

Lack of galectin-3 up-regulates IgA expression by peritoneal B1 lymphocytes during B cell differentiation

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Abstract Galectin-3 is a β -galactoside-binding protein with an inhibitory role in B cell differentiation into plasma cells in distinct lymphoid tissues. We use a model of chronic schistosomiasis, a well-characterized experimental disease hallmarked by polyclonal B cell activation, in order to investigate the role of galectin-3 in controlling IgA production through peritoneal B1 cells. Chronically infected, galectin-3-deficient mice (*Lgals3*^{-/-}) display peritoneal fluid hypercellularity, increased numbers of atypical peritoneal IgM⁺/IgA⁺ B1a and B1b lymphocytes and histological disturbances in plasma cell niches when compared with *Lgals3*^{+/+} mice. Similar to our infection model, peritoneal B1 cells from uninfected *Lgals3*^{-/-} mice show enhanced switching to IgA after in vitro

treatment with interleukin-5 plus transforming growth factor- β (IL-5 + TGF- β 1). A higher number of IgA⁺ B1a lymphocytes was found in the peritoneal cavity of *Lgals3*^{-/-}-uninfected mice at 1 week after i.p. injection of IL-5 + TGF- β 1; this correlates with the increased levels of secreted IgA detected in the peritoneal fluid of these mice after cytokine treatment. Interestingly, a higher number of degranulated mast cells is present in the peritoneal cavity of uninfected and *Schistosoma mansoni*-infected *Lgals3*^{-/-} mice, indicating that, at least in part, mast cells account for the enhanced differentiation of B1 into IgA-producing B cells found in the absence of galectin-3. Thus, a novel role is revealed for galectin-3 in controlling the expression of surface IgA by peritoneal B1 lymphocytes; this might have important implications for manipulating the mucosal immune response.

Felipe L. Oliveira and Emerson S. Bernardes contributed equally to this work.

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Introduction

Schistosomiasis represents a critical illness in developing countries and affects more than 207 million people. It is caused by the trematode *Schistosoma mansoni*, which promotes a chronic liver fibrotic disease (Utzing et al. 2009). The pathogenesis of schistosomiasis results from the penetration of early parasite larvae (cercariae) into the skin of humans or mice hosts and by egg-laying adult worms inhabiting the mesentery. The eggs are frequently drained to the hepatic portal system and liver parenchyma evoking an acute-to-chronic progressive fibrogranulomatous reaction in host tissues (Andrade 2009; Borojevic 1992). The hallmarks of chronic phase are liver fibrosis and hepatomegaly, splenomegaly correlated with polyclonal B cell activation, extramedullary

hematopoiesis and peritoneal responsiveness associated with macrophage hyperactivity (El-Cheikh et al. 1994). In addition, soluble schistosoma-antigens and increased circulating levels of cytokines continuously stimulate peritoneal B cells during schistosomiasis (Pinho et al. 2005; Weinberg et al. 1992). In particular, peritoneal B1 lymphocytes have been shown to switch from IgM to IgE during *S. mansoni* infection (Oliveira et al. 2005).

S. mansoni synthesizes immunogenic glycoproteins-containing poly-lactosamines that are recognized by endogenous glycan-binding proteins such as galectin-3 and elicits a robust humoral immune response (Nyame et al. 1999; Van de Vijver et al. 2006; van den Berg et al. 2004). Galectin-3, a 30-kDa protein characterized by having a carbohydrate recognition domain (CRD) and an N-terminal regulatory domain, is currently considered a biomarker in chronic diseases, including heart and liver fibrosis (de Boer et al. 2014; Henderson and Sethi 2009). Galectin-3 is highly expressed by liver cells located around the intrahepatic granulomas during chronic schistosomiasis. Recently, we demonstrated that *S. mansoni*-infected *Lgals3*^{-/-} mice exhibit abnormal plasmacytosis in the bone marrow, mesenteric lymph nodes and spleen followed by significant hyperglobulinemia (Brand et al. 2012; Oliveira et al. 2007, 2011). However, the involvement of galectin-3 in B cell physiology is far from being completely understood and only a few reports in the literature address the role of galectin-3 on B cell activation/differentiation. Kimata (2002) first reported that neutrophils could control IgE⁺ B cell differentiation via galectin-3 during IgE-associated atopic eczema/dermatitis syndrome. An interesting report on the role of galectin-3 in interleukin-4 (IL-4)-induced B cell commitment toward a memory phenotype was then published by Acosta-Rodríguez et al. (2004). Using *Lgals3*^{-/-} mice to study B1 cell physiology, we demonstrated that galectin-3 can act as an inhibitor of peritoneal and conventional B cell differentiation into plasma cells (Oliveira et al. 2007, 2009). Recently, by means of an autoantibody transgene model, Clark et al. (2013) revealed that the absence of galectin-3 leads to a significant increase in the number of Tg spleen B cells, thus interfering with B cell fate during autoantibody production and autoimmunity (Clark et al. 2013).

In the current study, we investigate whether galectin-3 controls B cell production of IgA during experimental chronic schistosomiasis and under treatment with cytokines that enhance IgA-class switching. Our data demonstrate that peritoneal B1 cells derived from both *S. mansoni*-infected or cytokine-treated *Lgals3*^{-/-} mice express higher levels of IgA on their surface in comparison with *Lgals3*^{+/+} control mice. In addition, the lack of galectin-3 also correlates with high levels of secretory IgA in the peritoneal fluid and increased numbers of degranulated mast cells in the peritoneal cavity. These data suggest a novel role for galectin-3 in regulating peritoneal B1

cell activation and differentiation into IgA-secreting B1 lymphocytes.

Materials and methods

Mice and infection with *Schistosoma mansoni*

Inbred wild-type (WT) C57/bl6 (*Lgals3*^{+/+}) and galectin-3-deficient mice (*Lgals3*^{-/-}; Hsu et al. 2000) that were age- and sex-matched were obtained from the colony bred at the Federal University of Rio de Janeiro, Brazil. All procedures with mice were performed in accordance with institutional guidelines (protocol no. DAHEICB 070, Federal University of Rio de Janeiro). Mice of 4–6 weeks of age were infected by the transcutaneous penetration of 40 *S. mansoni* cercariae (BH strain, Oswaldo Cruz Institute/RJ, Brazil) and killed under carbon dioxide in a chamber at 90–95 days after infection, corresponding to the chronic phase of the disease (Dutra et al. 1998). Uninfected mice were used as experimental controls during in vivo assays.

Peritoneal cellularity and cytospins

Resident peritoneal cells obtained from WT and *Lgals3*^{-/-} infected mice were harvested under sterile conditions after injection of 10 ml phosphate-buffered saline (PBS), pH 7.2, supplemented with 3 % fetal bovine serum into the abdominal cavity. Peritoneal leukocytes were counted by using a hemocytometer. Cytospins were performed with 2.0×10^4 cells in 300 μ l PBS, centrifuged at 36g for 3 min (Thermo Scientific Cytospin 4 Cyto centrifuge, Mass., USA), fixed in absolute methanol by 24 h and stained with May-Grünwald-Giemsa (El-Cheikh and Borojevic 1990). Morphological analysis was performed by using high-power microscopy (Zeiss-Axioplan, Germany). The images were acquired by bright field microscopy via a camera (Evolution MP 5.0 RTV-Color-Media Cybernetics, Canada).

Flow cytometry: DNA content and phenotypic analysis

Cell cycle analysis was performed according to Vindelov's protocol (Vindelov 1985). Briefly, peritoneal cells (5×10^5 cells per mouse) were resuspended in 500 μ l propidium iodide solution (PBS, 0.1 % Triton X-100, 0.1 % RNase, 50 μ g/ml propidium iodide; Sigma Aldrich, USA) and incubated on ice for 5 min. For the phenotypic analysis, approximately 1×10^6 cells/ml obtained from the peritoneal wash were incubated with Fc block (Clone 2.4G2; Cell Bank of Rio de Janeiro) for 20 min before the addition of the following monoclonal antibodies: fluorescein isothiocyanate (FITC) conjugated to anti-IgM and anti-CD3; phycoerythrin (PE) conjugated to

anti-IgA and anti-CD19; peridinin chlorophyll (PerCP) conjugated to anti-CD5 and anti-CD45; allophycocyanin (APC) conjugated to anti-B220 (BD Bioscience, USA). Samples were examined in a flow cytometer (FACSCalibur, BD Bioscience, USA) and analyzed by using CellQuestPro (BD Bioscience, USA). Total B cells were characterized as B220⁺CD19⁺CD45⁺CD3⁻ cells. Peritoneal B cells were phenotypically identified as B1a cells (B220^{low}CD5^{low}IgM^{high} cells), B1b (B220^{low}CD5^{neg}IgM^{high} cells) and B2 cells (B220^{high}CD5^{neg}IgM^{low} cells). Peritoneal B cells were also selected based on their morphometric features by using forward scatter (FSC) and side scatter (SSC) parameters.

