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RESEARCH PAPER

Deletion of galectin-3 in the host attenuates metastasis of murine melanoma by modulating tumor adhesion and NK cell activity

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Abstract Galectin-3, a β galactoside–binding lectin, plays an important role in the processes relevant to tumorigenesis such as malignant cell transformation, invasion and metastasis. We have investigated whether deletion of Galectin-3 in the host affects the metastasis of B16F1 malignant melanoma. Galectin-3-deficient (Gal-$3^{-/-}$) mice are more resistant to metastatic malignant melanoma as evaluated by number and size of metastatic colonies in the lung. In vitro assays showed lower number of attached malignant cells in the tissue section derived from Gal-$3^{-/-}$ mice. Furthermore, lack of Galectin-3 correlates with higher serum levels of IFN-γ and IL-17 in tumor bearing hosts. Interestingly, spleens of Gal-$3^{-/-}$ mice have lower number of $Foxp3^{+}$ T cells after injection of B16F1 melanoma cells. Finally, we found that while $CD8^{+}$ T cell and adherent cell cytotoxicity were similar, there was greater cytotoxic activity of splenic NK cells of Gal-$3^{-/-}$ mice compared with "wild-type" (Gal-$3^{+/+}$) mice. Despite the reduction in total number of $CD3\varepsilon^{-}NK1.1^{+}$, Gal-$3^{-/-}$ mice constitutively have a significantly higher percentage of effective cytotoxic $CD27^{high}CD11b^{high}$ NK cells as well as the percentage of immature $CD27^{high}CD11b^{low}$ NK cells. In contrast, $CD27^{low}CD11b^{high}$ less functionally exhausted NK cells and NK cells bearing inhibitory KLRG1 receptor were more numerous in Gal-$3^{+/+}$ mice. It appears that lack of Galectin-3 affects tumor metastasis by at least two independent mechanisms: by a decrease in binding of melanoma cells onto target tissue and by enhanced NK-mediated anti-tumor response suggesting that Galectin-3 may be considered as therapeutic target.

Keywords B16F1 · Galectin-3 · Malignant melanoma · Metastasis · NK cells

Abbreviations

B16F1	Murine skin melanoma cell line
Gal-3	Galectin-3
IFN-γ	Interferon-gamma
IL-17	Interleukin-17
IL-4	Interleukin-4
KLRG1	Killer cell lectin-like receptor G1
NK cells	Natural killer cells
TNF-α	Tumor necrosis-alpha

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Introduction

Galectin-3 is one of the 16 known members of the galectin family, which selectively binds β-galactoside. Depending on cell types and proliferative status, this molecule can be found in the cytoplasm, on the cell surface, within the nucleus, and in the extracellular compartment [1–3]. Galectin-3 binds and interacts with a numerous ligands in the intra- and extra-cellular environment and regulates many biological processes and signaling pathways in

normal and tumor cells, including cell proliferation, differentiation and apoptosis [4, 5]. When derived extracellularly, Galectin-3 acts as an adhesion molecule mediating cross-linking between adjacent cells, and cells and extracellular matrix proteins [4]. It is believed that Galectin-3 plays an important role in the processes relevant to tumorigenesis such as malignant cell transformation, invasion and metastasis [5, 6].

The tumor metastasis is a dynamic and multistep process. Although, the development of metastasis has random features, basically it is a strictly regulated process. It appears that one of the critical steps in hematogenous phase of metastasis is the adhesion of circulating tumor cells to the vascular endothelium in targeted organs that allows their selective survival [7, 8]. This process is thought to be regulated by various adhesion molecules and their ligands expressed on tumor cells and endothelial cells [9]. In this regard, some evidence indicates that Galectin-3 and its glycoconjugate ligands are engaged in this process [10–12]. It has been suggested that Galectin-3 on the vascular endothelium could serve as the first anchor for the circulating tumor cells [13]. Galectin-3 is also expressed in many different tumor types and, in general, its expression correlates with tumor cell transformation and acquisition of metastatic phenotype [14]. Clinical evidence has shown that overexpression of Galectin-3 in human melanoma correlates with metastatic progression and with poor clinical outcome [15]. In addition, the concentration of circulating Galectin-3 is markedly increased in the serum of patients with different tumor types including malignant melanoma [16, 17], and it is probably generated not only by tumor cells, but also by peritumoral inflammatory cells and stromal cells [3]. Although, the exact role of the Galectin-3 in tumor progression is yet to be determined, some studies have proposed that this molecule may facilitate metastasis by promoting tumor cell adhesion [18] and invasiveness [19], or immune escape [5, 20]. However, the role of Galectin-3 expression in the target tissue is not completely understood.

The network of immune cells involved in preventing tumor initiation and metastasis is complex, and is mediated by innate and acquired immune system [21, 22]. Although, Galectin-3 is expressed in many immunocompetent and inflammatory cells, including macrophages, dendritic cells, eosinophils, mast cells, and activated T and B cells [4], its role in innate and acquired immune response to tumor remains elusive. Data from Zubieta et al. [23] demonstrated that Galectin-3 expression correlated with apoptosis of tumor-associated lymphocytes in human melanoma biopsies. A recent study also suggest that tumor-associated Galectin-3 contributes to tumor immune escapes by killing tumor reactive $CD8^+$ T cells and promotes tumor growth in a mouse model of colorectal cancer [24]. Although, the expression of Galectin-3 on uterine NK cells has been suggested to downregulate their function [25, 26], little is known about the role of Galectin-3 on NK cells, in general. This is of potential importance because NK cells provide effective anti-melanoma activity, leading to reduced number of metastasis [27, 28].

Therefore, we studied whether deletion of Galectin-3 in the host affects development of experimental metastasis. It appears that the deletion of Galectin-3 in the host affects metastasis of malignant melanoma by at least two mechanisms: tumor cell attachment in the target tissue, and maturation and function of NK cells.

Materials and methods

Animals and cell culture

The experiments were approved by the Ethics Board of the Faculty of Medicine University of Kragujevac. We used Galectin-3-deficient ($Gal\text{-}3^{-/-}$) on C57BL/6 background mice (generated as previously detailed by Hsu et al. [29], kindly provided by Dr Daniel Hsu through Prof. FY. Liew, Glasgow, UK), and "wild-type" ($Gal\text{-}3^{+/+}$) C57BL/6 mice. Male and female mice 8–12 weeks old were used in all experiments.

