

January 2018

Lag-3 Mediates Acute Rejection & Memory In Mouse Transplantation

Jeffrey Mark Erfe

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LAG-3 MEDIATES ACUTE REJECTION & MEMORY IN MOUSE
TRANSPLANTATION

A Thesis Submitted to the
Yale University School of Medicine
In Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

By
Jeffrey Mark Erfe, MPH

2018

LAG-3 MEDIATES ACUTE REJECTION & MEMORY IN MOUSE TRANSPLANTATION

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ABSTRACT: Lymphocyte Activation Gene-3 (LAG-3) is a soluble protein and transmembrane protein receptor expressed on lymphocytes such as immunosuppressive regulatory T cells. Our goal was to evaluate the effect of LAG-3 on transplantation outcomes, specifically rejection and cellular memory response to donor antigen, by comparing graft survival and IFN- λ secretion to donor antigen in C57BL/6 LAG-3^{-/-} versus wild-type mouse recipients of DBA/2 grafts. We found that LAG-3 deletion accelerates rejection time and enhances IFN- λ secretion among heart or skin graft recipients. FACS analysis of memory T cells demonstrated disproportionate increases in effector T cell subsets, consistent with a heightened rejection response. Although the absence of LAG-3 enhanced rejection of heart and skin grafts, it did not abrogate tolerance of spontaneously accepted kidney allografts. To further understand the mechanism of LAG-3 signaling and the potential importance of dendritic cells, we cultured donor dendritic cells in a tolerogenic milieu with recipient T cells and found increased PD-1 and IL-10 expression among T cells. Lastly, we performed soluble LAG-3 injections and adoptive transfers of LAG-3^{+/+} cells into knock-out graft recipients. This demonstrated that the presence of LAG-3 on T cells is critical for mediation of rejection, while LAG-3 on dendritic cells downregulates donor-specific IFN- λ secretion. Our data suggest that in addition to LAG-3's effects on proliferation and activation, LAG-3 may also affect differentiation of precursor CD4⁺ T cells. Additionally, these data indicate the importance of dendritic cell-mediated control of the memory response in a LAG-3-dependent manner.

ACKNOWLEDGEMENTS

I would like to thank the numerous CTS faculty and staff whose instruction, particularly in a variety of surgical and laboratory methods, was essential for the completion of these projects. These data could not have been acquired without their guidance and help. I would in particular like to thank Alessandro Alessandrini and Joren Madsen for their scientific and professional mentorship. I would like to thank everyone at the Sarnoff Foundation, which funded my research and provided numerous role models for a career as an academic physician. In particular, I want to thank my Scientific Advisor James De Lemos and my Yale Sponsor Lauren Cohn. I would also like to thank my Yale Faculty Advisor, George Tellides, for his insights. Last but not least, I would like to thank my family, particularly my daughter Indra and my wife Betty, who has always provided strong support of my scientific interest in transplantation immunology.

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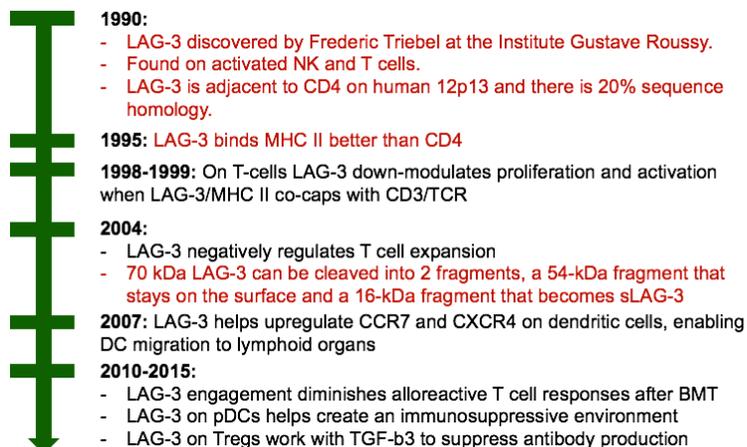
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Introduction

Heart transplantation has become a mainstay treatment for patients with severe end-stage heart failure despite maximum medical therapy. Since 1967, one-year survival rates have jumped from 30% to 85-90%, and current three-year survival approaches 75% (1, 2). A significant proportion of late mortality after the first year is attributable to the usage of broadly immunosuppressive drugs that enable systemic infections and cancers such as post-transplant lymphoproliferative disease (PTLD) or non-Hodgkin lymphoma (2). What is needed are new therapies that allow for the targeted induction, proliferation or activation of narrow cellular and non-cellular immune system elements that specifically enhance allograft protection while preserving systemic host defenses against infections and cancers. One possible solution involves the therapeutic alteration or introduction of glycoprotein moieties to increase the temporally- and locationally-specific proliferation or activation of regulatory cells. If donor allografts can be modified to express immunosuppressive glycoprotein moieties, it could downregulate local immune reactions while preserving systemic immune responses.

A. LAG-3 Structure & Function

Fig. 1: A History of LAG-3

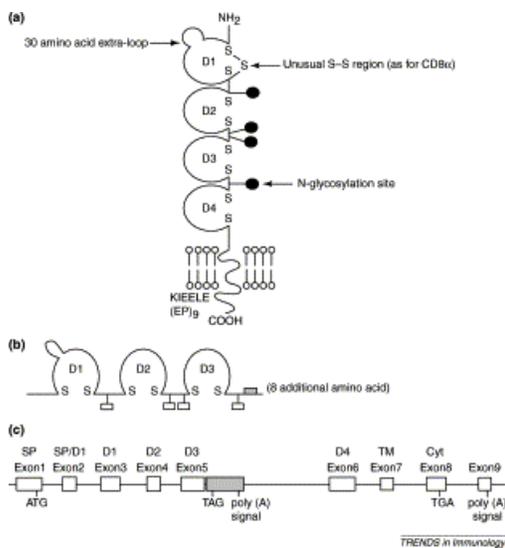


Discovered in 1990, LAG-3 is primarily known as a Type I cell-surface membrane receptor and on human chromosome 12p13 is adjacent to CD4, a gene with which it possesses 20% sequence homology (3). It is believed that LAG-3 and CD4 may have resulted from a gene duplication and likely share common regulatory elements (4). LAG-3 binds with greater avidity to the MHC-TCR complex, suggesting that it may block CD4 pathways and therefore full T cell activation (4, 5). Little is known about the direct intracellular effects of LAG-3, though the original paper wherein the protein was described demonstrated that LAG-3 modifies extracellular calcium influx in a CD3/TCR-dependent manner (6).

LAG-3 has at least one other splicing variant, a soluble protein (5).

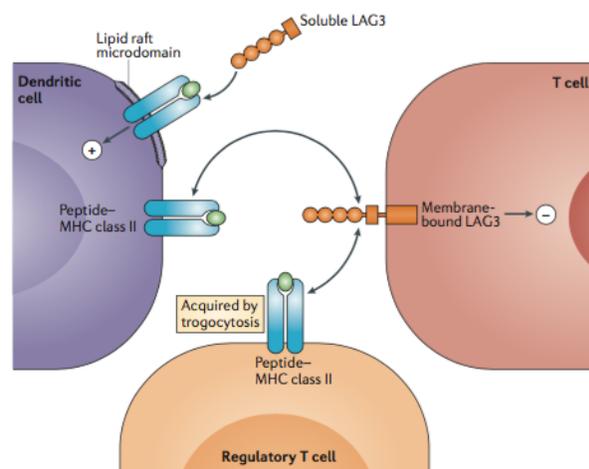
Transcriptional control governs whether LAG-3 is trafficked to the cell-surface as a receptor or extruded into the serum. Depicted below (Figs. 2, 3) is the structure of the protein, which is comprised of nine exons, including two stop codons.

Fig. 2: LAG-3 Structure



Trends in Immunology
Treibel, 2003

Fig. 3: LAG-3 Soluble vs. Membrane-Bound



Nature Review Immunology
Nguyen & Ohashi, 2015

Signaling through the cell-surface isoform occurs on effector T cells after binding MHC II, which negatively regulates T cell function as shown above (5). T cells signal downstream via cytoplasmic KIEELE motifs, resulting in decreased cellular proliferation, cytokine inhibition, and reduced cytotoxicity, but the intermediary pathway steps have not yet been described (7). Some DCs are also known to activate after binding LAG-3, either as a receptor or a soluble protein, using lipid raft microdomains. It has also been theorized that in addition to interacting with MHC II molecules on DCs, LAG-3 can bind MHC II that has been acquired by regulatory T cells via trogocytosis (5). LAG-3-MHC II interactions may be analogous in some ways to CD40L-CD40 interactions. Both CD40L and CD40 are necessary for IL-12 and IFN- γ production in antigen-presenting cells *in vitro* and upregulate LAG-3. Similarly, soluble LAG-3 can directly induce DCs to produce Th1 cytokines and chemokines, such as CCL22 and CCL17, which helps direct migration of maturing DCs to lymph nodes (3). LAG-3 may also modulate differentiation of DCs from monocyte precursors (8).

