Published in final edited form as:

Melanoma Res. 2016 October ; 26(5): 429–441. doi:10.1097/CMR.0000000000000281.

Galectin-9 modulates immunity by promoting Th2/M2 differentiation and impacts survival in patients with metastatic melanoma

Elizabeth Ann L. Enninga¹, Wendy K. Nevala², Shernan G. Holtan³, Alexey A. Leontovich⁴, and Svetomir N. Markovic^{2,5}

¹Mayo Graduate School, Mayo Clinic, Rochester MN

²Division of Hematology, Mayo Clinic, Rochester MN

³Department of Medicine, University of Minnesota, Minneapolis MN

⁴Health Sciences Research, Mayo Clinic, Rochester MN

⁵Division of Oncology, Mayo Clinic, Rochester MN

Abstract

PURPOSE—Galectin-9, a β -galactoside binding protein, is defined as a negative regulator of T helper 1 (Th1) immune responses, favoring Th2 bias. Systemic immunity in patients with metastatic melanoma is predominately Th2 biased. We hypothesized galectin-9 could modulate systemic immunity toward Th2 polarization in patients with advanced melanoma.

EXPERIMENTAL DESIGN—Presence/concentration of galectin-9 was assessed in tumors and plasma, respectively, in patients with metastatic melanoma. Immunomodulatory function of galectin-9 was determined by exposing human peripheral blood mononuclear cells (PBMC) to galectin-9 *in vitro*.

RESULTS—Galectin-9 was expressed in 57% of tumors and was significantly (3.6-fold) increased in the plasma of patients with advanced melanoma compared to healthy controls ($p < 0.001$). High plasma galectin-9 concentration was associated with systemic Th2 polarization and reduced two-year survival compared to low/no galectin-9 expression. *In vitro*, galectin-9 reduced proliferation of healthy PBMCs, promoted Th1 cell apoptosis, and encouraged Th2 biased cell phenotypes and cytokine secretion. Galectin-9 also stimulated monocyte differentiation towards an M2 macrophage phenotype, as assessed by chemokine/cytokine secretion and CD206 expression, observed both *in vitro* as well as in patients with metastatic melanoma.

CONCLUSION—Elevated galectin-9 in patient plasma correlates with Th2 systemic bias and less favorable clinical outcomes in metastatic melanoma. This Th2 bias appears to be not only a feature of the known mechanisms of Th1 apoptosis via Tim3 binding, but also mediated via myeloid cell

Corresponding Author: Svetomir N. Markovic, 200 1st St SW, Rochester, MN 55905, Phone: 507-538-0118, Fax: 507-284-1637, Markovic.svetomir@mayo.edu.

Conflict of interest: Authors state no conflict of interest

differentiation towards a M2 phenotype, potentially favoring tumor progression. These data support galectin-9 as a novel therapeutic target for patients with metastatic melanoma.

Keywords

galectin-9; melanoma; immunity; Tim3; macrophage

INTRODUCTION

Understanding immune regulation in the context of the tumor microenvironment is necessary and critical for effective advancements in cancer therapy. Immunotherapies, such as anti-CTLA4 (cytotoxic T-lymphocyte-associated protein 4) and anti-PD1 (program death 1), have revolutionized cancer therapeutics by blocking the ability of the tumor to impede T-cell activation, resulting in prolonged survival in metastatic melanoma (1, 2). Nonetheless, in order for these checkpoint inhibitors to significantly impact patient outcomes, these therapies require a functional endogenous immune system. However, patients with cancer exhibit systemic immune responses consistent with chronic inflammation (Th2 biased helper T-cells) which creates an optimal environment for tumor development (3, 4). The loss of Th1 type responses and the promotion of Th2 type immune bias has been shown to negatively affect clinical responses to therapy and patient outcomes (5, 6). Recognizing this problem, monoclonal antibodies against chronic inflammatory promoting proteins have been developed and shown to have clinical efficacy. For example, blocking vascular endothelial growth factor (VEGF) limits its ability to promote angiogenesis and lymphangiogenesis induced inflammation which improves survival (7, 8). Thus, investigating other pro-tumor targets of which blocking the effect might activate the immune system could lead to novel therapies.

One such protein that has been characterized as an inhibitor of Th1 immune responses is galectin-9. Galectins are carbohydrate binding proteins which are evolutionarily conserved and have pleiotropic roles in immunity (9). First defined as an eosinophil chemoattractant, galectin-9 has been implicated in regulation of Th2 type responses in allergy models (10, 11). Others have shown transgenic Th1 biased mice increase myeloid derived suppressor cells (MDSC) after addition of galectin-9 (12). T-cell immunoglobulin and mucin-domain containing-3 (Tim3) is the most well characterized binding partner of galectin-9, and their interaction was shown to negatively regulate Th1 type responses through apoptosis of activated CD4+ cells (13, 14). Remarkably, galectin-9/Tim3 regulation at the feto-maternal interface seems to be critical for the development and maintenance of healthy pregnancies (15, 16). Many similarities exist between tumor and trophoblastic development and regulation in promoting immune tolerance (17, 18). Since Th1 responses are correlated with a less favorable immune environment for tumor growth and fetal development, galectin-9 may be an important target that modulates immunity in support of the tumor. The Tim3/galectin-9 interaction shares the same outcome, specifically apoptosis and cell exhaustion, as the program death (PD)-1 and PD-ligand (L)-1 signaling pathway which has been extensively characterized and shown to be a clinically effective target in many tumor types (19, 20). However, galectin-9 and its function in human metastatic melanoma remains largely unknown. The present study was designed to define the relevance and role of

galectin-9 on immunity in human metastatic melanoma. Our results confirm galectin-9 as a prognostic marker in metastatic melanoma. These findings support the rationale for pre-clinical development of galectin-9 targeted immunotherapies.

