Expanded CD23⁺/CD21hi B Cells in Inflamed Lymph Nodes Are Associated with the Onset of Inflammatory-Erosive Arthritis in TNF-Transgenic Mice and Are Targets of Anti-CD20 Therapy

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Expanded CD23+/CD21hi B Cells in Inflamed Lymph Nodes Are Associated with the Onset of Inflammatory-Erosive Arthritis in TNF-Transgenic Mice and Are Targets of Anti-CD20 Therapy

Jie Li,*†,1 Igor Kuzin,*1 Safiekhkatoon Moshkani,†‡ Steven T. Proulx,* Lianping Xing,* Denise Skrombolas, † Robert Dunn,‡ Iñaki Sanz,‡ Edward M. Schwarz,*† and Andrea Bottaro†,‡

Anti-CD20 B cell depletion therapy (BCDT) is very effective for some patients with rheumatoid arthritis (RA); however the pathogenic role of B lymphocytes in RA and the primary targets of BCDT are unknown. The human TNF transgenic (hTNF-Tg) mouse model of RA displays a chronic, progressive disease that spreads from distal to proximal joints and is generally considered to be adaptive immune system independent. We have previously reported that knee arthritis in hTNF-Tg mice is accompanied by structural and functional changes of the adjoining popliteal lymph node (PLN), detectable by contrast-enhanced magnetic resonance imaging. To better understand these changes, in this paper we show that onset of knee synovitis and focal erosions are paralleled by PLN contraction and accumulation of large numbers of B cells in the lymphatic sinus spaces within the node. Flow cytometry from hTNF-Tg mice 2, 4–5, and 8–12 mo old demonstrated that B cell accumulation in the PLN follows ankle arthritis, but commences before knee disease, and involves early expansion of CD21hi, CD23+, IgMhi, CD1d+, activation marker-negative, polyclonal B cells that are found to be specifically restricted to lymph nodes draining inflamed, arthritic joints. The same B cell population also accumulates in PLNs of K/BxN mice with autoantigen-dependent arthritis. Strikingly, we show that BCDT ameliorates hTNF-Tg disease and clears follicular and CD21hi, CD23+ B cells from the PLNs. On the basis of these findings, we propose a model whereby B cells contribute to arthritis in mice, and possibly RA, by directly affecting the structure, composition, and function of joint-draining lymph nodes. The Journal of Immunology, 2010, 184: 6142–6150.

Rheumatoid arthritis (RA) is a chronic, progressive inflammatory-erosive autoimmune disease of the joints that affects as many as 1% of the population, predominantly females. Although major progress has been made in recent years in understanding the mechanisms of disease, many questions about RA pathogenesis remain unanswered. Clearly, autoimmunity and ultimately tissue destruction in RA are the result of the complex interaction of multiple contributing mechanisms. Proinflammatory cytokines, such as TNF-α, IL-1, and IL-6 play a critical, possibly primary, role in disease (1). In particular, TNF-α has emerged as a key cytokine exerting pleiotropic effects in driving the arthritis process by regulating other proinflammatory cytokines, promoting osteoclastogenesis, recruiting leukocytes to inflamed sites, and directly driving expression of enzymes responsible for tissue damage such as metalloproteinases and oxygenases (2, 3). As a result, TNF antagonists have become common in the clinical treatment of RA (4). T cell involvement in RA is highlighted by strong genetic associations with MHC haplotypes, synovial and joint infiltration by activated T cells, and the recognized role of T cells in murine models of disease, such as collagen-induced arthritis (5). In contrast, the contribution of B cells to disease has been more controversial: Although production of autoantibodies (rheumatoid factor, anti-cyclic citrullinated peptide Abs) and accumulation of immune complexes and of ectopic germinal center-like structures in the joint and synovium are common in RA patients, they are not universal features of the disease (6–8).

Despite these uncertainties, B cell depletion therapy (BCDT) with anti-CD20 Abs, originally developed for the treatment of B cell malignancies, has emerged in recent years as an effective strategy to ameliorate disease in patients who do not respond to more conventional therapy (9, 10). Although disease amelioration by BCDT underscores the importance of B cells in RA, clinical improvement does not always correlate with reduction of serum autoantibody levels, indicating that B cells may exert additional pathogenetic functions (reviewed in Ref. 11). B cells have the potential to promote autoimmune pathology by a number of Ab-independent effector mechanisms, in their role as APC, or by secreting cytokines

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The online version of this article contains supplemental material.

Abbreviations used in this paper: ALN, axillary lymph node; AU, arbitrary unit; BCDT, B cell depletion therapy; Bin, B cell in inflamed node; CE, contrast enhancement; FoB, follicular B cell; hTNF-Tg, human TNF transgenic; ILN, iliac lymph node; LN, lymph node; LNCap, LN capacity; μCT, microcomputed tomography; MLN, mesenteric LN; MRI, magnetic resonance imaging; MZB, marginal zone B; PLN, popliteal LN; RA, rheumatoid arthritis; RAG, recombination activating gene; SynVol, synovial volume; WT, wild-type.

