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Galectin-3 Mediates IL-4-Induced Survival and Differentiation of B Cells: Functional Cross-Talk and Implications during Trypanosoma cruzi Infection

Eva V. Acosta-Rodríguez,* Carolina L. Montes,† Claudia C. Motrán,‡ Elina I. Zuniga,† Fu-Tong Liu,‡ Gabriel A. Rabinovich,‡ and Adriana Gruppi*‡

The role of transcription factors in B cell survival and differentiation has been delineated during the last years. However, little is known about the intermediate signals and the intracellular pathways that control these events. In this study, we provide evidence both in vitro and in vivo, showing that galectin-3 (Gal-3), a β-galactoside-binding protein, is a critical mediator of B cell differentiation and survival. Although Gal-3 is not expressed in resting B cells from normal mice, its expression is markedly induced after activation with stimuli such as IL-4 and CD40 cross-linking. These signals promote survival and block the final differentiation of these cells, thus allowing the rising of a memory B cell phenotype. In addition, Gal-3 is expressed in B cells from Trypanosoma cruzi-infected mice, which received signals for activation and differentiation in vivo. By using an antisense strategy, we determined that Gal-3 is a critical signal mediating the effects of IL-4 on B cell fate. Blockade of intracellular Gal-3 in vitro abrogated IL-4-induced survival of activated B cells, favoring the differentiation toward a plasma cell pathway. Moreover, B cells with restrained endogenous Gal-3 expression failed to down-regulate the Blimp-1 transcription factor after IL-4 stimulation. Finally, inhibition of Gal-3 in vivo skewed the balance toward plasma cell differentiation, which resulted in increased Ig production and parasite clearance during T. cruzi infection. Thus, the present study provides evidence of a novel role for Gal-3 as an intracellular mediator of B cell survival and a checkpoint in IL-4-induced B cell commitment toward a memory phenotype. The Journal of Immunology, 2004, 172: 493–502.

During the development of an effective immune response against microorganisms, the immune system must accomplish two important aims to protect the host, which involve the generation of effector T and B lymphocytes able to eliminate the pathogen, and the ability to generate rapidly these effector lymphocytes after the pathogen is encountered again in the future. This function is achieved by memory cells, which are responsible for the rapid proliferation and differentiation into effector B and T lymphocytes after exposure to the same pathogen (1). Thus, memory lymphocytes allow a faster and more effective secondary response that is essential for the long-term protection of the host (2).

Within the B lymphocyte compartment, once cells encounter Ags, some of them undergo rapid clonal expansion and seed germinal centers, where somatic hypermutation, Ig isotype switching, and generation of high affinity Ag-specific B cells take place. Because the expression of high affinity Abs favors plasma cell differentiation rather than memory cell development (3), some mechanisms are required to prevent all the high affinity cells differentiating into plasma cells, allowing the generation of the memory B cell pool. In this regard, it has been reported that signals triggered by IL-4 or CD40 cross-linking induce prolonged B cell survival, while blocking plasma cell differentiation (4–6). To date, intensive research has been conducted at elucidating the intracellular pathways that regulate B cell differentiation, and several transcription factors have been reported to influence plasma cell commitment (7, 8). It has been demonstrated that the mechanism by which IL-4 and CD40 cross-linking induce differentiation involves down-regulation of Blimp-1, a transcription factor responsible for B cell apoptosis (9, 10) and essential for plasma cell commitment (7, 11). However, while some information is available about the transcription factors that play essential roles in B cell survival and differentiation and ultimately integrate signals from receptors, little is known about the intermediate signals involved in the intracellular pathways that control these events.

Recently, a family of carbohydrate-binding proteins, galectins, has attracted the attention of cell biologists and immunologists as master regulators of immune cell homeostasis and inflammation (12, 13). Based on their biochemical structure, galectins have been classified by Hirabayashi and Kasai (14) into prototype galectins (galectin (Gal)-1, -2, -5, -7, -10, -11, -13, and -14), chimera type (Gal-3), and tandem-repeat galectins (Gal-4, -6, -8, -9, and -12).

Abbreviations used in this paper: Gal, galectin; As, antisense; BI, B cells from T. cruzi-infected mice; BM, bone marrow; BN, B cells from normal mice; FCM, flow cytometry; MFI, mean fluorescence intensity; PI, propidium iodide; Synd-1, Syndecan-1.

