“Discovery consists of looking at the same thing as everyone else, and thinking something different”
Studies of bone and the immune system have converged in recent years under the banner of “osteoimmunology”. Osteoimmunology is an interdisciplinary research field combining the exciting fields of osteology and immunology. An observation that contributed enormously to the emergence of osteoimmunology was the accelerated bone loss caused by inflammatory diseases such as RA. Also, various factors produced during immune responses are capable of profoundly affecting regulation of bone. Mechanisms have evolved to prevent excessive interference by the immune system with bone homeostasis, yet pathologic bone loss is a common phenomena associated with autoimmunity. There are also developmental links, or parallels, between the bone and immune system. Cells that regulate bone turnover share a common precursor with inflammatory immune cells and may restrict themselves anatomically, in part by utilizing a signaling network analogous to lymphocyte costimulation. Efforts are currently under way to further characterize how these two organ systems overlap and to develop therapeutic strategies that benefit from this understanding.

RA is a chronic systemic disorder characterized by autoimmunity, infiltration of joint synovium by activated inflammatory cells, synovial hyperplasia, neo-angiogenesis, and progressive destruction of cartilage and bone. This disease affects 1–2% of the population worldwide, most commonly middle-aged women. In the chronic phase of the disease, a non-remitting activation of cells and expression of soluble mediators of especially the innate immune system dominates the inflammatory process. The resulting synovial inflammation is characterized by non-specific infiltration of both lymphocytes and innate immune cells, such as synoviocytes, macrophages and neutrophils.
Khapli et al, (2003) found that macrophages formed in the presence of IL-3 are resistant to RANKL action due to irreversible down-regulation of RANK expression. Yogesha et al, (2005) have shown that IL-3 inhibits TNF-α-induced osteoclast formation by down-regulation of TNFR1 and TNFR2. This suggests that IL-3 inhibits osteoclast differentiation by targeting the members of TNF receptor family. They also showed that IL-3 inhibits synergistic action of RANKL and other proinflammatory cytokines suggesting the potent inhibitory action of IL-3 on osteoclast differentiation. Interestingly, IL-3 prevented the development of arthritis in mice induced by a mixture of anti-type II collagen mAbs and LPS. IL-3 also prevented cartilage and bone destruction in the joint (Yogesha et al., 2009). It was also observed that IL-3 and GM-CSF had inhibitory effect on TNF-α-induced osteoclastogenesis and bone resorption in the presence of other pro-inflammatory cytokines such as IL-1α, TGF-β1, TGF-β3, IL-6 and PGE2. These results indicated the potent inhibitory activity of IL-3 in prevention of bone loss in mice. However, the mechanism(s) of anti-inflammatory role of IL-3 is not known.

Data obtained over the last years 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. These cells are critical in preventing autoimmune disease in animal models (Sakaguchi et al., 2005). Although Treg cells can be seen at the site of inflammation, their function is defective under various inflammatory conditions such as RA, contributing to chronicity (Chabaud et al., 1998). In this thesis, the role and mechanism(s) of IL-3 action on Treg and Th17 cell differentiation using both in vitro and in vivo systems has been investigated.
Studies over the past several years have identified Foxp3 as the key player in the biology of Treg cells. Stable expression of high amounts of Foxp3 is required 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 (Gavin et al., 2007; Lin et al., 2007). There exists a reciprocal relationship between Treg cells and Th17 cells, and IL-6 has a pivotal role in dictating the balance between these two cell populations (Bettelli et al., 2006). It has now been found that Rorγt and Rorα, the transcription factors for Th17 cells, and Foxp3, the transcription factor for Treg cells, can physically bind to each other and antagonize each other’s functions (Zhou et al., 2008). In line with this concept, conditional deletion of Foxp3 protein in ‘Treg cells’ in vivo resulted in an increase in Rorγt, IL-17 and IL-21 expression (Williams and Rudensky, 2007), further corroborating the reciprocal relationship between Th17 cells and Treg cells.

IL-3 exerts its biological activities by binding to specific high affinity receptors on cell surface. In this thesis it has been reported for the first time that both Treg and Th17 cells express IL3Ra. Also, IL-3 dose-dependently increases the proliferation of Treg cells by enhancing the expression of Foxp3 in both natural and induced Treg cells, and reciprocally regulates the development of IL-17+ Th17 cells by inhibiting the differentiation of Rorγt+ T cells.

Acquisition of suppressor activity by iTreg cells activated with TGF-β in vitro requires upregulation of CTLA-4 (Zheng et al., 2006). One member of the TNFRSF, the GITR (TNFRSF18), has been shown to play an important role in regulation of T-cell suppressor activity. Both a polyclonal antiserum and mAb against GITR appeared to be able to reverse suppression mediated by freshly isolated Treg cells (Shimizu et al., 2002). IL-3 treated Treg cells express both
CTLA-4 and GITR and thus are capable of modulating various immune responses mediated through CTLA-4 and GITR.

