

IL-17E (also called IL-25) signaling enhances production of Th2 cytokines, eosinophilia and B cell hyperplasia in mice [10, 11], and signaling of IL-17E depends on IL-17RA [7]. IL-17E signals through an IL-17RA-RB receptor complex [7]. The IL-17RB subunit has a TRAF6 binding motif, which is not present on the IL-17RA subunit [12, 13]. In T cell specific TRAF6 deficient mice, IL-4 production by T cells is greatly enhanced upon stimulation [14]. These data suggest a possible T cell-intrinsic negative feedback loop limiting the IL-17E induced response. In IL-17RA deficient mice, signaling by IL-17E through the IL-17RB is abrogated. It is possible that this negative feedback loop limiting IL-4 production upon T cell activation is therefore impaired. This would parallel the observed negative feedback loop present in T cells for IL-17A production [15].

Increased IL-4 production in CIA in IL-17RA KO mice could explain the discrepancies between the total absence of CIA in these mice compared to the 20% incidence of CIA in IL-17A KO mice [16]. Alternatively, signaling of other members of the IL-17 family may also be sufficient to induce CIA in IL-17A KO mice and there is a need for conditional knock-outs of cell specific IL-17 family members / signaling pathways to understand the individual role of these family members in the pathogenesis of autoimmune arthritis. To investigate the potential protective role of IL-4 produced by T cells in CIA pathogenesis, we attempted to block IL-4 in a T cell – fibroblast co-culture system with anti-IL-4 antibodies. In this assay, we cultured T cells from CIA immunized wild-type and IL-17RA KO mice with fibroblasts and determined the pathogenic capacity of the T cells by measuring IL-6 production by the fibroblasts. However, this experiment proved to be technically difficult because the fibroblasts could be activated by the anti-IL-4 antibodies, possibly through Fc receptors. Use of $F(ab)_2$ fragments could be a solution to this problem. To investigate this further, IL-4 blocking antibodies could be administered to IL-17RA KO mice in CIA to determine if blocking IL-4 can restore the phenotype observed in IL-17A KO mice. As administering antibodies to mice over a longer period of time could induce immune responses against these antibodies, IL-17RA KO mice could also be crossed onto IL-4 deficient mice.

A role for IL-17A and IL-17RA signaling has been indicated in B cell activation and germinal center reactions [17-20]. However we show in **chapter 2** that in IL-17RA-deficient mice in CIA, germinal center formation is not affected and mice even produce more non-auto-reactive IgG1

antibodies. Upon influenza infection, we observed no effects of loss of IL-17RA signaling on germinal center formation. Indeed in B6.lpr mice, loss of IL-17RA signaling enhanced plasma cell formation, associated with increased anti-HMGB1 titers (**chapter 8**). These data indicate that although IL-17RA may play a role in B cell immunity, other factors can compensate for the loss of IL-17RA signaling.

We show in **chapter 2** that blocking IL-17RA signaling has beneficial effects on arthritis severity even after priming of T cells. This is in line with data from a similar study in CIA [21] and points to IL-17RA as an interesting therapeutic target in rheumatic disease. Although blocking IL-17RA signaling in psoriasis patients reduces clinical symptoms [22], it had limited effects in RA patients [23]. However, IL-17RA blocking therapy was well tolerated when given short term. These data suggest a different approach to blocking IL-17RA signaling might be beneficial in human disease, for example using antibodies that have a higher binding affinity or a longer half life. In this context, it is unknown whether human T cells also shift to a Th2-like phenotype after IL-17RA inhibition. IL-4 could have immune modulatory effects in RA through inhibition of the Th17 pathway [24], but it may also induce other autoimmune symptoms. Furthermore, the long term effects of blocking signaling of most or all IL-17 family members is not clear, as the role of several family members is unknown. These cytokines are expressed by many different tissues in the body and may therefore have effects on homeostasis [25]. Current data suggests that of the IL-17 family, IL-17A is mostly associated with disease in RA patients. The additive effects of the other family members in RA are not known. This suggests that blocking IL-17A rather than IL-17RA signaling could be a safer therapeutic strategy.

IL-17(R)A and innate immunity

Interferon (IFN) type I plays a role in many systemic auto-immune diseases [26]. Overexpression of IFN type I can be detected in about half of all SLE patients and correlates with disease activity [27]. In addition, a pathogenic role for IL-17A and Th17 cells has been described in mouse models of SLE as well as in SLE patients [20, 28-30]. A recent study in MS patients showed that patients that responded poorly to IFN-β treatment had higher IL-17A levels at onset of treatment, suggesting a possible connection between the IFN type I pathway and the IL-17A/Th17 pathway [31]. In **chapter 7** we show for the first time a co-occurrence of the IFN type I signature and increased IL-17A producing memory CCR6+ Th cells in SLE patients. Data from several studies suggest these pathways may be regulated by similar factors. Furthermore, IFN type I may enhance Th17 differentiation.

We also find a correlation between IL-21 and BAFF expression. These downstream factors of the IL-17A/Th17 pathway and the IFN type I pathway, respectively, are both potent inducers of B cell activation, class switching and antibody production, and have been shown to work in synergy in human plasma cell differentiation [32]. IL-17A and BAFF have also been shown to synergize in

B cell survival and plasma cell formation in SLE [20]. The close connection between the IL-17A/Th17 pathway and the IFN type I pathway suggest that a therapeutic approach inhibiting both of these pathways or downstream factors simultaneously may be beneficial in SLE patients with increased activity of these pathways.

Increased levels of high mobility group box 1 (HMGB1) occur in SLE patients and correlate with disease severity [33-35]. This protein is involved in the folding of DNA and can be released from cells when they go into apoptosis [36]. In addition, it can be excreted by macrophages as a pro-inflammatory factor during tissue stress or damage [37]. High levels of HMGB1 therefore suggest a more pro-inflammatory state of the immune system. In line with this, we find that HMGB1 levels correlate with disease severity in B6.lpr mice (**chapter 8**). In mouse models of several auto-immune diseases, treatment with anti-HMGB1 antibodies ameliorated disease symptoms [38-40]. Treatment with anti-HMGB1 reduced differentiation of Th17 cells by lowering IL-6, TGFβ and IL-23 levels in two studies on heart disease [40, 41]. Also, HMGB1 is involved in pDC maturation and IFN type I production through TLR4 [42, 43]. These data suggest that HMGB1 could be the trigger for both IFN type I production and Th17 differentiation (figure 1), which is supported by our data in **chapter 7** showing that the IFN type I and Th17 pathways are strongly correlated. Culture experiments with human pDC could show whether this is indeed the case in humans. Also, it would be interesting to block HMGB1 in this SLE mouse model to determine the possible effect on IL-17A/Th17 and IFN type I and the potential of this treatment in SLE pathogenesis.

In **chapter 8** we investigate the role of IL-17RA signaling in the pathogenesis of SLE. As increased levels of IL-17A have been shown to correlate with disease activity in SLE patients, we were surprised to find that IL-17RA deficient B6.lpr mice had an enhanced SLE phenotype. This phenotype was characterized by enhanced lymphocyte proliferation, plasma cell formation and auto-antibody production. Interestingly, we also found enhanced HMGB1 levels in the serum of these mice compared to B6.lpr mice. This may be caused by a dysbalance in levels of cytokines inducing clearance of apoptotic debris by macrophages. IL-17A seems to have an important role in the induction of a subset of M2 macrophages that clear early apoptotic bodies [44]. However, in our mice this cytokine cannot signal. IFNγ and IL-4 on the other hand were shown to stimulate M1 macrophage maturation, which are pro-inflammatory [44]. This leads to increased presence of late apoptotic bodies and extracellular presence of nuclear structures like DNA [44]. IFNγ and IL-4 can signal in our mice, which could lead to a shift in macrophage phenotype and the higher levels of HMGB1 that we observe. These data suggest that IL-17A has a protective role in the pathogenesis of SLE in this mouse model, contradicting data from IL-23R deficient B6.lpr mice [45] (figure 1). However, it is likely that IL-17A required for the induction of M2 macrophages is not produced by Th17 cells but other cell types. Therefore, at present the role of IL-23 in this process is not clear. IL-17A produced by different cell types may have a regulatory role in early, innate phases of SLE pathogenesis, but a pro-inflammatory role in the T cell effector phase of

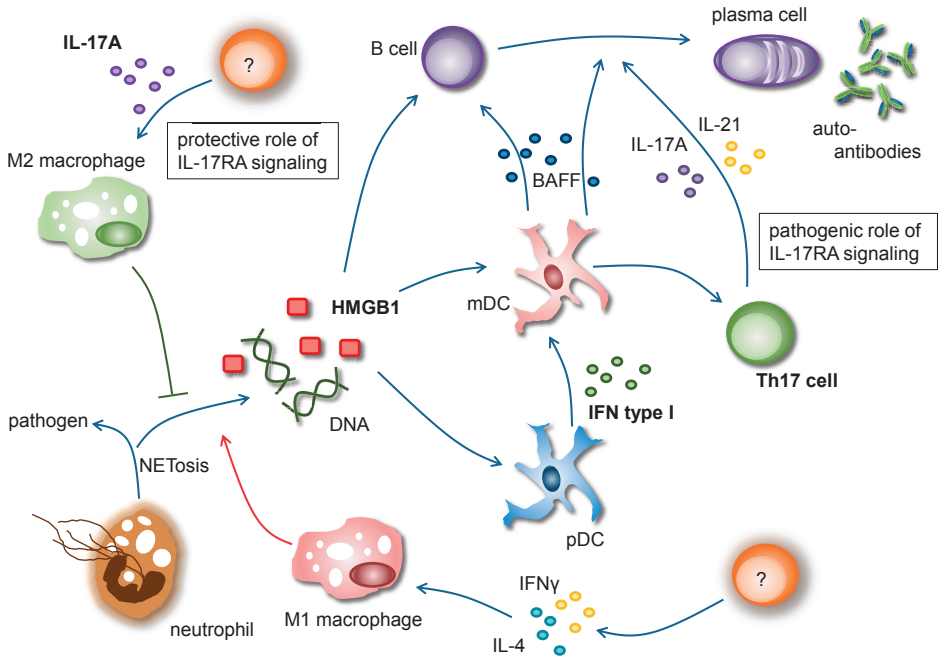

Figure 1. Possible differential roles for IL-17A / IL-17RA signaling in the pathogenesis of auto-immunity. IL-17A produced by an unidentified cell type, possibly innate cells like ILCs or NK cells, may induce a protective M2 phenotype in macrophages. This phenotype enhances clearance of apoptotic / NETosis debris. In contrast, IFNγ and IL-4 induce a pro-inflammatory M1 phenotype in macrophages, which could lead to insufficient clearance of apoptotic / NETosis debris. This process exposes nuclear proteins like DNA and HMGB1 to immune cells. DNA and HMGB1 activate B cells and DCs through TLRs. This induces IFN type I production, Th17 differentiation and auto-antibody production.

