A Soluble BAFF Antagonist, BR3-Fc, Decreases Peripheral Blood B Cells and Lymphoid Tissue Marginal Zone and Follicular B Cells in Cynomolgus Monkeys

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BAFF (also known as BlyS), a member of the tumor necrosis factor superfamily, plays a critical role in the maturation and development of B cells. BAFF has three receptors on B cells, the most crucial of which is BR3. In this study, we demonstrate the biological outcome of BAFF blockade in cynomolgus monkeys using a soluble fusion protein consisting of human BR3 and human IgG1 Fc. *In vitro*, BR3-Fc blocked BAFF-mediated survival and proliferation of cynomolgus monkey B cells. Weekly treatment of cynomolgus monkeys with BR3-Fc for 13 to 18 weeks resulted in significant B-cell reduction in the peripheral blood and in lymphoid organs. CD21^{high} B cells in lymphoid tissues, a subset analogous to human marginal zone B cells, expressed nearly twofold higher BR3 levels than did CD21^{med} B cells. Lymphoid tissue flow cytometric analysis showed that BR3-Fc reduced this CD21^{high} B-cell subset to a greater extent than it reduced CD21^{med} B cells. Dual-label immunohistochemistry and morphometric image analysis supported these results by demonstrating that BR3-Fc reduced a significant proportion of the B cells within the splenic inner and outer marginal zones. These findings should prove very useful in guiding the desired therapeutic use of BR3-Fc for autoimmune diseases in the clinic. (*Am J Pathol* 2006, 168:476–489; DOI: 10.2353/ajpath.2006.050600)

BAFF (also known as BlyS, THANK, TALL-1, TNFSF13B, and zTNF4) is a member of the tumor necrosis factor (TNF) ligand superfamily and has been shown to stimulate proliferation and to induce maturation of B lymphocytes.^{1–4} Mice deficient in BAFF have reduced B-cell numbers and antigenic responses.^{5,6} Conversely, transgenic mice overexpressing BAFF have a phenotype reminiscent of autoimmunity, and mice with autoimmune diseases exhibit increased serum BAFF levels.^{7–9} BAFF exists as both a membrane-bound and soluble form and is produced by a variety of cell types, including stromal cells, dendritic cells, neutrophils, and monocytes.^{10–13}

BAFF has three receptors on B cells: B-cell maturation antigen, transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), and BAFF-R or BR3.^{14–17} BR3 was identified as the crucial receptor for B-cell survival, because mice carrying a loss-of-function mutation in BR3, as well as BR3 knockout mice, have reduced numbers of peripheral B cells.^{15,18,19} In contrast, normal B-cell maturation is seen in mice deficient for B-cell maturation antigen,²⁰ whereas increased B-cell proliferation and autoimmunity is seen in TACI knockout mice.^{21,22} BR3 is expressed on a wide range of B-cell subsets, including immature, transitional, mature, memory, and germinal center B cells, as well as on plasma cells.^{23,24}

Several lines of evidence implicate BAFF signaling in autoimmunity. Autoantigen-binding B cells may have an increased dependence on the BAFF survival signal.²⁵ In addition, circulating levels of soluble BAFF are increased in many patients with autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Sjögren's syndrome.^{26–29} In patients with RA,

Accepted for publication October 28, 2005.

All authors are employed by either Genentech, Inc. or Biogen Idec, Inc. BR3-Fc is a therapeutic candidate that is being jointly developed by these two companies.

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BAFF levels in synovial fluid show even greater elevations than they do in serum.^{26,30} In transgenic mice, overexpression of BAFF leads to the expansion of marginal zone B cells, a cell type implicated in autoimmunity.³¹ Finally, BAFF binding to BR3 on T cells has been shown to costimulate T-cell proliferation both *in vitro* and *in vivo*.^{32,33}

Inhibition of BAFF signaling is a potential therapeutic option for treatment of B cell-mediated autoimmune conditions. Inhibition of BAFF in a chimeric mouse model of implanted human RA synovium with a TACI receptor fusion protein (TACI-Ig) led to collapse of the ectopic synovial lymphoid structures.³⁴ In addition, treatment with TACI-Ig in the mouse collagen-induced arthritis model of RA resulted in reduced numbers of B cells that coincided with decreased disease activity, whereas treatment with a soluble BR3-Fc fusion protein in a spontaneous mouse model of lupus nephritis also resulted in decreased disease activity.^{5,7,35} BAFF-targeting therapeutics have just recently entered clinical trials. A neutralizing anti-BAFF monoclonal antibody, belimumab, has recently completed a phase I trial in SLE and is now in a phase II trial for SLE and RA.³⁶ Belimumab demonstrated biological activity in humans, inducing a moderate decrease in peripheral blood B cells (11 to 47% on days 42 to 49).³⁶

In this study, we characterize the biological activity, both *in vitro* and *in vivo*, of a human BR3-Fc fusion protein³⁷ in nonhuman primates. Specifically, we describe in detail the time course and extent of B-cell subset reduction and subsequent recovery in the peripheral blood and within the lymphoid tissues of cynomolgus monkeys using both FACS analysis and dual-label immunohistochemistry (IHC). Furthermore, we show that BR3-Fc significantly decreases both marginal zone and total follicular B cells in lymphoid tissues. These results support the clinical potential of BR3-Fc as an autoimmune disease therapeutic.

Materials and Methods

Characterization of BR3-Fc

The BR3-Fc recombinant fusion protein was stably transfected and produced in a CHO cell line.³⁷ The protein consists of two polypeptide chains linked by disulfide bonds, with sequences from the extracellular domain of the human BAFF receptor, BR3, and the Fc domain of human IgG.³⁷ BR3-Fc has an apparent molecular weight of 127 kD and binds to mouse, cynomolgus monkey, and human BAFF (data not shown).

In Vitro Activity

B cells from cynomolgus monkey peripheral blood mononuclear cells (PBMCs) were isolated by positive selection using CD20 MACS beads (Miltenyi Biotec, Auburn, GA) according to manufacturer's instructions. For the proliferation assay, cells were then cultured in 96-well flat bottom plates coated with 10 μ g/ml goat anti-human α -IgM

Table 1. Study Design

Group	No./sex	Dose level (mg/kg)	Dose conc. (mg/mL)
1	8M, 8F	0	0
2	3M, 3F	2	0.62
3	8M, 8F	20	6.2

Interim necropsy: Two males (M) and two females (F) each from groups 1 and 3 were necropsied on week 13 (days 91 and 92; four per day).

Terminal necropsy: Three males and three females from each group (1 to 3) were necropsied on week 18 (days 126, 127, and 128; six per day).

Recovery necropsy: Three males and three females each from groups 1 and 3 were necropsied on week 41 (days 287, 288, and 289; four per day). Conc., concentration.

