Rescue of the mature B cell compartment in BAFF-deficient mice by treatment with recombinant Fc-BAFF

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Abstract

BAFF deficiency in mice impairs B cell development beyond transitional stage 1 in the spleen and thus severely reduces the size of follicular and marginal zone B cell compartments. Moreover, humoral immune responses in these mice are dramatically impaired. We now addressed the question whether the decrease in mature B cell numbers and the reduced humoral immune responses in BAFF-deficient mice could be overcome by the injection of recombinant BAFF. We therefore engineered a recombinant protein containing the human IgG1 Fc moiety fused to receptor binding domain of human BAFF (Fc-BAFF). At one week after the second injection of this fusion protein a complete rescue of the marginal zone B cell compartment and a 50% rescue of the follicular B cell compartment was observed. Moreover these mice mounted a T cell-dependent humoral immune response indistinguishable from wild type mice. By day 14 upon arrest of Fc-BAFF treatment mature B cell numbers in the blood dropped by 50%, indicating that the life span of mature B cells in the absence of BAFF is 14 days or less.

Collectively these findings demonstrate that injection of Fc-BAFF in BAFF-deficient mice results in a temporary rescue of a functional mature B cell compartment.

Introduction

The first steps of B cell development take place in the bone marrow, where cells are selected for productive rearrangement of the immunoglobulin heavy and light chain genes and subsequently for the surface expression of a proper non-autoreactive B cell receptor (BCR) [1-4]. These immature B cells then leave the bone marrow and differentiate further in the spleen through transitional phases 1, 2 and 3 (T1, T2 and T3). These stages can be distinguished by the expression of CD21, CD23, IgM and IgD, T1 being defined as $CD21^{-}CD23^{-}IgM^{high}IgD^{low}$, T2 $CD21^{+}CD23^{+}IgM^{high}IgD^{high}$ and T3 $CD21^{+}CD23^{+}IgM^{low}IgD^{high}$ [5-9]. Transitional B cells then differentiate into follicular or marginal zone mature B cells (FO B and MZB). The hallmark of the transition from immature to mature B cells is the loss of CD93 cell surface marker [6].

B cell activator factor of the TNF family (BAFF) [10], a member of the Tumor Necrosis Factor (TNF) family, has been identified as the key factor in the survival of immature B cells as they differentiate to mature B cells. BAFF-deficient mice were initially described as having a major block at the T1 stage of B cell differentiation and very low numbers of mature B cells [11]. It was shown later that B cells beyond the T1 stage do exist in BAFF-deficient mice, but that they are present in reduced number and fail to express CD21 and CD23 [Gorelik 2004, JI 172:762]. BAFF binds to three different receptors, BAFF-R, TACI and BCMA [12-14]. Among these receptors, only BAFF-R deficiency recapitulates the B cell lymphopenia observed in BAFF-deficient mice, demonstrating that BAFF binding to BAFF-R is the critical event in

mature B cell differentiation [15-18]. Upon binding, BAFF-R induces the activation of the NF-κB pathway, which then induces the expression of anti-apoptotic members of the Bcl-2 family [19, 20]. Transgenic expression of Bcl-2 in B cells of BAFF-deficient mice rescue mature FO B cells development but not MZB [21]. This suggests that at transitional stage survival signals are necessary and sufficient to allow differentiation into the FO B cell lineage, whereas it is not sufficient to induce MZB differentiation.

BAFF-mediated survival signals are not only required during the transition from immature to mature B cells but also during the entire life of naïve B cells. Inhibition of BAFF-R signalling by injection of TACI-Ig, BAFF-R-Ig or BAFF-R-specific antagonist antibodies in wild type mice results in the rapid loss of mature B cells [22-25].

We demonstrate here that injection of BAFF-deficient mice with a recombinant Fc-BAFF protein can rescue mature B cell development and leads to the formation of a normal splenic B cell zone architecture. Moreover, treatment completely restored the ability of BAFF-deficient mice to mount an antibody response against T-dependent antigen. When Fc-BAFF treatment was interrupted, mature B cell population decreased with an estimated half-life time of ≤ 14 days.

Altogether, these findings demonstrate that BAFF-deficiency can be almost completely corrected by exogenous administration of recombinant Fc-BAFF.

Results

Fc-BAFF recombinant protein forms oligomeric complexes, binds to mouse BAFF receptors and induces survival of sorted mouse mature B cells.

We generated a recombinant fusion protein consisting of the TNF homology domain of the human BAFF and the Fc part of human IgG1 (Fc-BAFF) (fig. 1a). This protein was produced in Chinese hamster ovary cells and purified from supernatants by affinity chromatography on Protein A. Fc-BAFF had apparent molecular weights of 50 kDa and 100 kDa by SDS-PAGE under reducing and non-reducing conditions, indicating that a disulfide bond between two Fc moieties led to the formation of dimers (fig. 1b).