Interleukin-5 and transforming growth factor- β 1 induction in vitro and in vivo

For the in vitro analysis, peritoneal cells (5×10^5 cells/ml) obtained from non-infected WT and galectin-3^{-/-} mice were cultured in DMEM (Dulbecco's modified Eagle medium) supplemented with 10 % fetal bovine serum and antibiotics (penicillin and streptomycin) at 37 °C under a 5 % CO₂ atmosphere. Interleukin-5 (IL-5) was obtained from a supernatant of a XR63-IL5 (Cell Bank of Rio de Janeiro) cell line transfected with IL-5 cDNA (Karasuyama and Melchers 1988). IL-5 and transforming growth factor- β 1 (TGF- β 1; 2 ng/ml; Sigma-Aldrich, USA) were used to induce IgA-class switching. For the in vivo analysis, a single dose of DMEM (0.5 ml) supplemented with IL-5 and TGF- β 1 was injected into the peritoneal cavity of WT and *Lgals3*^{-/-} mice. The phenotype and morphological analyses of cells (in vitro) and mice (in vivo) were performed by flow cytometry and optical microscopy, respectively, after 1 week of treatment with the cytokines under both experimental conditions. Control groups were cultured or injected with medium alone. Trypan blue exclusion was used to evaluate cell viability in the culture systems. Under all experimental conditions, cell viability was more than 90 % after 1 week of culture.

Immunocytochemistry

Anti-CD138 was used to investigate the presence of plasma cells after cytokine induction. At 1 week after stimulation, peritoneal cells from non-infected WT and galectin-3^{-/-} mice were collected by cytopsin on poly-L-lysine-coated coverslips. The samples were fixed for 15 min in 4 % paraformaldehyde in PBS pH 7.4 at room temperature. A permeabilization step was not required because CD138 is preferentially detected on the cell membrane. Cells were incubated with anti-CD138 (BD Bioscience, USA) and counterstained with Harris' hematoxylin. Images were acquired by bright field microscopy via a camera (Evolution MP 5.0 RTV-Color-Media Cybernetics, Canada).

IgA and IgM secretion

Wild-type and *Lgals3*^{-/-} mice were injected intraperitoneally with IL-5 (5 ng/ml) and TGF- β 1 (10 ng/ml), whereas PBS-injected mice were used as negative controls. The serum and peritoneal lavage fluid of WT and galectin-3^{-/-} mice were collected (7 and 14 days after stimulation) and analyzed individually by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Mabtech, Sweden). The optical density was measured by an ELISA microplate reader (Thermo Scientific, Mass., USA) and the absorbance values were directly proportional to the concentration of secretory IgA and IgM.

Mast cell count

Peritoneal cells were cytocentrifuged for 3 min (Thermo Scientific Cytospin 4 Cytocentrifuge) and fixed in absolute methanol for 24 h. Mast cells were identified after metachromatic staining with May-Grünwald Giemsa. Mast cells containing cytoplasmic granules were considered as non-activated cells, whereas degranulated mast cells were considered as activated cells. In total, 300 cells per sample/mouse were counted and the percentage value to mast cells was quantified.

Histological analysis of mesentery and omentum

For histological analysis of the omentum, the abdominal cavity of WT and *Lgals3*^{-/-} infected mice (5 animals per group) was exposed in order to remove the mesenteric membranes and omentum as described elsewhere (Pinho et al. 2005). Immediately after extirpation, the omentum was fixed for 24 h in 10 % buffered formalin, dehydrated and embedded in paraffin. Tissue sections (5 μ m thick) were stained with hematoxylin and eosin or processed for further immunohistochemistry analysis (Oliveira et al. 2011). The tissue was analyzed by a light microscope (Zeiss-Axioplan, Jena, Germany) and images were acquired by bright field microscopy via a camera (Evolution MP 5.0 RTV-Color-Media Cybernetics, Canada).

Immunohistochemistry

Paraffin-embedded sections of the omentum from WT and *Lgals3*^{-/-} infected mice (5 animals per group) were dewaxed and hydrated. After inhibition of endogenous peroxidase, sections were incubated for 1 h with 0.01 M PBS containing 5 % bovine serum albumin, 4 % skim milk, 0.1 % Triton X-100 (Sigma Aldrich, USA), 0.05 % Tween-20 and 10 % normal goat serum. The following purified antibodies were used: anti-galectin-3 (M3/38; ATCC, USA), anti-CD138 and Blimp-1 (Santa Cruz Biotechnology, USA). Bound antibodies were

detected by using a biotinylated anti-rat IgG (BA-4001, Vector Laboratories, USA) followed by incubation with avidin-peroxidase (Sigma-Aldrich, USA) and then revealed by using diaminobenzidine as the chromogen. Sections were counterstained with Harris' hematoxylin. The images were acquired by bright field microscopy via a camera (Evolution MP 5.0 RTV-Color-Media Cybernetics, Canada). As negative controls, sections of WT and knockout mice tissues were incubated with non-immune rat serum, instead of anti-galectin-3 antibody. Positive cells were quantified by using Image J software.

Statistical analysis

The statistical analyses were performed by using Tukey's multiple comparison test (*t*-test); the significance threshold was fixed at $P \leq 0.05$.

Results

Galectin-3 controls cellular proliferative status and decreases peritoneal B1b and B2 cell numbers during schistosomiasis

We previously reported that galectin-3 depletion caused a reduction in peritoneal cavity cellularity and favored B cell differentiation in non-infected mice (Oliveira et al. 2009). Therefore, *Lgals3*^{-/-} mice were considered an excellent model for studying the cellular events involving B cell responses. Here, we evaluated the peritoneal microenvironment in *Lgals3*^{-/-} mice that were chronically challenged with *S. mansoni*. During the chronic phase of infection, a marked polyclonal B cell activation occurred with enhanced peritoneal hyperresponsiveness (El-Cheikh et al. 1994). Since peritoneal cellularity was significantly reduced in uninfected *Lgals3*^{-/-} mice but substantially increased during infection with *S. mansoni* (Fig. 1a), we therefore investigated whether galectin-3 could modulate the proliferative status of peritoneal cells. The DNA content of peritoneal cells was analyzed by flow cytometry and we could observe a significant accumulation of cells in both the S and G2/M phases of the cell cycle in *Lgals3*^{-/-} infected mice in comparison with *Lgals3*^{+/+} infected mice (Fig. 1b). In *Lgals3*^{+/+} infected mice, cytospins of peritoneal cells revealed neutrophils, monocytes, lymphocytes, mast cells (Fig. 1c), eosinophils and macrophages (Fig. 1d) dispersed in the peritoneal fluid. In infected *Lgals3*^{-/-} mice, we could observe all the mitotic phases, including prophase (Fig. 1e), metaphase (Fig. 1f) and anaphase and telophase (karyokinesis and cytokinesis, Fig. 1g, h, respectively).

In order to identify a possible alteration in the balance of B cell subsets during chronic schistosomiasis in *Lgals3*^{-/-} mice,

we monitored the phenotype of the peritoneal B cells. B cell subpopulations of *Lgals3*^{+/+} and *Lgals3*^{-/-} infected mice were quantified according to their typical phenotype: B220^{low}CD5⁺ B1a lymphocytes (Fig. 2a, gate R2), B220^{low}CD5^{neg} B1b lymphocytes (Fig. 2a, gate R3) and B220^{high}CD5^{neg} conventional or B2 cells (Fig. 2a, gate R4). The number of B1a lymphocytes was significantly decreased, whereas both B1b and B2 lymphocytes were numerically increased in the peritoneal cavity of infected galectin-3^{-/-} mice (Fig. 2b). Noticeably, peritoneal cellularity and especially the B lymphocyte compartment of *S. mansoni*-infected *Lgals3*^{-/-} mice were affected by the absence of galectin-3 when compared with *Lgals3*^{+/+} mice. These results suggest that galectin-3 inhibits B cell proliferation/differentiation and exerts a negative effect on B1 cell accumulation in the peritoneal cavity during infection with *S. mansoni*.

