

IL-5 and IL-17A are critical for the chronic IgE response and differentiation of long-lived antibody-secreting cells in inflamed tissues

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ABSTRACT

Prolonged survival of long-lived antibody-secreting cells in the BM has been implicated as a key component of long-term humoral immunity. The current study was designed to uncover the extrinsic signals required for the generation and maintenance of ASC in several niches (peritoneum, spleen and bone-marrow). Our results show that protein mixture of the *Thalassophryne nattereri* venom induced a chronic Th2 humoral response that is characterized by splenic hyperplasia with GC formation and venom retention by follicular DCs. Retention of B1a in the BM were observed. In the late phase (120 d) of chronic venom-response the largest pool of ASC into the peritoneal cavity consisted of B220^{neg}CD43^{high} phenotype; the largest pool of ASC into spleen was constituted by B220^{high} and B220^{low}, whereas the largest pool of ASC into the BM was constituted by the B220^{high}CD43^{low} phenotype; and finally, terminally differentiated cells (B220^{neg}CD43^{high}) were only maintained in the inflamed peritoneal cavity in late phase. After 120 d a sustained production of cytokines (KC, IL-5, TNF- α , IL-6, IL-17A and IL-23) and leukocytes recruitment (eosinophils, mast cells, and neutrophils) were induced. IL-5- and IL-17A-producing CD4⁺ CD44⁺ CD40L⁺ Ly6C⁺ effector memory T cells were also observed in peritoneal cavity. Finally, treatment of venom-mice with anti-IL-5- and anti-IL17A-neutralizing mAbs abolished the synthesis of specific IgE, without modifying the splenic hyperplasia or GC formation. In addition, IL-5 and IL-17A negatively regulated the expansion of B1a in peritoneal cavity and BM, and promoted the differentiation of these cells in spleen. And more, IL-5 and IL-17A are sufficient for the generation of ASC B220^{neg} in the peritoneal cavity and negatively regulate the number of ASC B220^{pos}, confirming that the hierarchical process of ASC differentiation triggered by venom needs the signal derived from IL-5 and IL-17A.

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1. Introduction

Immunological memory is a hallmark of acquired immunity. The contribution of B cells for the memory Abs response is based on different population of cells: memory B cells and long-lived antibody-secreting cells – ASC. Memory B cells undergo rapid clonal expansion and differentiation to mount high affinity Ab responses upon exposure to antigens. ASC are terminally differentiated and continue secreting Abs without antigenic stimulation in the bone marrow (BM) that provides a special microenvironment for their longevity [1–5].

Both memory B cells and ASC can be generated during the first immune response from innate-like B cells as B1 and conventional B (B2) which differ in development, surface marker expression, tissue localization, and function. Peritoneal B1 cells are comprised of two different populations, B1a is CD5^{pos}, long-lived and

responsible for the production of low affinity and polyreactive IgM referred to as natural antibodies. Upon Ag encounter, they quickly migrate to lymphoid organs (e.g., the spleen) to become Ab-producing cells [6].

Numerous changes are associated with ASC differentiation which distinguished then from memory conventional B cells, including the loss of surface IgM, B220, CD19, and MHC class II, the production of secretory IgM, and the upregulation of the specific marker as CD43, CD138 (syndecan-1) and CD93 [7–9]. ASC express adhesion molecules like VLA-4, VLA-5, CD9, CD44 [8–10] and chemokines receptors as CXCR3 and CXCR4 [10–12], which could be involved in homing and selective survival in particular tissues or niches, mainly in the BM followed by spleen and sites of inflammation.

The intrinsic genetic program that drives the differentiation of ASC is becoming clear. ASC differentiation is largely controlled by the transcriptional repressor B lymphocyte-induced maturation protein-1 (Blimp-1) that neutralizes Pax5- and Bcl6-mediated repression, thus enabling the expression of the plasma cell – specific gene program, in collaboration with other essential regulators such as IFN regulatory factor (IRF) 4 and X-box-binding protein-1

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(XBP-1) [13]. Extrinsic factors such as IL-5, IL-6, SDF-1 α , TNF α , and a proliferation-inducing ligand (APRIL, also known as TNFSF13) have been associated with the survival of ASC [14,15]. Results from Cassese et al. [16] strongly support the concept that ASC survival depends on niches in which a combination of specific signals, including IL-5, IL-6, stromal cell-derived factor-1 α , TNF- α , and ligand for CD44, provides an environment required to mediate plasma cell longevity. Thus, the understanding of the mechanisms by which ASC are generated and maintained is of fundamental interest to reveal the basis of immunological memory.

The help that T cells provide to B cells allows the production of memory B cells and ASC. The Th17 lineage develops in a pathway independent from Th1 and Th2 differentiation [17,18]. A hallmark of Th17 cells is the production of IL-17a-f, the proinflammatory cytokines. These cytokines bind to multimeric IL-17 receptors comprised of two IL-17RA subunits and one IL-17RC subunit [19]. Work has focused attention on the pathogenic function of the IL-17A in several organ-specific autoimmune and chronic inflammatory diseases as SLE [20] and in particular, IL-17 has been reported as the central cytokine involved in the spontaneous development of germinal center (GC) B cell-derived autoantibodies in autoimmune BXD2 mice [21]. However, the involvement of IL-17A in the ASC differentiation and maintenance is still largely unknown.

Thalassophryne nattereri fish envenomation is commonly reported among fishermen and bathers in the Brazilian North and Northeast coast, estimated at hundreds of accidents per year [22]. Using a mouse model, we have reveal the mechanism of actions of venom and its toxin families, and recently we shown that low doses of *T. nattereri* venom mixed to alum for optimal conditions of T cell activation elicited a strong and sustained (for more than 6 months) active memory response with high levels of specific IgG1 and IgG2a, and total IgE, accompanied by a striking IL-5 production and a decreased B220 expression in splenic B cells [23]. Therefore, this model provides an interesting scenario for studying the signals allowing survival and differentiation of the memory B cells.

Thus in this work, we have used venom proteins of *T. nattereri* Brazilian fish to establish a model in which germinal center derived B cells and high affinity specific antibodies were permanently generated and to study the extrinsic signals required for the generation and maintenance of ASC in several niches (peritoneum, spleen and bone-marrow) as well as the signaling pathways that they engage.

2. Material and methods

2.1. Mice

Male BALB/c mice (5–6 weeks old) were obtained from a colony at the Butantan Institute, São Paulo, Brazil. Animals were housed in a laminar flow holding unit (Gelman Sciences, Sydney, Australia) in autoclaved cages on autoclaved bedding, in an air-conditioned room in a 12 h light/dark cycle. Irradiated food and acidified water were provided *ad libitum*. All procedures involving animals were in accordance with the guidelines provided by the Brazilian College of Animal Experimentation (666/09 and 25/84/02).

2.2. Induction of memory immune response and treatment of mice with neutralizing antibody

T. nattereri fish venom was obtained from fresh captured specimens at the Northeastern coast of Brazil (IBAMA 16221-1) in different months of the year according to Lopes-Ferreira et al. [24]. Endotoxin content was evaluated (resulting in a total dose

<0.8 pg/ml LPS) with QCL-1000 chromogenic Limulus amoebocyte lysate assay (Bio-Whittaker) according to the manufacturer's instructions. Groups of five mice were immunized with intraperitoneal injections of 10 μ g of *T. nattereri* venom adsorbed in Al(OH)₃ on day 0 and boosted on day 14 with the same dose of venom. Animals injected only with Al(OH)₃ were considered as control mice. All groups of animals (venom or control) were bled and killed at days 21, 28, 48, 74 and 120. In some experiments mice were injected i.v with 5 μ g of neutralizing anti-IL-5 and anti-IL-17A mAbs (R&D Systems Europe Ltd.) or control rat IgG mAb (GL113) 30 min before venom injection at days 0 and 14. Mice were killed at 28 and 74 d after the first immunization. For determination of leukocyte recruitment into peritoneal cavity and T cell analysis mice were immunized on day 0 with 10 μ g of venom adsorbed in alum and boosted on days 14, 20, 47 and 119 with 10 μ g of venom and killed at days 15, 21, 48 and 120 to detection of effector and memory CD4⁺ T cells.

2.3. Cell isolation

At time points indicated (21, 28, 48, 74, and 120 d) after venom immunization, animals were killed by CO₂ asphyxiation, and peritoneal cells were recovered by peritoneal flushing using 5 ml of ice-cold sterile PBS plus 0.1% EDTA (ethylenediaminetetraacetic acid). BM cells were isolated from femur bones and after centrifugation; the supernatant from both cell suspensions was collected for cytokine, chemokine, and FACS analysis. Spleens were removed aseptically and single-cell suspensions were prepared for FACS analysis and *in vitro* re-stimulation. Cells were cultured at 2×10^6 cells/ml in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 25 mM HEPES buffer, and 10% heat-inactivated FCS (all from Life Technologies, Paisley, UK). Cells were cultured with 10 μ g/ml of *T. nattereri* venom for 72 h, and the supernatants from parallel triplicate cultures were stored at -70°C until analysis of cytokine concentrations by ELISA.

2.4. Flow cytometry analysis

For surface staining single-cell suspensions (1×10^6) were treated with 3% mouse serum of naive mice to saturate Fc receptors followed by the staining by fluorescence conjugated Abs: Rat IgG2ak PE-anti-mouse CD5, Goat IgG2bk PE-anti-mouse Ig (specific for IgG1, IgG2a, IgG2b and IgG3), Rat IgG2ak PerCP-Cy5-anti-mouse CD45R/B220, Rat IgG2ak FITC-anti-mouse CD43, Rat IgG2ak PE-anti-mouse CD138, Rat IgG2bk FITC-anti-mouse CD4, Rat IgG2ak Biotin anti-mouse, second antibody goat PE anti-rat, Rat IgG2bk FITC-anti-mouse Ly6C, Goat IgG2bk PE-anti-mouse CD154 (CD40L), Rat IgG2bk PE-anti-mouse Ly6G, Rat IgG2ak FITC-anti-mouse CD117, Rabbit IgG2ak anti-mouse CCR3, goat FITC anti-rabbit for 30 min on ice. Cells were washed three times in PBS 1% BSA. For intracellular staining, cells were stimulated with 20 ng/ml acetate phorbol myristate, 1 μ M ionomycin (Sigma) e Golgi-stop solution (BD Biosciences) for 6 h. Cells were washed, fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) and stained with Rat PerCP-Cy5 anti-mouse IL-17A, Rat PerCP-Cy5 anti-mouse IFN- γ , Rat PerCP-Cy5 anti-mouse IL-4 and Rat PerCP-Cy5 anti-mouse IL-5. Cells were washed three times in PBS 1% BSA. Negative controls were used to set the flow cytometer photomultiplier tube voltages, and single-color positive controls were used to adjust instrument compensation settings. Cells were examined for viability by flow cytometry using forward/side scatter characteristics or dead cells were excluded by LIVE/DEAD fixable dead cell stain. Data from stained samples were acquired using a FACSCalibur flow cytometer equipped with CellQuest software

(BD Biosciences) and were analyzed using CellQuest Software (Becton–Dickinson, San Jose, CA).

