Sensitivity and Resistance to Regulation by IL-4 in Th17 Cells: Molecular Mechanisms and Significance in Autoimmune Disease

by

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Dedication

I would like to dedicate this to my friends and family, who have graciously tolerated my wide range of moods over the years and who have always been tremendous advocates.

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ABSTRACT

Th17 cells are highly pathogenic CD4+ T cells that promote many immune-mediated diseases, including rheumatoid arthritis, and a thorough understanding of the regulation and maturation of the Th17 response may prove therapeutically useful. We therefore chose to characterize the mechanisms of Th17 suppression by the Th2 cytokine IL-4.

After one week of *in vitro* differentiation or a two week immunization *in vivo*, IL-4 rapidly inhibits expression of several Th17-family genes, including IL-17A, IL-17F and RORγt, and this suppression overcomes stimulation by TGFβ, IL-6 and IL-23. However, suppression by IL-4 is unstable and does not induce Th2 conversion. The mechanism of suppression downstream of the IL-4R is dependent on STAT6 but independent of STAT5, IRS-2 and GATA-3. At the chromatin level, IL-4 up-regulates markers of active transcription at the *Il17a* locus, including acetylation of H3 and H4 and binding of PolII, despite the fact that IL-17A message levels are down-regulated. However, IL-4 also displaces the transcriptional inducer STAT3 from the *Il17a* promoter, suggesting that a transcriptional repressor may take its place.

We found that Th17 cells undergo a process of maturation, whereby *in vitro*-generated Th17 cells stimulated for three weeks or *in vivo*-generated Th17 cells re-stimulated for three days become resistant to suppression by IL-4. This transition depends on a combination of TCR and cytokine stimuli and results in desensitization of the IL-4R. Specifically, mature Th17 cells lose the ability to phosphorylate STAT6 in response to IL-4, despite normal expression of the IL-4R. The suppression of IL-4R signaling did not depend on SOCS5, but may be mediated by SOCS1.

To explore the regulation of IL-17 by IL-4 and IFNγ in CIA, we treated mice with cytokine-neutralizing antibodies *in vivo* during the initiation phase of disease. The results showed that IFNγ plays a protective role in CIA via down-regulation of IL-17. IL-4, once released from suppression by IFNγ, also plays a protective role, particularly in bone and cartilage erosion. However, the protective effect of IL-4 is not mediated by suppression of IL-17. Interestingly, when both IFNγ and IL-4 are neutralized, mice develop a severe arthritis that is independent of IL-17.

Chapter 1

Introduction

CD4+ T helper cells are the arbiters of the immune system and thus have the power to mediate both protective and pathogenic immune responses. Two decades ago Mossman and Coffman [1] proposed that CD4+ T cells differentiate into two subsets with reciprocal functions and patterns of cytokine secretion, termed T-helper 1 (Th1) and T-helper 2 (Th2). Th1 cells are characterized by production of interferon-γ (IFNγ) and induce cell-mediated immunity against intracellular pathogens, while Th2 cells produce interleukin-4 (IL-4) and stimulate humoral immunity against parasitic helminths. This paradigm was maintained until 2005, when a third T-cell subset, known as T-helper 17 (Th17), was identified [2, 3]. Th17 cells are characterized by production of interleukin-17 (IL-17) and may have evolved for host protection against microbes that Th1 and Th2 immunity are not well-suited for, such as extracellular bacteria and some fungi.

While Th17 cells were only recently recognized as a unique Th-cell subset, IL-17 has been known for much longer. Human IL-17 was originally cloned in 1995, and early reports demonstrated multiple inflammatory and hematopoietic effects on epithelial, endothelial and fibroblastic cells [4-6]. These initial studies set the stage for much of what is now known about IL-17. The over-riding theme is that IL-17 mediates powerful effects on stromal cells, resulting in production of inflammatory cytokines and recruitment of leukocytes, especially neutrophils, thus creating a link between innate and adaptive immunity. Although Th17 cells play an important role in host defense, they have received considerable attention in recent years primarily because they appear to be

the principle mediators of pathogenesis in several autoimmune and inflammatory disorders, including rheumatoid arthritis (RA), psoriasis, multiple sclerosis, asthma and inflammatory bowel disease. Thus a thorough understanding of the development and regulation of Th17 cells holds important prospects for the future of targeted clinical therapeutics.

Th17 differentiation

When a naïve T cell is activated, the local cytokine milieu plays an important role in determining which effector lineage that cell will assume by inducing lineage-specific transcription factors. Naïve $CD4^+$ T cells stimulated through the T cell receptor (TCR) in the presence of interleukin-12 (IL-12) become Th1 cells and express the transcription factor T-bet, while those stimulated in the presence of IL-4 become Th2 cells and express the transcription factor GATA-3 (reviewed in [7]). Initial studies in mice suggested that interleukin-23 (IL-23), a heterodimeric cytokine that shares a subunit with IL-12, induced IL-17 expression [2, 3, 8, 9]. However, subsequent studies demonstrated that the IL-23 receptor (IL-23R) is only expressed on T cells after activation, and therefore IL-23 can up-regulate IL-17 in memory T cells but cannot act on naïve T cells to induce Th17 differentiation [8]. Instead, three groups nearly simultaneously discovered that the key to Th17 differentiation in the mouse is the combination of transforming growth factor-β (TGFβ) and interleukin-6 (IL-6) [10-12]. In addition, tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β) can further enhance mouse Th17 differentiation, but only in the presence of TGFβ and IL-6 [12-14]. This discovery was a surprise because TGFβ is

well known to inhibit most T-cell responses and to induce differentiation of forkhead box protein 3 (FoxP3)-expressing regulatory T cells (Tregs) (reviewed in [15]).

One of the ways in which the anti-inflammatory role of TGFβ supports Th17 development is through inhibition of IFNγ and IL-4, both of which inhibit Th17 development. However, TGFβ must also have more direct roles in Th17 differentiation because it is required even in the absence of IFNγ and IL-4 [10-12]. TGFβ synergizes with IL-6 to induce expression of the transcription factor retinoid-related orphan receptor-γt (RORγt), a key regulator of Th17 differentiation. In mice, RORγt is both necessary and sufficient for IL-17 expression *in vitro* and *in vivo* [16]. In humans, RORγt is induced by the same cytokines that induce IL-17 and is only expressed by IL-17-producing clones [17-20]. Thus, just as T-bet controls the Th1 lineage and GATA-3 controls the Th2 lineage, RORγt appears to control the Th17 lineage. Still, there are a few unanswered questions. RORγt is an orphan nuclear receptor with a ligand-binding pocket, suggesting that its activity may be regulated by an unknown ligand. Also, RORγt has not yet been shown to directly bind to the *Il17a* promoter, although a potential binding site was identified [16]. Despite these issues, inhibition of RORγt may be therapeutically useful, and the ligand-binding pocket is an ideal pharmacological target. Similarly, evidence suggests that a related nuclear receptor, RORα, plays a role in Th17 development. RORα is induced by TGFβ and IL-6 and synergizes with RORγt in the induction of IL-17 [21].

In addition to RORγt and RORα, Th17 development in mice depends on the transcription factor signal transducer and activator of transcription 3 (STAT3), which is

activated by IL-6 and IL-23. STAT3 has multiple roles in Th17 development: in activated Th17 cells stimulated with IL-23, it binds directly to the *Il17a* promoter and induces IL-17 expression, and in naïve T cells stimulated with TGFβ and IL-6, it is required for induction of RORγt expression, although it is not yet known if STAT3 binds directly to the *Rorc* promoter [22-25]. Because both RORγt and STAT3 are required for IL-17 expression, there may be cooperation between the two transcription factors at the *Il17a* promoter. IL-23 also activates signal transducer and activator of transcription-4 (STAT4), which is the primary mediator of IL-12 signaling and is required for Th1 differentiation, yet is still important for IL-23-induced IL-17 production [23]. Thus, STAT4 may inhibit Th17 development downstream of IL-12, while also supporting IL-17 expression downstream of IL-23. In addition to STAT3 and STAT4, there are likely to be other transcription factors that are required for Th17 development, such as SMAD-2 or SMAD-3 downstream of TGFβ.

IL-23 plays an important role in Th17 effector function, but the mechanism is still under debate. IL-23 up-regulates IL-17 production and has been suggested to promote survival and expansion of Th17 cells *in vitro*, although it is not absolutely necessary and more thorough experiments are necessary [9, 12, 26]. Cua and colleagues recently reported interesting *in vivo* results showing that IL-23R positive and negative Th17 cells survive and produce IL-17 equally well, but only IL-23R-positive Th17 cells proliferate and migrate to the site of inflammation in a mouse model of multiple sclerosis [27]. Much more work is needed to fully understand how IL-23 supports Th17-mediated pathology, especially considering that neutralizing antibodies targeting the shared IL-

12/IL-23 subunit are already under clinical investigation in multiple inflammatory diseases.

Evidence also demonstrates the existence of IL-17-producing cells within the $CD8^+$, $CD4^-CD8^-$ and γδ T cell subsets, although less is known about their differentiation and regulation.

Cytokines expressed by Th17 cells

When T cells differentiate, they begin to express specific cytokines, such as IFNγ in Th1 and IL-4 in Th2, which act in an autocrine feedback loop to further promote differentiation, thus giving activated T cells self-sufficiency to move out of the lymphoid tissue and traffic to a site of inflammation while continuing to develop. Similarly, mouse Th17 cells specifically express interleukin-21 (IL-21) soon after activation, and autocrine IL-21 plays an important role in RORγt and IL-17 expression. IL-21 can also partially replace IL-6 during Th17 differentiation, giving established Th17 cells the ability to promote further Th17 development in neighboring cells. IL-23 in combination with TGFβ can also induce RORγt and IL-17 expression, but only after IL-6 or IL-21 induces IL-23R expression [28-32]. Thus IL-6, IL-21 and IL-23 act sequentially: first IL-6 up-regulates IL-21, then both IL-6 and IL-21 up-regulate IL-23R, and finally IL-23 appears to up-regulate effector function and pathogenicity in Th17 cells through an unknown mechanism.

Th17 cells are characterized by production of IL-17, but they also produce other inflammatory cytokines that can play an important role in disease. IL-17, or IL-17A, is

one member of a family of six cytokines known as IL-17A through F. Th17 cells specifically express IL-17F in addition to IL-17A. IL-17A and IL-17F are closely related, with 55% amino acid identity, as well as a common receptor [33]. In addition, the *Il17a* and *Il17f* genes are located side-by-side on the chromosome and thus may be subject to coordinate regulation. IL-17A and IL-17F are both homodimeric cytokines, but recent evidence shows that Th17 cells also produce an IL-17A/F heterodimer that has potent inflammatory effects [34, 35]. Currently, much less is known about the inflammatory effects of IL-17F than IL-17A, but given their high degree of similarity and possible redundancy, it may be important to measure, as well as to target, both IL-17A and IL-17F in disease.

Other pro-inflammatory cytokines produced by both mouse and human Th17 cells include TNFα, a well-known mediator of inflammatory disease, interleukin-22 (IL-22) and interleukin-26 (IL-26) [17, 18, 20, 36-39]. Much less is known about IL-22 and IL-26, members of the interleukin-10 (IL-10) family, which promote innate, non-specific immunity in cells outside of the immune system. Studies in keratinocytes and colonic myofibroblasts show that IL-22 induces anti-microbial proteins, defensins, acute-phase proteins, inflammatory cytokines, chemokines and hyperplasia [40-42]. Others have found, however, that IL-22 protects hepatocytes during acute liver inflammation [43]; thus, IL-22 produced by Th17 cells may enhance inflammation or limit tissue damage induced by IL-17, depending on the type of tissue.

Unexpectedly, a subset of Th17 cells co-expresses IFNγ, particularly in humans, in whom as many as half of all the IL-17-positive cells also express IFNγ [17, 18, 20, 36].

These double-positive cells seem to contradict the idea that Th17 cells are a unique subset distinct from Th1 cells, and these cells are particularly problematic to explain given that IFNγ has been shown to inhibit IL-17 expression. However, human Th cell differentiation is known to be more flexible than that in mouse, and it is not uncommon to see IL-4/IFNγ double-positive T cells in humans, although it is rare in mice. It is not yet clear if these cells represent a stable phenotype or a transitional phase, undergoing a switch from Th17 to Th1 or *vice versa*. IFNγ/IL-17 double-positive cells are likely to be highly inflammatory, and a recent paper showed that these cells are very common in the brains of multiple sclerosis patients and are preferentially recruited to the central nervous system in a mouse model of multiple sclerosis [44].

Another unexpected finding is the existence of many Th17 cells co-expressing IL-10. IL-10 is an anti-inflammatory cytokine produced by a number of different cell types. T-cell sources of IL-10 are generally thought to include Th2 cells and various types of Tregs, but Th1 cells have also been found to secrete IL-10 in certain conditions and to thereby limit their own inflammatory effects [45, 46]. In mice, the combination of TGFβ and IL-6, which synergize to induce IL-17 production, also synergize to induce IL-10, with the end result that half of the IL-17-positive cells co-express IL-10 and half of the IL-10-positive cells co-express IL-17 [47, 48]. IL-10 produced by Th17 cells may serve an important protective function by limiting inflammation and tissue damage normally caused by IL-17. In fact, Th17-derived IL-10 was found to play an important role in limiting Th17-driven inflammation in a mouse model of multiple sclerosis [48]. It is not yet known whether human Th17 cells ever co-express IL-10 or what role the double-positive cells might play human Th17-driven disease. Exogenous IL-10 has been shown

to inhibit IL-17 production by T cells from peripheral blood of RA patients and IL-10 is overexpressed in RA synovium, but the cellular source is unknown [49].

T helper cell cross-talk

Th1, Th2 and Th17 cells develop in response to very different cytokine milieus and each lineage is effective against a different class of microorganism. During an immune response, cells of the innate immune system recognize pathogen-associated molecular patterns and secrete cytokines to induce differentiation of the appropriate T helper cell lineage while preventing development of an inappropriate and potentially ineffective or pathogenic immune response. For example, IL-12, which induces Th1 development, can inhibit Th2 differentiation, while IL-4, which induces Th2 development, can inhibit Th1 differentiation. In addition, T helper cells themselves support lineage cross-regulation because Th1-derived IFNγ inhibits Th2 development and Th2-derived IL-4 further inhibits Th1 development. The respective differentiation programs are then reinforced in a cell-intrinsic manner through both positive and negative mechanisms, largely dependent on transcriptional regulation.

The master-regulator transcription factors T-bet and GATA-3 simultaneously instruct one differentiation pathway and inhibit the other. In developing Th2 cells, signal transducer and activator of transcription-6 (STAT6) and GATA-3 bind to DNA in the Th2 cytokine locus, triggering chromatin remodeling and inducing expression of IL-4, IL-5 and IL-13, as well as up-regulating GATA-3 expression in a positive feedback loop.

However, STAT6 and GATA-3 are simultaneously recruited to the *Ifng* locus and inhibit expression of IFNγ, IL-12Rβ2 and STAT4 [7, 50, 51]. In addition, Th2 cells express the transcription factor Ikaros, which binds to both the *Tbx21* and *Ifng* loci, inhibiting expression of T-bet and IFNγ [52, 53]. Similar mechanisms underlie suppression of Th2 genes in developing Th1 cells. T-bet and STAT4 up-regulate expression of IFNγ, STAT1 (the primary mediator of IFNγ receptor signaling), IL-12Rβ2 and Runx3. T-bet and Runx3 both bind to and suppress *Il4*, *Il5* and *Gata3* gene transcription [51, 54]. In addition, recent evidence suggests that there may be direct protein-protein interaction between GATA-3 and both T-bet and Runx3, leading to repression of GATA-3 activity [55, 56].

Much less is known about the cross-regulation between Th17 cells and the other T helper lineages. TGFβ can inhibit Th1 and Th2 development through a number of mechanisms, including inhibition of signaling through the IL-12R, IFNγR and IL-4R, as well as down-regulation of STAT4, T-bet and GATA-3 expression [57-59]. In addition, limited evidence suggests that IL-23 and IL-17 inhibit Th1 development by down-regulating T-bet and IL-12Rβ2, respectively [60, 61]. However, the general consensus seems to be that Th1 and Th2 cells suppress Th17 development much more potently than Th17 cells suppress Th1 or Th2 development. In fact, one plausible explanation for why Th17 cells went undiscovered for so long is that they rarely develop *in vitro* without the addition of neutralizing antibodies to both IL-4 and IFNγ [2, 3]. Mice lacking T-bet show enhanced Th17 development *in vitro* [62, 63], and forced expression of T-bet in naïve CD4+ T cells prevents IL-17 expression under Th17-polarizing conditions[64]. In addition, T cells from mice overexpressing c-Maf, a Th2 transcription factor important

for IL-4 expression, produce much less IL-17 [3]. The precise mechanisms by which Th1 and Th2 transcription factors inhibit IL-17 are currently unknown and are likely to depend on the relative expression levels or activities of a number of factors. For instance, T-bet can inhibit Th17 development in some settings, but in other settings cells expressing both T-bet and RORγt and producing both IFNγ and IL-17 have been identified [65]. Similarly, T cells from mice over-expressing GATA-3 showed a moderate decrease in Th17 differentiation and RORγt expression both *in vitro* and *in vivo* but only at certain time points or in certain disease models [66, 67]. In systems such as these it is often difficult to disentangle direct effects from indirect effects due to changes in cytokine production by Th1 and Th2 cells. Recent evidence has also demonstrated that IL-25 (IL-17E), a member of the IL-17 cytokine family, inhibits IL-17 expression indirectly by promoting Th2 differentiation [68, 69] and IL-13, a cytokine produced by Th2 cells, acts directly on Th17 cells to suppress IL-17 expression [70].

T helper cell maturation

In developing Th1 cells the intrinsic inhibition of Th2 development becomes more effective and the Th1 phenotype becomes more stable over time. In one important study, naïve CD4+ T cells were stimulated to induce Th1 differentiation and the suppressive effects of IL-4 were tested at various time points. The results showed that after 24 hours of Th1 stimulation, IL-4 treatment caused Th1 cells to down-regulate IFNγ and up-regulate IL-4, essentially reverting from a Th1 phenotype to a stable Th2 phenotype. However, after 96 hours of Th1 stimulation, IL-4 treatment caused a transient down-regulation of IFNγ without concomitant up-regulation of IL-4. Thus Th1 cells lost

the ability to induce IL-4. IL-4 receptor (IL-4R) signaling is primarily mediated by phosphorylation and activation of Janus-activated kinases-1 and -3 (JAK1 and JAK3), which then phosphorylate and activate STAT6. Activated STAT6 then dimerizes and goes to the nucleus to modify gene expression. At both time points IL-4 was capable of activating STAT6, but only in the early stage was GATA-3 up-regulated [71].

The results of this and other studies suggest that developing Th1 cells stabilize their phenotype by inhibiting the expression of GATA-3 through a mechanism dependent on IL-12 and STAT4 [72]. It also suggests that IFNγ expression can be down-regulated by IL-4-dependent signals other than GATA-3, possibly through STAT6. Additional studies have shown that with prolonged activation, Th1 cells lose the ability to phosphorylate JAK3 and STAT6 in response to IL-4, despite normal expression of the IL-4R [73]. This loss of IL-4R signaling was dependent on IFNγ [74]. In these mature Th1 cells, treatment with IL-4 no longer inhibited IFNγ production. Taken together these reports suggest that there are three stages of Th1 maturation: after one day of *in vitro* stimulation IL-4R signaling is unimpaired and induces complete reversion to a Th2 lineage, after three days of *in vitro* stimulation IL-4R signaling is blocked downstream of STAT6 and induces transient down-regulation of IFNγ production, and after seven days of *in vitro* stimulation IL-4R signaling is completely inhibited and IFNγ production is unaffected.

The mechanism mediating loss of IL-4R signaling in committed Th1 cells is not clear. Potential candidate mediators include the family of proteins known as suppressors of cytokine signaling (SOCS). SOCS proteins are typically thought to mediate classical

negative feed-back loops: they are up-regulated in response to cytokine stimulation, bind to phosphorylated cytokine receptors through an SH2 domain, and inhibit JAK activity through a KIR domain (reviewed in [75]). Both SOCS1 and SOCS5 have been shown to bind to the IL-4R and inhibit activation of STAT6 [76-80]. Thus, in maturing Th1 cells, IL-4-induced STAT6 activation may be inhibited by SOCS1, which can be induced by both IFNγ and IL-12, but there is also evidence of inhibition of recruitment of STAT6 to the phosphorylated IL-4R through a SOCS-independent mechanism [74].

There is no published evidence to date about the ability of Th2 cells to become resistant to IFNγ or IL-12. Similarly, nothing is known about how Th17 cells mature and become unresponsive to cross-regulation by Th1 and Th2 cytokines, but our data suggests a similar three stage process may exist in Th17 cells as in Th1 cells.

T helper cell plasticity

New evidence has emerged demonstrating a remarkable degree of plasticity among T helper lineages, which may seem at odds with the idea of maturation. While T helper cell subsets were initially thought to be stable lineages with unique cytokine profiles defined by discrete expression of a master regulator transcription factor, there are now many examples that expression of both cytokines and transcription factors is flexible. For example, IL-10, which was once thought to be Th2 cytokine, has been found to be produced by Th1, Th2, Treg and Th17 cells [45, 46, 48]. Similarly, Th17 cells that produce IFNγ are common *in vivo*, and IL-22, which is thought of as a Th17 cytokine, is often produced by cells that express IFNγ but not IL-17 [81, 82]. Also, IL-12 can suppress many Th17-specific genes and induce a phenotype that is almost completely

Th1 [83]. Even more surprising is the finding that *in vitro* differentiated, IL-4-producing Th2 cells can produce IFNγ when transferred *in vivo* [84]. These findings clearly suggest that T helper lineages cannot be defined strictly on the basis of expression of a single signature cytokine, particularly *in vivo*. However, it appears that expression of master regulator transcription factors is not fixed either. Tregs have been found to extinguish expression of FoxP3 and up-regulate pro-inflammatory cytokines, particularly in the setting of autoimmunity [85-88]. In addition, T cells have been found to co-express FoxP3 and T-bet [87, 89], FoxP3 and RORγt [90, 91], or T-bet and RORγt [65]. On the other hand, the lack of lineage commitment observed in many of these experiments could be attributed to defects in *in vitro* differentiation, as *in vivo*-derived Th17 cells have a very different gene expression profile and a more stable phenotype than *in vitro*-derived Th17 cells [92-94]. These observations raise many important questions about T helper cell lineage commitment and emphasize the importance of considering exactly when, where and for how long a T cell has undergone differentiation. It will also be essential to decipher the relationship between plasticity and maturation.

Chromatin regulation of T helper cell differentiation

Chromatin remodeling determines the heritability of gene expression patterns and plays an important role in T helper cell differentiation, as well as in the balance between plasticity and maturation. Active transcription requires an "open" chromatin conformation in order to allow binding of specific transcriptional activators to the DNA and recruitment of general transcriptional machinery. Some of the mechanisms that regulate DNA accessibility include DNA methylation, a number of different histone

modifications and three-dimensional chromatin structure. In particular, the histone code hypothesis posits that a combination of various post-translational modifications of histone tails, including acetylation, phosphorylation, methylation and others, creates an epigenetic mechanism for the fine-tuning of gene expression. The most well-characterized histone modifications associated with active transcription include tri-methylation of histone three on lysine four (H3K4me3), acetylation of histone three (H3Ac) and acetylation of histone four (H4Ac), while the most well-characterized histone modification associated with a repressive chromatin state is tri-methylation of histone three on lysine 27 (H3K27me3). Genes marked with bivalent methylation patterns (H3K4me3 and H3K27me3) or null methylation patterns (neither H3K4me3 nor H3K27me3) are thought to be poised for transcription (reviewed in [95]).

Data suggest that lineage-specific transcription factors regulate T helper cell fate in part through epigenetic processes. For example, chromatin-remodeling complexes displace nucleosomes and remodel chromatin at the *Ifng* promoter in Th1 cells in a STAT4-dependent manner, and these complexes are required for normal IFNγ expression [96]. T-bet binds to the *Ifng* promoter even when the DNA is repressively methylated, displaces histone deacetylases and recruits histone acetyltransferases and methyltransferases, thereby creating a highly permissive chromatin state [97-99]. These observations may explain how forced expression of T-bet can induce the expression of IFNγ even in committed Th2 cells [100]. Similarly, GATA-3 recruits chromatin-remodeling complexes to the Th2 cytokine locus, inducing permissive H3Ac, H4Ac and H3K4me3 marks while removing repressive H3K27me3 marks [101-105]. In Th17 cells, H3Ac is induced at the *Il17a* and *Il17f* promoters, which is dependent on STAT3 [106].

Recent technological advances leading to the combination of chromatin immunoprecipitation with high-throughput sequencing (ChIP-Seq) have allowed genome-wide examination of histone modifications in T helper subsets and provided insights into the mechanisms underlying T helper cell plasticity. A paper by Wei et al. characterized H3K27me3 and H3K4me3 levels at the genes for the signature cytokines and master regulator transcription factors in naïve, Th1, Th2, Th17 and Treg cells [87]. As predicted, naïve T cells displayed either null or bivalent marks across all the cytokine and transcription factor genes. Also as expected, the epigenetic marks found at the *Ifng*, *Il4* and *Il17a* loci correlated precisely with Th1, Th2 and Th17 loci, with *Ifng* marked by H3K4me3 in Th1 and H3K27me3 in Th2 and Th17, *Il4* marked by H3K4me3 in Th2 and H3K27me3 in Th1 and Th17 and *Il17a* marked by H3K4me3 in Th17 and H3K27me3 in Th1 and Th2. On the other hand, the loci for *Tbet*, *Gata3* and *Rorc*, while marked by H3K4me3 in the appropriate lineage, had more bivalent marks in the opposing lineages, suggestive of a state poised for subsequent activation or silencing. These findings imply a mechanism that may allow cells to adopt overlapping functional profiles or potentially to switch from one lineage to another.

Role of Th17 cells in RA

RA is an autoimmune disease characterized by chronic inflammation of synovial tissues in multiple joints associated with bone and cartilage damage. It affects almost 1% of the population and leads to enormous morbidity and accelerated mortality, despite recent improvements in its treatment. Several studies have evaluated the tissue distribution of IL-17 in RA. While there are some discrepancies regarding the serum

levels of IL-17 and the frequency of Th17 cells in the systemic circulation in RA, most reports agree that IL-17 is increased in the synovial fluid and synovial tissues of patients with RA. IL-17 is expressed in the T cell rich areas of the synovium and is primarily secreted by CD4+CD45RO+ memory T cells in the synovium and peripheral blood [107-115]. TGFβ, IL-6, IL-21, TNFα, IL-1β and IL-23, all of which are important in Th17 differentiation, are found in RA serum and synovial fluid, suggesting that the inflamed joint may provide the ideal cytokine milieu for the generation and maintenance of Th17 cells [116-120]. Interestingly, the frequency of Th17 cells correlates with markers of RA disease activity, such as C-reactive protein levels and tender joint count. In addition, a prospective study found that increased expression of IL-17 and TNFα mRNA in synovial tissue were independently associated with more severe joint damage progression, while expression of IFNγ was associated with protection from joint damage progression [121]. This evidence clearly points to a role for IL-17 in the pathogenesis of arthritis.

Further support for the pathogenic role of IL-17 in RA comes from *in vitro* studies that demonstrate robust and widespread inflammatory effects of IL-17 on cells of the joint. The IL-17 receptor (IL-17R) is ubiquitously expressed and initiates an inflammatory response in many cells types important to RA, including monocytes/macrophages, chondrocytes, osteoblasts and fibroblasts. IL-17 induces the production of inflammatory cytokines such as IL-1β, TNFα, IL-6 and IL-23 by a number of cell types, all of which promote inflammation and Th17 development (reviewed in [122]). Furthermore, IL-1β has been shown to induce the generation of Th17 cells from regulatory T cells [123]. Thus Th17 cells in the joint may initiate a positive feedback

loop, leading to the perpetual T-cell activation that is thought to be critical in the generation of autoimmunity.

IL-17 also induces an array of chemokines, including CXCL-1, -2, -5, -8, CCL-2 and CCL-20, leading to recruitment of T cells, B cells, monocytes and neutrophils, all of which populate the inflamed joint (reviewed in [122]). Leukocyte recruitment is further enhanced by IL-17-induced up-regulation of granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), inducers of granulopoiesis, as well as vascular endothelial growth factor (VEGF), an inducer of angiogenesis (reviewed in [122, 124]).

A significant body of evidence demonstrates that IL-17 can enhance the inflammation and cellular infiltration common in arthritis, but it can also mediate bone and cartilage damage, which cause pain and disability in RA patients. IL-17 up-regulates matrix metalloproteinases (MMPs), nitric oxide and receptor activator of nuclear factor κB (RANK)/RANK ligand (RANKL), as well as inflammatory cytokines and chemokines in chondrocytes and osteoblasts, cells of the cartilage and bone, respectively. Th17 cells can induce osteoclastogenesis indirectly by up-regulating RANKL on osteoblasts and also by directly expressing RANKL on their cell surface [110, 122, 125, 126]. All of these pro-inflammatory molecules are found in the RA synovium and can contribute to RA pathology by recruiting and activating inflammatory cells, maintaining the IL-17 response and mediating destruction of tissue and bone.

The arthritic joint presents a unique microenvironment, whereby autocrine and paracrine positive feedback loops mediated by TNFα, IL-1β, IL-6 and IL-23 promote

Th17 development and inflammation. Thus, interrupting one or more of these positive feedback loops may limit inflammation directly as well as inhibit maintenance and activation of Th17 cells in the joint, hopefully without paralyzing normal immune responses. Several studies have shown that TNFα and IL-1β, either together or separately, can induce the generation of Th17 cells and IL-17 can induce TNFα and IL-1β expression by synoviocytes as well [13, 17, 18, 32]. The precise mechanisms are still unclear, but IL-23 may be involved in the TNFα- and IL-1β-induced secretion of IL-17 [14, 127]. Additionally, there is synergy between IL-17, TNFα and IL-1β in mediating downstream effector functions. Thus it is not surprising that neutralization of TNFα in combination with IL-1β and IL-17 is most effective in suppressing IL-6 production and collagen degradation in *ex vivo* cultures of RA synoviocytes [128]. Similarly, combination blockade of TNFα and IL-17 suppressed ongoing collagen-induced arthritis and was more effective than neutralization of TNFα alone [129]. These results suggest that treatments designed to block IL-17 may be beneficial in combination with treatments that block TNFα or IL-1β, provided that this approach proved to be safe. IL-17 neutralizing therapies may also be particularly useful for the considerable number of patients who do not respond to TNFα blockade.

The value of regulating IL-17 or Th17 pathway cytokines is being tested in clinical studies of patients with inflammatory arthritis. Phase I/II clinical trials of anti-IL-17 in RA were recently completed and preliminary data suggest a therapeutic effect in at least one of these trials [130, 131]. In addition, two phase II trials of anti-IL-17 neutralizing antibody, one in psoriatic arthritis and the other in ankylosing spondylitis, are currently underway (NCT00809614 and NCT00809159). A phase II clinical trial in

psoriatic arthritis using anti-IL-12/IL-23 p40 was recently completed and results are pending (NCT00267956), and a trial with an oral IL-12/IL-23 inhibitor is ongoing in patients with RA (NCT00642629). In view of the existence of multiple IL-17 isoforms, the complexity of the IL-17 receptors, the various ways of inducing Th17 cells and the production of pro-inflammatory cytokines other than IL-17 by these cells, the best way to target the Th17 axis in human disease is far from obvious and may differ among the various forms of human inflammatory arthritis. It will likely require many years of clinical studies to sort this out, but these studies are also likely to offer further insights into the pathogenesis of human arthritis and the role of the Th17 pathway.

Role of Th17 cells in collagen-induced arthritis

Animal models of arthritis are important tools for understanding the etiology and underlying mechanisms of disease, as well as for discovering and testing new therapeutic targets. Many of these rodent models closely resemble RA pathologically, with infiltration of the joints by inflammatory cells, autoantibody production, synovial hyperplasia and erosion of cartilage and bone. Arthritis can be induced experimentally by systemic immunization with joint proteins mixed with adjuvant, local injection of microbial products or inflammatory mediators directly into the joint or genetic mutation leading to exaggerated immune responses and spontaneous joint inflammation. There are many similarities as well as important differences in the pathogenesis of these diverse animal models of arthritis, which possibly parallel the clinical, genetic and immunological subcategories of RA and other human arthritic syndromes.

One of the best characterized models of RA is collagen-induced arthritis (CIA). To induce CIA, DBA mice are immunized intradermally with type II collagen (cII) in complete Freund's adjuvant (CFA). Several weeks later, joints of the front and hind paws develop severe synovial inflammation and cellular infiltration, leading to destruction of both cartilage and bone. CIA is a T cell-dependent disease, and although Th1 cells were previously thought to be the key pathogenic subset, substantial evidence now demonstrates that Th17 cells are largely to blame. In CIA, serum IL-17 levels increase shortly after immunization, and IL-17 mRNA is up-regulated in the synovium after the onset of arthritis [132]. Many of the CD4+ T cells in the joint are IL-17 positive [133]. Several approaches have shown IL-17 to be both necessary and sufficient for joint inflammation. IL-17-deficient mice develop significantly less severe (although not completely absent) CIA, and IL-17 is important for priming collagen-specific T cells and for collagen-specific IgG2a production [134]. Administration of soluble IL-17R or neutralizing antibody to IL-17, either before or after the onset of disease, significantly reduces macroscopic joint swelling and the associated histological changes, including cellular infiltration, proteoglycan depletion, cartilage surface erosion and bone erosion [132, 135]. Conversely, local adenoviral over-expression of IL-17 in the knee of naïve or immunized mice results in aggravated joint inflammation, including increased cellular infiltration, synovial hyperplasia, RANK and RANKL expression, osteoclastogenesis, proteoglycan depletion, chondrocyte death and erosion of cartilage and bone [132, 136]. Thus IL-17 is important for both recruitment of inflammatory cells and for joint destruction. This conclusion is further supported by a multitude of *in vitro* studies which show that IL-17 can act on synovioctes to induce inflammatory cytokoines such as TNFα,

IL-1β and IL-6, chemokines such as IL-8, CXCL1 and CXCL2 and mediators of bone and cartilage loss such as RANKL and MMPs (reviewed in [122]). Interestingly, a recent paper showed that γδ T cells are major producers of IL-17 in the arthritic joints of mice with CIA [137].

Further evidence for the role of Th17 cells in CIA comes from studies on the effects of Th17-related cytokines, such as TGFβ, IL-6, IL-21 and IL-23. The actions of TGFβ are complex, with both pro- and anti-inflammatory effects. In mice, injecting TGFβ systemically inhibits CIA [138] and neutralizing antibody to TGFβ worsens disease [139], yet in rats injection of TGFβ directly into the joint results in accelerated arthritis and enhanced neutrophil recruitment, synovial inflammation and hyperplasia, while injection of blocking antibody to TGFβ inhibits acute and chronic synovial inflammation [140-143]. Thus, the precise role of TGFβ may vary greatly, depending on the species, the microenvironment or the timing.

IL-6, on the other hand, has robust and well-characterized inflammatory effects in multiple animal models. Injection of blocking antibody to the IL-6 receptor (IL-6R) at the time of immunization inhibits differentiation of Th17 cells and the development of CIA, even after a second booster immunization with collagen [133]. Soluble IL-6R or neutralizing antibody to IL-6 can also ameliorate disease [144, 145] and IL-6-deficient mice have reduced IL-17 expression and are completely resistant to CIA [29, 146].

IL-21 is produced by Th17 cells and can act in an autocrine manner to enhance Th17 development [29-32]. A role for IL-21 in a variety of autoimmune diseases and their animal models has been proposed (for review see [147]), but relatively little is

currently known about its role in arthritis. In CIA, mice treated with a soluble IL-21R-Fc fusion protein after the onset of disease demonstrate a modest but significant decrease in disease severity and down-regulation of IL-6 and IL-17 expression in spleen cell cultures, although more dramatic results were obtained in a rat model of arthritis [148].

Role of IL-4 and IFNγ in arthritis

The role of IFNγ in animal models of arthritis is complex, with evidence for both protective and pathogenic functions. CIA was originally considered to be a Th1-mediated disease due to the fact that deficiency of the p40 subunit of IL-12, the key Th1-inducing cytokine, conferred resistance to disease. However, the IL-12 p40 subunit is shared by IL-23, which supports the maintenance and pathogenicity of Th17 cells. A key observation concerning the relative roles of Th1 and Th17 cells in CIA was made by Dan Cua and colleagues, who demonstrated that IL-23, rather than IL-12, was critical for development of arthritis. Mice lacking the specific p19 subunit of IL-23 have significantly fewer Th17 cells and no joint or bone pathology, despite normal numbers of Th1 cells. Mice lacking the specific p35 subunit of IL-12, on the other hand, develop exacerbated arthritis and increased expression of many inflammatory cytokines in the joint, including TNFα, IL-1β, IL-6 and IL-17 [149]. Furthermore, multiple studies have found that mice deficient in either IFNγ or IFNγ receptor develop more severe CIA than wild type counterparts [150-153]. More recently, Chu et al. showed that deletion of the IFNγ gene from the CIA-resistant B6 strain of mice renders them susceptible to CIA and correlates with an increase in IL-6 and IL-17 expression [154].