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Gal-1, a prototype member of this family, has been reported to mediate important events in the development and effector function of B cells. This β-galactoside-binding protein has been reported to be essential for pre-BCR signaling during maturation in the bone marrow (BM) (15) and to mediate T cell apoptosis induced by activated B cells (16). In contrast, Gal-3 (29–33 kDa), the only chimeric-type member of the galectin family, has been reported to regulate the inflammatory state of different immune cell types. This lectin has been shown to potentiate LPS-induced IL-1 production by macrophages and to inhibit IL-5 gene expression in eosinophils (reviewed in Ref. 13). Moreover, expression of intracellular Gal-3, induced by TCR ligation and enhanced by cytokines such as IL-2, IL-4, and IL-7, is able to protect T cells from apoptosis (17–19). Interestingly, there is still no information about the role of Gal-3 within the B cell compartment. In the present study, we investigated the expression of Gal-3 in B cells and its role in survival and differentiation of B cells, using experimental Trypanosoma cruzi infection as a natural model of B cell activation and differentiation. The acute phase of T. cruzi infection is characterized by an extensive polyclonal activation of B cells that express high levels of class II MHC molecules, proliferate spontaneously, and secrete higher titers of Abs. Concomitant with B cell activation coexists an immunosuppressive state with enhanced apoptosis of B cells (20, 21). Using this experimental model, we have recently reported that IL-4 is not only able to rescue B cells from apoptosis, but also to block their differentiation into plasma cells (4). Therefore, this model provides an interesting scenario for studying the signals allowing survival and differentiation of the B cells.

In the present study, we provide evidence of a novel role for Gal-3 as a critical intermediate signal in IL-4-induced B cell survival, differentiation, and commitment to a memory cell phenotype.

Materials and Methods
Reagents
RPMI 1640, protease inhibitors, 2-ME, m.w. markers, LPS from Escherichia coli serotype 0127:B8, nitrocellulose membranes, and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO). Electro-phoretic reagents were from Bio-Rad (Richmond, CA). t-glutamine was purchased from Sigma-Aldrich (St. Louis, MO). Electro-

Cell preparations and culture
Splenocytes from infected or normal mice were obtained by homogenization in a tissue grinder. Erythrocytes were lysed in RBC lysis buffer (Sigma-Aldrich). For B cell purification, monocytes and T cells were depleted by magnetic cell sorting using rat IgG anti-mouse CD11b (Mac-1) Abs, followed by anti-rat IgG-coated magnetic beads and anti-Thy-1.2-coated magnetic beads (Dynal Biotech, Compiegne, France), as indicated by the manufacturer’s instructions. This procedure yielded an enriched B cell pop-

Parasitemia count
Blood was collected at day 15 postinfection. Erythrocytes were lysed in 0.87% ammonium chloride buffer, and viable trypomastigotes were counted in a Neubauer counting chamber.

In vitro antisense oligonucleotide treatment
Isolated B cells from normal (BN) or T. cruzi-infected (BI) mice were incubated in serum-free medium with a morpholino-oligonucleotide (5'-CGT TAA GCC AAA AGC TGT CTG CCA T-3') complementary to the 25 bp of mouse Gal-3 mRNA (antisense oligonucleotide) or a standard control oligonucleotide (5'-CCT CTT ACC TCA GTA ACA ATT TAT A-3') corresponding to β-globin pre-mRNA of thalassemia (control oligonucleotide) for 3 h at 37°C and 5% CO2, following the manufacturer’s instructions (www.morpholino.com). Then B cells were washed and cultured in the absence or presence of IL-4 for the indicated periods.

FCM analysis and apoptosis assays
BM cells or B cells freshly explanted or harvested after culture were washed twice in ice-cold FCM buffer (HBSS, 1% FBS, 0.1% NaN3) and preincubated with anti-mouse CD32/CD16 mAb (Fc block, clone 2.4G2) at 4°C for 30 min. Then, for surface staining, cells were incubated with PE-, FITC-, or biotin-conjugated Ab at 4°C for 30 min and washed with FCM buffer. When biotin-labeled Abs were used, a third step involving an extra 30-min incubation was performed with CyChrome-labeled streptavidin. Data were acquired on a Cytomor Absolute Absolute cytometer (Ortho Diagnostic System, Raritan, NJ) and analyzed using WinMDI 2.8 software (J. Trotter, Scripps Institute, La Jolla, CA). In all the cases, cell debris was eliminated through gating living cells from forward scatter vs side scatter dot plots and/or PI staining during the FCM analysis.

For intracellular Gal-3 detection, cells stained, as previously indicated, were fixed by incubation with CytoFix/CytoPerm solution (BD PharMingen) at 4°C for 30 min and permeabilized by two washes with Perm/Wash solution (BD PharMingen). Then cells were incubated with a 1/400 dilution of the FITC-conjugated anti-Gal-3 mAb in Perm/Wash solution at 4°C for 30 min and washed twice with FCM buffer. Data were acquired, as indicated above.

