

Kinetics of mobilization and differentiation of lymphohematopoietic cells during experimental murine schistosomiasis in galectin-3^{-/-} mice

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Abstract: Galectin-3 (gal-3), a β -galactoside-binding animal lectin, plays a role in cell-cell and cell-extracellular matrix interactions. Extracellular gal-3 modulates cell migration and adhesion in several physiological and pathological processes. Gal-3 is highly expressed in activated macrophages. *Schistosoma mansoni* eggs display a large amount of gal-3 ligands on their surface and elicit a well-characterized, macrophage-dependent, granulomatous, inflammatory reaction. Here, we have investigated the acute and chronic phases of *S. mansoni* infection in wild-type and gal-3^{-/-} mice. In the absence of gal-3, chronic-phase granulomas were smaller in diameter, displaying thinner collagen fibers with a loose orientation. Schistosoma-infected gal-3^{-/-} mice had remarkable changes in the monocyte/macrophage, eosinophil, and B lymphocyte subpopulations as compared with the infected wild-type mice. We observed a reduction of macrophage number, an increase in eosinophil absolute number, and a decrease in B lymphocyte subpopulation (B220^{+/high} cells) in the periphery during the evolution of the disease in gal-3^{-/-} mice. B lymphopenia was followed by an increase of plasma cell number in bone marrow, spleen, and mesenteric lymph nodes of the infected gal-3^{-/-} mice. The plasma IgG and IgE levels also increased in these mice. Gal-3 plays a role in the organization, collagen distribution, and mobilization of inflammatory cells to chronic-phase granulomas, niches for extramedullary myelopoiesis, besides interfering with monocyte-to-macrophage and B cell-to-plasma cell differentiation. *J. Leukoc. Biol.* 82: 000–000; 2007.

Key Words: *Schistosoma mansoni* · plasma cells · macrophages · granuloma

INTRODUCTION

Galectins are a family of conserved, β -galactoside-binding animal lectins. Galectins play crucial roles in cell-matrix in-

teractions, signaling, and inflammation [1, 2]. So far, 15 distinct members have been described in the galectin family. These molecules are classified according to their architecture in three distinct groups: proto, which has a single carbohydrate recognition domain (CRD); tandem, lectins with two CRD; and chimera. Galectin-3 (gal-3) is unique among galectins, as it is the only member of the chimeric group [1].

Gal-3 is found in the nucleus, cytoplasm, and on the cell surface of a variety of normal cells (e.g., inflammatory cells) and tumor cells [3, 4]. Gal-3 secretion follows a nonconventional pathway of exocytosis, and once secreted, extracellular gal-3 may function as a cytokine or as a modulator of cytokine function [5]. In the inflammatory process, such as in acute and allergic inflammation, extracellular gal-3 has been proposed to be a powerful, proinflammatory molecule, stimulating the superoxide production from neutrophils [6, 7], potentiating LPS-induced IL-1 production by monocytes, and promoting chemotaxis of this cell population [8–10]. In addition, in lymphoid differentiation, intracellular gal-3 was detected in activated T cells, and its nuclear accumulation was associated with cell proliferation and survival [11, 12]. In B cell development, down-regulation of gal-3 gene expression using antisense oligonucleotides led to an increase in the number of plasma cells in the inflammatory process caused by *Trypanosoma cruzi* [13].

Besides its potential role in leukocyte biology, extracellular gal-3 also binds to glycoconjugates synthesized by different parasites, such as *Schistosoma mansoni*, which is responsible for one of the most prevalent, infectious diseases in the world. The main structures produced by *S. mansoni*, which interact with gal-3 residues, are GalNAc β 1-4(Fuc α 1-3)GlcNAc (Lac-DiNAc) structures (*N*-acetylgalactosamine β 1-4 *N*-acetylglucosamine) [14], eliciting a well-characterized, humoral immune response toward the parasite [15]. In this regard, gal-3 has

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been implicated as a pathogen-associated molecular pattern receptor.

Murine schistosomiasis is an excellent model of a long-term, inflammatory process as a result of the following reasons: the permanent presence of worm antigens secreted in the mesenteric blood flow, continuously drained into the mesenteric lymph nodes (MLN) from the intestines; the presence of the egg antigens, which diffuse into liver tissue inducing the formation of hepatic granulomas; and the involvement of the spleen by the permanent stimulation of the immune system at the systemic level [16, 17].

Adult worms live in the mesenteric venous system, depositing their eggs in submucosal venules of the intestine. Part of the eggs elicits abscesses in the intestinal wall, part is expelled into the intestinal lumen, and a part is trapped in the mesenteric vessels or washed through the portal blood flow into the liver, where they elicit a well-characterized, macrophage-dependent, granulomatous, inflammatory reaction [18, 19]. The acute phase of the disease (from 45 to 50 days postinfection) is characterized by a typical Th2 response with high levels of IL-4 and IL-5. The evolution toward the chronic phase (from 85 to 95 days postinfection) is associated with a modified pattern of cytokine secretion, including down-regulation of IL-4 and IL-5 [17, 20] and remodeling of the granulomatous reaction, which shifts from an exudative to a fibrotic pattern, leading to a progressive decrease in the granuloma diameter [19]. Splenomegaly is observed frequently in all stages of the disease, and it is characterized by the activation of germinative centers and enhancement of antibody production with polyclonal B cell activation. A robust, humoral response is initiated after 40 days of infection, reaching the highest levels of Ig production in the chronic phase of the disease [21, 22]. The involvement of coelome-associated lymphoid tissues is also observed, and it is characterized by exacerbated macrophage reactivity and by extensive hyperplasia of myeloid and lymphoid lineages [23, 24].

Considering that granulomas represent a tissue reaction, which is orchestrated essentially by monocyte-macrophage activation; *S. mansoni* antigens bear glycosylation patterns, which are recognized by gal-3; studies of gal-3^{-/-} mice have provided a significant support for the proinflammatory role of this lectin [9, 25, 26]; and evidence has been provided for a role of gal-3 in the interface of innate and acquired immunity [27], a role for gal-3 in the course of *S. mansoni* infection is anticipated. As an initial characterization of this problem, here, we described the dynamics of inflammatory cell mobilization in the course of a *S. mansoni* infection, comparing wild-type and gal-3^{-/-} mice. Although no clinical-pathological, striking differences were observed in the course of infection between wild-type and the gal-3^{-/-} mice, a remarkable plasmacytogenesis took place in extramedullary compartments, including the MLN, intrahepatic granuloma, and the hepatic extramedullary haematopoiesis foci. Histological analysis of the hepatic granuloma in gal-3^{-/-} mice showed a decrease in periovular granuloma diameter and fibrosis deposition and depicted further an enhanced eosinophil recruitment and/or in situ proliferation.

MATERIALS AND METHODS

Schistosomal infection

Inbred C57/bl6 and gal-3^{-/-} mice [26] (backcrossed to C57BL/6 for 10 generations) of both sexes were obtained from the colony bred at the Federal University of Rio de Janeiro (Brazil) and infected by transcutaneous penetration of ca., 40 *S. mansoni* cercariae (BH strain, Oswaldo Cruz Institute, Rio de Janeiro, Brazil). Mice were killed after 40–50 and 90–95 days after infection, corresponding to the acute and the beginning of the chronic phase of the disease, respectively. Uninfected age- and sex-matched mice were used as controls.

Preparation of cell suspensions of spleen, MLN, and bone marrow

Cell suspensions from uninfected and infected mice were obtained *ex vivo* by standard mechanical dissociation from bone marrow, spleen, and MLN. Blood was obtained from cardiac puncture and stored in contact with EDTA solution. RBC and red splenic cells were lysed using Gey's solution. After lysis, the cell suspensions were washed twice with PBS, pH 7.2, containing 3% FBS and quantified, and their concentration was adjusted to 1×10^6 cells/mL.

