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## Pathogen stimulation history impacts donor-specific CD8<sup>+</sup> T cell susceptibility to costimulation/integrin blockade-based therapy

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### Abstract

Recent studies have shown that the quantity of donor-reactive memory T cells is an important factor in determining the relative heterologous immunity barrier posed during transplantation. Here, we hypothesized that the *quality* of T cell memory also potently influences the response to costimulation blockade-based immunosuppression. Using a murine skin graft model of CD8<sup>+</sup> memory T cell-mediated costimulation blockade resistance, we elicited donor-reactive memory T cells using three distinct types of pathogen infections. Strikingly, we observed differential efficacy of a costimulation and integrin blockade regimen based on the type of pathogen used to elicit the donor-reactive memory T cell response. Intriguingly, the most immunosuppression-sensitive memory T cell populations were composed primarily of central memory cells that possessed greater recall potential, exhibited a less differentiated phenotype, and contained more multi-cytokine producers. These data therefore demonstrate that the memory T cell barrier is dependent on the specific type of pathogen infection via which the donor-reactive memory T cells are elicited, and suggest that the immune stimulation history of a given transplant patient may profoundly influence the relative barrier posed by heterologous immunity during transplantation.

### Introduction

Costimulation blockade (CoB) with belatacept (a second generation CTLA4-Ig) in renal transplantation has the benefit of improved long-term renal allograft function and less metabolic toxicity (1, 2). However, belatacept has been associated with a higher incidence and severity of acute rejection. The mechanisms responsible for this CoB resistant rejection have not been clearly defined, but it has been increasingly recognized that the immune history and alloreactive memory T cell precursor frequency of a transplant recipient may be major determinants of the success or failure of more selective immunosuppressive strategies (3–6).

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#### Supporting Information

Additional Supporting Information may be found in the online version of this article.

There is abundant pre-clinical evidence that CoB alone can induce tolerance in mice (7, 8), but this strategy has been less capable of tolerance induction in more immunologically complex and antigen experienced nonhuman primates and humans (9–11). To underscore this point, while memory T cells comprise approximately 2% of the T cell compartment in specific pathogen free experimental mice, they comprise 40–50% of the T cell pool of nonhuman primates and adult humans (12–14). Thus antigen stimulation history and the pre-existing memory T cell repertoire may potentially play a central role in mediating CoB resistant rejection, as memory T cells possess reduced activation thresholds and decreased reliance on costimulatory signals (4, 5).

In transplant recipients, donor-reactive memory T cells arise from prior exposure to foreign MHC via prior blood transfusion, transplantation or pregnancy. Additionally, heterologous immune mechanisms whereby memory T cells generated in response to infectious pathogens become cross-reactive with donor antigens provide another potential source of CoB resistant alloreactive memory T cells in transplant recipients (15–18). Experimental evidence has implicated memory T cells as mediators of CoB resistant rejection (17, 19) and higher pre-transplant frequencies of donor-specific memory have been shown to correlate with inferior transplant outcomes (3, 20, 21). Furthermore, Nadazdin et al. recently showed that high alloreactive memory T cell precursor frequency impairs tolerance induction to kidney allografts in nonhuman primates (22).

In an effort to facilitate the use of CoB by selectively targeting donor-reactive memory T cells, our group has previously shown that neutralizing memory T cells by targeting integrin molecules that are differentially expressed on this subset of T cells could overcome the barrier of CoB resistant rejection (23, 24). Additionally, in a murine model of donor-specific memory CD8<sup>+</sup> T cells that mediate CoB resistance, rejection was abrogated when coupling either anti-LFA-1 or anti-VLA-4 therapy to costimulatory blockade (25), thus validating that a combined costimulation and integrin blockade approach specifically inhibits graft rejection mediated by donor-specific CD8<sup>+</sup> memory T cells.

It has become increasingly apparent that a large degree of heterogeneity exists amongst memory T cell phenotypes, function, distribution, longevity and protective capacity (26). For example, central (T<sub>CM</sub>) and effector (T<sub>EM</sub>) memory T cells have been classically characterized based on the differential expression of homing receptors (27), but analysis of actual post-activation populations illustrates much greater diversity in survival, recall potentials and subsets defined by other markers (28, 29). Moreover, current thinking holds that the route of exposure, dose, replication rate, recurrence, and tropism of the infectious challenge may impact qualitative aspects of memory T cell development (30). Therefore we hypothesized that the ultimate quality of T cell memory formed in response to pathogen stimulation can influence the host response to proven selective immunosuppressive strategies.

In this study we show that pathogen stimulation in the form of pre-transplant acute, latent, or persistent infections produces quantitatively similar but phenotypically and functionally distinct donor-reactive CD8<sup>+</sup> memory T cell populations. Importantly, genetically identical mice that experienced different “immune histories” (i.e. had been infected with different

pathogens) exhibited differential graft survival following combined costimulation/integrin blockade. Our data suggest that qualitative differences may account for the differences in allograft survival and altered susceptibility profiles observed in response to immunosuppression with combined CoB and anti-LFA-1 or anti-VLA-4 therapy. These findings highlight the influence of pathogen stimulation history on the differentiation of donor-reactive memory T cells and the generation of immune modulation strategy-dependent variability in allograft survival.

## Methods

### Mice

C57BL/6 mice were obtained from the National Cancer Institute and ovalbumin (OVA)-specific TCR transgenic OT-I mice (31) purchased from Taconic Farms were bred to Thy1.1<sup>+</sup> background. C57BL/6 mice that constitutively express membrane-bound OVA under the beta-actin promoter (mOVA mice) (32) were a gift from M. Jenkins (University of Minnesota). All animals were housed in pathogen-free facilities and maintained in accordance with Emory University Institutional Animal Care and Use Committee guidelines.

### T cell adoptive transfers and pathogen stimulation

For adoptive transfers of OVA-specific T cells, spleen and mesenteric lymph nodes isolated from Thy1.1<sup>+</sup> OT-I mice were stained with mAbs for CD4, CD8, Thy1.1, and V $\alpha$ 2 for quantification by TruCount bead analysis (BD Biosciences). After resuspension in PBS, 10<sup>4</sup> Thy1.1<sup>+</sup> OT-I T cells were injected intravenously into naïve C57BL/6 mice. OT-I TCR transgenic cells were used to standardize the T cell precursor frequency, TCR fine specificity, and TCR affinity for OVA antigen between animals and treatment arms. 48 hours after adoptive transfer, mice were inoculated with one of three recombinant pathogens engineered to express the OVA epitope: 10<sup>4</sup> CFU of *Listeria monocytogenes* (LM)-OVA (33), 10<sup>5</sup> PFU of murine gammaherpesvirus 68 (gHV)-OVA (34), or 10<sup>5</sup> PFU of mouse polyoma virus (PyV)-OVA (35). LM and gHV were given intraperitoneally (i.p.) and PyV via footpad injection.

### OT-I CD8<sup>+</sup> T cell depletion

Thirty days after OT-I adoptive transfer and pathogen stimulation, anti-Thy1.1 mAb (19E12, Bio X Cell) was administered i.p. for Thy1.1<sup>+</sup> T cell depletion (36). Mice were injected 7 (500  $\mu$ g), 4 (250  $\mu$ g), and 1 (250  $\mu$ g) day before skin transplantation, followed by skin grafting on day 0. Depletion was maintained with weekly 250  $\mu$ g injections. Anti-Thy1.1 antibody-mediated depletion of OT-I CD8<sup>+</sup>Thy1.1<sup>+</sup> T cells was robust and consistent between anatomic compartments (Figure 1S).

