



Explore what's possible with innovative
research tools

Discover the difference >



Fibroblast-Like Synoviocytes of Mesenchymal Origin Express Functional B Cell-Activating Factor of the TNF Family in Response to Proinflammatory Cytokines

This information is current as
of June 22, 2021.

Junko Ohata, Nathan J. Zvaifler, Mitsufumi Nishio, David L.
Boyle, Susan L. Kalled, Dennis A. Carson and Thomas J.
Kipps

J Immunol 2005; 174:864-870; ;
doi: 10.4049/jimmunol.174.2.864
<http://www.jimmunol.org/content/174/2/864>

References This article **cites 35 articles**, 15 of which you can access for free at:
<http://www.jimmunol.org/content/174/2/864.full#ref-list-1>

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2005 by The American Association of
Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Fibroblast-Like Synoviocytes of Mesenchymal Origin Express Functional B Cell-Activating Factor of the TNF Family in Response to Proinflammatory Cytokines¹

Junko Ohata,* Nathan J. Zvaifler,[†] Mitsufumi Nishio,* David L. Boyle,[†] Susan L. Kalled,[‡] Dennis A. Carson,[†] and Thomas J. Kipps^{2*}

Immunohistochemical analysis revealed that the intimal lining cells of synovial tissue of inflamed joints of patients with rheumatoid arthritis differed from that of normal joints or of diseased joints in osteoarthritis in that they stained with mAb specific for the B cell-activating factor of the TNF family (BAFF; also called BLYS). We generated fibroblast-like synoviocytes (FLS) cell lines that were bereft of myelomonocytic cells to examine whether mesenchymal-derived FLS could express this critical B cell survival factor. We found that FLS expressed low amounts of BAFF mRNA relative to that of myelomonocytic cells. However, when various cytokines/factors were added to such FLS cell lines, we found that IFN- γ or TNF- α were unique in that they could induce significant increases in BAFF mRNA and protein. Even minute amounts of IFN- γ primed FLS for TNF- α , allowing the latter to stimulate significantly higher levels of BAFF mRNA and protein than could TNF- α alone. Consistent with this, B cells cocultured with IFN- γ and/or TNF- α -treated FLS had a significantly greater viability than B cells cocultured with nontreated FLS. The enhanced protection of B cells afforded by IFN- γ /TNF- α -treated FLS was inhibited by the addition of BAFF-R:Fc fusion protein. We conclude that the proinflammatory cytokines IFN- γ and TNF- α can induce mesenchymal-derived FLS to express functional BAFF in vitro. The induced expression of BAFF on FLS by proinflammatory cytokines may enhance the capacity of such cells to protect B cells from apoptosis in inflammatory microenvironments in vivo. *The Journal of Immunology*, 2005, 174: 864–870.

The joint tissue (synovium) of patients with rheumatoid arthritis (RA)³ has a hyperplastic and redundant lining layer comprised of macrophages and fibroblast-like synoviocytes (FLS) (1). Adjacent to this layer are infiltrations of monocytes, macrophages, and T cells, which produce factors, such as TNF- α and IL-1 (2, 3), and trace amounts of IFN- γ (4). In addition, the inflamed synovium contains numerous B cells that often are organized in nodular pseudofollicles, which resemble germinal centers (5).

B cells are intimately involved in the pathogenesis of RA joint disease through the local production of Igs and autoantibodies, such as rheumatoid factors. They participate in the immune complex formation and complement activation that begets synovial inflammation (6). In addition, B cells produce important chemokines and cytokines, are efficient APCs, and provide cognate help for T cell activation (7). A recent study using RA articular tissue in the SCID mouse chimera model demonstrated that only B cells,

but no other APCs, support CD4⁺ T cell activation in synovial ectopic germinal centers (pseudofollicles) (8). This suggests that B cells can be significantly involved in the cellular immune responses in RA pathogenesis.

In the absence of follicular dendritic cells (FDC), FLS might play a central role in the generation of synovial pseudofollicles and in protecting B cells from apoptosis. FLS produce chemokines, such as stromal-derived factor-1 (SDF-1; CXCL12) (9) and B cell chemoattractant-1 (CXCL13) (10, 11), which likely helps attract B cells into the joint and contributes to B cell survival. Interactions between FLS and B cells via adhesion molecules, such as CD106 (VCAM-1) or CS-1 fibronectin (12), enhance B cell viability (9, 13). In short-term in vitro studies (e.g., hours), engagement of α_4 integrins and CXCR4 on B cells with their respective ligands, VCAM-1 (CD106) and SDF-1, on FLS, protects B cells from spontaneous apoptosis. However, the protective influence of FLS on long-term B cell survival (days) is only partially inhibited with reagents that specifically interfere with these interactions, suggesting that additional factors must be involved (9).

One factor known to play an important role in the survival of mature B cells is B cell-activating factor of the TNF family (BAFF; also called BLYS, TALL-1, THANK, zTNF4) (reviewed in Ref. 14). BAFF can interact with any one of three distinct B cell receptors: transmembrane activator and CAML-interactor, B cell maturation Ag, and BAFF-R (also called BR3). However, the interaction between BAFF-R on B cells and BAFF is highly specific. BAFF-null mice and mice with a naturally occurring mutation within the gene encoding BAFF-R exhibit a profound loss of mature B cells (14), demonstrating a critical role for BAFF↔BAFF-R interactions in mature B cell survival. Prior studies identified that expression of BAFF was restricted to myelomonocytic cells or blood-derived dendritic cells of hemopoietic origin (15–17). A recent study found that FDC, which may not be of hemopoietic

Divisions of *Hematology/Oncology, and [†]Rheumatology, Allergy, and Immunology, University of California, San Diego, La Jolla, CA 92093; and [‡]Department of Molecular and Cell Biology, Biogen Idec, Inc., Cambridge, MA 02142

Received for publication July 9, 2004. Accepted for publication October 25, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported in part by Grant 6217 from the Leukemia and Lymphoma Society.