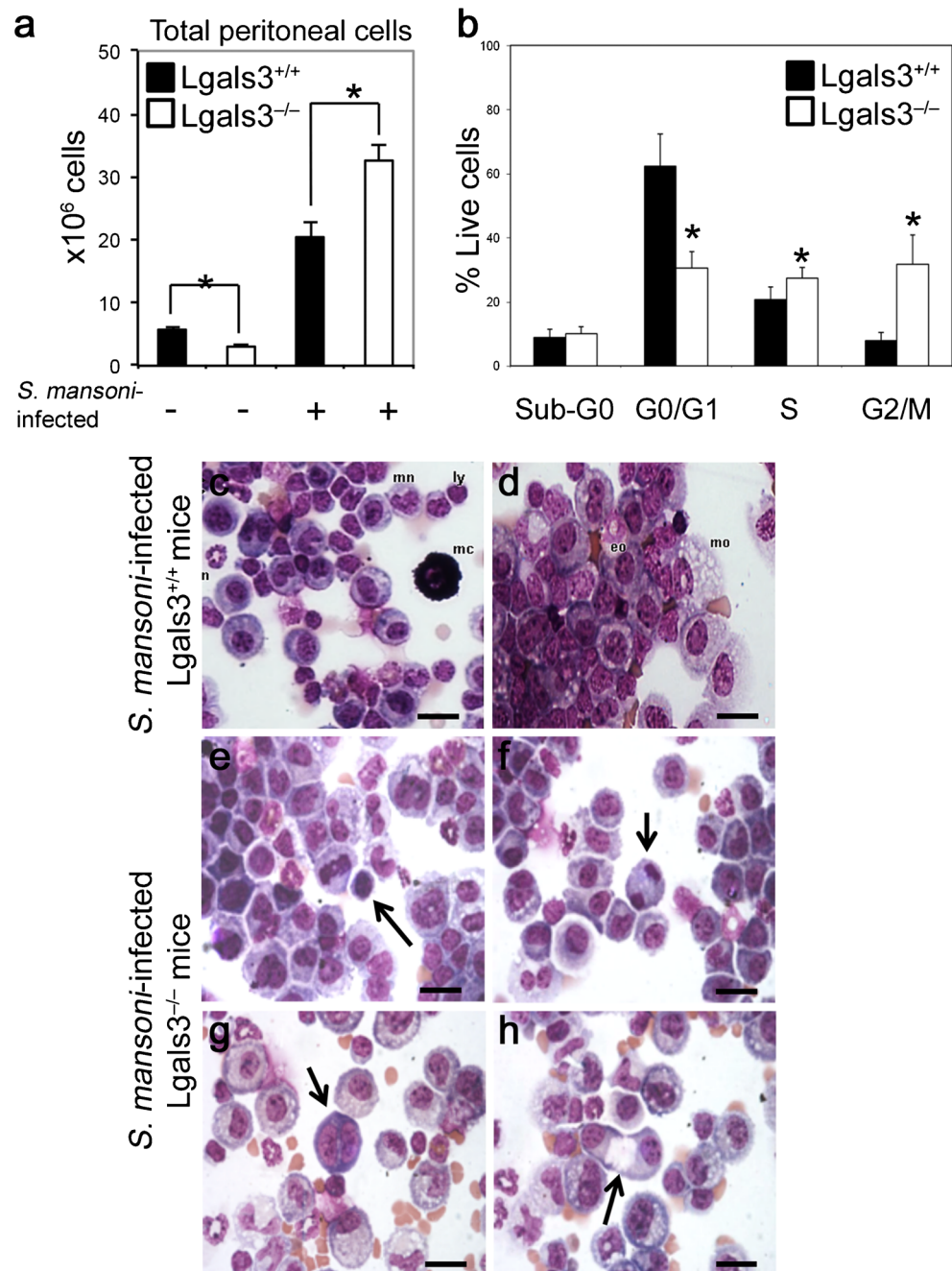
Galectin-3 inhibits differentiation of peritoneal B1 cells into IgA⁺ plasma cells during chronic schistosomiasis

Lgals3^{-/-} mice chronically infected by *S. mansoni* have previously been reported to exhibit high levels of serum immunoglobulins (Igs), suggesting a potential regulatory role for galectin-3 in Igs production (Oliveira et al. 2007). Therefore, we evaluated whether the absence of galectin-3 interferes with Ig expression on peritoneal B cell subpopulations during the chronic phase of the disease. As B1 cells in mucosa-associated tissues are an important source of IgA-producing cells (Bao et al. 1998; Roy et al. 2013), we monitored the expression of surface IgA (sIgA) and IgM (sIgM) on peritoneal B1 cells in our model of infection. We observed that peritoneal B1a lymphocytes from *Lgals3*^{-/-} infected mice expressed lower levels of sIgM (Fig. 2c) and higher levels of sIgA (Fig. 2d) in comparison with *Lgals3*^{+/+} infected mice. Indeed, we observed a four-fold increase in the number of IgA⁺ B1a cells in *Lgals3*^{-/-} infected mice compared with *Lgals3*^{+/+} infected mice (Fig. 2e). Taking into account that all cells were positive for IgM, we assumed that B1a IgA⁺ cells also expressed IgM on the membrane.

We also found that, in B1b lymphocytes from infected *Lgals3*^{-/-} mice, the levels of sIgM were reduced (Fig. 2f), whereas the expression of sIgA was significantly increased (Fig. 2g), in comparison with *Lgals3*^{+/+} infected mice. Moreover, the number of peritoneal IgA⁺ B1b cells was increased approximately 15-fold in the absence of galectin-3 (Fig. 2h). On the other hand, the expression of sIgM and sIgA by conventional B2 lymphocytes and the number of B2 IgA⁺ cells did not change in the absence of galectin-3 (Fig. 2i). These results indicate that galectin-3 negatively affects the terminal differentiation of B1a and B1b lymphocytes into IgA-producing plasma cells during chronic schistosomiasis.

Fig. 1 Cellularity and morphological analysis of peritoneal cells in *Lgals3^{+/+}* and *Lgals3^{-/-}* mice during schistosomiasis. *Lgals3^{+/+}* or *Lgals3^{-/-}* mice were infected with *Schistosoma mansoni* and (a) the total number or (b) the cell cycle of peritoneal cells was evaluated (black bars wild-type mice, white bars *Lgals3^{-/-}* mice). Data represent the mean of three experiments (error bars standard deviation). * $P < 0.05$.

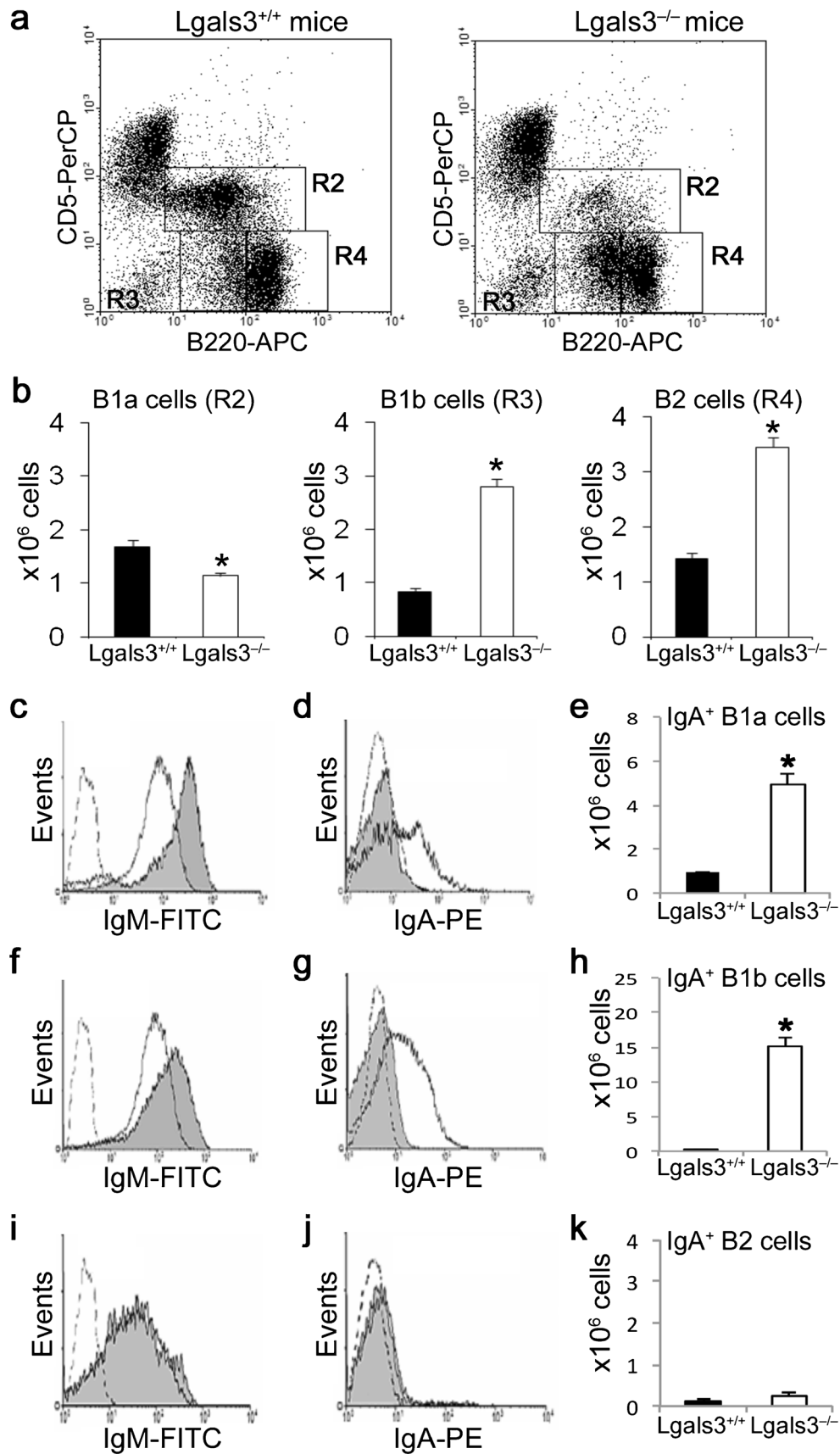
Morphological analysis of cytosmears of both infected *Lgals3^{+/+}* (c, d) and *Lgals3^{-/-}* mice (e–h) by light microscopy (arrows proliferative cells, mn monocyte, mc mast cell, ly lymphocyte, eo eosinophil, mo macrophage). Prophase (e), metaphase (f) anaphase and telophase, including karyokinesis (g) and cytokinesis (h). Magnification: $\times 400$. Bars 10 μm



Galectin-3 impairs IL-5/TGF- β -induced IgA-class switching in vitro

In order to verify whether B cells of uninfected *Lgals3^{-/-}* mice also display higher expression of surface IgA, we induced IgA-class switching by in vitro stimulation with IL-5 and TGF- β 1, two master cytokines that promote IgA synthesis (Stavnezer and Kang 2009). Peritoneal cells from *Lgals3^{+/+}* or *Lgals3^{-/-}* mice were stimulated in vitro with IL-5 and TGF- β 1 for 1 week. Flow cytometry analysis revealed that B220^{neg/low} IgA⁺ cells of galectin-3^{-/-} mice were significantly increased

after cytokine treatment when compared with cells of *Lgals3^{+/+}* mice (Fig. 3a, gate R2, lower dot plot). Although the absolute number of total B cells (total B220⁺ cells) from both *Lgals3^{+/+}* and *Lgals3^{-/-}* mice did not change after cytokine treatment (Fig. 3b), the number of B220^{neg/low} IgA⁺ cells increased approximately 4.5-fold in the presence of galectin-3 and about 9.8-fold in the absence of galectin-3 (Fig. 3c). Accordingly, the number of B220⁺ IgA^{neg} cells was proportionally reduced in both *Lgals3^{+/+}* and *Lgals3^{-/-}* cells after cytokine treatment (Fig. 3d). The expression of sIgM on cultured peritoneal cells was also monitored in control and cytokine-treated groups.