For apoptotic cell detection, PI staining was performed to analyze sub-
diploid DNA content. Briefly, harvested cells were washed twice with HBSS and fixed overnight in 1 ml 70% ethanol at 4°C. Cell pellets were gently resuspended in 1 ml hypotonic fluorochrome solution (50 μg/ml PI diluted in 4 mM sodium citrate plus 0.3% Nonidet P-40) and kept at 4°C for 18 h in the dark. Finally, cells were washed twice with FCM buffer and acquired, as indicated before.

Cell proliferation assay
B cells (2 x 10^6/well) purified from normal or T. cruzi-infected mice were cultured in quadruplicate in a volume of 200 μl in flat-bottom 96-well tissue culture plates (TPP, Trasadingen, Switzerland) for 48 or 72 h with medium alone, Gal-3 (concentrations ranging from 10 ng/ml to 20 μg/ml), or 25 ng/ml IL-4. Cells were harvested and proliferation was measured using a beta scintillation counter by incorporation of 1 μCi [3H]thymidine/well during the last 18 h of culture. Results are expressed as incorporation of radioactivity (cpm ± SD).
Measurements of Ab production

For the determination of Ig secretion, B cells were incubated in the condition indicated (see legends for figures) at a density of 2 × 10⁶ cells/ml in a volume of 2 ml. After 96 h of culture, supernatants were collected and assayed in an isotype-specific ELISA. Ninety-six-well ELISA plates were coated with 10 μg/ml of the isotype-specific goat anti-mouse Abs (IgM and IgG) diluted in PBS, overnight at 4°C, extensively washed, and blocked by the addition of 1% BSA for 1 h at room temperature. Plates were emptied, and supernatants from IL-4-stimulated or nonstimulated cell cultures were added in triplicate and incubated at 37°C for 2 h in a humidified atmosphere. After washing three times with PBS containing 0.05% Tween 20, peroxidase-conjugate anti-mouse IgG or anti-mouse IgM were added and incubated at 37°C for 1 h. The reaction was developed with o-phenylenediamine. For seric IgM and IgG determination, serum was diluted 1:100 and 1:200 in PBS, respectively, and ELISAs were performed, as above.

SDS-PAGE and Western blot

Cellular lysates were obtained following Santa Cruz Research Protocol, and nuclear extracts were obtained following the Clontech handbook (BD Biosciences, Mountain View, CA). SDS-PAGE was performed in a Mini-protein II electrophoresis apparatus (Bio-Rad). Briefly, 50 μg of cellular lysates was diluted in sample buffer and resolved on a 15 or 7.5% separating polyacrylamide slab gel for Gal-3 or IL-4R detection, respectively. A total of 100 μg of nuclear lysates was resolved on a 10% separating polyacrylamide slab gel for Blimp-1 detection. After electrophoresis, the separated proteins were transferred onto nitrocellulose membranes and probed with the corresponding primary Ab. Blots were then incubated with peroxidase-conjugated-secondary Ab and developed by using the ECL system. Prestained protein m.w. markers (Sigma-Aldrich) were run in parallel. Protein concentration was estimated by the method of Bradford, using the Bio-Rad protein assay.

In vivo antisense treatment

Mice were infected, as indicated above, and injected i.v. with the oligonucleotides (antisense and control) daily from day 8 up to 11 postinfection at doses of 7.5 mg/kg. The oligonucleotides were resuspended in saline, and a 0.2 ml final volume was injected in the mice tail vein. Infected mice injected with saline solution were assessed in parallel. Mice were sacrificed at day 15 postinfection.