Flow cytometry

To flow cytometry, these cells were incubated with Fc blocker (Clone 2.4G2) for 10 min before adding mAb. The cells were labeled with fluorescent mAb, anti-B220 FITC, anti-membrane-activated complex 1, anti-B220 PE, anti-CD5 Cy-chrome, and unlabeled anti-gal-3, obtained from the M3/38 hybridoma (American Type Culture Collection, Manassas, VA, USA). The secondary antibody was anti-rat IgG FITC. The sample was acquired in a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA) and analyzed using two specific softwares, CellQuest and WinMDI 2.8. Unless otherwise specified, all the antibodies were purchased from PharMingen (San Diego, CA, USA).

Cell suspension

Hepatic granulomas were obtained by homogenization of liver tissue, followed by repeated sedimentation. Granulomas were submitted to digestion with collagenase 1A (1 mg/mL in DMEM, 10% FBS) [28]. Cells were harvested after each digestion period, layered over a discontinuous Percoll gradient (25% and 70%), and centrifuged. The cells were harvested from the layer formed on the top of 70% Percoll.

Cytosmears and optical microscopy

The cell suspensions were adjusted to 2.0×10^4 cells/mL. The samples were centrifuged at 36 *g* during 3 min. The material was fixed in absolute methanol by 24 h. The samples were stained using the May-Grünwald Giemsa method, as described elsewhere [16]. The differential count was done in the high-power field by optical microscopy. For each experiment, samples of five animals/group were analyzed in triplicates.

Histological preparations

For histological analyses, animals were killed after 60 days ($n=10$) and 90 days of infection ($n=10$) concomitantly with sex- and age-matched, uninfected, wild-type and knockout animals (10 animals per group). Uninfected age- and sex-matched mice were killed at the same time and used as controls. Sacrifice was performed under anesthesia given *i.p.* (ketamine, 35 mg/kg; xylazine, 9 mg/kg, Sigma Chemical Co., St. Louis, MO, USA). Liver was removed, cut into 0.5 mm-thick slices, washed in cold saline, and fixed in Bouin's fixative. After 6 h of fixation, specimens were dehydrated in alcohol and embedded in paraffin. Sections of 5 μ m were obtained and stained with H&E, Masson's trichrome staining, Sirius red technique for eosinophils, and a modified Sirius red technique for collagen [29].

Immunohistochemistry for gal-3

The anti-gal-3 mAb produced by hybridoma M3/38 was used to detect gal-3-expressing cells in wild-type animals. Paraffin-embedded sections were de-

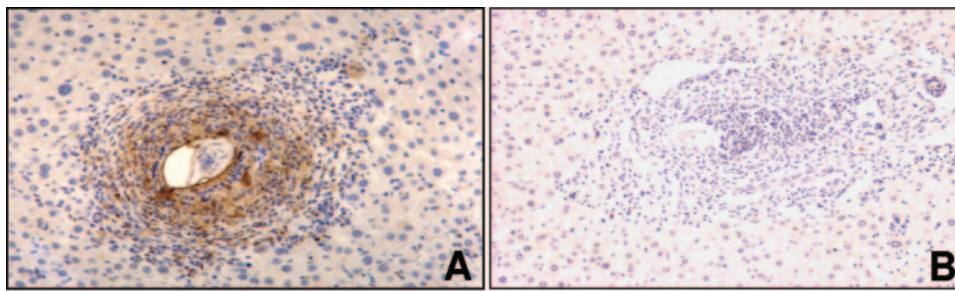


Fig. 1. Detection of gal-3 by immunoenzymatic reaction in *S. mansoni*-infected, wild-type and gal-3^{-/-} mice. (A) In wild-type mice, immunoreactivity for gal-3 was observed in macrophages of granulomas around schistosomal eggs. (B) Complete absence of gal-3 reactivity in infected gal-3^{-/-} mice. (A and B, Original magnification, ×100.)

waxed and hydrated. After inhibition of endogenous peroxidase, sections were incubated for 1 h with 0.01 M PBS containing 5% BSA, 4% skim milk, 0.1% Triton X-100 (Sigma Chemical Co.), 0.05% Tween-20, and 10% normal goat serum, followed by incubation of M3/38 (1:10 in PBS, -3% BSA, 1% normal goat serum) overnight at 4°C in a humid chamber. M3/38 was detected with a biotinylated anti-rat IgG (BA-4001, Vector Laboratories, Burlingame, CA, USA) and developed with avidin-peroxidase (1:50 in PBS, Sigma Chemical Co.; E-8386), using diaminobenzidine as chromogen. Sections were counterstained with Harris' hematoxylin. As negative controls, sections of wild-type and knockout mice tissues were incubated with nonimmune rat serum instead of anti-gal-3 antibody.

Histological analysis of liver granuloma

Periovarian granulomas are cell-mediated granulomas having epithelioid and giant multinucleated cells. Depending on the degree of maturation/death of *S. mansoni* eggs, periovarian granulomas were classified as necrotic-exsudative, exsudative-productive, and involutinal. Necrotic-exsudative granulomas were those with a central zone of necrosis associated with neutrophils, and eosinophils, although exsudative-productive ones, were characterized by the presence of some fibroblastoid cells concentrically arranged and with a reticular fibers network circumscribing granulomas. Involutinal granulomas were the fibrotic ones, with concentric deposition of collagen fibers. The collagen network of periovarian granulomas was observed in a laser confocal microscope (LSM 410, Zeiss) [30, 31]. Granuloma measurements and characterization of the collagen network were done with three animals per group at least three times.

Serum Ig dose

Approximately 1 ml blood was obtained by cardiac puncture from each animal. Sera Igs (IgM, IgG, and IgE) were quantified by ELISA "sandwich" assay [32]. ELISA plates (Corning, Corning, NY, USA) were coated with mAb anti-mouse-Ig (R35-72, PharMingen) diluted in PBS. The presence of Igs was measured by addition of biotin-labeled anti-Ig (R35-92, PharMingen), followed by addition of streptavidin-conjugated alkaline phosphatase (PharMingen). The antibody interactions were determined by addition of *p*-nitrophenylphosphate (Sigma Chemical Co.). The samples were acquired in an ELISA reader (BioRad, Boston, MA, USA) using a 405-nm filter.

Statistical analysis

The statistical tests were accomplished using the Tukey's multiple comparison test (*t*-test); significance threshold was fixed for $\alpha = 0.05$.

RESULTS

Gal-3 is involved in the recruitment of inflammatory cells induced by acute stimuli [33], allowing the fine-tuning of innate immunity. Here, we exploited a long-lasting infection model to characterize the role of gal-3 in the chronic inflammatory response induced by *S. mansoni*. Gal-3 was detected in