◀ **Fig. 2** Phenotype of peritoneal B lymphocytes. Peritoneal B subpopulations of control and *S. mansoni*-infected *Lgals3^{+/+}* and *Lgals3^{-/-}* mice were identified according to B220 and CD5 expression. **a** B1a lymphocytes (B220⁺CD5⁺ cells, gate R2), B1b lymphocytes (B220^{low}CD5⁻ cells, gate R3) and B2 lymphocytes (B220^{high}CD5⁻ cells, gate R3). **b** Bar graph representative of the absolute number of each B cell subtype. **c, d** Histograms represent expression of sIgM and sIgA by B1a cells. **e** Absolute number of B1a IgA⁺ cells. **f, g** Histograms represent expression of sIgM and sIgA by B1b cells. **h** Absolute number of B1b IgA⁺ cells. **i, j** Histograms represent expression of sIgM and sIgA by B2 cells. **k** Number of B2 IgA⁺ cells. *Black bars* represent *Lgals3^{+/+}* mice, *white bars* represent *Lgals3^{-/-}* mice, *gray histograms* represent *S. mansoni*-infected *Lgals3^{+/+}* mice, *empty histograms* indicate *S. mansoni*-infected *Lgals3^{-/-}* mice, *dashed histograms* represent isotype control. Data are representative of three experiments with three mice per group. **P*<0.05. Antibodies: allophycocyanin conjugated to anti-B220 (B220-APC), peridinin chlorophyll conjugated to anti-CD5 (CD5-PerCP), fluorescein isothiocyanate conjugated to anti-IgM (IgM-FITC) and phycoerythrin conjugated to anti-IgA (IgA-PE)

Lgals3^{+/+} and *Lgals3^{-/-}* B cells co-expressing B220 and sIgM were predominant in control groups (Fig. 3e, f, gates R2, R3, respectively). Interestingly, the numbers of sIgM^{Low}B220^{neg/Low} cells were higher in peritoneal *Lgals3^{-/-}* B cells before (Fig. 3e, f) and after (Fig. 3g, h) treatment with IL-5 and TGF- β when compared with peritoneal B cells from *Lgals3^{+/+}* mice. These data suggest that galectin-3 deletion accelerates IL-5/TGF- β -induced IgA-class switching.

Galectin-3 impairs IL-5/TGF- β -induced IgA-class switching in peritoneal B1a lymphocytes

In order to study the effects of IL-5 and TGF- β on peritoneal B cell differentiation into IgA⁺ cells in vivo, *Lgals3^{+/+}* and uninfected *Lgals3^{-/-}* mice were treated intraperitoneally with both cytokines. After 1 week, peritoneal cells were harvested and analyzed by flow cytometry. FSC x SSC parameters were used to distinguish small lymphocytes, large lymphocytes and granulocytes (Fig. 4a-d). Large lymphocytes were selected in R1 and B220⁺ cells were analyzed in the subsequent experiments. B220⁺IgA⁺ cells from *Lgals3^{+/+}* unstimulated control mice represented 4.4 \pm 0.9 % of the total peritoneal cells and increased to 10.3 \pm 1.4 % after cytokine stimulation (Fig. 4a, b). On the other hand, in *Lgals3^{-/-}* unstimulated control mice, we found a percentage of B220⁺IgA⁺ cells of 3.5 \pm 0.8 % of the total peritoneal cells (Fig. 4c); this increased to 26.9 \pm 2.1 % after IL-5 and TGF- β treatment (Fig. 4d). Treatment with IL-5 and TGF- β resulted in a 2.86-fold increase in the absolute number of sIgA⁺ peritoneal B cells in *Lgals3^{+/+}* mice and a 6.11-fold increase in *Lgals3^{-/-}* treated mice (Fig. 4e) compared with the untreated controls.

In order to calculate the percentage of B1a and B1b cells, we monitored the expression of CD5, which is present on B1a but not on B1b cells. In *Lgals3^{-/-}* mice, approximately 69 % of peritoneal cells co-expressed sIgA and CD5 (IgA⁺CD5^{Low}

cells), in comparison with 50 % of peritoneal cells from *Lgals3^{+/+}* mice (Fig. 4f, g). In *Lgals3^{-/-}*-treated mice, an approximately three-fold increase was seen in the number of peritoneal IgA⁺ B1a cells in comparison with *Lgals3^{+/+}* treated mice (Fig. 4h). These results indicate that galectin-3 deletion favors the differentiation of B1a cells into IgA-producing cells after in vivo stimulation with IL-5 and TGF- β .

Galectin-3 inhibits IgA and IgM secretion in peritoneal cavity

Since B cells of *Lgals3^{-/-}* mice expressed high levels of surface IgA, we sought to measure the levels of IgM and IgA in the serum and peritoneal fluid of both *Lgals3^{+/+}* and *Lgals3^{-/-}* mice in response to IL-5 and TGF- β stimulation. IgA serum levels did not change significantly in *Lgals3^{+/+}* and *Lgals3^{-/-}* mice, even after cytokine stimulation (Fig. 5a). On the other hand, *Lgals3^{-/-}* mice displayed higher levels of peritoneal IgA before and after treatment with IL-5 and TGF- β in comparison with *Lgals3^{+/+}* mice (Fig. 5b). The levels of IgM in the serum and peritoneal fluid were also increased in untreated *Lgals3^{-/-}* mice and did not change after the treatment with cytokines (Fig. 5c, d). On average, the levels of peritoneal IgA were 1.5-fold higher in untreated and cytokine-treated *Lgals3^{-/-}* mice (Fig. 5e). Untreated *Lgals3^{-/-}* mice showed a two-fold higher level of peritoneal IgM; however, no difference was detected in IgM levels between *Lgals3^{+/+}* and *Lgals3^{-/-}* mice at 7 days after cytokine treatment (Fig. 5f). Together, these data indicate that galectin-3 negatively modulates IgA secretion by peritoneal cavity B cells upon stimulation with IL-5 and TGF- β .

Galectin-3 negatively controls mast cell degranulation in peritoneal cavity

Mast cells have been previously demonstrated to enhance B cell expansion and to drive B cells toward differentiation into IgA-producing plasma cells (Merluzzi et al. 2010). Therefore, in order to investigate further the mechanism behind the enhanced production of sIgA found in *Lgals3^{-/-}* mice, we next quantified the number of degranulated mast cells present in the peritoneal cavity of *Lgals3^{-/-}* and *Lgals3^{+/+}* mice. Three times more degranulated mast cells were found in the peritoneal cavity of *S. mansoni*-infected *Lgals3^{-/-}* mice in comparison with *S. mansoni*-infected *Lgals3^{+/+}* mice (Fig. 6a, d, e). Interestingly, when cells from the peritoneal cavity from uninfected *Lgals3^{-/-}* and *Lgals3^{+/+}* mice were stimulated in vitro (Fig. 6b, f, g) or in vivo (Fig. 6c, h, i) with IL-5 + TGF- β 1, we still found increased numbers of degranulated mast cells in the absence of galectin-3. These data indicate that mast cell degranulation/activation accounts, at least in part, for the increased number of IgA-producing plasma cells found in *Lgals3^{-/-}* mice.

