

Tolerogenic property of B-1b cells in a model of allergic reaction[☆]

Beatriz.H.P. De Lorenzo^a, Ronni R.N. Brito^a, Luiz Claudio Godoy^b,
José Daniel Lopes^a, Mario Mariano^{a,*}

^a Discipline of Immunology, Department of Microbiology, Immunology and Parasitology, Federal University of São Paulo, São Paulo, Brazil

^b Department of Medicine, University of Massachusetts, Medical School, Worcester, MA, USA

Received 20 April 2007; received in revised form 20 September 2007; accepted 22 September 2007

Available online 31 October 2007

Abstract

Since B-1 cells were first described, their origin and function remain controversial. Given the ability to produce natural antibodies and large amounts of IL-10, there is a consensus about their role in innate immunity. More recently, however, B-1 cells have been associated to adaptive immunity as well, due to the demonstration of immunological memory and antigen presentation capability. Here we demonstrate that adoptive transfer of pre-sensitized B-1b cells (obtained from OVA-sensitized mice) to naïve B-1 deficient animals, drastically affects the ability of transplanted animals to mount an adaptive response upon immunization with OVA. In contrast to naïve B-1 populated mice, mice transplanted with sensitized B-1 exhibit lower anti-OVA antibody levels, milder footpad swelling in response to OVA subcutaneous injection and reduced granulomatous reaction to OVA-coated beads. Moreover, we show that these pre-sensitized B-1 cells, when acting as APCs, induce poor T cell proliferation *in vitro* when compared with macrophages or B-1 cells obtained from naïve mice. This property may be due in part to insufficient expression of the co-stimulatory molecule CD86, necessary for optimal antigen presentation. In conclusion, our data suggest a novel role for B-1 cells as part of suppressor mechanisms in the immune system.

© 2007 Elsevier B.V. All rights reserved.

Keywords: B cells; Tolerance; Suppression; Anergy; Allergy

1. Introduction

The observation that malignant cells from patients suffering from chronic lymphatic leukemia express both lymphoid and myeloid surface markers stimulated the search for cells bearing this phenotype physiologically. In 1980s Herzenberg and her group demonstrated that Ly-1 cells featured these characteristics and could be found in the mouse peritoneal and pleural cavities, but were rare in the spleen and virtually absent in lymph nodes [1,2]. These findings supported a new classification of B cells. In 1991, the division of B cells into three subpopulations was proposed: conventional B cells (B-2), which evolve to plasma cells, B-1a and B-1b cells [3].

B-1 cells have a peculiar morphology not compared with the morphology of B-2 cells [4]. B-1b cells express CD11b (Mac-1), CD19, CD45 (B220) and IgM surface markers, whereas B-1a cells have added to this repertoire the T cell-related CD5 molecule. The mixed myeloid and lymphoid characteristics of these cells have justified their designation as “promiscuous cells”, and may contribute to their remarkable plasticity. For instance, B-1b cells obtained from cultures of mouse peritoneal adherent cells differentiate into a mononuclear phagocyte [5]. In the course of such differentiation, the cells loose expression of lymphoid transcription factors, while maintaining the expression of myeloid transcription factors (personal communication). In addition, they are shown to migrate to inflammatory foci and to participate in giant cell formation [6] and to present antigen [7].

Despite the increasing volume of information about B-1 cells, the role played by them in innate and acquired immune response is not clear. As they are the major producers of self-reactive IgM [8,9], a number of reports have linked them with resistance to infection [10–12], tumor development [13] and autoimmune

[☆] Supported by grant from Fundação de Amparo à Pesquisa do Estado de São Paulo [FAPESP], nos. 99/11144-4 and CAPES.

* Corresponding author at: Rua Botucatu 862, 4th floor, 04023-9000 São Paulo, Brazil. Tel.: +55 11 5549 6073; fax: +55 11 5572 3328.

E-mail address: mariomu@uol.com.br (M. Mariano).

manifestations [14,15]. Paradoxically, Xid mice, which lack B-1 cells due to a mutation in the Bruton's tyrosine kinase [1,16], cope more efficiently with *Trypanosoma cruzi* infection as compared with wild type counterparts [17]. It was also demonstrated that depletion of B-1 cells by selective irradiation turns the animals significantly more resistant to growth and metastatization of melanoma cells. The same phenomenon was observed when Xid mice were inoculated with this neoplastic cell lineage (personal communication). These data point to a down-modulatory effect of B-1 cells in the immune response.

The putative suppressor mechanisms by which B-1 cells facilitate parasite infection, autoimmune manifestations and melanoma malignancy are not understood. In an attempt to link B-1 cells with acquired immunity, Alugupalli et al. [18] demonstrated that B-1 cells have immunological memory, since animals lacking B-1b lymphocytes are susceptible to *Borrelia hermsii* infection but become protected upon adoptive transfer of this cell subtype derived from convalescent mice. Seeking to understand the role that B-1 cells might play in acquired immune response, we adoptively transferred B-1 cells from ovalbumin immunized wild type mice to Xid mice and investigated the subsequent generation of response to the antigen. Results demonstrated that B-1 cells exert a potent tolerogenic property in this model. The significance of this finding for innate and acquired immunity is discussed.

2. Material and methods

2.1. Experimental design

BALB/Xid mice were adoptively transferred with B-1 cells obtained from either naïve or OVA-immunized BALB/c mice (immunization with hen egg white as described below), constituting NB-1 (naïve B-1) and IB-1 (immune B-1) groups, respectively. Non-transplanted BALB/c and BALB/Xid mice were used as controls. The four groups were immunized with OVA 24 h after adoptive transfer. Fourteen days post-immunization, the animals were submitted to one of the following challenge procedures to assess the magnitude of response to OVA: [1] footpad injection of purified OVA and measurement of swelling as a parameter of hypersensitivity response; [2] intrapulmonary injection of OVA-coated beads and assessment of granulomatous reaction by histology 14 days later. Anti-OVA humoral response was also evaluated.