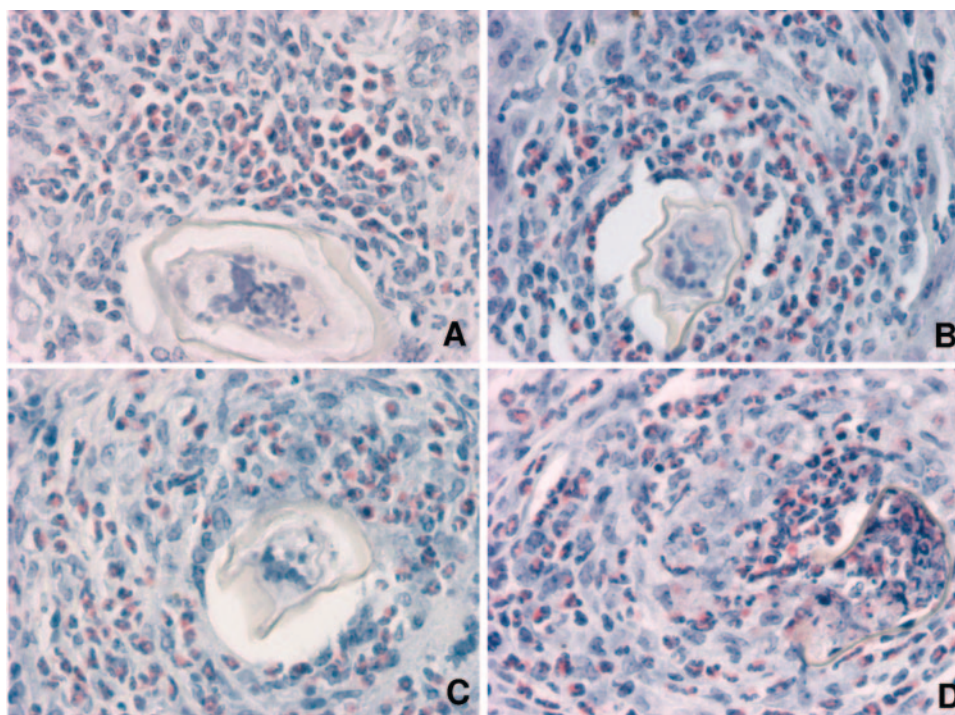


Fig. 2. Representative photomicrographs of eosinophils stained with Sirius red in *S. mansoni*-infected, wild-type and gal-3^{-/-} mice. (A) Exsudative granuloma showing eosinophils around the schistosomal egg in infected wild-type mice (acute phase). (B) Granuloma from an infected gal-3^{-/-} mice (acute phase) exhibiting a great amount of eosinophils circumscribing the schistosomal egg. (C) Eosinophils present in a schistosomal granuloma of chronic phase of infection in wild-type mice. (D) Abundant eosinophils are present around and inside the schistosomal egg; chronic phase of infection of gal-3^{-/-} mice. (A–D, Original magnification, ×200.)

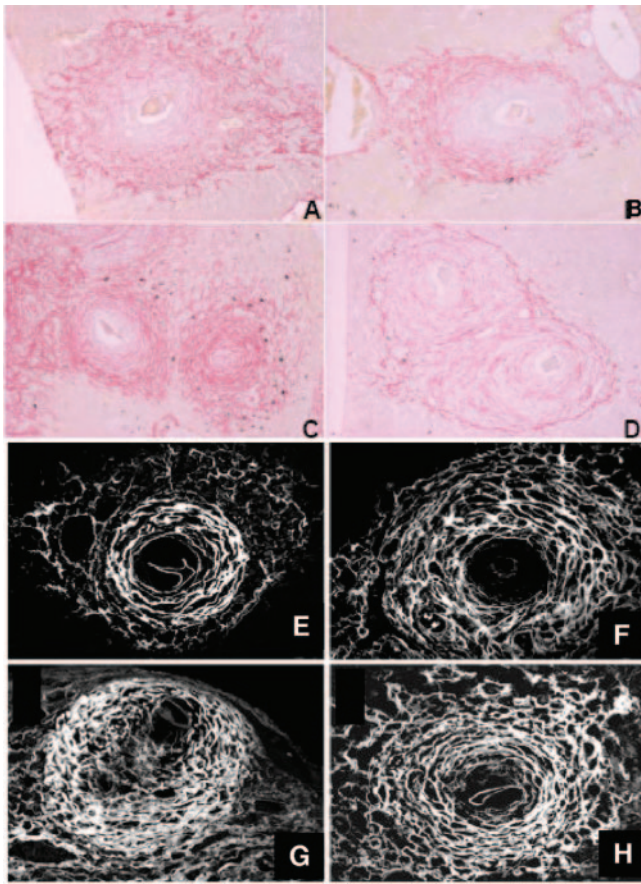


Fig. 3. Collagen network detection in infected wild-type and gal-3^{-/-} mice using Sirius red staining and observed in light microscopy and confocal laser microscopy. (A) Sirius red staining depicting rare fibers at the periphery of an exsudative granuloma of acute phase (wild-type animal). Collagen fibers of the portal space are present around granuloma. (B) Relative absence of collagen fibers in an exsudative granuloma of infected gal-3^{-/-} mice (acute phase). (C) Exsudative-productive and a productive-involutive granuloma exhibiting collagen fibers inside granulomas in an infected, wild-type animal (chronic phase). (D) Two exsudative-productive granulomas of infected gal-3^{-/-} mice (chronic phase) showing rare collagen fibers inside granulomas. (E) Confocal image of Sirius red-stained sections of an involutive granuloma of infected, wild-type mice (acute phase). Collagen fibers were disposed as a parallel network circumscribing the egg (→). (F) Confocal image of an exsudative-productive granuloma of an infected gal-3^{-/-} animal (acute phase) showing collagen fibers disposed sparsely inside the granuloma. (G) Confocal image of a productive-involutive schistosomal granuloma of chronic phase (wild-type mice) exhibiting a densely packed collagen network. (H) Confocal image of an involutive granuloma of an infected gal-3^{-/-} animal (chronic phase) showing a network of collagen fibers circumscribing the egg. In the periphery, collagen fibers were disposed loosely and oriented in several directions. (A–D, Original magnification, ×100; E–H, original magnification, ×400.)

some cells of granulomas, including giant and epithelioid cells, as well as in extramedullary myelopoietic foci and Kupffer cells of wild-type-infected animals. Gal-3 immunoreactivity was absent in gal-3^{-/-} mice, as expected (**Fig. 1, A and B**).

Livers of wild-type and gal-3^{-/-} animals with acute infection showed periovular granulomas, which contained cells of the monomacrophagic lineage in heterogeneous patterns. Granulomas in wild-type and gal-3^{-/-}-infected animals showed the presence of macrophages, giant multinucleated and epithelioid cells. Although in acutely infected, wild-type mice, granulomas were predominantly of the necrotic-exsudative pattern with some exsudative-productive ones, gal-3^{-/-} animals showed a majority of the productive type. In the chronic stage of infection, granulomas were smaller in wild-type and gal-3^{-/-} (data not shown). In the former group, they were predominantly of the productive and productive-exsudative type and in the latter one, of the involutive type. In addition, the granulomas in gal-3^{-/-} mice were less cellular and smaller than those in wild-type, infected animals (**Fig. 2, A–D**). Chronically infected gal-3^{-/-} animals had granulomas, which apparently were structured loosely, and inflammatory cells were interspersed among fibroblastoid cells in the exsudative-productive ones. However, eosinophils were more abundant than in wild-type animals in exsudative granulomas around schistosomal eggs (**Fig. 2, A–D**).

The fibrotic evolution was impaired in gal-3^{-/-} animals. This aspect was observed predominantly in chronically infected animals. Granulomas from gal-3^{-/-}-infected mice had less collagen depicted by Sirius red staining (**Fig. 3, A–D**) arranged in a loose pattern (**Fig. 3, E–H**), as could be seen clearly in confocal images.

During the chronic phase of infection, medullary mobilization is replaced gradually by in situ production of myeloid cells [34]. Therefore, granulomas elicited by *S. mansoni* are specialized microenvironments for sustained extramedullary myelopoiesis [24, 35, 36]. Here, we have followed the dynamics of production and mobilization of inflammatory cells in medullary and extramedullary compartments (spleen, MLN, and hepatic granulomas) in the course of *S. mansoni* infection in wild-type and gal-3^{-/-} mice.