METHODS & MATERIALS

Patient selection

276 patients with metastatic melanoma and 40 healthy controls were utilized for our study. Baseline blood specimens, prior to treatment, were collected from patients on three clinical trials: N057e, N0775 and N0879. All available specimens were run from each study. Phase II clinical trial N057e was conducted by the North Central Cancer Treatment Group (21). We obtained plasma from 51 patients enrolled in N057e for our studies. For the randomized Phase II trial N0775, we obtained samples from 82 patients on this study (22). N0879 was a randomized Phase II and plasma was utilized from 143 patients. Informed consent was obtained from all subjects to participate in N057e, N0775 and N0879 and studies were approved by the Mayo Clinic Institutional Review Board.

Protein determination by ELISA and multiplex

Plasma samples were isolated from the blood of metastatic melanoma patients and healthy donors, and then frozen until analysis. Concentration of galectin-9, interferon (IFN)- γ , inducible protein (IP)-10, interleukin (IL)-10 and monocyte chemoattractant protein (MCP)-1 was determined by enzyme linked immunosorbant assay (ELISA) using the human DuoSet antibodies (R&D Systems, Minneapolis, MN) as per manufactures' instructions. Protein concentration was determined using a 7-point standard curve at 450nm by a VersaMAX microplate reader (Molecular Devices, Sunnyvale, CA).

Supernatants from healthy PBMCs cultured with and without 2ug/mL galectin-9 were collected for multiplex analysis. Lymphocytes were isolated from those same PBMC samples through CD14 positive selection and monocytes were isolated through negative selection of CD14+ cells (Miltenyi Biotec, San Diego, CA). Proteins were measured using the MagPlex® Milliplex human 38 cytokine panel (Millipore, Billerica, MA) per manufacturer's instructions. This kit contains EGF, FGF-2, Eotaxin, TGF- α , G-CSF, Flt-3L, GM-CSF, Fractalkine, IFN α 2, IFN- γ , GRO, IL-10, MCP-3, IL-12p40, MCD, IL-12p70, IL-13, IL-15, sCD40L, IL-17A, IL-1RA, IL-1 α , IL-9, IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1 α , MIP-1 β , TNF α , TNF β and VEGF. Samples were run using Luminex xPONENT technology (Luminex, Austin, TX) and concentrations were determined using a standard curve from 1.6 to 5000pg/mL, using Milliplex Analyst software (VigeneTech, Carlisle, MA). All samples were run in duplicate and averaged to determine concentration.

Tissue histology

Tumor samples were retrieved from 118 patients with resected metastatic melanoma lesions as a part of routine clinical care from 1993 to 2004, as previously reported (23). Primary antibodies for galectin-9 and PD-L1 (BD Biosciences, San Jose, CA) were diluted according

to the manufacturer's instructions. Tumor microarray slide images were digitized using the Olympus BLISS HD Virtual Microscopy System™ (Olympus, Center Valley, Pennsylvania).

10 metastatic melanoma tumors were fixed in formalin and embedded in paraffin. Antibodies against galectin-9 (AbCam, Cambridge, MA), PD-L1, CD68 and S100 (Novus Biologics, Littleton, CO) were utilized for staining, along with isotype controls by Histoserv, Inc. (Germantown, MA). Stains were digitized and imported into IHCscore (Bacus Laboratories, Lombard, IL). Representative images are shown at 20×.

Proliferation assay

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors for measurement of proliferative capacity in the presence of recombinant human galectin-9 (R&D Systems, Minneapolis, MN). In three separate experiments, cells from two allogeneic healthy controls were mixed together: one of the PBMC samples having been irradiated (stimulator). Culture conditions included 20U/mL IL-2 in RPMI + 10% fetal bovine serum (FBS) and experimental wells received 2ug/mL galectin-9. In a 96-well plate, 100,000 responder cells in 100uL were added with 100uL of serial diluted irradiated cells in triplicate. Cultures were incubated for 3 days at 37°C and 5% CO₂ before addition of 1uCi/well of H³ thymidine overnight. The plate was washed and transferred to a 96-well filter plate. Scintillation fluid was added and counts per minute (cpm) were recorded on a scintillation counter. Averages and standard deviations were calculated from each replicate and combined between all three experiments.