B. Tr1 Regulatory Cells & Immunosuppression

While FOXP3⁺ regulatory T cells (Tregs) are the quintessential regulatory cells, other immunosuppressive cell types exist, such as FOXP3⁻ Tr1 cells generated from CD4⁺ memory T cells (9). LAG-3 has gained attention in the field of transplant immunology in part due to its presence on Tr1 cells, which may be beneficial in dampening the post-transplant immune response. Tr1 cells play an important role in reducing autoimmune colitis and encephalomyelitis in mice and in controlling reactive arthritis and multiple sclerosis in humans (10-13). Moreover, Tr1 cells may fill

immunological niches not served by Tregs. In contrast to FOXP3⁺ Tregs, Tr1 cells suppress NLRP3 inflammasome activation via an IL-10-dependent mechanism (14). It has also been proposed that while natural Tregs are critical early in an immune response for controlling the magnitude of inflammation, Tr1 cells become important later for maintaining tolerance (11). The strong potential for Tr1 cells to serve as an immunomodulatory clinical treatment relates to their higher propensity for inducing local tolerance against non-self antigens in the periphery, unlike thymus-derived or some peripherally-derived regulatory T cells (15). This characteristic makes Tr1 cells natural candidates for controlling inflammation against allogeneic transplants. In fact, recent studies demonstrate that antigen-specific Tr1 cells are critical for: 1) restoring insulin production in patients with Type 1 diabetes, 2) facilitating pancreatic islet transplant tolerance in PTPN22 deficiency, 3) promoting tolerance to mismatched HLA stem-cell transplants in SCID patients, and 4) preventing mouse skin allograft rejection (12, 15).

Tr1 cells in the periphery have long been characterized as CD4⁺FOXP3⁺CD226⁺ and by a cytokine production profile of IL-10⁺, IL-4⁺, TGF- β ⁺, IL-5⁺, IL-2^{low/+}, IFN- γ ^{+/-} (9, 15-17). These markers, however, were too broad to efficiently track Tr1 lineage and movement. In a 2013 *Nature* paper, Gagliani *et al.* used differential gene expression in human blood and immunohistochemical profiles of murine gut isolates to streamline the identification of Tr1 cells as IL-10-secreting cells that co-express CD49b and LAG-3 (9). LAG-3 can be expressed as either a transmembrane protein that downregulates TCR-mediated signal transduction in human and mouse lymphocytes or as a soluble molecule that activates dendritic cells (DCs) and enhances antigen-specific T cell responses (15). Interestingly, exogenously-induced expression on Tregs of LAG-3, like Tr1 induction,

requires IL-27 (18). The role of LAG-3 on Tr1 cells has not yet been specified, however. Moreover, its intracellular pathways have not fully been elucidated, apart from a description of its cytoplasmic tail receptors (19).

Tr1s generated *in vitro* using IL-27 and TGF- β co-expressed CD49b and LAG-3 for up to 12 days in culture and up to 13 days after *in vivo* transfer (9). Markers of Tr1 activation include CD28, CD69, CTLA4, CD25, IL-2R $\beta\gamma$, CD40L, and HLA-DR (11, 15). Tr1 cells can express FOXP3 but only transiently (15). Interestingly, CD4⁺CD49b⁺LAG-3⁺ differentially express certain receptors (low *epidermal growth-factor receptor 2 (Egr2)* but high *Aryl hydrocarbon receptor (Ahr)*), suggesting a different process of IL-10 production from that principally used by Tregs (9). Tr1 cells express AhR and require c-Maf, IL-21, and ICOS for IL-27-dependent activation (12, 15, 20, 21). AhR binds c-Maf in Tr1 cells and enhances transcription of IL-10 and IL-21, secretion of which is important in Tr1 induction as it maintains C-Maf expression through a feed-forward transcription loop (12). IL-6 has also been implicated in driving expression of c-Maf and AhR, as well as IRF-4, another critical transcription factor for IL-10 secretion and Tr1 differentiation, via a STAT3 promoter pathway (15, 22).

C. Galectin Signaling & Tr1 Cells

Galectins are a family of broadly-expressed mammalian carbohydrate-binding proteins defined by a common β -sandwich structure; they function in multiple compartments to regulate immune responses by binding glycan ligands, particularly β -galactosides (23, 24). It is plausible that variations in glycosylation patterns affect the

binding affinity of galectins, which control downstream transcription factors that alter the likelihood of transplant acceptance.

Galectin-3 (Gal-3) modulates TCR activation threshold on naïve T cells and galectin-1 (Gal-1) influences TCR signaling in developing thymocytes by altering negative selection via the ERK pathway (25). Of all known galectins, Gal-1 also has the strongest evidence for activating and proliferating Tr1 cells by binding CD45 so that TCR signaling is muted. This may be due to the prevention of Lck phosphorylation, allowing Tr1 induction via an intracellular mechanism that involves IL-27. However further clarification is required (26).

Interestingly, LAG-3, the transmembrane receptor that along with CD49b defines Tr1 cells, has been shown to bind galectin-3 *in vivo* to induce immunosuppression by blocking IFN- γ production and CD8⁺ cytotoxicity (27, 28). If LAG-3 serves a similar function on Tr1s as it does on T effectors, then it is plausible that LAG-3 on Tr1 cells may be used to selectively trigger Tr1-mediated graft protection. If true, this would suggest that existing rates of cardiac allograft tolerance can be further improved using galectins and possibly custom-designed therapeutics that mimic glycoproteins structures necessary for activating LAG-3 receptors.

D. *Accepting and Rejecting Mouse Allograft Models*

Because of its relatively short lifespan, the variety of MHC combinations that can be tested across strains, and the wide availability of commercially-available genetic knockouts, the mouse is a suitable model organism to measure the effects of donor-host incompatibility. It has been shown that the rejection pattern and timing can differ

markedly both across strain combinations and organ types (29). The particular MHC combination used in this study, H-2^b to H-2^d, has been reported to exhibit an average survival of 9.6 ± 0.4 days for cardiac allografts for C57BL/6 to BALB/c (29). In general, major allele differences across various strain combinations cause rejection in 7-10 days (30-32). For the purposes of this study, the heart and skin models are referred to as “rejection models” because without host immunosuppression they ultimately result in graft failure. In contrast, in some strain combinations of donors and recipients, kidney allografts do not undergo failure, even after an extended time period. For instance, DBA/2 (H-2^d) kidneys transplanted into C57BL/6 (H-2^b) recipients have been reported to maintain their function beyond 60 days, for unknown reasons (33). In this study, kidney allografts between these mouse strains are referred to as an “accepting model” because they do not typically reject in the long-term even without host immunosuppression.

Statement of Purpose

Though LAG-3 has been investigated in cancer and infectious diseases research, its role or lack thereof in transplantation needs to be clarified. Specifically, our main objective is to determine whether LAG-3 simply identifies Tr1 cells or whether it plays a functional role in mediating rejection or graft-protection. If LAG-3 does modulate rejection, it is important to investigate its effects in both the short-term (acute rejection) versus the long-term (immunologic memory). Additionally, characterization of LAG-3's disparate effects, if any, on a rejecting model of heart or skin allografts versus a spontaneously accepting model of kidney allograft is important. Another objective is to assess whether Gal-3, insofar as it has been reported to be a ligand for LAG-3, modulates

the function of LAG-3, particularly with respect to the expression of receptors theorized to be important for graft-protection, such as Foxp3 and IL-10. Finally, if LAG-3 deletion demonstrates a measurable transplant effect, it will be important to discriminate between effects mediated via its soluble form or its membrane-bound form (including its corresponding cell type). We therefore propose the following research aims, hypotheses, and methods:

Table 1: Research Aims, Hypotheses, & Methods

Research Aim	Hypothesis	Method(s)
1. To assess whether LAG-3 levels influence acute allograft rejection	LAG-3 mediates allograft rejection	A. Murine heart and skin transplantations B. Histologic examination
2. To assess whether LAG-3 is important for immunologic T cell memory	LAG-3 mediates T cell memory development	C. Murine heart and skin transplantations D. Splenocyte isolation and ELISPOT for IFN- γ secretion to donor antigen
3. To assess whether the function of LAG-3 differs between accepting and rejecting models	LAG-3 may function differently between model types	E. Murine heart and skin transplantations F. Murine kidney transplantations
4. To assess whether activation of LAG-3, via Gal-3, affects the	Gal-3 enhances the effects of LAG-3 on graft-	A. Co-culture of T cells and DCs B. Assessment of IL-10 secretion G. FACS assessment of Foxp3, PD-1, IL-10 expression

expression of Foxp3, PD-1, and IL-10	protective receptor expression	
5. To assess which form of LAG-3 (receptor or soluble protein) is most important for transplantation	LAG-3 surface receptor, due to its downstream effects on T cell effectors, is more likely to affect transplant outcomes than soluble LAG-3	A. Adoptive transfer with LAG-3-competent T-cells or DCs B. Pre-treatment with soluble LAG-3 C. Murine heart and skin transplantations

Methods

Bolded initials denote which author performed which methods.

