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ORIGINAL ARTICLE

The aggressiveness of murine lymphomas selected in vivo by growth rate correlates with galectin-1 expression and response to cyclophosphamide

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Abstract Although lymphomas account for almost half of blood-derived cancers that are diagnosed each year, the causes of new cases are poorly understood, as reflected by the relatively few risk factors established. Galectin-1, an immunoregulatory ß-galactoside-binding protein, has been widely associated with tumor-immune escape. The aim of the present work was to study the relationship between tumor growth rate, aggressiveness, and response to cyclophosphamide (Cy) therapy with regard to Gal-1 expression in murine T-cell lymphoma models. By means of a disruptive selection process for tumor growth rate, we generated two lymphoma variants from a parental T-cell lymphoma, which have unique characteristics in terms of tumor growth rate, spontaneous regression, metastatic capacity, Gal-1 expression and sensitivity to Cy therapy. Here, we show that Gal-1 expression strongly correlates with tumor growth rate, metastatic capacity and response to single-dose Cy therapy in T-cell lymphoma models; this association might have important consequences for evaluating prognosis and treatments of this type of tumors.

Keywords Lymphoma · Galectin-1 · Aggressiveness · Cyclophosphamide

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Introduction

Lymphomas account for almost half the blood-derived cancers that are diagnosed each year. The incidence of NHL is 19.6 per 100,000 persons [1]. The causes of new lymphoma cases are poorly understood, as reflected by the relatively few risk factors established [2].

Galectins are a family of β-galactoside-binding proteins characterized by the presence of at least one carbohydrate recognition domain (CRD) that recognizes *N*-acetyllactosamine structures on *N*- and *O*-glycans [3]. These lectins have emerged as regulators of immune cell homeostasis, inflammation and cancer [3]. Galectin-1 (Gal-1), a "prototype" member of this family, regulates adaptive immune responses by controlling T-cell survival, cytokine secretion, transendothelial migration and dendritic cell (DC) physiology [3–6]. In addition, Gal-1 has been regarded as a key effector of the immunosuppresive activity of $CD4^+CD25^+$ regulatory T cells [7]. Administration of Gal-1 suppresses chronic inflammation and skews the balance of the immune response toward a T_H2 cytokine profile by selectively

eliminating the T_H1 and T_H17 subsets [3, 8]. In addition, selective blockade of Gal-1 within tumor tissue results in increased T_H1-mediated antitumor responses, suggesting a potential role of this lectin in tumor-immune escape in models of melanoma, Hodgkin lymphoma and lung carcinoma [9–13]. Moreover, siRNA-mediated Gal-1 silencing sensitizes tumor cells to chemotherapeutic agents [14, 15].

We have previously demonstrated in the L-TACB rat lymphoma model that Gal-1 expression positively correlates with the primary tumor volume and that an anti-metastatic single low dose of cyclophosphamide (Cy) down-regulates Gal-1 expression to levels commonly found during early stages of tumor growth [16]. In addition, recent evidence indicates that AP1-dependent production of Gal-1 by Hodgkin lymphoma cells fosters an immune-privileged microenvironment by favoring the expansion of T_H2 and T regulatory cells [10]. In addition, the AP-1-dependent expression of Gal-1 serves as a diagnostic biomarker to discriminate between classical Hodgkin and anaplastic large cell lymphoma from other type of lymphoid malignancies [11].

Tumor growth rate is widely used for prognostic purposes and is indicative of therapeutic responses to different treatment modalities [17–23]. Theoretically, exponential growth is due to tumor cells duplicating during cell cycle. When a tumor becomes larger, its growth rate often decreases and changes to a non-exponential growth model, as illustrated by the Gompertzian model [24]. However, in clinical studies, volume estimations of non-treated tumors are usually available only for short measurement time intervals, and tumor growth may be well described by an exponential model. Tumor growth rate is usually characterized by the tumor volume doubling time (TvDT), a term that was introduced more than 50 years ago by Collins et al. [25].

The present work was conducted to study how Gal-1 expression and response to Cy therapy correlate with tumor growth rate and aggressiveness in murine lymphoma. For this, we generated by means of a disruptive selection process, two lymphoma variants from a parental T-cell lymphoma that differed in tumor growth rate and metastatic capacity. We found that Gal-1 expression is highly associated with tumor growth rate and metastatic capacity, thus emphasizing the relevance of the Gal-1-glycan axis as a therapeutic target in these tumor types.

Materials and methods

Animals

Ten-week-old female BALB/c mice were housed and cared at the animal facilities of the Institute of Experimental Genetics, School of Medical Sciences, National University of Rosario. Animals were fed with commercial chow and water ad libitum and were maintained in a 12-h light/dark cycle. All the experiments were performed during the first half of the light cycle in accordance with institutional animal care standards, which comply with guidelines issued by the Canadian Council on Animal Care.

Tumor

L-DGE is a poorly differentiated murine immunoblastic T-cell lymphoma that spontaneously arose in a BALB/c female mouse in 1985. It was maintained by serial s.c. implantation of 1 mm^3 tumor fragments in the right flank of syngeneic hosts. It metastasizes exclusively to regional lymph nodes (LN) at low frequency [26].

Protein extracts preparation

Protein extracts from primary tumors and the respective axillary LN metastases were prepared in lysis buffer [2 mM EDTA (Sigma), 4 mM EGTA (Sigma), 10 mM β-mercaptoethanol (BioRad), complete Protease Inhibitor Cocktail (Roche) in 20 mM Tris–HCI; pH 7.5 at 4°C]. Protein cell lysates were centrifuged at 2,500 × g for 10 min at 4°C. Protein concentration was determined using the Proti-ULC/R method in duplicates (Wiener Lab, Rosario, Argentina).

Western blot analysis

Equal amounts of protein (10 μg of protein per lane) from lysates of primary tumors or axillary LN metastases were resolved by SDS–PAGE as described elsewhere [9, 10]. Equal loading was checked by stripping membranes and probing them with anti-β-actin (1:5000, Sigma) or Ponceau S staining. Films were analyzed with Scion Image software. Results were expressed as relative densitometric values.

Drugs

Cyclophosphamide (Cy) (Laboratorios Filaxis, Argentina) was dissolved in sterile saline to a concentration of 20 mg/ml and injected at a dose of 250 mg/kg of body weight when tumors reached a volume of approximately 0.7 cm^3.

Tumor growth rate and exponential tumor growth and decay

Tumor size was measured thrice weekly from day 5, and tumor volume was estimated according to the formula [$V = (\text{minor diameter})^2 \times \text{major diameter} \times 0.4$]. The data

"Volume versus Time" were adjusted with an exponential curve [$V_t = V_i.e^{(k.t)}$], where V_i is the initial tumor volume, V_t is the tumor volume at a t time, t is the days of tumor growth and k is the exponential growth rate. The tumor volume doubling time (TvDT) was obtained as TvDT = 0.69/k. Cy-induced exponential tumor regression was fit to an exponential decay curve [$V_t = S.e^{(-k.t)}$], where S is a constant and k is the exponential decay constant.

Generation of two different variants of L-DGE mouse T-cell lymphoma by disruptive selection

L-DGE was s.c. implanted by trocar on 12 BALB/c mice (day 0). On day 14, the mouse showing the highest tumor growth rate was chosen as donor, the tumor excised and implanted into six mice. This criterion was maintained for 25 serial passages, leading to the generation of the L-DGE/M tumor variant. On day 21 of L-DGE tumor evolution, the mouse bearing the tumor with the lowest growth rate was chosen as donor and the tumor was excised and implanted into eight mice. This criterion was maintained for 16 serial passages, approximately every 24 days, leading to the generation of L-DGE/L tumor variant.

