Special Issue: Novel targeted therapies

Mini Review

Potential molecular targets for suppressing Th17 development

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Bone destruction associated with rheumatoid arthritis (RA) is caused by the enhanced activation of osteoclasts, which are terminally differentiated cells of monocyte/macrophage lineage that resorb bone matrix. Accumulating evidence lends support to the theory that interleukin (IL)-17-producing helper T (Th17) cells induce the expression of receptor activator of nuclear factor-κB ligand (RANKL) in synovial cells, which in turn stimulates the differentiation and activation of osteoclasts together with inflammatory cytokines. Thus, a better understanding of the mechanism of Th17 induction is important for the development of effective therapeutic strategies against RA. Our study indicates that cathepsin K, which was thought to be an osteoclast-specific enzyme, also functions as a regulator of TLR9 signaling in dendritic cells, and thus is a potential therapeutic target for the control of Th17-mediated autoimmune inflammation. Furthermore, we have explored the transcriptional program of Th17 development, and discovered that the transcriptional regulator IκBζ is essential for the development of Th17 cells. These findings comprise an important advance in our understanding of the mechanism of Th17 development, providing molecular basis for novel effective therapeutic approaches.

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Introduction

The immune and skeletal systems share a number of regulatory molecules including cytokines, receptors, signaling molecules and transcription factors. Furthermore, hematopoietic stem cells are maintained in the bone marrow where they interact with bone cells. The evidence that the physiology and pathology of
one system might affect the other is compelling, and the term osteoimmunology was coined to cover these overlapping scientific fields\(^1\). 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 rheumatoid arthritis (RA), which is characterized by progressive multiple joint destruction. Elucidating the relationship between osteoclast-mediated bone destruction and aberrant immune responses is now considered to be required for development of effective therapeutic strategies against RA.

**Osteoclasts and bone destruction in RA**

Osteoclasts are large, multinucleated cells formed by the fusion of precursor cells of monocyte/macrophage lineage\(^2\). Mature osteoclasts degrade bone matrix proteins by secreting proteolytic enzymes, such as cathepsin K and matrix metalloproteinase, and decalcify the inorganic components of bone by releasing hydrochloric acid. The essential cytokine for osteoclast differentiation is RANKL, which belongs to the tumor necrosis factor (TNF) family\(^2\). In bone, RANKL is expressed by osteoclastogenesis-supporting cells including osteoblasts, in response to osteoclastogenic factors, such as 1,25-dihydroxyvitamin D\(_3\), prostaglandin E\(_2\) and parathyroid hormone, and is a crucial determinant of the level of bone resorption \textit{in vivo}\(^2,\,3\). Under pathological conditions such as the bone destruction which occurs in RA, RANKL has been reported to be highly expressed\(^4\,-\,5\). Furthermore, our laboratory demonstrated efficient osteoclast formation in culture of synovial cells obtained from patients with RA\(^6\). Notably, inflammatory cytokines such as TNF-\(\alpha\), interleukin (IL)-1, and IL-6, which are also abundant in the synovial fluid and synovium of RA patients, have a potent capacity to induce RANKL expression on synovial fibroblasts/osteoblasts and to facilitate RANKL signaling, thus directly contributing to the bone destruction process. Furthermore, several studies using genetically modified mice have clearly demonstrated the essential role of RANKL and osteoclasts in the bone damage which takes place in arthritis\(^7,\,8\). Consistent with this, anti-RANKL and anti-osteoclast therapies have been shown to be beneficial in the treatment of bone damage in animal models of arthritis, as well as in human clinical trials\(^9,\,10\).