F(ab')₂, Fc fragment specific (Jackson ImmunoResearch Laboratories, West Grove, PA), at a density of 2×10^5 cells per well. Soluble recombinant mouse BAFF was added at a concentration of 5 μ g/ml for 5 days. Tritiated-thymidine (1 μ Ci) (Perkin Elmer, Boston, MA) was added during the last 6 hours of culture, and cells were harvested onto UNIFILTER plates (Perkin Elmer) and counted. For survival assays, purified B cells were cultured with 1 μ g/ml soluble recombinant CD40 Ligand (R & D Systems, Minneapolis, MN) and interleukin (IL)-2 (2 ng/ml) (R & D Systems) for 4 days at a density of 2×10^5 cells per well in six-well plates. Cells were then washed and cultured with soluble recombinant mouse FLAG-tagged BAFF (produced at Genentech using the murine BAFF extracellular domain cloned into a pCMV-FLAG vector; Sigma, St. Louis, MO), IL-2 (2 ng/ml) (R & D Systems), and indicated blocking reagents (50 μ g/ml) in six-well plates at a density of 1×10^5 cells per well. Cell viability was assayed at the indicated time points using a Coulter Viacell (Beckman Coulter, Fullerton, CA).

Animals

This study was conducted at Shin Nippon Biomedical Laboratories USA, Ltd. (SNBL USA, Everett, WA), according to their standard operating procedures and in compliance with applicable regulations concerning the use of laboratory animals. Three- to five-year-old male and female naïve cynomolgus monkeys (weight range, 2.26 to 3.26 kg for the females and 2.64 to 4.50 kg for the males) were used in the study. All animals were acclimated to the study room for 28 days before the initiation of dosing.

Cynomolgus Monkey Study Design

Nineteen male and 19 female cynomolgus monkeys were each given a slow intravenous bolus injection of BR3-Fc at either 2 or 20 mg/kg or the BR3-Fc vehicle control once weekly for 13 (interim necropsy) or 18 weeks (see Table 1 for a detailed summary of the study design). The interim necropsy was performed on four animals (two males and two females) from the control and 20-mg/kg group at week 13; the main necropsy was performed on six animals (three males and three females) from each treat-

ment group at week 18; whereas the recovery necropsy was performed on six animals (three males and three females) from only the control and 20-mg/kg group at week 41. All animals in the 2-mg/kg group were necropsied at week 18.

Sample Collection for Flow Cytometry and IHC

For flow cytometry, samples were collected at SNBL USA and were kept on ice until placed on cold packs for overnight transport to Genentech. Whole-blood samples were collected using a butterfly infusion set or syringe. The samples were taken from a femoral or alternate vein and collected into heparinized tubes. Tissue samples from spleen, inguinal, mesenteric, and mandibular lymph nodes (about 1 cm³) were trimmed of excess fat and placed in chilled media in Petri dishes. Tissues were mechanically disrupted, and suspended cells were aspirated from a Petri dish and placed into prechilled 15-ml tubes. This procedure had no significant impact on cell viability as determined by Trypan Blue exclusion (data not shown). For IHC on paraffin-embedded tissues, sections of spleen and three different lymph nodes (axillary, mesenteric, and mandibular) were fixed in 10% neutral buffered formalin, routinely processed and paraffin embedded, sectioned at 5 μ m, and mounted on charged glass microscope slides (Erie Scientific, Portsmouth, NH). For frozen-section IHC, fresh sections of spleen were placed into plastic cryomolds, covered with OCT media (Miles Laboratories, Elkhart, IN) and snap frozen by submersion into 2-methyl butane chilled to its freezing point in liquid nitrogen.

Flow Cytometry

Samples (whole-blood or tissue cell suspensions) were incubated with saturating concentrations of fluorescently conjugated monoclonal antibodies to human CD3e, CD4, CD8, CD16, CD14, CD20, CD21, and CD27 (all from BD Biosciences, San Jose, CA), all of which are known to cross-react with cynomolgus monkey leukocytes, as previously described.³⁸ The following antibody cocktails were used: CD20-fluorescein isothiocyanate (FITC)/CD27-PE/CD21-APC, CD14-FITC/CD16-PE, CD3-APC, and CD8-FITC/CD4-PE/CD3-APC. For the BR3 expression analysis, samples were first incubated with anti-BR3-biotin (Biogen Idec, Cambridge, MA),²⁴ followed by a CD20-FITC/streptavidin-PerCp/CD21-APC antibody cocktail. Appropriate isotype controls were also prepared on each day of analysis for each animal. All incubations were performed at room temperature in the dark for 30 $\pm$ 5 minutes. After incubation, a Lyse Wash Assistant (BD Biosciences) was used to lyse red cells and perform a series of wash steps. At the end of the automated sample processing sequence, the Lyse Wash Assistant resuspended the cells in a fixative buffer. Flow cytometric analysis was performed on a FACSCalibur (BD Biosciences). For B-cell subset analysis, 20,000 lymphocyte events were acquired using a forward scatter/side scatter plot. Lymphocyte subsets were expressed as percent-

age of gated lymphocytes, using analysis techniques described previously.³⁸ For each whole-blood sample at each time point for each lymphocyte subset, the absolute cell count was calculated based on cell count data provided by SNBL USA. Absolute counts were compared with the control group using statistical tests described below.

Unlike peripheral blood samples, tissue samples were not collected at baseline (pretreatment). In addition, tissue B cells were expressed as a fraction of total lymphocytes rather than as an absolute B-cell count. Estimates of absolute lymphocyte counts cannot be done in nonhuman primate studies, because only a fraction of the total tissue is available for assay, because each lymphoid tissue is also used for histopathological and immunohistochemical evaluation. Thus, lymphoid tissue data in this study are presented as the fraction of B cells within the total lymphocyte population, and these values are compared with control group values as opposed to baseline values. Because other lymphocyte subsets in lymphoid tissues were not likely to have been affected by BR3-Fc, the B-cell fraction (of lymphocytes) is a relatively good estimate of the extent of B-cell reduction. In addition, this method has been previously used in all studies on B-cell reducing agents in nonhuman primates.^{39–41} For BR3 expression analysis, 50,000 lymphocyte events were acquired using a forward scatter/side scatter plot, and BR3 mean fluorescence intensity (MFI) was determined for B-cell subsets specified in the results.

Statistical Analysis of Flow Cytometric Data

For peripheral blood B cells and B-cell subsets, statistical analysis was performed on the percentage of baseline absolute counts (absolute B count, relative to baseline). The average of the measurements on study days –28, –14, and 0 was used as the baseline for B cells and B-cell subsets, and the percentage of baseline absolute count was calculated for each animal. Analyses were performed on absolute counts for peripheral blood T cells, CD4⁺ T cells, CD8⁺ T cells, and natural killer (NK) cells. For tissue samples, analyses were performed separately on percentage-gated cells at the interim (day 92/week 13), terminal (day 127/week 18), and recovery (day 287/week 41) necropsies.

A one-way analysis of variance was performed in a model with dose group as the factor. Comparisons between each of the BR3-Fc groups with the vehicle control group were performed using Dunnett's test. The analysis of variance was reduced to a two-sample *t*-test for the interim and recovery tissue samples and for peripheral blood during the recovery period when there were only two groups (vehicle control and the 20-mg/kg BR3-Fc group). Test results cited as statistically significant were significant at the conventional 5% level ($P < 0.05$).