### Skin transplantation and immunosuppression

30 days after pathogen stimulation, full thickness tail skin grafts were transplanted onto the dorsal thorax of inoculated recipient mice (37). Skin graft recipients then received either no treatment, CoB consisting of human CTLA4-Ig (500  $\mu$ g, Bristol-Myers Squibb) and hamster anti-mouse CD154 mAb (500  $\mu$ g, MR-1, Bio X Cell), rat anti-LFA-1 mAb alone (250  $\mu$ g,

M17/4, Bio X Cell), rat anti-VLA-4 mAb alone (250 µg, PS/2, Bio X Cell), or CoB plus anti-LFA-1 or anti-VLA-4. All mAbs were administered i.p. on post-transplant days 0, 2, 4 and 6. Anti-LFA-1 and anti-VLA-4 mAbs were non-depleting (25).

### Flow cytometry and intracellular cytokine staining (ICCS)

For phenotypic analysis, peripheral blood lymphocytes were stained for CD8, Thy1.1, CD44, CD62L, CD27, CD43, OX40, CD137, ICOS, 2B4, PD-1, KLRG1, LFA-1 and VLA-4. For ICCS, lymphocytes from graft-draining axillary lymph nodes were stimulated with 10 nM OVA peptide 257–264 (SIINFEKL) (Emory University Core Facility) in the presence of 10 µg/ml of Brefeldin A for 4 hours. An ICCS kit was used according to the manufacturer's instructions and stained for IFN- $\gamma$ , TNF, and IL-2. All reagents were purchased from BD Biosciences. All samples were run on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo Software (Tree Star).

### Statistical Analysis

Survival data were plotted on Kaplan-Meier curves and the log-rank (Mantel-Cox) test was used for survival analyses. Wilcoxon matched-pairs signed rank test was used for fraction of total distribution analyses. The Mann-Whitney U nonparametric test was performed for all other analyses. Statistical significance was attributed to p values < 0.05.

## Results

### Antigen-specific CD8<sup>+</sup> T cell responses are similar in magnitude and tissue distribution following pathogen stimulation

We first set out to quantify the antigen-specific memory T cell response following pathogen stimulation with LM, gHV or PyV. While LM is an acutely cleared intracellular bacterium known to elicit potent memory responses (38), gHV (murine homologue of human Epstein-Barr virus) and PyV (mouse homologue of human BK and JC viruses) cause latent and persistent infections, respectively, and are controlled by effector and memory T cell subsets (39, 40). Since these pathogens primarily stimulate MHC class I restricted T cell responses (33–35) and CoB resistance is primarily mediated by CD8<sup>+</sup> T cells (17, 25, 37, 41), we studied this response by adoptively transferring congenically labeled Thy1.1<sup>+</sup> naïve CD8<sup>+</sup> OT-I T cells into naïve C57BL/6 mice (Figure 1A). Mice were infected with OVA-expressing LM, gHV, or PyV, and peripheral blood lymphocytes were analyzed for the magnitude of the CD8<sup>+</sup>Thy1.1<sup>+</sup> T cell response at days 8 and 30 post-infection. Both the 30–40% peak effector response observed on day 8 (Figures 1B, C) and the 3–4% contracted memory response on day 30 (Figures 1D, E) were quantitatively equivalent amongst the three groups. The tissue distribution of OT-I cells amongst the peripheral blood, spleen and bone marrow 30 days after pathogen infection was not significantly different between LM-, gHV- or PyV-infected mice (Figure 1F).

### Pathogen stimulation history influences donor-specific CD8<sup>+</sup> T cell susceptibility to a costimulation and LFA-1 blockade-based regimen

To test whether pathogen stimulation history impacts the susceptibility of CoB-resistant donor-reactive memory T cells to combined CD28/CD154/LFA-1 blockade, we re-evaluated

the efficacy of our previously effective regimen against LM-induced CD8<sup>+</sup> memory T cell-mediated CoB resistant rejection (25). Using the OT-I transgenic mouse system, naïve B6 mice were adoptively transferred OT-I T cells, infected with OVA-expressing LM, gHV or PyV, and then grafted with skin from mOVA mice 30 days after infection (Figure 2A). All three infected groups of mice received either CoB alone, anti-LFA-1 alone, or CoB plus anti-LFA-1 therapy. Skin graft survival was most prolonged in LM-infected mice treated with combined costimulation and LFA-1 blockade ( $p < 0.001$ , Figure 2B). CoB and anti-LFA-1 therapy more modestly prolonged allograft survival in PyV-infected mice ( $p = 0.003$ , Figure 2D), and failed to prolong skin graft survival in mice exposed to gHV ( $p = 0.125$ , Figure 2C). Donor-specific memory T cell mediated rejection was equally resistant to CoB alone and anti-LFA-1 therapy alone independent of infection history (Figures 2B–D). Group median survival times (MSTs) are outlined in Table 1.

### **Combined costimulation and integrin blockade against VLA-4 elicits an altered donor-specific memory T cell susceptibility profile**

In order to determine whether the impact of T cell stimulation history on allograft survival in response to CoB and anti-LFA-1 therapy was particular to LFA-1, we similarly targeted the integrin VLA-4. Mice with adoptively transferred OT-I T cells were infected with OVA-expressing LM, gHV or PyV and then transplanted mOVA skin grafts (Figure 3A). Immunosuppression consisted of either CoB alone, anti-VLA-4 alone, or combination CoB plus anti-VLA-4 therapy previously effective against LM-elicited CoB-resistant CD8<sup>+</sup> donor-specific T cells (25). CoB plus anti-VLA-4 exhibited an altered susceptibility profile compared to the anti-LFA-1-based regimen (Table 1, Figure 2). Similar to the results observed with CoB/anti-LFA-1, this combination regimen most effectively prolonged skin allograft survival in recipients that had been previously infected with LM ( $p < 0.001$ , Figure 3B); however in contrast to CoB/anti-LFA-1, CoB/anti-VLA-4 modestly prolonged graft survival in recipients that had been previously infected with gHV ( $p < 0.001$ , Figure 3C) but failed to prolong graft survival in recipients that had prior PyV infection ( $p = 0.232$ , Figure 3D). Group MSTs are outlined in Table 1.

### **Pathogen-primed OVA-specific CD8<sup>+</sup> OT-I memory T cells are required for differential resistance to immunosuppression**

To confirm whether OT-I memory T cells mediate differential immunosuppression resistance in this mOVA transplant model, we adoptively transferred OT-I T cells into naïve B6 mice and infected them with OVA-expressing LM, gHV or PyV. Thirty days post-infection, mice within each group served as either untreated controls or received a depleting anti-Thy1.1 mAb to eliminate all OVA-specific CD8<sup>+</sup>Thy1.1<sup>+</sup> memory T cells (Figure 4A). The anti-Thy1.1 mAb effectively depleted OT-I CD8<sup>+</sup> memory generated via infection with any of the three pathogens (Figures 4B, C). Following depletion, control and anti-Thy1.1-treated mice were transplanted mOVA skin grafts and treated with CoB/anti-LFA-1 therapy. The mice depleted of OVA-specific CD8<sup>+</sup> memory T cells following LM, gHV or PyV infection uniformly accepted the mOVA skin grafts (Figure 4D). As above, non-depleted control LM-infected mice also accepted their skin grafts under CoB/anti-LFA-1 therapy, but in stark contrast, both non-depleted control gHV- and PyV-infected mice rejected their mOVA skin grafts despite CoB/anti-LFA-1 therapy (Figure 4D).