Crystallographic studies demonstrated that the soluble form of hBAFF forms trimers [26]. We therefore expected Fc-BAFF protein to multimerize under native conditions. Indeed, when analyzed on a Superdex-200 gel filtration column, Fc-BAFF eluted inbetween the 440 and 232 kDa markers, in agreement with the predicted size of ~300 kDa for hexameric Fc-BAFF (fig. 1c). These data strongly suggest that Fc-BAFF indeed contains 2 trimeric hBAFF portions and 3 dimeric Fc parts assembled in a hexamer (fig. 1d). Previously it was shown that FasL-Fc forms a similar type of complex [27].

We next addressed the binding properties of Fc-BAFF to mouse BAFF receptors, BAFF-R, TACI and BCMA. Fc-BAFF binding could be detected on transfectants expressing BAFF-R and TACI but could not be observed on

cells expressing BCMA (fig. 1e) reflecting the different affinity of BAFF for its receptors.

We then tested Fc-BAFF biological activity in vitro. For this purpose, we addressed the ability of Fc-BAFF to induce survival of mature splenic B cells. As shown in figure 2, the vast majority of CD19+ B cells kept for 5 days in culture in the absence of survival signals were propidium iodide positive, but the addition of 1 to 100 ng/ml of Fc-BAFF induced B cell survival in a dose-dependent manner. Altogether, these data show that recombinant Fc-BAFF has similar receptor-binding properties and in vitro biological activity as BAFF.

Fc-BAFF treatment rescues B cell development in BAFF-deficient mice.

In BAFF deficient mice, B cells development beyond the transitional 1 stage is impaired due to lack of BAFF-mediated survival/differentiation signals (fig.3A and [11]). Having demonstrated Fc-BAFF biological activity on mouse mature B cells in vitro, we wondered whether the mature B cell pool could be rescued in BAFF-deficient mice by exogenous administration of the recombinant cytokine. As shown in figure 3, Fc-BAFF treatment rescued mature B cell development to a very large extent. Control BAFF-deficient mice had very low percentages of CD19+CD93- mature B cells and among them very few expressed CD21 and/or CD23 (fig 3a and b, middle panels). In contrast, Fc-BAFF-treated mice had a mature B cell compartment (CD19+CD93-), which was about 70% of that found in wild type controls (fig.3a and c). Moreover CD21 and CD23 expression on rescued mature B cells revealed that treatment resulted in the formation of a MZB compartment whose size was

indistinguishable from WT mice whereas the number of follicular B cells that could be rescued was about half that found in WT mice (fig. 3b and c).

Spleen sections from WT, BAFF-deficient control and BAFF-deficient mice treated with Fc-BAFF stained for IgM and CD90 (Thy1) revealed that Fc-BAFF treatment led to a complete normalization of B and T cell architecture (fig. 4a). Moreover, staining with anti-IgM and the anti-metalophilic macrophage-specific antibody Moma revealed that Fc-BAFF treatment also resulted in the formation of a correctly localized MZB cell compartment (fig.4b).

Fc-BAFF treatment rescues mature B cell function

Given the ability of Fc-BAFF injections to rescue B cell development, we wondered whether treatment of BAFF-deficient animals would improve their ability to mount an antibody response upon immunization with a T-dependent antigen. As shown in figure 5, humoral immune response in BAFF-deficient mice was significantly lower than in WT mice. In marked contrast, Fc-BAFF treatment significantly improved immune response in BAFF-deficient mice that became as efficient as in WT mice.

In conclusion, Fc-BAFF treatment not only rescues B cell development in BAFF-deficient mice but also entirely rescues an immune response measured by antibody production toward a T-dependent antigen.

Decrease of mature B cells upon Fc-BAFF withdrawal.

In order to investigate the fate of the mature B cells rescued by Fc-BAFF upon treatment arrest, we injected a cohort of BAFF-deficient mice at day 0 and 14 with recombinant Fc-BAFF and monitored the percentage of mature B cells in peripheral blood over time. As shown in figure 6a, one week after the last injection of Fc-BAFF about 20% CD19+CD93^{-} mature B cells could be detected in the blood, which is about half that found in the blood of wild type mice (data not shown). Two weeks later, this percentage had dropped to around 10%, and yet another two weeks later, percentages of mature B cells were identical to those found in untreated BAFF-deficient mice (fig 6a). These findings indicate that the half-life of mature B cells in the absence of BAFF is maximally 2 weeks. However, this is probably an overestimation since the half-life of the Fc-BAFF also has to be taken into account. In order to determine the half-life of Fc-BAFF in the serum we injected wild type mice with 50 μg of recombinant protein and measured its decay over time. As shown in figure 6b Fc-BAFF levels dropped rather quickly and became at the border of detection 96 hrs after injection. Based on these results, the half-life of Fc-BAFF in the serum was around 13 hrs. However, the half-life of Fc-BAFF biological activity might well be longer, as B cells from BAFF-deficient taken 96 hrs after Fc-BAFF administration still had very significant amounts of Fc-BAFF bound on their cell surface (fig. 6c).

