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Abstract

During the past decade, a better understanding of the cellular and molecular mechanisms underlying tumor immunity has provided the appropriate framework for the development of therapeutic strategies for cancer immunotherapy. Under this complex scenario, galectins have emerged as promising molecular targets for cancer therapy responsible of creating immunosuppressive microenvironments at sites of tumor growth and metastasis. Galectins, expressed in tumor, stromal, and endothelial

cells, contribute to thwart the development of immune responses by favoring the expansion of T regulatory cells and contributing to their immunosuppressive activity, driving the differentiation of tolerogenic dendritic cells, limiting T cell viability, and maintaining T cell anergy. The emerging data promise a future scenario in which the selective blockade of individual members of the galectin family, either alone or in combination with other therapeutic regimens, will contribute to halt tumor progression by counteracting tumor-immune escape. Here we describe a selection of methods used to investigate the role of galectin-1 in tumor-immune escape.

Keywords (separated by "-")	Galectin - Tumor immunity - Immunosuppressive - Tumor growth - Metastasis - T regulatory cells - Tolerogenic dendritic cells

Chapter 16

Study of Galectins in Tumor Immunity: Strategies and Methods

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Abstract

During the past decade, a better understanding of the cellular and molecular mechanisms underlying tumor immunity has provided the appropriate framework for the development of therapeutic strategies for cancer immunotherapy. Under this complex scenario, galectins have emerged as promising molecular targets for cancer therapy responsible of creating immunosuppressive microenvironments at sites of tumor growth and metastasis. Galectins, expressed in tumor, stromal, and endothelial cells, contribute to thwart the development of immune responses by favoring the expansion of T regulatory cells and contributing to their immunosuppressive activity, driving the differentiation of tolerogenic dendritic cells, limiting T cell viability, and maintaining T cell anergy. The emerging data promise a future scenario in which the selective blockade of individual members of the galectin family, either alone or in combination with other therapeutic regimens, will contribute to halt tumor progression by counteracting tumor-immune escape. Here we describe a selection of methods used to investigate the role of galectin-1 in tumor-immune escape.

Key words Galectin, Tumor immunity, Immunosuppressive, Tumor growth, Metastasis, T regulatory cells, Tolerogenic dendritic cells

1 Introduction

Despite major advances in understanding the mechanisms leading to tumor immunity, a number of obstacles hinder the successful translation of mechanistic insights into effective cancer immunotherapy [1]. Such obstacles include the ability of tumors to display multiple immunosuppressive mechanisms to avoid immune recognition or to disarm effector T cell function [2]. These mechanisms involve alterations of the antigen presentation machinery; secretion of immunosuppressive cytokines such as transforming growth factor-β (TGF-β) and interleukin-10 (IL-10);

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expression of inhibitory molecules such as programmed death ligand-1 (PD-L1), cytotoxic T-lymphocyte antigen-4 (CTLA-4), and indoleamine 2,3 dioxygenase (IDO); and specific recruitment of regulatory cell populations including FoxP3$^+$ T regulatory (Treg) cells, IL-10-producing type-1 T regulatory (Tr1) cells, tolerogenic dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs), and M2-type macrophages [2].

The prominent immunological phenotypes observed upon disruption of genes encoding components of the glycosylation machinery, including glycosyltransferases, glycosidases, and lectins, reflect the central role played by glycosylation in the control of immune tolerance and inflammation. In fact, glycosylation regulates a variety of immune cell processes including immune cell activation, differentiation, homing, and survival [3, 4]. In addition, aberrant expression of glycans during the transition from normal to inflamed or neoplastic tissue provides a vast potential for information display [5]. Although these alterations have been mostly documented in cancer cells during tumor progression, cell surface glycosylation is also dramatically altered in the tumor microenvironment, particularly in stromal and immune cell compartments. Endogenous glycan-binding proteins, including C-type lectins, siglecs, and galectins, can selectively recognize neo-glycoepitopes and convey this structural information into functional cellular responses, including the modulation of immunological and vascular signaling programs [6].

Galectins show prominent expression in inflammatory and tumor microenvironments [7]. Through regulation of cellular signaling programs, galectin–glycan interactions provide "on-and-off" signals that control the decisions between immune cell responsiveness and tolerance. Particularly, galectin-1 suppresses chronic inflammation, blunts Th1 and Th17 responses, and skews the immune response toward a Th2 profile [8]. In addition, this lectin instructs DCs to become tolerogenic [9], induces alternatively activated "M2-type" macrophages and microglia [10, 11], inhibits T cell trafficking [12, 13], and favors the expansion of FoxP3$^+$ Treg and FoxP3(−) Tr1 cells [14, 15] further limiting the magnitude of an effective immune response.

Galectin-1 expression correlates with tumor burden and adverse clinical features in several tumor types including laryngeal squamous cell carcinoma [16], prostate adenocarcinoma [17], colon adenocarcinoma [18, 19], ovarian carcinoma [20, 21], breast carcinoma [14, 22], melanoma [23], Hodgkin lymphoma [24, 25], cervical cancer [26], T cell lymphoma [27], pancreatic ductal adenocarcinoma [28], neuroblastoma [29], hepatocellular carcinoma [30, 31], chronic lymphocytic leukemia [32], glioblastomas [33, 34], MLL-rearranged B lymphoblastic leukemias [35], and thyroid carcinoma [36].

