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J Immunol 2012; 188:5944-5953; Prepublished online 16 May 2012;
doi: 10.4049/jimmunol.1103071
http://www.jimmunol.org/content/188/12/5944

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/05/16/jimmunol.1103071.DC1

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CD23^{+}CD21^{high}CD1d^{high} B Cells in Inflamed Lymph Nodes Are a Locally Differentiated Population with Increased Antigen Capture and Activation Potential

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CD23^{+}CD21^{high}CD1d^{high} B cells in inflamed nodes (Bin cells) accumulate in the lymph nodes (LNs) draining inflamed joints of the TNF-α-transgenic mouse model of rheumatoid arthritis and are primarily involved in the significant histological and functional LN alterations that accompany disease exacerbation in this strain. In this study, we investigate the origin and function of Bin cells. We show that adoptively transferred GFP⁺ sorted mature follicular B (FoB) cells home preferentially to inflamed LNs of TNF-alterations that accompany disease exacerbation in this strain. In this study, we investigate the origin and function of Bin cells. We show that adoptively transferred GFP⁺ sorted mature follicular B (FoB) cells home preferentially to inflamed LNs of TNF-α-transgenic mice where they rapidly differentiate into Bin cells, with a close correlation with the endogenous Bin fraction. Bin cells are also induced in wild-type LNs after immunization with T-dependent Ags and display a germinal center phenotype at higher rates compared with FoB cells. Furthermore, we show that Bin cells can capture and process Ag-immune complexes in a CD21-dependent manner more efficiently than can FoB cells, and they express greater levels of MHC class II and costimulatory Ags CD80 and CD86. We propose that Bin cells are a previously unrecognized inflammation-induced B cell population with increased Ag capture and activation potential, which may facilitate normal immune responses but may contribute to autoimmunity when chronic inflammation causes their accumulation and persistence in affected LNs. The Journal of Immunology, 2012, 188: 5944–5953.

The involvement of B cells in the pathogenesis of several autoimmune disorders, such as rheumatoid arthritis (RA), systemic lupus erythematosus, and autoimmune diabetes, is well established (1, 2, reviewed in Ref. 3). Although autoantibody production is still broadly considered to be the primary effector mechanism of B cells in these diseases, recent research highlighted other key B cell pathogenetic processes that contribute to the establishment and maintenance of autoimmune states, including Ag presentation, T cell costimulation, and secretion of cytokines (4–8). Sifting through this multiplicity of potential effects by B cells to identify the key mechanisms in each specific condition is daunting, but it is essential for a full understanding of disease pathogenesis and the design of targeted therapies.

RA was one of the first autoimmune diseases in which B cell involvement was postulated based on autoantibody production; however, this theory fell into disfavor later because of a lack of direct correlation between autoantibodies and pathogenesis (9–11). However, the recent success of B cell-depletion therapy in RA patients refractory to other therapies has rekindled interest in the role of B cells in this disease (12–14). The observation that clinical improvement in patients does not always correspond with a reduction in serum autoantibody levels has focused attention on the possibility of Ab-independent pathogenic function(s) for B cells in RA (15, 16).

The process of inflammation is intrinsically tied to autoimmune manifestations through recruitment and differentiation of effector cells, mediation of tissue necrosis and destruction by cytolytic and proteolytic enzymes, induction of tissue swelling, and pain (17–19). More interestingly, from an immunological perspective, inflammation has also been linked to the onset of autoimmunity by causing alterations in T cell (and possibly B cell) subset balance, affecting central and peripheral tolerance mechanisms, and directly inducing the differentiation of tertiary lymphoid structures at target organs, such as the pancreas β-islets or the rheumatoid synovium, via the process of lymphoid neogenesis (20–23). B cells can be active participants in inflammatory processes: in addition to causing autoantibody/immune complex (IC) deposition and autoantibody-mediated cell death, they can be primary effectors of lymphoid neogenesis (23–25), can capture and transport ICs to facilitate responses within lymphoid organs (26), and secrete both proinflammatory and regulatory cytokines (27–30).

In addition to actively contributing to inflammatory processes, B cells themselves can be profoundly affected by environmental changes during inflammation (31, 32). In particular, inflammatory cytokines were shown to induce significant, specific changes in B lymphopoiesis, as well as peripheral differentiation, which, in turn, may affect the tolerance versus autoimmunity balance. For instance, chronic inflammation can induce IL-2–mediated apoptosis in marginal zone B cells, altering Ag trafficking and IC clearance in the spleen; IFN-α was shown to provide an initial
trigger for the differentiation of proinflammatory B effector-1 cells; reduced production of bone marrow CXCL12 during inflammation results in mobilization of immature B cells to the spleen; and B cell-activating factor can directly alter peripheral tolerance checkpoints for autoreactive B cells in RA (33–36). In recent years, these and other findings led to the emergence of a new appreciation of the influence of the inflammatory microenvironment on B cell development, differentiation, mobilization, and survival (37).

