THE ROLE OF THE LECTIN BINDING PROTEIN, GALECTIN-3, ON
REGULATING THE TUMOR SPECIFIC CD8 T CELL RESPONSE AFTER GM-CSF
VACCINATION

By
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ABSTRACT

BACKGROUND: Galectin-3 is a 31 kD carbohydrate-binding lectin that is over-expressed by many human malignancies. It also modulates T cell responses through a diverse array of mechanisms including induction of apoptosis, TCR cross linking in CD8⁺ T cells, and T cell receptor (TCR) down regulation in CD4⁺ T cells. It is also seen as a major regulator protein of the innate immune response. However, very few studies exist that describe its overall role in regulating a tumor-specific immune response in a tolerogenic host.

METHODOLOGY/PRINCIPAL FINDINGS: We found that patients responding to a granulocyte-macrophage colony-stimulating factor (GM-CSF) secreting allogeneic pancreatic tumor vaccine developed post immunization antibody responses to galectin-3 on a proteomic screen. We used the HER-2/neu (neu-N) transgenic mouse model to study galectin-3 binding on adoptively transferred high avidity neu-specific CD8⁺ T cells derived from TCR transgenic mice. Here, we show that galectin-3 binds preferentially to activated antigen-committed CD8⁺ T cells only in the tumor microenvironment (TME). Galectin-3 deficient mice exhibit improved CD8⁺ T cell effector function and increased expression of several inflammatory genes when compared with wild type (WT) mice. We also show that galectin-3 complexes with LAG-3, and LAG-3 expression is necessary for galectin-3 mediated suppression of CD8⁺ T cells in vitro. Lastly, galectin-3 deficient mice have significantly elevated levels of circulating plasmacytoid dendritic cells (pDCs), which are superior to conventional dendritic cells (cDCs) in activating CD8⁺ T cells.
CONCLUSION/SIGNIFICANCE: Binding of galectin-3 to cell surface glycoproteins on immune cells suppresses the pro-inflammatory immune response. Thus, inhibiting galectin-3 in conjunction with CD8$^+$ T cell directed immunotherapies should enhance the tumor specific immune response.

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CHAPTER 1: INTRODUCTION

**Seroproteomics identifies galectin-3 as a protein of interest**

Pancreatic cancer is the fourth leading cause of death due to cancer and accounts for approximately 40,560 deaths per year with the incidence increasing on a yearly basis. Current treatment options for pancreatic cancer can prolong survival but seldom lead to complete remission or cure of the disease. The 5-year survival rate for pancreatic cancer is still only 7% for all stages combined (1). Our lab has developed an immunotherapeutic approach utilizing irradiated allogeneic pancreatic ductal adenocarcinoma (PDA) cell lines genetically modified to secrete granulocyte macrophage colony stimulating factor (GM-CSF) (2). In combination with chemotherapy, to deplete Treg cells, this approach has been shown to be successful in activating the immune response in mouse models of pancreatic cancer to eradicate the tumor.

Serum was collected from 60 patients in a Phase II clinical trial study at pre- and post-vaccination time points to examine whether a vaccine-induced antibody response occurs and, if so, what tumor associated antigens (TAA) are targeted. Patients were divided into >3 year disease free survival (DFS) and < 3 year DFS groups for analysis. The 3 year cut off was chosen because patients surviving disease free after 3 years are likely to remain disease free. These experiments identified several protein bands that patients in the > 3 year DFS group developed vaccine-induced antibody responses to versus patients in the < 3 year DFS group. In total, 11 different proteins, including galectin-3, were identified by this method.
Galectin-3 and its role in peripheral tolerance

One of the greatest hurdles in the development of a successful cancer vaccine is peripheral tolerance. Because TAAs are often recognized as self-antigens by the immune system, T cells that have strong reactivity against these self-antigens are often tolerized through various mechanisms before they have the opportunity to kill tumor cells. A great deal of research has focused on understanding the role of co-regulatory proteins on the surface of T cells; however, despite the fact that oligosaccharide chains exist on nearly every plasma membrane protein as well as occupying a large volume of surface area known as the glycocalyx, the role that glycan-lectin interactions play in the activation state of immune cells remains controversial.

Galectin-3 is a 31 kD lectin with a 1 mM affinity for lactose. It is a unique member of the galectin family due to the fact that it is a chimeric protein with an N-terminal domain that has a Bcl-2 like motif conferring anti-apoptotic properties to galectin-3 intracellularly. The N-terminal domain also allows galectin-3 to oligomerize into pentamers in order to form high avidity interactions with its ligand and cross-link glycoproteins (3). Galectin-3 has been shown to reduce the affinity of the TCR for its cognate MHC I-peptide ligand by forming TCR-galectin-3 lattices that sequester the TCR from its CD8+ co-receptor (4). Additionally, galectin-3 has also been shown to induce apoptosis in CD8+ T cells via interactions with CD29 and CD7 (5). In CD4 cells, galectin-3 has been demonstrated to induce internalization of the TCR leading to disruption of the immunological synapse (6). In addition to its role in the adaptive immune response, galectin-3 has been shown to be critical in the innate immune response as well. It has been shown to be necessary for β2-integrin independent recruitment of
neutrophils (7). In dendritic cells (DCs), galectin-3 has been shown to influence the strength of antigen activation (8). Galectin-3, therefore, is a ubiquitous protein that is involved in every step of the immune response.

**Galectin-3 and its role in the PDA tumor microenvironment**

Several studies have demonstrated a critical role for stromal elements in the development of cancer. Interactions between mesenchymal cells and epithelial cells through direct cell-to-cell contact and soluble factors are necessary for maintenance of regulated cell growth and tissue architecture. Perturbations of these mesenchymal cells have been shown to lead to tumorigenesis. The main mesenchymal cell in pancreatic cancer is the pancreatic stellate cell, which is believed to be central for the development of the desmoplastic reaction that predominates in pancreatic cancer. Pancreatic stellate cells can be activated by cancer cells, and secrete a variety of growth factors, proteases, and cytokines that promote tumor growth, immune evasion, and metastasis. Signaling through the TGF-β pathway in particular is responsible for the development of fibrosis and induction of T regulatory cells that suppress a tumor-specific immune response (9).

A role for galectin-3 in fibrosis has been demonstrated in mouse models of hepatic fibrosis as well as in human studies of fibrosis in heart disease (10, 11). In hepatic fibrosis, the activation of hepatic stellate cells (HSCs) to differentiate into extracellular matrix (ECM) secreting myofibroblasts is influenced heavily by the profibrinogenic cytokine TGF-β, and is believed to be dependent on the presence of extracellular galectin-3, which is rapidly internalized by HSCs. *Galectin-3<sup>−/−</sup>* HSCs are deficient in growth, smooth muscle actin expression, and pro-collagen expression. The addition of exogenous recombinant galectin-3 to *galectin 3<sup>−/−</sup>* primary HSCs restored a wild type
phenotype to galectin-3\(^{-}\) HSCs. Because galectin-3 has been shown to be an important factor in the development of fibrosis in various other organs, it is likely to be important in establishment of the tumor microenvironment (TME) in PDA, which is characterized by dense desmoplasia.

**HER-2/neu model and TCR transgenic mice**

HER-2/neu is a 185 kD protein, and a member of the erbB oncogene family. The erbB family consists of type I tyrosine kinase receptors that are related to but distinct from the epidermal growth factor receptor (EGFR) family. These proteins form homo and heterodimers upon ligand binding with HER-2 being the most favorable binding partner. HER-2/neu is overexpressed in 13-23\% of breast cancers resulting in prolonged activation of MAPK pathways (12). The HER-2/neu mouse model is on the FVB/N mouse strain with the rat HER-2/neu proto-oncogene driven by the mouse mammary tumor virus (MMTV) promoter. In this setting, female mice develop focal mammary tumors by 4 months of age, and are tolerant to HER-2/neu containing orthotopic tumors (NT2.5) and vaccination (13). However, supplementing vaccination with cyclophosphamide (CY) to deplete T regulatory cells (Tregs) allows for the expansion of neu-specific CD8\(^{+}\) T cells leading to tumor clearance in 10-20\% of tumor challenged mice.

Using overlapping peptide libraries, our lab identified the immunodominant epitope of HER-2/neu, RNEU\(_{420-429}\), against which the majority of tumor-specific CD8\(^{+}\) T cells were reactive after vaccination with 3T3NeuGM, a whole cell GM-CSF vaccine that overexpresses HER-2/neu (14). Furthermore, dilutional cloning allowed us to further differentiate this pool of tumor-specific CD8\(^{+}\) T cells into high avidity and low avidity
populations as measured by staining with MHC I tetramers containing the RNEU_{420-429} peptide (15). High avidity CD8$^+$ T cells have T cell receptors (TCRs) characterized by V$\beta$4 and V$\alpha$1.1 side chains while low avidity CD8$^+$ T cells have TCRs found to use the V$\beta$2 and V$\alpha$5 chains. For further analysis of these two populations in an adoptive transfer setting, we generated high avidity and low avidity TCR transgenic mouse lines on an FVB/N Thy1.2 background. The TCR transgenic mice contain pre-rearranged gene segments for the V$\alpha$ and the V$\beta$ TCR side chains so that all T cells in the high avidity TCR transgenic mouse should be a clonal population expressing the high avidity TCR, and likewise for the low avidity transgenic mouse. The Thy1.2 marker provides a convenient way to label adoptively transferred cells after they have been injected into recipient mice.

**Overview of Thesis Work**

The overall goal of this thesis is to understand the survival benefit of patients who developed high titers of anti-galectin-3 antibodies in response to whole-cell GM-CSF vaccination, and understand the effect of galectin-3 on modulating the anti-tumor immune response. This has been accomplished by:

1. Demonstrating the ability of purified IgG from post-vaccination patient serum to neutralize galectin-3’s inhibitory effect on IFN$\gamma$ production by maximally activated CD8$^+$ T cells *in vitro*.

2. Characterizing phenotypic and functional differences associated with the presence of surface galectin-3 on tumor-specific CD8$^+$ T cells through surface marker characterization, whole genome microarray studies, and tumor survival experiments between galectin-3 WT and galectin-3 KO mice.
3. *In vitro* studies identifying putative binding partners through which galectin-3 may mediate CD8\(^+\) T cell suppression.

4. Demonstrating expansion of the plasmacytoid dendritic cell population after deletion of galectin-3, and characterizing its influence on CD8\(^+\) T cell activation.
CHAPTER 2: CHARACTERIZATION OF POST-TREATMENT SEROLOGIC RESPONSES IN PDA PATIENTS WITH > 3 YEAR DFS SURVIVAL

Introduction

Our lab previously developed a functional proteomic approach to identify PDA associated proteins that might serve as targets of the immune response (16). This approach utilized pre- and post-treatment sera from patients who received two GM-CSF-secreting whole pancreatic tumor cell lines as vaccine and compared serologic reactive proteins between treatment responders DFS > 3 years and non-responders (early recurrence after a single vaccine or DFS < 3 years). Paired sera from 60 subjects treated on a recently reported phase 2 study were evaluated by western blot. Proteins that demonstrated an increase in post-treatment serologic responses were purified by 2-D gel and mass spectrometry. Eleven proteins were identified (Table 1), two of which were serologic targets recognized by multiple patient sera. The first protein, annexin A2, induces an epithelial to mesenchymal transition (EMT) in pancreatic tumor cells and promotes pancreatic tumor metastases (16). The second protein, galectin-3, is a galactoside-binding protein and a known cancer-associated protein secreted by a number of tumor types (17). Galectin-3, like annexinA2, induces post-treatment serologic responses that correlate with improved DFS and overall survival (OS) (Fig. 1A-C).

ELISA results from all 60 patients in the Phase II trial showed that 67% of patients in the > 3 year DFS group had a greater than 2-fold increase in anti-galectin-3 antibody titers, and only 9.5% of patients in the < 3 year DFS showed a similar increase. In contrast, vaccination does not alter immune responses against the positive control antigen, influenza A.
The humoral arm of the immune response is critical for aiding T-cell mediated tumor rejection (18-23). Antibodies are known to elicit their effects through a diverse array of mechanisms such as antigen neutralization, complement fixation, ADCC, and promotion of cross-presentation by dendritic cells (24). It was hypothesized that post-treatment serologic responses against galectin-3 improves DFS survival by enhancing CD8⁺ T cell function through neutralization of galectin-3 binding to the cell surface. The addition of exogenous galectin-3 to activated CD8⁺ T cells in vitro has previously been shown to induce apoptosis, and suppress both IFNγ production and cellular proliferation. Thus, the presence of anti-galectin-3 neutralizing antibodies was assessed by the ability of purified IgG form patient sera to block galectin-3 mediated suppression of CD8⁺ T cells in vitro.