IHC

For dual-label IHC on paraffin-embedded sections, 4- μ m sections of spleen and lymph node were deparaffinized

Figure 1. Histomorphology of a normal cynomolgus monkey splenic lymphoid follicle. Sections of cynomolgus splenic lymphoid follicle stained for the B-cell marker CD20 (blue) and α -SMA (brown) (**A**), CD20 (blue) and the T-cell marker CD3 (brown) (**B**), and IgD (green) and F-actin (red) (**C**). **A** and **B**: Serial paraffin-embedded sections; **C**: frozen section. **A**: The band of SMA⁺ myofibroblasts (MFs) divides the IMZ from the OMZ. **B**: Periaerterial lymphoid sheath (PALS) of CD3⁺ T cells that surrounds the central arteriole (CA) adjacent to the CD20⁺ lymphoid follicle. **C**: IgD expression differentiates between the GC⁺ IgD⁺ mantle zone (strongly IgD⁺) and the IMZ (weakly to moderately strongly IgD⁺). CA, central arteriole; MZ, mantle zone; PALS, periaerterial lymphoid sheath; PZ, peripheral zone. Bars = 100 μ m.

and then treated with Target Retrieval solution (Dakocytomation, Carpinteria, CA) heated to 99°C in a boiling water bath. Primary antibodies used in this study were mouse anti-human CD3 (clone SP34-2, used at 5 μ g/ml; BD/Pharmingen, San Diego, CA), mouse anti-human CD20 (clone L26, used at 1 μ g/ml; Dakocytomation), and mouse anti-human smooth muscle α -actin (clone 1A4, used at 0.1 μ g/ml; Dakocytomation). Isotype control antibodies were mouse IgG1 and mouse IgG2a (BD/Pharmingen). Sections were stained with the first primary antibody, then incubated with biotinylated horse anti-mouse IgG (Vector, Burlingame, CA), and finally incubated with avidin-biotin peroxidase complex (ABC-HRP Elite; Vector). The first primary antibody was detected with metal-enhanced diaminobenzidine (Pierce Chemical, St. Louis, MO). Slides were then subjected to a second round of

antigen retrieval, which served to denature and remove the first primary antibody complex. Slides were re-blocked for endogenous biotin and nonspecific protein interactions before incubation with mouse anti-human CD20. Slides were then incubated with biotinylated horse anti-mouse IgG followed by streptavidin alkaline phosphatase (Vector). Chromogenic detection of CD20 was performed using Alkaline Phosphatase Substrate kit III (Vector), producing a blue reaction product. Slides were washed, dehydrated in an alcohol series into a limonene-based clearing agent (Master Clear), and coverslipped using VectaMount (Vector).

For dual-label immunofluorescence, frozen sections of cynomolgus spleen were cut at 5 μ m. Frozen sections were blocked with 10% normal donkey serum and then incubated with either rabbit anti-human IgD (Dakocytomation, Carpinteria, CA) or rabbit anti-human CD20 (Dakocytomation, Carpinteria, CA).

mation) used at 10 $\mu\text{g/ml}$ or a mixture of rabbit anti-IgD and mouse anti-smooth muscle actin (clone 1A4; Dako) used at 5 $\mu\text{g/ml}$ for 1 hour at room temperature. Slides were washed twice and then incubated in either donkey anti-rabbit Cy2 (Jackson ImmunoResearch) or a mixture of donkey anti-rabbit Cy2 and donkey anti-mouse Cy3 at 2.5 $\mu\text{g/ml}$ for 30 minutes. Single-labeled slides were counterstained with Alexa Fluor 568 phalloidin (Molecular Probes, Eugene, OR) diluted 1:50 for 30 minutes. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (Molecular Probes), and sections were coverslipped with ProLong Gold fluorescence anti-fade mounting medium (Molecular Probes).

Fluorescently labeled sections were imaged on an Olympus BX-51 microscope equipped with filter cubes for 4',6-diamidino-2-phenylindole, FITC/Cy2, and tetramethyl rhodamine iso-thiocyanate/Cy3. Twelve-bit monochrome images were acquired using a Hamamatsu ORCA CCD camera driven by MetaMorph software (Universal Imaging Corporation, Downingtown, PA). Image files were transferred to Photoshop (Adobe, Mountain View, CA), adjusted for contrast, and merged to create 24-bit color images.

Histological Scoring of IHC Sections

Paraffin-embedded sections of spleen were scored for CD20⁺ B-cell follicle size, germinal center (GC) size, and outer marginal zone (OMZ) area based on the extent of follicular CD20⁺ staining outside of the smooth muscle α -actin (SMA)⁺ band of myofibroblasts surrounding each splenic lymphoid follicle, as described for human splenic white pulp by Steiniger et al.⁴² Representative paraffin-embedded sections of a normal cynomolgus monkey splenic lymphoid follicle double stained for CD20 and SMA (Figure 1A) and CD20 and CD3 (Figure 1B) are shown to demonstrate how the different splenic lymphoid follicle zones were determined using IHC and morphology. Paraffin-embedded sections of lymph node (LN) were scored for CD20⁺ B-cell follicle size and for the total extent of CD20⁺ staining throughout the LN. Semiquantitative IHC scores were assigned with the pathologist blinded to the treatment groups and were determined by examining several sections from each group, determining the largest and/or most extensive staining result for each category, and then assigning that result a score of 5. Then, each individual section was scored for each category with a score of 0 to 5, with 5 representing the largest or most extensive staining observed, and 0 being absent. Only spleen was examined for the interim necropsy group, whereas spleen and LNs were examined for both the main and recovery necropsy groups. CD3⁺ T-cell immunostaining was also assessed without scoring in each section of spleen and LN, and there were no differences observed between treatment groups. No staining was observed on matched control sections of spleen or lymph node treated with nonspecific murine IgG1 and mouse IgG2a antibodies.

For frozen-section dual-label immunofluorescence, sections of spleen were similarly scored 0 to 5 for total

lymphoid follicular IgD⁺ area and for the area between the strongly IgD⁺ zone (primarily mantle zone) and the SMA⁺ or F-actin⁺ band of myofibroblasts that demarcates the weakly to moderately strongly IgD⁺ inner marginal zone (IMZ) from the OMZ.^{42,43} A representative frozen section of a cynomolgus monkey splenic lymphoid follicle dual-fluorescently stained for IgD and F-actin is shown in Figure 1C to illustrate how IgD expression differentiates between the germinal center (IgD⁻), marginal zone (strongly IgD⁺) and the IMZ (weakly to moderately strongly IgD⁺).

Morphometric Image Analysis

Paraffin-embedded sections of spleen from all animals necropsied at week 18 were further evaluated for total lymphoid follicle size and OMZ area by computer-assisted image analysis using a calibrated MetaMorph Image Analysis System (Universal Imaging). Briefly, CD20/SMA dual-labeled IHC sections of spleen had representative 2 $\times$ images digitally captured. For each captured image, the CD20⁺ blue-staining lymphoid follicles and the CD20⁺ area outside of the brown staining SMA⁺ band of myofibroblasts that encircled each follicle (the OMZ area) were assessed for total area. The total follicle area and the OMZ area were each divided by the total number of follicles analyzed per section to derive an average total lymphoid follicle area and OMZ area for each animal.