### **LFA-1 and VLA-4 surface expression on donor-specific memory T cells does not account for differences in susceptibility profiles**

One possible explanation for the variable susceptibility patterns between LM-, gHV-, and PyV-primed animals could be differential expression of LFA-1 and/or VLA-4. To test this we measured the surface expression of LFA-1 and VLA-4 on CD8<sup>+</sup>Thy1.1<sup>+</sup> memory T cells. LFA-1 and VLA-4 were both increased on memory relative to naïve T cells (Figures 5A, B); but there were no differences in surface expression of LFA-1 or VLA-4 amongst all three pathogen-stimulated groups (Figures 5C, D).

### **Donor-specific memory T cell susceptibility to dual costimulation/integrin blockade correlates with a T<sub>CM</sub> phenotype, greater recall potential, and altered PD-1/KLRG1 expression**

To determine the mechanism underlying the variable immunosuppression-related susceptibility profiles amongst CD8<sup>+</sup> memory T cells following pathogen stimulation, we next sought to phenotypically characterize donor-reactive CD8<sup>+</sup> OT-I T cells generated by each pathogen. The most treatment-susceptible LM-stimulated memory T cells expressed a predominant T<sub>CM</sub> (CD44<sup>hi</sup>CD62L<sup>hi</sup>) phenotype, while the more resistant gHV- and PyV-stimulated memory T cells displayed majority T<sub>EM</sub> (CD44<sup>hi</sup>CD62L<sup>lo</sup>) phenotypes (Figure 6A). A reciprocal relationship between the T<sub>CM</sub> and T<sub>EM</sub> compartments was observed amongst all three groups (Figures 6B, C). LM-stimulated OT-I T cells were composed of more T<sub>CM</sub> and less T<sub>EM</sub> than gHV- and PyV-stimulated cells ( $p = 0.007$  and  $p < 0.001$ , respectively). Significant differences were also observed between gHV- and PyV-induced T<sub>CM</sub> and T<sub>EM</sub> compartments ( $p = 0.006$  and  $p = 0.024$ , respectively).

We then evaluated CD8<sup>+</sup>Thy1.1<sup>+</sup> memory T cells for expression of CD27 and CD43, activation markers that define subpopulations of memory T cells that differ in their capacities to mount recall responses (29). LM- and gHV-induced memory T cells contained the greatest frequency of CD43<sup>lo</sup>CD27<sup>hi</sup> cells (greatest recall potential) compared to PyV-stimulated T cells (Figures 6D, E). Conversely, PyV-stimulated memory consisted of the most CD43<sup>hi</sup>CD27<sup>hi</sup> cells (lower recall potential) when compared to T cell memory derived from LM and gHV infection. Differences between LM- and gHV-induced memory by CD27 and CD43 were not observed.

We did not observe any differences in OX40, ICOS or 2B4 expression on OT-I memory T cells between the three pathogen-infected groups (Figure 7A). We did observe differences in CD137, PD-1 and KLRG1. Analysis of PD-1 by KLRG1 on pathogen-primed memory T cells indicated that PD-1<sup>lo</sup>KLRG1<sup>lo/hi</sup> cells comprise the majority of LM-induced donor-specific memory, while PD-1<sup>hi</sup>KLRG1<sup>lo/hi</sup> cells comprise the majority of PyV-primed memory (Figures 7B, C). gHV-infected memory manifested intermediate compositions of these phenotypes. More specifically, LM-induced memory was almost entirely composed of PD-1<sup>lo</sup> cells with the greatest percentage of the least differentiated subset of PD-1<sup>lo</sup>KLRG1<sup>lo</sup> T cells amongst the pathogen-infected groups ( $p < 0.001$ , Figure 7C). In stark contrast, PyV-infected memory consisted almost entirely of PD-1<sup>hi</sup> cells, where PD-1<sup>hi</sup>KLRG1<sup>lo</sup> cells were the most dominant subset within PyV-primed memory and between all of the pathogen-infected groups ( $p < 0.001$ ).



## Pathogen stimulation history influences the functional characteristics of donor-specific CD8<sup>+</sup> memory T cells following skin transplantation

To evaluate for functional differences between the pathogen-induced donor-reactive memory T cells, analysis of their cytokine producing ability was performed. Mice were adoptively transferred OT-I T cells, infected with LM, gHV or PyV and then transplanted with mOVA skin grafts. Lymphocytes from graft-draining axillary lymph nodes harvested from recipient mice 7 days after transplant were then stimulated with cognate antigen and stained for intracellular cytokines. Flow cytometric analysis revealed that LM generated more donor-specific IFN- $\gamma$ <sup>+</sup>TNF<sup>+</sup> multi-cytokine producers than gHV ( $p = 0.008$ ) and PyV ( $p = 0.036$ ) (Figures 8A, B). The percentage of IFN- $\gamma$ <sup>+</sup>IL-2<sup>+</sup> multi-cytokine producers amongst CD8<sup>+</sup>Thy1.1<sup>+</sup> T cells following antigen stimulation was also greatest amongst LM-infected mice ( $p = 0.008$  and  $p = 0.036$ , respectively, Figures 8C, D). PyV infection led to fewer IFN- $\gamma$ <sup>+</sup>TNF<sup>+</sup> and IFN- $\gamma$ <sup>+</sup>IL-2<sup>+</sup> producers than gHV ( $p = 0.036$ ).

## Discussion

Immunologic memory has been recognized as a barrier to improved allograft outcomes and tolerance induction in transplantation – particularly with more selective agents like belatacept (3, 4, 6, 11). While it has been established that the *quantity* of donor-reactive memory can significantly impact tolerance induction (17, 22), the data presented here suggest that the actual *quality* of T cell memory may have an equally critical impact on the outcome of CoB-based immunosuppressive strategies. Determinants of memory T cell quality include factors related to the nature of the antigen, antigen-specific precursor frequency, and the surrounding inflammatory environment. Blair et al. have shown that the availability and competition for antigen, along with the duration of antigen presentation, can regulate memory T cell differentiation, with more prolonged antigen exposure favoring a T<sub>EM</sub> phenotype (42, 43). Moreover, initial antigen-specific precursor frequency can also impact the programming of memory T cells as well as their functional requirement for costimulatory signals (44). Still other studies have shown that T cell expansion and memory formation are critically dependent upon the cytokine milieu (45, 46), for example increased IL-12 exposure favors the development of KLRG1<sup>hi</sup> T<sub>EM</sub> (47). Based on these studies, we speculate that the differential generation of costimulation/integrin resistant memory T cells may be related to the ability of OVA-expressing LM, gHV and PyV to elicit a range of inflammatory cytokine levels with variable periods of antigen availability, as LM is an acutely cleared bacterial pathogen, and gHV and PyV cause latent and persistent viral infections, respectively (38–40). Overall, the quality of memory we observed to be generated by these pathogens is consistent with known effects of their individual attributes on memory T cell differentiation.