Skin, heart, and kidney transplants

Transplants were performed between allogeneic DBA/2 (H-2^d) donors and C57BL/6 (H-2^b) recipients as well as DBA/2 donors and LAG-3^{-/-} recipients on a C57BL/6 background. In some cases, experimental controls consisted of identical surgeries performed between syngeneic donor and recipient (C57BL/6 to C57BL/6). All mice were purchased from Jackson Labs. Shaved skin was harvested from the right dorsum of donors between the forearm and the hindleg and transplanted within a few hours to the same location on the recipient (**JME**). Donor hearts were transplanted heterotopically in the abdomen by dissecting out the mesentery to the right of the midline and anastomosing the donor aorta and pulmonary vein to the recipient aorta and inferior

vena cava, respectively (CY). Donor kidneys were transplanted into the peritoneal cavity near the midline after complete resection of both recipient kidneys, followed by attachment of the donor ureter to the recipient bladder (CY). Surgical tools were autoclaved prior to use, and donors and recipients were operated on using different sets of sterile tools (JME).

Graft survival assessment

Survival of skin grafts was assessed via visual inspection for evidence of desiccation and necrosis between 8-15 days post-transplant (JME). Heart grafts were assessed via abdominal palpation (JME). Grafts were harvested (JME) from different sets of mice at weeks 1 and 2 stained with H&E for pathologic analysis (RW, IR). Kidney grafts were evaluated by measuring spectrophotometrically the BUN levels of sera collected from cheek veins (JME). Grafts were categorized as rejected if BUN exceeded 100 mg/dL (JME).

Adoptive transfer & cytokine pre-treatment

In some experiments, T cells were isolated from spleens and bone marrow collected using mechanical separation and chemical isolation (Stem Cell Tech) from WT C57BL/6 animals (JME, DN). DCs were isolated from both spleens and bone marrow using the same kits (JME). Cells were injected on the same day into tail veins of either WT or LAG-3^{-/-} animals, one week prior to skin engraftment (JME). Other animals received soluble LAG-3 (sLAG-3, R&D Systems) injections via peritoneal injection one week prior to graft placement (JME).

ELISPOT assay

ELISPOT was performed using PVDF 96-well plates pre-washed with 70% ethanol, washed three times with PBS, then coated with IFN- γ primary antibody diluted 1:100 in PBS for at least 4 hours at room temperature (**JME, DN**). Isolated cells were seeded at 100,000 cells/well (**JME**), along with irradiated 250,000-cell aliquots from DBA/2, C57BL/6 or C3H mouse spleens (**DN**). Cells were cultured overnight at 37C in a CO₂ incubator (**JME**). After 24 hours, plates were washed three times with PBS 0.1% Tween 20 (**JME**). Each well received 100 μ L of biotinylated IFN- γ secondary antibody diluted 1:100 in PBS for 2 hours at room temperature (**JME**). After washing, wells were incubated with streptavidin-horseradish peroxidase (diluted 1:5000) for 1 hour at 37C (**JME**). Plates were washed with hydrogen peroxide substrate for 7-10 minutes at room temperature and dried 24-48 hours (**JME**). Spots were counted using an automated protocol in ImmunoSpot Suite 5.0 (**JME, AA**).

Plasmacytoid dendritic and T cell co-cultures

Splenic and bone marrow lymphocytes were isolated from DBA/2, C57BL/6, or LAG-3^{-/-} C57BL/6 mice using gentle mechanical techniques followed by red cell lysis buffer (**JME, DN**). Cells were resuspended in RPMI and processed using kits for pan-T or helper T cell isolation or DC purification (Stem Cell Tech) (**JME**). Cell cultures were seeded in triplicates on 96-well plates at 50,000 cells/well for DCs and 150,000 cells/well, first with T cells and irradiated DBA/2 splenocytes (for antigen presentation)

for 24-72 hours in complete growth medium (RPMI, FCS, streptomycin, penicillin, sodium pyruvate, and non-essential amino acids) at 37 C with 5% CO₂ (**JME**). Some wells were later combined with DCs after 72 hours, concurrently with the addition of combinations of various cytokines (Stem Cell Tech, R&D Systems) for an additional 120 hours in these concentrations: IL-15 (100 ng/mL), IL-27 (200 ng/mL), TGF- β (5 ng/mL), Galectin-1 (100 ng/mL), Galectin-3 (200 ng/mL) (**JME**). Cell media was changed every 24 hours for the first 72 hours, and then every 48 hours for the final 120 hours (**JME**). Cell isolates were analyzed for IL-10 secretion after 5 hours stimulation using PMA, Ionomycin, and Brefelden A (**JME**).

Flow Cytometric Staining & Analysis

Cellular staining was conducted with dyes stored at 4C with minimal light exposure. Prior to extracellular staining, mouse cells undergoing intracellular staining as well were pre-incubated with 1 ug of anti-mouse CD16/CD32 diluted in 100 uL of FACS buffer (PBS, 0.5% BSA, 0.1% sodium azide, DI water) for 15 minutes at 4C (**JME**). Cell isolates from organ separation and culture wells were transferred in aliquots of at least 50,000 cells in 1 mL of FACS buffer into 5 mL tubes and were washed at least twice using serial centrifugation at 1300 RPM for 8 minutes (**JME, DN**). Extracellular stains were diluted in aliquots of 1:100 in FACS buffer, and mixed with cells for 30 minutes at 4C with minimal light exposure. Isolates undergoing intracellular (nuclear) staining were then washed in 500 uL of fixation/permeabilization solution (eBioscience), then incubated for an additional 60 minutes at room temperature, with minimal light exposure (**JME**). Samples were washed twice in 1 mL of permeabilization buffer

(eBioscience) with centrifugation at 1300 RPM for 8 minutes at 4 C in between washes (JME). After resuspension in buffer, cells were stained for 30 minutes at room temperature with minimal light exposure using 1:100 dye aliquots (JME). Cells were rewashed twice in permeabilization buffer, followed by resuspension in FACS buffer prior to analysis on a 3-laser 8-color BD FACSVerser Analyzer (JME). Samples were stored as necessary prior to analysis for up to 48 hours in 10% paraformaldehyde (JME). Sample gating was conducted in FlowJo v8 (JME, AA).

Statistical Analysis

Kaplan-Meier analyses and Mann-Whitney U Tests were conducted in GraphPad Prism v7.0 (JME).

Results

Conclusion I: LAG-3 potentiates allograft survival and inhibits T cell memory generation in transplant rejection models

Transplant outcomes were compared between DBA/2 donor organs placed either with C57BL/6 or LAG-3^{-/-} C57BL/6 recipients (Figs. 4, 5). Heart grafts survived for an average of 7 days in LAG-3^{-/-} recipients (n=4) versus an average of 9 days in wild-type C57BL/6 (WT) recipients (n=3), a difference that was statistically significant via log-rank testing (P=0.010). Skin grafts survived for an average of 10 days on LAG-3^{-/-} recipients (n=4) while skin on WT animals (n=4) survived for 13 days on average, a difference that was statistically significant (P=0.009).

