Recipient LAG3 deficiency results in antibody-mediated rejection of mouse renal allografts

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Abstract

Lymphocyte activation gene-3 (LAG3) is a coinhibitory receptor expressed by a wide range of immune cells. While LAG3 is predominantly studied in T cells, its functions in humoral immune responses remain poorly characterized. The goal of this study was to test the role of recipient LAG3 in a mouse model of renal allograft rejection. Compared to WT animals, naïve B6.LAG3$^{-/-}$ mice have increased splenic cellularity and higher frequencies of CD44^{hi} memory T cells, CXCR5^{hi} follicular T cells, and B220^{+}CD138^{+} plasma cells yet do not develop spontaneous autoimmunity. Furthermore, naïve B6.LAG3$^{-/-}$ (H-2D^{b}) mice have increased frequencies of memory T cells recognizing H-2D^{d}, H-2D^{s}, H-2D^{q} and H-2D^{k} alloantigens. In addition, naïve non-transplanted B6.LAG3$^{-/-}$ mice have elevated levels of serum IgG reactive to allogenic class I and class II MHC molecules. Whereas 4/4 B6.WT recipients spontaneously accept fully MHC-mismatched C3H (H-2^{k}) renal allografts for longer than 60 d, recipient LAG3 deficiency led to rapid allograft rejection and elevated serum creatinine levels. Graft histology at rejection revealed minimal T cell infiltration, diffuse C4d staining, atrophic peritubular capillaries, endothelial swelling and edema characteristic of antibody mediated rejection (AMR). Compared to WT, LAG3$^{-/-}$ recipients had elevated frequencies of anti-donor IFNγ producing T cells and increased levels of anti-donor MHC antibodies. Recipient CD8^{+} T cell depletion did not alter the rejection kinetics in LAG3$^{-/-}$ recipients, while B cell depletion significantly extended C3H kidney allograft survival. Conditional knockout mice were used to determine the importance of LAG3 expression by T and B lymphocytes. The absence of LAG3 on either T or B lymphocytes had no impact on graft rejection with both groups accepting kidney allografts for longer than 30 days, suggesting that LAG3 expression on either T or B cells is sufficient to regulate immune responses to kidney allografts. These results suggest

that LAG3 is a regulator of both T and B cell responses to kidney allografts and is an attractive potential therapeutic target for AMR prevention and treatment.

Introduction

Lymphocyte activation gene 3 (LAG3, also known as 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[1-6], plasmacytoid dendritic cells[7] (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[8, 9]. LAG3 is a member of immunoglobulin superfamily and has high homology to CD4 coreceptor[1]. It is associated with the T cell receptor/CD3 complex in T cells[10] and, analogous to CD4, binds to major histocompatibility complex II molecules (MHC-II) on antigen presenting cells (APCs)[11, 12]. Additionally, LAG3 interacts with other ligands such as Galectin-3[13], liver sinusoidal endothelial cell lectin (LSECtin)[14], α-synuclein fibrils (α-syn)[15], and fibrinogen-like protein 1 (FGL1)[16]. In T cells, LAG3 is co-localized with immune synapse and inhibits TCR signaling leading to impaired cell activation, proliferation, differentiation, and effector functions in the context of autoimmunity, infection and tumor[17-22]. In addition, LAG3 plays an essential role in T regulatory cells (Treg)[6, 23, 24], and mice with Treg-specific LAG3 deficiency develop autoimmune diabetes[25]. Activation of murine splenocytes *in vitro* induces the endogenous expression of LAG3 on $CD19^+$ B cells in T cell dependent manner[3], however the functional consequences of LAG3 up-regulation in B cells during humoral immune responses remain to be determined.

Even though the relative importance of LAG3 on conventional T cells, Tregs, B cells and other cell types during immune responses still remains unclear it is a molecule of great interest for clinical interventions. A number of LAG3 monoclonal antibodies with either antagonistic, depleting, or agonistic activities have been developed and are currently in clinical trials for cancer and autoimmunity patients[26-35]. 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. A study by Lucas *et al*.[36] in a mouse model of allogeneic bone marrow transplantation reported that LAG3 blockade prevents tolerance induction in alloreactive

$CD8^+$ T cells, 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 the 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 pathway to alloimmune responses using a mouse model of life-supporting renal transplantation. We found that non-transplanted mice deficient in LAG3 expression have elevated cellular and humoral heterologous immunity against a panel of alloantigens. 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. Neither T cell- or B-cell specific LAG3 conditional knockout recipients rejected renal allografts suggesting that LAG3 is an important regulator of T/B cell interaction and alloantibody production in the context of transplantation. 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.

Materials and Methods

Animals

Male C3H/HeJ (C3H, H-2^k), C57BL/6J (B6, H-2^b), BALB/cJ (Balb/c, H-2^d) DBA1/J (DBA H-2^q) and SJL/J-*Pde6b*^{rd1} (SJL, H-2^s), B6.129P2(C)-Cd19 tm1(cre)Cgn/J (B6.$CD19^{Cre}$) aged 6-8 week, were purchased from The Jackson Laboratory (Bar Harbor,ME). B6.$LAG3^{-/-}$, B6.$CD4^{Cre}LAG3^{fl/fl,}$ and $LAG3^{fl/fl}$ mice were provided by Booki Min. All animal procedures were approved by the Institutional Animal Care and Use Committee at Cleveland Clinic.

