Chapter 2

Overview on role of IL-3 in osteoimmunology
2.1 Osteoimmunology: interplay between the immune system and bone metabolism

The close relationship between the immune and skeletal systems has long been appreciated, since the pioneering works in the early 1970s (Horton et al., 1972; Mundy et al., 1974; Horowitz et al., 1984; Dewhirst et al., 1985). Soluble factors secreted from antigen-stimulated immune cells were known as osteoclast-activating factors, one of which was revealed to be IL-1 (Dewhirst et al., 1985). Since then, accumulating evidence has indicated that the immune and skeletal systems share a number of regulatory molecules including cytokines, receptors, signalling molecules and transcription factors (Takayanagi et al., 2005; Walsh et al., 2006). Furthermore, immune cells are formed and haematopoietic stem cells (HSCs) are maintained in the bone marrow where they interact with bone cells. Therefore, the evidence that the physiology and pathology of one system might affect the other is compelling (Fig. 2.1) and the term osteoimmunology was coined to cover these overlapping scientific fields (Arron et al., 2000).

The most typical example of the interaction between the skeletal and immune systems is seen in the abnormal and/or prolonged activation of the immune system in autoimmune diseases, such as RA, that lead to bone destruction caused by bone-resorbing osteoclasts (Takayanagi et al., 2005; Sato et al., 2006). Osteoclast differentiation is regulated by M-CSF (Ross and Teitelbaum, 2005) and RANKL (also known as TNFSF11), a TNF-family cytokine (Asagiri and Takayanagi, 2007). A mutation in the M-CSF gene causes a defect in both macrophage and osteoclast formation, pointing to the notion that immune cells and bone cells are derived from the same origin (Yoshida et al., 1990).
RANKL is not only expressed by the bone forming osteoblasts that support osteoclastogenesis in bone tissue, but also by activated T cells, indicating that osteoclastic bone resorption is influenced by the immune system (Theill et al., 2002).

**Figure 2.1 The osteoimmune system.** The skeletal system is involved in the regulation of three main outputs that are related to calcium reserves, locomotion and haematopoiesis. To maintain homeostasis while responding to various inputs (such as nutrition, mechanical stress, ageing, and inflammation), the cells in the bone marrow are controlled by the immune systems in concert with other regulatory systems, such as the endocrine and neural systems. As depicted, the bone system and the immune system in the bone-marrow microenvironment are regulated as if they were integrated in the context of the osteoimmune system (adapted from Takayanagi, 2007).
M-CSF and RANKL are not the only factors linking the immune and skeletal systems. Targeted disruption of various immunomodulatory molecules has resulted in unexpected phenotypes in the skeletal system. Moreover, recent studies have indicated that osteoblasts and even osteoclasts are involved in the maintenance and regulation of HSCs, indicating the crucial role of skeletal cells in both the haematopoietic and immune systems (Kollet et al., 2006). Clinically, the effectiveness of TNF-specific antibody therapy against bone destruction in patients with RA also highlights the importance of the relationship between the immune and skeletal systems (Palladino et al., 2003). Therefore, osteoimmunology is becoming increasingly important for understanding the pathogenesis of, and developing new therapeutic strategies for, diseases affecting both systems.

2.2 Overview of the cells in the skeletal system

The bony skeleton, the essential component of the skeletal system, enables locomotive activity, the storage of calcium and the harbouring of HSCs from which immune cells are derived (Fig. 2.2). This multifunctional organ is characterized by calcified hard tissue composed of type I collagen and highly organized deposits of calcium phosphate (hydroxyapatite) (Seeman and Delmas, 2006). Although it seems to be metabolically inert, bone is restructured at such a high speed that approximately 10% of the total bone content is replaced per year in adult vertebrates. This process, called bone remodeling, is dependent on the dynamic balance of bone formation and resorption, which are mediated by osteoblasts and osteoclasts, respectively. A delicate regulation of this process is a prerequisite for normal bone homeostasis, and an imbalance is often linked to metabolic bone diseases in humans, such as osteoporosis and inflammatory bone loss (Harada et al., 2003).
Figure 2.2 Schematic diagram of the bone microenvironment as a loosely compartmentalized lymphoid organ: T* (memory T cells and circulating T cells), B* (B cells, differentiation of which occurs via interaction with stromal cells; memory B cells also interact with stromal cells; in addition, there are circulating mature B cells), stromal cells (bone marrow stromal cells are of mesenchymal origin, but not fully characterized), M* (monocyte and its derivatives), and osteoid [newly formed, but not yet calcified matrix, composed mostly of type I collagen (~90%) and noncollagenous proteins (~10%)], (adapted from Walsh, 2006).

