Chapter 1

Immunobiology of Treg and Th17 cells
1.1 Introduction

The immune system protects us against infectious pathogens and it has evolved to do this while minimizing damage to self tissues. These responses are potent enough to destroy not only pathogens but also the host. This implies that immunity, like the other major systems in the body, maintains essential regulatory mechanisms that prevent inappropriate harmful responses. These regulatory processes constitute the immunological tolerance.

Several key mechanisms have been described in the last three decades to explain how the immune system is capable of preventing the *Horror autotoxicus* i.e. auto-immunity (Silverstein, 2006). One of these major mechanisms is the clonal deletion that occurs in the thymus which leads to the destruction of most self reactive T cells (Kappler et al., 1987; Hengartner et al., 1988). Another key mechanism in the maintenance of tolerance is the activation induced cell death (AICD) of T cells (Liu and Janeway, 1990) through the interaction of Fas with its ligand Fas-L (Alderson et al., 1993).

It is now widely accepted that another mechanism involving T cells that actively suppress the activation and the proliferation of other immune cells is important in the maintenance of tolerance to self constituents and in the prevention of autoimmune diseases. Circulating T cells that have specificity to self antigens can be detected in healthy individuals but usually do not trigger clinically potent autoimmunity (Danke et al., 2004). This suggests that a dominant inhibitory phenomenon that controls these potentially harmful cells is operating permanently. It took a very long time for investigators to convince the scientific community that a distinct subset of CD4+ T cells, initially called suppressor T cells then rebaptized as regulatory T (Treg) cells was the
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mediator of such permanent suppression of autoimmune responses in the periphery. Studies over the past 5 years have identified the X-chromosome-encoded forkhead transcription factor (Foxp3), as the key player in the biology of CD4+CD25+ Treg and are required for the maintenance of Treg cell function. (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003).

Interleukin-3 (IL-3), a cytokine secreted by T helper (Th) cells, stimulates the proliferation, differentiation and survival of pluripotent hematopoietic stem cells (Schrader, 2003). It has been previously demonstrated that IL-3 is a potent inhibitor of osteoclastogenesis, and inhibits both receptor activator of NFκB ligand (RANKL)-and tumor necrosis factor-α (TNF-α) induced mouse osteoclast formation (Khapli et al., 2003; Yogesha et al., 2005). Recently, it was also demonstrated that IL-3 inhibits RANKL-induced human osteoclasts derived from blood monocytes and bone marrow cells of osteoporotic individuals (Gupta et al., 2010). IL-3 also inhibits TNF-α-induced pathological bone resorption in the presence of other proinflammatory cytokines such as IL-1α, TGF (transforming growth factor)-β1, TGF-β3, IL-6 and prostaglandin E2 (PGE2), and pretreatment with IL-3 prevents the development of inflammatory arthritis in mice, and protects cartilage and bone destruction in the joint (Yogesha et al., 2009). These results suggest that IL-3 has an anti-inflammatory activity. However, the mechanism(s) of anti-inflammatory role of IL-3 is not known.

In rheumatoid arthritis (RA) and collagen-induced arthritis (CIA) Treg cells are defective and there is increased osteoclastogenesis (Ehrenstein et al., 2004; Kelchtermans et al., 2005 and 2009). Also, Treg cells have been shown to inhibit osteoclastogenesis, and prevent development of CIA (Zaiss et al., 2007, Kelchtermans et al., 2009). Also IL-17 secreted from Th17 cells exacerbates joint destruction in CIA (Lubberts et al., 2004). The fact that Th17 and Treg
developmental programs are reciprocally interconnected led me to hypothesize that the anti-inflammatory activity of IL-3 may be through regulation of Treg and Th17 cell development.

In this thesis the role of IL-3 in regulation of differentiation of Treg and Th-17 cells in both in vitro and in vivo conditions has been investigated. The aims of the study are:

1. To investigate the role of IL-3 in modulation of Treg and Th17 cell differentiation in vitro.
2. To develop CIA in DBA/1J mice.
3. To examine the in vivo role of IL-3 on development of Treg and Th17 cells and prevention of CIA.

This thesis comprises of two introductory chapters, first chapter briefly reviews the immunobiology of Treg and Th17 cells with more detailed description on regulation of Treg and Th17 cells differentiation in various autoimmune diseases and in the second chapter IL-3 and the new field of osteoimmunology has been reviewed. This is followed by a chapter detailing the materials and methods used in this study. The experimental work has been described in the next three chapters followed by a chapter on general discussion and conclusions.

1.2 Discovery of Treg cells

Thymus-derived immune cells with suppressive capabilities were found to exist in the late 1960s. Various groups have demonstrated the ability, for mice that underwent thymectomy at day 3, to develop autoimmune disease. This
effect was not reproduced in mice that underwent thymectomy at day 7, suggesting that a tolerogenic subset of T cells develop in the thymus entering the systemic circulation between days 3 and 7 (Nishizuka and Sakakura, 1969). This was further substantiated when day 3 thymectomised mice failed to develop autoimmune disease when transplanted with thymocytes or splenocytes from normal adult mice (Kojima et al., 1976). The importance of antigen-specific suppressor T cells was demonstrated in the early 1970s (Gershon and Kondo, 1970). These cells, once activated, target CD4+ T helper cells, blocking activation and progression of both the humoral and cell-mediated immune systems. Over the ensuing years, the mechanism of suppression was found to be more complicated and the term ’suppressor’ T cells drifted out of use.

Sakaguchi et al. (1995) subsequently discovered a subset of CD4+ T cells that constitutively expressed high levels of IL-2 receptor α chain (CD25) and prevented autoimmune diseases in mice. In 1998, Shevach’s group and Sakaguchi’s group made one step forward to better characterizing the function of suppressor CD4+CD25+ T cells in vitro. They demonstrated that CD4+CD25+ T cells, that were anergic upon stimulation, could suppress the proliferation and the production of IL-2 of activated CD4+ T cells in vitro in a contact-dependent manner (Thornton et al., 2004). Thus, the characterization of CD25 as a reliable surface marker and the possibility to assess their function in vitro definitively pushed suppressor T cells out from oblivion. These cells, eventually named T ‘regulatory’ cells, have since been implicated in the development of autoimmunity, allergy, and rejection in transplant medicine and suppression of immune responses to cancer (Fig. 1.1).
1.3 Subsets of Treg cells

There are different types of CD4+ Treg cells that tend to be variable in their mechanism of action and origin. These cells make up 5–10% of naive CD4+ T cells in the periphery of healthy individuals. As yet, there is still much uncertainty with regard to the method of regulation of these Treg cell subsets (Fig. 1.2).

Naturally occurring Treg (nTreg) cells

Treg cells in mice constitutively expressing high levels of CD25 develop in the thymus and enter the periphery as CD4+CD25+ T cells and are described as...
natural Treg cells (Sakaguchi et al., 1995). Due to their distinctiveness, they are easily identified in mice and have been analysed in great depth. However, identifying nTreg cells as a distinct subset in humans is difficult since a quarter of CD4+ T cells express CD25 of which a large proportion have no regulatory properties and are in fact critical for immune function.

*In vitro* studies have shown that Treg cells need T cell receptor (TCR) activation to fulfil their regulatory attributes. This is usually mediated by major histocompatibility complex (MHC) class II receptors on antigen-presenting cells (APC), indicating their antigen specificity. However, there are also subtle differences between the activation of induced Treg cells (iTreg cells) (which need both TCR activation and co-stimulation) and nTreg cells (which only need TCR activation). Once activated, the suppressive activity of thymus-derived nTreg cells appears to be mediated entirely by direct cell-to-cell contact with effector T cells, as no suppressive activities were seen when nTreg cells were separated from the effector cells by a semi-permeable membrane. Furthermore, blocking antibodies to the cytokines IL-10 and TGF-β had little effect in reversing this suppression (Thornton and Shevach, 1998), highlighting the predominant effect of cell-to-cell contact.

**Adaptive Treg cells**

Generally adaptive Treg cells are formed from naive CD25- T cells in the periphery under specific antigen exposure and cytokine stimulation. A variety of adaptive Treg cells exist with differing patterns of cytokine secretion. There is still much discussion about the distinctive differences between the cell types within this subset of Treg cells, but broadly, there appear to be three groups.
Inducible Treg (iTreg) cells

iTreg cells are very similar in function to nTreg cells but derive from Foxp3-naive T cells in the periphery after stimulation by TGF-β. Once induced, these cells begin to express Foxp3, cytotoxic T lymphocyte antigen (CTLA-4) and secrete IL-10 and TGF-β. iTreg cells have a more contact-independent mechanism of action toward effector T cells. The release of IL-10 and TGF-β has a potent immunosuppressive effect on other effector T cells and APCs (Asseman et al. 1999).

Tr1 Treg cells

Tr1 cells differ from nTreg cells in their ability to produce IL-10 and TGF-β in large amounts, which both suppress naive and memory CD4+ T cell function in murine in vitro studies (Groux et al., 1997). Like iTreg cells, Tr1 cells are also induced by antigen-mediated TCR activation, but unlike iTreg cells, they are induced in the presence of IL-10 (Roncarolo et al., 2006).

Th3 Treg cells

Th3 cells are another adaptive Treg subset vital for the maintenance of oral tolerance in animal models. They achieve their suppressive effects by secreting IL-10, IL-4, and large amounts of TGF-β and indirectly by promoting the differentiation of antigen-specific Foxp3+ Treg cells peripherally. Th3 cells differ from Tr1 in their dependence on TGF-β for differentiation from CD4+CD25- T cells (Awasthi et al. 2007).

The Treg subsets are more distinct in mice than in humans where it is more difficult to differentiate between the subtypes. Foxp3 expression does help to
differentiate these adaptable Treg cells from naïve CD4+ T cells in the periphery but not from nTreg cells. As a result, targeting of a specific population of Treg cells in the therapeutic setting is still an uncertain strategy.

‘Other’ regulatory cells

It is increasingly apparent that the CD4+ T cell lineage of Treg cells is not the only immune cells to have exclusive regulatory properties. There exists a subset of CD8+ T cells with suppressive capabilities, which were initially described in oral tolerance to myelin basic protein (Chen et al., 1995). Furthermore, an *in vitro* study identified a subset of human CD8+ T cells (CD8+CD28-) that was able to confer tolerance by preventing up-regulation of the co-stimulatory markers CD80 and CD86 on APCs by CD4+ T cells (Liu et al., 1998). CD8+ T cells can also protect against exacerbation and recurrence of experimental autoimmune encephalitis in murine models (Sarantopoulos et al., 2004). Unlike CD4+ inducible Treg cells, CD8+ Treg cells are dependent on IFN-γ in order to secrete TGF-β as seen in one murine model (Myers et al, 2005). The difficulty with studying CD8+ Treg cells has been largely due to the lack of a discriminatory marker, though a lack of CD28 expression on CD8+ T cells is indicative of a regulatory phenotype.