the disease. To confirm this hypothesis, characterization of macrophages in these animals will have to be performed. In addition, the presence of late apoptotic or NETosis structures should be confirmed. Furthermore, to exclude that these findings are unique for the B6.lpr SLE mouse model, these experiments could also be performed in other SLE mouse models, like FcγRIIb deficient C57BL/6 mice [46].

Which cell type produces IL-17A or other IL-17 family members in macrophage skewing remains to be investigated. IL-17A produced by T cells was shown to induce M2 protective macrophage differentiation *in vitro*. However, these T cells have been associated with increased disease severity in SLE [47]. It is possible that Th17 or other IL-17A producing T cells have differential roles during SLE development, but it is more likely that another IL-17A producing cell type is involved. Innate lymphoid cells are important in the regulation of homeostasis. A subset of innate lymphoid cells (ILC3) has been shown to produce IL-17A. However, production of IL-17A

by these cells appears to be IL-23 driven, arguing against their role in this process [48], as IL-23R KO mice are protected against the lpr-induced SLE phenotype [45].

Interestingly, we do not see an increase in total ANA in IL-17RA KO.lpr mice compared with B6.lpr mice, suggesting a specific effect of loss of IL-17RA signaling on anti-HMGB1 levels. Indeed, serum HMGB1 levels are increased in IL-17RA KO.lpr mice compared with B6.lpr mice. It is possible that higher HMGB1 levels are not a consequence of enhanced apoptosis or NETosis, but that these levels rise because more cells excrete HMGB1 as a pro-inflammatory molecule. This would suggest a role for IL-17RA signaling in the regulation of macrophage and neutrophil effector function.

Clinical symptoms did not become apparent before 12 weeks of age in IL-17RA deficient B6.lpr mice, but HMGB1 levels were already increased at 8 weeks of age. This shows that increased HMGB1 levels precede clinical symptoms, perhaps making it an interesting biomarker for development of severe disease.

IL-22 in autoimmunity

IL-22 has been suggested to be a pathogenic cytokine in RA and mouse models of RA. Levels of IL-22 in serum of RA patients are correlated with disease severity [49]. In culture experiments, IL-22 was found to stimulate osteoclastogenesis in mice [50] and stimulate proliferation and MCP-1 production by RA synovial fibroblasts [51]. However, the concentrations of IL-22 used in these culture experiments greatly exceed concentrations found in the synovial fluid of RA patients [52]. It is therefore debatable whether these observations have physiological relevance. Although IL-22 producing T cells are increased in peripheral blood of RA patients [53], IL-23, the cytokine that drives induction of IL-22 expression by Th17 cells [54], is hardly present in a bioactive form in RA synovium [55]. Furthermore, IL-23 levels in serum of active RA patients are significantly higher than in synovial fluid [56]. In line with these data, Ikeuchi et al. have shown that IL-22 in RA synovium is mostly produced by RA synovial fibroblasts, and not by T cells [51]. To identify a possible role of IL-22 in joint inflammation in a more physiological setting, fibroblasts or osteoclasts could be cultured with syovial fluid from RA patients and healthy controls in the presence or absence of IL-22 blocking antibodies.

Indeed, we show in **chapter 3** that IL-22 produced by T cells has limited effects on pro-inflammatory cytokine and chemokine production by RA synovial fibroblasts. In fact, IL-17A appears to be the more pathogenic cytokine. We show in **chapter 4** that IL-22 deficient CCR6+ T cells are equally capable of inducing IL-6 production by synovial fibroblasts from mice. Together, these data show that the effect of IL-22 produced by T cells on synovial fibroblasts is limited, and that loss of IL-22 has no effect on pathogenic capacity of the T cells. It is possible that IL-22 produced by the synovial fibroblasts is effective in an autocrine manner to enhance the activation of the fibroblasts by other cells. However, we show in **chapter 3** that IL-22 deficient mice develop experimental arthritis in a T cell mediated model comparable to wild type mice,

suggesting that the effect of IL-22 on fibroblasts in arthritis is redundant. Culture experiments with IL-22 deficient fibroblasts could shed light on this issue.

A possible alternative explanation for the expression of IL-22 by synovial fibroblasts in RA patients but not in healthy controls is the role of IL-22 in tissue repair. IL-22 is involved in tissue repair and cell survival in murine hepatitis [57, 58], and enhances wound healing in an *in vitro* model [59-61]. It is possible that fibroblasts from RA patients upregulate IL-22 production because the tissue is damaged, explaining the absence of IL-22 expression in healthy controls.

We find that both AIA and DTH responses in IL-22 deficient mice are comparable to responses in wild type mice. However, we and others show that IL-22 is crucial for CIA development in mice ([50] and **chapter 4** of this thesis). This can be attributed to an impaired humoral response in these mice. Although Geboes et al reported an increase in collagen type II specific IgG2c antibodies, they did not report whether these were specific for chicken or mouse collagen. We find that anti-mouse collagen type II (CII) IgG2c serum levels are decreased in these mice, which is due to impaired germinal center maintenance. As the IL-22R is not expressed on immune cells [62, 63], it is likely that stromal cells involved in the structural organization of germinal centers are affected by IL-22 during immune responses. We found that IL-22R is expressed on stromal cells in lymph nodes, and that expression of the IL-22R by stromal cells is up-regulated by TNFα stimulation *in vitro* (Reijmers et al., unpublished data). In addition, IL-22 stimulated CXCL13 expression by lymph node stromal cells (Reijmers et al., unpublished data), suggesting a role for IL-22 in regulating B cell migration and structural organization of B cell follicles (figure 2).

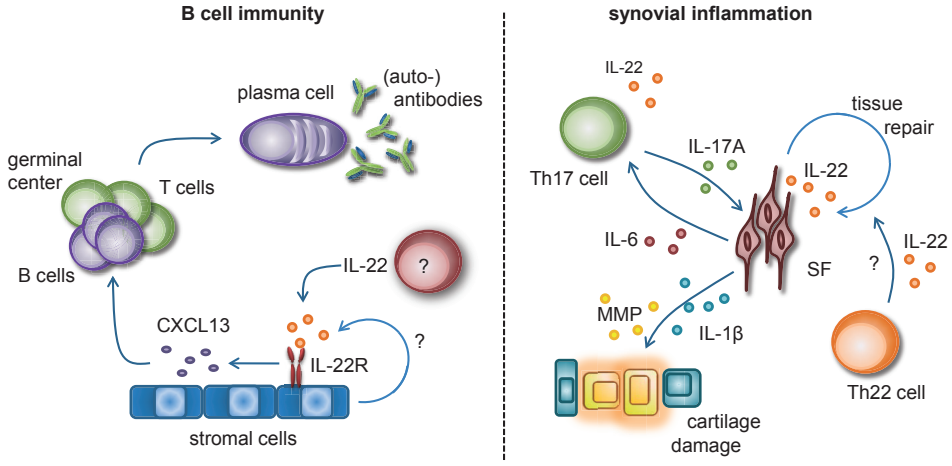

Figure 2. The potential role of IL-22 in RA. LEFT: IL-22 facilitates B cell immunity. IL-22 produced by an unidentified cell type induces CXCL13 expression by stromal cells, possibly FDC. CXCL13 expression is involved in structural organization of B cell follicles and germinal centers. RIGHT: The role of IL-22 in synovial inflammation is limited. Th17 mediated inflammation is IL-17A dependent. IL-22 upregulation by synovial fibroblasts (SF) could enhance tissue repair. It is unclear whether IL-22 produced by Th22 (or Th17 cells) could also facilitate this process.

Initial germinal center formation in IL-22KO mice in CIA is normal, however, already at day 21 the number of germinal centers is dramatically decreased. These data suggest that loss of IL-22 expression leads to a germinal center maintenance defect. This observation could explain the discrepancy between the higher anti-chicken CII IgG levels (immune) found by Geboes et al. [50] and the lower anti-mouse CII IgG levels (auto-immune) that we observed in our experiments. If germinal center function is normal during the short time that germinal centers are present (until 10-15 days after immunization), the immune response against the foreign antigen (chicken CII) will have resulted in chicken CII specific antibody production. However, loss of tolerance for mouse CII may require more time. Constructing a time line of immune and auto-immune antibody levels in serum during CIA in IL-22KO and wild-type mice will aid our understanding of loss of self-tolerance in CIA.

The question remains which cell type is responsible for IL-22 production in structural organization of B cell follicles and germinal centers. To further investigate this, bone marrow chimeras could be made to establish whether the cell type is of stromal or hematopoietic origin.