Osteoblasts

Osteoblasts are cells of mesenchymal origin that secrete bone-matrix proteins and promote mineralization (Harada et al., 2003; Seeman and Delmas, 2006). The proliferation and differentiation of osteoblasts are under the control of a number of soluble factors and transcription factors such as RUNX2 (runt-
related transcription factor 2) and OSTERIX (also known as SP7) (Nakashima et al., 2002). Differentiated osteoblasts embedded in the bone matrix are called osteocytes, and might have a specific but as-yet unclear role in mechano-transduction (Seeman and Delmas, 2006).

**Osteoclasts**

Osteoclasts are cells of haematopoietic origin that decalcify and degrade the bone matrix by acid decalcification and proteolytic degradation, respectively (Boyle et al., 2003). They are large, multinucleated cells formed by the fusion of precursor cells of the monocyte-macrophage lineage. In *vitro* osteoclast differentiation is supported by mesenchymal cells (bone-marrow stromal cells or osteoblasts) through cell–cell contact (Suda et al., 1999), although there has been little *in vivo* information about osteoclastogenesis-supporting cells. Osteoclastogenic signals are mediated by RANKL and its co-stimulatory signals, in addition to M-CSF, (Theill et al., 2002; Takayanagi et al., 2005; Asagiri and Takayanagi, 2007). The congenital lack of osteoclasts leads to osteopetrosis, which is characterized by a high bone mass and a defect in bone-marrow formation. Naturally occurring mutant mice or genetically modified mice with osteopetrosis have provided insights into the molecular mechanism of osteoclast differentiation and function. M-CSF and the transcription factor PU.1 are crucial for the proliferation and survival of osteoclast precursor cells; transcription factors such as cFOS, microphthalmia-associated transcription factor (MITF) and NF-κB have been shown to be essential for osteoclast differentiation; and factors such as cSRC, VAV3, β3-integrin, chloride-channel family member ClC7, vacuolar ATPase and cathepsin K are crucial for osteoclast function (Teitelbaum and Ross, 2003).
2.3 Direct influence of the immune system on bone

Immune and skeletal systems have several regulatory factors in common, such as cytokines, transcription factors and receptors. Consequently, these two systems interact with each other either in physiological or in pathological conditions. Pathological activation of one system affects the other, such as in the case of RA where abnormal activation of the immune system affects bone remodeling leading to pathological bone erosions. During chronic inflammation, the balance between bone formation and resorption is skewed towards osteoclast-mediated bone resorption. Moreover, in inflamed joints, osteoclasts are located in the interface between the inflamed synovium and the bone and, as in physiological conditions; the major player in bone resorption is the RANKL/RANK/OPG system (Herman et al., 2008).

RANKL is produced by several cell types besides osteoblasts, including monocytes, neutrophils, dendritic cells, B and T lymphocytes. In this way, immune cells have the ability to induce osteoclast differentiation and, consequently, bone resorption. Also, these cells are known for producing a variety of pro-inflammatory cytokines that also contribute to bone damage by potentiating the effects of the RANK–RANKL signalling (Herman et al., 2008). The cytokines TNF-α and IL-1, IL-6, IL-7, IL-11, IL-15 and IL-17 potentiate bone loss either by increasing osteoclast generation and activation or by inducing RANKL expression by the osteoblasts. On the other hand, IL-4, IL-5, IL-10, IL-12, IL-13, IL-18 and IFN-α, IFN-β and IFN-γ are inhibitors of osteoclastogenesis by blocking RANKL signalling, either directly or indirectly. Interestingly, IL-1 is a stimulator of TNF receptor-associated factor 6 (TRAF6) expression on the osteoclast, thereby potentiating RANK–RANKL signalling cascade, whereas IFN-γ is known to downregulate TRAF6 by proteosomal degradation aborting osteoclast formation (Datta et al., 2008).
In addition, other factors contribute to the complex regulation of osteoclast differentiation. For instance granulocyte-macrophage colony-stimulating factor (GM-CSF) suppresses the transcription factors c-fos and Fra-1, which are key factors for osteoclast development, while TGF-β can have both an inhibitory or stimulatory action over osteoclastogenesis, as on one hand TGF-β was described to down modulate RANKL expression in osteoblasts and on the other hand this cytokine potentiates RANKL expression in T cells (Datta et al., 2008).