Through galectin-1-driven inhibitory mechanisms, cancer cells can evade and thwart immune attack [37]. In several tumors

galectin-1 selectively blunts tumor-specific T cell responses through modulation of T cell apoptosis and skewing of the cytokine balance toward a Th2 profile [23, 28, 29, 38]. Furthermore, in breast adenocarcinoma, this lectin favors the differentiation and recruitment of FoxP3$^+$ Treg cells [14] or modulates the survival of effector T cells [38]. Moreover, galectin-1 promotes the differentiation of tolerogenic DCs in settings of melanoma, lung adenocarcinoma, and neuroblastoma [9, 29, 40]. Although we will focus here on galectin-1, it should be mentioned that other galectins, including galectin-3 and galectin-9, also influence tumor-immune escape mechanisms including T cell apoptosis, T cell anergy, NK cell activation, and expansion of myeloid-derived suppressor cells [41–44]. Thus, the spatiotemporal regulation of different galectins in conjunction with other immune escape mechanisms will dictate the decisions between immune cell responsiveness and tolerance in tumor microenvironments. Here we describe a selection of methods used to study the role of galectins, particularly galectin-1, in tumor immunity.

2 Materials

2.1 Methods to Study the Regulatory T Cell Compartment in the Tumor Microenvironment

2.1.1 Collection of Tumor Tissue, Draining Lymph Nodes, and Spleen from Tumor-Bearing Mice

1. 8- to 12-week-old Balb/c and C57Bl/6 tumor-bearing mice.
2. RPMI 1640 (GIBCO).
3. 1 ml syringe (Neojet).
4. Sterile scissors.
5. P60 Petri dishes (GBO).
6. Sterile 70 μm filter (BD Pharmingen).
7. Phosphate buffer saline (PBS): 136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4.
8. Sterile red blood lysis buffer (ACK buffer): 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in distilled H_2O.
9. Frosted microscope slides (BioTraza).
10. 15 ml conical tubes (BD Pharmingen)
11. FACS buffer I (PBS with 0.1 % BSA and 2 mM EDTA).

2.1.2 Staining and Purification of CD4$^+$ Treg Cells, Naïve T Cells, and Responder T Cells

1. Allophycocyanin (APC)-conjugated CD4 antibody (clone GK1.5), Alexa Fluor 488-conjugated CD25 antibody (clone PC61.5), Phycoerythrin (PE)-conjugated CD62L antibody (clone MEL-14), PE-conjugated Foxp3 antibody (clone FJK-16s) (all from eBiosciences).
2. Fix/Perm buffer (eBiosciences).
3. Permeabilization Buffer 10× (eBiosciences).
4. Dynal® Mouse CD4 Cell Negative Isolation Kit (Invitrogen).

5. Heat-inactivated fetal bovine serum (Gibco, FBS).
6. FACS buffer I: PBS with 0.1 % BSA and 2 mM EDTA.
7. Sorted cells collection medium: RPMI 1640 supplemented with 20 % FBS.
8. 15 ml conical tubes.
9. PBS (136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4).
10. Mouse spleen.
11. FACSaria sorter

2.1.3 Coating of 24- and 96-Well Plates with Anti-CD3 Antibodies

1. 96- and 24-well round bottom plates (GBO).
2. Purified NA/LE hamster anti-mouse CD3ε monoclonal antibody (clone 145-2C11, BD Pharmingen).
3. Phosphate buffer saline (PBS): 136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4.
4. Humidified incubator at 37 °C.

2.1.4 Differentiation of Treg Cells In Vitro in the Presence of Conditioned Media

1. Recombinant TGF-β (R&D Systems) diluted in phosphate buffer saline (PBS), pH 7.4 (30 μg/ml).
2. Incubator at 5 % CO_2 and 37 °C.
3. Recombinant IL-2 (R&D Systems) in PBS (10 μg/ml).
4. RPMI 1640 supplemented with 50 μM β-mercaptoethanol and antibiotic-antimycotic (Invitrogen).
5. Gal-1 wild type (WT) or Gal-1 knockdown (shRNA-Gal-1) tumor cells.
6. P60 dishes (GBO).
7. Twenty-four well plates (GBO).
8. 0.22 μm syringe filter (Millipore).
9. Naïve T cells.
10. Purified NA/LE Hamster anti-mouse CD28 monoclonal antibody (clone 37.51, BD Pharmingen).
11. Twenty-four well plates coated with anti-mouse CD3 monoclonal antibody (*see* **Note 1**).

2.1.5 Purification of Tumor-Associated Treg Cells and $CD3^+$ Responder T Cells (Tresp)

1. 8- to 12-week-old Balb/c and C57Bl/6 tumor-bearing and tumor-free mice.
2. RPMI 1640 (GIBCO) supplemented with 20 % FBS.
3. 5 ml polystyrene tubes.
4. 15 ml conical tubes (BD Pharmingen).
5. Allophycocyanin (APC)-conjugated CD4 antibody (clone GK1.5), Alexa Fluor 488-conjugated CD25 antibody (clone PC61.5), Phycoerythrin Cyanine-7 (PECy7)-conjugated FR4

antibody (clone eBio12A5), Fluorescein Isothiocyanate (FITC)-conjugated CD3 antibody (clone 145-2C11).

6. Phosphate buffer saline (PBS): 136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4.
7. FACS buffer I: PBS with 0.1 % BSA and 2 mM EDTA.
8. FACSAria flow cytometer.