Overall, data presented here demonstrate that the half-life of mature B cells in the absence of BAFF is maximally 14 days.

Discussion

BAFF and BAFF-R deficiency in mice results in impaired B cell development beyond the T1 B cell stage [11]. Therefore these mice have reduced number of mature B cells and their capacity to mount a humoral immune response is impaired [18] (Schiemann, 2001). Here we demonstrate that the BAFF deficiency can be almost completely corrected by administration of recombinant Fc-BAFF protein. Fc-BAFF treatment rescued the production of both mature follicular and marginal zone B cells and resulted in a completely normal splenic T and B cell zone architecture. Moreover, injected BAFF-deficient mice demonstrated an ability to mount a T-dependent immune response that was comparable to WT mice. The survival of mature B cells in these mice was strictly dependent on the constant injection of Fc-BAFF, because CD19+CD93- peripheral blood B cells dropped upon treatment interruption. The findings reported here indicate that the half-life time of mature B cells in the absence of BAFF is maximally 14 days. However, an accurate determination of the half-life of mature B cells in absence of BAFF-mediated signals would require more careful monitoring of the rate by which Fc-BAFF bioavailability decreases. By ELISA, we showed that the half-life of Fc-BAFF in the serum is about 13 hrs. However, these findings do not exclude that Fc-BAFF could still deliver survival signals even when undetectable in serum. Recently we showed that the half-life time of mature B cells in mice injected with an antagonistic anti-BAFF-R mAb is about 7 days (22). Furthermore it has been demonstrated that injection of TACI-Fc or BAFFR-Fc fusion proteins lead to the disappearance of mature B cells with

very similar kinetics [23, 24]. Therefore, the 14 days half-life measured here is most likely and overestimation.

Survival of mature B cells is also dependent on the expression of the BCR [28, 29]. Conditional ablation of BCR expression by mature B cells shortens their life span from 100 to 3 to 6 days [28, 29].

Recently it has been demonstrated that BCR signalling induces the expression of the non-canonical NF-κB factor p100, which is required for BAFF-R mediated survival signals [30]. Moreover it has been shown that BCR cross-linking on mature B cells results in the up regulation of BAFF-R expression [31]. Taken together these findings suggest a strong connection between BCR and BAFF-R signalling in mature B cell survival. This provides an explanation why BCR-less and BAFF-less mature B cells display similar half-lives.

Treatment of BAFF-deficient mice with Fc-BAFF induced a significant and reproducible bias toward marginal zone B cells generation. Previously, a similar bias was observed in B lymphopenic rodents [32-34]. Therefore, the more efficient generation of MZB cells over follicular B cells in BAFF-deficient mice upon Fc-BAFF treatment might just reflect the B lymphopenic situation. However, the fact that BAFF-transgenic [35-37] mice and mice treated with Fc-BAFF display similarly increased MZB to follicular B cell ratio offer other mutually not exclusive explanations for the MZB bias observed upon Fc-BAFF administration, arguing against the only effect of B cell lymphopenia [35, 36]. First, BAFF might be a growth factor for MZB and increased BAFF availability may enlarge this B cell compartment. Second, follicular B cells may differentiate into MZB under the influence of high BAFF concentrations. And,

third, the number of immature B cells that differentiate into the MZB compartment may be determined by the amount of BAFF available. Recently it was shown that ectopic expression of the anti-apoptotic Bcl-2 gene in B cells can rescue follicular B but not MZB cell development in BAFF-deficient mice [21]. Based on these findings it was argued that BAFF plays an instructive role in the generation of MZB cells. If true, the amount of available BAFF might indeed determine the size of the MZB compartment.

Recently it was shown that T and B cell deficiencies in mice lacking IL-7 could be overcome by the injection of IL-7 or even more efficiently by the treatment with IL-7/anti-IL-7 complexes [38]. Based on these findings recombinant cytokine administration, and in particular BAFF, might well be envisaged as a potential therapeutic strategy for patients with immunodeficiencies caused by deficient production of a certain cytokine.

Acknowlegments

BAFF-deficient mice were kindly provided by Susan Kalled (BiogenIdec, Cambridge).

Figures legends

Figure 1. Production of recombinant Fc-BAFF protein.