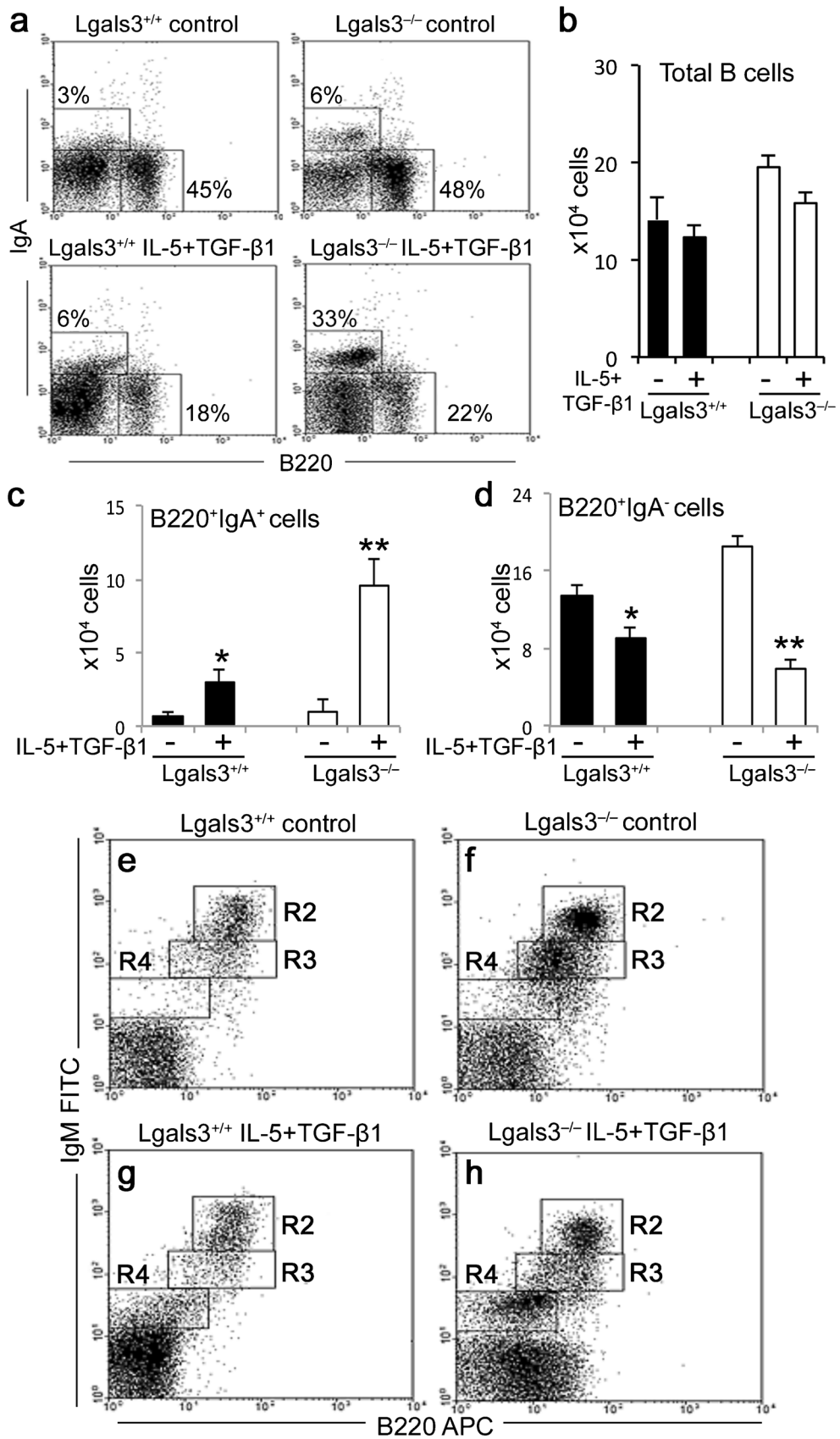


Fig. 3 IgA-class switching after interleukin-5 (*IL-5*) and transforming growth factor- β 1 (*TGF- β 1*) treatment in vitro. Peritoneal cells from *Lgals3*^{+/+} or *Lgals3*^{-/-} mice were cultured in the presence or absence of *IL-5* and *TGF- β 1* for 1 week. **a** B220 and IgA expression was evaluated by flow cytometry. **b** Absolute number of B cells (B220⁺ cells) was quantified. **c** Determination of number of B220⁺IgA⁺ cells of wild-type (*Lgals3*^{+/+}) and galectin-3^{-/-} (*Lgals3*^{-/-}) mice. **d** Determination of B220⁺IgA⁻ cells of *Lgals3*^{+/+} and *Lgals3*^{-/-} mice in control (-) and cytokine-treated (+) cells. **e–h** IgM and B220 expression on the peritoneum of (e) control *Lgals3*^{+/+} cells, (f) control *Lgals3*^{-/-} cells, (g) cytokine-treated *Lgals3*^{+/+} cells and (h) cytokine-treated *Lgals3*^{-/-} cells was analyzed by flow cytometry. Gate R2 includes B220⁺IgM^{high} cells, Gate R3 includes B220⁺IgM^{int} cells and Gate R4 includes B220^{low}-IgM^{low} cells. These data are representative of three independent experiments with three mice per group. **P*<0.05, ***P*<0.01

Galectin-3 restrains plasma cell response in mesentery and omentum following *S. mansoni* infection

Taking into account the results described so far, we decided to investigate the presence of plasma cell niches in the omentum and mesentery during chronic schistosomiasis. Plasma cells were only rarely found in the mesentery of *Lgals3*^{+/+} infected mice (Fig. 7a) but were found frequently attached to the mesentery of *Lgals3*^{-/-} infected mice (Fig. 7b). We next evaluated the histological architecture of the omentum, a coelomic-derived tissue involved in B cell responses against abdominal antigens (Rangel-Moreno et al. 2009) and receiving activated B cells migrating from the peritoneal cavity (Carlow et al. 2009). In *Lgals3*^{+/+} infected mice, the omentum was strongly

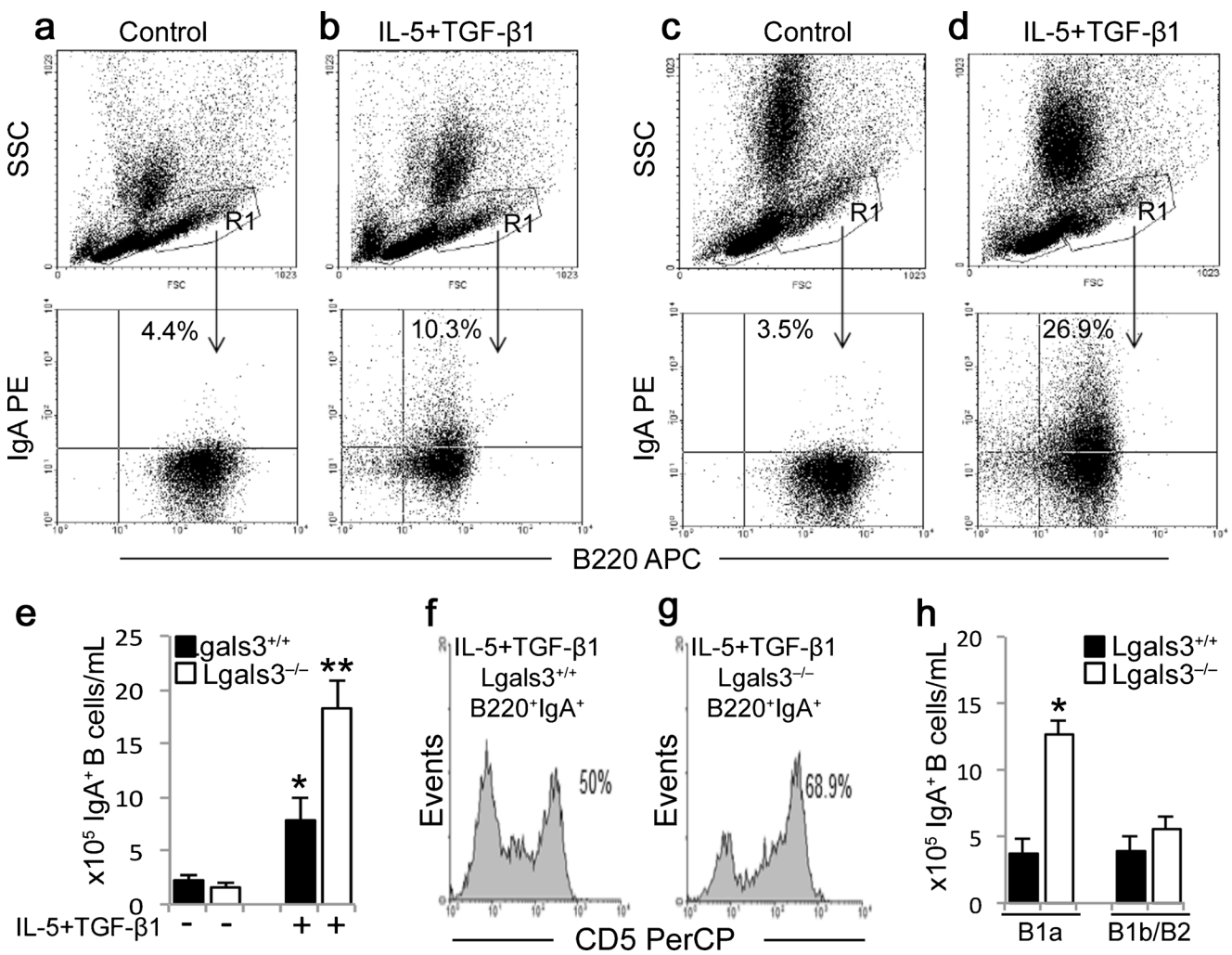
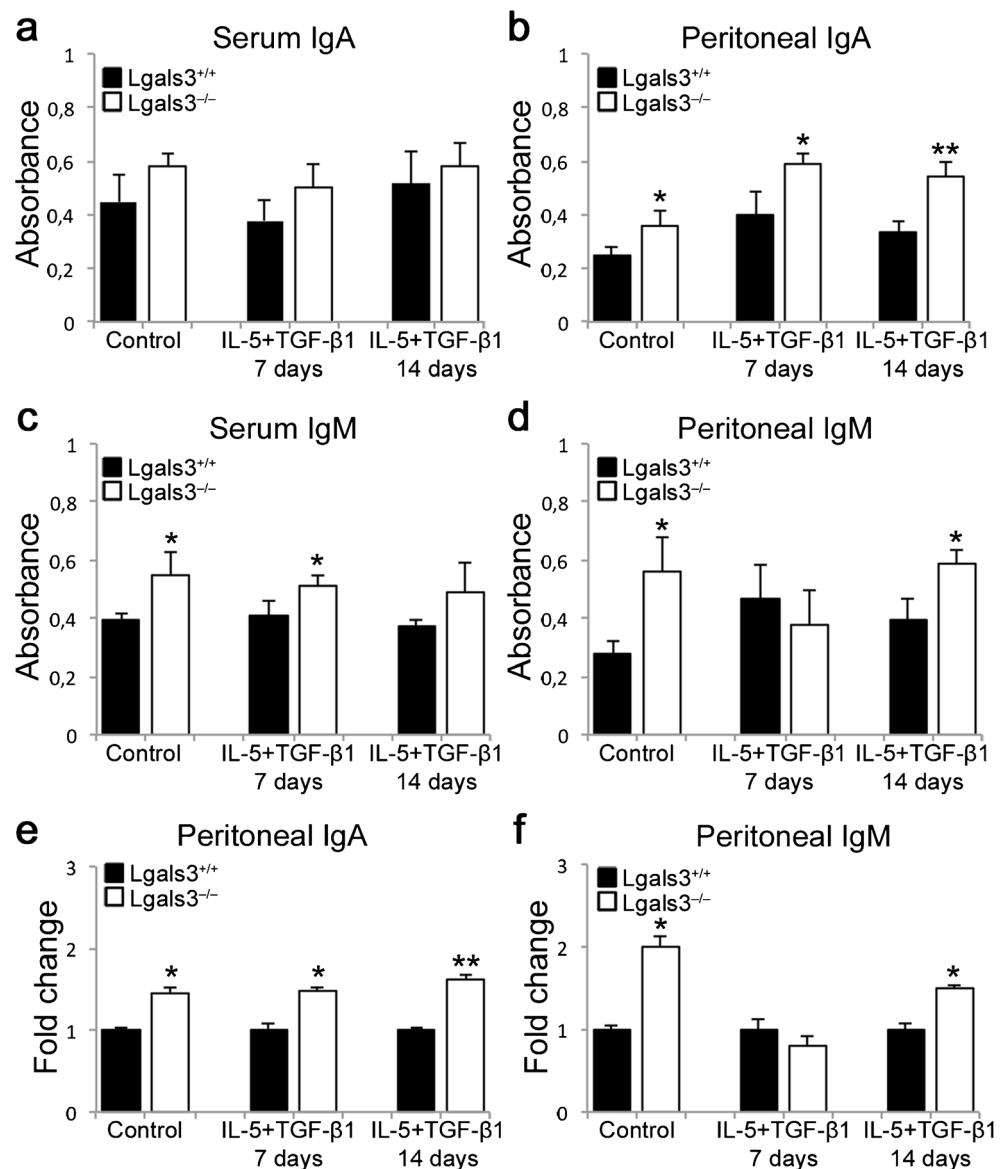