2.1.6 Assessment of the Suppressive Activity of Treg Cells

1. Tregs and Tresp purified as described in Subheading 3.1.5.
2. Ninety-six plates coated with anti-CD3 monoclonal antibody (as described in Subheading 2.1.3).
3. Recombinant mIL-2 (R&D Systems) in PBS (10 μg/ml).
4. Purified NA/LE Hamster anti-mouse CD28 monoclonal antibody (clone 37.51, BD Pharmingen).
5. RPMI supplemented with 5 % FBS, 50 μM β-mercaptoethanol, and 1 μg/ml CD28 mAb.
6. Incubator at 5 % CO_2 and 37 °C.
7. [^{3}H]-thymidine solution (PerkinElmer).
8. Direct β-counter.
9. 1 ml syringe (Neojet).
10. Cell harvester.
11. Scintillation liquid (Perkin Elmer).
12. Scintillation vials.
13. Absorbent glass filter paper (3 M).

2.1.7 Adoptive Transfer of Treg Cells

1. Treg cells from the desired source.
2. Phosphate buffer saline (PBS): 136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4.
3. Mice.
4. Anesthetic.
5. Warm water.
6. Sterile 27G needles.
7. 1 ml syringe (Neojet).

2.1.8 Tumor Antigen-Specific T Cell Proliferation

1. Tumor cell lines (B16, 4T1) as an antigenic source.
2. Liquid nitrogen.
3. 37 °C water bath.
4. Phosphate buffer saline (PBS): 136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4.
5. RPMI 1640, 10 % FBS supplemented with 50 μM β-mercaptoethanol and an antibiotics-antimycotics.
6. Single cell suspension of mouse spleen and draining lymph nodes.

7. 96-well round bottom plates.
8. [^{3}H]-thymidine solution (PerkinElmer).
9. Direct β-counter.
10. Scintillation liquid (Perkin Elmer).
11. Scintillation vials.
12. Absorbent glass filter paper (3 M).

2.1.9 Cytokine Determination by ELISA

1. DuoSet ELISA Mouse IFN-γ (R&D).
2. BD OptEIA™ Mouse IL-10 ELISA Set (BD Biosciences).
3. BD OptEIA™ Mouse IL-5 ELISA Set (BD Biosciences).

2.2 Study of Galectins in DC Compartment

2.2.1 Differentiation of Bone Marrow-Derived Tolerogenic DCs

1. Complete RPMI (cRPMI): RMPI 1640 (Invitrogen) medium with 10 % heat inactivated fetal bovine serum (FBS) (GIBCO), 40 μg/ml of gentamicin, 50 μM β-mercaptoethanol, 2 mM L-glutamine, and 10 mM HEPES. [AU3]
2. Recombinant mouse GM-CSF (rGM-CSF) (R&D System).
3. Recombinant human Gal-1 (rGal-1).
4. 8- to 12-week-old C57BL/6 mice.
5. P60 and P100 non-adherent Petri dishes (Greiner-GBO).
6. Sterile red blood lysis buffer (ACK buffer): 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in distilled H_2O.
7. rGal-1 (in-house production) as described [10].
8. Phosphate buffer saline (PBS): 136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4.
9. Sterile red blood lysis buffer (ACK buffer): 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in distilled H_2O.
10. rGM-CSF (R&D System).
11. 21- or 25-gauge needles (BD PrecisionGlide).
12. Scissors and scalpel.
13. 1 ml syringe (Neojet).

2.2.2 Determination of DC Markers by Flow Cytometry

1. FACS buffer II: PBS (136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4) with 0.1 % FBS (Gibco).
2. PE-conjugated anti-CD11c antibody (clone HL3), PE-conjugated anti-MHC II (I A^b) antibody (clone AF6-120.1), FITC-conjugated anti-CD86 antibody (clone GL1), FITC-conjugated anti-CD45RB antibody (clone 16A).
3. 1.5 ml tubes.

2.2.3 Determination of IL-27 by ELISA

1. ELISA for mouse IL-27 p28 (R&D).

2.2.4 Evaluation of STAT-3 Phosphorylation by Western Blot

1. Dendritic cells.
2. Protein Extraction Buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 10 mM EDTA; 1 % v/v NP-40) with protease and phosphatase inhibitors (Pierce).
3. Bradford reagent.
4. 2× Laemmli sample buffer (BioRad).
5. Amersham Hybond-ECL (GE Healthcare).
6. Tris-buffered saline (TBS): 150 mM NaCl, 50 mM Tris, pH 7.4.
7. tTBS (TBS with 0.05 % Tween 20).
8. Blocking buffer: tTBS with 5 % nonfat milk or BSA (Sigma).
9. Anti-phospho-STAT3 antibody (Santa Cruz Biotechnology, sc-8059).
10. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Vector Labs).
11. Immobilon chemiluminescent HRP substrate (WBKLS01-00, Millipore).
12. G-Box.

2.3 Profiling Galectin Expression in the Tumor Microenvironment

2.3.1 Galectins Immunostaining in Paraffin Embedded Tissues

1. Rabbit anti-galectin-1 (H-45), anti-galectin-8 (H-80), anti-galectin-3 (H-160), anti-galectin-12 (H-166), and goat anti-galectin-9 (C-20) (Santa Cruz Ref).
2. Saponin (Sigma).
3. PBS (136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4).
4. PBS-0.05 % w/v saponin.
5. Ethanol (Cicarelli).
6. Xylene (Cicarelli).
7. Vectastain Universal Elite ABC Kit (Vector).
8. Normal horse serum.
9. Dako DAB + substrate system (Dako).
10. Giemsa (Sigma).
11. Dako Ultramount Aqueous Permanent Mounting medium (Dako).
12. Cover Glass 24 × 40 mm.
13. H_2O_2 30 % solution (Cicarelli) (stored at 4 °C and protected from light).
14. Wet chamber.
15. ImmEdge Pen (Vector).
16. HRP-Label anti-goat (Sigma).
17. Humidified chamber.