Kidney Transplantation and Graft Evaluation

Transplantation of murine kidneys was performed as previously described[38]. In brief, the kidney and its vascular supply and ureter were harvested *en bloc*, the donor vasculature were anastomosed to the recipient abdominal aorta and inferior vena cava. Excised kidneys were

perfused with University of Wisconsin (UW) solution (320mOsm; Preservation Solutions, Elkorn, WI) and stored on ice for 0.5 hours before transplantation. Urinary reconstruction was performed as previously published[39]. The remaining native kidney was removed at the time of transplant, ensuring that survival of the mouse was dependent upon functionality of the transplanted kidney. Graft survival was assessed by daily examination of overall animal health. Recipients were euthanized when low and slow mobility in addition to a hunched posture indicated rejection. At time of euthanasia serum creatinine levels were measured using the VetScan i-STAT1 Analyzer (Abaxis, Union City, CA). Grafts were harvested, fixed in methanol before embedding in paraffin for staining with hematoxylin and eosin or Gomori's trichrome C, and the following antibodies: Rabbit monoclonal anti-mouse/human Mac-2 (clone M3/M8; Biolegend #125401), rabbit monoclonal anti-mouse CD4 (clone EPR19514; Abcam #ab183685), rabbit monoclonal anti-mouse CD8 (clone EPR21769; Abcam #ab217344), polyclonal rabbit anti-mouse C4d[40], rabbit polyclonal anti-Human/mouse vWf (Dako #A0082), rat monoclonal anti-mouse/rat FOXP3 (clone FJK-16s; eBioscience #14-5773) and rat monoclonal anti-mouse B220/CD45R (clone RA3-682; BD Pharmingen #550286). Each stain was evaluated based on two to three complete cross sections from 4-5 grafts per group.

Recipient Treatment

To deplete $CD8^+$ T cells recipients were treated with monoclonal anti-mouse CD8 antibodies, clones TIB105 and YTS169 (Bio X Cell, 0.2mg of each i.p. on days -3, -2, -1 prior to transplantation and every 5 days posttransplant for the duration of the experiment). For B cell depletion, recipients were treated with anti-mouse CD20 monoclonal antibody (mouse IgG2a, clone 18B12; Biogen Idec, San Diego,CA; 0.25mg i.p. on day 3 and 0.1mg i.p. on days 8, 19 and 25 posttransplant.)

Measurement of Serum Alloantibody

Alloreactive serum antibody titers were measures as previously published[41]. In brief, serum samples were collected via tail-vein bleeding from naïve 10 weeks old mice or from kidney allograft recipients at d. 14 posttransplant and stored at -20°C. Flat-bottomed 96-well plates (Thermo Fisher Scientific) were coated overnight at 4°C with biotinylated class 1 D^d (folded with RPGGRAFVTI peptide), class 1 D^k (folded with RRLGRTLLL peptide), class 2 I-A^d (folded with PVSKMRMATPLLMQA class 2-associated invariant chain peptide), or class 2 I-A^k (folded with PVSKMRMATPLLMQA class 2- associated invariant chain peptide) MHC monomers at 1μg/ml in PBS. Peptide/MHC monomers were provided by the National Institutes of Health Tetramer Core Facility at Emory University (Atlanta, GA). Plates were washed with PBS and blockade with 1% BSA-PBS solution for 1h at room temperature. A total of 100μl of serum diluted from 1:100-1:500

were added to the plates and incubated overnight at 4°C. Plates were washed with PBS and PBS-Tween (0.25%), and then incubated with goat anti-mouse IgG -horse radish peroxidase (HRP) conjugate (1:20000 dilution; Thermo Fisher Scientific) or IgG1, IgG2a, IgG2b, IgG3-horse radish peroxidase (HRP) conjugate (1:5000 dilution; Thermo Fisher Scientific) for 1 hour at room temperature. After incubation with conjugate-bound antibodies, plates were washed and developed with ABTS Peroxidase Substrate (KPL, Gaithersburgh, MD). Absorbance was measured at 415nm using the Bio-Rad iMark Microplate Reader (Bio-Rad Laboratories, Hercules, CA).

ELISPOT Assay

Interferon gamma (IFNγ) ELISPOT assays were performed as previously described using capture and detecting antibody from BD Pharmingen[42]. Spleens from recipients or naïve non-transplanted mice were stimulated with mitomycin C-treated donor C3H or BALB/c, SJL or DBA spleen cells for 24 hours. The resulting spots were analyzed using an ImmunoSpot Series 4 Analyzer (Cellular Technology, Cleveland, OH).

Flow Cytometry

Fluorochrome conjugated antibodies were purchased from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA). Cells were isolated from the spleen and stained as previously described[43], ≥100,000 events per sample were acquired on a BD Biosciences LSRII or a BD Biosciences LSRFortessa X-20 followed by data analysis using FlowJo software (TreeStar, Inc., Ashland, OR).