Although T cells express RANKL, they are classical anti-osteoclastogenic cells as they produce cytokines that inhibit bone resorption (especially IFN-γ, the most potent inhibitor). However, the Th cell subset involved in the production of IL-17 (Th17 cells) is considered to be the typical osteoclastogenic Th subset due to the fact that it expresses RANKL at higher levels than Th1 or Th2, does not produce high amounts of IFN-γ and produces pro-inflammatory cytokines (like TNF-α) that potentiate RANKL expression; in fact, Th1 and Th2 subpopulations suppress Th17 subset through the secretion of cytokines (Sato et al., 2006; Furuzawa-Carballeda et al., 2007). In addition, IL-17 induces the synthesis of matrix-degrading enzymes, such as MMPs, inducing bone and cartilage degradation (David, 2007). These effects are balanced by Treg cells which inhibit bone destruction through suppression of osteoclast formation by a cell contact-dependent manner that might be mediated by the expression of CTLA-4, which binds to B7-1 and B7-2 in the pre-osteoclasts (Kim et al., 2007). Treg cells also express cytokines, like IL-4, IL-10 and TGF-β, which not only have anti-inflammatory properties but also suppress osteoclastogenesis (Lan et al., 2005; Kelchtermans et al., 2008). Therefore, in pathological conditions, the effects of T cells on osteoclastogenesis should depend on the balance between positive and negative factors that they express.
Although RANKL and M-CSF are essential factors and key players in osteoclast differentiation, it was hypothesized that costimulatory molecules such as immunoglobulin-like receptors are the third essential factor required for osteoclastogenesis. Among these molecules are osteoclast-associated receptor (OSCAR) and paired immunoglobulin-like receptor (PIR)-A, that signal through FcRγ. Interestingly, OSCAR and PIRA trigger receptors expressed on myeloid cells (TREM)-2 and signal-regulatory protein (SIRP)-β1, which signal through DNAX-activation protein 12 (DAP12) (Datta et al., 2008), activating an intracellular calcium signalling cascade. These processes lead to the dephosphorylation of calcineurin, thereby activating the auto-amplification of nuclear factor of activated T cells cytoplasmic1 (NFATc1), which, consequently, promotes osteoclast differentiation. Therefore, FcRγ and DAP12 are adaptor molecules that associate with immunoglobulin-like receptors helping their expression and transducing signals through immunoreceptor tyrosine-based activation motif (ITAM) (Takayanagi et al., 2007). RANKL also interacts with ITAM by inducing its phosphorylation, thus increasing expression of immunoglobulin-like receptors and enhancing ITAM signal (Datta et al., 2008).

Further modulation of osteoclastogenesis is provided by TLR’s. TLR expression was detected on bone cells and direct signalling through TLR activates a signalling cascade, mediated by TRAF6, leading to the activation of transcription factors, such as NF-κB and AP-1 family factors and to the synthesis and release of pro-inflammatory cytokines (Walsh et al., 2006; Hurst et al., 2008). However, the outcome of TLR activation depends on the stage of differentiation of the osteoclast; in this way, in early precursor cells, TLR inhibit osteoclastogenesis, but in cells that have already started to develop into osteoclasts, TLR is a potent pro-osteoclastogenic factor. In mature osteoclasts, TLR signalling promotes cell survival. On the other hand, osteoblasts were
also found to express TLR, namely TLR-4, TLR-5 and TLR-9, and exposure of these cells to pathogen-associated molecular patterns (PAMP) induces the secretion of pro-inflammatory cytokines. Therefore, TLR contribute to the modulation of osteoclastogenesis by modulating the function of both osteoblasts and osteoclasts (Bar-Shavit et al., 2008).

2.4 Regulation of osteoclastogenesis by T cells

Activation of the immune system is essential for host defence against pathogens, but aberrant and/or prolonged activation under certain pathological conditions results in tissue damage owing to the activation of effector cells. In autoimmune arthritis, it has long been a challenging question as to how the abnormal T-cell activation (characterized by infiltration of CD4+ T cells) mechanistically induces bone damage (Takayanagi, 2005; Sato and Takayanagi, 2006).

As RANKL is expressed by activated T cells, T cells have the capacity to induce osteoclast differentiation by directly acting on osteoclast-precursor cells (Horwood et al., 1999). However, the IFNγ produced by T cells potently suppresses RANKL signalling through downregulation of TRAF6 (Takayanagi et al., 2000). T cells also secrete various cytokines such as IL-4 and IL-17 (Kotake et al., 1999), so the effects of T cells on osteoclastogenesis should be dependent on the balance between positive and negative factors expressed by the T cells (Table 2.1). As the well-known effector CD4+ T Th-cell subsets Th1 and Th2 produce IFNγ and IL-4, respectively, both of which are anti-osteoclastogenic, it has been a paradox that activated CD4+ T cells in arthritis enhance osteoclastogenesis in the presence of these cytokines.
Recent data from various laboratories indicate that an IL-17 producing Th17 cells represents the long-sought-after osteoclastogenic Th cell (ThOc cells) subset among the known CD4+ T cell lineages; whereas Th1 and Th2 cells have anti-osteoclastogenic effects (Sato et al., 2006). It has already been reported that IL-17 expression is increased in RA joints (Kotake et al., 1999). IL-17 is well known to induce local inflammation in autoimmune diseases through inflammatory cytokine production (Bailey et al., 2007; Van Beelen et al., 2007). Moreover, IL-17 induces RANKL on mesenchymal cells (Kotake et al., 1999). Also Th17 cells express higher levels of RANKL than Th1 and Th2 cells (Table 2.1) (Sato et al., 2006). Therefore, the infiltration of Th17 cells into the inflammatory lesion links the abnormal T-cell response to bone damage in arthritis, and the pathogenesis of RA should be reconsidered in the context of a Th17-type disease (Fig. 2.3). Clearly, this subset is an auspicious target for future therapies, and cytokines related to Th17-cell differentiation and
expansion, such as IL-6, TGF-β and IL-23, will be of great clinical importance (Iwakura and Ishigame, 2006).