2.1.1. Mice

BALB/c female mice and BALB/Xid mice were obtained from the Animal Facility of the Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, Brazil. The experimental groups of 5–6 animals were 5–7 weeks old and maintained in controlled light and temperature receiving water and food *ad libitum*. This study was approved by Research Ethical Committee from Federal University of São Paulo, Brazil.

2.1.2. B-1b cell purification and transfer

B-1b cells were obtained as described by Almeida et al. [5]. Briefly, peritoneal cells were collected from mouse abdomi-

nal cavity by repeated lavage with 5 ml of RPMI-1640 medium (Sigma). Cells (2×10^6 cells/ml) were dispensed on glass dishes and incubated at 37 °C for 60 min. The culture supernatants were then aspirated to remove the non-adherent cell fraction. Adherent cell monolayers were rinsed with RPMI and subsequently covered with supplemented medium. Cultures were maintained at 37 °C in 5% CO₂ for 5 days. B-1 cells were purified from free-floating cells in the supernatant of these cultures using a magnetic bead system MiniMACS (Miltenyi Biotec, Germany). Approximately, 10^7 cells were incubated with rat antibody anti-mouse CD19 (Pharmingen, San Diego, CA, USA), followed by incubation with iron-coupled anti-rat IgG (Miltenyi Biotec, Germany). Cells were passed through a 25MS positive selection column (Miltenyi Biotec), where B-1b cells were retained. The purity of population was evaluated by FACS as described below, using anti-mouse CD19 and anti-mouse CD11b (Mac-1) phycoerythrin and fluorescein-conjugated, respectively (Pharmingen). It was recently demonstrated in our laboratory that B-1a cells do not attach to the glass surface (Thies et al., in preparation). Mice from NB-1 and IB-1 groups received 5×10^5 naive and OVA-primed B-1 cells, intraperitoneally, respectively.

2.1.3. Immunization with heat-coagulated hen egg white (HEW)

HEW was prepared as previously described [19,20]. Briefly, hen eggs were boiled in water for 30 min. The solid egg white was separated, washed in distilled water, dehydrated in 100% ethanol for 24 h and cut into small fragments of 4 mm × 2 mm × 2 mm (approximately 40 ± 2 mg each). The fragments were re-hydrated by immersion in phosphate-buffered saline (PBS) for 2 h at room temperature and subcutaneously implanted in the dorsum of control and B-1-populated mice.

2.1.4. Detection of serum anti-OVA IgM and IgG

ELISA was performed to detect anti-OVA antibodies in mouse serum. Plates were coated with 5 µg chicken egg albumin (Sigma) and maintained at 37 °C for 2 h and overnight at 4 °C. The blockage was made with PBS containing 1% BSA for 1 h at room temperature. Plates were washed three times with PBS containing 0.05% Tween-20 (A.G. Scientific, San Diego, CA). Serum samples were added (50 µl, 1:100) in duplicate and serial dilutions were performed up to 1:12,800. Plates were incubated at room temperature for 2 h. After washing, wells were coated with peroxidase-conjugated anti-IgG or anti-IgM monoclonal antibodies, obtained in our laboratory, and incubated at room temperature for 1 h. Plates were then washed and *o*-phenylenediamine (Sigma) was added as a peroxidase substrate. Optical density (OD) was determined at 492 nm.

2.1.5. Heat-aggregated ovalbumin

Type II ovalbumin (Sigma, St. Louis, MO, USA) was diluted in PBS and heated in a dry bath for 5 min at 100 °C. After centrifugation at $3000 \times g$ for 10 min, the supernatant was discarded and the pellet resuspended in sterile PBS at a concentration of 20 mg/ml.

2.1.6. Footpad antigenic challenge

Fourteen days after immunization, animals were challenged with aggregated ovalbumin prepared as described above by injecting 50 μ l into the left hind footpad. Footpad swelling was measured at 0.5, 3, 6, 9, 24 and 48 h after the challenge with the aid of a gauge caliper (Mitutoyo, Japan). Swelling of footpads was calculated by subtracting the footpad diameter before the challenge from the footpad diameters at different times after challenge.

2.1.7. Challenge with OVA-coated sepharose beads

OVA was linked to CNBr-activated Sepharose 4B (Pharmacia Biotech, Piscataway, NJ, USA) following fabricant instructions. Beads were resuspended in PBS and adjusted to 10,000 beads per ml. Fourteen days after immunization by HEW implantation, 100 μ l of this suspension was intravenously injected in each animal through the retro-ocular plexus.

2.1.8. Spleen cells and bone marrow-derived macrophages

Fourteen days after OVA immunization, spleen cells were collected. Red blood cells were lysed with hemolytic buffer and leukocytes washed and resuspended in RPMI. Bone marrow-

derived macrophages were generated from bone marrow stem cells cultured for 7 days in RPMI medium supplemented with 20% fetal bovine serum and 30% L929 cell conditioned medium.

2.1.9. Cell proliferation assays

Carboxyfluorescein diacetate, succinimidyl ester (CFDA SE, Molecular Probes, Eugene, Oregon, USA) was used to access cell proliferation *in vitro*. Spleen cells were obtained as described above and resuspended in 100 μ l PBS containing 1% BSA. An equal volume of 2 μ M CFSE was added to the cell suspension. Cell suspensions were incubated at 37 °C for 15 min and washed twice with RPMI. The pellet was resuspended in RPMI supplemented with 10% FCS and the number of cells adjusted for proliferation assays as below.

