JCI The Journal of Clinical Investigation

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J Clin Invest. 2025. https://doi.org/10.1172/JCI172988.

Research In-Press Preview Immunology

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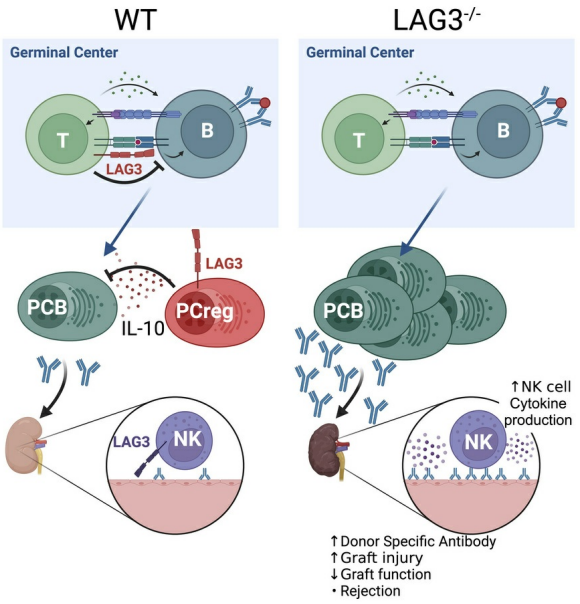

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LAG3 regulates antibody responses in a murine model of kidney transplantation.

Michael Nicosia[1*], Ran Fan[1], Juyeun Lee[2], Gabriella All[1], Victoria Gorbacheva[1], José I. Valenzuela[1], Yosuke Yamamoto[1], Ashley Beavers[1], Nina Dvorina[1], William M. Baldwin III[1], Eduardo Chuluyan[3,4], Motoo Araki[5], Brian T. Gaudette[1], Robert L. Fairchild[1], Booki Min[1,6#] & Anna Valujskikh[1#]

1 - Department of Inflammation and Immunity, Lerner Research Institute, Cleveland Clinic, Ohio

2 - Department of Cardiovascular and Metabolic Sciences, Lerner Research Institute, Cleveland Clinic, Ohio

3 - Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas, Centro de Estudios Farmacológicos y Botánicos (CEFYBO), Facultad de Medicina, Buenos Aires, Argentina

4 - Universidad de Buenos Aires, Facultad de Medicina, Departamento de Microbiología, Parasitología e Inmunología, Buenos Aires, Argentina

5 - Department of Urology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

6 - Current - Department of Microbiology and Immunology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois

** - Corresponding author*

 # - Contributed equally

Correspondence: Michael Nicosia, Department of Inflammation and Immunity, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Avenue, NB3-77, Cleveland, OH, 44195, USA. Email address: nicosim@ccf.org. Telephone:

Conflict of Interest Statement: The authors have declared that no conflict of interest exists.

Abstract

Lymphocyte activation gene-3 (LAG3) is a coinhibitory receptor expressed by various immune cells. While immunomodulatory potential of LAG3 is being explored in cancer and autoimmunity, there is no information on its role following organ transplantation. Our study investigated the functions of LAG3 in a mouse model of renal allograft rejection. $LAG3^{-/-}$ recipients rapidly reject MHC-mismatched renal allografts that are spontaneously accepted by WT recipients, with graft histology characteristic of antibody mediated rejection (ABMR). Depletion of recipient B cells but not $CD8^{+}$ T cells significantly extended kidney allograft survival in $LAG3^{-/-}$ recipients. Treatment of WT recipients with an antagonistic LAG3 antibody enhanced anti-donor immune responses and induced kidney damage associated with chronic rejection. The studies of conditional $LAG3^{-/-}$ recipients and mixed bone marrow chimeras demonstrated that LAG3 expression on either T or B cells is sufficient to regulate anti-donor humoral immunity but not to induce acute allograft rejection. The numbers and proinflammatory functions of graft-infiltrating NK cells were markedly increased in $LAG3^{-/-}$ recipients suggesting that LAG3 also regulates the effector stage of ABMR. These results are the first to identify LAG3 as a regulator of immune responses to kidney allografts and a potential therapeutic target for ABMR prevention and treatment.

Introduction

Lymphocyte activation gene 3 (LAG3, CD223) is an immune checkpoint inhibitor of the immunoglobulin superfamily, closely related to CD4 (1). LAG3 is expressed on a number of immune cells including $CD4^+$ and $CD8^+$ T cells, T regulatory cells, B lymphocytes (1-5), plasmacytoid dendritic cells (6) (pDCs) and NK cells(1). Similar to other coinhibitory receptors such as CTLA4 or PD-1, LAG3 is not expressed by naïve T cells, but its expression is induced on $CD4^+$ and $CD8^+$ T cells following TCR engagement (1). Murine LAG3 shares 69.9% sequence homology with the human protein preserving structure and function, and largely mirrors the expression profile of human LAG3 (7, 8). Analogous to CD4, LAG3 binds to major histocompatibility complex II molecules (MHC-II) on antigen presenting cells (APCs) (9, 10), and additionally interacts with other ligands such as Galectin-3 (11), liver sinusoidal endothelial cell lectin (LSECtin) (12), α-synuclein fibrils (α-syn) (13), and fibrinogen-like protein 1 (FGL1) (14).

In T cells, LAG3 is co-localized with T cell receptor/CD3 complex within immune synapse and inhibits TCR signaling leading to impaired cell activation, proliferation, differentiation, and effector functions in the context of autoimmunity, infection and tumor (15-20), recent studies have started to reveal the intricacies that underly the suppressive mechanism of LAG3 in T cells (21-23). In addition, LAG3 plays an essential role in T regulatory cells (Treg) (5, 24, 25), though data is conflicted dependent upon the model and setting (26).

In contrast, there is a paucity of studies investigating the role of LAG3 in B lymphocytes. Activation of murine splenocytes *in vitro* induces the endogenous expression of LAG3 on $CD19^+$ B cells in T cell and soluble factor dependent manner (2). However, the functional consequences of LAG3 up-regulation in B cells during humoral immune responses remain to be investigated. LAG3 expression has been shown to be a defining feature of an IL-10 producing subset of plasma cells (27). While it was demonstrated that these cells arise from BCR dependent signals and that their

IL-10 production is dependent upon innate signals such as TLRs, the role of LAG3 expression on this B cell subset is still poorly understood.

Even though the relative importance of LAG3 on conventional T cells, Tregs, B cells and other cell types during immune responses remains to be determined in different settings it is a molecule of great interest for clinical interventions. Several monoclonal antibodies targeting LAG3 with either antagonistic, depleting, or agonistic activities have been developed and are currently in clinical trials for cancer and autoimmunity patients (28-35). However, LAG3 is not commonly considered as a therapeutic target in organ transplantation, largely due to the paucity of information on the role of this pathway in experimental animal models. Lucas *et al*. (36) reported that LAG3 blockade prevents tolerance induction in alloreactive $CD8^+$ T cells in a mouse model of allogeneic bone marrow transplantation, but that this effect did not depend on LAG3 expression by $CD8^+$ T lymphocytes themselves. In a different study, the depletion of $LAG3^+$ T cells with monoclonal antibody extended rat cardiac allograft survival, yet prevented tolerance induction via donor-specific cell transfusion (37). These findings suggest LAG3 involvement in different aspects of allograft rejection and tolerance. Whereas the functions of CTLA4 and PD1 coinhibitory molecules in transplantation have been extensively studied, it is unclear whether and how LAG3 regulates alloimmune responses to solid organ transplants.

In the current study, we investigated the contribution of LAG3 to alloimmune responses using a mouse model of life-supporting renal transplantation and found that recipient LAG3 deficiency resulted in rapid rejection of fully MHC-mismatched renal allografts that are spontaneously accepted by wild type recipients. While $LAG3^{-/-}$ recipients developed increased cellular and humoral anti-donor immune responses, the renal graft tissue injury was characteristic of antibody-mediated rejection and was significantly diminished by the depletion of B cells but not of $CD8^+$ T lymphocytes. Analogous increase in IgG antibody responses were observed after immunization

of LAG3$^{-/-}$ mice with either T cell dependent or independent model antigens, whereas LAG3 cross-linking inhibited antibody secretion by plasma cells *in vitro* indicating B cell intrinsic role of LAG3 pathway. Nevertheless, while T cell- or B-cell specific LAG3 conditional knockout recipients and mixed bone marrow chimeras with LAG3 deficiency in both T and B cells had increased donor-specific alloantibody (DSA) production, they did not reject renal allografts suggesting that LAG3 additionally regulates antibody-mediated allograft injury. In support of this, the numbers and proinflammatory functions of graft infiltrating NK cells, important mediators of antibody-mediated injury, were markedly increased in LAG3$^{-/-}$ allograft recipients compared to wild type controls. These data present the first evidence for LAG3 involvement in regulation of humoral alloimmune responses and identify LAG3 as a potential target for diminishing pathogenic alloimmunity.

Results

Mice deficient in LAG3 have elevated heterologous alloimmune responses prior to transplantation.

As LAG3 was demonstrated to play critical role in immune tolerance, we initially evaluated the immune cells from naïve non-transplanted 10 week old B6.WT and B6.LAG3$^{-/-}$ mice. LAG3 deficiency resulted in modest increase in splenic cellularity (data not shown) and in the increased numbers of total and effector memory (TEff$_M$) CD4$^+$ and CD8$^+$ T cells, as well as Tregs compared to WT mice. In addition, mice deficient in LAG3 had elevated numbers of T follicular helper (TFh) and T follicular regulatory (TFreg) cells in the spleen (**Fig. 1A**, **Fig. S1**). The numbers of major spleen B cell subsets – follicular (FoB), marginal zone (MZB), and transitional (TrB) B cells – were not significantly different between WT and LAG3$^{-/-}$ mice. Notably, mice deficient in LAG3 had significantly increased numbers of germinal center B cells (GCB) and plasma cells (PCB) in the spleen suggesting ongoing B cell activation (**Fig. 1B**, **Fig. S2**). There was a trend towards the increase in numbers of regulatory B cells (Bregs) defined as CD19$^+$CD1d^{hi}CD5$^+$ cells. It should be noted that despite the observed shifts towards activated immune cell phenotypes, unmanipulated LAG3$^{-/-}$ mice did not exhibit signs of autoimmune disease up to 6 months of age.

To assess the impact of this phenotypic shift on alloimmunity, we probed both B6.WT and B6.LAG3$^{-/-}$ for T cell reactivity against a panel of allogeneic strains (**Fig. 1C**). IFNγ ELISPOT assay demonstrated that compared to WT mice, naïve LAG3$^{-/-}$ mice have increased frequencies of preexisting memory T cells reactive against C3H (H2-D^k) alloantigens. The numbers of BALB/c (H-2^d), SJL (H-2D^s) but not DBA (H2-D^q)-reactive T cells were elevated in some non-transplanted LAG3$^{-/-}$ mice, albeit these increases were not statistically significant (**Fig. 1C**). We also tested for the presence of alloreactive antibodies in the sera of non-transplanted mice (**Fig. 1D**). Some of

the tested naïve LAG3$^{-/-}$ mice (H2-D^{b}) contained elevated levels of IgG antibodies against class I and class II MHC molecules from BALB/c (H2-D^{d}, I-A^{d}) and C3H mice (H2-D^{k}, I-A^{k}). These results suggest that LAG3 is an important regulator of immune cell homeostasis and as such may play a crucial role during immune responses to transplanted organs.