Cellular apoptosis assay

PBMCs were plated at 100,000/well in a 96 well plate with 2ug/mL galectin-9, 10ug/mL anti-Tim3 neutralizing antibody (Clone 344801; R&D Systems, Minneapolis, MN) or both and incubated for 24 hours at 37°C and 5% CO₂. Blocking was determined by phospho-flow cytometry utilizing phospho-Y265 PE (Millipore, Billerica, MA) and Tim3 APC or secondary staining (R&D Systems, Minneapolis, MN). Cultures were assayed for apoptosis using the APO-DIRECT flow cytometry apoptosis method (Millipore, Billerica, MA) per manufacturers' instructions. In brief, extracellular staining was carried out for 30 minutes with CD4-PE, CD8-PE-Cy5 and CD14-PE-Cy7 (BD Biosciences, San Jose, CA). Cells were fixed and incubated overnight at -20°C in 70% ethanol. Cells were washed and stained for DNA breaks by FITC tagged nucleotides, resuspended in FACS buffer and read on a Guava easyCyte flow cytometer (Millipore, Billerica, MA). 20,000 events were counted and apoptotic, dead, and live cells were compared between groups with and without galectin-9.

Flow cytometry

Mononuclear cells from healthy donors (n=15) were isolated from buffy coats and incubated overnight at 37°C and 5% CO₂ with or without 2ug/mL of galectin-9. Cells were washed and four color flow cytometry was run to determine effects of galectin-9 on Tim3+ and Tim3- CD4 and CD8 cells. Permeabilization was carried out in 0.5% saponin. Antibodies were acquired from BD Bioscience (San Jose, CA) and T helper cells (CD3+CD4+), cytotoxic T-cells (CD3+CD8+), and T regulatory cells (CD4+CD25+FOXP3+), along with

CD294, PD-1 and Tim3, were probed. Experiments were run on a Guava easyCyte 8HT flow cytometer (Millipore, Billerica, MA) with 20,000 events counted.

Macrophage culture

THP-1 (TIB-202™) cells, a human monocytic cell line, was obtained within the last 2 months from ATCC (Manassas, VA) where it was authenticated using single tandem repeat profiling (STR). Healthy CD14⁺ monocytes were isolated out of fresh blood from healthy donors using a Ficoll-Paque gradient (GE HealthCare, Pittsburgh, PA) and the MACS Human Monocyte Isolation Kit (Miltenyi Biotech Inc., San Diego, CA). THP-1 were differentiated into macrophages with 150nM PMA for 24 hours, and then polarized to M2 phenotype for 72 hours with IL-4 and IL-13 using a previously published method (24) or 2ug/mL of galectin-9. CD14⁺ cells were differentiated into macrophages with 150nM PMA for 24 hours, and then polarized to a M2 phenotype for 48 hours with IL-4 and IL-13, or 2ug/mL galectin-9 as above. To determine M2 status, cells were stained for CD206-PE, CD68-FITC and CD14-PC7 (BD Biosciences, San Jose, CA) by flow cytometry. Supernatants were tested by ELISA for IFN- γ and IP-10 (M1) and IL-10 and MCP-1 (M2) secretion as above.

Data analysis

Experimental samples were averaged and statistical comparisons were determined using a two-tailed Student's t test. High versus low galectin-9 threshold was determined by recursive partitioning and compared using chi-squared tests (25). Probability of 2-year survival based on galectin-9 was estimated by Kaplan-Meier curves and compared by log-rank tests. Survival was defined as the date of initial blood draw to date of death or last follow up. Univariate analysis of patient characteristics between low/high galectin-9 cohorts were compared with a Chi-Square test. Multivariate analysis was assessed using the Cox proportional hazard regression model. JMP 10.0.0 (SAS Institute Inc. Cary, NC) was used for statistical analysis. All tests were two sided and a p-value less than 0.05 were considered statistically significant. For multiplex data, significance was defined by a p-value of less than 0.001 after utilizing the Holm-Sidak method to correct for multiple comparisons.

RESULTS

Patient characteristics

Two-hundred seventy six patients were included in this study. Date of death was missing for only three patients. The majority of patients were male, stage M1c and between 50–60 years old. Supplementary table S1 shows the clinic-pathologic differences between the high soluble galectin-9 (≥ 1532 pg/mL) and low soluble galectin-9 (<1532 pg/mL) patient groups determined by recursive partitioning. There were no significant differences between the two groups related to gender, age, LDH or clinical stage.

Galectin-9 is elevated in metastatic melanoma

By ELISA, plasma samples from patients with metastatic melanoma were compared to healthy controls for concentration of soluble galectin-9. Galectin-9 concentrations from healthy persons (n=16) had a mean of 1,192pg/mL (SEM ± 125.5) while patients with

metastatic melanoma plasmas (n=20) were an average of 3.6 fold higher, 4,283.78pg/mL (SEM $\pm$ 350.3; $p < 0.0001$) (Fig. 1A). Next, tumor microarrays of metastatic melanoma tumors were analyzed for expression of galectin-9 and PD-L1. Of the 118 tumors, 51% of them co-express galectin-9 and PD-L1, 34% were PD-L1 positive, 6% were galectin-9 positive and 9% were negative for both receptors (Fig. 1B). To determine where staining for galectin-9 show highest positivity, 10 stage IV tumor biopsy tissues were stained for S100, PD-L1 and galectin-9. Figure 1C is a representative stain for these bio-markers showing highest staining patterns for galectin-9 at the interface between the tumor and the non-cancerous stroma (S100 negative), which is comparable to known PD-L1 staining (19). Using IHCscore to determine the percentage of the tumor core, tumor edge and non-cancerous tissue which are positive for each marker, it was observed that although the tumors differed in their staining pattern, highest staining intensity for galectin-9 was on the edge ($p = 0.0003$) and in the tumor core ($p = 0.02$) compared to S100 negative stromal tissue (Fig. 1D). PD-L1 staining was significantly increased on the tumor edge ($p = 0.02$), but not the core compared to non-cancerous tissue.