² Address correspondence and reprint requests to Dr. Thomas J. Kipps, Division of Hematology/Oncology, Department of Medicine, University of California, San Diego, School of Medicine, 9500 Gilman Drive, La Jolla, CA 92093-0663. E-mail address: tkipps@ucsd.edu

³ Abbreviations used in this paper: RA, rheumatoid arthritis; OA, osteoarthritis; FLS, fibroblast-like synoviocyte; FDC, follicular dendritic cell; SDF-1, stromal-derived factor-1; BAFF, B cell-activating factor of the TNF family; APRIL, a proliferation-inducing ligand; s, soluble; DiOC₆, 3,3-dihexyloxycarbocyanine iodide; PI, propidium iodide.

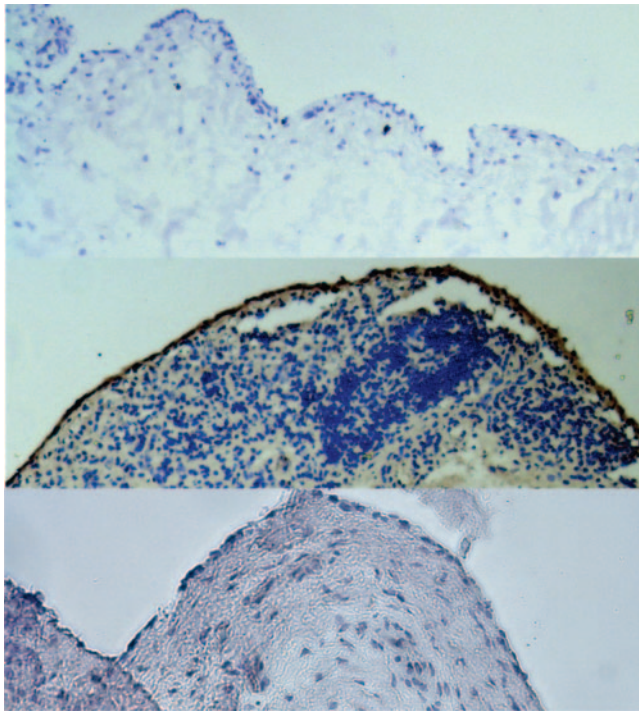


FIGURE 1. RA synovial tissues stain for BAFF. Immunoperoxidase staining of snap frozen synovial tissue sections. Mouse monoclonal anti-BAFF Ab was used at 1 $\mu\text{g/ml}$, and biotin labeled anti-mouse IgG1 Ab at 1/1000 dilution. Sections were counterstained with hematoxylin. The *upper panel* is normal synovium. The *middle panel* is synovium from a patient with RA, and the *lower panel* is synovium from a patient with OA.

origin, also may express BAFF (18). Although FLS are of mesenchymal origin and would not be expected to express this B cell survival factor, they may share with FDC the capacity to express BAFF and thereby promote B cell survival. Because of the functional significance of BAFF and the hypothetical role played by FLS in contributing to the survival of synovial B cells, we examined whether FLS could express this B cell survival factor, either spontaneously or following treatment with proinflammatory cytokines commonly found in the diseased joint of patients with RA.

Materials and Methods

Cells and cell preparation

Synovial tissues were collected with informed consent from RA or osteoarthritis (OA) patients who were undergoing joint replacement surgery. Normal synovial tissues were obtained by arthroscopic biopsy. Synovial cells were isolated by enzymatic digestion of the synovial tissues, as described (19). The cells were cultured in DMEM supplemented with 10% FBS and penicillin-streptomycin-glutamine (Invitrogen Life Technologies). Before use in these experiments, FLS were passaged three to six times (19). Blood mononuclear cells were isolated from the buffy coat of healthy donors that was obtained from the San Diego Blood Bank via density-gradient centrifugation using Ficoll-Hypaque (Pharmacia Biotech). Then CD19-positive B cells were purified using CD19-Dynabeads (Dyna) and Detach A Bead (Dyna), following manufacturer's instruction. The purity of the isolated B cells was >95%, as assessed by flow cytometry using fluorochrome-conjugated anti-CD19 mAb, which does not compete with the anti-CD19 mAb used for prior positive selection.

Reagents

Recombinant human IFN- γ , IL-1 β , IL-15, and IL-17 were purchased from BD Pharmingen. Recombinant human TNF- α and IL-18 were purchased from R&D Systems. Human BAFF-R:Fc was as previously described (20). Human CD40-ligand trimer (soluble (s)CD154) was obtained from Immunex.

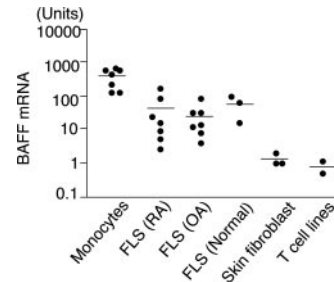


FIGURE 2. BAFF mRNA expression in FLS and in isolated cell types. Total RNA was isolated from blood monocytes, dermal fibroblasts, T cells, and FLS from various synovial tissues. Expression levels of BAFF mRNA were assessed and quantified by real-time RT-PCR. A monocyte cell line, U937, was used as a standard with which to compare the relative expression levels of BAFF mRNA in each sample. One thousand units in this assay are comparable to the amount of BAFF mRNA detected in equivalent numbers of U937 cells. The dots in the column marked T cell line indicates the amount of BAFF mRNA detected in Jurkat or MOLT-4 cells.

Immunohistochemistry

Frozen sections from synovial tissues were fixed in cold acetone for 10 min. The specimens were incubated with 1 $\mu\text{g/ml}$ anti-human BAFF mAb (Research Diagnostics) or irrelevant control Ab for 1 h at room temperature. The slides were then washed three times in PBS and incubated with biotinylated anti-mouse Ab. The slides were washed, incubated with avidin-biotin complex (Vector Laboratories), and the peroxidase was developed with diaminobenzidine and hydrogen peroxide (Vector Laboratories).