Fig. 4: Heart Life Span

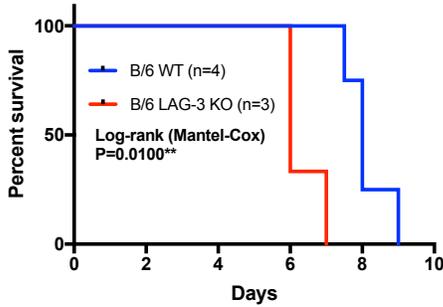
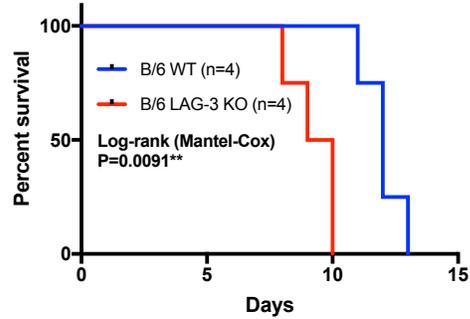
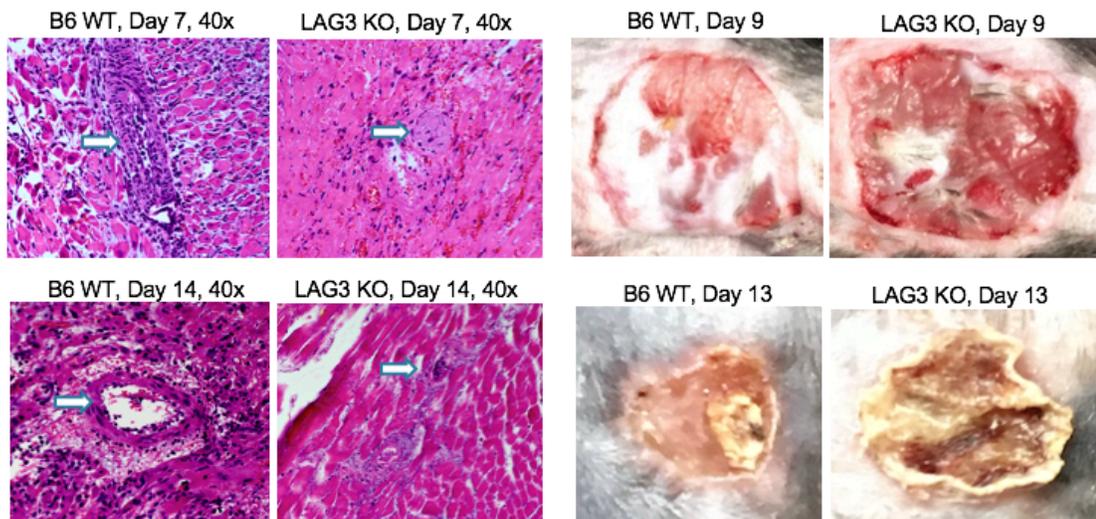


Fig. 5: Skin Life Span



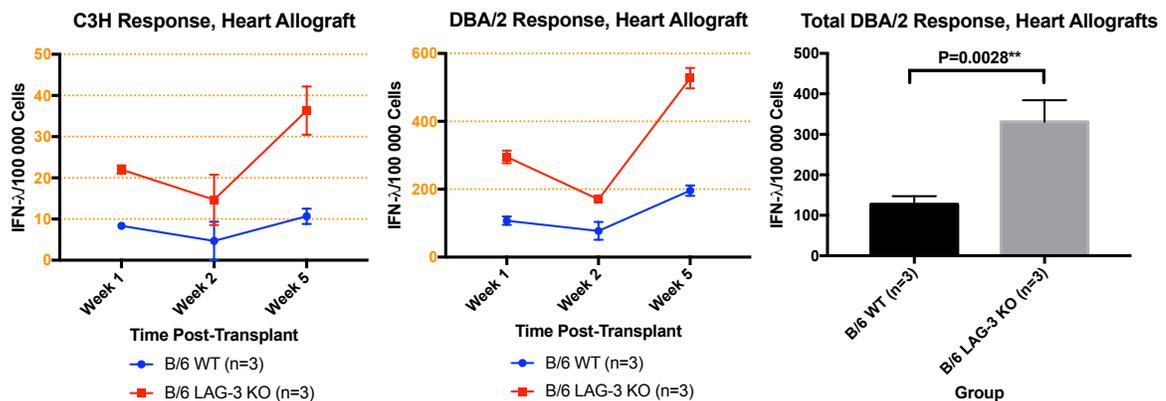
Pathology assessments of heart and skin grafts revealed earlier necrosis in both graft sets (Fig. 6). Although heart grafts placed in LAG-3^{-/-} animals stopped beating a few days earlier on average, they showed pathologic evidence of ischemia with preserved tissue architecture in contrast to diffuse disruptive necrosis shown among grafts placed in WT recipients (Fig. 6). Skin transplants displayed less epithelialization overall as well as faster necrosis and desiccation for up to two weeks after transplant.

Fig. 6: Heart & Skin Allograft Inspection



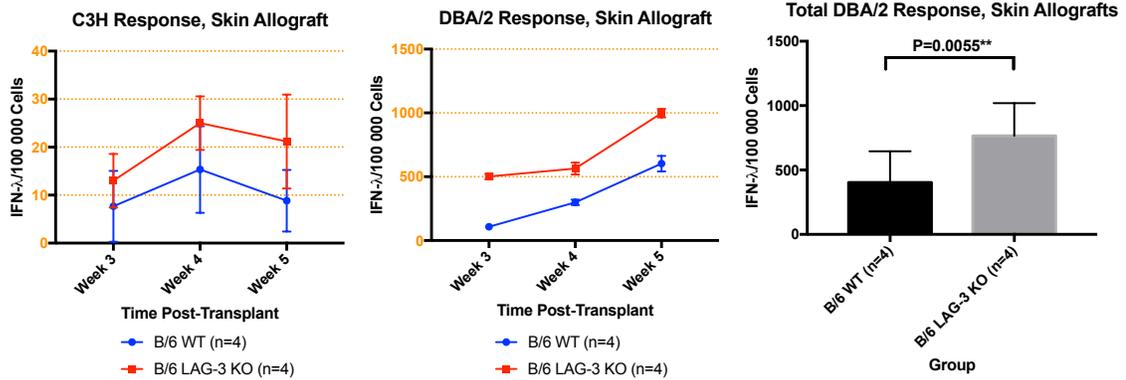
ELISPOT assays were performed for quantifying IFN- γ secretion response toward DBA/2 donor antigen, relative to the response to third-party C3H antigen against which recipients had not been sensitized (Fig. 7). T cells from LAG-3^{-/-} heart recipient spleens (n=3) doubled their response, relative to WT T cells (n=3), toward DBA/2 and C3H in the first two weeks after transplant. The relative LAG-3^{-/-} spot increase compared with WT cells reached almost ten-fold by the fifth week, for both DBA/2 and C3H antigens. By the fifth week, spot counts among LAG-3^{-/-} animals at least doubled from the second week post-transplant. Across all weeks, the difference between WT and LAG-3^{-/-} antigens was significant (P=0.003).

Fig. 7: IFN- γ Secretion from Splenic T Cells After Heart Transplant



The response to skin grafts followed a different pattern, though the increase in antigenic response to DBA/2 was also markedly increased among LAG-3^{-/-} (n=4) relative to WT (n=4) (Fig. 8). To provide further time for vascularization, assessments were made at weeks 3-5 post-transplant. As with hearts, response to DBA/2 grafts among LAG-3^{-/-} animals rose consistently through the fifth week. However, this pattern was not observed for C3H antigen (Fig. 8). Across all time points, the LAG-3^{-/-} cytokine response was higher than that of WT (P=0.006).