Recipient LAG3 deficiency enhances anti-donor alloimmune responses and elicits kidney allograft rejection

To test the functional consequences of LAG3 deletion, we used a previously described mouse model of kidney transplantation in which B6 (H-2^{b}) recipients undergo bilateral nephrectomy, and are then transplanted with a single fully MHC-mismatched kidney allograft (38). In this model the recipient survival is dependent upon allograft functionality, which can be measured by serum creatinine levels. Consistent with previously published studies that used different donor strains (38, 39), B6.WT recipients spontaneously accept C3H renal allografts for >60 days (**Fig. 2A&B**). In contrast, most B6.LAG3$^{-/-}$ recipients rapidly rejected kidney allografts (median survival time, MST, of 15d) (**Fig. 2B**) and had significantly increased serum creatinine levels at d. 14 (1.3 mg/dl versus 0.1 mg/dl in B6.WT controls and <0.4 mg/dl in non-transplanted mice, **Fig. 2C**). Immunohistochemistry of allografts recovered from LAG3$^{-/-}$ recipients around the time of rejection (d. 14) showed decreased numbers of infiltrating T cells compared to grafts from B6.WT recipients (**Fig. S3**). Analysis of the graft histology showed diffuse C4d deposition in both WT and LAG3$^{-/-}$ recipient kidney allografts (**Fig. 2D, Fig. S3 , Fig. S4A and Table S1**). However, LAG3$^{-/-}$ recipient grafts had significantly increased graft edema, dilatation of the peritubular capillaries of the inner and outer cortex, and endothelial swelling (**Fig. S4A & Table S1**). These findings are congruent with BANFF criteria for antibody mediated rejection (40) . Flow cytometry analysis of graft infiltrating cells on d. 10 posttransplant (**Fig. 2E-F**) revealed no significant changes in CD4^{+} or

176 $CD8^+$ T cell infiltration, with the only significant difference being in the increased numbers of
177 infiltrating $FoxP3^+$ T regulatory cells.

178 Analysis of the peripheral T cell pool revealed no significant differences in the proportion or
179 numbers of various spleen T cell subsets (**Fig. 3A** and **Fig. S5A**). Nevertheless, the absence of
180 recipient LAG3 resulted in elevated frequencies of donor specific T cells, as measured by IFNγ
181 ELIPSOT assay on d. 14 posttransplant (**Fig. 3D**). Analogous to the T cell compartment, spleen
182 subsets of follicular, marginal zone, transitional, and regulatory B cells were similar in WT and
183 $LAG3^{-/-}$ allograft recipients (**Fig. 3B** and **Fig. S5B**), whereas germinal center B cells and plasma
184 cells were increased in some but not all recipients. To assess anti-donor humoral immune
185 responses, recipient serum was collected at 14 d posttransplant and analyzed for the levels of
186 IgG antibodies against donor class I (D^k) and class II ($I\text{-}A^k$) MHC molecules. Consistent with our
187 published studies, WT renal allograft recipients had developed minimal donor specific
188 alloantibodies (DSA) at this timepoint. In contrast, $LAG3^{-/-}$ recipients had significantly elevated
189 levels of IgG against both D^k and $I\text{-}A^k$ (**Fig. 3C**). Isotype analysis showed that $LAG3^{-/-}$ recipients
190 had a trend towards increased levels of IFNγ induced T-cell dependent IgG2c, known to strongly
191 activate FcγR and enhance antibody dependent cellular cytotoxicity (ADCC) (**Fig. 3C**) (41-44) .
192 To understand the underlying mechanisms of rejection, we analyzed LAG3 expression in recipient
193 spleen cells on day 10 posttransplant. As anticipated, effector T cells and Tregs expressed LAG3
194 under these conditions (data not shown and **Fig. S10**). Given the observed graft pathology (**Fig.**
195 **2D**) and the serum DSA levels (**Fig. 3C**), we measured LAG3 expression on B and T cell subsets
196 critical to DSA generation. We found that follicular T cells and plasma cells both express LAG3
197 following transplantation (**Fig. 3E & Fig. S10**). Taken together these data demonstrate that LAG3
198 plays an essential role in regulating both cellular and humoral immune responses to transplanted
199 allografts and contributes to spontaneous renal allografts acceptance in wild type recipients.

LAG3 regulates de novo alloresponses.

Our data show that non-transplanted LAG3 deficient mice have increased alloreactive immune responses against C3H antigens (**Fig. 2A&B**). To establish whether LAG3 is regulating *de novo* immune responses, we treated WT kidney allograft recipients with a course of LAG3 blocking antibody and monitored mice for signs of rejection (**Fig. 4A**). LAG3 blockade resulted in rejection in 2 out 9 mice within 14 days posttransplant (**Fig. 4B**). Despite the long-term graft survival in the remaining recipients, we found that LAG3 blockade did induce graft damage, as evidenced by increased kidney injury markers NGAL and KIM1 in the urine, and blood urea nitrogen (BUN) in the serum (**Fig. 4C-E**). Blockade of LAG3 also increased DSA production (**Fig. 4F&G**) and frequencies of donor reactive T cells (**Fig. 4H**), indicating that LAG3 regulates *de novo* alloresponses following transplantation. The increase in alloresponses and markers of kidney injury suggested that LAG3 blockade may induce chronic injury of renal allografts. Indeed, histological analysis at d. 42 posttransplant (**Fig. 4I & Fig. S6**) revealed tubular atrophy, endothelial cell swelling and increased alpha-smooth muscle actin (αSMA) staining due to graft fibrosis, also reflected in the Trichrome staining (**Fig. S6**). While C4d staining showed C4d graft deposition in both control IgG and anti-LAG3 treated recipients, recipients that received LAG3 blockade had dilated peritubular capillaries with marginated mononuclear cells (red arrows, **Fig. 4I**). In addition, recipients that received LAG3 blockade had increased $CD4^+$ and $CD8^+$ T cell graft infiltrates. These findings identify LAG3 as an important regulator of *de novo* immune responses to a kidney allograft.

B cells, but not $CD8^+$T cells, are essential for kidney allograft rejection by $LAG3^{-/-}$ recipients.

Given that LAG3 is a well-studied regulator of T cell responses we anticipated that LAG3 deficiency primarily affects priming of alloreactive T cells and their pathogenic functions within the kidney graft. However, the modest increase in donor-reactive T cells and the graft histology (**Figs. 2-3 and Fig. S3)** suggested that alloantibody are the major mediators of graft tissue injury in LAG3$^{-/-}$ recipients. To formally test the contributions of cellular vs antibody mediated rejection, LAG3$^{-/-}$ recipients were treated with CD8^{+} T cell depleting antibody prior to transplantation of C3H kidney allografts (**Fig. 5A** and **Fig. S7B**). CD8^{+} T cell depletion antibodies failed to prolong kidney allograft survival in LAG3$^{-/-}$ recipients, with MST of 16 days (**Fig. 5B**). Anti-donor humoral immune responses were assessed at 14 d posttransplant and analyzed for the levels of IgG antibodies against donor class I and class II MHC molecules. WT allograft recipients depleted of CD8 T cells had a low-grade DSA response at this timepoint. In contrast, LAG3$^{-/-}$ recipients had significantly elevated levels of IgG against both donor class I and class II alloantigens (**Fig. 5C**). Although CD8 T cell depletion reduced the frequencies of IFNγ producing T cells in both groups, LAG3 deficient recipients still had modestly elevated frequencies of donor-specific T cells on d. 14 posttransplant compared to WT (**Fig. 5D**). This is likely due to a faster reconstitution of LAG3$^{-/-}$ cells following depletion, which has been previously reported in memory CD4^{+} T cells (45). Nevertheless, histological analysis at the time of rejection showed that grafts from CD8^{+} T cell depleted LAG3$^{-/-}$ recipients had significant damage to the kidney tubules such as tubular dilation, casts, tubular atrophy, and edema. C4d staining of these grafts showed dilated capillaries, endothelial cell swelling, and heavy damage to the tubules and to the glomeruli (**Fig. 5E & Fig. S8**). In summary, the grafts rejected by LAG3$^{-/-}$ recipients following CD8^{+} T cell depletion displayed the typical features of antibody mediated graft damage similar to or even exceeding those observed in non-depleted LAG3-deficient recipients.

To test the roles of B cells and antibodies in the observed rejection, LAG3$^{-/-}$ recipients of C3H kidney allografts were treated with anti-mouse CD19 and B220 B cell depleting antibodies starting

on d. 3 posttransplant and throughout the experiment (**Fig. 5F & Fig. S7A**). Remarkably, B cell depletion restored graft survival in the majority of LAG3$^{-/-}$ recipients (**Fig. 5G**). Depletion of recipient B cells lead to the abrogation of the DSA responses in both WT and LAG3$^{-/-}$ recipients (**Fig. 5H**). While B cell depletion reduced T cell alloresponses in both groups, LAG3$^{-/-}$ recipients still had increased frequencies of donor-reactive IFNγ producing T cells compared to WT (**Fig. 5I & Fig. 3D**). Histological analysis at d. 30 posttransplant showed that recipient B cell depletion reduced allograft damage with marked decrease in T cell infiltrates and antibody binding, demonstrated by the absence of C4d staining (**Fig. 5J & Fig. S9**). Together, these results demonstrate that the rejection observed in LAG3$^{-/-}$ mice is driven by B cell production of DSA and not by cytotoxic T cells, and that the generated DSA is a major effector mechanism of graft injury.

LAG3 deficiency on both T and B lymphocytes is required for kidney allograft rejection.

The absence of LAG3 on T cells often induces pathogenesis in other mouse models (14). To address whether increased T cell help or defective T regulatory cell function was a major driver of the observed allograft injury, we used T cell conditional knockout mice and littermate controls as renal allograft recipients (**Fig. 6A**). B6.CD4Cre$^{+/-}$LAG3$^{fl/fl}$ which lack LAG3 on CD4^{+} and CD8^{+} T cells (**Fig. S10B**) spontaneously accepted kidney allografts for > 60 days (**Fig. 6B**) indicating that LAG3 deficiency on T cells alone was not sufficient to induce rejection. Analysis of the immune response in these recipients showed elevated DSA responses (**Fig. 6C**), but no significant changes in the frequencies of IFNγ producing alloreactive T cells (**Fig. 6D**). Histological analysis of the allografts at four weeks posttransplant revealed no major impact on fibrosis or graft tissue injury Interestingly, despite the presence of serum DSA, T cell LAG3 deficiency resulted in

reduced C4d deposition within interstitial capillaries compared to LAG3$^{-/-}$ recipients (**Fig. 6E, Fig. S11, & Fig. 2D**).

We next investigated the role of LAG3 using a B cell conditional knockout recipient model (**Fig. 6F & Fig. S10**). Unexpectedly, B6.CD19Cre$^{+/-}$LAG3$^{fl/fl}$ recipients with specific LAG3 deficiency in B lymphocytes also failed to reject kidney allografts by d. 30 posttransplant (**Fig. 6G**). LAG3 deficiency on B cells of renal transplant recipients resulted in a modest increase in DSA levels by d14 posttransplant (**Fig. 6H**). In contrast, LAG3 deficiency on B cells had no impact on the frequencies of donor reactive T cells at d14 posttransplant (**Fig. 6I**), indicating that B cell LAG3 expression does not affect T cell priming. Graft histology at four weeks posttransplant showed no significant differences in T cell infiltration. C4d staining was less intense in the CD19Cre$^{+/-}$LAG3$^{fl/fl}$ recipients compared to the littermate controls but showed a rarefaction of peritubular capillaries, and endothelial cell swelling consistent with antibody mediated graft injury (**Fig. 6J** and **Fig. S12**) (46). Taken together these data show that LAG3 expression on either helper T cells or B cells is sufficient to induce spontaneous acceptance of murine kidney allografts.

