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The Role of Bone Marrow Edema and Lymphangiogenesis in Inflammatory-Erosive Arthritis

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Abstract

A common feature of autoimmune diseases is perpetual production of macrophage, dendritic and/or osteoclast effector cells, which mediate parenchymal tissue destruction in end organs. In support of this, we have demonstrated previously that patients and mice with inflammatory-erosive arthritis have a marked increase in circulating CD11b⁺ precursor cells, which are primed for osteoclastogenesis, and that this increase in osteoclast precursors (OCPs) is due to systemically increased TNF production. From these data, we proposed a unifying hypothesis to explain these osteoimmunologic findings during the pathogenesis of inflammatory-erosive arthritis, which has three postulates: 1) myelopoiesis chronically induced by TNF has profound effects on the bone marrow and joint tissues that should be evident from longitudinal MRI; 2) TNF alters the chemokine/chemokine receptor axis in the bone marrow to stimulate OCP release into the blood, and 3) OCP-mediated lymphangiogenesis occurs in the end organ as a compensatory mechanism to drain the inflammation and remove by-products of joint catabolism. Here, we describe our recent experimental findings that support these hypotheses and speculate on how this information can be used as diagnostic biomarkers and tools to discover novel therapies to treat patients with inflammatory-erosive arthritis.

Keywords

Inflammatory Arthritis; Lymphangiogenesis; In vivo Imaging; 3D-MRI

Introduction

Based on the remarkable success of anti-TNF therapy in patients with immune-mediated inflammatory disorders (IMID) [1, 2], which include rheumatoid arthritis (RA), psoriatic arthritis (PsA), ankylosing spondylitis, Crohn's disease and psoriasis, we have pursued a unifying myelopoiesis hypothesis to explain how these drugs are so effective in seemingly unrelated diseases [3–5]. This theory posits that TNF produced in the end organ (e.g. joint, gut, and skin), acts as an endocrine factor to stimulate the proliferation and release of CD11b⁺ myeloid precursors from the bone marrow into the circulation, where they home to sites of inflammation and differentiate into osteoclast, macrophage or dendritic effector cells to perpetuate autoimmune disease. In support of our theory, we have demonstrated that TNF-Tg mice [6, 7], and PsA patients [8, 9] with inflammatory-erosive arthritis have increased numbers of CD11b⁺ peripheral blood mononuclear cells (PBMC) that readily differentiate into bone resorbing osteoclasts, and that amelioration of their disease with anti-TNF therapy

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is associated with a dramatic decrease in this osteoclast precursor (OPC) frequency in PBMC. To further understand the underlining etiology of IMID and develop a translational approach to test our myelopoiesis model in animals and people, we developed longitudinal MRI outcome measures to study inflammatory-erosive arthritis in mice [10–12], and completed an exhaustive microarray analysis of mRNA isolated from CD11b+ cells in the bone marrow and blood of transgenic mice that over-express human TNF (TNF-Tg) [13], and their wild-type (WT) littermates [14–16]. These studies revealed several novel findings that provide a mechanistic understanding of how systemic TNF mediates macroscopic changes in the bone marrow to achieve perpetuating myelopoiesis, and the consequences this has on inflammatory-erosive arthritis. Here, we attempt to put these osteoimmunologic findings into context with respect to how this information can be utilized to diagnosis IMID and monitor responses to therapy, as well as now to pursue novel drug intervention strategies.

Translational MRI outcome measures of inflammatory-erosive arthritis identify bone marrow changes and draining lymph node function as key biomarkers of disease pathogenesis

One of the greatest limitations of animal models of inflammatory-erosive arthritis is the absence of translational outcome measures that can be replicated in humans. This presents three problems for pre-clinical investigations of drug effects on inflammation, joint erosion, and healing [6, 17]. These are: i) the absence of an objective assessment of disease severity prior to treatment, ii) the extensive reliance on outcome measures that cannot be performed in humans (i.e. histologic and *ex vivo* molecular analyses of joint tissues during all phases of inflammatory arthritis), and iii) inability to stratify animals randomized to drug and placebo groups to account for the significant differences in the incidence and severity of arthritis at treatment initiation, which occurs even in genetically identical littermates. Since magnetic resonance imaging (MRI) has become the “gold standard” for the assessment of joint inflammation and damage in inflammatory arthritis in humans [18–21], and several longitudinal biomarkers of human disease have been developed for this approach (e.g. synovial inflammation and bone marrow edema) [22–27], we aimed to develop this outcome measure in mice. The approach we chose was to develop longitudinal 3-dimensional (3-D) imaging in which a custom surface coil for the mouse knee was integrated into a 3 tesla clinical MRI, utilizing pulse sequences that were compatible with human studies [12]. In these experiments we measured inflammation using this contrast-enhanced MRI (CE-MRI), and bone erosion using *in vivo* microfocal computed tomography (micro-CT), to assess TNF-induced disease and its amelioration with effective therapy. The results of these studies validated these methods by demonstrating a significant relationship between changes in synovial and patellar volumes during the progression of inflammatory arthritis ($R^2 = 0.75$, $P < 0.01$). Moreover, the data demonstrated the highly variable nature of TNF-induced joint inflammation, and identified the popliteal lymph node (PLN) volume as the most sensitive biomarker of ankle and knee arthritis.