Fig. 4 B1a lymphocytes respond promptly to *IL-5* and *TGF- β 1* induction in vivo in the absence of galectin-3. *IL-5* and *TGF- β 1* cytokines were injected i.p. at 1 week before phenotypic analysis. Control *Lgals3*^{+/+} mice (a) exhibited 4.4 % of peritoneal cells positive to IgA, whereas cytokine-treated *Lgals3*^{+/+} mice (b) increased their number of IgA⁺ cells to 10.3 % of peritoneal cells. Control *Lgals3*^{-/-} mice (c) showed 3.5 % of peritoneal cells positive to IgA. Cytokine-treated *Lgals3*^{-/-} mice (d) increased to 26.9 % the peritoneal cells positive to IgA. Absolute number of cells expressing

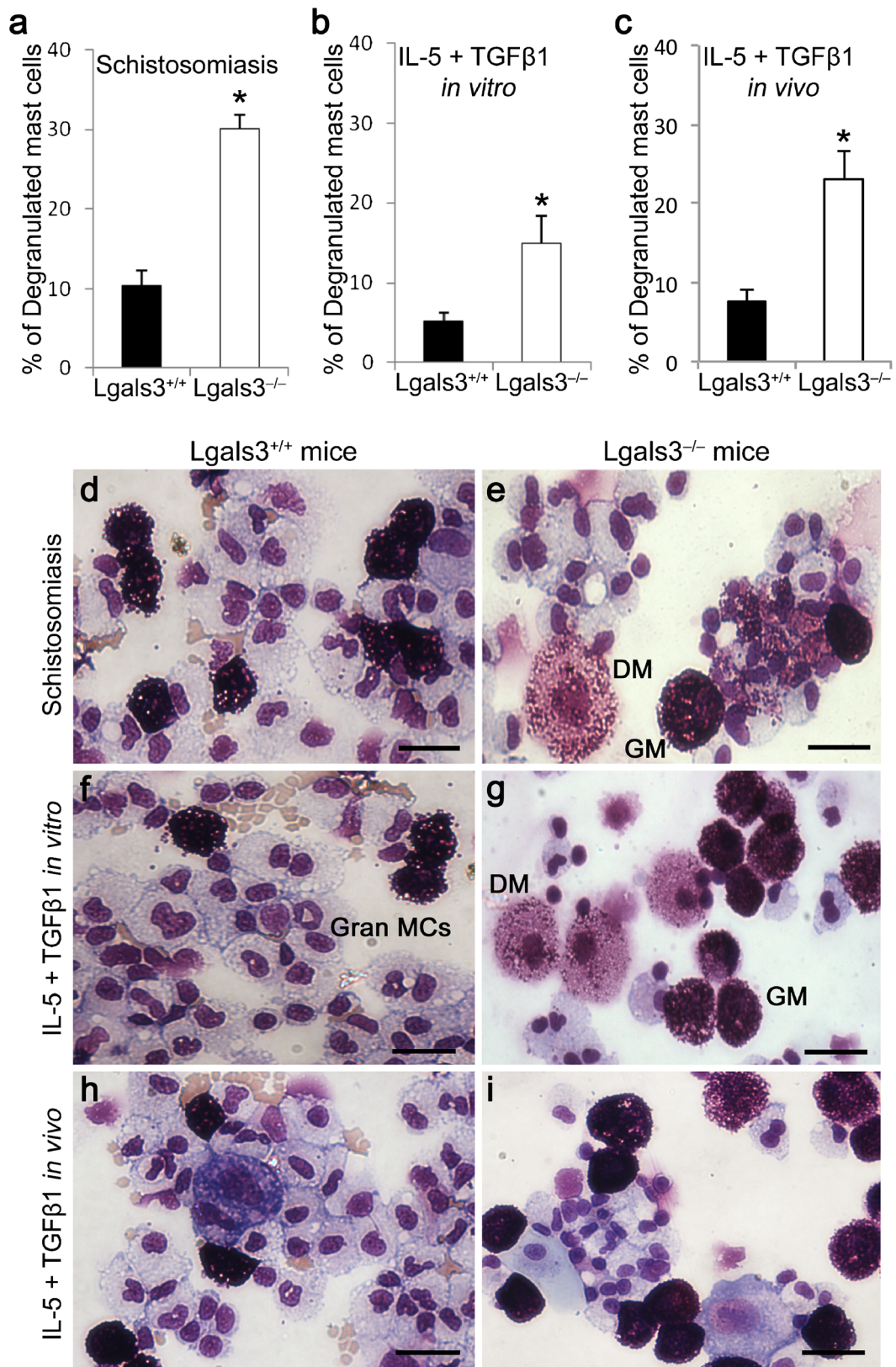
B220 and IgA (e). Flow cytometry of peritoneal cells derived from *Lgals3*^{+/+} or *Lgals3*^{-/-} mice treated with *IL-5* and *TGF- β 1* cytokines. Approximately 50 % of B220⁺ IgA⁺ cells of *Lgals3*^{+/+} mice (f) and 69 % of peritoneal B220⁺ IgA⁺ cells of *Lgals3*^{-/-} mice (g) were CD5⁺. Absolute number of B1 cells from *Lgals3*^{+/+} or *Lgals3*^{-/-} mice treated with *IL-5* and *TGF- β 1* cytokines (h). Black bars represent *Lgals3*^{+/+} mice, white bars represent *Lgals3*^{-/-} mice. Data are representative of three experiments with three mice per experimental group. **P*<0.05, ***P*<0.01

