Biochemical and Biophysical Research Communications xxx (2017) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

B-cell activating factor deficiency suppresses splenomegaly during *Leishmania donovani* infection

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ARTICLE INFO

Article history:
Received 24 May 2017
Accepted 1 June 2017
Available online xxx

Keywords:
Visceral leishmaniasis
BAFF
Hypergammaglobulinemia
Splenomegaly

ABSTRACT

B-cell activating factor (BAFF) is a critical regulator for B-cell development and differentiation. We previously reported elevation of serum BAFF levels in patients with visceral leishmaniasis (VL). In this study, we examined if BAFF is involved in pathologies during infection of *Leishmania donovani*. BALB/cA mice infected with *L. donovani* showed significant elevation in serum BAFF and IgG levels as seen in VL patients. In contrast, elevation of serum IgG by *L. donovani* infection was significantly suppressed in BAFF-deficient mice. The spleen weight of the BAFF-deficient mice after infection was significantly lower than that of the infected wild-type mice, whereas comparable degree of hepatomegaly and anemia were observed in those mice. In the enlarged spleen of *L. donovani*-infected wild-type mice, increase of $CD19^+$ lymphocytes was more prominent than that of $CD3^+$ cells, suggesting the contribution of B cell increase to splenomegaly during VL. Besides, increase of $CD19^+$ lymphocytes was not found in BAFF-deficient mice after *L. donovani* infection. Taken together, these results suggest that BAFF is involved in strong B cell activation, which has a pathological role in splenomegaly but not in hepatomegaly or anemia, during VL.

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1. Introduction

Visceral leishmaniasis (VL), also known as kala-azar, is a protozoan disease caused by *Leishmania donovani* in East-Africa, India, and parts of the Middle East and by *L. infantum* in Europe, North Africa, South and Central America [1]. The annual incidence of VL is estimated as 200,000 to 400,000 cases, with about 20,000 to 40,000 associated deaths [2]. Clinical manifestations of VL include fever, hepatosplenomegaly, anemia and weight loss, and it can be fatal if left untreated. Splenomegaly is one of the most typical symptoms in VL. Previous studies have reported its high prevalence among VL patients, 71% in Brazil [3], 77% in Uganda and Kenya [4], and 97% in India [5].

Immunological characteristics of active VL include strong humoral responses. In fact, we and others have reported hypergammaglobulinemia in VL patients [6,7]. Since high IgG antibodies and delayed type hypersensitivity to leishmanial antigens are dichotomic factors in VL [8], it is presumed that Th2-dominant immunity to the parasites is associated with the humoral immunity during VL. For example, serum levels of IL-4, the representative Th2 cytokine, are high in VL patients in India [9]. VL patients also have elevated levels of total and antigen-specific IgE [10], indicating the involvement of IL-4 in the humoral responses during the disease. In contrast, IL-4 has little effect on parasite burden or pathologies during *L. donovani* infection in mice [11–14], and there is no clear evidence that IL-4 is the critical regulator for hypergammaglobulinemia in experimental VL.

B-cell activating factor (BAFF), also known as BLyS, TALL-1 and TNFSF13B, is a critical regulator of B cell development and differentiation [15]. Although the molecule is indispensable in maintaining B cell functions, aberrant expression of BAFF is associated with hyper-activation of B cells leading to disorder. Mice overexpressing BAFF demonstrate hypergammaglobulinemia and increase in peripheral blood B cell counts [16,17]. These mice also represent autoimmune disease-like characteristics such as increased autoantibodies, immune complex deposition in the kidney, IgA-associated nephropathy, splenomegaly/lymphadenopathy

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http://dx.doi.org/10.1016/j.bbrc.2017.06.005
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Please cite this article in press as: S. Omachi, et al., B-cell activating factor deficiency suppresses splenomegaly during *Leishmania donovani* infection, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.06.005

with increased B cells and plasma cells, and decreased saliva flow [16–18]. Increased BAFF expression is also associated with human diseases such as systemic lupus erythematosus (SLE), Sjogren's syndrome (SS), rheumatoid arthritis (RA) and B cell malignancy [18–21]. Together, BAFF may have a pathological role in those diseases through hyper-activation of B cells. In fact, inhibitors of BAFF signaling such as belimumab and blisibimod are approved and/or being evaluated for treatment of SLE, SS and IgA nephropathy.

We have recently reported elevation of serum BAFF levels during VL [6]. The mean serum BAFF value in Brazilian VL patients was 4.3 times higher than that of healthy controls, and the magnitude of elevation was equivalent to or higher than those previously reported for other diseases such as SLE, RA and SS [20–23]. Although the majority of the VL patients had high levels of both BAFF and IgG, however, a clear positive correlation between those values within the patient group was not found. The reason may be because BAFF is a critical but not the sole factor for hyper IgG syndrome in a clinical setting. In fact, BAFF synergizes with other cytokines resulting in different outcomes on B cell activation compared with the sole molecule [24].

In order to elucidate the role of BAFF in induction of strong humoral response during VL, we produced BAFF-deficient mice and performed experimental infection with *L. donovani*. The aim of this study is to elucidate the involvement of BAFF signaling in immunological and pathological responses to *L. donovani* infection.