Figure 2. BR3-Fc blocks BAFF-mediated survival and proliferation of cynomolgus monkeys B cells *in vitro*. Cynomolgus monkey B cells were isolated from PBMCs by MACS sorting. **A:** B cells were cultured with human CD40L (1 $\mu\text{g/ml}$) and IL-2 (2 ng/ml) for 4 days. B cells were then washed and cultured for 0 to 8 days with human IL-2 in the presence or absence (as indicated) of soluble murine BAFF (5 $\mu\text{g/ml}$), BR3-Fc (50 $\mu\text{g/ml}$), or human IgG (50 $\mu\text{g/ml}$). Viability was assayed at the indicated time points by trypan blue exclusion. Data show average viability ($n = 3/\text{time point}$); error bars show $\pm\text{SD}$ in this value. **B:** B cells were cultured in the presence or absence of BAFF (5 $\mu\text{g/ml}$), anti-IgM (10 $\mu\text{g/ml}$), and BR3-Fc or human IgG, as indicated. Proliferation was assessed by incorporation of tritiated thymidine. Shown are mean cpm ($n = 3$) $\pm$ SD.

Figure 3. Flow cytometric analysis illustrates BR3-Fc-mediated reduction and subsequent recovery of peripheral blood B cells in cynomolgus monkeys. Cynomolgus monkeys were treated weekly with 0, 2, or 20 mg/kg BR3-Fc for 18 weeks (127 days) and allowed to recover until week 41 (day 287). The total absolute CD20⁺ B-cell count and B-cell subset counts (as indicated) were assessed by FACS at the specified number of days after the first dose, as described in the text. **A:** BR3-Fc induced a non-dose-dependent decrease in the peripheral blood absolute CD20⁺ B-cell count through dosing on day 127, with eventual gradual recovery by day 287. The reduction in total CD20⁺ B-cell counts relative to the control group was statistically significant from day 29 through the terminal necropsy at day 127 (week 18) for the 2-mg/kg dose group (all 2-mg/kg dose group animals were necropsied at week 18) and from day 64 through day 225 (14 weeks after the end of dosing) for the 20-mg/kg dose group. **B:** BR3-Fc had no effect on peripheral blood CD20⁺ CD21⁻ B cells. **C:** BR3-Fc had no effect on peripheral blood CD21⁺CD27⁺ (memory) B cells, compared with the control group. **D:** BR3-Fc induced a non-dose-dependent decrease in the peripheral blood CD21⁺CD27⁻ (naïve) B-cell count, with eventual gradual recovery by day 287, as was seen for the total CD20⁺ B cells in **A**. The reduction in peripheral blood CD21⁺CD27⁻ B cells was a statistically significant decrease relative to the control group from day 15 through the terminal necropsy at day 127 (week 18) for the 2-mg/kg dose group (all 2-mg/kg dose group animals were necropsied at week 18), and from days 15 to 211 for the 20-mg/kg dose group. Total absolute cell counts ($\times 10^3/\text{ml}$) $\pm$ SD are shown at each time point.

Results

BR3-Fc Blocks BAFF-Mediated Survival and Proliferation of Cynomolgus Monkey B Cells in Vitro

The *in vitro* biological activity of BR3-Fc on cynomolgus monkey B cells was established in two different *in vitro* assays before *in vivo* administration. In the CD40L/BAFF-mediated B-cell survival assay,²⁴ B cells were isolated from cynomolgus monkey PBMCs and cultured with CD40L and IL-2 for 4 days. Cells were then washed and cultured with IL-2 and soluble BAFF in the presence or absence of BR3-Fc for another 8 days. In this assay, BR3-Fc blocked BAFF-mediated survival of cynomolgus monkey B cells, as measured by trypan blue exclusion (Figure 2A).

In the anti-IgM/BAFF-mediated B-cell proliferation assay, B cells were isolated from cynomolgus monkey PB-

MCs and cultured with anti-IgM and soluble BAFF and with various concentrations of BR3-Fc. BR3-Fc inhibited anti-IgM/BAFF-mediated *in vitro* proliferation of cynomolgus monkey B cells in a dose-dependent manner (Figure 2B).

Effects of BR3-Fc on Cynomolgus Monkey Peripheral Blood B Cells and B-Cell Subsets

BR3-Fc was administered weekly to cynomolgus monkeys at 0, 2, and 20 mg/kg for 18 weeks (day 127). B cells and B-cell subsets were monitored by flow cytometry in peripheral blood throughout the dosing period (days -28 to 127) and through the end of recovery period at week 41 (day 287). In peripheral blood, B-cell reduction was evident within 4 weeks of dosing (Figure 3A). Reduction in total B-cell counts relative to the control group was statistically significant from day 29 through the terminal

Table 2. B-Cell Subsets in Lymphoid Tissues Expressed as a Percentage of the Control*

		Spleen			Inguinal LN		
		Week 13	Week 18	Week 41	Week 13	Week 18	Week 41
All CD20 ⁺	2 mg/kg	ND	67	ND	ND	67	ND
	20 mg/kg	52 (<0.0001) [†]	56 (0.04)	138	77	39	98
All CD21 ⁺	2 mg/kg	ND	61 (0.04)	ND	ND	66	ND
	20 mg/kg	47 (<0.0001)	52 (0.01)	142	76	39	98
CD21 ^{high}	2 mg/kg	ND	56	ND	ND	47	ND
	20 mg/kg	45 (0.02)	37 (0.02)	143	43 (0.008)	16 (0.009)	77
CD21 ^{med} CD27	2 mg/kg	ND	54 (0.03)	ND	ND	67	ND
	20 mg/kg	37 (<0.0001)	55 (0.03)	140	65	51	107
CD21 ^{med} CD27 ⁺	2 mg/kg	ND	83	ND	ND	78	ND
	20 mg/kg	77	150	158 (0.04)	108	34	81

*Percentage of control group value was calculated using the formula: 100% × average B-cell fraction in the treatment group/average B-cell fraction in the control group.

[†]Statistically significant versus the control group. The number in parentheses indicates the *P* value.

LN, lymph node; ND, not done.

necropsy at day 127 (week 18) for the 2-mg/kg dose group (all 2-mg/kg dose group animals were necropsied at week 18) and from days 64 through 225 (14 weeks after the end of dosing) for the 20-mg/kg dose group. The extent of B-cell reduction at the end of dosing period (week 18) varied from ~45 to 60% of baseline. There was no evidence of dose dependency in the degree of peripheral blood B-cell reduction at the doses examined.

