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Interaction between Synovial Inflammatory Tissue and Bone Marrow in Rheumatoid Arthritis

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Axel Wanivenhaus,‡ Andreas Chott,‡ Josef S. Smolen,* and Georg Schett2*

Rheumatoid arthritis (RA)1 is due to a chronic inflammatory process of the synovial membrane. This membrane, the innermost part of the joint capsule, is normally a fine strand of only a few cell layers. It bridges the two neighboring bones and inserts at periosteal regions close to the articular cartilage. This insertion site, where articular cartilage, periosteum, and synovial membrane come into close contact is also called the junction zone. In RA, the synovial membrane is transformed into a hypertrophic inflammatory tissue. This is based on an influx of inflammatory cells, such as monocytes, T cells, and B cells, from the blood stream as well as hyperplasia of resident synovial cells.

An important feature of synovial inflammatory tissue is its capacity to invade neighboring structures, such as cartilage and bone (1–3). This invasive synovial tissue is also termed “pannus.” This property makes RA the most disabling joint disease, since it leads to structural damage of the joint, which finally leads to loss of function and disability. Mineralized tissue, such as mineralized cartilage and bone, is considered as primary target of synovial inflammatory tissue, since at the aforementioned junction zone, it is directly located underneath the inflammatory tissue of the insertive synovial membrane and the periosteum (2). Resorption of subchondral bone, which appears in radiography, is a criterion for the classification of RA and assessment of radiographic bone damage has become an important tool for monitoring RA patients in clinical studies and daily practice (4, 5).

The ability of synovial tissue to invade bone is closely linked to the generation of osteoclasts (6–8). Differentiation of osteoclasts appears to be enhanced in the RA synovial membrane, since there is an increased influx of mononuclear cells serving as osteoclast precursors and a plethora of signals, such as TNF and receptor activator of NF-κB ligand, which stimulate osteoclast differentiation (9, 10). In animal models, osteoclasts are essential for arthritic bone resorption and blockade of osteoclasts has emerged as a powerful tool to interfere with structural bone damage in arthritis (11–13). Thus, osteoclasts are the tools of the inflamed synovial membrane to invade bone. Whether cortical bone can actually be completely penetrated by the inflamed synovial tissue, and what the consequences of such penetration into the marrow space might be, is less clear.

The cortical bone layer, which separates bone marrow from synovial tissue, is comparatively thin, suggesting that an attack by osteoclasts from the outside could penetrate cortical bone and open the marrow space for synovial tissue. Such interaction could entail profound changes of cellularity of synovial inflammatory tissue, since the marrow space harbors vast amounts of leukocytes and their precursors, and vice versa. To test this hypothesis, we studied specimens from RA patients subjected to joint replacement surgery, which contained not only synovial inflammatory tissue but also neighboring cartilage, bone, and bone marrow. We performed a histochemical and immunohistochemical study to search for and define the nature of penetration of synovial inflammatory tissue into the marrow space in human RA patients.

Materials and Methods

Patients and tissue specimens

Twelve patients, fulfilling the American College of Radiology criteria for diagnosis of RA were analyzed in this study (4). All 12 patients were routinely scheduled for joint replacement surgery because of refractory RA in the affected joint. All joints showed clinical signs for active synovitis (pain, swelling, and stiffness). Age, sex, and the years of disease duration were recorded from all subjects. In addition, the number of tender and...
swollen joints, the presence of radiographic bone erosions in the replaced joint, and the levels of C-reactive protein (mg/dl), erythrocyte sedimentation rate (ESR) (mm/h), and rheumatoid factor (U/L) were assessed in these patients. Moreover use of steroids and disease-modifying antirheumatic drugs (DMARDs) including biological agents was recorded. Resected material consisted of juxtaarticular bone, articular cartilage and synovial tissue attached to bone and cartilage in all 12 patients. In addition, four joints from healthy patients receiving amputation surgery of the lower limb after traumatic injury were collected. All participants gave written informed consent. After explanation, the material was immediately placed into 0.9% NaCl and fixed within 1 h in 4.0% formalin. Fixation lasted for at least 18 h.

