Chapter 6

In vivo role of IL-3 in CIA mice
6.1 Introduction

Animal models of autoimmune arthritis have proven to be valuable research tools for the study of pathogenic mechanisms of this disease as well as for testing new therapies. Several mouse models of arthritis have been established, including those that require immunization with antigen—proteoglycan-induced arthritis (PGIA) (Finnegan et al., 1999), streptococcal cell-wall arthritis (Koga et al., 1985), CIA (Courtenay et al., 1980) and antigen induced arthritis (Brackertz et al., 1977); those induced by chemical agents—oil-induced arthritis (Hopkins et al., 1984); and spontaneous models—TNF-α transgenic mouse (Butler et al., 1997) and K/BxN T-cell receptor transgenic mouse (Kouskoff et al., 1996). While each of these models has advantages and disadvantages, CIA has been the most widely studied model of human RA. The CIA model has been used extensively to identify potential pathogenic mechanisms of autoimmunity, including the role of individual cell types in disease onset and progression, as well as to design and test new therapeutics.

CIA is elicited in genetically susceptible strains of mice by immunization with CII emulsified in CFA. The ensuing pathogenesis shares several pathological features with RA, including synovial hyperplasia, mononuclear cell infiltration, cartilage degradation, and, like RA, susceptibility is linked to the expression of specific MHC class II genes. The original “gold standard” of the CIA model was the DBA/1 (H-2q) mouse strain; however, in recent years, several HLA-DR mouse models have been established in which transgenic expression of the HLADR1 or DR4 class II genes associated with susceptibility to RA confers susceptibility to CIA in the recipient mouse strain (Rosloniec et al., 1998).
In recent years, the CIA model has been instrumental in the testing and development of the new biologically based therapeutics, which can be used to address questions of pathogenic mechanisms and to screen candidate therapeutic agents, such as those that target TNFα, a cytokine produced by macrophages and T cells that is a dominant inflammatory mediator in the pathogenesis of RA. The development of these biologically based therapies has revolutionized the treatment of RA.

In previous chapters, it has been shown that IL-3 in a dose-dependent manner increases the percentage of Foxp3+ T reg cells and inhibits the differentiation of Rorγt+ Th17 cells in vitro. Therefore, I next examined the in vivo role of IL-3 in modulating the development of Treg-Th17 cells in CIA mice. Thus in this chapter the in vivo model for CIA in DBA/1J mice is standardized. And the in vivo effect of IL-3 on the development of Treg and Th17 cells was determined.
6.2 Results

6.2.1 Development and characterization of CIA model in DBA/1J mice

CIA is an experimental autoimmune disease that can be elicited in susceptible strains of rodents and nonhuman primates by immunization with CII, the major constituent of protein of articular cartilage. Following immunization, these animals develop an autoimmune-mediated polyarthritis that shares several clinical, histological and immunological features with human RA. Two strains of mice are most commonly used for CIA model in mice, since they are highly susceptible to CIA. These are DBA/1J (H-2q) mice which respond to chick, bovine, porcine and human type II collagen, and B10.RIII (H-2r) mice which respond to bovine and porcine type II collagen only.

In this study 8-10 weeks old DBA/1J mice were used for induction of CIA with protocol discussed under materials and methods. Assessment of arthritis was done by visual scoring. The most widely used system is a simple visual scoring of 0 to 3. Clinical arthritis was assessed using the following system: grade 0, no swelling; grade 1, slight swelling and erythema; 2, pronounced edema; 3, joint rigidity. Each limb was graded, giving a maximum possible score of 12 (4 X 3) per animal (Fig. 6.1A and B). To reduce the subjectivity and prevent bias, two separate investigators performed the examination, one of whom was unaware of the identity of the treatment groups.