On the other hand, administering recombinant IFNγ to mice exacerbates CIA [150, 152], and the mouse model proteoglycan-induced arthritis is dependent on IFNγ and independent of IL-17 [155, 156]. IFNγ clearly has the ability to induce inflammation in some settings, but it can also inhibit Th17 differentiation and thereby reduce inflammation in others. Thus the net effect of IFNγ may depend on the phase of disease and the location - such as the joint versus the spleen or lymph node. By administering neutralizing antibodies at different time points, one study suggested that IFNγ has pathogenic effects in the early phase of disease but protective effects in the later stages [157]. Although this study did not measure IL-17, one plausible interpretation of these results is that IFNγ takes on a protective role after Th17 cells become overabundant and highly pathogenic.

Similar to IFNγ, evidence for the role of IL-4 in arthritis is complex. IL-4-based interventions can prevent or alleviate joint inflammation and bone damage in multiple animal models of arthritis [158-160]. We have shown previously that systemic injection of dendritic cells genetically engineered to produce IL-4 (IL-4 DCs) attenuates CIA [160]. Further mechanistic studies revealed that IL-4 secreted from IL-4 DCs is a potent suppressor of IL-17 production by T cells from the early phase of CIA [161]. These results suggest that endogenous IL-4 could also play a protective role in arthritis by suppressing IL-17 in the early phase of disease. However, it leaves open the possibility that IL-4 could also have pathogenic effects by suppressing production of IFNγ, once IFNγ has taken on a protective role. In addition, IL-4 reduces bone damage in established CIA and is necessary for the development of arthritis, possibly due to the important role of IL-4 in B cell activation and antibody production [158, 162]. Thus, like

IFNγ, IL-4 may have both protective and pathogenic roles in CIA, depending on the stage of disease, location of IL-4 production and relative abundance of other cytokines. In human RA, a polymorphism of the IL-4R that results in decreased signaling is associated with more severe erosive disease, suggesting that IL-4 plays a protective role [163]. Although the underlying mechanism is unknown, work from our lab has shown that Th17 cells from healthy controls with the weak signaling IL-4R are less susceptible to down-regulation by IL-4 [164].

Chapter 2

Cytokine regulation of Th17 differentiation and re-stimulation

Regulation of *in vitro* Th17 differentiation

Naïve CD4+ T cells stimulated through the TCR can differentiate into a number of different subtypes depending on the cytokine milieu, including Th1, Th2, Th17, Treg and Tfh cells, with each subtype serving a unique functional role. To ensure that a singular, focused and appropriate type of response develops, each T helper cell lineage can suppress the development of other lineages, through both intra- and inter-cellular mechanisms. For example, previous work has shown that IFNγ, the prototypical Th1 cytokine, inhibits Th2 differentiation, while IL-4, the prototypical Th2 cytokine, inhibits Th1 differentiation. Thus we hypothesized that IFNγ and IL-4 would inhibit the differentiation of Th17 cells from naïve precursors.

To test this hypothesis we developed a system for *in vitro* Th17 differentiation. Naïve $CD4^{+}CD25^{-}CD62L^{+}CD44^{lo}$ T cells were isolated from BALB/c spleens by FACS, cultured with bone-marrow-derived dendritic cells (BM-DCs) and stimulated with anti-CD3 antibody in the presence of a Th17-skewing cytokine cocktail (TGFβ, IL-6, IL-23 and neutralizing antibodies to IL-4 and IFNγ). Th17 differentiation was measured by intracellular cytokine staining (ICS) and ELISA for IL-17A, as well as real-time PCR for IL-17A, IL-17F, IL-22, RORγt and IL-23R. Using this system we were able to induce robust Th17 differentiation, with 20 to 60% of the cells typically staining positive for IL-17A and less than 1% staining positive for IFNγ or IL-4 (following six-hour stimulation

with PMA, ionomycin and brefeldin A). Representative dot plots are shown in Figure 2.1. The results clearly demonstrate that adding IL-4 or IFNγ to Th17 cultures inhibits the expression of IL-17 protein, sometimes by as much as 95%, and with a more potent inhibition by IL-4 (Fig. 2.2). IL-4 and IFNγ also inhibited mRNA expression of IL-17A, IL-17F and RORγt (Fig. 2.3). Interestingly, however, IL-4 was able to suppress the expression of IL-23R but not IL-22, while IFNγ was able to suppress expression of IL-22 but not IL-23R (Fig. 2.3).

Multiple groups have begun to characterize the changes that occur at the level of the chromatin structure during Th17 differentiation. Naïve T cells cultured with TGFβ and IL-6 undergo histone H3 acetylation and K4 tri-methylation, two signals that mediate increased DNA accessibility and are associated with active transcription, at the promoter regions of the *Il17a* and *Il17f* genes [106]. Further work by O'Shea and colleagues has begun to examine the epigenetic regulation of the *Il17a/f* locus when Th17 differentiation is inhibited by IFNγ and IL-4. For example, they found that IL-4 inhibits H3 acetylation and K4 tri-methylation at the *Il17a* promoter (personal communication). These results suggest that the presence of IFNγ or IL-4 during the earliest stages of Th17 differentiation can supersede the Th17-skewing signals at the chromatin level and may instead push cells towards a Th1 or Th2 lineage, respectively. However, our observation that IL-23R and IL-22 are differentially regulated imply that the Th17 gene expression program is not completely reversed in the presence of IFNγ and IL-4, leaving open the possibility that these conflicting cytokine milieus may yield either a mixed population of cells or cells of a mixed phenotype. Further studies are needed to determine whether IL-22-positive/IL-17-negative "Th22" cells or IL-23R-expressing Th1 cells develop in these

circumstances and what the physiological relevance of these cells may be. Continued expression of IL-23R in Th1 cells, however, may contribute to the development of IFNγ/IL-17 double-positive cells and explain the observation that in some instances IL-23 can up-regulate IFNγ expression.

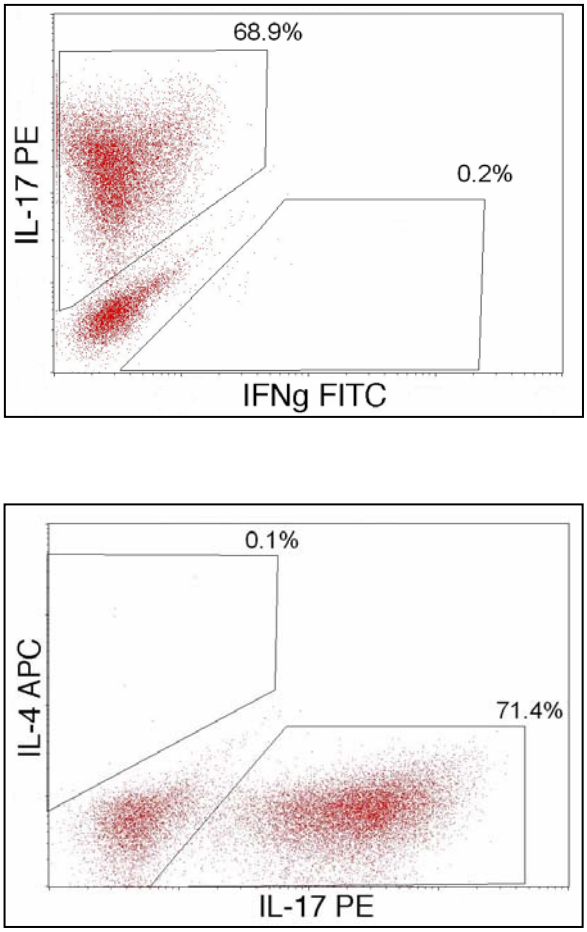
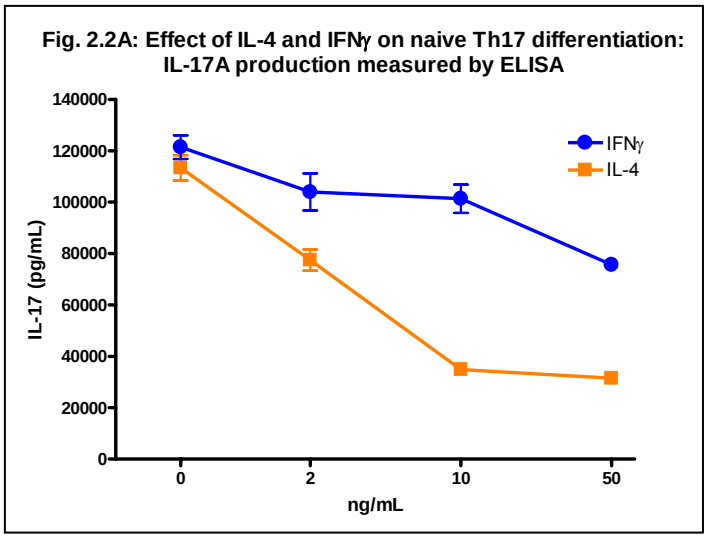


Figure 2.1: Flow cytometric analysis showing efficient *in vitro* Th17 differentiation.

Naïve $CD4^{+}CD25^{-}CD62L^{+}CD44^{lo}$ T cells were stimulated with anti-CD3 and a cocktail of Th17-skewing cytokines and neutralizing antibodies in the presence of BM-DCs for five days, rested for two days and re-stimulated with PMA, ionomycin and brefeldin A for six hours. The cells were then stained for CD4, IL-17, IFNγ and IL-4 and analyzed by flow cytometry. The results typically show 30-70% $IL\text{-}17^{+}$, with less than 0.5% $IFN\gamma^{+}$ or $IL\text{-}4^{+}$, indicating that the naïve T cells have effectively differentiated into Th17 cells.



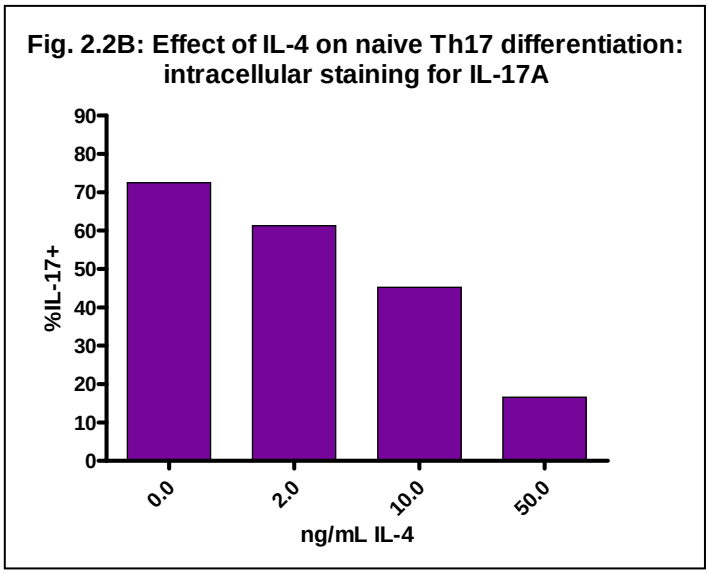
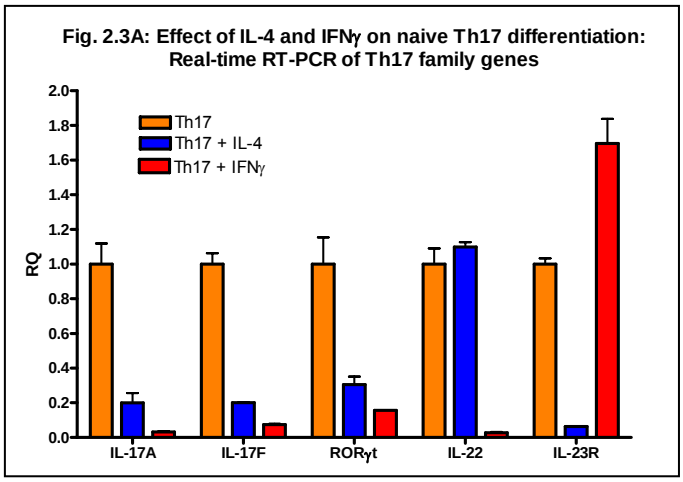


Figure 2.2: IL-4 inhibits IL-17A expression during *in vitro* Th17 differentiation.

Naïve T cells were stimulated to induce Th17 differentiation as described above, in the presence or absence of exogenous IL-4 or IFNγ (neutralizing antibodies to these cytokines were omitted from the Th17 cocktail as appropriate) and IL-17 expression was measured by ELISA (A) and ICS (B). Error bars represent SEM for triplicate cultures.



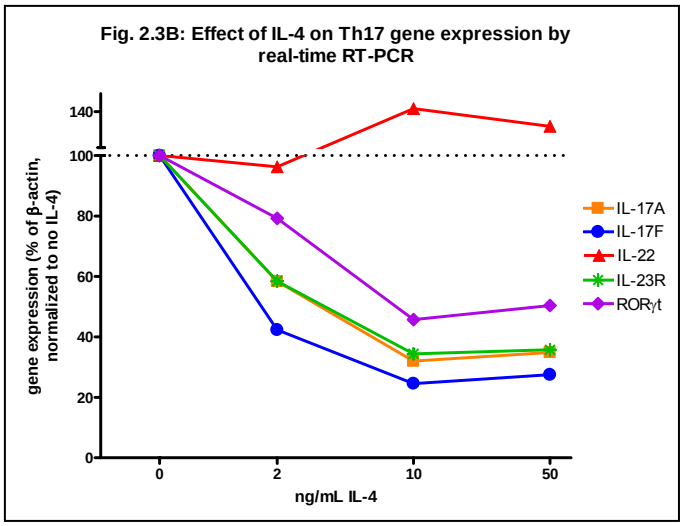


Figure 2.3: IL-4 and IFNγ inhibit expression of different subsets of Th17 genes.

Naïve T cells were cultured in Th17 conditions in the presence or absence of IL-4 or IFNγ for five days, followed by purification of RNA and analysis by Taqman-style real-time PCR for IL-17A, IL-17F, RORγt, IL-22 and IL-23R. A) IL-4 fails to suppress IL-22 expression, while IFNγ fails to suppress IL-23R expression. B) IL-4 dose response curve showing inhibition of IL-17A, IL-17F, IL-23R and RORγt but no effect on IL-22. Results were normalized first to β-actin (the internal control) and then to the untreated sample. Error bars represent the SEM of triplicate PCR reactions.

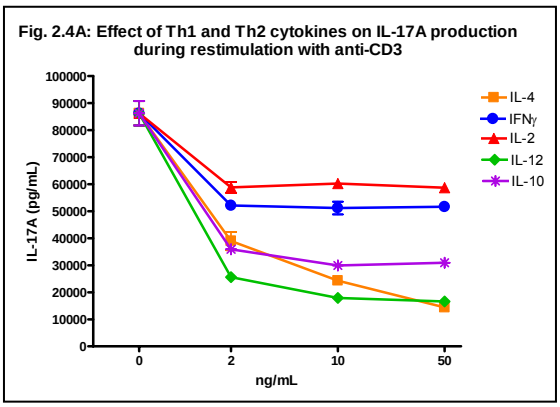
Regulation of *in vitro* Th17 re-stimulation

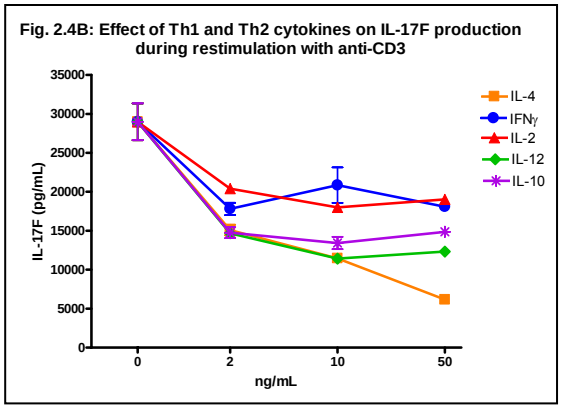
Most of what is known about the cross-regulation of T helper cells is limited to the earliest stage of differentiation, which occurs within the first few hours to days following the initial T cell activation. However, it is also important to address the regulation of T cells after differentiation is complete and upon secondary stimulation, which may correspond to what happens to a T cell that has become activated and differentiated in a lymph node in a particular cytokine milieu, and then exited the lymph node and traveled to a site of inflammation, such as an inflamed joint, where it is likely to encounter a significantly altered microenvironment. Understanding the regulation of pre-existing activated T cells may also have therapeutic applications in the formulation of strategies to control chronic T cell-mediated diseases such as RA. Knowledge of the immune system's intrinsic regulatory mechanisms may lead to a better understanding of the etiology of disease and to exploitation of these mechanisms therapeutically in order to restore a natural balance to the immune system.

To study the regulation of pre-existing Th17 cells, FACS-sorted naïve T cells were stimulated with BM-DCs, anti-CD3 and a Th17 cytokine cocktail, as described above. Th17 cells were allowed to differentiate for five or six days, washed to remove all cytokines and antibodies, and rested for two days. To test the effects of Th1 and Th2 cytokines on Th17 re-stimulation, we then re-plated the cells with increasing concentrations of recombinant IFNγ, IL-4, IL-2, IL-10 or IL-12 for two days, in the presence or absence of anti-CD3 stimulation or anti-CD3 plus the Th17-skewing cytokine cocktail. Using ELISA, we observed that each of these cytokines was able to suppress

expression of IL-17A, IL-17F and IL-22 even in the presence of strong pro-Th17 conditions, although to varying degrees (Fig. 2.4). These data imply that even after a substantial period of differentiation, Th17 cells are still susceptible to counter-regulation by opposing T helper lineages, which raises many interesting questions about the stability of lineage commitment, the mechanisms of suppression and the role of these regulatory pathways in chronic inflammation, some of which will be addressed in other chapters.

Using real-time PCR we carried out a time-course experiment to assess how quickly IL-4 and IFNγ could down-regulate the expression of IL-17A, IL-17F and RORγt in pre-differentiated Th17 cells. Interestingly, IL-4 acted very rapidly, suppressing Th17 gene expression after only four hours (Fig. 2.5). IFNγ, on the other hand, had no significant effect on IL-17 message after 6 hours, and changes were not visible even after as much as 24 hours in some experiments (data not shown). These results suggest that suppression of Th17 activation by IL-4 may be mediated by a much more direct mechanism than suppression by IFNγ. The molecular mechanisms of IL-4-mediated Th17 suppression will be explored in more detail in Chapter 3.





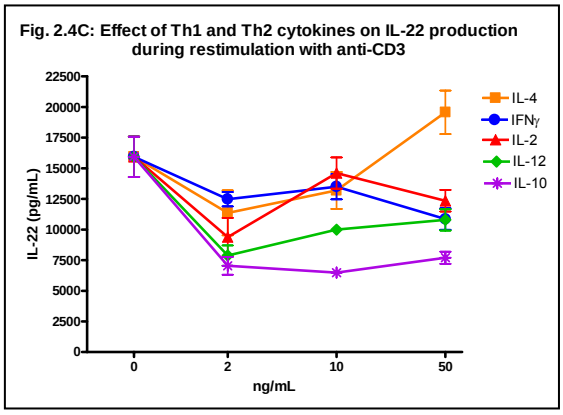


Figure 2.4: Th1 and Th2 cytokines inhibit Th17 cytokine production after differentiation.

Th17 cells were differentiated for five days, rested for two days, and then re-stimulated with anti-CD3 plus increasing concentrations of IL-4, IFNγ, IL-2, IL-12 or IL-10 for two days. Supernatants were collected and analyzed for IL-17A (A), IL-17F (B), and IL-22 (C) by ELISA. Error bars represent SEM of triplicate culture samples.

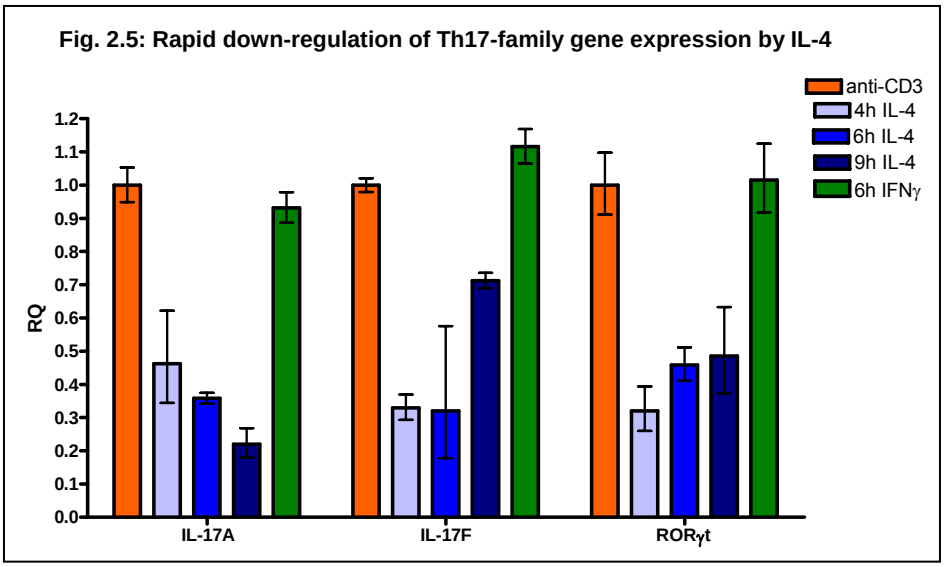


Figure 2.5: IL-4 rapidly down-regulates Th17 gene expression during re-stimulation.

Th17 cells were differentiated for five days and rested for two days and then re-stimulated with anti-CD3 and IL-4 or IFNγ. RNA was collected after four, six or nine hours and analyzed by real-time PCR using primers and probes for IL-17A, IL-17F and RORγt from Applied Biosystems. Results were normalized first to β-actin (the internal control) and then to the untreated sample. Error bars represent the SEM of triplicate PCR reactions.

Th17 plasticity

Several groups have recently demonstrated a high degree of plasticity in Th17 cells, such that stimulation with IL-12 up-regulates T-bet and IFNγ and induces a Th1-like phenotype, while stimulation with TGFβ up-regulates FoxP3 and induces a Treg-like phenotype [64, 83]. One report suggested that the Th17 phenotype is unstable, and Th17 cells will spontaneously convert to Th1 cells in lymphopenic hosts [165]. However, another group demonstrated that *in vitro*-generated Th17 cells quickly lose IL-17 expression unless IL-23 is added and opposing cytokines are blocked, while *in vivo*-generated Th17 cells continue to express IL-17 regardless of which cytokines are added [93]. Some Th17-to-Th1 conversion may not be surprising, considering the evidence for the close relationship between these two lineages. Th17 cells were first thought to be a subset of Th1 cells, and IL-17/IFNγ double-positive cells are quite common *in vivo*. Similarly, the shared dependence on TGFβ implies some Treg-Th17 commonality. There is no evidence, on the other hand, for any shared attributes between Th17 and Th2 cells, and there is no evidence for IL-17/IL-4 double-positive cells *in vivo*. However, we decided to address the question of whether prolonged culture with IL-4, the chief Th2-skewing cytokine, would induce Th17-to-Th2 conversion.

Th17 cells were generated *in vitro* as described above, with five days of differentiation followed by two days of rest. Following the rest period, the Th17 cells were re-stimulated in Th0 (anti-CD3, anti-IL-4, anti-IFNγ), Th2 (anti-CD3, IL-4, anti-IFNγ) or Th17 (anti-CD3, TGFβ, IL-6, IL-23, anti-IL-4, anti-IFNγ) conditions for two, four or six days. Th2 conversion was assessed by real-time PCR for IL-4, GATA-3 (the

master regulator transcription factor for Th2 development), IL-17 and RORγt (Fig. 2.6). The results showed that low levels of mRNA for IL-4 and GATA-3 were expressed in Th2-stimulated Th17 cell cultures, but similar levels of IL-4 and GATA-3 message were also expressed in Th17 cells stimulated with anti-CD3 alone. Thus there was no specific up-regulation in response to stimulation with IL-4, which we believe to be indicative of a small number of contaminating Th2 cells in the culture rather than induction of new Th2 differentiation. In addition, the levels of IL-4 mRNA expressed by Th17 cells re-stimulated in Th2 conditions were an order of magnitude lower than the levels of IL-4 mRNA expressed by Th2 cells (data not shown). We also looked at IL-4 expression in Th17 cells by ICS and found that less than two percent of the cells expressed IL-4 after two days re-stimulation in Th0 or Th2 conditions, which was extinguished after four or six days re-stimulation in either condition, and none co-expressed IL-4 and IL-17 (Fig. 2.7 and data not shown). Although these experiments could be refined by purifying IL-17 expressing cells to remove the possibility of inducing new Th2 differentiation rather than Th17-Th2 conversion, the results suggest that there is no Th17-to-Th2 conversion.

Although we concluded that IL-4-treated Th17 cells do not become Th2 cells, we were left wondering what it is that they do become. In other words, does encountering IL-4 render Th17 cells permanently inactivated, or is the suppression merely temporary and the Th17 cells will regain IL-17 expression upon removal of the IL-4? To answer this question, we again generated Th17 cells *in vitro* with five days differentiation and two days of rest and re-stimulated them in Th0 conditions (anti-CD3, anti-IL-4, anti-IFNγ) to maintain the existing Th17 cell population without inducing new differentiation or in Th2 conditions (anti-CD3, IL-4, anti-IFNγ) to suppress Th17 cell populations. After

five days of culture the cells were washed and rested for two days. At this point some cells were removed to assay the IL-17 and IL-4 expression by ICS, while others were put back into culture either with no stimulation or with anti-CD3 (without neutralizing antibodies). After two days Th17 function was measured by ICS for IL-17 and IL-4. The results in Fig. 2.8 show that five days of Th2 culture suppressed IL-17 production and induced IL-4 expression. Upon removal of the IL-4, however, IL-17 expression in resting cultures returned to the level of cells cultured without IL-4, suggesting that IL-4-mediated suppression of IL-17 is not stable and is reversed without continuous exposure to IL-4. On the other hand, when the IL-4-suppressed Th17 cells were put back into culture with anti-CD3, the newly differentiated Th2 cells proliferated and continued to suppress IL-17 due to the absence of IL-4-neutralizing antibodies in these tertiary cultures. We did not observe IL-17/IL-4 double-positive cells in any condition, suggesting that there was no Th17-Th2 conversion (data not shown). Although IL-17 expression appears to be easily suppressed by low doses of opposing cytokines, the rapid, stimulation-independent reversal of Th17 suppression upon removal of opposing cytokines suggests that these cells may be more stably committed than was previously appreciated.

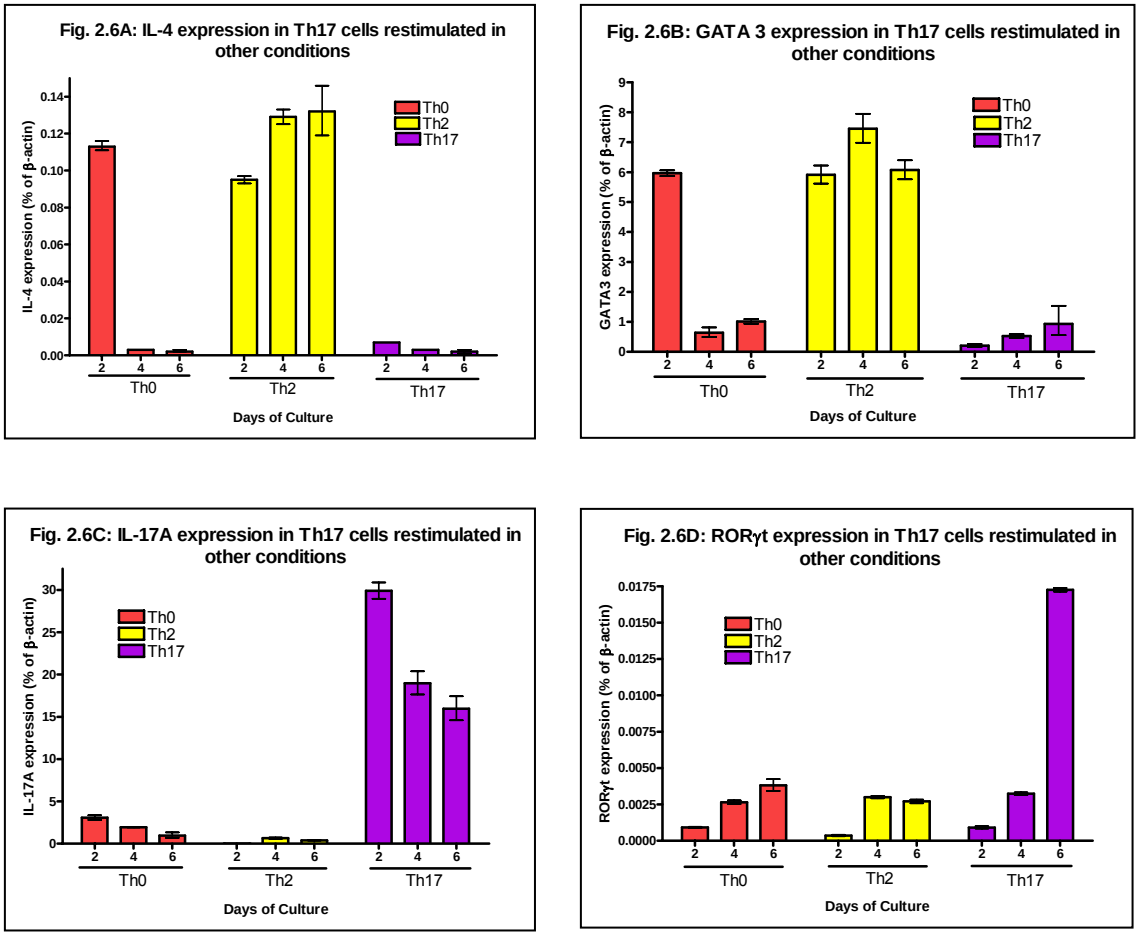
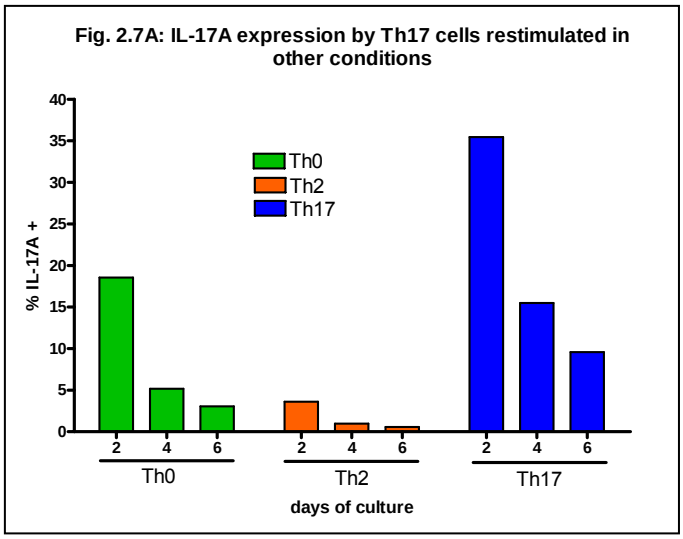


Figure 2.6: Th17 cells re-stimulated in Th2 conditions up-regulate low levels of Th2 genes.

Th17 cells were re-stimulated for two, four or six days in Th0, Th2 or Th17 conditions and expression of IL-4 (A), GATA-3 (B), IL-17A (C) and RORγt (D) were analyzed by real-time PCR. Results were normalized to β-actin expression. Error bars represent the SEM of triplicate PCRs.



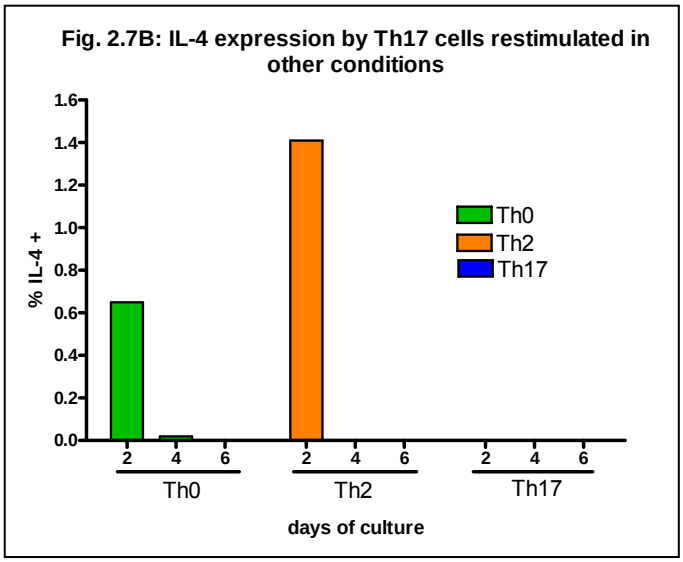


Figure 2.7: Th17 cells re-stimulated in Th2 conditions express IL-4 but it is quickly extinguished.

Th17 cells were re-stimulated for two, four or six days in Th0, Th2 or Th17 conditions and then treated with PMA, ionomycin and brefeldin A for six hours. IL-17A (A) and IL-4 (B) expression were measured by ICS.

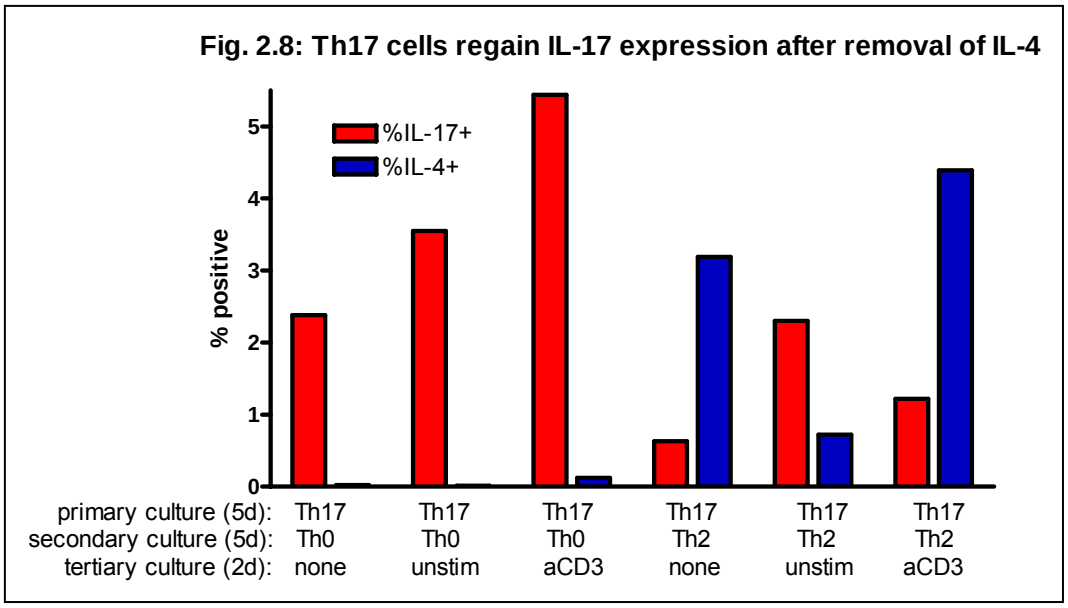


Figure 2.8: Th17 cells regain IL-17 expression after removal of IL-4

Th17 cells were generated *in vitro* as described. After five days differentiation and two days rest, the cells were re-stimulated for five days in Th0 conditions (anti-CD3, anti-IL-4, anti-IFNγ) to maintain the existing Th17 cells without inducing new differentiation or Th2 conditions (anti-CD3, 10ng/mL IL-4, anti-IFNγ) to suppress existing Th17 cells, followed by two days rest. After the second round of stimulation some cells were treated with PMA, ionomycin and brefeldin A and analyzed by ICS for IL-17 and IL-4. The remaining cells were put back into culture for two days with no stimulation or with anti-CD3 alone to allow Th17 cells to regain IL-17 expression without inducing new differentiation.

Regulation of *ex vivo* Th17 re-stimulation

We have established that many Th1 and Th2 cytokines, and most potently IL-4, can suppress the re-activation of *in vitro*-derived Th17 cells. However, these Th17 cells develop under highly un-physiological conditions and may have a very different phenotype than *in vivo*-derived, antigen-specific Th17 cells, particularly in the context of Th17-mediated inflammatory disease. In fact, *in vitro*-derived Th17 cells have been found to lack the chemokine receptor expression and EAE pathogenicity of *in vivo*-derived Th17 cells [92]. To examine the cytokine-mediated regulation of Th17 cells generated *in vivo* in the context of an IL-17-dependent autoimmune disease, we immunized DBA1/LacJ mice i.d. with chick type II collagen emulsified in CFA following our standard protocol for induction of collagen-induced arthritis and measured serum IL-17 levels by ELISA. The data in Figure 2.9 show that there is a very rapid and dramatic spike in serum IL-17 within the first few days after immunization, which likely comes from innate-like sources, including γδ T cells. After seven days the early peak subsides, and IL-17 levels remain stably and significantly elevated at about twice the baseline level throughout the course of disease, suggesting that systemic Th17 responses have developed by one week and persist long-term. Thus, rather than wait three to four weeks for arthritis to develop, we decided to assess collagen-specific Th17 responses in the spleen and draining lymph nodes (DLN) at two weeks post-immunization. Specifically, single-cell suspensions of spleens and DLNs were re-stimulated *in vitro* with collagen, with or without the Th17-skewing cocktail, and in the presence or absence of recombinant Th1 and Th2 cytokines or neutralizing antibodies. After five days IL-17 production was measured by ELISA and ICS.