2.1.10. T cell proliferation assay

At the indicated times, mouse spleen cells were collected and adjusted to 1.25×10^6 cells/ml. The final concentration of OVA with which APCs were pulsed *in vitro* was 1 mg/ml. After 1 h of incubation with the antigen at 37 °C, the APCs were gently washed with medium prior to the addi-

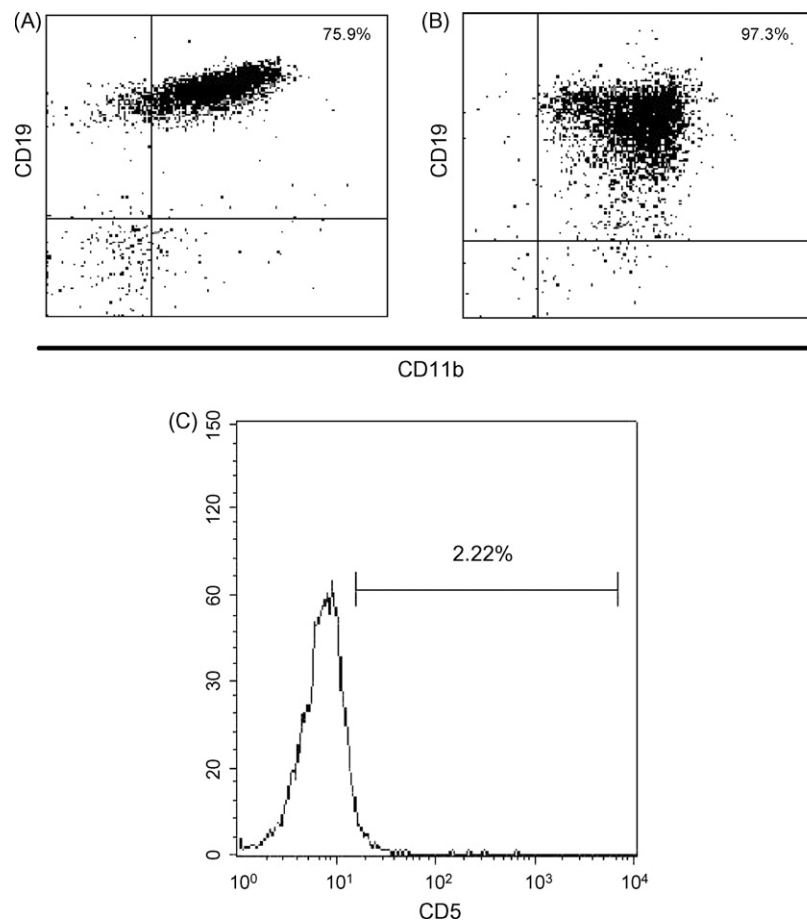


Fig. 1. Purification of mouse B-1b cells. Flow cytometry analysis of mouse peritoneal cellular subpopulations showing the percentage of B-1 cells (CD11b⁺CD19⁺). (A) A B-1b cell enriched population was generated from peritoneal adherent cells after 5 days in culture by collecting the free-floating cells in the supernatant as described in Section 2. (B) The cells described in (A) were further purified through a magnetic bead system, using an antibody anti-mouse CD19 for positive selection of B-1b cells. (C) The expression of CD5 was verified in the cells purified with magnetic beads to confirm the low contamination of the purified population by B-1a cells.

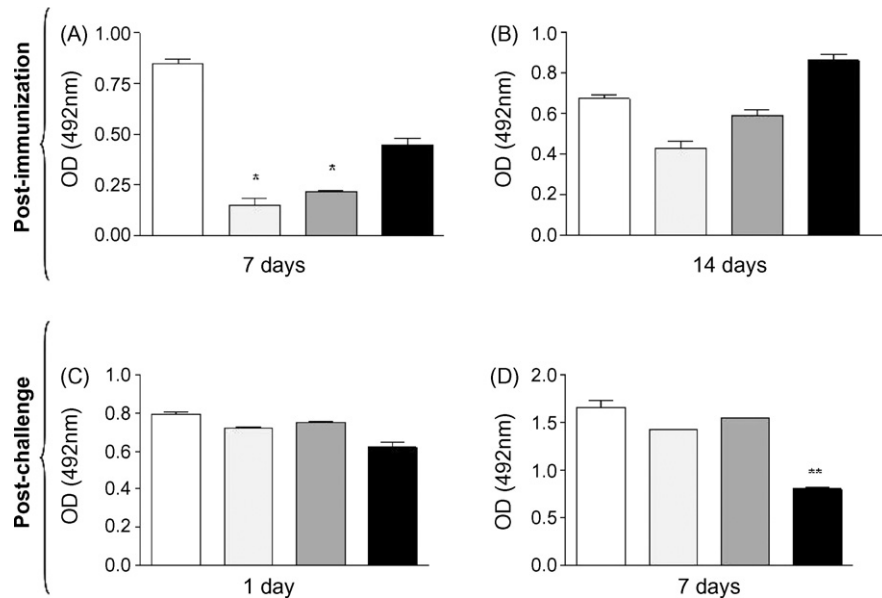


Fig. 2. Humoral response to antigen is compromised in presence of pre-sensitized B-1b cells. B-1b cells obtained from either naïve or OVA-immunized mice were adoptively transferred to Xid animals, following immunization by implantation of heat coagulated hen egg white in the dorsum. Serum was collected at the indicated times and analyzed by ELISA for the presence of anti-OVA antibodies. Primary response was verified on the 7th (9A) and 14th days post-immunization (B). Fourteen days post-immunization, animals were challenged subcutaneously with purified OVA and the secondary humoral response was analyzed 1 (C) and 7 days post-challenge. Animals not submitted to adoptive transfer of B-1 cells were used as controls. The bars represent the mean of absorbance values obtained from sera diluted 1:100 (white bar) BALB/c; (light gray bar) Xid; (dark gray bar) Xid transplanted with naïve B-1b cells; (black bar) Xid transplanted with pre-sensitized B-1b cells. * $p < 0.0001$ related to BALB/c group; ** $p < 0.01$ related to all groups.

tion of T cells. T cells were loaded with carboxyfluorescein diacetate succinimidyl ester as above and cultured in RPMI supplemented with 10% FCS in presence of APCs previously loaded with OVA (bone marrow macrophages, B-1 cells or immune B-1 cells). A negative control for antigen presentation consisted of bone marrow macrophages not loaded with OVA. The ratio APC:T cell was 1:4. Concanavalin A (Sigma) was used as positive control for T cell proliferation at a final concentration of $2 \mu\text{g/ml}$. After 3 days in culture, cells were stained with phycoerythrin-labeled anti-CD3 monoclonal antibodies (PharMingen) and lymphocyte proliferation was evaluated by FACS. Results were expressed as stimulation index mean fluorescence intensity of experimental groups divided by that of respective controls (T cells in absence of APC).