Evaluation of tumor growth rate, spontaneous tumor regression and metastatic capacity of L-DGE, L-DGE/M and L-DGE/L

BALB/c mice were s.c. challenged with L-DGE, L-DGE/M or L-DGE/L ($n = 20$/group). From day 5 onward, tumor size was measured thrice weekly, tumor volume was estimated and TvDT was calculated. The animals were euthanized when they reached the maximum tumor volume allowed (V_{MAX}). Occurrence of lymph node metastases was checked by palpation during tumor growth and histopathologically when mice were euthanized and metastases excised. Tumor and LN metastases were excised in order to assess Gal-1 expression. When mice showed spontaneous tumor regression, animals were euthanized 1 week after complete regression and the absence of tumor was confirmed during the necropsy.

Time-course study of Gal-1 expression during tumor progression

Kinetics of Gal-1 primary tumor expression was studied in L-DGE and its variants, L-DGE/M and L-DGE/L. Animals were challenged subcutaneously with the tumor on day 0 ($n = 15$/tumor variant) and were euthanized on day 7 and on the last day of tumor evolution (V_{MAX}). Primary tumors were excised, protein homogenates prepared from each sample and Gal-1 expression determined by Western blot analysis.

Quantitative Gal-1 mRNA expression in L-DGE and L-DGE/M primary tumor and homolateral lymph nodes by qRT-PCR

Mice bearing L-DGE or L-DGE/M ($n = 4$/group) were euthanized on day 14 of tumor evolution, and primary tumor and homolateral axillary LN were excised. Total RNA was prepared using TRIzol (Invitrogen). Quantification of Gal-1 mRNA was achieved using the absolute quantification method. Transcript expression was normalized to GAPDH in each sample. The real-time quantitative PCR was performed with the SYBR Green PCR Master Mix (Applied Biosystem) in an ABI PRISM 7500 Sequence Detection Software (Applied Biosystem). Primers used were the following: mouse *Lgals1* forward: 5′-TGA ACCTGGGAAAAGACAGC-3′; mouse *Lgals1* reverse: 5′-TCAGCCTGGTCAAAGGTGAT-3′.

Analysis of the frequency of $CD4^+CD25^+FoxP3^+$ T_{reg} cells in homolateral lymph nodes of L-DGE-, L-DGE/M- and L-DGE/L-bearing mice

On day 14 of tumor evolution, mice bearing L-DGE, L-DGE/M or L-DGE/L ($n = 4$/tumor variant) were euthanized, homolateral LN were excised and a cellular suspension was prepared. A total of 10^6 cells were stained with anti-CD4-FITC (eBioscience, San Diego, CA) and anti-CD25-PE (eBioscience). After permeabilization, anti-FoxP3-PE-Cy5 (eBioscience) was added. As a control, right flank lymph node cells of normal mice were used.

Effect of a non-toxic single dose of Cy on tumor evolution, metastatic capacity and Gal-1 serum levels

Mice were implanted s.c. with L-DGE, L-DGE/M or L-DGE/L ($n = 20$/group by trocar) on day 0. Half of the animals were i.p. inoculated with Cy (250 mg/kg) when the median tumor volume of each lymphoma variant was approximately 0.7 cm^3 (treated group) and the remaining mice received an i.p. saline injection (control group). Mice were euthanized when tumors reached the V_{MAX} or after spontaneous or Cy-induced tumor regression. Sera from both groups were obtained at day 0 and when mice were euthanized.

Gal-1 ELISA

Soluble Gal-1 was determined using an in-house ELISA. Briefly, high binding 96-well microplates (Corning Costar) were coated with capture antibody (2 μg/ml purified rabbit anti-Gal-1 polyclonal IgG) in 0.1 M sodium carbonate, pH 9.5. After incubation for 18 h at 4°C, wells were rinsed three times with wash buffer (0.05% Tween-20 in PBS)

and incubated for 1 h at RT with blocking solution (2% BSA in PBS). Samples and standards (100 µl) were diluted in 1% BSA and incubated for 18 h at 4°C. Plates were washed and incubated with 100 ng/ml biotinylated anti-Gal-1 polyclonal IgG for 1 h at RT and then rinsed 3 times. Finally, horseradish peroxidase-labeled streptavidin (0.33 µg/ml; Sigma) was added followed by TMB solution (0.1 mg/ml tetramethylbenzidine and 0.06% H_2O_2 in citrate–phosphate buffer pH 5.0) and 4 N H_2SO_4 to stop the reaction. A standard curve ranging from 2.5 to 160 ng/ml recombinant Gal-1 was run in parallel.

Histopathology

Primary tumors and LN node metastases were fixed in Bouin's solution, dehydrated, embedded in paraffin, sectioned at 5 µm thickness and stained with hematoxylin–eosin. Microscopic analysis allowed the detection of metastatic micro- and macro-colonies.

Statistical analysis

Statistical comparison between two groups was made using Mann–Whitney's *U* test. Goodness of fit was assessed by means of runs test. Fischer's exact test was used for the evaluation of contingency tables. Survival analyses were carried out with Kaplan–Meier curves and logrank test. Correlation studies were performed by means of Spearman's rank correlation test. All the statistical analyses were performed using GraphPad Prism® version 3.03 (Graph-Pad Software, San Diego, CA). Differences between groups were considered significant when $P < 0.05$.

Results

Establishment of lymphoma variants by a disruptive selection process

The selection process by which L-DGE/M and L-DGE/L variants of L-DGE lymphoma were generated lasted until these lymphomas showed stable growth rate following consecutive serial passages. Following this process, we characterized the growth of these tumors in syngeneic BALB/c mice in comparison with that of the parental L-DGE lymphoma. BALB/c mice were s.c. challenged with L-DGE, L-DGE/M or L-DGE/L (20 mice per group) to assess tumor growth rate, metastatic capacity and spontaneous tumor regression. Tumor growth was adjusted to an exponential growth curve, except for those that spontaneously regressed. The application of runs test confirmed non-deviation from the curve.

Tumor growth in selected lymphoma variants

L-DGE/M and L-DGE/L growth was more uniform, specifically had lower variance, than that observed in L-DGE (Fig 1a). L-DGE showed a median TvDT of 3.34 days (range: 1.90–5.31), whereas the faster growing variant L-DGE/M, as expected, showed a lower median TvDT of 2.49 days (range: 1.33–3.68) and L-DGE/L, the lymphoma selected for slower growth rate, showed a higher median TvDT of 4.29 days (range: 2.83–5.20), being these differences statistically significant (Kruskal–Wallis; $P < 0.0001$) (Fig. 1c).

Spontaneous tumor regression of different lymphoma variants selected by growth rates

L-DGE, when s.c. injected into BALB/c mice, followed two different growth patterns. This tumor either grew exponentially until reaching the V_{MAX} or spontaneously regressed, a process that ended with tumor elimination. When mice were s.c. challenged with L-DGE, L-DGE/M or L-DGE/L, tumors behaved in a different way. The most aggressive variant, L-DGE/M, showed the lowest regression percentage (13%), while 26 and 32% of L-DGE and L-DGE/L tumors, respectively, regressed ($P = 0.0054$) (Fig. 1d).

Comparison of the metastatic capacity of lymphoma variants selected by different growth rates

L-DGE is a T-cell lymphoma that metastasizes to draining LN at low frequency. The selection process that led to the generation of L-DGE/M, the faster growing lymphoma variant, also had an effect on its metastatic capacity. When mice were s.c. challenged with L-DGE/M, 75% developed LN metastases which, in many cases, affected not only the homolateral axillary LN (the primary metastasis site), but also contralateral axillary and inguinal LN. On the contrary, L-DGE was able to develop metastases in 25% of the animals, whereas L-DGE/L showed an even lower metastatic capacity (11%). Statistically, the three tumor variants differed in their metastatic capacity ($P < 0.0001$) (Fig. 1e). Figure 2 depicts the presence of metastatic cells in homolateral draining LN of mice bearing L-DGE, L-DGE/L and L-DGE/M.