Taken together, these data suggest that IL-22 produced by T cells has limited effects in the pathogenesis of RA. Instead IL-17A producing T cells appear to be pathogenic, regardless of whether they produce IL-22. However, IL-22 is involved in B cell immunity, although it does not directly affect B cells. It is therefore questionable whether treatment approaches to block IL-22 in RA patients will ameliorate RA symptoms. IL-22 does not seem to be pathogenic in the joints of RA patients, and the more generalized indirect effect of IL-22 on B cell immunity makes it a less suitable therapeutic target.

B cells as therapeutic targets in RA

The importance of B cells in the pathogenesis of RA is supported by the efficacy of B cell depleting therapy [64, 65]. Surprisingly, treatment of patients with anti-CD20 therapy has few side effects, even though peripheral B cells become virtually undetectable [66]. Unfortunately, after completion of the therapy, many patients develop relapses associated with the return of peripheral B cell numbers to normal levels [67]. Although this treatment does not lead to increased infection rates and appears mainly to affect auto-reactive memory B cells [67, 68], long term follow up or a study into the effects of repeated treatment have not yet been performed. Refining this strategy by specifically targeting pathogenic B cell subsets could limit possible side effects to allow long term use of medication.

Over 10 years ago a unique subset of peripheral B cells was described that expresses the surrogate light chain (SLC) [69]. These cells appear to have an autoreactive antibody repertoire and have been shown to accumulate in the joints of RA patients [69-71]. These data suggested that this subset of B cells may be an interesting new therapeutic target in RA. However, the contribution of these cells to RA pathogenesis was still unclear. We show in **chapter 5** that in fact

SLC expression in B cells partially protects mice from autoimmunity by inducing clonal deletion of B cells and anergy. These data suggest that SLC expressing B cells in RA patients do not contribute to the pathogenesis of RA and are therefore not a useful therapeutic target.

Dual roles of IL-27 in autoimmunity

In RA patients, serum levels of IL-27 are increased compared to healthy controls, correlating with disease severity [72, 73]. IL-27 activates synovial fibroblasts of RA patients and induces the production of pro-inflammatory cytokines by these cells [73] (figure 3). These data suggest that inhibition of IL-27 could be an interesting therapeutic strategy in RA. However, administration of IL-27 in CIA ameliorates disease [74]. Also, IL-27 was shown to limit Th17 differentiation [75, 76], although it does not affect already differentiated Th17 cells [77]. Furthermore, IL-27 limits RANKL expression by T cells [78] and directly inhibits osteoclastogenesis [79]. In contrast to RA, serum levels of IL-27 in SLE patients are significantly lower than in healthy controls, and inversely correlate with disease severity [80], suggesting administration of IL-27 could be beneficial.

IL-27 was initially described as a T cell stimulatory cytokine. It enhances susceptibility of naïve Th cells for Th1 differentiation and directly limits Th17 differentiation [81-84]. Furthermore, IL-27 can induce effector function of Treg cells [85], but also controls Treg development [86]. Several studies suggest that IL-27 also has an effect directly on human B cells, mostly activating naïve B cells [87-89]. In **chapter 6** we further studied the direct effects of IL-27R signaling on B cells, and the effect of loss of IL-27R signaling on B cell immunity *in vivo* in mice. We show that IL-27R signaling is essential for T cell dependent as well as T cell independent B cell immunity. It most prominently affects antibody production by B cells, enhancing the production of IgG antibodies, although these effects appear to be B cell independent but elicited through other cell types involved in B cell immunity. The effect of IL-27 on germinal center formation has been previously studied. Loss of germinal centers in IL-27R deficient mice can be attributed to a defect in Tfh cell effector function [90]. It was recently reported that IL-27 also plays a role in human Tfh cell differentiation [91]. We show in **chapter 6** that IL-27 is also essential for T cell independent responses, even though a direct effect on B cells was not found in mice. It is unclear which IL-27 responsive cells mediate this T cell independent response. Bone marrow chimeras can shed light on the stromal or hematopoietic origin of these cells. Furthermore, although we did not find an effect of loss of IL-27R signaling on B cells *in vitro*, it is still possible that B cell migration is affected in IL-27R deficient mice. B cell specific IL-27RKO mice could be used to investigate this option.

Auto-antibody production is the hallmark of many auto-immune diseases, including SLE. Administration of IL-27 could therefore enhance rather than ameliorate disease pathology (see figure 4 for role of IL-27 in SLE). However, in MRL/lpr mice, overexpression of the IL-27R protected mice against SLE pathology in a dose dependent fashion [92]. In fact, T cell specific overexpression of the IL-27R was sufficient to significantly reduce cutaneous lesions in MRL/lpr

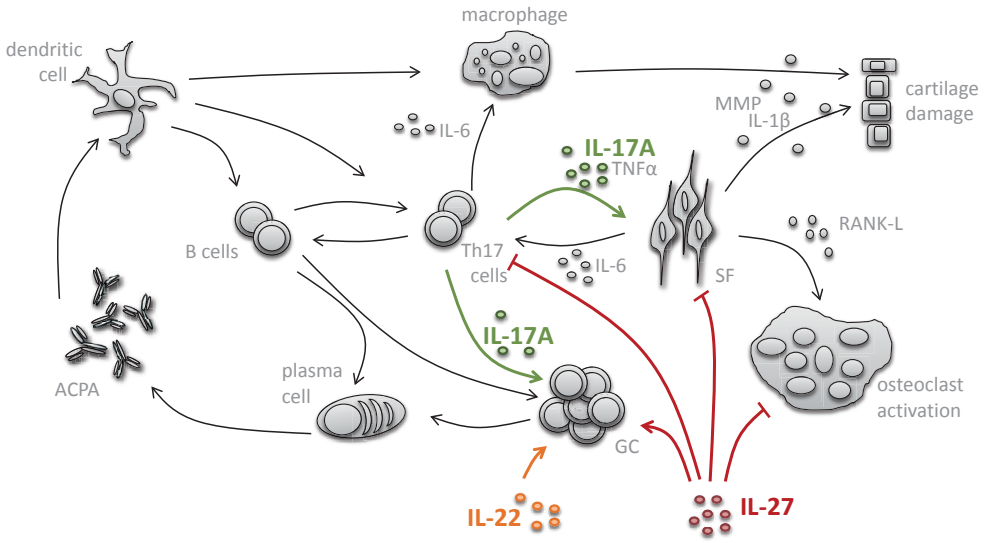

Figure 3. The (potential) role of Th17 related cytokines in RA pathogenesis. The effects of the cytokines studied in this thesis are indicated in colour. SF synovial fibroblast, GC germinal center.

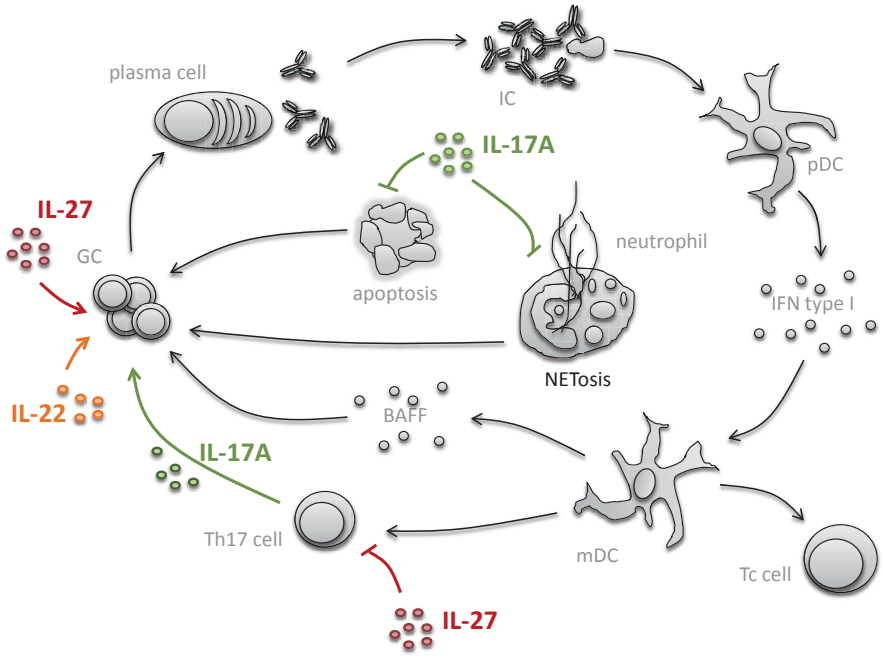

Figure 4. The (potential) role of Th17 related cytokines in SLE pathogenesis. The effects of the cytokines studied in this thesis are indicated in colour. pDC plasmacytoid DC, mDC myeloid derived DC, GC germinal center, Tc cytotoxic T cell.

mice [93]. These data suggest that, at least in mice, the effects of IL-27 and IL-27R signaling on T cells are dominant in SLE. In RA patients, it is possible that local effects of IL-27 in synovium are more pathogenic than protective. This balance of whether IL-27 has a more pro-inflammatory or inhibitory role may be tissue specific and different in SLE and RA (figure 3 and 4). Together, these observations show the extent of the influence of IL-27 on immune responses and suggest that these highly variable roles of IL-27 make it a difficult target for disease treatment.