Elucidating bone marrow edema and myelopoiesis in murine arthritis using contrast-enhanced magnetic resonance imaging

Given the dramatic effects of TNF on myelopoiesis, our model posits that macroscopic changes in the bone marrow must be evident in mice and patients with inflammatory-erosive arthritis. In support of these gross changes, it has long been realized that arthritis is associated with enigmatic bone marrow changes that are consistent with fluid signals on MRI, and thus have been generically referred to as bone marrow edema (BME). While several retrieval studies have been performed on human tissues obtained from patients

undergoing joint replacement surgery [28–31], and have grossly characterized the BME signal to be the conversion of adipocyte rich (yellow) to hematopoietic (red) marrow, the true essence of this MRI signal in terms of its: i) molecular and cellular composition, ii) natural history, and iii) value as a biomarker of disease pathogenesis and response to therapy, has remained elusive due to the absence of an animal model and the inability to serially sample affected human joints. As expected, these BME signals were readily apparent in our mouse models [11, 12]. Thus, we developed two quantitative measures of this MRI signal to assess BME [11]. The first is normalized bone marrow intensity (NBMI), which is derived from the inherent signal intensity of the bone marrow standardized to the adjacent muscle tissue on pre-contrast MRI. The second is normalized marrow contrast enhancement (NMCE), which is enhancement of the MRI signal following intravenous administration of gadolinium contrast agent in the marrow region of interest (ROI) divided by the enhancement of signal intensity of the adjacent muscle. In our studies, WT and TNF-Tg mice were scanned from 2 to 5 months of age, followed by histologic or fluorescence-activated cell sorting (FACS) analysis of marrow. We also performed efficacy studies in which TNF-Tg mice were treated with anti-TNF or placebo for 8 weeks, and then studied using bimonthly MRI and histologic analysis. The results demonstrated that while NBMI values were similar in WT and TNF-Tg mice at 2 months, NBMI in WT mice steadily decreased thereafter, while TNF-Tg mice retained their high values throughout life. Red to yellow marrow transformation occurred in WT, but not in TNF-Tg mice, as observed histologically at 5 months. Interestingly, the marrow of TNF-Tg mice treated effectively with anti-TNF therapy converted from red to yellow marrow, with lower NBMI values versus placebo at 6 weeks. To confirm that the hematopoietic cells in the marrow of untreated TNF-Tg mice were OCPs, we performed FACS analysis of bone marrow, which revealed a significant correlation between NBMI values and CD11b⁺ monocytes ($R^2 = 0.91$, $P = 0.0028$). Perhaps of most importance to the value of this biomarker for assessing the severity of arthritis is that we were able to demonstrate thresholds values for "normal" red marrow versus pathologic BME, and we also found that inflammatory marrow is highly permeable to contrast agent. Based on these findings, we conclude that BME signals in arthritic mice are caused by conversion of yellow to red marrow, with associated increased myelopoiesis and increased marrow permeability. The factors that mediate these changes are currently under investigation.

Given these remarkable MRI findings and their link to TNF-induced OCP frequency (OCPF) in PBMC and focal erosions in PsA [8], we performed a clinical pilot study in which OCPF and BME were assessed in 20 patients with active erosive disease before and after etanercept therapy [9]. We found that the median OCPF decreased from 24.5% to 9% ($p = 0.04$) and 7% ($p = 0.006$) after 3 months and 6 months of treatment, respectively. Thirteen of these 20 patients completed all of the MRI evaluations. Although we found that BME decreased in 47 involved sites in these patients at 6 months, it also increased in 31 other sites. Additionally, we found no correlation between OCPF, BME and clinical parameters, likely reflecting the small size of the study. These preliminary findings suggest that our myelopoiesis model of TNF-induced inflammatory-erosive arthritis is correct, but clearly they need to be assessed in an appropriately powered clinical trial. We also found that BME signals in the marrow of affected bones of PsA patients persist well after the inhibitory effects of anti-TNF therapy on OCPF are observed. This unexpected finding may suggest that there is ongoing subchondral inflammation or altered trabecular bone remodeling in adjacent to affected joints of patients with PsA, and warrants further investigation.