The human TNFα-transgenic (TNFtg) mouse strain Tg3647 carries a single human TNF transgene copy and develops a slowly progressing inflammatory joint disease very similar to RA (38, 39). In this model, a chronic and progressive inflammatory-erosive joint disease generally starts with ankle swelling and advances to the knee and forelimbs over time (38, 39). By using noninvasive small animal-imaging techniques, the progression of the disease from ankle to the knee and the onset of arthritic flares were shown to be accompanied by significant changes in the size, fluid content, and draining function of the adjoining lymph nodes (LNs). Moreover, both the disease and the associated LN changes are reversible upon anti-TNF treatment (40, 41). With further histological and flow cytometric analysis, we demonstrated that B cells are involved in the observed structural alterations of the affected TNFtg popliteal LNs (PLNs) and iliac LNs from the earliest stages of disease (42, 43). A unique B cell subset (B cells in inflamed nodes [or Bin cells]), characterized by a CD23+CD21highCD1dhigh surface phenotype, was found to dramatically accumulate specifically in the LNs draining TNFtg inflamed joints and to be primarily involved in the histological disruption of the affected LNs (42, 43). We also showed that B cell depletion ameliorates disease in TNFtg mice, highlighting a previously unrecognized role for B cells in this model (42).

The Bin population phenotype does not correspond to any of the main mature B cell subsets in the mouse periphery, but it shares similarities with some immature spleen subsets (44), as well as with B cells with regulatory functions (B-regulatory or B-10 cells) observed in several autoimmune models (45–48). The functional significance of high levels of CD21 (complement receptor 2; involved in IC capture, but also a receptor for CD23 and possibly other surface and secreted molecules) and/or of CD1d (involved in IC capture, but also a receptor for CD23 and possibly other surface and secreted molecules) and/or of CD1d (involved in presentation of glycolipid Ags) (49) on these cell types is still unclear. However, it is known that engagement of CD21 on B cells can lower the threshold of B cell activation (50–52, reviewed in 50). The Bin population phenotype does not correspond to any of the main mature B cell subsets in the mouse periphery, but it shares similarities with some immature spleen subsets (44), as well as with B cells with regulatory functions (B-regulatory or B-10 cells) observed in several autoimmune models (45–48). The functional significance of high levels of CD21 (complement receptor 2; involved in IC capture, but also a receptor for CD23 and possibly other surface and secreted molecules) and/or of CD1d (involved in presentation of glycolipid Ags) (49) on these cell types is still unclear. However, it is known that engagement of CD21 on B cells can lower the threshold of B cell activation (50–52, reviewed in 50).

Given the evidence linking Bin cells to the structural/functional alterations in TNFtg LNs, as well as to disease pathogenesis and progression in this model, it is important to identify their origin and functional characteristics. In this article, we describe the results of further experiments aimed at addressing these questions, which show that Bin cells can directly differentiate locally from mature conventional follicular B (FoB) cells within inflamed LNs, as well as that they display an enhanced ability to capture and process Ag via CD21 and to exhibit a germinal center (GC) phenotype during T cell-dependent immune responses. This suggests a new link between the inflammatory microenvironment and B cell function in peripheral lymphoid organs, with general relevance to immune responses in normal and autoimmune conditions.

Materials and Methods

Mice

All experimental procedures involving mice were performed under the approval of the University of Rochester Committee on Animal Resources and according to all applicable federal and state regulations. All of the mice used in this study were on the C57BL/6J genetic background. The 3647 line of human TNFtg mice (38) was originally obtained from Dr. George Kollias (Institute of Immunology, Alexander Fleming Biomedical Sciences Research Center, Vari, Greece) and maintained by breeding with wild-type (WT) C57BL/6 mice. TNFtg mice and age-matched WT littermates were used for all of the adoptive-transfer and immunization experiments. Transgenic mice with an enhanced GFP EDNA under the control of a chicken β-actin promoter (55) were maintained by breeding to WT mice. CD45.1 congenic mice were obtained from The Jackson Laboratory and maintained by sibling inbreeding. OT-II mice (56) were used as source of OVA peptide-specific CD4+ T cells.