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with proinflammatory activity (TNF, IFN-γ, IL-12p40, and so on) and chemotactic factors (MIP1-α and -β, CCL1, RANTES) (12, 13; reviewed in Ref. 14). B cells also play a critical role in the formation of ectopic lymphoid tissue structures, which are commonly observed in the inflamed synovium of RA patients and are thought to play a role in local pathogenesis (15, 16; reviewed in Ref. 17).

Human TNF-α transgenic (hTNF-Tg) mouse strains develop a disease that closely resembles RA, which, although variable in features and timing based on transgene type and expression levels, is characterized by spontaneous, chronic, progressive inflammatory-erosive joint disease, generally starting in the hind paws and advancing cephalically to the knees and fore limbs (18). The initiating role of TNF-α in this model, the lack of significant lymphocytic infiltration in the joints and synovium, the absence of detectable serum rheumatoid factor, and the finding that recombination activating gene 1 (RAG1)-deficient mice, in which the endogenous TNF gene was upregulated by gene targeting, develop ankle arthritis indistinguishable from that of their RAG-competent counterparts, has led to the conclusion that TNF-induced arthritis in Tg mice is likely B and T cell-independent (18–20; reviewed in Refs. 21, 22).

Using imaging techniques such as contrast-enhanced magnetic resonance imaging (CE-MRI) and microcomputerized tomography (μCT), we have previously shown that in the single-copy number hTNF-Tg strain Tg3647 disease progression is paralleled closely by changes in the popliteal lymph node (PLN) (23, 24). These studies developed precise metrics that allow the longitudinal study of disease progression patterns in vivo. Among the most striking of the biomarkers identified is the observation that disease progression from the ankle (starting at around 1–2 mo of age) to the knee (4–5 mo) is accompanied by a dramatic increase in PLN size and CE values (i.e., fluid content) (23, 24). Anti-TNF treatment in these mice reversed both arthritis and the associated changes in PLN structure, strongly suggesting a functional link between the two phenomena (23, 24).

In this paper, we extend these findings by analyzing later changes in PLN structure and organization associated with the progression of inflammatory-erosive disease in the knee; demonstrate an involvement of PLN B cells in these changes from the earliest stages of disease, and in particular of a CD21-high, CD23+, CD1d+ subset of B cells that accumulate specifically in inflamed nodes; identify similar B cell changes in the lymph nodes (LN)s of K/BxN mice; and show that, unexpectedly, B cell depletion significantly ameliorates disease in the hTNF-Tg model.

Materials and Methods

Animals and anti-CD20 treatment

The 3647 line of TNF-Tg mice in a C57BL/6 background were obtained from Dr. George Kollias (Institute of Immunology, Alexander Fleming Biomedical Sciences Research Center, Vari, Greece) (18). All animal studies were performed in accordance with University of Rochester Committee for Animal Resources. Starting at 3 mo of age, hTNF-Tg mice received CE-MRI bimonthly, as described (23, 24; see below), until PLN collapse was detected, at which time they received baseline μCT (23, 24). Mouse anti-mouse CD20 mAbs (18B12 IgG2a) or nonspecific placebo IgG2a Abs (2B8) (Biogen Idec, San Diego, CA) were dosed at 10 mg/kg i.p. every 2 wk for 6 wk, with continuous CE-MRI every 2 wk, followed by posttreatment μCT scan. Knee joints were subjected to histologic examination, and cells from PLN and iliac lymph node (ILN) were subjected to flow cytometry.

(KRN × NOD)F1 Tg mice were obtained by crossing KRN Tg males in a C57BL/6 genetic background (kindly provided by Dr. C. Benoist, Harvard Medical School, Boston, MA) (25) with female NOD mice (purchased from The Jackson Laboratory, Bar Harbor, ME). Offspring were bled at day 21, and those expressing the αV6-TCR KRN transgene were identified by flow cytometry. These TCR transgene-positive mice were named K/BxN mice, and non-TCR Tg littermates were used as controls. All K/BxN mice developed severe ankle joint inflammation around 1 mo of age, and the joint tissue damage progressed thereafter. The K/BxN mice and littermates used in this work were 1 y old.

CE-MRI and MR data analysis

Detailed methods of CE-MRI have been previously described (23, 24). Briefly, anesthetized mice were positioned with the knee inserted in a custom-designed mouse knee coil. MR images were obtained on a 3 Tesla Siemens Trio MRI (Siemens Medical Solutions, Erlangen, Germany). Amira 3.1 (TGS Unit, Mercury Computer Systems, San Diego, CA) was used for analysis of high-resolution CE-MRI data. For segmentation of the LN, regions of interest are manually drawn on a postcontrast three-dimensional stack of images and thresholded based on signal intensity ≥1500 arbitrary units to define the boundary between the LN and the fat pad surrounding the node. The Tissue Statistics module is used to quantify the volume of the LN and the value of CE of this tissue and of surrounding muscle. LN capacity (LNCap) is defined as the LN CE divided by muscle CE and multiplied by LN volume.