To characterize the CIA model in DBA/1J mice both histological examination and cytokine analysis were carried out. Cytokine analysis of serum samples obtained from normal and CIA DBA/1J mice at various time points viz. at day 30, 45 and 60 was done by CBA. There was significant increase in various pro-inflammatory cytokines mainly IL-6 (> 100 pg/ml), TNF-α (> 35 pg/ml) and
Figure 6.1 Development and characterization of CIA in DBA1/J mice. CIA was induced in 6-8 week DBA/1J mice by collagen type II emulsion as described in materials and methods. Control mice were either uninjected or injected s.c. with PBS (○) on day 0. In other group mice were injected s.c. with collagen (●) prepared in CFA (200 μg) on day 0 followed by s.c. injection of booster dose prepared in IFA (200 μg) on day 21. (A)The development of arthritis was monitored two to three times per week for arthritis incidence by two independent blind
observers and the qualitative visual scores assessed were as follows: figures (a-d) represent photographs of paw swelling in DBA/1J mice showing front views of hind paws, with severity scores from 0-3. (B) Graphical representation of clinical arthritic score. (C) Serum was collected from mice (n = 3 per group) at day 30, 45, and 60 from both control and arthritic mice and analyzed by CBA-mouse inflammation kit for various proinflammatory cytokines. (D) Mice were sacrificed on day 40, and knee joints were dissected, fixed, decalcified, and processed for histopathology. Five-micrometer sections were stained for H&E. Knee joint of CIA mice with arrows indicating increased influx of inflammatory cells in synovium with pannus formation. Original magnification, X10. Similar results were obtained in two independent experiments.

MCP-1 (> 75 pg/ml) with respect to their levels in normal mice (IL-6 < 5 pg/ml, TNF-α < 10 pg/ml and MCP-1 < 30 pg/ml). On the other hand the concentration of various anti-inflammatory cytokines was either negligible or absent in both control and arthritic mice (IFN-γ levels were < 5 pg/ml in both). Levels of IL-12 and IL-10 were undetected in both control and arthritic mice (Fig. 6.1C).

Histologically, mice CIA model is characterized by intense synovitis that corresponds precisely with the clinical onset of arthritis. In knee joint section of normal mice no infiltration of inflammatory cells in synovium was observed. From day 35-40 when the arthritis was severe a marked increase in infiltration of lymphocytes and mononuclear cells in the synovial tissue was observed. There was formation of pannus-like tissue in the arthritis joint. Also subsequent erosion of cartilage and subchondral bone was noticed (Fig. 6.1D).

The severity of the disease and cytokine profile correlated well with the kinetics of arthritis development, with maximum concentration of proinflammatory cytokines at 30 days and decreasing thereafter at both 45 and 60 days (Fig. 6.1C). Thus, the method of induction and characterization of CIA in DBA/1J mice was standardized and was used in all further studies.
6.2.2 IL-3 reduces arthritic score and inflammation, and prevents damage to bone and cartilage tissues in CIA mice

To investigate the in vivo role of IL-3 in regulation of Treg and Th17 cells, I used well-established CIA mouse model of human RA. DBA/1J mice were primed on day 0 with CII and then boosted on day 21. In treatment group, mice were injected for 15 days with rmIL-3 (1.5 µg/d i.p. in two divided doses at 12 hours interval) from day 21 at the time of booster. Arthritis was manifested by redness and swelling of the paws, including digits.