Our results suggest that susceptibility to dual blockade and improved skin allograft survival in the LM-infected mice could be related to the induction of a predominant T<sub>CM</sub> phenotype amongst allograft-specific memory T cells. T<sub>CM</sub> and T<sub>EM</sub> differ in their homing receptors and immediate effector function (27). T<sub>CM</sub> express more CD62L, produce more IL-2, and have greater proliferative capacity than their effector counterparts (48). T<sub>EM</sub> are generally considered to have a more limited lifespan with weaker proliferative potential but rapid

effector function (49). In our study, the most immunosuppression-sensitive LM-induced memory T cell compartment was primarily composed of CD44<sup>hi</sup>CD62L<sup>hi</sup> T<sub>CM</sub> and functionally exhibited the greatest TNF and IL-2 multi-cytokine production (Figures 6, 8). Consistent with this T<sub>CM</sub>-like phenotype, this treatment-susceptible LM-induced memory T cell population was predominantly composed of CD43<sup>lo</sup>CD27<sup>hi</sup> cells (Figure 6), a phenotype that has previously been associated with greater recall and proliferative potential (26, 29, 50).

Analysis of PD-1 and KLRG1 on pathogen-primed memory cells revealed differential pathogen-specific phenotypic signatures. While little published data exists describing the relationship between concomitant PD-1 and KLRG1 expression on CD8<sup>+</sup> memory T cells, their independent expression has been relatively well studied. PD-1 is considered a negative inhibitor of the immune response that is upregulated on activated T cells and is a marker of T cell exhaustion (51). PD-1 blockade restores CD8<sup>+</sup> T cell proliferation, cytokine secretion, and cytolytic activity (52). The co-inhibitory receptor KLRG1 is expressed on antigen-experienced T cells and has been postulated to be a marker of senescent cells that lack proliferative capacity. KLRG1 expression increases with age and differentiation; with the highest percentage of expression on memory cells and highly differentiated end stage cells (53, 54). PD-1 and KLRG1 expression levels generally parallel each other and are proportionally related to the degree of T cell differentiation (55), but the temporal relationship between these markers of exhaustion and senescence and how they relate to susceptibility to immunosuppression following transplantation are unknown. In the context of the differential allograft survival and memory T cell susceptibility to immunosuppression observed in this study, we posit that PD-1<sup>hi</sup>KLRG1<sup>lo</sup> memory cells possibly represent antigen-experienced cells that are intermediately differentiated – cells that have recently encountered antigen (PD-1<sup>hi</sup>) but are not yet senescent (KLRG1<sup>hi</sup>). This PD-1<sup>hi</sup>KLRG1<sup>lo</sup> phenotype may be associated with memory T cells that are differentiated enough to exhibit resistance to costimulation/integrin blockade, but not too differentiated to effectively mediate allograft rejection; while pathogen-primed PD-1<sup>lo</sup>KLRG1<sup>lo</sup> memory T cells may be more susceptible to blockade by virtue of their less differentiated state.

Taken together, we find that the most immunosuppression-sensitive memory was more polyfunctional and T<sub>CM</sub>-like, with greater recall potential and a less differentiated PD-1<sup>lo</sup>KLRG1<sup>lo</sup> phenotype. Conversely, the more immunosuppression-resistant memory T cells were less capable of cytokine production and majority T<sub>EM</sub>, with lower recall potential and a more differentiated PD-1<sup>hi</sup>KLRG1<sup>lo</sup> phenotype. We postulate that the degree of memory T cell maturity and activation along the differentiation pathway inversely confers the amount of reliance on costimulation “checkpoints” needed to mediate an immune response; and that this pathogen-dependent quality of T cell memory influences its susceptibility or resistance to the specific immunosuppression used.

An interesting facet of the data presented here is that the pathogen-dependent susceptibility profiles were different when anti-LFA-1 vs. anti-VLA-4 was employed (Table 1, Figures 2, 3). While the mechanism underlying this differential susceptibility to anti-LFA-1 vs. anti-VLA-4 therapy remains unknown, we showed that it is not related to differences in expression levels of LFA-1 or VLA-4 on T cells primed with the three different pathogens



(Figure 5), or to alterations in the physical distribution of memory OT-I T cells preceding LFA-1 vs. VLA-4 antagonism (Figure 1). However, we previously demonstrated that despite the fact that LFA-1 and VLA-4 both belong to the integrin family of adhesion molecules, targeting them in transplantation results in divergent effects on memory T cells. Specifically, while anti-VLA-4 in the setting of CoB dramatically suppressed trafficking of memory T cells to the allograft, LFA-1 antagonism both impaired trafficking and additionally attenuated donor-reactive T cell effector function during the memory recall response (25).

In this study we show that OVA-specific OT-I memory T cells are necessary for the observed differential resistance between mice infected with the three types of pathogens (Figure 4). It is probable that pathogen-specific factors impact both the quality of donor-specific memory and other immune parameters within the host that might be influencing graft outcome. It is also possible that pathogen-dependent differences in memory T cell quality engendered upon initial priming of OT-I T cells could lead to differential trafficking and distribution of memory subsets in our model. While we did not observe any differences in the distribution of total antigen-specific memory T cells between groups within secondary lymphoid organs (Figure 1), differences in the composition of memory subsets (e.g.  $T_{CM}$  vs.  $T_{EM}$ ) might suggest that the relative representation of circulating vs. resident memory T cells could differ between groups, and could impact memory T cell susceptibility to the immunosuppressive regimens used in this study. However, these possibilities would still support the concept that different pathogen infections can result in the generation of memory T cells that are qualitatively different in terms of their resistance to a costimulation/integrin blockade-based regimen following transplantation.

In summary, this study highlights that the quality of pathogen-derived donor-reactive  $CD8^+$  T cell memory has potential to impact the clinical response to alternative immunosuppressive regimens following organ transplantation. Our data focus on the pre-transplant status of donor-specific memory and support the conclusion that phenotypic and functional differences amongst T cell memory influence allograft survival and the susceptibility profiles to costimulation and integrin blockade-based therapies, with the clinical implication that the overall composition and character of a transplant recipient's pre-existing alloreactive T cell memory will ultimately influence the outcomes of novel immunosuppressive strategies, particularly belatacept-based therapies. We conceive that in the future, assessment of the quality, in addition to quantity, of a potential transplant recipient's donor-reactive memory T cell compartment could inform clinicians on the optimal immune modulation strategy to employ.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>CoB</b>	costimulation blockade
<b>TCM</b>	central memory T cells
<b>TEM</b>	effector memory T cells
<b>OVA</b>	ovalbumin
<b>LM</b>	<i>Listeria monocytogenes</i>
<b>gHV</b>	gammaherpesvirus 68
<b>PyV</b>	polyoma virus
<b>i.p.</b>	intraperitoneal
<b>ICCS</b>	intracellular cytokine staining
<b>SIINFEKL</b>	ovalbumin peptide 257–264
<b>MST</b>	median survival time