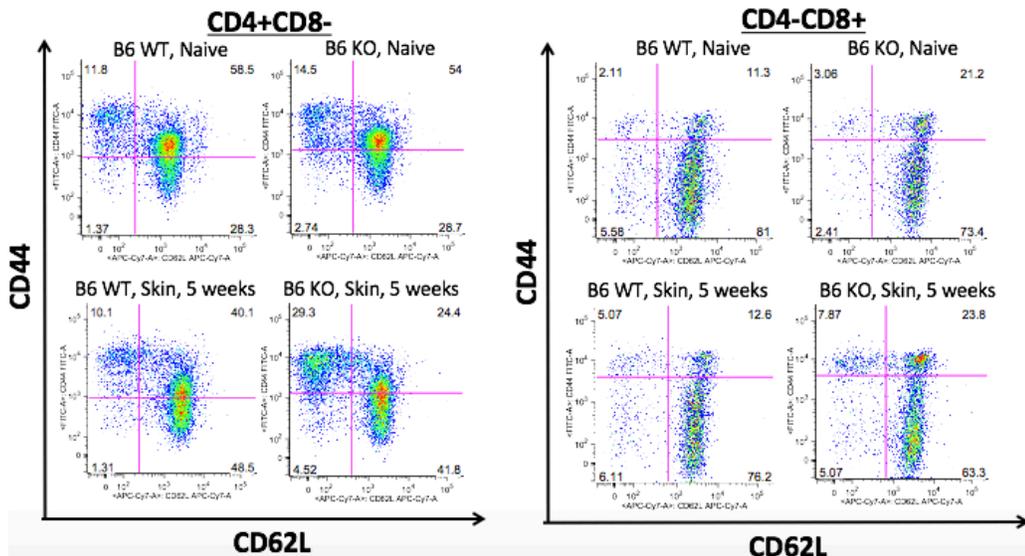
Fig. 8: IFN- γ Secretion from Splenic T Cells After Skin Transplant



Conclusion II: LAG-3 deletion alters memory cell distributions and promotes differentiation of CD44^{hi}CD62^{lo} CD4⁺ T cells post-transplant

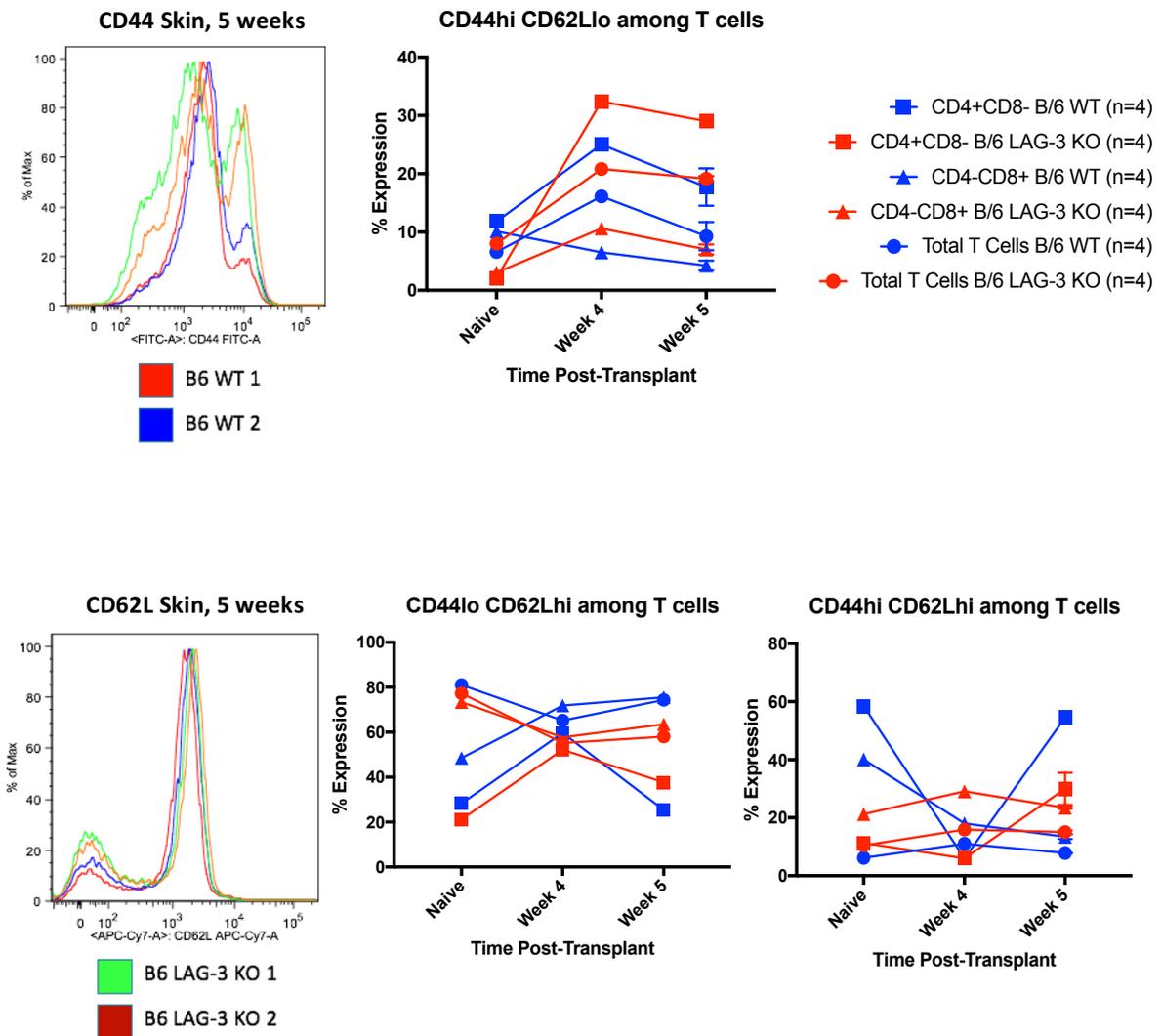
LAG-3^{-/-} skin graft recipients demonstrated altered CD4⁺ and CD8⁺ compartments relative to WT recipients (Fig. 9). While at five weeks post-transplant the proportion of CD44^{hi}CD62^{lo} effector memory cells showed relative stability in the WT animal, this proportion doubled in the LAG-3^{-/-} animal. The relative proportions of the memory cell subtypes were grossly unchanged for CD8⁺ cells (Fig. 9).

Fig. 9: Effector Memory T Cells, Naïve vs. Skin Transplant



The differences in memory compartments can be appreciated by examining the trends in CD4⁺ and CD8⁺ sub-types across weeks 3-5 for skin graft recipients (Fig. 10). The percent expression of CD44^{hi}CD62^{lo} memory cells reaches about 30 percent only for LAG-3^{-/-} CD4⁺ cells (Fig. 10). Notably, no other memory subtype increased to this degree, either for WT or LAG-3^{-/-} cells.

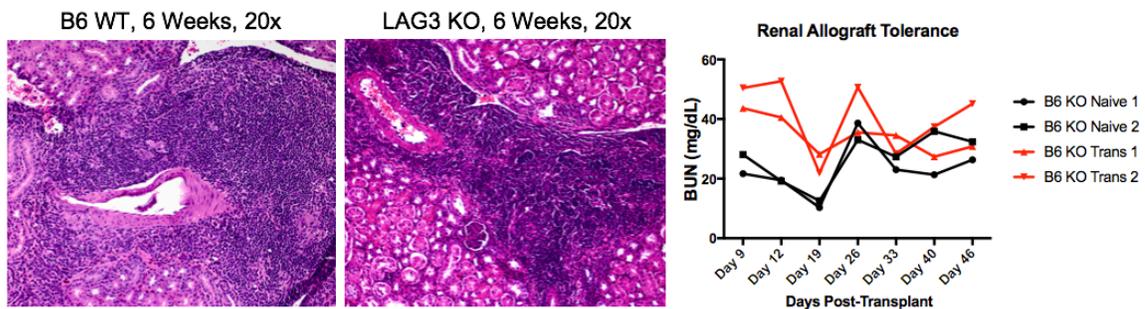
Fig. 10: Memory Cell Compartments, Naïve vs. Skin Transplant



Conclusion III: LAG-3 deletion causes fibrosis but does not inhibit tolerance induction of spontaneously accepted renal allografts

Next, we investigated the effect of LAG-3 deletion in an accepting model, given that the results from heart and skin graft rejection models were consistent with faster rejection in LAG-3^{-/-} recipients relative to WT recipients (Fig. 11). In certain cases, kidney transplants are spontaneously accepted despite MHC class differences between sub-species, notably DBA/2 donor and C57BL/6 recipient. DBA/2 kidney transplants into LAG-3^{-/-} C57BL/6 mice (n=2), compared against WT mice (n=2), did not show an appreciable increase in BUN levels, by which rejection was defined as exceeding 100 mg/dL (Fig. 11). Nevertheless, histologic examination of graft tissue revealed greater fibrosis interspersed throughout and surrounding smaller T cell-rich perivascular regions. The data also suggest that Tr1 cells may not play a role in the induction of tolerance, as seen by the allograft kidneys surviving up to 46 days. Whether Tr1 cells are needed for maintenance of the allograft will require further analysis and monitoring the recipients for a longer period of time.