2.5. Determination of cytokine and chemokine by ELISA

Cytokines and chemokines were measured in supernatant of peritoneal and BM flushing and splenic cell culture by a specific two-site ELISA using antibody pairs purchased from Pharmingen (BD, San Diego, CA, USA). Binding of biotinylated monoclonal antibodies was detected using streptavidin-horseradish peroxidase complex and TMB (3,3',5,5'-tetramethylbenzidine) substrate solution containing hydrogen peroxide. Results are expressed as picograms per milliliter in reference to commercially available standards. Detection limits were 7.8 pg/ml for IL-1 β , TNF α , IL-6, IL-5, IL-17A, IFN- γ and IL-10; for IL-23 was 39 pg/ml and for chemokine KC was 15.6 pg/ml. The results were expressed as the mean values and standard deviations from five animals.

2.6. Titration of total IgE and specific IgG1 and IgG2a by ELISA

Blood samples were obtained by the puncture of the right ventricle of immunized mice. ELISA for detection of Abs was performed as described [25]. Plasma was tested for IgG1 or IgG2a Abs using venom-coated 96-well plates and biotinylated goat anti-mouse IgG1 or IgG2a antiserum. The reactions were developed with streptavidin-horseradish peroxidase complex (Sigma), O-phenylenediamine (OPD) and H₂O₂ and the plates were read at 490 nm on an automated ELISA reader (Spectramax, Molecular Devices). The results were expressed as the mean $\pm$ SEM absorbance. An IgE-specific ELISA was used to quantitate total IgE Ab levels in plasma using matched Ab pairs (553413 Pharmingen and 1130-08 Southern Biotech.), according to the manufacturer's instructions. Samples were quantified by comparison with a standard curve of IgE (03131D, Pharmingen).

2.7. Passive cutaneous anaphylaxis (PCA)

For determination of the anaphylactic activity of IgE, PCA reactions were performed in rats using non-inactivated plasma, according to Mota and Wong [26]. After 18 h they were challenged i.v. with 100 μ g of venom +0.25% of Evans blue solution. All tests were made in triplicate and PCA titers were expressed as the reciprocal of the highest dilution that gave a lesion of >5 mm in diameter. The detection threshold of the technique was established at 1:5 dilutions.

2.8. Determination of peritoneal influx of cells

Groups of five mice were immunized i.p. with 10 μ g of *T. nattereri* venom adsorbed in Al(OH)₃ on day 0 and boosted on day 14 with the same dose of venom. Animals injected only with Al(OH)₃ were considered as control group. At 120 d after the first immunization, animals were killed by CO₂ inhalation and peritoneal cells were obtained by peritoneal flushing with 5 ml of sterile PBS/EDTA 10 mM. For the recovery of cellular contents, lavage fluid was centrifuged at 378g for 10 min and the cell pellet was resuspended in cold PBS/BSA 0.1% for leukocyte cell counts. Using a hemocytometer and cytocentrifuge, slides were prepared, air dried, fixed in methanol, and stained (Wright-Giemsa, Scientific Products, Chicago, IL). For differential cell counts, 300 leukocytes were enumerated and identified as macrophages, neutrophils, eosinophils or mast cells based on staining and morphologic characteristics using a light microscope (Nikon Eclipse E200). Representative photomicrographs of each group were acquired digitally using an Axio Imager A1 microscope (Carl Zeiss, Germany) with an AxioCam ICc1 digital camera (Carl Zeiss, Germany).

2.9. Histology and immunohistochemistry analysis of splenic sections

Spleens from control or venom-mice were collected and fixed (10% buffered formalin). The fragments were then dehydrated, cleared and embedded in paraffin. Serial sagittal sections of the whole spleen were made (3–4 μ m thick), stained with hematoxylin/eosin (H&E) to evaluate general morphology. Immunohistochemistry was performed using mAbs against the membrane Ig (Goat IgG2bk PE-anti-mouse Ig specific for IgG1, IgG2a, IgG2b and IgG3 – Southern Biotech) or polyclonal antibodies against *T. nattereri* venom obtained from pre-immunized mice. Endogenous peroxidase was inhibited with 3% H₂O₂ in PBS for 10 min at room temperature. Antibody binding in tissue sections for venom determination was visualized with a biotinylated secondary antibody (goat anti-mouse), streptavidin and biotinylated horseradish peroxidase complex (StreptABComplex/HPE) and diaminobenzidine (DAB) as chromogen. Sections were counterstained with hematoxylin/eosin. Negative control slides were processed in parallel with each batch of staining or normal serum. All slides were examined with light microscopy (Axio Imager A1, Carl Zeiss, Germany) calibrated with a reference micrometer slide. For each group of five mice, four stained spleen sections from each mouse were analyzed and it was performed three separate experiments.

2.10. Statistical analysis

All values were expressed as mean $\pm$ SEM. Parametric data were evaluated using an analysis of variance, followed by the Bonferroni test. Non-parametric data were assessed using the Mann–Whitney test. Differences were considered statistically significant at $p < 0.05$. Experiments were repeated at least three times.

3. Results

3.1. Venom induces a chronic Th2 antibody response

BALB/c mice with high susceptibility to the development of Th2-type immune response were immunized with proteins of *T. nattereri* venom in the presence of adjuvant. After a venom booster without adjuvant, they were left for a period of at least 7 d and up to 106 d in the absence of further antigenic stimulation for the induction of a chronic response in accordance with McHeyzer-Williams et al. [27] (Fig. 1A). First, we focus our investigation on the analysis of antibody memory response as a way to directly measure the activity of memory B cells, *ex vivo* (Fig. 1B) and the results show a high production of venom-specific IgG1 and IgG2a accompanied by high levels of total and specific IgE antibodies compared with control-mice. Until 74 d we observed an increase in the production of IgG2a that decrease at 120 d. In contrast, the levels of IgG1, total as well as specific IgE dramatically increase at 120 d compared with 21–74 d. These results indicate that the chronic interaction of venom proteins with host promotes a shift from Th1 for Th2 in the late phase of antibody responses.

Besides the ability to produce specific antibodies, another aspect that is discussed on the formation of immunological memory is the change in the spleen architecture, a lymphoid organ considered a center of lymphocyte proliferation and high-affinity antibody production. Here we observed that spleens of venom-mice appeared enlarged in size, and splenomegaly was accompanied by an increased number of splenocytes (Fig. 1C). We observed that typical B cells areas in lymphoid follicles of control-mice (Fig. 1D) were modified in spleen of venom-mice with chronic immune response at 120 d, showing an increased expansion of GC, largest and most populous than in control-mice (Fig. 1E). In addition, a significant enhancement of Ig^{pos} B cells was seen in the regions of GC

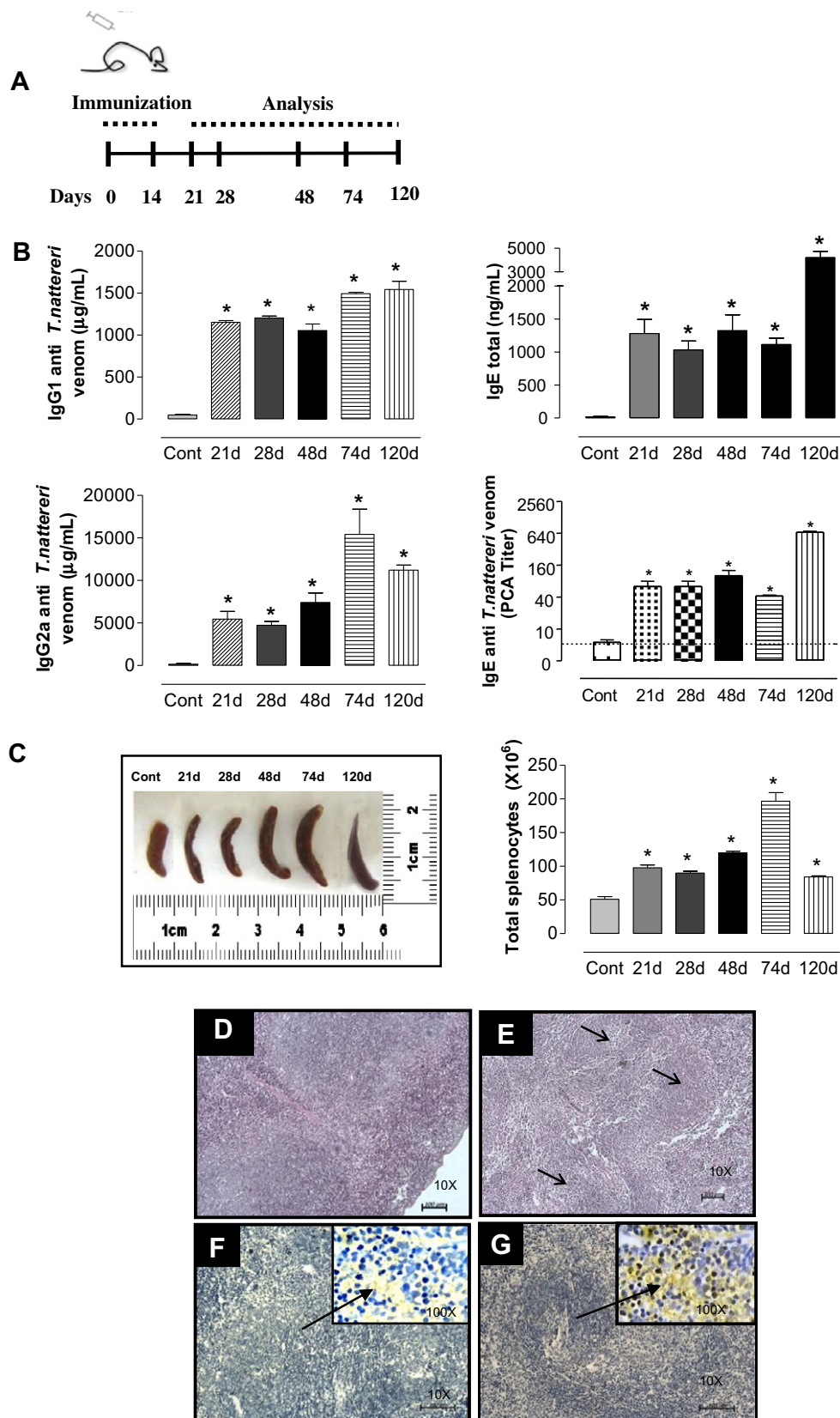


Fig. 1. Venom induces a chronic Th2 antibody response. BALB/c mice ($n = 5$) were immunized i.p. with $10 \mu\text{g}$ of *T. nattereri* venom adsorbed in $\text{Al}(\text{OH})_3$ on day 0 and boosted on day 14 with the same dose of venom alone. Animals injected only with $\text{Al}(\text{OH})_3$ were considered as control group. Mice were bled and killed at days 21, 28, 48, 74 and 120 (A). Plasma was tested for IgG1 or IgG2a antibodies using venom-coated 96-well plates and biotinylated goat anti-mouse IgG1 or IgG2a antiserum. Total IgE and venom-specific IgE were determined by sandwich ELISA or PCA, respectively (B). Spleens appeared enlarged in size and the splenomegaly was accompanied by an increased number of splenocytes (C). Typical B cell areas in lymphoid follicles were modified in venom-mice with chronic immune response (E, 120 d), showing an increased expansion of germinal centers, largest and most populous than control-mice (D). A significant enhancement of positive Ig B cells (Ig^{pos}) was seen in the regions of germinal center of venom-mice (G) compared with control-mice (F). Scale bar, $100 \mu\text{m}$. Mean $\pm$ SD of three independent experiments is shown. $^* p < 0.05$ compared to control-mice.

of these venom-mice until 120 d (Fig. 1G) as compared to regions of the GC of control-mice (Fig. 1F).