**Materials and Methods**

*In vitro activation and suppression of CD8⁺ T cells with exogenous galectin-3*

CD8⁺ T cells were negatively isolated from total splenocytes using Dynal CD8⁺ negative isolation kits (Invitrogen), and stimulated for 3 days with anti-CD3/CD28 (Invitrogen) beads at a T cell to bead ratio of 1:1 according to the manufacturer’s recommendations (25). For suppression studies, cells were incubated with the indicated amount of recombinant human galectin-3. Cells were also incubated with a 5 fold molar concentration of purified IgG from patient’s serum, as described in the figures. After 3 days of activation, CD8⁺ T cells were assessed for IFNγ production by ICS. Beads were removed by magnetic separation and cells were plated with fresh anti-CD3/CD28 beads in a 96-well assay plate at a T cell to bead ratio of 1:1 in the presence of monensin.
(GolgiStop, Invitrogen) for 5 hours at 37°C. Following the 5-hour incubation period, ICS studies were conducted as detailed in Chapter 3.

**Results**

*Patients develop neutralizing antibodies against galectin-3 after receiving whole cell GM-CSF PDA vaccine*

Galectin-3 is known to inhibit T cell activation by direct T cell binding (26). We therefore tested our hypothesis that post-treatment serologic responses against galectin-3 improves DFS survival by enhancing CD8+ T cell function through inhibition of galectin-3 binding to the T cell surface. First, we assessed the ability of recombinant human galectin-3 to inhibit IFNγ production by CD8+ T cells maximally stimulated *in vitro* with anti-CD3/CD28 beads. The minimal concentration necessary to observe a significant reduction in IFNγ production was 25 µg/mL. Next, we tested whether purified IgG from pre-vaccinated and post-vaccinated serum from patients with > 3 year DFS could prevent this reduction. We chose to compare sera from patient 3.027, who exhibited the highest post-vaccination anti-galectin-3 antibody titers in the DFS group, and patient 3.052 whose antibody titers were significantly lower. All antibodies were co-incubated with galectin-3 at a 5:1 molar ratio. For both patients, we found that IgG from post-vaccine serum but not pre-vaccine serum could attenuate the suppression observed at 25 µg/mL of galectin-3 (*Figure 2A-B*).

**Discussion**

These results provide a mechanism by which vaccine induced anti-galectin-3 antibodies may confer long-term DFS survival in patients with PDA by improving CD8+
T cell function; however, it remains to be elucidated whether these antibodies function in the same manner in vivo. Galectin-3 targeted therapies using small molecule inhibitors are already under investigation for the treatment of chronic organ failure (27-30) and cancer (31-34). Similar to neutralizing antibodies, small molecule inhibitors also provide a method to neutralize galectin-3 binding; however, antibodies possess the additional ability to mediate clearance of antibody-antigen complexes by macrophages via the Fc domain. Because extracellular galectin-3 exists in equilibrium with surface-bound galectin-3, persistently elevated titers of anti-galectin-3 antibodies could promote galectin-3 clearance and dissociation from the cell-surface (35-37). Multiple conformations of galectin-3 exist depending on phosphorylation of the N terminus as well as whether or not the CRD is engaged with a ligand. Evidence also demonstrates that the N-terminal domain is also involved in carbohydrate recognition (38, 39); therefore, immunization with recombinant galectin-3 alone may not be sufficient to develop effective neutralizing antibodies. Rather, immunization strategies involving galectin-3 in its native conformation with bound ligand, such as in whole cell GM-CSF vaccines, would be preferred.
Table 1. Antibody-reactive proteins identified by a functional seroproteomics approach

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein description</th>
<th>Protein accession</th>
<th>Gene symbol</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chaperonin (heat shock protein 60)</td>
<td>g0131542947</td>
<td>HPSD1</td>
<td>Essential for folding and assembly of newly imported proteins in mitochondria</td>
</tr>
<tr>
<td>2</td>
<td>Prolyl 4-hydroxylase, beta subunit presursor</td>
<td>g0120670125</td>
<td>P4HB</td>
<td>Subunit of a multifunctional protein disulfide isomerase</td>
</tr>
<tr>
<td>3</td>
<td>Calumenin isofrom A precursor</td>
<td>g014502551</td>
<td>CALU</td>
<td>Calcium-binding protein involving in protein folding and sorting in ER</td>
</tr>
<tr>
<td>4</td>
<td>Rho GDP dissociation inhibitor (GDI) alpha</td>
<td>g014757768</td>
<td>ARHGDI/A</td>
<td>Regulator of Ras related GTP-binding protein signaling pathways</td>
</tr>
<tr>
<td>5</td>
<td>Pyruvate kinase, muscle isoform M2</td>
<td>g0133286418</td>
<td>PKM2</td>
<td>Glycolytic enzyme</td>
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<td>6</td>
<td>Enolase 1 or α</td>
<td>g014503571</td>
<td>ENO1</td>
<td>Glycolytic enzyme</td>
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<tr>
<td>7</td>
<td>Annexin A2 isoform 2</td>
<td>g014757756</td>
<td>ANXA2</td>
<td>Calcium-dependent phospholipid-binding protein regulating cell growth, signal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>transduction, and membrane physiology</td>
</tr>
<tr>
<td>8</td>
<td>Lactate dehydrogenase A variant</td>
<td>g010269717</td>
<td>LDHA</td>
<td>LDH M isozyme in anaerobic glycolysis</td>
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<tr>
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<td>Cyclosporin-binding protein</td>
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<td>g014507989</td>
<td>TPT1</td>
<td>Anti-apoptotic and chaperon-like protein</td>
</tr>
<tr>
<td>11</td>
<td>Galectin 3</td>
<td>g0145728143</td>
<td>LGALS3</td>
<td>Galactoside-binding protein with pleiotropic functions including inhibition of T</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cell activation</td>
</tr>
</tbody>
</table>
Figure 1. Vaccine-induced galectin-3 antibody responses correlate with improved DFS (1:400 Dilution).

A) Pre-vaccine titers shown for patients who were either disease-free >3 years (DFS>3yr), disease-free less than 3 years (DFS<3yr), patients who received only a single vaccine before recurring (single Vac), or healthy donors (Donors). B) Post-vaccine titers for patients divided by DFS >3 years (left panel) or < 3 years (right panel) shown before the first vaccine (pre-Vac), 28 days after the first vaccine (Vac 1), after chemoradiation which was given after the first vaccine (post-Rx), and after two additional vaccines given after completing chemoradiation (Vac 3). C) Titers shown over time for 8 of the 12 patients demonstrated DFS>3 years. These patients received a total of 5 vaccinations (Vac 1, Vac 2, Vac 3, Vac 4, and Vac 5), the first one before chemoradiation, the 2nd, 3rd, and 4th each one month apart beginning one month after completing chemoradiation, and the 5th, 6 months after completing the 4th vaccination.
A) Pre-vaccine titers

B) Post-vaccine titers

C) Titers over time of DFS patients
Figure 2. In vitro neutralization of galectin-3 by purified IgG from post-vaccination patient serum.

Antibodies were purified from patient serum by Protein G isolation, and then incubated with healthy donor T cells and recombinant human galectin-3 as described in materials and methods. **A**) Gating of IFN$\gamma$+ CD8$^+$ T cells on histogram plots and percentages of IFN$\gamma$+ CD8$^+$ T cells after a 3 day anti-CD3/CD28 activation in the presence of various conditions as shown in the graph for patient 3.052. **B**) Percentages of IFN$\gamma$+ CD8$^+$ T cells after a 3 day anti-CD3/CD28 activation in the presence of various conditions as shown in the graph for patient 3.027.
A) Patient 3.052

![Graph showing IFN-γ levels for Patient 3.052](image)

B) Patient 3.027

![Graph showing IFN-γ levels for Patient 3.027](image)
Chapter 3: Characterization of Tumor Specific CD8+ T Cells with Surface Galectin-3

Introduction

Galectin-3 has been demonstrated to possess a diverse array of functions in tumor cells including intracellular signaling (40-44), apoptosis (45-48) trafficking and metastasis (49-51), and angiogenesis (52-54). The diversity of galectin-3’s many functions can be attributed to the ubiquity of oligosaccharide chains on nearly all plasma membrane associated proteins. Unlike the high degree of specificity needed for protein-protein interactions that govern the majority of receptor-ligand pairings, glycan-lectin interactions allow for a single lectin to interact with multiple different receptors at once. This phenomenon enables molecules such as galectin-3 to influence the function of a broad spectrum of different cell types.

Due to the many pleiotropic effects of galectin-3, it is also an attractive target for therapeutic intervention in a variety of human diseases. In addition to its involvement in tumorigenesis, galectin-3 also has immunomodulatory functions. Galectin-3 can reduce the affinity of the TCR for its cognate major histocompatibility complex (MHC) I-peptide ligand by forming TCR-galectin-3 lattices that sequester the T cell receptor (TCR) from its CD8+ co-receptor (4, 55). Furthermore, galectin-3 induces apoptosis in CD8+ T cells via interactions with CD29 and CD7 (56) and internalization of the TCR leading to disruption of the immunological synapse in CD4+ T cells (57). Galectin-3 also influences the strength of antigen activation in dendritic cells (DCs) (58, 59). In addition, galectin-3 is necessary for β2-integrin independent recruitment of neutrophils (60).
While these studies have been critical for the elucidation of galectin-3’s impact on immune cells, they were largely conducted *in vitro* by studying only a single population of cells in isolation with the addition of exogenous galectin-3 into the culture medium. However, because the immune response typically does not involve just one cell type acting in isolation, but rather multiple cell types acting upon one another, these studies still fail to present a complete picture of galectin-3’s role in the immune response. While several *in vivo* studies have looked at the effect of removing galectin-3 on the innate immune response to infectious diseases in galectin-3 knockout (KO) mice, very few have been able to evaluate the effect of removing galectin-3 on the adaptive immune response to tumors in a tolerogenic setting.

To elucidate the role of galectin-3 on CD8$^+$ cancer specific T cells, we crossed HER-2/neu transgenic (*neu*-N) mice which develop natural HER-2/neu (neu) expressing mammary tumors and high avidity CD8$^+$ TCR transgenic mice specific for the immunodominant epitope recognized on the tumors of these mice, with galectin-3 KO mice, and analyzed CD8$^+$ T cell responses following treatment with a neu targeted vaccine. Rather than focusing on galectin-3 signaling through individual cell surface receptors as has been done in the past, the ability to compare tumor-specific CD8$^+$ T cell responses in galectin-3 wild type versus galectin-3 KO mice in response to a whole cell GM-CSF vaccine enabled us to examine genome-wide changes in protein expression caused by galectin-3. Here, we demonstrate *in vivo* that removal of galectin-3 increases both the number of functional CD8$^+$ T cells found in the tumor microenvironment and the expression of inflammatory proteins by these T cells, leading to enhanced tumor rejection in galectin-3 KO mice when compared with galectin-3 WT mice. Further, we show that
these changes occur independently of improved TCR signaling (4), demonstrating the vast potential for lectin-glycan mediated regulation of CD8+ T cell responses.

**Materials and Methods**

**Mice**

HER-2/neu (neu-N) and LGALS3−/− (galectin-3 KO) mice were purchased from Jackson Laboratories (Bar Harbor, ME), bred and housed in the Johns Hopkins animal facility. High avidity T cell receptor (TCR) transgenic mice were generated as previously described, and avidity was confirmed by tetramer staining (61). Galectin-3 KO mice were backcrossed for 6 generations using a marker assisted selection (i.e. “speed congenic”) approach. Mouse genomes were assessed at the DartMouse™ Speed Congenic Core Facility at Dartmouth Medical School. Genetic background at the final backcross generation was determined to be 99.75% for the desired FVB/N background. Backcrossed galectin-3 KO mice were bred with neu-N and high avidity TCR transgenic mice to generate galectin-3 KO neu-N and galectin-3 KO high avidity TCR transgenic mouse lines. All experiments were conducted with female mice between 6-12 weeks of age according to protocols approved by the Johns Hopkins Animal Care and Use Committee.

**Cell lines and media**

The NT2.5 is a neu expressing tumor cell line generated from spontaneously arising tumors in female neu-N mice as previously reported (62, 63). The 3T3neuGM vaccine line is genetically modified from 3T3 fibroblast cells to secrete GM-CSF and express rat neu (64). The T2-D9 cell line is a TAP-2 deficient cell line that expresses the
D<sup>9</sup> MHC I allele, and was generated as previously reported (64). All cell lines are cultured and maintained according as previously reported (65).