Preparation of tissue sections
After fixation, joints were longitudinally cut into two equally sized pieces. One part was used to prepare decalcified paraffin-embedded tissue sections. Material was fixed in 4.0% formalin overnight and then decalcified in 14% EDTA (Sigma-Aldrich) at 4°C (pH adjusted to 7.2 by addition of ammonium hydroxide) until the bones were pliable. Ten paraffin sections (2 μm) were cut sequentially throughout the joint and used for the histochemical analyses. Twenty additional serial sections were cut for immunohistochemical analysis (see below). The other part was used for preparation of undecalcified plastic-embedded tissue material. Material was fixed in 70% ethanol, dehydrated in 100% methanol, and embedded in methylnepyrilate (K-Plast; MDS GmbH), as previously described (14, 15). Ten sequential sections 3-μm thick throughout this part of the joint were made on a Jung microtome (Jung).

Antibodies
Monoclonal Abs against the following Ags were used: CD3 (1:50, clone PS1; Novoceastra), CD5 (1.20, clone 4C7; Novoceastra), CD20 (1:200, clone L26; DakoCytomation), CD21 (1:20, clone 1F8; DakoCytomation), CD23 (1:40, clone 1B12; Novoceastra), CD27 (1:80, clone 137B4; Novoceastra), CD45RA (1.100, clone 4K85; DakoCytomation), CD68 (1:100, clone PG-M1; DakoCytomation), CD79a (1:25, clone JCB117; DakoCytomation), CD138 (=Syndecan-1, 1.40, clone B-B4; Serotec), CXCL13 (=B-CA-1, 1.30, clone 53610; R&D Systems), Ki-67 (1.50, clone MIB-1; DakoCytomation), and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (1:20, clone 355G8; Zymed Laboratories). Rabbit polyclonal Abs against the following Ags were used: IgG (1:100; DakoCytomation), IgM (1:100; DakoCytomation), κ L chain (1:1200; DakoCytomation), κ L chain (1:1200; DakoCytomation), and myeloperoxidase (1:1200; DakoCytomation). Goat polyclonal Abs were used for labeling BAFF (=Blys, 1:25; R&D Systems) and CCL21 (=6kCine, 1:20; R&D Systems).

Immunohistochemistry
All joint specimens were assessed by immunohistochemistry. For Ag retrieval, sections subjected to microwave in citrate buffer at 1 bar for Ag retrieval, sections were blocked with 500 W for 20 min (CD3, CD5, CD20, CD21, CD79a, CD138, CCL21, IgD, IgM, Syndecan-1, 1.40, clone B-B4; Serotec), CXCL13 (=B-CA-1, 1.30, clone 53610; R&D Systems), Ki-67 (1.50, clone MIB-1; DakoCytomation), and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (1:20, clone 355G8; Zymed Laboratories). Rabbit polyclonal Abs against the following Ags were used: IgG (1:100; DakoCytomation), IgM (1:100; DakoCytomation), κ L chain (1:1200; DakoCytomation), κ L chain (1:1200; DakoCytomation), and myeloperoxidase (1:1200; DakoCytomation). Goat polyclonal Abs were used for labeling BAFF (=Blys, 1:25; R&D Systems) and CCL21 (=6kCine, 1:20; R&D Systems).

Histochemistry
Paraffin-embedded tissue sections were stained by H&E staining, tartrate-resistant acid phosphatase (TRAP) staining (leukocyte acid phosphatase kit; Sigma-Aldrich) for identification of osteoclasts and toulidine blue for detecting cartilage proteoglycan content. Plastic embedded tissue sections were stained by Goldner trichrome, von Kossa and Movat pentachrome, according to standard protocols (15, 16).