Comparison of the survival rates of mice bearing tumors selected by different growth rates

In accordance with their increased tumor growth rate, enhanced metastatic capacity and lower spontaneous regression, L-DGE/M-bearing mice showed the shortest median survival time, 15 days, whereas L-DGE-bearing

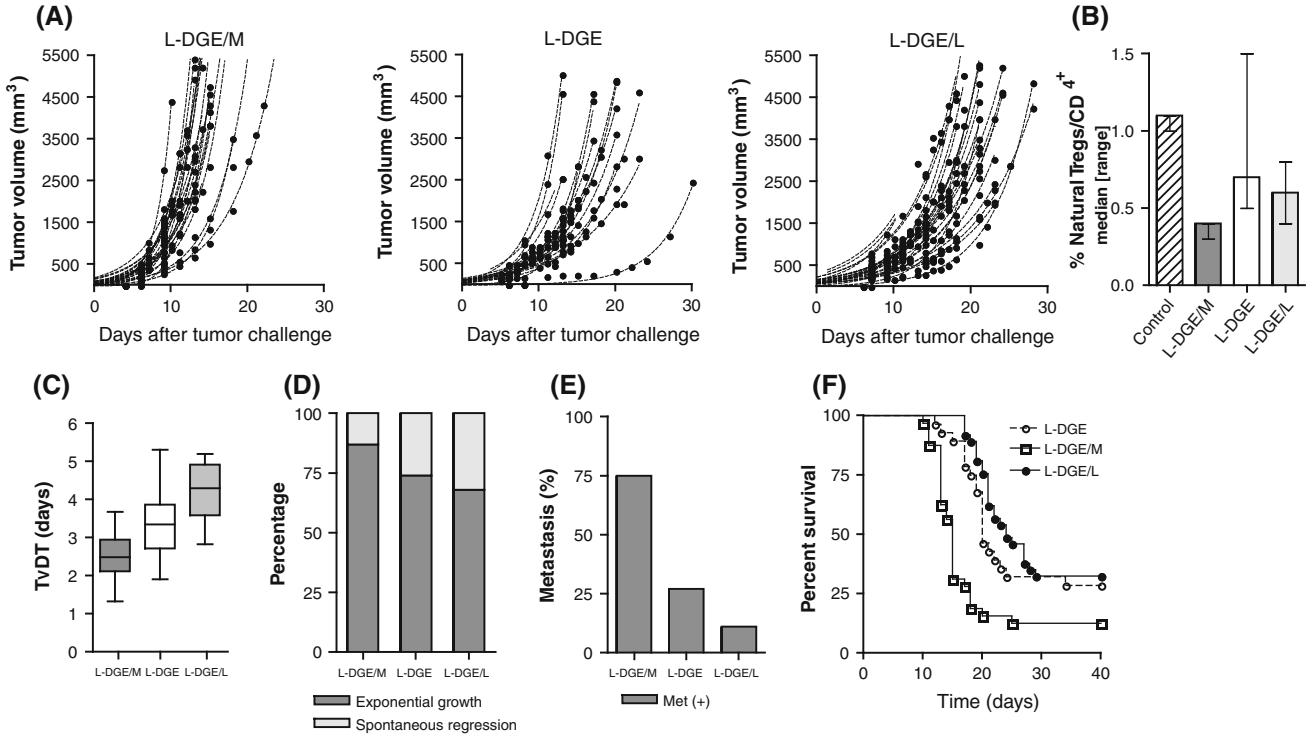

Fig. 1 Characterization of lymphoma L-DGE and its selection-derived variants, L-DGE/M and L-DGE/L. **a** Each tumor that grew until reaching the V_{MAX} was adjusted to an exponential curve. Spontaneously regressing tumors are not shown. **b** Percentage of T_{reg} cells with respect to total $CD4^+$ lymph node lymphocytes: normal versus lymphoma-bearing mice (Kruskal–Wallis, $P = 0.0539$; normal versus L-DGE/M, $P < 0.05$). **c** TvDT for each tumor was obtained from the exponential regression equation (Kruskal–Wallis, $P < 0.0001$; L-DGE versus L-DGE/M and L-DGE versus L-DGE/L, $P < 0.05$; L-DGE/M versus L-DGE/L, $P < 0.001$). **d** The proportion of tumors that grew or spontaneously regressed differed among the three lymphomas (contingency table, $P = 0.0054$). **e** Metastatic capacity: Percentage of mice that developed spontaneous metastases with respect to the number of mice with growing tumors (contingency table; $P < 0.0001$). **f** Survival time of lymphoma-bearing mice (logrank test, $P < 0.0001$)

mice had a survival time of 20 days and L-DGE/L-bearing mice survived for 24 days ($P < 0.0001$) (Fig. 1f).

Analysis of the frequency of $CD4^+CD25^+FoxP3^+$ T_{reg} cells in mice bearing selected tumor variants

T_{reg} cells, either naturally occurring or inducible, are $CD4^+$ T cells characterized by surface expression of CD25 and intracellular expression of the transcription factor FoxP3. These cells play an important role in peripheral tolerance and suppress T-cell proliferation and cytokine production [27]. As their modulation may influence tumor evolution and escape [28], we analyzed the frequency of T_{reg} cells in the three lymphoma variants. BALB/c mice were s.c. challenged with L-DGE, L-DGE/M or L-DGE/L and euthanized on day 14, when L-DGE/M tumors reach the V_{MAX}. Although we expected an expansion of LN T_{reg}-cell population during tumor evolution, the percentage of T_{reg} cells with respect to the total $CD4^+$ T-cell population was lower in lymphoma-bearing mice than in normal

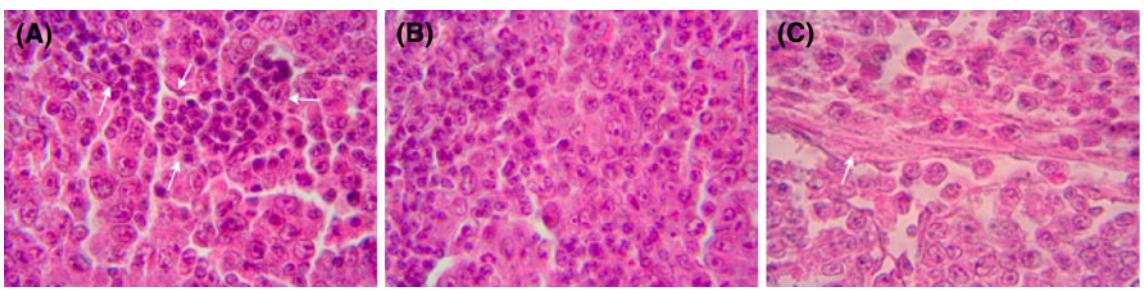

Fig. 2 Spontaneous axillary lymph node metastases (100X) of: **a** L-DGE: *the arrow* shows a group of normal lymphocytes surrounded by metastatic tissue, **b** L-DGE/L, **c** L-DGE/M: detail of the capsule (*arrow*) that encloses the lymph node, with metastatic cells on both sides

BALB/c mice (%T_{reg}/$CD4^+$: normal 1.1%, L-DGE/M 0.4%, L-DGE 0.7% and L-DGE/L 0.6%, Kruskal–Wallis; $P = 0.0539$) (Fig. 1b). The data in L-DGE tumor-model were highly variable, paralleling the high variance observed when analyzing L-DGE tumor growth, while such a behavior was less evident in L-DGE/M and L-DGE/L tumor variants.

Expression of Gal-1 in primary tumors is directly associated with tumor growth rate

Previous studies showed that Gal-1 expression correlates with growth, escape and aggressiveness of a variety of tumors [9, 10, 29]. Expression of Gal-1 in primary tumors and in L-DGE/M and L-DGE spontaneous LN metastases was determined. Gal-1 expression was assessed at V_{MAX} day on primary tumors. Expression of this endogenous lectin in in vivo-selected primary lymphomas correlated with tumor growth rate and aggressiveness; the highest expression was found in L-DGE/M, the lowest in L-DGE/L, while L-DGE showed intermediate values (Kruskal–Wallis, $P = 0.0001$) (Fig. 3a). Thus, the new lymphoma variants generated by disruptive selection were characterized by expressing Gal-1 at different levels in direct correlation with their growth rate.