We did not observe any significant differences in the total leukocyte number from uninfected, wild-type and gal-3^{-/-} mice in all of the analyzed compartments. However, comparing the wild-type and gal-3^{-/-}-infected mice, we observed a sig-

TABLE 1. Cellularity of Spleen and MLN from Wild-Type and Gal-3^{-/-} Mice

Spleen	Wild-type mice	Gal-3 null mice
Noninfected	7.2×10^7 cells/ml $\pm$ 1.9	8.6×10^7 cells/ml $\pm$ 2.5
Acute phase	38.2×10^7 cells/ml $\pm$ 8.8	25.5×10^7 cells/ml $\pm$ 3.8
Chronic phase	51.5×10^7 cells/ml $\pm$ 10.8	31.2×10^7 cells/ml $\pm$ 1.7
MLN	Wild-type mice	Gal-3 null mice
Noninfected	31.1×10^6 cells/ml $\pm$ 6.05	45.7×10^7 cells/ml $\pm$ 6.45
Acute phase	94.2×10^6 cells/ml $\pm$ 9.25	83.6×10^6 cells/ml $\pm$ 8.28
Chronic phase	76.6×10^6 cells/ml $\pm$ 9.42	57.2×10^6 cells/ml $\pm$ 6.35

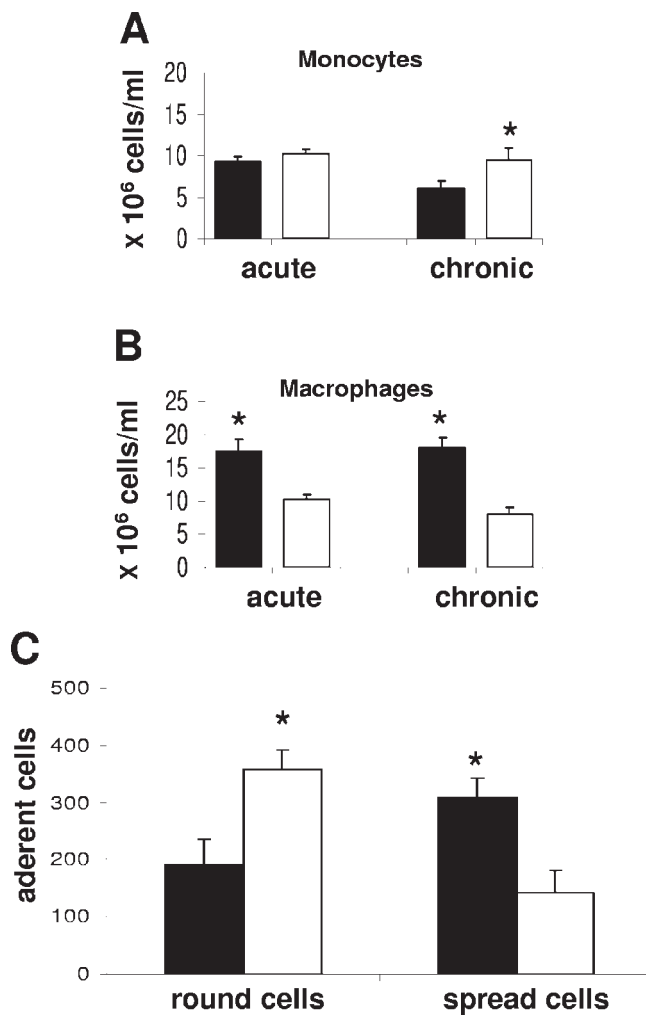


Fig. 4. Differential account of monocyte/macrophages obtained after enzymatic dissociation of hepatic granulomas. (A and B) We compared cells recovered from granulomas obtained from acute and chronic phases of infection by cytosmeares (May-Grunwald Giemsa staining). The solid bars indicate the wild-type mice, whereas the open bars represent the gal-3^{-/-} mice. (C) The mononuclear cells from chronic-phase granulomas from wild-type (solid bars) and gal-3^{-/-} mice (open bars) were then plated and evaluated using an adherence assay. Round cells represent monocytes, whereas spread cells represent macrophages. Data are reported as means $\pm$ SEM and are representative of five independent experiments, each carried out in three normal mice, five mice with acute infection, and five with chronic infection. Statistical analysis: Tukey's multiple comparison test (*, $P < 0.05$).

nificant decrease in the total leukocyte number in the spleen and MLN of gal-3^{-/-} mice during the acute and chronic phases of the disease (Table 1).

We quantified the cells obtained from the granulomas from acute and chronic phases of the disease and observed an increase of monocytes (Fig. 4A) and eosinophils (Fig. 5) in the infected gal-3^{-/-} mice, as compared with the wild-type, infected mice. The increase of monocyte numbers was concomitant with a drastic reduction of macrophages in granulomas (Fig. 4, A and B).

The fact that an increased number of monocytes observed in the circulation and within the granulomas of gal-3^{-/-} was not followed by a proportional increase in the population of mac-

rophages suggested a delay in the monocyte-macrophage differentiation in the absence of gal-3. Indeed, we observed that the mononuclear fraction from the granulomas of infected gal-3^{-/-} had little capacity to spread on the plastic flasks, and the majority stayed as rounded, attached cells in contrast to the wild-type cells, which spread efficiently on the plastic substrate (Fig. 4C). These data suggest that gal-3 plays a role in monocyte-macrophage differentiation and activation.

We observed an increase in the number of mature eosinophils in the bone marrow of the noninfected and infected gal-3^{-/-} mice (Fig. 5A). It is interesting to note that gal-3^{-/-}

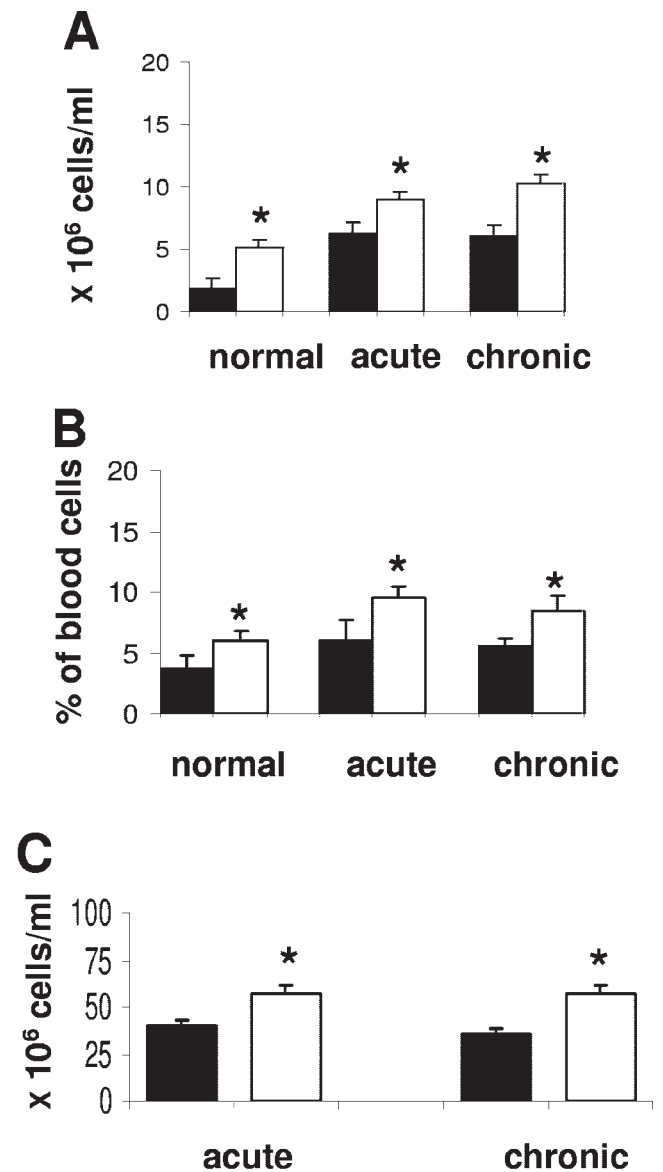


Fig. 5. Gal-3^{-/-} mice are eosinophilic. Eosinophil counts in bone marrow (A), peripheral blood (B), and *S. mansoni*-induced granuloma (C) from wild-type (solid bars) and gal-3^{-/-} mice (open bars). We compared the noninfected group (normal) with both infected groups of mice (acute and chronic phases). Data are reported as means $\pm$ SEM and are representative of five independent experiments, each carried out in three normal mice, five mice with acute infection, and five with chronic infection. Statistical analysis: Tukey's multiple comparison test (*, $P < 0.05$).