Histological assessments
Number and size of subcortical bone marrow cell aggregates associated with penetrated synovial tissue were determined from 10 sequential decalcified paraffin sections and 10 sequential plastic-embedded undecalcified sections. For assessing the size of these subcortical bone marrow cell aggregates, area of each single aggregate was measured by histomorphometry using OsteoMeasure system (OsteoMetrics) and results were summarized to define the total area of bone marrow affected by aggregates. For control purposes, number and size of bone marrow cell aggregates, which were not in association with synovial tissue penetration and were not directly localized subcortically, were assessed. Width of cortical defects at sites of synovial tissue penetration into the marrow space was also measured. All measurements were done by histomorphometry using OsteoMeasure system. Immunohistochemistry was done on 20 serial paraffin-embedded sections from each specimen. For each cell surface marker and each aggregate, the fraction of positively labeled cells was assessed and a mean ± SEM was calculated for all patients. These analyses were performed in subcortical bone marrow aggregates and for control purposes in cell aggregates within synovial tissue as well as in the inflamed synovial membrane. Osteoid deposits in the subcortical region were assessed on Movat-labeled sections of undecalcified tissue at two different sites, at the bone surface next to subcortical bone marrow aggregates and at a site devoid of such aggregates. The fraction of surface covered by osteoid from total bone surface was measured and calculated in both compartments by using histomorphometry. Finally, standard osteomorphometry measures were applied for assessing juxtaarticular trabecular bone in healthy normal subjects and RA patients. The following parameters were measured: the fraction of bone volume of total volume (BV/TV), trabecular thickness (Tb.Th), number (Tb.N), and separation (Tb.S), number of osteoclasts and osteoblasts per bone perimeter (N.Oc/B.Pm, N.Ob/B.Pm), and the fraction of bone surface covered by osteoclasts and osteoblasts (Oc/S/BS, Oa/S/BS).

Statistical analysis
Data are shown as means ± SEM. Number and size of subcortical bone marrow aggregates, size of cortical penetration, cortical width, and bone surface covered by osteoid were compared by Mann-Whitney U test. For correlating the size of subcortical bone marrow aggregates to markers of disease activity, Spearman’s correlation coefficients and Bonferroni correction were calculated. A value of p < 0.05 was regarded as statistically significant.

Results
Patients characteristics
Of the 12 patients studied, eight were female and four were male (Table I). Mean (±SEM) age was 60 (±3) years, and mean disease duration was 18.9 (±2.8) years, indicating long-standing RA. Most patients had active disease as revealed by the number of tender (mean ± SEM number of tender joints: 6.8 ± 2.9) and swollen joints (4.4 ± 1.3) and/or elevated acute phase response (C-reactive protein, 4.8 ± 2.7 mg/dl; ESR, 51 ± 10 mm/h). Eleven patients were rheumatoid factor positive and its mean (±SEM) level was 286 (±127) U/L. Ten patients received DMARD therapy with methotrexate, five of them as a monotherapy, one in combination with leflunomide, two with anakinra, a rIL-1 receptor antagonist, and two with TNF blockers (infliximab and etanercept). The remaining two patients received monotherapy with anakinra or leflunomide. Low dose oral glucocorticoids were taken by eight of 12 patients. Material originated from joint replacement surgery of the metacarpophalangeal and proximal interphalangeal finger joints in five patients, the metatarsofophalangeal joints in another five patients, and the knee as well as the wrist joint in each patient.

Cortical penetration leads to mononuclear cell aggregates in the adjacent bone marrow
To search for a possible cortical penetration of synovial inflammatory tissue into the bone marrow, we generated axial sections of the joints to allow a simultaneous assessment of cartilage, subchondral bone and bone marrow. Cortical penetration was found in nine of 12 patients and originated from subchondral bone erosions (Fig. 1). Areas with cortical penetration by the pannus were associated with a widespread resorption of subchondral mineralized cartilage and bone, separating unmineralized articular cartilage from underlying bone and characterized by small regions of bone marrow invasion (Fig. 1A). Articular cartilage covering these areas
Cortical penetration and bone marrow invasion in RA. Histological sections from metacarpal heads of patients with RA.

**FIGURE 1.** (Fig. 1, A–C). This interface was localized close to the inner layer of cortical bone and filled most of the marrow space, where cortical penetration had occurred (Fig. 1C). Mononuclear cell marrow aggregates were absent when bone marrow was covered by an intact cortical bone layer, even if eroded from the outside (Fig. 1, D and E). When cortical bone was still present, numerous osteoclasts associated with synovial inflammatory tissue were localized at the outer side, whereas the inner endosteal region was not affected (Fig. 1, E and F).