CD4+ and CD8+ Treg cells are not the only immune cells with regulatory capabilities and other immune cells with similar properties are continually being discovered. Recently the discovery that a small subset of B cells called as “regulatory B cells” powerfully regulates inflammatory immune responses has ignited the interest of immunologists in the field of autoimmune diseases from a different view point (Mauri and Blair, 2010). Further research needs to be undertaken to delineate the exact mechanism of regulation of these cells.
Figure 1.2 Thymic and peripheral generation of Foxp3+ Treg cells. nTreg cells differentiate in the thymus and migrate to peripheral tissues. Adaptive Foxp3+ iTreg cells differentiate in secondary lymphoid organs and tissues. The peripheral population of Foxp3+ Treg cells comprises both nTreg and iTreg cells (adapted from Curotto de Lafaille et al., 2009).

1.4 Foxp3 and Treg cell differentiation

Recent progress in the understanding of Treg cell biology came with the discovery of the X chromosome-encoded gene Foxp3 during efforts to identify the genetic basis for the autoimmune disorder in human patients suffering from immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome and in the spontaneous mouse mutant scurfy (Bennett et al., 2001). Mice and humans harboring a loss of function mutation in the Foxp3 gene are affected by fatal early onset lymphoproliferative immune-mediated disease affecting a variety of organs and tissues. Subsequent studies revealed stable expression of high amounts of Foxp3 restricted to Treg cells and its
requirement for Treg cell differentiation (Fontenot et al., 2003 and 2005; Hori et al., 2003; Khattri et al., 2003; Wan and Flavell, 2007) and for their suppressor function, proliferative potential, and metabolic fitness (Lin et al., 2007).

Furthermore, sustained Foxp3 expression in mature Treg cells is necessary for maintenance of the Treg cell phenotype and suppressor function; loss of Foxp3 or its diminished expression in Treg cells leads to acquisition of effector T cell properties including production of immune response-promoting cytokines such as IL-2, IL-4, IL-17, and interferon (IFN-γ) (Wan and Flavell, 2007; Williams and Rudensky, 2007). Together, these studies established a central role for Foxp3 in defining the Treg cell lineage.

**1.5 Mechanisms of Treg cell differentiation**

Although nTreg cells develop in a highly controlled thymic microenvironment, Foxp3+ iTreg cells differentiate under more varied conditions. Our understanding of the different microenvironments of iTreg cell development in vivo is still incomplete. However, the minimal program for Foxp3+ iTreg cell development has been defined: it requires TCR stimulation and the cytokines TGF-β and IL-2, for both in vitro and in vivo generated iTreg cells.

In contrast to the essential role of TGF-β in the differentiation of Foxp3+ iTreg cells, the role of TGF-β in the generation of nTreg cells is less clear. Studies with T cell-specific deletion of TGF-βRII reported that TGF-β was not involved in thymic nTreg cell development (Fahlen et al., 2005; Li et al., 2006; Marie et al., 2006); furthermore, young TGF-β1-deficient mice have normal number of thymic nTreg cells. However, a recent study offered a different interpretation, based upon the fact that between postnatal days 3 and 5 there is a severe
deficiency in nTreg cell generation in mice that have a conditional (lck-Cre driven) deletion of TGF-βRI (Liu et al., 2008). The authors attributed the late surge of nTreg cells in TGF-βRI-deficient mice to a heightened responsiveness of these T cells to IL-2 and showed that double deficiency in TGF-βRI and IL-2 abrogated nTreg generation (Liu et al., 2008).

Nevertheless, it is clear that IL-2 by itself is not necessary for Foxp3+ nTreg cell generation (Curotto de Lafaille et al., 2004). Two other studies used IL-2-deficient and IL-2Rα (CD25)-deficient mice to probe the role of IL-2 in Treg cell biology and also concluded that IL-2 was dispensable for the generation of nTreg cells in the thymus (Fontenot et al., 2005). Finally, neutralization of IL-2 with antibodies resulted in a strong reduction of Foxp3 expression in the spleen but a non-significant difference in the thymus (Setoguchi et al., 2005).

As indicated above, IL-2 appears to be essential for iTreg cell generation and/or homeostasis. In cultures of naive CD4+ T cells stimulated with anti-CD3 and TGF-β, IL-2 was required to release the TGF-β-mediated proliferation inhibition on Treg cells (Chen et al., 2003). Experiments utilizing IL-2 neutralization and IL-2-deficient T cells demonstrated that IL-2 is required in vitro for TGF-β induction of Foxp3 transcription and suppressor activity (Zheng, S. et al., 2007). IL-2, but not other common-γ chain signaling cytokines, could replace the requirement for CD28 costimulation for the induction of Foxp3 by anti-CD3 and TGF-β (Davidson et al., 2007). In vitro differentiated iTreg cells did not require IL-2 to maintain Foxp3 expression after transfer into in RAG-deficient recipient mice (Davidson et al., 2007). IL-2 signaling activates signal transducer and activator of transcription factor 5 (STAT5). STAT5 binds to the Foxp3 gene and may cooperate with STAT3 for Foxp3 induction (Burchill et al., 2007). Thus, as may be the case for TGF-β, the
requirement for IL-2 also differs between thymic-derived nTreg cells and peripheral iTreg cells.

Another important difference between nTreg and iTreg cell generation relates to CTLA-4. Expression of high amounts of Foxp3 and acquisition of suppressor activity by naive cells activated with TGF-β in vitro required upregulation of CTLA-4. In contrast, CTLA-4 is not necessary for the development of nTreg cells in the thymus (Zheng et al., 2006). Consistently, B7 expression in host cells was required for the conversion of CD4+CD25- adoptively transferred T cells into Foxp3+CD25+ cells (Liang et al., 2005). Also, strong CD28 costimulation suppresses induction of Treg cells from naïve precursors through Lck signaling (Semple et al., 2011).

1.6 Mechanisms of Foxp3+ Treg cell mediated suppression

It was thought that the development of an in vitro model system (Thornton and Shevach, 1998) for the analysis of Treg cell function would offer major insights into the mechanism of action of Treg cells in vivo. However, the in vitro model systems have identified a long list of molecules and processes that contribute to Treg cell suppressive activities and it remains unclear whether any of the conclusions drawn from these studies shed light on how Treg cells function in vivo. A detailed analysis of Treg cell function is further confounded by the large number of different cell types that are purported to be directly targeted by Foxp3+ Treg cell.

Although multiple T cell subsets (e.g., Tr1 cells, Th3 cells, Th1 and Th2 cells, etc.) can certainly exert negative immunoregulatory effects by producing immunomodulatory cytokines (IL-10, TGF-β), I will confine this discussion to CD4+Foxp3+ Treg cells that develop in both the thymus and periphery and
represent the major Treg cell populations that are critical for immune homeostasis. The precise molecular mechanisms of suppression by human Treg cells remains to be determined, although in vitro and in vivo mouse studies have implicated several mechanisms (Table 1.1). These include modulation of the cytokine microenvironment, metabolic disruption of the target cell, alteration of dendritic cell (DC) activating capacity and cytolysis (Fig. 1.3).

1.7 Treg cells and autoimmune diseases

Autoimmune disease may develop as a consequence of altered balance between Treg cells and self-reactive conventional T cells. Given the dynamic nature of this balance, it is conceivable that any genetic anomalies or environmental agents that tip the balance toward self-reactive conventional T cells can cause or predispose to autoimmune disease. Treg cells have a central role in protecting an individual from autoimmunity. This role was first identified in mice in which the absence of Treg cells, or the depletion of Treg cells, resulted in the development of autoimmune gastritis, thyroiditis, diabetes and inflammatory bowel disease (IBD) (Torgerson and Ochs, 2007).

Subsequently, numerous studies in animal models of autoimmunity showed that defects in CD4+CD25+Foxp3+ Treg cells can contribute to the development of autoimmunity and that the disease could be reversed by the adoptive transfer of Treg cells (Sakaguchi et al., 2006). This was followed by studies identifying the presence of Treg cells in human peripheral blood and their ability to suppress T cell proliferation in vitro (Baecher-Allan et al., 2001; Stephens et al., 2001 and Taams et al., 2002).
Figure 1.3 Depiction of the various Treg cell mechanisms centred around four basic modes of action. a) Inhibitory cytokines include IL-10, IL-35 and TGFβ. b) Cytolysis includes granzyme-A and granzyme-B dependent and perforin-dependent killing mechanisms. c) Metabolic disruption includes high-affinity CD25-dependent cytokine deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition, and CD39- and/or CD73-generated, adenosine receptor 2A (A2AR)-mediated immunosuppression. d) Targeting DCs includes mechanisms that modulate DC maturation and/or function such as lymphocyte-activation gene 3 (LAG)-MHC-class-II mediated suppression of DC maturation, and CTLA4–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule made by DCs (adapted from Vignali et al., 2008).

The importance of T cell regulation in human disease is highlighted by the severe inflammation and autoimmunity that occurs in individuals who suffer from IPEX. These individuals develop a broad range of autoantibodies, insulin-dependent diabetes, thyroiditis, eczema, haemolytic anaemia and IBD, and in the absence of a bone marrow transplant, these patients die at an early age (Torgerson and Ochs, 2007). These observations have driven a search for mechanisms of defective T cell regulation in human autoimmunity (Table 1.2).
Table 1.1 **Treg cell suppressive mechanisms** (adapted from Sakaguchi et al., 2010).

<table>
<thead>
<tr>
<th>Key molecule(s)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mechanisms of contact-dependent suppression</strong></td>
<td></td>
</tr>
<tr>
<td>CTLA4</td>
<td>Downregulation of APC co-stimulatory function</td>
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<tr>
<td></td>
<td>Interaction with CD80 and CD86 on conventional T cells</td>
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<tr>
<td>CD73–CD39</td>
<td>Hydrolysis of inflammatory extracellular ATP</td>
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<tr>
<td>LAG3</td>
<td>Induction of inhibitory signalling through MHC class II molecules</td>
</tr>
<tr>
<td>Granzyme B (mouse) and granzyme A (human)</td>
<td>Lysis of conventional T cells</td>
</tr>
<tr>
<td>CD95–CD95 ligand</td>
<td>Induction of apoptosis in conventional T cells</td>
</tr>
<tr>
<td><strong>Mechanisms of cytokine-mediated suppression</strong></td>
<td></td>
</tr>
<tr>
<td>TGFβ and LAP</td>
<td>Induction of FOXP3 in conventional T cells</td>
</tr>
<tr>
<td>IL-10</td>
<td>Attenuation of DC function</td>
</tr>
<tr>
<td></td>
<td>Conversion of conventional T cells to T&lt;sub&gt;r1&lt;/sub&gt; cells</td>
</tr>
<tr>
<td>Galectin 1</td>
<td>Cell cycle arrest and apoptosis in conventional T cells</td>
</tr>
<tr>
<td>CD25</td>
<td>Adsorption of IL-2</td>
</tr>
<tr>
<td>IL-35</td>
<td>Induction of conventional T cell expression of IL-35 by T&lt;sub&gt;reg&lt;/sub&gt; cells enhances suppression (IL-35 is not expressed by human T&lt;sub&gt;reg&lt;/sub&gt; cells)</td>
</tr>
</tbody>
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Table 1.2 **Overview of Treg cells in autoimmunity** (adapted from Buckner, 2010).