Concluding remarks

In this thesis we have explored the role of several Th17 related cytokines and cytokine receptors in the pathogenesis of RA and SLE. By using different models of disease we have found that these cytokines and cytokine receptors have differential roles in different stages of disease. These differential roles may be tissue specific and depend on the cytokine producing cell and receiving cell, explaining why the same cytokine may be pathogenic in one auto-immune disease but protective in another. For example, we have shown that lack of IL-17RA signaling in CIA in mice fully protects these mice against disease development, whereas in SLE prone mice, loss of this receptor enhances disease. It is therefore important to characterize the cell types responsible for pathogenic cytokine production. Chemokine and chemokine receptor expression profiles of lymphocytes at different stages of the disease will help to identify specific pathogenic subpopulations of cells, and may be useful in a clinical setting. Inhibiting a specific subset of pathogenic cells rather than a cytokine may limit side effects of medication.

Many new cytokine producing immune cell types have recently been described. ILCs are of particular interest, as they express similar cytokine profiles as Th cells, but do not express antigen specific receptors and appear to be involved in tissue homeostasis [94]. However, it is possible that cytokine production by these cells, e.g. IL-17A and IL-22 production, can induce pathogenic responses in the context of inflammation or autoimmunity.

Research on the auto-reactive BCR repertoire and B cell characteristics in RA and SLE may identify specific pathogenic B cell subsets and could help identify the antigens involved in autoimmune diseases. For example, it was recently reported that B cells with BCRs specific for citrullinated proteins are preferentially present in the joints of RA patients, suggesting local selection of auto-reactive BCRs [95]. Similar studies could be performed to assess whether specific auto-reactive TCR repertoires are preferentially involved in local auto-immunity. These data will enhance our understanding of the pathogenesis of autoimmune diseases and may further improve therapeutic strategies.

REFERENCES

1. Hemdan NY, Birkenmeier G, Wichmann G, Abu El-Saad AM, Krieger T, Conrad K, Sack U: **Interleukin-17-producing T helper cells in autoimmunity**. *Autoimmun Rev* 2010, **9**(11):785-792.
2. McInnes IB, Schett G: **The pathogenesis of rheumatoid arthritis**. *N Engl J Med* 2011, **365**(23):2205-2219.
3. Shin MS, Lee N, Kang I: **Effector T-cell subsets in systemic lupus erythematosus: update focusing on Th17 cells**. *Curr Opin Rheumatol* 2011, **23**(5):444-448.
4. Yamaguchi Y, Fujio K, Shoda H, Okamoto A, Tsuno NH, Takahashi K, Yamamoto K: **IL-17B and IL-17C are associated with TNF-alpha production and contribute to the exacerbation of inflammatory arthritis**. *J Immunol* 2007, **179**(10):7128-7136.
5. Hwang SY, Kim HY: **Expression of IL-17 homologs and their receptors in the synovial cells of rheumatoid arthritis patients**. *Mol Cells* 2005, **19**(2):180-184.
6. Chang SH, Reynolds JM, Pappu BP, Chen G, Martinez GJ, Dong C: **Interleukin-17C promotes Th17 cell responses and autoimmune disease via interleukin-17 receptor E**. *Immunity* 2011, **35**(4):611-621.
7. Rickel EA, Siegel LA, Yoon BR, Rottman JB, Kugler DG, Swart DA, Anders PM, Tocker JE, Comeau MR, Budelsky AL: **Identification of functional roles for both IL-17RB and IL-17RA in mediating IL-25-induced activities**. *J Immunol* 2008, **181**(6):4299-4310.
8. Kuestner RE, Taft DW, Haran A, Brandt CS, Brender T, Lum K, Harder B, Okada S, Ostrander CD, Kreindler JL *et al*: **Identification of the IL-17 receptor related molecule IL-17RC as the receptor for IL-17F**. *J Immunol* 2007, **179**(8):5462-5473.
9. Toy D, Kugler D, Wolfson M, Vanden Bos T, Gurgel J, Derry J, Tocker J, Peschon J: **Cutting edge: interleukin 17 signals through a heteromeric receptor complex**. *J Immunol* 2006, **177**(1):36-39.
10. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, Menon S, Clifford T, Hunte B, Lesley R *et al*: **IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo**. *Immunity* 2001, **15**(6):985-995.
11. Kim MR, Manoukian R, Yeh R, Silbiger SM, Danilenko DM, Scully S, Sun J, DeRose ML, Stolina M, Chang D *et al*: **Transgenic overexpression of human IL-17E results in eosinophilia, B-lymphocyte hyperplasia, and altered antibody production**. *Blood* 2002, **100**(7):2330-2340.
12. Maezawa Y, Nakajima H, Suzuki K, Tamachi T, Ikeda K, Inoue J, Saito Y, Iwamoto I: **Involvement of TNF receptor-associated factor 6 in IL-25 receptor signaling**. *J Immunol* 2006, **176**(2):1013-1018.
13. Schwandner R, Yamaguchi K, Cao Z: **Requirement of tumor necrosis factor receptor-associated factor (TRAF)6 in interleukin 17 signal transduction**. *J Exp Med* 2000, **191**(7):1233-1240.
14. King CG, Kobayashi T, Cejas PJ, Kim T, Yoon K, Kim GK, Chiffoleau E, Hickman SP, Walsh PT, Turka LA *et al*: **TRAF6 is a T cell-intrinsic negative regulator required for the maintenance of immune homeostasis**. *Nat Med* 2006, **12**(9):1088-1092.
15. Smith E, Stark MA, Zarbock A, Burcin TL, Bruce AC, Vaswani D, Foley P, Ley K: **IL-17A inhibits the expansion of IL-17A-producing T cells in mice through "short-loop" inhibition via IL-17 receptor**. *J Immunol* 2008, **181**(2):1357-1364.
16. Nakae S, Nambu A, Sudo K, Iwakura Y: **Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice**. *J Immunol* 2003, **171**(11):6173-6177.
17. Hsu HC, Yang P, Wang J, Wu Q, Myers R, Chen J, Yi J, Guentert T, Tousson A, Stanus AL *et al*: **Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice**. *Nat Immunol* 2008, **9**(2):166-175.
18. Mitsdoerffer M, Lee Y, Jager A, Kim HJ, Korn T, Kolls JK, Cantor H, Bettelli E, Kuchroo VK: **Proinflammatory T helper type 17 cells are effective B-cell helpers**. *Proc Natl Acad Sci U S A* 2010, **107**(32):14292-14297.
19. Pisitkun P, Ha HL, Wang H, Claudio E, Tivy CC, Zhou H, Mayadas TN, Illei GG, Siebenlist U: **Interleukin-17 cytokines are critical in development of fatal lupus glomerulonephritis**. *Immunity* 2012, **37**(6):1104-1115.
20. Doreau A, Belot A, Bastid J, Riche B, Trescol-Biemont MC, Ranchin B, Fabien N, Cochat P, Pouteil-Noble C, Trolliet P *et al*: **Interleukin 17 acts in synergy with B cell-activating factor to influence B cell biology and the**

pathophysiology of systemic lupus erythematosus. *Nat Immunol* 2009, **10**(7):778-785.

21. Lubberts E, Joosten LA, Oppers B, van den Bersselaar L, Coenen-de Roo CJ, Kolls JK, Schwarzenberger P, van de Loo FA, van den Berg WB: **IL-1-independent role of IL-17 in synovial inflammation and joint destruction during collagen-induced arthritis**. *J Immunol* 2001, **167**(2):1004-1013.
22. Papp KA, Leonardi C, Menter A, Ortonne JP, Krueger JG, Kricorian G, Aras G, Li J, Russell CB, Thompson EH *et al*: **Brodalumab, an anti-interleukin-17-receptor antibody for psoriasis**. *N Engl J Med* 2012, **366**(13):1181-1189.
23. **Abstracts of the American College of Rheumatology & Association of Rheumatology Health Professionals, Annual Scientific Meeting. November 9-14, 2012. Washington, D.C., USA**. *Arthritis Rheum* 2012, **64**(10 Suppl):S1-1216.
24. van Hamburg JP, Mus AM, de Bruijn MJ, de Vogel L, Boon L, Cornelissen F, Asmawidjaja P, Hendriks RW, Lubberts E: **GATA-3 protects against severe joint inflammation and bone erosion and reduces differentiation of Th17 cells during experimental arthritis**. *Arthritis and rheumatism* 2009, **60**(3):750-759.
25. Gaffen SL: **Structure and signalling in the IL-17 receptor family**. *Nat Rev Immunol* 2009, **9**(8):556-567.
26. Higgs BW, Liu Z, White B, Zhu W, White WI, Morehouse C, Brohawn P, Kiener PA, Richman L, Fiorentino D *et al*: **Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway**. *Ann Rheum Dis* 2011, **70**(11):2029-2036.
27. Kirou KA, Lee C, George S, Louca K, Peterson MG, Crow MK: **Activation of the interferon-alpha pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease**. *Arthritis Rheum* 2005, **52**(5):1491-1503.
28. Crispin JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, Kyttaris VC, Juang YT, Tsokos GC: **Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys**. *J Immunol* 2008, **181**(12):8761-8766.
29. Chen XQ, Yu YC, Deng HH, Sun JZ, Dai Z, Wu YW, Yang M: **Plasma IL-17A is increased in new-onset SLE patients and associated with disease activity**. *J Clin Immunol* 2010, **30**(2):221-225.
30. Espinosa A, Dardalhon V, Brauner S, Ambrosi A, Higgs R, Quintana FJ, Sjostrand M, Eloranta ML, Ni Gabhann J, Winqvist O *et al*: **Loss of the lupus autoantigen Ro52/Trim21 induces tissue inflammation and systemic autoimmunity by disregulating the IL-23-Th17 pathway**. *J Exp Med* 2009, **206**(8):1661-1671.
31. Axtell RC, de Jong BA, Boniface K, van der Voort LF, Bhat R, De Sarno P, Naves R, Han M, Zhong F, Castellanos JG *et al*: **T helper type 1 and 17 cells determine efficacy of interferon-beta in multiple sclerosis and experimental encephalomyelitis**. *Nature medicine* 2010, **16**(4):406-412.
32. Ettinger R, Sims GP, Robbins R, Withers D, Fischer RT, Grammer AC, Kuchen S, Lipsky PE: **IL-21 and BAFF/BLyS synergize in stimulating plasma cell differentiation from a unique population of human splenic memory B cells**. *J Immunol* 2007, **178**(5):2872-2882.
33. Abdulahad DA, Westra J, Bijzet J, Dolff S, van Dijk MC, Limburg PC, Kallenberg CG, Bijl M: **Urine levels of HMGB1 in Systemic Lupus Erythematosus patients with and without renal manifestations**. *Arthritis Res Ther* 2012, **14**(4):R184.
34. Abdulahad DA, Westra J, Bijzet J, Limburg PC, Kallenberg CG, Bijl M: **High mobility group box 1 (HMGB1) and anti-HMGB1 antibodies and their relation to disease characteristics in systemic lupus erythematosus**. *Arthritis Res Ther* 2011, **13**(3):R71.
35. Ma CY, Jiao YL, Zhang J, Yang QR, Zhang ZF, Shen YJ, Chen ZJ, Zhao YR: **Elevated plasma level of HMGB1 is associated with disease activity and combined alterations with IFN-alpha and TNF-alpha in systemic lupus erythematosus**. *Rheumatol Int* 2012, **32**(2):395-402.
36. Bonaldi T, Langst G, Strohner R, Becker PB, Bianchi ME: **The DNA chaperone HMGB1 facilitates ACF/CHRAC-dependent nucleosome sliding**. *EMBO J* 2002, **21**(24):6865-6873.
37. Yang D, Chen Q, Yang H, Tracey KJ, Bustin M, Oppenheim JJ: **High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin**. *J Leukoc Biol* 2007, **81**(1):59-66.
38. Kokkola R, Li J, Sundberg E, Aveberger AC, Palmblad K, Yang H, Tracey KJ, Andersson U, Harris HE: **Successful treatment of collagen-induced arthritis in mice and rats by targeting extracellular high mobility group box**