Real-time quantitative RT-PCR

Total RNA was isolated from indicated T cell lines, monocytes, FLS, or synovial tissues using RNeasy Mini kit (Qiagen). In other experiments, FLS or adherent cells from the blood mononuclear cells of healthy donors were cultured in the presence of the indicated molecules for 3 days before isolation of total RNA. The molecules added to the cultures were as follows: sCD154, 2.5 $\mu\text{g/ml}$; IL-15, 100 ng/ml; IL-17, 100 ng/ml; IL-18, 100 ng/ml; IL-1 β , 10 ng/ml; TNF- α , 10 ng/ml; and IFN- γ , 10 ng/ml. To generate conditioned FLS, 0.001–0.1 ng/ml IFN- γ was added to FLS 1 day before culture with 10 ng/ml TNF- α for 3 days. To remove DNA contamination in RNA samples, the isolated RNA was treated with RQ1 RNase-Free DNase (Promega) according to the manufacturer's instruction. First-strand cDNA synthesis was performed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies). For real-time PCR, SYBR Green PCR MasterMix (Applied Biosystems) was used with 300 nmol/L forward and reverse primers for a final volume of 50 μl for each reaction. Amplification primers for the human genes were as follows: BAFF, 5'-ACCGCGGACTGAAAATCT-3' and 5'-CACGCTTATTTCTGCTGTTCTGA-3'; a proliferation-inducing ligand (APRIL), 5'-CTGCACCTGGTTCCCATTAAC-3' and 5'-AAGAGCTGGTTGCACATCA-3'; and GAPDH, 5'-ACGGATTGGTCGTATTGGGC-3' and 5'-TTGACGGTGCCATGGAATTTG-3'. Each sample was run in duplicate. Real-time PCR were performed using GeneAmp 5700 Sequence Detection System (Applied Biosystems) with initial incubation at 95°C for 10 min, and thermal conditions followed 40 cycles of 95°C for 15 s, 60°C for 1 min. For each run, serially diluted cDNA of U937 cells were used for quantitative standards. We determined the cell equivalence (CE) numbers of BAFF and GAPDH mRNA in each sample according to the standard curve generated from values obtained with U937. The unit number showing relative BAFF mRNA level in each sample was determined as a value of BAFF CE normalized with GAPDH CE. Melting-curve analysis was performed to assess the specificity of PCR product. Following 40 cycles of PCR, samples were heated to 95°C for 30 s, and 60°C for 20 s, and then heated to 95°C at a ramp rate of 0.2°C/s. The melting curve for each sample was drawn with GeneAmp 5700 sequence detector system software (Applied Biosystems).

Immunoblot analyses

Whole-cell lysates were prepared from each sample using RIPA solution (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM NaF, 0.1% SDS, and 1% Nonidet P-40) containing protease inhibitors (10 $\mu\text{g/ml}$ aprotinin, 10 $\mu\text{g/ml}$ leupeptin, and 10 $\mu\text{g/ml}$ pepstatin). Cell lysates were normalized with total protein content, and 30

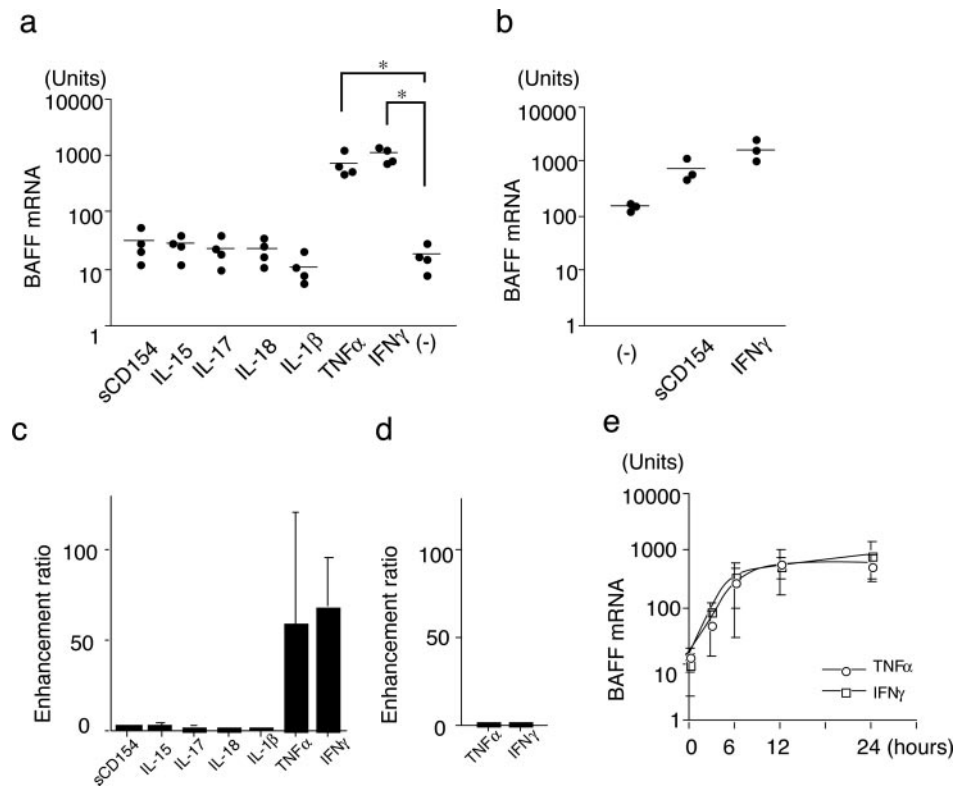


FIGURE 3. The effect of cytokines on expression of BAFF and APRIL mRNA. BAFF mRNA levels were measured using real-time RT-PCR on FLS (a), or blood monocytes (b) of healthy donors following culture with various factors or with medium alone (–), as indicated at the bottom of the figure. ●, Values obtained from individual cell lines are represented. The bar represents the average value for all cell lines tested. The lines are used to compare the mean values of BAFF mRNA obtained from FLS treated with TNF- α or IFN- γ vs that obtained from FLS cultured in medium alone. An asterisk indicates that this value is significantly different from that of the control nonstimulated FLS cultures (value of $p < 0.05$ using Mann-Whitney U test). c, Enhancement ratios of BAFF mRNA on samples cultured with each cytokine. d, APRIL mRNA levels were measured on FLS cultured with the cytokines that induced BAFF mRNA, and enhancement ratios were shown. The error bars indicate the SD about the mean. e, Data from a time course experiment measuring the levels of BAFF mRNA expressed by FLS treated with TNF- α (○) or IFN- γ (□) over time. Each data point represents the mean level of BAFF mRNA obtained from each of six different FLS (as indicated on the y-axis) at each time point, as indicated on the x-axis. The error bars indicate the SD about the mean.