A, Schematic representation of the fusion protein consisting of the human Fcγ1 moiety and the receptor binding part of human BAFF (amino acids 136 to 285). B, SDS-PAGE and Coomassie Blue staining analysis of Fc-BAFF under reducing (lane 1) and non-reducing conditions (lane 2). **XXX** µg of Fc-BAFF was loaded per lane. C, Gel permeation chromatography of Fc-BAFF on a Superdex-200 column. 1 ml fractions were collected, in which Fc-BAFF content was determined by an anti-human Fcγ1 ELISA. Broken lines represent the elution position of the standard proteins tyroglubuline (440 kDa) and catalase (232kDa). D, Hypothetic schematic structure of native Fc-BAFF. E, Binding of Fc-BAFF to mouse BAFF-R, mouse TACI and mouse BCMA transfected in SP2/0 cells. Grey filled histograms represent the negative controls.

Figure 2. Recombinant Fc-BAFF induces B cells survival in vitro.

CD19+ splenic WT B cells were sorted and incubated in medium alone or in the presence of the indicated concentrations of Fc-BAFF. At day 0 (“input”) or after 5 days, cells were harvested and stained for CD19 together with propidium iodide. Numbers in the dot plots represent the percentages of live cells recovered under the various culture conditions.

Figure 3. Recombinant Fc-BAFF treatment rescues mature B cell development in BAFF-deficient mice.

BAFF-deficient mice were injected at day 0 with 100 μg Fc-BAFF i.v. and at day 14 with 50 μg Fc-BAFF i.p.. At day 21, splenic B cell compartments of WT, control BAFF-deficient and Fc-BAFF-treated BAFF-deficient mice were analysed by flow cytometry. A, CD19 and CD93 expression by splenic cells from WT (left dot plot), control (middle dot plot) or injected BAFF-deficient mice (right dot plot). B, CD21 and CD23 expression of CD19+CD93- mature splenic B cells from WT (left dot plot), control (middle dot plot) or injected BAFF deficient mice (right dot plot). C, Absolute numbers (+/- SD) of immature and mature splenic B cell subpopulations from WT (black bars), control BAFF-deficient (light grey bars) and injected BAFF-deficient mice (dark grey bars).

Figure 4. Restoration of the splenic T and B cell architecture in Fc-BAFF-injected BAFF-deficient mice.

BAFF-deficient mice were injected at day 0 with 100 μg Fc-BAFF i.v. and at day 14 with 50 μg Fc-BAFF i.p.. At day 21, injected BAFF-deficient mice were sacrificed together with WT and control BAFF-deficient mice and splenic T and B cell architecture was analysed by fluorescence microscopy on cryosections. A, Spleen sections of WT (left picture), BAFF-deficient (middle picture) or injected BAFF-deficient mice (right picture) were stained for IgM (red) and CD90 (green). B, Spleen sections of the same mice were stained for IgM (green) and MOMA (red).

Figure 5. BAFF-deficient mice injected with Fc-BAFF mount normal T cell-dependent humoral immune responses.

BAFF-deficient mice were injected at day 0 with 100 μg of Fc-BAFF i.v., and at days 14 and 21 with 50 μg Fc-BAFF i.p.. At day 15, WT, BAFF-deficient or injected BAFF-deficient mice were immunized i.p. with NIP-CGG in alum. Anti-NIP IgG titers were measured by ELISA in the serum of WT, BAFF-deficient or injected BAFF-deficient mice at day 29 and compared to pre-bleed titers (day 14). *: p value ≤ 0.05 **: p value ≤ 0.01 on student T test.

Figure 6. Kinetics of mature B cell disappearance in the absence of a Fc-BAFF-mediated survival signal.

A, BAFF-deficient mice were injected at day 0 with 100 μg of Fc-BAFF i.v. and at day 14 with 50 μg of Fc-BAFF i.p.. Percentages of mature CD19+CD93- B cells were analysed in peripheral blood 1, 3 or 5 weeks after the last Fc-BAFF injection. B and C, WT mice were injected i.v. with 50 μg of Fc-BAFF. B, Fc-BAFF concentrations were measured in the serum at different time points by an anti-hBAFF ELISA. C, Mice were sacrificed after 96 hrs. The presence of Fc-BAFF at the surface of spleen cell of WT control or Fc-BAFF injected mice was analysed using a monoclonal antibody directed against human Fcγ1 together with CD19 staining.

Materials and Methods

Experiment animals

C56BL/6 mice were obtained from RCC (Füllinsdorf, Switzerland) or Harlan Netherlands (Horst, The Netherlands) or bred in an animal facility under pathogen-free conditions at the Center of Biomedicine of Basel or at the Biochemistry Department of the University of Lausanne. BAFF-deficient mice (Schiemann 2001) were bred at the Biochemistry Department of the University of Lausanne. All animal experiments were carried out within institutional guidelines with the permission of national or local authorities (authorization numbers 1370, 1886, 1887 and 1888).