Flow cytometry

Single-cell suspensions were prepared from lymphoid organs by mechanical disruption and stained with a mixture of fluorochrome-conjugated anti-mouse mAbs: B220 (RA3-6B2), IgM (11/41), CD80 (16-10A1), GL7 (GL7), PD-1 (343), CD62L (MEL-14), CD44 (IM7), CD11b (M1/70), CD11c (N418), CD45.1 (A20), and CD45.2 (104) from eBioscience; CD19 (6D5), CD21/35 (TE9), CD23 (B3B4), CD4 (GK1.5), and CD86 (GL-1) from BioLegend; and CD21/35 (7G6), CD1d (B3B4), I-A/E (M5/114.15.2), CD95 (J02), and CXC5R (2G8) from BD Pharmingen. This was followed by PE-Texas Red streptavidin (Invitrogen) staining if biotin-conjugated Ab was included in the staining panel. All of the samples were stained for dead cell exclusion using Live/Dead fixable violet dead cell stain kit (Invitrogen). Samples were run on a 12-color LSRII cytometer (Becton Dickinson Pharmingen) and analyzed by FlowJo software (Tree Star, Ashland, OR). Bin cells were defined as CD19+ or B220+, CD23+CD21highCD1dhigh and confirmed to be CD1d+ on separate gating. Gates for these markers were defined for each experiment based on their distribution on parallel samples of spleen B cell subsets (CD23−CD21−FoxB versus CD23+FoxB) and CD21highFoxB+ marginal zone B cells.

Adoptive-transfer experiments

Single-cell suspensions were generated from WT enhanced EGFP-transgenic mouse spleens, and after hypotonic RBC lysis, cells were stained with anti-B220, anti-CD19, CD80, I-A/E, 114.15.2, CD95 (JO2), and CXCR5 (2G8) from BD Pharmingen. This was followed by PE-Texas Red streptavidin (Invitrogen) staining if biotin-conjugated Ab was included in the staining panel. All of the samples were stained for dead cell exclusion using Live/Dead fixable violet dead cell stain kit (Invitrogen). Samples were run on a 12-color LSRII cytometer (Becton Dickinson Pharmingen) and analyzed by FlowJo software (Tree Star, Ashland, OR). Bin cells were defined as CD19+ or B220+, CD23+CD21highCD1dhigh and confirmed to be CD1d+ on separate gating. Gates for these markers were defined for each experiment based on their distribution on parallel samples of spleen B cell subsets (CD23−CD21−FoxB versus CD23+FoxB).

In vivo proliferation analysis

CD19+ cells were purified from spleen of CD45.1+ mice by positive selection by MACS (Miltenyi Biotec). The purified B cells were labeled with 1.25 μM CFSE (Cell Trace CFSE; Molecular Probes). A total of 18–20 × 10⁹ CFSE-loaded CD45.1+CD19+ B cells was transferred by tail vein injection into CD45.2 WT or TNFtg recipients. Seventy-two hours later, the cells from lymphoid tissues were harvested, live cells were analyzed for expression of CD19 and CD45.1, and the proliferation of CD19+CD45.1+ B cells was measured based on CFSE dilution. CFSE-loaded cells were tested for proliferation in parallel by in vitro stimulation with 20 μg/ml LPS in complete RPMI 1640 medium, 10% FBS.

Immunizations

WT and TNFtg mice (3–4 mo of age) were immunized in one footpad with 25 μg chicken OVA (Sigma Aldrich) in CFA (Sigma Aldrich). 20 μl final volume. Fifty microliter of a 50:50 (v/v) solution of 0.5 mg/ml FITC in acetone/dibutylphthalate (25 μg FITC total) was applied by painting to the immunized footpad skin at day 13 after immunization. On day 14, the animals were sacrificed, and PLN cells were harvested and stained for analysis by flow cytometry.

For detection of Ag capture by B cells (15-h immunization experiments), FITC-OVA conjugate (Molecular Probes) was mixed in CFA. 20 μl emulsion containing 50 μg Ag conjugate was injected into one footpad of each animal, and the draining LN cells were harvested 15 h later and stained for flow cytometry analysis.

For detection of Ag processing by B cells, OVA-DEA (Molecular Probes) was mixed with CFA, and 20 μl emulsion containing 50 μg OVA was injected into one footpad of each animal. Twenty hours after immunization, draining LN cells were harvested and analyzed by flow cytometry. Cells harboring processed OVA (Ex/Em: 505/515 nm) were detected by fluorescence in the FITC channel. In all experiments, PLNs from the contralateral unimmunized leg were used as paired controls.
Hyperimmune serum preparation

Hyperimmune serum against OVA was prepared by immunizing C57BL/6 mice with 50 μg OVA three times at 2-wk intervals (57). Alum (Injtech Alum; Pierce) was used as adjuvant in primary i.p. immunization, and IFA (Sigma Aldrich) was used for secondary and tertiary s.c. immunization. Ten days after the last immunization, sera were collected, pooled, and used for preparation of ICs.