Statistical Analyses

Kidney allograft survival was compared between groups by Kaplan-Meier analysis. All other results were analyzed by using a parametric unpaired t-test. 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.

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 (**Fig. S1A**) and in the increased numbers of total and effector memory ($TEff_M$) $CD4^+$ and $CD8^+$ T cells, as well as Tregs compared to B6.WT mice (**Fig. 1A**). In addition, mice deficient in LAG3 had elevated numbers of T follicular helper (Tfh) and T follicular regulatory (Tfr) cells in the spleen. The numbers of major spleen B cell subsets – follicular (FoB), marginal zone (MzB), and transitional (TrB) B cells – were not significantly different between B6.WT and B6.LAG3$^{-/-}$ mice (**Fig. 1B**). Notably, mice deficient in LAG3 had significantly increased numbers of germinal center B cells (GcB) and plasma cells (PC) in the spleen suggesting ongoing B cell activation. The was a non-significant 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 B6.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 B6.WT mice, naïve B6.LAG3$^{-/-}$ mice have increased frequencies of preexisting memory T cells reactive against BALB/c (H2-D^d), C3H (H2-D^k), and to a lesser extent, SJL (H-2D^s) and DBA (H2-D^q) alloantigens (**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 B6.LAG3-/- (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), although a great inter-individual variability was observed. 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.

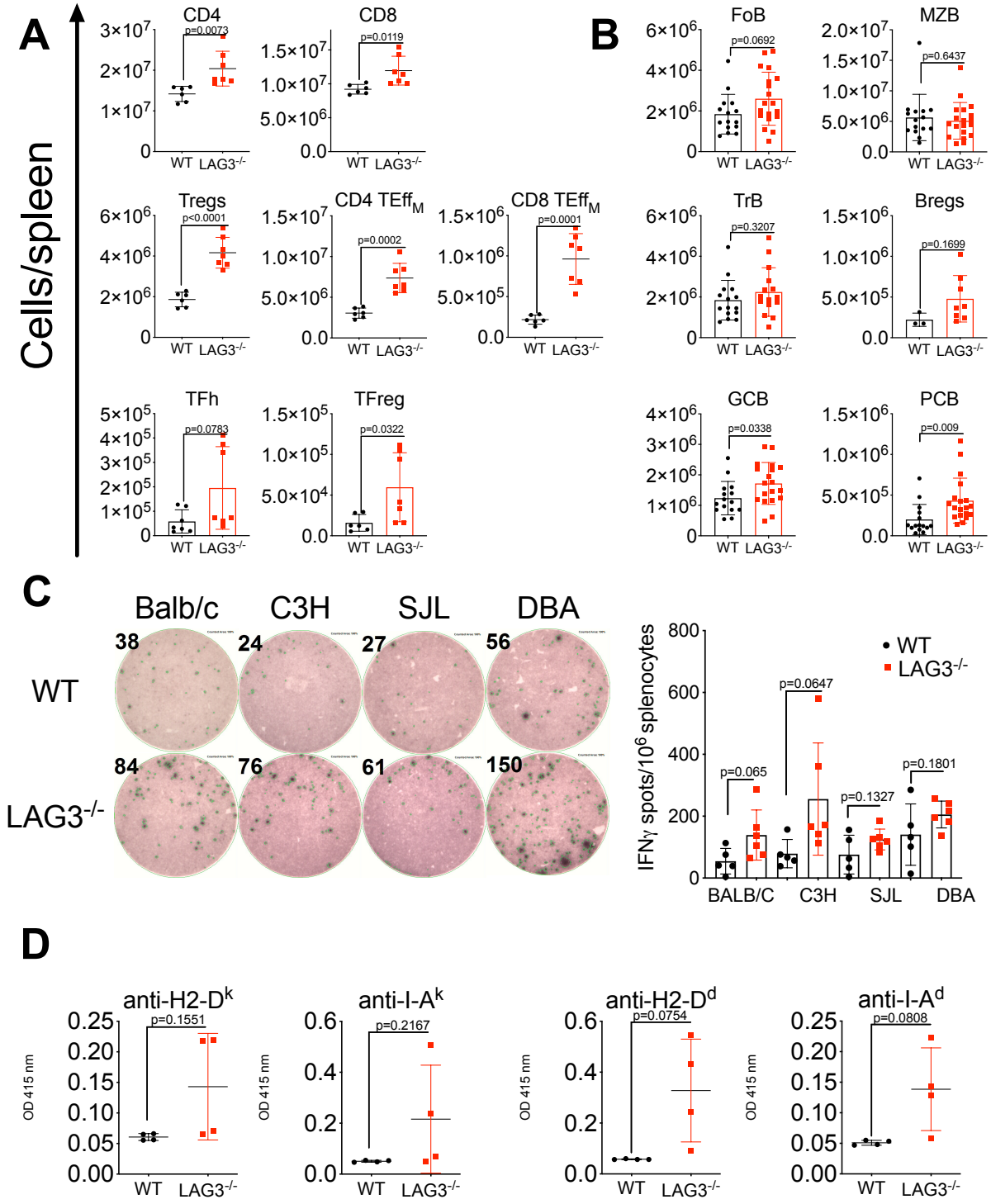

Figure 1. LAG3 deficient mice have elevated heterologous alloimmune responses. *Naïve none-transplanted B6.WT and B6.LAG3$^{-/-}$ mice were sacrificed at 10 weeks old.* ***A.*** *Splenic T cell composition: CD4 (CD3$^+$CD4$^+$), CD8 (CD3$^+$CD8$^+$), Tregs (CD3$^+$, CD4$^+$, FoxP3$^+$), CD4 TEff$_M$ (CD3$^+$,*