Recombinant Fc-BAFF

The Fc-BAFF expression plasmid encodes the hemagglutinin signal peptide, the Fc portion of human IgG1 (amino acids 108-338 of GenBank accession number AAC82527, excluding the stop codon), a linker sequence (RSPQPQPKPQPKPEPEGSLQVD) and the receptor-binding domain of human BAFF (amino acids 136-285) (Bossen 2007 JBC). An IRES-GFP EcoRI/SalI fragment of pMIG-IRES-GFP was cloned in the EcoRI/XbaI sites 3' of the Fc-BAFF construct. Chinese hamster ovary cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer's protocol and selected 24 h later with 0.5 mg/ml of G418 (Invitrogen). After 14 days of selection, GFP-bright cells were sorted using a FACS aria (BD Biosciences), amplified and grown in 2-liter roller bottles for 14 d at 37°C. Fc-BAFF was

purified on protein A-Sepharose, eluted with 100 mM citrate-NaOH (pH 2.8) and dialysed against PBS.

SDS-PAGE

Fc-BAFF was resolved by 12% SDS-PAGE in presence or absence of 50 mM DTT. Proteins were stained with Coomassie Blue.

Gel permeation chromatography

180 μg of Fc-BAFF was applied onto a Superdex 200 column (GE Healthcare) eluted in PBS at 0.5 ml/min. Fractions of 1 ml were collected of which 5 μl were analyzed by ELISA against human IgG1.

ELISA

Unlabeled goat anti-human IgG1 and alkaline phosphatase-coupled goat anti-human IgG1 were purchased form SouthernBiotech. An anti-human BAFF sandwich ELISA was developed in our laboratory. Unlabelled anti-human BAFF mAb 2.81.5 was used for capture. Human BAFF was subsequently revealed with biotinylated-anti-human BAFF mAb 4.62, followed by alkaline phosphatase-coupled streptavidin (Amersham Biosciences, Little Chalfont, UK).

Fc-BAFF treatment and immunization of mice

Unless specified otherwise in the figure legend, mice were injected i.v. at day 0 with 100 μg of Fc-BAFF in PBS, and then injected i.p. at day 14 with 50 μg of Fc-BAFF in PBS. In the immunization experiment, treated mice were immunized i.p. with 100 μg of NIP-CGG in alum at day 15, and administered with an additional 50 μg of Fc-BAFF i.p at day 21. Anti-NIP IgG titers were

measured by ELISA with coated NIP-BSA two weeks post-immunization (day 29).

Antibody and flow cytometry

Anti-CD19-PE-Cy7 (1D3) and anti-CD19-PECy5 (1D3) were purchased from BD Bioscience (BD Pharmingen). Anti-CD93 (PB493), anti-CD19 (1D3), anti-CD21 (7G6), anti-CD23 (B3B4), anti-CD90 (T24), and anti-IgM (M41) purified and labelled with biotin, Alexa (488) or Alexa (647) using standards procedures. Biotin-labelled antibodies were revealed by steptavidin-PE (SouthernBiotech), -PECy or -APC (BD Pharmingen). Staining of cells was performed as described previously. Propidium iodide was used at 0.5 μg/ml. Flow cytometry was performed using a FACS Calibur (BD Biosciences) and data were analyzed using the FlowJo software.

Immunohistochemistry

Spleens were snap frozen and embedded into OCT (Sakura, Zoetermeer, NL). Cryostat sections of 5 μm were prepared and fixed for 10 minutes. Sections were stained with anti-IgM^{biot} (M41) and anti-CD90^{fitc} (T24) or anti-IgM^{fitc} (M41) and MOMA^{biot}. Biotin-labelled antibodies were revealed with Fluorochrome-coupled streptavidin. Fluoresence was visualized using an Axioskop Immunofluorescence (Zeis, Feldbach, Switzerland) equipped with a Nikon digital camera.

In vitro B cell survival assay

CD19+ cells were sorted form the spleen of C57BL/6 WT mice using a FACS aria (BD Biosciences) and seeded at $2x10^5$ cells/wells in 96 wells plates in 100 µl medium, in the presence or absence of the indicated concentrations of Fc-BAFF. After 5 days of culture, percentages of living cells were estimated by flow cytometry using propidium iodide and anti-CD19 stainings.