**Figure 2.3 Mechanism of bone destruction in RA.** In RA, cells within the inflamed synovium proliferate and invade the bone while activating osteoclastic bone resorption. CD4+ T-cell infiltration is a hallmark of the pathogenesis of arthritis, and Th17 cells function as osteoclastogenic Th cells. Th17 cells do not produce IFN-γ, which suppresses RANKL signaling, but rather secrete a huge amount of IL-17, which induces RANKL on synovial fibroblasts. IL-17 also stimulates local inflammation and activates synovial macrophages to secrete proinflammatory cytokines, such as TNF, IL-1 and IL-6. These cytokines activate osteoclastogenesis by either directly acting on osteoclast precursor cells or inducing RANKL on synovial fibroblasts. Th17 cells also express RANKL on their membrane, which might further contribute to the enhanced osteoclastogenesis. The induction of Th17 cells is regulated by IL-6, TGF-β and IL-23 produced by dendritic cells. Th1 and Th2 cells inhibit osteoclastogenesis through the production of IFN-γ and IL-4, respectively. Treg cells have a suppressive role, but their target is not completely determined (adapted from Takayanagi, 2009).
2.5 Clinical implications

A detailed osteoimmunological understanding of the pathogenesis of bone destruction in many contexts will lead to novel strategies for the treatment of various diseases, including RA, periodontal disease, Paget’s disease, osteoporosis, osteoarthritis, multiple myeloma and metastatic bone tumors. Rheumatologists are now aware of the remarkable impact of TNFα-specific antibody therapy on the treatment of RA, and other cytokines will soon be targeted by similar strategies using biological agents (Palladino et al., 2003). The action of TNFα and other inflammatory cytokines is not limited to the induction of local inflammation, but is directly and indirectly involved in the activation of osteoclasts, as mentioned above. Such osteoimmunological pleiotropy might explain the dramatic efficacy of the biological agent to prevent or even ameliorate bone destruction. In addition, the efficacy of a RANKL-specific antibody for postmenopausal osteoporosis and RA in clinical trials has been reported (McClung, 2006), and its apparent preventive effect on bone metastasis is enormously promising (Jones et al., 2006).

Interestingly, severe adverse effects of RANKL inhibition on the immune system have never been reported. The clinical relevance of newly recognized cytokines such as IL-17 and IL-23 has yet to be established in humans, but IL-6 inhibition, which is also under clinical trial with successful results, might have a dual impact on Th17-mediated immune responses and bone (Nishimoto, 2006). Although it is difficult to specifically target transcription factors and signalling molecules in drug treatment, some of the anti-rheumatic drugs inhibit osteoclastogenesis by suppressing the induction of NFATc1 (Urushibara et al., 2004). Therefore, the osteoimmunological perspective has enabled us to gain profound insights into the understanding of the mode of action of therapeutic drugs as well as a novel strategy for drug design.
2.6 Interleukin-3 (IL-3)

IL-3 [(also called as multi-colony stimulating factor (CSF))] is a multipotent haematopoietic growth factor produced by activated Th cells, mast cells, eosinophils, monocytes/macrophages and stromal cells (Mangi and Newland, 1999). Ihle and co-workers (1981) first described IL-3 as a T-cell product, involved in the pathogenesis of moloney leukemia-virus-induced T-cell lymphomas. Since IL-3 acts on numerous cells of haematopoietic system, it was studied and discovered independently by a number of laboratories studying various biological activities in different cell types. A number of biological activities have been assigned to this molecule, and these activities go by different names including cell-stimulating factor, histamine producing cell-stimulating factor, multi-CSF, multilineage haematopoietic growth factor and mast cell growth factor. Biochemical purification (Ihle et al., 1983; Clark-Lewis et al., 1984), and molecular cloning and expression (Yokota et al., 1984) convincingly established that a single polypeptide mediates all of these bioactivities.