<table>
<thead>
<tr>
<th>Disease</th>
<th>T&lt;sub&gt;reg&lt;/sub&gt; cell number (percentage of CD4&lt;sup&gt;+&lt;/sup&gt;CD25&lt;sup&gt;hi&lt;/sup&gt; or CD4&lt;sup&gt;+&lt;/sup&gt;CD25&lt;sup&gt;+&lt;/sup&gt;FOXP3&lt;sup&gt;+&lt;/sup&gt; cells)</th>
<th>T&lt;sub&gt;reg&lt;/sub&gt; cell function</th>
<th>Effector T cell function</th>
<th>T cell resistance</th>
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<tbody>
<tr>
<td><strong>Peripheral blood</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1 diabetes</td>
<td>Normal; altered subsets of T&lt;sub&gt;reg&lt;/sub&gt; cells</td>
<td>Decreased</td>
<td>Increased</td>
<td></td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>Normal; altered subsets of T&lt;sub&gt;reg&lt;/sub&gt; cells</td>
<td>Increased in the CNS</td>
<td>Decreased</td>
<td>Normal</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>Decreased</td>
<td>ND</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>Increased</td>
<td>Increased in the synovial fluid of active disease</td>
<td>Decreased</td>
<td>Normal</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td>Decreased in active ulcerative colitis; normal in Crohn's disease</td>
<td>Increased in the lamina propria and mesenteric lymph nodes</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Psoriasis</td>
<td>Increased</td>
<td>Increased in the skin</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
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</table>
1.8 Impaired Treg cell regulation in autoimmunity

To address the question of whether immune suppression by Treg cells is impaired in the setting of human autoimmune disease, it is important to recognize the potential means by which such a defect may occur. Defects in the number and function of Treg cells, as well as a resistance of effector T cells to Treg cell-mediated suppression, could each contribute to failed T cell regulation. Each of these defects has been shown to contribute to the development of autoimmunity in various model systems. In these models, the underlying mechanisms by which these defects in regulation occur have also been investigated (Fig. 1.4).

Such studies indicate that cell-intrinsic defects in effector T cells, CD4⁺Foxp3⁺ T cells and APCs, as well as alterations in the composition of the inflammatory milieu, can contribute to failed tolerance to self.
1.8.1 Inadequate numbers of Treg cells

In mouse models, the concept that inadequate numbers of Treg cells may contribute to autoimmunity is supported by the occurrence of aggressive autoimmunity in scurfy mice and is indirectly implied by the successful treatment of autoimmunity in mice through the adoptive transfer of wild-type Treg cells (Sakaguchi et al., 1995 and Tang et al., 2004). Evidence that an inadequate number of Treg cells leads to autoimmunity in humans is most clearly shown in patients with IPEX, who completely lack Treg cells as a result of a mutation in Foxp3 (Wildin et al., 2002). However, most patients with autoimmune disease probably have a more modest reduction in Treg cells. In these common diseases, the challenge is to determine whether the number of Treg cells is inadequate at the site of inflammation and whether this is due to systemic factors or factors in the local tissue milieu.

The number of Treg cells found in individuals with autoimmune disease is influenced by Treg cell development, persistence and proliferation in the periphery and homing to the site of inflammation. Genetic factors are likely to have the strongest impact on thymic output of Treg cells. Maintenance of Treg cells in the periphery is a dynamic process, influenced in part by conditions that favour the induction of Treg cells in the periphery and support their proliferation and survival. Factors that favour the thymic development, peripheral growth and survival of Treg cells have been shown to have an effect on Foxp3 expression (Darrasse-Jeze et al., 2009). Such factors include CD28, TGFβ, DCs and the common cytokine receptor γ-chain (γc) cytokines IL-2, IL-4, IL-7 and IL-15, which signal through STAT5.
1.8.2 Defects in Treg cell function

Identifying defects in the function of Treg cells is made difficult both by the multiple mechanisms used by Treg cells to suppress inflammation (Workman et al., 2009) and by the manner in which suppression is measured. Treg cell dysfunction in autoimmune disease may be due to a defect in one of the many mechanisms through which Treg cells function (Vignali et al., 2008). This could occur through inadequate expression of cell surface molecules that are known to be involved in contact-dependent suppression (such as CTLA4, CD39, LAG3, granzyme A and CD95 (also known as FAS)) or as a result of a failure to produce the soluble factors (such as TGFβ, IL-10 and IL-35) that are involved in some aspects of suppression. Underlying genetic factors may influence these mechanisms. In addition, the composition of the local milieu, including the types of APCs and cytokines such as TNF, IL-6 (Korn et al., 2007), IL-4 (Thornton et al., 2004), IL-12 (King and Segal, 2005), IL-7, IL-15 (Ben Ahmed et al., 2009) and IL-21 (Clough et al., 2008), can influence Treg cell function.

1.8.3 Resistance of effector T cells to suppression

The resistance of effector T cells to Treg cells has been observed in several animal models of autoimmunity. In these models, inflammation and tissue destruction progress despite the presence of functional Treg cells at the site of inflammation. Such findings suggest that a resistance of effector T cells to Treg cells may contribute to disease progression. Multiple mechanisms by which effector T cells resist Treg cells have been proposed (Walker et al., 2009). Cell-intrinsic resistance to suppression has been shown to occur in CD4+ memory T cells and Th17 cells (Yang et al., 2007).
The cytokines IL-2, IL-4, IL-7 and IL-15 support the proliferation of effector T cells in the presence of Treg cells, indicating that despite the favourable roles of these cytokines in Treg cell homeostasis (Yates et al., 2007), the presence of these cytokines in the short term allows effector T cells to bypass suppression by Treg cells. In addition, several members of the TNF receptor family have been implicated in this process: antibody specific for OX40 (also known as TNFRSF4) abrogates suppression when bound to effector T cells (Takeda et al., 2004), and ligation of 4-1BB (also known as TNFRSF9) results in suppression-resistant effector T cells (Choi et al., 2004).

1.9 Treg cells and rheumatoid arthritis (RA)

RA is debilitating autoimmune disease of unknown etiology characterized by systemic inflammation of the joints and the ensuing destruction of cartilage and bone. While the exact destructive mechanism(s) of RA is unknown, clinical symptoms are paralleled by the production of pro-inflammatory cytokines and immune cell activation within the joints (Muller-Ladner and Pap, 2005). It is believed that a fundamental breakdown in the processes of self-tolerance is responsible for the initiation of RA and subsequent destruction of joint tissue. Data obtained over the last years, however, have shed new light on the role of T cells in regulation of the inflammatory response. This line of research started almost 15 years ago with the discovery of so-called Treg cells (Sakaguchi et al., 1995). This exciting discovery raised expectations for novel ways of treating arthritis by targeting these Treg cells.

As in other autoimmune diseases, CIA the experimental model of RA is exacerbated by depletion of Treg cells (Morgan et al., 2003). However, unlike other diseases, the target tissue in RA-synovium can be obtained from patients with disease. This has allowed investigators to analyse the number and
function of Treg cells not only in the peripheral blood of these patients but also in the diseased tissue.

1.9.1 Causes of Treg cell functional impairment in RA

Several analyses of Treg cell numbers in the peripheral blood of subjects with RA have produced differing results. In established disease, the CD4⁺CD25⁹ population has been shown to be no different from that of controls (Cao et al., 2003; Mottonen et al., 2005), whereas a modest decrease in Treg cells was reported for untreated patients with early stage RA (Lawson et al., 2008). These findings contrast with observations by Han et al. (2008), who reported an increase in the relative and absolute numbers of Treg cells (based on CD4⁺CD25⁹Foxp3⁺ staining) in the peripheral blood of patients with RA compared with numbers in controls. Despite these differences, there is a general agreement that the percentage of Treg cells is higher in the synovial fluid in patients with RA than in controls (Cao et al., 2003; Mottonen et al., 2005; Lawson et al., 2008).

Initial studies of the suppressive function of Treg cells isolated from both the peripheral blood and the synovium found no defects in suppression (Cao et al., 2003; Mottonen et al., 2005; Lawson et al., 2008). However, in later studies Ehrenstein et al. (2004) identified a focal defect in Treg cell function in RA with respect to the cells’ ability to suppress the production of IFNγ and TNFα in co-culture assays. They further established that this defect is intrinsic to the Treg cells of subjects with RA. Subsequent studies by this group have shown a defect in CTLA-4 mediated inhibition of TCR signalling in Treg cells from patients with RA. This defect can be reversed by overexpression of CTLA-4 in these Treg cells (Flores-Borja et al. 2008).
Resistance of effector T cells to suppression has not been tested exhaustively in RA and was not the reason for the impaired suppression observed by Ehrenstein et al. (2004). However, it has been shown that synovial macrophages may influence the responsiveness of effector T cells to Treg cells through their increased expression of MHC class II molecules and CD86 and increased production of TNF, IL-6 and IL-7, thereby altering the cytokine milieu and the stimulatory conditions in which suppression occurs in the joint (Van Amelsfort et al., 2007).

In summary, defects in immune regulation in RA do occur and are probably due to both Treg cell-intrinsic defects and the inflammatory milieu that is present in the rheumatoid joint.

1.9.2 Treg cells as therapeutic targets in RA

The ultimate goal of therapy for patients with active RA and other autoimmune diseases is to restore normal immune function rather than to achieve broad immunosuppression. In addition to being able to sample the target tissue, studies of RA benefit from the existence of well-established biological therapies, allowing the impact of these therapies on Treg cell number and function to be studied. Evidence has accumulated over the past decade that Treg cells could be an ideal target for therapies to induce durable remission of autoimmune and inflammatory disease. Treg cells are ideal for this purpose because they suppress inflammation in an antigen-specific manner. Furthermore, short-term therapy with Treg cells can lead to long-term inhibition of autoimmune disease in mouse models, and immunomodulatory agents can affect numbers and functioning of Treg cells in both mice and humans. Thus, approaches that bolster numbers or
functioning of Treg cells could achieve selective and durable inhibition of pathologic inflammation without blocking protective immune responses against infection.