*Tumor, vaccine, chemotherapy, adoptive transfer, and GCS-100 administration procedures*

For tumor clearance studies, mice were tumor challenged with the minimal tumorigenic dose of 5x10<sup>4</sup> NT2.5 tumor cells injected subcutaneously (s.c.) in the right upper mammary fat pad on day 0, given 100 mg/kg cyclophosphamide (CY) intraperitoneally (i.p.) on day 2, vaccinated with 3 simultaneous s.c. injections of 1x10<sup>6</sup> 3T3neuGM cells in the bottom and right limbs on day 3, and given 2x10<sup>6</sup> adoptively transferred high avidity CD8 T cells on day 4. GCS-100 was administered 3 times per week i.p at a dose of 20 mg/kg. For tumor infiltrating lymphocytes (TIL) and lymph node experiments, mice were given 2 simultaneous s.c. injections of 2x10<sup>6</sup> NT2.5 tumor cells in the right and left upper mammary fat pads on day 0, CY on day 8, vaccine on day 9, and 6x10<sup>6</sup> adoptively transferred CD8<sup>+</sup> T cells on day 10. High avidity Thy1.2<sup>+</sup>CD8<sup>+</sup> T cells were negatively isolated from spleens of female TCR transgenic mice and adoptively transferred as previously described (65)

*Peptides and antibodies*

RNEU<sub>420-429</sub> (PDSLRDLSVF) and the negative control peptide LCMV NP<sub>118–126</sub> (RPQASGVYM) peptides were produced in the Johns Hopkins Biosynthesis and Sequence Facility at a purity >95%. Antibodies used for flow cytometry studies were: anti-CD8-FITC (BD Biosciences), anti-CD8-PE (BD Biosciences), anti-CD8-PerCP (BD
Biosciences), anti-CD8-APC (BD Biosciences), anti-galectin-3-AF647 (Biolegend), anti-
galectin-PE (R&D), anti-PD1-PE (eBioscience), anti-LAG-3-PE (eBioscience), anti-
Thy1.2-PerCP (Biolegend), anti-CD44-Pacific Blue (eBioscience), anti-CD11b-PE (BD 
Biosciences), anti-CD11c-FITC (BD Biosciences), anti-B220-APC (BD Biosciences),
anti-Ly6C-PerCP-Cy5.5 (eBioscience), anti-IFNγ-PE (BD Biosciences), anti-IFNγ-
Pacific Blue (eBioscience), and anti-Granzyme B-APC (BD Biosciences). Cellular 
division was assessed by labeling of high avidity CD8+ T cells with 1.5 mM CellTrace 
CFSE cell proliferation kit (Invitrogen) prior to adoptive transfer (65). Apoptosis was 
determined using Live/Dead fixable aqua stain (Invitrogen) and Annexin V-Pacific Blue 
labeling (Biolegend) for phosphatidylserine. All antibody staining was conducted at 4°C 
for 20 minutes in FACS buffer (PBS, 5%FBS, 0.02% NaAzide). Samples were read by 
LSR-II and FACS Caliber (BD Biosciences) flow cytometers. Analysis was performed 
using FACS Diva (BD Biosciences) and Flow Jo (Tree Star, Inc).

Flow cytometry, intracellular cytokine staining (ICS) and tetramer analyses

Lymph nodes were dissected 3 and 5 days after adoptive transfer, and tumors 
were dissected 5 days after adoptive transfer, and homogenized by mashing through 40 
mM nylon cell strainers. Tumors were further processed by enzymatic digestion using 
collagenase (1mg/mL, Gibco) and hyaluronidase (25 mg/mL, Sigma). After digestion, 
cells were washed with RPMI before being trypsinized for 2 minutes with 0.25%
Trypsin-EDTA (Gibco). Lymphocytes were incubated for 5 hours at 37°C in CTL media 
with RNEU_{420–429} or NP_{118–126} peptide-pulsed T2-D4 target cells in the presence of 
monensin (GolgiStop, BD Biosciences) at a lymphocyte to target ratio of 4:1. Cells were
surface stained for Thy1.2 expression prior to fixation/permeabilization using a mouse ICS kit (BD Biosciences) to stain for intracellular IFN-g and Granzyme B. The percent of IFN-g and Granzyme B producing cells was determined by subtracting the percentage of cytokine producing cells in NP<sub>118–126</sub>-pulsed samples from the percentage of cytokine producing cells in RNEU<sub>420–429</sub>-pulsed samples. Absolute numbers were calculated from total cell counts performed on each sample prior to the 5-hour incubation.

Avidity was determined by dilutional tetramer analyses with the RNEU<sub>420–429/H-2D<sup>q</sup></sub> tetramer. Samples were stained at a starting concentration of 1.9 µM and dilutions of 1:100 were performed to assess avidity by flow cytometry.

*In vitro activation and suppression of CD8<sup>+</sup> T cells with exogenous galectin-3*

CD8<sup>+</sup> T cells were negatively isolated from total splenocytes using Dynal CD8<sup>+</sup> negative isolation kits (Invitrogen), and stimulated for 3 days with anti-CD3/CD28 (Invitrogen) beads at a T cell to bead ratio of 1:1 according to the manufacturer’s recommendations (25).

For suppression studies, cells were also incubated with the indicated amount of recombinant mouse galectin-3. After 3 days of activation, CD8<sup>+</sup> T cells were assessed for IFN-g production by ICS. Beads were removed by magnetic separation and cells were plated with fresh anti-CD3/CD28 beads in a 96-well assay plate at a T cell to bead ratio of 1:1 in the presence of monensin (GolgiStop, Invitrogen) for 5 hours at 37°C. Culture media was also supplemented with fresh galectin-3 at the indicated concentrations. Following the 5-hour incubation period, ICS studies were conducted as above.
**In vitro pZAP-70 dephosphorylation**

CD8\(^+\) T cells were isolated and stimulated in culture as previously described. Briefly, 2\(\times\)10\(^6\) cells were stimulated at a 1:1 ratio of cells:\(\alpha\)CD3/CD28 beads in 2 mL of XVivo-15 media (Lonza) with 30 U/mL rhIL-2. On Day 2 of stimulation, 1 mL of media was replaced with fresh XVivo-15 and rhIL-2. On Day 3 of stimulation, beads were removed from culture, and cells were allowed to rest for 24 hours with 0, 2, 5, 10, 20, 30 \(\mu\)g/mL rmGalectin-3. The next day, cells were collected, washed, and resuspended in 100 \(\mu\)L cold PBS. Cells were then incubated for 25 minutes with 20 \(\mu\)g/mL biotinylated anti-CD3 and anti-CD28, and then washed one with cold PBS. CD3 and CD28 were then cross-linked by the addition of 10 \(\mu\)g/mL streptavidin for 20 minutes. After cross-linking, stimulation was allowed to proceed at 37 C for the indicated time points prior to lysis in lysis buffer (RIPA, sodium orthovanadate, PMSF, NaF, Protease Inhibitor). Cell lysate was then quantified by BCA assay and western blotting was performed for pZAP70 and total ZAP70.

**Cloning and purification of recombinant mouse galectin-3**

Total RNA was isolated from in vitro activated high avidity CD8\(^+\) T cells using the RNEasy Mini Kit (Qiagen). Galectin-3 cDNA was amplified with Superscript III First Strand Synthesis System (Invitrogen) and galectin-3 specific primers containing BamHI and NdeI restriction sites: 5’-

\[
\text{GGAATTCCATATGGCAGACAGCTTTTCGCTTAACGATG} \quad (\text{Forward})
\]

\[
\text{CGGGATCCCTTAGATCATGGCGTGGTGTAGCGCTGGTGAGGG} \quad (\text{Reverse})
\]

The galectin-3 open reading frame (ORF) was then cloned into the pET-22B bacterial
expression vector (Novagen), which was transfected into BL21(DE3) chemically competent bacterial cells (Invitrogen) according to manufacturer’s recommendations.

BL21(DE3) cells were cultured in Luria Broth (LB) at 37°C and 200 rpm to an OD$_{600}$ of approximately 0.700 at which point protein expression was induced with the addition of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were incubated for an additional 4 hours before being pelleted at 4°C. Galectin-3 was purified from bacterial cell lysate material by binding to lactosyl-agarose beads (Sigma) and elution with 200 mM lactose. Purified material was dialyzed into PBS, and endotoxin was removed using the ToxinEraser Endotoxin Removal Kit (GenScript). Endotoxin was quantified to be less than 1.0 EU/mL by the LAL assay (Pierce).

Co-Immunoprecipitation of galectin-3 and LAG-3

10 mg LAG-3 (Clone 410C9) or galectin-3 (M3/38) specific antibody and corresponding isotype controls were conjugated to Protein G Dynabeads (Invitrogen) in PBS followed by cross-linking with 10mM BS3 (66). CD8$^+$ T cells were isolated and activated as previously described. Cell surface proteins were cross-linked with 10 mM BS(PEG)9 prior to cell lysis with CelLytic M (Sigma) supplemented with 100 mM lactose and protease inhibitor. Conjugated beads were incubated at 4°C overnight with CD8$^+$ T cell lysates. After washing beads with TBST (Tris-Buffered Saline + 0.1% Tween-20) the following day, bound proteins were eluted by boiling in sample buffer under reducing conditions. Standard western blotting procedures were followed and protein interactions were shown after developing membranes for 1 hour on high chemiluminescence film.
**Gene Expression Analysis**

RNA was extracted using the Stratagene Absolutely RNA Nanoprep Kit. Microarray hybridization and analyses were performed by the Johns Hopkins Deep Sequencing and Microarray Core Facility using the NuGen amplification system and an Affymetrix Exon 1.0 ST array. The data discussed in this publication are accessible through GEO Series accession number GSE59454.

**Statistical Analysis**

Student’s t tests were performed using GraphPad Prism software assuming equal variances. Log-rank tests were used for Kaplan-Meier plots. P values of less than 0.05 were considered to be statistically significant.

**Results**

*Galectin-3 binds specifically to tumor-specific CD8*<sup>+</sup> *T cells found in the tumor.*

A few studies have already implicated galectin-3 in the regulation of CD8<sup>+</sup> T cell responses through several different mechanisms including induction of apoptosis and TCR cross linking that results in preventing CD8<sup>+</sup> T cell function (4). We therefore employed the *neu*-N mouse model of mammary tumors for which we have developed neu-specific TCR transgenic mice to further evaluate the role galectin-3 plays in modulating neu-specific anti-mammary cancer T cell responses within the tumor microenvironment. We previously reported that our whole cell GM-CSF vaccine cell line, 3T3NeuGM, induces potent antitumor immune responses against the
immunodominant neu protein expressing CD8$^+$ T cell epitope, RNEU$_{420-429}$ in the parental non-tolerant FVB/N mice but not in the tolerant neu-N mice (63). Low doses of cyclophosphamide (CY) given with the vaccine results in the depletion of Tregs and allows for improved high avidity RNEU$_{420-429}$-specific CD8$^+$ T cell responses that are associated with the cure of pre-existing tumors in 30% of neu-N mice. Adoptive transfer of the high avidity T cells given together with CY and vaccine mediates long-term tumor control and produce significantly more cytokines in the majority mice suggesting that vaccine alone does not produce enough activated T cells to take on larger tumor burdens (65). CD8$^+$ T cells derived from these TCR transgenic mice express the Thy1.2 surface marker, which allowed us to differentiate galectin-3 binding on adoptively transferred high avidity CD8$^+$ T cells from endogenous CD8$^+$ T cells that express the Thy1.1 surface marker, and are thought to be tolerized and nonfunctional against the neu antigen. Thus, adoptive transfer of high avidity CD8$^+$ T cells into tolerized neu-N mice provides the opportunity to study a functionally well-defined population of T cells with uniform avidity upon which the effects of galectin-3 can be evaluated in vivo.

We first evaluated adoptively transferred high avidity CD8$^+$ T cells for expression of surface galectin-3. To optimize recovery of tumor infiltrating lymphocytes (TIL) in our adoptive transfer neu-N model, mice were first given a large tumor burden of two simultaneous subcutaneous injections of 2x10$^6$ neu-expressing NT2.5 tumor cells in both the right and left upper mammary fat pads on Day 0, followed by low dose CY (100 mg/kg) on Day 8, whole cell GM-CSF vaccine on Day 9, and adoptive transfer of 6X10$^6$ high avidity CD8$^+$ T cells on Day 10. TIL were isolated from the mammary tumors 5 days following adoptive transfer which is the optimal time point of T cell trafficking into
the tumor microenvironment (65). For an accurate sampling of different immune
environments, we isolated CD8$^+$ T cells from excised tumor tissue, lymph nodes, and
spleens. The largest number of T cells with expression of galectin-3 occurred on TILs,
with relatively negligible amounts by T cells isolated from the tumor draining node
(TDN) and spleen (Figure 3A). In addition, galectin-3 staining on TILs exhibited a
significant increase in mean fluorescence intensity (MFI) relative to what was observed
in the TDN and spleen suggesting that individual T cells bound more galectin-3 when in
the tumor microenvironment. To determine whether the increase in surface galectin-3
was due to an overall increase in extracellular galectin-3 from increased expression and
secretion by tumor cells, or due to increased expression of galectin-3 binding targets
specifically by high avidity CD8$^+$ T cells in the tumor microenvironment, we further
compared galectin-3 staining on endogenous non-specific CD8$^+$ T cells versus galectin-3
staining on high avidity CD8$^+$ T cells in the TIL. Endogenous CD8$^+$ T cells in TIL do not
appear to stain for surface galectin-3 to the same high levels as observed for adoptively
transferred high avidity CD8$^+$ T cells (Figure 3B). These data suggest that galectin-3
selectively binds T cells that traffic into the tumor microenvironment relative to other T
cell residing sites, and T cells that are tumor antigen committed rather than all T cells
within the tumor microenvironment. Taken together, these data suggest galectin-3 does
not simply bind indiscriminately to every cell it encounters, but that there is a certain
degree of specificity governing galectin-3 binding.