Figure 2.10A shows the effects of a number of Th1 and Th2 cytokines on IL-17 production from splenocytes stimulated with collagen. The results demonstrate that, much like the *in vitro*-derived Th17 cells, pre-existing, *in vivo*-derived Th17 cells are susceptible to counter-regulation by cytokines from opposing T helper cell lineages. In particular, IL-4 and IL-12 were potent suppressors and IFNγ was a weak suppressor of collagen-specific IL-17 production. IL-13, another important Th2 cytokine like IL-4, also inhibited IL-17 production, confirming recent data from Newcomb et al. and furthering the idea that Th2 cells suppress Th17 cells through multiple mechanisms [70]. Surprisingly, we found that collagen-specific IL-17 production was up-regulated by exogenous IL-10, which differs from our previous finding that IL-10 inhibits production of IL-17A, IL-17F and IL-22 by *in vitro*-derived Th17 cells (Fig. 2.4). This discrepancy may be attributed to differences between *in vitro*- and *in vivo*-derived Th17 cells (if IL-10 acts directly on the T cell) or to differences between the BM-DCs used to induce *in vitro* Th17 differentiation and the accessory cells present in the whole spleen *ex vivo* cultures (if IL-10 acts indirectly). Although we chose not to pursue this avenue of investigation, preliminary data suggests that up-regulation of IL-17 by IL-10 is accessory cell dependent (data not shown). In addition, neutralizing antibodies to IL-4, IFNγ or IL-12 up-regulated IL-17 production, implying that *in vivo*-derived Th17 cells are constrained by endogenous cytokine production much like their *in vitro*-derived counterparts (Fig. 2.10B).

Because IL-12 is a powerful inducer of Th1 development and IFNγ production, we wondered whether suppression of IL-17 by IL-12 was mediated by increased IFNγ. We measured IFNγ and IL-17 production by spleens stimulated with collagen and IL-12

or IL-23 and found that, as predicted, IL-12 induced large quantities of IFNγ in our cultures (Fig. 2.11A). IL-23, on the other hand, up-regulated IL-17 while down-regulating IFNγ. To determine if IFNγ was required for the suppressive effects of IL-12, we stimulated spleen cells in the presence of IL-12 and neutralizing antibody to IFNγ. IL-12 continued to inhibit IL-17 expression even in the presence of anti-IFNγ, suggesting that IL-12 exerts its suppressive effect by acting directly on the Th17 cells themselves, rather than indirectly through IFNγ-producing Th1 cells (Fig. 2.11B).

Although several groups have shown that the combination of TGFβ and IL-6 acts on naïve T cells to induce Th17 differentiation [10-12], it is not clear what effect TGFβ and IL-6 have on pre-existing Th17 cells. Therefore, we decided to re-stimulate collagen-immunized spleen cells in the presence of TGFβ and/or IL-6, with the assumption that two weeks after immunization most collagen-specific T cells have already differentiated, thus the TGFβ and IL-6 are more likely to be acting on activated or memory Th17 cells rather than inducing new Th17 differentiation in naïve T cells. The results showed that IL-6 alone, and to a much greater extent TGFβ alone, was able to up-regulate IL-17, possibly due to the presence of endogenous IL-6. However, the combination of TGFβ and IL-6 was significantly better at inducing IL-17 than either cytokine alone, and adding IL-23 to the cocktail enhanced IL-17 production even further (Fig. 2.12). In addition, TGFβ, IL-6 and IL-23, either alone or in combination, induced considerable IL-17 production even in the absence of exogenous collagen, suggesting that the right cytokine milieu can stimulate pre-existing Th17 cells without the need for concomitant TCR stimulation, assuming that antigen from the *in vivo* immunization does

not persist in these cultures. The potential for Th17 cells to become antigen-independent could have important implications for the development of chronic inflammation.

Given that TGFβ, IL-6 and IL-23 can greatly enhance the activation of pre-existing Th17 cells, we wondered how these positive signals might interact with negative signals coming from Th1 and Th2 cytokines. Thus we asked the question of whether IL-4, IFNγ and IL-12 would continue to suppress IL-17 production in the presence of TGFβ, IL-6 and IL-23. Looking first by ELISA of spleen cultures, we found that the suppressive signals from IL-4 superseded any combination of activating signals and even extremely large quantities of IL-17 were still potently down-regulated (Fig. 2.13A). Similarly, ICS showed that TGFβ, IL-6 and IL-23 up-regulated, while IL-4 down-regulated, the number of IL-17+ cells in DLN cultures (Fig. 2.13B). Interestingly, while IL-4 and IFNγ continued to suppress IL-17 in the presence of TGFβ, IL-6 and IL-23, IL-12 lost its suppressive capacity. We hypothesize that there may be competition between IL-12 and IL-23 for IL-12Rβ2, the subunit which is shared by both the IL-12 and IL-23 receptors, but further work is needed to address this question.

As the data shown thus far demonstrate, Th1 and Th2 cytokines are potent regulators of Th17 cells generated *in vitro*, as well as Th17 cells generated *in vivo* during the early stages of CIA. However, we wanted to confirm that these effects are not restricted to DBA mice, the collagen-specific response or an auto-inflammatory disease, but rather that similar patterns can be generalized to all *in vivo*-derived Th17 cells. Thus we decided to analyze the Th17 responses in the more commonly used BALB/c strain of

mice following i.p. immunization with the highly immunogenic protein antigen, keyhole limpet hemocyanin (KLH).

Two weeks after immunization, spleen cells were re-stimulated with KLH and recombinant cytokines, and IL-17 production was assessed by ELISA. Despite the well-known Th2 bias in BALB/c mice, immunization induced a prominent KLH-specific Th17 response in the spleen, and the results largely confirmed our previous findings in the collagen system. TGFβ, IL-6 and IL-23 all up-regulated IL-17, while IL-4, IFNγ and IL-12 all down-regulated IL-17, even in the presence of the Th17-skewing cytokine cocktail (Fig. 2.14). The only discrepancy between the two systems was that IL-12 continued to suppress IL-17 in the presence of TGFβ, IL-6 and IL-23. The reason for this difference remains a topic for future study.

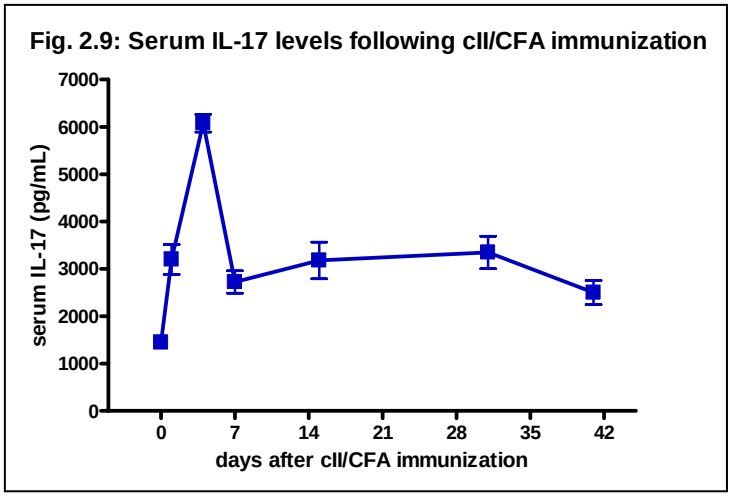
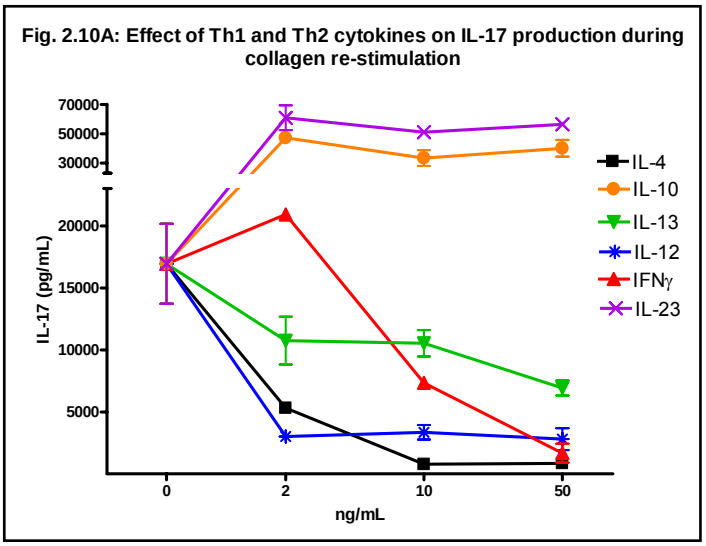


Figure 2.9: Collagen/CFA immunization induces two phases of systemic IL-17 production.

DBA mice were immunized i.d. with cII/CFA. Blood samples were collected from the tail into serum separator tubes prior to immunization and after 1, 4, 7, 15, 31 or 41 days. IL-17 levels in the serum were analyzed by ELISA. Error bars represent SEM of four individual mice at each time point.



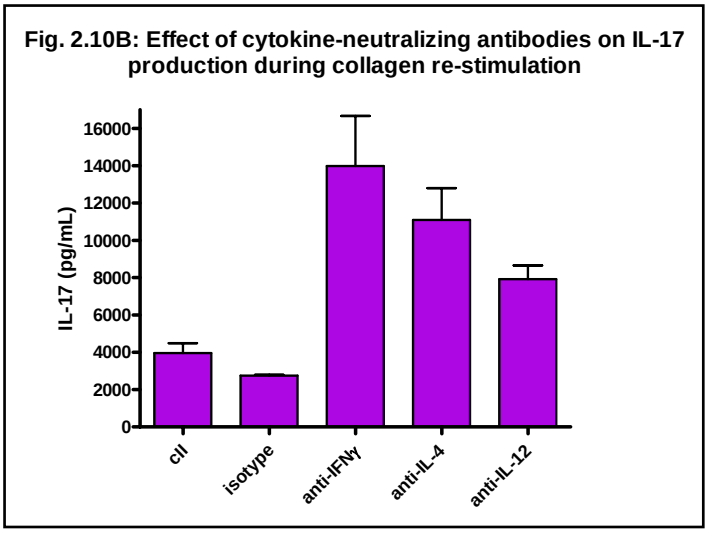
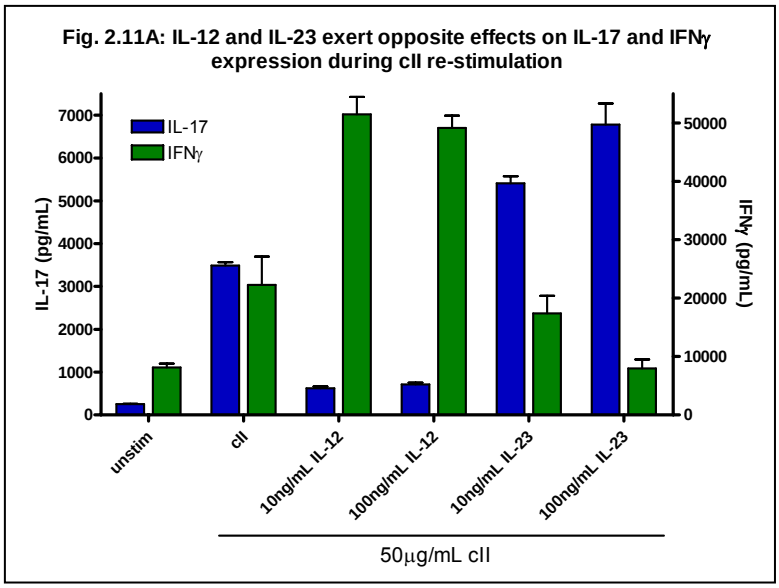


Figure 2.10: Collagen-specific IL-17 production is regulated by endogenous and exogenous Th1 and Th2 cytokines.

DBA mice were immunized i.d. with cII/CFA and spleens were collected two weeks later. Single cell suspensions were re-stimulated *in vitro* with heat-denatured collagen in the presence of recombinant cytokines (A) or purified cytokine-neutralizing antibodies (B) for five days. Supernatants were collected and IL-17 was measured by ELISA. Error bars represent SEM of triplicate culture samples.



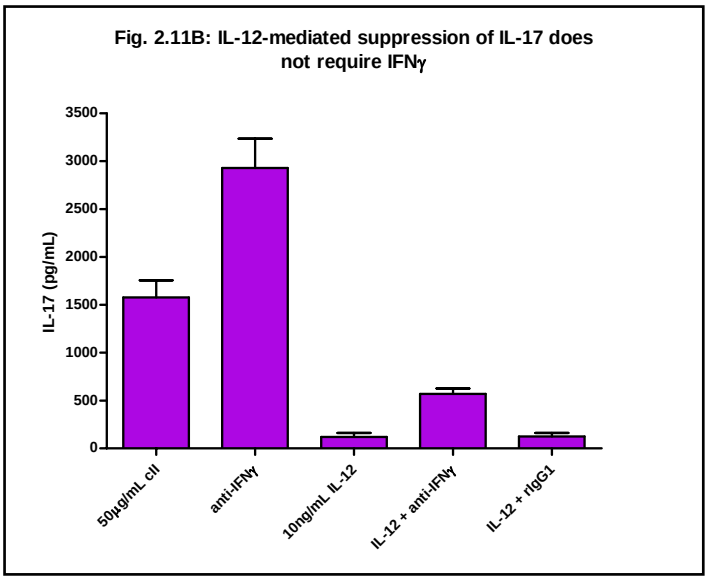


Figure 2.11: Down-regulation of IL-17 by IL-12 does not depend on up-regulation of IFNγ.

(A) Splenocytes from two-week collagen-immunized mice were re-stimulated with collagen plus recombinant IL-12 or IL-23. After five days IL-17 and IFNγ were measured by ELISA. (B) Splenocytes were stimulated with collagen in the presence of IL-12 and neutralizing antibody to IFNγ. After five days IL-17 was measured by ELISA. Error bars represent SEM of triplicate culture samples.

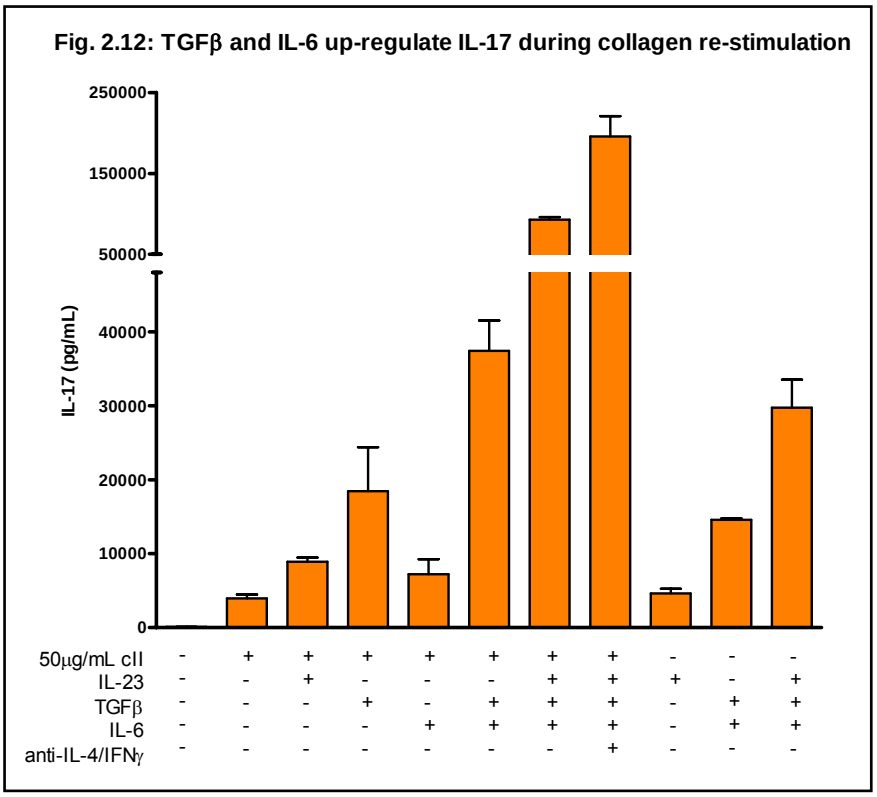


Figure 2.12: TGFβ, IL-6 and IL-23 synergistically up-regulate IL-17 production even in the absence of antigen.

Splenocytes from immunized mice were re-stimulated with or without collagen, IL-23, TGFβ, IL-6 or neutralizing antibodies to IL-4 and IFNγ. Supernatants were collected after five days and IL-17 was measured by ELISA. Error bars represent SEM of triplicate culture samples.

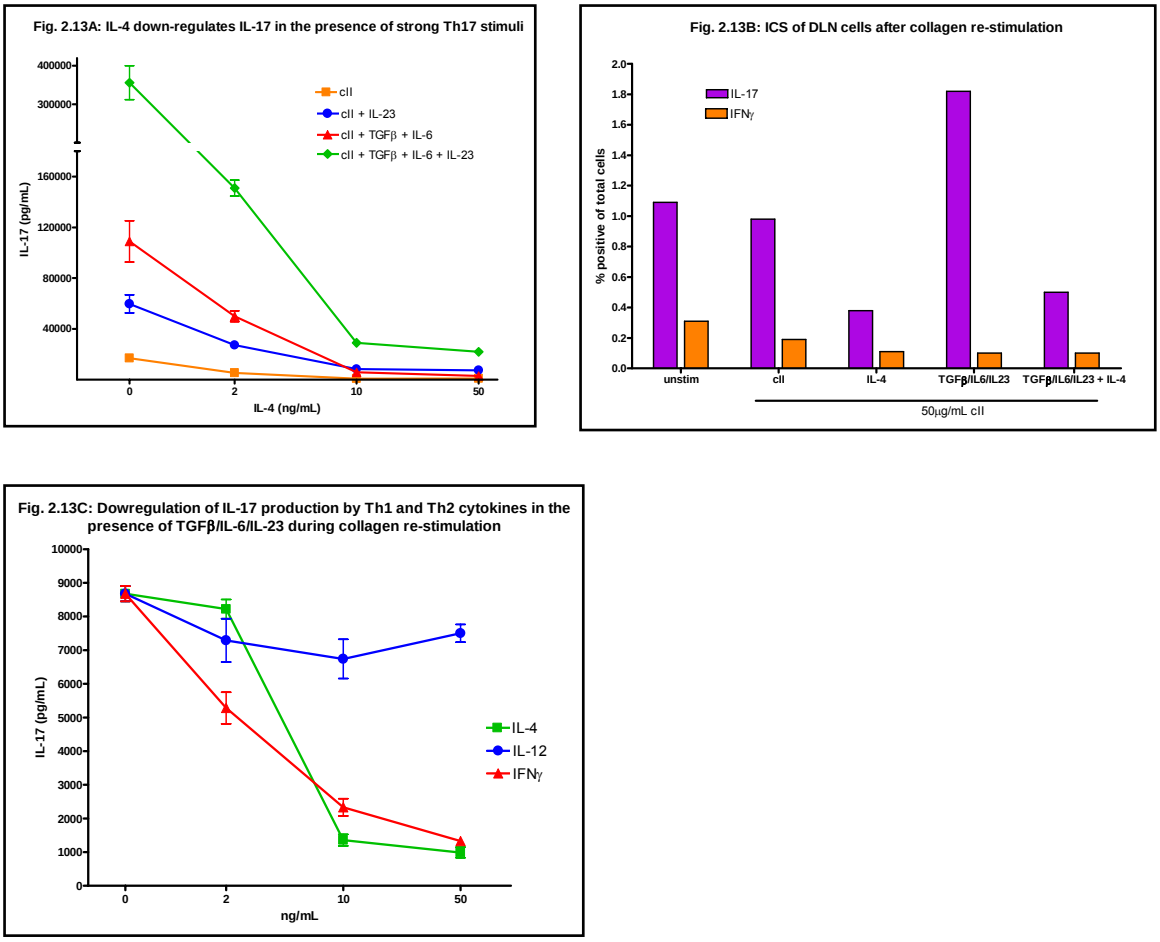
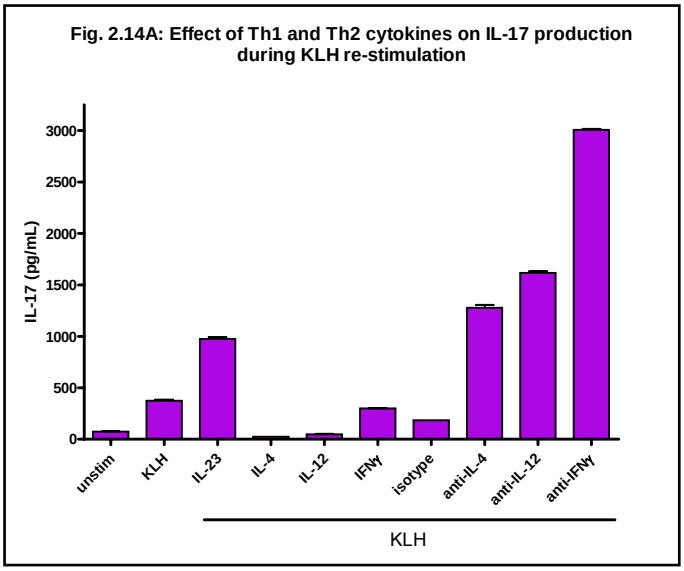


Figure 2.13: Suppression of IL-17 by IL-4 overpowers induction by TGFβ, IL-6 and IL-23.

(A) Splenocytes from two-week immunized mice were re-stimulated with collagen, TGFβ, IL-6 and IL-23 in the presence of increasing concentrations of IL-4. After five days IL-17 production was measured by ELISA. Error bars represent SEM of triplicate culture samples. (B) Inguinal lymph node cells were re-stimulated with collagen, TGFβ, IL-6 and IL-23 in the presence or absence of IL-4 for five days, followed by six hours with PMA, ionomycin and brefeldin A. IL-17 and IFNγ expression were measured by ICS. Data represents cytokine staining among total LN cells, of which approximately 25% are $CD4^+$. (C) Splenocytes were cultured with collagen, TGFβ, IL-6 and IL-23 in the presence of increasing concentrations of IL-4, IFNγ or IL-12. After five days IL-17 production was measured by ELISA. Error bars represent SEM of triplicate culture samples.



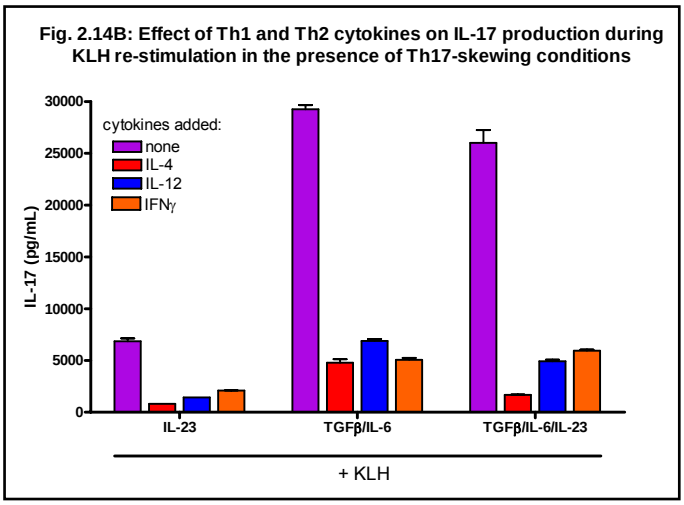


Figure 2.14: Endogenous and exogenous Th1 and Th2 cytokines regulate KLH-specific IL-17 production.

BALB/c mice were immunized i.p. with KLH in CFA. Splenocytes were collected two weeks later and re-stimulated *in vitro* with KLH plus IL-4, IFNγ, IL-12 or neutralizing antibodies to these cytokines (A). Alternatively, splenocytes were re-stimulated with KLH plus TGFβ, IL-6 or IL-23 in the presence or absence of IL-4, IFNγ or IL-12 (B). After five days IL-17 was measured by ELISA. Error bars represent SEM of triplicate culture samples.

Discussion

The data presented herein demonstrates a remarkable degree of complexity in the cytokine-mediated regulation of Th17 cells. Initial reports suggested that T helper cell cross-regulation is rather black and white: TGFβ, IL-6 and IL-23 promote Th17 cells while IL-4, IFNγ and IL-12 inhibit Th17 cells. Upon closer inspection, however, we see many shades of grey. For instance, Th17 cells developing in the presence of IL-4 may continue to express IL-22, while Th17 cells developing in the presence of IFNγ may continue to express IL-23R, suggesting that there may be an array of T helper cell subset hybrids. It is also particularly interesting that IL-12 can suppress Th17 development very potently, through both direct and indirect mechanisms, yet IFNγ and IL-4 continue to suppress Th17 activity in the presence of TGFβ, IL-6 and IL-23, while IL-12 does not. On the other hand, although IL-4 is a potent suppressor of Th17 development and cytokine expression even in the face of strong positive signals, our data suggests that IL-4-mediated inhibition of Th17 activity is unstable and short lived. This observation seems at odds with previous work from our lab demonstrating that a single injection of IL-4-transduced dendritic cells induces long-lasting protection from CIA, which is thought to be mediated by suppression of collagen-specific Th17 responses [160, 161]. One potential explanation may be that sustained suppression requires multiple negative signals, or that the negative signals must be received in the proper context – such as TCR stimulation or costimulation. However, the instability of *in vitro* suppression by IL-4 supports the fact that there is no evidence for Th17-Th2 conversion, despite the abundance of data demonstrating Th17-Th1 conversion.

These results suggest a fundamental difference in the mechanisms underlying regulation of Th17 cells by Th1 cytokines, which may be stable and induce conversion, versus Th2 cytokines, which may be unstable. How Th17 cells integrate a complex array of positive and negative signals is an interesting area for future research and likely depends on factors such as the ability of key transcription factors to bind to Th17 gene loci, which we will explore in further detail in the following chapter.

Although much attention has been given to the cytokines that regulate T helper differentiation from naïve T cells, there is a paucity of data on how cells are regulated beyond this early window. For instance, current dogma states that TGFβ and IL-6 act on naïve T cells to induce Th17 differentiation, while IL-23 acts on existing Th17 cells, but our observations suggest that TGBβ and IL-6 may be equally as important as IL-23 for augmenting the pathogenicity of effector and memory Th17 cells. In addition, recent observations demonstrate a remarkable degree of fluidity within T helper cell lineages *in vivo*, with cells converting from one lineage to another or stably expressing multiple cytokines characteristic of different lineages. Thus, much more work is needed to address the role of cytokine-mediated regulation throughout the full lifespan of each of the T helper cell subsets, as well as the ways in which our notions of divergent T helper lineages break down and the lines between distinct subsets become blurred.

Chapter 3

Signal transduction and chromatin remodeling downstream of the IL-4R

We have established that IL-4 is a potent suppressor of IL-17 expression and Th17 differentiation, which logically leads us to question the molecular mechanisms of suppression downstream of the IL-4 receptor. The IL-4R is composed of two subunits: the high affinity IL-4-binding subunit IL-4Rα and the pleiotropic cytokine receptor subunit known as the common γ chain. Binding of IL-4 to the IL-4R activates JAK1 and JAK3, which phosphorylate the IL-4Rα chain, leading to recruitment and phosphorylation of STAT6. Phosphorylated STAT6 then translocates to the nucleus, where it binds to DNA and modulates gene expression, particularly genes involved in Th2 differentiation. In addition, the phosphorylated IL-4Rα chain recruits IRS-2, which leads to activation of Akt and ERK, which are thought to play an important role in cell survival and proliferation (reviewed in [166]). Multiple reports have also shown that STAT5, the primary mediator of IL-2 receptor signaling, can be activated by IL-4 through the common γ chain, which is shared by both cytokine receptors [167-169].

Role of STAT6

We hypothesized that suppression of IL-17 by IL-4 was mediated by STAT6, and to address this question we repeated many of our previous *in vitro* experiments using STAT6-deficient Th17 cells. Wildtype or STAT6-deficient naïve T cells were stimulated with WT or STAT6-deficient BM-DCs, respectively, under Th17 conditions as described previously, with the one exception that bone marrow was cultured with GM-CSF alone,

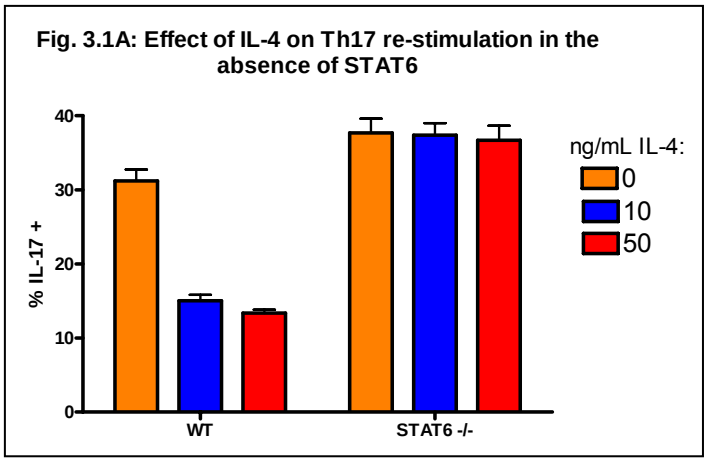
rather than IL-4 and GM-CSF, to minimize potential artifacts from differences in phenotype between WT and STAT6-deficient DCs. Looking first at suppression of re-stimulation of pre-existing Th17 cells, the results confirmed our hypothesis in that IL-4 had no effect on IL-17 expression in the absence of STAT6 (Fig. 3.1A). Similarly, by real-time RT-PCR, IL-4 failed to suppress IL-17A, IL-17F, RORγt and IL-23R expression in STAT6-deficient Th17 cells (Fig. 3.1B). Surprisingly, however, when testing inhibition of naïve Th17 differentiation, we found that IL-4 retained partial but significant suppressive effects even in the absence of STAT6 (Fig. 3.2A). To confirm these results in another culture system we stimulated whole splenocytes with anti-CD3 and measured IL-17 by ELISA, which in theory should induce a combination of naïve Th17 differentiation and memory Th17 re-stimulation. Again we found that IL-4 was able to modestly suppress IL-17 production in STAT6 knockout spleen cultures (Fig. 3.2B). To exclude potential artifacts due to an unforeseen leaky STAT6 deletion, we did Western blots of splenocytes and confirmed the absence of STAT6 in the knockout mice (Fig. 3.3).

In addition, the basal level of IL-17 production, both in culture supernatants and in the serum of un-manipulated mice, was greatly increased in the STAT6 knockouts (Fig. 3.2B and data not shown), suggesting that STAT6 mediates suppression of IL-17 by endogenous IL-4 or IL-13 (the only other cytokine known to signal via STAT6) in the steady state. In fact, a recent publication demonstrates that IL-13 can act directly on Th17 cells and inhibit IL-17 expression, which came as a surprise because previous data suggested that CD4+ T cells did not express the IL-13 receptor [70]. We found that there is also increased basal production of IL-17 in culture supernatants from IL-4Rα knockout

mice as compared to wildtype (see Figure 3.5A, below), confirming the role of endogenous IL-4 or IL-13 (the IL-4Rα chain is shared by the IL-13R) in suppressing Th17 development, but IL-17 levels are significantly higher in STAT6 knockouts than in IL-4Rα knockouts, suggesting that STAT6 may also suppress IL-17 through IL-4Rα-independent mechanisms.

Given that STAT6 is a transcription factor, we hypothesized that it may be playing a role in the regulation of IL-17 production by directly binding to DNA in the *Il17a*, *Il17f* and *Rorc* loci and inhibiting transcription. Sequence analysis of these loci identified potential STAT6 binding sites approximately 4 kb upstream of the *Il17a* locus and 6 kb upstream of the *Rorc* locus, as well as a couple sites in introns of both genes. While none of these sites are ideally situated (i.e., within the proximal promoter close to and directly upstream of the transcription start site), many transcription factors have been shown to alter gene expression from distant or intronic sites. Thus we decided to look for STAT6 binding at these sites in IL-4-treated Th17 cells by chromatin immunoprecipitation (ChIP). Unfortunately we were unable to detect any signal above the background in any of our attempts at STAT6 ChIP, even at positive control sites such as the IL-4 promoter in IL-4-stimulated Th2 cells, despite significant efforts at trouble-shooting (data not shown). Thus we cannot conclusively rule out a false-negative result and the possibility of STAT6 binding to Th17 loci. Based on personal communication with John O'Shea at the NIH, who has had success with STAT6 ChIP-seq, there is no evidence for STAT6 binding to the IL-17 promoter in Th2 cells. However, evidence also suggests that Th2 cells silence gene expression at the IL-17 locus by regulatory nucleosomes, which may occlude potential STAT6 binding sites in the DNA [87]. The

possibility remains that STAT6 binds to the IL-17 locus in Th17 cells (when the chromatin is in an open conformation) but not in Th2 cells (when the IL-17 locus is epigenetically silenced).



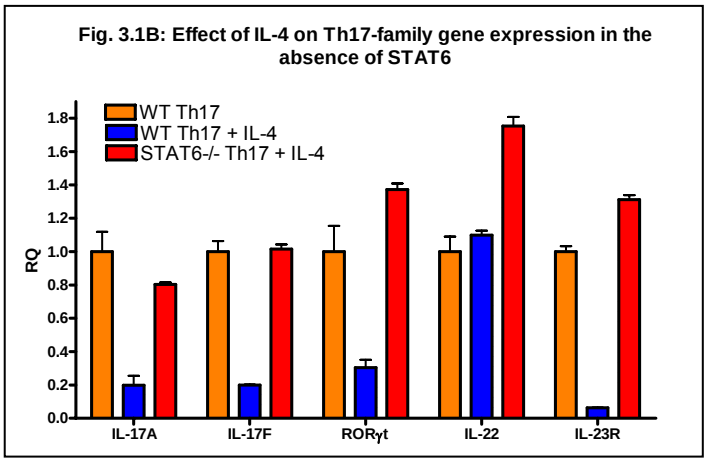
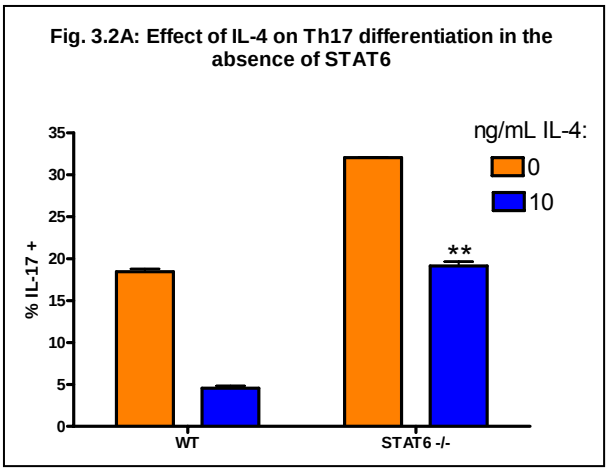
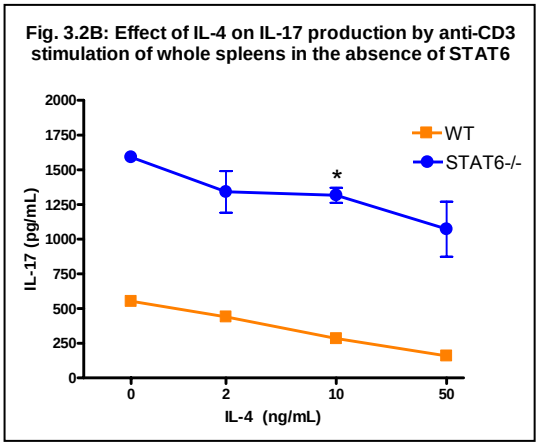


Figure 3.1: Suppression of Th17 re-stimulation by IL-4 depends on STAT6.

Wildtype or STAT6-deficient naïve T cells were cultured under Th17 conditions for five days, rested for two days and then re-stimulated with anti-CD3 in the presence or absence of IL-4 for two days. (A) Cells were treated for six hours with PMA, ionomycin and brefeldin A and IL-17 expression was measured by ICS. Error bars represent SEM of triplicate cultures. (B) RNA was collected and IL-17A, IL-17F, RORγt, IL-22 and IL-23R expression was measured by real-time PCR with primers and probes from Applied Biosystems. Data is normalized first to β-actin, the internal control, and then to the matched sample without IL-4. Error bars represent SEM of triplicate PCRs.





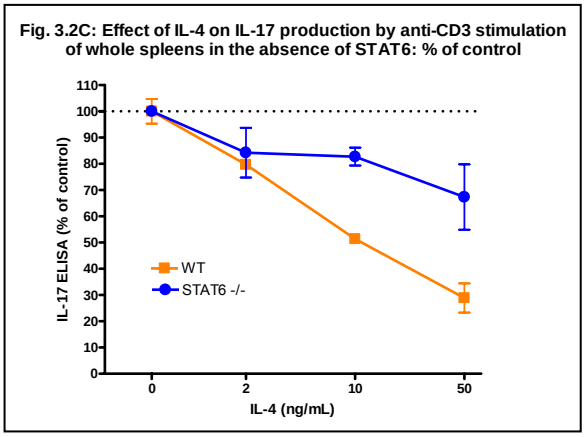


Figure 3.2: IL-4 maintains partial suppression of IL-17 in the absence of STAT6.