We next tested whether the loss of LAG3 on both T and B lymphocytes was sufficient to drive allograft rejection, or if another cell type was contributing to the rejection phenotype. To generate recipients in which both T and B cells lack LAG3 expression, RAG1$^{-/-}$ mice were lethally irradiated and injected with a mixture of 9x10^6 RAG1$^{-/-}$ and 1x10^6 WT or LAG3$^{-/-}$ bone marrow cells (**Fig. S13A**). 6 weeks after adoptive transfer, the reconstituted RAG1$^{-/-}$ chimeras received C3H kidney transplants. Successful generation of mice in which only T and B cells lack LAG3 was confirmed by PCR (**Fig. S13**). As with systemic LAG3$^{-/-}$ mice, the double conditional LAG3$^{-/-}$ had increased splenic cellularity (**Fig. 1** and data not shown). The control chimeric mice mimicked the WT recipient survival phenotype with 100% of mice surviving to 56 days (**Fig. S13A**), while 60% of double T and B cell conditional LAG3$^{-/-}$ chimeric recipients rejected between day 28-39

posttransplant. Transplant recipients with the double T and B cell LAG3 deficiency also demonstrated a trend toward increased graft damage as measure by serum BUN (**Fig. S13D**) and urine KIM1 (**Fig. S13E**). Analysis of immune responses showed no difference in serum DSA at day 21 posttransplant (**Fig. S13F**), but a significant increase in the frequency of donor reactive T cells from double T and B cell conditional LAG3$^{-/-}$ chimeric recipients (**Fig. S13G**). Together these data suggest that LAG3 expression on T and B cells is required to regulate alloimmune responses, and that the absence of LAG3 in these subsets alone is sufficient precipitate kidney allograft rejection.

Recipient LAG3 deficiency results in increased accumulation and effector functions of NK cells in renal allografts.

LAG3 was first discovered in NK cells (1), and the absence of LAG3 from NK cells may render them hyperresponsive. The critical role for NK cells during antibody-mediated tissue injury of renal allografts was recently demonstrated by Yagisawa and colleagues (47). Consistent with these findings, LAG3$^{-/-}$ recipients had increased infiltration of NK cells, predominantly of the NK1.1^{hi} phenotype, into the graft (**Fig. 7A** and data not shown). We next examined the effect of LAG3 deficiency on NK cell proinflammatory effector functions. LAG3$^{-/-}$ transplant recipients have elevated numbers of NK cells expressing degranulation marker CD107a at d. 10 posttransplant compared to WT controls (**Fig. 7B & C**). We also found a significant increase in the numbers of IFNγ producing NK cells, and a trend toward increased NK cell perforin and granzyme B production in LAG3$^{-/-}$ vs WT recipients (**Fig. 7D & E and Fig. S14**). Taken together our findings suggest that LAG3 regulates functions of several lymphocyte subsets at different stages of antibody-mediated rejection from DSA generation to graft tissue injury.

LAG3 regulates antibody responses to both T dependent and independent antigens.

To understand mechanistic aspects of LAG3 regulation of antibody responses, we next tested the impact of LAG3 deficiency on T cell dependent vs independent antibody responses. WT or LAG3$^{-/-}$ mice were immunized with T-dependent antigen 4-Hydroxy-3-nitrophenylacetyl (NP)-Keyhole Limpet Hemocyanin (NP-KLH) mixed with Alum adjuvant or T-independent antigen NP-AminoEthylCarboxtyMethyl-FICOLL (NP-FICOLL). No difference in anti-NP IgG was found between WT and LAG3$^{-/-}$ mice prior to immunization. In contrast, increased levels of low and high affinity anti-NP IgG was detected in LAG3$^{-/-}$ mice compared to WT controls at d. 14 after immunization with either T-dependent or T-independent antigen (**Fig. 8A-D**). 21 days after immunization with NP-KLH, LAG3$^{-/-}$ mice had modest increases in NP-specific GCBs and memory B cells compared to WT controls (**Fig. S15B**). No increase in splenic NP-specific plasma cells was observed at this timepoint (**Fig. S15B**). To assess the contribution of B cell LAG3 to antibody production in this model, immunization with NP-KLH or NP-FICOLL was performed in CD19CreLAG3$^{fl/fl}$ mice. Analogous to our findings in conditional knockout renal transplant recipients (**Fig. 6**), CD19CreLAG3$^{fl/fl}$ mice had only a modest increase in anti-NP serum antibody levels following immunization with T dependent (**Fig. 8E&F**) and T independent (**Fig. 8G&H**) antigens relative to littermate controls, and no significant changes were observed in various NP-specific splenic B cell populations (**Figs. S15C & S16C**). These data support the hypothesis that LAG3 mediated regulation of antibody responses is intrinsic to both T cells and B cells. The major T cell subsets influencing antibody production are TFh, TFreg and Treg cells. We saw no significant change in the production of IL-21, IL-4 or IL-6 by LAG3$^{-/-}$ vs WT TFh cells at d. 10 after immunization with NP-KLH (**Fig. 8I & Fig. S17A**). Furthermore, we observed only a slight decrease in IL-10 production by both Tregs and TFregs in LAG3$^{-/-}$ mice (**Fig. 8J & Fig. S17B & Fig. S17C**), suggesting changes in IL-10 production do not drive the observed increase in

antibody responses. One of the mechanisms by which TFreg cells regulate antibody responses is by suppressing cytotoxic TFh cells (48). In support of this possibility, there was a trend, albeit without reaching statistical significance, toward decreased granzyme B production in the LAG3$^{-/-}$ mice (**Fig. 8I & Fig. S17A**).

Plasma cell LAG3 regulates antibody production.

The intrinsic role of LAG3 on B cells is poorly understood. As B cells can serve as antigen presenting cells we sought to address whether LAG3 regulated antigen presentation by B cells, using complementary *in vitro* and *in vivo* approaches. First, purified B cells from WT and LAG3$^{-/-}$ mice were stimulated for 24 hours with either anti-IgM/anti-CD40 mAbs or with CpG/IL4/IL-5 combination (**Fig. 9A**). In these settings, the absence of LAG3 in B cells resulted in increased expression of costimulatory molecules CD40, CD80, and CD86 as well as class I and class II MHC molecules (**Fig. 9A**). However, we confirmed whether this impacted antigen presentation by using WT or LAG3$^{-/-}$ B cells as stimulator cells in an allogeneic T cell ELISPOT assay. LAG3$^{-/-}$ B cells were equally as capable APCs as WT B cells, eliciting an allogenic IFNγ response equal in magnitude (**Fig. 9B**). Finally, we assessed the splenic B cells in vivo 10 days after immunization with NP-KLH. In contrast to in vitro stimulation experiments, there was no significant difference between WT and LAG3$^{-/-}$ mice in the expression of CD40, CD80, CD86 and MHC-II in TrBs, MZBs, FoBs, and GCBs (**Fig. 9C**) indicating that possible changes in B cell APC functions are not likely to account for increased humoral immune responses.

Recent studies demonstrated that LAG3^{+} plasma cells represent an IL-10 producing natural B cell subset that can regulate antibody responses (27, 49). Consistent with these reports, we detected

LAG3 expression on plasma cells in renal allograft recipients (**Fig. 3E**). We investigated whether the absence of LAG3 results in the loss of IL-10 production by plasma cells. After immunization with NP-KLH, $LAG3^{-/-}$ mice demonstrated a significant reduction in the amount of IL-10 produced by plasma cells (**Fig. 9D**) and a modest decrease in the frequency of $IL\text{-}10^{+}$ splenocytes (**Fig. S17D**). We then tested whether LAG3 engagement affects antibody production by performing plasma cell ELISPOT measuring IgG production by preexisting PCBs from WT or $LAG3^{-/-}$ mice. To signal through LAG3, cells are pretreated with an anti-LAG3 antibody and a secondary cross-linking antibody, whereas controls were treated with secondary antibody only (**Fig. 9E & Fig. 9F**). LAG3 crosslinking (LAG3-XL) in WT splenocytes reduced the frequency of detectable IgG producing cells and the amount of IgG produced as measured by spot size (**Fig. 9E**). To rule out the possibility that the effect is mediated through other LAG3 expressing cells, such as Tregs, we repeated the assay using splenocytes from mice with B cell specific LAG3 deficiency. LAG3-XL had no impact on the frequency of IgG producing cells in $CD19Cre^{+/-}$ control littermates (**Fig.9F**). In absence of LAG3 on B cells alone, there was no effect of LAG3-XL on the frequency of IgG producing cells. It should be noted that in case of $CD19CreLAG3^{fl/fl}$ the frequency of IgG producing cells was higher than the CD19Cre control counterparts (**Fig. 9F**). LAG3-XL significantly reduced the amount of IgG produced per cell by CD19Cre controls, but had no impact on $CD19CreLAG3^{fl/fl}$ cells, further confirming that the effects of LAG3 engagement are plasma cell dependent (**Fig. 9F**). These findings indicate that LAG3 can regulate B cells in an intrinsic manner and that providing agonistic signals through LAG3 can diminish antibody production by pre-existing plasma cells.

Discussion

Coinhibitory molecule LAG3 is best studied in regulating effector and regulatory T cell functions in autoimmunity, infection and cancer (16-20, 50). However, little is known about the contribution of this pathway in regulating humoral immune responses. Our results definitively demonstrate that LAG3 regulates alloantibody generation in response to solid organ transplantation, and suggest that LAG3 expression on both T and B lymphocytes plays a role in this process.

The absence of LAG3 in T cells leads to enhanced effector T cell responses and increased formation of T cell memory (26). Consistent with this, naïve, non-transplanted LAG3-deficient mice have elevated levels of $CD44^{hi}$ memory T cells and enhanced response to a panel of alloantigens compared to wild type counterparts (**Fig. 1A**). In addition, we observed increased numbers of germinal center B cells and $CD138^{+}$ plasma cells in naïve 10-week-old $LAG3^{-/-}$ mice, which suggested ongoing B cell activation (**Fig. 1B**). Based on previous studies, we initially proposed that LAG3 deletion in the recipient will result in dysregulated alloimmunity characterized by exaggerated activation of alloreactive T cells and leading to T cell-mediated rejection.

The model of renal transplantation was chosen for these experiments as even fully MHC mismatched mouse kidney allograft typically do not undergo acute rejection, and are spontaneously accepted in many donor/recipients strain combinations (51). While functional C3H kidney allografts survive for > 60 days in B6.WT recipients, they are rapidly rejected by $B6.LAG3^{-/-}$ mice (**Fig. 2**). Long term survival and function of mouse renal allografts have been previously correlated with the numbers of graft-infiltrating regulatory T cells, as $FoxP3^{+}$ regulatory T cell depletion results in rapid graft rejection (52, 53). As anticipated, donor reactive T cell responses were increased in the absence of LAG3 (**Fig. 3D**), but strikingly donor specific antibody responses

were also elevated (**Fig. 3C**). Given that we demonstrated LAG3 is expressed by TF cells and PCBs (**Fig. 3E**), it suggested that dysfunction in these cells could result in enhanced humoral responses.

However, as stated previously, mice with systemic deficiencies in coinhibitory receptors have an array of immune dysfunction phenotypes (54-56). Our data utilizing LAG3 blockade in kidney transplant recipients (**Fig. 4**), supports the notion that LAG3 regulates de novo priming and the observed dysregulation in immune responses is not due to a loss in central tolerance in the systemic knockout mice.

It is important to note that, treatment of WT B6 recipients with a LAG3 blocking antibody induced a pathology consistent with chronic rejection (**Fig. 4**). This was evident in the increased kidney injury markers in the urine and serum (**Fig. 4C-E**), increased serum DSA levels (**Fig.4F&G**), and increased histological signs of injury (**Fig. 4I** and **Fig. S6**). There are a paucity of small animal chronic injury/rejection models in kidney transplantation, with some of the current models relying on repeated administration of anti-MHC-I antibodies (57), or repeat transfers of anti-donor antibody containing sera (58, 59). Our findings thus identify a new clinically relevant model for studying the mechanisms of chronic injury and graft rejection in renal transplantation.

In contrast to germline LAG3$^{-/-}$ mice, T cell specific LAG3 deletion did not result in acute graft rejection (**Fig. 6**). LAG3 is highly expressed by FoxP3^{+} Tregs and LAG3 is thought to play a role in regulatory T cell's optimal suppressor activity (5, 60, 61). Previous studies demonstrated that FoxP3 regulatory T cell depletion in mouse renal allograft recipients resulted in T cell mediated rejection without affecting DSA generation (53). Moreover, LAG3$^{-/-}$ mice have increased numbers of FoxP3^{+} T cells before and after transplantation (**Fig. 1 & Fig. 2**). However, it is unlikely that the augmented alloimmunity and graft rejection in our model are entirely due to dysfunctional Tregs

as specific deletion of LAG3 in recipient T cells (including Tregs) was not sufficient to induce rejection (**Fig. 6A-E**) (53).