*Increased binding of galectin-3 occurs on activated CD8 T cells with an exhausted
phenotype*
Due to the predominance of galectin-3 binding on CD8⁺ T cells only after they
have trafficked into the tumor microenvironment, and the increased binding observed on
high avidity CD8⁺ T cells over other CD8⁺ T cells in the tumor microenvironment which
were previously shown to be low avidity neu-specific, we next determined whether or not
galectin-3 binds preferentially to the most activated T cells. We found that CD8⁺ T cells
maximally activated in vitro with anti-CD3/CD28 beads show a significant increase in
galectin-3 MFI after 3 days in culture (Figure 4A). Because the majority of effector
CD8⁺ T cells found in the tumor have already been activated and undergone terminal
differentiation, we used lymphocytes isolated from TDNs, which contain populations of
T cells at several different stages of cellular division, to illustrate the differences in
galectin-3 binding during the various stages of T cell activation. Carboxyfluorescein
succinimidyl ester (CFSE) labeling of adoptively transferred T cells demonstrated that
the presence of surface galectin-3 was associated with only the most CFSE dilute cell
populations, further establishing a link between galectin-3 binding and cellular
differentiation. In addition, co-staining of galectin-3 with CD44, a cellular adhesion
molecule highly expressed only on effector/memory CD8⁺ T cells, revealed that galectin-3
surface binding occurs only on cells that co-express CD44 indicating that galectin-3
binds preferentially to terminally differentiated effector T cells (Figure 4B).

Next, we evaluated TIL for the phenotypic differences of T cells associated with
increased galectin-3 binding. First, we separated high avidity CD8⁺ T cells in TIL into
galectin-3 high (galectin-3hi) versus galectin-3 low (galectin-3lo) T cells and analyzed
each population for the co-expression of other known regulatory molecules, specifically
programmed death 1 (PD-1) and lymphocyte activation gene 3 (LAG-3) because these
two markers are inhibitory receptors often involved in T cell tolerance (67). PD-1 expression often increases in a tolerogenic setting when CD8$^+$ T cells fail to receive the appropriate co-stimulatory signals. Several tumor cell types express the PD-1 ligand B7H1, and signaling through PD-1 is one mechanism by which tumor cells can evade the immune response (68). The role of LAG-3 is still controversial, but its expression has been shown to increase during antigen stimulation and is thought to play a homeostatic role in T cell expansion through the regulation of calcium flux (69). We observed an increase in the expression of the inhibitory markers PD-1 and LAG-3 only on galectin-3$^{hi}$ CD8$^+$ T cells, with the majority of galectin-3$^{hi}$ T cells being PD-1 and LAG-3 double positive when compared with galectin-3$^{lo}$ cells (Figure 4C). In addition, galectin-3$^{hi}$ cells also have higher levels of Annexin V staining when compared with galectin-3$^{lo}$ cells (Figure 4D). Despite the increase in expression of PD-1 and LAG-3, we did not observe any differences in interferon gamma (IFN$\gamma$) expression associated with galectin-3 binding in vivo (data not shown). Thus, galectin-3 binding on CD8$^+$ T cells occurs almost exclusively on differentiated effector T cells. Furthermore, increased binding is observed on T cells displaying an exhausted or tolerized phenotype with elevated expression of PD-1 and LAG-3, and higher Annexin V labeling.

*LAG-3 is necessary for galectin-3 mediated suppression of IFN$\gamma$ in vitro*

These data so far provide a link between elevated galectin-3 binding and increased PD-1 and LAG-3 expression on high avidity neu-specific CD8$^+$ T cells. Next, we evaluated whether PD-1 and LAG-3 surface proteins are directly involved in galectin-3 associated downstream effects on T cell function. Both these molecules are known to
be heavily glycosylated and possess several potential binding sites for galectin-3 (70, 71). We first confirmed previously reported findings that addition of exogenous galectin-3 inhibits IFNγ production in CD8+ T cells in vitro (26). Consistent with previously published reports, we also demonstrated inhibition starting at 50 mg/mL of galectin-3 after a 3-day stimulation of CD8+ T cells with anti-CD3/CD28 beads (data not shown).

Next we determined whether galectin-3 can inhibit IFNγ production in PD-1 knockout and LAG-3 knockout CD8+ T cells when compared with wild type controls. Although exogenous galectin-3 did not significantly alter IFNγ production of PD-1 knockout T cells, LAG-3 knockout T cells produced maximum IFNγ even at the highest concentrations of exogenous galectin-3. In contrast, wild type CD8+ T cells had a 20% reduction in IFNγ production at concentrations of 150 mg/mL or higher of exogenous galectin-3 (Figure 5A and 5B). Furthermore, co-immunoprecipitation studies demonstrated a physical interaction between LAG-3 and galectin-3. Using whole cell lysates of activated CD8+ T cells, we were able to isolate LAG-3 with an anti-galectin-3 antibody, and vice versa (Figure 5C). Next, cross-linking of cell surface proteins prior to cell lysis allowed us to immunoprecipitate a LAG-3/galectin-3 complex migrating between 150 and 250 kD with a LAG-3 specific antibody (Figure 5D). Importantly, we were not able to co-immunoprecipitate PD-1 with galectin-3. These data establish a link between LAG-3 and galectin-3 on antigen-committed CD8+ T cells, and suggests a new mechanism by which galectin-3 may regulate CD8+ T cell function.

These data establish for the first time a link between LAG-3 and galectin-3 expression on antigen-committed CD8+ T cells, and suggests a new mechanism by which galectin-3 may regulate CD8+ T cell function. Conversely, this data also provides a
possible mechanism through which LAG-3 can still regulate CD8+ T cell function
independent of its known ligand MHC II.

Expression of CEACAM1 and ST6GAL1 are increased in galectin-3hi CD8+ T cells

We examined changes in gene expression of adoptively transferred high avidity
galectin-3hi CD8+ TILs versus high avidity galectin-3lo CD8+ TILs with Affymetrix
cDNA hybridization arrays (Figure 6). We chose to focus our attention on CD8+ TILs
due to the high percentage of these cells that are galectin-3hi relative to T cells seen in
peripheral lymphoid organs. The expression of CEACAM1 (CD66) was found to be two-
fold higher in galectin-3hi cells. CD66 is of particular interest because it has been
identified as the major receptor for galectin-3 in human neutrophils (72). Furthermore,
the cross-linking of CD66 has been demonstrated to result in recruitment of phosphatases
that dephosphorylate the TCR signaling molecule Zap-70 (73). CD66 expression was
verified by flow cytometry staining, and remains a potential binding partner of interest
due to a strong linear correlation with galectin-3 staining (Figure 7A-C). However,
western blot studies performed to demonstrate Zap-70 dephosphorylation with increasing
amounts of exogenous galectin-3 added to galectin-3 -/- CD8+ T cells were inconclusive,
and no significant differences were observed. The addition of 5 μg/mL exogenous
galectin-3 may have lead to a decrease in Zap-70 phosphorylation observable at the 6 to 8
minute time points; however, these results were minimal and could not be repeated
(Figure 8).

The expression of an additional gene of interest, ST6GAL1, was found to be two-
fold lower in galectin-3hi cells. ST6GAL1 encodes the enzyme, beta-galactoside alpha-
2,6-sialyltransferase 1, which catalyzes the addition of sialic acid to galactose containing residues. It has previously been shown that modification of galactose residues with sialic acid might inhibit binding of galectin-3 (74). Indeed, labeling of T cells with Sambucus nigra lectin to detect α2,6-linked sialic acid residues on terminal galactose residues revealed higher staining of T cells isolated from peripheral lymphoid organs than from TIL, which corroborates the observation that the percentage of galectin-3hi CD8+ T cells in the TIL is significantly higher than observed in the tumor draining lymph node or the spleen (Figure 9A). In addition, removal of sialic acid residues with sialidase increases the amount of galectin-3 detected on the surface of CD8+ T cells after a 5 hour stimulation with T2-D3 cells pulsed with RNEU420–429 (Figure 9B). Interestingly, antigen exposure appears to increase surface galectin-3, which suggests a role for autocrine secretion from the CD8+ T cell.

**Determination of factors influencing α2,6-sialylation of CD8+ T cells**

Previous work has demonstrated the importance of cell surface glycan chains, and their impact on T cell function by influencing receptor signaling on the T cell surface (75, 76). Further, activation of CD8+ and CD4+ T cells can lead to extensive remodeling of cell surface glycoproteins by altering expression of glycan transferases. Notably, the amount of sialylated glycans was dramatically reduced (77). Our observations thus far that terminal sialylation of glycan chains appears to inhibit galectin-3 binding are consistent with what has been described in the literature. However, this does not explain why activated, terminally differentiated CD8+ T cells in the periphery remain sialylated while those in the TME do not. To test whether or not desialylation of CD8+ T cells and
galectin-3 binding is specific to the TME or a result of antigen exposure, we used a mouse model of acute graft versus host disease where adoptively transferred H-2b CD8+ T cells develop an alloresponse against H-2d splenocytes in the host mouse (78). At 8 weeks post-adoptive transfer we observed marked splenomegaly reflecting the degree of engraftment by adoptively transferred cells (Figure 10A). If galectin-3 binding were due solely to antigen exposure and glycan remodeling, then one would expect to see increased surface galectin-3 on engrafted H-2b CD8+ T cells; however, the amount of surface galectin-3 detected by flow cytometry was similar to the level seen in our neu-N mouse model (Figure 10B). Therefore, we reasoned that the TME must provide conditions that promote galectin-3 binding that are not satisfied elsewhere. Because of low sialylation observed in the TME, we wondered if a sialidase could be secreted either by tumor cells or other cells in the TME. Using the neu-N mouse model, we tested to see if directly injecting a sialidase inhibitor into mouse tumors could reduce the amount of galectin-3 binding observed on adoptively transferred high avidity CD8+ TILs (Figure 11). However, we observed no difference between treated and untreated controls indicating that either the drug treatment did not penetrate into the TME as we had hoped or that other factors in the TME must be responsible for glycan remodeling and galectin-3 binding in and in vivo setting.

Depletion of extracellular galectin-3 leads to improved T cell function

Despite our findings that galectin-3 suppresses IFNγ production by high avidity antigen-specific CD8+ T cells in vitro, we were not able to observe a similar effect on TILs in vivo when we sorted on galectin-3hi and galectin-3lo T cells. We reasoned that this
discrepancy may be due in part to the observation that galectin-3 binds to the majority of CD8+ T cells that traffic into the TIL, and that a truly galectin-3 negative T cell population does not exist in the TIL. While categorizing cells into galectin-3 hi or galectin-3 lo may be sufficient to study MFI shifts in surface protein expression, it may not be sufficient for examining functional differences. Further, galectin-3 can be expressed and secreted by a variety of cell types, making it difficult to study the impact of its absence using the current model (79). Thus, in order to accurately determine a functional role for galectin-3 in vivo, we bred the neu-N and high avidity TCR transgenic mice onto a galectin-3 genetic knockout background.

We first evaluated the genetic knockout of galectin-3 in only the neu-N mouse and found that it was not sufficient to completely abolish galectin-3 binding on the surface of high avidity CD8+ T cells, although it did result in an overall decrease in galectin-3 expression (Figure 12A). However, galectin-3 knock out in both the high avidity CD8+ T cell and the neu-N recipient mouse completely abolished galectin-3 binding on high avidity CD8+ T cells (Figure 12B). This model allows the removal of galectin-3 from TILs without disrupting galectin-3 expression in the transplantable tumor cells themselves, thus demonstrating that the major source for galectin-3 are tumor-reactive CD8+ T cells and cells belonging to the tumor stroma rather than the tumor itself.

Based on prior reports, galectin-3 can mediate CD8+ T cell anergy by sequestering the TCR from the CD8 co-receptor, raising the threshold of activation (62). We therefore evaluated the outcome of reduced galectin-3 on TCR avidity by tetramer staining. As expected, removal of galectin-3 from the surface of high avidity CD8+ T cells did not
result in a significant change in tetramer binding, confirming that any observed changes in T cell function is not a result of increased TCR signaling (Figure 12C).

Next, we evaluated the effect on CD8+ T cell function in TIL populations after genetic removal of galectin-3 from the neu-N recipient alone, in the T cell alone, or in combination. We observed an increase in the total number of IFNγ and Granzyme B producing T cells only when galectin-3 is knocked out in both the T cell and the recipient mouse. Depletion of galectin-3 in CD8+ T cells alone or in the recipient mouse alone was insufficient to cause an improvement in CD8+ T cell effector function as measured by cytokine production (Figure 13A-B). This observation is consistent with our earlier finding that elimination of galectin-3 expression is required in both the recipient mouse and the adoptively transferred T cell to completely remove galectin-3 from the cell surface.