High galectin-9 correlates with worse 2-year survival

276 plasma samples from patients with metastatic melanoma at baseline prior to participation in three different clinical trials were measured for galectin-9 concentration by ELISA. Concentrations were then divided into high (≥ 1532 pg/mL) or low (< 1532 pg/mL) galectin-9 using recursive partitioning, as in glioblastoma (25). At two years after blood draw date, patients with high levels of galectin-9 had poor survival (Fig. 2A), with a median of 340 days (95% CI 370–621 days) compared to those with low galectin-9 in plasma (510 days; 95% CI 293–391 days; $p = 0.02$). In a multivariate model, high galectin-9 remained significantly associated with poor survival after adjustment for age, sex, and LDH (supplementary table S2). As expected, LDH also remained predictive to worse survival in our model (risk ratio 1.8, 95% CI 1.3–2.4; $p = 0.0002$).

To determine systemic immunological impact of high versus low galectin-9 with known immune phenotypes, we looked at the Th1:Th2 ratio in PBMCs defined as cellular CD4+Tim3+ (Th1):CD4+CD294+ (Th2), the concentration of Th1 biasing cytokine IFN- γ and the concentration of Th2 biasing cytokines IL-4 and IL-10 in plasma from this same patient population by flow cytometry and multiplex analysis. The Th1:Th2 ratio was significantly lower, thus more Th2 polarized, in the high galectin-9 group compared to the low (0.96 vs. 1.39, $p = 0.01$) (Fig. 2B). IFN- γ was lower in patients that corresponded with the high galectin-9 group (18.2pg/mL vs. 5.3pg/mL, $p = 0.02$) (Fig. 2C). Th2 biasing cytokines IL-4 and IL-10 were significantly increased in the plasma of patients who had high galectin-9 levels (1.9pg/mL vs. 0.7pg/mL, $p = 0.03$; 7.8pg/mL vs. 1.5pg/mL, $p = 0.03$; respectively) (figure 2D and E). Interestingly, the percentage of CD4+Tim3+ cells did not differ ($p = 0.35$) between the high versus low galectin-9 cohort (data not shown).

Galectin-9 modulates immunity

Using irradiated allogeneic blood cells, healthy PBMCs were stimulated to induce cellular expansion to foreign antigen in the presence and absence of galectin-9. Irradiated cells were serially diluted from 100,000 cells down to 50 cells per 100,000 viable PBMCs. In the

presence of 2ug/mL galectin-9, proliferation was damped up to a dilution of 1:8 responders to stimulators (Fig. 3A). After that, the allogeneic stimulus was strong enough to override galectin-9 effects and initiate proliferation similar to the control.

To assess the role of galectin-9 and its receptor Tim3 on apoptosis, TUNEL staining was used to assess viability of T helper (CD4), T cytotoxic (CD8), and monocytic (CD14) cell populations. Effectiveness of Tim3 blocking was determined by phospho-flow cytometry and Tim3+ p-Y265+ signal was measured (supplemental figure S1). After 24 hours with galectin-9, there was a significant increase in percentage of cellular apoptosis in CD4+, CD8+ and CD14+ cells compared to untreated controls (36% vs. 20.5%, $p=0.001$; 37.8% vs. 9.7%, $p=0.0007$; 45.6% vs. 20%, $p=0.03$; respectively)(Fig. 3B). With the addition of a Tim3 blocking antibody, there was no difference in the percentage of apoptosis initiated by galectin-9 in the CD8+ and CD14+ populations. However, there was a significant difference in apoptosis between CD4+ cells treated with galectin-9 and the anti-Tim3 antibody compared to those treated with galectin-9 alone (21.76% vs. 36%, $p=0.003$). This data suggests CD4 apoptosis is dependent on Tim3 binding, whereas galectin-9 initiates CD8 and CD14 apoptosis independent of Tim3.