1.10 Effects of approved drugs on Treg cells

Although much still remains to be clarified about how Treg cell defects might contribute to the pathogenesis of RA, approaches that specifically boost Treg cell activity could be useful in the treatment of RA (Fig. 1.5). High concentrations of TNF can block the immunosuppressive functions of Treg in vitro (Valencia et al., 2006). Patients treated with anti-TNF agent infliximab were found to have an increase in the number of peripheral Treg cells, and this correlated with changes in the level of C-reactive protein, a marker of disease activity and inflammation (Ehrenstein et al., 2004). This increase in Treg cells was not a result of expansion of the nTreg cell population but was due to the induction of TGFβ-producing Treg cells (Ehrenstein et al., 2004).

Tocilizumab, an antibody that blocks the human IL-6 receptor, has shown efficacy in the treatment of RA (Genovese et al., 2008). IL-6 can block the immunosuppressive activity of Treg cells in mice (Pasare and Medzhitov, 2003). By contrast, blockade of the IL-6 receptor with a monoclonal antibody in mice attenuates the severity of graft-versus-host disease and increases the absolute number of Treg cells in the spleens of treated mice through conversion of peripheral CD4+ T cells to Treg cells (Chen et al., 2009). IL-6 prevents Foxp3 upregulation in human T cells in vitro (Yang et al., 2008-a). Thus, blockade of the IL-6 receptor may benefit patients with RA, at least in part, through augmenting the conversion of peripheral effector T cells to Treg, perhaps by preventing IL-6 from driving CD4+ cells toward a Th17 phenotype.
Importantly, IL-6 reportedly has no effect on the immunosuppressive capacity of Treg cells from the synovial fluid of patients with RA (Van Amelsfort et al., 2007). More work needs to be done, therefore, to elucidate the effects of IL-6 receptor blockade on the balance between Treg and Th17 cell differentiation.

Another class of drug that may affect Treg cell function in patients with RA includes the FDA-approved CD28 co-stimulation blocker, abatacept (a CTLA4–Ig fusion protein), and its higher-affinity derivative, belatacept. These drugs bind to the CD28 ligands CD80 and CD86 with a higher affinity than does the CD28 receptor, which prevents T-cell co-stimulation and interferes with T-cell-driven autoimmune processes (Linsley and Nadler, 2009). Determining the optimal dose of CTLA4–Ig in patients with RA and other diseases will be critical if effector T cells are to be selectively inhibited while Treg cell function is preserved.

Rapamycin is an immunosuppressive small-molecule drug with a wide variety of effects on cells of both the innate and adaptive immune systems (Thomson et al., 2009). Importantly, rapamycin and other mTOR (mammalian target of rapamycin) inhibitors promote human Treg cell survival and differentiation and block effector T cell proliferation (Battaglia et al., 2006; Strauss et al., 2009). Patients with RA who were treated with the rapamycin derivative, everolimus, and methotrexate showed a greater response rate than patients treated with methotrexate alone. Thus, mTOR inhibition may ameliorate autoimmunity in part by promoting Treg cell function. However, to what extent the clinical efficacy of rapamycin results from its effects on Treg cells is not known.
1.11 Experimental drugs targeting Treg cells

Toll-like receptors (TLRs) are expressed on cells of the immune system and respond to the presence of microbial products as well as to human self-molecules such as RNA and DNA (Iwasaki and Medzhitov, 2004). Treg cells express multiple TLRs, and different TLR ligands regulate the immunosuppressive capacity of murine Treg cells in vitro and in vivo (Lee et al., 2008). Mice deficient in TLR9 have elevated numbers of Treg cells in gut-associated lymphoid tissue, which highlights the importance of TLRs to Treg homeostasis (Hall et al., 2008). Furthermore, synovial fluid from patients with RA contains TLR3 ligands, which promote inflammatory cytokine production in RA synovial fibroblasts (Brentano et al., 2005). In fact, hydroxychloroquine, a drug currently used to treat RA, may work in part by blocking TLR signaling (Kyburz et al., 2006). These results suggest that TLR antagonists could have therapeutic effects in patients with RA as a result of directly or indirectly boosting Treg cell function.

Trichostatin-A, a small-molecule inhibitor of histone deacetylases (HDACs) increases the number of Treg cells in normal mice by increasing thymic output of Treg cells (Tao et al., 2007). A recent study demonstrated that the HDAC inhibitors MS-275 and vorinostat (suberoylanilide hydroxamic acid) induce Foxp3 expression and immunosuppressive capacity in human T cells activated in vitro (Lucas et al., 2009). Trichostatin A may work by increasing the acetylation and functioning of Foxp3 (Tao et al., 2007), or by preventing Treg cells from producing IL-17 (Koenen et al., 2008). Thus, promotion of Treg cell differentiation might be a general result of HDAC inhibition, which suggests that HDAC inhibitors may, in future, have a role in therapy for autoimmune and inflammatory diseases. One HDAC inhibitor, vorinostat, has been
IL-2 is a principal survival factor for Treg cells (Setoguchi et al., 2005) and, in mice, its absence may contribute to defective Treg cell function in inflamed tissues (Tang et al., 2008). Mice treated with a stabilized form of IL-2 demonstrate increased Treg cell proliferation and function (Tang et al., 2008; Webster et al., 2009). IL-2, also known as aldesleukin, is currently used in cancer therapy, and some evidence suggests that it might also boost Treg cell numbers in humans (Wei, S. et al., 2007). Thus, administration of IL-2 could prove useful in the treatment of human autoimmune disease.

Figure 1.5 Treg mediated therapies for RA. Several immunomodulatory agents that are or may be effective in the treatment of RA boost numbers or function of Treg cells. Cytokine-based therapies, such as IL-2 (aldesleukin) or agents that block TNF or the IL-6 receptor, may restore the regulatory capacity of Treg cells and increase peripheral Treg cell production and/or survival. TLR antagonists (for example, IRS 954) may restore the regulatory capacity of Treg cells by decreasing production of inflammatory cytokines from APCs. Administration...
of CTLA4-Ig also increases the percentage of Treg cells in inflamed tissue. HDAC inhibitors (for example, MS-275, vorinostat) may promote Treg cell stability by increasing Foxp3 expression (adapted from Esensten et al., 2009).

1.12 Direct approaches to enhance Treg cell function

There are several methods available to directly target Treg cells for the treatment of autoimmune disease. These include expansion and induction of Treg cells in vitro followed by reinfusion into the patient, or in vivo by immunomodulatory compounds (Fig. 1.6).

1.12.1 Ex vivo expansion of Treg cells

Treg cells can be isolated and expanded ex vivo by anti-CD3/anti-CD28 stimulation in the presence of IL-2 (Hoffmann et al., 2004). With this protocol up to 3000-fold expansion can be reached without loss of suppressive function. Moreover, the cells have a higher inhibitory potential compared with directly isolated Treg cells, even in co-cultures with pre-activated effector cells (Hoffmann et al., 2004). Therefore, expanded Treg cells could have enhanced suppressive capacity in ongoing immune responses in vivo and be useful in the treatment of autoimmune disease.

1.12.2 In vitro induction of Treg cells

In addition to expansion of already existing Treg cells, Treg cells can also be induced in vitro from non-Treg cells. This method circumvents the difficulty of obtaining high numbers of natural Treg cells required for expansion. Treg cell induction works well in mice in which CD4+CD25- cells activated in the presence of TGF-β develop into Foxp3-expressing cells with suppressive capacity that is maintained after transfer in vivo (Chen et al., 2003).
However, TCR stimulation of human CD4⁺CD25⁻ cells can also result in transient expression of Foxp3 (Wang and Davies, 2007). Furthermore, activation-induced expression of Foxp3 in humans does not confirm a regulatory phenotype and can even coincide with IL-2 and IFN-γ production (Tran et al., 2007). Therefore, in vitro induction of Treg cells is far more complicated in humans compared with mice and it still needs to be established which culture conditions reinforce stable Foxp3 expression and suppressive function.

1.12.3 In vivo expansion and induction of Treg cells

Next to expanding and inducing Treg cells in vitro, several immunoactive agents can be used to enhance Treg cell function in vivo.

Anti-CD3 antibodies

The immunosuppressive efficiency of mAbs against CD3 was initially established in the transplantation field, where they prevented allograft rejection. In new-onset Type 1 diabetes patients, treatment with humanized CD3 antibodies led to preserved β-cell function and reduced insulin need (Keymeulen et al., 2005). Also in rheumatic disease, efficacy of anti-CD3 treatment was confirmed (Utset et al., 2002). Thus, CD3-specific antibodies are capable of inducing Treg cells and have already been proved to be safe and effective in patients with autoimmune disease. As such, they may provide a valuable treatment option for RA and juvenile idiopathic arthritis (JIA) as well, which should be further investigated.
Neuropeptides

Vasoactive intestinal peptide (VIP), is capable of enhancing Treg numbers and suppressive function, presumably via the induction of tolerogenic dendritic cells (Delgado et al., 2005). In CIA, administration of VIP increased both the absolute number and percentage of Treg cells, leading to lower arthritis scores (Gonzalez-Rey et al., 2006). Another neuropeptide, urocortin, also reduced disease severity in this model via the induction of Treg cells (Gonzalez-Rey et al., 2007). Although clinical trials in human autoimmune disease are still awaiting, neuropeptides could be of therapeutic value, due to their Treg-enhancing capacity.

Retinoic acid

All-trans retinoic acid (ATRA) an active metabolite of vitamin A induces Treg cell generation, while simultaneously inhibiting Th17 development (Mucida et al., 2007; Pino-Lagos et al., 2008). Thus ATRA might be able to restore the balance between Treg and pathogenic Th17 cells thought to be disturbed in various autoimmune diseases (Nistala and Wedderburn, 2009). ATRA administration has been shown to reduce severity and incidence of CIA. This beneficial effect was accompanied by a decrease in pro-inflammatory cytokines and collagen-specific antibodies (Nozaki et al., 2006). In addition, Treg cells induced in vitro in the presence of ATRA are resistant to conversion into Foxp3- cells (Benson et al., 2007) and have enhanced suppressive capacity (Wang et al., 2009). Therefore, ATRA can also be used as a potential candidate to optimize protocols for the in vitro expansion and induction of Treg cells.
Histone deacetylase inhibitors

HDAC inhibitors increase Foxp3 gene transcription and prevent protein degradation, thereby enhancing and stabilizing Foxp3 expression. Two HDAC inhibitors, MS-275 and suberoylanilide hydroxamic acid (SAHA), have been shown to induce Foxp3 expression and suppressive function in human CD4+CD25- cells \textit{in vitro} (Lucas et al., 2009). Another HDAC inhibitor, nicotinamide, increases the number of Foxp3+ cells in CD4+ cell cultures as well as the amount of Foxp3 per cell and the suppressive capacity of CD4+CD25+ cells (Van Loosdregt et al., 2009). Also \textit{in vivo}, administration of HDAC inhibitors leads to increased numbers of Foxp3+ T cells with enhanced suppressive capacity. Moreover, treatment with HDAC inhibitors reduces pathology in experimental arthritis (Saouaf et al., 2009), by enhancing Treg function.