After demonstrating improved effector function after removal of galectin-3, we next tested the ability of high avidity CD8+ T cells to promote long-term tumor control in a galectin-3 null environment. We previously determined that a minimum of 4x10^6 high avidity CD8+ T cells was required to achieve long-term tumor control in >75% of neu-N mice (ref). Thus, to illustrate the increase in efficacy of CD8+ T cells in galectin-3 null mice, we wanted to see if galectin-3 knockout (KO) high avidity CD8+ T cells could mediate a similar level of clearance using half the amount of T cells required for clearing tumor in galectin-3 wild type (WT) mice. We compared long-term tumor control in WT neu-N mice receiving 2x10^6 WT high avidity CD8+ T cells, WT neu-N mice receiving 2x10^6 WT high avidity CD8+ T cells in addition to treatment 3 times a week with the galectin-3 inhibitor GCS-100, galectin-3 KO neu-N mice receiving 2x10^6 galectin-3 KO
high avidity CD8$^+$ T cells, and galectin-3 KO neu-N mice without adoptive transfer (Figure 14A). In addition, we also compared long-term tumor control in WT neu-N mice receiving adoptive transfer of either 2x10$^6$ galectin-3 WT or KO high avidity CD8$^+$ T cells (Figure 14B). All mice were given the minimal tumorigenic dose of 5x10$^4$ NT2.5 cells on Day 0 followed by CY on Day 1, whole cell GM-CSF vaccine on Day 2, and adoptive transfer on Day 3. Consistent with our functional data, we only observed improved tumor-free survival in the galectin-3 KO mice that had received galectin-3 KO CD8$^+$ T cells with 90% of mice tumor free at the end of 60 days versus 50% of the control mice. Galectin-3 WT mice that received galectin-3 KO CD8$^+$ T cells did not show any survival advantage over control mice. Interestingly, treatment with the galectin-3 inhibitor, GCS-100, resulted in an initial delay in tumor growth. Furthermore, because 100% of galectin-3 KO mice that did not receive adoptive transfer of high avidity CD8$^+$ T cells rapidly developed tumors by Day 20, we show that tumor clearance in these mice is mediated by tumor-specific high avidity CD8$^+$ T cells, which may not be present at high enough numbers in the endogenous host without adoptive transfer.

To confirm the more general role of galectin-3 in regulating antigen-specific T cell responses, we used the transplantable Panc02 tumor model to compare endogenous anti-tumor T cell responses in galectin-3 KO mice versus WT mice. The Panc02 tumor cell line has many similarities to human PDA, and has been used previously to study PDA specific vaccine induced in vivo T cell responses and the role of regulatory T cells (Tregs) in preventing antitumor immunity (80). Mice were given a tumor burden of 2.5x10$^5$ Panc02 tumor cells injected subcutaneously in the right flank on Day 0 followed by CY (100 mg/kg) and an anti-CD25 antibody, PC61 (50 mg) on Day 2 (to deplete
Tregs), and a whole cell GM-CSF vaccine on Day 3. Mice were then followed for 60 days for tumor growth. We observed that at the end of the 60 day period, a significantly higher percentage of galectin-3 KO mice were tumor free versus control WT mice (Figure 14C). Thus, these data provide for the first time evidence that galectin-3 expression by tumor-specific high avidity CD8+ T cells inhibits their function in vivo, and that the source of galectin-3 is provided by both the T cells and the tumor stromal cells.

Galectin-3 removal leads to increased expression of pro-inflammatory genes in CD8+ T cells

Due to the increase in the numbers of IFNγ and Granzyme B producing high avidity CD8+ T cells observed when galectin-3 is knocked out, we were interested in identifying other molecules whose expression may also be affected by the presence of galectin-3 on the cell surface. We used whole genome microarray analyses of galectin-3 WT CD8+ TILs isolated from galectin-3 WT neu-N mice (WT/WT), galectin-3 WT CD8+ TILs isolated from galectin-3 KO neu-N mice (WT/KO), and galectin-3 KO CD8+ TILs isolated from galectin-3 KO neu-N mice (KO/KO). Adoptively transferred high avidity CD8+ T cells in TILs were purified from the endogenous T cell population by FACS sorting on Thy1.2, and global gene expression patterns were compared between groups. We found that in both KO/KO and WT/KO CD8+ T cells, molecules associated with inflammatory response processes were increased relative to WT/WT cells. Specifically, CCL8, CXCL16, Gp49a/Lilrb4, Lyz1/Lyz2, RNASE3, CCL13, Chi3l3/Chi3l4, CYBB, SHC1, TYROBP, CD86 were upregulated at least 2-fold or higher in in WT/KO CD8+ T cells. Some similar and some additional genes were upregulated 2-fold or higher in
KO/KO CD8⁺ T cells including CCL8, CXCL16, Gp49a/Lilrb4, Lyz1/Lyz2, RNASE3, C3AR1, C5AR1, CXCL9, S100A8, S100A9, SPI1, IRF1, Tlr13, GAB2, Sirpb1a (Figure 15). Analyses of upstream regulator pathways predicted activation of the TNFα, IFNγ, and IL-6 signaling pathways in the KO/KO CD8⁺ T cells but not in WT/KO CD8⁺ T cells, suggesting a possible mechanism behind why increased effector function is observed only in a completely galectin-3 null mouse (see Appendix A for Table 2).

Increased expression of the genes CCL8, CXCL16, Gp49a/Lilrb4, Lyz1/Lyz2, and RNASE3 are observed in both WT/KO and KO/KO groups in comparison with WT/WT mice, suggesting the expression of these genes are influenced by the presence of galectin-3 specifically in the recipient mouse but are independent of whether or not galectin-3 is expressed in CD8⁺ T cells. In contrast, C3AR1, C5AR1, CXCL9, S100A8, S100A9, SPI1, IRF1, Tlr13, GAB2, and Sirpb1a are only increased in expression when galectin-3 expression is completely eliminated. A comparison between WT/KO and KO/KO CD8⁺ T cells reveal very few changes with respect to inflammatory response genes with the exception of S100A9, which is often found to be co-expressed with S100A8, and has also been shown to stabilize S100A8 expression via a posttranscriptional mechanism (81). S100A8 and S100A9 are EF-hand Ca²⁺ binding proteins that are heavily involved in several inflammatory processes and possess tumoricidal properties in vitro. These inflammatory response genes have been shown to be expressed by neutrophils, but not yet by T cells (82). We show for the first time that S100A8 expression is increased almost 7-fold in galectin-3 KO/KO CD8⁺ T cells when compared with WT/WT cells (Figure 16).
Discussion

Our data describe three novel findings supporting galectin-3 as a regulator of antigen-specific T cell activation within the TME. First, galectin-3 selectively targets T cells only after they have been activated and traffic into the TME. In addition, the T cells themselves and host derived cells within the TME, but not the tumor cells, are the major sources of galectin-3 that mediate galectin-3 dependent T cell suppression. Second, galectin-3 regulation of activated T cells leads to genome wide changes in inflammatory gene expression. Third, galectin-3 co-expresses with LAG-3 on activated and terminally differentiated T cells, and functional LAG-3 is required for galectin-3 mediated T cell suppression.

We show for the first time that galectin-3 regulation of T cells occurs primarily in the TME, and selectively targets only tumor-specific T cells. In addition, this is the first example that galectin-3 mediates T cell suppression as a result of autocrine secretion of galectin-3 by the T cells themselves, as well as by non-tumor cells likely residing in the tumor stroma. Other groups have also observed that activated CD8^+ T cells readily bind more galectin-3 than naïve T cells (83). One hypothesis proposed by Demotte et.al. and others is that activation of CD8^+ T cells leads to changes in glycosylation machinery that increases the number and accessibility of LacNAc motifs available for galectin-3 binding (84). However, glycan remodeling resulting from activation still fails to fully explain why only a small percentage of activated, effector CD8^+ T cells in peripheral lymphoid tissue stain positive for galectin-3 binding while the majority of activated CD8^+ T cells in the tumor do bind galectin-3. Thus, the TME must provide other conditions that are not satisfied elsewhere to promote galectin-3 binding in order to promote an
immunosuppressive environment. Preliminary experiments with sialidase inhibitors were not successful in either reducing surface galectin-3 or increasing glycan sialylation. These findings are consistent with microarray results demonstrating 2-fold downregulation of \textit{ST6GAL1} in galectin-3\textsuperscript{hi} staining CD8\textsuperscript{+} T cells. Future work should focus on clarifying regulation of glycan remodeling in CD8\textsuperscript{+} T cells that reside in peripheral lymphoid tissues versus in the TME.

The leading assumption has always been that tumor cells are a major source for galectin-3 (85). While this may be the case for certain tumor types, our data demonstrate for the first time that tumor cells are not always the source of extracellular galectin-3. Rather, galectin-3 expression by tumor-specific CD8\textsuperscript{+} T cells and stromal cells within the TME, result in cancer-specific CD8\textsuperscript{+} T cell suppression. A likely candidate in the stromal compartment could be fibroblasts as they have been shown in models of fibrosis to secrete large amounts of galectin-3, and are also known to be important for establishing the TME (86). Our findings highlight the importance of targeting not just tumor cells, but also stromal cells for effective cancer immunotherapy. These data also imply that T cells may regulate their own activity via the autocrine secretion of galectin-3, but interestingly, this only appears to occur in the TME. Future studies should focus on identifying the specific tumor stromal cell types that secrete galectin-3 as well as what other additional factors provided by the TME promote galectin-3 binding to T cells.

This is also the first study to conduct a genome wide analysis evaluating changes that occur in tumor-specific CD8\textsuperscript{+} T cells resulting from binding of extracellular galectin-3 in an \textit{in vivo} setting. The data demonstrates that removal of galectin-3 leads to increased expression of proinflammatory genes within CD8\textsuperscript{+} T cells providing additional
evidence that galectin-3 is a member of a suppressive network of signals. Recent data from several groups demonstrate the capacity for galectin-3 to suppress T cell function by inducing T cell anergy via TCR clustering, and that these T cells can be rescued by removing surface galectin-3 (55). Our gene array findings demonstrate that the mechanisms of T cell regulation by galectin-3 extends beyond TCR signaling, and includes altering T cell fate at the gene expression level. These findings are not surprising given the extent of glycosylation occurring on cell surface proteins, and the importance of T cell co-receptors for overall T cell function. Furthermore, while these previous studies were able to reduce the amount of galectin-3 bound to the surface of T cells using antibodies or inhibitors, they were not able to completely eliminate galectin-3 binding from the surface of the T cell. We show that after optimizing for TCR avidity, further improvements in T cell function were still attainable after complete elimination of galectin-3 from the cell surface. Taken together, these observations suggest multiple layers of T cell inhibition by galectin-3, and that TCR avidity is just one mechanism in the broader scheme of galectin-3 regulation. Future research should focus on improving galectin-3 targeted therapies to increase elimination of bound galectin-3 in order to maximize T cell function. Additional studies should further elucidate the role of the S100A8/9 signalizing pathway as a potential mediator of T cell lytic activity.

PD-1 and LAG-3 are two major co-receptors that have been shown to modulate T cell function, and their co-expression has been shown to be associated with regulating terminal T cell activation/exhaustion (67). Our finding that LAG-3 expression is associated with galectin-3 binding, and further, is required for galectin-3 suppression in vitro provides a new mechanism through which LAG-3 can regulate CD8+ T cells. LAG-
3 is capable of negatively regulating the function of CD8\textsuperscript{+} T cells despite the fact that CD8\textsuperscript{+} T cells do not interact with LAG-3’s known ligand, MHC II. Anti-LAG-3 antibody therapy was shown to reverse this effect in CD4\textsuperscript{+} depleted mice, which indicates a direct role for LAG-3 on CD8\textsuperscript{+} T cells (87). The mechanism by which LAG-3 mediates CD8\textsuperscript{+} T cell activity is unknown. LAG-3 can be extensively glycosylated, and as a result, would be a likely target for galectin-3 binding. Our data provide the first evidence that LAG-3 signaling can be induced by galectin-3 cross-linking, and this may be one of the primary means by which galectin-3 regulates T cell function. Furthermore, our co-immunoprecipitation studies of LAG-3 and galectin-3 support a direct physical interaction. Lastly, it is interesting to point out that while increased PD-1 expression was also associated with galectin-3 binding, we did not observe any functional relationship between the two. Thus, it is possible that either galectin-3 does not bind PD-1 at all or galectin-3/PD-1 interactions are irrelevant. The latter highlights the fact that not all lectin-glycan interactions are functionally consequential. Further studies are required to connect galectin-3 surface binding with signaling downstream of LAG-3.