2.1.11. FACS analysis

Direct staining for flow cytometry analysis was performed by incubating viable cells (1×10^6 cells) with phycoerythrin-labeled anti-IgM and B220, as well as FITC-labeled anti-CD5 and Mac-1 (PharMingen, San Diego, CA) for B-1 cells analysis. For spleen cells analysis, biotin-conjugated anti-CD3 and fluorochrome-conjugated streptavidin were used. Fluorochrome-conjugated anti-MHC class-II, anti-CD80 and anti-CD86 were utilized for analysis of cell surface receptors expression. Cells were analyzed in a FACSCalibur (Becton Dickinson, Mountain View, CA). A total of 10,000 cells were counted and the frequency of each cell-surface marker determined using Cell Quest Software (Becton Dickinson).

2.1.12. Statistical analysis

Statistics were performed using ANOVA or the paired two-tailed Student's *t*-test, and $p < 0.05$ was adopted as the significance level.

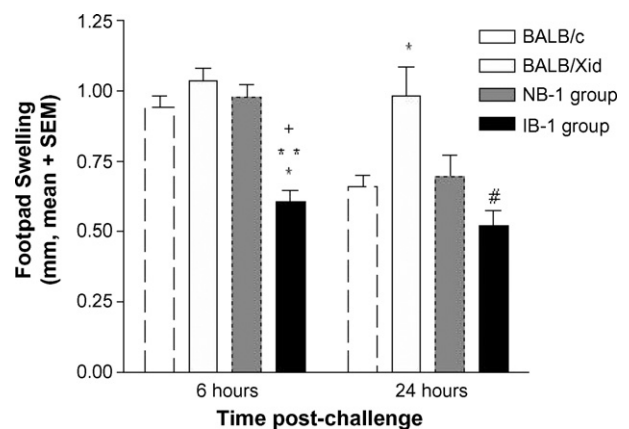


Fig. 3. Pre-sensitized B-1b cells decrease immediate cellular response. After transplantation of B-1b cells obtained from naïve or OVA-immunized mice, Xid animals was immunized by implantation of heat coagulated hen egg white in the dorsum. Mice not submitted to adoptive transfer of B-1b cells were used as controls. Fourteen days post-immunization, animals were challenged by subcutaneous injection of purified OVA in the footpad and the inflammatory reaction quantified by measuring the local swelling. Bars represent the variation of footpad related to the measure before the antigenic challenge (mean + S.E.M). (White bar) BALB/c; (light gray bar) Xid; (dark gray bar) Xid transplanted with naïve B-1b cells; (black bar) Xid transplanted with pre-sensitized B-1b cells. (*) $p < 0.01$ related to BALB/c group; (***) $p < 0.001$ related to NB-1 group; (+) $p < 0.001$ related to Xid group and (#) $p < 0.01$ related to Xid group.

3. Results

3.1. Purification and transfer of B-1b cells

B-1b cells for adoptive transfer were obtained from mouse peritoneal cells as described in Section 2. The 5-day B-1 enriched cell culture (Fig. 1A) was further purified with magnetic beads to render a cell population constituted of >97% B-1b cells (Fig. 1B). The lack of CD5 expression ensured that the purified population was virtually free of the B-1a subtype (Fig. 1C). B1-b cells obtained by this procedure were used to populate Xid mice, which were subsequently submitted to immunization and challenges as described below.

3.2. Anti-OVA humoral response is compromised in presence of OVA-sensitized B-1b cells

We first verified whether the adoptive transfer of B-1b cells obtained from OVA-immunized mice (IB-1) to naïve animals would affect the development of humoral response upon immunization of these transplanted animals with OVA. Anti-OVA IgG serum levels were measured by ELISA. As shown in Fig. 2A and B, the level of anti-OVA IgG produced by Xid mice is lower than in BALB/c 7 days post-immunization, but tends to be in the same range as BALB/c at 14 days. The secondary response to the antigen was also evaluated by challenging the animals with OVA subcutaneously 14 days after immunization. The groups did not differ regarding early