IL-3 and GM-CSF synergise for the differentiation and function of myeloid cells, and are of particular importance in allergic inflammation and protective immunity and also in the pathophysiology of diseases (Arai et al., 1990; Bazan, 1990-a; Nicola, 1994-b; Rozwarski et al., 1994). Recent studies have also demonstrated a role for these cytokines in modulating Th1 and Th2 immune responses and playing role in modulating osteoclasts. Thus unraveling the biology of IL-3 will help to elucidate the process of haematopoiesis, osteoclast regulation, mechanisms of protective and pathologic immune responses, and molecular targets for modulation of these cytokine-mediated responses. This thesis deals with the regulation of Treg and Th-17 cells differentiation by IL-3, so in this chapter biology of IL-3 is briefly reviewed.
2.7 Sources of IL-3

IL-3 is mainly produced by activated T lymphocytes (Schrader and Nossal, 1980; Schrader, 1981). Although CD4+ T cells can mainly be divided into Th1 and Th2 type, which are responsible for cellular and humoral immunity, respectively, both subsets also have potential to produce IL-3 (Razin et al., 1981). This implies that any kind of T cell response would thus always be associated with concomitant production of IL-3. Crosslinking of mast cells with IgG FcRs also leads to the production of IL-3 (Burd et al., 1989; Ishizuka et al., 1999), but the physiological significance of this phenomenon is not known. It may serve to prime other cells in vicinity of an allergic response including mast cells themselves as well as macrophages and other haematopoietic cells. Secretion of IL-3 by activated mast cells may account for the rapid increase in histamine production. Eosinophils, when activated through cross-linking of FcR and adherence to fibronectin also produce IL-3 (Moqbel et al., 1994).

2.8 Structure of IL-3

In mouse, the IL-3 gene is located on chromosome 11 at bands A5 to B1, and displays similar structural characteristics to the human gene (Webb et al., 1989). It is made up of five exons interrupted by four introns. Campbell and his group (1985) have showed that single gene codes for IL-3 in the haploid murine genome. Their studies in activated T cells have shown that the expression IL-3 appears to be controlled at transcriptional level (Campbell et al., 1985). The full-length murine IL-3 mRNA encodes for a molecule of 140 amino acids containing a 26 amino acid hydrophobic leader sequence that, upon cleavage, results in the production of a core polypeptide with a molecular weight of 15 kDa, two intramolecular disulphide bonds (Cys 17-80 and Cys 79-140) of which only the former is crucial for biological activity, and
four potential N-glycosylation sites at Asn 16, Asn 44, Asn 51, and Asn 86 (Fung et al., 1984; Yokota et al., 1984; Clark-Lewis et al., 1988). The native mouse protein has a molecular weight of 22–32 kDa, presumably due to various degrees of N-linked glycosylation; however, glycosylation is not required for biological activity. Human and murine IL-3 coding sequences show only 45% identity at the nucleotide level and 29% identity at the amino acid level and do not cross-react in their biological activities. Nuclear magnetic resonance (NMR) studies have revealed that IL-3 has the basic 4-helix bundle in three-dimensional structure, which is a characteristic feature of the members of cytokine and hormone family. IL-3 was the first protein of its size to be successfully synthesised by automated chemical methods (Clark-Lewis et al., 1986).

The functional structure of haematopoietic cytokines is characterised by orientation of their 4α-helices in an up-up-down-down antiparallel conformation that is enabled by loop sequences between each of the helices (Bazan, 1990-a; Rozwarski et al., 1994). IL-3 can assume this functional conformation by means of the intramolecular folding of a single polypeptide chain (Rozwarski et al., 1994). The domains within IL-3 that engage their cognate Rα and the βc have also been elucidated.

2.9 Receptors of IL-3

All the biological effects of IL-3 are mediated via IL-3R, members of the gp140 family of the type I cytokine receptor group. These receptors are comprised of two distinct chains, a ligand specific α subunit and a common β subunit (βc) (Fig. 2.4). The α subunit does not transduce any biological activity ascribed in haematopoietic cells (Muto et al., 1995; Robb et al., 1995). The α chain associates with low affinity with IL-3 (kDa 50–100 nM) and shows rapid dissociation kinetics (Gearing et al., 1989; Park et al., 1992; Rasko et al., 1995).
Although β chain does not display intrinsic binding affinity, it is necessary for signal transduction and mediates the high affinity binding of IL-3 (kDa 100–300 pM) together with the α receptor in a complex that displays slow dissociation kinetics (Chiba et al., 1990; Park et al., 1992). Curiously mice has two β subunits, a βc which can be activated by GM-CSF/IL-3, and the IL-3-specific β subunit, βIL-3, which binds IL-3 with low affinity and only forms a high-affinity receptor with IL-3Rα (Hara and Miyajima, 1992; Nicola et al., 1997).