In contrast to down-regulation of Gal-1 previously found in spontaneous LN metastases of L-TACB rat B-cell lymphoma [16], expression of this lectin in metastases of L-DGE and L-DGE/M did not differ from that found in the corresponding primary tumors (Fig. 3b). To further analyze this regulation, we also studied Gal-1 mRNA in L-DGE and L-DGE/M primary tumors and LN metastases. Surprisingly, we found that Gal-1 mRNA levels were 0.34 times lower in primary tumors of L-DGE/M when compared to those found in L-DGE, whereas in draining LN, Gal-1 mRNA levels were 2.49 times higher (Fig. 3c), suggesting changes at both transcriptional and transductional levels in Gal-1 expression in both primary tumor and

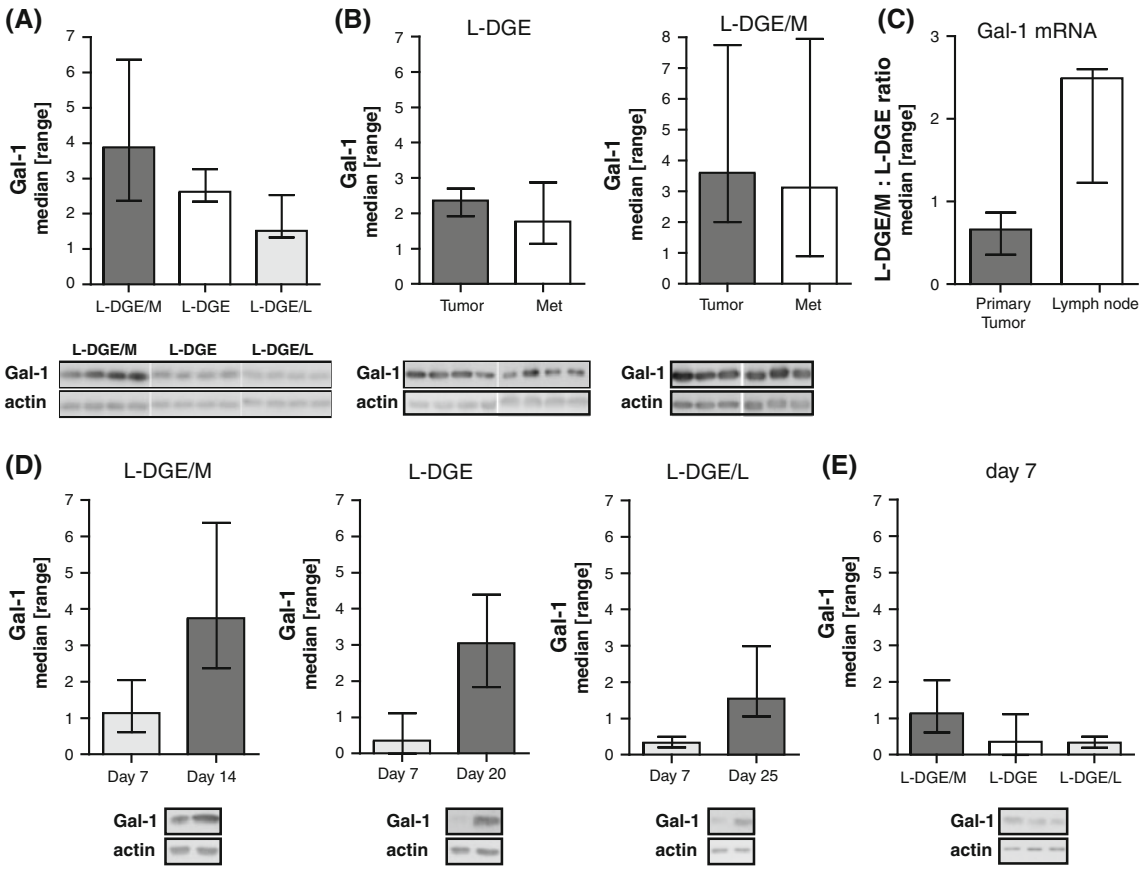

Fig. 3 Gal-1 expression in primary tumor and lymph node metastases of L-DGE, L-DGE/M and L-DGE/L. **a** Gal-1 expression is different in the three lymphomas analyzed (Kruskal–Wallis, $P = 0.001$); L-DGE versus L-DGE/M and versus L-DGE/L ($P < 0.05$); L-DGE/M versus L-DGE/L ($P < 0.01$). **b** Gal-1 expression in primary tumor and lymph node metastases (not significant). **c** L-DGE/M:L-DGE Gal-1 mRNA levels ratio in primary tumor and lymph nodes. **d** Gal-1 expression augments from day 7 to the final day of tumor evolution in the three lymphomas (day 7 vs. V_{MAX} day: L-DGE and L-DGE/L, $P = 0.0143$; L-DGE/M, $P = 0.001$). **e** Gal-1 expression in primary tumor on day 7 ($P = 0.0548$). Gal-1 expression is indicated as relative densitometric value (median [range]) and was normalized to β-actin quantification

metastasis in tumor variants selected by different tumor growth rates.

Time-dependent Gal-1 expression in tumor variants selected by growth rates

To determine whether differential Gal-1 expression was an early or late event in tumor evolution, we assessed Gal-1 on day 7 and on V_{MAX} day. In the three lymphomas analyzed, Gal-1 expression significantly augmented from day 7 until the last day of tumor evolution (L-DGE and L-DGE/L, $P = 0.0143$; L-DGE/M, $P = 0.001$) (Fig. 3d). The early expression of Gal-1, on day 7, followed the same pattern as evidenced on V_{MAX} day, showing higher levels in L-DGE/M, intermediate in L-DGE and lower in L-DGE/L ($P = 0.0548$) (Fig. 3e).

Non-toxic single dose of Cy impacts differently on tumor evolution and survival of mice bearing different lymphoma variants

Our as well as others' experience on the efficacy of a single dose of Cy on lymphoma growth [16] prompted us to study the effects of this treatment on selected lymphoma variants to analyze how the efficacy of this therapy is influenced by the intrinsic growth capacity of tumors. Considering that the three lymphomas' growth rates are different, we decided to administer a single dose of Cy at a fixed tumor volume, instead of a fixed day of tumor evolution. Also, given the aggressiveness of L-DGE/M, the administered Cy dose was higher than the antimetastatic one previously reported, but still non-toxic, as white blood cell count remained within the normal range, with no body weight losses, when administered to tumor-bearing BALB/c mice (data not shown).

BALB/c mice were s.c. challenged with L-DGE, L-DGE/M or L-DGE/L ($n = 30$/per lymphoma variant), and when the median tumor volume/variant was 0.7 cm^3, mice were distributed in two groups and treated with a single dose of 250 mg Cy/kg of body weight (Cy-treated group) or saline (control group). Tumor volume was determined daily until it reached the V_{MAX} or until complete tumor regression was observed. According to different tumor growth rates, L-DGE reached an optimal volume for Cy administration on day 10 while L-DGE/M reached this volume on day 7 and L-DGE/L on day 9.

Exponential tumor regression induced by Cy treatment in different lymphoma variants

The administration of Cy had an immediate effect on tumor evolution, causing regression of the three variants. This effect was sustained in 100% L-DGE/L-bearing mice while was only beneficial for 66.7% (10/15) L-DGE- and 53.3% (8/15) L-DGE/M-bearing animals (Fig. 4a). The regression and recurrence percentages were different among the three tumors ($P < 0.0001$; Fig. 4b). In the remaining L-DGE- and L-DGE/M-bearing mice, tumor regained exponential growth after the regression period until reaching the V_{MAX} (Fig. 4a). Following Cy administration, all tumors decreased its volume exponentially, yet the exponential tumor volume decay rate (ETvDR) among the three tumors was significantly different. Accordingly, the more aggressive lymphoma variant, L-DGE/M, decreased its volume more slowly after Cy administration, showing the lowest ETvDR, which differed significantly from those of L-DGE and L-DGE/L (Fig. 4c).

