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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 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; 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-1α, TNFα, 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-1α, 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 μg 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 μg 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 μg of venom adsorbed in alum and boosted on days 14, 20, 47 and 119 with 10 μg 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 CO_2 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 μg/ml streptomycin, 25 mM HEPES buffer, and 10% heat-inactivated FCS (all from Life Technologies, Paisley, UK). Cells were cultured with 10 μg/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 μM iomycin (Sigma) e Golgi-stop solution (BD Biosciences) for 6 h. Cells were washed, fixed and permeabilized with Cytofix/Cytoperm solution (BD Biociences) 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

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