Next we sought to understand how galectin-9 affects the phenotype of cells that are positive for Tim3 compared to those that do not express Tim3 by flow cytometry (Fig. 3C). After a 48 hour incubation with galectin-9, PD-1 and Tim3 receptors were measured on CD4+ cells and the result was a decrease in the CD4+Tim3+ population (413 vs. 139, $p=0.02$), the double positive population did not change (120 vs. 91, $p=0.47$), and the Tim3-PD-1+ population increased in the presence of galectin-9 (487 vs. 861, $p=0.0008$). CD294, a receptor indicative of a Th2 biased cell, significantly increased in number of CD4+Tim3- cells after galectin-9 incubation compared to untreated (1270 vs. 733, $p=0.002$), but did not affect the double positive population (120 vs. 122, $p=0.97$). In the cytotoxic T-cell population, there was no observable change in the number of CD8+Tim3+ cells (520 vs. 693, $p=0.47$), a decrease was observed in the Tim3+ PD-1+ population (79 vs. 265, $p=0.04$), and an increase was seen in the Tim3- PD-1+ population (591 vs. 198, $p=0.01$) following galectin-9 incubation. Lastly, T-regulatory cells (CD4+ CD25+FOXP3+) treated with galectin-9 and resulted in Tim3+ FOXP3- cells being slightly diminished (38 vs. 122, $p=0.08$), the Tim3+ FOXP3+ cells remaining unchanged (45 vs. 67, $p=0.14$) and the Tim3- FOXP3+ cells were found to be expanding (231 vs 114, $p=0.001$). This confirms our earlier findings that CD4 cells positive for Tim3 are undergoing apoptosis, Tim3+ CD8 cells are minimally affected by galectin-9 incubation and all the cells, which are Tim3 negative, display increased expression of immune tolerant markers after galectin-9 exposure relative to control.

Finally, cytokines secreted by PBMCs and CD14 depleted lymphocytes after a 12 hour exposure to galectin-9 were evaluated to determine the immune milieu induced by galectin-9. PBMCs cultures secreted high amounts tumor necrosis factor (TNF)- α ($p=0.003$) and IL-1 β (<0.0001) in the presence of galectin-9 (Fig. 3D). Th2 biasing cytokines IL-4 ($p=0.001$), IL-6 ($p=0.003$) and IL-10 (0.02) also increased significantly after galectin-9 incubation. Strikingly, when the CD14+ monocytes were removed, overall cytokine secretion decreased 30-fold, although IL-10 ($p<0.0010$) and IL-6 (0.005) remained elevated

(Fig. 3E). Results from other chemokines and cytokines measured were non-significant or outside the limit of detection (supplementary table S3 and S4). These effects suggest that galectin-9 induces a chronic inflammatory state which is driven predominantly by the myeloid population.

Galectin-9 promotes the M2 phenotype

CD68+ macrophages also stain strongest around the edge of the tumor and were most abundant at the tumor/stromal interface ($p=0.005$) compared to non-cancerous tissue (S100 negative) and tumor core (Fig. 4A). To determine galectin-9 dependent function of macrophages, which are abundant in the tumor microenvironment, we assessed changes in chemokine and cytokine secretion with the addition of galectin-9 to healthy human CD14+ monocytes and incubated them with or without galectin-9 for 12 hours. Results demonstrate that M2 biasing chemokines MCP-1 (4567 vs. 615pg/mL; $p<0.0001$) and MIP-1 α (2138 vs. 12pg/mL; $p<0.0001$) were increased after galectin-9 incubation; however, M1 promoting IP-10 (97 vs. 75pg/mL; $p=0.64$) was not significantly changed (Fig. 4B). Cytokines were measured for M1 or M2 phenotype after galectin-9 addition. M2 cytokines IL-10 (1594 vs. 11.6pg/mL; $p=0.002$), IL-4 (7.3 vs. 4.9pg/mL; $p<0.0001$) and IL-6 (3914 vs. 5.3pg/mL; $p<0.0001$) were also increased in the presence of galectin-9, whereas M1 cytokine IFN- γ (4.7 vs. 2.7; $p=0.27$) was not affected by treatment (Fig. 4C). Interestingly, TNF- α (4427 vs. 6.84pg/mL; $p<0.0001$) and IL-1 β (2122 vs. 3.5pg/mL, $p=0.0004$) increased with galectin-9. This suggests that the M2 phenotype could possibly be further defined as M2b, which secrete IL-10, TNF- α , IL-1 β and IL-6 (26). Other chemokines and cytokines measured were non-significant or outside the limit of detection of the assay (supplementary table S5). Together, our data suggests that galectin-9 biases monocytes towards an M2, or possibly M2b, immunosuppressive phenotype.

In order to more clearly define the effect galectin-9 has on promoting an M2 phenotype, we utilized the THP-1 human monocyte cell line to study CD206 expression and cytokine/chemokine secretion *in vitro*. Macrophage differentiation and polarization to M2, defined by CD206 expression, was completed using published methods (24). After 72 hours, surface expression of CD206 was increased on the CD68+ macrophage population treated with galectin-9 (MFI 102; $p=0.037$), similar to IL-4/IL-13 (MFI 101; $p=0.047$) compared to the control (MFI 68) (Fig. 5A). Supernatants from THP-1 cultured cells were measured for M1 (IP-10 and IFN- γ) and M2 (MCP-1 and IL-10) markers after 72 hours with galectin-9, IL-4/IL-13 or PMA by ELISA. M1 markers IP-10 and IFN- γ were low for all treatments, whereas M2 chemokine MCP-1 was significantly increased in both IL-4/IL-13 and galectin-9 culture compared to control (163 vs. 9pg/mL, $p=0.0004$; 165 vs. 9pg/mL, $p<0.0001$; respectively) (Fig. 5A). IL-10 was also elevated in the supernatants of macrophages treated with both IL-4/IL-13 or galectin-9 compared to controls (65 vs. 18pg/mL, $p<0.0001$; 58 vs. 18pg/mL, $p=0.001$; respectively). To determine if this effect can be reproduced in primary monocytes, we incubated CD14+ cells for 48 hours in IL-4/IL-13 or galectin-9 and measured CD206 expression. Although more heterogeneous, galectin-9 incubation with healthy CD14+ monocytes resulted in an increase of CD206 expression with galectin-9 incubation (MFI=104; $p=0.005$) similar to IL-4/IL-13 (MFI=116, $p=0.001$) compared to PMA only control (MFI=79) (Fig. 5B). Primary CD14 differentiated