1.13 Indirect approaches to enhance Treg cell function

In addition to the above-described strategies that target the Treg cell population directly, indirect approaches can also be used to enhance Treg function in patients with various autoimmune diseases. These include \textit{viz.} reducing the proinflammatory environment and enhancing responsiveness of effector cells to suppression.

1.13.1 Inhibition of pro-inflammatory cytokines

The \textit{in vivo} pro-inflammatory environment at the site of inflammation in patients with autoimmune disease can have profound negative effects on Treg cell function. Therefore, dampening the ongoing inflammation, for instance by
inhibiting pro-inflammatory cytokines, can indirectly lead to better Treg-cell mediated suppression. This is clearly shown by two studies that examined Treg cell function in RA patients before and after anti-TNF-α (infliximab) therapy. Both studies reported impaired Treg cell function before therapy, which was completely restored after infliximab treatment (Ehrenstein et al., 2004; Valencia et al., 2006). Probably, neutralizing the high TNF-α levels in these patients directly reduced the down-regulating effect of TNF-α on Treg cells (Valencia et al., 2006), thereby restoring their suppressive function.

1.13.2 Enhancing the responsiveness of effector cells to suppression

Indirect improvement of Treg cell function can also be achieved by enhancing responsiveness of effector cells to suppression. Several studies suggest that this can, at least partially, be achieved by blocking the production of pro-inflammatory cytokines. The increased resistance of effector cells at the site of inflammation in experimental autoimmune encephalomyelitis (EAE) mice is caused by TNF-α and IL-6 produced by these cells (Korn et al., 2007). IL-6 acts on effector cells rather than on Treg cells (Koenen et al., 2008). Similarly IL-7, known to reduce Treg-mediated suppression, is expected to target effector cells as well (Van Amelsfort., 2007). Therefore, blocking these pro-inflammatory cytokines will reduce the resistance of effector cells to suppression and thereby enhance control of inflammation by Treg cells.

1.13.3 Combination therapy

Clinical outcome can be enhanced by a combination of both direct and indirect strategies. Both nasal administration of heat shock protein (HSP)-60 peptide as well as a single dose of anti-TNF-α (etanercept) treatment, led to a small and
insignificant reduction in arthritis scores. However, combining the two therapies resulted in a highly significant improvement of disease, as shown by lower arthritis scores and reduced joint destruction (Roord et al., 2006). Also in humans there is evidence for enhanced effectiveness of Treg cell induction, when combined with anti-inflammatory treatment. Together, these data clearly demonstrate that combining Treg induction with anti-inflammatory treatment enhances clinical outcome. In addition to increased effectiveness, dampening the ongoing inflammation might also be crucial in preventing adverse effects, as it has been shown that in a pro-inflammatory environment TGF-β produced by Treg cells drives Th17 differentiation (Veldhoen and Stockinger, 2006; Xu et al., 2007) and Treg cells can convert into Th17 cells themselves (Xu et al., 2007; Koenen et al., 2008; Radhakrishnan et al., 2008).

**Figure 1.6 Methods of enhancing Treg cell function in arthritic patients.** Treg can be enhanced in arthritic patients via different methods: (A) isolation and ex vivo expansion of natural Treg or (B) in vitro induction of Treg from non-Treg (n-Tr), followed by reinfusion into the patient; (C) in vivo induction and expansion of Treg by anti-CD3 antibodies (aCD3), HADCI inhibitors (HADCi) and neuropetides, such as VIP; (D) mucosal tolerization with self-antigen, preferably HSP; (E) indirect improvement of Treg function by enhancing the responsiveness of effector cells to suppression and blocking pro-inflammatory cytokines (adapted from Wehrens et al., 2010).
1.14 Pitfalls of cellular therapy

Several pitfalls are possible in the treatment of patients with Treg cells. First, incomplete lineage commitment enables some human CD4⁺Foxp3⁺ cells to express nuclear factor receptor-related orphan receptor (Ror)-γt and to develop into IL-17-producing Th17 effector cells (Koenen et al., 2008; Beriou et al., 2009). Moreover, recent data from mice suggest that some fully differentiated Treg cells may be unstable and could be able to trans-differentiate in vivo into effector memory T cells that produce pathogenic cytokines such as IFN-γ, and lead to exacerbation rather than attenuation of the autoimmune process. Second, Treg cell may be less effective in suppressing effector T cells in an autoimmune setting, and may even augment IL-17 production in vitro (Evans et al., 2007). Third, it is unclear whether a large polyclonal population of Treg cell would be able to interrupt an autoimmune process that targets antigens found in specific tissues. Fourth, polyclonal Treg cell therapy could result in generalized immuno-suppression and increased susceptibility to various infections. Finally, some patients with autoimmune disease may have poorly appreciated intrinsic defects in Treg cell function that cannot be overcome by infusion of large numbers of these defective cells. In spite of these potential concerns, however, clinical trials are underway to test the therapeutic potential of Treg in graft-versus-host disease (Riley et al., 2009).

1.15 Future of Treg cell therapy

Although existing drugs significantly reduce the morbidity and mortality associated with RA, these therapies are not curative. Several drugs that affect Treg cell numbers or function have shown efficacy in the treatment of RA.
These results imply that direct administration of Treg cell could be an ideal therapy to induce durable remission of RA, as these cells persist in vivo and act in an antigen-specific manner. Thus, approaches that bolster Treg cell numbers and functions could be a fruitful means of selectively and durably inhibiting pathologic inflammation without blocking protective immune responses against infection.
1.16 Immunobiology of Th17 cells

Effective immunologic homeostasis relies on a continual balance between a number of factors, including Th cell activation and Treg cell suppression. When homeostasis is disrupted and the immune system tips in favour of activation, the host becomes susceptible to autoimmunity. Although it was originally believed that many autoimmune disorders, including RA and multiple sclerosis, partially resulted from an aberrant Th1 response, several studies in mice that demonstrated the deleterious effects of IFN-γ deficiency called the role of the Th1 lineage in autoimmune pathogenesis into question (Sato et al., 2006). The recent discovery of another subset of helper T cells, termed Th17, breaks the long-accepted paradigms about T cell subsets in autoimmunity and has the potential to resolve the contradictory evidence regarding the role of Th1 cells.

Identified originally by their expression of the proinflammatory cytokine IL-17A, Th17 (Fig. 1.7) cells have gained wide acceptance as a distinct subset of CD4+ cells with expression of unique master regulatory transcription factors retinoic acid Rorγt and Rora (Harrington et al., 2006; Yang et al., 2008-b). Th17 cells are induced by a number of pro-inflammatory cytokines including IL-1, IL-6, and IL-23, and in turn these cells secrete anti-microbial peptides and proinflammatory cytokines such as TNF, IL-21, and IL-22.
1.17 Differentiation of Th17 cells

Th17 cells were established as an independent subset of Th cells by the identification of differentiation factors and transcription factors that are unique to Th17 cells. Three independent studies found that a combination of the immunoregulatory cytokine TGF-β and the proinflammatory and pleiotropic cytokine IL-6 is required to induce IL-17 in naive T cells (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen and Stockinger, 2006). Interestingly, two cytokines with opposing effects have to cooperate to induce the differentiation of Th17 cells, suggesting that signaling molecules and
transcription factors involved downstream of the TGF-β and IL-6 receptors work together to induce Th17 differentiation. Also, three independent groups reported simultaneously that IL-21, a member of the IL-2 family of cytokines, is produced in overwhelming amounts by Th17 cells and could, in combination with TGF-β, induce Th17-differentiation (Korn et al., 2007; Ivanov et al., 2007). These findings point to a relevant function of IL-21, produced by newly generated Th17 cells, in amplifying the precursor frequency of differentiating Th17 cells. In addition to IL-21, other cytokines such as TNFα and IL-1, which are not specifically produced by Th17 cells, have been proposed to have an additional role in the amplification of Th17 responses (Sutton et al., 2006; Veldhoen and Stockinger, 2006).

Recently it has been shown that T cells cultured in the presence of TGF-β plus IL-6 did not induce tissue inflammation unless they are further cultured in the presence of IL-23, which could decrease the secretion of IL-10 by these cells (McGeachy et al., 2007). Also, both IL-6 and IL-21 are strong inducers of IL-23R on Th17 cells (Ivanov et al., 2007). Furthermore, IL-23 can enhance the expression of its own receptor through an autocrine or paracrine feedback loop in mouse (Langrish et al., 2005) and human (Chen and Wood, 2007) T cells. The maintenance of already committed Th17 cells in vitro requires IL-23 (Veldhoen and Stockinger, 2006) which supports a role for IL-23 not in the differentiation but rather in the expansion/stabilization of Th17 cells.

Thus the full differentiation of Th17 cells requires three different steps: induction, amplification and stabilization/maintenance. (1) The differentiation is initiated by the combined actions of IL-6 and TGF-β; (2) the amplification of the Th17 response is driven through the production of IL-21 by Th17 cells; (3) the stabilization/maintenance of the Th17 phenotype is achieved by IL-23. Whereas the first two steps in the development of Th17 cells seem to be
distinct, it is possible that the stabilization and the amplification phases overlap or take place simultaneously (Bettelli et al., 2008) (Fig. 1.8).

Figure 1.8 Steps in the differentiation of Th17 cells. Different factors control the initial differentiation of Th17 cells from naive T cells, the amplification of Th17 precursor cells, and finally the stabilization and effector phenotype of Th17 cells. Whereas TGF-β together with IL-6 are the differentiation factors for Th17 cells, IL-21, which is produced by Th17 cells themselves, acts in a positive feedback loop to increase the frequency of Th17 cells. STAT3 is the essential signalling molecule for the differentiation of Th17 cells because the induction of IL-21 is absolutely dependent on STAT3, and STAT3 is also critical in the signal transduction cascades of IL-6, IL-21 and IL-23 receptors. IL-23 expands and stabilizes Th17 cells to produce their effector cytokines IL-17, IL-17F and IL-22 (adapted from Bettelli et al., 2008).