2.4 Lentiviral-Mediated Silencing of Galectin Expression

2.4.1 Silencing Galectin Expression. Lentiviral Production (See **Note 2**)

1. HEK 293 T cell line (ATCC).
2. Vector—(pLVTHM-shRNA).
3. Packaging plasmid—pMD2.G (Addgene).
4. Envelope plasmid—pCMVR8.74 (Addgene).
5. P100 Petri dishes (Greiner, GBO).
6. PBS (136 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4).
7. DMEM (Gibco) supplemented with 10 % heat-inactivated FBS (Gibco).
8. 2× HBSS solution (for 500 ml final solution include 8 g NaCl; 0.38 g KCl; 0.1 g Na_2HPO_4; 5 g HEPES; 1 g glucose in 400 ml of bi-distilled water. pH: 7.05–7.12, complete to 500 ml bi-distilled water) (*see* **Note 3**).
9. 2.5 M $CaCl_2$ (Sigma) (in bi-distilled water).
10. Incubator at 5 % CO_2 and 37 °C.
11. Sterile RNAse-free DNAse (Invitrogen) in bi-distilled water.
12. 15 ml conical tubes (BD Falcon).
13. Syringe filters 0.45 μm (MilliPore).

2.4.2 Titration of Lentiviral Vectors and Transduction of Target Cells

1. HEK 293 T cells.
2. 24-well plates.
3. DMEM (Gibco) supplemented with 10 % heat-inactivated FBS (Gibco).
4. Polybrene (4 % solution).
5. Thawed virus solution collected (Subheading 3.4.1).
6. FACSAria flow cytometer.
7. Snap lock 1.5 ml tubes (Axygen).

3 Methods

3.1 Methods to Study the Regulatory T Cell Compartment in the Tumor Microenvironment

3.1.1 Collection of Tumor Tissue, Draining Lymph Nodes, and Spleen from Tumor-Bearing Mice

1. Euthanize tumor-bearing mice and harvest spleen, draining lymph nodes, peripheral lymph nodes, and irrigated tumor tissue.
2. Disrupt the spleen and draining lymph nodes (both axilar and inguinal) with the plunger of a 1 ml syringe against a 70 μm filter in a P60 Petri dish filled with 2 ml RPMI.
3. Cut the tumor tissue with sterile scissors and grind it using the frosted sides of two microscope slides in a P60 Petri dish with 2 ml of RPMI. Filter the suspension with a 70 μm filter. Centrifuge single cell suspensions in 15 ml conical tubes for 8 min at no more than 300 × *g*.

4. Resuspend splenocytes with 5 ml of ACK buffer and incubate for 5 min at RT. Dilute it with PBS and centrifuge for 8 min at no more than $300 \times g$.
5. Resuspend the cell pellets in FACS buffer I or RPMI.

3.1.2 Staining and Purification of CD4+ Treg Cells, Naïve T Cells, and Responder T Cells

1. Staining of CD4 and CD25 molecules is performed for 30 min in the dark at 4 °C. For 2×10^6 cells, use 0.03 μg of APC-conjugated anti-CD4 antibody and 0.075 μg of Alexa Fluor 488-conjugated anti-CD25 antibody in 100 μl of FACS buffer I.
2. Wash cells with PBS and centrifuge for 8 min at no more than $300 \times g$. Fix and permeabilize cells using Fix & Perm buffer in 100 μl for 30 min to 18 h in the dark at 4 °C.
3. Wash cells with Permeabilization Buffer 1×. Foxp3 staining is performed in 100 μl Permeabilization Buffer 1× using 0.225 μg PE-conjugated anti-Foxp3 antibody for 1 h at 4 °C in the dark.
4. Wash cells with Permeabilization buffer 1×, centrifuge for 10 min at $300 \times g$, and resuspend in FACS buffer I.
5. For flow cytometry analysis a two-laser cytometer must be used and five additional tubes containing the appropriate compensation samples should be considered (*see* **Note 4**).
6. For isolation of $CD4^+CD62L^+$ naïve T cells prepare a single cell suspension from mouse spleens.
7. Purification of $CD4^+$ T cells is performed by negative selection using Dynal® Mouse CD4 Cell Negative Isolation Kit (Invitrogen). This procedure is thoroughly detailed in the protocol provided by manufacturer (*see* **Note 5**). Protocol yield is usually 20–25 % of spleen cells.
8. After purification of $CD4^+$ T cells adjust the cell concentration to 4×10^7/ml in FACS buffer I and proceed to CD4 and CD62L surface immunostaining.
9. Use 0.2 μg APC-conjugated anti-CD4 antibody and 0.3 μg PE-conjugated anti-CD62L antibody per 200 μl of lymphocyte suspension. Incubate for 30 min at 4 °C in the dark.
10. Wash cells with FACS buffer I, centrifuge for 8 min at no more than $300 \times g$, and resuspend cell pellet at a concentration of 3×10^7/ml.
11. Using a FACSAria cell sorter proceed with the selection and sorting of the $CD4^+CD62L^{high}$ population (*see* **Note 6**).
12. Use 15 ml conical tubes to collect sorted population with 2.5 ml of collection medium. Prior to use, vortex the tubes (*see* **Note 7**).
13. Keep the sorted population on ice.