**Th17 cells function as an osteoclastogenic Th cell subset**

As infiltration of T cells into the synovium is a pathological hallmark of RA, it is vital to address how T-cell immunity is linked to the enhanced expression of RANKL and eventual osteoclastic bone resorption. More specifically, RANKL is known to be also expressed in activated T cells, suggesting that osteoclastic bone resorption is influenced by T cells\(^2,\,9,\,11\). However, it is important to note that T cells produce various cytokines, including interferon (IFN)-\(\gamma\), IL-4 and IL-10, which exert potent inhibitory effects on osteoclast differentiation. Upon activation, naïve CD4\(^+\) T cells differentiate into different lineages of helper T (Th) cells, depending on the cytokine milieu\(^12\). Th1 and Th2 cells are traditionally thought to be the major subsets generated upon antigenic stimulation. Th1 cells, which are induced by IL-12, produce mainly IFN-\(\gamma\) and are involved in cellular immunity; Th2 cells mainly produce IL-4, IL-5 and IL-10, and contribute to humoral immunity. RA was previously considered to be a disease in which the Th1-Th2 balance is skewed towards Th1. However, in the early 2000s, Th17 cells have been identified as a new effector Th cell subset characterized by the production of proinflammatory cytokines including IL-17, IL-17F, IL-21 and IL-22\(^13\). Th17 cell differentiation is induced by the combination of IL-6 and transforming growth factor (TGF)-\(\beta\). IL-23 is indispensable for the lineage commitment of Th17 cells, but is required for the growth, survival, and effector functions of Th17 cells\(^13,\,14\). This unique subset plays a critical role in host defense against certain extracellular pathogens, and also contributes to the pathogenesis of various autoimmune diseases including RA, multiple sclerosis and psoriasis\(^15\). Moreover, our laboratory found that Th17 cells function as the exclusive osteoclastogenic T-cell subset\(^16\). IL-17 induces RANKL on osteoclastogenesis-supporting mesenchymal cells such as osteoblasts and synovial fibroblasts\(^16\). IL-17 also enhances local inflammation and increases the production of inflammatory cytokines, which further promote RANKL expression and activity. Thus, the infiltration of Th17 cells into the inflammatory lesion is the link between the abnormal T-cell response and bone damage (Fig.1).

**Molecular mechanisms underlying Th17 development**

Th17 cells have emerged as attractive therapeutic targets for both inflammation and bone destruction. It is therefore important to understand the molecular mechanism underlying Th17 development in order to develop novel therapeutic strategies.

1)A novel role of cathepsin K in autoimmunity

Cathepsin K is a lysosomal cysteine protease that plays a pivotal role in osteoclast-mediated degradation of the bone matrix\(^17\). Thus, cathepsin K has been considered as a potential therapeutic target for the treatment of bone diseases such as os-
Interleukin (IL)-17-producing helper T (Th17) cells have stimulatory effects on osteoclastogenesis and play an important role in the pathogenesis of rheumatoid arthritis through IL-17, while Th1 and Th2 cells have inhibitory effects on osteoclastogenesis through interferon (IFN)-γ and IL-4, respectively. IL-17 not only induces receptor activator of nuclear factor-κB ligand (RANKL) on synovial fibroblasts, but also activates local inflammation, leading to the upregulation of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, 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 cellular membrane, which partly contributes to the enhanced osteoclastogenesis.

teoporosis. We developed a new orally active cathepsin K inhibitor named NC-2300, which suppresses osteoclastic bone resorption both in vitro and in vivo. Oral administration of NC-2300 markedly inhibited both bone erosion in the inflamed joints and periarticular osteoporosis in adjuvant-induced arthritis. Interestingly, we observed the unexpected result that NC-2300 reduced inflammation even when administered after the onset of disease. Cathepsin K, despite a low expression level in dendritic cells, plays an important role in the activation of Toll-like receptor (TLR) 9 signaling. Unmethylated CpG-containing DNA (a TLR9 ligand)-induced production of cytokines such as IL-6 and IL-23 was found to be impaired in cathepsin K inhibitor-treated or cathepsin K-deficient dendritic cells. The immune function of cathepsin K was further determined in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis, and the severity of the disease was markedly suppressed in cathepsin K-deficient mice. Interestingly, the suppression of inflammation was associated with reduced induction of Th17 cell polarization, indicating that cathepsin K contributes to autoimmune inflammation by inducing Th17 cells, possibly through cytokines such as IL-6 and IL-23 in dendritic cells (Fig.2).