CD4$^+$, CD62L^{lo}, CD44^{hi}), CD8 TEff$_M$ (CD3$^+$, CD8$^+$, CD62L^{lo}, CD44^{hi}), Tfh (TCRb$^+$, CD4$^+$, FoxP3$^-$, PD-1$^+$, CXCR5$^+$) and Tfr cells (TCRb$^+$, CD4$^+$, FoxP3$^+$, PD-1$^+$, CXCR5$^+$). ***B.*** *The composition of splenic B cell subsets. Subsets were defined as follows; 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}, and PC –B220$^-$, CD138^{hi}.* ***C.*** *Left: Representative ELISPOT wells demonstrating 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. Right: Quantification of basal frequencies of alloreactivity in B6.WT and B6.LAG3$^{-/-}$ mice.* ***D.*** *ELISA quantification of serum IgG (D) reactive to allogenic MHC I and II molecules. The data represents at least 2 pooled experiments where each symbol represents an individual mouse.*

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[41] 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. 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[41, 44], B6.WT recipients spontaneously accept C3H renal allografts for >60 days (**Fig. 2A**). In contrast, most B6.LAG3$^{-/-}$ recipients rapidly rejected kidney allografts (median survival time, MST, of 15d) (**Fig. 2A**) 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. 2B**). Immunohistochemistry of allografts recovered from B6.LAG3$^{-/-}$ recipients around the time of rejection (d. 14) showed decreased numbers of infiltrating T cells compared to grafts from B6.WT recipients (**Fig. 2C**). Instead, the rejecting grafts showed characteristic signs of antibody mediated rejection including such as diffuse C4d staining, atrophic peritubular capillaries, endothelial cell swelling and edema (**Fig. 2C**). Flow cytometry analysis of graft infiltrating cells on d. 10 posttransplant (**Fig. 2D-E**) revealed no significant changes in CD4$^+$ or CD8$^+$ T cell infiltration, with the only significant difference being in the increased numbers of infiltrating FoxP3$^+$ T regulatory cells. B6.LAG3$^{-/-}$ recipients did demonstrate a modest increase in the infiltration of NK cells, the majority of which were of the NK1.1^{hi} phenotype (**Fig. 2D-E** and data not shown). This finding is consistent with antibody-mediated tissue injury of renal allografts as recently demonstrated by Yagisawa and colleagues[45].