Together these results show that the protein mixture of the venom of *T. nattereri* induced a chronic humoral immune response with a predominant production of IgG2a antibodies that decline after 74 d privileging the production of IgE antibodies. The venom induced the development of splenic hyperplasia with GC formation and proliferation of positive Ig B lymphocytes.

3.2. Bone marrow maintenance of B1a cells induced by venom

Although B1 cells are classified as B cells responding to T cell-independent Ags, adaptive response and T cells can influence many aspects of B1 cell activation and differentiation [28–30]. Next, we analyzed B1a cells (B220/CD45R^{low}CD5^{pos}) using antibodies against B220 (anti-B cell isoform of CD45R) and CD5 in a negative CD4 cells gate. The results present in Fig. 2 show that there was an expansion of peritoneal B1a cells between 28 and 48 d (2.46% and 3.05%, respectively – Fig. 2A) that returned to control levels after 74 d. An elevated number of these cells were seen in spleen at 21, 28 and 74 d (9.72%, 8.48% and 5.45%, respectively – Fig. 2B); we can observe an almost disappearance of B1a cells in spleen at 48 d and 120 d. Although few B1a cells were observed in BM of venom-mice compared with control-mice, this environment sustained the chronic maintenance of B1a cells until 120 d (Fig. 2C).

Thus, these results demonstrate that venom induced the emergence of B1a in the BM and spleen, followed by the mobilization into the peritoneal cavity and retention in the BM.

3.3. Venom antigens promote the final differentiation of ASC in inflamed tissue

The cellular differentiation of B memory cells into ASC has not been completely elucidated, but a hierarchical model of differentiation has been proposed: activated B cells progressively acquire increasing levels of CD138 and decreasing levels of CD45R/B220 to finally arrive at B220^{neg} ASC phenotype, which are either CD138^{int} or CD138^{high} [9,31]. CD43 (leukosialin or sialophorin) is a cell surface sialoglycoprotein implicated in cell adhesion and proliferation whose tightly regulated expression in B lymphocytes is likely important for their normal development and/or function [10].

In Fig. 3A we observed that the expression of B220 and CD43 divides this population into four subsets that persist at different levels into all compartments. In peritoneal cavity venom induced the formation of B220^{high}CD43^{high}, B220^{high}CD43^{low}, and B220^{neg}CD43^{high}. In spleen: B220^{high}CD43^{high}, B220^{low}CD43^{high}, and B220^{neg}CD43^{high}; and finally in the BM: B220^{high}CD43^{high}, B220^{high}CD43^{low}, and B220^{neg}CD43^{high}.

In Fig. 3B, analyzing the expression of syndecan-1 – CD138 (histogram) in gated cells of the four subsets we show that venom induced into the peritoneal cavity until 120 d the differentiation and maintenance of B220^{high}CD43^{high}CD138^{pos}, B220^{high}CD43^{low}CD138^{pos}, and a bigger percentage of B220^{neg}CD43^{high}CD138^{pos}. Also, B220^{high}CD43^{high}CD138^{pos} positive cells were observed at 21 d; B220^{high}CD43^{low}CD138^{pos} positive cells were detected at 21 d and 48 d; and B220^{neg}CD43^{high}CD138^{pos} were detected at 21 d and 74 d.

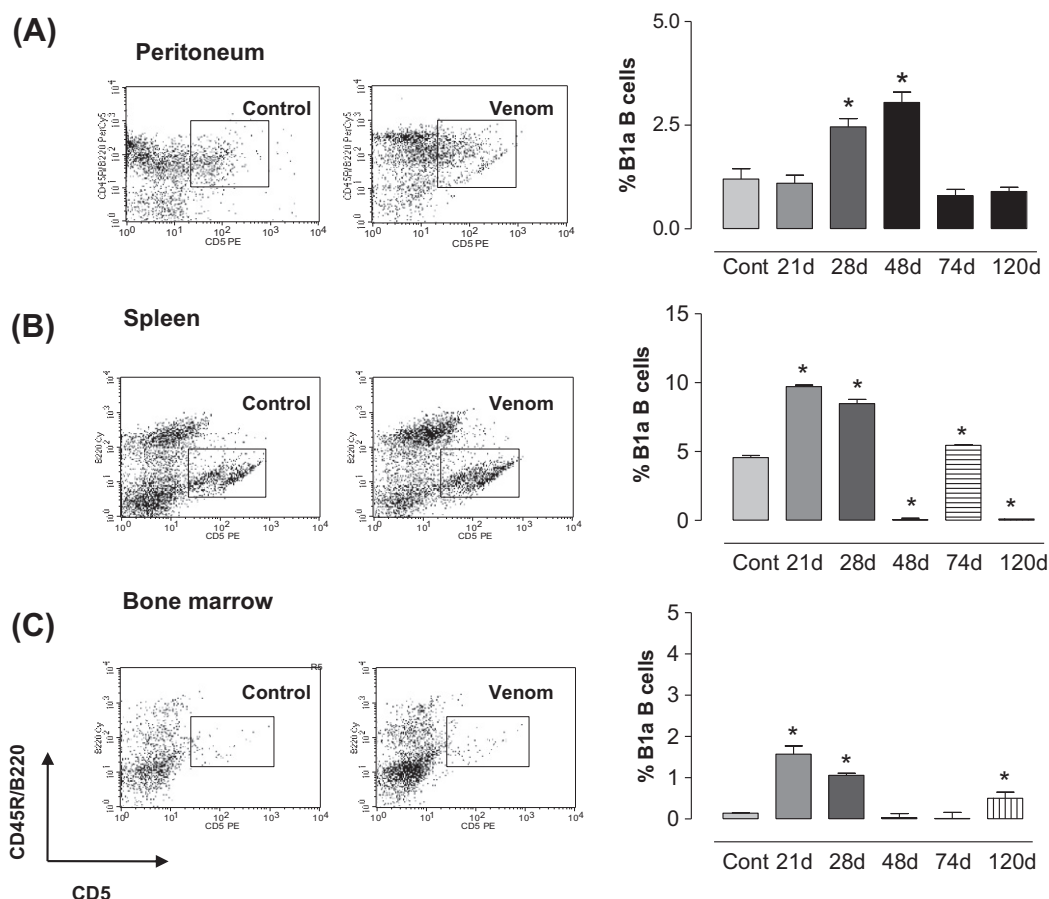


Fig. 2. Accumulation of B1a cells subset. Determination of B1a by flow cytometry using B220 co-staining with CD5 in total cells of peritoneal cavity (A), spleen (B) and BM (C) from immunized or control mice at various time points after immunization. Percentages of cells indicate for each bar represent the mean $\pm$ SEM value from determinations from pooled compartments each from five individual mice and three independent experiments. * $p < 0.05$ compared to control-mice.

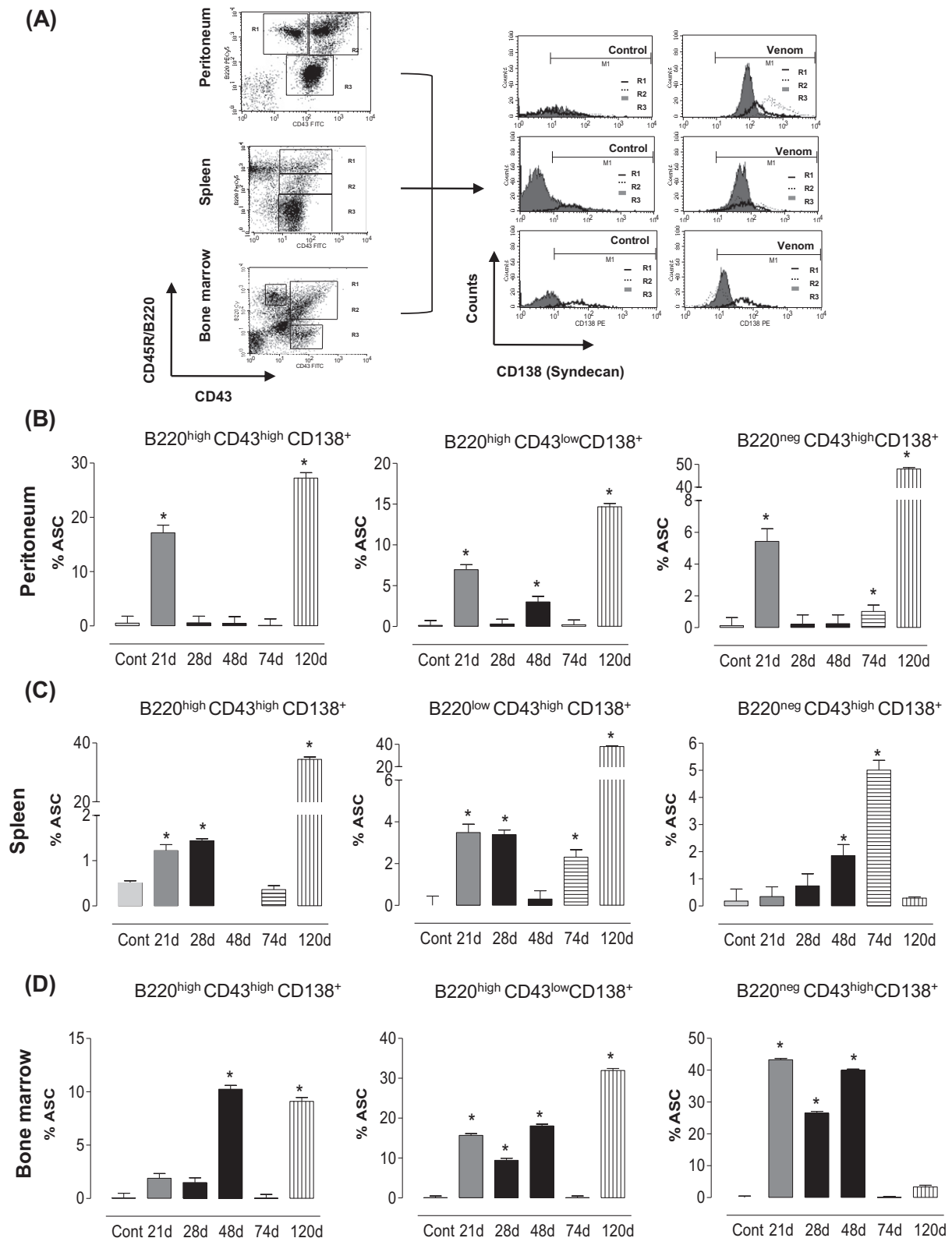


Fig. 3. Venom promotes the differentiation of ASC subtypes. The expression of B220 and CD43 divides the ASC population into 4 subsets that persist at different levels into all compartments. In peritoneal cavity (Fig. 3A) venom induced the formation of B220^{high}CD43^{high}, B220^{high}CD43^{low}, and B220^{neg}CD43^{high}. In spleen (Fig. 3B): B220^{high}CD43^{high}, B220^{low}CD43^{high}, and B220^{neg}CD43^{high}; and finally in the BM (Fig. 3C): B220^{high}CD43^{high}, B220^{high}CD43^{low}, and B220^{neg}CD43^{high}. Representative expression of high levels of CD138 in gated cells from population of peritoneal cavity (D), spleen (E) and BM (F) were determined. Mean $\pm$ SEM of three independent experiments is shown. * $p < 0.05$ compared to control-mice.