While LAG3 expression in B cells was reported in 2005 (2), its contribution to their functions has not been extensively studied. Our results demonstrate that the lack of recipient LAG3 leads to increased titers of IgG DSA antibodies and allograft rejection with characteristic features of antibody-mediated injury. Furthermore, depletion experiments confirmed that B cells are required for acute rejection of kidney allografts (**Fig. 5F-J**), whereas the rejection still occurred with the same kinetics in CD8 T cells-depleted LAG3$^{-/-}$ recipients (**Fig. 5A-E**). To date, there are only a few studies addressing the role of LAG3 in humoral immune response. Butler and colleagues (62), reported that the combination treatment with anti-PD-L1 and anti-LAG3 mAb during established malaria enhances TFh responses, plasma cell formation and protective antibody generation in a mouse model of malaria. Our results investigating antibody production following immunizations offer key insights into the mechanisms of regulation (**Fig. 8 & Figs. S15-17**). Notably, the absence of LAG3 systemically, resulted in increased antibody responses to both T-dependent (**Fig. 8A-B**) and T-independent antigens (**Fig. 8C-D**), despite the absence of increased NP-specific plasma cells (**Figs. S15B & S16B**) suggesting the loss of LAG3 did not drive proliferation of antigen specific plasma cells, but rather increased antibody production by plasma cells. This finding was corroborated by our *in vitro* studies showing that cross-linking of LAG3 on plasma cells diminished the amount of antibody produced by individual cells, while only modestly diminishing the frequency of antibody secreting cells (**Figs. 9E & F**).

While a novel subset of LAG3^{+} IL-10 secreting plasma cells with regulatory properties has been recently reported (27), the requirement of LAG3 for IL-10 production remained to be tested. To this end, we found that IL-10 production by plasma cells was lost in immunized LAG3$^{-/-}$ mice, suggesting LAG3 signaling on plasma cells induces the production of IL-10. Further studies are

ongoing in our laboratory to determine the relative importance and the molecular mechanisms of regulation by LAG3 on helper T cells vs regulatory plasma cells during the initiation of B cell activation, germinal center formation and immunoglobulin secretion.

To understand whether LAG3 regulation of pathogenic responses leading to ABMR is entirely T and B cell dependent we used a bone marrow chimera system to generate double conditional $LAG3^{-/-}$, wherein only T and B cell lineages do not express LAG3 (**Fig. S13**). These recipients demonstrated no increased DSA responses (**Fig. S13F**), but did demonstrate increased T cell responses (**Fig. S13G**), likely due to a shift in immune cell composition following reconstitution (**Fig. S13H & I**). Kidney transplants in these recipients were rejected demonstrating that the absence of LAG3 on both T and B cells was sufficient to precipitate rejection.

Our study does not entirely rule out the contribution of LAG3 expressed by cells other than T and B lymphocytes. NK cells express LAG3 and are important mediators of antibody-mediated injury of renal allografts (47). While the effects of LAG3 on various NK cell functions is highly controversial (reviewed in (63)), it is possible that dysregulated NK cells mediate rejection in $LAG3^{-/-}$ recipients, secondary to enhanced T cell activation and DSA generation. Our findings support this, in that NK cells contribute toward rejection as $LAG3^{-/-}$ NK cells have increased degranulation and IFNγ production suggestive of an increased cytotoxic state (**Fig. 7B&C**) and increased IFNγ production (**Fig. 7D&E**). Indeed, this corroborates findings that show the loss of LAG3 signaling in human NK cells leads to increased cytokine production (64). Taken together these findings indicate that LAG3 on NK cells could be a target for future agonistic therapies (18), in mitigating the effector phase of the alloantibody response, which could have downstream impacts on fibrosis progression and ultimately graft outcomes.

The absence of LAG3 on recipient antigen presenting cells (APCs) may also contribute to elevated T cell responses. Initial analyses of LAG3 expression in dendritic cell (DC) subsets showed high LAG3 expression by plasmacytoid but not lymphoid or myeloid conventional DCs (6). However, a more recent study demonstrated a role for LAG3 on bone marrow derived DCs in optimal T cell priming (65). Due to these controversies, the impact of LAG3 deficiency in recipient APCs needs to be carefully dissected in future studies.

To our knowledge, our study is one of the first to address the role of LAG3 during alloimmune responses. Lucas *et al*. reported that blocking LAG3 with mAb prevents tolerization of CD8 T cells following allogeneic bone marrow transplantation in mice (36). In an earlier study, the investigators used depleting anti-LAG3 mAb as an induction therapy in a rat model of cardiac transplantation. Interestingly, the treatment extended heart allograft survival, yet abrogated the tolerogenic effects of donor specific cell transfusion, which the authors attributed to Treg depletion (37).

Despite many gaps and controversies in LAG3 biology, it is a molecule of therapeutic interest, particularly in the cancer field. Antagonistic antibodies such as Relatlimab are given to patients in combination with anti-PD-1 therapy in phase II clinical trials (66). Another promising reagent undergoing phase III trials is a bispecific antibody against LAG3 and PD-1, MGD013(28). Anti-LAG3 depleting antibodies have been developed and validated in non-human primates to target overactivated immune cells during undesired immune response (67). An agonistic anti-LAG3 antibody (IMP761) is currently under development for T cell mediated autoimmunity, and this approach could have potential for clinical transplantation (18).

In conclusion, our study demonstrates for the first time the importance of LAG3 in regulating pathogenic humoral immune responses to a transplanted solid organ. The results provide

524 rationale for investigating LAG3 impact on B cell activation, survival and differentiation, and
525 suggest LAG3 as a potential target for future therapeutic interventions for the prevention and
526 treatment of antibody-mediated rejection.
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Methods

Sex as a biological variable

Due to technical challenges in female recipients, we predominantly used male mice for our studies. However, we examined male and female animals, and similar findings were found for both sexes.

Experimental Methods

For extended experimental methods, please refer to extended methods in the supplement.

Statistical Analyses

Kidney allograft survival was compared between groups by Kaplan-Meier analysis. Other results were analyzed by using a parametric unpaired t-test (two-tailed), one way ANOVA with Tukey's multiple comparisons test, or multiple unpaired T tests with Benjamini, Krieger and Yekutieli false discovery approaches. The difference between groups was considered significant if the p value was <0.05. Unless noted otherwise, the data are presented as mean ± SD values. Total numbers of animals in each experimental group are indicated in respective figure legends.

Study Approval

All animal studies were conducted on mice between 8 and 12 weeks of age and were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

Data Availability

All data generated in this study are available through the Supporting data values file.

Author Contributions

Design of research study: MN, JL, WMB, EC, MA, RLF, BG, BM & AV

Conducting Experiments: MN, RF, JL, GA, VG, JIV, AB, & ND

Acquiring data: MN, JL, VG, JIV, & AB

Analyzing data: MN, JL, VG, JIV, & WMB

Providing reagents/mice: BM

Writing the manuscript: MN, WB, BM, & AV

Acknowledgements

Graphics were created with Biorender.com.

This work was supported by funding from the American Society of Transplantation (gCDX-221C0MN - MN), The Sheikha Fatima bint Mubarak Global Center for Pathogen and Human Health Research at the Cleveland Clinic (MN), and the National Institutes of Health (R01AI165513 – WMB, R01AI-74740 – RLF, R01AI125247 – BM, R01AI60740 & R01AI113142-AV).

Figures and Figure Legends

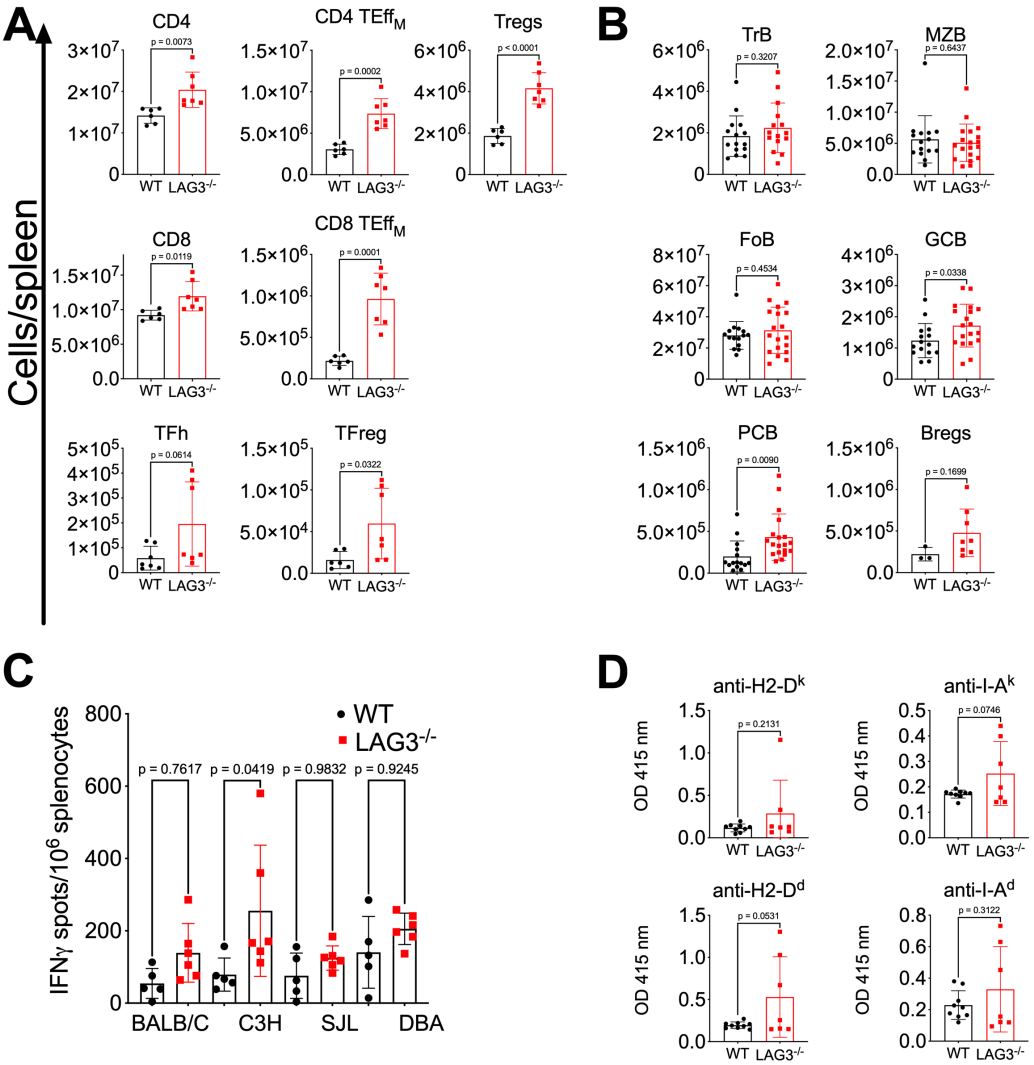


Figure 1. LAG3 deficient mice had expanded lymphocyte subsets and a modest increase in alloreactivity. Naïve non-transplanted B6.WT and B6. LAG3$^{-/-}$ mice were sacrificed at 10 weeks of age. **A**. Splenic T cell composition: CD4 (CD3$^+$CD4$^+$), CD8 (CD3$^+$CD8$^+$), Tregs (CD3$^+$CD4$^+$Foxp3$^+$), CD4 TEff$_M$ (CD3$^+$CD4$^+$CD62L^{lo}CD44^{hi}), CD8 TEff$_M$ (CD3$^+$CD8$^+$CD62L^{lo}CD44^{hi}), TFh (TCRb$^+$CD4$^+$FoxP3$^-$PD-1$^+$CXCR5$^+$), TFreg (TCRb$^+$CD4$^+$FoxP3$^+$PD-1$^+$CXCR5$^+$). **B**. The composition of splenic B cell subsets: FoB (B220$^+$IgM^{int}CD21/35^{int}), MZB (B220$^+$IgM^{hi}CD21/35^{hi}), TrB (B220$^+$IgM^{hi}CD21/35^{lo}), Bregs (CD19$^+$CD1d^{hi}CD5$^+$), GCB (B220$^+$GL7$^+$CD38^{lo}), PC (B220$^-$CD138^{hi}). **C**. ELISPOT quantification of the frequencies of alloreactive IFNγ-secreting splenic T cells in naïve non-transplanted B6.WT and B6. LAG3$^{-/-}$ mice against, BALB/c, C3H, SJL and DBA stimulator cells. **D**. ELISA of serum IgG reactive to allogenic MHC-I and II molecules. The data represents at least two pooled experiments where each symbol represents an individual mouse. Analysis of ELISPOT data utilized one way ANOVA with Tukey's multiple comparisons test, all others used student's T tests and for all analyses $p<0.05$ were considered significant.