macrophages secreted higher levels of M2 cytokine IL-10 (122.4 vs. 36.6pg/mL, $p=0.007$) and M2 chemokine MCP-1 (747.2 vs. 136.2pg/mL, $p=0.035$) compared to control and relative to M2 biasing conditions (137.7pg/mL and 672.2pg/mL, respectively) (Fig. 5B). No difference was observed between IFN- γ (9.67 vs. 11.6pg/mL, $p=0.17$; 9.63 vs. 11.6pg/mL, $p=0.18$; respectively) and IP-10 (14.6 vs. 14.2pg/mL, $p=0.98$; 12.1 vs. 14.2pg/mL, $p=0.84$; respectively) after galectin-9 or IL-4/IL-13 addition compared to control. The results indicate the galectin-9 acts on both THP-1 culture cells and primary monocytes to promote a M2 phenotype. Considering that galectin-9 upregulated CD206 on macrophages *in vitro*, we analyzed CD206 expression on circulating CD14+ PBMCs from 276 pre-treatment metastatic melanoma patients and 270 healthy, age-matched controls. There was an observed increase in the frequency of CD14+CD206+ monocytes in the melanoma population compared to healthy controls (8.94 vs. 6.47; $p<0.0001$) indicating that high levels of galectin-9 may be acting on the myeloid population to promote the immunosuppressive phenotype seen in cancer patients (Fig. 5C).

DISCUSSION

Recent advancements in cancer immunotherapy have improved survival of patients with metastatic melanoma; still, the majority of patients will still succumb to their disease. New therapies that rationally target other aspects of the host immunologic response to melanoma remain necessary, especially for patients who do not respond to existing immunotherapies. This study characterizes soluble galectin-9 and survival in metastatic melanoma, as well as defines immunoregulatory mechanisms of galectin-9 on human lymphoid and myeloid cells (Fig. 6). Our data suggests that galectin-9 (1) is highly expressed at the melanoma tumor edge, (2) is found in high concentrations in the plasma of metastatic melanoma patients, where it is associated worse survival, independent of clinical factors such as LDH, (3) suppresses the Th1 phenotype through Tim3 dependent apoptosis, (4) promotes T-cell tolerance independent of Tim3 and (5) enhances M2 macrophage polarization *in vitro*. Our results suggest neutralizing galectin-9 functions may be an effective way to recover a Th1, tumor targeting immune state.

The immune system is highly redundant; thus, it is not surprising that the galectin-9/Tim3 interaction shares many of the same characteristics and functionalities of the PD-1/PD-L1 interaction, although the pathways by which they signal are different (27). Tumors express highest levels of galectin-9 at their edge, similar to PD-L1 staining (19). This is advantageous for the tumor as it can interact more effectively to block activated tumor specific T-cells and evade immune cell killing. We observed high levels of circulating galectin-9 in the blood of melanoma patients compared to healthy controls. Similarly, soluble PD-L1 has been observed in patients with renal cell cancer and it has been shown to cause apoptosis of T-cells (28). Previously, melanoma tumors expressing galectin-9 were found correlate with improved patient survival (29). However, this data did not include any measure of soluble galectin-9. Our analysis demonstrates that those patients who have high levels of circulating galectin-9 have a worse 2-year survival than those with low levels of galectin-9. Galectin-9 may be both a biomarker and have therapeutic potential in metastatic melanoma.

We have also demonstrated the importance of the Tim3 binding partner, galectin-9, in regulating immunity by Tim3-independent and dependent mechanisms. Just as galectin-9 was first shown to interact with Tim3, the data also revealed that removing Tim3 did not completely abolish the ability of galectin-9 to induce apoptosis of CD4⁺ T-cells (13). Cells from Tim3 knockout mice treated with galectin-9 underwent apoptosis and secreted IFN- γ at the same rates as wild-type cells (30). We observed that CD4⁺ apoptosis was dependent on Tim3, but not for CD8⁺ and CD14⁺ cells, which underwent apoptosis regardless of this interaction. These studies suggest an alternative binding partner for galectin-9 that has not yet been discovered. CD44 has been demonstrated to interact with galectin-9 in an allergic inflammation model (11), and more recently this relationship has been described as a major promotor of regulatory T-cells (T-regs) through Smad3 (31). Both studies were done in murine models, so future experiments to confirm and expanded upon these results using human samples are justified.