μg of protein was loaded to each lane. SDS-PAGE/immunoblot analyses were performed using 1/1000 dilution of first Abs (anti-BLYS rabbit Abs; BD Pharmingen) and secondary HRP-conjugated anti-rabbit Ab (1/3000 (v/v) dilution; Santa Cruz Biotechnology) with detection accomplished using ECL (Detection Reagent 1 & 2; Amersham Biosciences).

Flow cytometry

Single-cell suspensions of FLS samples were stained with anti-human BAFF mAb (Research Diagnostics) or isotype control mouse IgG1 (MOPC-21) and PE-conjugated anti-mouse IgG1 (BD Pharmingen) using a standard protocol, and analyzed on FACSCalibur (BD Immunocytometry Systems). Flow cytometry data were analyzed using FlowJo 3.7.1 software (Tree Star).

Viability assay

A total of 1.5×10^5 cells per well of FLS was seeded into 24-well plates. FLS were cultured in the presence of either 10 ng/ml TNF- α , or medium alone for 3 days, or were conditioned by pretreatment with IFN- γ and then TNF- α . We removed the supernatant from the adherent FLS, which then were washed twice with fresh medium. One-half million normal B cells were suspended in RPMI 1640 medium supplemented with 10% FBS and penicillin-streptomycin-glutamine, and then added to plastic wells with or without FLS. The cells were cocultured with 1 $\mu\text{g}/\text{ml}$ BAFF-R:Fc or control Ig for ≤ 5 days as indicated. The suspended cells were collected from the cultures. The adherent FLS were detached using Cell Dissociation Buffer (Enzyme-free, PBS-based; Invitrogen Life Technologies). The cells were stained with 3,3-dihexyloxycarbocyanine iodide (DiOC₆), to assess the mitochondrial transmembrane potential (ψ_m), and with propidium iodide (PI) to assess membrane permeability, as described (21, 22). Briefly, cell suspensions were incubated with 40 nmol/L DiOC₆ and 1 $\mu\text{g}/\text{ml}$ PI for

15 min at 37°C, and then analyzed on FACSCalibur (BD Biosciences). A lymphocyte gate was set using forward-angle and side-angle light scatter characteristics of lymphocytes. The vital B cells were brightly positive when stained with DiOC₆ and excluded PI.

Data analysis and statistics

Results are shown as mean \pm SD or SEM. For statistics comparison, Mann-Whitney U test, Wilcoxon matched paired test, or Bonferroni t test were used for comparisons between multiple groups, as indicated in the text. Analyses were performed using GraphPad Prism (GraphPad Software).

Results

Synovial tissues from RA patients have high-level expression of BAFF

Immunohistochemical analysis revealed that the intimal lining cells of synovial tissue of inflamed joints of patients with RA ($n = 3$) differed from that of normal joints or the joints of patients with OA in that they stained with mAb specific for BAFF (Fig. 1). The staining of synovial tissue appeared specific in that these cells did not stain when the primary anti-BAFF Ab was omitted or irrelevant isotype control mAb were used. Typical synovial inflammation was seen in the RA samples, including lymphocytic infiltrates, scattered follicle formation, increased vascularity, and hyperplasia of synovial lining cells (Fig. 1, middle panel). Diffuse anti-BAFF staining occurred throughout the subintimal connective tissues, around the follicles, and was most marked in the adjacent synovial

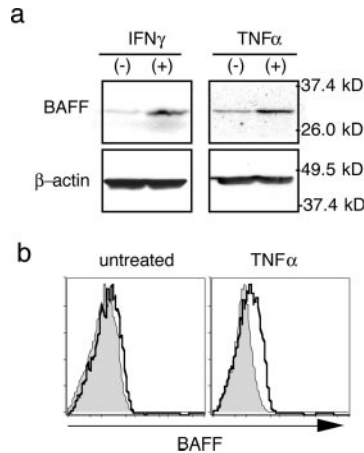


FIGURE 4. IFN- γ or TNF- α induce FLS expression of BAFF protein. *a*, An immunoblot analysis for BAFF (top panels, labeled “BAFF” on the left margin) or β -actin (bottom panels, labeled β -actin on the left margin) using lysates prepared from FLS cultured for 3 days in medium without (-) or with (+) the cytokine (10 ng/ml) indicated at the top of the panels. The size markers are shown on the right margin. *b* provides the fluorescence histograms of FLS stained with mAbs specific for BAFF (dark lines) or an isotype control mAb (shaded histograms) after culture in medium alone (untreated) or in culture medium with TNF- α , as indicated at the top of each histogram.

lining cells. Such staining was not observed in the synovial lining of normal joint tissue (Fig. 1, upper panel) or the joint tissue of patients with OA (lower panel). Although the staining for BAFF in subintimal tissues was anticipated for myelomonocytic cells, the staining of synovial lining cells that appeared like FLS was unexpected. To evaluate whether isolated FLS bereft of myelomonocytic cells actually could express BAFF, we generated FLS cell lines and examined them for expression of this B cell survival factor.

Expression of BAFF mRNA by FLS

We examined isolated FLS for expression of BAFF mRNA using quantitative real-time PCR. Serially passaged FLS from inflamed joints of patients with RA were found to express levels of BAFF mRNA that were significantly greater than that of negative-control T cell lines (e.g., Jurkat) or skin fibroblasts. Surprisingly, the serially passaged FLS from the joints of OA patients also were found to have detectable BAFF mRNA. However, the levels of BAFF mRNA expressed by such FLS were significantly less than that found in blood monocytes, which are known to express high levels of BAFF (Fig. 2).