(A) Wildtype or STAT6-deficient naïve T cells were stimulated with Th17-skewing conditions in the presence or absence of IL-4 for five days, rested for two days and re-stimulated with PMA, ionomycin and brefeldin A. IL-17 was measured by ICS. Error bars represent SEM of triplicate culture samples. ** $p<0.01$ versus no IL-4 (B) Whole spleen cells from wildtype or STAT6-deficient mice were stimulated with anti-CD3 and increasing concentrations of IL-4 for five days and IL-17 was measured by ELISA. Error bars represent SEM of triplicate culture samples. * $p<0.05$ versus no IL-4. In (C) the data from (B) was normalized to the IL-17 expression in the sample with no IL-4 to more clearly show the efficiency of suppression by IL-4.

Role of STAT5

As mentioned above, published reports suggest that the IL-4R can activate STAT5 in addition to STAT6 [167-169]. Previous work from O'Shea and colleagues also demonstrates that STAT5, downstream of the IL-2 receptor, can bind to the IL-17 promoter and inhibit Th17 differentiation [170]. Thus we hypothesized that partial suppression of IL-17 by IL-4 in the absence of STAT6 was mediated by STAT5. As a proof of principle we confirmed that IL-4 induces STAT5 phosphorylation in WT and STAT6-deficient T cells by Western blot (Fig. 3.3). The results show that IL-4 does, in fact, activate STAT5, albeit to a much lesser extent than IL-2. In addition, we observed enhanced STAT5 activation in STAT6 knockouts, suggesting that there may be competition between STAT5 and STAT6 for binding sites on IL-4Rα and/or compensation for the lack of STAT6 by STAT5.

To directly determine the role of STAT5, we obtained spleens from mice with CD4+ T cell-specific deletion of STAT5 (*Stat5a/b*$^{fl/fl}$:*CD4-Cre*) from Dr. John O'Shea (NIH) and tested the effect of IL-4 on IL-17 expression during stimulation with anti-CD3 (Fig. 3.4) or Th17-polarizing conditions (Fig 3.5). In both conditions, the IL-4 dose-response curve of the *Stat5a/b*$^{fl/fl}$:*CD4-Cre* spleen cell cultures closely recapitulated that of the WT cultures, indicating that STAT5 is not required for suppression of IL-17 by IL-4. Although the basal level of IL-17 production is greatly increased in the *Stat5a/b*$^{fl/fl}$:*CD4-Cre* cultures, this difference could be attributed to a defect in Th17 suppression downstream of IL-2 [170]. However, a potentially confounding factor with these mutant mice is that the majority of the CD4+ T cells in the periphery appear to be

previously activated Th17 cells. To address the effect of IL-4 on differentiation and re-stimulation of Th17 cells that more closely resemble WT, we obtained *Stat5a/b*$^{fl/fl}$:*CD4-Cre* thymi and repeated the experiment using purified CD4 single-positive thymocytes. The results of the thymocyte cultures recapitulated the spleen cultures: the CD4 single-positive thymocytes efficiently differentiated into Th17 cells *in vitro* and were potently suppressed by IL-4, regardless of the presence or absence of STAT5 (Fig. 3.6A). As one might expect given the predominance of Th17 cells in the periphery of *Stat5a/b*$^{fl/fl}$:*CD4-Cre* mice, the baseline levels of IL-17 production are considerably higher in the *Stat5a/b*$^{fl/fl}$:*CD4-Cre* spleen cultures than in the WT spleen cultures (Fig. 3.4 and 3.5). Also as predicted, when we circumvented this problem by purifying single-positive thymocytes, the basal levels of Th17 differentiation equalize, and the levels are slightly higher in the WT than in the *Stat5a/b*$^{fl/fl}$:*CD4-Cre*, possibly due to decreased survival or proliferation of the *Stat5a/b*$^{fl/fl}$:*CD4-Cre* cells as a result of the defect in IL-2 receptor signaling (Fig. 3.6A). Confirming the functional absence of STAT5 in the *Stat5a/b*$^{fl/fl}$:*CD4-Cre* thymocytes, Figure 3.6B reproduces the data from O'Shea and colleagues showing a lack of Th17 suppression by IL-2.

The possibility remains that STAT5 only mediates suppression of IL-17 downstream of IL-4 as a compensatory mechanism in the absence of STAT6, which is supported by the enhanced STAT5 activation in the STAT6 knockout (Fig. 3.3). Testing this hypothesis would require either breeding STAT5/STAT6 double-deficient mice or using siRNA to knock down STAT5 expression in STAT6-deficient T cells and is an area for future study.

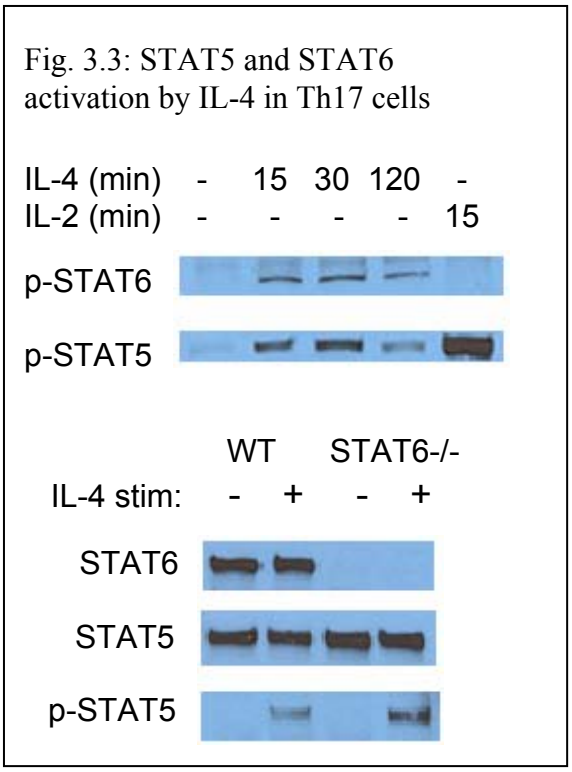
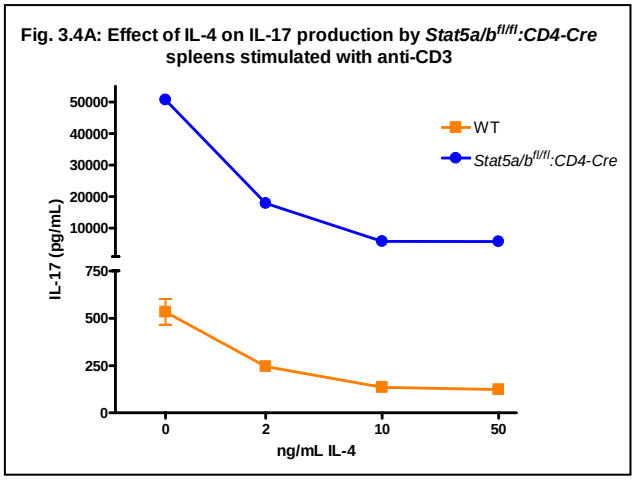


Figure 3.3: IL-4 activates STAT5 and STAT6 in Th17 cells.

Wildtype or STAT6-deficient Th17 cells were generated *in vitro* as described and rested overnight in low-serum media with cytokine-neutralizing antibodies to reduce the background level of STAT activation. Cells were then washed and stimulated with 50ng/mL IL-4 or IL-2 for 15min, unless stated otherwise, and lysed with PhosphoSafe Lysis Buffer (Novagen). Lysates were reduced and run on 10% SDS-PAGE gels and stained with antibodies for phospho- or total STAT5 or STAT6.



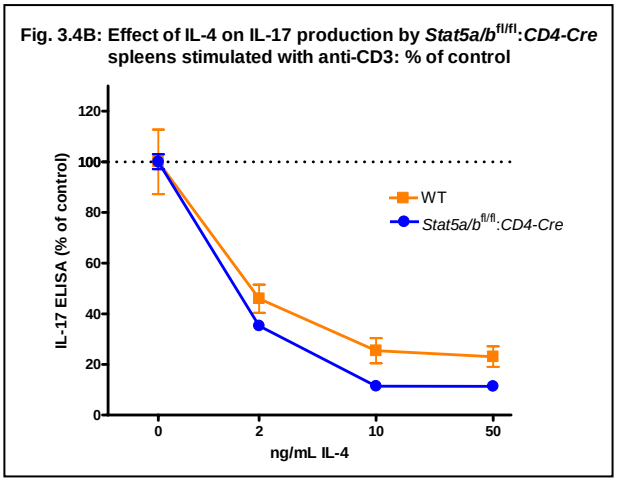
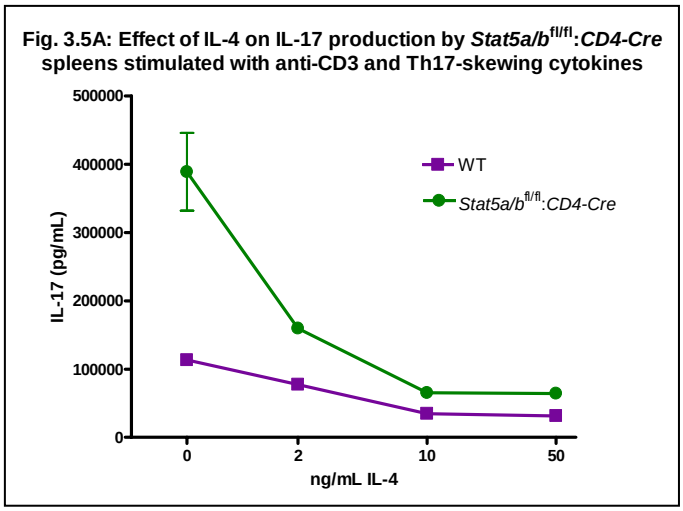


Figure 3.4: STAT5 is not required for suppression of IL-17 production by anti-CD3-stimulated splenocytes.

(A) Spleen cells from wildtype or *Stat5a/b*$^{fl/fl}$:*CD4-Cre* mice were stimulated with anti-CD3 with increasing concentrations of IL-4. After five days IL-17 expression was measured by ELISA. Error bars represent SEM of triplicate culture samples. (B) The same results as in (A) with IL-17 expression normalized to the sample with no IL-4 to more clearly show the efficiency of suppression by IL-4.



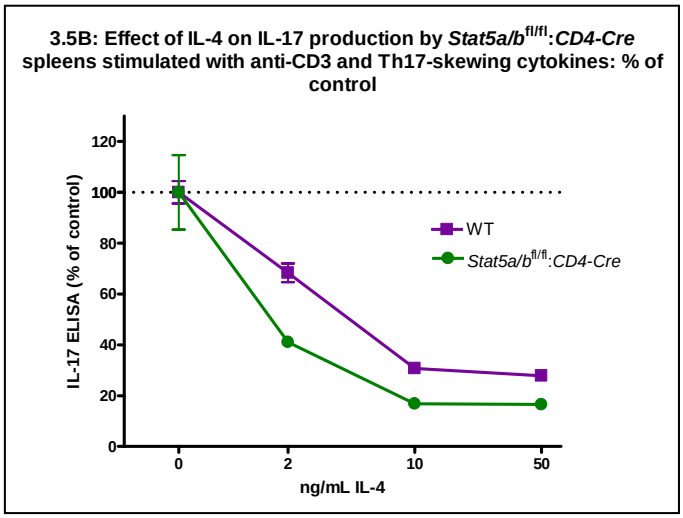
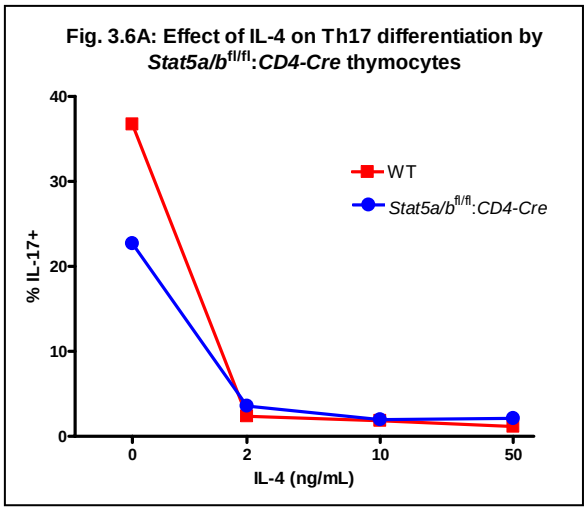


Figure 3.5: STAT5 is not required for suppression of IL-17 production by Th17-stimulated splenocytes.

(A) Spleen cells from wildtype or *Stat5a/b*$^{fl/fl}$:*CD4-Cre* mice were stimulated with anti-CD3 and the Th17-skewing cocktail plus increasing concentrations of IL-4. After five days IL-17 expression was measured by ELISA. Error bars represent SEM of triplicate culture samples. (B) The same results as in (A) with IL-17 expression normalized to the sample with no IL-4 to more clearly show the efficiency of suppression by IL-4.



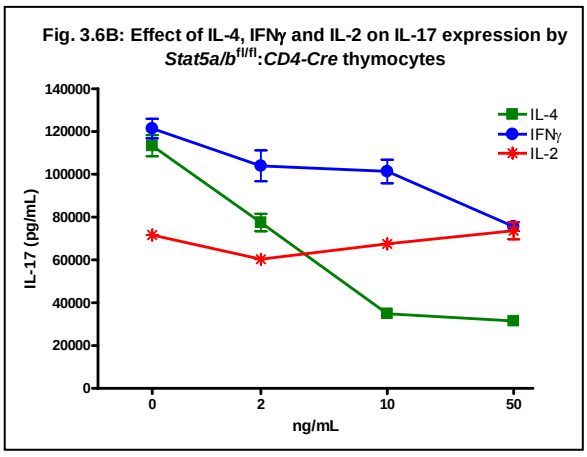


Figure 3.6: IL-4 and IFNγ but not IL-2 inhibit IL-17 expression by *Stat5a/b*$^{fl/fl}$:*CD4-Cre* thymocytes.

CD4 single-positive thymocytes were isolated by negative selection MACS from wildtype or STAT5-deficient thymi and stimulated with BM-DCs under Th17-polarizing conditions, in the presence or absence of IL-4, IFNγ or IL-2, for five days. Th17 differentiation was measured by ICS (A) or ELISA (B). Part (B) depicts only the STAT5-deficient cultures. Error bars represent SEM of triplicate culture samples.

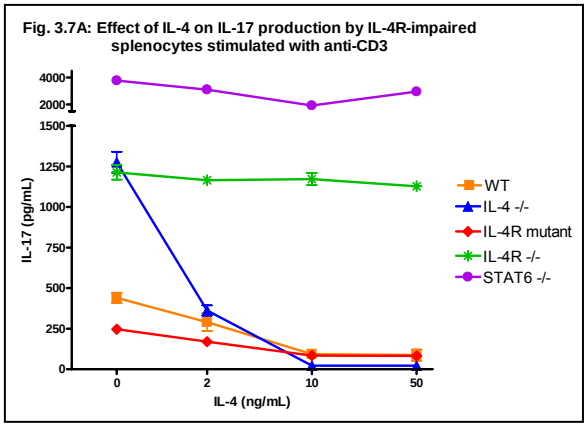
Role of IRS-2

In addition to activating the STAT pathways, phosphorylation of the IL-4Rα chain leads to recruitment of IRS-2, which serves as an adaptor for a number of other signaling proteins, including the p85 subunit of PI3K and Grb2. Activated PI3K then activates Akt, while Grb2 recruits SOS/Raf, which activates MEK1/2 and ERK1/2. Detailed structure-function analyses of the IL-4Rα chain initially led to the conclusion that signals generated through STAT6 primarily activate differentiation-specific gene programs, while IRS-2 mediates mitogenic and anti-apoptotic signals (reviewed in [166]). However, more recent data using primary T cells from STAT6 and IRS-2 deficient mice demonstrated that both STAT6 and IRS-2 are required for the mitogenic response to IL-4 and that IRS-2 plays a previously unrecognized role in the development of Th2 cells [171].

To address the role of IRS-2 in IL-4-mediated Th17 regulation we obtained mice bearing a mutation in the IL-4Rα chain that prevents recruitment and activation of IRS-2 without affecting STAT6 activation. As controls we included STAT6 knockout mice, as well as mice deficient in either IL-4 or the IL-4Rα chain. Total splenocytes were stimulated with anti-CD3 alone or anti-CD3 plus Th17 conditions in the presence or absence of IL-4, and IL-17 production was measured by ELISA (Fig. 3.7). The results from stimulation with anti-CD3 alone show that the STAT6 knockout, IL-4 knockout and IL-4Rα knockout all had a higher baseline levels of IL-17 production than WT. Addition of IL-4 had no effect on IL-17 production in the IL-4Rα knockout, a moderate effect in the STAT6 knockout and a potent effect in the IL-4 knockout and WT. The effect of IL-4 in the presence of Th17-skewing conditions was comparable to the effect in cultures

stimulated with anti-CD3 alone. The only notable discrepancy between the two stimuli was that the differences between the baseline level of IL-17 in the WT, IL-4 knockout, IL-4Rα knockout and STAT6 knockout that were evident with anti-CD3 alone were masked in the Th17-skewing conditions, possibly due to the addition of neutralizing antibody to IL-4 or the fact that IL-17 production reached a maximal level.

Interestingly, the IL-4Rα mutant cultures, which cannot activate IRS-2 in response to IL-4, produce less IL-17 than WT with either anti-CD3 or Th17 stimulation but respond to exogenous IL-4 equally or slightly better than WT. These results suggest that IRS-2 activation is not required for suppression of IL-17 expression in response to IL-4 but that there may be an unanticipated role for IL-4-induced IRS-2 signaling in promoting Th17 responses. Similarly, although IL-4 is commonly known to inhibit Th1 responses, there is some evidence that IL-4 can also support Th1 differentiation, possibly at a very early stage or indirectly through effects on APCs. IL-4-induced IRS-2 signaling may promote survival or proliferation of Th17-precursors or memory cells rather than having a direct effect on Th17 differentiation. Alternatively, IRS-2 may support Th17 development indirectly, via survival, proliferation or activation of splenic antigen presenting cells, for example. Further studies are needed to determine the key cellular targets and the relative contributions of Akt and ERK to this pathway.



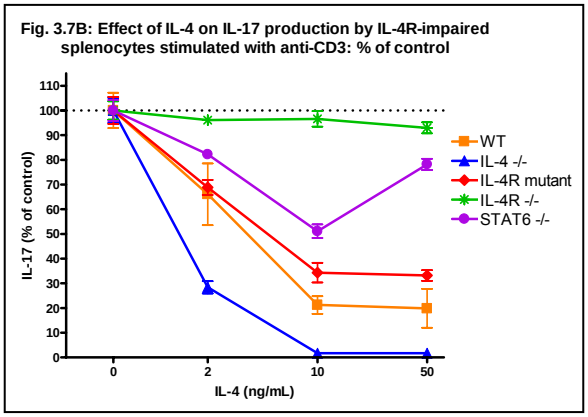
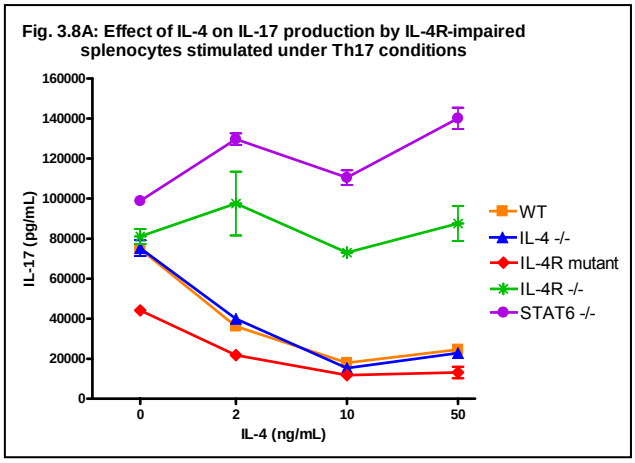


Figure 3.7: Effect of IL-4 on IL-17 production by IL-4R-impaired splenoctyes stimulated with anti-CD3.

Spleen cells from wildtype, IL-4-deficient, IL-4R-deficient, IL-4R-mutant and STAT6-deficient mice were stimulated with anti-CD3 and increasing concentrations of IL-4 for five days. Supernatants were anyalzyed by IL-17 ELISA. Error bars represent SEM of triplicate culture samples. In (B), the data from (A) was normalized to the IL-17 expression in the condition with no IL-4.



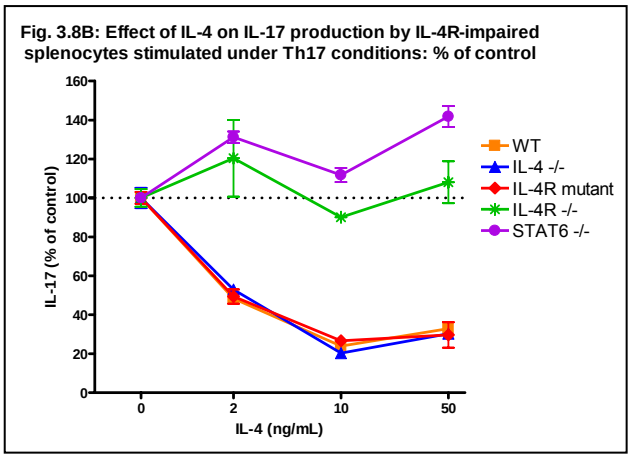


Figure 3.8: Effect of IL-4 on IL-17 production by IL-4R-impaired splenocytes stimulated under Th17 conditions

Spleen cells from wildtype, IL-4-deficient, IL-4R-deficient, IL-4R-mutant and STAT6-deficient mice were stimulated with anti-CD3 and Th17-polarizing cytokines with increasing concentrations of IL-4 for five days. Supernatants were anyalzyed by IL-17 ELISA. Error bars represent SEM of triplicate culture samples. In (B), the data from (A) was normalized to the IL-17 expression in the condition with no IL-4.

Role of GATA3

Our studies on the role of STAT6 in suppression of IL-17 by IL-4 demonstrate that STAT6 is activated downstream of the IL-4R in Th17 cells, but we were unable to demonstrate direct binding of STAT6 to the *Il17a* locus. Previous data from other groups, however, suggests that suppression of Th1 development by IL-4 is mediated by GATA-3, the transcription factor induced by IL-4 that acts as the master regulator of Th2 differentiation [51, 55, 72, 101]. Using real-time PCR, we looked at the very early effects of IL-4 on GATA-3 expression in Th17 cells stimulated with anti-CD3. The results in Figure 3.9 show that GATA-3 is up-regulated as early as two hours after the addition of IL-4, prior to down-regulation of IL-17. We also found many potential GATA-3 binding sites near the *Il17a* and *Rorc* transcriptional start sites. Thus we hypothesized that IL-4 activates STAT6, which goes to the nucleus to induce GATA-3 expression, and GATA-3 binds to DNA in the *Il17a/f* and *Rorc* loci, inhibiting Th17 activity.

To confirm our hypothesis that STAT6 acts indirectly, we attempted to determine if IL-4-induced suppression of Th17 gene expression requires synthesis of new proteins by adding the protein synthesis inhibitor cycloheximide. These experiments were inconclusive, however, because we found the effects of cycloheximide to be highly variable, and the addition of cycloheximide alone often significantly down-regulated IL-17 expression, regardless of the presence or absence of IL-4, despite very brief exposure (data not shown). Further experiments are necessary to determine if it is possible to find the right balance between efficacy and toxicity. However, we decided to move forward with the working hypothesis that STAT6 acts indirectly and look for direct binding of

GATA-3 to Th17 loci by ChIP. Although we consistently saw an increased signal at multiple loci when Th17 cells were treated with IL-4, the expression levels were not high enough above the background (i.e., ChIP with rat IgG) to allow us to draw any conclusions (data not shown). Like the STAT6 ChIP, these experiments require further trouble-shooting for the immunoprecipitation step, and without a strong positive control it is difficult to rule out a false-negative result due to technical issues with the antibody to GATA-3.

To address the role of GATA-3 in suppression of IL-17 expression downstream of IL-4, we received spleens from mice with an induced GATA-3 deletion as a kind gift from the laboratory of Dr. J.D. Engel at the University of Michigan. Because GATA-3 plays multiple roles in embryonic development in addition to the role in Th2 differentiation, mice bearing a germline *Gata3* deletion are non-viable. To create conditional knockouts, mice bearing a floxed *Gata3* gene (flanked by loxP sites) were crossed to mice expressing Cre recombinase under the control of the interferon-inducible *Mx1* promoter [172-174]. The resulting $Gata3^{fl/fl}$:Tg^{Mx1cre} mice were injected three times i.p. with synthetic double-stranded RNA [polyinosinic-polycytidylic acid (pI-pC)] to induce IFN expression, leading to up-regulation of Cre recombinase and excision of the *Gata3* gene. Spleen cells from these mice and control mice (bearing one wildtype *Gata3* allele without loxP sites, lacking *Mx1cre* or untreated with pI-pC) were cultured with anti-CD3 in the presence or absence of Th17-skewing cytokines, and the effect of IL-4 on IL-17 expression was measured by ICS and real-time PCR. The results show that deletion of *Gata3* had no effect on suppression of IL-17 by IL-4, in both anti-CD3 and Th17-stimulated cultures, as measured by both ICS (Fig. 3.10) and qRT-PCR (Fig. 3.11).

The data suggests that there is a slight increase in IL-17 expression in spleens lacking GATA-3, which may be an indirect result of the fact that in the absence of GATA-3 Th2 differentiation is impaired and more naïve T cells can differentiate into Th17 cells.

To confirm that the inducible deletion was successful, we measured GATA-3 mRNA expression in these spleen cultures by real-time PCR. The results in Figure 3.12 demonstrate that GATA-3 expression was reduced by approximately 70% under all culture conditions. Although this level of deletion may be less than ideal, it is not outside the expected range for *in vivo* conditional deletion. Despite the incomplete deletion, we believe that the fact that there was clearly no change in the dose-response of IL-4 in these cultures implies that GATA-3 is not required for suppression of IL-17. However, to confirm these results in a cleaner system we plan on repeating the experiments using purified CD4+ T cells rather than whole spleens to get a more accurate measurement of GATA-3 expression specifically within our cells of interest. If necessary, potential future experiments to improve *Gata3* deletion include crossing *Gata3*$^{fl/fl}$ mice with a strain carrying a more effective inducible Cre, such as the tamoxifen-inducible system, or transfecting *Gata3*$^{fl/fl}$ T cells with retroviral Cre *in vitro* and sorting for transfectants based on expression of a co-transfected marker.

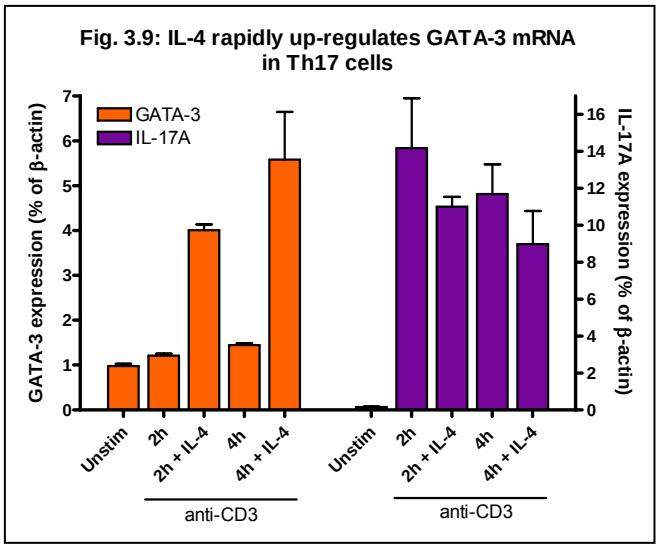
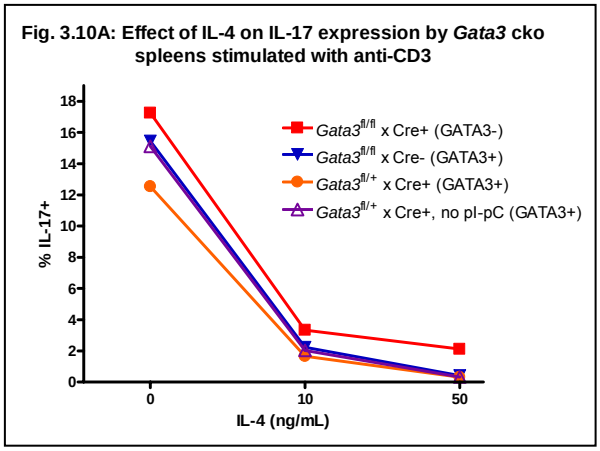


Figure 3.9: IL-4 rapidly up-regulates GATA-3 mRNA in Th17 cells.

Th17 cells were generated *in vitro* as described. After a week the cells were re-stimulated with anti-CD3 in the presence or absence of 50ng/mL IL-4 for two or four hours. RNA was collected and message levels for IL-17 and GATA-3 were analyzed by real-time PCR with primers and probes from Applied Biosystems. Expression is normalized to β-actin. Error bars represent SEM of triplicate PCR reactions.



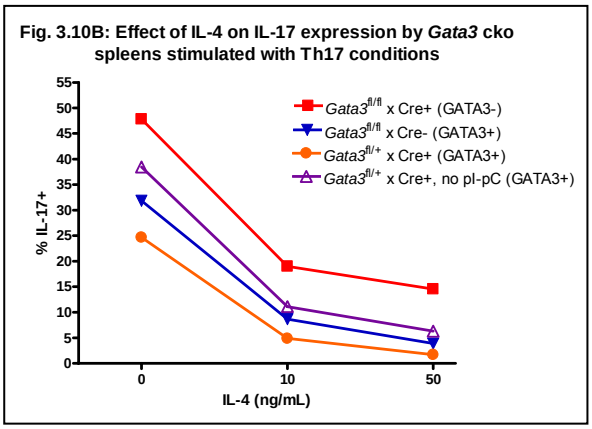
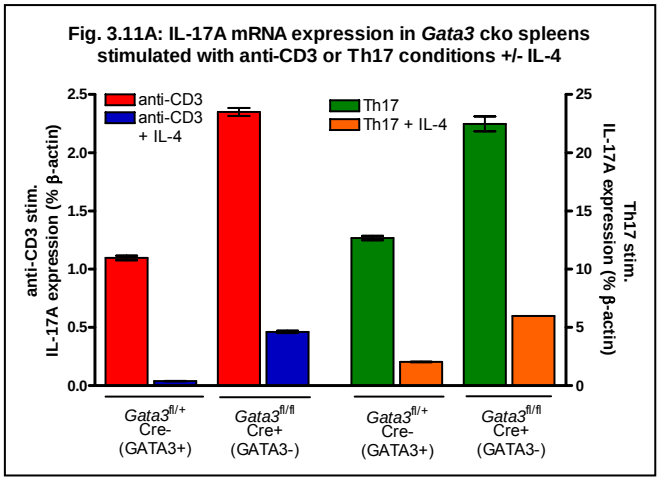


Figure 3.10: Effect of IL-4 on IL-17 production by *Gata3* cko spleens.

Spleen cells from mice with inducible *Gata3* deletion or controls without deletion were stimulated with anti-CD3 (A) or anti-CD3 plus Th17-polarizing cytokines (B) with increasing concentrations of IL-4. After five days cells were treated with PMA, ionomycin and brefeldin A for six hours and IL-17 expression was measured by ICS.



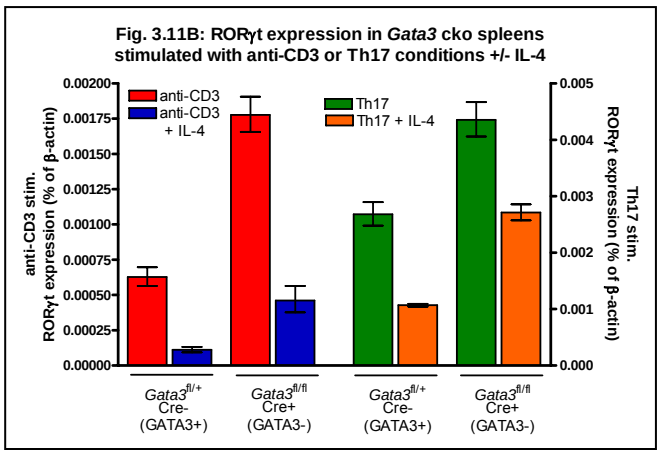


Figure 3.11: Effect of IL-4 on IL-17 and RORγt mRNA expression by *Gata3* cko spleens.

Spleens from mice with inducible *Gata3* deletion or controls without deletion were stimulated with anti-CD3 or anti-CD3 plus Th17-polarizing cytokines, in the presence or absence of 50ng/mL IL-4. After three days RNA was collected and analyzed for IL-17 (A) and RORγt (B) expression by real time PCR. The left y-axis refers to the samples treated with anti-CD3, while the right y-axis refers to samples treated with anti-CD3 plus Th17-polarizing cytokines. Error bars represent SEM of triplicate PCR reactions.

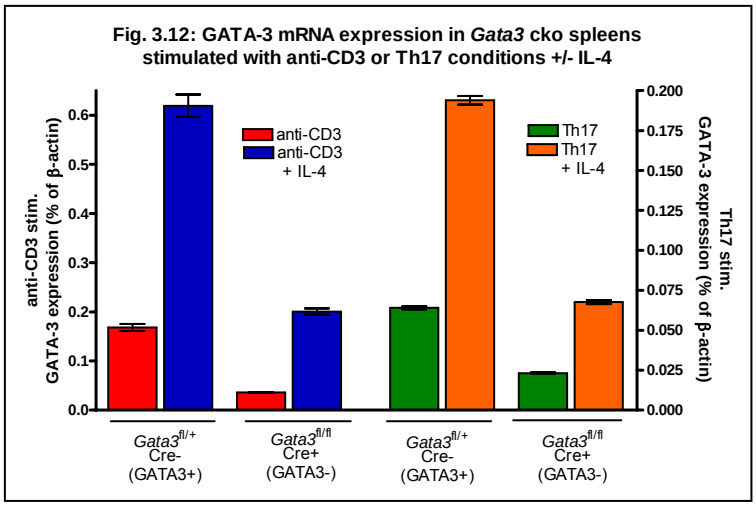


Figure 3.12: GATA-3 mRNA expression by *Gata3* cko spleens.

Spleens from mice with inducible *Gata3* deletion or controls without deletion were stimulated with anti-CD3 or anti-CD3 plus Th17-polarizing cytokines, in the presence or absence of 50ng/mL IL-4. After three days RNA was collected and analyzed for GATA-3 expression by real time PCR. The left y-axis refers to the samples treated with anti-CD3, while the right y-axis refers to samples treated with anti-CD3 plus Th17-polarizing cytokines. Error bars represent SEM of triplicate PCR reactions.

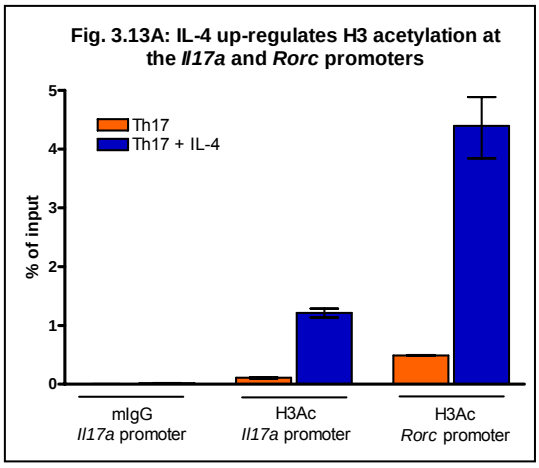
Effect of IL-4 on chromatin structure at Th17 loci

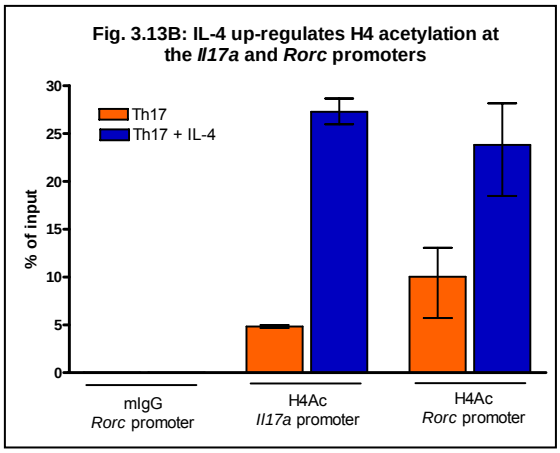
Similar to the natural progression of the signaling cascade, the focus of our experiments has gradually moved inward, starting near the cell membrane with events proximal to the IL-4R and ultimately leading to the nucleus with changes at the level of the chromatin. The literature suggests that T helper cell differentiation and cross-regulation are associated with both activating and inhibitory epigenetic changes at cytokine and transcription factor loci. For example, STAT6 and GATA-3 silence IFNγ in Th2 cells by recruiting the histone methyltransferase EZH2 and inducing H3K27me3, a marker of inactive chromatin [50, 175]. In addition, GATA-3 binds to histone de-acetylase enzymes and may mediate gene silencing via de-acetylation of H3 and H4 [97, 101]. Recent reports have shown that the *Il17* locus undergoes H3Ac and H3K4me3, two markers of transcriptionally active chromatin, in Th17 cells, versus H3K27me3 in Th1, Th2 and Tregs [87, 106]. The addition of IL-4 during Th17 differentiation prevents H3Ac and H3K4me3 and induces H3K27me3 at the *Il17a* promoter (John O'Shea, personal communication). However, these observations represent stable changes associated with heritable lineage commitment, and nothing is currently known about epigenetic changes induced by transient suppression of previously differentiated T helper cells. Given that adding IL-4 during Th17 differentiation likely results in permanent skewing towards a Th2 phenotype, while we have shown that adding IL-4 to pre-existing Th17 cells inhibits IL-17 expression without inducing de-differentiation or IL-4 expression, the associated epigenetic changes are almost certain to be different. Another important factor to consider is that inhibition of Th17 differentiation occurs in a naïve T cell, where the chromatin at the *Il17* locus exists in a relatively neutral state, while

inhibition of Th17 activity requires reversal of a highly active chromatin state at the *Il17* locus.