In PBS injected control mice, no signs of inflammation were seen, whereas CIA mice developed severe inflammation as evidenced by marked swelling and erythema of the hind paws. In contrast, mice treated with IL-3 displayed a significant reduction in paw thickness (Fig. 6.2A) and mean arthritic score (Fig. 6.2B). Photographs of hind paws in Fig. 6.2C show significant reduction of inflammation in mice treated with IL-3. In presence of IL-3, thickness of the inflammatory soft tissues was also decreased when examined by radiological soft X-rays (Fig. 6.2D). RA is a chronic inflammatory disorder that ultimately leads to the destruction of joint architecture. By radiological examination it was observed that IL-3 treatment prevented damage to articular cartilage (Fig. 6.2D, enlarged regions). CIA mice had markedly enlarged spleen and inguinal lymph nodes which were normal in size in IL-3 treated mice (Fig. 6.2E). By histological examinations of knee joints on day 36, no infiltration of inflammatory cells and no damage to the articular cartilage of control mice was observed. The knee joints in CIA mice showed massive infiltration of polymorphonuclear, and other inflammatory cells, and there was multiple superficial cartilage erosion. In contrast, mice treated with IL-3 showed infiltration of few inflammatory cells, and erosion of articular cartilage was not observed (Fig. 6.2F).
Figure 6.2 IL-3 suppresses inflammation and protects joint destruction in CIA mice. CIA was induced in 8-10 week old DBA/1J mice by CII emulsion as described in Materials and Methods. Control mice were injected s.c. with PBS on day 0 and day 21. In another group mice were injected s.c. with CII prepared in CFA (200 μg/mice) on day 0 followed by s.c.
injection on day 21 with booster dose of CII prepared in IFA (200 μg/mice). In third group mice were treated with IL-3 i.p. (1.5 μg/mice per day in two divided doses) for 15 days starting from day 21 along with booster dose of CII. Two independent blind observers assessed the severity of arthritis every fifth day after booster injection for hind paw thickness (A) and clinical arthritic score (B). Data is represented as mean ± SEM, n = 5 mice per group. (C) Photographs of paw swelling in DBA/1J mice showing front and side views of hind paws, and X-rays of soft tissues showing inflammation of surrounding tissue. (D) X-ray radiographs of knee joints showing bone and cartilage structure. (E) Appearance of spleen and lymph nodes from control, CIA and IL-3 treated mice. (F) Mice were sacrificed on day 36, and knee joints were dissected, fixed, decalcified, and processed for histopathology. Five-micrometer sections were stained for H&E. Original magnification, X10. Similar results were obtained in two independent experiments.

6.2.3 IL-3 maintains normal bone structure in mice

To determine the effect of IL-3 on bone morphology, I next assessed the trabecular structure of tibiae by microcomputed tomography (μ-CT). In CIA mice there was significant loss of trabecular and cortical bones, which was prevented in IL-3 treated mice (Fig. 6.3A). Also, significant increase in trabecular thickness (Tb. Th.), trabecular number (Tb. N.), bone volume fraction [bone volume (BV)/tissue volume (TV)] connectivity density (Conn. Dn.) and cortical thickness (Ct. Th.) was observed in IL-3 treated mice with respect to CIA mice (Fig. 6.3B). Bone architecture denoted by the structure model index (SMI) was not altered. These results suggest that IL-3 maintains normal bone structure in mice. Thus, IL-3 treatment reduces arthritic score and inflammation, and prevents damage to bone and cartilage tissues in knee joints in CIA mice.
Figure 6.3 IL-3 prevents bone loss in CIA mice. Mice were sacrificed on day 36 and tibias were analyzed for bone parameters. (A) $\mu$-CT reconstructions showing trabecular and cortical bone structures of representative tibias in control, CIA and IL-3 treated CIA mice. (B) Morphological measurements of trabecular and cortical bone indices such as BV/TV, Tb. N, Tb. Th, SMI, Conn. Dn. and Ct. Th. were computed from $\mu$-CT reconstructions of control, CIA and IL-3 treated CIA mice tibias. Similar results were obtained in two independent experiments.
6.2.4 IL-3 reduces severity of arthritis by promoting *in vivo* expansion of Foxp3⁺ Treg cells in CIA mice

To investigate the *in vivo* mechanism of IL-3 action in prevention of CIA the effect of IL-3 on Foxp3⁺ Treg cell development was analyzed. Mice were sacrificed on day 36 and total lymphocyte population derived from lymph nodes, spleen and thymus tissues were analysed for percentage of CD4⁺Foxp3⁺ Treg cells by FACS. As compared to control mice the percentage of Foxp3⁺ Treg cells in CIA mice was drastically decreased in thymus, lymph nodes and spleen. There was more than 50% decrease in percentage of Foxp3⁺ Treg cells in lymph nodes and around 70% decrease in spleen and thymus. Interestingly, in IL-3 treated mice the percentage of CD4⁺Foxp3⁺ Treg cells in all the three tissues was restored to near normal levels (Fig. 6.4A). Fig. 6.4B represents the average percentage of Foxp3⁺ Treg cells in lymph nodes, spleen and thymus of mice treated with or without IL-3. These results suggest that IL-3 also has a potential to augment the percentage of Foxp3⁺Treg cells *in vivo*.