3.1.3 Coating of 24- and 96-Well Plates with Anti-CD3 Antibody

1. Prepare a 5 μg/ml solution from the stock of CD3ε antibody (1 mg/ml) in sterile PBS and vortex. For 24-well and 96-well round bottom plates use 150 μl and 40 μl, respectively.
2. Incubate at 37 °C in a humidified atmosphere for at least 2 h.
3. Before use rinse wells with PBS and aspirate twice.

3.1.4 Differentiation of Treg Cells In Vitro in the Presence of Conditioned Media

1. For preparing conditioned media (CM) from wild-type or Gal-1 knockdown tumor cells, plate wild-type (WT) or Gal-1 knockdown tumor cells in P60 dishes at 50 % confluence with 2 ml of serum-free RPMI. Incubate for 18 h at 37 °C with 5 % CO_2 and then collect CM. Filter with 0.22 μm syringe filter, aliquot, and store at −70 °C.
2. The stimuli required for Treg cell differentiation in vitro are TGF-β and IL-2. To assess the role of Gal-1 in Treg cell differentiation, it is important to use a limiting concentration of TGF-β. Adjust the number of naïve T cells to 1×10^6/ml in serum-free RPMI supplemented with 1–2 ng/ml hTGFβ, 100 U/ml mIL-2, 1 μg/ml CD28 mAb, and a combination of antibiotic-antimycotic.
3. Plate 1 ml of a suspension of naïve T cells per well in 24-well plates coated with anti-CD3 monoclonal antibody (obtained in Subheading 3.1.2).
4. Add CM from WT or Gal-1 knockdown tumor cells (*see* **Note 8**).
5. Incubate at 37 °C with 5 % CO_2 for 4 days (*see* **Note 9**).
6. Asses Treg cell frequency by flow cytometry after staining of CD4, CD25, and FoxP3.

3.1.5 Purification of Tumor-Associated Treg Cells and $CD3^+$ Responder T Cells

1. For Treg cell purification, prepare a lymphocyte suspension from the tumor, draining lymph nodes, or spleen collected from Balb/c or C57BL/6 tumor-bearing mice. Adjust lymphocyte number to 4×10^7/ml cells in FACS buffer I.
2. Regulatory T cells are characterized by surface expression of CD4, CD25, and $FR4^{high}$ [45]. Use 0.2 μg of APC-conjugated anti-CD4 antibody, 0.5 μg of Alexa Fluor 488-conjugated anti-CD25 antibody and 0.4 μg of PECy7-conjugated anti-FR4 antibody per 0.2 ml cells. Incubate for 30 min at 4 °C in the dark.
3. Wash cells with FACS buffer I, centrifuge for 8 min at no more than $300 \times g$, and resuspend cell pellet at a concentration of 3×10^7 cells/ml.
4. Using a FACSAria cell sorter, select within the lymphocyte gate the $CD4^+CD25^+FR4^{high}$ population (*see* **Note 10**).
5. Collect in 5 ml polystyrene round bottom tube containing 2 ml collection medium. Vortex tube prior to use (*see* **Note 7**).
6. Keep the sorted population on ice.

7. For T responder cells, prepare a single cell suspension of mouse spleen from tumor-free mice as described (Subheading 3.1.1).
8. Adjust splenocyte concentration to 4×10^7/ml with FACS buffer I and stain for the CD3 surface marker using 0.2 μg of FITC-conjugated anti-CD3 antibody per 200 μl. Incubate for 30 min at 4 °C in the dark.
9. Wash cells and resuspend the cell pellet in FACS buffer I at a concentration of 3×10^7/ml.
10. Using a FACSAria cell sorter select within the lymphocyte gate the $CD3^+$ population (*see* **Note 10**).
11. Collect cells in 15 ml conical tubes containing 2.5 ml of collection buffer. Vortex tubes prior to use (*see* **Note 7**).
12. Keep the sorted population on ice.

3.1.6 Assessment of the Suppressive Activity of Treg Cells

1. Purify Treg cells and T responder cells from the desired source.
2. Count Treg and T responder cells and adjust to 5×10^5 cells/ml in RPMI 5 % FBS supplemented with 50 μM β-mercaptoethanol and 1 μg/ml anti-CD28 monoclonal antibody. Authors have reported that sorted Treg cells remain partially anergic after purification [46]. It is therefore recommended to supplement culture medium with 20 U/ml IL-2.
3. In 96-well round bottom plates coated with anti-CD3 monoclonal antibody, add Treg cells and prepare twofold serial dilutions of these cells. It is recommended that at least three serial dilutions are performed. Treg cell proliferation should be also evaluated.
4. Add 50 μl of T responder cells to all the required wells. Make sure to evaluate proliferation of T responder cells in the absence of Treg cells. The Tresp:Treg ratio should be 1:1, 1:0.5, 1:0.25, 1:0.125, etc.
5. Incubate plates at 37 °C, 5 % CO_2 for 4 days.
6. Pulse plates with 1 μCi [^{3}H]-thymidine per well 18 h prior to completion of the experiment. Since proliferation by [^{3}H]-thymidine incorporation is often variable, wells must be processed in triplicate.
7. Harvest cultures with a commercial cell harvester and determine counts per minute (cpm) with a direct β-counter.
8. Data are reported as cpm or percent of suppression considering T responder cells alone as 100 % of proliferation.

3.1.7 Adoptive Transfer of Treg Cells

1. Purify Treg cells from the desired source and resuspend cells in sterile PBS at a concentration of 6×10^6/ml.
2. Anesthetize mice and gently warm the tail vein that is located laterally.
3. Inoculate 50 μl of Treg cells intravenously with 27G needles using a 1 ml syringe.