To address possible epigenetic mechanisms for IL-4-induced silencing of IL-17 expression in pre-existing Th17 cells, we generated Th17 cells *in vitro* as described above, re-stimulated them with anti-CD3 and Th17-skewing cytokines in the presence or absence of IL-4 for 6 or 24 hours, and measured levels of H3Ac, H4Ac, H3K4me3 and H3K27me3, as well as PolII and STAT3 binding at the *Il17a* promoter, *Il17a/f* intergenic region and *Rorc* promoter by ChIP. Given the complexity of the histone code and how little we currently understand about transcriptional regulation, it is not surprising that our results show a confusing combination of signals expected to both promote and inhibit gene expression. Acetylation of H3 and H4, which are typically associated with active chromatin, were both up-regulated at the *Il17a* and *Rorc* promoters by IL-4 (Fig. 3.13A&B). IL-4 also increased binding of PolII at the *Il17a* and *Rorc* promoters (Fig. 3.13C), another marker typically associated with increased transcriptional activity, despite the fact that we clearly see decreased expression of IL-17 and RORγt message. ChIPs for H3K4me3 and H3K27me3, which are associated with active and inactive chromatin, respectively, were inconclusive because neither produced a significant signal over background (data not shown). Further experiments are needed to optimize these assays. ChIPs for STAT3, on the other hand, demonstrated very strong signals at the *Il17a* promoter, as expected based on the published role of STAT3 in IL-17 expression, but surprisingly also showed strong binding at the *Il17a/f* intergenic region and the *Rorc* promoter, which has not been previously described (Fig. 3.14). Interestingly, IL-4

potently inhibited STAT3 binding at the *Il17*a promoter but had no effect at the *Il17a/f* intergenic region or the *Rorc* promoter.





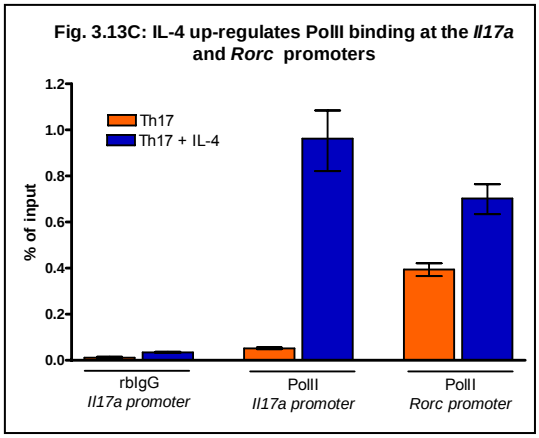


Figure 3.13: IL-4 up-regulates markers of active chromatin at Th17 loci.

Th17 cells were generated *in vitro* as described. After one week the cells were re-stimulated for 24 hours with anti-CD3 and Th17-polarizing cytokines in the presence or absence of 50ng/mL IL-4. ChIPs were carried out using the EZ-ChIP kit according to manufacturer's instructions (Millipore), with antibodies for acetylated histone 3 (A), acetylated histone 4 (B), RNA polymerase II (C) or isotype control. Eluted DNA was quantitated by SYBR green real time PCR with primers specific for the *Il17a* or *Rorc* promoters. Data is normalized to the corrected Ct values of the 1% input sample. Error bars represent SEM of triplicate PCR reactions.

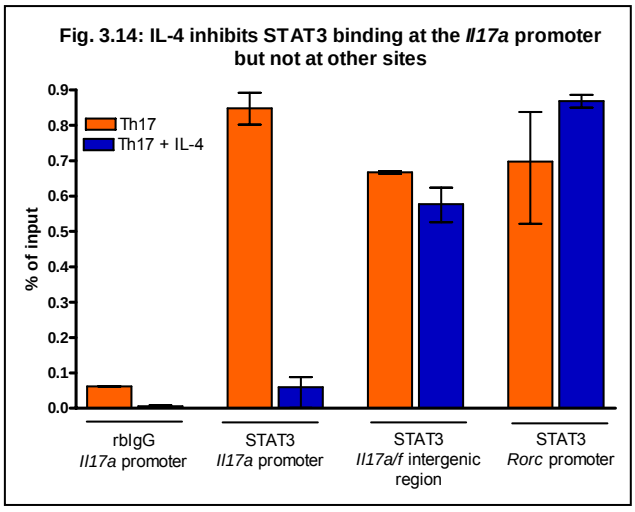


Figure 3.14: IL-4 inhibits STAT3 binding at the *Il17a* promoter but not at other sites.

Th17 cells were generated *in vitro* as described. After one week the cells were re-stimulated for 6 hours with anti-CD3 and Th17-polarizing cytokines in the presence or absence of 50ng/mL IL-4. ChIPs were carried out using the EZ-ChIP kit according to manufacturer's instructions (Millipore), with antibody specific for STAT3 or isotype control. Eluted DNA was quantitated by SYBR green real time PCR with primers specific for the *Il17a* promoter, *Il17a/f* intergenic region or *Rorc* promoter. Data is normalized to the corrected Ct values of the 1% input sample. Error bars represent SEM of triplicate PCR reactions.

Discussion

Our initial hypotheses about the molecular mechanisms of suppression of Th17 responses by IL-4 were simple and direct: IL-4 binds to the IL-4R and activates STAT6, STAT6 goes to the nucleus and inhibits transcription of IL-17A, IL-17F and RORγt. Instead what we found was several added layers of complexity, which has prompted us to broaden our view, delve deep into exciting new territories and ultimately expand the way we think about T helper cell regulation.

To start with, we have shown that the well-established dogma that the IL-4R signals through STAT6, while true, overlooks potential secondary effects mediated by STAT5. Given that the IL-4R is a heterodimer composed of the specific IL-4Rα subunit and the common γ chain, which is found in a number of other cytokine receptors, including the STAT5-activating IL-2R, it is not at all surprising that there may be some "leaky" STAT5 activation by IL-4. Although we found that STAT5 was dispensable for inhibition of IL-17 expression by IL-4, there are many other effects of IL-4, possibly in other IL-4R-positive cell types, which may depend on STAT5.

Despite a number of technical hurdles that make it difficult to draw firm conclusions about STAT6 binding to the *Il17a* promoter and about the requirement for protein synthesis, the limited precedent for direct inhibition of gene transcription by STAT family molecules led us to hypothesize that suppression of IL-17 downstream of STAT6 is most likely indirect, or at least a combination of direct and indirect effects. Thus we have begun to search for factors up-regulated by IL-4 and/or capable of silencing IL-17 expression, both in the literature and by microarray. From the literature

we identified a number of candidate transcription factors and looked for early up-regulation of their gene expression in Th17 cells in response to IL-4. Our microarray data has also returned a number of interesting transcription factor genes either up-regulated or down-regulated by IL-4, and future experiments exploring these leads are planned. Although without convincing results from the cycloheximide experiments, the possibility remains that suppression of IL-17 depends on IL-4-induced changes in protein activity rather than expression.

Our initial findings suggested that GATA-3 was a highly promising candidate: it has been shown to mediate both positive and negative effects on gene expression in T helper cells though multiple mechanisms, there are several potential binding sites in the *Il17a* and *Rorc* promoters, and the message is rapidly up-regulated in Th17 cells in response to IL-4. However, induced *Gata3* deletion, leading to a 70% decrease in average splenic mRNA expression, had no effect on suppression of IL-17. It may be necessary to confirm these results both by looking at GATA-3 expression at the protein level and by enhancing deletion efficiency, but the data strongly suggests that GATA-3 is not the transcription factor we have been searching for. This conclusion is supported by published data showing that *Gata3* knock-in mice still develop Th17 cells *in vitro* and *in vivo* [66, 67].

While the search for the missing link in this pathway continues, we moved our focus further downstream and addressed changes in histone modifications and binding of the transcriptional machinery at multiple Th17 gene loci. It has already been shown that activation of the *Il17a* locus in Th17 cells is associated with H3 acetylation and H3K4 tri-

methylation, which is inhibited by IL-4, and silencing of the *Il17a* locus in Th1, Th2 and Tregs is associated with H3K27 tri-methylation ([87, 106], O'Shea personal communication). However, these experiments address epigenetic changes associated with stable lineage commitment, and much less is known about the role of histone modifications in inducible gene expression, such as when an effector T cell integrates a combination of positive and negative stimuli resulting in rapid and short-lived changes in cytokine expression.

A recent paper from Medzhitov and colleagues greatly illuminated the mechanisms underlying inducible gene expression in macrophages treated with LPS and raised many new questions in our minds [176]. This paper described two types of inducible gene expression with very different mechanisms: broadly classified as primary response genes and secondary response genes. Primary response genes are maintained in a poised state by co-repressors associated with DNA-binding transcription factors, which are dismissed upon stimulation, allowing for very rapid induction of gene expression. Secondary response genes, on the other hand, are maintained in an inactive state by regulatory nucleosomes, which may occlude DNA binding sites, resulting in a much slower induction of gene expression following stimulation. The surprising finding was that primary response genes have many markers associated with active gene expression, including H3K4 tri-methylation, H3 acetylation and PolII binding, prior to stimulation. The key stimulation-induced epigenetic switch that activates the pre-assembled transcriptional machinery at primary response genes appears to be H4 acetylation, which recruits factors that phosphorylate PolII, releasing it from suppression, and allowing it to move along the gene. Although these findings have yet to be extended to T cells or to the

chromatin changes underlying repression of active gene expression, it importantly demonstrates the fallibility of our oversimplified assumptions about the landscape of active versus inactive chromatin.

With this in mind, we hypothesized that chromatin at the Th17 loci may transition along a continuum between repression and activation: neutral in naïve T cells; stably suppressed in Th1, Th2 and Tregs; poised in resting Th17 cells; active in stimulated Th17 cells; and transiently suppressed in Th17 cells treated with IL-4. It will be interesting to determine exactly how far along the continuum IL-4 pushes Th17 cells and how this relates to the stability of suppression. Our ChIP results surprisingly demonstrated an increase in H3 and H4 acetylation, in addition to an increase in PolII binding. Although the acetylation is difficult to explain, the increase in PolII binding at the promoter may represent an accumulation of inactive polymerase, suggesting that IL-4 may simply induce a poised state, similar to a resting Th17 cell, rather than a stably suppressed state. Our STAT3 ChIP data, showing decreased binding to the *Il17a* promoter following treatment with IL-4, are consistent with gene silencing, suggesting that STAT3 binding may provide the necessary activation signal at a locus that is otherwise poised for transcription. However, it is currently unknown if STAT3 binds to the *Il17a* promoter constitutively or only in Th17 cells actively transcribing *Il17a.*

The great number of surprises we encountered during this work points out exactly how little we understand about the signaling and epigenetic mechanisms mediating regulation of T cell cytokine production and raises many interesting questions for future research. Particularly in the area of transcriptional regulation and the histone code, we

are likely in the early stages of a great flood of new understanding, facilitated by recent advances in the ChIP-seq method.

Chapter 4

Th17 maturation

Previous reports from other groups have demonstrated that developing Th1 cells progress through several stages of maturation, gradually stabilizing their phenotype in response to cytokine stimulation. Early in the culture, IL-4 suppresses Th1 differentiation and IFNγ expression, but after prolonged stimulation, Th1 cells lose the ability to respond to IL-4 and become resistant to suppression [72-74]. While these initial reports are highly intriguing and raise many questions, much remains unknown, including the mechanism underlying desensitization of the IL-4R, the physiological significance and the applicability to other T helper lineages. Our preliminary data on the effect of IL-4-transduced dendritic cells on collagen-specific IL-17 production from cII/CFA immunized spleen cells suggested that Th17 cells become less susceptible to regulation after the onset of arthritis [161]. Thus we decided to pursue the maturation of Th17 cells *in vitro* and *in vivo*, as measured by development of resistance to suppression by IL-4.

Th17 maturation *in vitro*

We generated Th17 cells *in vitro* by stimulating naïve T cells with BM-DCs, anti-CD3 and the Th17 cytokine cocktail for five days, followed by two days of rest, as described previously. To induce maturation we repeated this process of five-day stimulation and two-day rest two times, for a total of three weeks of culture, and then assayed the effect of IL-4 on IL-17 expression during a two-day re-stimulation with anti-

CD3 followed by ICS. Figure 4.1 shows representative dot plots of ICS for IL-17 in Th17 cells treated with IL-4 after one week or three weeks of culture. The results show that after three rounds of Th17 stimulation, IL-17 expression is largely resistant to suppression by IL-4. Figure 4.2A demonstrates that the development of resistance is reproducible. Interestingly, Th17 cells cultured for three weeks become resistant to suppression by IL-4 and IFNγ but remain sensitive to suppression by IL-12 (Fig. 4.2B).

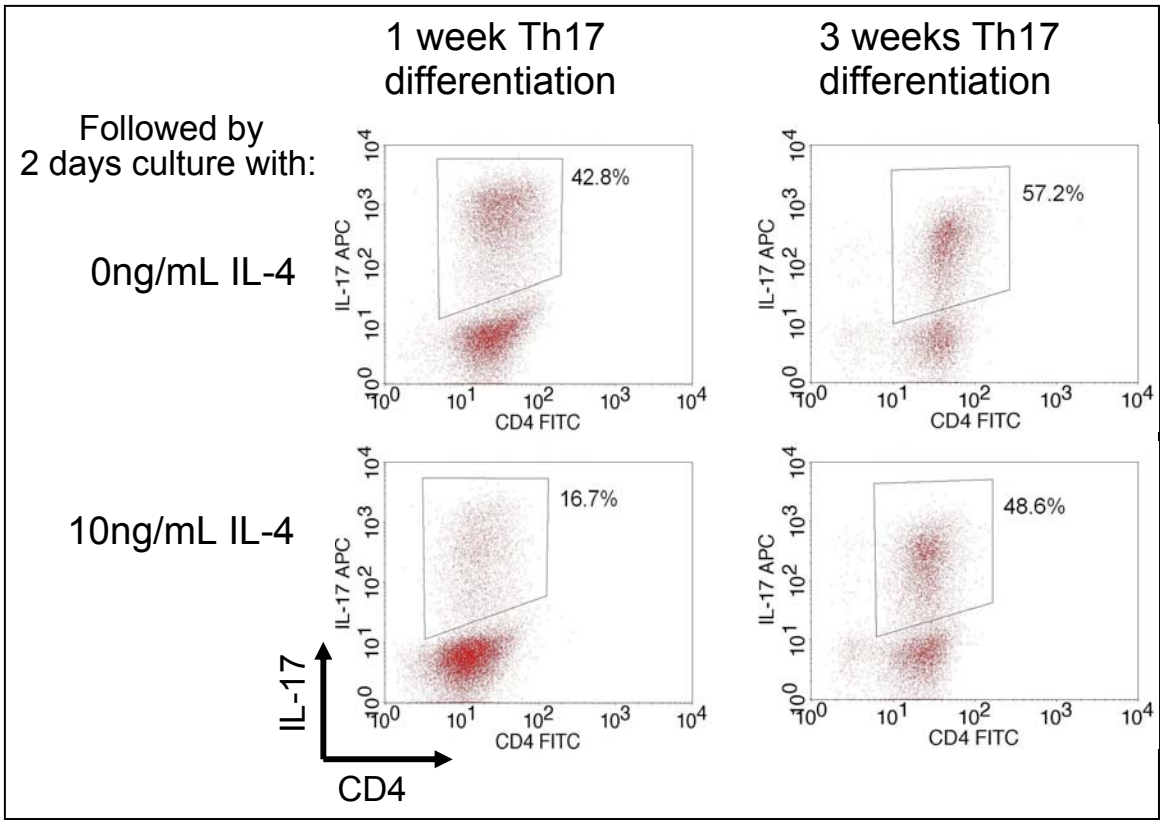
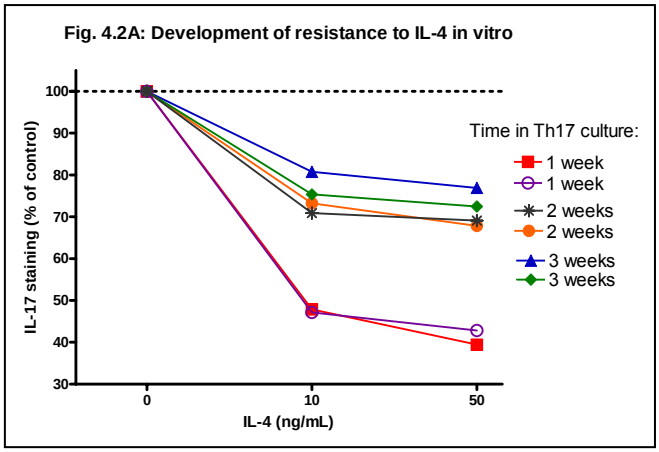


Figure 4.1: Three weeks culture renders Th17 cells resistant to suppression by IL-4.

Naïve T cells were stimulated with BM-DCs, anti-CD3 and the Th17-polarizing cocktail for five days, followed by two days rest. At this point some cells were re-stimulated with anti-CD3 in the presence or absence of IL-4, followed by six hours with PMA, ionomycin and brefeldin A and ICS for IL-17. The remaining cells were cultured for two more rounds of five days Th17 stimulation and two days rest, for a total of three weeks of culture. After three weeks, the cells were re-stimulated for two days with anti-CD3 in the presence or absence of IL-4, followed by six hours PMA, ionomycin and brefeldin A and ICS for IL-17. Numbers represent the percent IL-17+ in the total sample, which was almost exclusively CD4+.



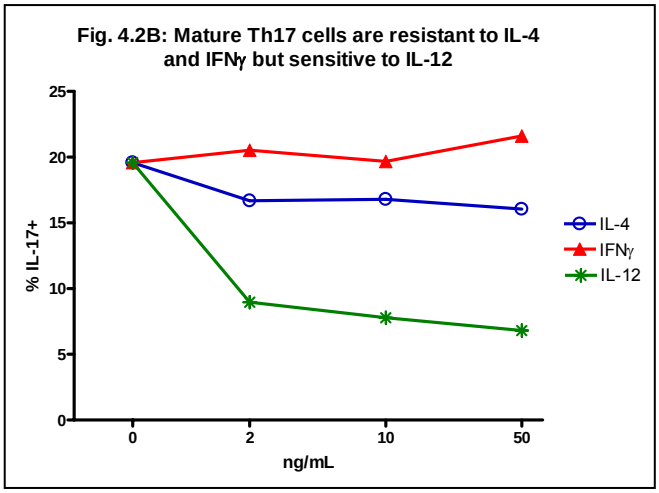


Figure 4.2: Th17 cells develop selective resistance *in vitro*.

(A) Naïve T cells were cultured for one, two or three rounds of five days Th17 stimulation and two days rest and then re-stimulated with anti-CD3 and increasing concentrations of IL-4 for two days, followed by PMA, ionomycin and brefeldin A and ICS for IL-17. Data represent compiled results from several experiments. Results are normalized to the IL-17 expression in the sample with no IL-4. (B) Th17 cells cultured for three weeks to induce maturation were re-stimulated for two days with anti-CD3 and increasing concentrations of IL-4, IFNγ or IL-12, followed by six hours PMA, ionomycin and brefeldin A and ICS for IL-17.

Th17 maturation *ex vivo* and *in vivo*

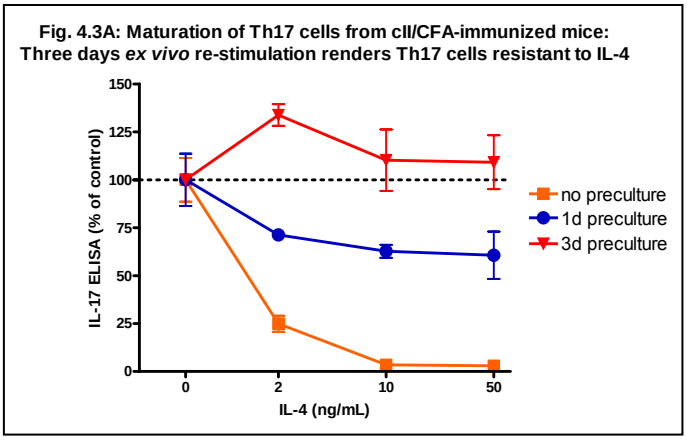
We have already shown that IL-4 suppresses IL-17 expression by collagen- or KLH-immunized splenocytes, thus demonstrating that antigen-specific Th17 cells in the spleen two weeks after immunization have not fully matured. However, we decided to look for *ex vivo* maturation of immunized splenocytes in two ways: initially we simply delayed the addition of IL-4 to our spleen cell cultures by one or three days, collected supernatants on day five and measured IL-17 by ELISA; alternatively, to avoid confounding effects from IL-17 in the supernatant produced prior to the addition of IL-4, we stimulated the spleen cells with antigen for one or three days and then washed and re-stimulated them in the presence or absence of IL-4 for two days, followed by IL-17 ELISA of the supernatants. As the results in Figure 4.2 show, we found that three days of *ex vivo* re-stimulation with antigen was sufficient to induce Th17 maturation and resistance to suppression by IL-4 in whole spleen cultures from two-week immunized mice. Similar results were observed for collagen-immunized DBA and KLH-immunized BALB/c.

The ability of Th17 cells to become resistant to suppression could have important implications for the development of autoimmune disease. Thus we asked whether Th17 maturation correlated with disease progression or severity in CIA. To address this question we collected spleens from mice at different time points after immunization with cII/CFA and assessed the Th17 sensitivity to suppression by IL-4 on day zero, one or three of culture. We were surprised to find that even six weeks after immunization, splenic Th17 cells were not fully mature directly *ex vivo*. However, we did observe an

inverse relationship between the time since immunization and the duration of *in vitro* stimulation required to induce maturation (Figure 4.4 and Table 4.1).

We hypothesized that collagen-specific Th17 cells may receive more cytokine and/or TCR stimulation closer to the site of inflammation, and thus cells in the DLN may be more mature than cells in the spleen, and cells in the arthritic joint may be more mature than cells in the DLN. We developed a protocol to isolate cells from inflamed joints by collagenase digestion and the resulting cells were analyzed by IL-17 ELISpot. We were able to measure IL-17-producing cells in the paws of arthritic mice by ELISpot following treatment with PMA and ionomycin, and IL-4 had no effect on the number or size of IL-17 spots (data not shown). However, because PMA and ionomycin is such a strong and unphysiological stimulus, we felt that this data may not represent an accurate measurement of the effect of IL-4 on IL-17. Alternatives include stimulating with anti-CD3 and anti-CD28, which may interfere with the ELISpot, or collagen, which we found was not sufficient to induce significant IL-17 production, possibly because the cells were too few or the cultures were too brief to allow for adequate antigen processing and presentation. These techniques require further exploration.

To look for differences in the maturation status of Th17 cells from spleens versus LNs and the relationship between maturation and disease severity, we compared the IL-4 dose-response curves of collagen-stimulated spleen and DLN cultures from mice with varying degrees of arthritis. The results in Figure 4.5 show that there is a trend towards decreased responsiveness to IL-4 in DLNs versus spleens and that there does not appear to be a significant correlation between disease severity and Th17 sensitivity to IL-4.



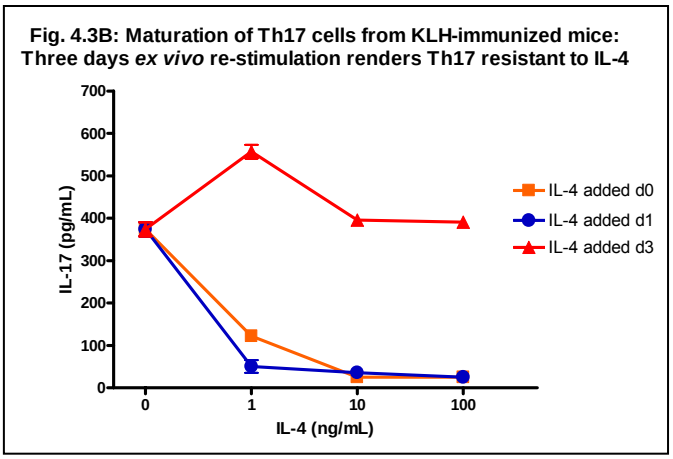


Figure 4.3: Th17 cells from immunized mice mature *ex vivo*.

(A) DBA mice were immunized i.d. with cII/CFA and spleens were collected two weeks later. Total spleen cells were re-stimulated with heat-denatured collagen for zero, one or three days, and then collected, washed and re-plated with collagen and increasing concentrations of IL-4 for two days. Supernatants were collected and IL-17 was measured by ELISA. (B) BALB/c mice were immunized i.p. with KLH/CFA and spleens were collected two weeks later. Total spleen cells were re-stimulated with KLH and increasing concentrations of IL-4 were added to the culture on day zero, one or three. Supernatants were collected on day five and IL-17 was measured by ELISA. Error bars represent SEM of triplicate culture samples.

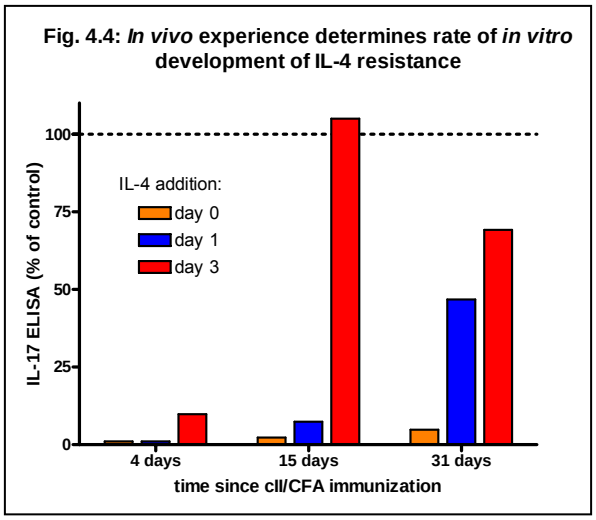


Figure 4.4: *In vivo* experience determines rate of *in vitro* development of IL-4 resistance.

DBA mice were immunized with cII/CFA and spleens were collected 4, 15 or 31 days laters. Spleen cells were re-stimulated with collagen and 50ng/mL IL-4 was added on day 0, 1 or 3. Supernatants were collect on day five and IL-17 was measured by ELISA. IL-17 expression is normalized to the sample with no IL-4.

Weeks since immunization	Days culture until IL-4 resistance
1	>3
2	2
4	1
6	0-1

Table 4.1: *In vivo* experience correlates with *in vitro* development of resistance.

Compiled data from several experiments as described in Figure 4.4. DBA mice were immunized with cII/CFA and spleens were collected after one, two, four or six weeks. Spleen cells were re-stimulated with collagen and IL-4 was added on day zero, one or three of culture. IL-17 was measured by ELISA on day five. A designation of resistance required that IL-17 production in the presence of IL-4 was at least 70% of IL-17 production in the absence of IL-4. Spleens from one week immunized mice continued to respond to IL-4 when it was added after three days of culture, while spleens from six week immunized mice failed to respond when IL-4 was added on day zero or one.

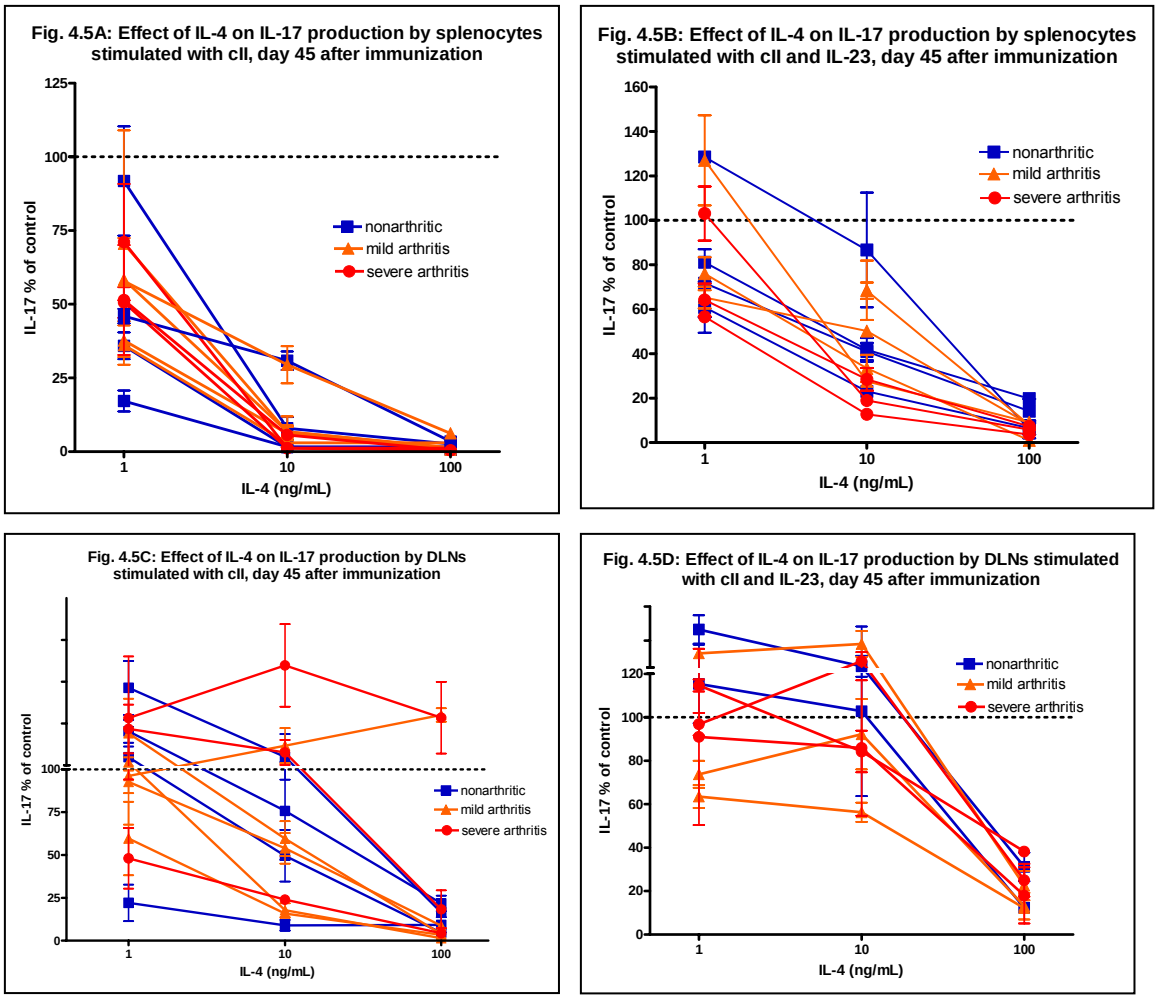
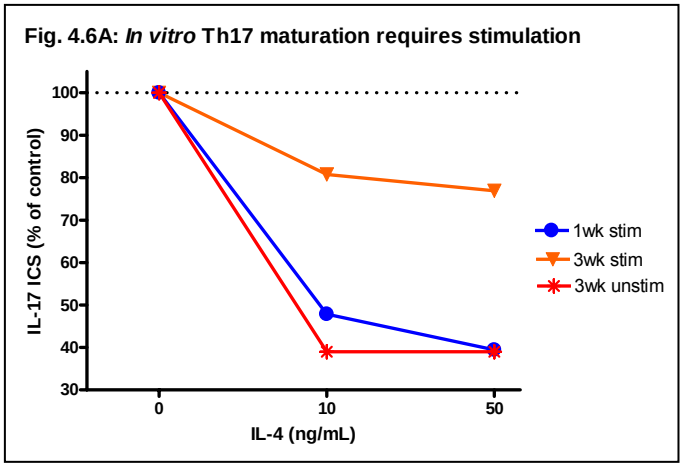


Figure 4.5: IL-4-sensitivity of spleens and lymph nodes from arthritic mice.

DBA mice were immunized with cII/CFA i.d.; 45 days after immunization, at the peak of disease, spleens (A and B) and DLNs (C and D) were collected. Single cell suspensions were cultured with heat-denatured collagen, with (B and D) or without (A and C) 10ng/mL IL-23, and increasing concentrations of IL-4. On day five the supernatants were analyzed for IL-17 by ELISA. IL-17 expression was normalized to the matched sample with no IL-4. Error bars represent SEM of triplicate culture samples. Mice with a score of four in at least one paw were considered severely arthritic, mice with scores of one, two or three were considered mildly arthritic, and mice with no visible signs of paw redness or swelling were considered nonarthritic.

Stimulation required to induce Th17 maturation

We established that *in vitro*-derived Th17 cells mature during stimulation with anti-CD3, TGFβ, IL-6 and IL-23, and *in vivo*-derived Th17 cells mature during re-stimulation with antigen, but we wanted to determine which specific signals are necessary and sufficient to induce Th17 maturation. Given that maturation of Th1 cells has been shown to depend on IL-12 and IFNγ [72-74] and that IL-23 confers a more pathogenic phenotype upon Th17 cells through an unknown mechanism [27], we hypothesized that IL-23 was responsible for inducing Th17 maturation. The results in Fig. 4.6A show that Th17 cells left completely un-stimulated fail to mature, but no one signal seems to be absolutely necessary or sufficient, as removing any one of the components of the cocktail or the anti-CD3 results in an intermediate degree of IL-4 responsiveness (Fig. 4.6B). The role of IL-23 in *ex vivo* Th17 maturation varied from one system to another and from one experiment to another, possibly due to heterogeneity in the expression of key cytokines or costimulatory molecules by endogenous APCs. For example, some experiments with cII-immunized splenocytes implied that exogenous IL-23 is required, while KLH-immunized splenocytes could mature even in the presence of neutralizing antibody to IL-23 (Fig. 4.7). The results in Fig. 4.8 show that, during *ex vivo* re-stimulation of collagen-immunized spleens, cells cultured without antigen did not mature, which could be partially rescued by exogenous IL-23 but not by the combination of TGFβ and IL-6. These results suggest that IL-23 plays a more important role in maturation of the collagen-specific response than the KLH-specific response, but in both systems Th17 cells likely require a combination of signals for full maturation.



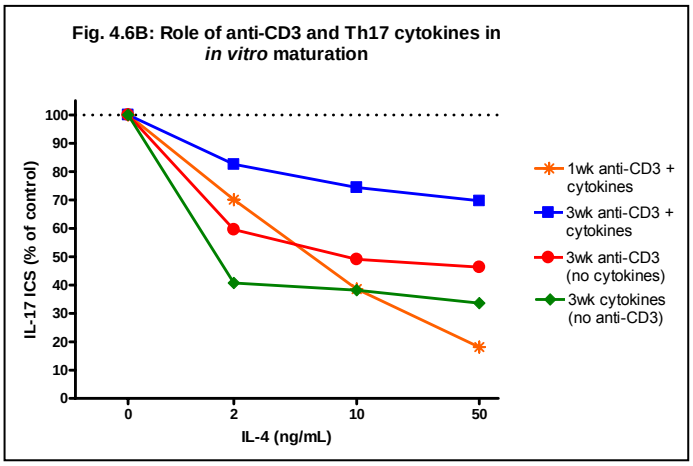
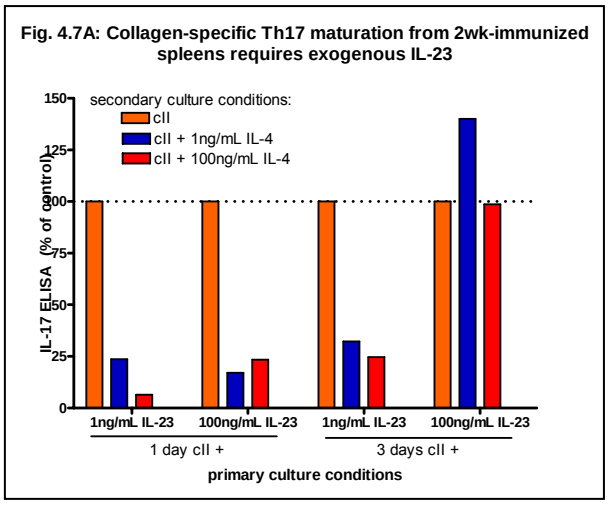


Figure 4.6: Stimulation requirements for *in vitro* Th17 maturation.

Naïve T cells were cultured with BM-DCs and Th17-polarizing cytokines for one week to induce Th17 differentiation. (A) Following differentiation, cells were cultured for two more weeks with anti-CD3 and Th17 cytokines or with neither. Alternatively, in (B), cells were cultured for two more weeks with anti-CD3 alone, Th17 cytokines alone or the combination of both. After one or three weeks of culture cells were washed and re-stimulated with anti-CD3 and IL-4 for two days and IL-17 was measured by ICS. IL-17 expression is normalized to the matched sample with no IL-4.