MR imaging and quantification of draining lymph node function in inflammatory arthritis

Based on the surprising observations we made using CE-MRI of murine arthritis, which identified the PLN as a powerful biomarker of disease, we followed up on these findings with prospective studies to assess the potential of CME-MRI in our model [10]. First, we demonstrated that the volumes of PLNs of TNF-Tg mice aged 5 months are significantly larger and have greater contrast enhancement after intravenous gadolinium administration than WT control mice. This difference correlated with the abundance of dilated intranodal sinuses that were immuno-positive for the lymphatic vessel-specific marker, LYVE-1. Furthermore, we utilized dynamic CE-MRI to demonstrate differences in the kinetics of PLN enhancement between TNF-Tg and WT mice, and established a LN capacity (LNcap) measurement, which is a function of both lymph node volume and CE. With this new outcome measure, we demonstrated that 5 month-old TNF-Tg mice have a 15-fold increase in lymphatic capacity over WT mice ($P < 0.001$). Interestingly, amelioration of arthritis with anti-TNF therapy resulted in a significant decrease in LNcap ($P < 0.0001$) that approached WT levels within 4 weeks of the start of treatment. However, this functional decrease was not associated with a reduction in lymphatic vessel area or numbers, which persist after therapy in both PLN and synovium. Finally, we demonstrated that there is a significant negative relationship between draining PLN function (LNcap) and synovial volume ($R^2 = 0.63$, $P = 0.01$), and that TNF-Tg mice with a lower LNcap display an accelerated progression of synovitis and focal joint erosion. Collectively, these results suggest that draining lymph nodes serve a function to protect joints from inflammatory arthritis, and that the initiation of joint erosion occurs when the amount of joint inflammation exceeds the maximal LNcap, or that a breakdown in LNcap may occur as a result of lymph node dysfunction or ‘shutdown’ triggered by a local immune response.

TNF increases bone marrow myelopoiesis by increasing proliferation through up-regulation of c-Fms expression on OCPs

To elucidate the mechanisms of TNF-induced increased myelopoiesis, we have focused on M-CSF and its receptor, c-Fms, because this signaling system plays a critical role in survival and proliferation of myeloid lineage cells [32]. We found that there is a two-fold increase in the percentage S-phase cells in bone marrow OCPs of TNF-Tg mice compared to their wild type littermates, suggesting increased proliferation of this cell population. *In vitro*, TNF induces the differentiation of bone marrow CD11b⁺/cFms^{-/lo} to CD11b^{hi}/cFms^{hi} cells [33] and these CD11b^{hi}/cFms^{hi} cells become highly proliferative in response to M-CSF, but not to TNF [33]. Along with the recent report that TNF stimulates M-CSF production by bone marrow stromal cells [32] and our findings, we believe that two of the molecular mechanisms responsible for increased marrow myelopoiesis in IMID are TNF directly targeting OCPs to increase their c-Fms expression and stromal cells to produce M-CSF.

TNF inhibits production of SDF-1 by bone marrow stromal cells and increases osteoclast precursor mobilization from bone marrow to peripheral blood

As mentioned above, one of the central goals of our research on myelopoiesis in IMID, is the molecular mechanism by which systemic TNF mediates the release of OCPs from the bone marrow to the circulation. To address this issue, we completed a detailed investigation of the potential chemokines that could be expressed in bone marrow stroma, and their cognate receptors on OCP, since these could be involved in the translocation of OCPs from

the bone marrow to the blood [15]. This investigation also focused on stromal cell derived factor-1 (SDF-1/CXCL-12) and its receptor, CXCR4, because SDF-1 is the master chemokine that modulates trafficking of hematopoietic stem cells and precursors and other cell types in the bone marrow. SDF-1 is primarily produced by bone marrow stromal cells, such as osteoblasts, and endothelial cells [34]. Its expression has also been identified in synoviocytes in RA [35, 36] and it has been shown to be a potent chemotactic factor for OCPs [37]. We found that bone marrow stromal cells in TNF-Tg mice have reduced levels of SDF-1 expression and that TNF injection into wild type mice significantly reduced the expression of SDF-1 by stromal cells. This is associated with increase OCP frequency in the peripheral blood. Because TNF treatment did not affect the percentage of bone marrow CXCR4⁺ OCPs or the expression levels of CXCR4, we believe that down-regulation of SDF-1 expression by bone marrow stromal cells may be an important mechanism to explain the elevated circulating myeloid cells in IMID.