3.1.8 Tumor Antigen-Specific Proliferation

1. Prepare cell lysates from cultured tumor cell lines (B16, 4T1) as an antigenic source by four freeze-thaw cycles (liquid nitrogen and 37 °C water bath) at a concentration of 2×10^7 cells/ml in PBS.
2. For ex vivo antigen stimulation prepare a working solution by diluting the tumor lysates 1:400 in RPMI 1640, 10 % FBS supplemented with 50 μM β-mercaptoethanol and a mixture of antibiotics-antimycotics.
3. Prepare a single cell suspension of mouse spleen and draining lymph nodes as described in Subheading 3.1.5.
4. Adjust cells concentration to 2×10^6/ml in RPMI 10 % FBS supplemented with 50 μM β-mercaptoethanol and antibiotic-antimycotic.
5. In 96-well round bottom plates add 50 μl of cells and 50 μl of the working dilution of tumor antigen or 50 μl of RPMI as a control. Calculate three wells for proliferation and two wells for determination of each cytokine by ELISA.
6. For proliferation assay, incubate plates at 37 °C, 5 % CO_2 for 4 days and pulse plates with 1 μCi [^{3}H]-thymidine per well for 18 h.
7. For cytokine determination, incubate plates at 37 °C, 5 % CO_2 for 24–48 h, harvest culture supernatants in 100 μl aliquots, and keep at −70 °C until use.

3.1.9 Cytokine Determination by ELISA

1. ELISAs for mouse IFN-γ, IL-10, IL-5 were performed according to the manufacturer's instructions.

3.2 Study of the Role of Galectins in the DC Compartment

3.2.1 Differentiation of Bone Marrow-Derived Tolerogenic DCs

1. Remove both femurs and tibias from C57Bl/6 mice and place them in a P60 Petri dish with cRPMI.
2. Remove excess muscle with forceps and scalpel. Cut bone's epiphysis.
3. Load 1 ml syringe with cRPMI.
4. Insert 21- or 25G needle into the bone marrow cavity. Flush the bone cavity with 2 ml cRPMI until the cavity is emptied.
5. Homogenize marrow suspension vigorously to disaggregate clusters that may be present in the suspension with a 21G needle.
6. Centrifuge cells for 10 min at 200 × *g*.
7. Discard supernatant. Resuspend cells with 5 ml ACK lysis buffer and incubate for 10 min. Dilute with 20 ml PBS.
8. Centrifuge cells for 10 min at 200 × *g*.
9. Discard supernatant and resuspend cells (10^6 cells/ml) in 10 ml cRPMI medium supplemented with 20 ng/ml rGM-CSF and 3 μM rGal-1 in P100 petri dish.

10. Feed cultures on days 2, 5, and 7 without discarding any cells: swirl plates and aspirate 75 % of the medium. Add fresh medium containing rGM-CSF and rGal-1.
11. After 8–9 days purify the non-adherent bone marrow-derived cells obtained in the supernatant.

*3.2.2 Determination of DC Markers by Flow Cytometry (See **Note 11**)*

1. Add cell suspension (5×10^5 cells) to a 1.5 ml tube. An isotype control antibody for each marker should be included.
2. Centrifuge cells at $200 \times g$ for 10 min at 4 °C and discard the supernatant.
3. Wash cells with 1 ml FACS buffer II.
4. Centrifuge cells at $200 \times g$ for 10 min at 4 °C and discard the supernatant.
5. Resuspend cells in 100 µl FACS buffer II and add 10 µl of a cocktail of antibodies (MHC II, CD11c, CD86, CD45RB). All the antibodies must be diluted with FACS buffer II (0.2 µg/tube).
6. Incubate for 30 min at 4 °C.
7. Wash cells with 1 ml FACS buffer II.
8. Centrifuge at $200 \times g$ for 10 min at 4 °C; discard the supernatant and resuspend the stained cells pellet in 500 µl PBS.
9. Analyze the sample(s) using a flow cytometer.

3.2.3 Determination of IL-27 by ELISA

1. IL-27 p28 ELISA protocol is thoroughly detailed in the data sheet provided by the manufacturer (*see* **Note 12**).

3.2.4 Evaluation of STAT-3 Phosphorylation by Western Blot

1. To prepare cell lysates, centrifuge DCs (1×10^7) at $200 \times g$ for 10 min at 4 °C. Discard the supernatant.
2. Resuspend cells in 200 µl ice-cold protein extraction buffer (200 µl per 10^7 cells).
3. Keep stirring for 30 min at 4 °C.
4. Centrifuge at $16{,}000 \times g$ for 20 min in a 4 °C pre-cooled centrifuge.
5. Transfer the supernatant to a fresh tube on ice and discard the pellet.
6. Remove a small volume (10 µl) of cell lysate to perform Bradford assay.
7. Determine the protein concentration for each cell lysate.
8. Prepare 30 µg of total protein from cell lysate with 2× Laemmli Sample Buffer.
9. Incubate each cell lysate at 100 °C for 5 min.
10. Load samples on an SDS-PAGE gel.
11. Run the gel for 1–2 h at 100 V.