RNA isolation and RT-PCR

Total RNA was extracted from B cells using TRIzol reagents (Life Technologies), according to the manufacturer’s recommendation, and resuspended in 20 μl of diethyl pyrocarbonate-treated water. The synthesis of first strand cDNA suitable for PCR amplification was performed using First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania). Briefly, a reaction mixture containing 4 μg of total RNA (template), 0.5 μg of oligo(dT)18 primer, and nuclelease-deionized water up to 11 μl was prepared on ice. The mixture was incubated at 70°C for 5 min and chilled on ice. Then the following components were added: 4 μl of 5× reaction buffer, 20 μl RNase inhibitor, 2 μl of 10 mM dNTP mix. This mixture was incubated at 37°C for 5 min. Then 40 U of Moloney murine leukemia virus reverse transcriptase was added, and the mixture was incubated at 37°C for 1 h. Finally, the reaction was stopped by heating at 70°C for 10 min. The relative quantity of cDNA of each sample was first normalized after semiquantitative PCR for β-actin. PCR mixture (25 μl) contained: 12.5 μl of 2× PCR Master Mix (TaqDNA polymerase (0.05 U/μl) in reaction buffer, 4 mM MgCl, and 0.4 mM each dNTPs) (MBI Fermentas), 0.2 μM forward and reverse primers, RNA template, and deionized water up to 25 μl. PCRs were performed on a PE 9600 (PerkinElmer, Wellesley, MA). For β-actin amplification, PCR consisted of a denaturation step of 94°C for 2 min, followed by 22 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min. For Blimp-1 and isoforms, reactions were conducted as follows: denaturation step 94°C for 2 min and 30 cycles of 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s. The following primer sequences were as follows: Blimp-1 forward and reverse primers, 5’-GAA GAA ACA ACG TGG CAA GA-3’ and 5’-AAG ACA CTT TCA GAC TGG TGC T-3’; β-actin, 5’-CCA GGT CAT CAC TAT TGG CAA CGA-3’ and 5’-GAG CAA TAA TCT CCT TCT GCA TCC-3’.

Statistical analysis

The statistical significance was analyzed using unpaired Student’s t test. Differences were considered to be significant at p < 0.05.

Results

Gal-3 is expressed in normal and infected B cells activated in the presence of differentiating stimuli

To study the role of Gal-3 on B cell survival and differentiation, we first explored expression of this lectin in resting and activated B cells previously exposed to antipapoptotic or prosurvival stimuli. For this purpose, BN or BI were cultured for 18 h in the presence of 25 ng/ml IL-4, a cytokine with known ability to promote BN and BI survival (4, 25). Cellular lysates were then obtained and Gal-3 expression was assessed by Western blot analysis. As shown in Fig. 1A, resting BN did not express Gal-3 even when cultured with IL-4. In contrast, highly activated BI expressed Gal-3 when cultured with medium alone, and, interestingly, this expression was markedly up-regulated after IL-4 stimulation. The high activation of BI was assessed by their high expression of class II MHC molecules and spontaneous proliferation (21) (data not shown). These results were further confirmed by intracelullar Gal-3 staining and FCM analysis (Fig. 1B).

Because Gal-3 is expressed in B cells from infected mice, which have naturally undergone processes of B cell activation and differentiation in vivo, we then attempted to determine whether BN could express this lectin after in vitro activation. Therefore, we determined intracelullar Gal-3 expression in BN cultured in the presence of mitogenic stimuli, such as LPS and F(ab’), anti-μ, and activation/differentiation stimuli, such as anti-CD40 and IL-4, or in the absence of any stimulus for periods ranging from 24 to 120 h. Nonstimulated BN did not express Gal-3 throughout the whole culture period (Fig. 1C). In contrast, mitogenic stimuli such as LPS and F(ab’), anti-μ were able to induce a slight increase in Gal-3 expression with a peak at 96 and 48 h, respectively. Interestingly, the highest expression of this lectin was found in BN upon culture with activation/differentiation stimuli (IL-4 and CD40 cross-linking) and at later time periods. As shown in Fig. 1C, after 2 days of IL-4 stimulation, BN expressed this lectin and its expression remained persistently high during, at least, the next 48–72 h. Similarly, anti-CD40 stimulation induced a marked up-regulation of Gal-3 expression in BN, which reached its maximum levels after 120 h of cell culture.