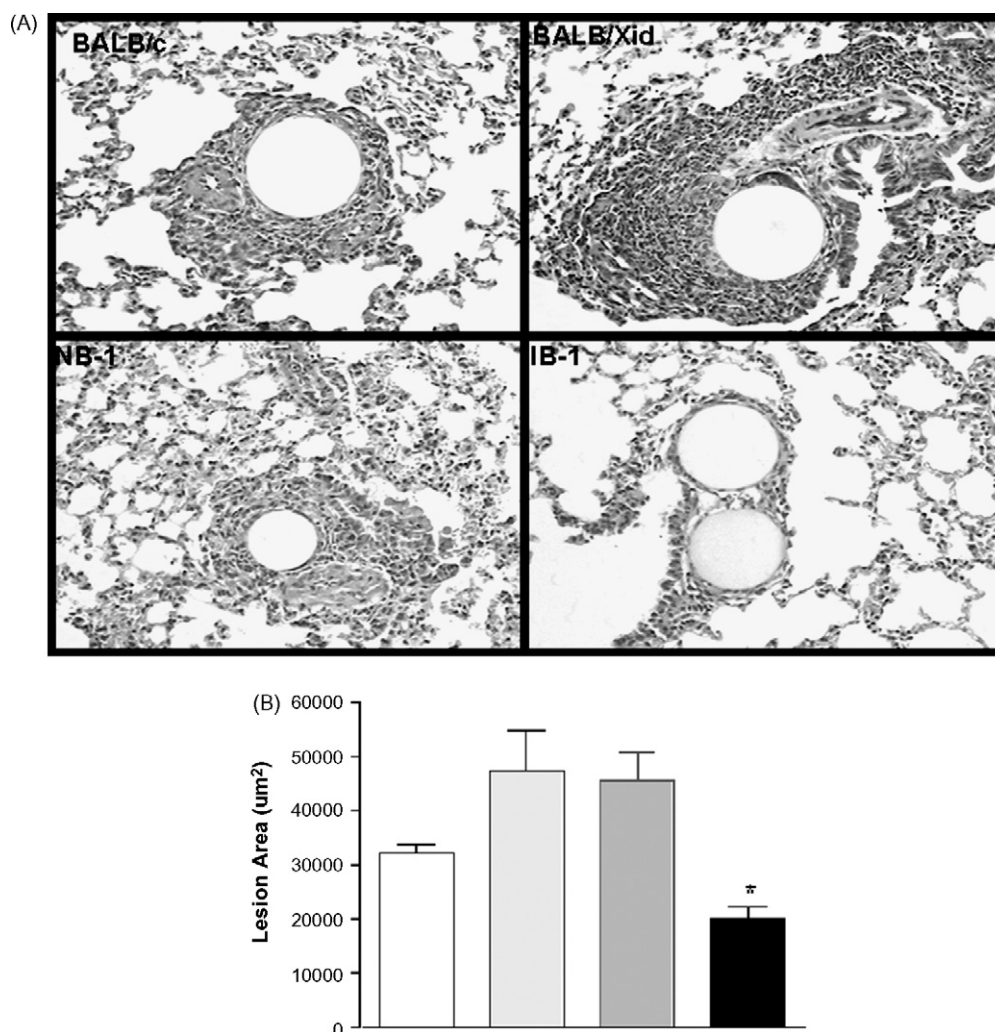


Fig. 4. Chronic cellular immunity is suppressed by pre-sensitized B-1b cells. B-1b cells were obtained from naïve or OVA-immunized mice and adoptively transferred to B-1-deficient Xid animals, following immunization with ovalbumin. Mice not submitted to adoptive transfer of cells were used as controls. Evaluation of chronic cellular immune response was carried out by analysis of granulomatous reactions developed against OVA-coated beads sequestered in the pulmonary parenchyma. Beads were injected intravenously 14 days after mice were immunized with ovalbumin. (A) HE stained histological sections of lung tissue showing granulomatous reactions around OVA-coated Sepharose beads 14 days after injection. (B) Area quantification of granulomatous reactions (mean + S.E.M. of 10 lesions in 5 animals). (White bar) BALB/c; (light gray bar) Xid; (dark gray bar) Xid transplanted with naïve B-1b cells; (black bar) Xid transplanted with pre-sensitized B-1b cells. (*) Indicates statistical difference between Xid transplanted with pre-sensitized B-1b cells and IB-1 and Xid group.

antibody response to the challenge (Fig. 2C). However, in contrast to the other groups, animals transplanted with IB-1 cells were not able to increase antibody levels 7 days after OVA injection. To assess whether this phenomenon was antigen-specific, other animals were submitted to the adoptive transfer of OVA-sensitized B-1b cells and then immunized with human fibronectin. When challenged with fibronectin, these animals exhibited exacerbated secondary humoral response similar to the other groups (data not shown). These results suggest that B-1b cells that have previously encountered the antigen may somehow exert a regulatory effect on the development of further immune response to the same antigen.

3.3. Immune B-1b cells decrease cellular response to OVA

To investigate whether the suppressive effect of IB-1 cells in humoral response was also observed in the cellular immunity to OVA, the animals were submitted to a cutaneous hypersensitivity test. Thus, after cell transfer and immunization with OVA, mice were challenged by injection of purified OVA into the footpad and the evolution of local swelling was monitored. Both BALB/c and NB-1-transplanted mice exhibited a similar inflammatory response, while BALB/Xid mice presented the highest swelling (Fig. 3). In contrast, IB-1-transplanted animals displayed the lowest footpad swelling when compared with the other groups.

The occurrence of IB-1 cells-mediated suppression of cellular immunity was also tested by evaluating the ability to mount a pulmonary granulomatous reaction to OVA-coated beads. As shown in Fig. 4A and B, 14 days after beads injection, BALB/Xid mice exhibited the largest granulomatous reactions, while granulomas in Xid mice previously populated with NB-1 cells were milder and comparable to the observed in BALB/c. Once more, animals that had been transplanted with IB-1 cells before immunization with OVA developed the least pronounced lesions, as seen by smaller granulomas and mild or no infiltration of inflammatory cells. These observations corroborate the results above, pointing to the suppressor activity of pre-sensitized B-1b cells in the development of adaptive immunity.

3.4. IB-1 cells induce poor T cell proliferation *in vitro*

Considering the well known participation of T cells in the model of reaction used in the above experiments [21,22], we evaluated whether the suppressive effects of IB-1 cells in antibody production and hypersensitivity reactions could be related with a down regulation of T cell response. For this purpose, the *in vitro* proliferative response of T cells from OVA-immunized animals was studied, using NB-1 cells and IB-1 cells as APCs. Macrophages and NB-1 cells-induced T cell proliferation in response to OVA (Fig. 5). In contrast, when IB-1 cells were tested as antigen presenting cells, T cells significantly failed to proliferate in response to the antigen. This finding points to relatively a low or absent efficiency of sensitized B-1 cells to act as antigen presenting cells.

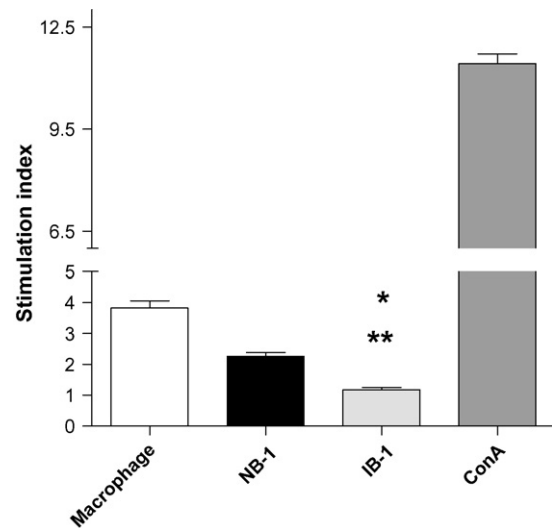


Fig. 5. Pre-sensitized B-1b cells exhibit low efficiency antigen presentation capacity. The antigen presentation capacity of naïve and pre-sensitized B-1b cells was compared in T cell proliferation assays. Splenic T lymphocytes isolated from mice immunized with ovalbumin were loaded with the proliferation assessment probe CFSE and cultured for 72 h in presence of OVA-loaded APC cells consisting of naïve B-1b cells, pre-sensitized B-1b cells or bone marrow macrophages. Proliferation was evaluated by FACS analysis. Bars represent the stimulation index, calculated as the mean fluorescence intensity of experimental groups divided by that of respective controls (T cells in absence of APC). Mean + S.E.M. of three experiments. (*) $p < 0.05$ related to NB-1 group and (**) $p < 0.01$ related to macrophages.