The most striking property of the Treg cells is their ability to suppress proliferative responses of both CD4+ and CD8+CD25- T cells (Thornton et al., 1998). The CD25+ T cells must be activated via their TCR to suppress. No suppression was seen when CD25+ Treg cells were separated by semipermeable membrane from the CD25- T cells. This demonstrates that cell contact between CD25+Treg cells and CD25- T cells is required. Neutralization of the suppressor cytokines IL-4, IL-10, and TGF-β individually or in combination also had no effect on the CD25 mediated suppression. Treg cells have the capacity to inhibit the activation of other T cells, especially autoreactive T cells (Paul, 2008). IL-3 expanded induced and natural Treg cells inhibit the proliferation of responder T cells in a cell density-dependent manner and are bonafide Treg cells endowed with the classical ability of Treg cells to suppress the proliferation of effector T 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 and IL-17F have similar functions. In addition to IL-17A and IL-17F, Th17 cells produce other effector cytokines, namely IL-21, IL-22 and TNFα (Liang et al., 2006; Korn et al., 2007; Lubberts et al., 2008). They induce the production of proinflammatory cytokines, chemokines and metalloproteinases from various tissues and cell types. As a result, they recruit neutrophils to tissues. Interestingly, IL-3 directly modulated in vitro Th17 polarization by inhibiting its development, accompanied with suppression of IL-17, TNF-α, and IL-21 production.
Studies using IL-2-deficient and IL-2Rα (CD25)-deficient mice to probe the role of IL-2 in Treg cell biology concluded that IL-2 was dispensable for the generation of nTreg cells in the thymus (D’Cruz and Klein, 2005; Fontenot et al., 2005). Also, 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). Thus, IL-2 appears to be essential for iTreg cell generation and/or homeostasis and is required in vitro for TGF-β induction of Foxp3 transcription and suppressor activity (Zheng, S. et al., 2007). IL-3 in a dose-dependent manner increased the percentage of IL-2 producing CD4+CD25lowFoxp3- cells and induces the development of Treg cells indirectly through secretion of IL-2 by these non Treg cells.

STAT5, activated downstream of IL-2R and other common γ-chain cytokine receptors, represents a likely candidate transcription factor for direct regulation of Foxp3 expression (Burchill et al., 2008), and promotes the generation of inducible Treg cells (Laurence et al., 2007). Here in this thesis I report that IL-3 independently increased the phosphorylation of STAT5 in a dose-dependent manner and also synergies with IL-2 for enhanced activation of STAT5 in T cells.

In RA and CIA Treg cells are defective and there is increased osteoclastogenesis (Ehrenstein et al., 2004; Kelchtermans et al., 2005 and 2009). Suppression of Treg cell function exacerbates arthritis, whereas increasing the number of Treg cells ameliorates inflammation and bone destruction (Frey et al., 2005), suggesting that modulation of Treg cells is potentially beneficial in the prevention of bone destruction. Some reports suggest that the bone-protective effects of Treg cells are mediated by direct inhibitory effects on osteoclastogenesis thereby preventing development of CIA
Here I report that IL-3 expanded Treg cells are little better than iTreg cells generated with TGF-β and IL-2 in inhibiting osteoclastogenesis under in vitro conditions. In further investigation on the in vivo role of IL-3 on bone loss by μ-CT, it was found that IL-3 treatment inhibits bone loss and cartilage damage in CIA mice and thus maintains normal bone structure.

In further investigation the in vivo mechanism of inhibitory action of IL-3 in CIA, it was found that IL-3 reduces severity of arthritis by promoting in vivo expansion of Foxp3+ Treg cells together with inhibition of Rorγt+ Th17 cells in CIA mice. Also, IL-3 significantly increases the levels of IL-10, IL-2, IL-5 and IFNγ and decreases the levels of IL-6, IL-17A, TNF-α and IL-1 in CIA mice. These results suggest that IL-3 has a potential to inhibit production of pro-inflammatory cytokines and induce anti-inflammatory cytokines in vivo.

Treg cells play a critical role in controlling autoimmune disease and several strategies are now being explored to target these cells for therapeutic purposes. For patients with RA, Treg cells provide a valuable new treatment option, since current therapies, such as anti-TNF-α therapy, cause a rather general immune suppression and do not induce sustained remission. As a result, side effects occur and life-long treatment is required. To enhance Treg cell function, the cells can be expanded and induced in vitro followed by adoptive transfer. However, these protocols have severe drawbacks, especially the risks associated with conversion of Treg cells into effector cells, and the costs and complexities associated with cellular therapy. Alternatively, Treg cells can be induced in vivo by immunomodulatory compounds and some of these agents have already been tested in patients.
Thus in this thesis I reveal for the first time that IL-3 may be useful to expand Treg cells and inhibit Th17 cells under both \emph{in vitro} and \emph{in vivo} conditions, and it may act as an important therapeutic agent to treat RA and various other autoimmune diseases (Fig. 7.1).

\emph{Figure 7.1 Modulation of Treg-Th17 balance by IL-3.} Naïve T cell differentiates either into Treg or Th17 cell depending upon the cytokine milieu encountered. IL-3 modulated this balance by promoting induction of both nTreg and iTreg cells and inhibiting the development of Th17 cell.