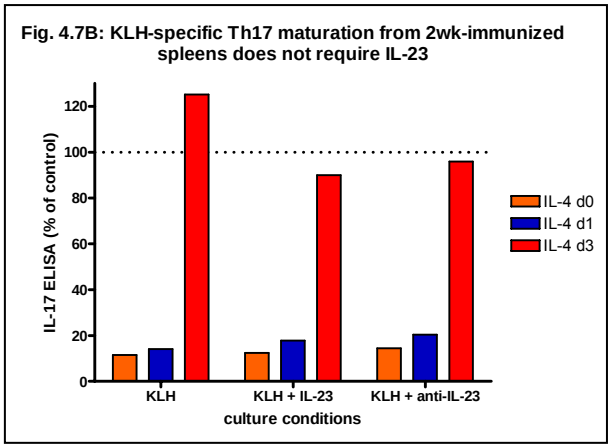


Figure 4.7: Role of IL-23 in *ex vivo* Th17 maturation.

(A) DBA mice were immunized i.d. with cII/CFA and spleens were collected two weeks later. Total spleen cells were re-stimulated with heat-denatured collagen and varying concentrations of IL-23 for one or three days, and then collected, washed and re-plated with collagen and varying concentrations of IL-4 for two days. Supernatants were collected and IL-17 was measured by ELISA. (B) BALB/c mice were immunized i.p. with KLH/CFA and spleens were collected two weeks later. Total spleen cells were re-stimulated with KLH, KLH plus 10ng/mL IL-23, or KLH plus 10µg/mL IL-23 neutralizing antibody and varying concentrations of IL-4 were added to the culture on day zero, one or three. Supernatants were collected on day five and IL-17 was measured by ELISA. IL-17 expression is normalized to the expression in cultures with no IL-4.

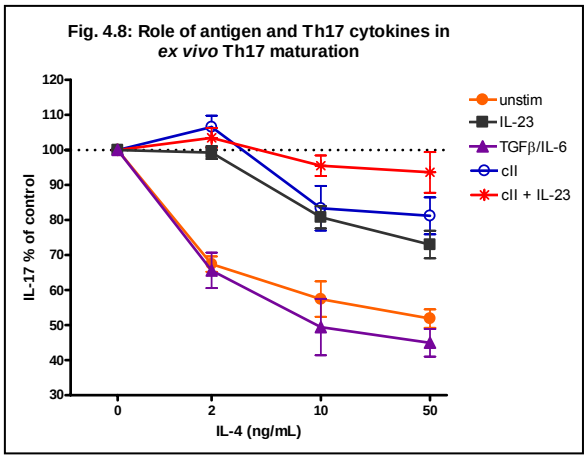


Figure 4.8: Role of antigen and Th17 cytokines in *ex vivo* Th17 maturation.

DBA mice were immunized i.d. with cII/CFA and spleens were collected two weeks later. Total spleen cells were cultured with cII, cII + 10ng/mL IL-23, 10ng/mL IL-23 alone, 2ng/mL TGFβ + 20ng/mL IL-6 or with no stimulation. After three days cells were washed and re-plated with collagen and varying concentrations of IL-4 for two days. Supernatants were collected and IL-17 was measured by ELISA. IL-17 expression is normalized to expression in cultures with no IL-4. Error bars represent SEM of triplicate culture samples.

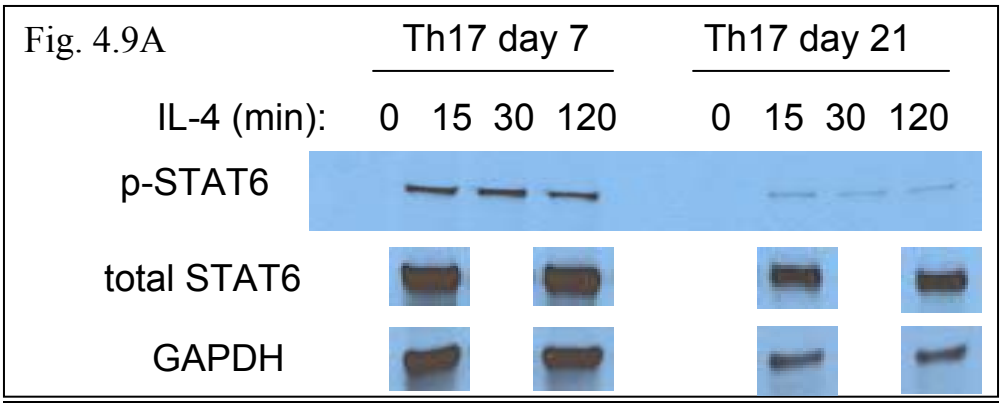
Loss of IL-4R signaling

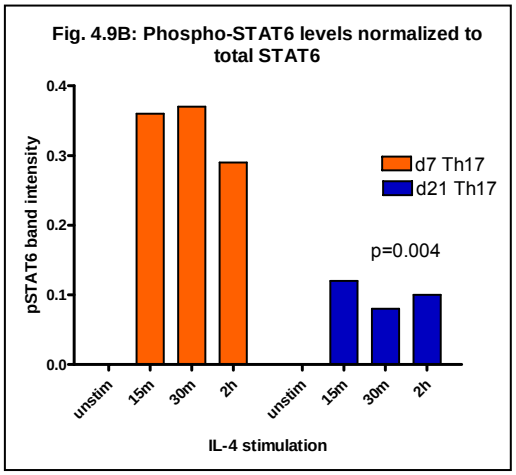
Our observation of desensitization of Th17 cells to challenge with IL-4 begged the question of whether signaling through the IL-4R remained intact following maturation. To measure IL-4R signaling in mature Th17 cells, we rested the cells in low serum media with cytokine neutralizing antibodies overnight to minimize the background level of activation, stimulated with IL-4 for 15, 30 or 120 min, harvested lysate in the presence of protein phosphatase inhibitors and measured STAT6 activation by Western blot with phospho-STAT6 specific antibodies. The results in Fig. 4.9A show that IL-4 induces significantly less STAT6 activation in mature Th17 cells cultured for three weeks versus immature Th17 cells cultured for one week, regardless of whether phospho-STAT6 levels are normalized to total STAT6 (Fig. 4.9B) or to the loading control GAPDH (Fig. 4.9C). In addition, when total STAT6 expression is normalized to GAPDH, we find that STAT6 is actually up-regulated in mature Th17 cells (data not shown). Thus the loss of STAT6 activation is not simply due to down-regulation of STAT6 expression.

Because we have also shown that IL-4 activates STAT5 in Th17 cells, we confirmed the loss of IL-4R signaling in mature Th17 cells by Western blot for phospho-STAT5. The results in Fig. 4.10 show that there is a similar loss of STAT5 activation in response to IL-4 in mature Th17 cells, although this is not due to a global defect in cytokine signaling, as mature Th17 cells induce normal levels of STAT5 phosphorylation in response to IL-2.

One plausible explanation for the loss in STAT5 and STAT6 activation could be down-regulation of the IL-4R or other proteins involved in the proximal IL-4R signaling cascade, such as JAK1 and JAK3. Therefore we decided to measure expression of these molecules at different stages of Th17 maturation by Western blot and qRT-PCR. The results show that mRNA and protein levels of IL-4Rα, JAK1 and JAK3 are not down-regulated in three-week Th17 cultures versus one-week cultures (Fig. 4.11).

One potential drawback to RT-PCR and Western blot, however, is that the results show the average expression level for all cells in culture, but Th17 cells are only a fraction of the culture, and mature Th17 cells make up an even smaller fraction. To look more specifically at IL-4R protein expression on Th17 cells by flow cytometry, we co-stained for IL-17 and IL-4R, but we discovered that treatment with brefeldin A, which is commonly used prior to ICS to increase intracellular cytokine levels by blocking secretion through the Golgi apparatus, had the unintended side-effect of down-regulating surface IL-4R staining (data not shown). Similarly, we looked for STAT6 expression and phosphorylation by intracellular staining, but the signal was very weak even in immature Th17 cells, making it difficult to determine if expression decreased in mature Th17 cells, particularly given that three weeks of culture tends to give mature Th17 cells higher levels of background staining (data not shown). One potential solution would be to isolate mature Th17 cells from contaminating cells in the culture prior to Western or qRT-PCR, but we have not yet been fortunate enough to find a surface marker for mature Th17 cells.





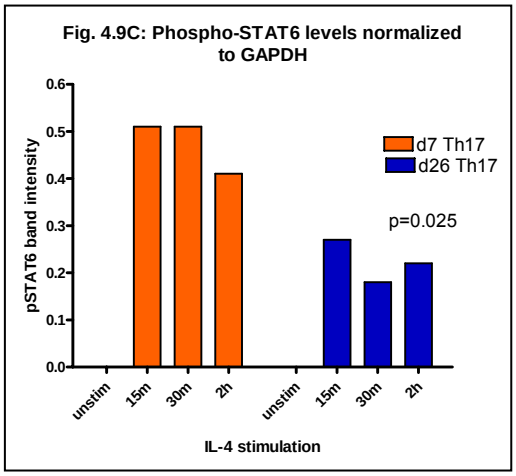


Figure 4.9: Decreased activation of STAT6 by IL-4 in mature Th17 cells.

(A) Th17 cells were generated *in vitro* with one or three weeks of stimulation and rested overnight in low-serum media with cytokine-neutralizing antibodies to reduce the background level of STAT activation. Cells were then washed and stimulated with 50ng/mL IL-4 for 15, 30 or 120 min and lysed with PhosphoSafe Lysis Buffer (Novagen). Lysates were reduced and run on 10% SDS-PAGE gels and stained with antibodies for phospho-STAT6, total STAT6 or GAPDH. Band intensities in part (A) were quantitated with Kodak software and phospho-STAT6 intensity was normalized either to total STAT6 intensity (B) or GAPDH intensity (C).

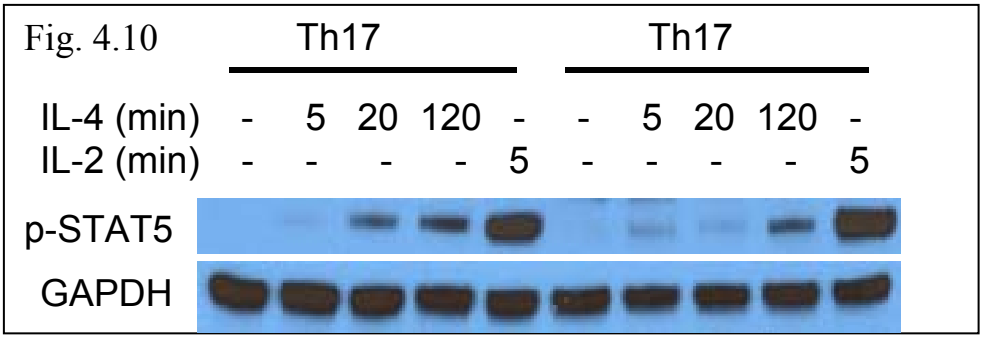
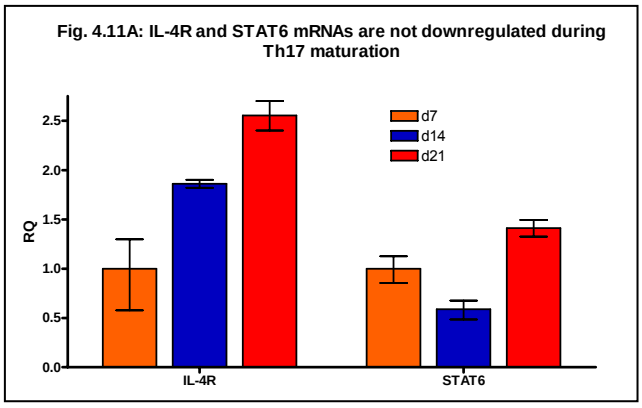


Figure 4.10: Decreased activation of STAT5 by IL-4 in mature Th17 cells.

Th17 cells were generated *in vitro* with one or three weeks of stimulation and rested overnight in low-serum media with cytokine-neutralizing antibodies to reduce the background level of STAT activation. Cells were then washed and stimulated with 50ng/mL IL-4 or IL-2 for 5, 20 or 120 min and lysed with PhosphoSafe Lysis Buffer (Novagen). Lysates were reduced and run on 10% SDS-PAGE gels and stained with antibodies for phospho-STAT5 or GAPDH.



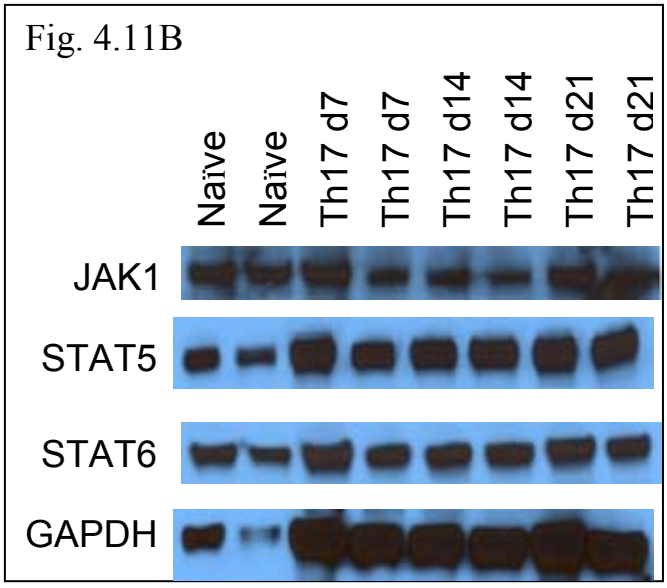


Figure 4.11: Mature Th17 cells maintain expression of IL-4R components.

(A) Th17 cells were generated *in vitro* with one, two or three weeks of stimulation. RNA was collected and levels of IL-4R and STAT6 were measured by real time PCR with primers and probes from Applied Biosystems. Data are normalized first to the internal control β-actin and second to the expression level in Th17 cells after one week of culture. Error bars represent SEM of triplicate PCR reactions. (B) Naïve T cells or Th17 cells after one, two or three weeks of culture were lysed with PhosphoSafe Lysis Buffer (Novagen). Lysates were reduced and run on 10% SDS-PAGE gels and stained with antibodies for JAK1, STAT5, STAT6 or GAPDH. Blots combine samples from multiple experiments.

Role of SOCS5

One potential mechanism for loss of STAT5 and STAT6 activation in mature Th17 cells is up-regulation of a member of the suppressor of cytokine signaling (SOCS) family of proteins. Most SOCS family proteins preferentially interact with a different cytokine receptor, thereby specifically inhibiting activation of unique STAT molecules. SOCS3, for example, has been shown to suppress Th17 differentiation by binding to the IL-6R and inhibiting STAT3 activation, while SOCS5 has been shown to bind to the IL-4R and inhibit STAT6 activation in Th1 cells [77]. There can be some redundancy, however, because previous work has also demonstrated that inhibition of IL-4R signaling in Th1 cells may be mediated by SOCS1 [78, 177]. Thus we decided to look for up-regulation of SOCS1 and SOCS5 expression in Th17 cells cultured for one, two or three weeks by qRT-PCR and Western blot (Fig. 4.12). Although the magnitude of the change varied from one experiment to another, there was consistent evidence for up-regulation of both message and protein for SOCS5 and to a lesser extent for SOCS1, suggesting that SOCS5 may be responsible for inhibition of IL-4R signaling in mature Th17 cells.

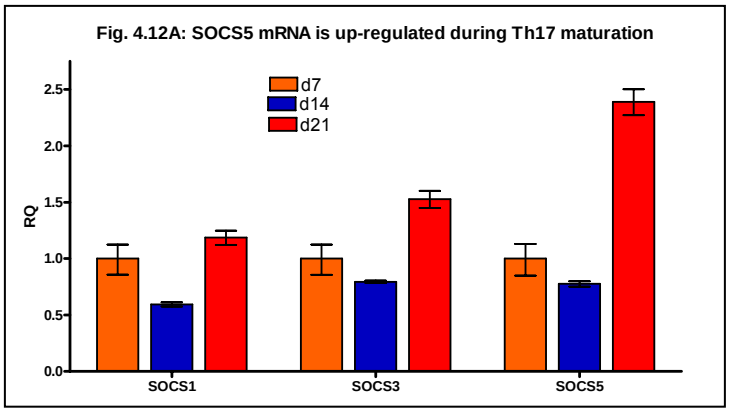
To address the role of SOCS5 in Th17 maturation, we received SOCS5 knockout mice on a C57BL/6 background as a kind gift from the lab of Dr. Sandra Nicholson at the Walter and Eliza Hall Institute in Australia. Given that our Western blots and qRT-PCR data showed some SOCS5 expression even in immature Th17 cells, we started by testing the effect of IL-4 on SOCS5-deficient Th17 cells after one week of *in vitro* differentiation, and to distinguish between potential effects of SOCS5 in T cells versus DCs we did the mix-and-match experiment with wildtype T cells and SOCS5-knockout DCs or vice versa. The data in Fig. 4.13 show that SOCS5-deficiency, in either the T

cells or the DCs, had absolutely no effect on suppression of IL-17 by IL-4. These results were no surprise, however, because we did not expect SOCS5 to play a role in IL-4R signaling until after three weeks of culture, when the Th17 cells have reached maturity. We hypothesized that SOCS5-deficient Th17 cells cultured for three weeks would fail to mature and IL-4 would maintain its suppressive capacity. However, numerous attempts at inducing *in vitro* Th17 maturation with T cells from wildtype littermate controls were unsuccessful, and we decided to go back to our system of *ex vivo* maturation via one-day versus three-day antigen re-stimulation of whole splenocytes following immunization with KLH. Initial attempts at inducing *ex vivo* Th17 maturation from wildtype littermate controls also failed, suggesting that Th17 maturation may be slower or more difficult to induce in the C57BL/6 strain than in the BALB/c or DBA strains used in previous experiments. However, we were ultimately successful at inducing Th17 maturation by *ex vivo* re-stimulation once we waited until four weeks after immunization, rather than harvesting spleens at two weeks as in previous experiments. The results, shown in Fig. 4.14, demonstrate that SOCS5 is not required for resistance to suppression by IL-4.

One interesting observation from these experiments, however, is that splenocytes from SOCS-knockout mice produced significantly more IL-17 than SOCS5-heterozygous mice, despite the fact that we saw no difference in naïve Th17 differentiation *in vitro*. How SOCS5 might inhibit IL-17 expression is not clear, and future experiments are needed to determine the cause of this discrepancy. Because we did not see a difference in the baseline IL-17 expression from splenocyte cultures of two-week immunized mice (data not shown), we hypothesize that SOCS5 plays a role in long-term maintenance of

memory Th17 cells *in vivo*. However, these effects very well may not be Th17-specific or T cell intrinsic.

Given that SOCS1 and SOCS5 both inhibit IL-4R signaling and that we found both SOCS1 and SOCS5 were up-regulated in mature Th17 cells, it seems likely that they are functionally redundant, and SOCS1 compensates for the absence of SOCS5 in the knockout mice. To address this question would require mice deficient in both SOCS1 and SOCS5 or to knockdown SOCS1 expression in SOCS5-deficient Th17 cells, both of which we are currently exploring.



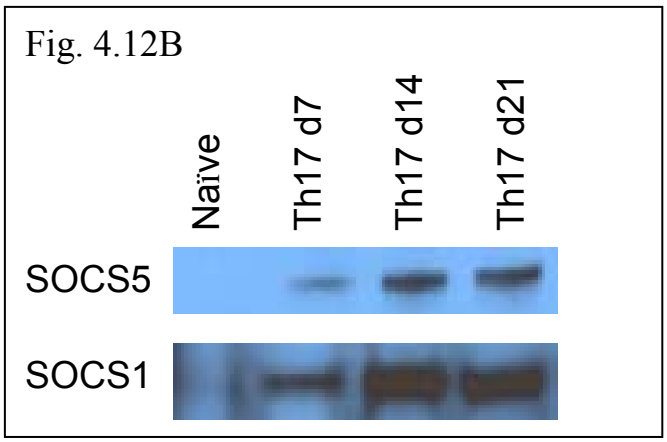


Figure 4.12: Mature Th17 cells up-regulate SOCS5.

(A) Th17 cells were generated *in vitro* with one, two or three weeks of stimulation. RNA was collected and levels of SOCS1, SOCS3 and SOCS5 were measured by real time PCR with primers and probes from Applied Biosystems. Data are normalized first to the internal control β-actin and second to the expression level in Th17 cells after one week of culture. Error bars represent SEM of triplicate PCR reactions. (B) Naïve T cells or Th17 cells after one, two or three weeks of culture were lysed with PhosphoSafe Lysis Buffer (Novagen). Lysates were reduced and run on 10% SDS-PAGE gels and stained with antibodies for SOCS5 or SOCS1.

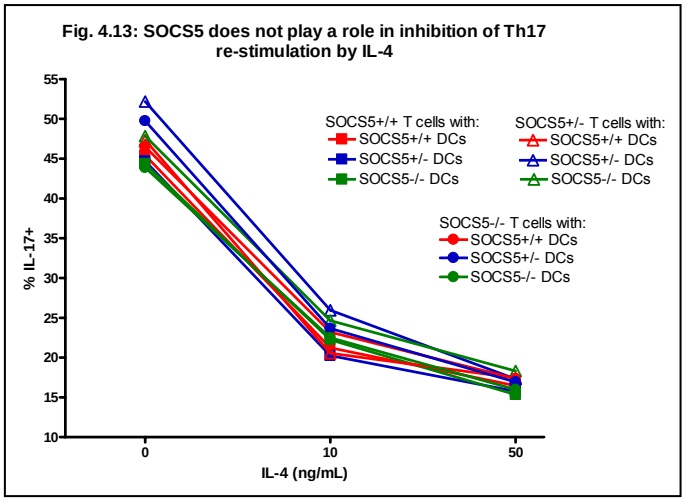
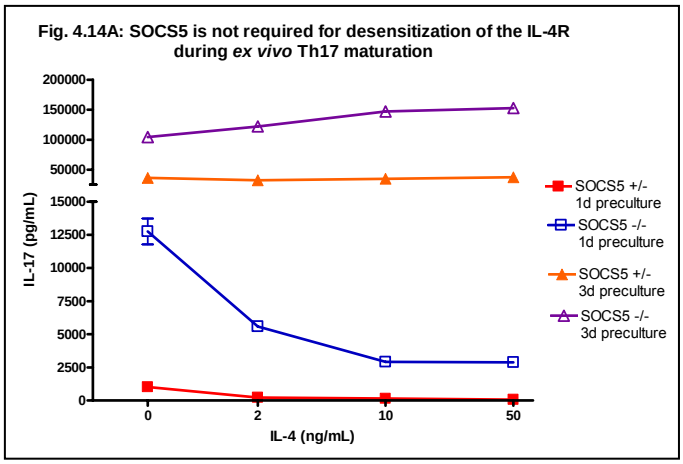


Figure 4.13: SOCS5 does not play a role in inhibition of Th17 re-stimulation by IL-4.

Naïve T cells from SOCS5 knockout, heterozygous or wildtype mice were cultured under Th17-polarizing conditions with BM-DCs from knockout, heterozygous or wildtype mice. After five days stimulation and two days rest cells were washed and re-stimulated with anti-CD3 and increasing concentrations of IL-4 for two days. IL-17 was measured by ICS.



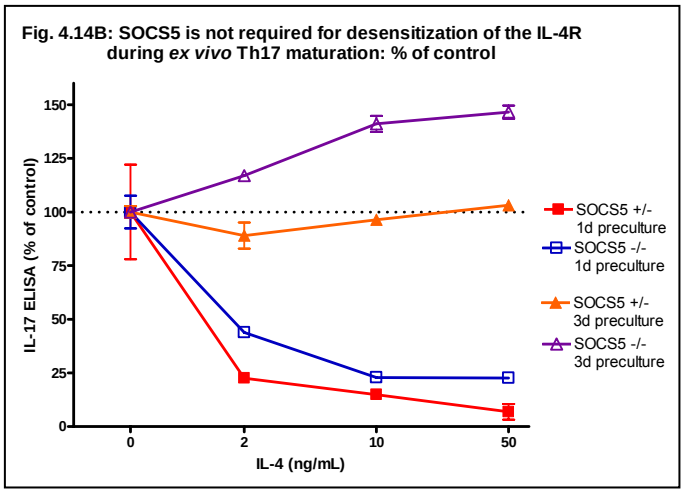


Figure 4.14: SOCS5 is not required for desensitization of the IL-4R during *ex vivo* Th17 maturation.

SOCS5 knockout or heterozygous mice were immunized i.p. with KLH/CFA. After four weeks spleen cells were collected and stimulated with KLH for one or three days, washed and replated with KLH and increasing concentrations of IL-4 for two days. IL-17 levels in the supernatant were measured by ELISA. Error bars represent SEM of triplicate culture samples. In (B), the IL-17 expression data from (A) was normalized to the expression level in the sample with no IL-4.

Discussion

We have observed that Th17 cells, generated both *in vitro* and *in vivo*, become resistant to suppression by IL-4 after re-stimulation. The maturation kinetics closely reproduce what has been demonstrated for Th1 cells, suggesting that this process is not Th17-specific, but rather may be a universal property of chronically activated T helper cells. There is no evidence to date either supporting or refuting the possibility of Th2 maturation and desensitization of the IFNγ receptor. However, given our observations that the stimulation required to induce maturation can be variable and ambiguous, T helper cell maturation may depend on a complex combination or sequence of as yet undefined signals, making it difficult to reproduce *in vitro* when each lab uses a slightly different method. Conflicting results between our group and others, who have shown Th17 plasticity even after three weeks of culture, may be due to differences in culture conditions or APC populations [83, 93]. For example, they used peptide plus irradiated spleens cells while we used anti-CD3 and BM-DCs. In addition, the difficulty we encountered demonstrating that Th17 cells from arthritic mice are fully mature without requiring short *in vitro* re-stimulation, begs the question of whether maturation is simply an *in vitro* artifact. Several groups have already shown that *in vitro*-derived Th17 cells differ greatly from *in vivo*-derived Th17 cells [92-94]. However, one of these reports actually suggests that *in vivo*-derived memory Th17 cells are more resistant to suppression and conversion than *in vitro*-derived Th17 cells, suggesting that our culture conditions may better approximate the natural setting.

Similar to published data on mature Th1 cells, we were able to demonstrate a loss of STAT6 activation in response to IL-4 in mature Th17 cells, despite normal levels of all

of the IL-4R signaling components [73, 77, 178]. However, there is disagreement on the underlying mechanisms of IL-4R desensitization: Seki et al. demonstrated selective up-regulation of SOCS5 in Th1 cells and SOCS5-dependent inhibition of IL-4R signaling, while Huang et al. found no increase in expression of SOCS1, SOCS3 or SOCS5 and suggested that STAT6 recruitment to the IL-4R was impaired through an unknown mechanism. In our experiments there was an up-regulation of SOCS5, and possibly SOCS1, leading us to hypothesize that IL-4R-desensitization in Th17 cells may be mediated by SOCS5. However, the data from SOCS5-deficient mice shows no loss of IL-4R desensitization in mature Th17 cells in the absence of SOCS5. Given the possibly redundancy between SOCS1 and SOCS5 and that both were expressed in mature Th17 cells, future experiments are planned to address the role of SOCS1 in Th17 maturation, with the knowledge that we may need to generate Th17 cells deficient in both SOCS1 and SOCS5, either by crossing the individual knockout mice or by *in vitro* siRNA-mediated knockdown.

Chapter 5

Control of inflammatory arthritis by cytokine balance

We and others have established that IL-4 and IFNγ suppress Th17 differentiation and re-stimulation *in vitro*, but the potential immunoregulatory roles of these cytokines *in vivo* are uncertain. While IL-17 has consistently been shown to play a pathogenic role in CIA, the effects of IL-4 and IFNγ are more complex, with evidence for both protective and pathogenic functions depending on phase of disease, location and relative abundance of other cytokines. Thus we asked what role the balance between IL-4, IFNγ and IL-17 plays in the development of CIA, taking advantage of a system in which collagen-immunization results in limited disease incidence and a wide range of severity. To answer this question we measured systemic cytokine levels in the serum at several time points after immunization, as well as *in vitro* cytokine production from lymphoid organs and inflamed joints during the peak of disease. We also administered neutralizing antibodies to IFNγ, IL-4 and IL-17 to perturb the cytokine balance and monitored the resulting changes in collagen-specific cytokine responses and disease pathogenesis.

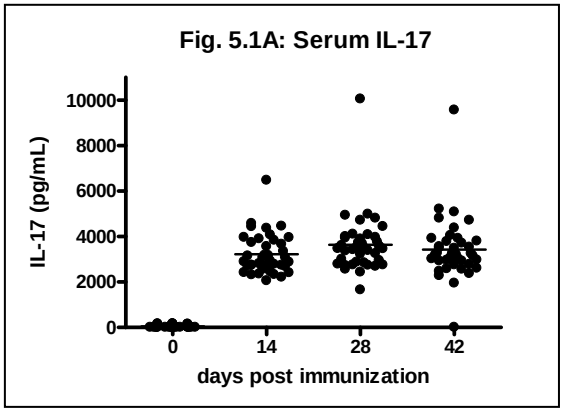
IL-17/IFNγ balance correlates with disease

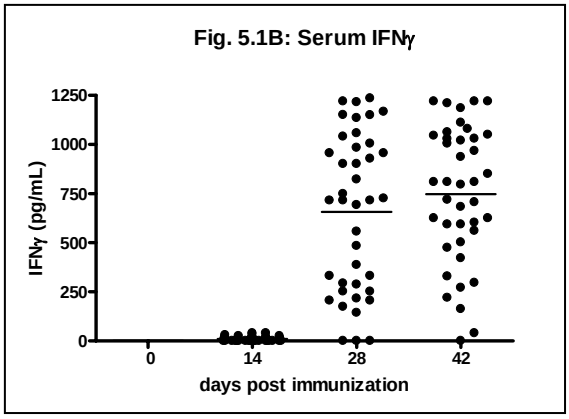
Previous reports suggest that susceptibility to arthritis in various mouse strains correlates with high levels of IL-17 and low levels of IFNγ. Similarly, we hypothesized that among individual collagen-immunized DBA mice, those with a Th17-biased response develop more severe arthritis than those with a Th1-biased response. To test this hypothesis we collected serial serum samples every two weeks, beginning on the day

of collagen immunization, and assessed cytokine levels by ELISA. The results in Fig. 5.1A show that serum IL-17 was markedly elevated by day 14 and remained elevated until at least day 42. All mice developed long-lasting elevation of serum IL-17, regardless of whether or not they developed arthritis. Serum IFNγ, on the other hand, did not peak until day 28, and there was a much wider range in its absolute level (Fig. 5.1B). IL-4 was not detectable in the serum at any time point (data not shown). In order to correlate disease outcome with a composite measurement of Th17 and Th1 responses, we calculated the ratio of IL-17 to IFNγ concentrations. The results, shown in Fig. 5.1C, demonstrate that mice that developed arthritis had a significantly higher serum IL-17/IFNγ ratio on day 28 than mice that did not develop arthritis, despite receiving the same immunization.

To quantify antigen-specific Th1 and Th17 responses in mice with or without arthritis on day 28, spleen and draining lymph node cells were re-stimulated *in vitro* with collagen, and IFNγ and IL-17 were measured in the supernatant by ELISA (Fig. 5.2). IL-4 was not detectable (data not shown). Although serum IL-17 levels were fairly uniform among arthritic and non-arthritic mice, the collagen-specific Th17 responses in the lymphoid organs were much more variable and there was a trend towards increased IL-17 in arthritic mice, which did not reach statistical significance (Fig. 5.2A). Consistent with the serum cytokine ratios, however, arthritic mice had a significantly higher IL-17/IFNγ ratio in culture supernatants than non-arthritic mice (Fig. 5.2C). These results suggest that both Th1 and Th17 responses are initiated in response to immunization with collagen and CFA and that disease progression depends on the balance between the two competing lineages rather than the absolute strength of either alone.

We next examined the correlation between clinical disease scores and cytokine responses in the target organ. IL-17, IFNγ and IL-4 were measured in paws by mincing and culturing them overnight, without exogenous stimulation, followed by ELISA of the supernatants. As the results in Fig. 5.3 show, the Th17 response in the joint was distinct from the systemic response, in that only paws from arthritic mice produced IL-17. Interestingly, non-arthritic mice did not have measurable IL-17 in the paw cultures even though they had similar levels of serum IL-17 compared with arthritic mice. Furthermore, IL-4 and IFNγ were only detectable at significant levels in arthritic paws, and the levels of IL-4 and IFNγ correlated positively with the level of IL-17 (Fig. 5.3). These results suggest that inflammatory responses in the secondary lymphoid tissues are distinct from those in the target organ and that the recruitment of cytokine-producing cells from lymphoid organs to target tissue may represent a key step in the development of inflammation.





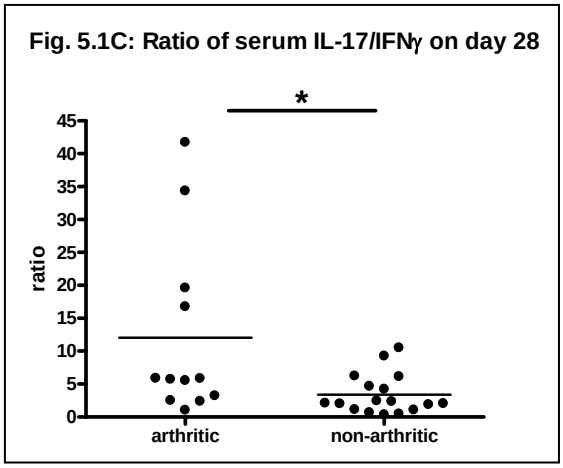
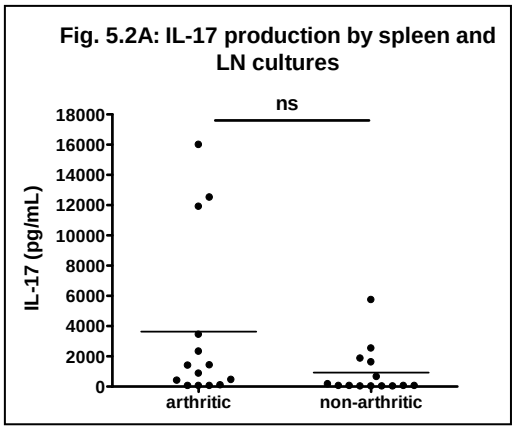
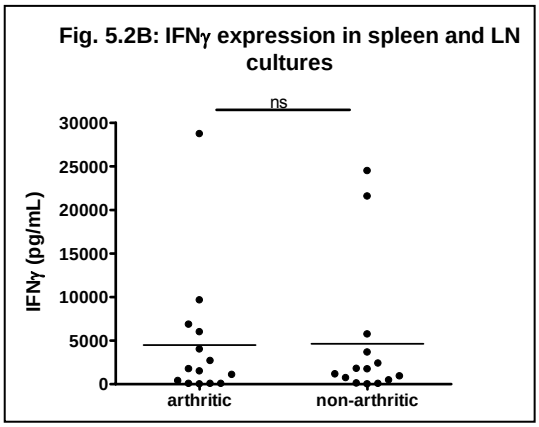


Figure 5.1: Serum IL-17 and IFNγ levels during CIA**.**

DBA mice were immunized i.d. with cII/CFA on day 0. Serum was collected by serial tail bleeds on days 0, 14, 28 and 42. IL-17 (A) and IFNγ (B) were measured by ELISA in triplicate. (C) Arthritis was scored visually every other day from day 20. Mice with a clinical score of two in at least one paw were considered arthritic. IL-17/IFNγ is the ratio of the absolute level of IL-17 to the level of IFNγ in serum on d28. Data represent the mean of 30 mice from two experiments. * $p<0.05$





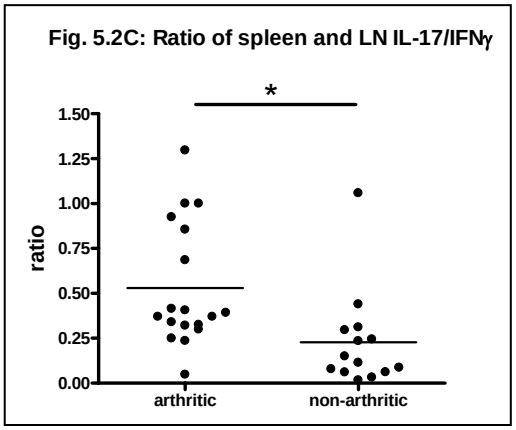
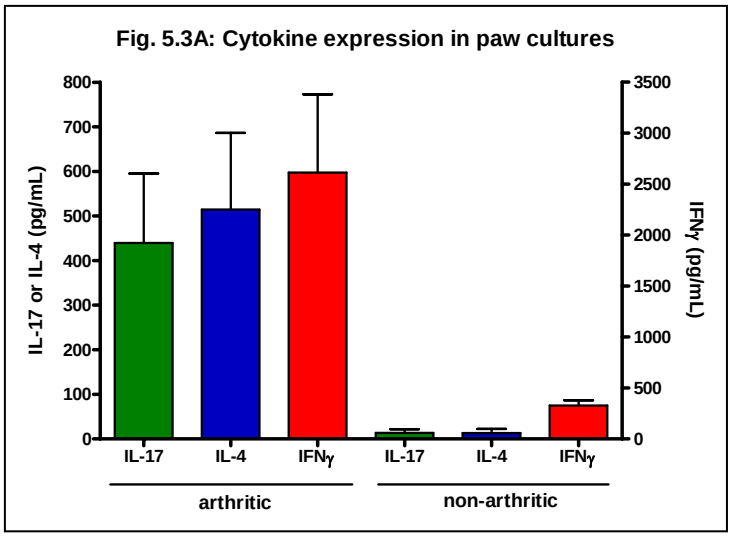


Figure 5.2: Collagen-specific IL-17 and IFNγ responses in spleen and DLN during CIA.