μCT and data analysis

Bone volume analysis was performed by scanning the knee joint in a Viva micro-CT 40 imaging system (Scanco, Bassersdorf, Switzerland). Patellar bone volume determination and three-dimensional reconstruction of the knee joint were performed using Amira 3.1, as previously described (23, 24).

Histology and immunohistochemistry

Knee joints were fixed in 4.5% phosphate-buffered formalin and decalcified in 14% EDTA for 7 d. Histology sections were stained with Orange G/Alcian Blue (H&E). LNs were processed using two different protocols. For immunohistochemistry, PLNs were dissected and fixed in 10% neutralized formalin. Tissues were embedded in paraffin wax, and deparaffined sections were quenched with 3% hydrogen peroxide and treated for Ag retrieval for 30 min. Sections were then stained with anti-B220 Ab (BioLegend, San Diego, CA). For multicolor immunofluorescence microscopy, fresh-frozen PLNs were cut into 7-μm-thick sections. PLN sections were fixed with 4% paraformaldehyde, rehydrated in PBS, blocked with rat serum, and stained with PE-conjugated anti-IgM (eBioscience, San Diego, CA) and FITC-conjugated anti-CD3ε (BioLegend).

Flow cytometry

Single-cell suspensions were prepared from lymphoid organs at defined stages of disease, and were analyzed for expression of surface markers with combinations of the following fluorochrome-labeled Abs: APC-Alexa 750 anti-B220 (clone RA3-6B2; eBioscience); PE-anti-IgM (clone II/41; eBioscience); Alexa Fluor 700 anti-CD19 (clone 6D5; BioLegend); Alexa Fluor 647 anti-IgD (clone 11-26c.2a; BioLegend); FITC anti-CD93 (clone AA4.1; eBioscience); Pacific Blue anti-CD21/35 (clone 7E9; BioLegend); PE-Cy7 anti-CD23 (clone B3B4; BioLegend); biotin anti-CD24 (clone M1/69; eBioscience); PE-anti-CD1d (clone 18B1; BD Pharmingen, San Diego, CA); PE-Cy5 anti-CD5 (clone 53-7.3; BioLegend); PE-Cy5 anti-CD80 (clone 16-10A1; BioLegend); Pacific Blue anti-CD86 (clone GL-1; BioLegend); biotin anti-CD40 (clone H1.2F3; BD Pharmingen); PerCP-Cy5.5 anti-CD25e (clone 7D4; BioLegend); FITC anti-IgD (clone PC61; BioLegend); PE-anti-CD4 (clone GK1.5; eBioscience); FITC anti-CD3ε (clone 17A2A; BioLegend); PE-Cy5 anti-IgD (clone 53-6.7; BioLegend); Alexa Fluor 647 anti-CCR6 (clone 140706; BD Pharmingen); PE anti-CXCR3 (clone 220803; R&D Systems, Minneapolis, MN); biotin anti-CXCR5 (clone 2G8; BD Pharmingen); PE-Cy5 anti-CR7 (clone 4B12; BioLegend); rabbit anti-mouse Ki-67 (clone SP6; Epitomics, Burlingame, CA), followed by secondary Ab PE-GOat-anti-rabbit IgH (Invitrogen, Carlsbad, CA); and PE-Texas Red streptavidin (Invitrogen). Samples were run on an LSRII cytometer and analyzed by FlowJo software (BD Pharmingen). To control for nonspecific Ab binding, isotype control experiments were conducted and resulted in nonsignificant background stains.

C D R 3 spectratyping

Total RNA from the indicated sources was isolated using TRIzol reagent (Invitrogen), and cDNAs were generated using random primers and Superscript III M-MLV reverse transcriptase (Invitrogen). The cDNA samples were subjected to PCR to amplify the CDR3 region, using a VH primer (26) and the CµR primer listed below, which maps at the 3' end of the Cµ1 exon. The PCR products were subjected to seminested PCR using VH primer and an internal FAM (6FAM)-labeled CµP primer. The seminested PCR products were run on an Applied Biosystems 3730 Genetic Analyzer at the University of Rochester Functional Genomic Center, and the resulting chromatograms were analyzed by Peak Scanner software version 1 (Applied Biosystems).
Primers sequences (5’−3’) are as follows: CαP, CAGCCCATGGC-CACCAGTTCATCATCAGAC (5’tFAM labeled); CαR, AATGTTGC-TGGCCAGGAACT; VHALL, AGTGSMARCTGCAGSAGTCWGG.