Fig. 5 IgA and IgM levels in serum and in peritoneal fluid from *Lgals3^{+/+}* and *Lgals3^{-/-}* mice treated with IL-5 and TGF- β 1. The levels of IgA were evaluated in the serum (a) and peritoneal liquid (b) of *Lgals3^{+/+}* and *Lgals3^{-/-}* mice following treatment with IL-5 and TGF- β 1 cytokines for 7 and 14 days. Additionally, the levels of IgM were quantified in the serum (c) and peritoneal fluid (d). Graphs showing the relative increase in the peritoneal levels of (e) IgM and (f) IgA. Data are illustrated as fold-change relative to *Lgals3^{+/+}* mice. Black bars represent *Lgals3^{+/+}* mice, white bars represent *Lgals3^{-/-}* mice. Data are representative of two experiments with five mice per experimental group. * $P < 0.05$, ** $P < 0.01$



cellularized and various types of cells could be identified, such as granulocytes, lymphocytes and plasma cells (Fig. 7c). The omentum of *Lgals3^{-/-}* infected displayed fewer cells in comparison with *Lgals3^{+/+}* infected mice (Fig. 7d, k). In order to quantify the number of plasma cells in the omentum of *Lgals3^{+/+}* and *Lgals3^{-/-}* infected mice, we stained tissue sections with anti-CD138 and anti-Blimp-1 antibodies. Both CD138 and Blimp-1 are plasma cell markers but the latter identifies Ig-secreting plasma cells. CD138⁺ plasma cells were preferentially confined to perivascular spaces in *Lgals3^{+/+}* infected mice (Fig. 7e). On the other hand, a higher number of CD138⁺ plasma cells was found randomly dispersed throughout the omental parenchyma of *Lgals3^{-/-}* infected mice in comparison with *Lgals3^{+/+}* infected mice (Fig. 7f, l). Blimp-1⁺ plasma cells were dominantly observed in well-defined milky spot-like structures in *Lgals3^{+/+}* infected mice

Fig. 6 Mast cell degranulation in vivo and in vitro in the absence of galectin-3. Peritoneal mast cells were identified according to the cytoplasm degranulation in the chronic phase of schistosomiasis. Data are shown as the percentage of degranulated mast cells under control conditions (a) and after IL-5 and TGF- β 1 treatment in vitro (b) and in vivo (c). In all situations, more activated mast cells were found in the absence of galectin-3. Black bars indicate *Lgals3^{+/+}* mice, white bars represent *Lgals3^{-/-}* mice. These data are representative of three independent experiments with three mice per group. * $P < 0.05$. Cytosmears showed granulated mast cells (GM) and degranulated mast cells (DM) in the peritoneal fluid of wild-type (d) and *Lgals3^{-/-}* mice (e) infected with *S. mansoni*. After 1 week of cytokine treatment in vitro, non-activated and activated mast cells derived from *Lgals3^{+/+}* (f) and *Lgals3^{-/-}* (g) mice could be identified. The same pattern was observed in cytosmears of peritoneal fluid obtained from *Lgals3^{+/+}* (h) and *Lgals3^{-/-}* (i) mice. Magnification: $\times 400$. Bars 10 μ m



(Fig. 7g), whereas in *Lgals3*^{-/-} infected mice, we found a higher number of Blimp-1⁺ plasma cells in distinct histological regions of the omentum (Fig. 7h, m). Interestingly,

galectin-3 was expressed in a wide range of cells types in omental tissue, predominantly at perivascular niches and inside blood vessels (Fig. 7i, insert). As expected, galectin-3-

expressing cells were not detected in the omentum of *Lgals3*^{-/-} infected mice (Fig. 7j).

The number of cells per area in the omentum of both *Lgals3*^{+/+} and *Lgals3*^{-/-} infected mice was quantified according to the expression of CD138 and Blimp-1. In the absence of galectin-3, the omentum exhibited a reduced number of cells per 100 μm^2 (Fig. 7k). On the other hand, the number of CD138+ cells (Fig. 7l) and Blimp-1+ cells (Fig. 7m) was significantly increased in these *Lgals3*^{-/-} infected mice. Taken together, our data demonstrate that galectin-3 deletion favors the accumulation of plasma cells in the mesentery and omentum.

Discussion

The inhibitory effect of galectin-3 on B lymphocyte differentiation was originally demonstrated by Acosta-Rodriguez and colleagues using a *Trypanosoma cruzi* infection experimental model (Acosta-Rodríguez et al. 2004). Subsequently, we demonstrated that *S. mansoni*-infected *Lgals3*^{-/-} mice have hyperglobulinemia and intense plasmacytogenesis in the bone marrow, mesenteric lymph nodes and spleen (Brand et al. 2012; Oliveira et al. 2007, 2011). Here, we propose a new regulatory role for galectin-3 in IgA⁺ plasma cell differentiation in the peritoneal cavity during chronic schistosomiasis inflammation and during IL-5 and TGF- β 1 treatment in vitro and in vivo.

The absence of galectin-3 in the peritoneal cavity is favorable to the generation of a quantitative and qualitative B1a/B1b imbalance. During schistosomiasis, nearly 65 % of peritoneal cells display a DNA content compatible with a proliferative status in the absence of galectin-3 (accumulating in the S and G2/M phases of cell cycle), whereas only 25 % of *Lgals3*^{+/+} peritoneal cells have been detected in the proliferative phase. On the other hand, a possible cell cycle arrest (Rieder 2011) and consequently more propensity to apoptosis cannot be excluded because galectin-3 is considered an anti-apoptotic molecule in various cell types (Hoyer et al. 2004; Matarrese et al. 2000; Nangia-Makker et al. 2007). Indeed, the peritoneal hypercellularity in *Lgals3*^{-/-} mice indicates a disorder in the proliferative status of these cells during chronic schistosomiasis but not after cytokine induction.

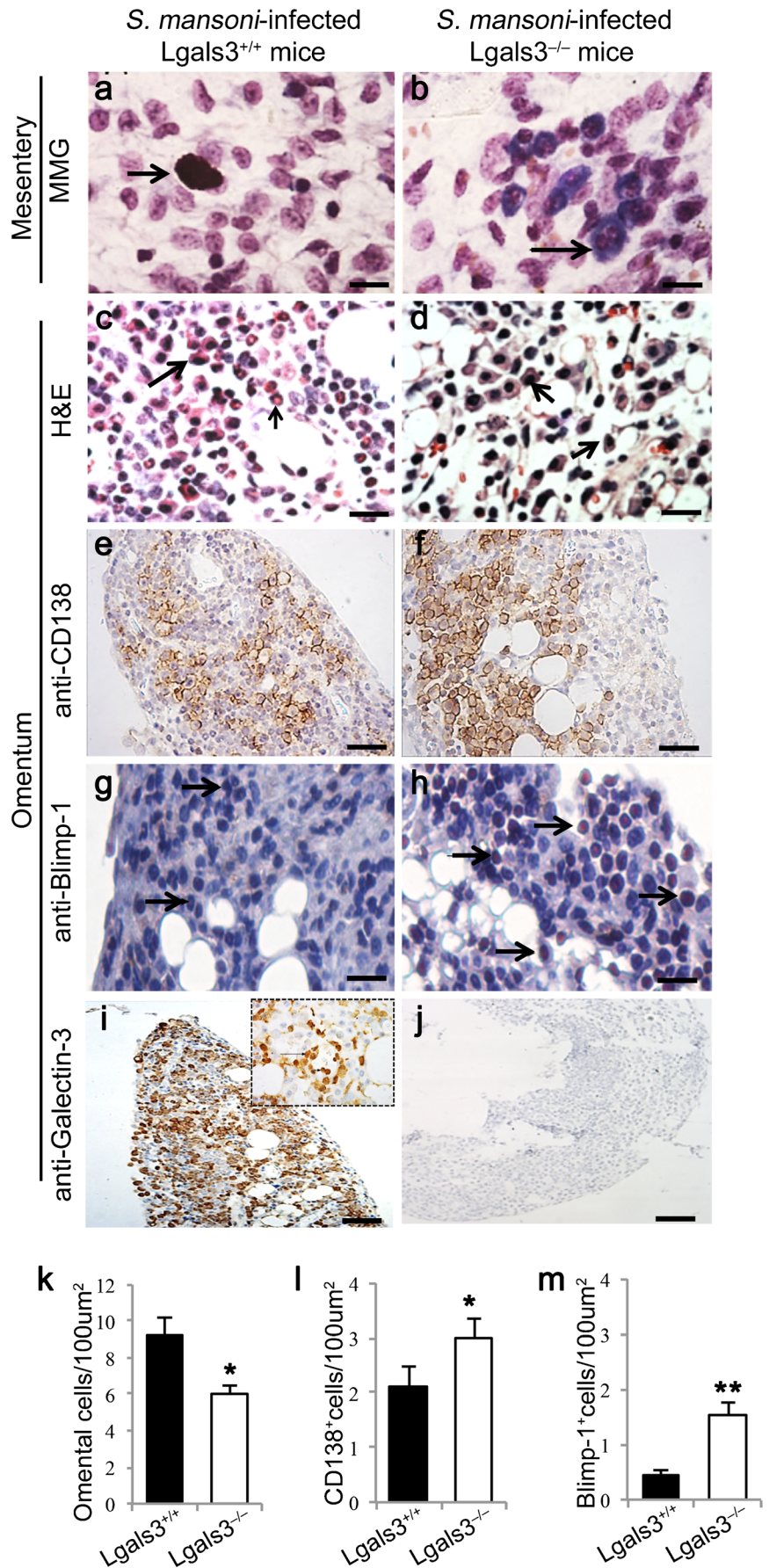
Previously, we have shown that peritoneal B1 cells from *S. mansoni*-infected mice switch from an IgM class to an IgE class (Oliveira et al. 2005) and that infected *Lgals3*^{-/-} mice present high levels of serum IgE during schistosomiasis (Oliveira et al. 2007). However, we found no differences in IgE⁺ B cells of *Lgals3*^{+/+} and *Lgals3*^{-/-} mice (data not shown). As peritoneal B cells are committed to an IgA repertoire (Roy et al. 2013) and as high levels of IgA are found in the serum during schistosomiasis (Gaber et al. 2010), we monitored sIgA expression by peritoneal B cells and conclude that a

significant correlation exists between galectin-3 and IgA⁺ differentiating plasma cells in the peritoneal cavity. Kracker and colleagues demonstrated that IgA/IgM co-expression is a remarkable event in the course of immunoglobulin class switching (Kracker and Durandy 2011) and our data indicate that galectin-3 is a potent regulator of IgA expression on B1 cells during chronic schistosomiasis and cytokine induction.