6.2.5 IL-3 inhibits the differentiation of CD4⁺ Rorγt⁺ Th17 cells in CIA mice

The development of Treg and Th17 cells is reciprocally regulated in normal immune system to prevent unwanted inflammatory reaction and to maintain normal immune homeostasis (Littman and Rudensky, 2010). Since, IL-3 inhibits the generation of Th17 cells under *in vitro* conditions. Thus here the *in vivo* mechanism of IL-3 action on Th17 cell development was investigated. Mice were sacrificed on day 36 and total lymphocyte population derived from lymph nodes, spleen and thymus tissues were analyzed for expression of CD4⁺ Rorγt⁺ Th17 cells by FACS. As compared to control mice the number of Rorγt⁺ Th17 cells in CIA mice was drastically increased in lymph nodes and spleen. There was more than 30% increase in percentage of Rorγt⁺ Th17 cells in
Figure 6.4 IL-3 prevents CIA by enhancing the percentage of Foxp3^+ Treg cells in vivo. (A) Mice were sacrificed on day 36 and total cells derived from lymph nodes, spleen and thymus tissues were analyzed for percentage of CD4^+Foxp3^+ Treg cells by FACS. Isotype controls of Foxp3 for each group are shown. (B) Average percentage of CD4^+Foxp3^+ cells from lymph nodes, spleen and thymus tissues of 3 mice. Mean ± SEM. *p < 0.05 in all the groups.
Figure 6.5 IL-3 inhibits the differentiation of CD4+ Rorγt+ Th17 cells in CIA mice. (A) Mice were sacrificed on day 36 and total cells derived from lymph nodes and spleen tissues were analyzed for percentage of CD4+Rorγt+Th17 cells by FACS. Isotype control of Rorγt is shown. (B) Average percentage of CD4+Rorγt+Th17 cells from lymph nodes and spleen tissues of 3 mice. Mean ± SEM. p < 0.05.
lymph nodes and around 40% increase in spleen. Interestingly, in IL-3 treated mice the percentage of CD4⁺ Rorγt⁺ Th17 cells in both the tissues was significantly decreased (Fig. 6.5A). Fig. 6.5B represents the average percentage of Rorγt⁺ Th17 cells in lymph nodes and spleen of mice treated with or without IL-3. These results suggest that IL-3 inhibits the differentiation of Rorγt⁺ Th17 cells in vivo and maintains normal immune homeostasis by regulating the Treg-Th17 cell axis.

6.2.6 IL-3 decreases production of pro-inflammatory cytokines and increases anti-inflammatory cytokines in CIA mice

The pathogenic events that lead to the development of human RA are not fully understood, although the pivotal role of proinflammatory cytokines such as TNF-α, IL-1β, IL-6 etc. in the induction and maintenance of RA is well documented (Feldmann, 2002). These cytokines promotes the deleterious imbalance in bone metabolism and contribute to enhanced bone destruction. Treg cells in active RA are defective in controlling production of proinflammatory cytokines (Ehrenstein et al., 2004). Treg cells suppress immune response through numerous mechanisms including the production of anti-inflammatory cytokines, direct cell to cell contact, and by modulating the activation state and function of APCs (Shevach, 2009). Therefore, next the effect of IL-3 on the levels of various pro-inflammatory and anti-inflammatory cytokines in the serum of control, CIA and IL-3 treated mice was determined.