Proinflammatory cytokines enhance expression of BAFF by FLS

We examined whether proinflammatory cytokines found in inflamed synovial tissues could influence the levels of BAFF mRNA expressed by FLS. We cultured FLS from each of four different

Table I. Pretreatment of FLS with IFN- γ significantly enhances TNF- α -induced expression of BAFF mRNA

IFN- γ (ng/ml)	TNF- α (ng/ml)	BAFF mRNA (U \pm SD)	n
0	0	32 \pm 16	8
0	10	714 \pm 224	8
0.001	10	1439 \pm 544	5
0.01	10	1480 \pm 945	6
0.1	10	2760 \pm 3260	8

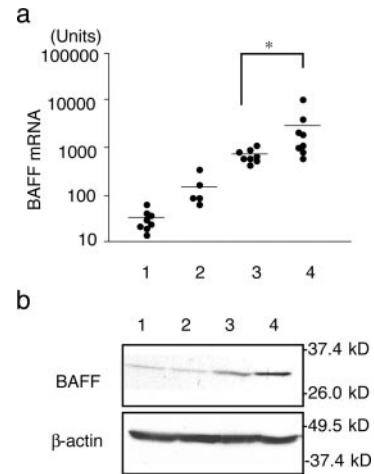


FIGURE 5. IFN- γ enhances the levels of FLS BAFF mRNA induced by subsequent treatment with TNF- α . *a*, ●, Level of BAFF mRNA measured in each FLS cell line following treatment with either medium alone (column 1), 0.1 ng/ml IFN- γ (column 2), 10 ng/ml TNF- α alone (column 3), or 0.1 ng/ml IFN- γ followed by treatment with 10 ng/ml TNF- α (column 4). The horizontal bars indicate the mean levels of BAFF mRNA detected. The lines are used to compare the mean values of BAFF mRNA obtained from FLS treated with TNF- α alone (column 3) vs that of FLS treated with IFN- γ and TNF- α (column 4). An asterisk indicates there is a significant difference between groups 3 and 4 as indicated by the lines ($p < 0.05$ using Mann-Whitney *U* test). An asterisk indicates there is a significant difference between groups 3 and 4 as indicated by the lines ($p < 0.05$ using Mann-Whitney *U* test). *b*, Immunoblot analyses for BAFF (top row) vs β -actin (bottom row) detected in electrophoretically size-separated FLS lysates. The number on the top of each column represents the same treatment, as discussed in *a*.

RA or OA synovial tissues with any one of several cytokines and then evaluated the cells for expression of BAFF mRNA. Two of the seven molecules tested, namely IFN- γ and TNF- α , were unique in their capacity to induce BAFF expression by FLS. Optimal induction of BAFF mRNA was observed at cytokine levels of 10 ng/ml for each cytokine (Fig. 3*a*). However, the other cytokines (e.g., IL-1 β , IL-15, IL-17, or IL-18) did not induce FLS to express increased levels of BAFF at any of the examined cytokine concentrations. Furthermore, sCD40L (sCD154) also did not induce FLS to express higher levels of BAFF mRNA (Fig. 3*a*). In contrast, sCD154 could induce monocytes to express increased amounts of BAFF mRNA (Fig. 3*b*). IFN- γ also could induce monocytes to express higher levels of BAFF mRNA (Fig. 3*b*), in agreement with earlier studies (15).

To compare the relative capacity of each cytokine to induce enhanced expression of BAFF, we calculated the stimulation index, which was the ratio of the number of BAFF mRNA units measured in FLS treated with cytokine/factor divided by the number of BAFF mRNA units measured in FLS cultured in medium alone. The BAFF stimulation index for FLS treated with IFN- γ or TNF- α was 67.8 or 58.7, respectively. The BAFF stimulation index for FLS treated with any other cytokine/factor was <2.1 (Fig. 3*c*). Similarly, we determined the stimulation index for APRIL. In contrast to BAFF, we found that the APRIL stimulation index for FLS treated with IFN- γ or TNF- α was <1.7 , indicating that these cytokines did not influence the expression levels of APRIL by FLS (Fig. 3*d*).

A time course study evaluated the levels of BAFF mRNA in FLS following treatment with exogenous IFN- γ or TNF- α . This revealed increased FLS expression of BAFF mRNA in as early as 3 h after the addition of either cytokine. The levels of BAFF

mRNA expressed by cytokine-treated FLS increased over time, reaching a plateau at ~24 h (Fig. 3e), persisting for at least 2 days thereafter.

Treatment of FLS with IFN- γ or TNF- α induced these cells to express increased amounts of BAFF protein (Fig. 4a). Flow cytometric studies demonstrated that the entire population of cytokine-treated FLS expressed increased levels of surface BAFF relative to that of untreated FLS (Fig. 4b), arguing against the possibility that only a subset of FLS or contaminating cells expressed BAFF in response to these cytokines.

IFN- γ and TNF- α function synergistically to enhance FLS expression of BAFF

Because TNF- α and IFN- γ frequently are found together at inflammatory sites in RA synovium, we examined whether the combination of these two cytokines could act synergistically to influence the expression of BAFF by FLS. FLS treated with minute concentrations of IFN- γ (e.g., 0.1 ng/ml), which by itself could not induce significant increases in BAFF mRNA, could nonetheless prime FLS for mounting an enhanced response to TNF- α . For example, conditioned FLS that were treated with IFN- γ at 0.001 ng/ml, and then treated with TNF- α at 10 ng/ml, had significantly higher levels of BAFF mRNA than FLS treated with either IFN- γ or TNF- α alone ($p < 0.05$, Mann-Whitney U test; Table I). IFN- γ at higher concentrations also could prime FLS to express higher levels of BAFF mRNA and protein than could treatment with TNF- α alone (Table I and Fig. 5).

FLS enhance survival of blood B cells in vitro

We isolated CD19⁺ B cells from the blood of healthy donors and cultured them with or without FLS. B cells cultured with medium alone survived poorly, such that $\leq 10\%$ were alive after 5 days in culture (Fig. 6, a and b). B cells cultured with untreated FLS maintained higher viability than did B cells cultured alone (Fig. 6, a and b). However, B cells cultured on conditioned FLS had significantly higher viability than B cells cultured with untreated FLS. After 5 days in culture, the overall viability of B cells was $52 \pm 10\%$ with untreated FLS and $66 \pm 8\%$ with conditioned FLS ($p < 0.005$,

$n = 11$, Mann-Whitney U test; Fig. 6b). B cells cocultured with FLS that had been treated with TNF- α (10 ng/ml) alone also had significantly higher viability than that of B cells cocultured with untreated FLS ($64 \pm 8\%$; $p < 0.01$, $n = 11$, Mann-Whitney U test).