3.5. IB-1 cells express lower levels of CD86 co-stimulatory molecule

To elucidate the possible mechanisms leading to the observed poor T cell response to IB-1, the expression of molecules involved in antigen presentation was investigated by flow cytometry. As shown in Fig. 6A and B, IB-1 cells unexpectedly express higher levels of MHC II when compared with NB-1 cells, while CD80 expression was similar in the NB-1 and IB-1 cells (Fig. 6C and D). On the other hand, the level of CD86 expression was reduced in IB-1 when compared with NB-1 cells (Fig. 6E and F). This finding suggests that when the antigen is presented by B-1b cells that have previously had contact with the antigen, a suboptimal signaling for T cell activation takes place, possibly due to insufficient expression of co-stimulatory molecules.

4. Discussion

A number of reports have linked B-1 cells with immunological phenomena suggesting these cells are a component of the innate immune response. For instance, B-1 cells produce and secrete substantial amounts of autoreactive IgM, which may function as a restriction factor for parasite infections [18,23]. These cells also participate in the genesis of foreign body giant cells [6]. In addition, it was demonstrated that B-1 cells differentiate into mononuclear phagocytes *in vitro* and that they migrate to inflammatory sites, thus resembling a macrophage-like cell [5]. However, although these cells are functional phagocytes and spontaneously secrete nitric oxide, they present

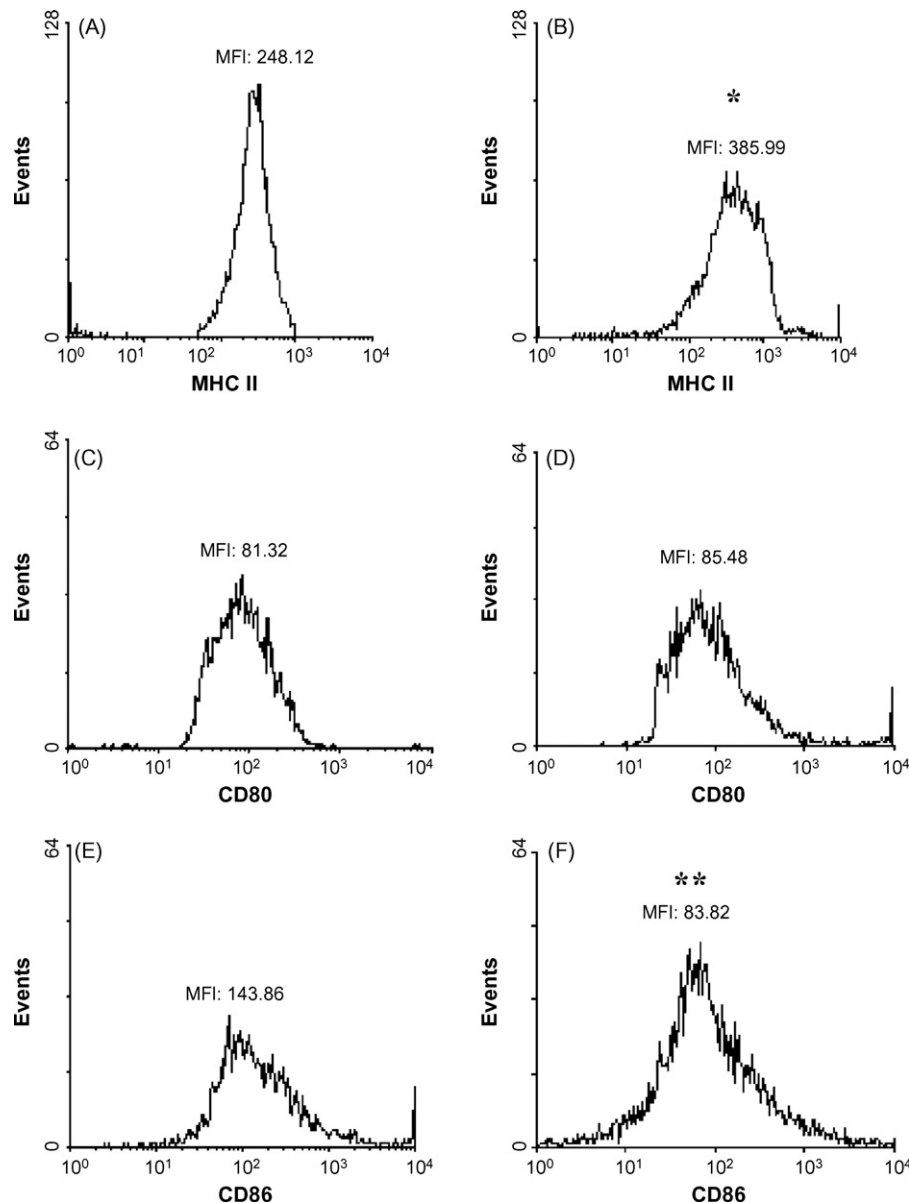


Fig. 6. Pre-sensitized B-1b cells express low levels of co-stimulatory molecules. The expression of molecules involved in antigen presentation was compared between pre-sensitized (A, C and E) and naïve B-1b cells (B, D and F). For this experiment cells were labeled for CD19, Mac-1 and IgM and only the cells expressing these three markers were further evaluated for expression of co-stimulatory molecules. Cells were purified as described in Section 2 and labeled with antibodies against MHC II and the co-stimulatory molecules CD80 and CD86. Cells were analyzed by FACS. MFI, mean fluorescence intensity. * $p < 0.0001$ related to NB-1; ** $p < 0.0001$ related to NB-1.

a low capacity to kill *Coxiella burnetii* *in vitro* when compared to bone marrow-derived macrophages (personal communication). Therefore, whether these phagocytes participate in innate mechanisms is still object of debate, as well as the possibility that B-1 cells may play a role in acquired immunity.