The murine skin melanoma cell line B16F1 was purchased from the American Type Culture Collection (CRL-6323; ATCC, USA). The cells were routinely cultured in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 2 mmol/l L-glutamine, 1 mmol/l penicillin/streptomycin, 1 mmol/l mixed non-essential amino acids (PAA Laboratories GmbH), in a humidified incubator at 37°C at 5% CO_2. Cells were routinely subcultured as subconfluent monolayers every 3 days and were not kept in culture for more than five passages.

Experimental metastasis assay

For inoculation, B16F1 melanoma cells were harvested upon ~90% confluency using 0.25% trypsin and 0.02% EDTA in phosphate buffered saline (PBS; PAA Laboratories GmbH). Cells were washed once in complete medium and twice in DMEM just before inoculation. The number of viable tumor cells was determined by the trypan blue and only cell suspensions with ≥95% viable cells were used. Experimental metastasis assay was performed by the intravenous injection (lateral tail vein) of 5×10^4 cells, in a volume 0.2 ml of medium, into syngeneic $Gal\text{-}3^{+/+}$ and $Gal\text{-}3^{-/-}$ mice as described previously [30]. 21 days after tumor cell injection, lung tissues were removed and examined histologically.

Histopathological analysis of metastatic lung

Hematoxylin-eosin staining was performed using 4 μm paraffin-embedded tumor bearing lung sections. To avoid missing micrometastasis, stained sections from at least three different levels were examined for the presence of lung metastasis. The number and size of metastatic colonies was examined with light microscope by an independent observer.

Adhesion assay

Adhesion assay were performed as previously described [13, 31]. Prior to adhesion experiments, the frozen sections of lungs were air dried at room temperature for 45 min. The sections were incubated at 4°C with 2% bovine serum albumin in PBS for 2 h. Subsequently, they were layered with 5×10^4 B16F1 cells suspended in 100 μl of PBS and incubated at 37°C in a humidified CO_2 incubator. After incubation, the sections were gently washed with PBS to remove non-adherent cells. The bound cells were fixed in methanol for 15 min at 4°C, stained with Mayer's hematoxylin & eosin. Number of attached cells was determined by counting 100 non-overlapping microscopic fields at an ×100 magnification. Each experiment was repeated at least three times (3 section per mouse was counted, four mice per group).

Measurement of cytokines

Sera from individual mice were collected and stored at −20°C until thawed for the assay. Serum levels of IL-17, IFN-γ, IL-4 and TNF-α were measured using highly sensitive enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Minneapolis, MN), specific for the mouse cytokines according to the manufacturer's instruction.

Cell preparation

Twelve days after tumor cells injection, mice were sacrificed and single-cell suspensions from spleen were obtained by mechanical dispersion through cell strainer (BD Pharmingen, USA) in complete growth medium. Pellets were resuspended in red blood cell lyses solution, washed three times and resuspended in complete growth medium.

Immunophenotyping of splenocytes

The following anti-mouse mAbs were used CD3, CD4, CD8, CD11c, CD80, CD86, CD3ε, CD19, NK1.1, CD27, CD11b, CD69, KLRG1 (BD Pharmingen/eBioscience). Appropriate isotype control antibodies were used to assess the level of specific labeling. Dead cells were excluded by gating out propidium iodide-positive cells. For intracellular staining of Foxp3, cells were fixed and permeabilized with permeabilization buffer (BD Pharmingen). Permeabilized cells were stained with anti-mouse Foxp3 mAbs (BD Pharmingen). Stained cells were analyzed subsequently using a FACSAria instrument (BD). Data were analyzed using CELLQUEST software and DiVa (BD).

Adherent cell separation

Single-cell suspensions of the spleens were incubated for 2 h in complete media on plastic Petri dishes, previously covered with FBS. The non-adherent cells were rinsed off by vigorously washing with DMEM and the adherent cells were collected by gentle scraping with rubber policemen.

NK cell and $CD8^+$ T cell separation

NK cells were isolated from spleen cells by magnetic cell sorting. Single-cell suspensions of splenocytes were labeled using microBeads conjugated to monoclonal anti-mouse CD49b (DX5) antibodies (Miltenyi Biotec) and positively selected using MidiMACS separator (Miltenyi Biotec). CD49b labeled cells that retained in the magnetic column were highly enriched NK cells, and were used in the cytotoxicity study as purified NK cells. In addition, $CD8^+$ T cell were negatively selected from single-cell suspensions of splenocytes using a Dynal mouse T cell negative isolation kit (Invitrogen) which depletes B cells, NK cells, monocytes/macrophages, dendritic cells (DCs), granulocytes, and erythrocytes using a mixture of rat mAbs for mouse CD45R, CD11b, Ter-119, and CD16/32. Cell suspensions containing $CD8^+$ T cells were then used in the cytotoxicity assays.

Cytotoxicity assay

Cytotoxic activity of splenocytes, adherent cells, $CD8^+$ T cells and NK cells were measured using the 4 h MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Isolated splenocytes, adherent cells, $CD8^+$ T cells and NK cells were used as effector cells (E), and B16F1 melanoma cells were used as target cells (T). Effector cells were plated at varying ratios (T:E) in 96-well flat bottom plates, preincubated for 24 h with 1×10^4 target cells/well. MTT cytotoxicity assays were performed as previously described [32]. The percentage of cytotoxity was calculated as: cytotoxity (%) = [1-(experimental group (OD)/control group (OD))] × 100. Data were expressed as the mean of triplicate wells ±SD. Cytotoxic capacity was also presented by lytic units, $LU_{20}/10^7$ cells, calculated from means of triplicates percentages of killing obtained in four different T:E ratios. Estimated numbers represent the mean values.

Statistical analysis

The data were analyzed using SPSS version 13, statistical package. The two-tailed Student's t test or nonparametric Mann–Whitney Rank Sum test were used. The normal data distribution was evaluated by Kolmogorov–Smirnov test. The results were considered significantly different when $P < 0.05$ and highly significantly different when $P < 0.01$.