To determine whether the improved B cell survival on conditioned FLS was due to BAFF, we treated cocultures of B cells and FLS with a soluble antagonist of BAFF \rightleftharpoons BAFF-R interactions, namely BAFF-R:Fc. Addition of BAFF-R:Fc had little influence on the survival of B cells cocultured with untreated FLS. However, BAFF-R:Fc significantly reduced survival of B cells cocultured with conditioned FLS (Fig. 6c).

Discussion

This study provides the first direct demonstration that cells of mesenchymal origin, namely FLS, can express BAFF, a critical molecule involved in maintaining B cell survival. Initially, the only identified cells capable of expressing BAFF were hemopoietic cells of myelomonocytic origin (14). However, a recent study found that FDC also may express this B cell survival factor (18). Nevertheless, we assumed that the BAFF protein found in synovial fluid of patients with RA (23) and the BAFF mRNA detected in RA synovial tissue were produced by macrophages resident in the inflamed synovium. However, the unanticipated finding that fibroblast-like synovial lining cells stained with an anti-BAFF mAb prompted us to examine mesenchymal-derived FLS for expression of BAFF. We detected significant levels of BAFF mRNA in FLS lines that had been passaged more than three times. Such passaged FLS lines were bereft of CD14⁺ cells, such as monocytes or macrophages (19, 24) (data not shown). Further evidence that the FLS samples did not contain monocytes or macrophages was provided in the response of such cultures to sCD154. Although monocytes/macrophages and FLS can share expression of CD40 (25, 26), the FLS cultures could not be induced to express enhanced levels of BAFF by sCD154, which is in contrast to monocytes or macrophages. As such, it is unlikely that the BAFF mRNA detected in our serial passaged FLS lines was derived from contaminating myelomonocytic cells.

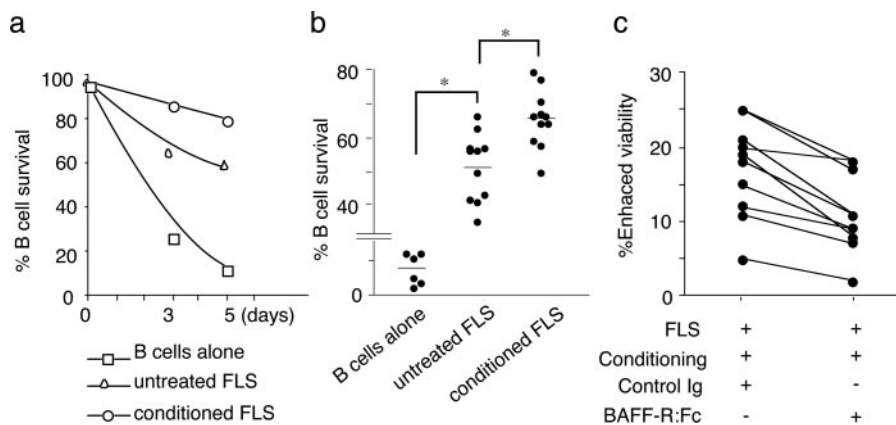


FIGURE 6. FLS and conditioned FLS support B cell survival in vitro. *a*, Mean cell viability (\pm SEM, as indicated by the error bars) of isolated blood B cells from healthy adults at various times in culture by themselves (\square), or with untreated FLS (\triangle) or FLS conditioned with 0.1 ng/ml IFN- γ and 10 ng/ml TNF- α (\circ), as indicated at the bottom of the figure. *b*, Viability of B cells cultured alone or cocultured with untreated FLS or conditioned, as described. Each dot represents a separate sample with the percent viability indicated on the ordinate. The horizontal lines represent the average B cell viability for all samples in a group. An asterisk represents a significant difference in survival between the compared groups ($p < 0.005$ using Mann-Whitney U test). ($p < 0.005$ using Mann-Whitney U test). *c*, The FLS were treated with IFN- γ followed by TNF- α (conditioned). The lines connect the viability readings of the same B cell population on cytokine-treated FLS relative to untreated FLS with control Ig vs BAFF-R:Fc, allowing for paired statistical analyses that revealed $p = 0.001$ using Wilcoxon matched paired test. To calculate the Δ B cell viability, the viability of B cells cocultured on untreated FLS with BAFF-R:Fc was subtracted from the viability of the same B cell population cocultured on cytokine-treated FLS with either control Ig or BAFF-R:Fc, respectively.

Our finding that nonhemopoietic cell can express BAFF is supported by a recently published study on reciprocal bone marrow chimeras with wild-type and BAFF-deficient mice (27). Gorelik et al. (27) found that normal B cell homeostasis required BAFF expression by radiation-resistant stromal cells, which challenges the notion that only myelomonocytic cells are capable of expressing this important B cell survival factor. In addition, FDC that may be derived from nonhemopoietic cells also have been found to express BAFF (18). The direct demonstration that cells of mesenchymal origin can express BAFF suggests that nonhemopoietic stromal cells can support B cell homeostasis, especially in response to certain cytokines in the inflammatory microenvironment. Conceivably, FDC also can be induced to express higher levels of this B cell survival factor in response to such proinflammatory cytokines.

In this regard, it is important to note that FLS expressed higher levels of BAFF than resting FLS after treatment with either IFN- γ and/or TNF- α , regardless of whether they were derived from either RA or OA synovium. As such, the capacity of FLS to protect mature B cells from apoptosis in the inflamed RA joint probably reflects the influence of the microenvironment more than any intrinsic property unique to RA FLS.

Prior studies found that TNF- α and IFN- γ act synergistically on dermal fibroblasts to enhance collagen synthesis (27) and on lung fibroblasts to synthesize glycosaminoglycan (28). In contrast, TNF- α and IFN- γ are mutually antagonistic in inducing synovial fibroblasts to proliferate, to express HLA-DR, or to produce collagenase (19). Because IFN- γ concentrations are low in the RA joint relative to that found in other inflammatory states or in T cell-mediated immune responses (4), it was suggested that unopposed TNF- α stimulation might be more important than IFN- γ in RA pathogenesis (29). However, the findings of current study imply that trace amounts of IFN- γ can prime FLS for enhanced sensitivity to TNF- α , at least in their capacity to express BAFF. Conceivably, trace amounts of IFN- γ can influence other changes in FLS, allowing these cells to contribute to the inflammatory reactions that characterize the RA synovium.