Our work indicates that CEACAM1 (CD66) should remain a molecule of interest given the strong correlation between galectin-3 surface binding and CD66 expression. Work done in Jurkat T cells has demonstrated that cross-linking of CEACAM1 with antibody was sufficient to lead to phosphorylation of ITIM motifs, which then allow for the recruitment of phosphatases that dephosphorylate ZAP-70 and result in downregulation of TCR signaling (73). The fact that we were not able to successfully repeat these experiments with primary mouse T cells stimulated \textit{in vitro}, and using galectin-3 as a cross-linker for CEACAM1 rather than antibody could be due to several
reasons. First, mouse T cells express a variety of different glycoforms of CEACAM1 that are not expressed by human T cells. Second, CEACAM1 exists in both long (CEACAM1-L) and short (CEACAM1-S) isoforms in mouse cells, whereas it exists almost exclusively in the long isoform in human cells (88). The cytoplasmic tail of the long isoform is required for ZAP-70 dephosphorylation. As a result, ZAP-70 dephosphorylation may not be detectable in mouse cells due to the presence of CEACAM1-S, and the variety of different glycoforms that exist which may or may not be amenable to galectin-3 cross-linking. Therefore, future studies examining the relationship between CEACAM1 and galectin-3 should utilize human cells rather than mouse cells.
Figure 3. Increased surface galectin-3 on high avidity CD8+ T cells in the tumor microenvironment.

A) Tissues were processed according to the procedure outlined in the materials and methods section 5 days after adoptive transfer into neu-N mice. Galectin-3 surface staining is shown for CD8+ T cells gated on Thy1.2 expression. B) Histogram overlays show comparisons of galectin-3 MFI on adoptively transferred high avidity CD8+ T cells (Thy1.2+) versus on endogenous CD8+ T cells (Thy1.2-) found in the tumor microenvironment.
Figure 4. Activation of CD8+ T cells increases surface bound galectin-3.

A) CD8+ T cells were negatively isolated from splenocytes and stimulated in the presence of 30 U/mL rhIL-2 with anti-CD3/CD28 beads. Galectin-3 surface staining is shown after 3 days of stimulation in comparison with naïve cells. B) High avidity CD8+ T cells were labeled with 1.5 μM CFSE prior to adoptive transfer into neu-N mice. The tumor draining node (TDN) was dissected 3 days after adoptive transfer and processed according to the materials and methods section. Galectin-3 surface staining is shown with CFSE dilution and CD44 expression for cells gated on Thy1.2. C+D) Tumor infiltrating lymphocytes (TIL) were extracted from tumors 5 days after adoptive transfer according to the materials and methods section. Cells were gated on Thy1.2 and divided into galectin-3hi and galectin-3lo staining populations based on isotype staining controls. A comparison of PD-1 and LAG-3 co-expression is shown for galectin-3hi and galectin-3lo populations in C) and Annexin V staining in D).
Figure 5. LAG-3 but not PD-1 expression is required for galectin-3 suppression of IFNγ production by T cells.

The effect of increasing concentrations of extracellular galectin-3 on the percentage of IFNγ producing CD8⁺ T cells is shown for A) PD-1 knockout versus wild type T cells in and B) LAG-3 knockout versus wild type T cells. Experiments performed at least 2 independent times with 3 replicates per sample with errors bars representing SD. C) Co-immunoprecipitation of LAG-3 and galectin-3 from activated CD8⁺ T cell lysates with either a galectin-3 specific antibody (M3/38) or a LAG-3 specific antibody (410C9). D) Co-immunoprecipitation of a LAG-3/galectin-3 complex with LAG-3 specific antibody after cross-linking of cell surface proteins on activated CD8⁺ T cells prior to cell lysis as described in materials and methods.
Figure 6. Ingenuity Pathway Analysis of gene expression differences between galectin-3hi CD8+ TILs vs. galectin-3lo CD8+ TILs identified CD66 and ST6GAL1 as genes of interest.

Adoptively transferred high avidity CD8+ T cells were isolated from tumor tissue as described in materials and methods and then sorted based on staining for Thy1.2 and galectin-3. Galectin-3^hi versus galectin-3^lo cells were separated based on the top 5% and bottom 5% of galectin-3 MFI. Gene expression changes were filtered based on fold change > 2 and p > .05.
Figure 7. Co-staining of CD66 and Galectin-3 on high avidity CD8+ TILs.

A) Adoptively transferred high avidity CD8+ T cells were isolated from tumor, and stained for surface galectin-3 as detailed in materials and methods. Cells were partitioned into separate populations based on galectin-3 MFI. B) Histogram overlays comparing CD66 MFI among cell populations with increasing galectin-3 MFI. C) Logarithmic transformation of galectin-3 MFI vs. CD66 MFI demonstrate a linear correlation between galectin-3 surface staining and CD66 expression.
A) Galectin-3 → CD66

B) **Gal-3 vs. CD66 Correlation**

\[
y = 3.1732x - 6.8422 \\
R^2 = 0.99288
\]
Figure 8. In vitro dephosphorylation of ZAP-70 after cross-linking of CD66 with galectin-3.

Galectin-3 KO CD8⁺ T cells were isolated and treated according to procedures outlined in materials and methods. Cells were incubated with 5 µg/mL galectin-3 for cross-linking. After cell lysis, 7.5 µg of total protein was loaded for each time point. Chemiluminescent film was exposed for 3 minutes prior to development.
Figure 9. Galectin-3 binding to the surface of CD8+ T cells is determined by α2,6-sialylation.

A) Bar graph demonstrating percent of adoptively transferred high avidity CD8+ T cells with galectin-3 MFI greater than isotype control reveals majority of galectin-3 binding occurs in the TME. Inset demonstrates inverse relationship between galectin-3 binding and α2,6–sialic acid as reflected by staining with FITC-Sambucus nigra lectin. B) Pretreatment of high avidity CD8+ T cells isolated from TDN 3 days post adoptive transfer with sialidase prior to 5 hour stimulation with either T2-Dq pulsed with NP or specific peptide, RNEU420–429 increases binding capacity for galectin-3 on the T cell surface.
Figure 10. Galectin-3 binding on adoptively transferred alloreactive CD8+ T cells in a model of graft versus host disease.

6 x 10^7 H2-K^b splenocytes isolated from female C57Bl/6 mice were adoptively transferred into female B6D2F1 mice, which possess the H2-K^b/d allotype. Spleens were harvested at 8 weeks post-adoptive transfer. **A)** Percent of CD8+ T cell engraftment in spleens demonstrated by gating on CD8^+ H2-K^b/d- T cells. Inset – size of spleens reflecting degree of engraftment. **B)** Histogram overlays demonstrate surface galectin-3 MFI on alloreactive CD8^+ T cells isolated from B6D2F1 spleens, high avidity CD8^+ T cells from *neu*-N spleens, and high avidity CD8^+ T cells from *neu*-N TIL.
Figure 11. Pretreatment of mouse tumors with methyl ester sialidase inhibitor does not increase surface galectin-3.

A) Mouse tumors were directly injected with 100 μM of a 10 mM solution of methyl-ester conjugated sialidase inhibitor in DMSO on days 3 and days 4 post adoptive transfer prior to TILs being extracted on day 5 and analyzed for surface galectin-3 by flow cytometry. Histogram overlays comparing untreated vs. treated mice are shown. B) SNA-FITC staining demonstrating no increase in sialylation as a result of sialidase inhibitor treatment.
Figure 12. Removal of galectin-3 from the surface of high avidity CD8+ T cells requires inhibition of galectin-3 expression in the tumor stroma and the T cell.

Tumor infiltrating lymphocytes (TIL) were isolated 5 days post adoptive transfer according to the materials and methods section. Cells were gated for Thy1.2 expression. 

A) Galectin-3 wildtype (WT) high avidity CD8+ T cells were adoptively transferred into either galectin-3 WT (red) or galectin-3 KO neu-N (blue) recipient mice. Histogram overlays show differences in galectin-3 MFI between the two groups with respect to isotype control staining (orange). 

B) Galectin-3 staining is now shown on galectin-3 KO high avidity CD8+ T cells adoptively transferred into a galectin-3 KO neu-N recipient (blue) with respect to an isotype control (red). 

C) TCR avidity is compared between galectin-3 WT high avidity CD8+ T cells from a galectin-3 WT neu-N recipient and galectin-3 KO high avidity CD8+ T cells from a galectin-3 KO neu-N recipient. TCR avidity was assessed by staining with MHC I D9 tetramer loaded with the RNEU420-429 (P50) peptide.
Figure 13. Removal of surface galectin-3 from high avidity CD8+ T cells leads to improved effector function.

Effector function of TILs was assayed after elimination of galectin-3 in the CD8 T cell, the tumor stroma, or both. Tumors were removed 5 days after adoptive transfer of high avidity CD8+ T cells, and TILs were isolated according to the protocol outlined in materials and methods. Cytokine expression was assessed by intracellular cytokine staining (ICS) after a 5 hour incubation with a TAP-2 deficient cell line (T2-D9) expressing MHC I Dq loaded with either RNEU_{420-429} (P50) or LCMV NP (NP). The absolute number is shown for Thy1.2^+ CD8^+ T cells / mg tumor expressing either A) IFN-γ or B) Granzyme B.
Figure 14. Galectin-3 knockout mice have improved tumor free clearance over wild type mice in both adoptive transfer and endogenous tumor vaccine models.

A) All neu-N recipient mice received the minimal tumorigenic dose of $5 \times 10^4$ NT2.5 cells on day 0, followed by 100 mg/kg CY on day 2, and whole cell GM-CSF vaccine on day 3. Adoptive transfer groups (n=10) received $2 \times 10^6$ galectin-3 WT or KO high avidity CD8$^+$ T cells as indicated above on day 4. A control group of galectin-3 KO mice (n=7) did not receive adoptive transfer in order to assess the endogenous anti-tumor response. Mice were followed every 5 days for development of palpable tumor formation at the injection site. One tailed p value is shown for KO/KO vs. WT/WT groups.

B) Mice were treated as in A), and galectin-3 WT neu-N mice received adoptive transfer of either $2 \times 10^6$ galectin-3 WT high avidity CD8$^+$ T cells (n=10) or $2 \times 10^6$ galectin-3 KO high avidity CD8$^+$ T cells (n=10).

C) WT C57Bl/6 mice (n=15) or galectin-3 KO C57Bl/6 mice (n=15) received $2.5 \times 10^5$ Panc02 tumor cells on day 0, 100 mg/kg CY and 50 μg PC61 on day 2, and whole-cell GM-CSF vaccine on day 3. Mice were followed every 5 days for development of palpable tumor formation at the injection site.
A) Tumor free survival in neu-N mice

B) Tumor free survival in Gal-3 WT neu-N mice

C) Panc02 Tumor free survival
Figure 15. Removal of galectin-3 increases expression of inflammatory molecules in high avidity CD8+ T cells.

This figure summarizes microarray findings comparing high avidity galectin-3 WT CD8+ TILs from galectin-3 WT neu-N mice (WT/WT) (n=4), high avidity galectin-3 WT CD8+ TILs from galectin-3 KO neu-N mice (WT/KO) (n=4), and high avidity galectin-3 KO CD8+ TILs from galectin-3 KO neu-N mice (KO/KO) (n=4). Analysis was performed using Ingenuity Pathway Analysis (IPA) software using a filter for linear fold changes ≥ 2, and p ≤ .05. Molecules listed above the arrows between each group are immune-associated genes found to be upregulated between groups. Molecules listed below each group are immune-associated genes found to be upregulated in comparison to the WT/WT control. CCL8, CXCL16, Gp49a/Lilrb4, Lyz1/Lyz2, and RNASE3 were found to be upregulated in both WT/KO and KO/KO groups in comparison with WT/WT. (*) denotes transcription factors.
Figure 16. Western blot analysis of TIL lysates confirm S100A8 expression in Gal-3 KO/KO TIL but not Gal-3 WT/WT TIL. Adoptively transferred galectin-3 WT high avidity CD8$^+$ T cells and galectin-3 KO high avidity CD8$^+$ T cells were purified by FACS sorting on cells expressing the Thy1.2 surface marker. Cells were then lysed and total protein concentration quantified by measuring absorbance at 280 nm. 6 μg of total protein was loaded per sample.
CHAPTER 4: DETERMINING THE ROLE OF GALECTIN-3 IN THE INFLAMMATORY RESPONSE

Introduction

The major aim of cancer immunotherapy is to induce an immune response against tumor cells akin to the immune response observed against foreign antigens such as viruses and bacteria, which are rapidly cleared by the immune system. In non-tolerized FVB/N mice, GM-CSF vaccination against NT2.5 tumor cells can induce potent tumor-specific high avidity CD8+ T cells that produce IFNγ, TNFα, and IL-2 (15, 65). Unlike foreign antigens, tumor cells are recognized by the immune response as “self” antigens, and thus several tolerogenic mechanisms exist in the periphery and the TME to prevent the induction of a potent anti-tumor immune response. These mechanisms include T-regulatory cells (Tregs), tolerogenic antigen-presenting cells, myeloid-derived suppressor cells (MDSCs), immunoregulatory molecules, and immunosuppressive cytokines (89-92).