**Figure 2.4 The IL-3 receptor.** The IL-3R represents the gp140 family of non-PTK cytokine receptor family. IL-3R comprises a unique ligand-binding α-subunit that is not shared by other members of the gp140 family. The signal transduction function is performed by the common β-subunit which is shared by the receptors for GM-CSF and IL-5. Both the proximal and distal portion of the signal transducing β-subunit recruits multiple downstream signaling intermediates which elicit the IL-3 response (adapted from Reddy et al., 2000).
The specific receptor α-subunits for IL-3 as well as βc are members of the type I cytokine receptor superfamily and contain conserved 200 amino acids extracellular domains termed cytokine receptor modules (Bazan, 1990-b). Each of these modules consists of two repeats of a fibronectin type III-like domain. These repeats carry two sets of conserved motifs typical of this family of receptors. The first repeat contains four cysteines with conserved spacing, while the second repeat contains a WSXWS motif. Mutagenesis studies have suggested that WSXWS motif is important for correct folding of the extracellular domain of cytokine receptors (Baumgartner et al., 1994; Hilton et al., 1996).

Recently solved crystal structure of the complete βc extracellular domain surprisingly revealed an interdigitating homodimer with an arch-like conformation (Carr et al., 2001; Gustin et al., 2001). The βc interlocking homodimer structure is also distinct from that of monomeric cytokine receptors that are homodimerised by ligand. Because the cytoplasmic domains of the homodimeric βc are too far apart to activate each other for signalling, βc activation would require proximate association of either monomer within βc with a ligand-bound IL-3Rα (Carr et al., 2001; Gustin et al., 2001). Although crystallisation of the βc external domain yielded a homodimeric structure with exposed residues predicted as binding sites for IL-3, immunoprecipitation of βc from cell membranes reveals both dimer and monomer forms of βc (Muto et al., 1996). Therefore, it is possible that βc can also exist as a monomer with a predicted structure that is capable of engaging IL-3.

2.10 Intracellular signalling by IL-3

Similar to all cytokines and growth factors, IL-3 exert their biologic responses by first binding to their cognate receptors on the surface of target cells (Adachi and Alam, 1998; Woodcock et al., 1999; Kisseleva et al., 2002). Specific ligand
binding of \( \alpha \) chain recruits the \( \beta_c \) chain, which leads to heteromeric receptor assembly and culminates in the initiation of signal transduction. It is the activation of intracellular signalling that rapidly reprograms gene expression of the target cell to alter its behavior. To date, at least three principal signalling pathways have been described for these cytokines: the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, the MAPK pathways, and the PI3-K pathway (Adachi and Alam, 1998; Woodcock et al., 1999).

IL-3 receptor binding leads to the activation of receptor-associated JAK2 and JAK1 kinases by means of transphosphorylation of the two kinases after oligomerisation of the receptor subunits (Adachi and Alam, 1998; Woodcock et al., 1999). Although JAK2 is the predominant \( \beta_c \)-activating kinase, members of the Src family of kinases, such as Lyn, Fyn, Syk, Hck, and Btk, have also been reported to be activated by these three cytokines (Yousefi et al., 1996). JAK activation results in tyrosine phosphorylation of \( \beta_c \) on six critical tyrosine (Y) residues at Y577, Y612, Y695, Y750, Y806, and Y866 (Adachi and Alam, 1998; Woodcock et al., 1999). Of these phosphorylated residues, Y612, Y695, and Y750 serve as docking sites for the Src homology 2 (SH2) domains of two members of the STAT family, STAT1 and STAT5. The two STATs recruited to the receptor complex by their SH2 domains, become tyrosine phosphorylated on the critical residues in their carboxyl termini, and subsequently homodimerise or heterodimerise by means of reciprocal SH2 and phosphotyrosine interactions. Once the STATs dimerise, they translocate to the nucleus and bind to specific enhancer sequences in the promoters of activated genes. IL-3 action leads to activation of multiple STATs, which includes STAT1, STAT3, STAT5 and STAT6. The nature of STAT that is activated is cell type specific.
It has been shown that in myeloid cells IL-3 induces rapid phosphorylation of JAK1 and JAK2 followed by activation and nuclear translocation of STAT1, STAT3 and STAT5. Expression of dominant negative mutant of Src in these cells result in blocking of IL-3 mediated phosphorylation of STAT3, and results in dramatic inhibition of cell proliferation mediated by IL-3. This indicates that JAK and STAT phosphorylation events are mediated by two independent pathways. Src mediated phosphorylation of STAT3 plays a critical role in myeloid cell proliferation (Reddy et al., 2000).