chromosomal protein 1 activity. *Arthritis Rheum* 2003, **48**(7):2052-2058.

39. Uzawa A, Mori M, Taniguchi J, Masuda S, Muto M, Kuwabara S: **Anti-high mobility group box 1 monoclonal antibody ameliorates experimental autoimmune encephalomyelitis**. *Clin Exp Immunol* 2013, **172**(1):37-43.
40. Su Z, Sun C, Zhou C, Liu Y, Zhu H, Sandoghchian S, Zheng D, Peng T, Zhang Y, Jiao Z *et al*: **HMGB1 blockade attenuates experimental autoimmune myocarditis and suppresses Th17-cell expansion**. *Eur J Immunol* 2011, **41**(12):3586-3595.
41. Zhu H, Li J, Wang S, Liu K, Wang L, Huang L: **Hmgb1-TLR4-IL-23-IL-17A Axis Promote Ischemia-Reperfusion Injury in a Cardiac Transplantation Model**. *Transplantation* 2013, **95**(12):1448-1454.
42. Dumitriu IE, Baruah P, Bianchi ME, Manfredi AA, Rovere-Querini P: **Requirement of HMGB1 and RAGE for the maturation of human plasmacytoid dendritic cells**. *Eur J Immunol* 2005, **35**(7):2184-2190.
43. Yanai H, Ban T, Wang Z, Choi MK, Kawamura T, Negishi H, Nakasato M, Lu Y, Hangai S, Koshiba R *et al*: **HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses**. *Nature* 2009, **462**(7269):99-103.
44. Zizzo G, Cohen PL: **IL-17 stimulates differentiation of human anti-inflammatory macrophages and phagocytosis of apoptotic neutrophils in response to IL-10 and glucocorticoids**. *J Immunol* 2013, **190**(10):5237-5246.
45. Kyttaris VC, Zhang Z, Kuchroo VK, Oukka M, Tsokos GC: **Cutting edge: IL-23 receptor deficiency prevents the development of lupus nephritis in C57BL/6-lpr/lpr mice**. *J Immunol* 2010, **184**(9):4605-4609.
46. Bolland S, Ravetch JV: **Spontaneous autoimmune disease in Fc(gamma)RIIB-deficient mice results from strain-specific epistasis**. *Immunity* 2000, **13**(2):277-285.
47. Shah K, Lee WW, Lee SH, Kim SH, Kang SW, Craft J, Kang I: **Dysregulated balance of Th17 and Th1 cells in systemic lupus erythematosus**. *Arthritis research & therapy* 2010, **12**(2):R53.
48. Takatori H, Kanno Y, Watford WT, Tato CM, Weiss G, Ivanov, II, Littman DR, O'Shea JJ: **Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22**. *The Journal of experimental medicine* 2009, **206**(1):35-41.
49. Leipe J, Schramm MA, Grunke M, Baeuerle M, Dechant C, Nigg AP, Witt MN, Vielhauer V, Reindl CS, Schulze-Koops H *et al*: **Interleukin 22 serum levels are associated with radiographic progression in rheumatoid arthritis**. *Ann Rheum Dis* 2011, **70**(8):1453-1457.
50. Geboes L, Dumoutier L, Kelchtermans H, Schurgers E, Mitera T, Renauld JC, Matthys P: **Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice**. *Arthritis and rheumatism* 2009, **60**(2):390-395.
51. Ikeuchi H, Kuroiwa T, Hiramatsu N, Kaneko Y, Hiromura K, Ueki K, Nojima Y: **Expression of interleukin-22 in rheumatoid arthritis: potential role as a proinflammatory cytokine**. *Arthritis Rheum* 2005, **52**(4):1037-1046.
52. Mitra A, Raychaudhuri SK, Raychaudhuri SP: **Functional role of IL-22 in psoriatic arthritis**. *Arthritis Res Ther* 2012, **14**(2):R65.
53. Zhang L, Li YG, Li YH, Qi L, Liu XG, Yuan CZ, Hu NW, Ma DX, Li ZF, Yang Q *et al*: **Increased frequencies of Th22 cells as well as Th17 cells in the peripheral blood of patients with ankylosing spondylitis and rheumatoid arthritis**. *PLoS ONE [Electronic Resource]* 2012, **7**(4):e31000.
54. Zheng Y, Danilenko DM, Valdez P, Kasman I, Eastham-Anderson J, Wu J, Ouyang W: **Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis**. *Nature* 2007, **445**(7128):648-651.
55. Brentano F, Ospelt C, Stanczyk J, Gay RE, Gay S, Kyburz D: **Abundant expression of the interleukin (IL)23 subunit p19, but low levels of bioactive IL23 in the rheumatoid synovium: differential expression and Toll-like receptor-(TLR) dependent regulation of the IL23 subunits, p19 and p40, in rheumatoid arthritis**. *Ann Rheum Dis* 2009, **68**(1):143-150.
56. Mrabet D, Laadhar L, Sahli H, Zouari B, Haouet S, Makni S, Sellami S: **Synovial fluid and serum levels of IL-17, IL-23, and CCL-20 in rheumatoid arthritis and psoriatic arthritis: a Tunisian cross-sectional study**. *Rheumatol Int* 2013, **33**(1):265-266.
57. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Karow M, Flavell RA: **Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation**. *Immunity* 2007, **27**(4):647-659.

58. Radaeva S, Sun R, Pan HN, Hong F, Gao B: **Interleukin 22 (IL-22) plays a protective role in T cell-mediated murine hepatitis: IL-22 is a survival factor for hepatocytes via STAT3 activation**. *Hepatology* 2004, **39**(5):1332-1342.

59. Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, Cianfarani F, Odorisio T, Traidl-Hoffmann C, Behrendt H *et al*: **Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling**. *J Clin Invest* 2009, **119**(12):3573-3585.

60. Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, Reinhart TA, McAllister F, Edeal J, Gaus K *et al*: **IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia**. *Nat Med* 2008, **14**(3):275-281.

61. Sonnenberg GF, Nair MG, Kirn TJ, Zaph C, Fouser LA, Artis D: **Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A**. *J Exp Med* 2010, **207**(6):1293-1305.

62. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R: **IL-22 increases the innate immunity of tissues**. *Immunity* 2004, **21**(2):241-254.

63. Lecart S, Morel F, Noraz N, Pene J, Garcia M, Boniface K, Lecron JC, Yssel H: **IL-22, in contrast to IL-10, does not induce Ig production, due to absence of a functional IL-22 receptor on activated human B cells**. *Int Immunol* 2002, **14**(11):1351-1356.

64. Edwards JC, Cambridge G: **Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes**. *Rheumatology (Oxford)* 2001, **40**(2):205-211.

65. De Vita S, Zaja F, Sacco S, De Candia A, Fanin R, Ferraccioli G: **Efficacy of selective B cell blockade in the treatment of rheumatoid arthritis: evidence for a pathogenetic role of B cells**. *Arthritis Rheum* 2002, **46**(8):2029-2033.

66. Hainsworth JD: **Safety of rituximab in the treatment of B cell malignancies: implications for rheumatoid arthritis**. *Arthritis Res Ther* 2003, **5 Suppl 4**:S12-16.

67. Cambridge G, Stohl W, Leandro MJ, Migone TS, Hilbert DM, Edwards JC: **Circulating levels of B lymphocyte stimulator in patients with rheumatoid arthritis following rituximab treatment: relationships with B cell depletion, circulating antibodies, and clinical relapse**. *Arthritis Rheum* 2006, **54**(3):723-732.