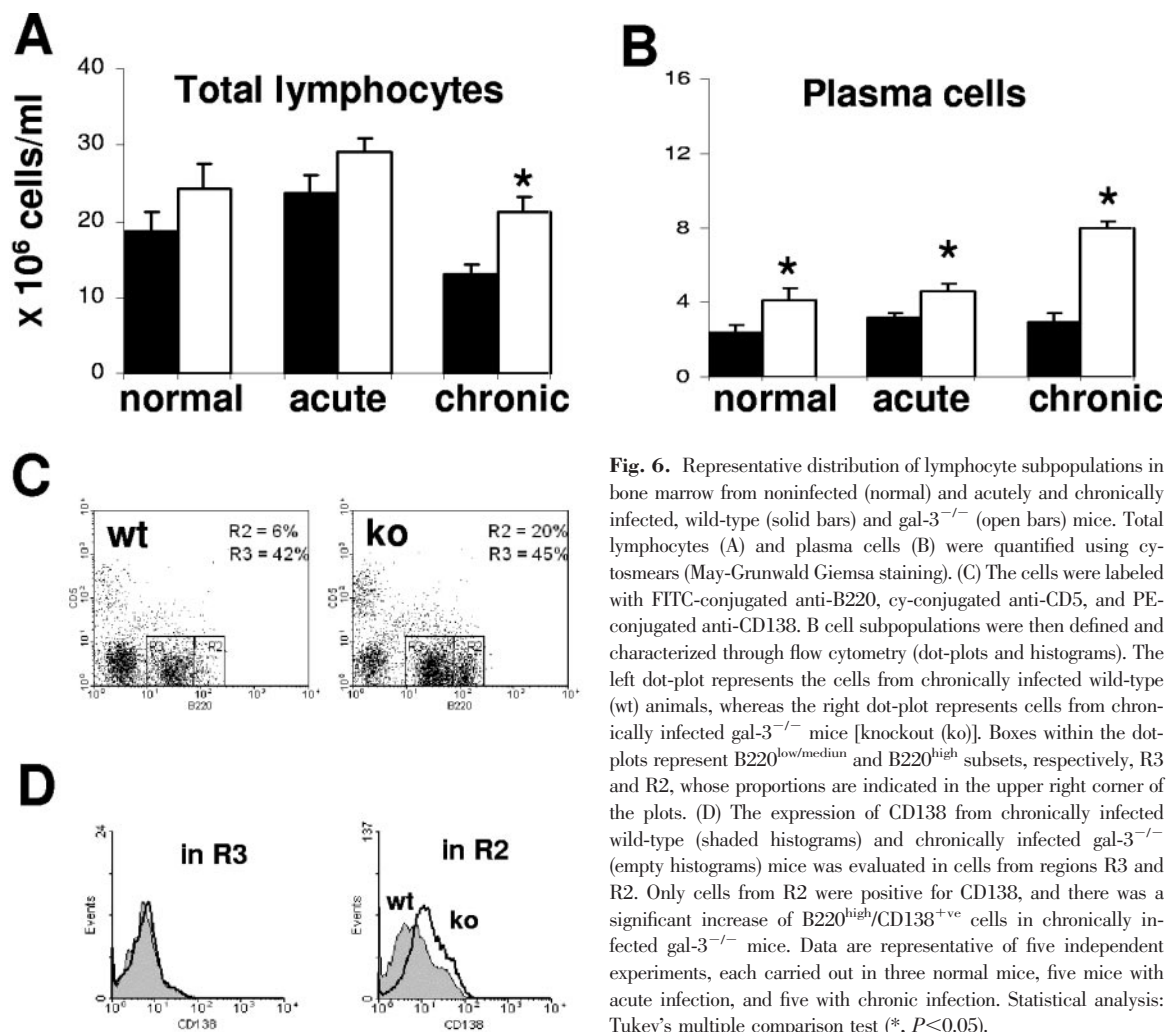


Fig. 6. Representative distribution of lymphocyte subpopulations in bone marrow from noninfected (normal) and acutely and chronically infected, wild-type (solid bars) and gal-3^{-/-} (open bars) mice. Total lymphocytes (A) and plasma cells (B) were quantified using cytospins (May-Grunwald Giemsa staining). (C) The cells were labeled with FITC-conjugated anti-B220, cy-conjugated anti-CD5, and PE-conjugated anti-CD138. B cell subpopulations were then defined and characterized through flow cytometry (dot-plots and histograms). The left dot-plot represents the cells from chronically infected wild-type (wt) animals, whereas the right dot-plot represents cells from chronically infected gal-3^{-/-} mice [knockout (ko)]. Boxes within the dot-plots represent B220^{low/medium} and B220^{high} subsets, respectively, R3 and R2, whose proportions are indicated in the upper right corner of the plots. (D) The expression of CD138 from chronically infected wild-type (shaded histograms) and chronically infected gal-3^{-/-} (empty histograms) mice was evaluated in cells from regions R3 and R2. Only cells from R2 were positive for CD138, and there was a significant increase of B220^{high}/CD138⁺ cells in chronically infected gal-3^{-/-} mice. Data are representative of five independent experiments, each carried out in three normal mice, five mice with acute infection, and five with chronic infection. Statistical analysis: Tukey's multiple comparison test (*, $P < 0.05$).

animals already presented an increased production of eosinophils and that upon infection, there was further amplification of this phenomenon. The same pattern was found in peripheral blood (Fig. 5B). Eosinophils were also increased in the infected gal-3^{-/-} mice as compared with the wild-type-infected mice (Fig. 5C).

Lymphocytes are largely involved in the chronic inflammatory reactions at the systemic and local levels. In this study, we observed an increase in the total number of lymphocytes in the bone marrow harvested from gal-3^{-/-} mice in the chronic phase of the disease (Fig. 6A) as well as in the blood of the same animals (data not shown). Phenotypic analysis of lymphocytes from chronically infected, wild-type (on the left) and gal-3^{-/-} (on the right) mice is shown in Figure 6C. Region R3 depicts the immature B cells, and Region R2 depicts the mature B cells. The increase in bone marrow-derived lymphocytes was specifically a result of the B lymphocyte subsets characterized as B220^{high} and therefore, mature B cells (Fig. 6C in R2, region at the right in both dot-plots). In parallel to the increase of B220^{high} lymphocytes, there was also a remarkable increase of plasma cells in uninfected and infected gal-3^{-/-} mice (Fig. 6B). The morphological analysis was confirmed by flow cytometry after labeling lymphocytes from chronically

infected gal-3^{-/-} mice with anti-CD138 and -B220 antibodies (Fig. 6D). The B220^{high} (cells from Region R2), but not the B220^{low/medium} (cells from Region R3) subset, expressed measurable amounts of CD138. Taken together, these results suggest the differentiation of B cells to plasma cells in the gal-3^{-/-} mice in the bone marrow microenvironment. The same pattern was observed in the noninfected gal-3^{-/-} mice (data not shown), although it was exacerbated upon infection. These results suggest that in the absence of gal-3, the bone marrow compartment harbored an increased number of B lymphocytes and plasma cells, which in turn, were associated with an increase in these cells in the periphery (see Figs. 7B and 8B).