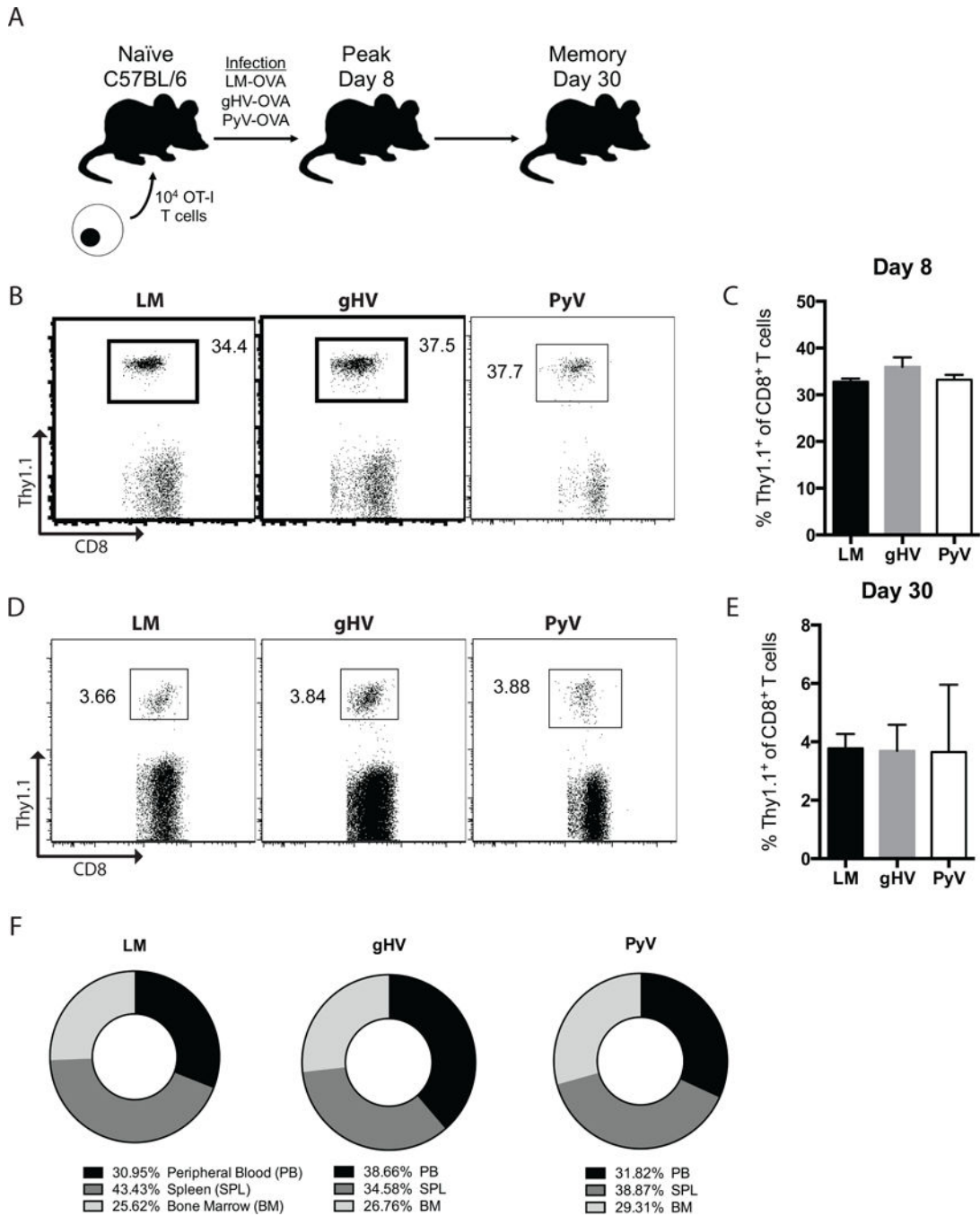
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**Figure 1. The magnitude and distribution of the antigen-specific T cell response is equivalent after pathogen stimulation with LM, gHV, or PyV**

(A) Schematic of the mouse system used.  $10^4$  OT-I CD8<sup>+</sup> T cells were adoptively transferred into naïve C57BL/6 mice that were infected 48 hours later with OVA-expressing LM, gHV, or PyV. Peripheral blood lymphocytes were then analyzed for antigen-specific CD8<sup>+</sup>Thy1.1<sup>+</sup> T cells to measure the peak effector and memory responses 8 and 30 days after infection, respectively. (B) Frequencies of antigen-specific CD8<sup>+</sup>Thy1.1<sup>+</sup> effector T cells 8 days after infection. Flow cytometry plots are representative and gated on CD8<sup>+</sup> lymphocytes. (C) Summary data of the frequency of antigen-specific CD8<sup>+</sup>Thy1.1<sup>+</sup> T cells following pathogen



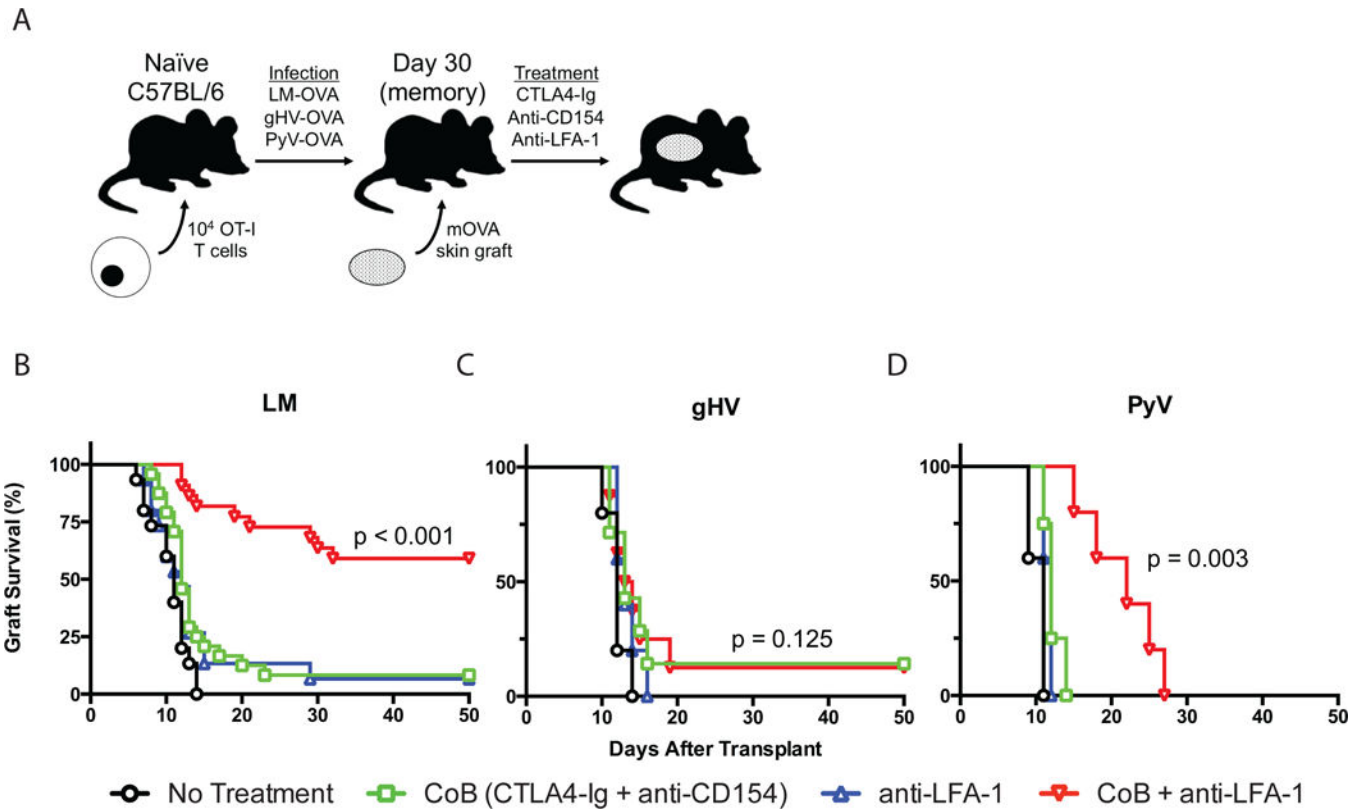
stimulation (n = 10 per group). (D) Frequencies of antigen-specific CD8<sup>+</sup>Thy1.1<sup>+</sup> memory T cells 30 days after infection. Flow cytometry plots are representative and gated on CD8<sup>+</sup> lymphocytes. (E) Summary data of the frequency of antigen-specific CD8<sup>+</sup>Thy1.1<sup>+</sup> T cells following pathogen stimulation (n = 10 per group). (F) Mean percent distribution of total pathogen-primed OT-I memory cells in each designated anatomic compartment 30 days after infection (n = 3 per group, p > 0.999 between all groups). Summary data represent mean (SE).