2. Materials and methods

2.1. Animals and experimental infection with L. donovani

BALB/cA mice were purchased from Japan Clea, Tokyo, Japan, and were maintained under specific pathogen-free conditions. BAFF-deficient BALB/cA mice were bred in the animal facility at the Graduate School of Agricultural and Life Sciences, The University of Tokyo. The animal experiments were reviewed and approved by an institutional animal research committee (No. P15-80 and No. P14-930) and an institutional committee on genetically modified organisms (No. 830-2630) at the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

BAFF-deficient BALB/cA mouse was generated by offset-nicking method of CRISPR/Cas system according to previous report [25]. Briefly, exon 1 to exon 2 of BAFF locus, which includes transmembrane region and proteolytic cleavage site [26], was deleted by using the four gRNAs (Supplementary Fig. 1). The mice were made specific pathogen-free at Central Institute for Experimental Animals, Kawasaki, Japan.

Culture of L. *donovani* promastigtotes, experimental infection, collection of the spleen, liver and blood/serum, and counting of Leishman-Donovan Units (LDU) on Giemsa-stained tissue stamps were performed as previously described [27].

2.2. Quantification of serum BAFF levels and immunoglobulin levels

Quantification of serum levels of BAFF, IgG, IgM and IgA in mice was performed by using Mouse BAFF/BLyS/TNFSF13B DuoSet ELISA (R&D Systems, Inc., Minneapolis, MN), Mouse total IgG ELISA Ready-Set-Go! ELISA kit, Mouse total IgM ELISA Ready-Set-Go! ELISA kit and Mouse total IgA ELISA Ready-Set-Go! ELISA kit (eBioscience, Inc., San Diego, CA), respectively, according to the manufacturers' instructions.

2.3. Flow cytometric analysis of splenocytes

Splenocytes of naive and infected mice were isolated through cell strainer (BD Pharmingen, USA) and red blood cells were lysed by Red Blood Cell Lysing Buffer (Sigma-Aldrich, USA). The cells were washed three times with PBS + 1% heat-inactivated fetal bovine serum, and incubated with FITC-conjugated anti-CD3 monoclonal antibody and PE-conjugated anti-CD19 monoclonal antibody (BD Pharmingen). After washing, the cells were fixed by BD Cytofix/Cytoperm Fixation/Permeabilization solution (BD Pharmingen), and then washed again. At least 10,000 cells per sample were analyzed on the BD FACSVerse™, and data analysis was performed using BD FACSuite™ software. The lymphocytes were gated in the FSC-SSC dot plots, and the number of lymphocyte-gated $CD19^+$ cells and $CD3^+$ cells were analyzed by quadrant analysis of 2-dimensional dot plots.

2.4. Antibody ELISA

Soluble *Leishmania* antigen (SLA, 1 μg/well) was used for coating Nunc MaxiSorp 96-well plates (Thermo Fisher Scientific, Waltham, MA) for 4 h at room temperature followed by blocking with 200 μl of PBS containing 0.05% Tween-20 (PBS-T) plus 1% BSA over night at 4 °C. The plates were washed 5 times with PBS-T and once with PBS, and were incubated with 100 μl of serum samples diluted at 1:100 with antibody dilution buffer (PBS-T containing 0.1% BSA) followed by fivefold serial dilutions to 1:7,812,500 for 2 h at room temperature. After washing, the plates were then incubated with 100 μl of HRP-conjugated goat anti-mouse IgG (1:2000 dilution with antibody dilution buffer; Southern Biotech, Birmingham, AL) for 1 h at room temperature. After washing, the plates were developed with tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 2 min. The development was stopped by adding 50 μl of 1 N H_2SO_4 and the plates were read by a microplate reader (SpectraMax Paradigm, Molecular Device, Sunnyvale, CA) at 450 nm. Reciprocal endpoint titers were calculated with the GraphPad Prism 6 software program (GraphPad Software, Inc., La Jolla, CA) by using the OD of 0.1 as a cutoff value.

2.5. Statistical analysis

Differences in BAFF levels among groups were analyzed by one-way ANOVA followed by Bonferroni multiple comparisons test and differences between wild-type mice and BAFF-deficient mice were analyzed by two-way ANOVA followed by Bonferroni multiple comparisons test. *P* values less than 0.05 were considered significantly different.

3. Results

3.1. Suppression of IgG production in BAFF-deficient mice during L. donovani infection

As previously described [27], BALB/cA mice infected with *L. donovani* developed progressive hepatosplenomegaly over 24 weeks of infection; the spleen and liver of the infected mice became significantly larger in size over time than those of naive mice. The progressive hepatosplenomegaly was accompanied with increase in tissue parasite burden during the 24-week infection in both the spleen and the liver. Besides, decline in hematocrit was also evident at 24 weeks of infection.

To examine if those infected mice also demonstrate elevation in serum BAFF and immunoglobulins as seen in human VL patients, the infected mice were first examined for serum BAFF levels. Means ± SD of serum BAFF levels in mice at 12 and 24 weeks post infection were 8.59 ± 0.90 ng/ml and 11.65 ± 1.43 ng/ml, respectively, and were statistically higher than that of naive mice (5.34 ± 1.19 ng/ml) (Fig. 1A). The BAFF levels at 24 weeks post

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