Single cell suspension of spleens and DLNs, from day 28 after collagen and CFA immunization, were re-stimulated with collagen for five days. Supernatants were analyzed for IL-17 (A) and IFNγ (B) by ELISA. (C) Arthritis was scored visually every other day from day 20. Mice with a clinical score of two in at least one paw were considered arthritic. IL-17/IFNγ is the ratio of the absolute level of IL-17 to the level of IFNγ in serum on d28. Data represent the mean of 30 mice from two experiments. * $p<0.05$, ns = not significant.



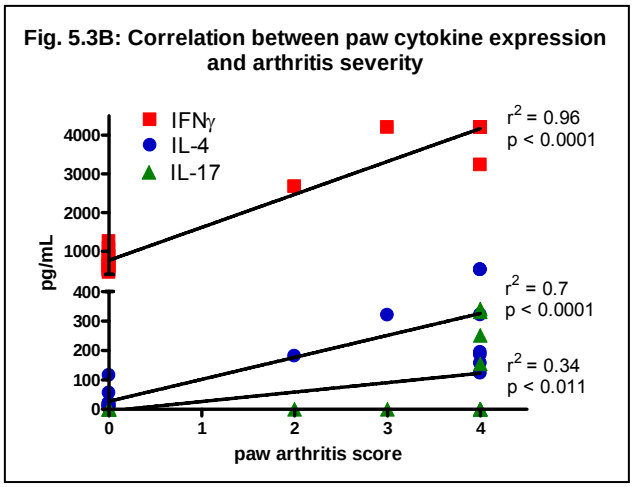


Figure 5.3: Expression of IL-17, IFNγ and IL-4 in paw cultures.

Paws were collected from mice 28 to 32 days after collagen immunization. The skin was removed and paws were minced and cultured overnight. Supernatants were collected for measurement of IL-17, IL-4 and IFNγ by ELISA (A). Error bars represent the SEM of 20 mice from two experiments. (B) Correlation analysis between individual paw cytokine levels and arthritis score. All three cytokines had a significant positive correlation with arthritis score.

Regulation of IL-17 and IL-4 by endogenous IFNγ during the initiation phase of arthritis

As the absolute level of IL-17 was not predictive of arthritis, but the balance of endogenous IL-17 and IFNγ appeared to be important, we chose to perturb this balance by neutralizing endogenous IFNγ from day 10 to 20 after collagen immunization, thus targeting the initiation phase of CIA. As shown in Fig. 5.4, treatment with neutralizing antibodies to IFNγ resulted in an accelerated course of arthritis. However, the effect of anti-IFNγ did not persist long after the end of treatment, and the incidence and severity at day 40 were the same amongst the different groups. These results are consistent with previously reported studies [161], and suggest that IFNγ has a protective role in the early response to collagen immunization.

In the absence of endogenous IFNγ, arthritis peaked by day 20, at which point the severity was significantly different from the control groups. Hence we chose to evaluate systemic and articular immune events around day 20. Mice that received neutralizing antibody to IFNγ had higher levels of IL-17 and IL-4 in the serum (Fig. 5.5), but there was no effect on IL-17 and IL-4 responses in collagen-stimulated spleen and lymph node cultures (data not shown). Consistent with previous data depicting the differences between the systemic and joint specific responses, the levels of IL-17, IL-4 and IFNγ were significantly higher in the paws of mice that received anti-IFNγ neutralizing antibodies versus control mice (Fig. 5.6) and correlated with disease severity (data not shown).

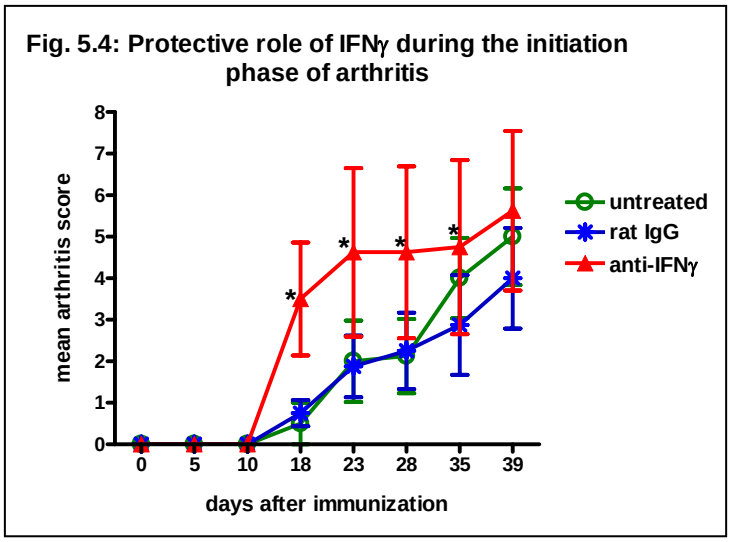
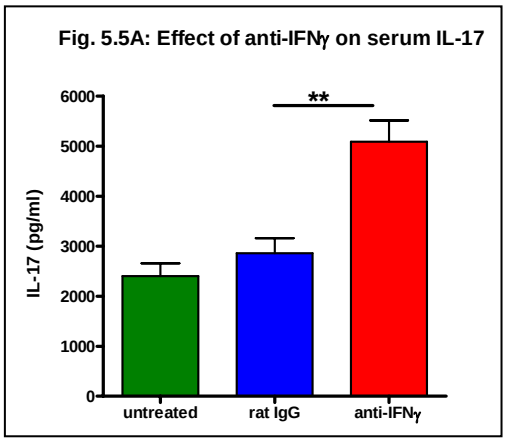


Figure 5.4: Protective role of IFNγ during the initiation phase of arthritis.

Neutralizing antibody to IFNγ (clone R46A2, 100ug/mouse/day) was administered i.p. from day 10 to 20 after immunization with cII/CFA. Rat IgG was used as isotype control. Arthritis was scored visually from day 10 onwards. Data are representative of two experiments, with n = 7 in each group in each experiment. * $p<0.05$ versus isotype control.



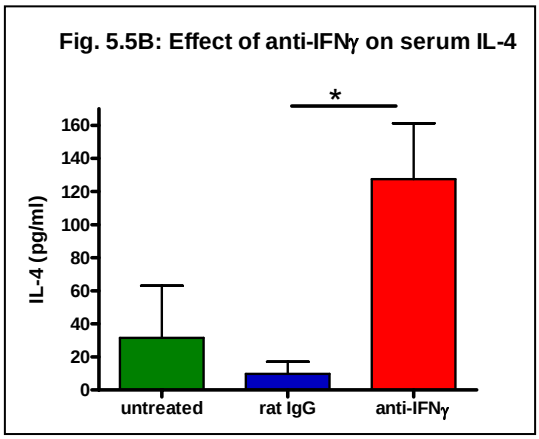


Figure 5.5: Effect of anti-IFNγ on serum IL-17 and IL-4 expression during CIA.

Mice were treated with anti-IFNγ (clone R46A2, 100ug/mouse/day) from day 10 to 20 after cII/CFA immunization. Serum from day 21 to 23 after collagen immunization was analyzed for IL-17 (A) and IL-4 (B) by ELISA. Error bars represent SEM. Data are representative of two experiments, with n = 7 in each group. * $p<0.05$ and ** $p<0.001$.

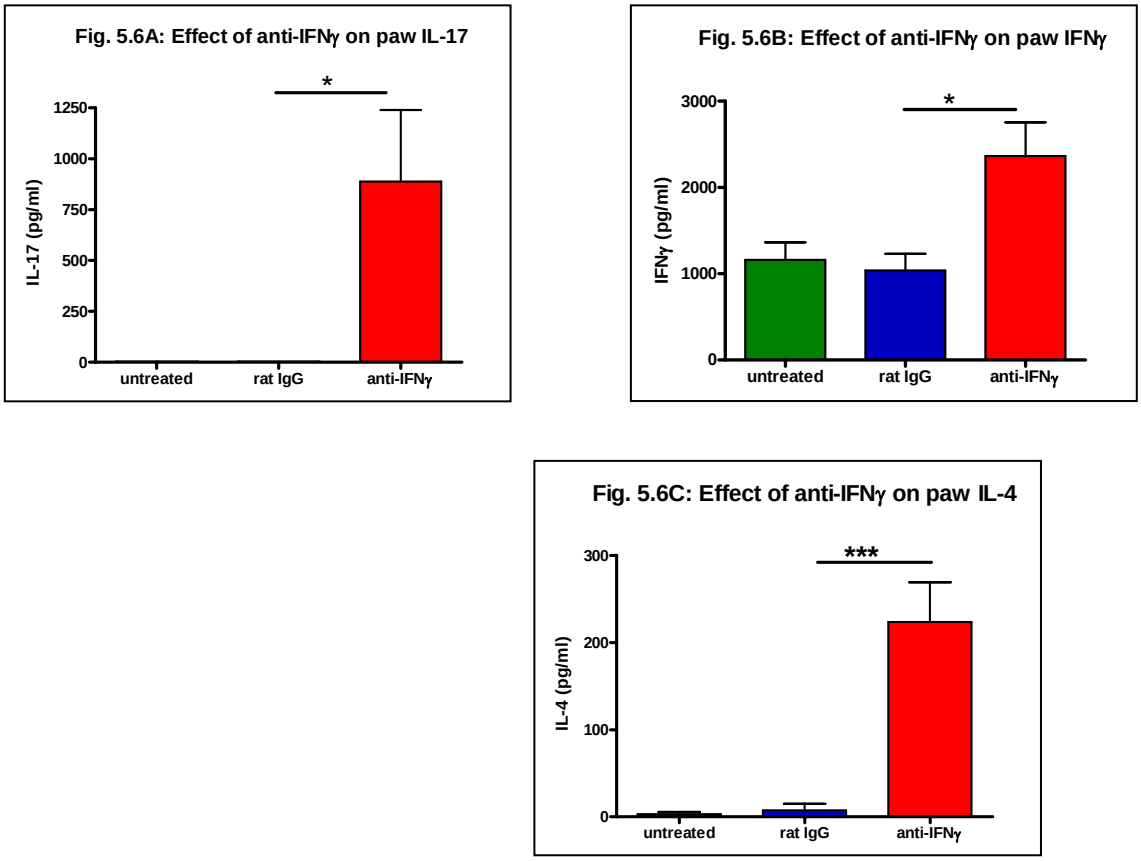


Figure 5.6: Effect of anti-IFNγ on paw IL-17, IFNγ and IL-4 expression during CIA.

Mice were treated with anti-IFNγ (clone R46A2, 100ug/mouse/day) from day 10 to 20 after cII/CFA immunization. On day 21-23 paws were minced and cultured overnight. The supernatants were analyzed for IL-17 (A), IL-4 (B), and IFNγ (C) by ELISA. Error bars represent SEM. Data are representative of two experiments, with n = 7 in each group. *** $p<0.0001$. *$p<0.05$.

Role of endogenous IL-4 in regulation of CIA

IL-4 has been shown to suppress IL-17 production *in vitro* during immune responses to collagen; thus it was plausible that the increased levels of IL-4 observed in mice treated with neutralizing antibody to IFNγ might fulfill a regulatory role. However, it has also been suggested that IL-4 might have pathogenic effects during the early phase of arthritis [27]. Additional experiments were therefore performed to assess the role of IL-4 in mice that were treated with neutralizing antibody to IFNγ by neutralizing both IFNγ and IL-4 during the early phase of arthritis.

Anti-IFNγ or anti-IL-4 antibodies were administered, either alone or in combination, from day 10 to 20 after immunization with collagen and CFA. As shown in Fig. 5.7, mice that received neutralizing antibodies to IFNγ alone developed an accelerated course of arthritis, and the group that received neutralizing antibodies to both IL-4 and IFNγ had significantly more severe arthritis than the anti-IFNγ alone group. Treatment with neutralizing antibody to IL-4 alone had no affect on arthritis, consistent with previous findings [162]. These results suggest that IFNγ plays a more prominent protective role in CIA than IL-4 but that IL-4 can play a regulatory (and not a pathogenic) role in the absence of IFNγ.

As both IFNγ and IL-4 have been shown to suppress IL-17 production, we asked whether the increased severity of arthritis seen in the absence of both IFNγ and IL-4, in comparison to the absence of IFNγ only, was secondary to higher levels of IL-17. Thus we measured serum IL-17 levels on day 22, immediately after completion of the 10-day

course of neutralizing antibody administration. The results in Fig. 5.8 demonstrate that neutralizing IL-4 and IFNγ did not up-regulate IL-17 above the level in mice that received neutralizing antibody to IFNγ alone. Therefore the increased incidence of arthritis in the absence of IFNγ and IL-4, versus IFNγ alone, cannot be attributed to systemic elevation of IL-17. Additionally, while neutralization of IFNγ resulted in elevation of serum IL-4, neutralization of endogenous IL-4 did not result in any change in serum IFNγ levels (data not shown). Consistent with our previous findings, we did not observe augmented IL-17 responses in collagen re-stimulation cultures of spleen and draining lymph nodes (data not shown). Thus the mechanisms underlying the protective role of IFNγ seem to differ from the mechanisms mediating the protective role of IL-4, and the increase in disease following treatment with anti-IL-4 may be dependent on a mechanism distinct from IL-17.

Levels of IFNγ, IL-4 and IL-17 in the target organ were measured by ELISA of supernatants from overnight paw cultures. Consistent with our previous findings, IFNγ, IL-4 and IL-17 were elevated in the paws of arthritic mice from the anti-IFNγ group (Fig. 5.9A,B&C). Interestingly, mice that received anti-IFNγ + anti-IL-4 had similar levels of IFNγ and IL-4 but lower levels of IL-17 in their paws compared to mice that received anti-IFNγ alone, even though they had more severe arthritis. A plausible explanation for this discrepancy could be that joints are more sensitive to Th17-mediated inflammation in the absence of systemic protective Th1 and Th2 responses or that inflammation in these paws is mediated by a factor other than IL-17.

Although we have shown that both endogenous and exogenous IL-4 down-regulate IL-17 production *in vitro* and *ex vivo*, a similar effect of endogenous IL-4 was not seen *in vivo*, as neither neutralizing antibodies to IL-4 alone nor the combination of antibodies to IL-4 and IFNγ up-regulated IL-17 in serum or culture supernatants of spleens, lymph nodes or paws. Thus endogenous IL-4 does not appear to play a major role in the regulation of IL-17 in the early phase of CIA. IL-4 likely exerts a protective function in the absence of IFNγ by some other mechanism, possibly by inducing other regulatory cytokines such as IL-10 and/or by direct effects on APCs or synovial cells.

As IL-10 has been found to be associated with a less pathogenic phenotype of Th17 cells in the mouse model of multiple sclerosis [162, 179], we evaluated IL-10 responses in mice that received neutralizing antibodies to IL-4 and/or IFNγ. Arthritic paws from mice that received neutralizing antibodies to IFNγ or IL-4 + IFNγ had increased levels of IL-10, which correlated positively with disease (Figure 5.9D). IL-10 was not detectable in collagen re-challenge cultures of lymphoid organs (data not shown). This suggests that in CIA, endogenous regulatory effects of IL-4 are not mediated through systemic production of IL-10. The elevated levels of IL-10 in the arthritic joints could in part reflect IL-10 production by synovial cells. Much like the other cytokines, paw IL-10 levels correlated positively with disease severity (data not shown). Interestingly, IL-10 is also highly expressed in RA synovium, but evidence suggests that IL-10R signaling in the synovium is blunted, limiting the potentially anti-inflammatory effects [180, 181]. It remains to be determined what role IL-10 plays in the arthritic joint in CIA and if the IL-10 receptor is competent.

Previous studies have shown that administration of IL-4 can protect against bone damage in CIA, and IL-4 is known to have direct inhibitory effects on osteoclastogenesis distinct from its effects on T and B cells [48]. We assessed the effects of neutralizing antibodies to IFNγ and IL-4 on joint pathology by staining paw sections with H&E, followed by visual scoring by two independent, blinded observers for several parameters, including inflammatory infiltrate, synovitis, cartilage destruction and bone destruction. Interestingly, we found that the increased severity of arthritis following neutralization of IFNγ and IL-4 was associated with increased bone and cartilage damage compared to the anti-IFNγ only group, despite the fact that both groups showed a similar degree of synovitis and inflammatory infiltrate (Fig. 5.10). The anti-IL-4 only group did not show any increased bone or cartilage damage over baseline. Fig. 5.11 illustrates the degree of inflammatory infiltrate and bone and cartilage damage associated with the neutralization of IFNγ compared with IFNγ plus IL-4.

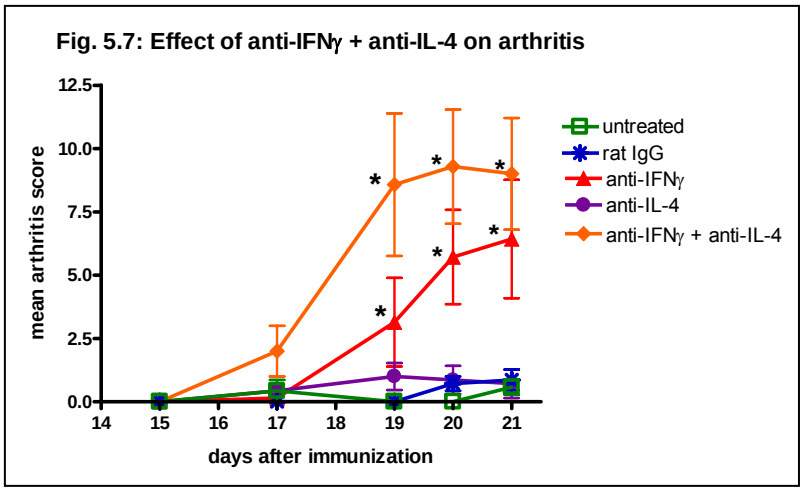
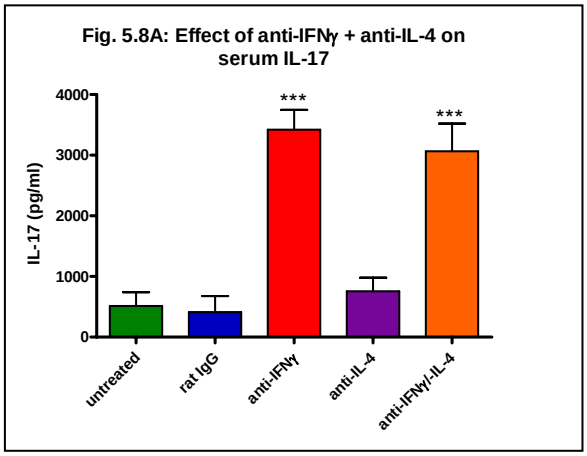
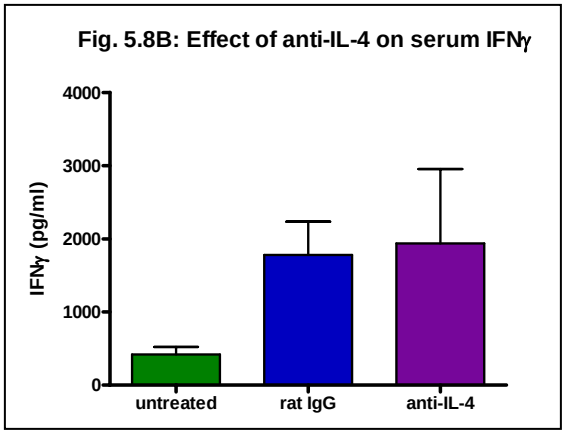


Figure 5.7: Protective role of IL-4 in the absence of IFNγ during CIA.

Neutralizing antibody to IFNγ (clone R46A2, 100 μg/mouse/day), and/or neutralizing antibody to IL-4 (clone 11B11, 100 μg/mouse/day) was administered i.p. from day 10 to 20 after cII/CFA immunization. Rat IgG was used as isotype control. Arthritis was scored visually from day 10 onward. Data are representative of two experiments, with n = 8 in each group. * $p<0.05$ versus rat IgG.





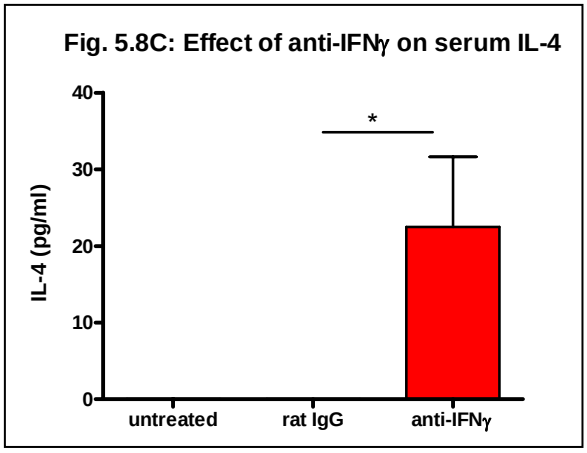


Figure 5.8: Effect of anti-IFNγ and anti-IL-4 on serum cytokine expression during CIA.

Mice were treated with neutralizing antibodies to IL-4 and/or IFNγ from day 10 to 20 after cII/CFA immunization. Serum samples were collected on day 22 and analyzed for IL-17 (A), IL-4 (B), and IFNγ (C) by ELISA. Error bars represent SEM. Data are representative of two experiments, with n = 8 in each group. *$p<0.05$, *** $p<0.001$ vs. rat IgG

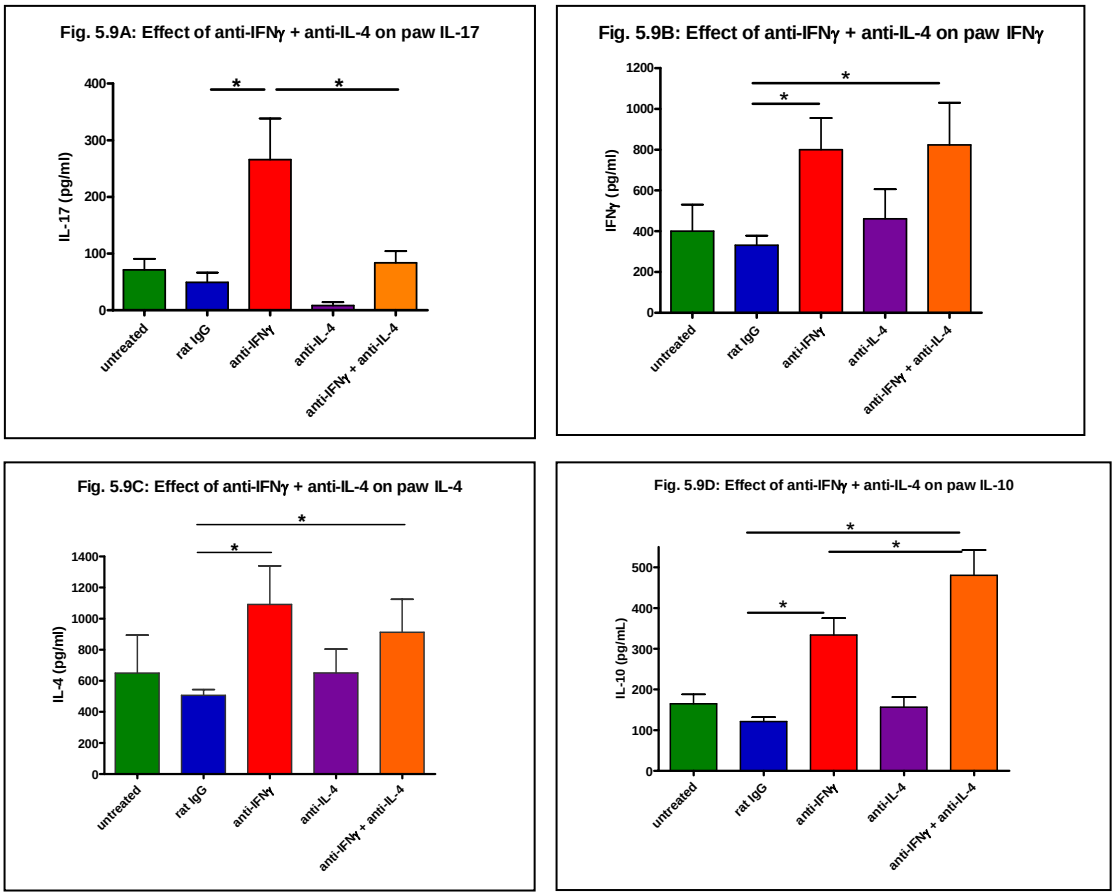


Figure 5.9: Effect of anti-IFNγ and anti-IL-4 on paw cytokine expression during CIA.

Mice were treated with anti-IFNγ and/or anti-IL-4 from day 10 to 20 after cII/CFA immunization. On day 21-23 paws were minced and cultured overnight. The supernatants were analyzed for IL-17 (A), IL-4 (B), IFNγ (C) and IL-10 (D) by ELISA. Error bars represent SEM. Data are representative of two experiments, with n = 8 in each group. *$p<0.05$.

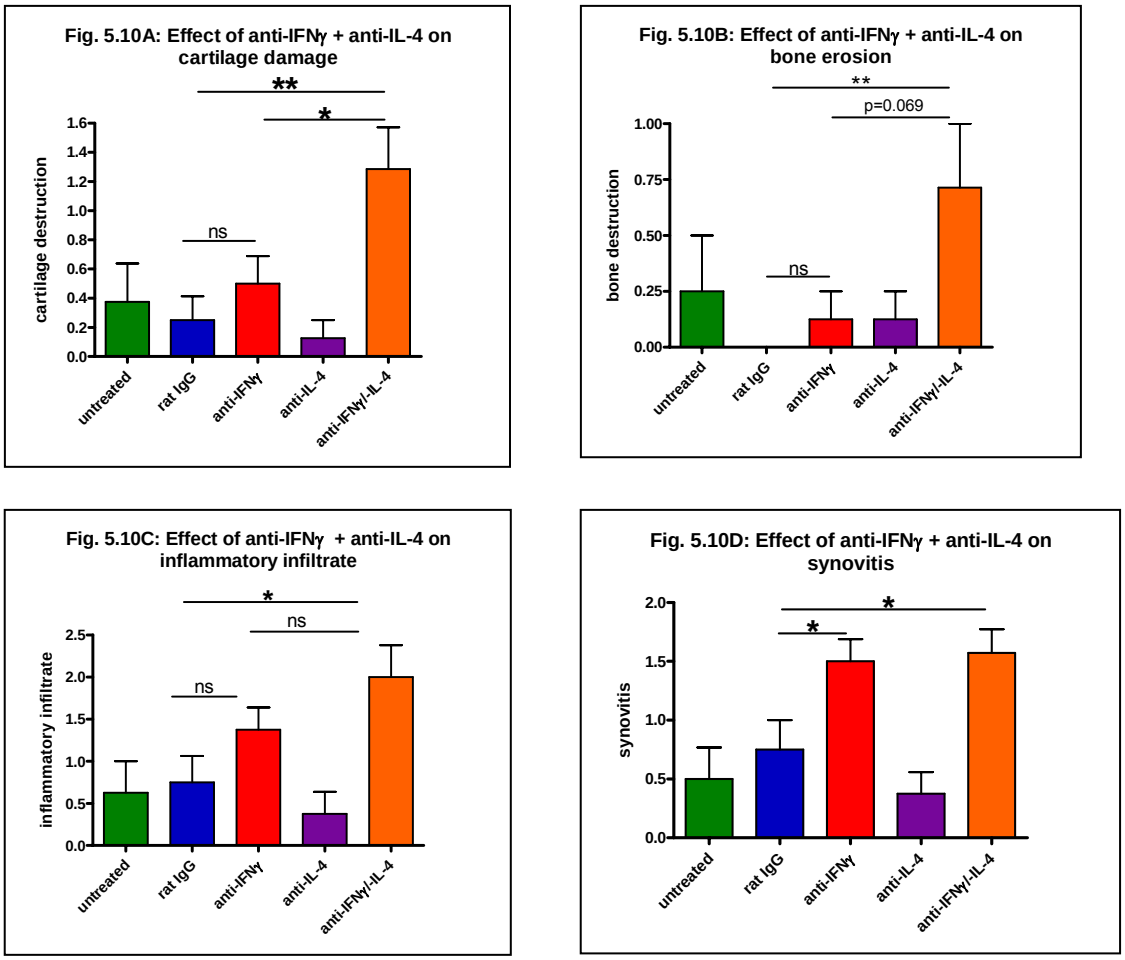


Figure 5.10: Joint pathology after treatment with anti-IFNγ and anti-IL-4.

Mice received neutralizing antibodies to IFNγ and IL-4 from day 10 to 20 after cII/CFA immunization. One hind paw was collected from each mouse on day 21-23, sectioned and stained with hematoxylin and eosin. The sections were scored on a scale of 0-3 for cartilage damage (A), bone erosion (B), inflammatory infiltrate (C) and synovitis (D) by two blinded observers. Error bars represent SEM of 8 mice per group. * $p<0.05$, **$p<0.01$, ns = not significant

Fig. 5.11

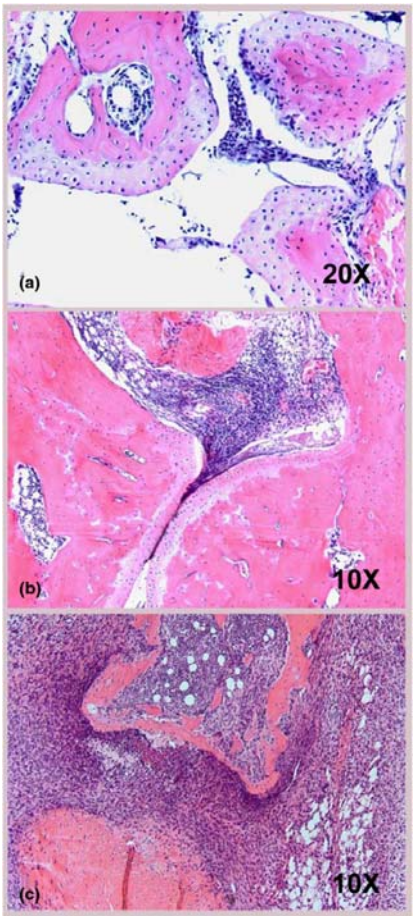


Figure 5.11: H&E staining of joints from mice treated with anti-IFNγ and anti-IL-4.

(A) Ankle joint (clinical score 0) from a mouse that received rat IgG demonstrates a mild inflammatory infiltrate within a non-distorted joint space. A mild degree of synovial hyperplasia is also present. No significant cartilage or bone destruction is seen (H&E, 20x). (B) Arthritic joint (clinical score 4) from a mouse that received anti-IFNγ, demonstrating inflammatory infiltrate, with partial filling of the joint space. Mild synovial hyperplasia is present along with early, minimal alteration of cartilage. Inflammatory changes extend into the adjacent soft tissue. No significant bony changes are present and the joint space is otherwise intact (H&E, 10x). (C) Arthritic joint (clinical score 4) from a mouse that received neutralizing antibodies to IFNγ + IL-4, demonstrating severe inflammatory changes including complete filling of the joint space and extension to the soft tissue. Cartilage is significantly destroyed and the bone shows a substantial amount of destruction and remodeling (H&E, 10x).

Relative contribution of IFNγ and IL-4 in the regulation of IL-17 *in vivo*

Because neutralizing antibodies to IFNγ and/or anti-IL-4 were associated with differential regulation of IL-17 responses *in vivo*, we wanted to confirm and evaluate the role of IL-17 in disease pathogenesis in these mice. Neutralizing anti-IL-17 antibody was administered in combination with anti-IFNγ and/or anti-IL-4 antibodies from day 10 to 20 after immunization with collagen and CFA. Consistent with our previous findings, mice that received anti-IFNγ + anti-IL-4 had more severe arthritis than the anti-IFNγ alone group (Fig. 5.12). Interestingly, neutralizing antibody to IL-17 completely abrogated disease in the anti-IFNγ alone group, whereas anti-IL-17 had only a minor effect on arthritis in the mice that received anti-IFNγ + anti-IL-4. These results suggest that treatment with neutralizing antibodies to both IFNγ and IL-4 prompts the development of joint inflammation that is partially independent of IL-17.

To further elucidate the relative contribution of IL-17 to disease in mice receiving anti-IFNγ versus anti-IFNγ + anti-IL-4, we evaluated the correlation between serum IL-17 and arthritis severity (Fig. 5.13). Although the absolute levels of serum IL-17 were comparable between the groups, there was a significant correlation between IL-17 and arthritis severity in the anti-IFNγ group but not in the anti-IFNγ + anti-IL-4 group (we could not address the IL-17/IFNγ ratio in these mice, as treatment with anti-IFNγ precludes ELISA for IFNγ in the serum). The expression of IFNγ, IL-4 and IL-17 in the supernatants of paws, spleen and lymph node cultures was consistent with previous experiments (data not shown).

Neutralization of IFNγ *in vivo* was associated with elevated serum IL-17, with no further up-regulation in IL-17 after combined neutralization of IFNγ and IL-4, while numerous studies have consistently shown that neutralization of IFNγ and IL-4 is required for the optimal differentiation of Th17 cells *in vitro***.** Furthermore, some studies, mostly in humans, have reported the presence of Th17 cells expressing both IFNγ and IL-17. Hence we wanted to investigate whether the IL-17 and IFNγ responses in our experiments were associated with double-positive Th1/Th17 cells and/or if the increase in systemic IL-17 levels was associated with an increase in the generation of Th17 cells *in vivo*. Splenocytes from the various groups were stimulated with PMA, ionomycin and brefeldin A and analyzed for IL-17, IFNγ and IL-4 production by intracellular flow cytometry. The results show that IFNγ and IL-17 were produced by a discrete population of T cells and that neutralization of both IFNγ and IL-4 was associated with increased differentiation of Th17 cells *in vivo* (Fig. 5.14). IL-4-producing T cells were not detectable. Surprisingly, neutralization of IFNγ alone was not associated with an increase in the number of Th17 cells, even though this group of mice had the highest levels of serum IL-17. On the other hand, neutralization of both IFNγ and IL-4 was associated with an increase in the number of Th17 cells, despite lower levels of serum and paw IL-17 than the anti-IFNγ alone group. Thus, Th17 differentiation and IL-17 production may be differentially regulated *in vivo*, with IFNγ primarily suppressing IL-17 production and IL-4 primarily suppressing Th17 differentiation. Alternatively, the Th17 cells that differentiate in the presence of anti-IFNγ + anti-IL-4 may be differently activated, resulting in a more pathogenic phenotype despite reduced IL-17 production.

Tissue sections of inflamed joints were again stained with H&E and analyzed for inflammatory infiltrate, synovitis, cartilage destruction and bone erosion. Consistent with arthritis severity scores, neutralizing antibody to IL-17 protected the joints of mice treated with anti-IFNγ but had no effect on the severe joint inflammation and tissue destruction observed in mice treated with anti-IFNγ + anti-IL-4 (Fig. 5.15). These results further support the supposition that the exacerbated disease induced by neutralizing IFNγ and IL-4 is mediated by an IL-17-independent mechanism.

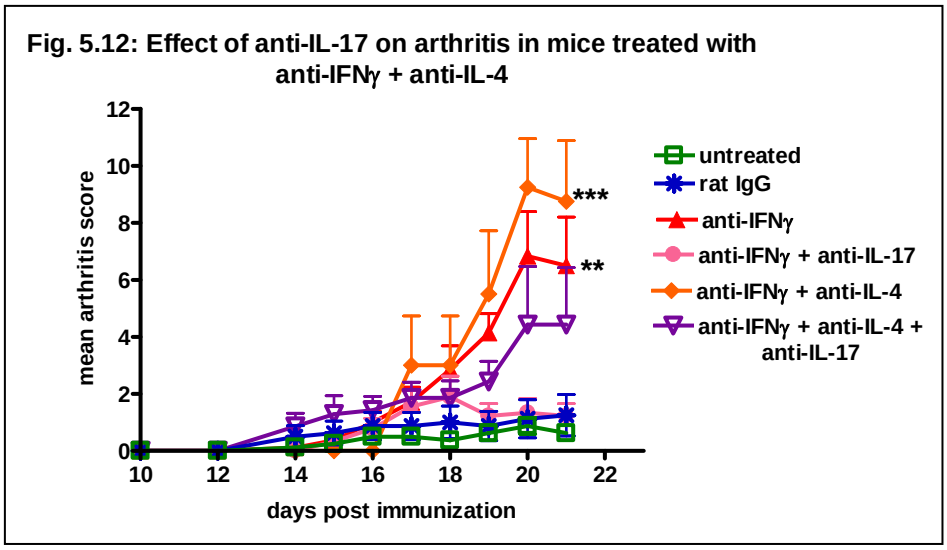


Figure 5.12: Effect of anti-IL-17 during treatment with anti-IFNγ and/or anti-IL-4 at the initiation phase of arthritis.

Neutralizing antibody to IFNγ (clone R46A2, 100 μg/mouse/day), and/or neutralizing antibody to IL-4 (clone 11B11, 100 μg/mouse/day), and neutralizing antibody to IL-17 (clone M210, 100 μg/mouse/day) was administered i.p. from day 10 to 20 after immunization with collagen and CFA. Rat IgG was used as isotype control. Arthritis was scored visually from day 10 onward. n = 8 to 9/group. Error bars represent SEM of 7-8 mice per group. ** $p<0.01$ *** $p<0.001$ versus rat IgG. Also, anti-IFNγ versus anti-IFNγ + anti-IL-17: $p<0.01$. Anti-IFNγ + anti-IL-4 versus anti-IFNγ + anti-IL-4 + anti-IL-17: not significant.