Statistical analysis

Linear mixed-effects regression models, with mouse as a random effect and time (treated as a continuous covariate) as a fixed effect, were used to assess changes over time based on longitudinal data. Differences between groups in synovial volume, LN volume, LNcap, and disease progression over time were tested by two-sided t test. The p values <0.05 were considered significant.

Results

**PLN changes associated with knee arthritis progression in hTNF-Tg mice**

We have previously shown how progression of knee synovitis in hTNF-Tg mice can be followed noninvasively via CE-MRI to quantify synovial volume (SynVol), and correlated these findings with μCT and histological results (23, 24). Although this work largely corroborated findings from cross-sectional studies demonstrating that arthritis initiates in the distal joints (e.g., ankle) and spreads to proximal joints (e.g., knee) over time (18–20), we discovered that increased knee SynVol is paralleled by an increase in volume and CE (i.e., fluid content) of the adjacent PLN, yielding parameters LNvol and LN-CE, respectively, which can be combined in a single functional biomarker, LN capacitance (LNcap = LNvol × LN-CE) (23, 24), which correlates with lymphatic flow through a LN. However, to our surprise, we found that knee synovitis in some hTNF-Tg mice is asymmetrical. Moreover, this dichotomy was associated with distinct PLN phenotypes determined by CE-MRI in which the unaffected knee drains to an expanded-contrast-enhancing PLN, whereas the contralateral knee with severe inflammatory-erosive arthritis is adjacent to a much smaller PLN that fails to take up gadolinium diethylenetriamine pentaacetic acid (Fig. 1A–1H).

To further investigate these findings, we performed a prospective study in which hTNF-Tg mice with bilateral ankle arthritis were followed with CE-MRI every 2 wk until they presented with knee synovitis, which revealed two distinct phases of disease progression (Fig. 1I). The first, characterized as the PLN “expansion” phase, is associated with increased, but relatively stable, synovial volumes without bone erosions, and large LNcap values, which indicate an expanded, fluid-filled node (exemplified in Fig. 1A, 1C, 1E). Subsequently, a yet to be identified event triggers the PLN “collapse” phase, in which LNcap values decrease rapidly owing to parallel reductions in both PLN volume and CE, whereas synovitis worsens, as highlighted by higher SynVol values (Fig. 1B, 1D, 1F). Consistent with synovitis presentation, knees that drain to an expanding PLN have no evidence of focal erosions (Fig. 1G), whereas knees adjacent to collapsed PLN display extensive bone loss (Fig. 1H). Thus, we aimed to elucidate the cellular changes in hTNF-Tg PLN, and hereafter refer to the initial PLN phase as “expansion” and the later stage as “collapse.”

![FIGURE 1.](image-url)

Asymmetrical inflammatory-erosive arthritis in hTNF-Tg knees is associated with changes in ipsilateral PLN. The knees of a 5-mo-old hTNF-Tg mouse were analyzed by two-dimensional CE-MRI of the stifle joints (A, B) and PLNs (C, D), E and F, three-dimensional MRI of the synovium (yellow) and PLN (red) with their volume; G and H, three-dimensional μCT of the proximal femur and distal tibia (aqua) with patella (yellow) volume; and I and J, ×10 magnification H&E-stained histological specimen. Asymmetrical arthritis is evident from unilateral synovitis (arrow in B), accounting for the 5-fold increase in synovial volume, and extensive focal erosions (moth-eaten bone in H) that account for the 45% loss in patellar bone volume. This asymmetrical arthropathy is confirmed by histological examination, in which only mild synovitis (arrows in J) was detected between the patella (p) and femoral chondyle (f) of the nonarthritic knee, whereas the patella of the grossly affected knee was replaced by pannus tissue. The other remarkable difference is the large, bright (expanding) PLN of the unaffected knee (C) versus the smaller, dark (collapsed) PLN in the contralateral leg (D). K. Disease progression in the knees of 3-mo-old TNF-Tg mice (n = 5) was assessed by longitudinal CE-MRI at 2-wk intervals. LNcap in arbitrary units (AU) and synovial volumes (SynVol, in mm⁴) for each CE-MRI scan were calculated, and the data are presented as the mean ± SEM. *p < 0.05 versus 2 wk before PLN collapse. Note that SynVol remains constant during the PLN expansion phase and significantly increases when LNcap greatly decreases during the PLN collapse phase of arthritic progression.
FIGURE 2. Transition from expanded to collapsed PLN phenotype is associated with B cell translocation into paracortical sinuses. A–D, Immunohistochemistry with anti-B220 Abs was performed on paraffin-embedded histological sections of the PLN from the hTNF-Tg mouse described in Fig. 1A–J, and representative images are shown at ×10 (A, B) and ×20 (C, D) original magnification. Note that the B220+ cells (brown) in the expanding PLN are primarily located in the follicles surrounding the node and the large sinus spaces are devoid of cells, whereas many B220+ cells in the collapsed PLN reside in congested sinuses. E and H, Fluorescent immunohistochemistry with anti-IgM PE (red) and anti-CD3 FITC (green) Abs was performed on fresh-frozen sections of WT PLN (E), collapsed PLN (F), and expanding PLN (G, H), and representative micrographs were taken at ×10 (E–G) and ×20 (H) magnification. Note the clearly defined B cell (b) and T cell (t) zones in WT and expanding PLN, and the vacant sinusoidal space in expanding PLN (box in G and H), which were both absent in the collapsed PLN.