Subcortical bone marrow aggregates in RA are linked to penetration of cortical bone

To characterize bone marrow changes in RA in more detail, we quantitatively assessed the number and size of subcortical bone marrow aggregates. An average number of three (mean ± SEM 3.0 ± 0.9) sites of cortical penetration with consecutive bone marrow aggregates were observed in each joint (Fig. 2A). In healthy joints, no such lesions were found. The average size of subcortical bone marrow aggregates was 0.06 mm², whereas no region of the subcortical bone marrow was covered by such aggregates in normal joints (Fig. 2B). The mean diameter of the cortical destruction sites was 236 ± 27 μm, which is big enough to allow a meaningful interaction between cells from synovial tissue and bone marrow (Fig. 2C). Moreover, cortical width was significantly diminished in the vicinity of cortical penetration sites (mean ± SEM, 99 ± 5 μm) compared with normal cortical width (162 ± 13 μm), suggesting that bone resorption had significantly weakened the cortical barrier (Fig. 2D).

Interestingly, distant from the site of penetration, bone marrow cell aggregates, when found, were of much smaller size than those at the penetration site.

Subcortical bone marrow aggregates are associated with high disease activity

To clarify whether clinical features of RA influence cortical bone penetration and formation of bone marrow aggregates, we next correlated clinical parameters of RA with the area of subcortical penetration (black arrow, E). The same regions stained by TRAP showing osteoclasts (purple staining, black arrow) resorbing cortical bone from the outside. F, Close-up view of osteoclasts stained for TRAP (black arrow). A, B, D, and E, Original magnification, ×50; C and F, original magnification, ×400.

### Table I. Characteristics of rheumatoid arthritis patients

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*DD, Disease duration (years); SJC, swollen joint count (n from 28 joints); TJC, tender joint count (n from 28 joints); RF, rheumatoid factor (U/L); GC, glucocorticoids; MTX, methotrexate; LEF, leflunomide; BIOL, biological drug; ANA, anakinra; IFX, infliximab; ETA, etanercept; MTP, metatarsophalangeal joint; MCP, metacarpophalangeal joint; ND, not determined; PIP, proximal interphalangeal joint.*
clinical parameters of disease

Table II. Correlation of subcortical bone marrow aggregates with
to cortical penetration or synovial tissue.

For comparative purposes clinical pa-

Table II. Correlation of subcortical bone marrow aggregates with
clinical parameters of disease

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<sup>a</sup> Area of subcortical bone marrow aggregates linked to penetration of cortical bone and attached to synovial inflammatory tissue.

B lymphocytes dominate subcortical bone marrow aggregates

Next, we turned to characterize cell-specific surface marker expression in subcortical bone marrow aggregates by immunohistochemical analysis. Interestingly, B cells, as detected by CD20 expression, were by far the most frequent cell type (mean ± SEM: 55 ± 8%) (Fig. 4, A and E, and Fig. 5A). This was confirmed by concomitant expression of other pan-B cell markers, such as CD45RA (Figs. 4J and 5A) and CD79a (Fig. 5A), both of which were found in a similar frequency in subcortical bone marrow aggregates. T cells, as detected by positive labeling for CD3 (35 ± 4%) and CD5 (37 ± 3%) were found in considerable lower frequency and only few macrophages were present in subcortical bone marrow aggregates (8 ± 1%) (Figs. 4, B, C, F, and G, and 5A). Follicular dendritic cells, as detected by CD21 expression (3 ± 1%) and neutrophils, as detected by labeling for myeloperoxidase (0.8 ± 0.6%), were almost completely absent (Figs. 4K and 5A). This pattern of cellular composition of subcortical aggregates was very similar among all patients investigated and was also not different among the various DMARD therapies.