CD21⁺ B cells in both BR3-Fc treatment groups were decreased in a manner similar to total B cells (data not shown). In contrast, no consistent change in CD21[−] B cells, a subpopulation that comprises a larger fraction of peripheral blood B cells in cynomolgus monkeys than in

humans,⁴⁴ was observed in either treatment group (Figure 3B). CD21⁺ B cells in peripheral blood can be subtyped into CD21⁺CD27⁺ and CD21⁺CD27[−] B cells. Based on expression of phenotypic markers, CD21⁺CD27⁺ and CD21⁺CD27[−] subsets are thought to be analogous to human memory and naïve cells, respectively.^{38,45} No consistent drug-induced change in peripheral blood CD21⁺CD27⁺ B cells was observed in the 2- and 20-mg/kg dose groups compared with the control group (Figure 3C). In contrast to CD21⁺CD27⁺ B cells, peripheral blood CD21⁺CD27[−] B cells showed a decrease very similar to that seen in total B cells (Figure 3D), with a statistically significant decrease relative to the control group from day 15 through the terminal necropsy at day 127 (week 18) for the 2-mg/kg dose group (all 2-mg/kg dose group animals were necropsied at week 18), and from days 15 to 211 for the 20-mg/kg dose group. At the end of the dosing period (week 18, day 127), the average CD21⁺CD27[−] B-cell count was reduced to 44% of baseline in the 2-mg/kg group and to 66% of baseline in the 20-mg/kg group. Therefore, in peripheral blood, the reduction in B cells induced by BR3-Fc was mainly due to a reduction in CD21⁺ B cells and, in particular, to a reduction in CD21⁺CD27[−] B cells (phenotypically similar to naïve B cells in humans). These findings differ from those seen in lymphoid tissues, where BR3-Fc induced a decrease in both CD21⁺CD27⁺ (memory) and CD21⁺CD27[−] (naïve) B cells (see below).

Clear evidence of peripheral blood B-cell, CD21⁺ B-cell, and CD21⁺CD27[−] B-cell recovery was observed in each of the six recovery animals in the 20-mg/kg group by the end of the 23-week recovery period (Figure 3).

Figure 4. FACS analysis illustrates BR3-Fc-mediated reduction and subsequent recovery of total B cells (CD20⁺) in lymphoid tissue of cynomolgus monkeys. Cynomolgus monkeys were treated weekly with 0, 2, or 20 mg/kg BR3-Fc for 18 weeks (127 days) and allowed to recover until week 41 (day 287). The B-cell fraction of tissue lymphocytes was assessed by FACS at the specified number of weeks after the first dose, as described in the text. CD20⁺ B cells in the bone marrow (BM); spleen; and inguinal (LN-Ing), mandibular (LN-Man), and mesenteric (LN-Mesen) lymph nodes were analyzed by FACS at week 13 (A), week 18 (B), and week 41 (C) after the first dose. **A:** BR3-Fc at 20 mg/kg induced a decrease in the total B-cell fraction in the spleen and the inguinal and mandibular lymph nodes; this decrease was statistically significant in the spleen (*P* = 0.0003). **B:** Both doses of BR3-Fc induced a decrease in the total B-cell fraction in all tissues examined without a clear dose-dependent effect at week 18. Statistically significant decreases are noted with *P* values. **C:** By the end of recovery period at week 41, all tissue B cells had essentially returned to baseline levels. The mean total B-cell fraction (CD20⁺) of lymphocytes ± SD is shown.

Effects of BR3-Fc on B Cells and B-Cell Subsets in Lymphoid Tissues

Lymphoid tissue B cells were examined on days 92 (week 13; interim necropsy), 127 (week 18; terminal necropsy), and 287 (week 41; recovery necropsy) by both flow cytometry and IHC. Flow cytometric analysis was performed on single cell suspensions obtained from the bone marrow, spleen, inguinal, submandibular, and mesenteric lymph nodes (LNs). B-cell subsets, T cells, and NK cells were measured as fraction of lymphocytes. IHC

Table 2. *Continued*

Mandibular LN			Mesenteric LN		Bone marrow	
Week 13	Week 18	Week 41	Week 18	Week 41	Week 18	Week 41
ND	33 (<0.0001)	ND	55 (0.02)	ND	64	ND
69	38 (<0.0001)	131	39 (0.003)	82	71	74
ND	32 (<0.0001)	ND	55 (0.02)	ND	56	ND
68	37 (<0.0001)	130	39 (0.003)	82	62	65
ND	22 (0.004)	ND	36 (0.02)	ND	ND	ND
43 (0.02)	20 (0.003)	198	25 (0.008)	82	ND	ND
ND	30 (<0.0001)	ND	53 (0.02)	ND	54	ND
55 (0.01)	33 (<0.0001)	130	41 (0.003)	77	57	67
ND	36 (<0.0001)	ND	64	ND	84	ND
88	46 (0.001)	127	43 (0.009)	90	127	47

was performed on tissue sections of spleen and axillary, mandibular, and mesenteric LNs.

B-cell fractions in both BR3-Fc-treated groups as measured by flow cytometry were reduced in all organs at weeks 13 and 18 (Figure 4). Differences between the BR3-Fc-treated groups and the control group were not always statistically significant; however, the sample sizes were small. At week 13, B-cell fractions in the 20-mg/kg group were 52 to 77% of control values for the spleen and LNs; the reduction was statistically significant only for the spleen (Figure 4A; Table 2). B cells in lymphoid tissues of both treatment groups at week 18 ranged from 33 to 72% of control with no good evidence of dose dependency; this reduction was statistically significant in the spleen and LNs (Figure 4B; Table 2). For the splenic B cells, the extent of reduction in the 20-mg/kg group was comparable at weeks 13 and 18 (~50 to 55% of control). LN B cells were reduced to a somewhat greater extent at week 18 compared with week 13: ~38 and 69% in the mandibular LNs at weeks 18 and 13, respectively. At week 18, the degree of B-cell reduction was the highest in the mesenteric and mandibular LNs, followed by the spleen, blood, and bone marrow.

Evaluation of B cells in lymphoid tissues by IHC at weeks 13 and 18 was generally in good agreement with the flow cytometric results, and the results are detailed below. As was the case with fluorescent activated cell sorter (FACS) analysis, the BR3-Fc-induced decreases in B-cell IHC staining were not clearly dose dependent for the dose levels examined.

After recovery at week 41, B-cell levels by FACS analyses and by IHC staining were essentially the same in animals from the control and the 20-mg/kg BR3-Fc-treated groups (Tables 2, 3, and 4). In addition, lymphoid follicle morphology as detected by CD20 IHC was the same in the control and the 20-mg/kg BR3-Fc-treated groups (data not shown).