The lymphatic growth factor, VEGF-C, is a RANKL target gene that is induced during osteoclastogenesis and contributes to increased lymphangiogenesis in joints of mice with inflammatory-erosive arthritis

In subsequent analyses of the aforementioned microarrays [15], we looked for cytokines that were differentially expressed in OCP from TNF-Tg and WT mice [14]. To our surprise, we found that expression of vascular endothelial growth factor-C (VEGF-C), whose primary function is considered to be regulation of lymphangiogenesis [38], was markedly increased in TNF-Tg mice OCPs. While it was known that VEGF-C is highly expressed by numerous cancer cells, inflammatory macrophages, and synoviocytes [39–41], the factors that control VEGF-C expression have not been studied in detail. Therefore, we investigated the effects of TNF, IL-1 and RANKL on the expression VEGF family members in OCPs. We found that RANKL increased *vegfc* mRNA expression 12-fold, while TNF and IL-1 increased its expression only 5-fold. Moreover, this effect of RANKL was specific for VEGF-C, as this was the only VEGF family member that was increased. These data provided the first evidence that osteoclastogenesis and lymphangiogenesis are concomitant events and suggest that these physiological events may be linked in the pathogenesis of inflammatory-erosive arthritis.

In order to examine the physiological significance of this VEGF-C induction in inflammatory arthritis, we assessed the effects of TNF on VEGF-C expression and lymphangiogenesis in TNF-Tg mice and in mice with serum-induced arthritis [16]. We used immunostaining and MRI to quantify lymphatic vessels and the volumes of synovium and draining lymph nodes. We found that TNF stimulated VEGF-C expression by OCPs in a NF- κ B-dependent manner. Moreover, OCPs from joints of TNF-Tg mice express high levels of VEGF-C, and this VEGF-C had autocrine effects to induce osteoclastic bone resorption through activation of the c-Src signaling pathway. Lymphatic vessel numbers and size were markedly increased in joint sections of TNF-Tg mice and in mice with serum-induced arthritis. Additionally, the severity of synovitis correlated with draining lymph node size. This led us to conclude that TNF induces OCPs to produce VEGF-C, which significantly increased lymphangiogenesis in joints of arthritic mice, and supports our emerging hypothesis that the lymphatic system may play an important role in the pathogenesis of inflammatory arthritis.

Conclusions

Based on our recent findings and the established literature, we now propose a comprehensive model to explain the mechanisms whereby TNF produced in arthritic joints

stimulates OCP production and release from the bone marrow to propagate a vicious cycle, which is manifested as inflammatory-erosive arthritis (Figure 1). The pathogenesis commences with an unknown event that leads to chronic TNF over expression in affected joints. TNF has endocrine effects on the bone marrow where it stimulates M-CSF production by stromal cells and differentiation of hematopoietic progenitors from CD11b^{hi}/cFms^{-/lo} to CD11b^{hi}/cFms^{hi} OCPs. This leads to increased proliferation of CD11b^{hi}/cFms^{hi} OCPs in response to M-CSF and enhanced bone marrow myelopoiesis. In the meantime, TNF reduces SDF-1 production by bone marrow stromal cells and increases the mobilization of OCPs from bone marrow to the peripheral blood. Joint inflammation then recruits circulating OCPs to the inflamed synovium where these cells are stimulated by RANKL and TNF to become bone resorbing osteoclasts and also produce angiogenic and lymphangiogenic factors, such as VEGF-C, to stimulate vasculogenesis.

Based on TNF's pleiotropic inflammatory effects on virtually all cell types in the body, it is not surprising that it is a central factor in autoimmune diseases. However, its dominant role in perpetuating the release of myeloid effector cells from the bone marrow now appears to be a common link in IMID. Thus, a current challenge is to further understand the molecular mechanisms of this osteoimmunologic phenomenon, which includes elucidation of the factors that mediate TNF-induced yellow to red bone marrow conversion, and the generation of novel interventions that are less immunosuppressive and more cost-effective than anti-TNF therapy.

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Figure 1. The TNF-induced OCP vicious cycle in inflammatory-erosive arthritis

(1) The pathogenesis commences with an unknown event that leads to chronic TNF over expression in the joint. (2) This TNF has endocrine effects on the bone marrow where it stimulates M-CSF production by stromal cells and differentiation of hematopoietic progenitors to CD11b^{hi}/cFms^{hi} OCPs. (3) M-CSF stimulates the proliferation of CD11b^{hi}/cFms^{hi} OCP, which (4) leads to their migration from the bone marrow to the blood via a mechanism that involves the down-regulation of stromal cell derived factor -1 (CXCL12). (5) PBMC/OCPs are recruited into the inflamed synovium, and are stimulated by RANKL to become bone resorbing osteoclasts. (6) This RANKL stimulation also induces the expression of lymphangiogenic factor, VEGF-C and perhaps other yet to be defined angiogenic and lymphangiogenic factors that complete the vicious cycle through paracrine/endocrine effects on inflammation and myelopoiesis.