A more detailed analysis of B cell markers revealed abundance of CD27, indicating that the main proportion of B cells are mature B cells (53 ± 2%) (Figs. 4J and 5A). In contrast, expression of IgM (18 ± 4%), representing immature and mature naive B cells, as well as IgD (5 ± 1%), representing mature naive B cells was much less frequent (Fig. 5A). CD23 expression was virtually absent (1 ± 0.5%) in subcortical bone marrow aggregates. Plasma cells, as detected by CD138 labeling were especially found at the periphery of subcortical bone marrow aggregates and were usually found in between aggregate and synovial inflammatory tissue (Fig. 4, D and H). Plasma cell attributed to 8 ± 4% of total cells in aggregates (Fig. 5A). Less than 5% of cells showed signs of proliferation, as detected by expression Ki-67 Ag (Fig. 5A).

Compared with subcortical bone marrow aggregates, synovial inflammatory tissue showed a different cellular composition (Fig. 5B). The proportion of B cells was significantly smaller and B cells only attributed to up to 10% of synovial cells. The number of T cells in synovial tissue was comparable to subcortical bone marrow aggregates (24 ± 6%), whereas macrophages (27 ± 4%) were much more frequent. Plasma cell content was very scarce (1 ± 1%). In seven patients, mononuclear aggregates were present within synovial tissue, whereas no such aggregates were found in the remaining five patients. All of these aggregates were localized in the synovial tissue of the joint space and were distant from subchondral bone erosions and sites of cortical penetration. This cellular composition showed some similarities with subcortical bone erosions, although their proportion of B cells (20 ± 4%) and plasma cells (2 ± 2%) was lower (Fig. 5C). Composition of B cells was very similar with a majority of mature B cells. T cells were found in a similar frequency, whereas the fraction of macrophages was more prominent in synovial aggregates than subcortical bone marrow aggregates.

Expression of molecules for B cell chemotaxis, homing, and activation in the vicinity of subcortical bone marrow aggregates

Hypothesizing that invading synovial inflammatory tissue expresses molecules, which facilitate accumulation of B cells in the neighboring bone marrow, we next stained for molecules responsible for B cell chemotaxis, homing, and activation (Fig. 6). Chemotactants for B cells, such as CXCL-13, also termed B cell
chemoattractant protein-1, as well as CCL-21 were found expressed at the interface of synovial inflammatory and subcortical bone marrow aggregates (Fig. 6, A and B). Moreover, numerous MAdCAM-1-positive blood vessels, resembling high endothelial venules were found within these aggregates (Fig. 6C). BAFF, also termed BlyS, a molecule important for B cell survival, was also expressed within inflammatory tissue next to bone marrow aggregates, suggesting that accumulation of B cells is triggered by local expression of molecules involved in B cell migration, homing, and survival (Fig. 6D).

FIGURE 3. Subcortical bone marrow aggregates are associated with surrogate markers for disease activity. The area covered by subcortical bone marrow aggregates linked to cortical penetration of synovial inflammatory tissue (A, C, E) as well as the area covered by small aggregates in the bone marrow, which are not linked to cortical penetration (B, D, F) was correlated to the number of swollen joints (A, B), the serum level of C-reactive protein (C, D) and rheumatoid factor (E, F).

FIGURE 4. Immunophenotypical characterization of subcortical bone marrow aggregates. Serial histological section from the specimen shown in Fig. 1, A and B, stained by Abs against CD20 for detection of B cells (A and E), CD3 for T cells (B and F), CD68 for macrophages (C and G) and CD138 for plasma cells (D and H). The images depicted in E–H show close-up views of the respective images depicted in A–D. I. Staining by Abs against CD45RO as a pan-B cell marker; J, CD27 for mature B cells; and K, CD21 for follicular dendritic cells. L, Control staining with normal mouse Ig. Note abundance of CD20 expression in subcortical bone marrow aggregates as well as the focused expression of CD138 at the interface of synovial inflammatory tissue and aggregates. A–D and I–L, original magnification ×100; E–H, original magnification ×400.
Bone marrow penetration and formation of subcortical bone marrow aggregates leads to endosteal bone formation