The delayed and highest expression of Gal-3 observed with stimuli that favor memory B cell differentiation suggested that the up-regulation of Gal-3 might be associated with late differentiation events. To test this hypothesis, BI were cultured for 96 h with IL-4 to favor differentiation to memory B cells (26, 27). In addition, to enhance plasma cell differentiation, we cultured BI with IL-2, which has been reported to induce plasma cell differentiation in BCR-activated B cells (28). After culture, expression of Synd-1 (marker of plasma cells) and intracellular Gal-3 were determined by FCM analysis. As previously reported (4), the percentage of Synd-1+ cells was greatly reduced after IL-4 stimulation relative to BI cultured with medium alone, whereas it was slightly increased after culture with IL-2 (data not shown). In this study, we observed that the percentage of BI that expressed Gal-3 increased from 10.0% when cultured in the absence of any stimulus (open dark gray histogram) up to 27.2% when stimulated with IL-4 (filled histogram) (Fig. 1D; left panel). In contrast, as shown in Fig. 1D (right panel), after IL-2 stimulation (filled histogram) only a slight increase in the percentage of Gal-3-expressing BI was observed (from 10.0 up to 16.0%). Hence, Gal-3 expression seems to be preferentially induced by a signal (i.e., IL-4) that favors memory B cell development and reduces the percentage of Synd-1+ cells.
Differential expression of Gal-3 in BN and BI in the absence or presence of IL-4 was assessed by Western blot (A) or by FCM analysis after intracellular staining (B). In A, the densitometric profile of Gal-3 expression is shown in relative arbitrary units. In B, staining with isotype control is shown as an open light gray histogram. BN were cultured for 24 up to 120 h in the presence of different stimuli and processed for intracellular Gal-3 detection by FCM. The graphics show the mean fluorescence intensity (MFI) values ± SD. MFI values of BN cultured with medium are shown as gray lines. (*, p < 0.05 and #, p < 0.01 vs BN cultured with medium). D. BI were cultured for 96 h in the presence of IL-2 or IL-4, and then stained for intracellular Gal-3 detection. The staining of B cells cultured with medium alone (light gray open histograms) is shown for comparison. Staining with isotype control is shown as black line histograms. Results are representative of five independent experiments.
the percentage of CD38<sup>B7.2</sup> memory B cells and reduce the percentage of Synd-1<sup>+</sup> plasma cells in the control oligonucleotide-treated B cells (Fig. 3A). However, when endogenous Gal-3 was inhibited by the antisense strategy, the percentage of the CD38<sup>B7.2</sup> population did not show a marked increase after IL-4 stimulation. In addition, the percentage of Synd-1<sup>+</sup> cells was not dramatically reduced after IL-4 stimulation of BN or BI treated with the Gal-3 antisense oligonucleotide. Accordingly, the absolute number of Synd-1<sup>+</sup> plasma cells was also reflected by an increase in Ig secretion, we analyzed the levels of IgM and IgG isotypes. Consistent with the increased plasma cell number, the concentrations of IgM and IgG determined in supernatants from IL-4-stimulated and antisense-treated BN and BI were significantly higher (p < 0.05) than those corresponding to their control-treated counterparts (Fig. 3B, right axis). To examine whether the rise in the absolute number of plasma cells was also reflected by an increase in Ig secretion, we analyzed the levels of IgM and IgG isotypes.

Table I. Effects of rGal-3 on BN and BI fate

<table>
<thead>
<tr>
<th></th>
<th>% Apoptotic Cells</th>
<th>% CD38&lt;sup&gt;+&lt;/sup&gt;B7.2&lt;sup&gt;+&lt;/sup&gt; Cells</th>
<th>% Synd&lt;sup&gt;+&lt;/sup&gt; Cells</th>
<th>&lt;sup&gt;3&lt;/sup&gt;H-Tm Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BN</td>
<td>BI</td>
<td>BN</td>
<td>BI</td>
</tr>
<tr>
<td>+ Medium alone</td>
<td>11.5 ± 0.2</td>
<td>29.6 ± 0.6</td>
<td>1.7 ± 0.2</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>+ rGal-3 (10 ng/ml)</td>
<td>14.7 ± 0.4</td>
<td>28.6 ± 0.5</td>
<td>NA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>+ rGal-3 (25 ng/ml)</td>
<td>12.1 ± 0.2</td>
<td>29.1 ± 0.6</td>
<td>2.1 ± 0.3</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>+ rGal-3 (50 ng/ml)</td>
<td>18.5 ± 0.3</td>
<td>29.0 ± 0.5</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>+ IL-4 (25 ng/ml)</td>
<td>5.9 ± 0.5</td>
<td>16.9 ± 0.4</td>
<td>6.0 ± 0.5</td>
<td>11.2 ± 0.4</td>
</tr>
<tr>
<td>+ rGal-3 (10 ng/ml) + IL-4 (25 ng/ml)</td>
<td>14.7 ± 0.4</td>
<td>28.6 ± 0.5</td>
<td>NA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>+ rGal-3 (25 ng/ml) + IL-4 (25 ng/ml)</td>
<td>12.1 ± 0.2</td>
<td>29.1 ± 0.6</td>
<td>2.1 ± 0.3</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>+ rGal-3 (50 ng/ml) + IL-4 (25 ng/ml)</td>
<td>18.5 ± 0.3</td>
<td>29.0 ± 0.5</td>
<td>NA</td>
<td>NA</td>
</tr>
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</table>

<sup>a</sup> NA, Not assayed.