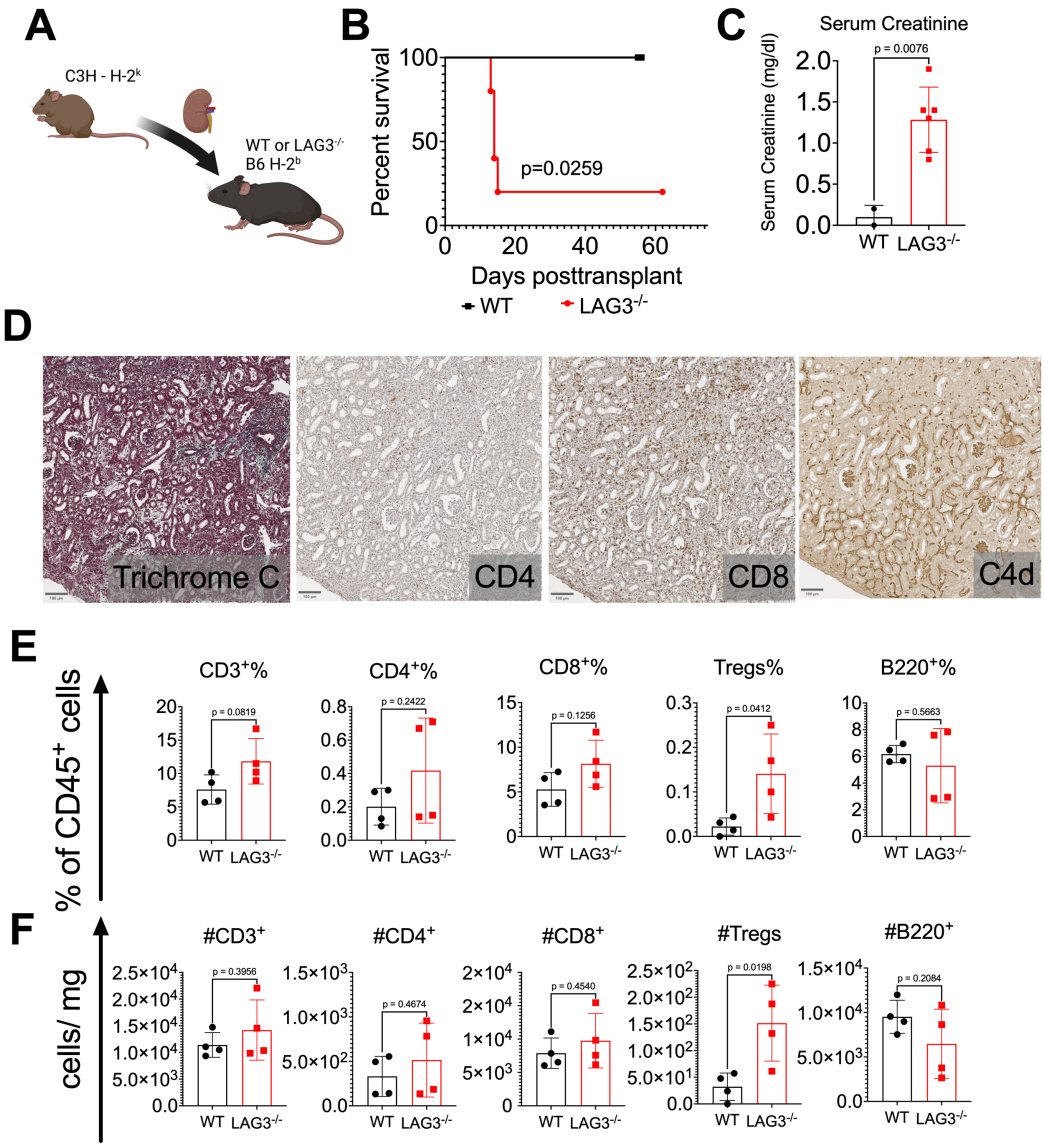


Figure 2. LAG3-deficient recipients acutely reject kidney allografts. Groups of B6.WT and B6.LAG3$^{-/-}$ were transplanted with complete major histocompatibility complex-mismatched C3H kidney allografts (n=4-5/group). **A**. Kidney Transplant Model. **B.** Renal allograft survival. **C.** Serum creatinine levels at d. 14 posttransplant. **D.** Renal allografts analyzed at the time of rejection (B6.LAG3$^{-/-}$) or on d. 14 posttransplant (B6.WT) by Trichrome C and immunoperoxidase staining for CD4, CD8 and complement component C4d. The photographs were taken at 200x and are representative of 4-5 animals in each group. **E & F.** Flow cytometry analysis of graft infiltrating immune cells: CD3$^+$ (CD45$^+$ CD3$^+$), CD4$^+$ (CD45$^+$ CD3$^+$ CD4$^+$), CD8$^+$ (CD45$^+$ CD3$^+$ CD8$^+$), Tregs (CD45$^+$, CD3$^+$ CD4$^+$ FoxP3$^+$), and B220$^+$ (CD45$^+$ B220$^+$) at d10 following transplantation of C3H kidney allografts to B6.WT or B6.LAG3$^{-/-}$ recipients. The data represents one of two experiments where each symbol represents an individual mouse. Statistical analysis of allograft survival was measured using Mantel-Cox log-rank test and for other analyses student's T tests were performed and $p<0.05$ were considered significant.

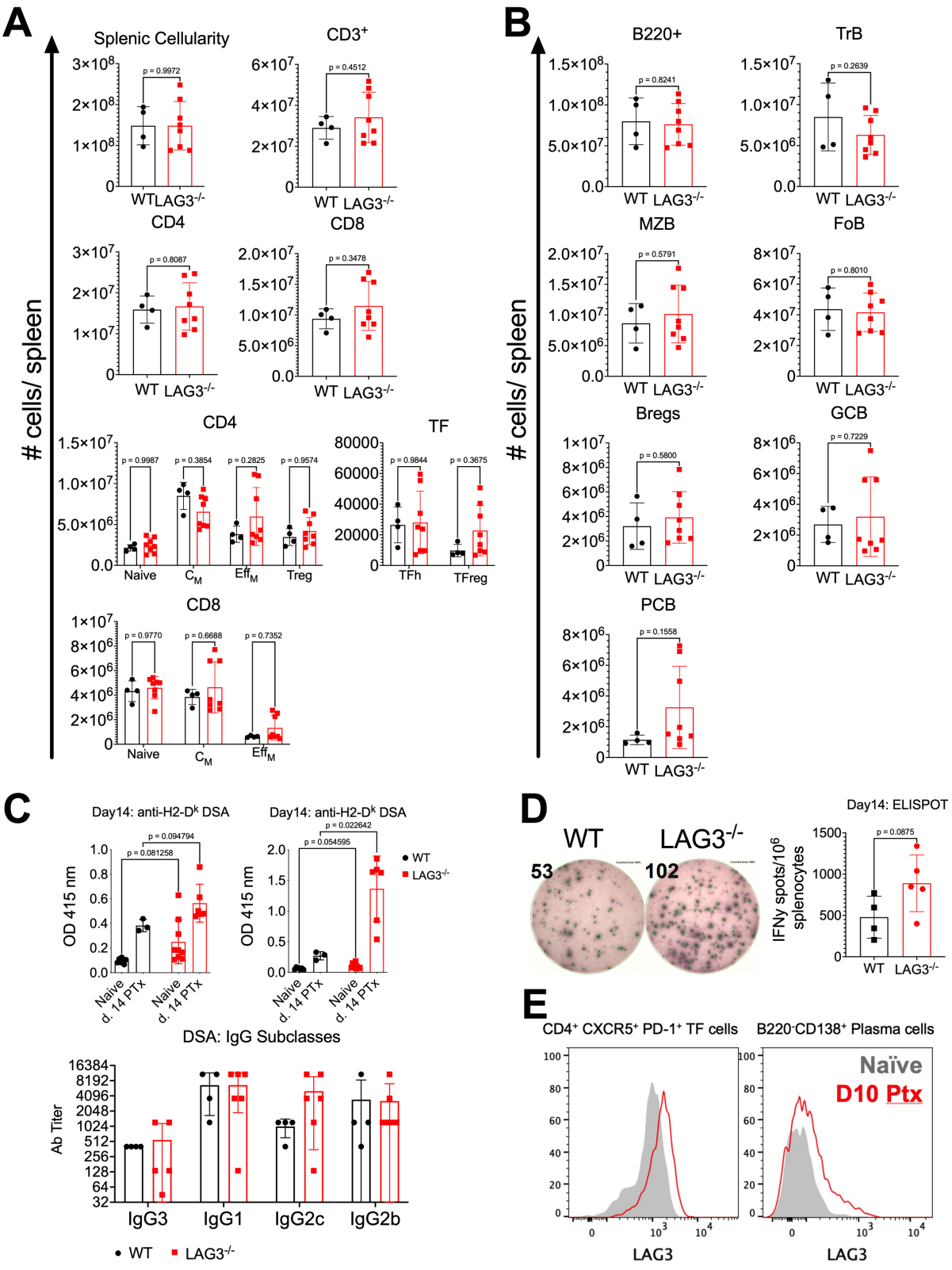


Figure 3. Recipient LAG3 deficiency enhances anti-donor immune responses. Analyses of donor-reactive immunity in B6.WT and B6.LAG3$^{-/-}$ allograft recipients were performed at d. 10 posttransplant. **A.** The composition of spleen T cell subsets was

defined as follows: CD3 – $CD3^+$, CD4 – $CD3^+CD4^+$, CD8 - $CD3^+CD8^+$, Tregs - $CD3^+CD4^+$ $FoxP3^+$, CD4 Naïve - $CD3^+CD4^+CD62L^{hi}CD44^{lo}$, CD4 C_M - $CD3^+CD4^+CD62L^{hi}CD44^{hi}$, CD4 Eff_M - $CD3^+CD4^+CD62L^{lo}CD44^{hi}$, CD8 Naïve - $CD3^+CD8^+CD62L^{hi}CD44^{lo}$, CD8 C_M - $CD3^+CD8^+CD62L^{hi}CD44^{hi}$, CD8 Eff_M - $CD3^+CD8^+CD62L^{lo}CD44^{hi}$, TFh - $TCRb^+CD4^+FoxP3^-PD\text{-}1^+CXCR5^+$ and TFreg - $TCRb^+CD4^+FoxP3^+PD\text{-}1^+CXCR5^+$. **B.** The composition of splenic B cell was defined as follows: B220 – $B220^+$, FoB - $B220^+IgM^{int}CD21/35^{int}$, MZB - $B220^+IgM^{hi}CD21/35^{hi}$, TrB - $B220^+IgM^{hi}CD21/35^{lo}$, Bregs - $CD19^+CD1d^{hi}CD5^+$, GCB - $B220^+GL7^+CD38^{lo}$, PCB - $B220^-$ $CD138^{hi}$. **C.** Top - Serum levels of IgG against donor MHC-I ($H2\text{-}D^k$) and MHC-II ($I\text{-}A^k$). Bottom – IgG subclass analysis of serum titers of IgG3, IgG1, IgG2c and IgG2b from WT and LAG3-/- recipients at d. 14 posttransplant. The data are pooled from two-three experiments, and each symbol represents an individual mouse. **D.** The frequencies of donor reactive IFNγ-secreting splenocytes on d. 14 posttransplant. **E**. Representative histograms of LAG3 expression by $CD4^+CXCR5^+PD\text{-}1^+$ follicular T cells and $B220^-CD138^+$ plasma cells. Analysis of DSA responses utilized multiple unpaired T tests with Benjamini, Krieger and Yekutieli false discovery approaches. For all other analysis, student's T tests were performed and for all analyses $p<0.05$ were considered significant.