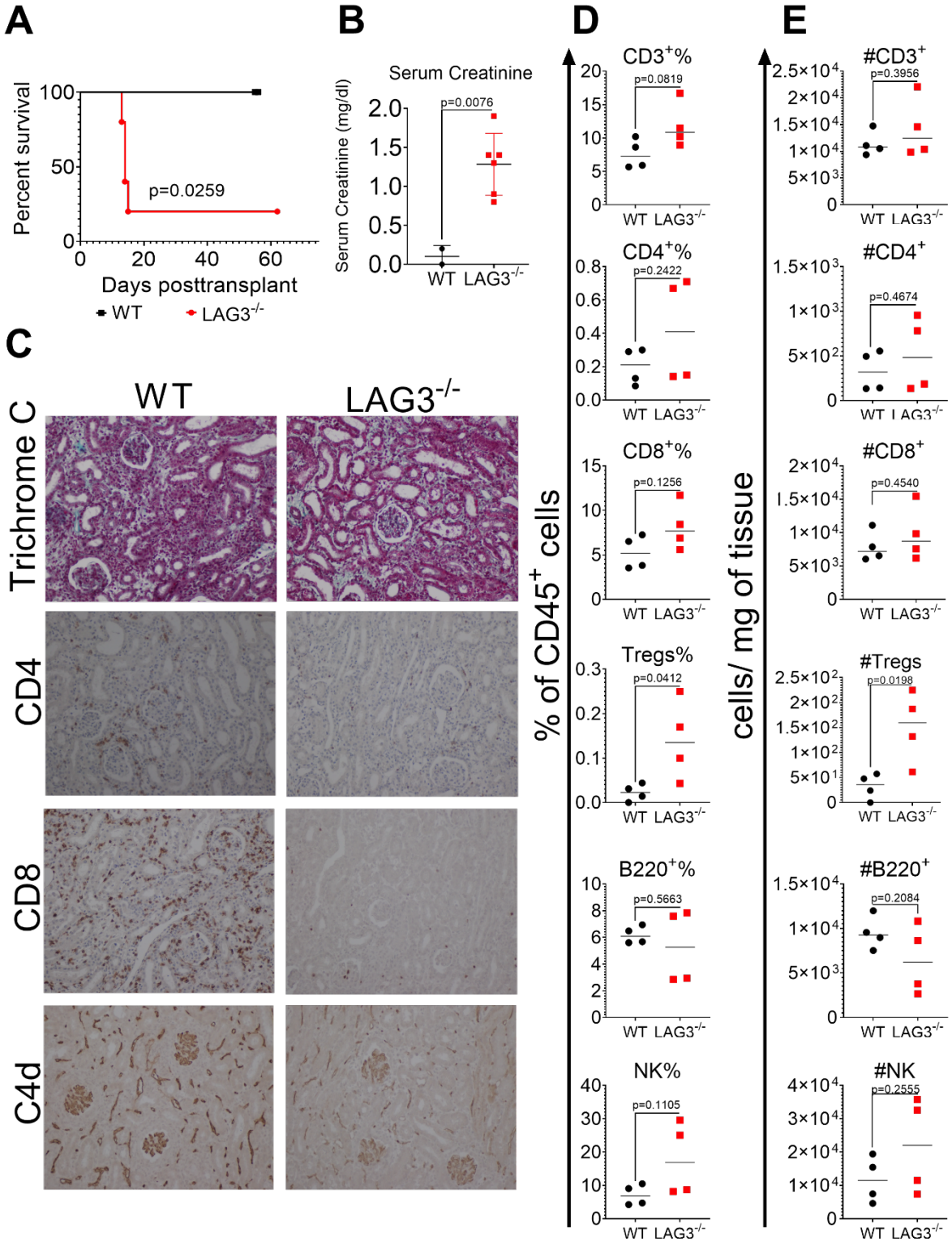

Figure 2. LAG3-deficient recipients acutely reject kidney allografts. *Groups of B6.WT and B6.LAG3$^{-/-}$ received complete major histocompatibility complex-mismatched C3H kidney allografts (n=4-5/group).* ***A.*** *Renal allograft survival.* ***B.*** *Serum creatinine levels at d14*

posttransplant. ***C.*** *Renal allografts harvested at the time of rejection (B6.LAG3$^{-/-}$) or on d. 14 posttransplant (B6.WT) and analyzed 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.* ***D & E.*** *Flow cytometry analysis of graft infiltrating immune cells: CD3^{+} (CD45^{+} CD3^{+}), CD4^{+} (CD45^{+} CD3^{+} CD4^{+}), CD8^{+} (CD45^{+} CD3^{+} CD8^{+}), Tregs (CD45^{+}, CD3^{+} CD4^{+} FoxP3^{+}), B220^{+} (CD45^{+} B220^{+}), and NK (CD45^{+} CD3^{-} DX1^{+}) 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.*

The analysis of the peripheral T cell pool revealed no significant differences in the proportion or numbers of various spleen T cell subsets (**Fig. 3A** and **Fig. S4A**). Nevertheless, the absence of recipient LAG3 resulted in elevated frequencies of donor specific T cells, as measured by IFNγ ELIPSOT assay on d. 14 posttransplant (**Fig. 3C**). Analogous to the T cell compartment, spleen subsets of follicular, marginal zone, transitional, and regulatory B cells were similar in B6.WT and B6.LAG3$^{-/-}$ allograft recipients (**Fig. 3B** and **Fig. S4B**), whereas germinal center B cells and plasma cells were increased in some but not all recipients. To assess anti-donor humoral immune responses, recipient serum was collected at 14 d posttransplant and analyzed for the levels of IgG antibodies against donor class I (D^{k}) and class II (I-A^{k}) MHC molecules. Consistent with our published studies, B6.WT renal allograft recipients had developed minimal donor specific alloantbodies (DSA) response at this timepoint. In contrast, B6.LAG3$^{-/-}$ recipients had significantly elevated levels of IgG against both D^{k} and I-A^{k} (**Fig. 3D**). Taken together these data demonstrate that LAG3 plays an essential role in regulating both cellular and humoral immune responses to transplanted allografts and contributes to spontaneous renal allografts acceptance in wild type recipients.