Results

Gal-$3^{-/-}$ mice are relatively resistant to metastatic growth of B16F1 melanoma cells

Galectin-3-deficient (Gal-$3^{-/-}$) mice are more resistant to metastatic malignant melanoma as evaluated by the number of metastasis and the cellularity of lung metastatic colonies (Fig. 1). In fact, the average number of lung metastasis was higher in the Gal-$3^{+/+}$ mice than in the Gal-$3^{-/-}$ mice, (M ± SEM: 5.92 ± 1.93 vs. 1.00 ± 1.00; $P < 0.05$; Fig. 1a). Eleven out of twelve Gal-$3^{+/+}$ mice (11/12; 92%) had numerous lung metastatic colonies, while only two out of eight Gal-$3^{-/-}$ mice (2/8; 25%) developed lung metastatic colonies ($P < 0.05$). Further, cellularity of lung colonies in Gal-$3^{+/+}$ mice is higher compared with lung colonies of Gal-$3^{-/-}$ mice ($P < 0.05$; Fig. 1b, c). Metastatic colonies were not observed in the other parenchymal organs (data not shown).

In vitro adhesion of malignant cells is decreased in lung tissue of Gal-$3^{-/-}$ mice

Experimental metastasis assay suggest that deletion of Galectin-3 renders mice resistant to melanoma metastasis. To address whether this observation is related to adhesive interactions of melanoma cells to lung parenchyma, we examined in vitro role of the Galectin-3 in the adhesion of the malignant cells onto lung tissue (Fig 2). Adhesion assays were performed on the frozen lung sections from healthy mice. Consistent with in vivo study, our results showed lower binding of malignant cells onto lung tissue of Gal-$3^{-/-}$ mice in comparison with Gal-$3^{+/+}$ mice (M ± SEM: 209.17 ± 39.49 vs. 623.67 ± 76.55; $P < 0.05$).

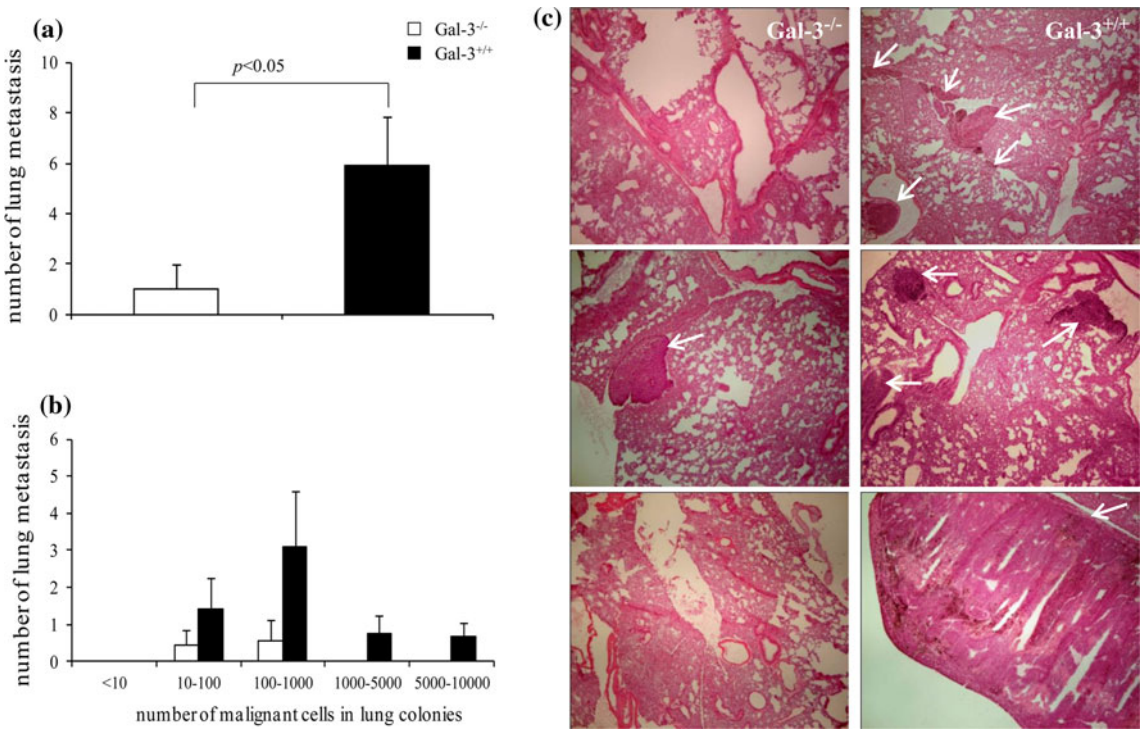

Fig. 1 Lung metastasis and cellularity of metastatic colonies in Gal-$3^{-/-}$ and Gal-$3^{+/+}$ mice 21 days after inoculation of 5×10^4 B16F1 per mouse. **a** The number of lung metastasis was significantly lower in Gal-$3^{-/-}$ mice ($P < 0.05$) when compared with the Gal-$3^{+/+}$ mice. **b** In addition, there was statistically significant differences in the number of malignant cells in the colonies ($P < 0.05$). **c** Representative histology of the mouse lung bearing B16F1 melanoma cells in Gal-$3^{-/-}$ and Gal-$3^{+/+}$ mice (H&E; original magnification 10×) showing metastatic colonies in the lung (*arrows*)

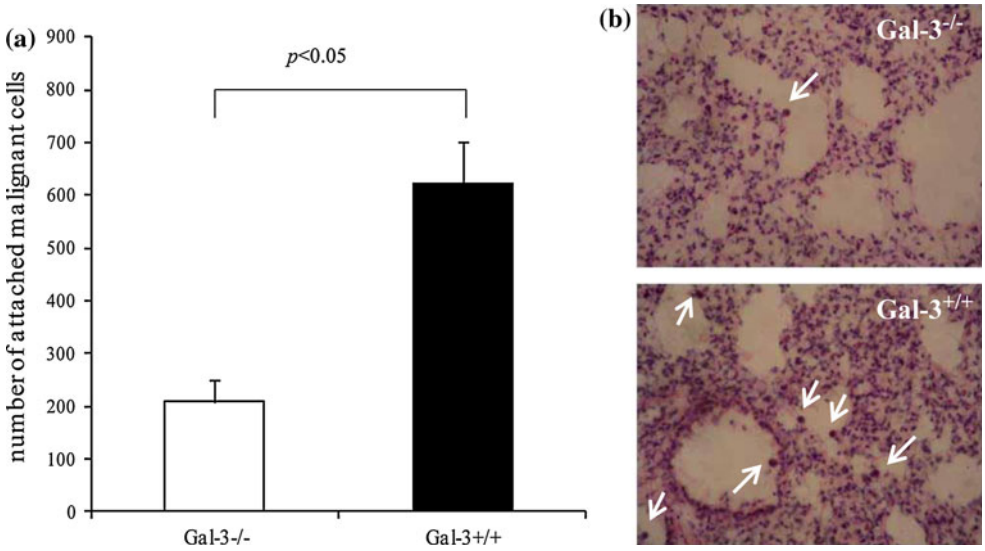

Fig. 2 Adhesion assay. **a** Average number of attached B16F1 melanoma cells was significantly lower in the tissue section derived from Gal-$3^{-/-}$ mice ($P < 0.05$) compared with Gal-$3^{+/+}$ mice. The mean number of attached malignant cells ± SEM is shown. **b** H&E staining images showing attached B16F1 melanoma cells in the lung (*arrows*; original magnification 40×)