In spleen, B220^{high}CD43^{high}CD138^{pos} and B220^{low}CD43^{high}CD138^{pos} appeared at 21 d until 28 d and were maintained in chronic

phase of venom response (120 d), but B220^{neg}CD43^{high}CD138^{pos} were only detected at 48 d and 74 d after immunization (Fig. 3C).

In the BM (Fig. 3D) B220^{high}CD43^{high}CD138^{pos} and B220^{high}CD43^{low}CD138^{pos} were maintained in chronic phase of venom response (120 d), but B220^{neg}CD43^{high}CD138^{pos} were only observed until 48 d.

Together, these results show that: (1) all compartments were constituted by B220^{high}CD43^{high} and B220^{neg}CD43^{high}, but the subpopulations B220^{high}CD43^{low} and B220^{low}CD43^{high} were only observed in peritoneal cavity, and spleen, respectively; (2) in the late phase (120 d) of chronic venom-response the largest pool of ASC into the peritoneal cavity consist of B220^{neg}CD43^{high} phenotype; the largest pool of ASC into spleen was constituted by B220 positive cells (B220^{high} and B220^{low}), whereas the largest pool of ASC into in the BM was constituted by the B220^{high}CD43^{low} phenotype; (3) and finally, terminally differentiated cells (B220^{neg}CD43^{high}) were only maintained in the inflamed peritoneal cavity in late phase of chronic immune response induced by venom (120 d).

3.4. Inflammatory as well T-derived cytokines are responsible for ASC maintenance

A current concept of ASC homeostasis suggests that survival of individual cells depends on specific signals like cytokines produced in a limited number of ecological niches and, most of them present in the BM, but also in inflamed tissues. The active mediators induced by the venom until 120 d in the peritoneal cavity were KC (Fig. 4A) and IL-5 (Fig. 4E), and produced by splenic cells were TNF- α (Fig. 5C), IL-6 (Fig. 5D), IL-5 (Fig. 5E), IL-17A (Fig. 5F), and IFN- γ (Fig. 5H). In the BM, only IL-23 was produced at 120 d (Fig. 6H).

Also, it was observed in peritoneal cavity the production of IL-1 β until 48 d (Fig. 4B), and TNF- α , until 28 d (Fig. 4C); the production of IL-17A was observed only at days 48 and 74 (Fig. 4F); and the production of IFN- γ in this compartment was only observed at 21 d (Fig. 4H). Splenic cells produced KC only at 28 d (Fig. 5A); IL-1 β was produced until 48 d (Fig. 5B); and IL-10 until 74 d (Fig. 5G). Finally, in the BM venom induced the production of KC until 28 d (Fig. 6A); IL-1 β (Fig. 6B) and TNF- α (Fig. 6C) until 48 d; IL-5 was induced until 74 d (Fig. 6E), and IL-17A and IL-10 were detected only at 48 d (Fig. 6G).

Together the results from Figs. 3 and 4–6 suggest that the combination of described positive regulators as KC, IL-5, and TNF- α , and also IL-17A and IL-23 with negative regulators as IFN- γ and IL-10 could regulate the differentiation and the movement of cells into and across the compartments analyzed. Also, the maintenance of terminally differentiated cells (B220^{neg}CD43^{high}) only in the inflamed peritoneal cavity in late phase of chronic immune response induced by venom could be related with the persistent production of time KC, IL-5, TNF- α , IL-6, and IL-17A and IL-23.

Next we hypothesized that the resultant high level of peritoneal KC and IL-5, and possibly systemic IL-6, TNF- α , and IL-17A could contribute to leukocyte production and mobilization in the BM and high peripheral cell numbers in venom-mice. We confirm this hypothesis with the demonstration that chronic humoral immune response induced by *T. nattereri* venom is accompanied by a persistent inflammatory reaction in the peritoneal cavity compared with control animals (Fig. 7A). At 21 d an influx of neutrophils and an exuberant influx of eosinophils to the peritoneal cavity of venom-mice were observed. At 48 d, venom induced an increase in the number of neutrophils and a decrease in the number of eosinophils recruited, but induced the influx of macrophages. The late phase of memory response (120 d) induced by venom was characterized by a persistent Th2-mediated inflammation, with the influx of eosinophils, and mainly mast cells and neutrophils. At 120 d, control animals show no inflammatory cells in the peritoneal cavity.

The most popular and well characterized hypothesis of long-term antibody production is that memory B cells are stimulated by persisting antigen that is retained in the form of antigen–antibody complexes on the surface of follicular dendritic cells – fDC [32]. We next asked whether splenic cells of immunized mice retained venom antigens. For this, we used specific serum produced by previously venom-immunized mice according to Piran-Soares et al. [33]. Immunohistochemistry analysis indicated that venom proteins were detected in the clear zone of GC at days 21–48 (Fig. 7C–E) compared with control-mice (Fig. 7B). At 74 d (Fig. 7F) and 120 d (Fig. 7G) after immunization, few amounts of venom were seen retained by splenic cells, indicative of residual antigen elimination from the cellular environment.

Taken together, these results demonstrate that the persistence of venom antigens retained in fDCs of the spleen and the active inflammation in peritoneal cavity generated the chronic production of extrinsic factors capable to support the differentiation and survival of ASC.

3.5. Peritoneum provides survival signals to sustain ASC differentiation

The idea that CD4 memory T cells (CD44^{hi}CD62L^{neg}) can provide accelerated help for antibody responses is well established. To define the location of antigen-specific memory CD4⁺ T cells generated in a chronic response induced by venom, we have analyzed memory CD4⁺ T cells expressing Ly6C and CD40L markers. Our results in Fig. 8A show that the venom induced a formation of memory CD4⁺ CD44⁺ T cells expressing high levels of both Ly6C (top panel) and CD40L (bottom panel) in peritoneal cavity until 120 d. In spleen persistent formation of CD4⁺ CD44⁺ T cells expressing Ly6C was also observed until 49 d. In this compartment, CD4⁺ CD44⁺ T cells expressing CD40L were also seen in an early period after immunization (15 d). Finally, in BM, only CD4⁺ CD44⁺ T cells expressing high levels of Ly6C were detected at 49 d and 120 d. Together, these results show that the late phase of chronic response (120 d) induced by the venom is characterized by the presence of memory CD4⁺ CD44⁺ T cells expressing high levels of both Ly6C and CD40L in inflamed tissue and memory CD4⁺ CD44⁺ T cells expressing Ly6C in the BM.

In Fig. 8B we confirm the activation status of memory CD4 T cells by the demonstration of the intracellular content of cytokines 14 d after immunization. In peritoneal cavity an elevated development of IL-17A-, IL-5-, IL-4-, and IFN- γ -producing CD4⁺ CD44⁺ memory T cells were observed compared to control-mice (an increase of 3.3-fold, 3.02-fold, 2.86-fold, and 3.28-fold, respectively). Also, in spleen venom induced the development of IL-5-producing CD4⁺ CD44⁺ T cells 1.68-fold compared to control-mice, and IL-4- and IL-5-producing CD4⁺ CD44⁺ T cells in BM (1.64-fold and 1.5-fold, respectively). This result identifies memory CD4⁺ CD44⁺ T cells clones as a source of critical cytokines involved in Th2 shift during the late phase of venom memory response.

Together, our results suggest that venom induced a Th2-mediated inflammation in inflamed tissue with infiltration of eosinophils, mast cells, neutrophils, and CD4⁺ CD44⁺ CD40L⁺ Ly6C⁺ effector memory T cells. In addition, these results confirm the participation of memory CD4 T cells in the sustained production of survival factors as IL-5 in all compartments and IL-17A only in inflamed peritoneal cavity.

3.6. IL-5 and IL-17A cytokines positively regulate the production of specific-IgE

We show that the resultant high level of peritoneal KC and IL-5, and possibly systemic IL-5, IL-6, TNF- α , and IL-17A and bone-marrow production of IL-23 could contribute to regulation of ASC differentiation and maintenance. ASC survival is largely controlled

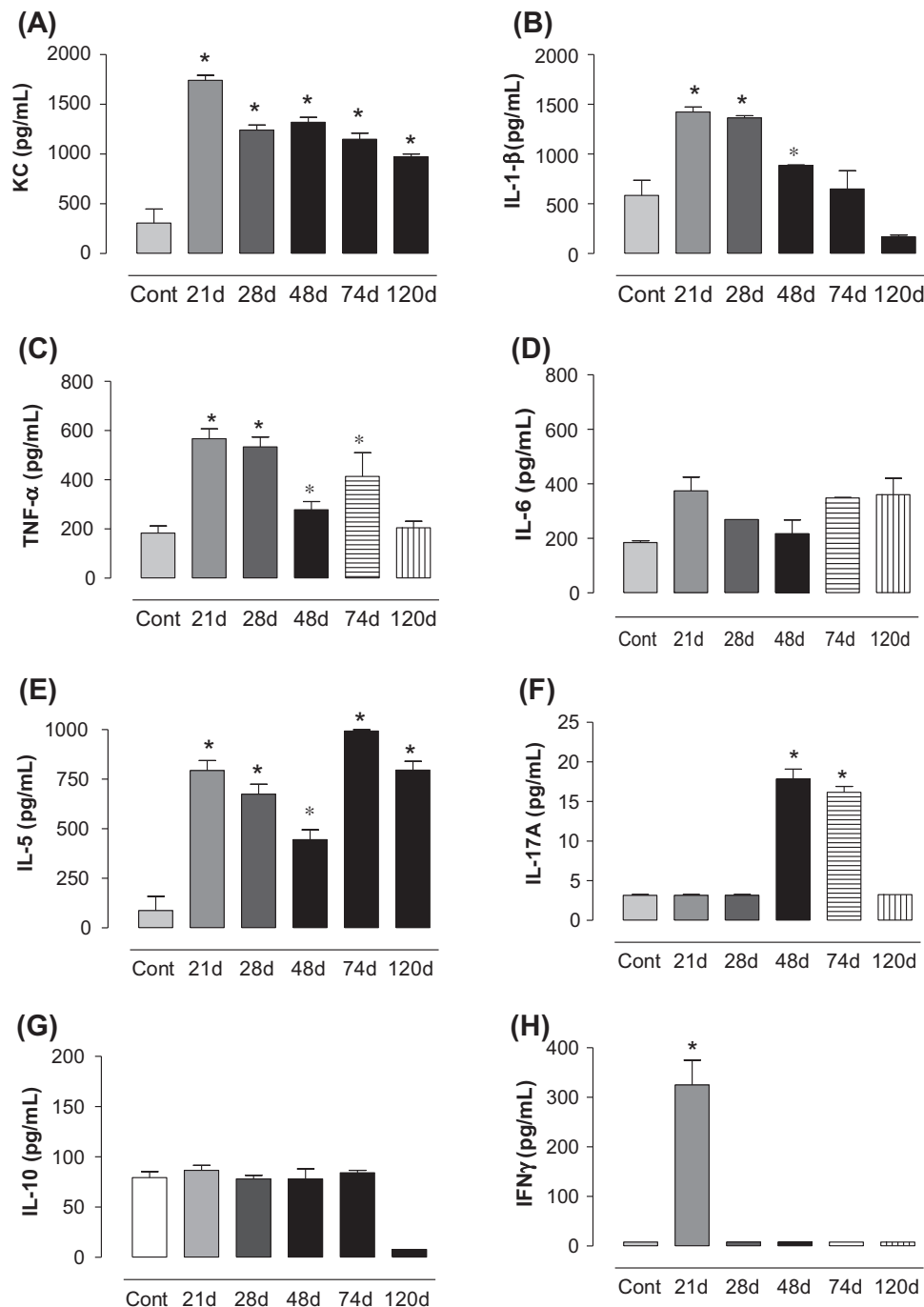


Fig. 4. Inflammatory mediators as well T-derived cytokines produced by peritoneal cavity cells. The levels of KC (A), IL-1 β (B), TNF- α (C), IL-6 (D), and IL-5 (E), IL-17A (F), IL-10 (G) and IFN- γ (H) were determined by ELISA in the supernatant of peritoneal cavity of mice. * $p < 0.05$ compared to control-mice.

by extrinsic factors such as interleukin IL-5, IL-6, SDF-1 α , and TNF α [16], but the direct or indirect influence of IL-5 and IL-17A in the *in vivo* differentiation or maintenance of ASC is not known.