Effects of Cy treatment on the survival of tumor-bearing hosts

As expected from the observed tumor regression, the effect of Cy treatment on lymphomas had a significant impact on survival median time in lymphoma-bearing mice (Fig. 4d). The median survival time of Cy-treated L-DGE/M-bearing mice was 27 days, while those of L-DGE and L-DGE/L were undefined, because at no point, during the experimental period, mice reached the 50% survival.

Effect of Cy treatment on the metastatic capacity of lymphoma variants

The effect of a single-dose Cy on the metastatic capacities of L-DGE, L-DGE/M and L-DGE/L showed significant differences among the three variants (Kruskal–Wallis; $P < 0.0001$). Histopathologic examination of paraffin-embedded hematoxylin–eosin-stained axillary LN showed that a single-dose Cy treatment completely inhibited metastasis growth in L-DGE- and L-DGE/L-bearing mice, whereas it showed only a partial anti-metastatic effect on L-DGE/M. Thus, 25% of mice whose L-DGE/M primary tumors had permanently regressed, and 100% of mice which experimented L-DGE/M recurrence, developed LN metastases, which totalize 60% (9/15) of mice belonging to this group.

Lymphadenomegaly in mice bearing different lymphoma variants

Homolateral (HL) axillary LN's weight was measured on day 14 and on V_{MAX} day in control mice and at the final day of the experiment on Cy-treated tumor-free mice. On day 14 of tumor evolution, the weight of HL axillary LN from mice bearing any of the three variants was significantly higher ($P < 0.001$) than that of normal mice (Fig. 5a). Moreover, the weight of axillary LN from

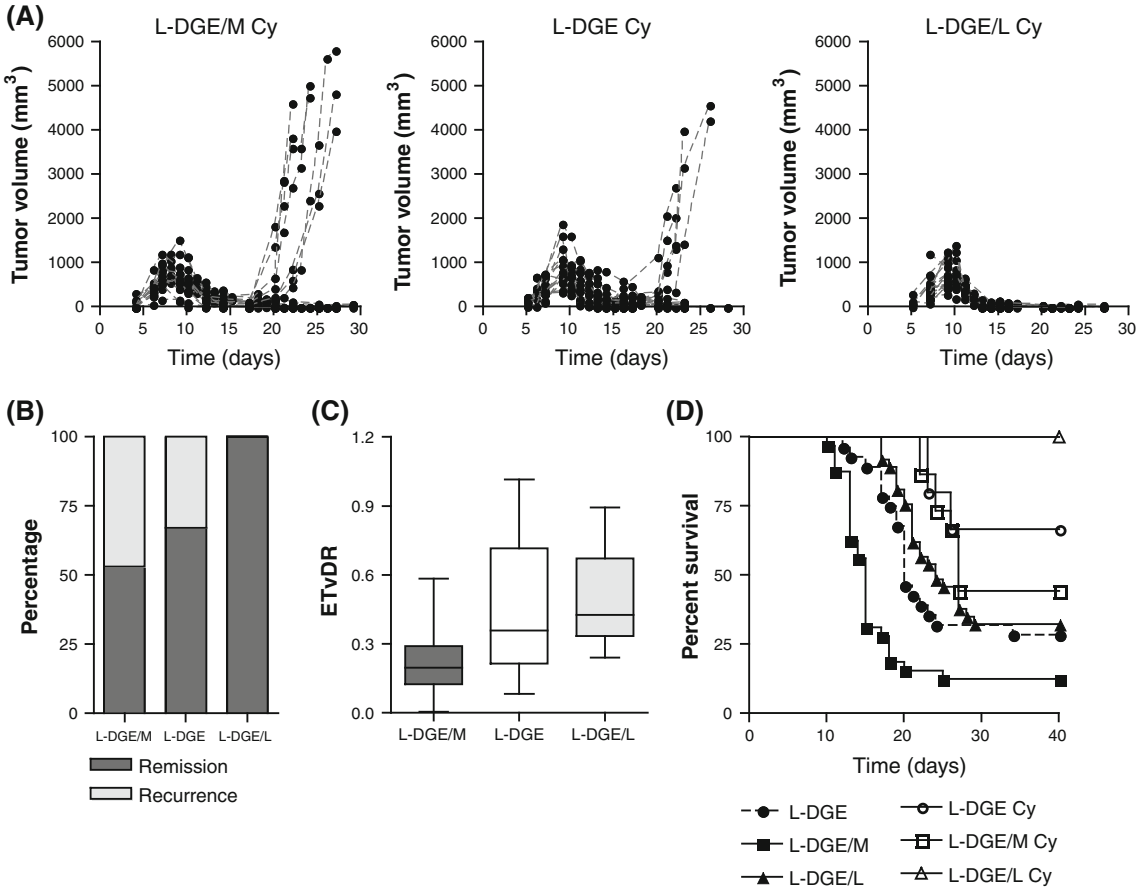

Fig. 4 **a** Individual tumor growth curves of mice treated with Cy. **b** Treatment with Cy caused permanent tumor regression of 100% L-DGE/L, 66.7% of L-DGE and 53.3% of L-DGE/M tumors (X^2, $P < 0.0001$). **c** The ETvDR (exponential tumor volume decay rate) differed among the three variants (Kruskal–Wallis, $P = 0.0006$), being that of L-DGE/M (median [range]: 0.19 [0.003–0.58]) lower than those of L-DGE (0.36 [0.008–1.0]), ($P < 0.05$) and L-DGE/L (0.43 [0.24–0.89]), ($P < 0.001$) (Dunn's test). **d** Cy treatment prolonged the survival time of lymphoma-bearing mice (logrank test: $P < 0.0001$). Median survival times, control: L-DGE/M 15 days, L-DGE 20 days, L-DGE/L 24 days; Cy-treated: L-DGE/M 27 days, L-DGE and L-DGE/L, undefined

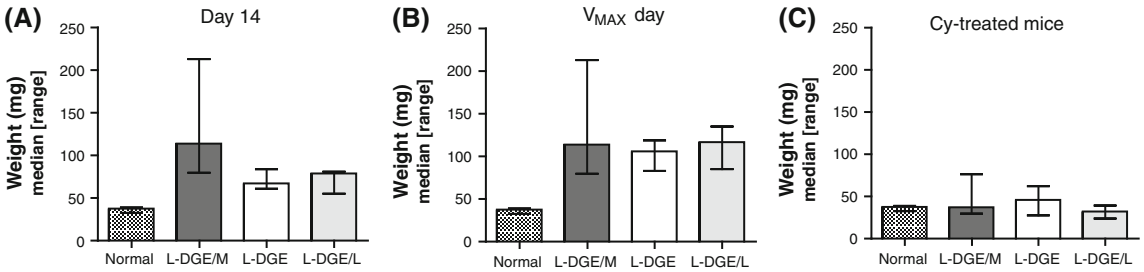

Fig. 5 Homolateral axillary lymph node weight in lymphoma-bearing mice. **a** HL axillary LN weight on day 14 of tumor evolution (Kruskal–Wallis, $P = 0.0063$). The weight of HL axillary LN from L-DGE-, L-DGE/M- and L-DGE/L-bearing mice versus normal mice (L-DGE/M: $P < 0.0001$; L-DGE and L-DGE/L: $P < 0.001$); L-DGE/M versus L-DGE and L-DGE/L-bearing mice ($P < 0.05$ in both cases). **b** HL axillary LN weight on V_{MAX} day (Kruskal–Wallis, $P = 0.0242$; L-DGE and L-DGE/L vs. normal, $P < 0.01$; L-DGE/M vs. normal, $P < 0.001$). **c** HL axillary LN weight in Cy-treated tumor-free mice did not differ from that of normal mice (Kruskal–Wallis, $P = 0.32$). L-DGE/L-Cy (median weight: 32.80 mg) versus L-DGE-Cy (46.20 mg), $P < 0.05$