The detailed mechanism by which cathepsin K regulates TLR9 signaling remains elusive, but recent studies have demonstrated that TLR9 requires proteolytic processing in the endolysosomal compartments to elicit the signaling pathway in response to the ligands. This processing occurs through a multistep process. Asparagine endopeptidase or cathepsin family members initially remove the majority of the ectodomain, and then cathepsins induce the cleavage of the processed receptor, resulting in the generation of the fully mature form of TLR9. Although it remains unclear how receptor proteolysis permits TLR activation, NC-2300 might inhibit optimal receptor function by interfering with the processing of TLR9. Furthermore, as cathepsin K is now known to be expressed by other cell types including synovial
2) ROR nuclear receptors in Th17 development

Th cell differentiation is initiated by the T cell receptor signal in combination with other cytokine receptor signals. These signals elicit the activation of specific transcription factors to promote lineage-specific cytokine production\(^1\). The T-box-containing protein expressed in T cells, which is activated by IL-12 and IFN-\(\gamma\), is required for Th1 cell differentiation. Th2 cell differentiation requires the function of the GATA binding protein 3, which is induced by the IL-4-activated signal transducer and activator of transcription (Stat) 6. Soon after the discovery of Th17 cells, Littman’s group reported that retinoid-related orphan receptor (ROR)\(_{\gamma}\) is selectively expressed in Th17 cells and is required for Th17 cell differentiation\(^2\). ROR\(_{\gamma}\) expression is induced by the combination of IL-6 and TGF-\(\beta\) through Stat3. Furthermore, ROR\(_{\gamma}\) deficiency led to an impairment of Th17 cell differentiation both in vitro and in vivo. Subsequent studies by Dong’s group demonstrated that another ROR family member, ROR\(_{a}\), is also highly induced during Th17 cell differentiation\(^3\). Although ROR\(_{a}\) deletion in mice had only a minimal effect on IL-17 production, the deficiency of both ROR\(_{a}\) and ROR\(_{\gamma}\) completely abolished IL-17 production and protected mice from EAE. Thus, ROR\(_{\gamma}\) and ROR\(_{a}\) have redundant functions, but ROR\(_{\gamma}\) seems to be the major player in Th17 cell differentiation.

3) A role of I\(\kappa\)B\(_{\zeta}\) in Th17 development

Although ROR nuclear receptors have been proposed as essential regulators for Th17 development, several groups have reported that the ectopic expression of ROR\(_{\gamma}\) or ROR\(_{a}\) leads to only modest IL-17 production in the absence of IL-6 and TGF-\(\beta\)\(^4,5\). It is thus important to elucidate the mechanisms by which the ROR receptors drive Th17 development and the production of Th17-related cytokines such as IL-17. Our recent study showed that a transcriptional regulator I\(\kappa\)B\(_{\zeta}\) was most highly expressed in Th17 cells among the helper T cell subsets\(^6\). I\(\kappa\)B\(_{\zeta}\) is a nuclear I\(\kappa\)B family member that is rapidly induced by TLR ligands or IL-1 in peritoneal macrophages and fibroblasts. Although Yamamoto et al. demonstrated using I\(\kappa\)B\(_{\zeta}\)-deficient mice that I\(\kappa\)B\(_{\zeta}\) is essential for the LPS induction of a subset of secondary response genes including IL-6 and the IL-12 p40 subunit in macrophages\(^7\), no attempt to determine the function of I\(\kappa\)B\(_{\zeta}\) in T cells...
was reported in their study.