Galectin-3-deficient mice showed lower number of splenic $CD4^+Foxp3^+$ T cells after tumor cell inoculation

Splenocyte populations may be altered in tumor bearing host [33]. By day 12 after tumor cell inoculation we estimated the cellular make up of the spleen. Total number of mononuclear cells was not significantly affected in Gal-$3^{-/-}$ and Gal-$3^{+/+}$ mice (data not shown).In addition, the percentage of $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ cells in Gal-$3^{-/-}$ mice did not significantly differ from that in Gal-$3^{+/+}$ mice. Nevertheless, we found that total number and percentage of T cells and $CD8^+$ cells were increased in both Gal-$3^{-/-}$ mice and Gal-$3^{+/+}$ mice after tumor cell inoculation, while total number and percentage of $CD19^+$ cells were decreased (data not shown). Our results suggest that the significantly reduced lung metastasis in the Gal-$3^{-/-}$ mice were not due to the differences in T cell compartment.

However, in Gal-$3^{+/+}$ mice injection of melanoma cells resulted in a significant increase in the percentage and total number of $CD4^+Foxp3^+$ T cells ($P < 0.05$), but not in Gal-$3^{-/-}$ mice which was not significantly affected by tumor cell inoculation (Fig. 3). It appears that Galectin-3 signaling may facilitate tumor metastasis by promoting the formation of the immunosuppressive environment.

Gal-$3^{-/-}$ mice have increased serum levels of IFN-γ and IL-17 during anti-melanoma response

We examined the serum levels of IFN-γ, TNF-α, IL-17 and IL-4 in Gal-$3^{-/-}$ and Gal-$3^{+/+}$ mice. The measurements were performed before and on days 21 after i.v. injection of melanoma cells. As shown in Fig. 4, we found higher serum level of IFN-γ in Gal-$3^{-/-}$ mice challenged with B16F1 melanoma cells compared with Gal-$3^{+/+}$ mice and compared with baseline ($P < 0.05$). We also noticed higher serum level of IL-17 in Gal-$3^{-/-}$ mice ($P < 0.05$) in tumor bearing hosts. The serum levels of IL-4 and TNF-α was also increased after tumor cell inoculation, but did not reach statistically significant difference between Gal-$3^{-/-}$ and Gal-$3^{+/+}$ mice.

Galectin-3 deletion correlates with greater NK-mediated anti-tumor cytotoxicity

We examined in vitro cytotoxic activity of splenocytes, adherent cells, $CD8^+$ T cells and NK cells against tumor cells. These cells were isolated before and on days 12 after i.v. injections of B16F1 melanoma cells, and were tested for cytotoxic activity against the melanoma cells using MTT assay. Compared with Gal-$3^{+/+}$ mice, Gal-3-deficient mice have a greater spontaneous cytotoxicity of total splenic cells ($P < 0.05$), and this is maintained after tumor injection ($P < 0.01$), as seen in Fig. 5a, b. We also noticed that after tumor cell injection cytotoxicity of splenocytes was diminished in Gal-$3^{-/-}$ ($P < 0.05$), but more in Gal-$3^{+/+}$ mice ($P < 0.01$). Further, we did not find any differences in the cytotoxicity of adherent cells, as seen in Fig. 5c, d, as well as in the cytotoxicity of $CD8^+$ T cells between Gal-$3^{-/-}$ and Gal-$3^{+/+}$ mice before and after tumor cell injection ($P > 0.05$; Fig. 5e, f). However, Galectin-3 deletion led to greater NK cell mediated anti-tumor cytotoxicity. In fact, we found higher cytotoxic capacity of splenic NK cells derived from Gal-$3^{-/-}$ mice in both, tumor cell-inoculated ($P < 0.01$) and naive animals ($P < 0.01$; Fig. 5g, h). Additionally, cytotoxic activity of NK cells in the spleen was diminished after tumor cell injection in Gal-$3^{+/+}$ mice but not in Gal-3-deficient mice ($P < 0.01$).