Galectin-9 promotes the secretion of Th2 cytokines IL-4, IL-6 and IL-10; however, it also consistently caused an increase in TNF- α and IL-1 β . Although defined as pro-inflammatory, the role of TNF- α in suppressing and/or promoting cancer remains controversial. Recently, Ras was defined as a critical regulator in diverting TNF- α function in each direction (32). IL-1 β , which is produced by myeloid cells, has also been shown to promote tumor growth and angiogenesis in a lung carcinoma model (33). Human astrocytes stimulated with IL-1 β caused an increase in galectin-9 expression (34). Combined with our data, feedback regulation between galectin-9 and IL-1 β is plausible. Interestingly, macrophages from an arthritis mouse model studied after treatment with galectin-9 showed decreased TNF- α and IL-1 β , and increased IL-10 compared to controls (35). Since this arthritis model is considered Th1 biased, these contrary results parallel our cytokine findings in cancer, a Th2 biased disease. Thus, TNF- α and IL-1 β , in the context of our study, may be promoting tumor growth through galectin-9 dependent mechanisms.

Tumor associated macrophages can make up 5–40% of the solid tumor mass and can be defined as having an M1, anti-cancer phenotype or an M2, tumor promoting phenotype. The M2 phenotype has further been defined based on initial activation signal and chemokines/cytokines secreted (36). Still, macrophage function and mechanism in malignancy remains incompletely understood due to difficulty in translating animal results to humans and myeloid plasticity in fluid microenvironments inside the body (37). High numbers of macrophages in tumors are typically associated with a poor prognosis (38). Additionally, M2 polarized macrophages have been shown to be indicative of poor survival in lung and cervical cancer (39, 40). In the context of galectin-9, THP-1 monocytes modified to over express intracellular galectin-9 were shown to promote inflammatory cytokine production through NF-IL6 and AP-1 (41). We found that extracellular galectin-9 caused a dramatic increase in cytokine production by PBMCs, specifically TNF- α , IL-10, IL-4, IL-1 β and IL-6. The result was lost after removal of the CD14 population, suggesting an important interaction between myeloid cells and galectin-9. Cytokines secreted by the CD14⁺ population had a similar milieu as all PBMCs, and these cytokines have been defined in the context of the M2 phenotype (42). Using gene expression analysis and western blots, TNF- α and IL-1 β which are typically considered M1, were found to be upregulated after lipopolysaccharide and immune complex addition and are characterized to be secreted by

M2b regulatory macrophages (26, 43). This phenotype is similar to an M1, but IL-4 instead of IFN- γ is the predominant secreted cytokine. Galectin-9 induced secretion of TNF- α and IL-1 β , along with M2 cytokines, suggesting it could be another activator of an M2b regulatory phenotype. Further research into these specific M2 populations and galectin-9 is warranted, along with further development of myeloid markers to more clearly define each subset *in vitro*.

Incubating THP-1 cells, a human monocyte cell line derived from acute monocytic leukemia, with IL-4 and IL-13 has been shown to induce a M2 phenotype *in vitro* (24). Using this condition as a positive control, galectin-9 promoted the same M2 phenotype in THP-1 cells with increased expression of CD206 and secretion of MCP-1 and IL-10. CD206 is a C-type mannose receptor that binds and internalizes collagen and glycoproteins (42). This bias toward an M2 phenotype was also observed in healthy human CD14⁺ monocytes cultured in the presence of galectin-9. The result was even higher levels of IL-10 and MCP-1 secretion compared to THP-1 cells in the presence of galectin-9. This added further confirmation as to the role of galectin-9 in modulating immunity towards a state that is favorable for the tumor.

The data indicates that galectin-9 may be a targetable molecule with clinical relevance for patients with metastatic melanoma. The ability of galectin-9 to regulate the immune system seems to be only partially dependent on Tim3 binding. Anti-Tim3 monoclonal antibodies (mAb) have had positive pre-clinical results in tumor models. For example, tumor bearing mice treated with anti-Tim3 antibodies had more IFN- γ producing T-cells as well as increased tumor infiltrating cells which correlated with tumor reduction (44). Administration of an anti-Tim3 antibody also caused an increase in Th1 cells and cytokines in a murine model (45). Phagocytes were also inhibited by immunization with anti-Tim3 mAb in C57BL/6 model (46). These studies demonstrate the importance of Tim3 to peripheral tolerance; however, translation to humans has yet to be reported. Researchers are seeing promising results using Tim3 in combination with other inhibitory molecules. Fourcade et al. utilized a blockade of PD-1 and Tim3 in a cancer vaccine for melanoma patients and observed an increase in CD8⁺ T-cells and anti-tumor cytokine production (47). In a pre-clinical melanoma model, inhibiting both Tim3 and Tim4 resulted in an increase of anti-tumor immune responses (48). Unfortunately, these studies do not include any measure of galectin-9. Consistently, our data showed that addition of galectin-9 to human T-cells caused the upregulation of PD-1 on the cell surface that was Tim3 negative. T-regs and CD4⁺ T-cells expressing CD294, a marker of Th2 polarization, also increased in the same fashion. This result suggests that galectin-9 drives tumor specific immune cell tolerance in a Tim3-independent manner. Thus, more research needs to be completed to understand the galectin-9, Tim3-independent mechanisms of immune tolerance.