L-DGE/M-bearing mice was significantly higher than those of L-DGE and L-DGE/L-bearing mice (Fig. 5a). Also, on V_{MAX} day, HL axillary LN's weight of L-DGE-, L-DGE/M- and L-DGE/L-bearing mice was significantly higher than that of normal mice (Fig. 5b). Finally, in Cy-treated tumor-free mice, HL axillary LN's weight did not differ from those of normal mice when analyzed together, although a significant difference was found between the

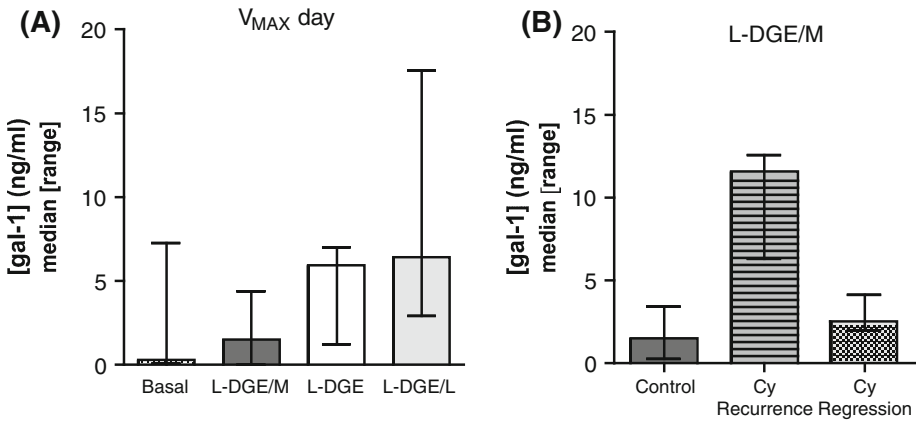

Fig. 6 Gal-1 serum levels in different lymphoma variants selected by tumor growth rates. **a** Gal-1 serum concentration did not differ on V_{MAX} day from normal levels (Kruskal–Wallis, $P = 0.20$). **b** L-DGE/M-bearing mice: Gal-1 concentration in control versus treated mice (Kruskal–Wallis, $P = 0.038$); control versus recurrence ($P < 0.05$)

weight of HL axillary LN from L-DGE and L-DGE/L-bearing mice (Fig. 5c).

Gal-1 serum levels in different lymphoma variants

As overexpression of Gal-1 has been widely associated with tumor aggressiveness [31], we investigated whether the differential expression of Gal-1 in primary tumors found in the three lymphomas correlated with circulating levels of this lectin. Unexpectedly, the concentration of Gal-1 in sera did not differ among the three variants analyzed, and with respect to normal levels (Fig. 6a). Data in each group showed high variability, a fact that may account for the lack of significant differences. Yet Gal-1 serum levels differed between control and Cy-treated L-DGE/M-bearing mice ($P < 0.05$) (Fig. 6b). This difference was mainly due to the higher Gal-1 serum levels in mice with tumor recurrence, which differed from those of control mice (Fig. 6b). In Cy-treated tumor-free mice, Gal-1 concentration did not differ among the three tumor-bearing animals, and also with respect to the levels obtained on V_{MAX} day in control mice (data not shown). Thus, differences in Gal-1 expression locally in primary tumors do not necessarily correlate with systemic Gal-1 levels in tumor-bearing hosts.

Discussion

Selection on a continuous character can be classified into three main types: directional, stabilizing and disruptive [30]. The latter occurs when two or more different phenotypes are favored simultaneously by selection within a single population [31]. In order to determine whether lymphoma aggressiveness and Gal-1 expression were directly associated, we carried out an artificial disruptive selection to favor the extreme growth rates showed by L-DGE lymphoma.

Gal-1 is an endogenous β-galactoside-binding protein that plays an important role in immune cell homeostasis [3]. A wide variety of tumors up-regulate this lectin as a mean of evading T-cell responses [32] and promoting angiogenesis [33]. In addition, expression of this lectin triggers a tolerogenic circuit on dendritic cells via mechanisms involving IL-27 and IL-10 [6].

The selection process was successful in generating two new lymphoma variants that were different in many aspects. L-DGE/M, the aggressive lymphoma variant, showed lower TvDT and spontaneous tumor regression and higher metastatic capacity than L-DGE, the original lymphoma. On the contrary, L-DGE/L showed higher TvDT and spontaneous tumor regression along with decreased metastatic capacity when compared to L-DGE. Interestingly, the selection process for higher and lower growth rates involved, indirectly, the selection of tumor cells with higher and lower metastatic capacity, respectively.

The modulation of the T_{reg}-cell population found during growth of a rat B-cell lymphoma [Rico MJ, personal communication] prompted us to determine the percentage of $CD4^+CD25^+FoxP3^+$ natural T_{reg} cells in draining lymph nodes of mice bearing L-DGE- or its variants. Surprisingly, the T_{reg}-cell population did not expand in response to tumor growth, at least at day 14 of tumor evolution. Moreover, L-DGE/M-bearing mice showed a lower percentage of T_{reg} cells than L-DGE (data not shown). The higher percentage of metastases in draining lymph nodes of L-DGE/M-bearing mice could account for this result. The lack of modulation of T_{reg} cells in L-DGE and L-DGE/L-bearing mice may indicate that this cell population does not play a major role in tumor-immune escape in this particular tumor type. Accordingly, Tiemessen et al. [34] found no differences in peripheral T_{reg} cells in patients with Sezary syndrome, whereas Prabhala et al. [35] showed that $CD4^+CD25^+FoxP3^+$ cells are decreased in multiple myeloma with respect to healthy donors.

Gal-1 expression was determined in primary tumor and metastasis. Confirming our hypothesis, the aggressive variant L-DGE/M overexpressed Gal-1 in the primary tumor, while in L-DGE/L, Gal-1 was down-regulated when compared to the parental L-DGE tumor. Thus, Gal-1 expression in primary tumor was modulated by the disruptive selection for tumor growth rate, suggesting that this lectin could directly influence tumor aggressiveness in T-cell lymphomas. Interestingly, acquisition of metastatic potential by L-DGE and L-DGE/M cells did not involve changes in Gal-1 expression at the protein level, as its expression in metastases was found to be similar to that found in the respective primary tumor. In classical Hodgkin lymphoma, it was demonstrated that Reed-Sternberg cells, the typical cells of this disease, selectively overexpress Gal-1, to favor the secretion of T_H2-type cytokines and to negatively regulate tumor-associated EBV-specific $CD8^+$ T cells, thus enabling these cells to evade immune attack [10, 36]. Juszczynski et al. [10] also found an expansion of the T_{reg}-cell population in vitro. Differences in lymphoma types could account for these divergent results.

In an experimental metastatic T-cell lymphoma model, and by means of an artificial selection for metastatic capacity, Demers et al. [37] found that overexpression of Gal-7 but not of Gal-1 was associated with the metastatic capacity of these tumors. Matrix metalloproteinase-9 (MMP-9) and intercellular adhesion molecule-1 (ICAM-1) were also necessary for liver or spleen metastases [38, 39]. Although apparently discordant, these findings do not oppose our present results, given that, in our system, the selection pressure was exerted on primary tumor cells and not on metastatic cells.

We have previously demonstrated that a single low dose of Cy, a treatment completely devoid of toxicity, inhibits the growth of spontaneous and experimental metastases of the rat B-cell lymphoma L-TACB, while it does not affect primary tumor growth, through modulation of the antitumor responses [40–42]. The same effect was achieved on L-DGE lymphoma using a similar chemotherapeutic schedule [Matar, personal communication]. Based on the aggressiveness of L-DGE/M tumor, we decided to modify both the dose and the schedule of Cy administration and injected Cy with a higher non-toxic dose on day 7, when the median tumor volume of L-DGE/M was 0.7 cm^3 and treated L-DGE and L-DGE/L-bearing mice at the day these tumors reached the same median volume. The response of the three lymphomas to Cy was, as expected, different as we observed complete and permanent tumor regression in L-DGE/L, whereas in L-DGE and L-DGE/M this effect was incomplete and transient. Moreover, this treatment prevented spontaneous metastatic seeding to draining lymph nodes in L-DGE- and L-DGE/L-bearing mice, but not in L-DGE/M. The overall effect was a significant increase in median survival time for the three lymphomas, having L-DGE/M the shortest one.