B cell accumulation and migration during PLN expansion and collapse

Because the close correlation of PLN changes and hTNF-Tg arthritis progression suggests a direct link with pathogenesis, we examined the histological and cellular features of PLNs at both the expanded and the collapsed stages. As we had previously reported, expanded PLNs display dramatically enlarged and mostly acellular paracortical sinusoidal spaces, which likely account for their increased fluid content and CE, and at least in part, their size; staining with anti-B220 indicates that most B cells reside in the follicles, although some clusters of B220-positive cells are present in the sinusoidal expansion area. (Fig. 2A, 2B). In contrast, collapsed PLNs display a strikingly different structure: The sinusoidal spaces are mostly completely filled with B220+ cells, although a portion of B cells are still clearly detectable in the follicular areas; B220+ cells also infiltrate more medullary areas of the node and the T cell areas (Fig. 2C, 2D).

To better characterize these changes, we stained frozen sections from wild type (WT), expanding, and collapsed PLN with fluorescently labeled Abs to CD3e and IgM (Fig. 2E–2H). Consistent with the immunohistochemical analysis above, we noticed relatively normal follicular and T cell zone areas in the expanded

Table 1. B cell populations in hTNF-Tg peripheral lymphoid organs

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Abbreviations and cohorts are as described in the text; n = 4–13 for each group.

* p < 0.05; † p < 0.01; ‡ p < 0.001 compared with WT; \( \beta \) p < 0.05; \( \gamma \) p < 0.01; \( \delta \) p < 0.001 compared with young; \( \varepsilon \) p < 0.05, *p < 0.01, \( \iota \) p < 0.001 compared with old (two-tailed t test).

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PLNs, with occasional clusters of IgM-bright cells in both the follicles and the paracortical area (Fig. 2G–2H). However, in collapsed PLNs, the node structure was completely disrupted. IgM-high cells have extensively invaded the central areas of the node, and the integrity of the T cell zone is lost (Fig. 2F). Together, these findings strongly point to B cells as key participants in the dramatic structural and histological changes observed during PLN expansion and collapse, which accompany arthritis progression.

Changes in LN B cell populations during arthritic progression

To elucidate the nature of the B cell populations involved in the observed PLN changes, we conducted an extensive analysis of PLNs and other peripheral lymphoid organs (spleen, as well as axillary, iliac, and mesenteric LNs—ALNs, ILNs, and MLNs, respectively) from hTNF-Tg mice and WT controls by flow cytometry (summarized in Table I). hTNF-Tg mice were selected from several age groups corresponding to different stages of disease: “Young,” 4–8 wk old, displayed initial signs of ankle arthritis, but no detectable changes in PLNs or knees by CE-MRI; “expanded” samples were from mice with abnormally large (>5 mm³) PLNs with high CE values (>3), as described above (in mice with asymmetrical PLNs, the ipsilateral ILNs draining the same leg were also included in the “expanded” category for statistical analysis); “collapsed” samples were PLNs from mice in which a remarkable decrease in LNvol (<1 mm³) and LNCap were observed over 2 wk via CE-MRI, usually accompanied by exacerbation of knee arthritis (ipsilateral ILNs, spleens, MLNs, and ALNs from mice with at least one collapsed PLN were also included in the “collapsed” category for statistical analysis); and “old” Tg mice were 8–12 mo of age, with advanced hind limb disease and detectable signs of ongoing arthritis in the forepaws.

The samples were analyzed by 11-color flow cytometry with a large panel of Abs to B cell markers, as well as markers to other cell types (see Materials and Methods). Fig. 3A shows the result of a representative set of flow cytometry plots for the key markers B220, IgM, CD21, and CD23 obtained from PLNs of a cohort of mice at the various age/disease groups. The complete set of data for these markers in all examined organs is summarized in Table I. The results indicate a clear expansion of B220+ B cells, the vast majority of which are IgM+, starting from the young Tg PLN samples. The absolute numbers of PLN total B cells are, on average, 3- to 5-fold higher in hTNF-Tg mice compared with WT controls, accounting for an increase in total cellularity of the node from 1.5 to >2.2-fold. When the B220+ cells were analyzed for expression of CD23 and CD21, it became apparent that an abundant subset of B cells, coexpressing high levels of CD21 and CD23, were selectively expanded in the PLNs of hTNF-Tg mice.

Analysis of the other lymphoid organs revealed a similar picture in the ILNs, which are known to also drain the posterior leg (27) (L. Xing, E.M. Schwarz, and A. Bottaro, unpublished observations), but not in...