BR3-Fc Decreases CD20⁺ Follicular and Marginal Zone B Cells in Tissues

Both BR3-Fc treatment groups showed a decrease in CD20⁺ follicular B cells in the spleen and LNs (axillary, mandibular, and mesenteric) compared with the control group (Tables 3 and 4). This decrease in CD20⁺ B cells was evident as a decrease in the total CD20⁺ B-cell

Table 3. Summary of Semiquantitative IHC Analysis: Spleen

		Week 13	Week 18	Week 41
Follicle size	Control	4.5 ± 0.5	4.2 ± 0.7	4.2 ± 0.7
	BR3-Fc 2 mg/kg	—	2.8 ± 0.8*	—
	BR3-Fc 20 mg/kg	3.0 ± 0.0*	2.5 ± 0.8*	4.5 ± 0.5
IgD ⁺ area	Control	—	4.8 ± 0.4	—
	BR3-Fc 2 mg/kg	—	3.2 ± 0.8*	—
	BR3-Fc 20 mg/kg	—	2.8 ± 0.8*	—
OMZ area	Control	4.8 ± 0.4	4.0 ± 0.6	4.7 ± 0.5
	BR3-Fc 2 mg/kg	—	2.7 ± 0.8*	—
	BR3-Fc 20 mg/kg	2.8 ± 0.5*	2.0 ± 0.6*	4.3 ± 0.8
GC area	Control	4.5 ± 0.5	4.5 ± 0.8	4.3 ± 0.7
	BR3-Fc 2 mg/kg	—	3.5 ± 0.6	—
	BR3-Fc 20 mg/kg	3.5 ± 0.6	3.2 ± 0.4	4.7 ± 0.5
Area between IgD ⁺ zone and SMA ⁺ band	Control	—	1.8 ± 0.9	—
	BR3-Fc 2 mg/kg	—	1.2 ± 0.4	—
	BR3-Fc 20 mg/kg	—	2.2 ± 0.8	—

*P < 0.05 versus control.

Sections were scored 0 to 5 semiquantitatively in a blinded fashion with 0 = absent and 5 = most extensive or largest.

Table 4. Summary of Semiquantitative IHC Analysis: Lymph Nodes

		Axillary LN			Mandibular LN			Mesenteric LN		
		Week 13	Week 18	Week 41	Week 13	Week 18	Week 41	Week 13	Week 18	Week 41
Follicle size	Control	—	4.5 ± 0.5	3.8 ± 0.4	—	4.5 ± 0.5	4.3 ± 0.8	—	4.2 ± 0.8	4.5 ± 0.5
	BR3-Fc 2 mg/kg	—	2.8 ± 0.8*	—	—	3.3 ± 1.4	—	—	3.2 ± 1.0	—
	BR3-Fc 20 mg/kg	—	2.3 ± 0.5*	3.7 ± 0.5	—	3.5 ± 0.5	4.2 ± 0.8	—	3.5 ± 1.0	4.2 ± 0.4
CD20 ⁺ area	Control	—	4.2 ± 0.8	3.8 ± 0.8	—	4.5 ± 0.8	4.3 ± 0.5	—	4.3 ± 0.8	4.3 ± 0.5
	BR3-Fc 2 mg/kg	—	2.5 ± 0.5*	—	—	2.8 ± 1.2*	—	—	2.3 ± 0.8*	—
	BR3-Fc 20 mg/kg	—	2.3 ± 1.0*	4.2 ± 0.8	—	3.0 ± 0.0*	4.5 ± 0.5	—	2.5 ± 0.5*	4.0 ± 0.6

**P* < 0.05 versus control.

Sections were scored 0 to 5 semiquantitatively in a blinded fashion with 0 = absent and 5 = most extensive or largest.

follicle size in both the spleen and in the LNs (Figure 5; Tables 3 and 4) and as a reduction in total CD20⁺ staining in LNs. The BR3-Fc effect on B-cell follicles in the spleen was more consistent and more pronounced than it was in the three different LNs examined. Computer-assisted image analysis results corresponded very strongly with the blinded semiquantitative scoring results, and showed an approximately 40% decrease in total CD20⁺ splenic follicular area, which consisted of GCs, the mantle zone, the IMZ, and the OMZ. The decrease in splenic follicular GC CD20⁺ B cells was much less pronounced (and did not reach statistical significance) than was the decrease in total follicular B cells within the SMA⁺ band of myofibroblasts, indicating that BR3-Fc more specifically targeted the mantle zone, the IMZ, or both (Figure 5, double-headed arrows). To better address the effects of BR3-Fc on this region of the splenic lymphoid follicle, frozen-section dual-label immunofluorescence for IgD⁺ and SMA or F-actin was performed. Both BR3-Fc treatment groups induced a pronounced reduction in the IgD⁺ area, but no clear difference in the area between the strongly IgD⁺ lymphocytes in the mantle zone, and the SMA⁺ or F-actin⁺ myofibroblasts that delineated the OMZ from the rest of the follicle were evident (Figure 6), most likely because of the variability in IgD expression by B cells in the IMZ.^{42,43} Because IgD is strongly expressed by mantle zone B cells but is also variably (diffusely weakly to sometimes strongly) expressed by B cells in the IMZ,^{42,43} a decrease in the IgD⁺ area can be interpreted as a decrease in both mantle zone and IMZ B cells. The OMZ is clearly delineated from the IMZ by a SMA⁺ band of myofibroblasts that surrounds every splenic follicle (illustrated in Figure 1A).⁴² Both BR3-Fc treatment groups also demonstrated a clear reduction in their splenic lymphoid follicle OMZ area (Figure 6, asterisks). Computer-assisted image analysis results again confirmed the semiquantitative scoring results and showed an approximately 30 to 35% decrease in the OMZ area (Tables 3, 4, and 5). Again, these results were not clearly dose dependent (Table 5).

The BR3-Fc-mediated decrease in marginal zone B cells was also evident by FACS analysis. CD21⁺ B cells in tissues have a distinct subset of CD21^{high} B cells that have relatively high CD21 levels compared with peripheral blood CD21^{med+} B cells and with the rest of the CD21^{med+} B cells in lymphoid tissues. These CD21^{high} B cells are phenotypically similar to human marginal zone B cells^{38,45} and have an approximately twofold increase in

BR3 expression (*P* < 0.001) compared with CD21^{med} B cells (Figure 7, A and B). CD21^{high} B cells were reduced to 16 to 56% of control values for weeks 13 and 18 (Figure 7C; Table 2). Specifically, at week 18, CD21^{high} B cells in the 20-mg/kg dose group were 37, 16, 20, and 25% of control values for the spleen and inguinal, mandibular, and mesenteric LNs, respectively, whereas those in the 2-mg/kg dose group were 56% of control values in the spleen and 22 to 47% of control values in the LNs. After recovery at week 41, CD21^{high} B cells in the 20-mg/kg group were essentially the same as those in the control group.

BR3-Fc Decreases Both CD21^{med}CD27⁺ and CD21^{med}CD27[−] B Cells in Lymphoid Tissues

CD21^{med+} B cells in lymphoid tissues can be subtyped into CD21^{med}CD27⁺ and CD21^{med}CD27[−] B cells by FACS, as is the case in peripheral blood. Consistent with findings in peripheral blood, CD21^{med}CD27⁺ (analogous to memory) B cells in lymphoid tissues were not significantly reduced at week 13 by FACS analysis (Table 2). However, in contrast to results in peripheral blood, by week 18, CD21^{med}CD27⁺ B cells were reduced to between ~35 and 50% of control in the LNs and spleen of the 20-mg/kg group; this reduction was statistically significant in the mandibular and mesenteric LNs. CD21^{med}CD27⁺⁺ B cells were also reduced in the LNs and in the spleen of the 2-mg/kg dose group at week 18, as assessed by FACS analysis. CD21^{med}CD27[−] (analogous to naïve) B cells in lymphoid tissues were also significantly reduced at weeks 13 and 18 (Table 2). At week 18, CD21^{med}CD27[−] B cells were reduced the most in the mesenteric and mandibular LNs (to between 30 and 53% of control), followed by the blood (to ~40% of control), and then by spleen and bone marrow (to ~55% of control). After recovery at week 41, both CD21^{med}CD27⁺ and CD21^{med}CD27[−] B cells in the 20-mg/kg group were essentially the same as those in the control group (Table 2).