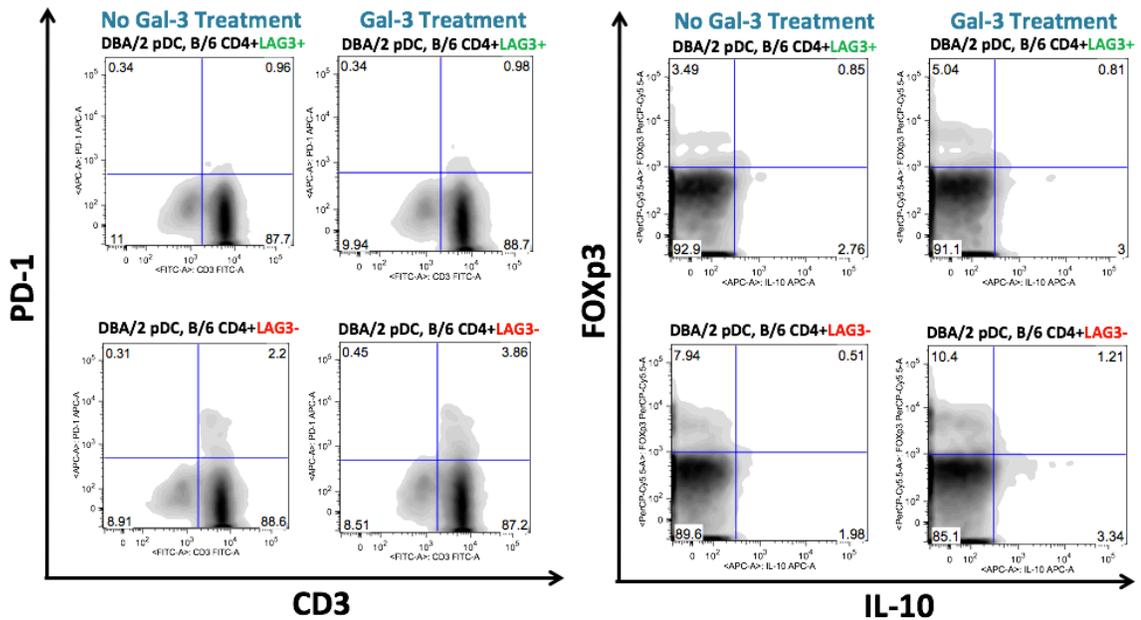
Fig. 11: Kidney Fibrosis & BUN, Naïve vs. Kidney Transplant



Conclusion IV: LAG-3 deletion in a tolerogenic *in vitro* milieu upregulates PD-1, FOXP3, and IL-10 on regulatory T cells

To further investigate the results found in the kidney transplants, an *in vitro* model of the tolerogenic kidney environment was developed using donor plasmacytoid dendritic cells and recipient CD4 T cells. Experimental cultures varied by treatment with Gal-3 and either WT or LAG-3^{-/-} to investigate the role of LAG-3 in tolerogenesis as well as the interplay LAG-3 and its reported ligand, Gal-3 (Fig. 12). Deletion of LAG-3 on CD4+ cells promoted expression of both PD-1 and FOXP3, inhibitory markers associated with regulatory T cells. Notably, Gal-3 treatment enhanced PD-1 expression only on LAG-3^{-/-} cells (Fig. 12). Gal-3 was also independently associated with increased expression of IL-10.

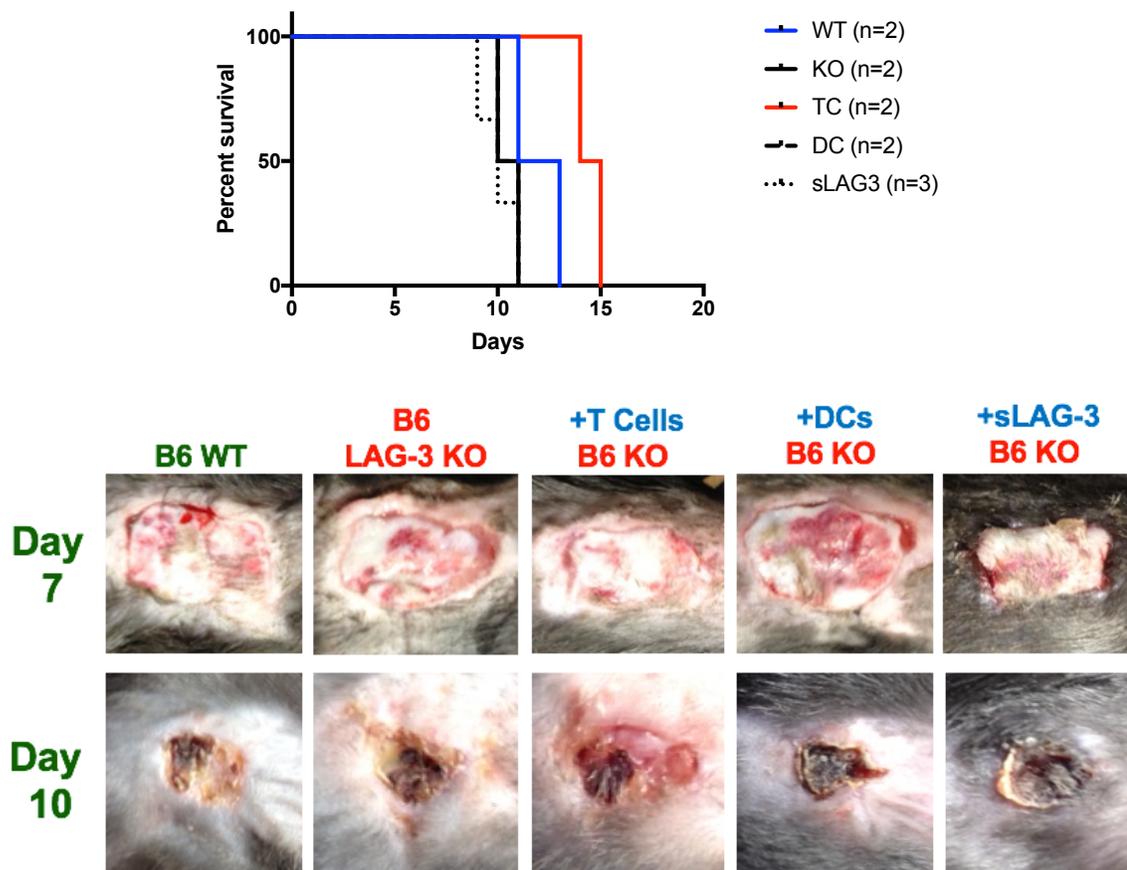
Fig. 12: Gal-3 & LAG-3 Effects on CD4 Cell Expression of Foxp3, PD-1, IL-10



Conclusion V: LAG-3 deletion on T cells are specifically responsible for enhanced graft rejection

Inspection of skin grafts on days 7 and 10 after transplant showed faster eschar development in LAG-3^{-/-} versus WT recipients from days 7-10, despite similar levels of epithelialization and granulation tissue on day 7 (Fig. 13). LAG-3^{-/-} pre-treated with either dendritic cells or soluble LAG-3 one week prior to transplant also showed greater eschar formation compared with WT animals. LAG-3^{-/-} animals that received T cells, however, showed delayed eschar formation and persistence of granulation tissue up to day 10 (Fig. 13).

Fig. 13: Skin Transplant Survival



Conclusion VI: Dendritic cells, not T cells, are responsible for heightened memory response as measured by IFN- γ secretion

Measurement of skin allograft responses again demonstrated higher DBA/2 and C3H cytokine secretion against DBA/2 and C3H antigens in LAG-3^{-/-} relative to WT recipients (Figs. 14, 15). LAG-3^{-/-} animals that did not receive pre-treatment, as well as those injected with T cells or soluble LAG-3, showed marked increases in IFN- γ responses, consistent with the results demonstrated in heart graft recipients (see Conclusion I). In contrast, LAG-3^{-/-} animals that received dendritic cell pre-treatment one week prior to transplant did not show IFN- γ increases and instead demonstrated levels similar to those of WT animals (Figs. 14, 15).