Next, we examined the ability of neutralizing mAbs against both cytokines to modulate specific Ab responses generated by *T. nattereri* venom (Fig. 9A). Mice treated with anti-IL-5 or anti-IL-17A produced at 28 d after immunization diminished levels of specific-IgG1 and undetectable amounts of specific-IgG2a compared with mice injected with venom plus isotype control. However, 74 d after immunization, IgG1 and IgG2a antibody levels were equally high in anti-IL-17A treated-mice, and elevated levels of IgG2a were observed in anti-IL-5 treated-mice, but in contrast a

diminished level of specific-IgG1 was observed in these mice (Fig. 9B). These results show that the production of specific-IgG1 response against venom is controlled only by IL-5. We show that the elevated total IgE levels observed in venom-mice were abolished after anti-IL-5 treatment at 28 d, but not after anti-IL-17A neutralization. Both treatments did not affect total IgE production in later period (74 d) and moreover, the absence of both cytokines virtually abolished the production of specific-IgE antibodies (Fig. 9B), suggesting that IL-5 and IL-17A is crucial for the maintaining of specific-IgE secreting cell functions.

Overall, these results suggest that IL-5 and IL-17A regulate the production of specific-IgE, without modifying the production of

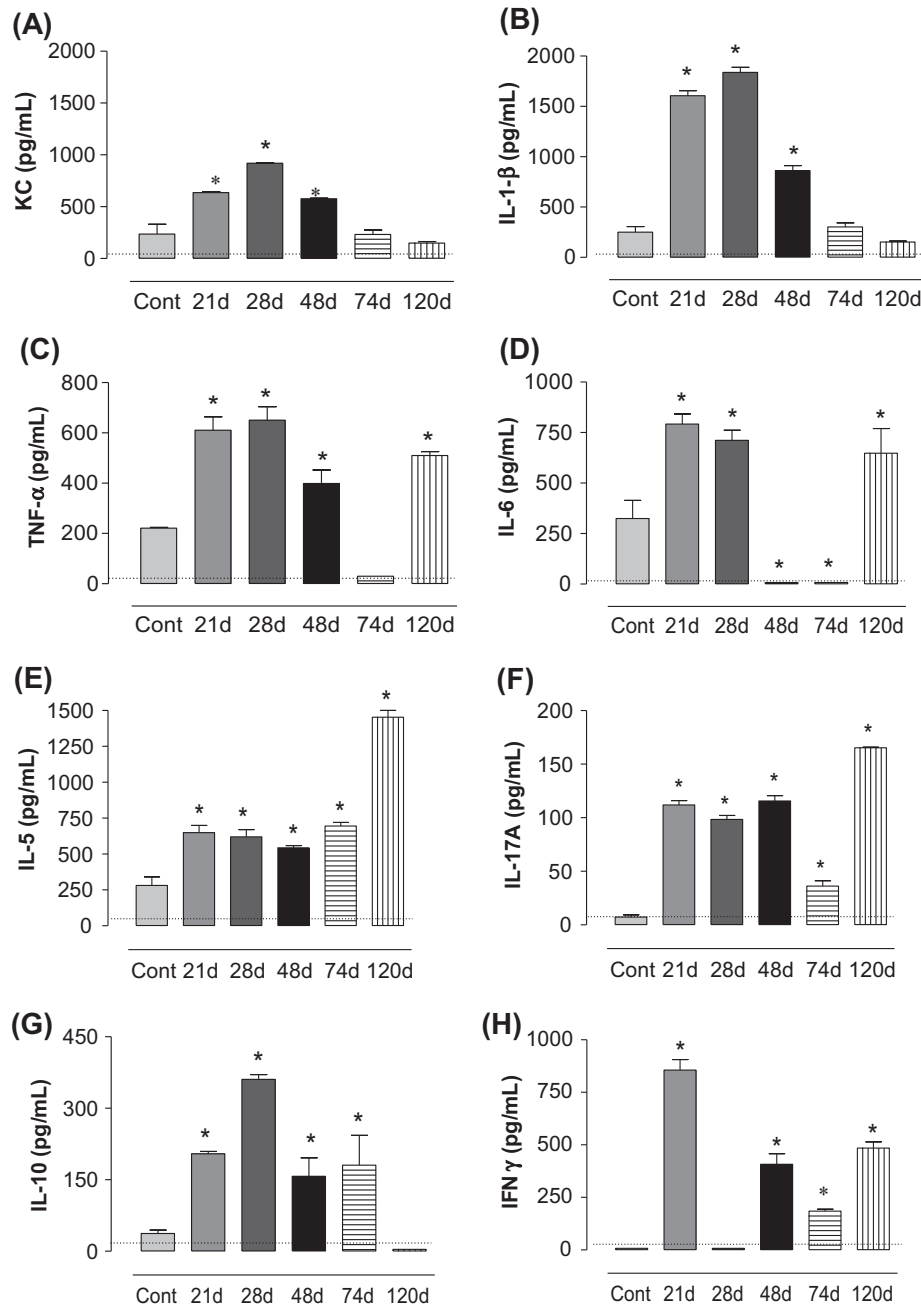


Fig. 5. Mediators produced by splenic cells. The levels of KC (A), IL-1 β (B), TNF- α (C), IL-6 (D), and IL-5 (E), IL-17A (F), IL-10 (G) and IFN- γ (H) were determined by ELISA in the supernatant of *in vitro* re-stimulated splenic cells. * $p < 0.05$ compared to control-mice.

specific-IgG2a or the total production of IgE. Also, IL-5 seems to be important for the chronic production of IgG1.

Nevertheless the treatment with neutralizing antibodies was not able to reverse the splenomegaly (Fig. 9C) or the GC formation (Fig. 9D), the absence of these cytokines during the immunization period of time induced a decrease in total number of splenocytes in 74 d compared with both animals injected only with venom or with venom plus control antibody GL113 (Fig. 9C). In addition, the blockade of IL-17A impairs partially the increase of Ig^{pos} B cells induced by the venom (lower panel, Fig. 9E).

Thus, although IL-5 and IL-17A regulate the synthesis of IgE antibodies, both cytokines do not modify the splenic hyperplasia or the formations of GC provoked by the venom; and also positively regulate the proliferation of splenocytes.

3.7. IL-5 and IL-17A regulate the maintenance of B1a in spleen

In the Fig. 10A, we observed that the treatment of venom-mice with blocking antibodies induced an expansion in the number of B1a in the peritoneal cavity and in the BM at days 28 and 74, suggesting a negative regulation of both cytokines in peripheral compartments as inflamed peritoneal cavity and BM. But in contrast, the splenic B1a population induced by the venom decreased dramatically after both treatments, suggesting a positive regulation of both cytokines in B1a in a secondary lymphoid organ as spleen.

Thus, IL-5 and IL-17A negatively regulate the expansion of B1a in the peritoneal cavity and in BM, and in contrast, they control the differentiation of these cells in spleen.

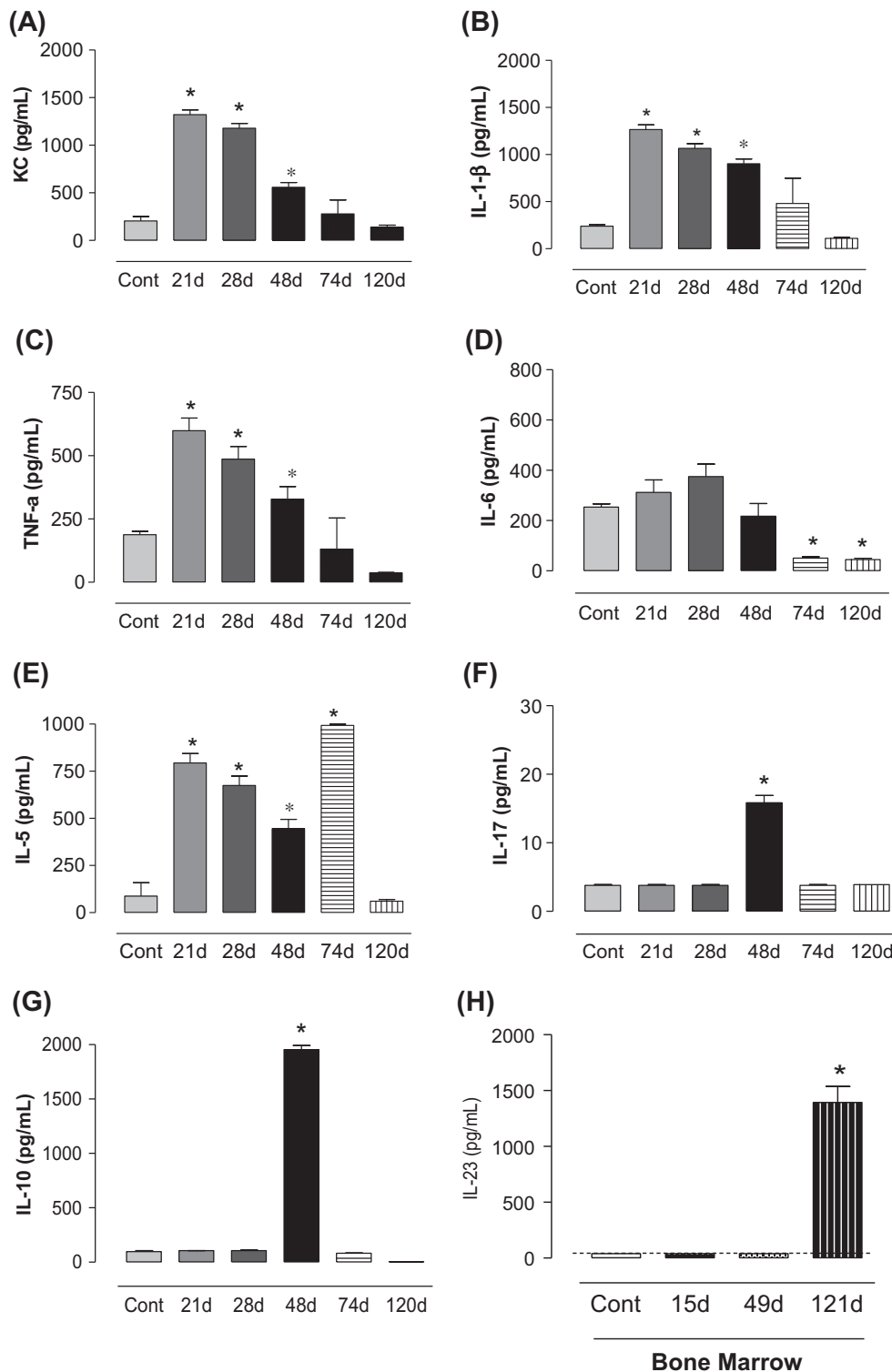


Fig. 6. Mediator produced by bone marrow cells. The levels of KC (A), IL-1 β (B), TNF- α (C), IL-6 (D), and IL-5 (E), IL-17A (F), IL-10 (G) and IL-23 (H) were determined by ELISA in supernatant of BM cells. * $p < 0.05$ compared to control-mice.