In vitro IC preparation and cell loading

Insoluble ICs were prepared in a U-bottom 96-well plate by incubation of FITC-OVA with the pooled hyperimmune serum at 37°C for 2 h (57), followed by two washes in RPMI 1640 medium (Invitrogen) and centrifugation at 1000 × g, 4°C for 10 min to eliminate unbound materials. Inactivation of serum complement in control experiments was carried out by incubation at 56°C for 1 h prior to use in IC preparation. Total PLN cells were harvested from TNFtg mice and left untreated or pretreated with 10 μg/ml CD21-blocking Ab (anti-CD21/35, clone 7G6; BD Pharmingen) (50) or isotype control (clone A95-1; BD Pharmingen) for 1 h. A total of 0.5 × 10⁶ cells was added to the generated ICs in 96-well plates, incubated for 30 min, washed extensively, and stained with fluorochrome-conjugated Abs to B220, CD3, CD23, and CD21/35 (clone 7E9, not competing with the 7G6 clone epitope) for flow cytometry analysis.

Adjuvant-mediated Bin induction

A 50:50 (v/v) of Alum, IFA in PBS, or 10 μg CPG (ODN 2006: 5'-TCG TCG TTT TGT CTT TGT GTC GTT-3') in PBS was prepared, and 20–25 μl each preparation was used for footpad injection. The same volume of PBS was injected into contralateral footpads as control. On day 10 after injection, PLN cells were harvested for flow cytometry analysis.

ELISA

Immulon 1B 96-well plates (Thermo Labsystems) were coated with 5 μg/ml OVA and blocked with 5% BSA. Serum samples were diluted in 1% BSA and transferred to the plate in duplicates. Alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotech) and Sigma 104 phosphatase substrate (Sigma) were used to detect anti-OVA IgG. IFN-γ in B:T coculture supernatants was measured using an AN-18 mouse IFN-γ ELISA kit (BD Biosciences), according to the manufacturer’s instructions.

Th1 polarization

Total lymph node cells were harvested from OT-II mice and cultured with 1 μg/ml OVA323–339 peptide (InvivoGen) and 0.1 ng/ml IL-12p70 (Peprotech) to activate and polarize naive CD4 T cells toward Th1. Cells were checked daily to prevent overgrowth. IL-7 (1 ng/ml) (Peprotech) was added to the culture from days 4 to 7; on day 14, resting cells were used for coculture experiments.

B:T coculture assay

Sorted FoB (CD23⁺CD1⁺low) or Bin cells (CD23⁺CD1⁺high) were cocultured with resting Th1 polarized OT-II T cells (1:10) in 50 μl well in triplicates, with or without 5 μg/ml OVA323–339. After 36 h, the culture supernatants were harvested, and IFN-γ secretion was measured by ELISA.

Statistical analysis

Linear regression using Pearson’s coefficient was used to analyze the correlation between exogenous Bin (GFP⁺) and endogenous Bin cells in adoptive-transfer experiments. The nonparametric two-tailed Mann–Whitney U test was used for unpaired comparisons, and the two-tailed Wilcoxon matched-pairs signed-rank test was used for paired variable groups. ANOVA with the Bonferroni posttest was used for multiple group comparisons. Graphs were generated with GraphPad Prism 5 or Microsoft Excel.

Results

B cells are preferentially recruited to TNFtg inflamed LNs and quantitatively acquire a Bin phenotype

Accumulation of Bin cells in large numbers in inflamed LNs of TNFtg mice could be due to the migration of a specific B cell subset/lineage or their in situ differentiation from precursor cells of a different phenotype, including conventional CD23⁺CD21low FoB cells, or transitional/marginal zone precursor cells with a CD23⁺CD21high phenotype. To test the hypothesis that Bin cells may differentiate from conventional FoB cells, GFP⁺CD23⁺CD21low B cells were sorted by FACs from the spleen of non-TNFtg GTP-transgenic mice (Supplemental Fig. 1A) and adoptively transferred into WT and TNFtg recipients. Seventy-two hours later, single-cell suspensions were generated from spleens, PLNs, mesenteric lymph nodes (MLNs), and pooled axillary LNs (ALNs) and brachial lymph nodes (BLNs) of recipients, and the resident cells were analyzed by flow cytometry. A larger fraction of GFP⁺ B cells was consistently recovered from inflamed LNs (PLNs, ALNs+BLNs) compared with their WT counterparts and TNFtg noninflamed sites (Fig. 1A).