As previously mentioned in Chapter 1, galectin-3 has been shown to be involved in the regulation of both the innate and the adaptive immune response, and functions as a regulatory molecule during every step of the continuum from acute inflammation to chronic inflammation and fibrosis (93). Several published reports have suggested a pro-inflammatory role for galectin-3. It has been shown to be critical for neutrophil recruitment and migration in mouse models of S. pneumoniae infection (94, 95), and triggering the oxidative burst (96, 97). However, its impact on monocytes and macrophages, which lie at the intersection between the innate and adaptive immune response appears to be less clear. While some papers have shown galectin-3 to be important for activation and recruitment of monocytes (98, 99), more recent papers have
shown the exact opposite, and that it inhibits monocyte to dendritic cell differentiation \((100, 101)\). Our microarray findings from galectin-3 KO mice discussed in Chapter 3 revealed upregulation of inflammatory pathways involving TNF\(\alpha\), IFN\(\gamma\), and IL-6. Because many of the aforementioned studies involving galectin-3 and inflammation were done in infectious disease models, we were interested in elucidating the impact of galectin-3 deficiency on inflammation in the neu-N mouse tumor model where the immune response is overwhelmingly tolerogenic.

**Materials and Methods**

**Cytokine expression arrays**

Mice were treated as in tumor challenge experiments, but did not receive cyclophosphamide or adoptive transfer. Four days after vaccination, mice were euthanized and cytokine expression in whole tissue lysates from VDNs was evaluated using cytokine arrays. Briefly, VDNs from each experimental group were pooled and homogenized as previously described in Chapter 3. Cells were lysed according to manufacturer’s instructions and lysate was blotted with the Mouse Cytokine Antibody Array, Panel A (R&D Systems, Minneapolis, MN). The dot blot was then scanned and analyzed using VisionWorks LS (UVP, LLC, Upland, CA) to determine density per pixel and fold-change in expression in galectin-3 KO cells in comparison with galectin-3 WT cells.

**Direct Ex Vivo Antigen Detection Assay**

Mice were treated as in tumor challenge experiments, but did not receive cyclophosphamide or adoptive transfer. Four days after vaccination, CD8\(^+\) dendritic cells
and plasmacytoid dendritic cells were isolated from spleen tissue using CD8+ dendritic cell and plasmacytoid dendritic cell isolation kits (Miltenyi). CD8+ T cells were negatively isolated from high avidity TCR transgenic mice and labeled with CFSE as mentioned above. All cells were co-cultured at a 1:1 ratio in CTL media for 3 days before evaluating CFSE dilution and cytokine production by FACS.

**Results**

*Galectin-3 KO mice have increased expression of inflammatory cytokines in vaccine draining nodes (VDNs) 4 days after receiving a GM-CSF vaccine*

The microarray results discussed in Chapter 3 comparing high avidity galectin-3 KO/KO CD8+ TILs with high avidity galectin-3 WT/WT CD8+ TILs revealed upregulation of inflammatory pathway genes associated with TNFα, IFNγ, and IL-6. Furthermore, increased expression of several inflammatory-associated molecules in galectin-3 WT CD8+ TILs adoptively transferred into galectin-3 deficient hosts indicates involvement of the host’s innate immune response in shaping the adaptive immune response. Previous work has demonstrated the importance of the cytokine milieu generated by the innate immune response in establishing an inflammatory or suppressive environment for CD8+ T cells (102).

To confirm these findings and to begin to identify any differences in the innate immune response to GM-CSF vaccination between galectin-3 KO and galectin-3 WT mice, we analyzed tissue lysates of VDNs for differences in cytokine expression 4 days after mice had received the GM-CSF vaccine. Four days post vaccination was chosen because this is the time point that has been shown to coincide with trafficking of dendritic cells into the draining lymph nodes (24). We found increases in TNFα and IL-1 in the
lysates of galectin-3 KO VDNs when compared with galectin-3 WT VDNs (Figure 17). IL-6 was not included in the array; however, IL-1 is also a pro-inflammatory cytokine, and is capable of increasing IL-6 production (103, 104).

*Inhibition of galectin-3 expression leads to an increase in plasmacytoid dendritic cells (pDC) in galectin-3 KO mice*

To begin to examine the impact of galectin-3 on cells of the innate immune response, we first looked broadly for differences in CD11b+ and CD11c+ cell populations in the lymph nodes of naïve galectin-3 KO and galectin-3 WT neu-N mice. Although not specific to any single cell population, these markers help to differentiate dendritic cells from macrophages and neutrophils. While we did not see significant differences in cells expressing CD11b+, we did note a large increase in CD11c+ cell types. Because CD11c+ is predominantly expressed on dendritic cells, we further characterized this population of cells for expression of B220 and Ly6C to distinguish between plasmacytoid dendritic cells (pDC), which express CD11c+ B220+ Ly6C+, and conventional dendritic cells (cDC), which express CD11c+ B220− Ly6C− (Figure 18). We observed a substantial increase in numbers of CD11c+ B220+ Ly6C+ cells in the galectin-3 KO versus the galectin-3 WT neu-N mice. Furthermore, the overall number of pDCs found in the lymph node did not seem to vary significantly from the naïve mouse in response to tumor challenge alone, or tumor challenge in addition to treatment with CY and whole cell GM-CSF vaccine (Figure 19A). This finding indicates that galectin-3 may instead play an intrinsic role in pDC homeostasis rather than solely during immune stimulation, as was the case for CD8+ T cells. To test this possibility, we compared pDC populations in
untreated female galectin-3 KO neu-N mice, untreated female galectin-3 WT FVB/N mice and untreated female galectin-3 WT neu-N mice. We found that pDCs were also present in galectin-3 WT FVB/N mice, which would suggest, not surprisingly, that other factors must also be involved in pDC regulation (Figure 19C).

A reciprocal relationship has been shown to exist between pDCs and myeloid derived suppressor cells (MDSCs). Ablation of pDCs with the depletion antibody 120G8 leads to increased myelopoiesis and mobilization of MDSCs into peripheral lymphoid organs, resulting in inhibition of autoimmunity (105). Because pDCs appear to be significantly elevated in the absence of galectin-3, we looked to see if there was a corresponding decrease in the number of MDSCs. In contrast to what was observed with pDCs in galectin-3 KO mice, the number of MDSCs found in the lymph nodes is minimal until immune activation with the whole cell GM-CSF vaccine. As expected, however, the number of MDSCs found in galectin-3 KO mice is significantly lower than what was observed in galectin-3 WT mice (Figure 19B).

Next, we determined whether or not pDCs directly activate T cells. Due to the known plasticity of pDCs, their role in antitumor immunity remains controversial. While pDCs are known to be involved in the promotion of inflammatory processes important for reversing microbial infections (106), they are also known to be immunosuppressive in the tumor microenvironment when galectin-3 is present (natural environment) (107-109).

To clarify the function of pDCs following a GM-CSF vaccination in the absence of galectin-3, we evaluated their capacity to induce proliferation of naïve high avidity CD8+ T cells in vitro using a direct ex-vivo antigen detection (DEAD) assay. Our data indicate that naïve T cells proliferate to a greater extent and produce more IFNγ when co-cultured
with both pDCs and conventional DCs than when co-cultured with either DC subtype alone. Further, co-culture of naïve T cells with just pDCs alone activated a significantly greater number of T cells than with cDCs alone (Figure 20A-C). These findings support a new role for pDCs in the promotion of an anticancer inflammatory response resulting in enhanced CD8⁺ T cell stimulation in vaccinated galectin-3 KO mice.

**Discussion**

In addition to galectin-3’s direct effect on T cells, several reports have also demonstrated galectin-3’s regulation of conventional dendritic cells, which would indirectly impact T cells by influencing the strength of their activation as well as the cytokine milieu they are activated in (59, 101, 110). We are the first to show a direct correlation between inhibition of galectin-3 expression, and the expansion of pDCs. Interestingly, LAG-3 has been reported to negatively regulate pDC homeostasis. Thus, steady state levels of extracellular galectin-3 may be necessary to regulate pDC expansion via LAG-3, without which, pDCs are allowed to proliferate unchecked. Galectin-3 has also been shown to be a negative regulator of monocyte differentiation into cDCs (101); therefore, it may be possible that a similar mechanism exists for pDC differentiation from its precursor. Further, because pDCs are either directly or indirectly involved with the homeostasis of both MDSC and Treg, dysregulation of pDCs may have downstream consequences on these cell types (105). Indeed, our findings that MDSCs proliferate significantly less in response to GM-CSF vaccination in galectin-3 KO mice are consistent with the notion of reciprocal regulation between pDCs and MDSCs.

The observation that pDCs are present in untreated galectin-3 WT FVB/N mice but are absent in untreated galectin-3 WT neu-N mice is puzzling. While these
observations would make sense if the *neu*-N mice had initially received vaccine or a tumor challenge to induce a state of tolerance that might suppress pDC expansion, these mice were untouched. It may be possible that a low level of inflammation may already exist due to the presence of microtumors in the *neu*-N mice, which could over time lead to a state of tolerance even before the mice are tumor challenged or receive a neu-specific vaccine. Expression of the MMTV promoter is increased during pregnancy and lactation, but they are also expressed during embryogenesis and development of the virgin mammary gland. Further, expression of the promoter is selective, but not specific, and is expressed in lungs, kidneys, salivary glands, seminal vesicles, T-cells, testes, and prostate (111).

The role of pDCs in tumor immunity remains unclear. While they are important for antiviral immunity, they have also largely been associated with tolerance induction, and poor prognosis in cancer. It may be possible that pDCs take on a stimulatory role in the absence of extracellular galectin-3. Our microarray finding that the expression of several inflammatory genes in T cells is increased as a result of galectin-3 deficiency in only host-derived cells may help to support this hypothesis. Although these results may be due simply to a reduction of surface galectin-3 on the T cell, a more compelling argument is that the expansion of host-derived pDCs in the absence of galectin-3 helps to skew the immune response toward inflammation. Galectin-3 binds preferentially to “self” glycans, and thus, has been theorized to provide a dampening mechanism that keeps the immune system at bay by raising the threshold of activation for immune cells (112). Further studies will be necessary to characterize differences in cytokine production and functionality of pDCs in the presence and absence of galectin-3.
Because pDCs are largely considered to be critical components of the innate immune response rather than the adaptive response, and furthermore, have demonstrated immunostimulatory capacities in the context of bacterial and viral infections, our data illustrates the importance of properly evoking both arms of the immune system in the appropriate context to combat cancerous cells. Indeed, cells associated with innate immunity such as neutrophils and macrophages have increasingly been shown to be protumorigenic by causing rampant inflammation when allowed to accumulate unchecked (113-115). This chronic inflammation in the TME then leads to development of an immunosuppressive environment that is unfavorable for immunotherapy. However, with further research, it may be possible to one day harness the inflammatory abilities of these cells in a more controlled manner.
Figure 17. TNFα and IL-1 are increased in whole tissue lysates of galectin-3 KO VDNs when compared with galectin-3 WT VDNs.

VDNs were isolated from mice 4 days after receiving GM-CSF vaccination, and processed according to materials and methods.
Figure 18. CD11c+ cells are increased in galectin-3 KO mice in comparison with galectin-3 WT mice.

Inguinal and axillary lymph nodes were dissected from naïve galectin-3 KO and galectin-3 WT neu-N mice. Lymph nodes were processed according to the materials and methods section. Cells were co-stained for CD11b and CD11c expression, and analyzed by FACS.
Figure 19. An increase in pDCs in galectin-3 KO neu-N mice is associated with lower numbers of MDSCs.