To address the question of what would be the role of B-1b cells in adaptive immune response, if any, we used an *in vivo* protocol of immediate hypersensitivity to ovalbumin, classically employed as a model of asthma and characterized by a Th2 profile [21,22]. The impact of B-1b cells on the ability to mount primary and secondary response to OVA was evaluated by adoptive transfer of these cells to B-1 deficient mice (BALB/Xid). When transplanted cells were obtained from

OVA-immunized BALB/c mice, they rendered BALB/Xid less responsive to antigen challenge in terms of humoral and cellular immune mechanisms, what strongly suggests that B-1b cells exhibit tolerogenic properties in this model.

The impact of Xid mutation in mouse immunity is not yet fully understood. Some studies have shown that the mutation confers protection in infection models [11,12], possibly due to lack of IL-10 secreted in high amounts by these cells, what could favor Th1 responses [10] and augmented production of IFN- γ [24]. Under the present experimental conditions, however, IL-10 levels in culture supernatants of naïve and pre-sensitized B-1b cells were similar (data not shown), what indicates that other mechanisms than this soluble suppressor factor might be

involved in the low response induced by pre-sensitized B-1b cells. Contrariwise, other authors show that mice carrying Xid mutation are more prone to filarial worm infections because they fail to mount Th2 responses as a consequence of lack of IL-10 production [23], a cytokine mainly derived from B-1b cells [25,26]. Considering that in our model the introduction of immune B-1 cells into the system did not induce Th2 responses, we assume that pre-sensitized B-1 cells with previous memory to a given antigen may not act as enhancers of Th2 immunity.

Our results confirm the previous observation that B-1b cells obtained from naïve animals are capable of satisfactorily present antigen to and induce proliferation of T lymphocytes, what has been proposed as a possible link between innate and acquired immunity [7]. On the other hand, we showed that once B-1b cells have had a previous encounter with the antigen, they will somehow decrease the ability of mice to develop an immune response to that same antigen, which points to a specific mechanism of tolerance induction. Other investigators have shown the existence of immunological memory in B-1b cells, albeit in a different context. Animals lacking B-1b cells are susceptible to *B. hermsii* infection and the adoptive transfer of this cell subtype obtained from convalescent mice confers protection against the bacteria [22]. Therefore, it is clear that B-1b cells can act in an antigen-specific manner, though the factors modulating their stimulating or suppressor effects remain unclear.

Since suboptimal conditions for antigen presentation lead to impairment of T cell activation [27], we hypothesized that the mechanism by which B-1 cells downregulate T cell function could be at the antigen presentation level. In fact, unlike macrophages and naïve B-1 cells, pre-sensitized B-1 cells failed to promote T cell proliferation. Interestingly, even though pre-sensitized B-1 cells were found to express higher levels of Class-II MHC when compared to naïve B-1, they in turn exhibited significantly reduced levels of the co-stimulatory molecule CD86. CD86 mRNA is known to be upregulated when B cells are in culture [28–30], particularly when in presence of antigenic stimuli [28–31]. Therefore, our data suggest that the failure to upregulate CD86 by B-1 cells after antigen exposure, in opposition to naïve B-1, may account for the non-responsive state induced by these cells.

CD86 molecule is closely related to humoral response and T cell priming [30,32] and the blockade of this co-stimulatory molecule in early stages of an infection are shown to deviate the immune response to a Th1 profile [33]. Thus, the fact that sensitized B-1b cells failed to increase CD86 expression in our model may in part explain the low levels of anti-OVA IgG produced after the challenge, as well as the inability of these cells to induce *in vitro* T cell proliferation and allergic reactions in the footpad and lung. Possibly, as B-2 lymphocyte activation is dependent on the interaction with T cells, pre-sensitized B-1 may indirectly downregulate B-2 function, leading to lower production of anti-OVA antibodies in pre-sensitized B-1-injected animals.

Altogether, our data contribute to the understanding of how B-1 cells may play a regulatory role in the development of acquired immunity in mice. As reported before, naïve B-1 cells are competent APCs and trigger T cell proliferation *in vitro* [7], while a

previous encounter with the antigen under given conditions may lead to the generation of suppressor or tolerogenic properties, as proposed here. The uncovering of the factors involved in the induction of such tolerogenic state of B-1 cells may open a new field in the investigation of immunosuppression.

Acknowledgement

We thank Ana Flávia Popi for critical reading of this manuscript.