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Figure 1

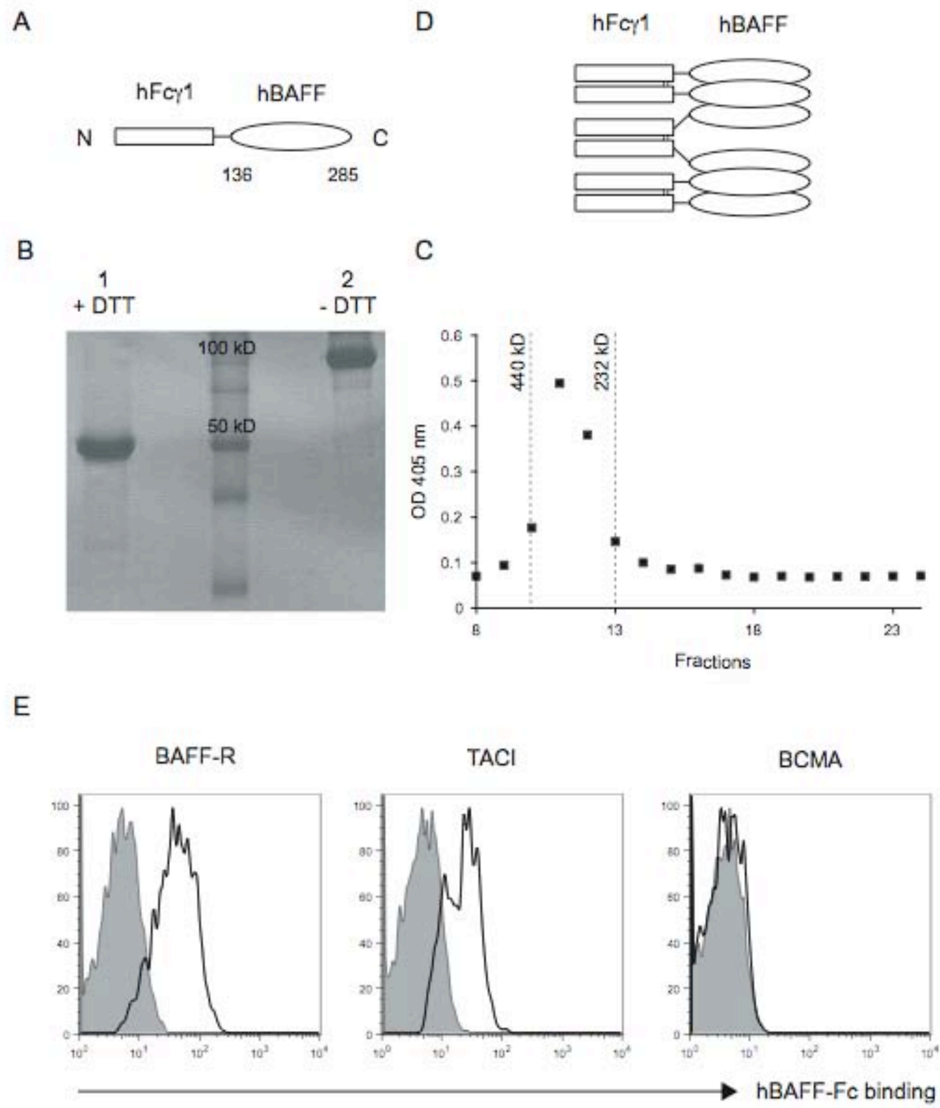
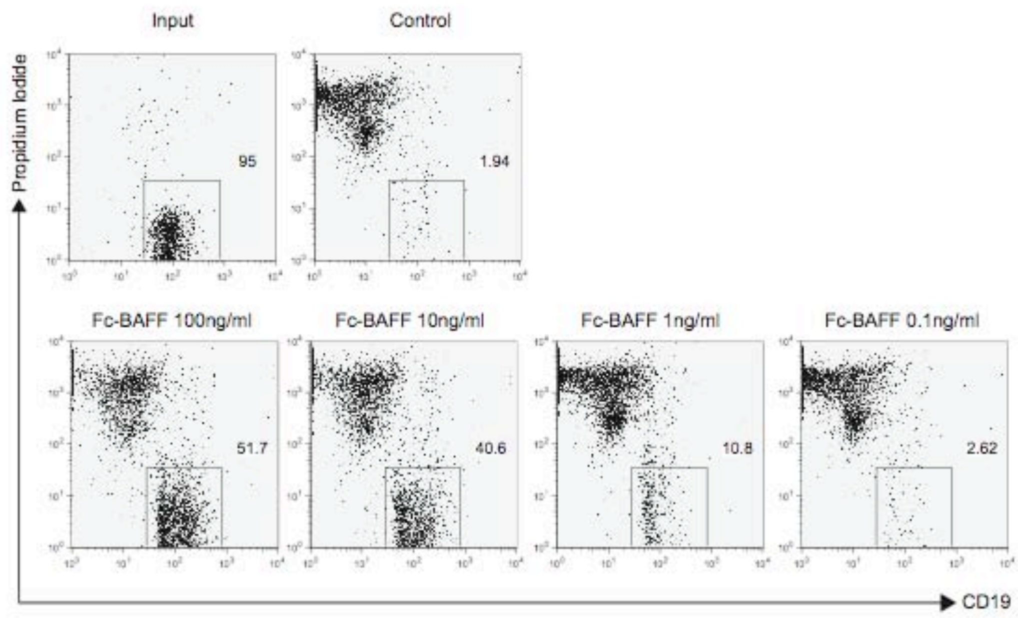