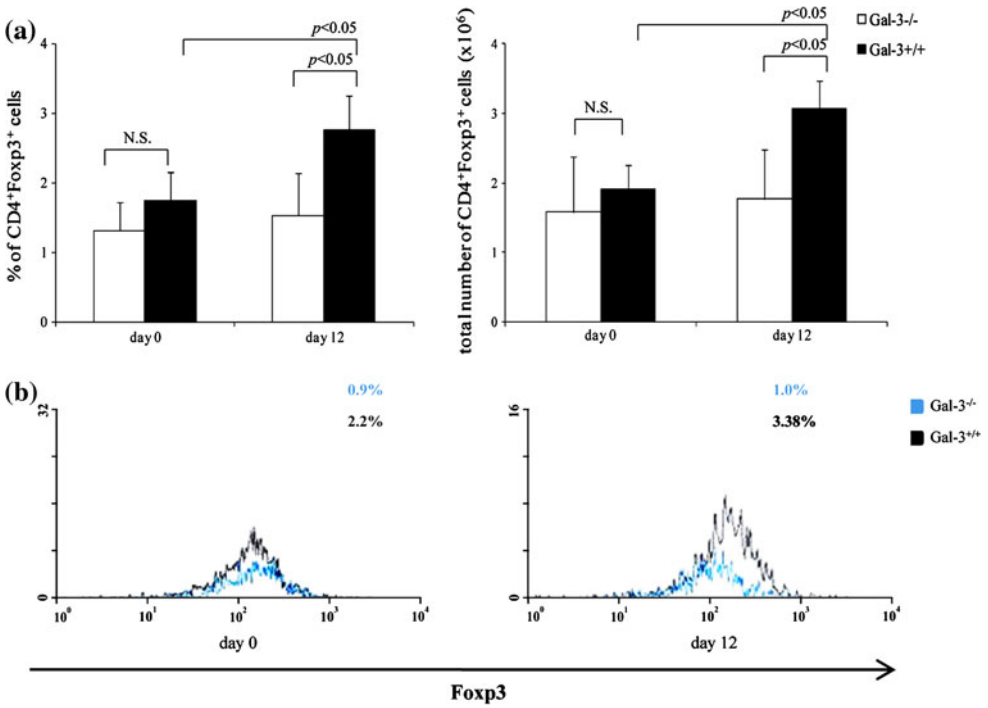

Fig. 3 Gal-$3^{-/-}$ mice have significantly lower percentage and total number of $CD4^+Foxp3^+$ Treg cells derived from spleen after tumor cell injection. **a** Gal-$3^{-/-}$ mice have a significantly lower percentage and number of $CD4^+Foxp3^+$ Treg cells compared with Gal-$3^{+/+}$ mice after tumor cell injection ($P < 0.05$). Further, Gal-$3^{+/+}$ mice have greater percentage and total number of $CD4^+Foxp3^+$ Treg cells compared with naive mice ($P < 0.05$; four mice per group). **b** Diagrams illustrating percentage of $CD4^+Foxp3^+$ Treg cells on 0-th and 12-th day after i.v. injection of B16F1 cells in Gal-$3^{-/-}$ mice (*blue*) and Gal-$3^{+/+}$ mice (*black*)

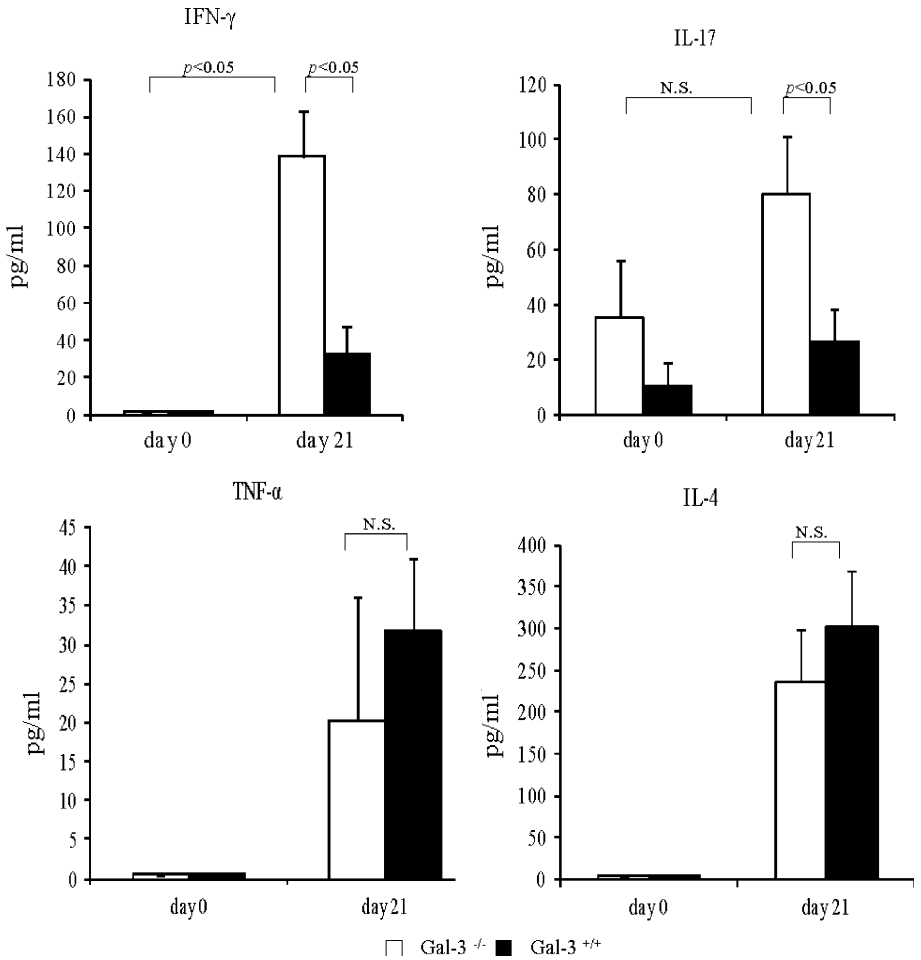

Fig. 4 Serum levels of IFN-γ, IL-17, TNF-α and IL-4 in Gal-$3^{-/-}$ and Gal-$3^{+/+}$ mice at 0 and 21-th day after i.v. injection of 5×10^4 B16F1 melanoma cells (five mice per group). When compared with the Gal-$3^{+/+}$ mice (M ± SD: 32.61 ± 15.21 pg/ml), serum level of IFN-γ was higher in the Gal-$3^{-/-}$ mice by day 21 after tumor cell inoculation (138.95 ± 24.58 pg/ml; $P < 0.05$). Also, serum level of IL-17 was higher in Gal-$3^{-/-}$ mice (80.31 ± 21.07 pg/ml; $P < 0.05$) compared with the Gal-$3^{+/+}$ mice (26.46 ± 11.47 pg/ml) in tumor bearing hosts