Analysis of the recovered GFP⁺ fractions in individual recipients showed that a significant fraction of transferred GFP⁺ cells in TNFtg PLNs had acquired a Bin phenotype. Strikingly, there was a highly significant correlation (r = +0.97, p < 0.0001) between the fraction of GFP⁺ exogenous Bin cells and that of the host endogenous Bin cells in the same recipients’ PLNs (Fig. 1B). Although the fraction of GFP⁺ Bin varied by organ, a similar correlation with endogenous Bin was found in other inflamed LNs (ALNs+BLNs, r = +0.9, p < 0.01, Supplemental Fig. 1B), and, to a lesser extent, in the spleen (r = +0.8, p = 0.02, Supplemental Fig. 1C), whereas the correlation was close but did not reach the significance threshold for MLNs (r = +0.7, p = 0.06, Supplemental Fig. 1D). Similar results were obtained after transfer of total CD19⁺ WT LN cells into TNFtg or WT recipients, regardless of the initial fraction of Bin-like cells (data not shown). Thus, Bin cells can differentiate locally from mature FoB cells within a proinflammatory LN microenvironment, rather than being a distinct subset migrating into inflamed nodes, and their phenotype induction is the result of local signals acting quantitatively on the Bin resident cells.

In addition to the differentiation of Bin cell, another notable change observed in TNFtg PLNs is the massive accumulation of B cell numbers as disease progresses (42). This could be due to local proliferation, preferential homing or retention of B cells in the affected nodes, or a combination thereof. As noted above (Fig. 1A), a larger fraction of the transferred GFP⁺ B cells was recovered from inflamed LN into TNFtg mice compared with other sites or with WT recipients at 72 h after adoptive transfer. This paralleled the accumulation of endogenous B cells in inflamed LNs. Because trafficking studies showed that the average time of permanence of B cells within a LN is ~24 h after entrance (58), a new set of adoptive-transfer experiments was performed over a shorter time (20 h) to distinguish between preferential migration/recruitment into the inflamed nodes and cell retention. In this shorter adoptive-transfer period, only the first wave of immigrant B cells would be expected to be detectable in each examined LN. The results showed that, by 20 h, a significantly higher number of transferred GFP⁺ FoB cell had entered the TNFtg LNs compared with WT LNs, suggesting that preferential homing is a primary mechanism for B cell accumulation in the inflamed nodes (Fig. 1C), consistent with previous results on inflammation-mediated lymphocyte recruitment to LNs (59). Interestingly, a significant fraction of the transferred cells was shown to already display a Bin phenotype at this earlier time point after their entrance into the inflamed LNs (4.2 ± 6.0% GFP⁺ Bin in WT versus 8.3 ± 1.8% GFP⁺ Bin in TNFtg PLNs, p = 0.02, Fig. 1D).

To rule out a contribution of proliferation in the local accumulation of B cells in the inflamed nodes of TNFtg mice, magnetically purified CD19⁺ splenic B cells from CD45.1⁺ congenic WT mice were loaded with CFSE and transferred into either WT or TNFtg CD45.2⁺ recipients. Seventy-two hours later, the CFSE
dilution on transferred CD45.1+ B cells was analyzed by flow cytometry. No detectable proliferation of CD45.1+-gated B cells was found in either recipient strain (Fig. 1E).

Taken together, these findings indicate that TNFtg LNbs draining arthritic joints are able to recruit higher numbers of recirculating B cells, as well as that the milieu in the node quantitatively induces the immigrant B cells to acquire the Bin phenotype in the absence of proliferation. Thus, the massive accumulation of total B and Bin cells responsible for TNFtg structural and functional PLN alterations is a consequence of processes intrinsic to the inflamed nodes.

Bin cells and immune responses in TNFtg and WT LNbs

Our previous findings showed that TNFtg LNbs do not appear to be undergoing Ag-specific immune responses and that Bin cells within the nodes do not show signs of activation or Ag-dependent clonal expansion (42). However, we wondered whether Bin cells would be able to engage in normal immune responses when appropriately challenged, as well as whether the massive expansion of Bin cells in TNFtg LNbs would affect the node’s ability to respond to immunization. To address these questions, TNFtg and WT mice were immunized in one footpad with OVA in CFA; 2 wk later, the B and T cell subsets in immunized and nonimmunized contralateral PLNbs were analyzed by flow cytometry.