Mechanisms underlying the role of Gal-3 on IL-4-induced B cell differentiation: participation of the Blimp-1 transcription factor

Because blockade of endogenous Gal-3 was able to prevent IL-4-induced B cell differentiation, we next analyzed the mechanisms underlying this effect. First, we tested whether inhibition of endogenous Gal-3 resulted in down-regulation of IL-4R. To address this issue, BN and BI were first exposed to control or Gal-3 antisense oligonucleotide and then cultured for 48 h with medium alone or in the presence of IL-4. Cellular lysates were then analyzed for IL-4R detection by Western blot analysis. As shown in Fig. 4A, untreated BN expressed IL-4R, and this expression was up-regulated after IL-4 stimulation. Moreover, the same effect was observed in BN treated with control or Gal-3 antisense oligonucleotide. In contrast, untreated BI expressed higher levels of IL-4R than BN, but this expression was not clearly up-regulated after IL-4 stimulation. A possible explanation for this result is that, in activated BI, the transcription of the IL-4R gene is intrinsically so high that it cannot be further enhanced even after IL-4 stimulation. Importantly, however, treatment of BI with control or Gal-3 antisense oligonucleotide did not affect IL-4R expression in nonstimulated or IL-4-stimulated conditions. Thus, down-regulation of the IL-4R does not seem to be an evident mechanism underlying the attenuated effects of IL-4 on B cells with abolished Gal-3 expression.

Because IL-4 is able to block plasma cell differentiation of activated cells by down-regulating Blimp-1 expression (9), we next studied whether inhibition of endogenous Gal-3 could influence IL-4-induced down-regulation of Blimp-1. Nuclear lysates of BI left untreated or treated with control or Gal-3 antisense oligonucleotides were obtained and assessed for Blimp-1 detection by Western blot. In agreement with a previous report in activated B cells (9), we determined that untreated BI express Blimp-1 and that IL-4 down-regulates this expression (data not shown). Similar results were observed in control-treated BI, which readily expressed Blimp-1 when cultured with medium alone, and this expression was down-regulated after culture in the presence of IL-4. More importantly, reduction in Blimp-1 expression after IL-4 stimulation was no longer observed in BI exposed to the Gal-3 antisense treatment (Fig. 4B). Furthermore, these results were confirmed by studying Blimp-1 mRNA levels by RT-PCR (Fig. 4C).

Therefore, the inability of BI to down-regulate Blimp-1 expression upon IL-4 stimulation following treatment with Gal-3 antisense oligonucleotide may explain why IL-4 is unable to block plasma cell differentiation in these cells.

Functional cross-talk between IL-4 and Gal-3 during in vivo T. cruzi infection

Our results in vitro clearly argue for a role of Gal-3 in IL-4-induced survival and differentiation of B cells. These results prompted us to study whether Gal-3 plays such a decisive role in vivo. For this purpose, we took advantage of experimental T. cruzi infection, an animal model of Chagas’ disease, in which several signals different from IL-4 also take place. Mice were divided into four groups of: 1) normal noninfected mice (numbers 1 and 2); 2) infected mice treated with PBS (numbers 3–6); 3) infected mice treated with a control oligonucleotide (numbers 7 and 8); and 4) infected mice treated with Gal-3 antisense oligonucleotide (numbers 9–12). The injection schedule (daily from days 8 and 11 postinfection) was selected to match the peak of the germinal center reaction (usually between days 9 and 10 postinfection) (30) (our unpublished observations). In all the experiments, mice were sacrificed at day 15 postinfection.

By Western blot analysis, we observed decreased Gal-3 expression in splenic B cells from mice treated with the Gal-3 antisense oligonucleotide (Fig. 5A). To assess the functional significance of the down-regulated Gal-3 expression, we next determined by FCM the percentage of Synd-1<sup>+</sup> cells in the spleen and BM of the different groups of mice. Although we observed no differences in the percentages of splenic plasma cells among the different groups of mice (data not shown), the percentage of Synd-1<sup>+</sup> cells in the BM from infected mice treated with the Gal-3 antisense oligonucleotide was augmented >2-fold compared with the population of infected mice that received control oligonucleotides or PBS (16.7% vs 6.9 and 6.0%) (Fig. 5B). Because the absolute numbers of splenic and BM cells were the same in all infected groups independent of the oligonucleotide injected (data not shown), our data indicate that the antisense treatment induced an increase in the absolute number of plasma cells in BM. These results suggest that the inhibition of endogenous Gal-3 by antisense treatment during...
acute T. cruzi infection favors plasma cell formation, leading to the accumulation of these cells in plasma cell-homing sites (e.g., BM).