3.8. IL-17A positively regulates the generation of terminally differentiated ASC B220^{neg} in inflamed tissue

The selection of allergen-specific B cells into ASC compartment is a critical step in the immune deregulation that leads to the production of IgE in allergic diseases [34]. We show that IL-5 and IL-17A positively control the production of specific IgE in venom-mice.

Next we examined the ability of neutralizing mAbs against both cytokines to modulate the differentiation of ASC (Fig. 10B). We also show that the treatment of venom-immunized mice with both blocking antibodies resulted in almost complete depletion of ASC with the phenotype B220^{neg}CD43^{high} in peritoneal cavity at 74 d. Also, both treatments promoted augmented percentage of ASC with B220^{high}CD43^{high} phenotype. The normal percentage of the

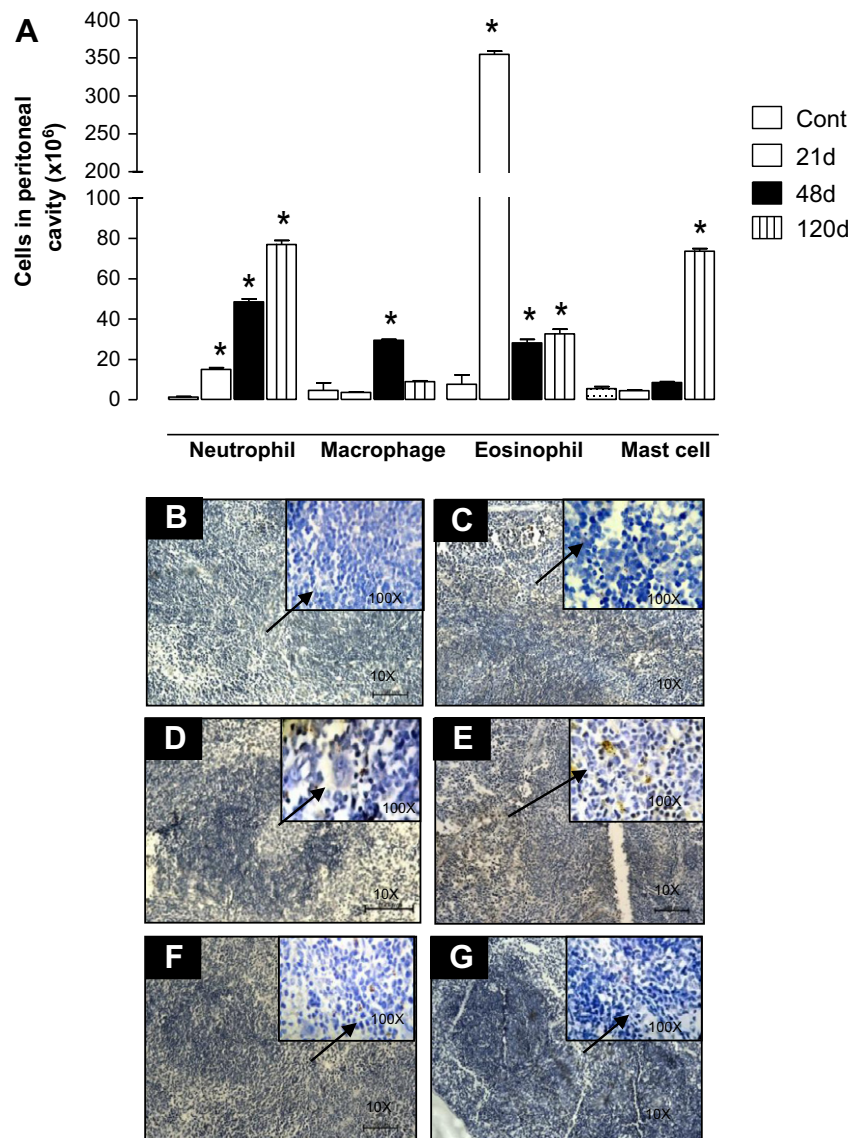


Fig. 7. Chronic humoral immune response induced by *T. nattereri* venom is accompanied by a persistent inflammatory reaction in the peritoneal cavity. The late phase of memory response (120 d) induced by venom was characterized by Th2-mediated inflammation, with the influx of eosinophils, and mainly mast cells and neutrophils. Control animals show no inflammatory cells in the peritoneal cavity (A). Immunohistochemistry analysis indicated that venom could be detectable in the clear zone of germinal center at 21 d until 48 d (C–E) compared with control-mice (B). Few amounts of venom were seen retained by splenic cells at 74 d (F) and 120 d (G) after immunization. * $p < 0.05$ compared to control-mice.

subpopulation B220^{high}CD43^{low} was not modified by neutralizing antibodies.

In the spleen, both blocking neutralizing antibodies promoted a higher percentage of ASC B220^{high}CD43^{high} (28 and 74 d) and B220^{low}CD43^{high} (74 d), however, they reduced the percentage of ASC with B220^{low}CD43^{high} phenotype at 28 d and B220^{neg}CD43^{high} phenotype at 74 d.

Concerning to ASC in the BM, treatment with both anti-IL-5 and anti-IL-17A mAbs promoted an increase in ASC B220^{high}CD43^{high} at 28 d, and in contrast, treatment with both blocking antibodies resulted in almost complete depletion of ASC with the phenotype B220^{neg}CD43^{high} in BM at this time. Furthermore, anti-IL-5 mAbs treatment induced at days 28 and 74 d an increased percentage of ASC B220^{high}CD43^{low}. These experiments indicate that IL-5 and IL-17A signals are sufficient for the generation of ASC with the B220^{neg} phenotype, controlling negatively the rate of survival of ASC with high B220 expression.

Thus, IL-5 and IL-17A are sufficient for the generation of ASC B220^{neg} in the peritoneal cavity and negatively regulates the number of ASC B220^{pos}, confirming that the hierarchical process of ASC differentiation triggered by venom needs the signal derived from IL-5 and IL-17A.

4. Discussion

The aim of this study was to investigate the impact of IL-5 and IL-17A on the development and maintenance of subtype of memory B cells in chronic humoral Th2 immune response. To this end, we used chronic immunized mice with *T. nattereri* venom proteins that were left for a period of 120 d in the absence of further antigenic stimulation. In order to distinguish the differential subtypes of memory B cells, the expression of B220 and CD43 markers was evaluated in these cells in several niches (peritoneum, spleen

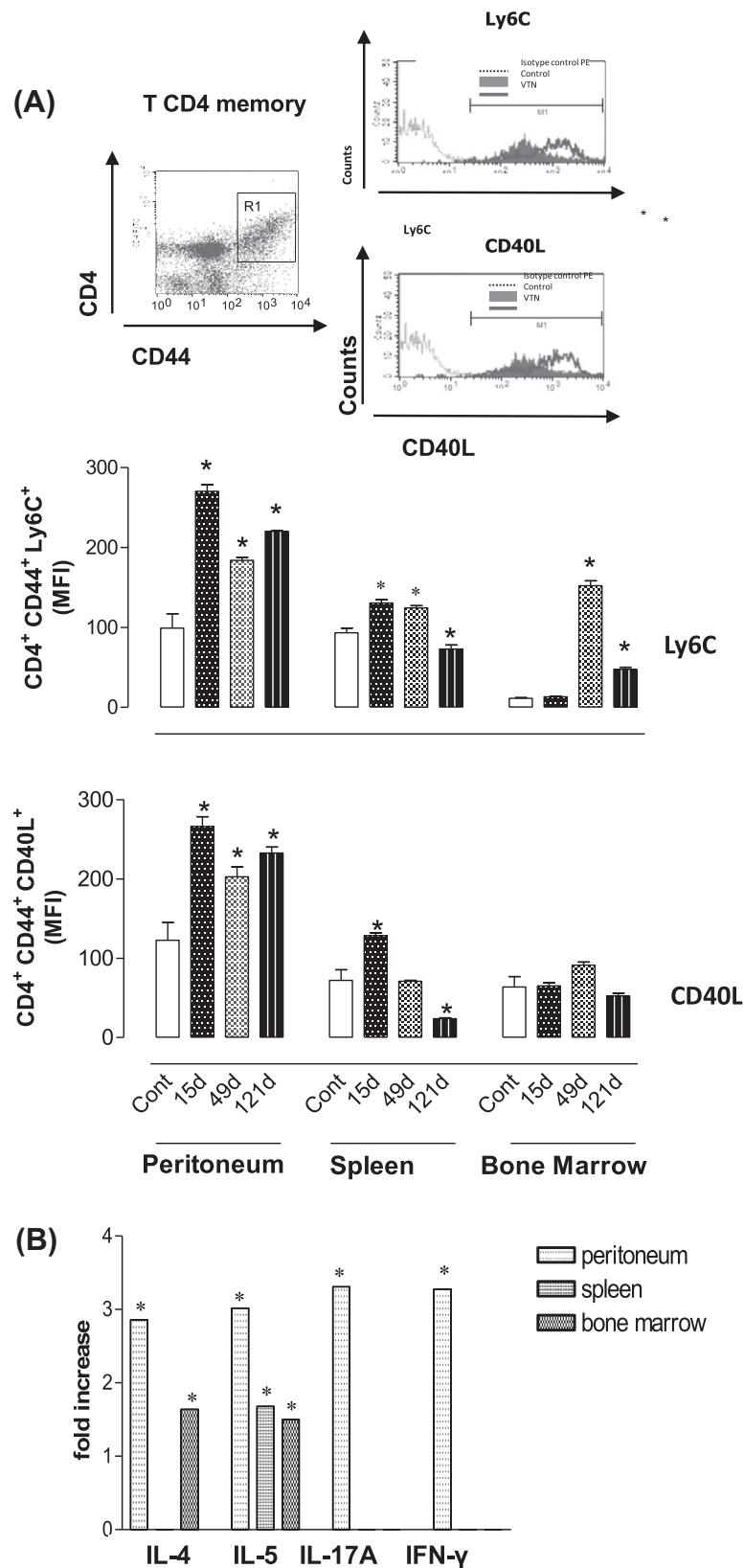


Fig. 8. Memory CD4 T cells were induced in response to *T. nattereri* venom. Representative dot plots showing 3-color staining for memory T cell identification: gated double positive CD4⁺CD44^{high} cells were identified by the co-expression of Ly6C (Fig. 8A, top) or CD40L (Fig. 8A, bottom) in total of peritoneal cavity, spleen and BM from immunized or control-mice at various time point after challenge. MFI were obtained via by flow cytometry and each bar represents the mean $\pm$ SEM value from determinations from pooled compartments each from five individual mice and three independent experiments. (B) In peritoneal cavity, spleen and BM of immunized or control-mice at 15 d, CD4⁺CD44^{high} T cells were analyzed for intracellular cytokines content using mAbs against IL-4, IL-5, IL-17A, and IFN- γ . The production of memory T cell-derived cytokines was calculated as fold increase induced by venom over the control group. * $p < 0.05$ compared to control mice.