The absolute number of B cells in immunized PLNbs of both TNFtg and WT mice increased significantly compared with their unimmunized counterparts (Fig. 2A), as did the relative percentage of B cells within the nodes (18.4 ± 7.5% in immunized to 34.2 ± 9% in immunized PLNbs, p = 0.002 for WT and 37.1 ± 6.5% to 44 ± 11%, p = 0.0005 for TNFtg PLNbs). Unexpectedly, the percentage of Bin in immunized WT PLNbs also increased significantly (27 ± 10% versus 7 ± 4% in unimmunized contralateral PLNbs, p = 0.002, Fig. 2B). As a result, despite the dramatic differences in the baseline numbers of Bin cells, 2-wk immunized PLNbs from both TNFtg and WT mice harbored similar numbers of CD23+CD21highCD1dhigh Bin cells (Fig. 2C). This previously unrecognized increase in Bin-like B cells in the immunized LNbs of WT mice is consistent with the adoptive-transfer findings above, which suggest that environmental cues in the nodes’ inflammatory milieu can induce the Bin phenotype in normal FoB cells. Similarly to adoptively transferred cells, induction of Bin cells after immunization occurs rapidly, with increased Bin cells detectable in immunized WT PLNbs as early as 20 h postimmunization compared with
unimmunized contralateral nodes (from 2.7 ± 0.9% to 4.2 ± 1.4%, p = 0.03).

To test whether inflammatory signals alone could induce Bin cell formation, WT mice were injected with Alum, CpG DNA, or IFA in one footpad and PBS in the contralateral footpad. Ten days after injection, the PLNs draining the adjuvant-injected sites were compared with the contralateral footpads. Increased frequency and absolute numbers of Bin phenotype cells were found in PLNs draining adjuvant-injected sites (Supplemental Fig. 2A, 2B). Together with the finding of Bin cells in TNFtg LNs in the absence of significant B or T cell activation, these observations suggest that Bin cell induction is primarily dependent on inflammation and not antigenic stimulation.

The numbers of GC B cells in 2-wk immunized PLNs, based on coexpression of CD95 and the GC-specific marker GL7, was similar in TNFtg and WT mice (Fig. 3A, 3B). However, when B cells were gated based on their FoB and Bin phenotype and analyzed for GC marker expression, a larger fraction of Bin cells within both WT and TNFtg immunized LNs displayed the GC phenotype compared with FoB cells in the same node (Fig. 3C). Thus, Bin cells not only can participate actively in T-dependent immune responses but, in fact, may be more readily involved in the GC reaction. Analysis of surface markers on GC+ B cell subsets showed that Bin cells stain more intensely with an anti-CD80 Ab compared with their FoB counterparts (Fig. 3D). Furthermore, MHC class II (MHCII) expression was higher on Bin

**FIGURE 2.** B cell numbers and subsets in WT and TNFtg PLNs 2 wk after footpad immunization. (A) Absolute numbers of live B220+ B cells in immunized (filled symbols) and contralateral unimmunized (open symbols) PLNs of WT (circles) and TNFtg (squares) mice. Although larger numbers of B cells are present in the TNFtg LNs at baseline, the values in WT and TNFtg equalize after immunization. (B) Representative flow cytometry plot comparing B cell subsets in unimmunized (left panel) and immunized (right panel) PLNs from a WT mouse. Note the increased percentage of Bin after immunization. (C) Absolute numbers of CD23+CD21+CD1d+ Bin cells in immunized and unimmunized PLNs from WT and TNFtg mice. Combined results of three independent experiments: n = 10 for WT and n = 12 for TNFtg. **p < 0.01, ***p < 0.001, Mann–Whitney U test for analysis between groups (WT versus TNFtg) or two-tailed Wilcoxon matched-pairs signed-rank test for analysis within a group (WT or TNFtg). Im-Tg, Immunized TNFtg PLN; Im-WT, immunized WT PLN; Tg, unimmunized contralateral TNFtg PLN; WT, unimmunized contralateral WT PLN.