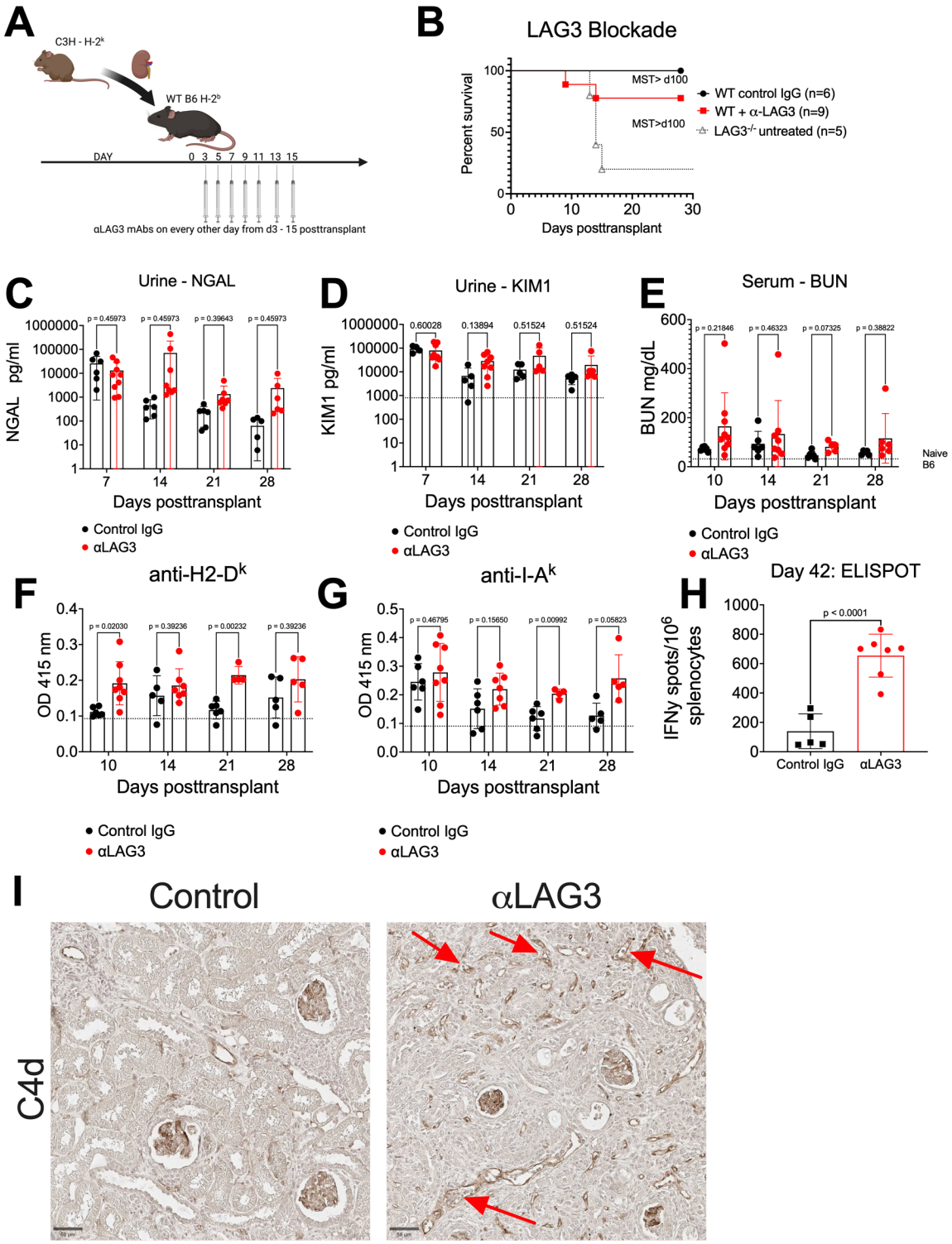
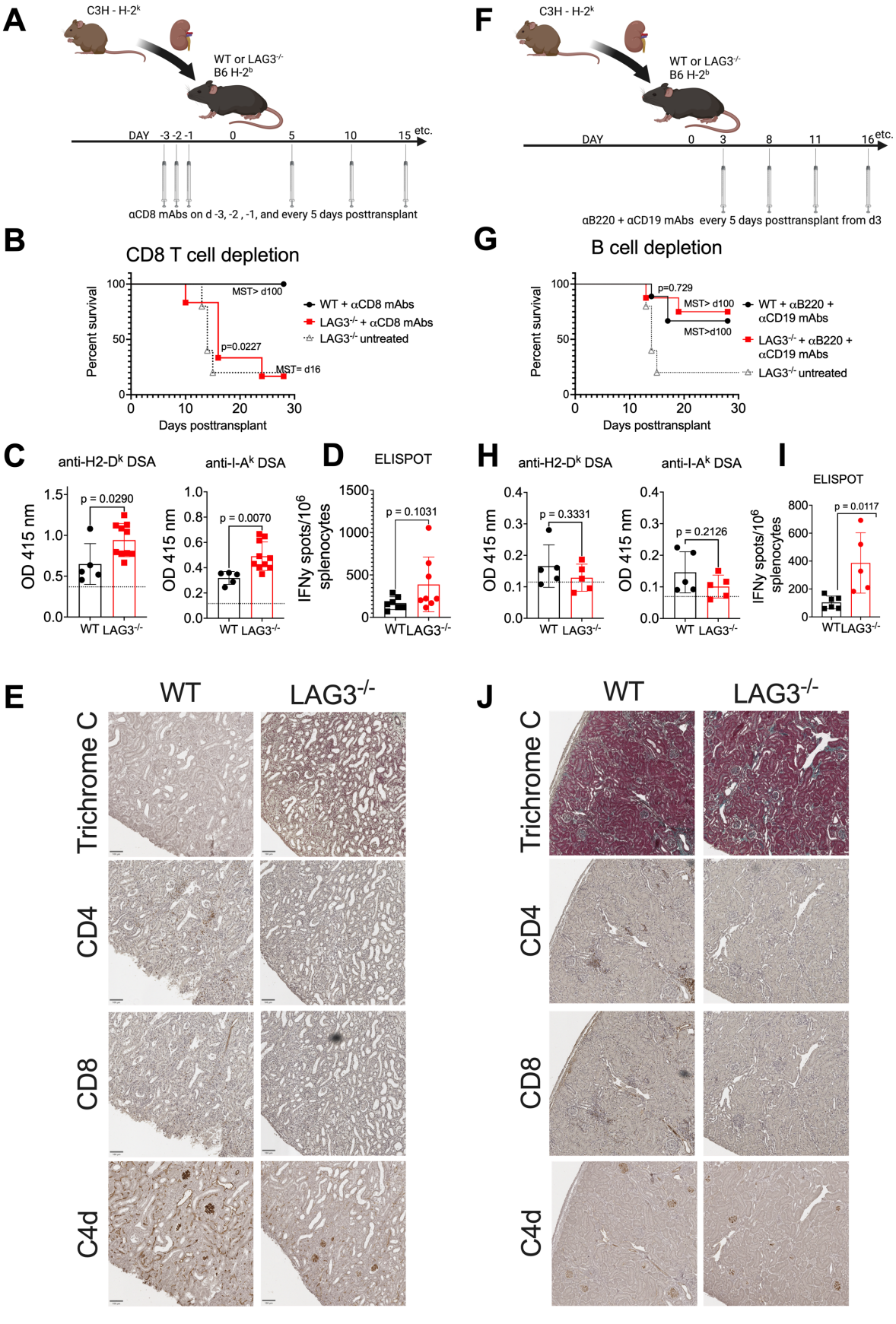


Figure 4. LAG3 blockade enhances de novo alloresponses following kidney transplantation leading to chronic antibody mediated graft injury. A. B6.WT treated with anti-LAG3 mAb (Clone C9B7W) or control IgG after transplantation of C3H renal allografts. **B.** Survival of renal allografts (n=6-9/group). **C-D**. NGAL and KIM1 levels in the urine collected from kidney allograft recipients. **E**. Serum Blood Urea Nitrogen (BUN)

levels. **F-G.** Serum levels of IgG against donor MHC-I ($H2\text{-}D^k$) and MHC-II ($I\text{-}A^k$). **H.** The frequencies of donor reactive IFNγ-secreting splenocytes on d. 42 posttransplant. **I.** Renal allografts harvested on d. 42 posttransplant and analyzed by immunoperoxidase staining for complement component C4d. Images were taken at 400x and are representative of 4-5 animals in each group. The data are pooled from two-three experiments, and each symbol represents an individual mouse. Statistical analysis of allograft survival was measured using Mantel-Cox log-rank test, for the time-course analysis of kidney injury markers and for DSA one-way ANOVA with Tukey's multiple comparison was performed and for ELISPOT analysis student's T tests were performed and $p<0.05$ were considered significant.


A
C3H - H-2k
WT or LAG3-/-
B6 H-2b
DAY -3 -2 -1 0 5 10 15 etc.
αCD8 mAbs on d -3, -2 , -1, and every 5 days posttransplant
B
CD8 T cell depletion
Percent survival
Days posttransplant
MST> d100
p=0.0227
MST= d16
WT + αCD8 mAbs
LAG3-/- + αCD8 mAbs
LAG3-/- untreated
C
anti-H2-Dk DSA
anti-I-Ak DSA
OD 415 nm
p = 0.0290
p = 0.0070
WT LAG3-/-
D
ELISPOT
IFNγ spots/10^6 splenocytes
p = 0.1031
F
C3H - H-2k
WT or LAG3-/-
B6 H-2b
DAY 0 3 8 11 16 etc.
αB220 + αCD19 mAbs every 5 days posttransplant from d3
G
B cell depletion
p=0.729
MST> d100
MST>d100
WT + αB220 + αCD19 mAbs
LAG3-/- + αB220 + αCD19 mAbs
LAG3-/- untreated
H
anti-H2-Dk DSA
anti-I-Ak DSA
p = 0.3331
p = 0.2126
I
ELISPOT
p = 0.0117
E
WT
LAG3-/-
Trichrome C
CD4
CD8
C4d
J
WT
LAG3-/-
Trichrome C
CD4
CD8
C4d

709

Figure 5. Kidney allograft rejection in LAG3 deficient recipients is dependent on B cells not CD8 T cells. A. B6.WT or B6.LAG3$^{-/-}$ recipients depleted of CD8^{+} T cells prior to transplantation of C3H renal allografts. **B.** Survival of renal allografts (n=5-6/group). **C.** Serum levels of IgG against donor MHC-I (H2-D^{k}) and MHC-II (I-A^{k}) in B6.WT and B6.LAG3$^{-/-}$ kidney allograft recipients. **D.** The frequencies of donor reactive IFNγ-secreting splenocytes on d. 14 posttransplant. **E.** Renal allografts harvested at the time of rejection and analyzed by Trichrome C and immunoperoxidase staining for CD4, CD8 and complement component C4d. Images were taken at 200x and are representative of 4-5 animals in each group. **F**. B cells were depleted in B6.WT or B6.LAG3$^{-/-}$ recipients after transplantation of C3H renal allografts. **G.** Survival of renal allografts (n=5-6/group). **H.** Serum levels of IgG against donor MHC-I (H2-D^{k}) and MHC-II (I-A^{k}). **I.** The frequencies of donor reactive IFNγ-secreting splenocytes on d. 14 posttransplant. **J.** Renal allografts harvested at the time of rejection and analyzed by Trichrome C and immunoperoxidase staining for CD4, CD8 and complement component C4d. Images were taken at 200x and are representative of 4-5 animals in each group. The data are pooled from two-three experiments, and each symbol represents an individual mouse. Statistical analysis of allograft survival was measured using Mantel-Cox log-rank test and for other analyses student's T tests were performed and $p<0.05$ were considered significant.