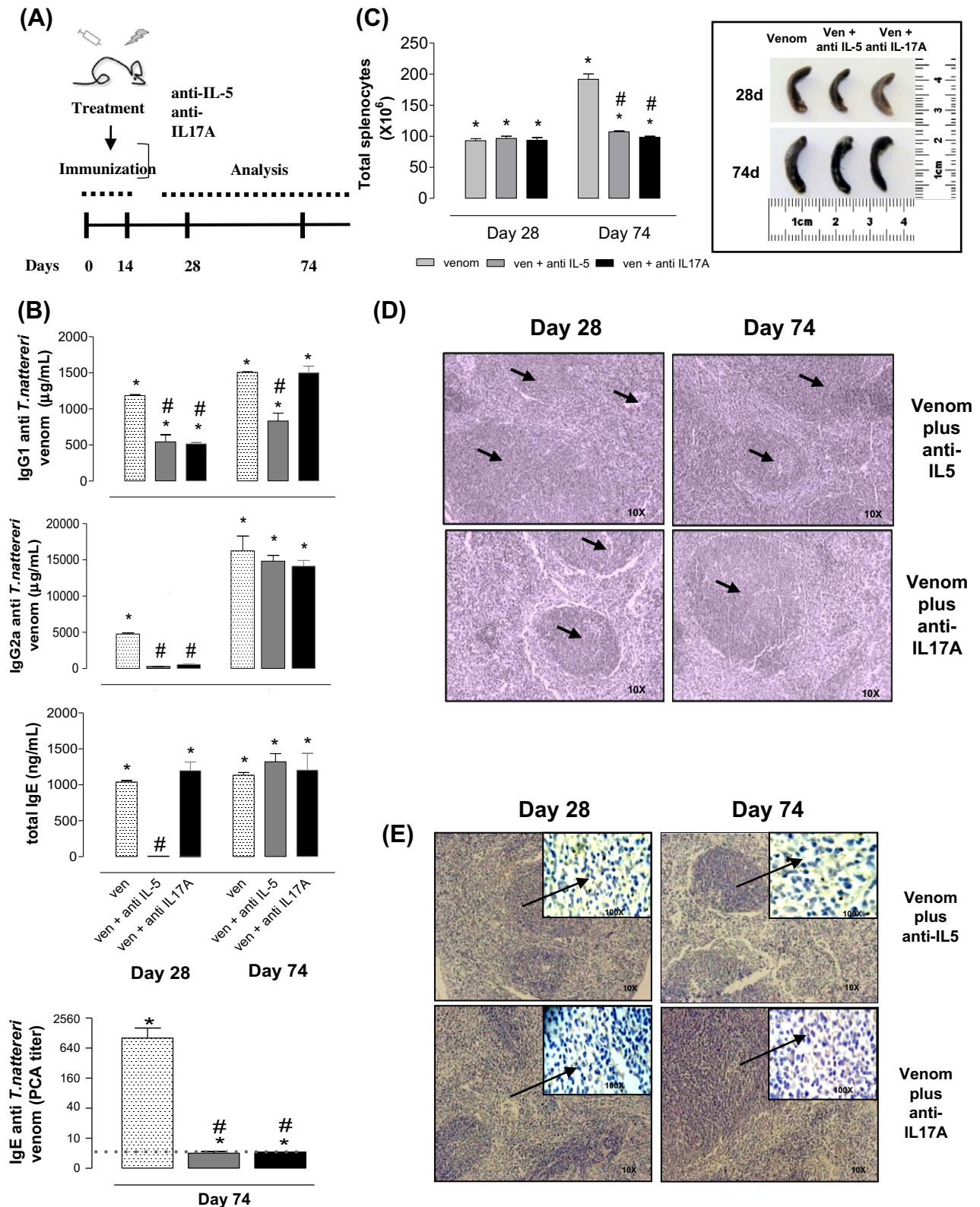


Fig. 9. IL-5 and IL-17A cytokines positively regulate the production of specific-IgE. BALB/c mice were injected with neutralizing anti-IL-5 or anti-IL-17A mAbs or matched isotype control 30 min before venom injection at days 0 and 14 (A). Plasma samples and spleen were isolated at 28 and 74 d after immunization. The isotypes of venom-specific IgG and total or specific IgE present in the plasma of treated animals were determined by ELISA or PCA, respectively (B). The treatment with neutralizing antibodies was not able to reverse the splenomegaly (C) or the germinal centers formation (D), but decreased the total number of splenocytes in 74 d compared with both animals injected only with venom or with venom plus control antibody GL113 (C). The blockade of IL-17A impairs partially the increase of Ig^{pos} B cells induced by the venom (bottom, E). **p* < 0.05 compared to control-mice, # *p* < 0.05 compared to untreated venom-mice.

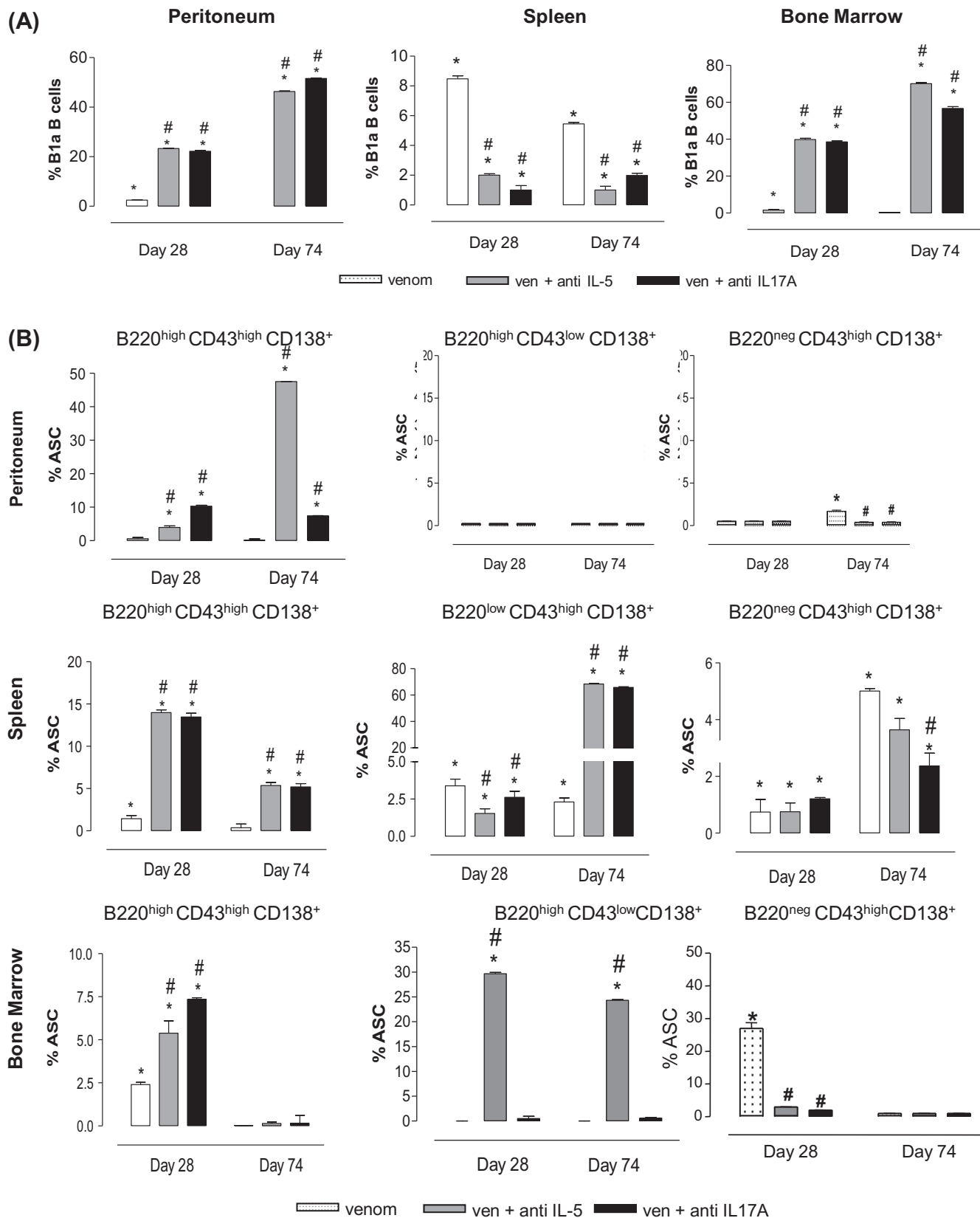


Fig. 10. IL-5 and IL-17A positively regulate the maintenance of terminally differentiated ASC B220^{neg} in inflamed tissue. Mice were injected with neutralizing anti-IL-5, anti-IL-17A or GL113 control mAbs 30 min before venom injection at days 0 and 14. Modulation of B1a cells by neutralizing antibodies treatment (A). Treatment with both blocking mAbs resulted in almost complete depletion of ASC with the phenotype B220^{neg} in peritoneal cavity and negatively regulated the differentiation of ASC with the phenotype B220^{pos} in all compartments (B). **p* < 0.05 compared to control-mice, # *p* < 0.05 compared to untreated venom-mice.

and BM); and extrinsic survival factors as cytokines produced by CD4 memory T cells and venom depot were also determined. Finally, venom-immunized mice were treated before the immunization processes with anti-IL-5- and anti-IL17A neutralizing antibodies and the Abs production and differentiation of the subtypes of ASC were investigated.

Two key transcriptional regulators, Blimp-1 and XBP-1, have been shown to be necessary for the terminal differentiation of B cells into long-lived antibody-secreting cells [6]. Little is known about signals that induce Blimp-1 and XBP-1 although it has been shown that the expression of Blimp-1 is induced by IL-5 [35]. IL-5 specifically binds to IL-5R α and induces the recruitment of β c to IL-5R, and at least three different signaling pathways including JAK2/STAT5 pathway, Btk pathway, and Ras/ERK pathway are activated. IL-5 critically regulates homeostatic proliferation and survival of B1 cells and the natural antibody production by these cells, and enhances the AID and Blimp-1 expression in activated B2 cells leading to induce μ to γ 1 class switch recombination and terminal differentiation to IgM- and IgG1-secreting plasma cells, respectively [36].

Since the demonstration the role of IL-23 in autoimmunity, it was discovered that Th17 subset has been linked to the pathogenesis of many inflammatory diseases, including arthritis, experimental autoimmune encephalitis, inflammatory bowel disease and airway inflammation [20,37,38]. The differentiation of this subtype of Th cell is dependent on IL-6–STAT3 activation of the transcriptional regulator retinoic acid receptor-related orphan receptor- γ t (ROR γ t); and these cells produce IL-17A, IL-17F, IL-21 and IL-22 and are highly responsive to IL-1 receptor 1 (IL-1R1) and IL-23R signaling [17,39]. IL-17 has been reported as the central cytokine involved in the spontaneous development of germinal center B cell-derived autoantibodies in autoimmune BXD2 mice; and promotes the prolonged retention of murine B cells in the GC [21].

Nevertheless such data established a link between IL-5 and IL-17 and B cells, the direct or indirect influence of IL-17 as well as IL-5 in the *in vivo* differentiation or maintenance of subtype of ASC is not known.