In CIA mice there was significant increase in production of IL-6, IL-17A, TNF-α, IL-1 and IFNγ (p < 0.05), and decrease in secretion of IL-5 and IL-10. IL-2 production was also decreased in CIA mice. Surprisingly, it was observed that 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 (Fig. 6.6). These results suggest that IL-3 has a potential to inhibit production of pro-inflammatory cytokines and induce anti-inflammatory cytokines.

**Figure 6.6 Effects of IL-3 on production of various cytokines in CIA mice.** Serum samples of mice were analyzed on day 36 for secretion of various pro-inflammatory and anti-inflammatory cytokines by CBA. Data is represented as mean ± SEM. n = 5. Similar results were obtained in two independent experiments.
Deficiency of Treg cells leads to breakdown of tolerance in various human autoimmune diseases including type-1 diabetes and RA (Nadkarni et al., 2007). Also, depletion of Treg cells exacerbates various experimental autoimmune diseases including CIA (Morgan et al., 2003). Foxp3+ Treg cells constitute 5 to 15% of peripheral CD4+ T cells (Brusko and Bluestone, 2009; Shevach, 2008) and this proportion appears to be reduced in mice genetically prone to autoimmune diseases such as diabetes and CIA (Morgan et al., 2003; Wu et al., 2002). In the present study it was found that therapeutic treatment of CIA mice with IL-3 led to a significant rise in the percentage of both natural and peripheral Foxp3+ Treg cells in thymus, spleen and lymph nodes with a simultaneous decrease in the percentage of Rorγt+ Th17 cells in spleen and lymph nodes.

Increased percentage of Treg cells improved clinical signs of arthritis and suppressed local and systemic bone destruction. Also, it was observed that IL-3 treated Treg cells are slightly better in suppressing osteoclast formation in vitro when compared with Treg cells generated with TGF-β and IL-2. Thus, the increased percentage of Foxp3+ Treg cells in CIA mice by IL-3 may lead to the inhibition of osteoclastogenesis and bone loss, thereby attenuating CIA.

Similar to human RA, various proinflammatory cytokines including TNF-α and IL-1β are involved in CIA, a rodent model of human RA. IL-3 significantly suppresses the production of most pro-inflammatory cytokines (viz. IL-6, IL-17A, TNF-α, and IL-1) and induces the production of various anti-inflammatory cytokines (viz. IL-10, IL-2, IL-5 and IFNγ) in vivo. Since inflammation and bone loss are two frequently occurring and tightly linked...
disorders, next the effect of IL-3 on bone loss was determined with the help of µ-CT analysis. Interestingly µ-CT measurements of tibias revealed that IL-3 prevent bone loss in CIA mice.

Cellular therapy based on ex vivo development of Treg cells and their subsequent transfer to patients is currently the focus of intense research in the treatment of various autoimmune diseases (Sakaguchi et al., 2010). Also, transfer of Treg cells have already been shown to prevent a wide range of experimental autoimmune diseases including diabetes, experimental encephalomyelitis, and colitis (Kohm et al., 2002; Wu et al., 2002; Read et al., 2000). Several, preclinical studies have shown that either freshly isolated or ex-vivo expanded Treg cells can prevent both local and systemic organ and tissue destruction (Riley et al., 2009). Two broad therapeutic approaches have been considered, first, to expand Treg cells *in vitro* with the intension of infusing these cells into patients, and second, to manipulate the immune system *in vivo* resulting in an increase in Treg cells.

Importantly, IL-3 appears to increase percentage of Treg cells and simultaneously inhibits the development of Th17 cells under both *in vitro* and *in vivo* conditions. Moreover, inhibition of most proinflammatory cytokines by IL-3 may results in increase in percentage of Treg cells with a simultaneous decrease in percentage of Th17 cells.

Thus, my study clearly demonstrates that IL-3 modulates the Treg-Th17 developmental axis *in vivo* by skewing the balance towards Treg cells. Furthermore, my data suggest that IL-3 may represent a novel therapeutic strategy to reduce immune responses, inflammation and bone loss in autoimmune diseases.