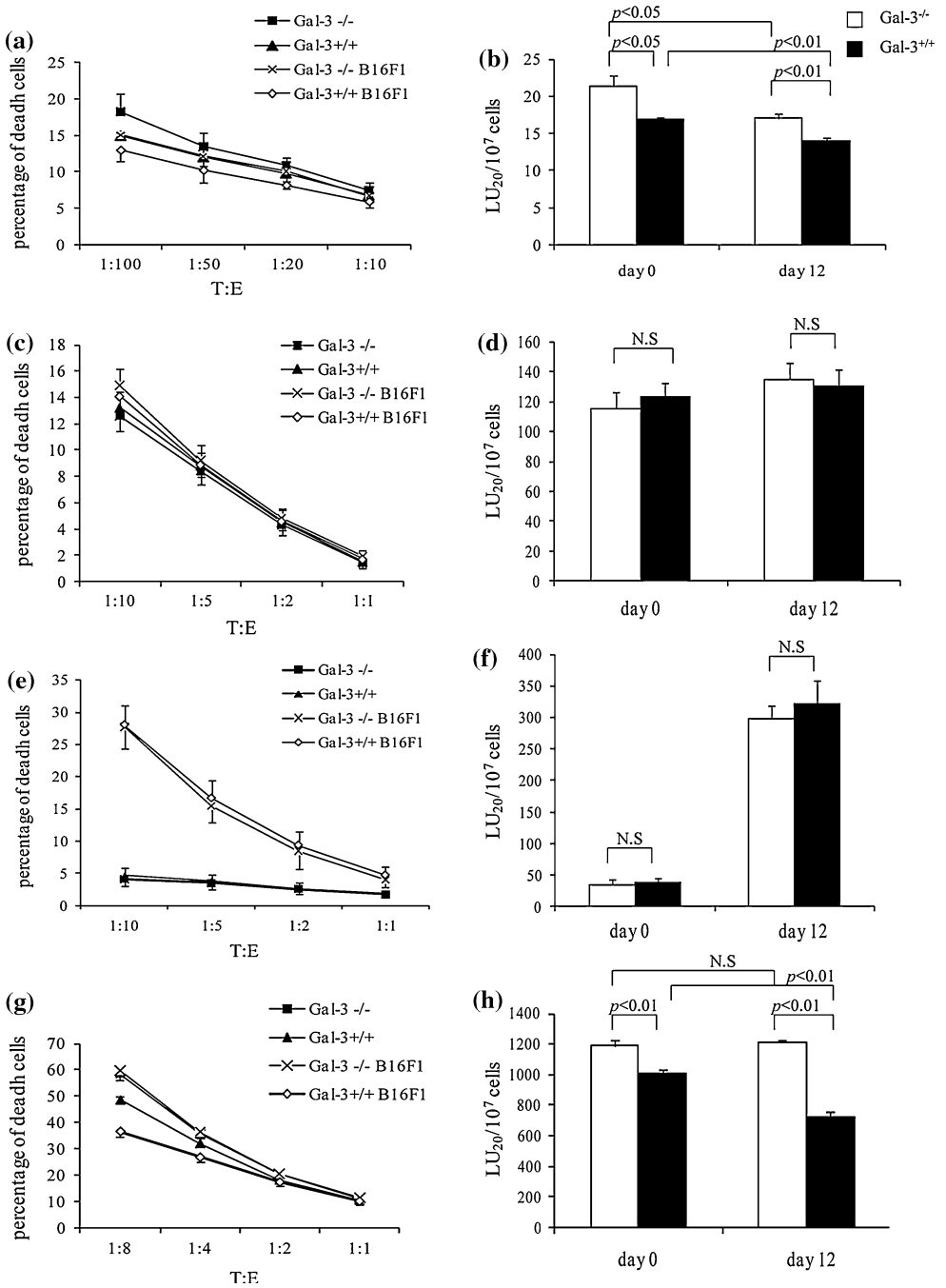

Fig. 5 Cytotoxicity of splenocytes, splenic adherent and NK cells (4 mice per group). **a**, **b** Cytotoxic activity of total splenic cells from Gal-3-deficient mice was higher in both tumor cell-inoculated groups ($P < 0.01$) and naive animals ($P < 0.05$). **c**, **d**, **e** and **f** There was no difference in the cytotoxicity of adherent cells, as well as in the cytotoxicity of $CD8^+$ T cells between Gal-$3^{-/-}$ and Gal-$3^{+/+}$ mice. 5 g and 5 h: Remarkably, we noticed higher cytotoxic activity of NK cells from Gal-3-deficient mice compared with Gal-$3^{+/+}$ mice in both tumor cell-inoculated groups ($P < 0.01$) and naive mice ($P < 0.01$)

Galectin-3-deficient mice showed higher percentage of effective cytotoxic $CD3\varepsilon^-NK1.1^+$ cell phenotype and their faster turnover

In order to define possible mechanisms of enhanced NK cell activity in Gal-$3^{-/-}$ mice, we also estimated the number and functional phenotype of NK cells in the spleen of naive Gal-$3^{-/-}$ and Gal-$3^{+/+}$ mice. Our results indicate that Gal-$3^{-/-}$ mice have a lower total number of $CD3\varepsilon^-NK1.1^+$ cells derived from spleen compared to Gal-$3^{+/+}$ mice ($P < 0.01$; Fig. 6a). Despite the reduction in total $CD3\varepsilon^-NK1.1^+$, Gal-$3^{-/-}$ mice constitutively have a

significantly higher percentage of effective tumoricidal $CD27^{high}CD11b^{high}$ NK cells ($P < 0.01$), as well as the percentage of immature $CD27^{high}CD11b^{low}$ NK cells (Fig. 6b and dot plots in Fig. 6d, left panels). This greater percentage of immature cells may indicate that Gal-$3^{-/-}$ mice display faster turnover of NK cells than Gal-$3^{+/+}$ mice ($P < 0.01$; Fig. 6b). In contrast, $CD27^{low}CD11b^{high}$ less functionally exhausted NK cells were more numerous in Gal-$3^{+/+}$ mice ($P < 0.01$). Remarkably, we noticed significantly lower percentage of inhibitory KLRG1 receptor expressing NK cells of Gal-$3^{-/-}$ mice as compared to Gal-$3^{+/+}$ control mice ($P < 0.01$; Fig. 6c and dot plots in Fig. 6d, right panels).

Discussion

Here we demonstrated that the deletion of Galectin-3 gene in the host significantly suppress lung metastasis of melanoma cells (B16F1). Our data reveals that the Galectin-3 deficiency in the host affects metastasis of malignant melanoma by at least two mechanisms: tumor cell attachment in target tissue, and maturation and function of NK cells.

Although, many studies have suggested that Galectin-3 plays important roles in various processes such as cell adhesion [34, 35], resistance of metastatic tumor cells to apoptosis [36, 37], malignant transformation [38–41], and tumor progression [15, 42], there is no information on the role of Galectin-3 expressed on the host cells in regulating metastastic process in vivo. The present study is the first demonstration that lack of Galectin-3 renders mice resistant to melanoma metastasis: in vivo study showed that Gal-$3^{-/-}$ mice exhibited relative resistance to lung colonization of melanoma cells as evaluated by number of lung metastatic colonies (Fig. 1a) and the higher cellularity of lung colonies in Gal-$3^{+/+}$ mice compared with Gal-$3^{-/-}$ mice (Fig. 1b). Complementary to our findings, Abdel-Aziz et al. [43] recently showed that the incidence of lung tumors was significantly lower in Gal-$3^{-/-}$ mice after intraperitoneal injection of chemical carcinogen 4-(methylnitrosamino)-1-(3-pyridyle)-1-butanone. Thus, Galectin-3 in the host could be important in lung tumorigenesis as well as in metastasis. Likewise, clinical evidences have shown that increased serum level of Galectin-3 in patients with malignant melanoma, reflects metastatic phenotype [16, 44].