Galectin-3 KO and WT neu-N mice were left untreated (n=3), challenged with 5x10⁴ NT2.5 tumor cells (n=3), or challenged with 5x10⁴ NT2.5 tumor cells and treated with CY on day 2 and whole cell GM-CSF vaccine on day 3 (n=4). All mice were sacrificed on day 6, and axillary and inguinal lymph nodes were dissected and pooled for each mouse. Tissues were processed according to the Materials and Methods section, and cells stained for expression of CD11b, CD11c, B220, and Ly6C. The total number of cells was averaged for each lymph node, and is shown for A) pDCs (CD11c⁺ B220⁺ Ly6C⁺) and for B) MDSCs (CD11b⁺ Ly6C⁺). C) pDC populations in untreated, naïve 8-10 week old female galectin-3 KO neu-N, galectin-3 WT FVB/N, and galectin-3 WT neu-N. Cells are gated on B220⁺.
A) Plasmacytoid Dendritic Cells

<table>
<thead>
<tr>
<th>Condition</th>
<th># of cells / lymph node</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galectin-3 KO naive</td>
<td>p=0.0006</td>
</tr>
<tr>
<td>Galectin-3 WT naive only</td>
<td>p=0.0001</td>
</tr>
<tr>
<td>Galectin-3 KO tumor only</td>
<td>p=0.0008</td>
</tr>
<tr>
<td>Galectin-3 WT tumor + CY + vaccine</td>
<td></td>
</tr>
<tr>
<td>Galectin-3 WT tumor + CY + vaccine</td>
<td></td>
</tr>
</tbody>
</table>

B) MDSC

<table>
<thead>
<tr>
<th>Condition</th>
<th># of cells / lymph node</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galectin-3 KO naive</td>
<td>p=0.0507</td>
</tr>
<tr>
<td>Galectin-3 WT naive only</td>
<td>p=0.0201</td>
</tr>
<tr>
<td>Galectin-3 KO tumor only</td>
<td></td>
</tr>
<tr>
<td>Galectin-3 WT tumor + CY + vaccine</td>
<td></td>
</tr>
<tr>
<td>Galectin-3 WT tumor + CY + vaccine</td>
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C) Galectin-3 KO neu-N

<table>
<thead>
<tr>
<th>Condition</th>
<th>Ly6C</th>
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<tr>
<td>Galectin-3 KO neu-N</td>
<td>0.24%</td>
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Galectin-3 WT FVB/N

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<td>Galectin-3 WT FVB/N</td>
<td>0.31%</td>
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Galectin-3 WT neu-N

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<th>Condition</th>
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</thead>
<tbody>
<tr>
<td>Galectin-3 WT neu-N</td>
<td>0.00372%</td>
</tr>
</tbody>
</table>
Figure 20. Plasmacytoid DCs from vaccinated galectin-3 KO mice are capable of activating more antigen-specific naïve CD8+ T-cells than conventional dendritic cells from vaccinated galectin-3 KO mice.

pDCs, cDCs, and high avidity Thy1.2+ CD8+ T cells were isolated according to the procedures listed in the Materials and Methods section. Cells were co-cultured at a 1:1 ratio as specified in the figure prior to being assessed for either A) CFSE dilution or B) IFNγ production by flow cytometry. Cytokine expression was assessed by intracellular cytokine staining (ICS) after a 5 hour incubation with a TAP-2 deficient cell line (T2-D9) expressing MHC I D9 loaded with RNEU420-429 (P50). C) Statistical comparison of cell division in T cells + pDC vs. T cells + pDC + cDC.
A) T cells alone  T cells + cDC  T cells + pDC  T cells + cDC + pDC

B) T cells alone  T cells + cDC  T cells + pDC  T cells + cDC + pDC

C) % of CD8 T cells divided

p = 0.0032
CHAPTER 5: SUMMARY

Development of an anti-tumor immune response is a constant balance between pro-inflammatory and anti-inflammatory signals. Activation of tumor-specific CD8\(^+\) T cells involves a complex interplay amongst not only stimulatory and inhibitory receptors expressed by the CD8\(^+\) T cell during priming, but also dendritic cells, CD4\(^+\) T cells, and the cytokine milieu that surrounds them. The complex signals that are involved in T cell activation are commonly simplified into 3 signals. Signal 1 refers to the TCR-MHCI-peptide interaction. Signal 2 refers to the interaction between stimulatory and inhibitory co-receptors, such as CD28, PD-1, and LAG-3, with their ligands. Signal 3 refers to the cytokine milieu in which T cell priming occurs. The cytokines IL-2, TNF\(\alpha\), and IFN\(\gamma\) are considered “inflammatory cytokines” often associated with T cell activation while IL-4, IL-10, and TGF\(\beta\) are “anti-inflammatory cytokines” associated with suppression. All three of these signals are in some way or another influenced by the degree and structure of sugar chains, or glycosylation, attached to the cell surface receptors that mediate these “signals”. Glycosylation of cell surface proteins occurs to such an extent that it forms a thick coat known as the glycocalyx, which is responsible for many of the cell’s “social” functions. In this thesis, we have explored the intricate role that galectin-3 plays in modulating the immune response from its impact on CD8\(^+\) T cell function to its global role in establishing a pro-inflammatory environment.

In Chapter 2, we described elevated anti-galectin-3 antibody titers in response to GM-CSF vaccination in PDA patients with > 3 year DFS when compared with PDA patients with < 3 year DFS. Using serum collected from the > 3 year DFS patients, we found that purified IgG from post-vaccination serum but not pre-vaccination serum could
neutralize galectin-3 inhibition of CD8\(^+\) T cells \textit{in vitro}. Future work should now focus on developing effective vaccination strategies targeting galectin-3. Galectin-3 can exist in multiple conformations, and neutralizing antibodies \textit{in vitro} may not necessarily have the same effect \textit{in vivo}. Furthermore, considering the role galectin-3 has been demonstrated to play in regulating the innate arm of the immune response, it would be worth evaluating the effectiveness of galectin-3 targeted therapy even prior to delivery of the GM-CSF vaccine. Last, it would be interesting to elucidate what allowed these patients to break tolerance against a secreted, and ubiquitously expressed protein like galectin-3. Given its involvement in wound healing and inflammation, it is highly unlikely that the whole-cell GM-CSF vaccine was the first time that the patient’s immune system was introduced to galectin-3 in the context of a “danger” signal.

In Chapter 3, we evaluated the effect of galectin-3 on tumor-specific high avidity CD8\(^+\) T cells in the tolerogenic \textit{neu-N} mouse model of breast cancer. We identified several new findings. First, galectin-3 appears to bind selectively to terminally differentiated, activated CD8\(^+\) T cells, and only in the TME. Second, galectin-3 can be secreted from antigen-experienced CD8\(^+\) T cells in an autocrine fashion, and it is also secreted by stromal cells in the TME. Third, lack of surface galetin-3 is associated with improved CD8\(^+\) T cell effector function, tumor free survival, and upregulation of inflammatory pathway molecules. Fourth, sialylation blocks galectin-3 binding, and trafficking of CD8\(^+\) T cells into the TME results in glycan remodeling and removal of \(\alpha_2\)-6-sialic acid. Lastly, \textit{in vitro} inhibition assays with galectin-3 identified LAG-3 as a potential binding partner for galectin-3, which was verified by co-immunoprecipitation studies of a LAG-3/galectin-3 complex. Microarray comparisons of galectin-3\textsuperscript{hi} and
galectin-3\textsuperscript{lo} also identified CEACAM1(CD66) as a potential binding partner of galectin-3. Flow cytometry also confirmed these findings, and demonstrated a strong correlation between CD66 expression and galectin-3 surface binding. While functional studies assessing ZAP-70 dephosphorylation as a potential mechanism were unrevealing, experiments were incorrectly performed using mouse cells, and should have been done using human cells due to reasons that were discussed in Chapter 3. From our data, it is clear that galectin-3 has an extensive impact on CD8\textsuperscript{+} T cell function. Whether or not it exerts its effects directly by cross-linking cell surface glycoproteins or indirectly through its effect on other cell populations remains controversial. Future studies should now focus on demonstrating inhibition of CD8\textsuperscript{+} T cells by galectin-3 via cross-linking of glycoproteins \textit{in vivo}, and verifying the LAG-3/galectin-3 interaction \textit{in vivo} as well.

In Chapter 4, we examined the effect of galectin-3 deficiency on tipping the balance between pro-inflammatory signals and anti-inflammatory signals. As previously described, galectin-3 is thought to raise the threshold of immune cell activation by sequestering surface receptors from interacting with their ligands. Microarray data in Chapter 3 revealed upregulation of inflammatory pathways involving TNF\textgreek{a}, IFN\textgreek{y}, and IL-6 in galectin-3 KO/KO CD8\textsuperscript{+} TILs versus galectin-3 WT/WT CD8\textsuperscript{+} TILs. We confirmed this using western blotting of cytokine arrays using whole tissue lysates of VDNs 4 days after GM-CSF vaccination, and found increased expression of TNF\textgreek{a} and IL-1. Because cells of the innate immune response are often the first to establish the cytokine milieu, we compared the neutrophil and monocyte populations in lymph nodes of untreated galectin-3 WT \textit{neu}-N and galectin-3 KO \textit{neu}-N mice by assessing differences in CD11b\textsuperscript{+} and CD11c\textsuperscript{+} populations by flow cytometry. We found that pDCs
were significantly increased in galectin-3 KO mice, and that these cells were superior to cDCs at activating CD8+ T cells. Because pDCs are also present in galectin-3 WT FVB/N mice at the same level but are completely absent in neu-N mice, expression of the neu-N oncogene must be exerting an effect on pDCs even in virgin mice. Low level expression of the neu-N oncogene in virgin mice, and development of low grade inflammation resulting from immunosurveillance of dysplastic cells may initiate the development of tolerance even at a very young age. Thus, future work should now focus on inflammation as it relates to tumorigenesis, and how galectin-3 mediates this process. The Pdx-1-Cre, LSL-Trp53R172H/−, LSL-KrasG12D/− (KPC) mouse model of PDA development is ideal for these experiments. These mice contain a pancreatic tissue-specific knock-in allele of activated KRasG12D mutant as well as a knock-in allele of p53R172H mutant. These mice recapitulate the progression of PanIN lesions to PDA observed in human patients, but at an accelerated rate. We have already crossed these mice onto a galectin-3 KO background.

In summary, these findings develop for the first time a comprehensive profile of the extensive impact that galectin-3 plays at every stage of an anti-tumor immune response in a tolerogenic mouse model. To our knowledge, we are the first to develop such a model in a galectin-3 deficient background, which provided us with the unique ability to evaluate in vivo the role of galectin-3 after immune priming with a whole-cell GM-CSF secreting cancer vaccine in a mouse model that accurately recapitulates the tolerance mechanisms of human patients. We propose that patients who develop anti-galectin-3 antibody titers in response to vaccination are able to neutralize galectin-3’s immunosuppressive effects either directly or by disruption of galectin-3/receptor lattices,
and as a result, mount a more effective cytotoxic CD8\(^+\) T cell response against tumor cells. TCR engagement, co-receptor activation, and the cytokine milieu have long been considered the three signals that determine T cell fate. However, consideration of protein glycosylation as a “Signal 4” may now help to uncover a new class of molecules for immunomodulation (Figure 21).
Figure 21. N-glycosylation of co-regulatory molecules serves as the signal 4 required for optimal T cell activation.

In the vaccine draining node (VDN) where naïve T cell priming occurs, activating signals are delivered via TCR recognition of peptide-MHC I complexes presented by dendritic cells (Signal 1) and interactions between co-stimulatory molecules and their ligands (Signal 2). Furthermore, cytokines secreted by regulatory cell populations also impact cellular differentiation (Signal 3). Plasmacytoid dendritic cells (pDCs) are increased in number in the absence of extracellular galectin-3, and the cytokines they secrete may skew T cell differentiation in the direction of a more pro-inflammatory phenotype.

Activation of CD8 T cells leads to increased expression of the co-regulatory molecules PD-1 and LAG-3 by terminally differentiated effector CD8 T cells. In the tumor microenvironment, expression of PD-L1 by tumor cells can suppress T cell function via PD-1. Changes in glycosylation of LAG-3 (Signal 4) make it a more favorable target for galectin-3 binding, which in turn leads to cross-linking and activation of the LAG-3 signaling complex. Cross-linking of LAG-3 results in suppression of effector CD8 T cell function. Lack of galectin-3 increases S100A8/9 expression, which may also lead to optimization of lytic function.
APPENDICES

Appendix A

See file Table 2.xls in supplemental files.
LIST OF REFERENCES


Histopathology 56, 560-572 (2010); published online EpubApr (10.1111/j.1365-2559.2010.03494.x).


44. S. Song, N. Mazurek, C. Liu, Y. Sun, Q. Q. Ding, K. Liu, M. C. Hung, R. S. Bresalier, Galectin-3 mediates nuclear beta-catenin accumulation and Wnt


50. J. R. Newton-Northup, M. T. Dickerson, L. Ma, C. L. Besch-Williford, S. L. Deutscher, Inhibition of metastatic tumor formation in vivo by a bacteriophage


Cancer Res **70**, 7476-7488 (2010); published online EpubOct 1 (10.1158/0008-5472.CAN-10-0761).


71. E. Baixeras, B. Huard, C. Miossec, S. Jitsukawa, M. Martin, T. Hercend, C. Auffray, F. Triebel, D. Piatier-Tonneau, Characterization of the lymphocyte


88. B. B. Singer, I. Scheffrahn, R. Heymann, K. Sigmundsson, R. Kammerer, B. Obrink, Carcinoembryonic antigen-related cell adhesion molecule 1 expression and signaling in human, mouse, and rat leukocytes: evidence for replacement of the short cytoplasmic domain isoform by glycosylphosphatidylinositol-linked


94. S. Sato, N. Ouellet, I. Pelletier, M. Simard, A. Rancourt, M. G. Bergeron, Role of galectin-3 as an adhesion molecule for neutrophil extravasation during


97. A. Karlsson, P. Follin, H. Leffler, C. Dahlgren, Galectin-3 activates the NADPH-oxidase in exudated but not peripheral blood neutrophils. *Blood* **91**, 3430-3438 (1998); published online EpubMay 1 (  


104. G. Tosato, K. D. Jones, Interleukin-1 induces interleukin-6 production in peripheral blood monocytes. *Blood* **75**, 1305-1310 (1990); published online EpubMar 15 (  


cells are crucial for the initiation of inflammation and T cell immunity in vivo. *Immunity* **35**, 958-971 (2011); published online EpubDec 23 (10.1016/j.immuni.2011.10.014).


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