12. Transfer proteins from the gel to a nitrocellulose or PVDF membrane.
13. Block the membrane for 1 h at room temperature using blocking buffer.
14. Incubate the membrane with the anti-phospho-STAT3 primary antibody (0.2 μg/ml) in blocking solution overnight at 4 °C.
15. Wash the membrane with tTBS for 5 min three times.
16. Incubate the membrane with the HRP-conjugated anti-rabbit IgG diluted 1/3,000 in tTBS at RT for 1 h.
17. Wash the membrane with tTBS for 5 min three times.
18. Incubate with Immobilon chemiluminescent HRP substrate and capture the luminescent image in a GBOX incubator.

3.3 Profiling Galectin Expression in Tumor Microenvironments

3.3.1 Galectins Immunostaining in Paraffin-Embedded Tissues

Deparaffinization of tissue sections

1. 30 min in xylene at RT.
2. 10 min in 100 % ethanol at RT.
3. 10 min in 95 % ethanol at RT.
4. 10 min in 75 % ethanol at RT.
5. 5 min in distilled H_2O three times.

Quenching of endogenous peroxidase activity

6. 10 min in 1 % H_2O_2 in H_2O.

Blocking and antigen retrieval

7. Incubate tissue samples with normal horse serum (2 drops in 40 ml PBS 0.05 % saponin) overnight at 4 °C.
8. Circumscribe the tissue section with the ImmEdge Pen.
9. Incubate with antibody dilutions for 1 h at RT in a humidified atmosphere. Antibodies are diluted 1:100 in PBS-saponin (in the case of the anti-galectin-9 antibody, dilution should be 1:50). Volume = 100 μl/condition. Antibodies should be centrifuged for 2 min at 10,000 rpm before use.
10. Wash twice in PBS-0.05 % saponin (5 min each).
11. Incubate with biotinylated antibodies (1 drop/1 ml in PBS-saponin) for 1 h at RT in a humidified chamber. Volume = 100 μl/condition. Biotinylated antibodies (anti-rabbit or anti-mouse are used). In case of galectin-9 staining, use HRP-labeled anti-goat dilution 1/100. Incubate for 1 h at RT in a humidified atmosphere.
12. Wash twice in PBS-0.05 % saponin for 5 min.

Amplification reaction: Avidin-Peroxidase-Biotyn system

13. Incubate with ABC reagent for 1 h at RT in a humidified atmosphere (1 drop of reagent A in 1 ml PBS-0.05 % saponin, incubate for 5 min. Add 1 drop of reagent B, vortex, and incubate for 5 min). Volume = 100 µl/condition.
14. Wash twice in PBS-0.05 % saponin for 5 min.
15. Add 100 µl/condition of DAKO substrate system. Prepare substrate adding 2 drops of chromogen in 2 ml buffer. Incubate for 5 min at RT (*see* **Note 13**).
16. Stop reaction by rinsing with distilled H_2O.
17. Incubate with Giemsa for 30 min at RT (30 drops in 10 ml of distilled H_2O).
18. Mount by using Dako Ultramount aqueous mounting medium (*see* **Note 13**).

3.4 Lentiviral-Mediated Silencing of Galectin Expression

3.4.1 Silencing Galectin Expression. Lentiviral Production (See **Note 2**)

Day 1: Plate 2–2.5 × 10^6 of HEK 293T cells (low passage) per P100 Petri dish in 10 ml of DMEM medium supplemented with 10 % FBS.

Day 2: Transfection

1. Change culture medium at least 2 h before transfection.
2. Prepare calcium-phosphate precipitate (1 ml/P100 Petri dish):
   (a) Transfer vector—(pLVTHM-shRNA): 20 µg [47].
   (b) Packaging plasmid pMD2.G (Addgene #12259) (plasmid encoding capsid and polymerase genes) 15 µg.
   (c) Envelope plasmid—pCMVR8.74 (Addgene #22036) (plasmid encoding amphotropic envelop VSVG) 6 µg.
   (d) Complete to 500 µl with bi-distilled water, and then add 50 µl of 2.5 M $CaCl_2$ (prepared in bi-distilled water). Add dropwise 500 µl of 2× HBSS while gently vortexing. Incubate at RT for 15–25 min.
   (e) Add dropwise on a plate and mix gently with culture medium.
3. After 6–8 h of culture in CO_2-controlled incubator at 37 °C, change medium; wash cells two times with pre-warmed PBS and add 6 ml/plate of fresh complete medium (*see* **Note 14**).
4. At day 4 collect medium containing virus particles in 15 ml conical tubes.
5. Spin at 200 × *g* for 5 min at RT to remove all cells and contamination.
6. Filter supernatant with a 0.45 µm syringe filter. Virus can then be used for transduction or stored at −70 °C until use.

3.4.2 Titration of Lentiviral Vectors and Transduction of Target Cells (See **Note 15**)

1. Day 1: Plate 30,000 HEK 293T cells in 24-well plates in 1 ml of complete DMEM.
2. Day 2:
   (a) Count cells: To count cells, evaluate the cell number in one well to evaluate the number of cells at the day of infection. In typical culture conditions, this number should be around 60,000–80,000 cells.
   (b) Infection cells: The infection of cells should be performed in DMEM complete medium—4 % of polybrene solution (250 μl final volume) with six serial dilutions of virus solution; use for example 10–200 μl of thawed virus solution and complete to 250 μl of DMEM complete medium in 1.5 snap lock tubes.
3. Day 3: Add 1 ml of complete DMEM.
4. Day 4: Split cells and assess transduction efficiency as the percentage of green fluorescent protein $(GFP)^+$ cells (transfer vector contains a GFP-coding sequence as a marker of viral integration) and analyze fluorescence by FACS. Read the percentage from linear values (usually 5–10 % to no more than 20 % of GFP^+ cells is considered as linear values) (*see* **Note 16**).
5. To infect your target cells with a ratio cells/virus between 1 and 10, follow the steps in **step 2**, and then wait two passages of transduced cells before analyzing the transduction efficiency (*see* **Note 17**).
6. Transduced cells should be amplified to allow purification of GFP^+ cells by FACS (*see* **Note 18**).