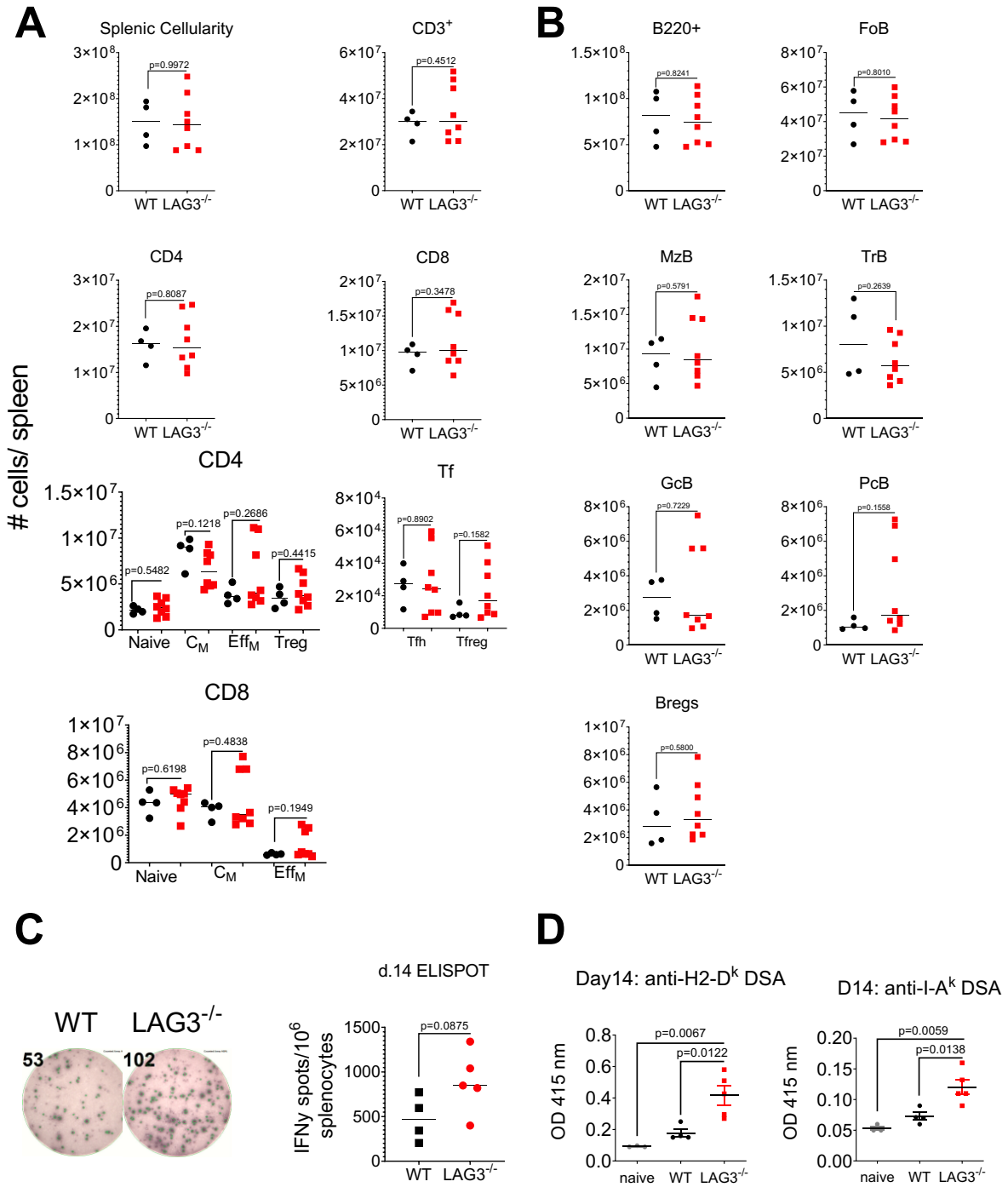

Figure 3. Recipient LAG3 deficiency enhances anti-donor immune responses. *Analyses of donor-reactive immunity in B6.WT and B6.LAG3$^{-/-}$ allograft recipients were performed at d. 10 posttranplant.* ***A.*** *The composition of spleen T cell subsets defined as follows: CD3 – CD3^{+}, CD4 – CD3^{+}CD4^{+}, 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 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.*** *The frequencies of donor reactive IFNγ-secreting splenocytes on d. 14 posttransplant.* ***D.*** *Serum levels of IgG against donor MHC-I ($H2\text{-}D^k$) and MHC-II ($I\text{-}A^k$) in B6.WT and $B6.LAG3^{-/-}$ kidney allograft recipients. The data are pooled from two-three experiments, and each symbol represents an individual mouse.*

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)** suggested that alloantibody are the major mechanism of graft tissue injury in $B6.LAG3^{-/-}$ recipients. To formally test the contributions of cellular vs antibody mediated rejection, $B6.LAG3^{-/-}$ recipients were treated with $CD8^+$ T cell depleting antibody prior to transplantation of C3H kidney allografts (**Fig. 4A**). $CD8^+$ T cell depletion antibodies failed to prolong kidney allograft survival in $B6.LAG3^{-/-}$ recipients, with MST of 16 days (**Fig. 4B**). Histological analysis at the time of rejection showed that grafts from $CD8^+$ T cell depleted $B6.LAG3^{-/-}$ 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. 4C**). In summary, the grafts rejected by $B6.LAG3^{-/-}$ recipients following $CD8^+$ T cell depletion had typical features of antibody mediated graft damage similar to or even exceeding those observed in non-depleted LAG3-deficient recipients. To test the importance of B cells and antibody in the observed rejection, we transplanted $B6.LAG3^{-/-}$ mice with C3H kidney allografts and treated them with anti-mouse CD20 B cell depleting antibody starting on d. 3 posttransplant and throughout the experiment (**Fig. 4D**). Remarkably, B cell depletion restored graft survival in the majority of $B6.LAG3^{-/-}$ recipients (**Fig. 4E**). These results demonstrate that the rejection observed in $LAG3^{-/-}$ mice is driven by B cell production of DSA and not by cytotoxic T cells.