![FIGURE 3](http://www.jimmunol.org/) Expansion of a CD21-high CD23+ B cell population in hTNF-Tg PLNs at various stages of disease. A. PLN cells were harvested from controls and hTNF-Tg mice at defined stages of PLN progression and were analyzed by multicolor flow cytometry using Abs to B20, IgM, IgD, CD1d, CD5, CD19, CD21, CD23, CD24, and CD93 (AA4.1). Initial gating was performed on live, B220+ cells (left panels), and the resulting B cell population was then subdivided into three regions (lower left panel) based on CD21 versus CD23 staining (right panels). These three regions correspond to CD21-low/CD23+ follicular B cells (FoB, region 1); CD21-high/CD23+ MZB-like cells (MZB, region 2); and CD21-high/CD23+ Bin cells (region 3). Numbers indicate the percentage of gated cells in each region. B. Cells gated through the B cell subset regions listed above were analyzed for expression of the additional indicated B cell markers, and the results are displayed as histograms.
the MLNs or spleens of hTNF-Tg mice (Table I). Interestingly, ALNs showed significant accumulation of CD21-high, CD23+ B cells only in older mice, in which disease had spread to the fore limbs, but not in younger hTNF-Tgs, regardless of knee disease stage. Thus, CD21-high, CD23+ B cells appear to selectively accumulate in LN draining sites of arthritic inflammation, but not other nodes, and hereafter are referred to as B cells in inflamed nodes (Bin).

We then analyzed marker expression profiles on B cells gated according to CD21 and CD23 expression: CD21-low, CD23+ conventional follicular B cells (FoB), CD21-high, CD23-low marginal zone B cell (MZB)–like cells (this region was defined based on gating of MZB cells in the spleen, although these cells are virtually absent from normal LNs), and the expanded CD23+, CD21-high Bin population (Fig. 3B). The Bin population differs from FoB cells because of higher expression of CD1d, IgM, CD5, and CD24, and from MZB-like cells because of lower IgM and CD1d expression, but higher IgD (Fig. 3B). According to Allison’s classification of peripheral B cell subsets (28), these cells do not match the phenotype of the T1–T3 transitional subsets, owing to their lack of AA4.1/CD93 expression, and appear more similar, although not identical, because of lower IgM levels, to the MZB-precursor population that is normally restricted to the spleen (28, 29; reviewed in Ref. 30).

Because of their extrafollicular localization and high IgM expression levels, we tested the possibility that Bin may correspond to an expanded, activated plasmablast-like population. However, we found that they do not express significant levels of any typical activation, germinal center, or plasma cell markers, including CD80, CD86, CD69, GL7, CD138, CD27, and CD25, or any typical activation, germinal center, or plasma cell markers, including CD80, CD86, CD69, GL7, CD138, CD27, and CD25a, and they do not appear proliferative based on Ki-67 expression (Supplemental Figs. 1A, 1B, 2). In addition, no significant consistent increase in expression of Blimp-1 or AID is observed in PLNs of hTNF-Tg mice, arguing against ongoing B cell activation (Supplemental Fig. 1C). Analysis of IgH CDR3-segment lengths using spectratyping also showed that no mono- or oligoclonal expansion is observed in mRNA from total B cells or sorted Bin from hTNF-Tg PLNs, indicating that the rapid, early accumulation of these cells is unlikely to be driven by reactivity to one or a few autoantigens (Fig. 4).

Accumulation of Bin and LN structure disruption are also observed in K/BxN LNs

An important question regarding significance of Bin is whether they represent a unique population restricted to the hTNF-Tg model or whether they are more generally associated with autoimmune inflammatory arthritis. To answer this question, we characterized B cell populations in the PLNs, ILNs, MLNs, and spleen of K/BxN mice, another mouse model that develops spontaneous B cell- and T cell-dependent arthritis within 2 mo of age, owing to expression of a self-reactive I-A–restricted TCR transgene to a peptide from the glucose-6-phosphate-isomerase enzyme (25, 31, 32). We analyzed samples from four arthritic K/BxN mice, together with four littersmates with no detectable arthritis in their hind legs. As in the hTNF-Tg mice, a very significant increase in cellularity, B cell and Bin absolute numbers, and frequency was observed in the PLNs of diseased K/BxN mice compared with their healthy littersmates (Fig. 5, Supplemental Table I). A similar tendency was observed in the ILNs, but not in the spleen, whereas MLNs from arthritic animals displayed a small relative decrease in Bin cells compared with those from healthy littersmates (Supplemental Table I). Consistent with published data (33), comparison of the structure of PLNs in WT, diseased K/BxN, and hTNF-Tg mice by immunofluorescence also showed a significant expansion and distortion of the node’s histologic structure in K/BxN mice similar to that observed in hTNF-Tg PLNs, although not as severe (not shown).