To address whether cortical bone reacts upon penetration and aggregate formation in the bone marrow, we investigated undecalcified plastic sections of the same joints (Fig. 7, A–H). Interestingly, the inner endosteal surface of cortical bone revealed wide areas covered by osteoblasts (Fig. 7, E–G), whereas osteoclasts were absent. Moreover, underneath these osteoblast-covered areas, new bone formation, as visualized by the presence of osteoid seams could be detected (Fig. 7, A, C, E–G). These areas were only found in the vicinity of subcortical bone marrow aggregates, whereas the endosteal surface distant from such sites was largely unaffected (Fig. 7, B and D). Quantification of these areas revealed that <5% of bone surface at sites without subcortical bone marrow aggregates was covered by osteoid seams, whereas up to 40% of endosteal bone surface was covered at sites close to the aggregates (Fig. 7H).

Severe loss of periarticular bone in RA is associated with high bone turnover

Considering the radiographic signs of periarticular bone loss in RA, we also investigated the structure of periarticular bone in this group of RA patients and for comparative purpose also in healthy controls. Histomorphometric analysis of undecalcified sections revealed massive loss of juxtaarticular trabecular bone as evident from a 4-fold decrease of bone volume in RA patients compared with normal controls (Fig. 8A). Bone loss was based on a more
than 2-fold decrease of the trabecular thickness leading to an almost 3-fold increased trabecular separation (Fig. 8, B–D). Whereas dynamic histomorphometry revealed only very low bone turnover in periarticular bone of normal individuals, as evident from the scarcity of osteoclasts and osteoblasts, both cell types were dramatically increased in trabecular bone adjacent to inflamed joints (Fig. 8, E–H).

Discussion

In this study, we describe the nature of bone marrow involvement in RA. We show that inflammatory synovial tissue can completely disrupt the cortical bone barrier resulting in a direct exposure of the underlying bone marrow to the inflammatory infiltrate. The consequence is a profound change of the adjacent bone marrow, which is characterized by the replacement of fat-rich bone marrow by a B cell-rich mononuclear cell aggregate. Such aggregates appear at the interface between invading synovial tissue and bone marrow. Aggregates were only present if cortical bone had been completely penetrated, but not underneath an intact cortical barrier. Moreover, penetration of cortical bone and bone marrow B cell aggregates were associated with an increased bone formation at the endosteum, suggesting an attempt to repair the defect from the inside (Fig. 9).

Although, destruction of juxtaarticular bone is a well known feature of RA, which has been described since the 19th century, the question, whether RA can affect the bone marrow space next to inflamed joints has never been entirely clarified (17). This is primarily based on the limited accessibility of material from human joints, which besides the synovial membrane contains cartilage, bone, and, most importantly, bone marrow. Most information from the nature of synovitis comes from needle biopsies and synovectomy, which however, does not include material from synovial tissue invading bone or cartilage. Material from joint replacement surgery is the only source containing sufficient amounts of material to allow investigation of processes in deeper region of the joint. In fact, studies on histological characterization of the junction zone have elaborated that the synovial membrane has the potential to invade subchondral regions of the joint (9, 18, 19). Moreover, there is one case report of the occurrence of a T and B cell-rich aggregate in this subchondral space (20). The subchondral region, which represents the primary target of invading synovial tissue, consists of wider areas of mineralized cartilage, which adjoins the superficial unmineralized cartilage at its distal end (also called tide mark) and the lamella of cortical bone at its proximal end. The layer of cortical bone is comparatively thin and separates articular cartilage from bone marrow. Thus, most of subchondral damage seen histologically and radiologically is, in fact, due to loss of mineralized cartilage, which in contrast to the unmineralized superficial region of articular cartilage is susceptible to degradation by osteoclasts. Whether the cortical barrier can be completely disrupted and whether marrow space can be directly exposed to synovial tissue had not been formally studied. Recent evidence from magnetic resonance imaging studies, however, suggested the possibility of a bone marrow involvement in RA. Thus, bone marrow alterations commonly termed as “bone marrow edema,” with high signal intensity on STIR or T2-weighted fat suppressed images and low signal intensity on T1-weighted images, are present in patients with RA (21, 22). Extending these observations, our study provides direct histological evidence for cortical penetration and bone marrow changes in RA.