68. Teng YK, Wheater G, Hogan VE, Stocks P, Levarht EW, Huizinga TW, Toes RE, van Laar JM: **Induction of long-term B-cell depletion in refractory rheumatoid arthritis patients preferentially affects autoreactive more than protective humoral immunity**. *Arthritis Res Ther* 2012, **14**(2):R57.

69. Meffre E, Davis E, Schiff C, Cunningham-Rundles C, Ivashkiv LB, Staudt LM, Young JW, Nussenzweig MC: **Circulating human B cells that express surrogate light chains and edited receptors**. *Nat Immunol* 2000, **1**(3):207-213.

70. Meffre E, Schaefer A, Wardemann H, Wilson P, Davis E, Nussenzweig MC: **Surrogate light chain expressing human peripheral B cells produce self-reactive antibodies**. *J Exp Med* 2004, **199**(1):145-150.

71. Meffre E, Chiorazzi M, Nussenzweig MC: **Circulating human B cells that express surrogate light chains display a unique antibody repertoire**. *J Immunol* 2001, **167**(4):2151-2156.

72. Shen H, Xia L, Xiao W, Lu J: **Increased levels of interleukin-27 in patients with rheumatoid arthritis**. *Arthritis Rheum* 2011, **63**(3):860-861.

73. Wong CK, Chen da P, Tam LS, Li EK, Yin YB, Lam CW: **Effects of inflammatory cytokine IL-27 on the activation of fibroblast-like synoviocytes in rheumatoid arthritis**. *Arthritis Res Ther* 2010, **12**(4):R129.

74. Niedbala W, Cai B, Wei X, Patakas A, Leung BP, McInnes IB, Liew FY: **Interleukin 27 attenuates collagen-induced arthritis**. *Ann Rheum Dis* 2008, **67**(10):1474-1479.

75. Batten M, Li J, Yi S, Kljavin NM, Danilenko DM, Lucas S, Lee J, de Sauvage FJ, Ghilardi N: **Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells**. *Nat Immunol* 2006, **7**(9):929-936.

76. Stumhofer JS, Laurence A, Wilson EH, Huang E, Tato CM, Johnson LM, Villarino AV, Huang Q, Yoshimura A, Sehy D *et al*: **Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system**. *Nat Immunol* 2006, **7**(9):937-945.

77. El-behi M, Ciric B, Yu S, Zhang GX, Fitzgerald DC, Rostami A: **Differential effect of IL-27 on developing versus committed Th17 cells**. *J Immunol* 2009, **183**(8):4957-4967.

78. Kamiya S, Okumura M, Chiba Y, Fukawa T, Nakamura C, Nimura N, Mizuguchi J, Wada S, Yoshimoto T: **IL-27 suppresses RANKL expression in CD4+ T cells in part through STAT3**. *Immunol Lett* 2011, **138**(1):47-53.

79. Furukawa M, Takaishi H, Takito J, Yoda M, Sakai S, Hikata T, Hakozaki A, Uchikawa S, Matsumoto M, Chiba K *et al*: **IL-27 abrogates receptor activator of NF-kappa B ligand-mediated osteoclastogenesis of human granulocyte-macrophage colony-forming unit cells through STAT1-dependent inhibition of c-Fos**. *J Immunol* 2009, **183**(4):2397-2406.

80. Li TT, Zhang T, Chen GM, Zhu QQ, Tao JH, Pan HF, Ye DQ: **Low level of serum interleukin 27 in patients with systemic lupus erythematosus**. *J Investig Med* 2010, **58**(5):737-739.

81. Chen Q, Ghilardi N, Wang H, Baker T, Xie MH, Gurney A, Grewal IS, de Sauvage FJ: **Development of Th1-type immune responses requires the type I cytokine receptor TCCR**. *Nature* 2000, **407**(6806):916-920.

82. Yoshida H, Hamano S, Senaldi G, Covey T, Faggioni R, Mu S, Xia M, Wakeham AC, Nishina H, Potter J *et al*: **WSX-1 is required for the initiation of Th1 responses and resistance to L. major infection**. *Immunity* 2001, **15**(4):569-578.

83. Diveu C, McGeachy MJ, Boniface K, Stumhofer JS, Sathe M, Joyce-Shaikh B, Chen Y, Tato CM, McClanahan TK, de Waal Malefyt R *et al*: **IL-27 blocks RORc expression to inhibit lineage commitment of Th17 cells**. *J Immunol* 2009, **182**(9):5748-5756.

84. Yang J, Yang M, Htut TM, Ouyang X, Hanidu A, Li X, Sellati R, Jiang H, Zhang S, Li H *et al*: **Epstein-Barr virus-induced gene 3 negatively regulates IL-17, IL-22 and RORgamma t**. *Eur J Immunol* 2008, **38**(5):1204-1214.

85. Hall AO, Beiting DP, Tato C, John B, Oldenhove G, Lombana CG, Pritchard GH, Silver JS, Bouladoux N, Stumhofer JS *et al*: **The cytokines interleukin 27 and interferon-gamma promote distinct Treg cell populations required to limit infection-induced pathology**. *Immunity* 2012, **37**(3):511-523.

86. Neufert C, Becker C, Wirtz S, Fantini MC, Weigmann B, Galle PR, Neurath MF: **IL-27 controls the development of inducible regulatory T cells and Th17 cells via differential effects on STAT1**. *Eur J Immunol* 2007, **37**(7):1809-1816.

87. Larousserie F, Charlot P, Bardel E, Froger J, Kastelein RA, Devergne O: **Differential effects of IL-27 on human B cell subsets**. *J Immunol* 2006, **176**(10):5890-5897.

88. Charlot-Rabiega P, Bardel E, Dietrich C, Kastelein R, Devergne O: **Signaling events involved in interleukin 27 (IL-27)-induced proliferation of human naive CD4+ T cells and B cells**. *J Biol Chem* 2011, **286**(31):27350-27362.

89. Boumendjel A, Tawk L, Malefijt Rde W, Boulay V, Yssel H, Pene J: **IL-27 induces the production of IgG1 by human B cells**. *Eur Cytokine Netw* 2006, **17**(4):281-289.

90. Batten M, Ramamoorthi N, Kljavin NM, Ma CS, Cox JH, Dengler HS, Danilenko DM, Caplazi P, Wong M, Fulcher DA *et al*: **IL-27 supports germinal center function by enhancing IL-21 production and the function of T follicular helper cells**. *J Exp Med* 2010, **207**(13):2895-2906.

91. Ysebrant de Lendonck L, Eddahri F, Delmarcelle Y, Nguyen M, Leo O, Goriely S, Marchant A: **STAT3 Signaling Induces the Differentiation of Human ICOS(+) CD4 T Cells Helping B lymphocytes**. *PLoS ONE [Electronic Resource]* 2013, **8**(7):e71029.

92. Sugiyama N, Nakashima H, Yoshimura T, Sadanaga A, Shimizu S, Masutani K, Igawa T, Akahoshi M, Miyake K, Takeda A *et al*: **Amelioration of human lupus-like phenotypes in MRL/lpr mice by overexpression of interleukin 27 receptor alpha (WSX-1)**. *Annals of the rheumatic diseases* 2008, **67**(10):1461-1467.

93. Kido M, Takeuchi S, Sugiyama N, Esaki H, Nakashima H, Yoshida H, Furue M: **T cell-specific overexpression of interleukin-27 receptor alpha subunit (WSX-1) prevents spontaneous skin inflammation in MRL/lpr mice**. *The British journal of dermatology* 2011, **164**(6):1214-1220.

94. Vonarbourg C, Diefenbach A: **Multifaceted roles of interleukin-7 signaling for the development and function of innate lymphoid cells**. *Semin Immunol* 2012, **24**(3):165-174.

95. Amara K, Steen J, Murray F, Morbach H, Fernandez-Rodriguez BM, Joshua V, Engstrom M, Snir O, Israelsson L, Catrina AI *et al*: **Monoclonal IgG antibodies generated from joint-derived B cells of RA patients have a strong bias toward citrullinated autoantigen recognition**. *J Exp Med* 2013, **210**(3):445-455.


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10

Addendum

SUMMARY

RA and SLE affect millions of people worldwide. Although therapeutic strategies have significantly improved over the last two decades, at present there is no cure available. In 2005, a new T helper subset was described that appears to play an important role in the development of auto-immune diseases. These Th17 cells are characterized by the production of IL-17A, IL-17F, TNFα, IL-21 and IL-22. In this thesis we have further investigated the role of several Th17 related cytokines in the activation and differentiation of lymphocytes in the pathogenesis of RA and SLE. For these studies we have made use of mouse models of these diseases and materials obtained from RA and SLE patients.

The role of the IL-17 cytokine family in RA has been studied extensively. The IL-17 cytokine family consists of IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25) and IL-17F. IL-17A and to a lesser extent IL-17F appear to be mostly involved in the pathogenesis of RA. IL-17 family members signal through distinct receptor heterodimers, but the IL-17 receptor A (IL-17RA) subunit appears to be the common subunit for all IL-17 cytokines. In chapter 2 we explore the effect of loss of IL-17RA signaling, and therefore loss of signaling of all IL-17 family cytokines, in the pathogenesis of collagen induced arthritis (CIA), a mouse model for RA. We find that IL-17RA signaling is essential for CIA development and loss of this receptor induces a Th2-like phenotype in splenic CD4+ T cells.

The pathogenic role of IL-17A and Th17 cells in synovial inflammation has been shown by many studies. In addition, several studies have suggested a pro-inflammatory role for IL-22 in joint inflammation. However, other studies have shown a role for IL-22 in mucosal barrier homeostasis, and that IL-22 is protective in autoimmunity. We therefore investigated the differential effects of Th17 cells, which produce both IL-17A and IL-22, and Th22 cells, which only produce IL-22 but not IL-17A, in RA.