Considering that the spleen is the most important site of activation and differentiation of B lymphocytes into plasma cells and that it is continuously stimulated by *S. mansoni* antigens, we analyzed the distribution of the lymphocyte subpopulations in this organ. In contrast to the bone marrow, we detected a reduction in total lymphocyte counts in the gal-3^{-/-} mice in the acute and chronic phases of the disease (Fig. 7A). By flow cytometry, we have demonstrated that the T lymphocyte pool (CD5⁺ cells) was not modified upon infection, whereas the B lymphocyte pool, defined as B220⁺ cells, was largely altered. In gal-3^{-/-}-infected mice in acute and chronic

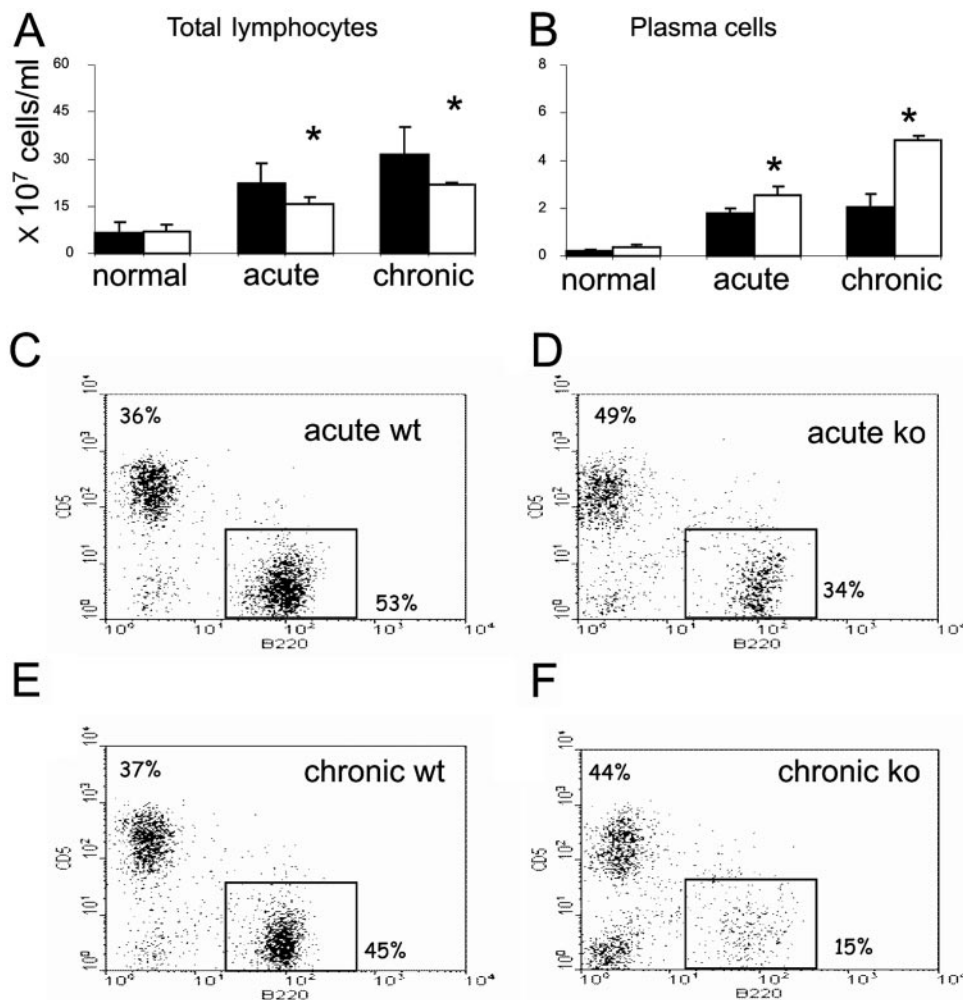


Fig. 7. Representative distribution of lymphocyte subpopulations from spleen from noninfected (normal) and acutely and chronically infected, wild-type (solid bars) and gal-3^{-/-} (open bars) mice. Total lymphocytes (A) and plasma cells (B) were quantified using cytomears. (C–F) Spleen cells from infected animals were labeled with FITC-conjugated anti-B220 and cy-conjugated anti-CD5. The splenic B cell population was defined and characterized through flow cytometry. Boxes in each dot plot circumscribe the region of B220^{high}CD5^{low} cells in all experimental groups indicated. Numbers indicate the relative proportion of the lymphocytes. Data are reported as means $\pm$ SEM and are representative of five independent experiments, each carried out in three normal mice, five mice with acute infection, and five with chronic infection. Statistical analysis: Tukey's multiple comparison test (*, $P < 0.05$).

phases, we have observed a more than twofold decrease in the B220⁺ lymphocyte population as compared with the wild-type, infected mice (Fig. 7, C–F). This decrease was concomitant with an increase of plasma cells within the spleen in both phases of the disease (Fig. 7B).

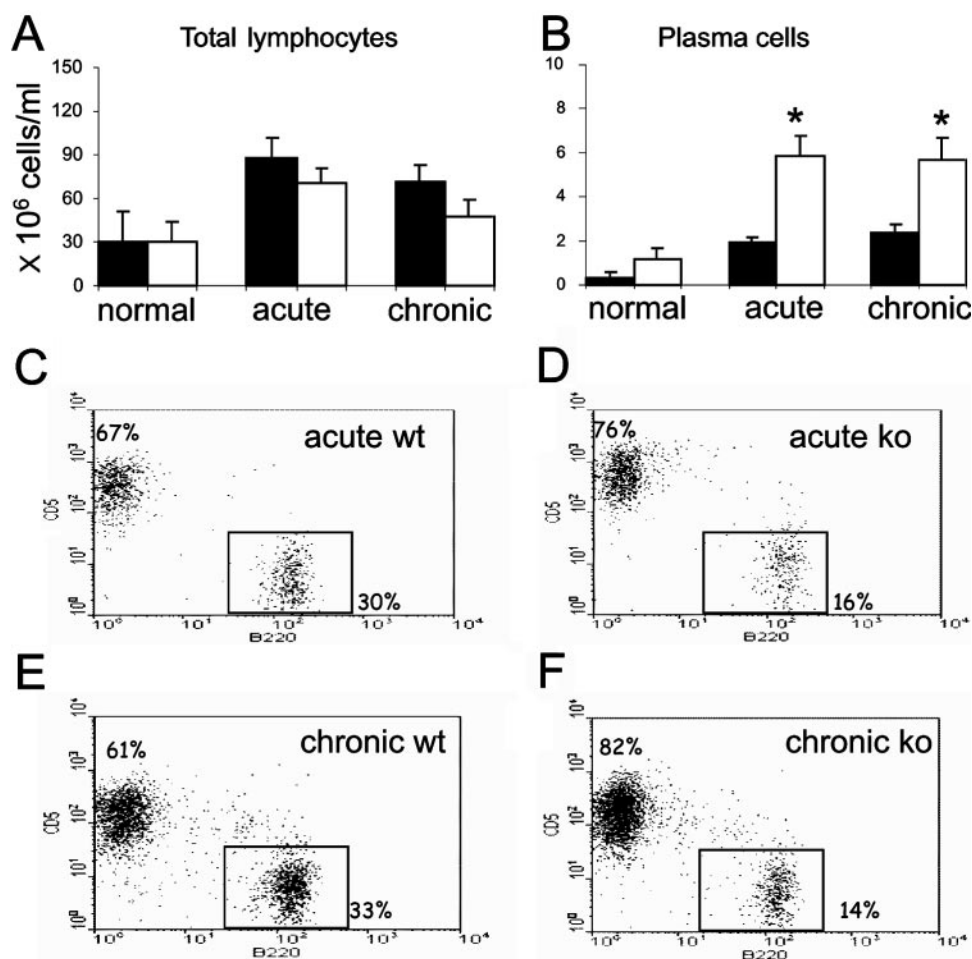
The same pattern of lymphocytes distribution was observed in MLN (Fig. 8A). There was an approximate twofold decrease in the number of B cells (Fig. 8, C–F), followed by an increase in the number of plasma cells harvested from gal-3^{-/-}-infected mice (Fig. 8B). No changes were observed in the T cell compartment, similar to the splenic compartment.