We found localized mononuclear cell aggregates in the bone marrow at sites of inflammatory tissue invasion. Surprisingly, B cells were the leading cell type in these lesions. T cells were far less frequent, and macrophages, granulocytes, and follicular dendritic cells were almost completely absent in these lesions. Thus, these subcortical bone marrow aggregates constitute a subcompartment with a unique cellular composition. Inflamed synovial tissue, for example, has a completely distinct cellular composition with abundant macrophages but only a small fraction of B cells. Even, mononuclear cell aggregates within synovial inflammatory tissue were different in cellular composition, showing a lower proportion of B cells but more macrophages than bone marrow aggregates. The majority of cells of the latter were mature B cells expressing markers such as CD20, CD27, CD45RA, and CD79.

Although it has been known for many years that synovial tissue of RA patients harbors B cells, the observation that the juxtaarticular bone marrow serves as a pool of B cells in the direct neighborhood of arthritis is interesting. It is known that B cells in the

![FIGURE 7. Accumulation of osteoblasts and new bone formation at the endosteal bone surface next to subcortical bone marrow aggregates. A–D, Undecalcified plastic embedded tissue sections stained by Movat pentachrome showing osteoid deposition (red, arrowhead) at the endosteal bone surface next to subcortical bone marrow aggregates (A) and distant from aggregates (B). C and D show the respective close-up views. Endosteal surface from undecalcified plastic embedded tissue sections stained according to Goldner trichrome (E), Movat pentachrome (F) and von Kossa (G) showing accumulation of osteoblasts (black arrow) and osteoid deposition underneath (black arrowhead) close to subcortical bone marrow aggregates. A and C. Original magnification ×100; B and D and E–G. original magnification ×400; C and F; original magnification ×400. H. Fraction of bone surface covered by osteoid at endosteal sites with and without aggregates. Values are means ± SEM; *, significant difference (p < 0.05).](https://www.jimmunol.org/content/2585/5/2586/F7)
such as IL-4 and IL-10 (29, 30). In support of T cell activation and are producers of regulatory cytokines, cells are not only a source of rheumatoid factor production but also somatic hypermutation, and terminal differentiation (23–28). B cells in the synovial membrane of RA patients undergo affinity maturation, somatic hypermutation, and terminal differentiation (23–28). B cells are not only a source of rheumatoid factor production but also support T cell activation and are producers of regulatory cytokines such as IL-4 and IL-10 (29, 30). In ~60% of RA patients, aggregates consisting of T and B cells are a major source of B cells in the synovial tissue (23, 30). However, such aggregates are not completely specific for RA; they are also found in ankylosing spondylitis and even sometimes in osteoarthritis (31, 32). Synovial aggregates develop early in RA and increase in frequency with disease duration (33–35). Although, there are clear similarities between bone marrow aggregates and synovial aggregates, the former do not represent classical germinal centers, as synovial aggregates are less frequent than one third of RA patients do (30, 36, 37).

Importantly, bone marrow aggregates did not contain a significant number of follicular dendritic cells, which are a typical feature of germinal centers.

At the interface between synovial inflammatory tissue and subcortical bone marrow aggregates, plasma cell concentrations were maximal. In contrast, only very limited numbers of plasma cells were present in other compartments of the synovial membrane. Plasma cells have been described to accumulate around germinal center-like aggregates. This reflects the distribution as found in subcortical aggregates, when synovial tissue comes in contact with B cell rich bone marrow aggregates (24). As suggested by previous experimental data, synovial tissue and neighboring mononuclear aggregates appear to have an intensive cross talk. Thus, synovial fibroblasts can support B cell survival by molecules such as CXCL-12 (SDF-1) and VCAM-1, which is termed pseudoperiostitis (38–42). Other chemokines expressed by synoviocytes promote B cell recruitment, such as CXCL-13 (also termed BCA-1) and CCL-21 (also termed 6Ckine) (30, 43–45). In fact, expression of the two latter chemokines could be detected at the front of synovial tissue invasion in the vicinity of subcortical aggregates, and numerous MAdCAM-1-positive high endothelial venules, which are important for B cell homing, were found within aggregates (46). Moreover, synovial tissue is also a source of B cell survival factors, such as BAFF (also termed BlyS), expression of which was also found in synovial tissue close to aggregates (47). These observations suggest that synovial tissue provide signals of B cell chemotaxis, homing and activation, which allow the formation of bone marrow aggregates. Interestingly, as previously observed in synovial B cells (48), B cells of subcortical bone marrow aggregates were EBV-negative (data not shown).