We show in chapter 3 that Th22 cells are not essential in synovial inflammation. IL-22 has limited effects on production of IL-6, IL-8 and MMP-1. Furthermore, antigen induced arthritis development, which is Th17 dependent, is not effected by loss of IL-22 in IL-22-deficient mice. These data show that Th17 mediated synovial inflammation does not require IL-22. In addition, in chapter 4 we investigate the role of IL-22 in B cell immunity. Although the IL-22 receptor is not expressed on hematopoietic cells, lack of IL-22 had profound effects on germinal center formation in CIA. We propose that this effect is mediated through stromal cells present in lymphoid follicles, which express certain chemokines that induce migration of B cells into these follicles.

B cells play an important role in RA and many RA patients are (anti-citrullinated protein antibody) ACPA positive. How this loss of tolerance is established is unknown. A small population of B cells has been found in the periphery that expresses both the surrogate light chain (SLC) as well as the immunoglobulin light (IgL) chain. These cells accumulate in RA patient synovium. These cells display an auto-reactive B cell receptor (BCR) repertoire. However, their contribution

to autoimmune disease is not clear. We show in chapter 5 that SLC and IgL chain co-expression on B cells in mice does not enhance autoimmunity in CIA. Instead, SLC chain expression induced clonal deletion and anergy in B cells, suggesting a protective role for the SLC in autoimmunity.

In chapter 6, we explore the role of IL-27R signaling in B cell immunity. The IL-27R is expressed on many cells in the body, including hematopoietic cells and epithelial cells. IL-27 enhances susceptibility of naïve T helper cells for Th1 differentiation and induces IL-10 production. In addition, IL-27 reduces Th17 differentiation. In IL-27R KO mice, more T cells express IL-17A. Also, follicular T helper cells, which are crucial for germinal center formation and function, depend on IL-27. The IL-27R is also expressed on B cells, but little is known about the direct effects on these cells. In chapter 6 we investigated the direct effect of IL-27R signaling on B cells, and the effect on B cell immunity. We find that IL-27R signaling is essential for both T cell dependent as well as T cell independent immunity, although the direct effect on B cells is limited, suggesting a role for IL-27 on cells directing B cells.

Both the IFN type I pathway and IL-17A producing cells have been implicated in SLE pathogenesis. Half of all patients display the IFN type I signature. Several studies show possible links between IFN type I and IL-17A producing cells, but so far no studies have been performed to confirm the association of IFN type I with IL-17A in SLE patients. In chapter 7, we show for the first time a co-occurrence of CCR6+IL-17A+ cells and the IFN type I signature. Furthermore, downstream factors of these pathways were also correlated (B cell activating factor (BAFF) and IL-21). Several studies suggest that these pathways are regulated through similar regulatory systems. Together with our data these studies suggest that targeting both pathways might be beneficial in the treatment of SLE.

The pathogenic role of Th17 cells in SLE has been suggested in both humans and mice. IL-17A is involved in germinal center formation and works in synergy with BAFF to enhance B cell survival and activation in an SLE mouse model. B6.lpr mice deficient for IL-23R expression are protected against development of SLE symptoms. Intriguingly, we find a strongly enhanced SLE phenotype in mice deficient for IL-17RA signaling, suggesting a protective role for IL-17 family members in this model. These data provide valuable insight in the differential effects of IL-17 cytokines and other cytokines produced by Th17 cells.

The data presented in this thesis shed light on the contribution of the studied cytokines in different stages of RA and SLE disease development and the potential of these cytokines as (novel) therapeutic targets.

SAMENVATTING

Reumatoïde artritis (RA) en systemische lupus erythematosus (SLE) treffen miljoenen mensen wereldwijd. Hoewel de behandel mogelijkheden de afgelopen twintig jaar enorm zijn toegenomen is genezing van deze ziekten nog steeds niet mogelijk. In 2005 is een nieuw T helper subtype beschreven. Deze Th17 cellen worden gekenmerkt door productie van onder andere IL-17A, IL-17F, TNFα, IL-21 en IL-22 en lijken een belangrijke rol te spelen in auto-immuniteit. In dit proefschrift hebben wij de rol van verschillende Th17 gerelateerde cytokines in de activatie en differentiatie van lymfocyten in de pathogenese van RA en SLE verder onderzocht. Voor deze studies hebben we gebruik gemaakt van muis modellen van deze ziekten en humaan materiaal verkregen van RA en SLE patiënten en gezonde controles.

De rol van de IL-17 cytokine familie in RA wordt intensief onderzocht. De IL-17 familie bestaat uit IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (ook wel IL-25 genoemd) en IL-17F. IL-17A en in mindere mate IL-17F lijken het meest betrokken bij de pathogenese van RA. IL-17 cytokines signaleren door verschillende receptor heterodimeren, waarbij de IL-17 receptor A (IL-17RA) de gemeenschappelijke *subunit* lijkt te zijn. In hoofdstuk 2 onderzoeken we het effect van verlies van IL-17RA expressie, en dus van signalering van alle IL-17 cytokines, in de pathogenese van *collagen induced arthritis* (CIA), een muismodel voor RA. Wij vinden dat IL-17RA signalering essentieel is voor het ontstaan van artritis in CIA en dat verlies van deze receptor een Th2 fenotype induceert in CD4+ T cellen in de milt.

De pathogene rol van IL-17A en Th17 cellen in synoviale inflammatie is in meerdere studies aangetoond. Daarentegen hebben verschillende studies een rol gesuggereerd voor IL-22 in gewrichtsontsteking. In tegenstelling hebben andere studies laten zien dat IL-22 een rol speelt in immuun homeostase van mucosale oppervlakken en dat IL-22 een beschermende werking heeft in auto-immuniteit. Daarom hebben wij verder onderzocht wat de effecten zijn in RA van Th17 cellen, die zowel IL-17A als IL-22 produceren, ten opzichte van Th22 cellen, die alleen IL-22 maar geen IL-17A produceren.

In hoofdstuk 3 laten wij zien dat Th22 niet essentieel zijn voor synoviale inflammatie. IL-22 heeft slechts beperkt effect op de productie van IL-6, IL-8 en MMP-1. Daarnaast is antigen geïnduceerde artritis ontwikkeling, een Th17 gemedieerd model, niet verminderd door verlies van IL-22 in IL-22 deficiënte muizen. Deze data laten zien dat Th17 gemedieerde synoviale inflammatie onafhankelijk is van IL-22. In hoofdstuk 4 laten we daarnaast zien dat IL-22 en rol speelt in B cel immuniteit. Hoewel de IL-22 receptor niet tot expressie komt op hematopoiëtische cellen, heeft het verlies van IL-22 sterke effecten op de formatie van *germinal centers* in CIA. Wij stellen voor dat dit effect van IL-22 gemedieerd wordt door stromale cellen die aanwezig zijn in de lymfoïde follikels. Deze cellen brengen bepaalde chemokines tot expressie die de migratie van B cellen naar de follikels reguleren.

B cellen spelen een belangrijke rol in RA en veel RA patiënten hebben antilichamen in hun bloed tegen lichaamseigen gecitrullinereerde eiwitten (ACPA). Hoe dit verlies van zelf tolerantie ontstaat is niet bekend. Bij RA patiënten is een kleine populatie perifere B cellen gevonden die zowel de surrogaat lichte keten (SLC) als de lichte keten (IgL) tot expressie brengen. Deze cellen accumuleren in het synovium van deze patiënten en brengen een auto-reactieve B cel receptor (BCR) tot expressie, maar hun bijdrage in de auto-immuun ontsteking van het gewricht was onduidelijk. Wij laten in hoofdstuk 5 zien dat co-expressie van de SLC en de IgL op B cellen in muizen niet leidt tot een toename van auto-immuniteit in CIA. In tegenstelling laat onze data zien dat SLC expressie door perifere B cellen klonale deletie en anergie induceren. Dit suggereert een beschermend effect van SLC expressie in auto-immuniteit.

In hoofdstuk 6 onderzoeken wij de rol van IL-27 receptor (IL-27R) signalering in B cel immuniteit. De IL-27R komt tot expressie op veel verschillende cellen van het lichaam, waaronder hematopoiëtische cellen en epitheliale cellen. IL-27 verhoogt de gevoeligheid voor naïeve Th cellen voor differentiatie naar Th1 cellen en induceert IL-10 productie. Daarnaast remt IL-27 Th17 differentiatie. In IL-27R deficiënte muizen brengen meer T cellen IL-17A tot expressie. Ook zijn folliculaire Th cellen, die essentieel zijn voor *germinal center* ontwikkeling, afhankelijk van IL-27. De IL-27R komt tot expressie op B cellen, maar er is weinig bekend over de directe effecten IL-27 op deze cellen. In hoofdstuk 6 onderzoeken wij het directe effect van IL-27R signalering op B cellen en het effect op B cel immuniteit. We vinden dat iL-27R signalering essentieel is voor zowel T cel afhankelijke als T cel onafhankelijke B cel immuniteit, hoewel er geen direct effect lijkt te zijn op B cellen zelf. Dit suggereert dat IL-27 een rol speelt in de regulering van B cellen via B cel non-intrinsieke mechanismen.

Zowel interferon (IFN) type I als IL-17A producerende cellen spelen mogelijk een rol in de pathogenese van SLE. Ongeveer de helft van alle SLE patiënten heeft de IFN type I handtekening. Meerdere studies suggereren een mogelijke link tussen IFN type I en IL-17A producerende cellen, maar tot dusverre zijn er nog geen studies uitgevoerd om de associatie tussen IFN type I en IL-17A/Th17 cellen in SLE patiënten te bevestigen. In hoofdstuk 7 laten wij voor het eerst zien dat de IFN type I handtekening en verhoogde IL-17A producerende cellen bij dezelfde SLE patiënten voorkomt. Daarnaast laten wij ook zien dat *downstream* factoren van deze *pathways* aan elkaar gecorreleerd zijn (*B cell activating factor* (BAFF) en IL-21). Enkele studies suggereren dat IFN type I en IL-17A expressie gereguleerd worden door dezelfde regulatoire mechanismen. Samen met onze data ondersteunt het idee dat het remmen van zowel IFN type I als IL-17A een gunstig effect kan hebben voor SLE patiënten.