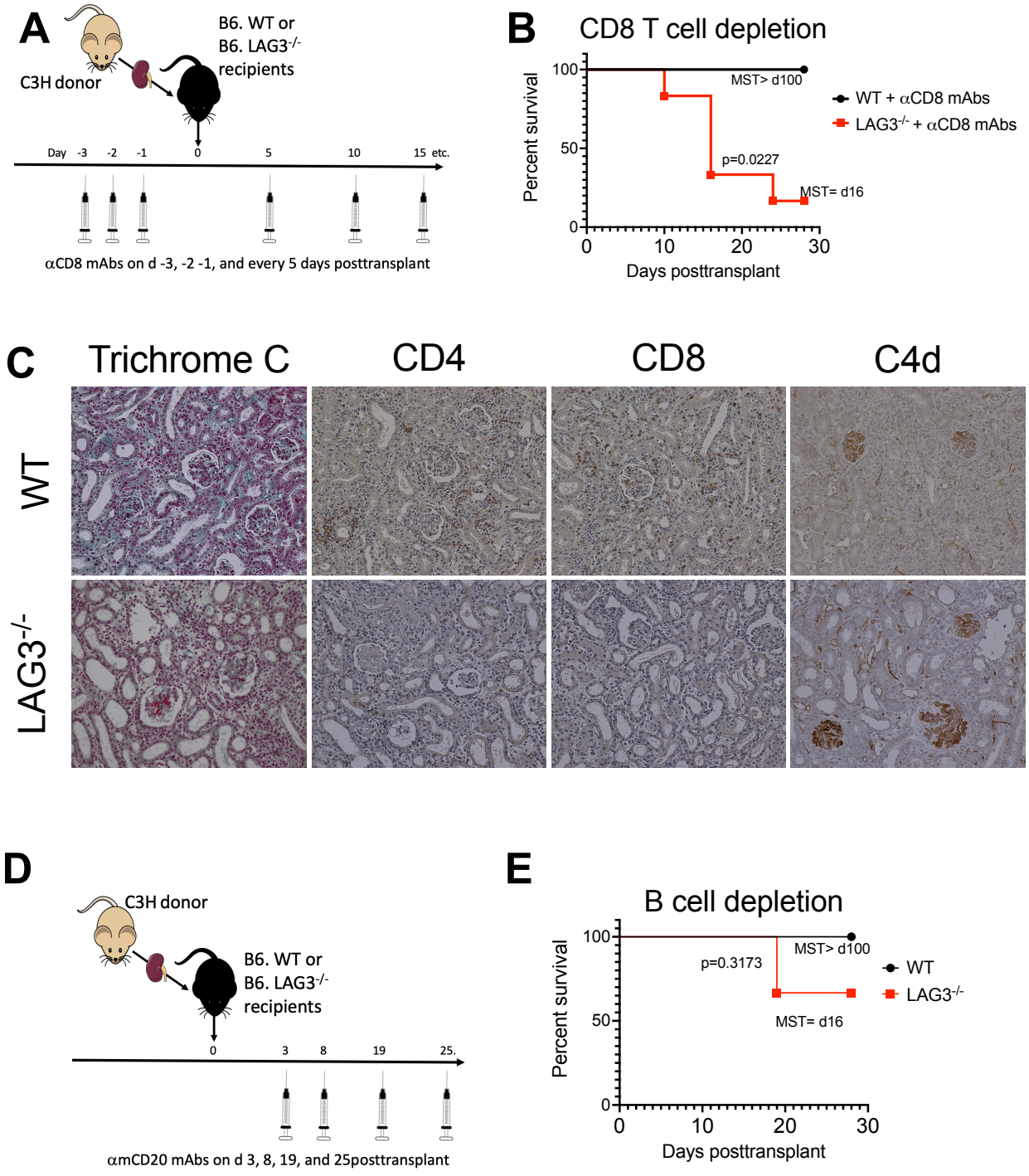

Figure 4. Kidney allograft rejection by LAG3 deficient recipients is mediated by antibody rather than by CD8 T cells. **A.** *B6.WT or B6.LAG3$^{-/-}$ recipients depleted of CD8 T cells prior to transplantation of C3H renal allografts.* ***B.*** *Survival of renal allografts (n=4-6/group).* ***C.*** *Renal allografts harvested at the time of rejection and analyzed 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. ***D.*** *B6.WT or B6.LAG3$^{-/-}$ recipients were treated with B cell depleting antibody starting on d. 3 after transplantation of C3H kidney allografts.* ***E.*** *Allograft survival, n=3/group.*

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

The absence of LAG3 on T cells in other mouse models often induces pathogenesis[16]. One possible reason behind the observed rejection is that the absence of LAG3 on T cells results in augmented help to B cells, thus resulting in elevated DSA production.

To determine the contribution of LAG3 deficiency on T vs B lymphocytes, we used conditional knockout mice and littermate controls as renal allograft recipients. B6.CD4-Cre$^{+/-}$LAG3$^{fl/fl}$ which lack LAG3 on CD4^{+} and CD8^{+} T cells spontaneously accepted kidney allografts for > 60 days (**Fig. 5A**) indicating that LAG3 deficiency on T cells alone was not sufficient to induce rejection. Unexpectedly, B6.CD19-Cre$^{+/-}$LAG3$^{fl/fl}$ recipients with specific LAG3 deficiency in B lymphocytes also failed to reject kidney allografts by d. 30 posttransplant (**Fig. 5B**). Thus, LAG3 expression on either helper T cells or B cells is sufficient to regulate the antibody response following kidney transplantation.