The adhesive interaction of metastatic tumor cells appears to be obligatory for successful creation of metastatic foci in the distant organs [45]. There is evidence to suggest that Galectin-3 promotes tumor cell adhesion in vitro. For instance, elevated expression of Galectin-3 markedly enhances tumor cell adhesion to common extracellular matrix proteins [18] and increases the incidence of lung metastasis [35]. This is in agreement with the data presented in this work showing resistance of Galectin-3-deficient mice to melanoma metastasis. Additionally, our in vitro adhesion assay showed lower binding of malignant cells to onto lung tissue of Gal-$3^{-/-}$ mice suggesting that lack of Galectin-3 may contribute to the resistance of mice to melanoma metastasis (Fig. 2a). This assumption may be supported by findings that Galectin-3 binds specifically the poly-*N*-acetyllactosamine residues of tumor cells [46–48] including melanoma cells [13]. Thus, endothelial Galectin-3 might function to promote tumor cell adhesion to blood vessel walls by interacting with numerous carbohydrate ligands expressed on tumor cells [12, 13, 49]. Furthermore, interaction between free circulating Galectin-3 and transmembrane mucin protein MUC1 promotes embolus formation and survival of disseminating tumor cells in the circulation [50]. An increase of tumor cell aggregation as a results of the increased interaction between circulating Galectin-3 and cancer-associated MUC1 in cancer patients provides a survival advantage to the disseminating tumor cells in the circulation [50].

Anti-tumor immunity is controlled by innate and adaptive immune system cells which play a major role in regulating tumor growth and metastasis. The resistance of Galectin-3-deficient mice to melanoma metastasis prompted us to investigate the role of Galectin-3 in anti-melanoma immunity. We found clear difference in the number of $CD4^+Foxp3^+$ T cells which was not accompanied with differences in the number of $CD4^+$ and $CD8^+$ cells. Obviously, it does not exclude the possibility that the number of tumor specific Th1 cells and $CD8^+$ cytotoxic cells is different. This assumption is strengthen by the clear difference in the serum IL-17 and IFN-γ in the tumor bearing host (Fig. 4). However, it is also possible that most relevant role of Treg cells in our tumor system is suppression of NK function [51, 52]. In fact, we observed significant increase in the NK cell cytotoxicity and maturation of cytotoxic NK cells in Gal-$3^{-/-}$ mice. Accordingly, increased serum levels of IL-17 and IFN-γ may be due to the stimulation of the different NK subset ratios [53, 54].

Recently, it has been indicated that the tumor cells actively interact with the immune cells to foster an immunosuppressive environment comprised of T_{reg} cells, cytokines and growth factors, all of which may promote tumor growth and metastasis [55]. After tumor cell injection Gal-$3^{-/-}$ mice showed lower percentage and total number of $CD4^+Foxp3^+$ T cells compared with Gal-$3^{+/+}$ mice. In fact, in Gal-$3^{+/+}$ mice but not in Gal-3-deficient mice, injection of melanoma cells resulted in a significant increase in the percentage and total number of $CD4^+Foxp3^+$ T cells in the spleen (Fig. 3). It appears that Galectin-3 signaling may facilitate tumor metastasis by