To correlate the parameters analyzed, we further analyzed the effect of Cy on serum levels of Gal-1. Although Gal-1 expression has been extensively associated with tumor aggressiveness and immune escape, the role of circulating Gal-1 in tumor evolution is, so far, elusive. In the present work, we could find no significant association between Gal-1 serum concentration and tumor evolution or aggressiveness. The highly variable results prevented us from reaching a clear conclusion. As Gal-1 levels tended to decrease toward the end of tumor evolution in L-DGE and L-DGE/L and that after Cy-induced complete tumor regression the levels were kept high, it could be hypothesized that circulating Gal-1 may derive from activated lymphocytes, dendritic cells or macrophages, which are known to secret Gal-1 upon activation [3, 43, 44]. The levels of circulating Gal-1 upon Cy treatment could be explained by the presence of a Cy-induced active T-cell response, while lower levels of circulating Gal-1 at the end of tumor evolution could reflect a dampened anti-tumor response and lack of tumor's Gal-1 shedding into the circulation. Up to date, there are few studies in the literature that address this issue. Saussez et al. [45] determined a threshold value of 13.5 ng/ml for Gal-1 serum concentration, in patients with head and neck squamous cell carcinomas (HNSCC). They showed that Gal-1 serum levels significantly decreased after the combination of radiotherapy and chemotherapy or surgery and radiotherapy in a group of stage IV HNSCC patients. Also, and opposite to our results, increased levels of circulating Gal-1 were found in malignant thyroid disease compared to benign disorders but no cutoff concentration could be determined [46]. The relevance of serum Gal-1 as a biomarker still remains to be elucidated, given the sensitivity of this lectin to oxidative inactivation and functional degradation [3]. However, recent studies, using proteomic-based strategies, demonstrated a predictive value for Gal-1 in tissue from patients with relapsed/refractory disease in classical Hodgkin lymphoma [47].

Moreover, it should be highlighted that in addition to its role in tumor growth, angiogenesis and immune escape, Gal-1 has been shown to play key roles in tumor cell migration [48, 49], suggesting that fluctuations in its levels might also indicate changes in the migratory capacity of these cells. Interestingly, another relevant molecule that could be modulated by Gal-1 in the tumor microenvironment is vascular endothelial growth factor (VEGF) that plays pleiotropic roles in tumor growth, angiogenesis, migration and immunity [32]. Our preliminary data show that VEGF serum concentration is not directly associated with tumor growth rate in these lymphoma models. This is due to the fact that while on V_{MAX} day, L-DGE/M showed

higher VEGF serum levels than L-DGE the concentration for L-DGE/L was intermediate between those of the other two lymphomas (data not shown). After Cy-induced complete tumor regression, VEGF levels decreased, but not to basal levels, a fact that could be due to its secretion by other non-tumor-stimulated cell types, a matter that needs further clarification (data not shown).

Thus, through a reverse approach with respect to that carried out in a murine melanoma model [9], we were able to demonstrate the relationship between Gal-1 expression and lymphoma aggressiveness. The present results show evidence for a relationship between T-cell lymphoma growth rate, metastatic capacity, Gal-1 expression and response to Cy treatment in primary lymphomas. Additional studies are warranted to further elaborate on the mechanisms underlying this association, particularly the critical requirement of Gal-1 for Cy treatment in vivo and the essential role of endogenous Gal-1 in the growth of primary lymphomas, with the ultimate goal of improving anti-lymphoma therapies.

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References

1. Non-Hodgkin Lymphoma, The Leukemia & Lymphoma Society. http://www.leukemia-lymphoma.org
2. Fisher SG, Fisher RI (2004) The epidemiology of non-Hodgkin's lymphoma. Oncogene 23:6524–6534
3. Camby I, Le Mercier M, Lefranc F, Kiss R (2006) Galectin-1: a small protein with major functions. Glycobiology 16:137R–157R
4. He J, Baum LG (2006) Endothelial cell expression of galectin-1 induced by prostate cancer cells inhibits T-cell trans-endothelial migration. Lab Invest 86:578–590
5. Van der Leij J, van den Berg A, Harms G, Eschbach H, Vos H, Zwiers P, van Weeghel R, Groen H, Poppema S, Visser L (2007) Strongly enhanced IL-10 production using stable galectin-1 homodimers. Mol Immunol 44:506–513
6. Ilarregui JM, Croci DO, Bianco GA, Toscano MA, Salatino M, Vermeulen ME, Geffner JR, Rabinovich GA (2009) Tolerogenic signals delivered by dendritic cells to T cells through a galectin-1-driven immunoregulatory circuit involving interleukin 27 and interleukin 10. Nat Immunol 10:981–991
7. Garin MI, Chu C–C, Golshayan D, Cernuda-Morollón E, Wait R, Lechler RI (2007) Galectin-1: a key effector of regulation mediated by CD4 + CD25 + T cells. Blood 109:2058–2065
8. Toscano MA, Bianco GA, Ilarregui JM, Croci DO, Correale J, Zwirner NW, Hernandez JD, Poirier F, Riley EM, Baum LG, Rabinovich GA (2007) Differential glycosylation of Th1, Th2 and Th17 effector cells selectively regulates susceptibility to cell death. Nat Immunol 8:825–834
9. Rubinstein N, Alvarez M, Zwirner NW, Toscano MA, Ilarregui JM, Bravo A, Mordoh J, Fainboim L, Podhajcer OL, Rabinovich GA (2004) Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection. A potential mechanism of tumor-immune privilege. Cancer Cell 5:241–251
10. Juszczynski P, Ouyang J, Monti S, Rodig SJ, Takeyama K, Abramson J, Chen W, Kutok JL, Rabinovich GA, Shipp MA (2007) The AP1-dependent secretion of galectin-1 by Reed Sternberg cells fosters immune privilege in classical Hodgkin lymphoma. Proc Natl Acad Sci USA 104:13134–13139
11. Rodig SJ, Ouyang J, Juszczynski P, Currie T, Law K, Neuberg DS, Rabinovich GA, Shipp MA, Kutok JL (2008) AP1-dependent galectin-1 expression delineates classical hodgkin and anaplastic large cell lymphomas from other lymphoid malignancies with shared molecular features. Clin Cancer Res 14:3338–3344
12. Kuo PL, Hung JY, Huang SK, Chou SH, Cheng DE, Jong YJ, Hung CH, Yang CJ, Tsai YM, Hsu YL, Huang MS (2011) Lung cancer-derived galectin-1 mediates dendritic cell anergy through inhibitor of DNA binding 3/IL-10 signaling pathway. J Immunol 186:1521–1530
13. Banh A, Zhang J, Cao H, Bouley DM, Kwok S, Kong C, Giaccia AJ, Koong AC, Le QT (2011) Tumor galectin-1 mediates tumor growth and metastasis through regulation of T-cell apoptosis. Cancer Res 71:4423–4431
14. Mathieu V, Le Mercier M, De Neve N, Sauvage S, Gras T, Roland I, Lefranc F, Kiss R (2007) Galectin-1 knockdown increases sensitivity to temozolomide in a B16F10 mouse metastatic melanoma model. J Invest Dermatol 127:2399–2410
15. Le Mercier M, Lefranc F, Mijatovic T, Debeir O, Haibe-Kains B, Bontempi G, Decaestecker C, Kiss R, Mathieu V (2008) Evidence of galectin-1 involvement in glioma chemoresistance. Toxicol Appl Pharmacol 229:172–183
16. Zacarias Fluck MF, Rico MJ, Gervasoni SI, Ilarregui JM, Toscano MA, Rabinovich GA, Scharovsky OG (2007) Low-dose cyclophosphamide modulates galectin-1 expression and function in an experimental rat lymphoma model. Cancer Immunol Immunother 56:237–248
17. Mehrara E, Forssell-Aronsson E, Ahlman H, Bernhardt P (2007) Specific growth rate versus doubling time for quantitative characterization of tumor growth rate. Cancer Res 67:3970–3975
18. Furukawa H, Iwata R, Moriyama N (2001) Growth rate of pancreatic adenocarcinoma: initial clinical experience. Pancreas 22:366–369
19. Kuroishi T, Tominaga S, Morimoto T, Tashiro H, Itoh S, Watanabe H, Fukuda M, Ota J, Horino T, Ishida T, Yokoe T, Enomoto K et al (1990) Tumor growth rate and prognosis of breast cancer mainly detected by mass screening. Jpn J Cancer Res 81:454–462
20. Shackney SE, McCormack GW, Cuchural GJ (1978) Growth rate patterns of solid tumors and their relation to responsiveness to therapy: an analytical review. Ann Intern Med 89:107–121
21. Spratt JS, Spratt TL (1964) Rates of growth of pulmonary metastases and host survival. Ann Surg 159:161–171
22. Szereday Z, Schally AV, Nagy A, Plonowski A, Bajo AM, Halmos G, Szepeshazi K, Groot K (2002) Effective treatment of experimental U-87MG human glioblastoma in nude mice with a targeted cytotoxic bombesin analogue, AN-215. Br J Cancer 86:1322–1327
23. Usuda K, Saito Y, Sagawa M, Sato M, Kanma K, Takahashi S, Endo C, Chen Y, Sakurada A, Fujimura S (1994) Tumor doubling time and prognostic assessment of patients with primary lung cancer. Cancer 74:2239–2244
24. Bajzer Z (1999) Gompertzian growth as a self-similar and allometric process. Growth Dev Aging 63:3–11
25. Collins VP, Loeffler RK, Tivey H (1956) Observations on growth rates of human tumors. Am J Roentgenol Radium Ther Nucl Med 76:988–1000
26. Scharovsky OG, Celoria GC, Racca AL, Font MT (1991) Association between immune response and body conformation in the mouse (Mus musculus). Rev Brasil Genet 14:373–380