As far as human T cells are concerned, the situation for the differentiation of Th17 cells has been confusing. In 2007, several studies claimed that TGF-β was dispensable for the differentiation of human Th17 cells (Acosta-Rodriguez et al., 2007; Wilson et al., 2007). Whereas naive mouse CD4+ T cells differentiate into Th17 cells upon exposure to TGF-β plus IL-6 or TGF-β plus IL-21, the combinations of IL-1β plus IL-6 (Acosta-Rodriguez et al., 2007) or IL-1β plus IL-23 (Wilson et al., 2007) were proposed to be the differentiation factors for human Th17 cells. Thus, a rather disturbing problem was raised by the claim
that human Th17 cells could develop in the absence of TGF-β, suggesting that the requirements for human and mice Th17 differentiations are essentially different. A series of three new reports tackled these problems, proving that TGF-β is indeed essential for the differentiation of human Th17 cells from naïve T cells (Manel et al., 2008; Yang et al., 2008-a) as well. TGF-β is absolutely required to induce Rorc (the human homolog of Rorγt), but its expression and function are inhibited by excess TGF-β. Only when additional cytokines such as IL-6 plus IL-23 or IL-21 are present is Rorc relieved from inhibition, and then naive T cells can begin transcribing IL-17 (Manel et al., 2008). Thus, at molecular level, the differentiation conditions of mouse and human Th17 cells do not appear to be different.

1.18 Transcription factors in Th17 differentiation

The steroid receptor–type nuclear receptor Rorγt, which is a splice variant of Rorγ expressed in T cells (He and Malek, 1998), is selectively expressed in vitro differentiated Th17 cells and in IL-17+ T cells present in the lamina propria of naive mice (Ivanov et al., 2006). Rorγt appears to be required for IL-17 production, as mice reconstituted with the bone marrow of Rorγt deficient mice show an impaired Th17 differentiation (Ivanov et al., 2006). Furthermore, transduction of naive T cells with a retroviral vector containing Rorγt induces the development of IL-17 producing T cells (Ivanov et al., 2006). Another member of the retinoid nuclear receptor family, Rorα, is also selectively expressed in Th17 cells—very similarly to Rorγt. A recent study reported that Rorα could fulfill a similar but not identical role to Rorγt in the differentiation of Th17 cells, suggesting that Th17 cell differentiation could be dictated by two lineage-specific transcription factors (Yang et al., 2008-b) (Fig. 1.9).
The mechanisms by which Rorγt and possibly Rorα regulate IL-17 production have not yet been fully elucidated. Although there is a potential Ror-binding site in the IL-17 promoter, it is not clear whether Rorγt binds directly to this promoter. Rorγt and Rorα are both strongly induced by IL-6 or IL-21 in the presence of low amounts of TGF-β. The induction of Rorγt is dependent on STAT3, which is preferentially activated by IL-6, IL-21, and IL-23 and plays an important role in the regulation of IL-17 production in T cells (Chen et al., 2006; Mathur et al., 2007; Yang et al., 2007; Zhou et al., 2007).

Figure 1.9 Transcriptional regulation of Th17-cell differentiation. The differentiation of Th17 cells is initiated by STAT3, downstream of IL-6 and IL21-induced signalling. Activation of STAT3 induces the expression of RORα and RORγt. These two factors establish the Th17-cell-associated gene-expression programme, leading to the production of IL-17, IL-17F and IL-22. STAT5, which is downstream of IL-2 signalling, and STAT1, which is downstream of IL-27 signalling, as well as ETS1, are negative regulators of Th17-cell differentiation. In addition, the transcription factor Foxp3, which is induced by TGFβ signalling, antagonizes the Th17-cell developmental programme (adapted from Dong, 2008).
Mice with conditional deficiency of STAT3 in T cells have impaired Th17 differentiation, and overexpression of a constitutively active form of STAT3 can increase IL-17 production (Harris et al., 2007; Yang et al., 2007). Thus, STAT3 might affect the expression of IL-17 by increasing the expression of Rorγt and Rora, which are upstream of IL-17 (Yang et al., 2007; Yang et al., 2008-b). However, STAT3 also binds directly to the IL-17 and IL-21 promoters (Chen et al., 2006; Wei, L. et al., 2007). Therefore, STAT3 and Rorγt seem also to cooperate, and competent production of IL-17 depends on the presence of both transcription factors.

Rorγt cooperates with other transcription factors in the induction of Th17 cells. For example, the interferon regulatory factor 4 (IRF4), which was previously associated with the differentiation of the Th1 and Th2 subsets (Rengarajan et al., 2002), is required for the differentiation of Th17 cells as well (Brustle et al., 2007). IRF4 knockout mice failed to mount a Th17 response and were resistant to EAE. Consistent with this observation, IRF4-deficient T cells failed to upregulate Rorγt upon stimulation in the presence of TGF-β plus IL-6 and could not be differentiated into Th17 cells (Brustle et al., 2007). However, overexpression of Rorγt in IRF4-deficient T cells failed to fully restore the induction of IL-17, again suggesting that IRF4 or its downstream targets may have to cooperate with Rorγt for full commitment of T cells to the Th17 lineage.

1.19 Effector cytokines of Th17 cells

Th17 cells are characterized by the production of IL-17A (also called IL-17), IL-17F and IL-22 and are thought to clear extracellular pathogens not effectively handled by either Th1 or Th2 cells. Because Th17 cells produce large quantities of IL-17A, most Th17-mediated effects are attributed to this cytokine. IL-17A is
the prototypic cytokine of the IL-17 family, which includes six members: IL-17A, B, C, D, E and F4. IL-17 is a phylogenetically old cytokine that is also detected in non-mammalian vertebrates (Gunimaladevi et al., 2006). In addition to IL-17A, Th17 cells co-produce IL-17F (Langrish et al., 2005; Liang et al., 2006). IL-17A and IL-17F have similar functions and closely related, with 55% amino acid identity as well as a common receptor (Kuestner et al., 2007). They induce the production of proinflammatory cytokines, chemokines and metalloproteinases from various tissues and cell types. As a result, they recruit neutrophils to tissues. Although there is often a coordinated expression of IL-17A and IL-17F in Th17 cells and other cell types, it is now clear that there are Th cells expressing only IL-17A, IL-17F or an IL-17A–IL-17F heterodimer (Chang and Dong, 2007) that has potent inflammatory effects (Liang et al., 2007). In addition to IL-17A and IL-17F, Th17 cells in both mouse and human produce other effector cytokines, namely IL-21, IL-22 and TNF-α (Acosta-Rodriguez et al., 2007; Korn et al., 2007; Liang et al., 2007; Zhou et al., 2007). Neither of them are Th17-exclusive cytokines, but are preferentially expressed by Th17 cells.

IL-22 is a member of the IL-10 family of cytokines, produced by Th17 cells, activated T cells and natural killer cells. It mediates its effects through a receptor complex composed of the IL-10R2 and the IL-22R chains. IL-22 induces antimicrobial proteins, defensins, acute-phase proteins, inflammatory cytokines, chemokines, and hyperplasia (Wolk et al., 2006). However, IL-22 protects hepatocytes during acute liver inflammation (Zenewicz et al., 2007); thus IL-22 may either enhance inflammation or limit tissue damage induced by IL-17, depending on the type of tissue. Interestingly, high concentrations of TGF-β can inhibit IL-6-induced IL-22 expression (Zheng, Y. et al., 2007). Furthermore, whereas the combination of TGF-β plus IL-6 induces large quantities of IL-17A and IL-17F by Th17 cells, the secretion of large amounts of
IL-22 by Th17 cells requires the addition of IL-23 in vitro (Liang et al., 2006; Zheng, Y. et al., 2007). This suggests that IL-22 could represent an end point effector cytokine produced by terminally differentiated Th17 cells.

Unexpectedly, a subset of Th17 cells coexpresses IFN-γ, particularly in humans, where as many as half of all the IL-17 positive cells also express IFN-γ (Acosta-Rodriguez et al., 2007; Chen and Wood, 2007; Wilson et al., 2007). These double positive cells complicate the idea that Th17 cells are a unique subset distinct from Th1 cells, and are particularly hard to explain given that IFN-γ has been shown to inhibit IL-17 expression. It is not clear yet whether these cells represent a stable phenotype or a transitional phase from Th17 to Th1 or vice versa. Although there are no data on the specific role of these double positive cells, both IFN-γ and IL-17 are important mediators of inflammation, and cells that produce both cytokines are likely to contribute to pathogenesis in certain environments.

Also unexpected is that Th17 cells coexpress IL-10, an anti-inflammatory cytokine. T-cell sources of IL-10 include Th2 cells and various types of Treg cells, but Th1 cells also secrete IL-10 in certain conditions (Anderson et al., 2007; Jankovic et al., 2007). In mice the combination of TGF-β and IL-6, which synergize to induce IL-17 production, also synergize to induce IL-10, such that half of the IL-17 positive cells coexpress IL-10 (McGeachy et al., 2007; Stumhofer et al., 2007). IL-10 produced by mouse Th17 cells may serve an important protective function by limiting inflammation and tissue damage normally caused by IL-17, through antagonistic effects on target tissues (McGeachy et al., 2007). It is not yet known whether human Th17 cells ever coexpress IL-10.
1.20 Reciprocal relationships between Th17 and Treg cells

Th17 and Treg developmental programs of T cells are reciprocally interconnected. This discovery was initially based on the observation that upon TCR stimulation, a naive T cell can be driven to express Foxp3 and become a Treg cell in the presence of TGF-β. However, in the presence of TGF-β plus IL-6 or IL-21, the Treg developmental pathway is abrogated, and instead T cells develop into Th17 cells. Only the combination of TGF-β plus IL-6/IL-21, but neither of them alone, induced robust production of IL-17 in naive T cells (Fig. 1.10).

In vitro, there is a true reciprocity between the Th17 and Treg developmental programs on the single-cell level. However, evidence is still incomplete as to whether this reciprocal developmental decision at the single-cell level is also relevant in vivo. Active TGF-β is a cytokine produced by various cell types, including nTreg and cells of the innate immune system. TGF-β has broad inhibitory effects on the entire immune system. In addition, TGF-β induces the Treg-specific transcription factor Foxp3, which is required for the induction and maintenance of iTreg in the peripheral immune compartment (Chen et al., 2003; Kretschmer et al., 2005). However, addition of IL-6 to TGF-β inhibits the generation of Treg cells and induces Th17 cells.