Fig. 14: DBA/2 Response, Skin Allograft

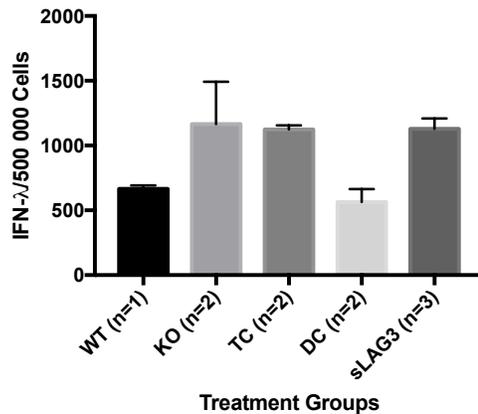
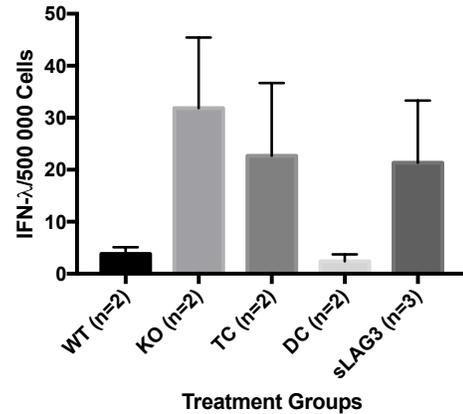


Fig. 15: C3H Response, Skin Allograft



Discussion

LAG-3 is a soluble and transmembrane protein whose significance in solid organ transplantation has not been fully characterized. As a classificatory marker for Tr1s, a

subset of inducible peripheral regulatory T cells, an important aim of this study is to determine whether LAG-3 does not functionally mediate the rejection process and therefore simply identifies specific classes of regulatory cells, or whether LAG-3 directly mediates allograft rejection. Because rejection can take multiple forms, most applicably either acute or chronic rejection, subsequently parsing out the time-dependent effect of LAG-3 deletion becomes a necessary goal. This we achieved by examining LAG-3 deletion on short-term graft survival as well as long-term immunological memory, as measured by IFN- λ secretion in response to donor antigen.

An important corollary is that to the extent there is a measurable effect of LAG-3 on transplant outcomes, these effects likely depend on a specific isoform, either soluble or membrane-bound, and/or cell type. This we investigated by re-introducing LAG-3 either as a soluble protein or as a membrane-bound protein attached to either T cells or dendritic cells and trending transplant and memory outcomes. In the context of investigating the effects of LAG-3, it became clear that LAG-3's effects may differ based on whether the mouse transplant model of choice was a rejecting model (heart or skin) or an accepting model (kidney). Due to an ambiguous effect of LAG-3 deletion in the accepting model, we created a T cell-dendritic cell co-culture environment mimicking that in the accepting model and noted the effect of LAG-3 deletion on immunological surface receptors.

Given the aims outlined above, this study generated six conclusions (see Results) which have implications for our understanding of how LAG-3 functions immunologically in a transplantation context. Additionally, the findings of the study delineate a clearer role for LAG-3 and its molecular functions with respect to important inhibitory markers,

particularly PD-1, FOXP3, and IL-10, as well as dendritic cells. These conclusions and their implications will be discussed in turn.

Effects of LAG-3 on allograft survival and T cell memory generation in rejection models

Our first results demonstrate that LAG-3 deletion accelerates rejection of cardiac and skin allografts by a few days in either case. H&E staining of rejected cardiac allografts shows greater intercellular lymphocytic infiltration and distortion of graft parenchyma. Grafts in LAG-3^{-/-} recipients, on the other hand, show relatively preserved architecture but appear to develop greater vascular occlusion, suggesting that these grafts reject faster, possibly due to an ischemic T cell-mediated process. Published evidence suggests that memory T cells directly mediate cardiac allograft vasculopathy in RAG-1^{-/-} B/6 (H-2^b) recipients of Balb/c (H-2^d) cardiac allografts in an OX40/OX40L-dependent manner (34). OX40L blockade in this study was associated with impairment of T-cell-mediated vascular injury, a finding reflected in human patients with CAV. Activated memory T cells present in human coronary arteries generate a significant proportion of infiltrating mononuclear cells contributing to vascular inflammation (35). Furthermore, the causality may be bidirectional: while inflammation can lead to ischemia so can ischemia lead to inflammation. In a separate study of allogeneic cardiac transplants among rats, the degree of vessel injury in ischemic injured allografts at 90 days post-transplant was significantly greater than that in non-ischemic injured allografts (36). It is possible therefore that ischemia can lead to greater degrees of inflammation and vice-versa, generating a positive feedback mechanism leading ultimately to chronic rejection. Our findings corroborate the theory of T-cell-mediated vascular injury but with the

important distinction that these findings occurred in the knockout animals on a faster timescale, suggesting that LAG-3 inhibits the ischemic and inflammatory effects of T cells to a graft. This is consistent with broadly accepted notions of LAG-3, namely that it retards T cell activation, proliferation, and inflammatory cytokine generation (5, 19, 37-40).

Consistent with the findings in heart, the model of skin engraftment showed that LAG-3^{-/-} animals accelerated rejection as evidenced by greater epithelial disruption by day 9 post-transplant and older scar formation by day 13. This additional evidence from a separate type of rejection model supports the theory that the absence of LAG-3 plays a measurable role in accelerating graft rejection. Both the heart and skin models also generated significantly higher levels of IFN- γ by T cells isolated from spleen in response to donor antigen (1.5-2.5x higher) at least three weeks out from surgery. While the cytokine production of LAG-3^{-/-} T cells in response to third-party C3H stimulators also exceeded that of wild-type T cells, suggesting a baseline level of LAG-3-dependent inhibition of T cell activity, the cytokine effect was proportionally higher for heart allografts at week 5. Thus, the blanket effect on all cellular responses due to LAG-3 deletion, consistent with the literature as described above, is shown by the effect on third-party. However, one novel finding of this study is the increased effect in LAG-3 knockout animals, which cannot be accounted for by increased activity alone, but rather a combination of either increased differentiation or increased cellular generation.

The second conclusion suggests that LAG-3 affects production of memory T cell differentiation. In the inflammatory milieu after skin and heart transplants, LAG-3 deletion increases splenic memory T cells as measured by IFN- γ ELISPOT secretion.

FACS analysis of naïve WT and *LAG-3^{-/-}* animals compared with skin transplant recipients 5 weeks later shows that transplantation increases the differentiation of CD44^{hi}CD62L^{lo} CD4⁺ memory T cells, a type of effector memory cells. Memory T cells originate from naïve thymic CD25⁻ T cells that can develop into CD25⁺ effector T cells (41). In mice some of these T cells can become memory T cells, as distinguished by CD62L (L-selectin) and CD44 (H-CAM) (42). Memory cells express low levels of L-selectin in contrast to naïve cells; however, naïve cells express low levels of HCAM relative to memory cells. Effector memory cells have been broadly associated with inflammation secondary to numerous causes, such as infection, allergens, and chemical irritants (42, 43). Recently, the field has also highlighted the importance of memory T cells in mediating graft rejection and their role as a significant barrier to tolerance induction in clinical transplantation, since these alloreactive cells reside in and recirculate among peripheral non-lymphoid tissues associated with the graft (15, 44, 45). Our findings in this study suggest that *LAG-3* dampens the effect of memory T cell generation, which occurs at an exceedingly higher rate when *LAG-3* is deleted. This novel finding is particularly important for transplantation because co-stimulation blockade, a staple of immunological therapies, does not adequately control the responses of memory T cells (45). These results are mutually substantiated by those mentioned earlier, specifically that memory cells have been associated with chronic allograft vasculopathy. It is possible that the same mechanism that upregulates acute cellular graft rejection at the vascular level also produces a greater level of memory T cells in the long-term.

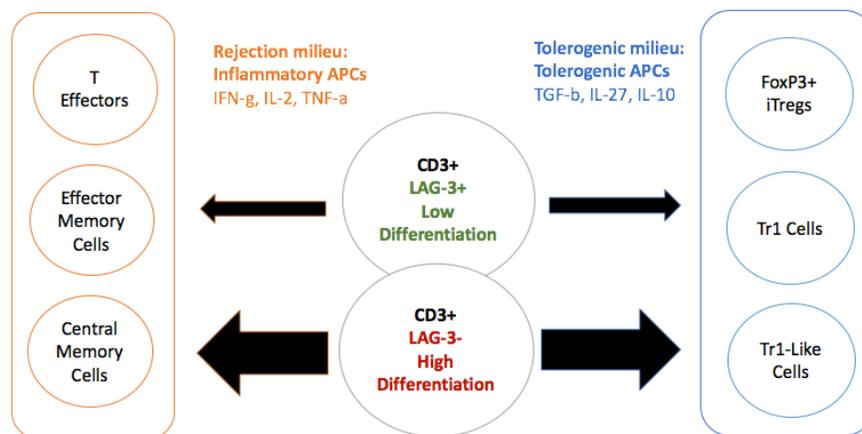
Effects of LAG-3 on allograft survival and cell differentiation in an accepting model

Spontaneously accepted renal allografts show smaller Treg-rich regions with higher fibrosis, but BUN levels do not reach a rejection level (>100 mg/dL) by 6 weeks. Beyond this time period, long-term graft follow-up and analysis may reveal a different pathology. The difference in these observed allograft acceptance results could in part be dependent on the varying roles of LAG-3 on different T cell populations in either a rejection or tolerogenic milieu. It has been established that T cell fates are highly dependent on the cytokine microenvironment to which they are exposed at various stages, and that ultimately graft survival in a host depends on whether its T cells largely take on a pro-inflammatory or pro-tolerant phenotype (46). While the BUN findings did not suggest a rejection process, cellular histology did demonstrate increased parenchymal fibrosis indicative of a chronic inflammatory process. One explanation for this finding could be that while LAG-3 does indeed accelerate inflammation, it has a countervailing and separate effect in a microenvironment of cytokines that are tolerogenic, or at least graft-protective.