Our results demonstrated a possible acceleration in B cell differentiation to plasma cells in bone marrow, spleen, and MLN of *S. mansoni*-infected gal-3^{-/-} mice. It is interesting to point out that this increase in plasma cells in the periphery was accompanied by higher levels of seric IgG and IgE, but not IgM, in the course of infection (Fig. 9). Gal-3 was found on the surface of the B220^{high} subset but not on the B220^{low} cells harvested from bone marrow (Fig. 10A), spleen (Fig. 10B), and MLN (Fig. 10C) from wild-type mice. Negative controls included the staining of cells from gal-3^{-/-} mice (Fig. 10, D–F).

DISCUSSION

Gal-3^{-/-} mice are viable, they have no overt abnormalities, and they can survive under conventional conditions [25, 26]. The availability of the gal-3^{-/-} mice permits us to study the role of this lectin using different stimuli. In the acute inflammatory process, mutant mice had a greatly reduced number of granulocytes on Day 4 after thioglycolate injection, but no differences were observed at earlier time-points, and the mutant granulocytes did not exhibit an accelerated rate of apoptosis nor an increased clearance by inflammatory macrophages. These data indicate that gal-3 can play a role in the maintenance of the granulocyte number in the inflamed peritoneal cavity or in the extravasation of granulocytes in inflamed tissues [25]. We have taken advantage of the availability of gal-3^{-/-} mice to investigate the role of this molecule in a model of a long-lasting, inflammatory reaction [16, 35, 37]. In this work, we have challenged the animals with *S. mansoni*, as the host response to the eggs of this worm involves monocyte/macrophage-centered reactions, leading to the formation of granulomas [19, 38]. Furthermore, *S. mansoni* produces high amounts of LacDiNAc-containing antigens, which are ligands for gal-3 [14]. It is interesting that beads coated only with

Fig. 8. Representative distribution of lymphocyte subpopulations from MLN from noninfected (normal) and acutely and chronically infected, wild-type (solid bars) and gal-3^{-/-} (open bars) mice. Total lymphocytes (A) and plasma cells (B) were quantified using cytosmears. (C–F) Cells from infected animals were labeled with FITC-conjugated anti-B220 and cy-conjugated anti-CD5. The LN B cell population was defined and characterized through flow cytometry. Boxes in each dot-plot circumscribe the region of B220^{high}CD5^{ve} cells in all experimental groups indicated. Numbers indicate the relative proportion of the lymphocytes. Data are reported as means ± SEM and are representative of five independent experiments, each carried out in three normal mice, five mice with acute infection, and five with chronic infection. Statistical analysis: Tukey's multiple comparison test (*, *P* < 0.05).



LacDiNAc structures are sufficient stimuli for the mobilization of inflammatory cells and can induce a granulomatous reaction around the coated bead in liver parenchyma [39]. As depicted in Figure 1 and discussed in the literature, the granulomas formed in response to *S. mansoni* eggs accumulate high amounts of gal-3 [39].

Gal-3 expression is highly up-regulated when monocytes differentiate into macrophages [40] and is down-regulated when these cells differentiate into dendritic cells [41], suggesting that this protein has a role in distinct stages of monocytic differentiation. Accordingly, our results showed a deficient monocytes/macrophage activation and differentiation, as measured by adherence assays, in gal-3^{-/-} mice. Gal-3 plays an important role in critical macrophage functions, such as phagocytosis, and participates in innate and adaptive immunity through clearance of microorganisms and apoptotic cells [42].

Our histological analysis demonstrated that although gal-3 is considered an important molecule for several macrophage functions, knockout mice are still able to form periovular granulomas, a cell-mediated, hypersensitivity response. It has been demonstrated already that some schistosome glycoconjugates, specifically those containing terminal LacNAc or LacDiNAc, are able to induce formation of granulomas [39]. Our results indicate that granuloma formation in schistosomiasis is quite complex and that immunological activation per se is capable of inducing granuloma, even in the absence of gal-3

binding. These results corroborate the notion suggested by Van de Vijver and colleagues [39] that other lectins, e.g., Gal/GalNAc-binding lectins, of the host may be involved in hepatic granuloma formation. The positive identification of the animal lectin involved in the initial steps of granuloma formation needs to be addressed. These studies certainly will be useful for the rational design of drugs, which may control granulomagenesis.

Recently, Bickle and Helmby [43] reported similar findings, focusing their analysis in granulomas formed at 8 weeks postinfection, which corresponds to the acute phase of *S. mansoni* infection. Furthermore, formed granulomas presented giant multinucleate cells or epithelioid cells, indicating that macrophages in gal-3^{-/-} mice were still capable of responding to cytokines involved in their differentiation [44].

The increased bone marrow and liver eosinopoiesis seen in knockout animals could explain the increase in this population inside the granulomas. Eosinophils are the key cells involved in egg death [45], and that is involved in granulomas size reduction as a result of the involution with rapid collagen deposition [46]. In fact, this was evident in our infected gal-3^{-/-} animals. Even in acute phase, the predominant granulomas are the productive and involutinal ones, suggesting that eggs are killed rapidly by the increased amount of eosinophils of granulomas. Recent evidence points to a critical role of gal-3 in the regulation of myofibroblast activation, which is a central

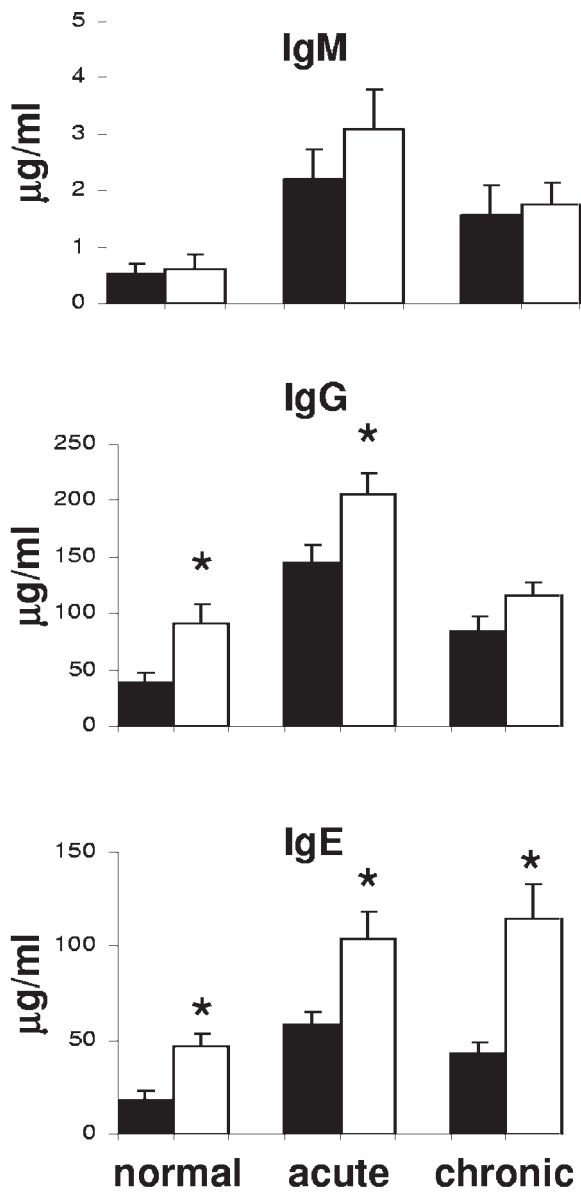


Fig. 9. The Ig levels were determined by ELISA. IgM, IgG, and IgE. The solid bars indicate the wild-type mice, whereas the open bars represent the $gal-3^{-/-}$ mice. We compared the noninfected group (normal) with both infected groups of wild-type and $gal-3^{-/-}$ mice (acute and chronic phases). Data are reported as means $\pm$ SEM and are representative of five independent experiments, each carried out in three normal mice, five mice with acute infection, and five with chronic infection. Statistical analysis: Tukey's multiple comparison test (*, $P < 0.05$).

control of hepatic fibrogenesis [47] and was also altered in the granuloma of $gal-3^{-/-}$ mice. Our data raise a potentially antiapoptotic effect of gal-3. Chronic-phase granulomas from wild-type mice were significantly larger in size than those obtained from $gal-3^{-/-}$ animals. Size reduction was not dependent on the degree of organization of the extracellular matrix but apparently, on the number of infiltrating, viable cells present in the granuloma.