The mechanism by which IL-6 and IL-21 act as switch factors relies on the control of the Foxp3/Rorγt balance (Zhou et al., 2008). In line with this concept, conditional deletion of Foxp3 in adult mice results in an increase in both Rorγt and IL-17 expression (Li MO et al., 2007). It is clear now that TGF-β is required for the expression of both Foxp3 and Rorγt, although the signaling cascades downstream of the TGF-β receptor might be different for the induction of Foxp3 versus Rorγt. In the presence of TGF-β, IL-6 and IL-21 play
TGF-β is ubiquitous although its most relevant source in regulating immune reactions is still unclear. Other factors such as retinoic acid or cytokines such as IL-6, IL-1, IL-23 or IL-27 are provided by cells of the innate immune system (immature or activated dendritic cells DCs, respectively) and dictate whether a naive T cell develops into a FOXP31 Treg cell, a Th17 cell or an IL-10-secreting T cell of the Tr-1 phenotype. IL-6R, IL-6 receptor; IL-21R, IL-21 receptor; IL-23R, IL-23 receptor; RAR, retinoic acid receptor; TGF-βR, TGF-β receptor; WSX-1, IL-27 receptor (adapted from Bettelli et al., 2008).

an important role in Th17 differentiation by inhibiting TGF-β-driven Foxp3 expression. Both Rorγt and Rorα physically associate with Foxp3 to antagonize each other’s functions (Zhou et al., 2008). This association is likely the molecular basis for the reciprocal relationship between Treg cells and Th17 cells. Foxp3 may also have to bind both Rorγt and additional transcription factors such as Runx1, which also cooperates with Rorγt, to fully inhibit the Th17 transcriptional program (Zhang et al., 2008).
The reciprocal relationship between Treg cells and Th17 cells is further supported by the results that IL-2, which is a growth factor for Treg cells, inhibits the generation of Th17 cells and promotes the generation of Treg cells (Laurence et al., 2007), suggesting that IL-2 receptor signaling promotes the generation of Treg cells but inhibits Th17 cells in the peripheral immune compartment in vivo.

Additional evidence for a reciprocal developmental relationship between Foxp3+ Treg cells and Th17 cells comes from studying the effects of retinoic acid, a vitamin A metabolite, on T cell differentiation. Retinoic acid could drive the generation of Treg cells while abrogating the differentiation of Th17 cells, but not of Th1 cells (Mucida et al., 2007). In the presence of both TGF-β and IL-6, retinoic acid suppresses the upregulation of IRF4 and IL-23R, resulting in decreased generation of Th17 cells (Xiao et al., 2008). It is clear that retinoic acid cannot induce Treg cells on its own, but rather needs to cooperate with TGF-β to mediate its effects. In an inflammatory setting, retinoic acid inhibits the generation of Th17 cells rather than enhancing de novo generation of Treg cells (Mucida et al., 2007; Xiao et al., 2008). Collectively, these findings indicate that a common metabolite like retinoic acid can regulate the balance between proinflammatory Th17 cells and anti-inflammatory Treg cells.

The question of whether reprogramming of either precommitted iTreg cells or Th17 cells is possible was recently addressed in a study from Chen Dong’s laboratory. iTreg cells could be reprogrammed to the Th17 phenotype in the presence of TGF-β plus IL-6 up to five days after differentiation. Foxp3 is downregulated upon restimulation in the presence of TGF-β plus IL-6; however, in the presence of retinoic acid, IL-6 was unable to induce IL-17 from Foxp3+ T cells. Even naturally occurring Treg cells begin to express IL-17 under inflammatory conditions such as, for example, in the target tissue of an
autoimmune reaction. Foxp3+ T cells begin to express IL-17 in the central nervous system (CNS) at the peak of disease during EAE. IL-6 may be the most crucial factor in mediating this conversion of Foxp3+ T cells into Th17 cells *in vitro* and *in vivo* (Yang et al., 2008-a). The re-expression of the Th17 program in Foxp3+ T cells appears to be a two-step process that includes downregulation of Foxp3 and release of Rorγt and Rora from Foxp3-mediated inhibition (Fig. 1.11).

![Diagram](image)

**Figure 1.11 Competitive antagonism between Foxp3 and RoR family members dictates iTreg versus Th17 cell development.** Naive CD4+ T cells activated by antigen in the presence of TGFβ are induced to express Rorγt, Rora, and FoxP3. These cells can differentiate into either Th17 cells or iTreg cells depending on the dominance of IL-6 or retinoic acid, respectively. In the presence of retinoic acid, and absence of IL-6, FoxP3 can bind, and inhibit the activity of Ror family factors, thereby promoting iTreg cell specification. IL-6, and other STAT3-activating Th17 cell-inducing cytokines (such as IL-21 and IL-23) seem to promote Th17 cell development at least in part by antagonizing the Foxp3-mediated inhibition of Ror family members through an undefined mechanism. Ror factors might also inhibit FoxP3 expression through direct or indirect mechanisms (adapted from Weaver and Hatton, 2009).
1.21 Th17 cells in autoimmune diseases

Although it was originally believed that many autoimmune disorders, including RA and multiple sclerosis, partially resulted from an aberrant Th1 response, several studies in mice that demonstrated the deleterious effects of IFN-γ deficiency called the role of the Th1 lineage in autoimmune pathogenesis into question (Takayanagi et al., 2000; Sato et al., 2006). The recent discovery of another subset of helper T cells, termed Th17, broke the long-accepted paradigms about T cell subsets in autoimmunity.

The idea that Th17 cells are an important part of the autoimmune reaction has emerged in light of the following observations: first, mice deficient for the Th1 effector cytokine IFN-γ develop enhanced EAE (Becher et al., 2002); second, deficiency in the IL-12p35 subunit (specific for IL-12) does not alter the progression of EAE, but deficiency in either p40 or p19, which form IL-23, results in a decreased number of Th17 cells and protection from EAE and CIA (Becher et al., 2002; Cua et al., 2003); third, the transfer of myelin-reactive IL-17-producing T cells expanded with IL-23 in vitro induces severe EAE (Langrish et al., 2005); and fourth, IL-17 has profound pro-inflammatory effects and induces tissue damage during the course of various autoimmune diseases.

Indeed, IL-17 can directly or indirectly promote cartilage and bone destruction (Sato et al., 2006). Conversely, IL-17-deficient mice develop attenuated CIA (Nakae et al., 2003) and EAE (Komiyama et al., 2006). Increased levels of IL-17 have been observed in patients with RA (Chabaud et al., 1999), multiple sclerosis (Lock et al., 2002), inflammatory bowel disease (Fujino et al., 2003) and psoriasis (Wilson et al., 2007). Furthermore, IL-22 produced by Th17 cells
mediates IL-23-induced acanthosis and dermal inflammation (Zheng, Y. et al., 2007). These data indicate a pathogenic role of Th17-associated cytokines and Th17 cells in inducing autoimmune tissue inflammation both in experimental animals and in humans.

1.22 Th17 cells and autoimmune arthritis

The importance of IL-17 in the pathogenesis of arthritis has been shown in a variety of animal models, both genetic and adaptive. Studies in IL-17 knock-out mice demonstrated that IL-17 is indispensable for spontaneous disease development through the priming of autoantigen-specific T cells as well as the generation of a pro-inflammatory cytokine milieu in joints (Nakae et al., 2003; Hirota et al., 2007). Also, IL-17 knockout mice develop significantly less arthritis, and treatment with neutralizing antibodies to IL-17 or soluble IL-17 receptor alleviates joint inflammation (Bush et al., 2002; Nakae et al., 2003; Lubberts et al., 2004). In TNF-deficient mice, IL-17 can drive cartilage and bone destruction (Joosten et al., 2008). In concurrence with these findings, local over-expression of IL-17 exacerbates joint destruction in CIA and chronic streptococcal cell wall arthritis (Lubberts et al., 2003; Lubberts et al., 2004). IL-17 is also required for the development of spontaneous arthritis in other mouse models of RA, such as IL-1Rα deficient mice and SKG (spontaneous autoimmune arthritis model) mice (Nakae et al., 2003; Hirota et al., 2007). Analysis of RA synovial fluid also demonstrated elevated levels of IL-17 (Chabaud et al., 1999; Ziolkowska et al., 2000). Thus, mounting evidence implicates IL-17 as a crucial cytokine in both the induction of local inflammation and the joint destruction that characterizes the synovial tissue of autoimmune arthritis.
1.23 Mechanisms of IL-17 driven joint destruction

Inflammation

Th17-driven inflammation during host defense markedly resembles the inflamed synovial tissue in RA and other forms of autoimmune arthritis. The RA synovium is characterized by elevated levels of IL-6, TNF, and IL-1β along with nitric oxide (NO) and PGE₂ (Afzali et al., 2007). Th17 cells, and IL-17 in particular, have been shown to synergize with or upregulate each of these pro-inflammatory factors. IL-17 mediates the induction of IL-6 and IL-8 in both adult RA and juvenile idiopathic arthritis (Hwang et al., 2004; Agarwal et al., 2008); associated with inflammation in synovial fluid and activation of fibroblast-like synoviocytes through the phosphatidylinositol 3-kinase/Akt and NF-κB pathways (Georganas et al., 2000; Hwang et al., 2004).

Additionally, IL-17 induces the expression of cyclooxygenase-2 (COX-2) in synoviocytes, a stress response molecule conducive to the high levels of PGE₂ observed during inflammation (Stamp et al., 2004) (Fig. 1.11 and 1.12). Through synergy with TNF, IL-17 has also been proposed to induce the alternative complement pathway proteins C3 and factor B, both of which are upregulated in RA synovial tissue (Katz et al., 2000). Abnormalities in the activation of the alternative complement pathway have been observed in RA synovium (Mollnes et al., 1986) and have been implicated in pathogenesis in autoimmune arthritis models (Banda et al., 2006; Katschke et al., 2007).

In addition, IL-17 activates RA synovial fibroblasts through the PI2K/Akt, p38 MAPK, and NFκB signaling pathways, inducing the IL-23-specific subunit, IL-23p19, in a probable positive feedback loop (Kim et al., 2007).
Figure 1.12 Opposing roles of IL-17 in bone turnover. IL-17 is produced by T cells (particularly memory T cells), and acts on a wide variety of target cells to trigger expression of inflammatory effectors. Most of these effectors have been shown to have an impact on bone metabolism. Those factors that promote osteoclastogenesis indirectly favor bone destruction. Conversely, chemotactic factors promote neutrophil recruitment and activation, which can exert both bone protective and bone destructive effects. G-CSF, granulocyte colony-stimulating factor; ICAM, intercellular adhesion molecule; IFN, interferon; IL, interleukin; LIX, LPS-inducible CXC chemokine; MCP, monocyte chemotactic protein; PGE2, prostaglandin E2; RANKL, receptor activator of nuclear factor-κB ligand; TNF, tumor necrosis factor (adapted from Gaffen, 2004).