**FIGURE 3.** B cell activation in immunized WT and TNFtg PLNs. (A) Plots show gated live/B220+ B cells from representative unimmunized (top row) and immunized PLNs (bottom row) of WT (left panels) and TNFtg (right panels) mice, plotted for expression of GC markers (CD95, GL7). (B) Absolute numbers of CD95+GL7+ B cells in immunized and unimmunized PLNs of WT and TNFtg mice. Similar numbers are observed after immunization in WT and TNFtg mice. Combined results of three independent experiments are shown. (C) B cells in immunized WT and TNFtg PLNs were gated for Bin and FoB phenotypes, and the percentage of cells of each subset that are CD95+GL7+ is shown (combined data from three independent experiments). In both WT and TNFtg PLNs, on average, Bin cells are twice as likely to display a GC phenotype as are FoB cells. (D) Mean fluorescence intensity of CD80 on the surface of Bin and FoB cells with a GC phenotype in immunized PLNs. In both WT and TNFtg, GC Bin cells express higher levels of CD80 than do GC FoBs. (E) Mean fluorescence intensity of MHCII on the surface of Bin and FoB cells harvested from unimmunized and immunized PLNs in TNFtg mice. Bin cells express more MHCII on their surface than do FoB cells both at baseline and after immunization. (F) IFN-γ secretion in B:T coculture supernatants after 36 h as measured by ELISA. Combined results of two independent experiments (A–E) or three independent experiments (F). (A–C) n = 10 for WT and n = 12 for TNFtg. (D) n = 8 for WT and n = 9 for TNFtg. (E) n = 9 for TNFtg. *p < 0.05, **p < 0.01, ***p < 0.001, two-tailed Mann–Whitney U test for analysis between the groups (WT versus TNFtg) or two-tailed Wilcoxon matched-pairs signed-rank test for analysis within a group (WT or TNFtg). Two-tailed paired t test was used for ELISA.
cells than FoB cells in both unimmunized and immunized PLNs of TNFtg mice (Fig. 3E). To test whether Bin cells are intrinsically more capable of stimulating T cells, sorted Bin and FoB cells were cocultured for 36 h with Th1-primed OT-II cells in the presence or absence of OVA peptide, and the supernatants were subjected to ELISA for IFN-γ. T cells stimulated by Bin cells in this manner secreted significantly higher levels of IFN-γ than did those stimulated by FoB cells (Fig. 3F). In vivo evaluation of T cell subsets revealed similar percentages and absolute numbers of total CD4+ and follicular Th cells, as well as of both CD62LhighCD44high central memory and CD62LlowCD44high effector/memory CD4+ T cells in immunized WT and TNFtg PLNs (data not shown). Finally, consistent with the comparable levels of activation observed in TNFtg and WT B cells, serum titers of anti-OVA IgG in the absence of OVA peptide, and the supernatants were subjected to ELISA for IFN-γ. T cells stimulated by Bin cells in this manner secreted significantly higher levels of IFN-γ than did those stimulated by FoB cells (Fig. 3F).

To confirm these results in a more specific system, we repeated the experiments using FITC-conjugated OVA for footpad immunization showed significant upregulation of CD80, CD86, and MHCIi (Fig. 5E–G). Again, Bin cells displayed higher levels of expression of these markers compared with FoB cells (Fig. 5E–G). Combined with the greater fraction of DQ-OVA–processing cells in the Bin compartment, and the evidence above describing enhanced T costimulatory ability by Bin cells, these results suggest that Bin cells represent the major subset of B cells with CD4+ T cell-activation potential in the early stages of the immune response.

Discussion

The scale, specificity, and timing of accumulation of Bin cells in LNs draining arthritic joints of TNFtg mice, their involvement in the histological and functional node alterations that accompany the onset of arthritic flares, and the unexpected effectiveness of B cell-depletion therapy in this model strongly implicate this new B cell population in the pathogenetic process (42, 43). In the studies presented in this article, we set out to characterize the origin and potential function of Bin cells in TNFtg mice. Our results not only contribute to clarify the picture regarding the original findings in TNFtg mice but also extend their relevance to the more general, and, incompletely understood, effects of inflammation on B cell responses.

The differentiation of Bin cells from adoptively transferred normal FoB cells clearly indicates that Bin cells are neither a distinct lineage nor belong to the transitional/precursors subsets found in the mouse spleen with which they share some phenotypic
Consistent with previous studies demonstrating the recruitment of nondividing naive lymphocytes into inflamed lymph nodes (59), we also showed that normal FoB cells are preferentially recruited to the LN draining arthritic joints in TNFtg mice, where they rapidly complete their phenotypic transition to Bin cells. Together with our observation that Bin cells are induced in draining LNs by adjuvant-only injections, these data suggest that, in TNFtg mice, the proinflammatory LN microenvironment is primarily responsible for both B cell accumulation from the circulating pool and for the generation of Bin cells. The specific signals that mediate this phenotypic transition remain to be determined, but the close correlation between the fraction of endogenous Bin cells and the rate of differentiation of adoptively transferred FoB cells into Bins clearly indicates that the effect is microenvironmental, specific, and quantitative.