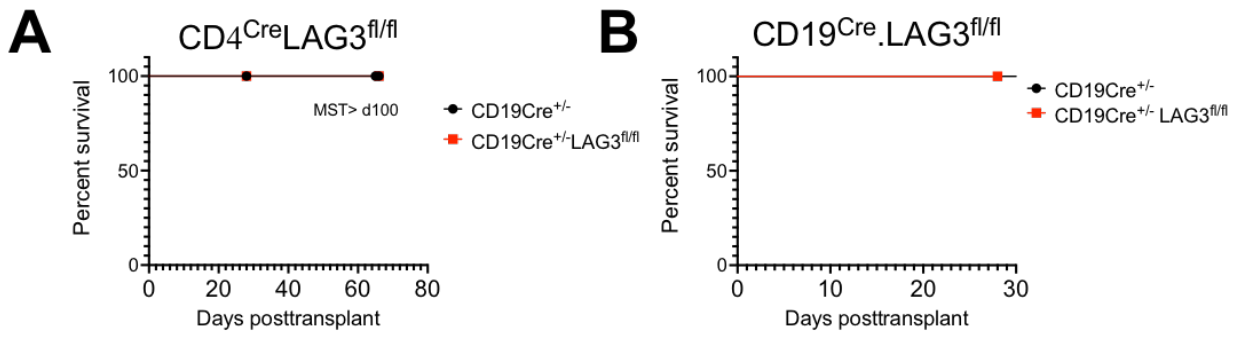

Figure 5. LAG3 deficiency on both T and B lymphocytes is required for kidney allograft rejection. A. *Survival of C3H renal allografts transplanted into B6.CD4-Cre$^{+/-}$LAG3$^{fl/fl}$ or control B6.CD4-Cre$^{+/-}$ recipients, n=5-7/group.* ***B.*** *Survival of C3H renal allografts transplanted into B6.CD19-Cre$^{+/-}$LAG3$^{fl/fl}$ or control B6.CD19-Cre$^{+/-}$ recipients n=6-8/group.*

Discussion

Coinhibitory molecule LAG3 is best studied in regulating effector and regulatory T cell functions in autoimmunity, infection and cancer[17-22]. 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[25]. 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. 1). 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. 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[46]. 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, whereas the depletion of $FoxP3^{+}$ resulted in rapid rejection (28-30). LAG3 is highly expressed by $FoxP3^{+}$ Tregs and is required for their optimal suppressor activity[6, 47, 48]. While $B6.LAG3^{-/-}$ mice have increased numbers of $FoxP3^{+}$ T cells before and after transplantation, including those in the graft infiltrate (**Figs. 1-3**), these Tregs may not have adequate regulatory properties to suppress anti-donor immune responses. 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. 5**). Moreover, in contrast to our data, previously reported depletion of FoxP3 Tregs in mouse renal allograft recipients did not affect DSA generation and resulted in T cell mediated rejection[49].

While LAG3 expression in B cells was reported in 2005[3], its contribution to B lymphocyte functions has not been extensively studied. Our results demonstrate that the lack of recipient LAG3 results in 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, whereas the rejection occurred with the same kinetics in CD8 T cells-depleted B6.LAG3$^{-/-}$ recipients (**Fig. 4**). To date, there are only few studies addressing the role of LAG3 in humoral immune response. Butler and colleagues 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. However, the individual contributions of PD-L1 and LAG3 blockade were not evaluated in this study. Another recent study identified a novel subset of LAG3^{+} IL-10 secreting plasma cells with regulatory properties. However, the relative importance of LAG3 on helper T cells vs B cells during the initiation of B cell activation, germinal center formation and differentiation into antibody-secreting cells remains to be determined and is the focus of ongoing studies in our laboratory.

Our data to 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[45]. While the effects of LAG3 on various NK cell functions is highly controversial (reviewed in[50]), it is possible that dysregulated NK cells mediate rejection in B6.LAG3$^{-/-}$ recipients, secondary to enhanced T cell activation and DSA generation. The absence of LAG3 on recipients 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[7]. However, a more recent study demonstrated a role for LAG3 on bone marrow derived DCs in optimal T cell priming[51]. 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 this model, CD8 T cells underwent tolerance induction regardless of LAG3 expression indicating regulation by other LAG3-expressing immune cells[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, but 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 cancer field. Antagonistic antibodies such as Relatlimab are given to patients in combination with anti-PD-1 therapy in phase II clinical trials[52, 53]. Another promising reagent undergoing phase III trials is a bispecific antibody against LAG3 and PD-1, MGD013[26]. Anti-LAG3 depleting antibodies have been developed and validated in non-human primates to target overactivated immune cells during undesired immune response[54]. Interestingly, an agonistic anti-LAG3 antibody (IMP761) is currently under development for T cell mediated autoimmunity, and this approach could have potential for clinical transplantation[20].

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

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