Several models have been proposed to account for the heterogeneity in memory B cell populations [3,10,27]. In contrast to models of memory induced by acute infection with live pathogens that have impaired long-term memory development, in this study, we have used venom proteins of *T. nattereri* Brazilian fish to establish a model in which germinal center derived B cells and high affinity specific antibodies were permanently generated. In particular, memory response to venom induced a chronic expansion of B1a cells in BM, while retains venom proteins by splenic cells and maintains in the peritoneal cavity a Th2-mediated inflammation with infiltration of eosinophils, mast cells, neutrophils and IL-17A-producing CD4⁺ CD44⁺ CD40L⁺ Ly6C⁺ effector memory T cells (TeM).

Furthermore, we have provided evidence that IL-17A as well as IL-5 produced in a context of chronic inflammatory response against venom proteins directly influence the production of specific IgE antibodies and the maintenance of B1a cells in the BM from the spleen. Both cytokines negatively regulate the maintenance of ASC B220^{pos} in different sites of response. A striking finding in the current study was that IL-5 and IL-17A are critical for the differentiation and maintenance of ASC with B220^{neg} phenotype in inflamed peritoneal cavity.

Some authors believe that the persistence of antigen, for months or even years, in the form of immune complexes on the surface fDC is an essential factor for the maintenance of memory B cells and production of specific antibodies [32,40,41]. However, even though antigen occupancy is apparently not required for the maintenance of memory B cells as reported by Maruyama

et al. [42], our data here do not exclude the possibility that a BCR signal is required for the maintenance of memory B cells and ASC induced by the venom. Presumably these signals are similar to that needed for the survival of naive B cells [43].

It is also conceivable that the B1a cells are required to initiate T-independent B cell differentiation and Ig production. These cells emigrate from the peritoneal cavity into spleen where they complete their differentiation program and switch from IgM to IgA/IgG-secreting B cells [44]. Furthermore, Zhong et al. [45] described that B1 cells induced T cells to express IL-17 and IFN- γ to a much greater extent than observed with either B2 cells. Our data showed here support the view that B1a cells emigrate from the peritoneal cavity into spleen, where they are regulated by IL-5 and IL-17A to become ASC.

Mitsdoerffer et al. [46] have shown that Th17 cells are able to promote class switching to IgG1, IgG2a, IgG2b, and IgG3. Finally B1a cells encounter a survival niche into BM, where they may contribute to activate IL-17A-producing CD4⁺ CD44⁺ Ly6C⁺ central memory T cells (TcM) present in this compartment.

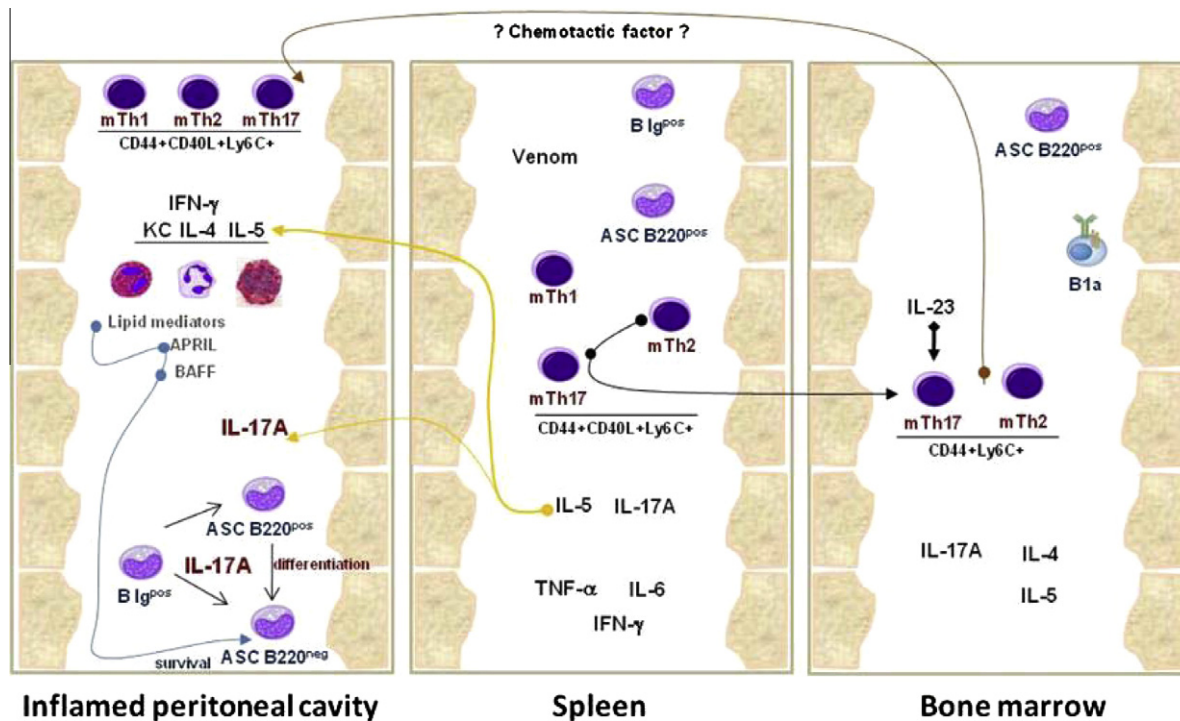
Class switched IgG1+ GC memory B cells were present in equivalent percentage during all time in anti-IL-5- and anti-IL-17A-treated venom-mice compared with non-treated venom-mice (data not shown) as well the treatments did not reverse the splenomegaly or the GC formation (Fig. 9), further highlighting that signals delivered via IL-5R and IL-17R are not required for GC maintenance. Consistent with this we observed an elevated relative number of ASC with both phenotypes (B220^{high/low} and in lesser extent B220^{neg}) in 74 d postimmunization in spleens obtained from anti-IL-5- and anti-IL-17A-treated mice, indicating that in the splenic niche, IL-5R and IL-17R signaling is not required for final differentiation into terminally-differentiated end stage cells with negative expression of B220.

In addition, our studies define that signals delivered via the IL-5R/IL-17R may directly support the differentiation of ASC B220^{neg} outside the GC, into a rich microenvironment necessary for long-lasting survival as chronic inflamed tissues. This is supported by data demonstrating that the treatment of venom-immunized mice with anti-IL-5 and anti-IL-17A mAbs promotes the complete depletion of ASC B220^{neg} in peritoneal cavity. Also in untreated venom-immunized mice we found that the presence of Th2 inflammation with neutrophil, eosinophil and mast cells migrated to KC, IL-4 and IL-5 chemokine gradient may be responsible for the attraction and maintenance of IL-17A-producing effector memory CD4⁺ Th17 cells, which control the direct differentiation of ASC B220^{neg}. Future studies will need to characterize the direct action of IL-17A and IL-5 in differentiation of ASC from memory B cells, and if the different subsets of ASC induced by *T. nattereri* venom possess differential functional subspecialties.

In addition, our results depicted here support a view [47] that the physical co-localization in the inflamed peritoneal cavity of leukocytes which induce the generation of BAFF/APRIL and possible COX₂-derived lipidic mediators regulate the activation and differentiation of ASC B220^{neg}. Corroborating this view, it is also demonstrated that non-haematopoietic cells as epithelial or endothelial cells in inflamed peritoneal cavity contribute to ASC survival by the production of BAFF and APRIL [15,48].

The population expansion and survival of Th17 cells depend on IL-23 [49]. Interestingly, our data show a positive correlation of the BM production of IL-23 and memory Th17 differentiation process. Presumably, IL-23 in BM participates in the activating of CD4⁺ CD44⁺ Ly6C⁺ central memory T cells into effector memory cells expressing CD40L (CD154) that may be mobilized from BM into the inflamed tissue, an ideal niche for interaction with B cells [50–53], amplifying the processes of differentiation and survival of ASC.

Antigen-specific IgE is the central event in the pathogenesis of chronic atopic disorders (in atopic dermatitis, followed by atopic



Scheme 1. Possible scenario for the effect of IL-17A on differentiation and survival of ASC in inflamed tissue. We postulate that during chronic response to *T. nattereri* venom a persistent peritoneal Th2-mediated inflammation consisting of the innate cells and inflammatory mediators as well T-derived cytokines produced by memory Th2 and Th17 cells promote a rich microenvironment that differentiates and maintains terminally differentiated cells (B220^{neg}CD43^{high}CD138^{pos}). The reservoir of splenic ASC was constituted by B220 positive cells; in the BM IL-23 support TcM cells; and ASC B220^{pos} were the major phenotype found in this compartment.

asthma, perennial allergic rhinitis, and seasonal allergic rhinitis) and is dependent on Th2 cytokines. It is also important to discuss that our study showed that specific IgE responses to venom were ablated in anti-IL-5- and anti-IL-17-treated-mice, suggesting that (i) IL-17A as well as IL-4 and IL-13 are key regulators of switching to IgE, and (ii) combined therapies with anti-IL-5- and anti-IL-17-monoclonal antibodies could be implemented. Moreover, our findings demonstrate that (iii) IL-17A is not involving in the IgG1 switch, in contrast to partial dependence of IgG1 on IL-5. Our study produced results which corroborate the provocative findings of Milovanovic et al. [54], showing that IL-17A has an important functional implication in IgE production. Furthermore, they demonstrated that blocking IL-17A affected only IgE and not IgG, IgM, or IgA production in peripheral blood mononuclear cells (PBMCs) from an atopic donor. Finally, our results enable us to propose a mechanism in which IL-5 and IL-17A produced by CD4⁺ CD44⁺ CD40L⁺ Ly6C⁺ effector memory T cells induce memory B-cell IgE class switching and differentiation into ASC.

Collectively, the observation described here that the production of survival factors for the innate cells as neutrophils, eosinophils and mast cells, and cytokines as IL-5 and IL-17A for activated effector memory T cells (CD40L^{pos}) guarantees the survival of the ASC B220^{neg} in the inflamed peritoneal cavity. IL-23 promotes in the BM an ideal niche for retention of TcM in rest state (CD40L^{neg}) that demonstrate fast capacity of mobilization for the inflamed tissue where they are activated and will help ASC in survival. Thus, we proposed that in our model, IL-5 and IL-17A is required for the differentiation and maintenance of ASC with B220^{neg} phenotype in inflamed tissue as peritoneal cavity, regulating the down-expression of B220 molecules in these cells (see Scheme 1).

5. Conclusion

In conclusion, by using venom proteins of *T. nattereri* Brazilian fish we have highlighted the importance of extrinsic mediators

as cytokines in Th2 chronic response development, demonstrating that IL-5 and IL-17A are crucial for the maintaining of specific-IgE secreting cell functions. We demonstrated that IL-5 and IL-17A exert positive regulation in B1a cells in a secondary lymphoid organ as spleen and are crucial for the maintenance of specific-IgE production. We found that IL-5 and IL-17A are required for the differentiation and maintenance of ASC with B220^{neg} phenotype in inflamed peritoneal cavity, whereas neutralizing anti-IL-5- and anti-IL-17A antibodies abolish this subpopulation. Thus, IL-5 and IL-17A are sufficient for the generation of ASC B220^{neg} in the peritoneal cavity and negatively regulate the number of ASC B220^{pos}, confirming that the hierarchical process of ASC differentiation triggered by venom needs the signal derived from IL-5/IL-5R and IL-17A/IL-17R.

Acknowledgments

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