The heretofore unnoticed appearance of Bin phenotype cells after immunization or adjuvant injection in normal LNs is consistent with the inducing factor(s) not being restricted to arthritis, but representing more general inflammatory signals present in reactive LNs. The relationship between inflammation and B cell function has been the focus of significant interest because of its implications for normal immune responses, the onset and amplification of autoimmune states, and the design of novel immune modulators and adjuvants (32, 35, 70). Inflammatory signals can have significant effects on B cell development and were shown to affect peripheral B cell homeostasis by modulating BCR repertoire and selection/tolerance (33, 34, 36). In addition, B cells can have active roles in regulating inflammatory states by their ability to secrete pro- or anti-inflammatory cytokines, as well as to influence T cell subset differentiation (7, 29, 48, 71–74).
Less well characterized are the direct effects of inflammation on B lymphocyte activation. Our results show that the inflammation-inducible Bin state corresponds to a heightened ability by B cells to capture and process ICs and to display GC and costimulatory markers. Although we cannot discriminate at this stage between increased Bin entrance into GCs and higher proliferation, survival, or persistence in the compartment, our findings indicate that these cells participate in the GC reaction more actively than do FoB. Also, together with the IC capture data, these observations point to a specific role for CD21 in this process.

CD21 expression on B cells was shown to play a critical role in Ab responses to T-dependent Ags (50, 75) and to provide a cognate Ag-independent signal required for GC B cell survival (76). C3d (the C component ligand for CD21) can function as a molecular adjuvant, and its attachment to a foreign Ag is 100-fold more effective than is CFA in lowering the threshold for activation of an adaptive immune response; this augmentation is CD21 dependent (60).

RA patients also display significant alterations in both peripheral blood and lymphoid organ B cell subset composition, which are reversible by anti-inflammatory therapy (77, 78), although it is unknown whether any human Bin-equivalent population exists. However, a subset of RA patients was reported to harbor a population of anergic, autoreactive CD21−/low B cells, which suggests that CD21 downregulation may contribute to tolerance in some situations (79). Paradoxically, however, increased CD21 ligation may provide new insights into effective mechanisms to enhance the immune response (e.g., to vaccination).

Bin cells share some phenotype similarities with B cell populations with regulatory functions identified in various inflammation and autoimmune models (45–48, 54, 72, 74, 85–87). Although we have been unable to detect significant secretion of IL-10 or other cytokines by Bin cells in vitro (42), it is interesting to speculate that Bin cells, arising early during inflammation, may represent the originating population for these regulatory subsets, thus explaining their phenotypic features.

Our data suggest that upregulation of CD21 as part of the Bin phenotype by both normal and TNFtg B cells results in enhanced Ag capture and processing potential and may contribute to T cell stimulation and to increased Bin cell involvement in the GC reaction. Therefore, we hypothesize that Bin differentiation may be part of the physiological response to inflammation, aiming to generate an expanded local cohort of B cells poised for efficient activation. Furthermore, increased Ag-processing ability and expression of co-stimulatory molecules within 1 day of immunization is suggestive of noncognate Ag presentation and, thus, that Bin differentiation might also support an early wave of priming and activation of Ag-specific T cells before cognate B cell clones become sufficiently expanded.

Noncognate Ag uptake and presentation by B cells were demonstrated in a number of experimental systems, but their physiological relevance is unclear, and the issue remains theoretically controversial (61, 64–67). Although our data are intriguingly consistent with this possibility, a formal test of the hypothesis will require detailed analyses of the dynamics and timing of Bin differentiation, Ag capture and processing, triggering, and activation; their effects on T cell activation in the context of normal immune responses; as well as a genetic dissection of the role of CD21 and BCR specificity and complement in the process. If confirmed, this may provide new insights into effective mechanisms to enhance the immune response (e.g., to vaccination).
The picture differs in chronic inflammation, as is the case for TNF\(\gamma\) mice, where, in contrast, Bin differentiation may play a pathogenetic role. The persistent presence of a large pool of B cells poised for activation and potentially capable of noncognate Ag presentation may lead to an increased risk for tolerance breakdown and may contribute to the established correlation between local chronic inflammation and subsequent development of autoimmunity (17–19). Even when Ag-specific autoimmunity is not apparent, as is the case for TNF\(\gamma\) mice (although the issue may warrant revisiting in light of these and our previous findings, particularly in older animals), Bin cells may display aberrant behavior upon specific triggering, such as mobilization from the follicles, sinusoidal space invasion, and LN histological and functional disruption, which, in themselves, may contribute to disease exacerbation by interfering with other homeostatic processes (e.g., lymphatic drainage) (42, 43). In these instances, specific inhibition of the signals involved in Bin differentiation or their later mobilization within the LN may provide the basis for new therapeutic strategies to locally prevent or control arthritis progression and flare without the undesirable effects of systemic immunosuppression.

Acknowledgments

We thank Drs. Jen Anolik, Edith Lord, Andrea Sant, Jim Miller, Alexandra Livingstone, Deborah Fowell, and Lianping Xing and their laboratories for advice, protocols, and reagents, as well as the University of Rochester Flow Cytometry Core facility and the Center for Vaccine Biology and Immunology cell sorting facility for expert technical support.

Disclosures

The authors have no financial interests of interest.

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