BR3-Fc Effects on Plasma Cells, T Cells, and NK Cells

IHC for plasma cells was performed on sections of spleen using an anti-plasma cell antibody (clone VS38c; Dako-

Figure 5. Dual-label IHC for CD20 (blue) and smooth muscle actin (SMA) (brown) on week-18 spleens from BR3-Fc-treated cynomolgus monkeys illustrates a decrease in follicular CD20⁺ immunostaining as well as in the OMZ of BR3-Fc-treated spleens. Representative paraffin-embedded sections of spleen dual-labeled for CD20 (blue) and SMA (brown) from two untreated control animals (**left**), two animals treated for 18 weeks with 2 mg/kg BR3-Fc (**middle**), and two animals treated with 20 mg/kg BR3-Fc (**right**). These panels illustrate that both doses of BR3-Fc induced a decrease in both total follicle size, particularly in the CD20⁺ follicular lymphocytes between the GC and the SMA⁺ band, indicated by **double-headed arrows**, and in the outer marginal zone area (CD20⁺ lymphocytes outside the SMA⁺ band identified by **asterisks**). Bars = 200 μ m.

Figure 6. Dual-label fluorescent IHC for IgD (green) and F-actin (red) on week-18 spleens from BR3-Fc-treated cynomolgus monkeys illustrates a decrease in IgD⁺ lymphocytes in BR3-Fc-treated spleens. Representative frozen sections of spleen dual-labeled for IgD (green) and F-actin (red) from two untreated control animals (**left**), two animals treated for 18 weeks with 2 mg/kg BR3-Fc (**middle**), and two animals treated with 20 mg/kg BR3-Fc (**right**). These panels illustrate that both doses of BR3-Fc induced a decrease in the follicular area expressing IgD, particularly in the lymphocytes that were strongly IgD⁺ (the mantle zone). In addition, BR3-Fc also induced a decrease in the IgD⁺ lymphocytes in the weakly to moderately strongly IgD⁺ lymphocytes in the IMZ (illustrated by **double-headed arrows** as the area between the strongly IgD⁺ mantle zone and the red band of myofibroblasts). MF, myofibroblasts. Bars = 100 μ m.

Table 5. Summary of Week 18 Quantitative Splenic IHC Analysis

	Control	BR3-Fc 2 mg/kg	BR3-Fc 20 mg/kg
Total follicle area (μm^2)	4927 $\pm$ 1190	3119 $\pm$ 1023*	3088 $\pm$ 730*
OMZ area (μm^2)	1329 $\pm$ 336	903 $\pm$ 506*	921 $\pm$ 195

* $P < 0.05$ versus control.

Follicle and OMZ areas were measured using a MetaMorph image analyzer with the user blinded to the treatment groups.

cytometry), but no differences in plasma cell numbers were evident between treatment groups, possibly because of the low numbers of tissue plasma cells present in normal lymphoid tissues (data not shown). No consistent differences between groups were observed in natural killer cells, CD8⁺ T cells, and CD4⁺ T cells in the peripheral blood (data not shown). In tissues, T-cell and NK-cell fractions were, in general, similar or somewhat higher in the BR3-Fc treatment groups compared with control (data not shown). Because no corresponding increases in peripheral blood T and NK cells were observed, the increase in the T-cell fraction in spleen and LNs was most likely the result of B-cell reduction in these tissues. These flow cytometric findings were supported by IHC staining for CD3, which also showed no effect on CD3⁺ T cells in either the spleen or in the LNs (data not shown).

Figure 7. Higher BR3 expression in CD21^{high} (marginal zone) B cells and BR3-Fc-mediated targeting of these CD21^{high} B cells in the spleen and lymph nodes of cynomolgus monkeys. Cynomolgus monkeys were treated weekly with 0, 2, or 20 mg/kg BR3-Fc for 18 weeks. **A** and **B**: Spleen samples from animals in the 0-mg/kg control group were stained with antibodies to CD20, CD21, and BR3 and assessed for expression of BR3. **A**: Representative FACS expression of BR3 for B cells expressing CD21^{med} (dotted line) or CD21^{high} (bold solid line). **B**: A summary of BR3 MFI for the two different B-cell subsets. Lines correspond to the average BR3 MFI for each subset (CD21^{med} and CD21^{high}) and demonstrate that the CD21^{high} B cells have a higher level of BR3 expression than do CD21^{med} B cells. **C**: The CD21^{high} (marginal zone) B-cell fraction of lymphocytes was assessed by flow cytometry at 18 weeks after the first dose, as described in the text. The mean CD21^{high} B-cell fraction $\pm$ SD is shown. LN-Ing, LN-Man, and LN-Mesen are inguinal, mandibular, and mesenteric lymph nodes, respectively. P values denote decreases that are statistically different from control.

Discussion

In this study, we have demonstrated that weekly treatment of cynomolgus monkeys with a soluble BAFF-blocking agent, BR3-Fc, for 13 to 18 weeks resulted in significant B-cell reduction in both the peripheral blood and within lymphoid organs, with a full return to baseline levels after a 23-week recovery period. This reduction in B cells had a functional consequence, because the 2-mg/kg dose of BR3-Fc induced a modest but statistically significant decrease in the IgG titer to a repeat immunization with tetanus toxoid (data not shown). We further demonstrate that BR3-Fc had direct biological effects on cynomolgus monkey B cells, because it blocked the BAFF-mediated survival and proliferation of cynomolgus monkey B cells in two different *in vitro* assays. The BR3-Fc-mediated reduction in B cells both in the peripheral blood and in lymphoid organs was not clearly dose dependent, suggesting that even the lower dose of 2 mg/kg was at or near a biologically maximally effective dose.

FACS analysis demonstrated that BR3-Fc reduced CD21^{med}CD27[−] (analogous to naïve) B cells in all secondary lymphoid tissues evaluated (spleen and inguinal, mandibular, and mesenteric lymph nodes). BR3-Fc also reduced CD21^{med}CD27⁺ (analogous to memory) B cells in lymphoid tissues by FACS analysis, but to a lesser extent. As was the case in the peripheral blood, these reductions were not clearly dose dependent. In addition, by FACS analysis, BR3-Fc significantly reduced CD21^{high} B cells, a B-cell subset that has phenotypic features of human marginal zone B cells.^{38,45,46} Consistent with these FACS results, dual-label IHC and image analysis of lymphoid organs demonstrated that BR3-Fc decreased follicular B cells outside of the germinal center and the B cells within the OMZ outside of the SMA⁺ myofibroblast layer. Immunofluorescence for IgD further demonstrated a reduction in IgD⁺ B cells, a population that resides within both the mantle zone and the IMZ.⁴² These results, together with the observation that CD21^{high} B cells (phenotypically analogous to marginal zone B cells) have a nearly twofold higher level of BR3 expression than do CD21^{med} B cells, are consistent with the conclusion that the BR3-Fc reduction of lymphoid tissue B cells was most specific for the marginal zone (both the IMZ and the OMZ), as well as for the lymphoid follicle exclusive of the germinal center.