Figure 2



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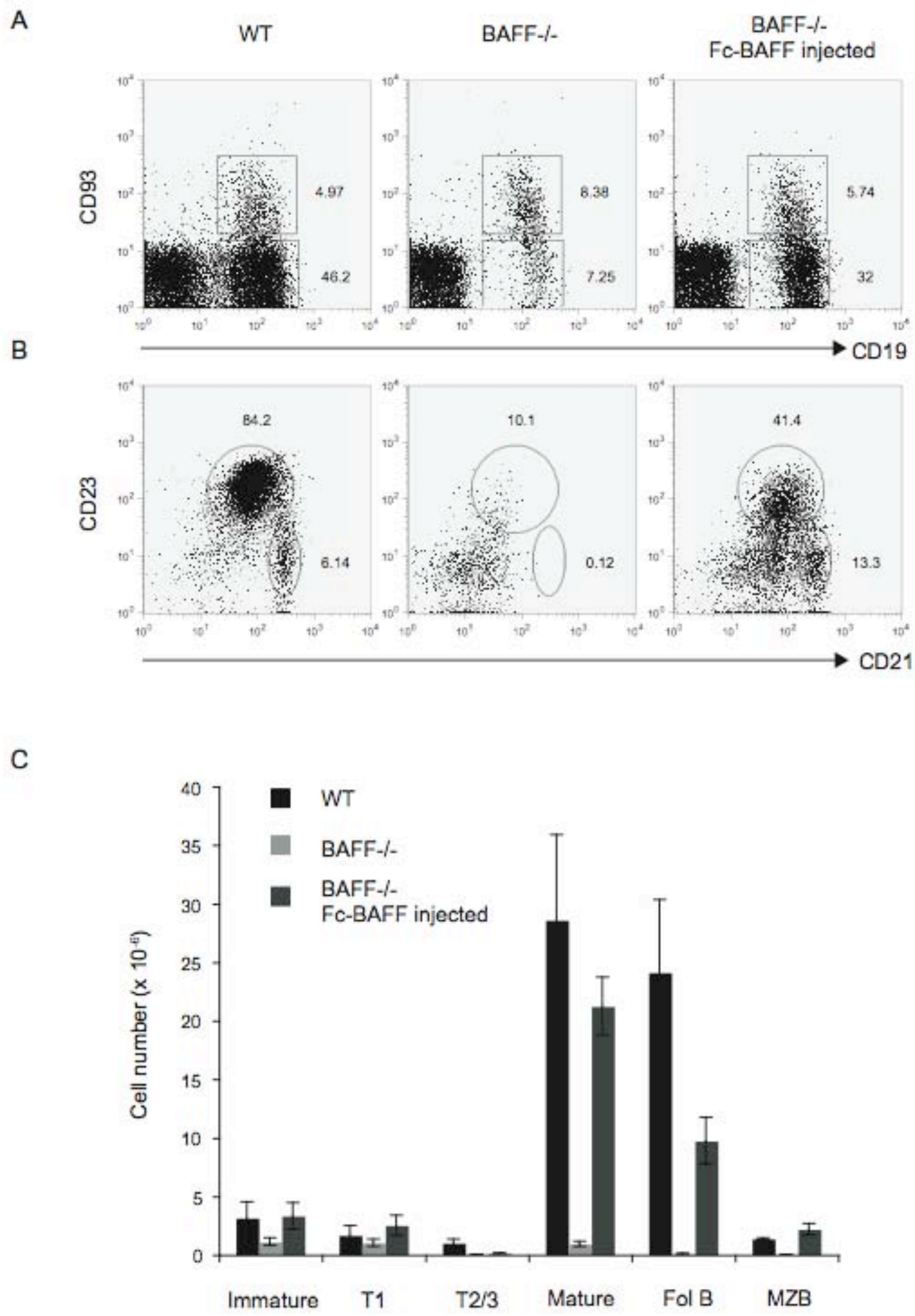