To conclude, this work suggests that galectin-9 could be a targetable molecule, as well as a possible biomarker of disease prognosis in melanoma. Although galectin-9 has been studied in the context of Tim3 in many animal models, translation to human disease has only just begun. We and others have found that galectin-9 generates many different consequences based on the immune cell being studied (49). It was observed that galectin-9 promotes apoptosis dependent and independent of Tim3 based on cell type, as well as drives M2

differentiation of monocytes. There is a clear Tim3 independent effect which has not yet been defined due to challenges determining additional receptors, developing effective antibodies, as well as the fact that galectins can bind multiple surface proteins which elicit similar downstream effects (30, 50). We noted that cells which were Tim3 negative upregulated tolerance receptors and promoted Th2 chemokine/cytokine secretion. Continued research into galectin-9 interactions and mechanisms to modulate immunity, promoting tumor growth are justified. Together, these data indicate that therapeutic targeting of galectin-9 on tumors and in plasma is a logical next step in the development of immunotherapeutics in metastatic melanoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support: This publication was supported by CTSA Grant Number TL1 TR000137 (E.A.L.E) from the National Center for Advancing Translational Science (NCATS). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

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Figure 1. Relevance of galectin-9 in metastatic melanoma

A) Concentration of galectin-9 in the plasma of healthy donors (n=16), and stage IV melanoma patients (n=24). B) Percentage of stage IV melanoma tumors (n=118) expressing PD-L1 and/or galectin-9. C) Metastatic melanoma tumors stained for galectin-9 or PD-L1. D) Total percentage of the area on a tumor core, edge or healthy tissue that expresses galectin-9 or PD-L1 (20× magnification). * denotes a p-value 0.05, ** denotes a p-value 0.01, *** denotes a p-value 0.001 and **** denotes a p-value 0.0001.

Figure 2. High levels of galectin-9 correlate negatively with 2 year survival and positively with Th2 biasing in metastatic melanoma

A) Patients with high (≥ 1532 pg/mL) galectin-9 have worse 2-year survival compared to patients with low (< 1532 pg/mL) galectin-9 ($n=276$). B) Average Th1:Th2 ratio defined as cellular CD4+Tim3+ (Th1) and CD4+CD294+ (Th2) positive populations in stage IV melanoma patients with high (>1532 pg/mL) and low levels of galectin-9 (< 1532 pg/mL; $p=0.01$). C) Average plasma concentration of IFN- γ among melanoma patients with high and low galectin-9 levels ($p=0.02$). D) Average plasma concentration of IL-4 in patients with high and low galectin-9 levels ($p=0.03$). E) Average plasma concentration of IL-10 compared between patients with high and low levels of galectin-9 ($p=0.03$).

Figure 3. Galectin-9 modulates immunity promoting a suppressive immune environment *in vitro*

A) Average and standard deviation of three independent autologous mixed lymphocyte reactions using PBMCs isolated from healthy donors with or without exogenous galectin-9 (2ug/mL). B) Percentage of CD4+, CD8+ and CD14+ cells undergoing apoptosis after a 24 hour incubation with 2ug/mL galectin-9 and a 10ug/mL Tim3 neutralizing antibody (n=9). C) Changes in T-cell phenotype after a 48 hour incubation with 2ug/mL galectin-9 (n=15) *p-value 0.05. D) Cytokine secretion of all PBMCs after a 12 hour incubation with 2ug/mL galectin-9 (3 independent experiments; n=5). E) Cytokine secretion of CD14- depleted lymphocytes incubated with 2ug/mL galectin-9 (3 independent experiments; n=5) *p-value 0.001.

Figure 4. CD68 cells are found at the tumor edge and galectin-9 induces chemokine and cytokine secretion

A) Melanoma biopsy (20× magnification) stained for CD68+ macrophages and the percentage of the area staining positive in the tumor core, edge and non-cancerous stroma (n=10). B) Average concentration of chemokines secreted by CD14+ cells after a 12 hour incubation with 2µg/mL galectin-9 (3 independent experiments; n=5). C) Average concentration of cytokines released by CD14+ cells after a 12 hour incubation with 2µg/mL galectin-9 (3 independent experiments; n=5).

Figure 5. Galectin-9 promotes macrophage differentiation into M2

A) Cell surface expression of CD206 gated on CD68+ THP-1 cells and cytokines/chemokines secreted after 72hrs with galectin-9, IL-4+IL-13 or PMA control (n=4 independent experiments). B) Cell surface expression of CD206 from CD14+ cells isolated from healthy donors and gated on CD68+, as well as cytokines/chemokines secreted after 48hrs with galectin-9, IL-4+IL-13 or PMA control (n=3 independent experiments). C) Percentage of positive CD206 staining on monocytes by flow cytometry between melanoma patients (n=115) and healthy controls (n=120). * indicates a p-value less than 0.05.

Figure 6. Proposed mechanism of galectin-9 immune modulation

Galectin-9 eliminates Th1 responses through apoptosis of Tim3+ T helper 1 cells while promoting immune tolerance through the increase of PD-1, CD294 and T regulatory cells (T-regs). Galectin-9 also modulates macrophages by stimulating an M2, pro-tumor phenotype with the upregulation of CD206 and secretion of M2 chemokines and cytokines.