4 Notes

1. Store antibodies in aliquots at −70 °C. Avoid repeated freeze-thaw cycles as it may lead to loss of activity.
2. *WARNING*! Production of lentivirus is not a simple and 100 % safe procedure. You should always keep in mind that the production process allows you to produce high titers of mammal's unreplicative but infectious virus solutions. Thus, viral production should be done following safety instructions, in authorized locations. It is essential to follow safety and security guidelines of your institution.
3. As transfection efficiency depends on the cell type, solutions at different pH should be tested to optimize transfection efficiency).
4. For three-color flow cytometry including APC, FITC, and PE staining, three individual additional tubes (each with a different fluorochrome-conjugated antibody) are needed in order to properly compensate the experiment. This is because FITC

usually bleeds considerably into PE channel. For FoxP3 staining as well as for detection of intracellular cytokines it is highly recommended to add a FM1 tube. Briefly cells are only stained with surface antibodies. After fixation and permeabilization add PE-conjugated isotype antibody (corresponding to PE-conjugated Foxp3 antibody) in permeabilization buffer 1×. This allows the correct definition of $Foxp3^-$ populations. It is highly recommended to exclude cell doublets using FSC-H vs. FSC-W and SSC-H vs. SSC-W dot plots.

5. Invitrogen's protocol is detailed for 1×10^7 leukocytes in 100 µl. Both antibody mix and Dynabeads yield up to three times the values that are specified. It is therefore recommended to use one-third of the reagent's volume corresponding to 1×10^7 leukocytes. Then, for a mouse spleen (usually around 10×10^7 leukocytes) one should use 70 µl antibody mix and 700 µl Dynabeads.
6. Exclude cell doublets using FSC-H vs. FSC-W and SSC-H vs. SSC-W dot plots. Percent of total cells should range between 60 and 70 % for Balb/c mice and 50–60 % for C57Bl/6 mice. Flow rate is recommended to be adjusted to around 1–3. Sort precision could be set to "yield."
7. Vortexing tubes will ensure that the tube will be covered by a thin layer of fluid to avoid cell death when cells are deflected to the tube.
8. It is advised to perform a dose-dependent curve using CM at different dilutions. Dilutions ranging from 1:10 to 1:100 are recommended.
9. Incubation beyond 4 days will only result in increased cell death.
10. Percentage of total cells depends on the tumor model and the time of tumor burden. Sort precision should be set to "purity" and flow rate around 1–3.
11. Surface markers of tolerogenic DCs are evaluated by flow cytometry. The typical markers are CD11c, MHC II ($I\text{-}A^b$), CD86, and CD45RB.
12. Use this approach to evaluate the secretion of IL-27 by tolerogenic vs. immunogenic DCs.
13. Avoid using buffers and solutions with sodium azide since this compound inhibits peroxidase activity.
14. All material used after transfection of HEK 293T cells should be washed twice in 2 % bleach solution to avoid viral contamination and prevent subsequent health risk for the personnel and the environment.
15. As transduction of cells lines with lentivirus is a rather efficient technique and allows integration of high number of copies of viral genome, target cells should be infected with low number of virus particles to ensure no more than two or three copies of

integrated shRNA cassette. There are important reasons for this. First, less of the target cell's genome will be modified, fewer side effects will be caused by virus integration, and fewer shRNA molecules will be produced to ensure no saturation of the miRNA natural program.

16. Titer corresponds to the percentage of cells (GFP^+ cells) transduced by a given virus suspension volume used on day 2, e.g., 50 μl of a dilution 5 leads to 10 % of positive cells, and the number of cells on day 2 is 70,000, then the titer of the viral solution is 5,000 TU/μl > 1.4×10^5 TU/ml.
17. Avoiding more than 10 % of infection allows one to keep the line heterogeneity, and no more than 15 % ensures minimum high copy integration number and thus genome integrity.
18. In this chapter we detail some of the strategies used to study the role of galectins, particularly galectin-1 in tumor immunity, including the study of the differentiation and frequency of Treg cells and tolerogenic DCs, the profile of galectin expression in the tumor microenvironment, and the production of lentiviral vectors to manipulate galectin expression selectively in different cell types (tumor, stromal, and immune compartments). Other methods including promotion of T cell apoptosis, cytokine detection, and T cell trafficking are described in detail in recent papers and excellent review articles [8–14, 17]. During the past decade, a better understanding of the cellular and molecular mechanisms underlying tumor immunity has provided the appropriate framework for the development of novel therapeutic strategies in cancer. Under this complex scenario, galectins and their glycosylated ligands have emerged as promising molecular targets and galectin antagonists have the potential to be used as anti-tumor and anti-metastatic agents in those cases in which galectins are up-regulated in tumor microenvironments. The emerging data promise a future scenario in which the selective blockade of galectin-1, either alone or in combination with other therapeutic regimens, will contribute to halt tumor progression by counteracting cancer immunosuppression [48, 49]. Blockade of galectin-1–glycan interactions may also influence the efficacy of tumor vaccines, and other immunotherapeutic approaches. We hope that the strategies and methods described here will facilitate and encourage scientists to further evaluate the role of galectins in tumor immunity.

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