References

- [1] Hayakawa K, Hardy RR, Parks DR, Herzenberg LA. The “Ly-1 B” cell subpopulation in normal immunodeficient, and autoimmune mice. *J Exp Med* 1983;157:202–18.
- [2] Manohar V, Brown E, Leiserson WM, Chused TM. Expression of Lyt-1 by a subset of B lymphocytes. *J Immunol* 1982;129:532–8.
- [3] Kantor AB. The development and repertoire of B-1 cells (CD5 B cells). *Immunol Today* 1991;12:389–91.
- [4] Abrahao TB, Freymuller E, Mortara RA, Lopes JD, Mariano M. Morphological characterization of mouse B-1 cells. *Immunobiology* 2003;208:401–11.
- [5] Almeida SR, Aroeira LS, Frymuller E, Dias MA, Bogsan CS, Lopes JD, et al. Mouse B-1 cell-derived mononuclear phagocyte, a novel cellular component of acute non-specific inflammatory exudate. *Int Immunol* 2001;13:1193–201.
- [6] Bogsan CS, Novaes e Brito RR, Palos MC, Mortara RA, Almeida SR, Lopes JD, et al. B-1 cells are pivotal for in vivo inflammatory giant cell formation. *Int J Exp Pathol* 2005;86:257–65.
- [7] Vigna AF, Godoy LC, Rogerio dA, Mariano M, Lopes JD. Characterization of B-1b cells as antigen presenting cells in the immune response to gp43 from *Paracoccidioides brasiliensis in vitro*. *Immunol Lett* 2002;83:61–6.
- [8] Hayakawa K, Hardy RR, Honda M, Herzenberg LA, Steinberg AD. Ly-1 B cells: functionally distinct lymphocytes that secrete IgM autoantibodies. *Proc Natl Acad Sci USA* 1984;81:2494–8.
- [9] Herzenberg LA, Stall AM, Lal or PA, Sidman C, Moore WA, Parks DR. The Ly-1 B cell lineage. *Immunol Rev* 1986;93:81–102.
- [10] Hoerauf A, Solbach W, Lohoff M, Rollinghoff M. The Xid defect determines an improved clinical course of murine leishmaniasis in susceptible mice. *Int Immunol* 1994;6:1117–24.
- [11] Paciorkowski N, Porte P, Shultz LD, Rajan TV. B1B lymphocytes play a critical role in host protection against lymphatic filarial parasites. *J Exp Med* 2000;191:731–6.
- [12] Tang Y, Kim WK, Holmes KL, Hugin AW, Kenny JJ, Chattopadhyay SK, et al. Contribution of B cell subsets to delayed development of MAIDS in xid mice. *Cell Immunol* 1995;165:1–6.
- [13] Chow DA, Bennet RD. Low natural antibody and low in vivo tumor resistance, in xid-bearing B cell deficient mice. *J Immunol* 1989;142:3702–6.
- [14] Murakami M, Yoshioka H, Shirai T, Tsubata T, Honjo T. Prevention of autoimmune symptoms in autoimmune-prone mice by elimination of B-1 cells. *Int Immunol* 1995;7:877–82.
- [15] Watanabe N, Ikuta K, Nisitani S, Chiba T, Honjo T. Activation and differentiation of autoreactive B-1 cells by interleukin 10 induce autoimmune hemolytic anemia in Fas-deficient anti-erythrocyte immunoglobulin transgenic mice. *J Exp Med* 2002;196:141–6.
- [16] Khan WN, Alt FW, Gerstein RM, Malynn BA, Larsson I, Rathbun G, et al. Defective B cell development and function in Btk-deficient mice. *Immunity* 1995;3:283–99.
- [17] Santos-Lima EC, Vasconcellos R, Reina-San-Martin B, Fesel C, Cordeiro-Da-Silva A, Berneman A, et al. Significant association between the skewed natural antibody repertoire of Xid mice and resistance to *Trypanosoma cruzi* infection. *Eur J Immunol* 2001;31:634–45.
- [18] Alugupalli KR, Leong JM, Woodland RT, Muramatsu M, Honjo T, Gerstein RM. B1b lymphocytes confer T cell-independent long-lasting immunity. *Immunity* 2004;21:379–90.

- [19] Carvalho C, Jancar S, Mariano M, Sirois P. A rat model presenting eosinophilia in the airways, lung eosinophil activation, and pulmonary hyperreactivity. *Exp Lung Res* 1999;25:303–16.
- [20] Russo M, Mariano M, Jancar S. A new murine model of persistent lung eosinophilic inflammation. *Mem Inst Oswaldo Cruz* 1997;92:215–8.
- [21] Cohn L, Elias JA, Chupp GL. Asthma: mechanisms of disease persistence and progression. *Annu Rev Immunol* 2004;22:789–815.
- [22] Kelly-Welch AE, Melo ME, Smith E, Ford AQ, Haudenschild C, Noben-Trauth N, et al. Complex role of the IL-4 receptor α in a murine model of airway inflammation: expression of the IL-4 receptor α on non-lymphoid cells of bone marrow origin contributes to severity of inflammation. *J Immunol* 2004;172:4545–55.
- [23] Al Qaoud KM, Fleischer B, Hoerauf A. The Xid defect imparts susceptibility to experimental murine filariasis—association with a lack of antibody and IL-10 production by B cells in response to phosphorylcholine. *Int Immunol* 1998;10:17–25.
- [24] Minoprio P, el Cheikh MC, Murphy E, Hontebeyrie-Joskowicz M, Coffman R, Coutinho A, et al. Xid-associated resistance to experimental Chagas' disease is IFN- γ dependent. *J Immunol* 1993;151:4200–8.
- [25] O'Garra A, Chang R, Go N, Hastings R, Haughton G, Howard M. Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. *Eur J Immunol* 1992;22:711–7.
- [26] Popi AF, Lopes JD, Mariano M. Interleukin-10 secreted by B-1 cells modulates the phagocytic activity of murine macrophages *in vitro*. *Immunology* 2004;113:348–54.
- [27] Thompson CB, Allison JP. The emerging role of CTLA-4 as an immune attenuator. *Immunity* 1997;7:445–50.
- [28] Freeman GJ, Gray GS, Gimmi CD, Lombard DB, Zhou LJ, White M, et al. Structure, expression, and T cell co-stimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7. *J Exp Med* 1991;174:625–31.
- [29] Guerder S, Meyerhoff J, Flavell R. The role of the T cell co-stimulator B7-1 in autoimmunity and the induction and maintenance of tolerance to peripheral antigen. *Immunity* 1994;1:155–66.
- [30] Hathcock KS, Laszlo G, Pucillo C, Linsley P, Hodes RJ. Comparative analysis of B7-1 and B7-2 co-stimulatory ligands: expression and function. *J Exp Med* 1994;180:631–40.
- [31] Chen C, Faherty DA, Gault A, Connaughton SE, Powers GD, Godfrey DI, et al. Monoclonal antibody 2D10 recognizes a novel T cell co-stimulatory molecule on activated murine B lymphocytes. *J Immunol* 1994;152:2105–14.
- [32] Han S, Hathcock K, Zheng B, Kepler TB, Hodes R, Kelsoe G. Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. *J Immunol* 1995;155:556–67.
- [33] Kuchroo VK, Das MP, Brown JA, Ranger AM, Zamvil SS, Sobel RA, et al. B7-1 and B7-2 co-stimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* 1995;80:707–18.