27. Tang Q, Bluestone JA (2008) The Foxp3 + regulatory T cell: a jack of all trades, master of regulation. Nat Immunol 9:239–244
28. Croci DO, Zacarías Fluck MF, Rico MJ, Matar P, Rabinovich GA, Scharovsky OG (2007) Dynamic cross-talk between tumor and immune cells in orchestrating the immunosuppressive network at the tumor microenvironment. Cancer Immunol Immunother 56:1687–1700
29. Demydenko D, Berest I (2009) Expression of galectin-1 in malignant tumors. Exp Oncol 31:74–79
30. Mather K (1953) The genetical structure of populations. Symp Soc Exp Biol 7:66–95
31. Skibinski DOF, Thoday JM (1979) Disruptive selection with fixed optima. Heredity 42:327–335
32. Rabinovich GA, Gabrilovich D, Sotomayor EM (2007) Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol 25:267–296
33. Thijssen VL, Postel R, Brandwijk RJ, Dings RP, Nesmelova I, Satijn S, Verhofstad N, Nakabeppu Y, Baum LG, Bakkers J, Mayo KH, Poirier F et al (2006) Galectin-1 is essential in tumor angiogenesis and is a target for antiangiogenesis therapy. Proc Natl Acad Sci USA 103:15975–15980
34. Tiemessen MM, Mitchell TJ, Hendry L, Whittaker SJ, Taams LS, John S (2006) Lack of suppressive CD4 + CD25 + FOXP3 + T cells in advanced stages of primary cutaneous T-cell lymphoma. J Invest Dermatol 126:2217–22123
35. Prabhala RH, Neri P, Bae JE, Tassone P, Shammas MA, Allam CK, Daley JF, Chauhan D, Blanchard E, Thatte HS, Anderson KC, Munshi NC (2006) Dysfunctional T regulatory cells in multiple myeloma. Blood 107:301–304
36. Gandhi MK, Moll G, Smith C, Dua U, Lambley E, Ramuz O, Gill D, Marlton P, Seymour JF, Khanna R (2007) Galectin-1 mediated suppression of Epstein-Barr virus specific T-cell immunity in classic Hodgkin lymphoma. Blood 110:1326–1329
37. Demers M, Couillard J, Giglia-Mari G, Magnaldo T, St-Pierre Y (2009) Increased galectin-7 gene expression in lymphoma cells is under the control of DNA methylation. Biochem Biophys Res Commun 387:425–429
38. Demers M, Biron-Pain K, Hébert J, Lamarre A, Magnaldo T, St-Pierre Y (2007) Galectin-7 in lymphoma: elevated expression in human lymphoid malignancies and decreased lymphoma dissemination by antisense strategies in experimental model. Cancer Res 67:2824–2829
39. St-Pierre Y, Aoudjit F, Lalancette M, Potworowski EF (1999) Dissemination of T cell lymphoma to target organs: a post-homing event implicating ICAM-1 and matrix metalloproteinases. Leuk Lymphoma 34:53–61
40. Matar P, Rozados VR, González AD, Dlugovitzky DG, Bonffl RD, Scharovsky OG (2000) Mechanism of antimetastatic immunopotentiation by low-dose cyclophosphamide. Eur J Cancer 36:1060–1066
41. Matar P, Rozados VR, Gervasoni SI, Scharovsky OG (2001) Downregulation of T-cell derived IL-10 production by low-dose cyclophosphamide treatment in tumor-bearing rats restores in vitro normal lymphoproliferative response. Int Immunopharmacol 1:307–319
42. Matar P, Rozados VR, Gervasoni SI, Scharovsky OG (2002) Th2/Th1 switch induced by a single-low dose cyclophosphamide in a rat metastatic lymphoma model. Cancer Immunol Immunother 50:588–596
43. Blaser C, Kaufmann M, Müller C, Zimmermann C, Wells V, Mallucci L, Pircher H (1998) β-galactoside-binding protein secreted by activated T cells inhibits antigen-induced proliferation of T cells. Eur J Immunol 28:2311–2319
44. Zuñiga E, Rabinovich GA, Iglesias MM, Gruppi A (2001) Regulated expression of galectin-1 during B-cell activation and implications for T-cell apoptosis. J Leukoc Biol 70:73–79
45. Saussez S, Lorfevre F, Lequeux T, Laurent G, Chantrain G, Vertongen F, Toubeau G, Decaestecker C, Kiss R (2008) The determination of the levels of circulating galectin-1 and -3 in HNSCC patients could be used to monitor tumor progression and/or responses to therapy. Oral Oncol 44:86–93
46. Saussez S, Glinoer D, Chantrain G, Pattou F, Carnaille B, André S, Gabius HJ, Laurent G (2008) Serum galectin-1 and galectin-3 levels in benign and malignant nodular thyroid disease. Thyroid 18:705–712
47. Kamper P, Ludvigsen M, Bendix K, Hamilton-Dutoit S, Rabinovich GA, Møller MB, Nyengaard JR, Honoré B, d'Amore F (2011) Proteomic analysis identifies galectin-1 as a predictive biomarker for relapsed/refractory disease in classical Hodgkin lymphoma. Blood 117:6638–6664
48. Le Mercier M, Fortin S, Mathieu V, Kiss R, Lefranc F (2010) Galectins and gliomas. Brain Pathol 20:17–27
49. Verschuere T, De Vleeschouwer S, Lefranc F, Kiss R, Van Gool SW (2011) Galectin-1 and immunotherapy for brain cancer. Expert Rev Neurother 11:533–543