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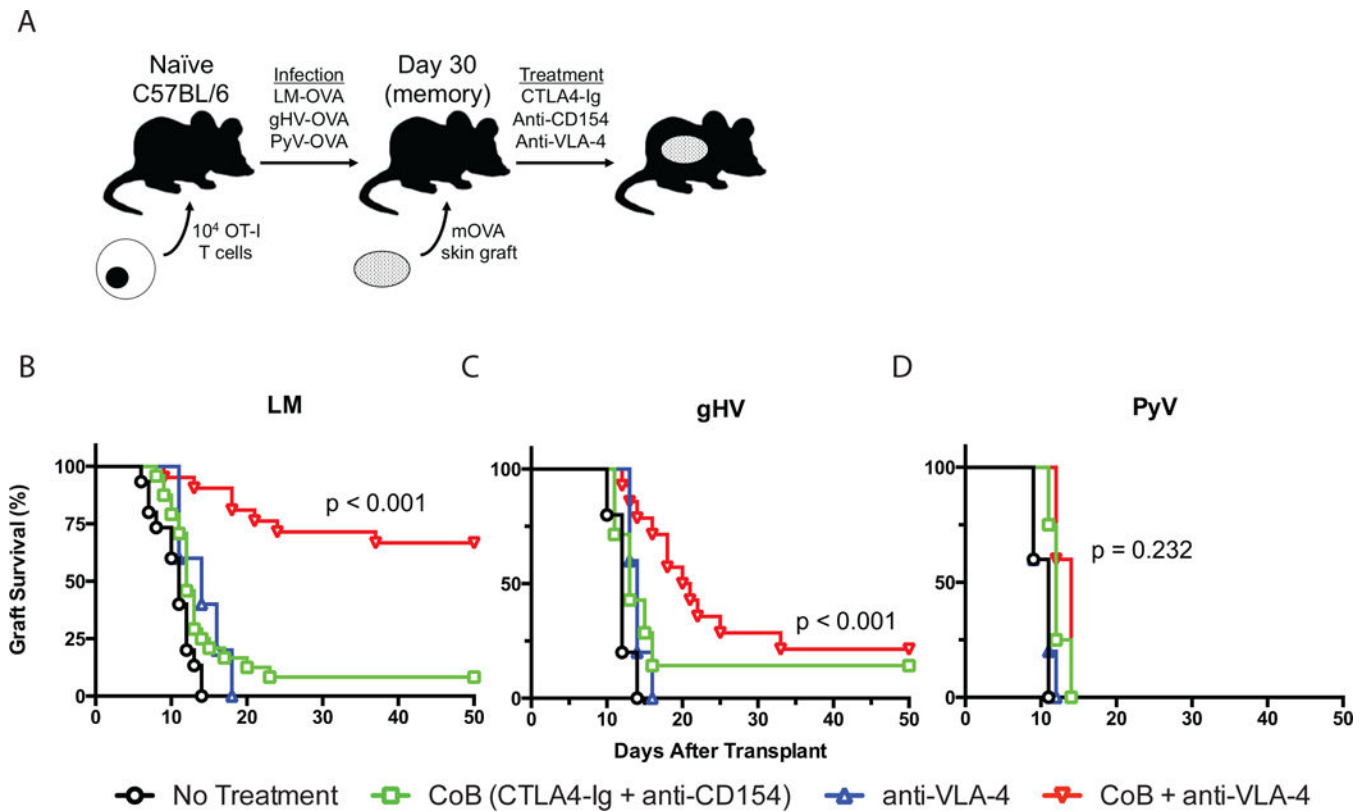
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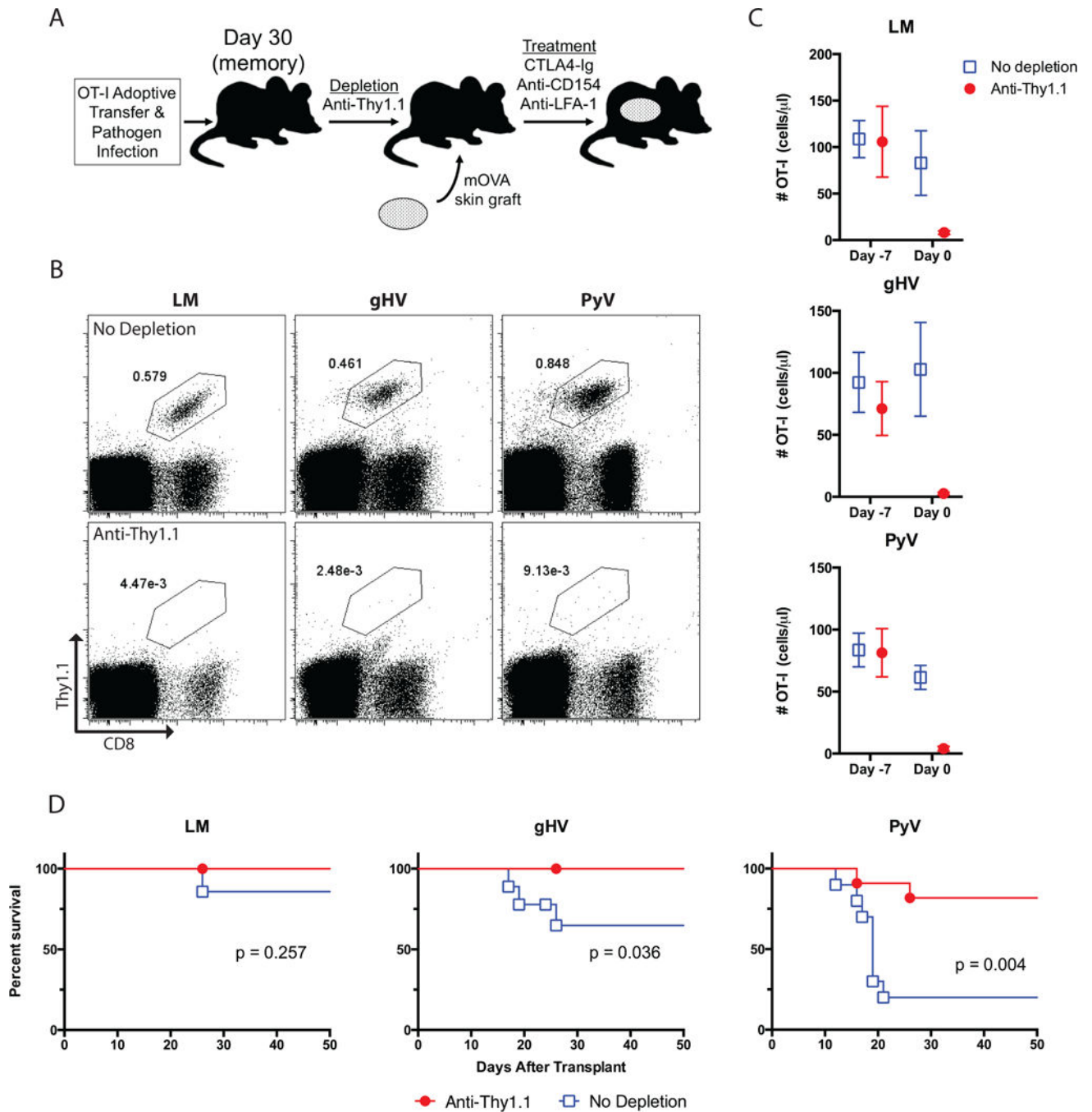
**Figure 2. Pathogen stimulation history influences skin allograft survival and donor-specific CD8<sup>+</sup> T cell susceptibility to CoB/anti-LFA-1 therapy**