As a rule, explanations for antagonism or synergy between cytokines invoke modulation of receptor numbers or affinity. For instance, in the human hepatocellular carcinoma cell line (HEP G2), IFN- γ down-modulates both TNF receptor numbers and receptor affinity (30). The opposite has been described in FLS (31). Using Scatchard plots to analyze the effect of adding full doses of radiolabeled cytokines (100 ng/ml-TNF- α or 100 IFN- γ U) to unstimulated synovial fibroblasts showed a 49.5 and 28.9% increase in binding sites for TNF- α and IFN- γ , respectively, but neither cytokine interfered directly with the binding of the other, and the K_d for each receptor remained the same. FLS treated with varying concentrations of IFN- γ for 24 h had negligible changes in surface expression of type I or type II TNF-Rs (data not shown), which seems insufficient to account for the synergy with TNF- α evoked by very small concentrations of IFN- γ . Instead, it appears that IFN- γ enhances the sensitivity or qualitative signaling capacity of the TNF-R on FLS, allowing TNF- α to induce greater expression of BAFF by IFN- γ -treated FLS than by untreated FLS.

The increased expression of BAFF on cytokine-treated FLS has functional significance in promoting B cell survival. This study corroborates prior studies demonstrating that FLS can protect blood B cells from spontaneous apoptosis in vitro (9, 13). In addition, we found that FLS treated with IFN- γ and/or TNF- α had an even greater capacity to support B cells survival than did untreated FLS. The greater capacity of conditioned FLS relative to untreated FLS to support B cell survival could be inhibited by BAFF-R:Fc, indicating that BAFF \rightleftharpoons BAFF-R interactions were involved.

The success of rituximab in the treatment of patients with RA has led investigators to reassess the role of B cells in RA pathogenesis (32). Synoviocytes support the terminal differentiation of activated B cells, which in turn could enhance plasma cell accumulation in RA synovial tissues and local autoantibody production (33). However, the origin of the plasma cells found in chronically inflamed tissues, like the joint, is disputed. Recent studies by Cassese et al. (34) favors the idea that plasma cells once formed can persist for months to years in specific survival niches. These niches have in common stromal elements that provide cytokines (IL-5, IL-6, TNF- α), chemokines (SDF-1), and ligands for CD44 (34). BlyS/BAFF appears also to be required (35). Although all factors seem necessary, the relative need for each in RA pathogenesis remains to be defined. Nevertheless, the finding that synovial fibroblasts can provide all of these elements required for B cell survival indicates that such cells can be conscripted to play an important role in the pathogenesis of this disease.

Acknowledgments

Synovial tissues and FLS samples were provided by the University of California San Diego-Rheumatic Disease Core Center. We are grateful to Kathleen S. Picha for providing human CD40L trimer (sCD154). We are also grateful to Todd A. Johnson for technical assistance in the preparation of this manuscript.

References

- Zvaifler, N. J., D. Boyle, and G. S. Firestein. 1994. Early synovitis—synoviocytes and mononuclear cells. *Semin. Arthritis Rheum.* 23:11.
- Feldmann, M., F. M. Brennan, and R. N. Maini. 1996. Role of cytokines in rheumatoid arthritis. *Annu. Rev. Immunol.* 14:397.
- Kinne, R. W., R. Brauer, B. Stuhlmüller, E. Palombo-Kinne, and G. R. Burmester. 2000. Macrophages in rheumatoid arthritis. *Arthritis Res.* 2:189.
- Firestein, G. S., and N. J. Zvaifler. 1987. Peripheral blood and synovial fluid monocyte activation in inflammatory arthritis. II. Low levels of synovial fluid and synovial tissue interferon suggest that γ -interferon is not the primary macrophage activating factor. *Arthritis Rheum.* 30:864.
- Weyand, C. M., and J. J. Goronzy. 2003. Ectopic germinal center formation in rheumatoid synovitis. *Ann. NY Acad. Sci.* 987:140.
- Zvaifler, N. J. 1974. Rheumatoid synovitis: an extravascular immune complex disease. *Arthritis Rheum.* 17:297.
- Dorner, T., and G. R. Burmester. 2003. The role of B cells in rheumatoid arthritis: mechanisms and therapeutic targets. *Curr. Opin. Rheumatol.* 15:246.
- Takemura, S., P. A. Klimiuk, A. Braun, J. J. Goronzy, and C. M. Weyand. 2001. T cell activation in rheumatoid synovium is B cell dependent. *J. Immunol.* 167:4710.
- Burger, J. A., N. J. Zvaifler, N. Tsukada, G. S. Firestein, and T. J. Kipps. 2001. Fibroblast-like synoviocytes support B-cell pseudoemperipolesis via a stromal cell-derived factor-1- and CD106 (VCAM-1)-dependent mechanism. *J. Clin. Invest.* 107:305.
- Shi, K., K. Hayashida, M. Kaneko, J. Hashimoto, T. Tomita, P. E. Lipsky, H. Yoshikawa, and T. Ochi. 2001. Lymphoid chemokine B cell-attracting chemokine-1 (CXCL13) is expressed in germinal center of ectopic lymphoid follicles within the synovium of chronic arthritis patients. *J. Immunol.* 166:650.
- Takemura, S., A. Braun, C. Crowson, P. J. Kurtin, R. H. Cofield, W. M. O'Fallon, J. J. Goronzy, and C. M. Weyand. 2001. Lymphoid neogenesis in rheumatoid synovitis. *J. Immunol.* 167:1072.
- Morales-Ducet, J., E. Wayner, M. J. Elices, J. M. Alvaro-Gracia, N. J. Zvaifler, and G. S. Firestein. 1992. $\alpha_4\beta_1$ integrin (VLA-4) ligands in arthritis: vascular cell adhesion molecule-1 expression in synovium and on fibroblast-like synoviocytes. *J. Immunol.* 149:1424.
- Lindhout, E., M. van Eijk, M. van Pel, J. Lindeman, H. J. Dinant, and C. de Groot. 1999. Fibroblast-like synoviocytes from rheumatoid arthritis patients have intrinsic properties of follicular dendritic cells. *J. Immunol.* 162:5949.
- Mackay, F., P. Schneider, P. Rennert, and J. Browning. 2003. BAFF AND APRIL: a tutorial on B cell survival. *Annu. Rev. Immunol.* 21:231.
- Nardelli, B., O. Belvedere, V. Roschke, P. A. Moore, H. S. Olsen, T. S. Migone, S. Sosnovtseva, J. A. Carrell, P. Feng, J. G. Giri, and D. M. Hilbert. 2001. Synthesis and release of B-lymphocyte stimulator from myeloid cells. *Blood* 97:198.
- Scapini, P., B. Nardelli, G. Nadali, F. Calzetti, G. Pizzolo, C. Montecucco, and M. A. Cassatella. 2003. G-CSF-stimulated neutrophils are a prominent source of functional BlyS. *J. Exp. Med.* 197:297.
- Craxton, A., D. Magaletti, E. J. Ryan, and E. A. Clark. 2003. Macrophage- and dendritic cell-dependent regulation of human B-cell proliferation requires the TNF family ligand BAFF. *Blood* 101:4464.
- Hase, H., Y. Kanno, M. Kojima, K. Hasegawa, D. Sakurai, H. Kojima, N. Tsuchiya, K. Tokunaga, N. Masawa, M. Azuma, et al. 2004. BAFF/BlyS can potentiate B-cell selection with the B-cell coreceptor complex. *Blood* 103:2257.