2.11 Biological activities of IL-3

IL-3 is undetectable in the blood of normal animals (Craper et al., 1984b), however, 25-40 pg found in 16% of the samples observed (Verhoef et al., 1992). This is in part due to the short half-life of IL-3 in circulation. IL-3 intravenously injected into mice was degraded in about 20 to 40 minutes (Craper et al., 1984a; Metcalf et al., 1986). This clearance occurs mainly through the kidney, with most of the filtered IL-3 resorbed and destroyed in the renal tubules with only degraded material passing through the urine (Schrader, 2003). A significant portion of injected IL-3 also accumulates in the liver where it appears to be cleared through the action of the parenchymal cells (Metcalf and Nicola, 1995). In addition to these metabolic degradation mechanisms, receptor-mediated internalisation and degradation regulate the IL-3 activity. In vitro experiments indicate that this internalisation process occurs rapidly and is followed by a fairly rapid intracellular degradation process (Gesner et al., 1988; Peleraux et al., 1990). Animals undergoing immune responses have undetectable IL-3 in their serum; however local production of IL-3 at the sites of immunological activation can be found (Craper et al., 1984a). The local release of IL-3 at the site of T cell activation results in characteristic histological “foot-print’ where there is local
accumulation of mast cells generated by the action of IL-3 on undifferentiated precursors (Crapper and Schrader, 1983). In case of graft-versus-host disease where there is massive activation of T cells, small amount of IL-3 can be detected in serum (Crapper and Schrader, 1986). Similarly immunised animals challenged with parasite antigen show the presence of IL-3 in serum (Abbud et al., 1983).

IL-3 is very much important for the cell proliferation and differentiation and share many common functions. IL-3 promotes the development of early haematopoietic cells into cells of the myeloid, lymphoid, and erythroid lineages (Blalock et al., 1999). IL-3 targets the earliest progenitors even though significant overlap exists with GM-CSF, which acts on a slightly more mature subset (Koike et al., 1987). IL-3 stimulates the differentiation of macrophages, neutrophils, eosinophils, basophils, mast cells, megakaryocytes and erythroid cells (Schrader et al., 1988). IL-3 stimulates the generation of DCs in mice (Storozynsky et al., 1999), and also from human CD34+ cells in the presence of TNF-α (Caux et al., 1996). Under physiologic conditions IL-3 is necessary for supporting increased numbers of tissue mast cells, enhanced basophil production, and protective immunity to parasites, although not essential for the development.

IL-3 acts on more primitive pluripotent stem cells, stimulates the growth of colonies containing a mixture of myeloid and erythroid cells, and stimulate in vitro and in vivo division of cells that form splenic colonies in irradiated mice (Iscove et al., 1989). IL-3 also stimulates the growth of human haematopoietic stem cells (Brugger et al., 1993). In mice subcutaneous injection of IL-3 results in increase in splenic weight with increase in number of mast cells and their progenitors, neutrophils and macrophages (Schrader et al., 1986). The administration of human IL-3 to primates and humans results in effects that is
broadly similar to those seen in mice (Mayer et al., 1989). IL-3 also potentiate the mobilisation of stem cells into peripheral blood induced by G-CSF (Geissler et al., 1996). The administration of IL-3 has been shown to result in an increase of stem cells in the circulation (Geissler et al., 1996). IL-3 not only affects immature haematopoietic cells but also acts on mature cells of other lineages. For example, IL-3 is responsible for survival of mast cell associated with mucosal surface (Crapper et al., 1984b; Schrader et al., 1988). IL-3 stimulation of macrophages results in increase levels of Class II major histocompatibility complex and LFA1 molecules (Frendl and Beller, 1990). IL-3 also blocks the rapid apoptosis of CD4⁺, CD3⁻ and CD11c⁻ cells present in secondary lymphoid tissues, and in the presence of IL-3 and CD40 ligand these cells differentiate into DCs (Grouard et al., 1997). Murine megakaryocytes differentiate in vitro in the presence of IL-3 (Ishibashi and Brustein, 1986).

IL-3 is implicated in the activation of inflammatory and immune responses (Arai et al., 1990; Nicola, 1994-a). It was thus surprising that the most striking phenotype observed following ablation of IL-3 by a gene KO approach was a lung defect (Nishinakamura et al., 1996). Double KO mice obtained by crossing murine βc⁻/⁻ mice with IL-3⁻/⁻ mice were viable and fertile (Nishinakamura et al., 1996). The βc/IL-3 double-KO mice exhibited apparently normal steady-state haematopoiesis with the exception of reduced numbers of eosinophils. However, these mice exhibited a lung defect very similar to a condition observed in humans, termed “pulmonary alveolar proteinosis” (PAP).

Detection of IL-3 in postmortem brain tissue from patients with Alzheimer’s disease suggests that IL-3 might mediate increased activation of microglial cells and the subsequent development of neurodegeneration (Araujo and
Lapchak, 1994). The expression of IL-3Rα on a wide range of haematologic malignancies is of potential importance as a marker of tumour load (Testa et al., 2002). Although not normally expressed by B cells, increased expression of IL-3Rα is observed in approximately 40% of patients with B cell-acute lymphocytic leukemia or acute myeloid leukemia leading to enhanced blast proliferation, increased cellularity, and a poor prognosis (Testa et al., 2002). Whether IL-3Rα is a potential target for therapeutic intervention in these malignancies remains to be determined.