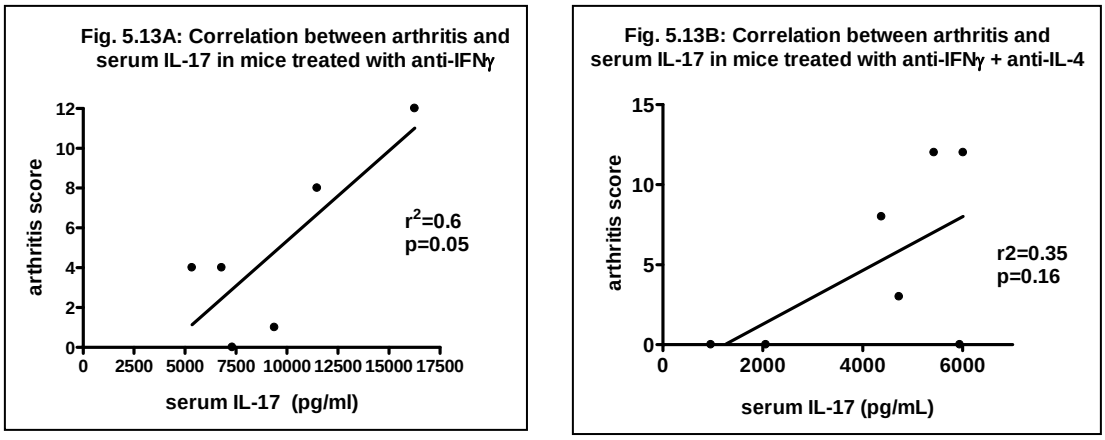


Figure 5.13: Serum IL-17 correlates with disease in mice treated with anti-IFNγ.

Mice were treated with anti-IFNγ and anti-IL-4 from day 10 to 20 after cII/CFA immunization. Serum cytokine levels were analyzed by ELISA on day 22 or 23. Analysis shows a significant correlation between IL-17 and disease in the anti-IFNγ group but not in the anti-IFNγ + anti-IL-4 group.

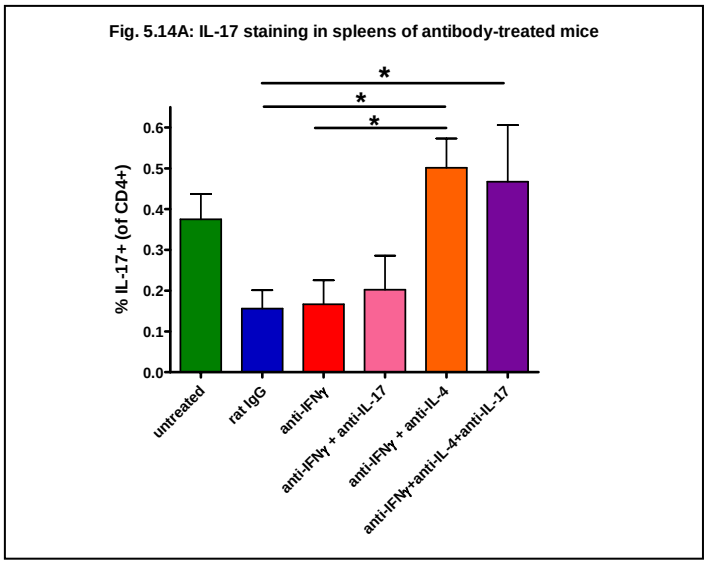


Figure 5.14: Th17 cells in the spleens of mice treated with cytokine neutralizing antibodies during CIA.

Mice were treated with neutralizing antibody to IFNγ, IL-4 and IL-17 from day 10 to 20 after cII/CFA immunization. Splenocytes were collected on day 22 or 23 and cultured overnight with collagen. Following six-hour stimulation with PMA, ionomycin and brefeldin A, cells were stained with anti-CD4, anti-IL-17, anti-IFNγ and anti-IL-4 antibodies and analyzed on a FACS Calibur using Cell Quest software. Data represent the percent of CD4-gated cells staining positive for IL-17A. Error bars represent SEM of 8-9 mice per group. * $p<0.05$.

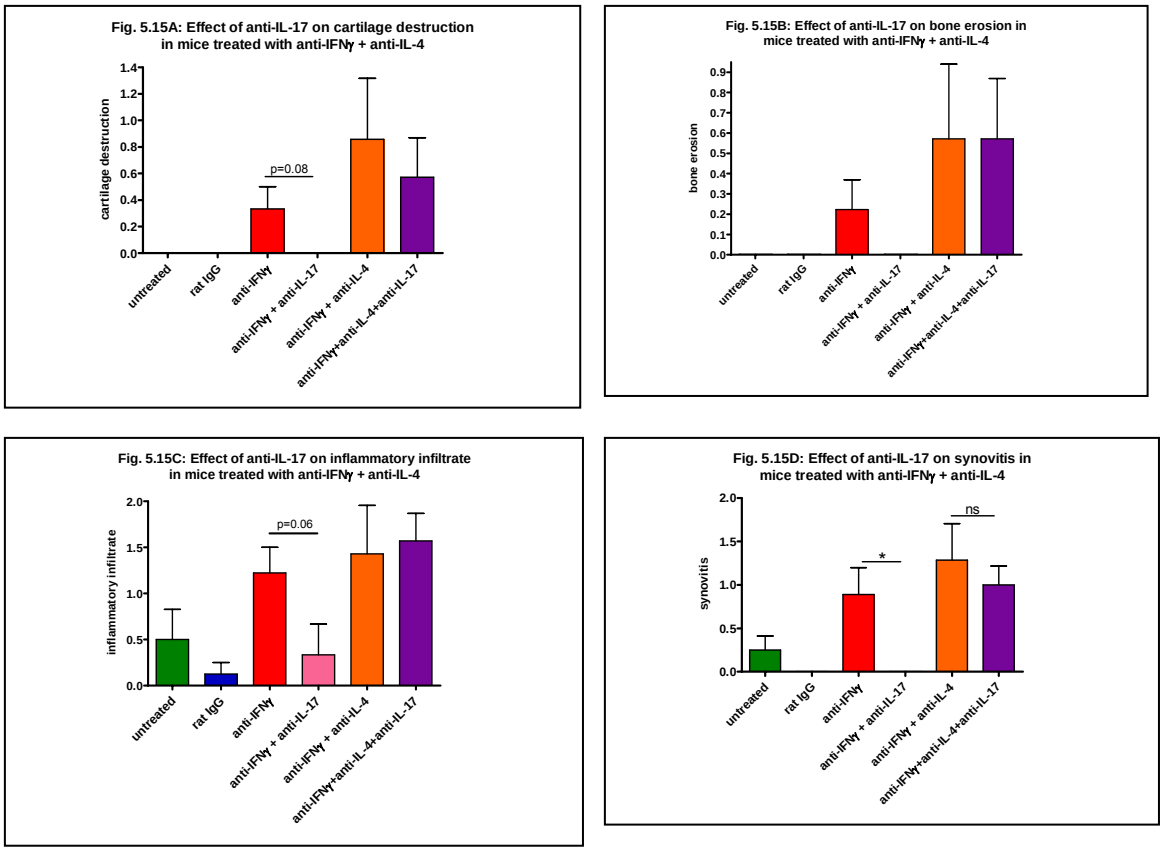


Figure 5.15: Effect of cytokine neutralizing antibodies on joint pathology during CIA.

Mice were treated with neutralizing antibodies to IFNγ, IL-4 and IL-17 from day 10 to 20 after cII/CFA immunization. One hind paw was collected from each mouse on day 22 or 23, sectioned and stained with hematoxylin and eosin. The sections were scored on a scale of 0-3 for cartilage damage (A), bone erosion (B), inflammatory infiltrate (C) and synovitis (D) by two blinded observers. Error bars represent SEM of 8-9 mice per group. *$p<0.05$, ns = not significant.

Discussion

Various antigenic stimuli can trigger IL-17 responses *in vivo* and not all of them will result in systemic or organ specific autoimmunity in animal models, implying that endogenous regulation of IL-17 responses is important in the prevention or attenuation of autoimmunity. Our studies show that the ratio of systemic IL-17/IFNγ is a better predictor of joint inflammation than the level of IL-17 alone, suggesting that disease outcome is not determined solely by the absolute level of the pathogenic cytokine, but rather by the balance between pathogenic and protective signals. How these competing signals regulate disease pathogenesis at the molecular level is not clear. Given our observation that only arthritic joints produced IL-17, one possibility is that these signals modulate trafficking of Th17 cells to the joint, either by altering expression of chemokines by cells of the synovium or expression of chemokine receptors by T cells. Once in the joint, Th17 cells may then induce inflammation and recruitment of other inflammatory cells. Interestingly, arthritic joints had higher levels of IFNγ, and IL-4 than non-arthritic joints, suggesting that once target organ inflammation is initiated there is recruitment of both inflammatory and anti-inflammatory cell types. Further studies are needed to determine the effect of the systemic Th1/Th17 balance on T cell homing and recruitment to the joint.

Our results imply that the balance between Th1 and Th17 cells plays an important role in disease outcome, so neutralizing antibody to IFNγ was administered to perturb this balance. Consistent with previous data suggesting that IFNγ negatively regulates IL-17 responses and clinical arthritis, mice that received anti-IFNγ antibody had accelerated

arthritis associated with elevated levels of IL-17. We also observed an increase in systemic IL-4, which was potentially surprising in view of the therapeutic effect of IL-4 on arthritis and the ability of IL-4 to suppress IL-17. In this situation, however, IL-4 could represent another mechanism for immune regulation that only emerged with neutralization of IFNγ. We found that treatment wth neutralizing antibody to IL-4 exacerbated arthritis, but only in mice that also received neutralizing antibodies to IFNγ, suggesting that IFNγ plays a more prominent role than IL-4 in down-regulating arthritis. This could be due to the fact that the immune response to collagen in DBA mice is primarily Th1 and Th17, with very little Th2 response unless the IFNγ-mediated constraint is removed.

Th17 cells that did not co-express IL-10 were found to have a higher pathogenic potential in a mouse model of multiple sclerosis than Th17 cells that co-expressed IL-10 [182]. It is possible that the increased arthritis in the presence of anti-IFNγ + anti-IL-4 is associated with the generation of a more aggressive phenotype of Th17 cells, one that may be associated with reduced levels of IL-10. However, IL-10 levels were higher in the paws of mice that received anti-IFNγ + anti-IL-4 than in paws of mice that received anti-IFNγ alone. This would suggest that the phenotype of Th17 responses in CIA in the absence of IL-4 is independent of IL-10.

There was a similar degree of inflammatory infiltrate and synovitis in the absence of IFNγ alone or in the absence of IFNγ + IL-4. However, there was more bone and cartilage destruction in the absence of IFNγ + IL-4, suggesting that IL-4 could have direct protective effects on bone and cartilage, independent of regulation of IL-17. This

supports finding by other groups showing that intra-articular delivery of IL-4 slowed bone and cartilage damage in rat adjuvant arthritis [158, 159] and that IL-4 can directly down-regulate osteoclastogenesis through inhibition of RANKL activity [48]. Interestingly, in patients with RA a polymorphism in the IL-4 receptor that results in reduced responsiveness to IL-4 is associated with rapidly erosive disease, suggesting that IL-4 plays a protective role in joint destruction in RA [183, 184].

As both IFNγ and IL-4 suppress IL-17 *in vitro*, one would expect that the increased arthritis with anti-IFNγ + anti-IL-4 would be associated with increased IL-17. However, there was no further increase in the serum levels of IL-17 in the anti-IFNγ + anti-IL-4 groups versus the anti-IFNγ alone group. In addition, the arthritis in the anti-IFNγ group was associated with significantly elevated IFNγ, IL-4 and IL-17 levels in the joints, whereas the arthritis in the anti-IFNγ + anti-IL-4 group was associated with a significant increase in IFNγ and IL-4 but only a modest increase in IL-17. While administration of anti-IL-17 antibody completely abrogated the arthritis associated with anti-IFNγ alone, it only partially suppressed the arthritis associated with anti-IFNγ + anti-IL-4. These results suggest that neutralization of IFNγ versus IFNγ + IL-4 lead to joint inflammation by distinct pathways, one completely dependent on IL-17 and the other only partially mediated by IL-17, but it remains unknown what mechanisms could be mediating inflammation in the absence of IFNγ, IL-4 and IL-17.

Chapter 6

Conclusions

The experiments outlined herein have answered many questions about the regulation of Th17 cells by Th1 and Th2 cytokines, while also raising many new questions. We have shown a remarkable degree of continuity between the regulation of *in vitro* Th17 differentiation, *in vitro* Th17 re-stimulation and *ex vivo* Th17 re-stimulation. In all three systems IL-17 expression is up-regulated by TGFβ, IL-6 and IL-23 and down-regulated by IL-4, IFNγ and IL-12. However, a few interesting differences do emerge. For example, IL-4 inhibits expression of IL-23R but not IL-22, while IFNγ inhibits expression of IL-22 but not IL-23R. Also, IL-4 and IFNγ continue to suppress IL-17 production in the presence of TGFβ, IL-6 and IL-23 while IL-12 does not. These discrepancies point to important differences in the downstream mechanisms, which remain largely unknown. We showed that IL-4-mediated suppression is dependent on STAT6 and independent of STAT5, IRS1/2 and GATA-3. Although we have been unable to identify the direct target of STAT6 that ultimately mediates IL-17 silencing as of yet, the search continues, and the answer will likely come from careful and thorough screening of a large number of candidates. Looking further downstream, we went on to demonstrate that IL-4 induces a loss of STAT3 binding at the *Il17a* promoter but also a surprising increase in PolII binding and acetylation of H3 and H4. The results of these experiments fly in the face of our assumptions about the relationship between chromatin modifications and gene expression, but they also suggest that an "open" chromatin conformation may facilitate binding of transcriptional repressors as well as inducers.

In other experiments that conflict with previously published data, we found Th17 cells generated both *in vitro* and *in vivo* to be stable and resistant to suppression. Published data suggest that *in vitro*-derived Th17 cells quickly lose their IL-17 expression and can be converted to other lineages, even after three rounds of stimulation. However, *in vivo*-derived Th17 cells maintained their phenotype and were resistant to suppression [83, 93, 165]. The results of these and other groups highlight the differences between *in vitro*- and *in vivo*-derived Th17 cells and suggest that our culture system may more closely resemble the natural setting, possibly due to the fact that we use BM-DCs as APCs rather than irradiated splenic feeder cells [92, 94]. In our experiments, Th17 cells cultured under Th2 conditions did not take on a Th2 phenotype and were able to re-express IL-17 after the suppressive signals were removed. We also found that three rounds of *in vitro* stimulation rendered Th17 cells resistant to suppression by IL-4 as a result of desensitization of the IL-4R. Importantly, we observed a similar maturation process in Th17 cells generated *in vivo*, and maturation state correlated with disease progression. The simple observation that inflamed joints from arthritic mice co-expressed large quantities of IL-17, IFNγ and IL-4 suggests that Th17 cells at the site of inflammation are resistant to suppression.

Although we showed that IL-4R desensitization does not depend on SOCS5, further studies are necessary to determine the role of SOCS1. Assuming a specific factor such as SOCS1 is found to mediate loss of IL-4R signaling, experiments using knockout mice to address the role of Th17 maturation in CIA will be extremely alluring.

Based on observations from our lab and others, we propose that differentiation and maturation of Th17 cells can be divided into three phases: initiation, commitment and stabilization, which can be distinguished based on sensitivity to IL-4 (see Fig. 6.1). Initiation consists of the first few hours to days following stimulation of a naïve T cell with antigen and Th17-skewing cytokines, and during this time T cells may have a Th0 phenotype and express cytokines and transcription factors specific for multiple lineages. Encountering IL-4 during initiation presumably activates STAT6 and up-regulates GATA-3, which prevents H3 acetylation and H3K4 tri-methylation and induces H3K27 tri-methylation at the *Il17a* locus, resulting in silencing of IL-17 expression and conversion to a Th2 phenotype. Commitment refers to the period of a few days to a few weeks after initial antigen encounter and may be mediated by a combination of TCR stimulation, TGFβ, IL-6, IL-23 and IL-21. During this time Th17 cells up-regulate lineage-specific cytokines and transcription factors and down-regulate lineage-inappropriate factors. As shown in our studies on Th17 suppression following one week of *in vitro* differentiation or two weeks of immunization, treatment with IL-4 at this stage activates STAT6, resulting in a loss of STAT3 DNA-binding activity and transient down-regulation of IL-17 expression through an unknown, GATA-3-independent mechanism, without concomitant up-regulation of IL-4 and conversion to a Th2 phenotype. Lastly, stabilization is a slow process that may take many weeks and leads to resistance to suppression by opposing cytokines. At this stage there is inhibition of IL-4-induced STAT6 phosphorylation, which is independent of SOCS5 but may be mediated by SOCS1. This transition may also result in more permanent mechanisms of silencing of lineage-inappropriate cytokines and transcription factors, such as condensation into

centromeric heterochromatin, which has been found to occur at the *Il4* and *Gata3* loci in Th1 cells [185-187].

We have drawn these lines in the sand between pre-Th17, immature Th17 and mature Th17 cells based on changes in the response to IL-4, but we have also found that Th17 cells respond differently to IL-4 versus IFNγ and IL-12, which raises the question of how applicable our model is to other systems. For example, immature Th17 cells treated with IL-4 do not assume a Th2 phenotype, but other groups have found that Th17 cells treated with IL-12 take on a Th1 phenotype [83]. In addition, mature Th17 cells are resistant to suppression by IL-4 and IFNγ but not by IL-12. One possibility is that the timeline of Th17 maturation in the context of IL-12 is simply slower than Th17 maturation in the context of IL-4. Alternatively, Th17 cells may never become resistant to IL-12, which may be more likely given that IL-12 and IL-23 share a receptor subunit and Th1-like Th17 cells are fairly common *in vivo*. However, reports from different groups have shown that forced expression of T-bet in naïve T cells prevents IL-17 expression in Th17-polarizing conditions, yet T-bet/RORγt double-positive cells that express both IFNγ and IL-17 have been found *in vivo*, suggesting that there must be mechanisms that overcome T-bet-mediated inhibition of IL-17 expression [188, 189]. Further support for the universality of our model comes from literature suggesting that Th1 cells undergo a similar three-stage maturation process of decreasing responsiveness to IL-4. One possibility is that our model is specific to regulation by IL-4 but not specific to Th17 cells. Thus it will be interesting to determine if Th2 and Th17 cells undergo a similar process of desensitization to suppression by IFNγ.

Th17 cells have been shown to play an important role in multiple inflammatory diseases, including rheumatoid arthritis, psoriasis, multiple sclerosis, asthma and inflammatory bowel disease, and a thorough understanding of the mechanisms leading to expansion and regulation of the Th17 response is of great clinical importance. Our studies have focused on the mechanisms of suppression of Th17 cells by IL-4, a cytokine with known significance in the immunopathology of allergy and asthma, but largely thought to be absent from the immune response in RA. Recent evidence suggests, however, that IL-4 may have an underappreciated protective role in RA. A polymorphism in the IL-4R that confers decreased signaling capacity is associated with more erosive RA, and work from our lab has shown that this allele results in impaired suppression of IL-17 by IL-4 [164]. Thus IL-4 may suppress bone and cartilage destruction in RA via inhibition of IL-17. Similarly, our studies with *in vivo* neutralizing antibody treatment in CIA described here suggest that endogenous IL-4 protects against joint destruction in mice. Although these studies did not support the hypothesis that the protective function of IL-4 is mediated by suppression of IL-17, this conclusion is based on the effects of neutralizing antibodies that were only administered during the early phase of disease and are unlikely to penetrate the joint. Given that IL-4 is highly expressed in the joint but not in the spleen or lymph node, it is plausible that IL-4 limits joint destruction by local inhibition of IL-17 at the site of inflammation without affecting IL-17 production in secondary lymphoid organs. However, this idea is at odds with our hypothesis that Th17 cells in the inflamed joint are mature and resistant to suppression by IL-4. Unfortunately, the experiments we would most like to do, namely modulating and analyzing joint-specific immune events during CIA, are fraught with technical challenges

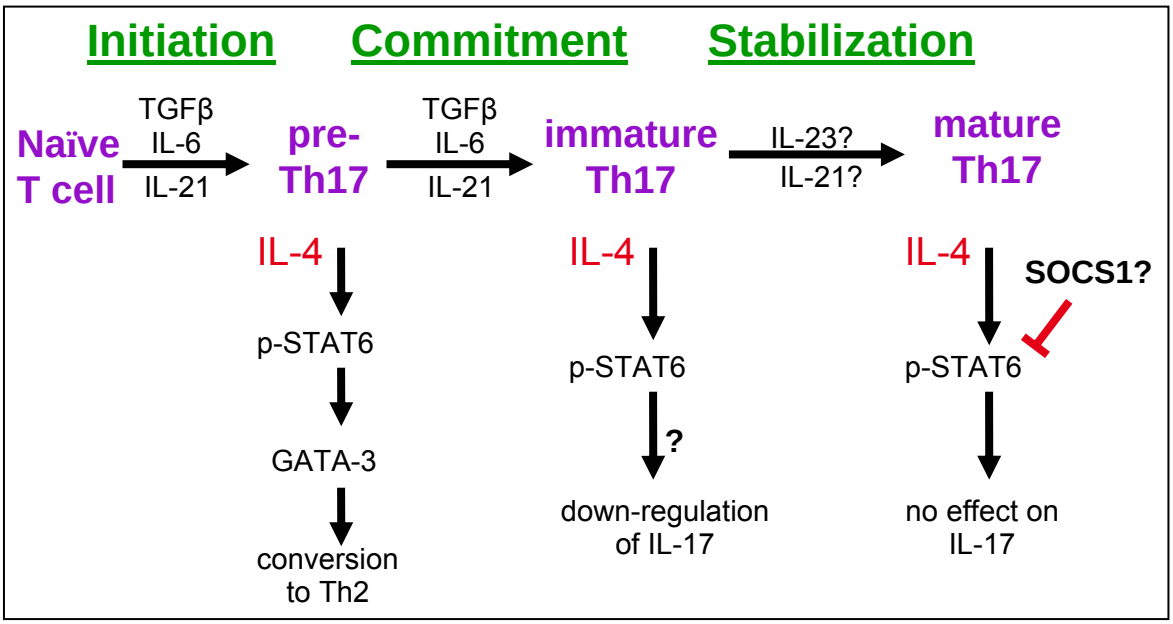
inherent in studying an autoimmune disease with such an inaccessible target organ. While we have had some success with ELISpots of joint-derived cells, flow cytometry of these populations has been more difficult and requires further refinement.

One technique for skewing both the systemic and joint-specific cytokine responses that we are currently exploring is adoptive transfer of collagen-specific Th1, Th2 and Th17 cells into arthritic mice, which we hope will traffic to the inflamed joint and contribute their prototypical cytokines to the milieu. These experiments would also be an ideal way to test the relative pathogenicity of immature versus mature Th17 cells and the stability of IL-4-mediated suppression of IL-17. We hypothesize that adoptive transfer of Th1 or Th2 cells would limit joint inflammation; adoptive transfer of immature Th17 cells would have a mild or delayed pathogenic effect; and adoptive transfer of mature Th17 cells would have a rapid, highly pathogenic effect and possibly induce arthritis in the absence of immunization. However, further work is needed to optimize the *in vitro* differentiation and maturation of T cells from mice bearing a transgenic collagen-specific TCR. Alternatively, the relative pathogenicity of immature versus mature Th17 cells may be easier to address in EAE, the mouse model of multiple sclerosis, because there are well-established techniques for adoptive-transfer-induced disease and adoptively transferred cells can be more easily isolated from the inflamed central nervous system (CNS). It will also be interesting to know if our *in vitro* and *ex vivo* observations of Th17 maturation can be extended to other disease models by testing the effects of IL-4 on Th17 cells isolated from the inflamed CNS of mice with EAE. Similarly, much work is needed to assess the sensitivity and resistance to suppression by IL-4 in human Th17 cells from patients with various immune-mediated diseases. The

possibility of human Th17 maturation raises many exciting new questions and ideas about the etiology of Th17-mediated disease, and a better understanding of the molecular mechanisms mediating stabilization of committed cytokine production may lead to new approaches for targeted therapies.

Our work on the regulation and maturation of Th17 cells is particularly timely, as the recent identification of several new T helper cell subsets has led to a surge in the interest and understanding of the mechanisms underlying lineage commitment and plasticity of CD4+ T cells. Technological advancements and increasing collaborations between scientists of diverse backgrounds have led us away from reductionist approaches and discrete, linear pathways and towards systems analyses and dynamic, relativistic models. In this vein, we have shown that joint inflammation depends more on the balance between cytokines than on the absolute concentration of any cytokine alone and that traditional markers of active gene transcription can go up even as gene expression is going down. It is also important to remember that many of our ideas about T helper cell differentiation are based on cells grown in highly biased conditions *in vitro*, and yet T helper cells are still able to differentiate and co-exist *in vivo*. The simple fact that *in vitro* Th17 differentiation requires strict inhibition of Th1 and Th2 differentiation, but Th17 cells clearly exist amidst a sea of cytokines *in vivo*, suggests that we are missing something significant. The lesson here is that, rather than thinking in terms of absolutes (transcription is either on or off, cytokines are either inflammatory or anti-inflammatory, external stimuli either activate or inhibit), it will be more fruitful to think in terms of probabilities and ratios. Our knowledge about T cell dynamics is continually expanding, ultimately leading to models that are more complicated, but also more inherently truthful.

Figure 6.1: Three-phase model of Th17 maturation and progressive desensitization to suppression by IL-4



Appendix

Materials and Methods

Mice

For *in vitro* Th17 differentiation and KLH-immunization, male 6- to 8-week old BALB/c mice were obtained from Jackson Laboratories. For cII/CFA immunization and CIA studies, male 8- to 10-week-old DBA1 mice were obtained from Jackson Laboratories. STAT6-deficient, IL-4-deficient, IL-4R-deficient and IL-4R mutant mice on a BALB/c background were obtained from Jackson Laboratories. SOCS5-deficient mice on a C57BL/6 background were obtained from the lab of Sandra Nicholson at the Walter and Eliza Hall Institute of Molecular Medicine (Melbourne, Australia) and bred in our facilities. All animals were housed in specific pathogen free conditions and all procedures were approved by the University Committee for the Use and Care of Animals of the University of Michigan. Single-cell suspensions from spleens and thymi of CD4-Cre/STAT5a/b^{flox} mice on a C57BL/6 background were collected in the lab of Dr. John O'Shea at the NIH and were shipped overnight on ice. Freshly isolated spleens from GATA3 conditional knockout mice were collected in the lab of Dr. James Engel at the University of Michigan.

Generation of BM-DC

Bone marrow was isolated from femurs and tibias, treated with ACK, and cultured for 6 days at 1x10^6 cells/mL with 10ng/mL recombinant mouse IL-4 and GM-CSF (Peprotech) in basic RPMI (10% FCS, 2% L-glutamine, 1% penicillin/streptomycin,

1X β-mercaptoethanol). The cells were then collected using a cell scraper and CD11c+ cells were positively selected by two rounds of MACS (Miltenyi Biotech).

Purification of naïve T cells

Spleens were collected and CD4+ T cells were magnetically isolated by negative selection using the EasySep kit from Stem Cell Technologies. The purified CD4+ T cells were then labeled with CD4 FITC, CD25 PE, CD44 PE-Cy7 and CD62L APC (Biolegend). The CD4+CD25-CD44loCD62L+ cells were sorted on a FACS Vantage, Aria or Diva.

Th17 differentiation

BM-DCs and naïve T cells were plated in 6 well plates in basic RPMI at $0.125x10^6$ BM-DCs and $0.25x10^6$ naïve T cells per mL with 4 μg/mL anti-CD3 (145-2C11), 10 μg/mL anti-IL-4 (11B11), 10 μg/mL anti-IFNγ (R4-6A2), 1 ng/mL recombinant human TGF-β1 (Peprotech), 20ng/mL recombinant mouse IL-6 (Peprotech) and 10 ng/mL recombinant mouse IL-23 (eBioscience). For inhibition of Th17 differentiation anti-IL-4 was omitted from the culture and recombinant mouse IL-4 (Peprotech) was added at 10 ng/mL, unless stated otherwise. Alternatively, anti-IFNγ was omitted from the culture and recombinant mouse IFNγ (Peprotech) was added at 10 ng/mL, unless stated otherwise. Cells were stimulated for six days and then collected, washed twice with cold 2% NCS/PBS and put back into culture in the same volume without stimulation for two days. For inhibition of Th17 re-stimulation IL-4 was added

to the culture during the two-day rest period or during a two-day restimulation with anti-CD3 following the rest period.

Th17 maturation

Naïve T cells underwent six days of Th17 differentiation followed by two days of rest, according to the protocol described above. To induce maturation the cells were then expanded two fold with the addition of fresh BM-DCs, re-stimulated with the same cytokine and neutralizing antibody cocktail for five days, and then washed and rested for two days. This cycle of five days of stimulation and two days of rest was repeated, for a total of three weeks of culture. At the end of the three weeks the Th17 cells were restimulated for two days with anti-CD3 and recombinant IL-4.

ELISA

In various experiments IL-17A, IL-17F, IL-22, IFNγ, IL-4 and IL-10 were measured by ELISA. Plates were coated with purified anti-IL-17A (clone TC11-18H10.1, Biolegend), anti-IFNγ (clone R46A2 or XMG1.2, Biolegend, San Diego, CA, USA) or anti-IL-4 (clone11B11). Plates were blocked and then loaded with tissue culture supernatants or serum. The plates were washed and treated with biotin-conjugated anti-IL-17A (clone TC11-8H4, Biolegend), anti-IFNγ (clone XMG1.2 or R4-6A2 Biolegend) or anti-IL-4 (clone BVD6-24G2, BD) mixed with streptavidin horseradish peroxidase (Biolegend). Lastly the plates were developed with OptEIA TMB substrate (BD) and absorbance at 450 nm was quantitated with a Biorad (Hercules, CA, USA) plate reader using KC4 software (Biotek, Winooski, VT, USA). IL-17F ELISA was performed using

a kit from R&D Systems according to the manufacturer's protocol. IL-22 ELISA was performed using a kit from Antigenix according to the manufacturer's protocol. IL-10 ELISA was performed using a kit from BD Pharmingen (San Jose, CA, USA), according to the manufacturer's protocol.

Flow cytometry

For ICS, cells were stimulated for 6 h with 5 ng/mL PMA and 500 ng/mL ionomycin, with 10 μg/mL Brefeldin A added for the last 5 h (all chemicals from Sigma). Cells were then treated with mouse FcBlock anti-CD16/32, stained with FITC- or PE-conjugated anti-CD4 (clone GK1.5) and fixed overnight. The next day cells were permeabilized with saponin and stained with fluorescent labeled anti-IL-17 (clone TC11-18H10.1), anti-IFNγ (clone XMG 1.2), and anti-IL-4 (clone 11B11) or the appropriate isotype control (all antibodies from Biolegend). Staining was measured with a FACS Calibur and data was analyzed using Cell Quest software (BD).

Real-time PCR

Gene expression at the mRNA level was analyzed by Taqman-based real time PCR with specific primers and probes. First, RNA was collected from frozen cell pellets with the RNEasy Mini kit and treated with DNase (Qiagen). cDNA was generated using the High Capacity cDNA archive kit (Applied Biosystems). Relative quantification using the comparative C_T method was carried out using TaqMan Universal PCR Master Mix or Gene Expression Master Mix (Applied Biosystems) and run on an AB7500 machine. The following primer and probe sets were obtained from Applied Biosystems: IL-17A,

IL-17F, IL-22, RORγ, IL-23R, IFNγ, IL-4, IL-4R, STAT6, SOCS1, SOCS3, SOCS5, GATA3, T-bet, β-Actin and GAPDH.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was carried out according to the EZ ChIP protocol (Upstate). Briefly, Th17 cells were fixed with formaldehyde and lysed with SDS. Lysates were sonicated to shear DNA and immunoprecipitated with protein G and antibodies to STAT3, STAT6, GATA3, H3K4me3, H3K27me3, H3Ac, H4Ac, PolII. Eluted DNA was quantitated by real-time PCR with SYBR green master mix (Applied Biosystems) and the following primers:

Il17a promoter forward: AGGGAGAGCTTCATCTGTGG
Il17a promoter reverse: AGATTCATGGACCCCAACAG
Il17a/f intergenic region forward: CAGACTCCAAGCACATCATG
Il17a/f intergenic region reverse: GACTGACCTACATTGTGGGC
Rorc promoter forward: AGGCTCCTGACCTTTGATTG
Rorc promoter reverse: AGGGGGTGCTGAGTAATCAC

Western blot

Th17 cells were washed and rested overnight in RPMI with 2% FCS plus cytokine-neutralizing antibodies to minimize background levels of STAT activation. The cells were then re-stimulated with 50 ng/mL IL-4 or IL-2 for various time periods. The reaction was stopped with cold PBS plus 1 mM Na_3VO_4, and the cells were lysed with Phosphosafe extraction reagent (Novagen) supplemented with protease inhibitor cocktail (Calbiochem). Lysates were reduced and denatured by boiling with SDS loading buffer

with 100 mM DTT. Sample were run on a 10% Precise Tris-Hepes-SDS gel (Pierce) and transferred to nitrocellulose membrane (Millipore). Membranes were stained with the following primary antibodies at 1:1000 unless noted otherwise: anti-STAT6 (Cell Signaling), anti-phospho-Tyr641 STAT6 (Calbiochem), anti-STAT5 (Cell Signaling), anti-phospho-Tyr694 STAT5 (Cell Signaling), anti-JAK1 (Cell Signaling), anti-JAK3 (1:200, Santa Cruz), anti-SOCS1 (1:200, Santa Cruz), anti-SOCS5 (1:200, Santa Cruz), and anti-GAPDH (1:400, Biolegend). Secondary antibodies were goat anti-rabbit IgG HRP (1:1000, Cell Signaling) or rabbit anti-goat IgG HRP (1:10,000, Abcam). Chemiluminescence was developed with Pierce ECL Western blotting substrate and detected on blue autoradiography film (MidSci). Band intensities were quantified using Kodak 1d 3.6 software.

Immunizations

Complete Freund's adjuvant (CFA) was prepared by mixing heat inactivated mycobacterial strain H37Ra in incomplete Freund's adjuvant at 4 mg/ml. For KLH immunization, Imject® mcKLH subunits (Pierce, Rockford, IL) were diluted in PBS to 2mg/mL and mixed at a 1:1 ratio with CFA. Mice were immunized intraperitoneally with 100μg KLH. For collagen immunization, lyophilized chicken collagen (Chondrex, Redmond, WA, USA) was dissolved overnight in acetic acid at 4 mg/ml. CFA and collagen were mixed at a 1:1 ratio to form an emulsion and 100 μg of collagen was injected intradermally at the base of the tail.

Arthritis scoring

Mice were scored for arthritis every other day from day 15 after immunization. Scoring was performed as follows: 0 = no swelling or redness of paws or digits; 1 = swelling and redness in one to two digits; 2 = swelling and redness over ankle or three or more digits or midfoot; 3 = swelling and redness over ankle and midfoot or digits and midfoot; 4 = swelling and redness over entire foot or ankylosis.

In vivo neutralizing antibody

For *in vivo* cytokine neutralization in cII/CFA-immunized mice, neutralizing rat antibodies to mouse IFNγ (clone R46A2) or IL-4 (clone 11B11) were purified from hybridomas (ATCC, Manassas, VA, USA) and used at 100 μg/mouse/ day. Neutralizing antibody to IL-17 (clone M210) was a kind gift from Amgen (Thousand Oaks, CA, USA). The antibodies were injected intraperitoneally from day 10 to 20. Rat IgG at 100 μg/mouse/day was used as a control.

Tissue collection and culture from immunized mice

For CIA experiments, blood was collected by cardiac puncture into serum separator tubes, and serum was frozen at –80^{o}C for cytokine assays to be performed at a later date. For some assays 100 μl of blood was collected serially from tail bleeds on days 0, 14, 28 and 42. Spleens and inguinal lymph nodes were collected and single-cell suspensions were restimulated with 100 μg/ml of heat-denatured chicken collagen. Cells were collected after overnight culture for ICS or supernatants were collected at day five of culture for ELISA. Paws were harvested by incising at the fur line, removing the skin, mincing and culturing overnight in 1 mL of media. Supernatants were collected for

ELISA. All cultures were in basic RPMI 1640 (10% FCS, 2% L-glutamine, 1% penicillin/streptomycin and 1X β-mercaptoethanol).

Histologic scoring

Mouse hind paws were used for histology scoring. The paraffin-embedded tissue was sectioned in an axis longitudinal to the tibia. Three sections from the center of each paw were stained with H&E and scored by two independent blinded observers. Inflammatory infiltrate, synovitis (synovial hyperplasia), cartilage destruction and bone involvement were each scored on a scale of 0 to 3: 0 = no change, 1 = mild, 2 = moderate and 3 = severe.

Statistical analysis

P values were calculated by students t test, one sample t test and one-way ANOVA using Prism, with a p value less than 0.05 representing statistical significance.

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