IL-18 expression was upregulated by the combination of IL-6 and TGF-β. Stat3 signaling is required for IL-18 induction in Th1 cells, but RORγt is dispensable. IL-18-deficient dendritic cells still normally supported Th17 cells differentiation in vitro. Not only IL-18-deficient mice but also Rag2-deficient mice transferred with IL-18-deficient CD4+ T cells were highly resistant to EAE. IL-18-deficient naive CD4+ T cells had the capacity to differentiate into Th1 and Th2 cells. In contrast, when activated under Th17-polarizing conditions, IL-17 production in IL-18-deficient T cells was markedly reduced compared to wild-type T cells. Since the expression of RORγt and RORα was shown to be normal in IL-18-deficient T cells, it is unlikely that ROR nuclear receptors function downstream of IL-18 or vice versa. Importantly, even in the absence of IL-6 and TGF-β, the ectopic expression of IL-18, together with RORγt or RORα, potently induced IL-17 production. Consistent with this, IL-18 moderately activated the promoter of the mouse II17a gene, but could highly activate the II17a promoter in combination with the ROR nuclear receptor. Dong’s group previously showed that an evolutionarily conserved noncoding sequences (CNS) 2 region in the II17a locus is associated with histone H3 acetylation in a Th17 lineage-specific manner, and that the ROR nuclear receptor is recruited to the CNS2 region during Th17 development24, 28. IL-18 was also recruited to the CNS2 region in Th17 cells, and recruitment of IL-18 to the CNS2 region was dependent on RORγt function (Fig.3). Moreover, the expression of IL-17F, IL-21 and IL-23 receptor was impaired in IL-18-deficient T cells. IL-18 also bound to the promoter or the enhancer region of these genes in Th17 cells. These findings indicate that IL-18 is critical for the transcriptional program in Th17 cell lineage commitment28.

Conclusion

Th17 cell subset is an auspicious target for future therapeutic investigation, and cytokines related to Th17 cell differentiation and function will be of great clinical importance. Antibodies against IL-17 or IL-23 would be expected to exert beneficial effects in autoimmune diseases, and antibodies targeting the IL-6 receptor might also inhibit Th17 development in RA, in addition to effecting a direct inhibition of local inflammation and osteoclastogenesis29, 30. Furthermore, it was recently reported that the cardiac glycoside digoxin and SR1001, a derivative of the benzenesulphonamide drug T0901317, act as specific RORγ inhibitors that potently block Th17 cell differentiation and reduce the severity of EAE31, 32. Therefore, not only antibodies that neutralize Th17-related cytokines, but also chemical compounds targeting molecules essential for Th17 development might be of therapeutic value. In this regard, our results raise the possibility that the targeting of IL-18 may prove effective in the treatment of autoimmune diseases. IRF4, BATF, Ahr and Runx1 have also been identified as transcriptional regulators of Th17 development to date33-36. Further studies are required to determine how IL-18 synergizes with other transcriptional regulators of Th17.

Fig.3  IL-18 and ROR nuclear receptors synergistically promote Th17 cell differentiation

Interleukin (IL)-6 and transforming growth factor (TGF)-β induce Th17 cell differentiation, in which the ROR nuclear receptors have an indispensable role. IL-18 expression is induced by the combination of IL-6 and TGF-β through signal transducer and activator of transcription (Stat) 3, but not RORγt. IL-18 and ROR nuclear receptor bind directly to the CNS2 region of the II17a promoter, and cooperatively activate the II17a promoter. Furthermore, recruitment of IL-18 to the CNS2 region was dependent on RORγt, suggesting that the binding of both IL-18 and ROR nuclear receptors to the II17a promoter leads to an efficient recruitment of transcriptional coactivators having histone acetylase activity.
cells. Although attention should be paid to the side effects in other systems including bone, developing therapeutic compounds to specifically block the function of \( \text{IxB}_{\gamma} \) or the upregulation of \( \text{IxB}_{\delta} \) expression in T cells may offer attractive strategies for autoimmune diseases, including RA.

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