Two other members of the IL-17 cytokine family, IL-17B and IL-17C, have also been implicated in chronic inflammation in an experimental model of arthritis mediated through expression of IL-1β, IL-6, TNF, and IL-23 (Yamaguchi et al., 2007). Taken together, these data are consistent with the localization of CD4+ T cells to the inflammatory pannus tissue formed during CIA (Ju et al., 2008).
**Cellular infiltrates**

IL-17 may intensify local inflammation by promoting angiogenesis and subsequently recruiting innate immune cells to the joint. IL-17 dose-dependently increases the production of vascular endothelial growth factor (VEGF) in RA fibroblast-like synovial cells *in vitro*, and stimulates this expression additively with TNFα, suggesting that these cytokines act through independent pathways (Cho et al., 2006; Ryu et al., 2006). Additionally, IL-17 can upregulate the constitutive release of other angiogenic factors from synovial fibroblasts, including keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), and heparin-binding epidermal growth factor-like growth factor (HB-EGF), all of which are involved in the proliferation of endothelial cells (Honorati et al., 2006).

IL-17-induced angiogenesis lays the foundation for the recruitment of neutrophils, macrophages and T cells to the joint, and local over-expression of IL-17 has been shown to augment the influx of polymorphonuclear leukocytes into the joints in CIA (Ottonello et al., 2002). IL-17 regulates neutrophil activity through a combination of granulopoeisis, chemokine induction, and neutrophil survival during host defense (Priebe et al., 2008) and may likewise be responsible for the influx of inflammatory neutrophils that occurs in the RA synovium.

IL-17 has also been implicated in the recruitment of CD4+ T cells from peripheral blood, in part through induction of IL-8 and IL-16 (Cho et al., 2008). In addition, TNFα and IL-1β, both of which synergize with IL-17, enhance IL-15 production from RA fibroblast-like synoviocyte cell lines, and IL-15 appears to promote the proliferation of lymphocytes in models of pannus (Wakisaka et al., 2000). The synergy between monocyte derived IL-1β and
TNFα and T cell-derived IL-17 also causes the upregulation from RA synoviocytes of CCL20, a protein involved in the chemotaxis of T cells and immature dendritic cells (Hirota et al., 2007). IL-17 thus appears to enhance inflammation in the joint through multiple pathways. In addition to inducing a highly inflammatory cytokine milieu, it drives angiogenesis and the subsequent recruitment of innate immune cells that further amplify inflammation.

**Cartilage destruction**

Local inflammation has long been implicated in triggering and exacerbating cartilage destruction in rheumatic joints. Two downstream mechanisms by which such degradation occurs have been elucidated: the simultaneous inhibition of proteoglycan and collagen synthesis and the catabolism of the extracellular matrix. It is thought that inflammation in the adjacent synovial tissue and fluids evokes changes in the metabolic activity of chondrocytes (Goldring et al., 2003) (Fig. 1.12).

Multiple studies have identified the capacity of IL-17 to disrupt such anabolism (Lubberts et al., 2000; Cai et al., 2001; Pacquelet et al., 2002), although the mechanisms by which this occurs remain unclear. The emerging picture suggests that IL-17-driven suppression of matrix synthesis involves a unique pathway from the one initiated by IL-1β (Cai et al., 2001; Pacquelet et al., 2002) and may involve the upregulation of leukemia inhibitory factor (Chabaud et al., 1998). IL-17 also appears to play an active role in the induction of cartilage matrix breakdown through the dysregulation of chondrocyte metabolism (Chabaud et al., 2001). Shalom-Barak et al. (1998) described the prolonged activation of NF-κB and all three subgroups of MAP kinases—ERK, JNK, and p38—in chondrocytes following exposure to IL-17.
These pathways regulate the expression of genes associated with joint inflammation and damage, such as matrix metalloproteinases (MMP), which degrade extracellular matrix and are implicated in RA pathogenesis (Burrage et al., 2006). In particular, IL-17 has been reported to induce the expression of MMP-2, MMP-3, MMP-9, and MMP-13 (Cai et al., 2001; Sylvester et al., 2004). In addition, IL-17 alone or in concert with IL-1\(\beta\) can induce or synergize with other degradative enzymes and molecules, including NO, PGE\(_2\), and oncostatin M to further damage articular tissue (Chabaud et al., 2000; Pacquelet et al., 2002).

**Bone erosion**

The joint destruction that follows in the wake of synovial inflammation and cartilage degradation is primarily brought about by osteoclasts following stimulation by RANKL and macrophage-colony stimulating factor (M-CSF) (Väänänen et al., 2008). Th17 cells and IL-17 in particular have been increasingly implicated in the bone degradation that occurs in inflamed joints. IL-17 enhances osteoclast differentiation and its functional activity through the upregulation of RANKL and other osteoclastogenic factors (Sato et al., 2006; Nakashima and Takayanagi, 2008). Local over-expression of IL-17 in mice with CIA significantly elevates expression of RANKL and its receptor, thereby altering the synovial ratio of RANKL to its decoy ligand, osteoprotegerin (OPG) (Lubberts et al., 2003). Also, IL-17 up-regulates osteoblast-derived PGE\(_2\), a known stimulus of osteoclastogenesis, and in cultured RA synoviocytes, inhibition of IL-17 reduces osteoclast differentiation by 80% (Lubberts et al., 2004). It also seems likely that IL-17 synergizes with other cytokines, such as TNF\(\alpha\), IL-6, and IL-23, all of which have been implicated in enhanced osteoclastogenesis (Yago et al., 2007). Thus it appears that IL-17 results in bone erosion by elevating the expression (directly and indirectly) of
RANKL on osteoclast-supporting cells to facilitate local formation of osteoclasts (Fig. 1.12).

1.24 Current and emerging therapies

Cytokines involved in the Th17 network, including IL-6, IL-1β and TNFα, have been targeted in therapies for RA, although to date no clinical trials have tested the efficacy of anti-IL-17 treatment directly. The synergy between IL-17 and TNFα may partially explain the efficacy of TNFα inhibitors in attenuating the symptoms of RA (Feldmann et al., 2001) (Table 1.3). Although two separate studies reported that infliximab does not lower the gene expression or serum levels of IL-17, the administration of TNFα inhibitors has been demonstrated to reduce serum levels of IL-6 and IL-15, both of which induce IL-17 (Kageyama et al., 2007). Furthermore, TNFα blockade in CIA increases the absolute number of pathogenic Th1 and Th17 cells but inhibits their accumulation in the joint, thus abrogating inflammation and attenuating disease (Notley et al., 2008).

Direct approaches to blocking IL-17 action include neutralization of IL-17 or the IL-17 receptor. Although animal data and in vitro studies suggest that this would be effective in RA, the clinical results available are only preliminary and are clouded by high response rates in placebo groups. At least 2 isoforms of IL-17 that are proinflammatory, IL-17A and IL-17F, appear to play a role in RA. It is possible that effective blockade of IL-17 will need to neutralize all of these molecules. Although worrisome toxicities have not yet been reported, the role of IL-17 in host defences against gram-negative bacteria and fungi is a reason for caution. The IL-17 receptor is also complex, employs several subunits, and may exist in more than one type of molecular assembly; the
optimal subunit to target in therapeutic blockade by an antibody is currently uncertain.

Figure 1.13 Schematic representation of the involvement of Th17 cells in RA. Infiltrating Th17 cells produce IL-17, which synergizes with other pro-inflammatory cytokines present in the synovial fluid, leading to (a) the activation of synovial fibroblasts and dendritic cells, leading to a local milieu conducive to Th17 development and further upregulating inflammation, (b) phenotypical modification of chondrocytes, resulting in the production of aggrecanases and MMPs that participate in cartilage destruction and (c) the upregulation of RANKL on osteoclast-supporting cells and synovial fibroblasts, which promotes bone erosion (adapted from Peck and Mellins, 2009).
Indirect approaches to lowering IL-17 levels involve inhibition of the differentiation or activation of Th17 cells. In view of the role of cytokines at several steps in these processes, a variety of targets are available, such as IL-6, IL-23, IL-21, IL-1 and others. Other possible approaches could be the development of inhibitors of key transcription factors in Th17 cells (especially Rorc of Rorγt), or inhibitors of signal transduction through either the IL-17 receptor itself or through receptors for cytokines that are important in the development of Th17 cells. Nutritional approaches may also be relevant. For example, suppressive effects of vitamin D on human Th17 differentiation have been documented (Colin et al., 2010). A vegetable-derived isothiocyanate termed sulforaphane inhibits IL-17 production by RA T cells and suppresses CIA (Kong et al., 2010).

**Table 1.3 Approaches in treatment of RA.**

<table>
<thead>
<tr>
<th>Target</th>
<th>Agent</th>
<th>Mechanism: Known or Postulated</th>
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<tbody>
<tr>
<td>IL-17 (A, F, or A/F)</td>
<td>Anti–IL-17</td>
<td>Neutralization of IL-17</td>
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<tr>
<td>IL-17 receptor</td>
<td>Anti–IL-17R</td>
<td>Receptor blockade</td>
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<tr>
<td>IL-6 receptor</td>
<td>Tocilizumab</td>
<td>Inhibition of Th17 differentiation</td>
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<td>IL-23</td>
<td>Ustekinumab</td>
<td>Inhibition of Th17 differentiation</td>
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<tr>
<td>IL-1</td>
<td>Anakinra</td>
<td>Inhibition of Th17 differentiation</td>
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<tr>
<td></td>
<td>Anti–IL-1</td>
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<tr>
<td>CD28 ligands</td>
<td>Abatacept</td>
<td>Blockade of costimulation required for Th17 cell activation</td>
</tr>
<tr>
<td>B lymphocytes</td>
<td>Rituximab</td>
<td>Depletion of B cells that can assist in Th17 cell differentiation by presenting antigen, engaging costimulatory receptors and secreting IL-6</td>
</tr>
<tr>
<td>Th17 cells</td>
<td>Vitamin D</td>
<td>Inhibition of Th17 cell differentiation</td>
</tr>
<tr>
<td>Th17 cells</td>
<td>Sulforaphane</td>
<td>Inhibition of IL-17 secretion by Th17 cells</td>
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Looking ahead, further elucidation of the Th17 network may result in novel therapies specifically designed to redress the imbalance between Th17 cells and Treg cells that is characteristic of diseased synovium (Nistala and Wedderburn, 2009). Mouse models suggest that the vitamin A metabolite all-trans retinoic acid may be one such factor (Elias et al., 2008; Xiao et al., 2008), and treatment of murine experimental autoimmune encephalomyelitis with retinoic acid suppresses disease by lowering the ratio of Th17 to Treg cells in draining lymph nodes (Xiao et al., 2008).

In view of the complexity of the Th17 axis and the many areas of uncertainty, careful testing of a variety of approaches is logical. It is likely that clinical trials of various anti–IL-17 strategies have the potential to fortify basic understanding of the regulation of IL-17 production and action in humans, as well as the roles of IL-17 and Th17 cells in RA and other immune-mediated diseases.