Compelling evidence for new bone formation was found at sites of bone marrow aggregates. Osteoblasts accumulated and covered large areas of the endosteal bone with osteoid deposits. In contrast, bone distant from these lesions showed only few scattered bone formation sites. The exact same pattern of skeletal effects has been observed in human TNF transgenic mice (15). There were no osteoclasts attached to the endosteum; however, numerous osteoclasts were embedded in synovial tissue attacking bone from the subchondral side of compact bone. This observation indicates that the synovial and subchondral side of the cortical bone is affected by resorption, whereas formation, as an attempt to repair bone comes from the inner endosteal area of bone. This observation also supports the hypothesis that bone erosion starts from the outside due to synovial inflammation rather than from the bone marrow.

The B cell-rich bone marrow aggregates, which occur exclusively at sites of pannus penetration through cortical bone into the bone marrow space, appear to “shield” bone marrow from the invading synovial tissue by forming a physical barrier and attempting to elicit new bone formation.

Currently targeted therapy of B cells has become a promising new tool for the treatment of RA. Rituximab, an Ab directed against CD20 leads to depletion of B cells and has shown efficacy in inhibiting signs and symptoms of RA (49, 50). Due to the striking accumulation of CD20-positive B cells, the subcortical bone marrow aggregates will represent a target of rituximab therapy. Although rituximab could thus eliminate a presumably protective process, direct or systemic synovial effects of anti-CD20 may counterbalance and exceed such potentially negative aspects of

**FIGURE 8.** Histomorphometric evidence for bone loss and high bone turnover in periarticular bone. Periarticular trabecular bone was assessed by histomorphometry in a specimen from healthy normal individuals (normal, left bars) and patients with RA (right bars). The following parameters were measured: percentage of bone volume of total volume (BV/TV) (A), trabecular thickness (Tb.Th) (B), trabecular number (Tb.N) (C), trabecular separation (Tb.S) (D), percentage of osteoclast covered surface of bone surface (Oc.S/BS) (E), percentage of osteoblast covered surface of bone surface (Ob.S/BS) (F), number of osteoclasts per bone perimeter (N.Oc/B.Pm) (G), and number of osteoblasts per bone perimeter (N.Ob/B.Pm) (H). Values are means ± SEM; *p < 0.05.
this therapy, it will be interesting to learn about the effects of rituximab on structural damage.

In summary, bone marrow can be regarded as a compartment, which is actively involved in the disease process of RA. It harbors cell aggregates if the cortical barrier is disrupted and is a source for bone repair.

Disclosures
The authors have no financial conflict of interest.

References

FIGURE 9. Scheme of bone marrow invasion in RA. A. Synovial inflammation starts at the junction zone (yellow, fibroblasts; green, macrophages; red, B cells; blue, T cells). Bone (yellow), cartilage (light blue and dark blue), and bone marrow are intact. B. Synovial inflammation leads to resorption of the mineralized cartilage (dark blue) and cortical bone, which is typically known as local bone erosions. The unmineralized cartilage remains intact. This invasion is due to osteoclast formation (purple cells) driven by synovial inflammatory tissue. In addition, attachment of synovial tissue to articular cartilage is followed by cartilage invasion. C. Cortical penetration leads to bone marrow invasion of synovial inflammatory tissue and to formation of a B cell-rich aggregate, which replaces bone marrow adipocytes. Plasma cells (larger red cells) dominate at the interface between inflammatory synovial tissue and aggregate. This infiltrate is associated with increased accumulation of osteoblasts (blue cells) and bone formation at the endosteum.