The concomitant expression of IgA and IgM on peritoneal B cells has been demonstrated preferentially in B1b cells (Roy et al. 2009). Here, we found, during chronic schistosomiasis, a higher number of peritoneal B1a- and B1b-IgA⁺ lymphocytes coexpressing sIgM in *Lgals3*^{-/-} mice. Nevertheless, the concomitant expression of IgA and IgM is also associated with IgA deficiency, as shown by Conley and Cooper (1981) who described that 90 % of patients presenting an IgA⁺IgM⁺ phenotype have a pathological blocker on IgA⁺ B cell differentiation during the IgA-class switch. Selective IgA deficiency is associated with a higher prevalence of infections and a variety of concomitant autoimmune diseases, including Graves' disease, systemic lupus erythematosus, type 1 diabetes, celiac disease, myasthenia gravis and rheumatoid arthritis (Wang et al. 2011). Galectin-3 plays a pro-inflammatory role exacerbating the effects in autoimmune disorders (Radosavljevic et al. 2012), however the involvement of galectin-3 in IgA physiology is not fully understood. The absence of galectin-3 favors IgA accumulation on the membrane of B1a and B1b lymphocytes but the exact significance of these findings remains unclear.

Our results indicate that galectin-3 negatively regulates IgA expression or that galectin-3 inhibits the proliferation of peritoneal IgA⁺ B1 cells when challenged by appropriate factors. Our in vitro and in vivo data suggest that, at least in part, peritoneal B1 cells of *Lgals3*^{+/+} and *Lgals3*^{-/-} mice do not proliferate after IL-5 and TGF- β 1 treatment but that they differentiate into IgA⁺ cells. Interestingly, in the absence of galectin-3, the number of IgA⁺ B1 cells is significantly increased under both experimental conditions. IL-5 and TGF- β 1 are essentials for IgA-class switching (Cerutti and Rescigno 2008) and the expression/synthesis of these cytokines can be modulated by galectin-3. IL-5 gene expression is down-regulated by galectin-3 in distinct cell types (Cortegano et al. 1998), whereas *Lgals3*^{-/-} mice present TGF- β -mediated myofibroblast activation and matrix production strongly inhibited after chronic CCL₄-induced liver fibrosis (Henderson et al. 2006). Accordingly, TD139 (inhibitor of galectin-3) blocks TGF- β -induced β -catenin activation and attenuates progressive lung fibrosis after experimental bleomycin induction (Mackinnon et al. 2012). Based on data from the literature, we hypothesized that, in the absence of galectin-3, increased levels of IL-5 and TGF- β 1 could account, at least in part, for the higher number of IgA⁺ B1 cells found in *Lgals3*^{-/-} mice.

Fig. 7 Photomicrographs of mesentery and omentum reveal disturbances on plasma cell niches in the absence of galectin-3 during chronic schistosomiasis. Mesentery of *Lgals3^{+/+}* mice (a) with mast cells (arrow), monocytes/macrophages (arrowhead) and lymphocytes (asterisk) and of *Lgals3^{-/-}* mice (b) predominantly occupied by lymphocytes (asterisk) and plasma cells (arrows) as stained with May-Grünwald Giemsa (MGG). Omentum sections of *Lgals3^{+/+}* and *Lgals3^{-/-}* mice were stained with hematoxylin and eosin (H&E; c, d, respectively) or immunostained for CD138 (e, f, respectively), Blimp-1 (g, h, respectively) and galectin-3 (i, j, respectively). As expected, omental cells did not express galectin-3 in *Lgals3^{-/-}* mice. Number of cells per area (cells/ μm^2 ; k), number of CD138-positive plasma cells (l) and number of Blimp-1-positive Ig-secreting cells (m) counted in the omentum. Black bars represent *Lgals3^{+/+}* mice, white bars represent *Lgals3^{-/-}* mice. Data are representative of three independent experiments with three mice per experimental group. * $P < 0.05$



Peritoneal B1 lymphocytes express IL-5R constitutively (Hitoshi et al. 1990) and higher levels of IL-5 in *Lgals3*^{-/-} mice (Oliveira et al. 2009) and promptly synthesize IgA (Takatsu et al. 2009). On the other hand, peritoneal B1 cells switch more readily to IgA than do splenic follicular or peritoneal B2 cells after B lymphocyte stimulator/lipopolysaccharide/TGF- β in vitro (Kaminski and Stavnezer 2006) and specifically, peritoneal B1b cells switch to IgA under the influence of the synergistic effect of TGF- β and retinoic acid (Roy et al. 2013). Indeed, we have arguments to suggest that galectin-3 plays a regulatory role on IgA⁺ cell differentiation controlling IL-5 and TGF- β functions in the peritoneal cavity.

Lgals3^{+/+} and *Lgals3*^{-/-} mice intraperitoneally treated with IL-5 and TGF- β do not modify the IgA and IgM levels in their serum, suggesting a poor systemic effect on the IgA/IgM secretion dependent on galectin-3. On the other hand, the lack of galectin-3 significantly disturbs the peritoneal levels of IgA and IgM, indicating a potent local effect of galectin-3 during IgA and IgM secretion. Our experimental data show that IL-5 and TGF- β induce IgA secretion by peritoneal cells after 1 week of injection and normal levels of IgA can be detected 14 days post-induction. In *Lgals3*^{-/-} mice, cytokine induction modifies the kinetics of IgA secretion and after 14 days of treatment, the IgA levels remain extremely high in comparison with *Lgals3*^{+/+} mice. As the IgA⁺ B1a cell subpopulation is markedly increased in *Lgals3*^{-/-} mice after cytokine induction, one can conclude that peritoneal B1a cell differentiation into IgA-secreting plasma cells is regulated by galectin-3, although the mechanisms remain unclear.

In vivo and in vitro experimental conditions revealed an intense plasmacytogenesis strictly associated with mast cell degranulation. Merluzzi and colleagues (2010) have recently shown that mast cells control B cell proliferation and regulate IgA⁺ plasma cell differentiation in secondary lymphoid organs. In parallel, Chen and colleagues (2006) proposed that galectin-3 has an important role in mast cell biology and revealed that mast cells of *Lgals3*^{-/-} secrete lower amounts of histamines and exhibit a reduced cutaneous anaphylaxis reaction when compared with *Lgals3*^{+/+} mice. Although our data are not conclusive concerning the role of mast cells from *Lgals3*^{-/-} mice on peritoneal B1 lymphocyte activation, we have evidence that indicates mast cell degranulation, at least in part, as a possible cellular mechanism disturbed in the absence of galectin-3 and responsible for exacerbated IgM- to IgA-class switching on peritoneal B1 lymphocytes of *Lgals3*^{-/-} mice.

During schistosomiasis, peritoneal cells react against *S. mansoni* antigens (Panasco et al. 2010). The peritoneal cavity is a reservoir of circulating leukocytes, including monocytes, T and B lymphocytes and granulocytes frequently detected attached in the omentum and mesentery (Carlow et al. 2009; Lenzi et al. 1996). Since B1a and B1b lymphocytes were directly associated with high plasmacytogenesis in

Lgals3^{-/-} infected mice, intraperitoneal plasma cell niches were studied and we found that the mesentery and omentum are widely disorganized in *Lgals3*^{-/-} mice chronically infected by *S. mansoni*. Whether the absence of galectin-3 damages the dynamics of peritoneal cell homing is uncertain but the high number of CD138⁺ plasma cells and Blimp-1⁺ immunoglobulin-secreting plasma cells indicates that galectin-3 has a relevant participation on peritoneal cell migration during inflammatory responses. In agreement with this hypothesis, perivascular galectin-3⁺ cells are frequently found in this organ and we sometimes observed cells expressing galectin-3 inside blood vessels. At least in part, we have evidence that the lack of galectin-3 modifies the scenario of moving peritoneal B cell and provokes an abnormal accumulation of plasma cells inside of the peritoneal cavity during chronic schistosomiasis.

In conclusion, we propose a novel regulatory role for galectin-3 on peritoneal B cell differentiation. Our results indicate that galectin-3 regulates a specific step of peritoneal B cell differentiation into IgA-secreting plasma cells during schistosomiasis and after IL-5 and TGF- β induction. Moreover, these data indicate that increased mast cell degranulation accounts for the higher number of IgA-producing plasma cells found in *Lgals3*^{-/-} mice. Thus, we suggest that both cell types should be investigated in future studies in order to determine the exact role of galectin-3 in regulating the cellular and molecular mechanisms for the induction of the mucosal immune response.

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