Altogether, we conclude that the key observations regarding hTNF-Tg PLN structure and cellular composition are shared with the K/BxN model.

BCDT effectively clears B cells from hTNF-Tg PLNs and ameliorates knee disease

It was previously reported that onset of ankle arthritis in another strain of TNF-overexpressing (gene-targeted TNF ΔARE × RAG1−/−) animals does not require the presence of B or T lymphocytes (19), although inflammatory arthritis in the proximal joints of these mice was not noted. The results discussed above, however, clearly implicate B cells in the dramatic PLN changes associated with disease progression in the hTNF-Tg strain we used in our studies. We therefore tested the hypothesis that Bin cells are targets of anti-CD20 BCDT in hTNF-Tg mice experiencing knee flare due to collapse of the draining PLN, and whether this treatment effectively ameliorates arthritic progression.
We first established that Bin cells indeed express CD20 based on flow cytometry analysis (Supplemental Fig. 3). To test BCDT efficacy, a cohort of 10 hTNF-Tg mice with established ankle arthritis and collapsed PLN were treated with anti-CD20 Abs every 2 wk for 6 wk, and the progression of knee synovitis in these animals during the treatment period was compared with progression in a cohort of four hTNF-Tg animals treated with a placebo Ab. Fig. 6A shows the extent of B cell depletion in PLN, ILN, and spleen of a representative hTNF-Tg mouse that completed BCDT. B cells were significantly depleted from the PLNs (>85% decrease in absolute numbers compared with controls), although at somewhat lower level than in spleen (>95% reduction). Interestingly, both the FoB and the Bin populations were equally reduced, whereas the MZB-like CD23+/CD21-high cells represented the bulk of the residual cells after treatment. Strikingly, disease progression was essentially arrested in the BCDT cohort, with SynVol stabilizing over the 6 wk. In contrast, we observed a significant increase in SynVol over time (1 mm³/wk; p < 0.002) in the untreated control group, which culminated in a significant (p < 0.05) increase versus the BCDT cohort at 6 wk (Fig. 6L). Fig. 6C illustrates an extreme case in which SynVol actually decreased following BCDT; note that the increase in CE and LNcap for the PLN suggests a “reopening” of the lymphatic flow through the node. These results show that BCDT is effective in the treatment of arthritic flare in the hTNF-Tg mouse, strongly suggesting a pathogenetic role for B cells in disease progression.

Discussion

Although the identification of autoantibodies in the serum of RA patients dates back to the 1950s, the role these autoantibodies and B cells may play in the pathogenetic processes of the disease is still poorly understood. One of the main reasons for this uncertainty is the underlying heterogeneity of the human patient population, which provides a strong rationale for the use of genetically and etiologically homogeneous mouse models of disease to tease out possible contributing factors. Among the many available arthritis models, the hTNF-Tg strain stands out for sharing several important characteristics with human RA, including the spontaneous, progressive nature of the disease and the well-recognized pathogenetic role of TNF-α. Although experiments with the TNF-overexpressing TNF ΔARE strain in a RAG-deficient background indicated that B and T lymphocytes are not required for arthritis onset (19), the experiments detailed above highlight several key features accompanying arthritis progression in hTNF-Tg mice that implicate B cells in at least some aspects of pathogenesis.

First, we show that onset of arthritic disease is paralleled by a dramatic increase in the B cell component of the draining LNs, which involves most markedly a population with a unique CD23⁺, CD21-high, IgM-high, IgD⁺, CD1d⁺ phenotype. These B cells are preferentially restricted to LNs draining arthritic tissues, suggesting that their accumulation is dependent on signals coming from the affected joints. However, expansion of the same population in K/BxN mice clearly indicates that this Bin population is not a unique feature of the TNF-Tg microenvironment. The rapid and significant expansion of B cells, and particularly of the Bin population, in the early stages of disease in hTNF-Tg mice does not appear to be dependent on one or a few autoantigens. Whether Bin cell expansion is equally polyclonal in K/BxN mice, in which a large proportion of LN B cells are known to be expressing anti-gpi Abs (33), remains to be determined. Our clonality analysis cannot rule out the possibility that hTNF-Tg PLN B cells are more broadly autoreactive (beyond the limit of detection of oligoclonal expansion by spectratyping) or that clonal populations may be selected as disease progresses, but the lack of expression of activation and plasma cell markers on these cells implies that, regardless of their Ag specificity, they are not directly involved in conventional immune responses within the node. Thus, it seems more likely that Bin cells arise as a polyclonal, possibly Ag-independent, population that is associated with arthritis, regardless of the primary cause and nature of autoantigen, and may exert additional roles in the context of disease progression.