Although the total number of myeloid precursors was not modified in noninfected and infected knockout mice, a significant increase in bone marrow-derived and circulating eosin-

ophils was observed in the course of the disease, as expected for *S. mansoni* infection [16, 37]. In the absence of gal-3, this response was amplified. The high level of IgE in the blood of the noninfected and infected knockout mice, associated with eosinophilia, suggests to us a predominant pattern of Th2 responses, in contrast to the exacerbated Th1 responses observed in the asthma model [10] and *Toxoplasma gondii* infection [27]. These results may indicate that gal-3 does not shift the character of Th1/Th2 responses but controls the intensity of a programmed response. It has been demonstrated that gal-3 acts in the nucleus inhibiting IL-5 transcription [48], the main cytokine involved in eosinophil proliferation and differentiation [49]. The high level of IL-5 production at the systemic and local levels possibly could explain the increased number of eosinophils in the granulomatous inflammatory reaction. The stroma of fibrogranulomatous-hepatic tissue, which produces cytokines such as stem cell factor, IL-5, and GM-CSF, maintains at least part of the production of myeloid cells, besides serving as a maturation site of bone marrow-derived myeloid precursors [50, 51]. The inflammatory hepatic stromal cells of $gal-3^{-/-}$ mice would produce larger amounts of IL-5, thus serving as an even more appropriate niche for eosinopoiesis.

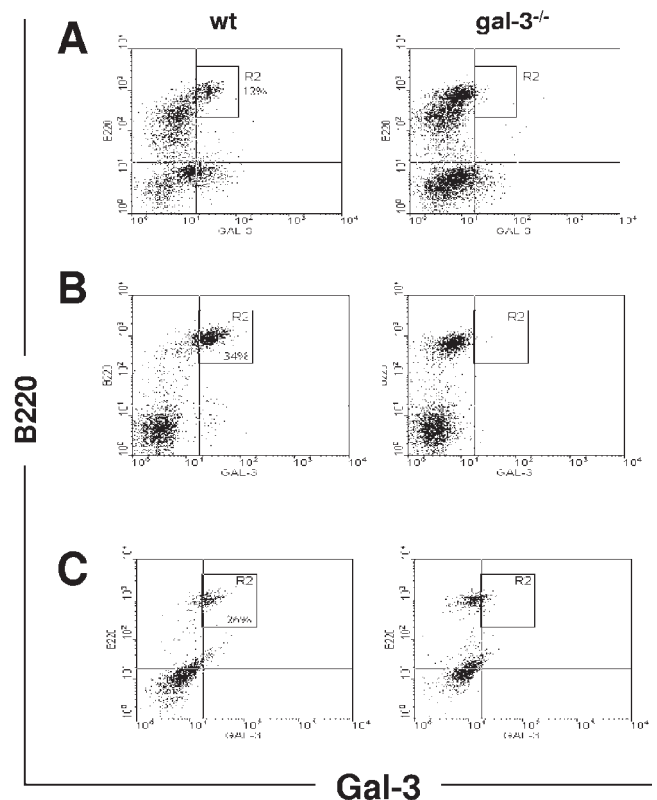


Fig. 10. Surface expression of B220, CD5, and gal-3 on cells from bone marrow (A), spleen (B), and MLN (C). Only cells with forward-scatter versus side-scatter parameters compatible with lymphocytes were selected for analysis. The R2 region represents mature B cells, which express gal-3 on the cell surface. The left dot-plots represent the wild-type mice, whereas the right dot-plots represent the $gal-3^{-/-}$ mice. Data are reported as means and are representative of three independent experiments, each carried out in three normal mice, five mice with acute infection, and five with chronic infection. Statistical analysis: Tukey's multiple comparison test.

Besides its involvement with the myeloid cell population, our results suggest an important role for gal-3 in B cell differentiation. In gal-3^{-/-} mice, the accelerated process of plasma cell generation was concomitant with the reduction of B220⁺ cells (B lymphocytes) in secondary lymphoid tissues, such as spleen and MLN. Activation of plasma cells was functionally confirmed by the higher levels of serum IgE and total IgG in infected knockout mice. Bickle and Helmbly [43] had shown recently that soluble egg antigen-specific IgG1 levels were significantly higher in the acute phase of infection in gal-3^{-/-} mice. Their finding would justify the increase of IgE levels in the chronic phase of the disease, as IgG1 production precedes IgE class-switch [52].

We observed an increase of a CD138⁺/B220^{low} subpopulation in the spleen but not in MLN of gal-3^{-/-} mice. The results of Acosta-Rodriguez et al. [13], using the acute phase of *T. cruzi* infection as a model of an extensive polyclonal B cell activation, strongly suggest that gal-3 is a critical signal for B cell survival, differentiation, and commitment to a memory cell phenotype, modulating the blimp-1 transcription factor, essential for plasma cell commitment [53, 54]. The results obtained by the authors using the antisense strategy were confirmed by the gal-3^{-/-} mice *in vivo* model, whose phenotype was strongly exacerbated by the *S. mansoni* infection. In both systems, gal-3 is an important regulatory molecule involved in the B cell differentiation process.

Our phenotypic analysis by flow cytometry demonstrated that B220^{high} lymphocytes presented gal-3 on the cell surface under control conditions, contrasting with the results described by Acosta-Rodriguez et al. [13]. This subpopulation of B cells represents the mature and circulating lymphocytes. These cells are capable of exiting from the bone marrow and entering into the bloodstream, homing to secondary lymphoid organs. The majority of mature B cells expresses surface gal-3 in all the studied tissues, such as bone marrow, spleen, and LN, regardless of whether the animals were infected. Although mature B cells were positive to gal-3, we cannot affirm if this lectin was expressed by the B cell itself or if the gal-3 was produced by another cell type, secreted, and then engaged to a B cell surface receptor. Acosta-Rodriguez and colleagues [13] used an antisense strategy to knock-down gal-3 expression in activated B cells from *T. cruzi*-infected mice. Therefore, at least part of the gal-3, which modulates B cell activation or differentiation, may be expressed by the B cell itself. Whether extracellular gal-3 interferes with B cell function remains to be elucidated. The high account of plasma cell could be generated by specific failure in check-points of B cell differentiation. Our data are in accordance with Katrina et al. [55], who demonstrated an aberrant increase of the gal-3 associated with a malignant B cell development as a result of the antiapoptotic role of gal-3. It is interesting to point out that in our model, the absence of gal-3 induced a more accelerated B cell differentiation by passing other cell-death controls, including the apoptotic programs, as demonstrated by Katrina et al. [55].

In this scenario, self-reactive, clonal cells would be generated, and self-antibodies could lead to autoimmune diseases. Altogether, our data highlight the involvement of gal-3 in key differentiation pathways of myelopoiesis and B lymphopoiesis.

Understanding how gal-3 interferes with monocytes/macrophage activation and B cell differentiation may have implications for the control of innate and acquired immunity.

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