(A) Schematic of the mOVA transplant mouse system used.  $10^4$  OT-I CD8<sup>+</sup> T cells were adoptively transferred into naïve C57BL/6 mice that were infected 48 hours later with OVA-expressing LM, gHV, or PyV. 30 days after infection, mice were transplanted mOVA skin grafts and treated with CoB (CTLA4-Ig + anti-CD154), anti-LFA-1, or CoB + anti-LFA-1. (B-D) Kaplan-Meier curves of mOVA skin allograft survival following infection with LM (B), gHV (C), or PyV (D). CoB + anti-LFA-1 therapy significantly prolonged skin allograft survival in LM-infected mice (MST 101 days,  $p < 0.001$ )(B) and PyV-infected mice (MST 22 days,  $p = 0.003$ )(D), but not in gHV-infected mice (MST 13.5 days,  $p = 0.125$ )(C).  $N = 4-24$  animals per group.



**Figure 3. Donor-specific CD8<sup>+</sup> T cells exhibit an altered susceptibility profile to CoB/VLA-4 therapy after pathogen stimulation**

(A) Schematic of the mOVA transplant mouse system used.  $10^4$  OT-I CD8<sup>+</sup> T cells were adoptively transferred into naïve C57BL/6 mice that were infected 48 hours later with OVA-expressing LM, gHV, or PyV. 30 days after infection, mice were transplanted mOVA skin grafts and treated with CoB (CTLA4-Ig + anti-CD154), anti-VLA-4, or CoB + anti-VLA-4. (B-D) Kaplan-Meier curves of mOVA skin allograft survival following infection with LM (B), gHV (C), or PyV (D). CoB + anti-VLA-4 therapy significantly prolonged skin allograft survival in LM-infected mice (MST 100 days,  $p < 0.001$ )(B) and gHV-infected mice (MST 20.5 days,  $p < 0.001$ )(C), but not in PyV-infected mice (MST 14 days,  $p = 0.232$ )(D).  $N = 4-24$  animals per group.



**Figure 4. Pathogen-primed CD8<sup>+</sup> OT-I T cells are required for differential CoB/anti-integrin resistance**

(A) Schematic of depletion regimen. OT-I T cells were adoptively transferred into naïve B6 mice and infected with OVA-expressing LM, gHV or PyV. Thirty days post-infection, mice within each group either received depleting anti-Thy1.1 mAb or were left untreated as controls. Mice were then transplanted mOVA skin grafts seven days after initiation of depletion and treated with CoB/anti-LFA-1 therapy. (B) Frequency of antigen-specific OT-I memory T cells on day 0 pre-transplant in control and anti-Thy1.1 depleted mice. Flow cytometry plots are representative and gated on lymphocytes. (C) Summary data of the

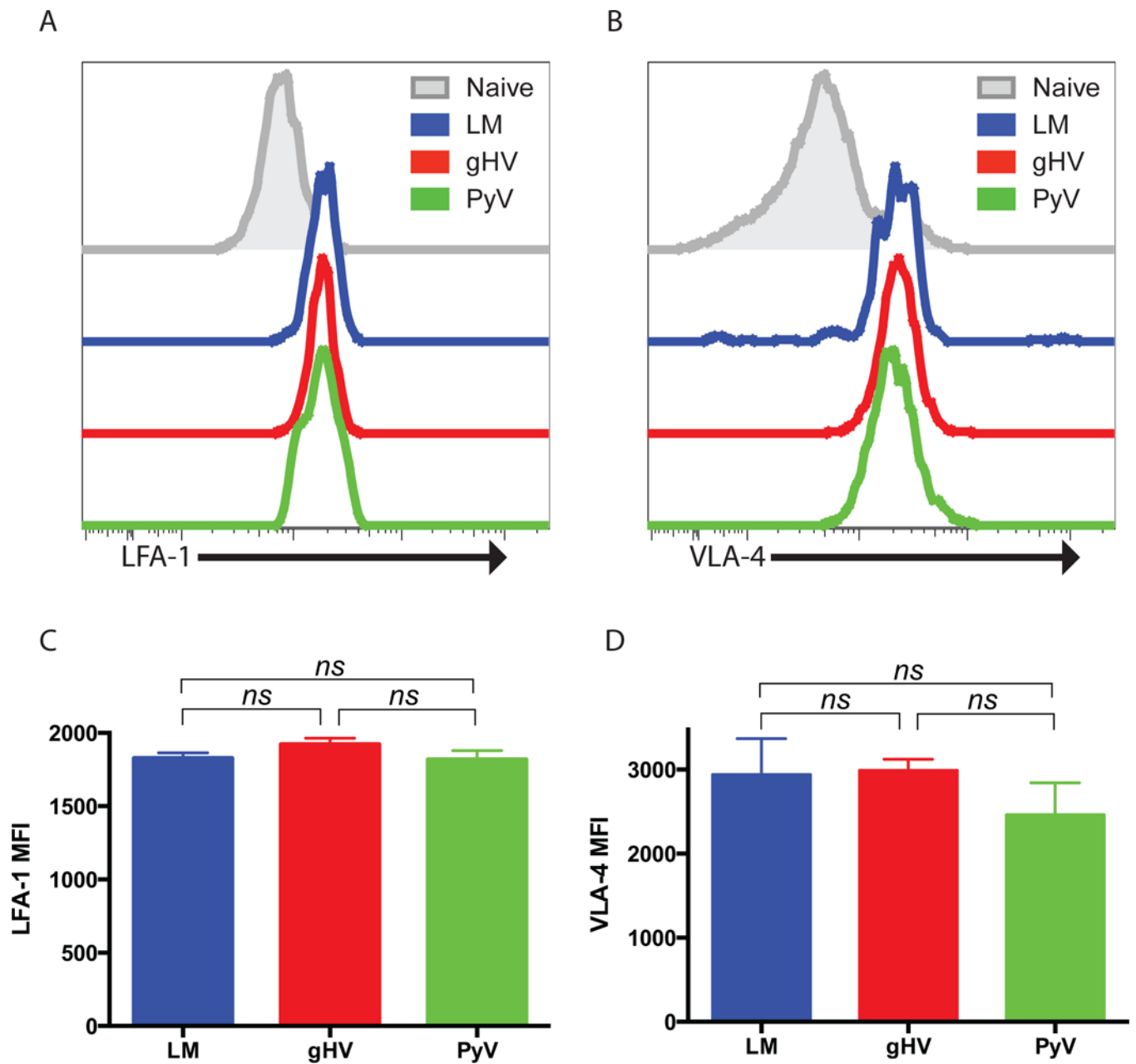
number of antigen-specific OT-I T cells before (day -7) and after (day 0) administration of anti-Thy1.1 mAb in the peripheral blood (n = 8–12 per group). (D) Kaplan-Meier curves of mOVA skin allograft survival following infection with LM, gHV or PyV. CoB + anti-LFA-1 therapy significantly prolonged skin allograft survival in anti-Thy1.1-depleted gHV- and PyV-infected mice compared to non-depleted controls (p = 0.036 and p = 0.004, respectively). N = 8–12 animals per group.