2.12 Role of IL-3 in osteoclast differentiation and bone resorption

In various studies IL-3 has been shown to stimulate and inhibit osteoclastogenesis. Barton and Mayer, (1989) showed that IL-3 increases osteoclast formation in whole bone marrow cultures as well as induces fusion of tartarate-resistant acid phosphatase (TRAP) positive cells to form polykaryons. They demonstrated that IL-3 induces TRAP-positive cell formation even in the absence of 1,25(OH)2D3. Hattersley and Chambers, (1990) compared the activities of CSFs such as M-CSF, GM-CSF and IL-3 on bone resorption and found that none of the CSFs induced bone resorption when added alone. However, in presence of 1,25(OH)2D3, M-CSF strongly inhibited the effect of 1,25(OH)2D3 on induction of calcitonin receptor (CTR)-positive cells formation and bone resorption. GM-CSF was found to reduce bone resorption at all the concentrations used and also reduced the number of CTR-positive cells, whereas cultures incubated with IL-3 alone showed considerable number of CTR-positive mononuclear cells but did not induced bone resorption. In the presence of 1,25(OH)2D3 and IL-3 increase number of CTR-positive cells with increased bone resorption. It has also been shown that IL-3 does not influence bone resorption by osteoclasts isolated from rat long bones (Hattersley et al., 1988). IL-3 has been shown to induce osteoclast fusion
in human blood monocytes cultures (Fujikawa et al., 2001; Toyosaki-Maeda et al., 2001; Lee et al., 2004). Mice lacking M-CSF are osteopetrotic. Aged osteopetrotic mice undergo haematopoietic recovery resulting in increased number of osteoclasts and macrophages with subsequent resolution of osteopetrosis and expansion of bone marrow cavity. This indicates a possible alternative mechanism used by haemopoietic system to compensate for absence of M-CSF. These aged mice exhibit elevated levels of IL-3 and GM-CSF in their serum as compared to young and wild type mice. Daily administration of low doses of IL-3 and GM-CSF to these mice corrected osteopetrosis (Myint et al., 1999).

Some contradictory reports also suggest that IL-3 inhibits osteoclastogenesis in vitro. Shinar et al, (1990) showed that IL-3 inhibits osteoclast formation in whole bone marrow cultures in the presence of 1,25(OH)2D3. This inhibitory effect of IL-3 was observed at all cell densities. CSFs have been shown to act differently in different culture systems. In the one-step mouse marrow cultures, none of the CSFs stimulated the formation of TRAP-positive cells. When marrow cells were first cultured in semisolid methylcellulose in the presence of a CSF, and the recovered marrow cells from the semisolid cultures were subsequently co-cultured with primary osteoblastic cells in the presence of 1,25(OH)2D3 numerous TRAP-positive MNCs were formed. This suggests that these CSFs may have different activities at different stages of differentiation (Takahashi et al., 1991). Recently it has been demonstrated that IL-3 inhibits RANKL (Khapli et al., 2003), and TNF-α-induced osteoclast differentiation in purified mouse osteoclast precursors (Yogesha et al., 2005). Also, it was demonstrated that IL-3 potently and irreversibly inhibits TNF-α-induced bone resorption in vitro, and prevents development of inflammatory arthritis, and cartilage and bone loss in mice (Yogesha et al., 2009). Very recently we have shown that IL-3 inhibits RANKL-induced human osteoclasts
derived from blood monocytes and bone marrow cells of osteoporotic individuals (Gupta et al., 2010). These results indicated the potent inhibitory nature of IL-3 on mouse osteoclast differentiation. IL-3 is well-known to regulate the formation of myeloid cells and their precursors. IL-3 also regulates lineage commitment of hematopoietic cells as well as maintains the proliferation and survival of these cells. However, little is known about the mechanisms of action of IL-3 in regulating the formation of either osteoclasts or osteoclast precursors. Thus, further dissection of IL-3 signaling pathway regulating the mechanism of action of IL-3 would help in better understanding the role of IL-3 in bone marrow microenvironment and osteoclast differentiation.

2.14 IL-3 and RA

Nevertheless, in contrast to other T cell derived cytokines such as IFN-γ, IL-2 and IL-4, little is known about the role of T cell derived IL-3 in the peripheral immune system, particularly in various autoimmune disease such as RA. Little is known about the role of IL-3 in arthritis. In an early study, IL-3 messenger RNA was not detected in the synovium of patients with RA (Firestein et al., 1998), and no effect of IL-3 on cultured fibroblast-like synoviocytes was observed (Alvaro-Gracia et al., 1990). Nevertheless, genetic analysis revealed an association between a single-nucleotide polymorphism in the IL-3 promoter and RA (Yamada et al., 2001). 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.