In addition to decreasing CD21^{high} marginal zone B cells in lymphoid tissues, BR3-Fc also decreased CD21^{med}CD27[−] (naïve) tissue B cells, a population that most likely resided within the mantle zone, because human mantle zone B cells are naïve by virtue of not having mutated Ig V-region genes.^{47,48} The decrease in IgD⁺ B cells demonstrated by immunofluorescence also supports the contention that BR3-Fc decreases B cells in the mantle zone in addition to those in the marginal zone and follicle.

These findings greatly expand on the findings in a previous study that analyzed the *in vivo* effects of a blocking anti-BAFF antibody on cynomolgus monkey B cells.⁴¹ In that study, an anti-BAFF antibody, belimumab, admin-

istered weekly to cynomolgus monkeys for 4 weeks led to a decrease in the percentage of both total CD20⁺ B cells and CD20⁺CD21⁺ B cells in the spleen and mesenteric lymph nodes that was similar in magnitude to that seen in the current study. B-cell subset analysis and IHC was not performed in that study, nor were any changes seen in peripheral blood B cells. In contrast, in the current study, we have performed a detailed B-cell subset FACS analysis in blood and lymphoid tissues and confirmed those findings using dual-label IHC, including quantitative morphometric analysis.

The BR3-Fc-induced decrease in marginal zone B cells that was observed in cynomolgus monkeys in this study was also seen in hCD20⁺ transgenic mice (mice expressing human CD20 on their lymphocytes), in which a similar reduction in splenic marginal zone B cells was seen after treatment with BR3-Fc.⁴⁹ This decrease in marginal zone B cells by BR3-Fc is of potential therapeutic significance, because there has been speculation that these cells might play a role in human autoimmune diseases.^{31,46,50} Marginal zone B cells are the predominant B-cell subset in the thyroid gland lymphocytic infiltrate of patients with Grave's disease,⁵¹ as well as in the salivary gland of BAFF transgenic mice that develop a Sjögren's syndrome-like sialadenitis.²⁷ In addition, patients with Sjögren's syndrome frequently develop a form of B-cell lymphoma with a marginal zone phenotype.^{52,53} Lastly, marginal zone B cells are much more efficient at antigen presentation and the delivery of co-stimulatory signals to T cells than are follicular B cells,^{46,54} and are also one of the primary B-cell populations (along with B1 cells) responsible for the generation of a T-independent immune response.^{46,55} Thus, the previously reported literature suggests that the targeting of marginal zone B cells by BR3-Fc may be of therapeutic significance.

Interestingly, mice are much more rapidly and profoundly affected after BR3-Fc treatment than are cynomolgus monkeys, with C57BL/6 mice showing marked reduction of splenic B cells within 8 days after a single dose of BR3-Fc,³⁷ and the previously described hCD20⁺ transgenic mice showing a similar marked reduction in splenic B cells within 4 days of a single BR3-Fc injection.⁴⁹ Because BR3-Fc had robust *in vitro* biological activity on cynomolgus monkey B cells in two different assay systems, this difference was not very likely due to low activity of BR3-Fc on cynomolgus monkey B cells. Another possibility is that the different kinetics and degree of BR3-Fc-mediated B-cell reduction in nonhuman primates and mice are due to the differences in B-cell follicles, and in particular the morphology of the marginal zone, between the two species. Mouse splenic white pulp is anatomically very different from primate splenic white pulp, consisting of a large T-cell zone surrounding the central arteriole (the periarteriolar lymphoid sheath zone) capped by smaller peripheral lymphoid follicles. The lymphoid follicles are surrounded by a marginal sinus containing marginal metallophilic macrophages, which serves to delineate the follicular B cells from the marginal zone B cells.^{45,46} In contrast, primate splenic white pulp lacks marginal zone sinuses and has large B-cell lymphoid follicles with a prominent SMA⁺ band of myofibroblasts that divides the outer marginal zone from the rest of the follicle.⁴²

In addition, mouse marginal zone B cells do not recirculate, are naïve B cells, and are found only in the spleen; whereas human, and very likely all primate, marginal zone B cells recirculate, consist primarily of memory B cells, and are found in other anatomical sites in addition to the spleen.^{45–47} These differences in anatomical localization and recirculation of marginal zone B cells between mice and primates, as well as the microanatomic differences in lymphoid follicle morphology, may at least partially explain why mouse B cells were much more rapidly decreased by BR3-Fc than were primate B cells. In addition, primate B cells may be less dependent on BAFF signaling for survival than are murine B cells and therefore require a much longer period of BAFF blockade to achieve substantial reduction in their numbers. Based on these findings, further investigation into the mechanisms of BAFF-mediated B-cell survival in mice versus primates is warranted.

There is significant evidence linking elevations in serum BAFF to autoimmunity. Like most members of the TNF superfamily, BAFF exists in both a soluble and a membrane-bound form.⁵⁶ Many patients with Sjögren's syndrome, SLE, and RA have elevated levels of the soluble form of BAFF in their serum.^{26,27,29} In RA patients, synovial fluid was found to contain even higher levels of soluble BAFF than did the serum from the same patients.³⁰ In addition, BAFF-overexpressing transgenic mice develop marked B-cell hyperplasia and an autoimmune phenotype that includes a lupus-like glomerulonephritis^{8,9,31} and the previously mentioned salivary gland inflammation that resembles Sjögren's syndrome.²⁷

Evidence that BAFF blockade can ameliorate disease symptoms in a variety of autoimmune diseases comes from mouse animal models of SLE and RA. Treatment of lupus-prone NZB/WF1 mice with both BR3-Fc³⁵ and TACI-Ig⁷ significantly decreased the proteinuria and glomerular damage seen in these mice, whereas treatment of mice with the collagen-induced arthritis model of RA also significantly reduced both their paw swelling and degree of histological joint damage.⁵ These findings in animal models in conjunction with the evidence of serum and joint BAFF elevations in patients with autoimmune disease offer further support to the contention that blockade of BAFF signaling may be of therapeutic benefit in a variety of autoimmune diseases.

In summary, we have characterized in detail the effects of a soluble BAFF receptor fusion protein, BR3-Fc, on cynomolgus monkey B-cell subsets in peripheral blood and in lymphoid tissues, as well as on the lymphoid histomorphology and immunophenotype of secondary lymphoid organs. We have demonstrated a clear reduction of total B cells both in the peripheral blood and in lymphoid organs, with a decrease in both marginal zone and follicular B cells in tissues, an effect that appears to be unique to BR3-Fc. These findings should prove very useful in guiding the potential therapeutic use of BR3-Fc for autoimmune diseases in the clinic.

Acknowledgments

We thank Julio Ramirez and the entire Genentech Histology Laboratory for their very capable technical assistance with the processing, sectioning, and staining of histological specimens. We also thank Clarissa Flores and Olivia Hwang for technical assistance with the FACS analysis, and Susan Palmieri for technical assistance with the morphometric image analysis.

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