In spontaneously accepted renal allografts, in which regulatory cytokines or proteins such as IL-27, TGF- β , and Gal-1 are upregulated or conserved (unpublished observations based on RNA transcript analysis), stimulation of CD4+ naïve cells with tolerogenic allogeneic DCs depends in part on LAG-3. In *in vitro* assays that replicate this tolerogenic milieu, LAG-3 deletion increases expression of PD-1 and IL-10 on regulatory T cells analyzed by FACS staining. At first glance, this finding contrasts with past research that shows that the development of naïve CD4+ cells can be shunted toward a regulatory phenotype, most notably Tr1 cells, in LAG-3 dependent processes in the gut

and in tumors (18, 47, 48). However, it may be that with germline deletion of LAG-3 an earlier T cell development stage is obviated or altered such that a greater proportion of T cell precursors ultimately become graft-protective. This theory is consistent with the evidence presented previously LAG-3 deletion functionally produces a higher proportion of memory T cells. The principal difference in these two cases is the cytokine milieu. In a pro-rejection microenvironment such as that of mouse heart or skin allotransplantation, then the net effect of germline LAG-3 deletion is to produce an anti-graft effect; whereas, in the tolerant microenvironment of kidney allotransplantation, then the corresponding net effect is a sustained immunoprotective response. This theory is encapsulated by the overarching model presented below:

Fig. 16: Suggested Model of LAG-3 Control of T Cell Differentiation



One theory by which this may occur is that LAG-3 may depress differentiation of T cells by blocking co-stimulatory or activating MHC or glycoprotein signals. Without LAG-3, cells are more likely to differentiate. This model explains how an absence of LAG-3 in the *in vitro* experiments in this study can generate relatively higher expression of PD-1, IL-10, and FOXP3 on FACS analysis. The relative proportions of certain cellular subtypes, primarily FOXP3 regulatory T cells and peripheral Tr1 cells, are greatly

enhanced in the absence of LAG-3 if LAG-3 exerts control through an undescribed mechanism over the differentiation of CD4⁺ precursors.

Gal-3 and LAG-3 interact to affect cellular phenotype and properties

An interesting result that further contextualizes what has been previously published is that the glycoprotein Gal-3 functions as a ligand for LAG-3. Although the binding of these two molecules was not directly tested in this study, we found evidence from co-culture FACS analysis that the level of Gal-3-dependent IL-10 and Foxp3 expression is affected by the presence or absence of LAG-3. Relative to LAG-3^{+/+} cell cultures, knockout cell cultures saw the relative expressions of IL-10 and Foxp3 increase from 3.49% and 5.04%, respectively, to 7.94% and 10.40%. The highest expression (10.40%) occurred when LAG-3 was absent and Gal-3 was present, suggesting that LAG-3 may be inhibiting receptor expression induced by Gal-3 and that the absence of LAG-3 accelerates differentiation, as discussed earlier. Interestingly, LAG-3 showed an even stronger effect in the case of PD-1 expression: Gal-3-dependent induction of PD-1 on CD4 cells only occurred in the absence of LAG-3. While it has been previously reported that Gal-3 interacts with LAG-3 to suppress lymphocytes (27, 28), it also appears based on this work that LAG-3 can block the downstream effects of Gal-3 in this cytokine environment.

Distinguishing the isoform and cellular types responsible for LAG-3's effects

The adoptive transfer experiments further demonstrate that the effects of surface-bound LAG-3 appear to eclipse those of soluble LAG-3 for transplant outcomes.

Furthermore, even the cell type on which LAG-3 is expressed appears to influence the both skin graft rejection speed and memory response. Mice with LAG-3-competent T cells from adoptive transfer injections experienced prolonged graft survival compared with LAG-3^{-/-} graft recipients that did not receive injections. However, it was LAG-3-competent dendritic cells, rather than T cells, that were responsible for reduced anti-donor response more than one month after skin engraftment.

An area that has not yet been fully explored in the literature is the role of tolerant dendritic cell subsets in producing graft-protective microenvironments. As master controllers and activators of cell classes, including T cells (49), dendritic cells are prime candidates for the cell type that would exert a very powerful effect on differentiation of CD4⁺ T cell precursors using LAG-3. One subtype of dendritic cells that are DNGR-1⁺ has been shown to be important for cross-priming of naïve CD8⁺ T cells and the optimal production of tissue resident memory cells (50). It is possible that another subset of dendritic cells analogously controls differentiation of naïve CD4⁺ T cells in a LAG-3 dependent process that modulates transplantation outcomes. In fact, LAG-3 has also been shown to be important for the development and homeostasis of dendritic cells and macrophages (8, 51), and LAG-3 is used by dendritic cells to influence the phenotypes and properties of other cells (5). Interestingly, Gal-3, shown in this and prior studies to interact with LAG-3, has also been reported to modulate the function and expansion of dendritic cells and the CD8 T cells they activate (28, 52).

While prior studies have demonstrated that LAG-3 is important for downregulating T cell activation and cellular proliferation (1-5), our results suggest that LAG-3 may also play an additional role in T cell biology by governing the differentiation

of naïve CD4+ T cells, both in rejection transplant models and tolerogenic transplant models. Additionally, LAG-3's role as a modulator of transplantation must also include its effect via dendritic cells, whose biological pathways with respect to effects downstream of LAG-3 require further exploration. Past studies have shown that LAG-3 is critical for dendritic cell activation and migration in some cases, but it is interesting that LAG-3 in this study depressed the anti-donor response, which would be consistent with either inhibition of dendritic cell activity or activation of an inhibitory dendritic cell subset.

Overall, the primary contribution of this research to the area of transplantation and the broader field of immunology is the suggestion that LAG-3 may have a role in promoting T cell differentiation in a way that is separate from its roles in promoting the activation and proliferation of T cells. This theory should be further substantiated and explored in related immunological research areas, such as tumor biology and infectious diseases. An additional contribution of this research to the field is the finding that targeting LAG-3 alone is likely to be insufficient in adequately mediating either tolerance or rejection, because of its competing properties. Rather, LAG-3 should be explored as a complementary therapy that eases the burden of broad immunosuppressives or other drugs that are aggressively antagonistic to the patient's systemic health.

Study Limitations & Future Directions

While the six conclusions generated by this study have produced novel results that can further illuminate our collective understanding of LAG-3 and its role in transplantation, there are aspects of the study results that need to be explored further.

First, the histologic results demonstrating vasculopathy should be stained to quantify the extent to which that antibody- or complement-mediated rejection are contributing to graft failure, as this may suggest alternative molecular mechanisms corresponding to LAG-3 deletion. Second, the role of Tr1 cells should be more definitively explored in more precise culturing experiments. Due to time and resource constraints, we were not able to definitively identify the cultured T cells as Tr1 cells, due to a lack of CD49b expression. Because CD49b is a widespread cellular integrin alpha subunit, one would expect that it would not be broadly expressed on T cells cultured *in vitro* because they lack the appropriate microenvironment stimuli necessary for promoting expression of migration ligands and receptors. It does appear that the expression of other functional Tr1 markers, LAG-3 (present) and Foxp3+ (absent), was consistent in the cell populations identified in our study as with those published in the literature (9). Third, the interaction of Gal-3 and LAG-3 should be further explored to determine whether one molecule directly affects the induction of the other, possibly by using FACS sorting analysis. Fourth, the adoptive transfer experiments used isolated dendritic cells, which were the basis for our study conclusions that dendritic cells are responsible for LAG-3-mediated memory control, but the isolation kits used do not reliably distinguish between dendritic cells and other antigen-presenting cells such as B cells. Experiments should be conducted to tease apart the separate contributions of each.

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