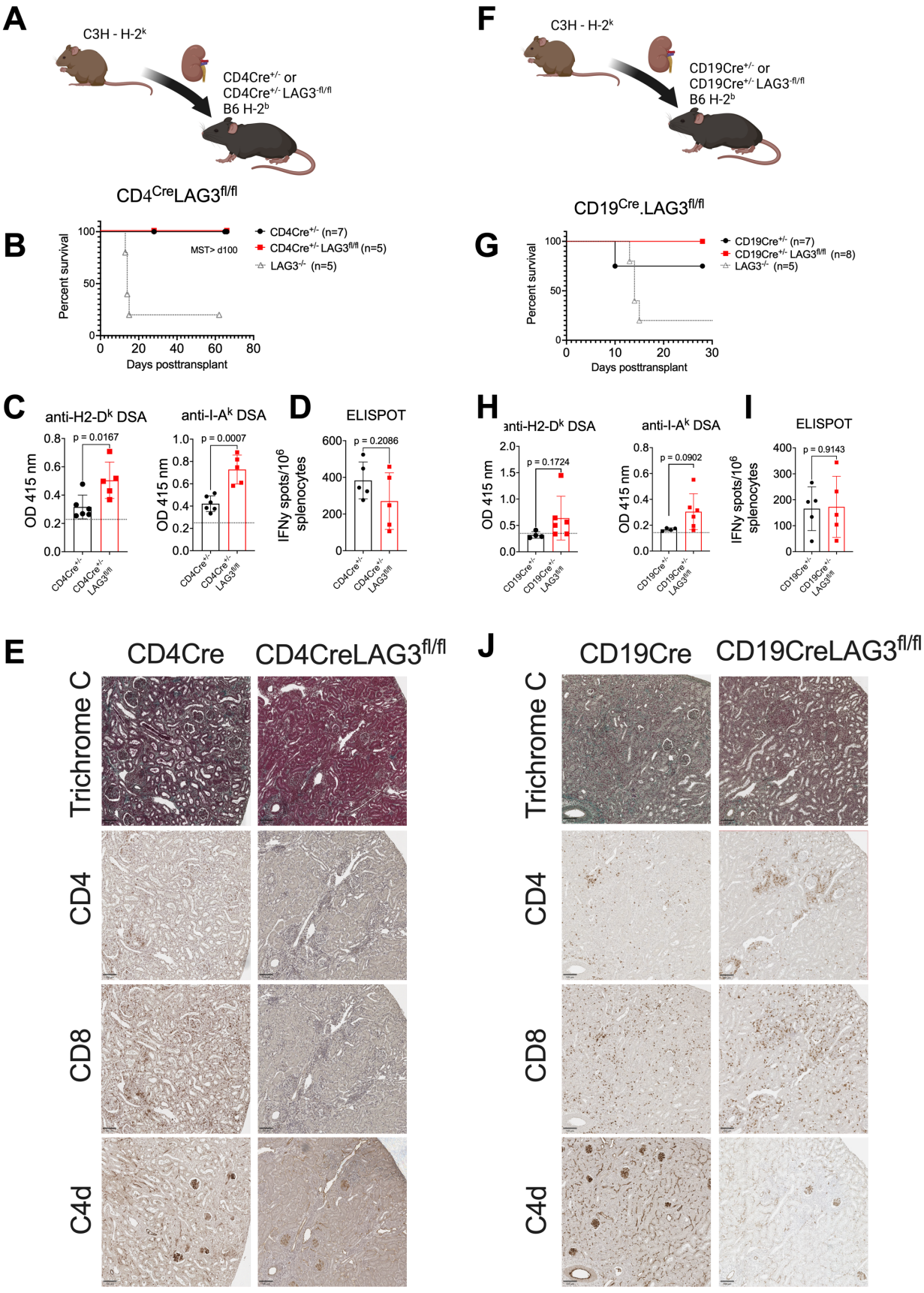

A
C3H - H-2k
CD4Cre+/- or
CD4Cre+/- LAG3-fl/fl
B6 H-2b
CD4CreLAG3fl/fl
B
Percent survival
Days posttransplant
MST> d100
CD4Cre+/- (n=7)
CD4Cre+/- LAG3fl/fl (n=5)
LAG3-/- (n=5)
C
anti-H2-Dk DSA
anti-I-Ak DSA
OD 415 nm
p = 0.0167
p = 0.0007
CD4Cre+/-
CD4Cre+/- LAG3fl/fl
D
ELISPOT
IFNγ spots/10^6 splenocytes
p = 0.2086
E
CD4Cre
CD4CreLAG3fl/fl
Trichrome C
CD4
CD8
C4d
F
C3H - H-2k
CD19Cre+/- or
CD19Cre+/- LAG3-fl/fl
B6 H-2b
CD19Cre.LAG3fl/fl
G
Percent survival
Days posttransplant
CD19Cre+/- (n=7)
CD19Cre+/- LAG3fl/fl (n=8)
LAG3-/- (n=5)
H
anti-H2-Dk DSA
anti-I-Ak DSA
OD 415 nm
p = 0.1724
p = 0.0902
CD19Cre+/-
CD19Cre+/- LAG3fl/fl
I
ELISPOT
IFNγ spots/10^6 splenocytes
p = 0.9143
J
CD19Cre
CD19CreLAG3fl/fl
Trichrome C
CD4
CD8
C4d

Figure 6. Loss of LAG3 expression on either T or B cells is not sufficient to mediate kidney allograft rejection. A. B6.CD4Cre$^{+/-}$ or B6.CD4Cre$^{+/-}$LAG3$^{fl/fl}$ recipients were transplanted with C3H renal allografts. **B.** Survival of renal allografts (n=5-7/group). **C.** Serum levels of IgG against donor MHC-I (H2-D^{k}) and MHC-II (I-A^{k}) in B6.CD4Cre$^{+/-}$ LAG3$^{fl/fl}$ or B6.CD4Cre$^{+/-}$ littermate control kidney allograft recipients. **D.** The frequencies of donor reactive IFNγ-secreting splenocytes on d. 14 posttransplant. **E.** Renal allografts harvested at the time of rejection and analyzed by Trichrome C and immunoperoxidase staining for CD4, CD8 and complement component C4d. **F.** B6.CD19Cre$^{+/-}$ or B6.CD19Cre$^{+/-}$LAG3$^{fl/fl}$ recipients were transplanted with C3H renal allografts. **G.** Survival of renal allografts (n=7-8/group). **H.** Serum levels of IgG against donor MHC-I (H2-D^{k}) and MHC-II (I-A^{k}) in B6.CD19Cre$^{+/-}$ littermate controls or B6.CD19Cre$^{+/-}$LAG3$^{fl/fl}$ kidney allograft recipients. **I.** The frequencies of donor reactive IFNγ-secreting splenocytes on d. 14 posttransplant. **J.** Renal allografts harvested at the time of rejection and analyzed by Trichrome C and immunoperoxidase staining for CD4, CD8 and complement component C4d. Images were taken at 200x and are representative of 4-5 animals in each group. The data are pooled from two-three experiments, and each symbol represents an individual mouse. Statistical analysis of allograft survival was measured using Mantel-Cox log-rank test and for other analyses student's T tests were performed and $p<0.05$ were considered significant.

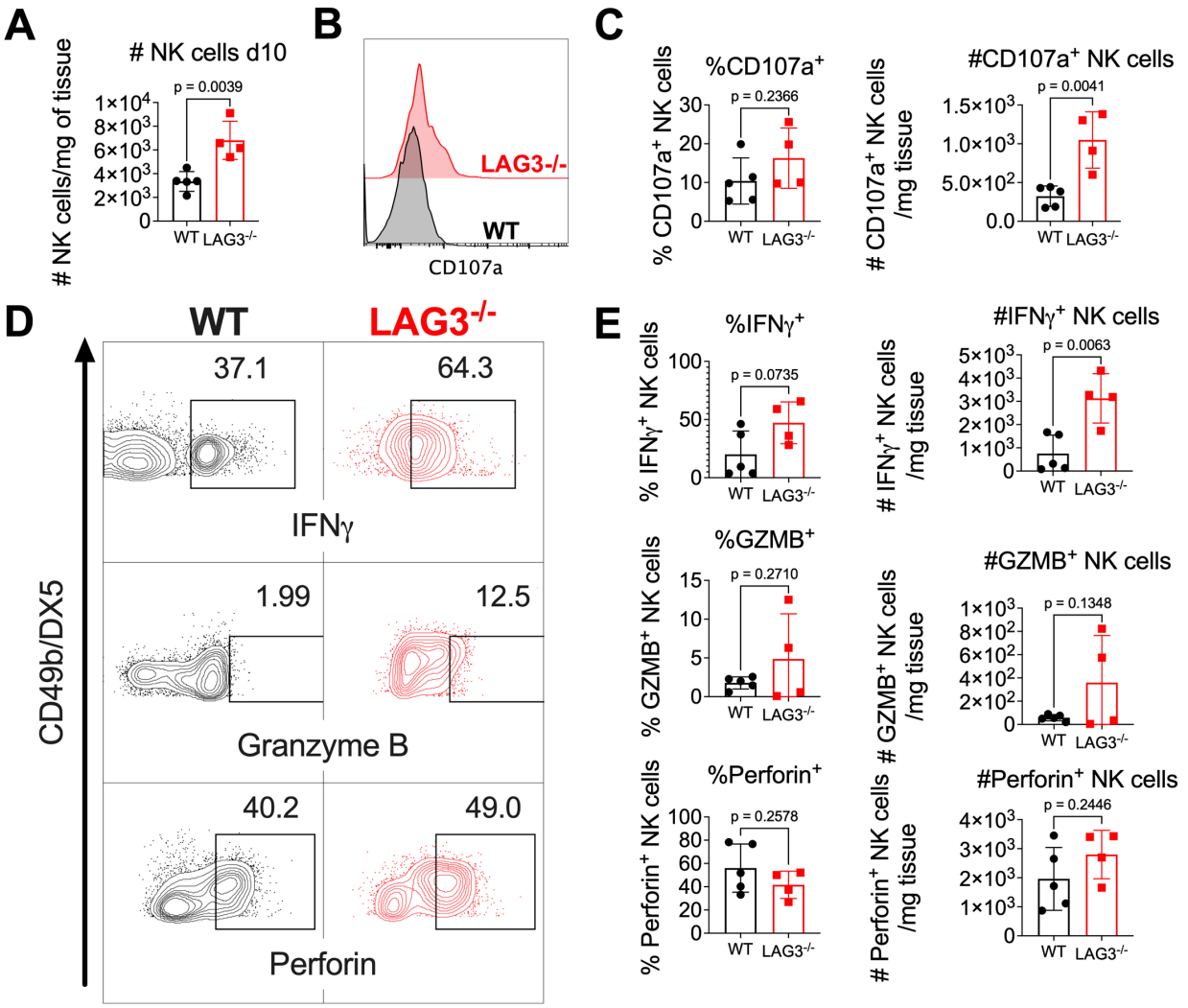


Figure 7. Recipient LAG3 deficiency results in increased accumulation and effector functions of NK cells in renal allografts

A. Quantification of allograft infiltrating NK cells in WT or LAG3$^{-/-}$ recipients on day 10 posttransplant. **B.** Representative histogram and **C** quantification of CD107a expression by graft infiltrating NK cells. **D.** Representative contour plots of graft infiltrating NK cells from WT and LAG3$^{-/-}$ kidney allograft recipient at d. 10 posttransplant. **E**. Quantification of IFNγ, Granzyme B (GZMB) and Perforin producing graft infiltrating NK cells. The gating strategy is shown in Supplementary Figure 13A. The data are pooled from two experiments, and each symbol represents an individual mouse. Student's T tests were performed and $p<0.05$ were considered significant.