19. Alvaro-Gracia, J. M., N. J. Zvaifler, and G. S. Firestein. 1990. Cytokines in chronic inflammatory arthritis. V. Mutual antagonism between interferon- γ and tumor necrosis factor- α on HLA-DR expression, proliferation, collagenase production, and granulocyte macrophage colony-stimulating factor production by rheumatoid arthritis synoviocytes. *J. Clin. Invest.* 86:1790.
20. Pelletier, M., J. S. Thompson, F. Qian, S. A. Bixler, D. Gong, T. Cachero, K. Gilbride, E. Day, M. Zafari, C. Benjamin, et al. 2003. Comparison of soluble decoy IgG fusion proteins of BAFF-R and BCMA as antagonists for BAFF. *J. Biol. Chem.* 278:33127.
21. Zamzami, N., P. Marchetti, M. Castedo, C. Zanin, J. L. Vayssiere, P. X. Petit, and G. Kroemer. 1995. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J. Exp. Med.* 181:1661.
22. Hu, D., and T. J. Kipps. 1999. Reduction in mitochondrial membrane potential is an early event in Fas-independent CTL-mediated apoptosis. *Cell. Immunol.* 195:43.
23. Tan, S. M., D. Xu, V. Roschke, J. W. Perry, D. G. Arkfeld, G. R. Ehresmann, T. S. Migone, D. M. Hilbert, and W. Stohl. 2003. Local production of B lymphocyte stimulator protein and APRIL in arthritic joints of patients with inflammatory arthritis. *Arthritis Rheum.* 48:982.
24. Dayer, J. M., S. M. Krane, R. G. Russell, and D. R. Robinson. 1976. Production of collagenase and prostaglandins by isolated adherent rheumatoid synovial cells. *Proc. Natl. Acad. Sci. USA* 73:945.
25. Lambert, N., P. L. Lescoulié, B. Yassine-Diab, G. Enault, B. Mazieres, C. De Preval, and A. Cantagrel. 1998. Substance P enhances cytokine-induced vascular cell adhesion molecule-1 (VCAM-1) expression on cultured rheumatoid fibroblast-like synoviocytes. *Clin. Exp. Immunol.* 113:269.
26. Miyazawa, K., A. Mori, and H. Okudaira. 1998. Establishment and characterization of a novel human rheumatoid fibroblast-like synoviocyte line, MH7A, immortalized with SV40 T antigen. *J. Biochem.* 124:1153.
27. Gorelik, L., K. Gilbride, M. Dobles, S. L. Kalled, D. Zandman, and M. L. Scott. 2003. Normal B cell homeostasis requires B cell activation factor production by radiation-resistant cells. *J. Exp. Med.* 198:937.
28. Elias, J. A., R. C. Krol, B. Freundlich, and P. M. Sampson. 1988. Regulation of human lung fibroblast glycosaminoglycan production by recombinant interferons, tumor necrosis factor, and lymphotoxin. *J. Clin. Invest.* 81:325.
29. Firestein, G. S., and N. J. Zvaifler. 1990. How important are T cells in chronic rheumatoid synovitis? *Arthritis Rheum.* 33:768.
30. Aggarwal, B. B., and R. Pandita. 1994. Both type I and type II interferons down-regulate human tumor necrosis factor receptors in human hepatocellular carcinoma cell line Hep G2: role of protein kinase C. *FEBS Lett.* 337:99.
31. Alvaro-Gracia, J. M., C. Yu, N. J. Zvaifler, and G. S. Firestein. 1993. Mutual antagonism between interferon- γ and tumor necrosis factor- α on fibroblast-like synoviocytes: paradoxical induction of IFN- γ and TNF- α receptor expression. *J. Clin. Immunol.* 13:212.
32. Edwards, J. C., L. Szczepanski, J. Szechinski, A. Filipowicz-Sosnowska, P. Emery, D. R. Close, R. M. Stevens, and T. Shaw. 2004. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *N. Engl. J. Med.* 350:2572.
33. Dechanet, J., P. Merville, I. Durand, J. Banchereau, and P. Miossec. 1995. The ability of synoviocytes to support terminal differentiation of activated B cells may explain plasma cell accumulation in rheumatoid synovium. *J. Clin. Invest.* 95:456.
34. Cassese, G., S. Arce, A. E. Hauser, K. Lehnert, B. Moewes, M. Mostarac, G. Muehlinghaus, M. Szyska, A. Radbruch, and R. A. Manz. 2003. Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *J. Immunol.* 171:1684.
35. O'Connor, B. P., V. S. Raman, L. D. Erickson, W. J. Cook, L. K. Weaver, C. Ahonen, L. L. Lin, G. T. Mantchev, R. J. Bram, and R. J. Noelle. 2004. BCMA is essential for the survival of long-lived bone marrow plasma cells. *J. Exp. Med.* 199:91.