Interestingly, B cells with a range of phenotypes that resemble marginal zone precursors, are CD1d⁺, and in some cases CD21-high have been defined as a regulatory, anti-inflammatory subset in...
a number of murine autoimmune conditions, including arthritis models (34–37; reviewed in Refs. 38, 39). The feature common to these B-regulatory subsets is their ability to produce IL-10, but according to our preliminary observations, hTNF-Tg CD23^{+}CD21-high B cells do not seem to be capable to prominently express this cytokine or proinflammatory cytokines such as TNF-α (human or mouse) and IFN-γ (Supplemental Fig. 3). Thus, although it is tempting to speculate that Bin cells may be a regulatory subset specifically recruited/differentiated at sites of ongoing inflammation, further analysis will be required to directly address this possibility. Interestingly, it was recently reported that T-regulatory cells are inherently unstable and can transition to a pathogenetic state and accumulate at inflammation sites (40), highlighting the fluid nature of regulatory populations and their potential to contribute to pathogenesis. If Bin cells do play a regulatory role in pathogenesis, however, a central function in the progression of inflammatory processes at the LN level seems more likely than local effects at the inflamed sites, because minimal, if any, lymphoid infiltrates are known to be present in the arthritic joints of these mice (20, 41, 42).

The second key observation we have made in this paper is that a close correlation exists between the exacerbation of knee disease in the hTNF-Tg strain and significant changes in the structure of the ipsilateral PLN, with a marked reduction in node capacitance and a massive migration of B cells into the expanded lymphatic spaces in the node ("collapse" phase). In support of this correlation, we have observed hTNF-Tg knees of 1.5-y-old mice with expanding PLN ≥20mm^{3} (10× WT) that never developed inflammatory-erosive arthritis. However, this correlation is not absolute, as we have also observed some hTNF-Tg knees (~20%) with expanding PLN and inflammatory-erosive arthritis. Thus, PLN collapse appears to be a prominent, but not necessary, component for the initiation of inflammatory-erosive arthritis of the knee in mice. To better understand lymphatics in this model, we have reported that arthritis in mice is accompanied by an increase in lymphangiogenesis and that lymphangiogenesis and lymphatic drainage are reciprocally related to the severity of joint lesions during the development of chronic arthritis (43, 44). These results are consistent with expansion of the sinusoids in the draining LNs and the higher PLNcap that we have shown by CE-MRI and histologic study, which is associated with earlier stages of disease (23) (Fig. 1). Thus, we hypothesize that the reduction in LNcap and an open sinusoidal space caused by B cell migration would correlate with a reduction in the lymphatic flow capacity of the draining LNs, with resulting reduction in the clearance of inflammatory cells and factors from the drained sites. In the case of knee arthritis in hTNF-Tg mice, this would result in the "flare" in synovitis and bone erosion that is observed in the PLN collapse phase. Because human RA is well known to alternate between moderate stages of inflammation and acute flares, the origin of which is yet unexplained, this finding may also represent an intriguing candidate for a more general mechanism of disease behavior.

Two critical issues with regard to the collapse process are the nature of the signals that induce migration of B cells from the follicular sites to the sinusoidal spaces, and whether B cell migration is causal to the collapse or simply associated with it. On the basis of immunohistological analysis, the migrating cells are preferentially of a IgM-high phenotype, suggesting they may be the same CD23^{+}, CD21-high cells that are observed accumulating during the expansion stage. However, only adoptive transfer experiments of purified, identifiable cells of the various subsets can answer the question of direct lineage relationship. Regardless, the distinct phenotype of the migrating population renders it amenable to specific functional studies aimed at identifying the potential chemotactic signals responsible for their unusual localization.

Finally, we have shown that BCDT is effective in ameliorating disease in hTNF-Tg mice. This is a startling observation, because of the commonly accepted paradigm that arthritis in TNF-overexpressing mouse models does not require adaptive immunity. Several possibilities can reconcile these findings. First, it is likely that the levels of TNF overexpression in the Tg3647 strain used in our study are lower than those in the TNF ΔARE mice used by Kontoyannis and coworkers (19), making disease in Tg3647 mice more dependent on additional mechanisms. Certainly, TNF ΔARE mice display a far more aggressive disease phenotype than do Tg3647 mice, and die by 3 mo of age (18). An additional and more interesting possibility is that we are looking at two different stages of disease, with potentially different proximal causal mechanisms. Both Tg3647 and TNF ΔARE first develop arthritis in the ankle, where disease commences with infiltration of the tendon sheaths by granulocytes and macrophages, and the formation of osteoclasts next to the inflamed tendon sheaths (20). Then, the tenosynovitis rapidly progresses into pannus-like tissue largely devoid of lymphocytes, with osteoclasts mediating focal erosions. Although this earlier stage is dominated by innate immunity components, we would like to suggest in this paper that there exists a second stage, associated with knee "flare" and PLN collapse, which is B cell dependent. If this is the case, the variability in clinical effectiveness of BCDT in RA patients may be in part due to the type/stage of disease primarily responsible for that patient's symptoms. Future preclinical and clinical studies prospectively designed to assess the cause–effect relationship of BCDT on lymphatic flow are warranted to test this hypothesis.

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References