De pathogene rol van Th17 cellen in SLE is zowel in patiënten als in muismodellen gesuggereerd. IL-17A is betrokken bij het ontstaan van *germinal centers* en heeft een synergistische werking met BAFF in het verhogen van B cel overleving en activatie in een muismodel voor SLE. B6.lpr muizen die deficiënt zijn voor IL-23R expressie zijn beschermd voor de ontwikkeling van het SLE fenotype. Wij beschrijven in hoofdstuk 7 een onverwacht sterke toename van het SLE fenotype

in IL-17RA deficiënte muizen, hetgeen een beschermende rol suggereert voor de IL-17 cytokine familie in dit model.

De data in dit proefschrift dragen bij aan het begrip van de rol van de onderzochte cytokines in de verschillende stadia van RA en SLE ziekte ontwikkeling, en de potentie die deze cytokines hebben als mogelijk (nieuwe) therapeutische targets in deze ziekten.

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toch zonde om niet ALLES in 1 keer mee te nemen. Gelukkig woonde jij om de hoek van de uni en kon jij het dan afmaken als ik mijn laatste trein dreigde te missen. Bedankt voor al je hulp, niet alleen voor het werk, maar ook bij DCO stress. Heel veel succes met alles wat de toekomst brengt, maar laten we eerst een feestje doen! Marjolein, bedankt voor al je hulp bij FACSen, ELISA en als ik weer eens een antilichaam of ander stofje kwam scoren. Ingrid, jouw hulp bij sort problemen was onmisbaar voor mijn boekje. Je hebt zelfs een keer een sort die bij de immu niet afkwam tot in de avonduren voor mij afgemaakt en daarmee het experiment gered. Super bedankt daarvoor!

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Zana en Marjan, we hebben in een hele korte tijd toch 2 mooie stukken weten te schrijven. Ik had nooit gedacht dat ik nog een stuk met SLE patientenmateriaal in mijn boekje zou krijgen, echt super dat dat met jullie zo snel nog kon! Zana, het schrijven ging echt top-speed. We waren echt een super team! Bedankt daarvoor!

Fleur en Hannie, onze samenwerking gaat als een trein! We hebben onze expertises perfect kunnen matchen, ik denk dat we er een super mooi stuk van gaan maken! Fleur, heel veel succes ook met jouw boekje!

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Britt, sorry voor de miljoenmiljard gemiste lunch afspraken, ik ga het goedmaken! Hopelijk lukt het vanaf nu om vaker naar het theater te gaan. Veel succes ook met jouw boekje, dat komt helemaal goed! Gwenny en Timon, we hebben een heleboel films moeten missen, maar gelukkig lijkt de bron van slechte films onuitputtelijk. Er komt vast een 4e/5e(?) Transformers film waar

we heen kunnen! Juul, onze dates zijn onmisbaar, ik kan met niet voorstellen hoe het zou zijn zonder de ‘en-de-jochemettes’. Bedankt voor al je steun de afgelopen tijd! Ons jaarlijkse ‘lange weekend’ naar Madrid kon nu niet doorgaan, maar volgend jaar gaan we zeker weer! Heel veel geluk samen met Saber!

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CURRICULUM VITAE

Odilia Corneth was born on the 20th of April 1986 in Leidschendam, The Netherlands. When she was four, her family moved to Brunei for six years. After this they moved to Woking, England, where Odilia attended Reed's School in Cobham. She finished her secondary education at Christelijk Gymnasium Sorghvliet where she graduated in 2004. She then studied Medicine at the Erasmus University in Rotterdam, for which she obtained her 'doctoraal' in 2008. She also studied Molecular Medicine, for which she obtained a MSc degree in 2009, graduating on the research project 'Surrogate light chain expression on mature B cells in autoimmunity' at the department of Pulmonary Medicine. After het PhD training at the department of Rheumatology, she will resume her medical studies.

PORTFOLIO

Name PhD student:	Odilia Barbara Jeannette Corneth
Erasmus MC Department:	Rheumatology
Research School:	Molecular Medicine
PhD period:	September 2009 – August 2013
Promotores:	Prof. Dr J.M.W. Hazes and Prof. Dr R.W. Hendriks
PhD Supervisor:	Dr E. Lubberts

Courses:

-	Biomedical Research Techniques	2008
-	Molecular Immunology	2009
-	Laboratory animal science	2009
-	NIBI course Project Management	2010
-	Statistics (Classical Methods for Data Analysis)	2011

(Inter)national scientific meetings:

-	NVVI annual meeting Noordwijkerhout	2009
-	EWRR annual meeting Bamberg (poster)	2010
-	NVVI annual meeting Lunteren	2010
-	ACR annual meeting Atlanta (oral presentation, poster)	2010
-	NVVI annual meeting Noordwijkerhout (poster)	2010
-	EWRR annual meeting Amsterdam (poster)	2011
-	NVVI annual meeting Lunteren	2011
-	ACR annual meeting Chicago (d.n.a.) (oral presentation)	2011
-	Th17 meeting Keystone (poster)	2012
-	Biology of B lymphocytes, Kloster Banz (poster)	2012
-	EWRR annual meeting Prague (poster)	2013

- 3rd int. Lymphoid Tissue meeting Rotterdam 2013
- ACR annual meeting San Diego
  (poster) 2013

Teaching:

- HLO student (HLO Leiden) 10 weeks 2010
- HLO student (HLO Leiden) 10 weeks 2011
- HLO student (HLO Leiden) 7 months 2011/ 2012

Master thesis supervision:

- Master student Toxicology and Environmental Health (University Utrecht)
  3 months 2012
- Master student Infection and Immunity (Erasmus University)
  10 months 2012/2013

Other activities:

- Active member of the PhD committee, department of Immunology 2011

LIST OF PUBLICATIONS

2012 Btk levels set the threshold for B-cell activation and negative selection of autoreactive B cells in mice
*Laurens P. Kil, Marjolein J. W. de Bruijn, Menno van Nimwegen, **Odilia B. J. Corneth**, Jan Piet van Hamburg, Gemma M. Dingjan, Friedrich Thaiss, Guus F. Rimmelzwaan, Dirk Elewaut, Dianne Delsing, Pieter Fokko van Loo and Rudi W. Hendriks*
Blood, 2012 119: 3744-3756

2013 Lack of IL-17 receptor A signaling prevents autoimmune inflammation of the joint and gives rise to a Th2-like phenotype in collagen induced arthritis
***Odilia B.J. Corneth**, Adriana M.C. Mus, Patrick S. Asmawidjaja, Roel G. Klein Wolterink, Menno van Nimwegen, Maarten D. Brem, Yara Hofman, Rudi W. Hendriks, Erik Lubberts*
Accepted for publication in Arthritis and Rheumatism

2013 IL-17/Th17 mediated synovial inflammation is IL-22 independent
*Jan Piet van Hamburg, **Odilia B.J. Corneth**, Sandra M.J. Paulissen, Nadine Davelaar, Patrick S. Asmawidjaja, Adriana M.C. Mus and Erik Lubberts*
Annals of the Rheumatic Diseases, 2013 Oct 1;72(10):1700-7

2013 IL-22 is essential for terminal B cell differentiation in collagen induced arthritis
***Odilia B.J. Corneth**, Adriana M.C. Mus, Patrick S. Asmawidjaja, R.W. Hendriks and Erik Lubberts*
Submitted for publication

2013 Surrogate light chain expression beyond the pre-B cell stage promotes tolerance in a dose-dependent fashion
*Laurens P. Kil, **Odilia B.J. Corneth**, Marjolein J.W. de Bruijn, Patrick Asmawidjaja, Arndt Krause, Erik Lubberts, Pieter Fokko van Loo and Rudi W. Hendriks*
Submitted for publication

2013 IL-27R signaling is essential for B cell immunity
***Odilia B.J. Corneth**, Patrick S. Asmawidjaja, Laurens P. Kil, Adriana M.C. Mus, R.W. Hendriks and Erik Lubberts*
Manuscript in preparation

2013 Th17 cytokines and Interferon type I: partners in crime in SLE?
***Odilia B.J. Corneth**, Zana Brkic, Cornelia G. van Helden-Meeuwsen, Radboud J.E.M. Dolhain, Naomi I. Maria, Sandra M.J. Paulissen, Nadine Davelaar, Jan Piet van Hamburg, Paul L. van Daele, Virgil A. Dalm, P. Martin van Hagen, Johanna M.W. Hazes, Marjan A. Versnel and Erik Lubberts*
Submitted for publication

2013 Protective role of IL-17 receptor signaling in SLE pathology in B6.lpr mice
***Odilia B.J. Corneth**, Fleur Schaper, Franka Luk, Patrick S. Asmawidjaja, Adriana M.C. Mus, Maarten D. Brem, Peter Heeringa, Rudi W. Hendriks, Johanna Westra and Erik Lubberts*
Manuscript in preparation

2013 IL-23 dependent and independent stages of experimental arthritis: no clinical effect of therapeutic IL-23p19 inhibition in collagen-induced arthritis
*Ferry Cornelissen, Adriana M.C. Mus, Patrick S. Asmawidjaja, **Odilia B.J. Corneth** and Erik Lubberts*
PLoS One. 2013; 8(2)


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