Looking for the mechanism underlying the increase in plasma cell number, we next studied by RT-PCR the mRNA levels of Blimp-1. Even though we observed no increase in the number of splenic Synd-1 cells in infected mice that were treated with the Gal-3 antisense oligonucleotide, Blimp-1 mRNA expression was greatly enhanced (both in native and Δ7 isoforms) in splenic B cells from the same mice (Fig. 5C). This result is not surprising because it has been reported that populations of Blimp-1− Synd-1− cells (plasma cell committed precursors) are present in splenic germinal centers, but once formed they do not remain in these sites (7). Hence, up-regulation of Blimp-1 mRNA in splenic B cells would further support the enhanced plasma cell differentiation observed in antisense (As)-treated mice.

Considering the results about plasma cell numbers, we next tested whether Gal-3 antisense treatment resulted in increased Ig levels. For that, the concentration of IgM or IgG was determined by ELISA in the serum of each mouse. As expected for infected mice, the levels of IgM or IgG in infected mice treated with control oligonucleotide were significantly higher than in normal mice (Fig. 5D). Moreover, no differences were found between PBS-treated infected mice and infected mice injected with control oligonucleotides. Furthermore, and accordingly with their higher percentage of Synd-1− cells in BM, infected mice treated with Gal-3 antisense oligonucleotide presented significantly increased concentrations of IgM and IgG compared with the other two groups of infected mice (Fig. 5D). Considering these results and the fact that parasite clearance in T. cruzi-infected mice largely depends on Abs, we finally studied the effect of antisense treatment in the levels of parasitemia. As expected, we observed no significant differences in the parasitemia of infected mice treated with PBS or control oligonucleotides (Fig. 5E). However, parasite levels in sera from infected mice treated with Gal-3 antisense oligonucleotide were markedly decreased in comparison with infected mice that received control oligonucleotides or PBS (p < 0.05).

Data presented in this work indicate that inhibition of Gal-3 in vivo by antisense treatment induces an up-regulation of Blimp-1 mRNA expression in splenic B cells from infected mice. Up-regulation of this transcription factor might promote plasma cell differentiation and may be responsible for the increased number of plasma cells in the BM of infected mice. Moreover, the consequence of enhanced plasma cell differentiation by Gal-3 inhibition may be reflected by higher Ig secretion and accelerated parasite clearance.

Discussion

Understanding the mechanisms of lymphocyte differentiation is of vital importance for immune regulation. We have previously described that IL-4 is able to trigger signals, which modulate two different aspects of B cell fate. While this cytokine reduces the high degree of apoptosis observed in B cells from parasite-infected mice, it also blocks the differentiation of these cells to plasma cells, favoring the development of a memory B cell phenotype (4). As well as for CD40 cross-linking (10), a down-regulation of the transcription factor Blimp-1 has been proposed to be a mechanism underlying IL-4-induced blockade of plasma cell differentiation (9). Moreover, because Blimp-1 is also a proapoptotic molecule (31), its down-regulation may also be responsible for IL-4-induced B cell survival. Even though several surface receptors and intracellular transcription factors that regulate B cell fate have been described in the last decades, it still represents a challenge for B cell immunologists to elucidate the way membrane-derived signals can integrate and trigger different intracellular pathways that, in
FIGURE 3. In vitro treatment with Gal-3 antisense oligonucleotides inhibits IL-4-induced differentiation of BN or BI. BN or BI were treated with control or Gal-3 As oligonucleotide for 3 h and then cultured with medium alone or in the presence of IL-4. After 96-h culture, cells were stained anti-mouse CD38-FITC, B7.2-Cy, and Synd-1-PE. The regions in the two-color dot-plot graphics show the percentage of the memory (CD38 high B7.2 high) cells. In the histograms, M1 shows the percentage of plasma (Synd-1 +) cells. Results are representative of five independent experiments (B). IgM (light gray bars) and IgG (dark gray bars) concentrations were determined by ELISA in 96-h culture supernatants. The graphics show the triplicate mean OD ± SD. The absolute numbers of Synd-1 + cells (see text for details) are also shown (•) (∗, p < 0.05 vs control oligonucleotide-treated B cells). Data are representative of three independent experiments.
turn, regulate activation of specific transcription factors that control B cell survival and differentiation.