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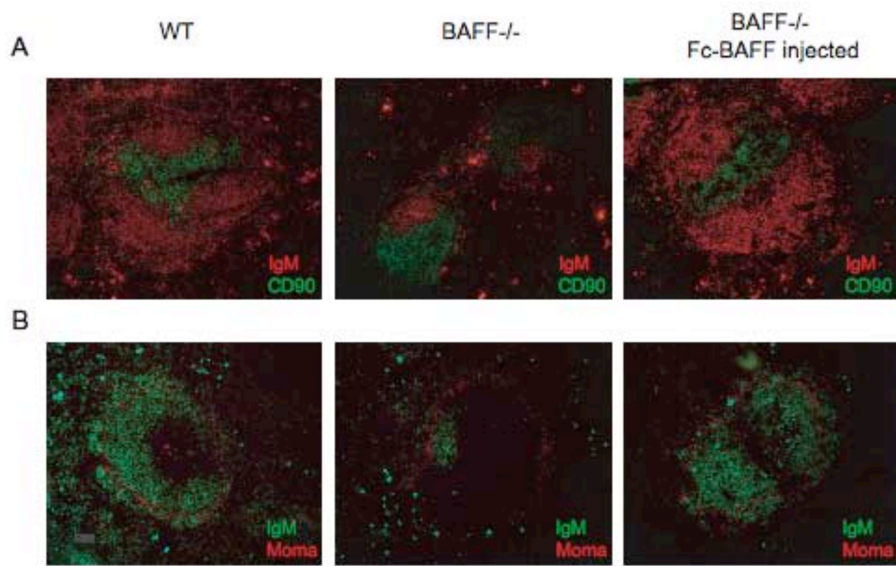

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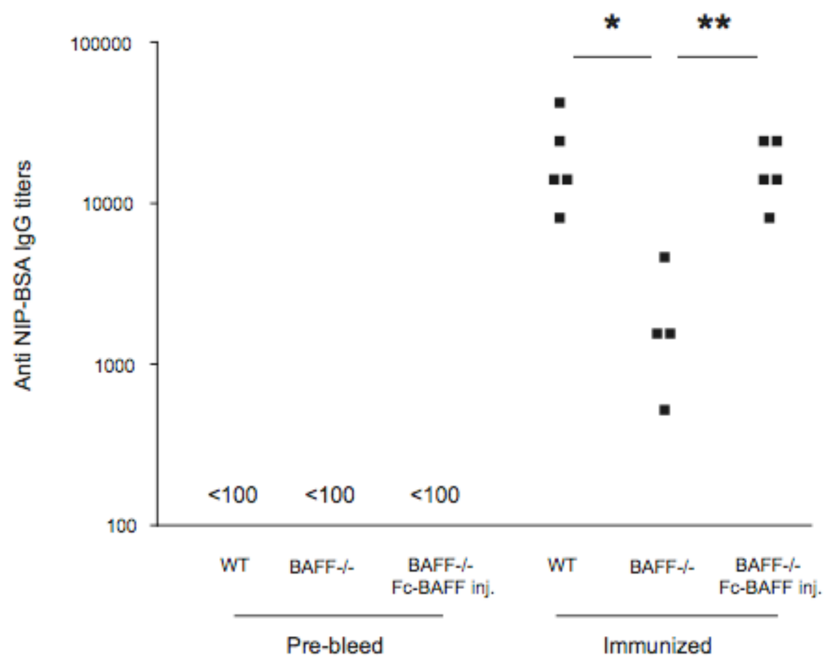
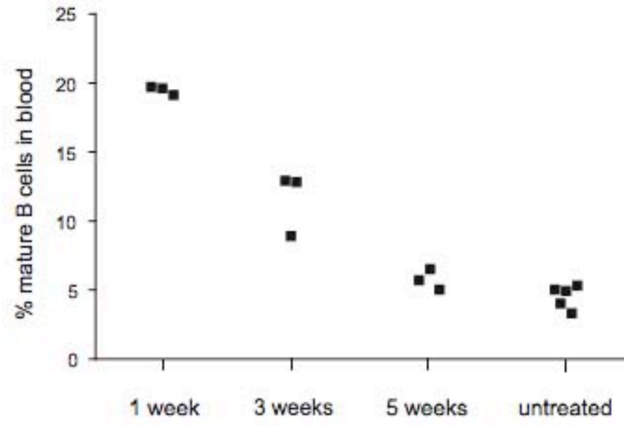


Figure 6

A



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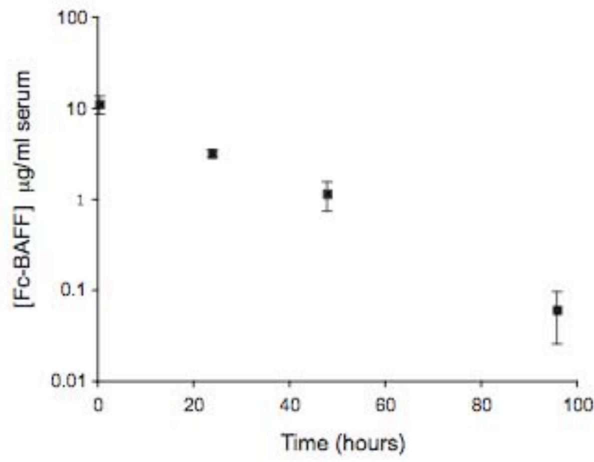

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