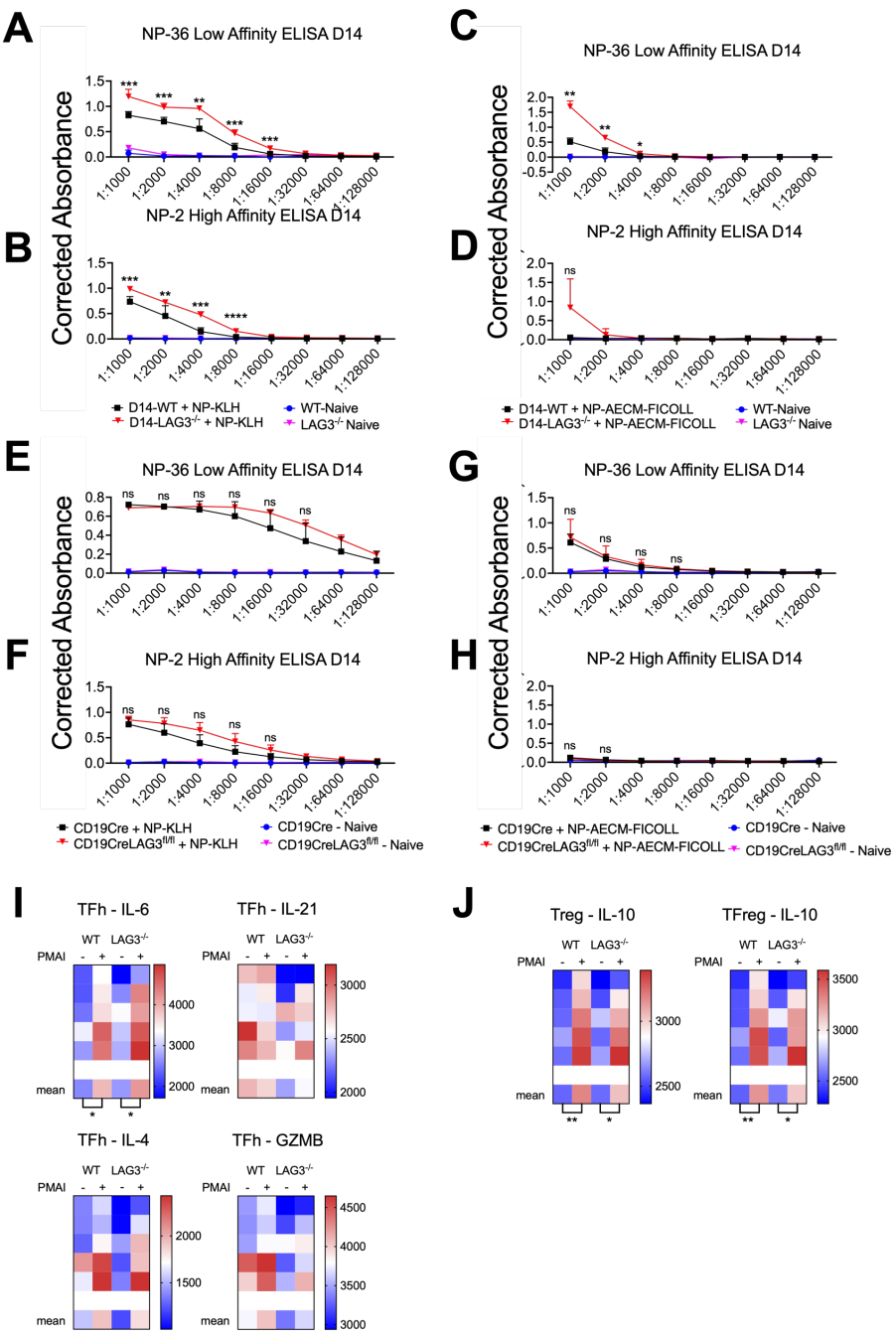


Figure 8. LAG3 regulates antibody responses to both T dependent and independent antigens. **A**. Serum ELISA for low affinity NP-specific antibody was performed on sera taken at day 14 postimmunization of WT and LAG3$^{-/-}$ with NP-KLH. **B**. Serum ELISA for high affinity NP-specific antibody was performed on sera taken at day 14 postimmunization of WT and LAG3$^{-/-}$ with NP-KLH. **C**. Serum ELISA for low affinity NP-specific antibody was performed on sera taken at day 14 postimmunization of WT and LAG3$^{-/-}$ with NP-AECM-FICOLL. **D**. Serum ELISA for high affinity NP-specific antibody was performed on sera taken at day 14 postimmunization of WT and LAG3$^{-/-}$ with NP-AECM-FICOLL. **E**. Serum ELISA for low affinity NP-specific antibody was performed on sera taken at day 14 postimmunization of CD19Cre$^{+/-}$ and CD19Cre$^{+/-}$LAG3$^{fl/fl}$ with NP-

KLH. **F**. ELISA for high affinity NP-specific antibody was performed on sera taken at day 14 postimmunization of CD19Cre$^{+/-}$ and CD19Cre$^{+/-}$LAG3$^{fl/fl}$ with NP-KLH. **G**. ELISA for low affinity NP-specific antibody was performed on sera taken at day 14 postimmunization of CD19Cre$^{+/-}$ and CD19Cre$^{+/-}$LAG3$^{fl/fl}$ with NP-AECM-FICOLL. **H**. ELISA for high affinity NP-specific antibody was performed on sera taken at day 14 postimmunization of CD19Cre$^{+/-}$ and CD19Cre$^{+/-}$LAG3$^{fl/fl}$ with NP-AECM-FICOLL. **I**. Heatmaps of MFIs of splenic Tfh staining for IL-4, IL-21, IL-6 and Granzyme B (GZMB) from WT or LAG3$^{-/-}$ mice on d.10 postimmunization with NP-KLH. **J**. Heatmaps of MFIs of splenic Treg and Tfreg staining for IL-10 from WT or LAG3$^{-/-}$ mice on d.10 postimmunization with NP-KLH. For heatmaps individual mice are shown above the indicated mean MFI values. Antibody dilution curves were analyzed with multiple unpaired T tests with Benjamini, Krieger and Yekutieli false discovery approaches. For all other analysis, student's T tests were performed and for all analyses $p<0.05$ were considered significant. For dilution curves and heatmaps – Blank- not significant $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

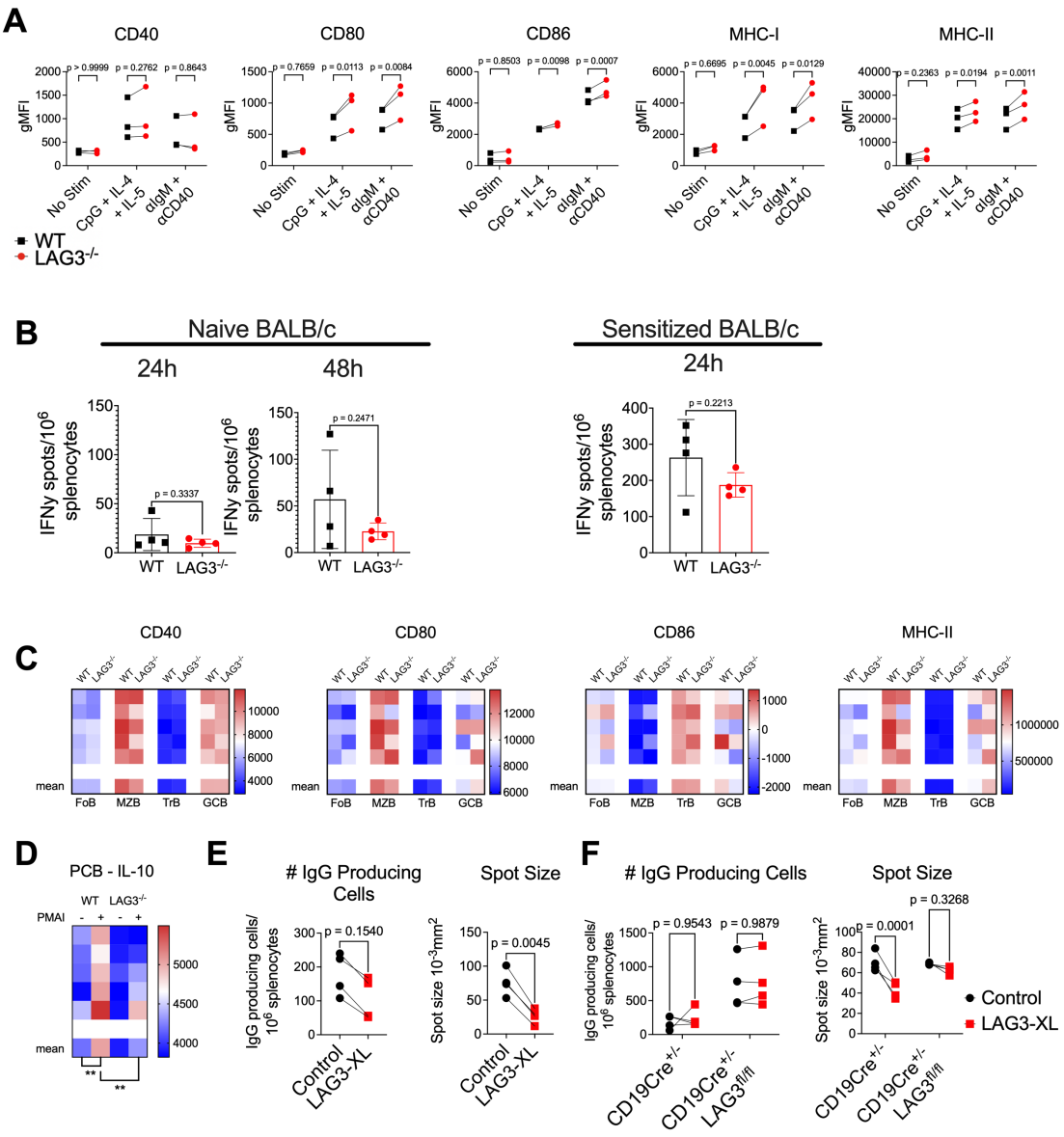


Figure 9. LAG3 is required for IL-10 production by plasma cell and regulates plasma cells intrinsically. A. Analysis of MHC-I, MHC-II, CD40, CD80 and CD86 expression by isolated follicular B cells from WT and LAG3$^{-/-}$ after stimulation for 24 hours with either anti-IgM and anti-CD40/CpG, IL-4 and IL-5 by flow cytometry. **B**. ELISPOT analysis of BALB/c T cell IFNγ responses to stimulation by either B6.WT or B6.LAG3$^{-/-}$ isolated B cells. **C**. Heatmaps MFIs of CD40, CD80, CD86 and MHC-II expression by follicular B cells (FoB), marginal zone B cells (MZB), transitional B cells (TrB), and germinal center B cells (GCBs) from the spleens of WT and LAG3$^{-/-}$ mice 10 days after immunization with NP-KLH. **D**. Heatmaps of MFIs of IL-10, production by splenic plasma cells (PCBs) of WT and LAG3$^{-/-}$ mice 10 days after immunization with NP-KLH. **E**. Quantification of frequency of IgG producing cells and IgG spot size from plasma cell ELISPOTs of WT and LAG3$^{-/-}$ splenocytes ± LAG3 cross-linking (LAG3-XL). **F**. Quantification of frequency of IgG producing cells and IgG spot size from plasma cell ELISPOTs of CD19Cre$^{+/-}$ and

CD19Cre$^{+/-}$LAG3$^{fl/fl}$ splenocytes ± LAG3 cross-linking (LAG3-XL). For heatmaps individual mice are shown above the indicated mean MFI values. For **A** two-way ANOVA with Sidaks multiple comparison test. Was performed. For **B-E**, Student's T tests were performed, and in **F** one-way ANOVA with Tukey's multiple comparison was performed and for all analyses $p<0.05$ were considered significant. For dilution curves and heatmaps – Blank- not significant $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

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