We report in this work that intracellular Gal-3, which has been previously implicated in several immunoregulatory processes, is expressed in BI that received important signals for activation and differentiation in vivo. In addition, while Gal-3 is not expressed in resting BN, it is markedly up-regulated after the cells are activated with stimuli such as IL-4 and CD40 cross-linking. Interestingly, both of these stimuli have been shown to induce B cell survival and block the final differentiation of these cells, allowing the rising of the memory B cell pool (4, 9, 26). Furthermore, by inhibiting endogenous Gal-3 with an antisense approach, we determined that this lectin is a critical mediator of the effects of IL-4 on B cell fate. Thus, the antiapoptotic effect of IL-4 was markedly blunted on BI that were treated to have their Gal-3 expression suppressed, and IL-4 was no longer able to drive these cells into a memory B cell phenotype or to block their plasma cell commitment. Moreover, the fact that IL-4 stimulation failed to down-regulate Blimp-1 in B cells with restrained endogenous Gal-3 expression argues for a role of endogenous Gal-3 in IL-4-induced B cell differentiation. In this regard, recent reports suggest a novel function for nuclear Gal-3 in the regulation of gene transcription (32). Hence, it is likely that this lectin, which has been reported to bind ssDNA and RNA (33, 34), may enhance and/or stabilize DNA-nuclear protein complex formation in various promoters, allowing their positive (32, 35) or negative (36) regulation. Therefore, we cannot exclude the possibility that similar mechanisms may take place in our system; however, the precise mechanisms by which Gal-3 regulates Blimp-1 expression are subject of current research.

Because Blimp-1 is also a proapoptotic transcription factor, the effect of Gal-3 in mediating IL-4-induced Blimp-1 down-regulation could argue for its antiapoptotic or prosurvival effect. However, we cannot rule out that other mechanisms may be involved in this effect. In this regard, Gal-3 has been reported to inhibit the apoptosis of several cell populations through diverse mechanisms. Moreover, it has been reported that this lectin can interact with Bcl-2 (18) and stabilize mitochondrial membrane integrity. Accordingly, recent reports (19, 37) indicate that this lectin prevents mitochondria damage and cytochrome c release. In contrast, because inhibition of Gal-3 by antisense oligonucleotides only reduced, but did not abolish IL-4-induced B cell survival in vitro, we postulate that other mediators or intermediate signals also may be involved in the antiapoptotic effect triggered by IL-4 (38). In this line, our in vivo results showed no differences in the levels of apoptosis between T. cruzi-infected mice treated with antisense or control oligonucleotides (data not shown), suggesting that, in vivo, other antiapoptotic molecules might compensate for Gal-3 inhibition.

In summary, we showed in this study for the first time that Gal-3 is expressed in activated B cells and is up-regulated after specific stimuli. Moreover, we determined that this lectin plays an important role in IL-4-induced survival and differentiation of B cell from mice infected with the protozoan T. cruzi. In addition, the inhibition of Gal-3 during the acute phase of T. cruzi infection allowed an enhanced plasma cell development that resulted in higher levels of Igs and more effective parasite clearance. Finally, even when much work remains to be done, the present study provided some insights into the complex intracellular pathways governing B cell fate decisions. Further understanding of these processes will provide a rational basis for designing novel vaccination protocols and therapeutic approaches for a more effective immunity against pathogens.
FIGURE 5. Blockade of Gal-3 in vivo alters B cell differentiation, plasma cell number, Ig secretion, and parasite clearance in an experimental model of T. cruzi infection. T. cruzi-infected mice were treated in vivo, as described in Materials and Methods, with PBS (mouse 3–6), control oligonucleotides (mouse 7 and 8), or antisense Gal-3 oligonucleotides (mouse 9–12). Normal mice (mouse 1 and 2) were assessed in parallel. On day 15 postinfection, mice were sacrificed and processed, as follows (A): cell lysates of splenic B cells from each mouse were assessed for Gal-3 expression by Western blot. The densitometric profile of Gal-3 expression is shown in relative arbitrary units. B, BM from each mouse was obtained, stained for Synd-1 expression, and analyzed by FCM. The histogram of one representative mouse of each group is shown, and M1 indicates Synd-1<sup>+</sup> cells. Mean percentages of Synd-1<sup>+</sup> cells and the MFI were calculated for each group and are shown together with their SD. C, Total RNA was obtained from splenic B cells isolated from each mouse and analyzed for the presence of Blimp-1 mRNA and its corresponding isoforms by RT-PCR. β-actin was used as internal control of RNA integrity and equal loading. D, Sera from different groups of mice were collected for determination of IgM (upper panel) and IgG (lower panel) concentrations by ELISA. (IgM: #, p < 0.005 vs normal; *, p = 0.00005 vs normal; †, p = 0.00002 vs infected + control oligonucleotide; IgG: ##, p < 0.03 vs normal; **, p = 0.0001 vs normal; ††, p = 0.00001 vs infected + control oligonucleotide.) E, Viable parasites (trypomastigotes/ml) in blood of infected mice were counted in a Neubauer camera (#, NS vs infected + PBS; *, p < 0.05 vs infected + PBS; †, p < 0.05 vs infected + control oligonucleotide).
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References