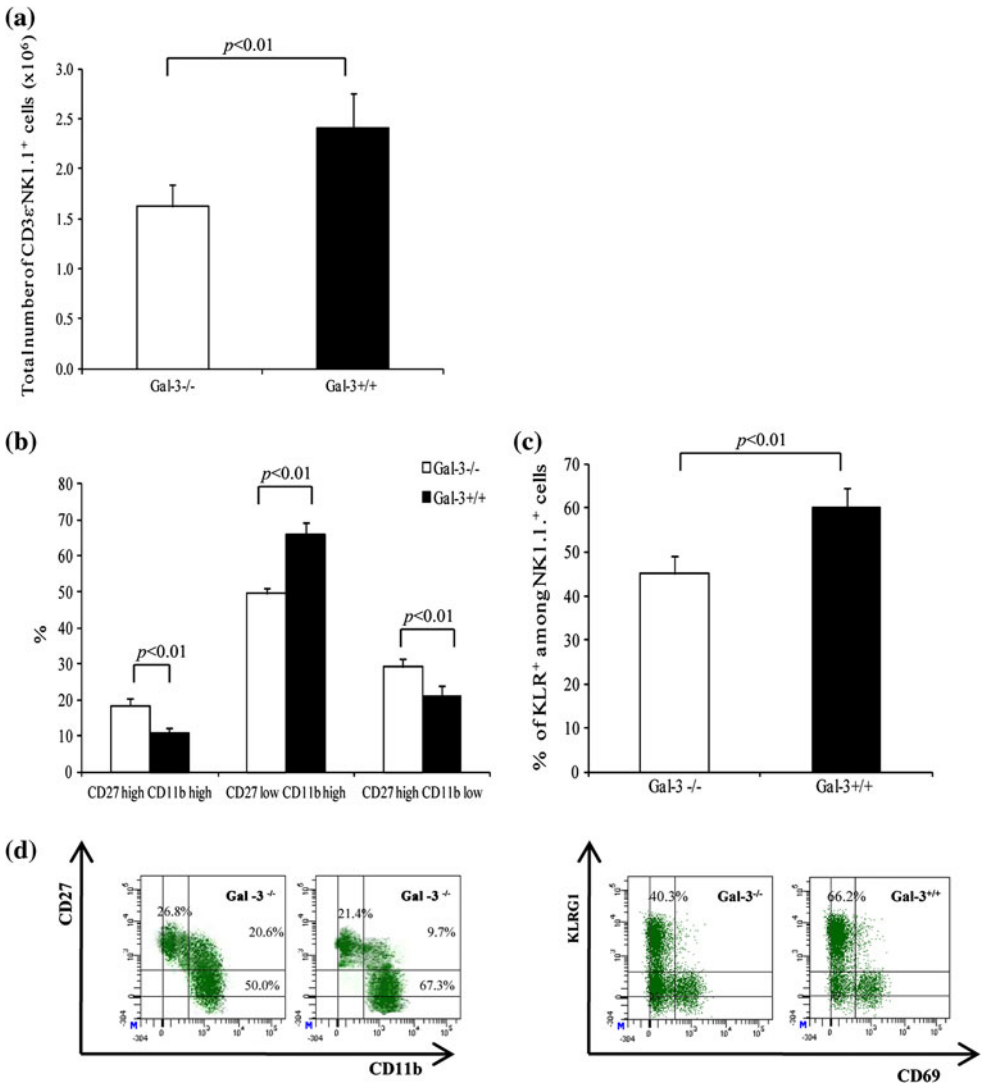

Fig. 6 Functional phenotype of CD3ε^{-}NK1.1^{+} cells derived from spleen (four mice per group). **a**: Gal-3$^{-/-}$ mice have a lower total number of CD3ε^{-}NK1.1^{+} cells derived from spleens compared to Gal-3$^{+/+}$ mice (M ± SD: 1.63 × 10^{6} ± 0.22 vs. 2.41 × 10^{6} ± 0.22; $P < 0.01$). **b** In addition, Gal-3$^{-/-}$ mice have a significantly greater percentage of CD11b^{high}CD27^{high} NK cells ($P < 0.01$) as well as the percentage of immature CD27^{high}CD11b^{low} cells ($P < 0.01$) compared to Gal-3$^{+/+}$ mice. In contrast, CD27^{low} less functionally exhausted NK cells were more numerous in Gal-3$^{+/+}$ mice ($P < 0.01$). **c** We also noticed significantly higher percentage of inhibitory KLRG1 receptor expressing NK cells in Gal-3$^{+/+}$ mice compared with Gal-3$^{-/-}$ mice ($P < 0.01$). There was no significant difference in the percentage of CD69^{+}NK cells between Gal-3$^{-/-}$ and Gal-3$^{+/+}$ animals. **d** *Dot plots* illustrate significant increase in the percentage of CD27^{high}CD11b^{high} (highly active, tumoricidal) NK cells (*left panel*) and lower percentage of KLRG1^{+}NK cells (senescent NK cells; *right panel*) in Gal-3$^{-/-}$ mice

promoting the formation of the immunosuppressive environment. Many studies of T_{reg} cells in human cancer including malignant melanoma have shown elevated number of T_{reg} cells in the peripheral blood and tumor microenvironment [56, 57]. Likewise, in animals' model removal of T_{reg} cells enhance anti-tumor immune response, implying that these cells suppress immune response against tumor cells [58]. In the current study, we found higher serum level of IFN-γ in Gal-3$^{-/-}$ mice challenged with B16F1 melanoma cells compared with Gal-3$^{+/+}$ mice. We also noticed higher serum level of IL-17 in Gal-3$^{-/-}$ mice. In line with our study, Kryczek et al. [59] suggested that IL-17-deficient mice exhibit accelerated tumor growth and lung metastasis. However, other data suggest that IL-17 support angiogenesis in tumor system [60]. On the other hand, the serum levels of IL-4 and TNF-α was also increased after tumor cell inoculation, but did not reach statistically significant difference between Gal-3$^{-/-}$ and Gal-3$^{+/+}$ mice (Fig. 4).

Next, we assess the cytotoxic capacity of splenocytes against B16F1 melanoma cells as targets, using MTT assay. Gal-3$^{-/-}$ mice have enhanced cytotoxicity of splenocytes when compared Gal-3$^{+/+}$ mice (Fig. 5a). Several studies have provided evidence that high number of tumor-infiltrating NK cells correlate with a good prognosis and increased patient's survival in many different tumor types [61, 62]. Also, NK activity might be important in control of metastasis, and patients with advanced metastatic disease often have abnormalities in NK cell function [63]. Remarkably in the present study, naive Gal-3$^{-/-}$ mice have a lower total number of splenic CD3ε^{-}NK1.1^{+} cells compared to Gal-3$^{+/+}$ mice (Fig. 6a), but greater constitutive NK cell mediated anti-tumor cytotoxicity, prior and after tumor cell injection (Fig. 5g, h).

Recently, in a murine system, NK cell pool divided into functionally distinct subsets, as defined by expression levels of CD27 [64]. Hayakawa et al. [64] reported that $CD27^{high}$ NK cells displayed higher cytolytic activity compared with $CD27^{low}$ NK cells. Previous studies have reported that KLRG1 engagement can inhibit NK cell cytotoxicity and cytokine production [65, 66]. For instance, $CD11b^{+}CD27^{-}$ $KLRG1^{+}$ cells produced low levels of IFN-γ following stimulation with IL-12 and IL-18 [67]. In addition, in the early phase of viral infection, KLRG1 expression on NK cells inversely correlates with ability of NK cells to produce IFN-γ [68]. It has been reported that KLRG1 expression may terminate NK cell response, although the mechanisms of this function is yet to be elucidated [69]. Additionally, expression of KLRG1 is also associated with slower in vivo turnover, and following homeostatic proliferation as most NK cells acquire KLRG1 expression in vivo [69]. In this regard, we estimated functional phenotype of NK cells by multicolor cytometry. Gal-$3^{-/-}$ mice constitutively have a significantly higher percentage of $CD27^{high}CD11b^{high}$ NK cells, as well as the percentage of immature NK cells (Fig. 6b). In contrast, $CD27^{low}CD11b^{high}$ less functionally exhausted NK cells were more numerous in Gal-$3^{+/+}$ mice. The fact that there was no significant difference in the percentage of $CD69^{+}$NK cells seems to be apparent contrast with finding that NK contribution to cell mediated cytotoxicity is higher in Gal-$3^{-/-}$ mice. We can only assume that Gal-$3^{-/-}$ mice have higher cytotoxic activity (Fig. 6c). Additionally, we noticed significantly lower percentage of inhibitory KLRG1 receptor expressing NK cells of Gal-$3^{-/-}$ mice as compared to Gal-$3^{+/+}$ control mice (Fig. 6c). As KLRG1 is expressed on $CD27^{low}CD11b^{high}$ NK cells, this finding is in accordance with the previous observation [69].

Taken together, our data indicate that attenuation of Galectin-3 signaling may facilitate melanoma metastasis by affecting tumor cell adhesion and NK cell mediated anti-tumor activity, suggesting that blockade of Galectin-3 might result in therapeutic benefits.

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