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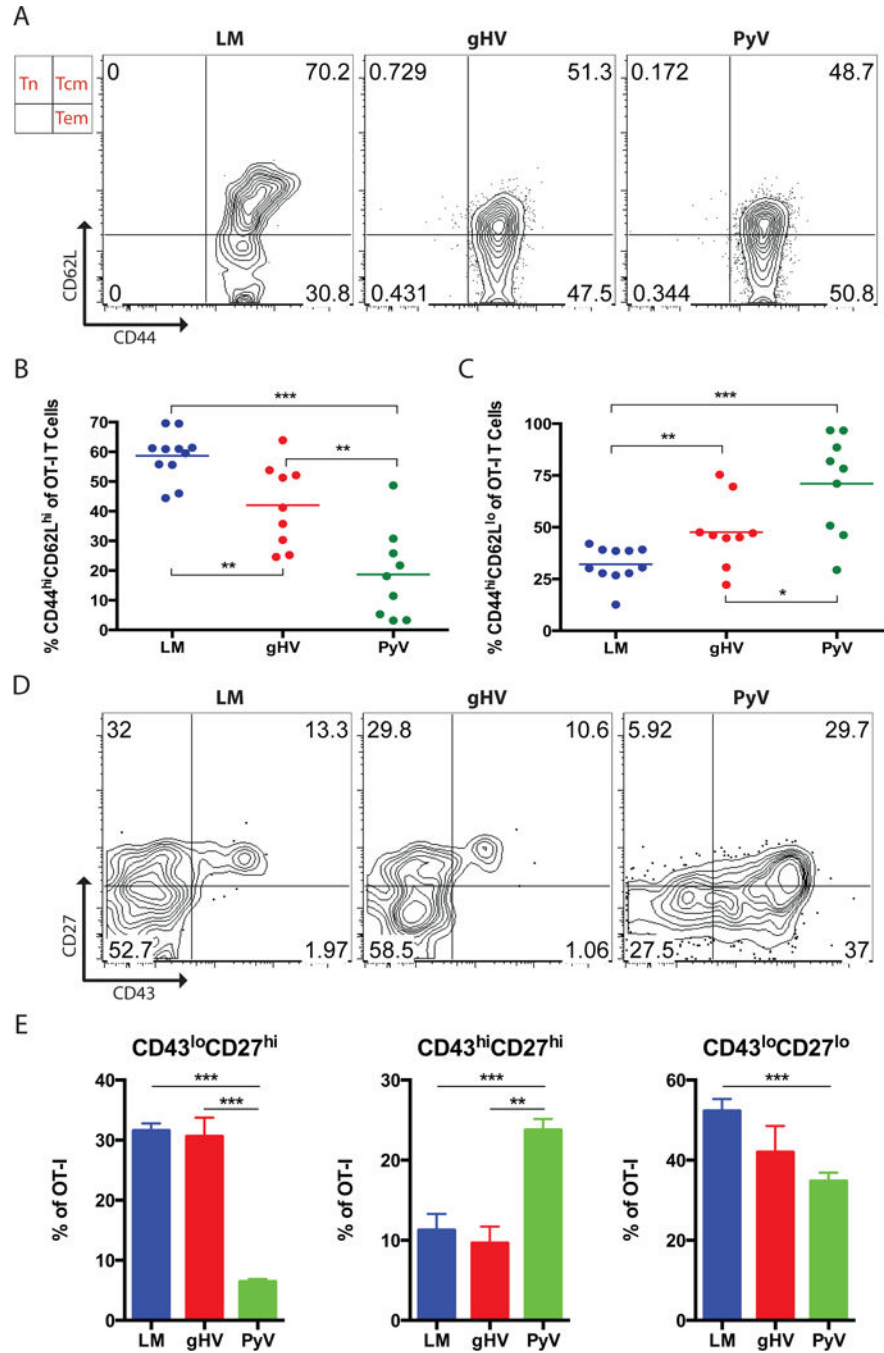
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**Figure 5. Donor-specific CD8<sup>+</sup> T cell surface expression of LFA-1 and VLA-4 does not explain altered susceptibility profiles to CoB/integrin therapy**

Naïve C57BL/6 mice were adoptively transferred  $10^4$  OT-I T cells, infected with OVA-expressing LM, gHV or PyV, and peripheral blood lymphocytes analyzed for surface expression of LFA-1 and VLA-4 30 days after infection. (A & B) Representative surface expression of LFA-1 (A) and VLA-4 (B) on CD8<sup>+</sup>Thy1.1<sup>+</sup> memory T cells compared to naïve T cells. (C & D) Summary data of LFA-1 (C) and VLA-4 (D) MFI on CD8<sup>+</sup>Thy1.1<sup>+</sup> T cells (n = 8–10 per group). Summary data represent mean (SE).





**Figure 6. Donor-specific CD8<sup>+</sup> memory T cells most susceptible to CoB/integrin blockade exhibit a T<sub>CM</sub> phenotype and are CD43<sup>lo</sup>CD27<sup>hi</sup>**

Naive C57BL/6 mice were adoptively transferred 10<sup>4</sup> OT-I T cells and then infected with OVA-expressing LM, gHV or PyV. Peripheral blood lymphocytes were then characterized 30 days after infection. (A) Analysis of CD44 and CD62L expression on antigen-specific memory T cells. Flow cytometry plots are representative and gated on CD8<sup>+</sup>Thy1.1<sup>+</sup> T cells. (B & C) Summary data of the frequency of CD8<sup>+</sup> antigen-specific CD44<sup>hi</sup>CD62L<sup>hi</sup> T<sub>CM</sub> (B) and CD44<sup>hi</sup>CD62L<sup>lo</sup> T<sub>EM</sub> (C) T cells (n = 8–10 per group). (D) Analysis of CD43 and CD27 expression on antigen-specific memory T cells. Flow cytometry plots are representative and

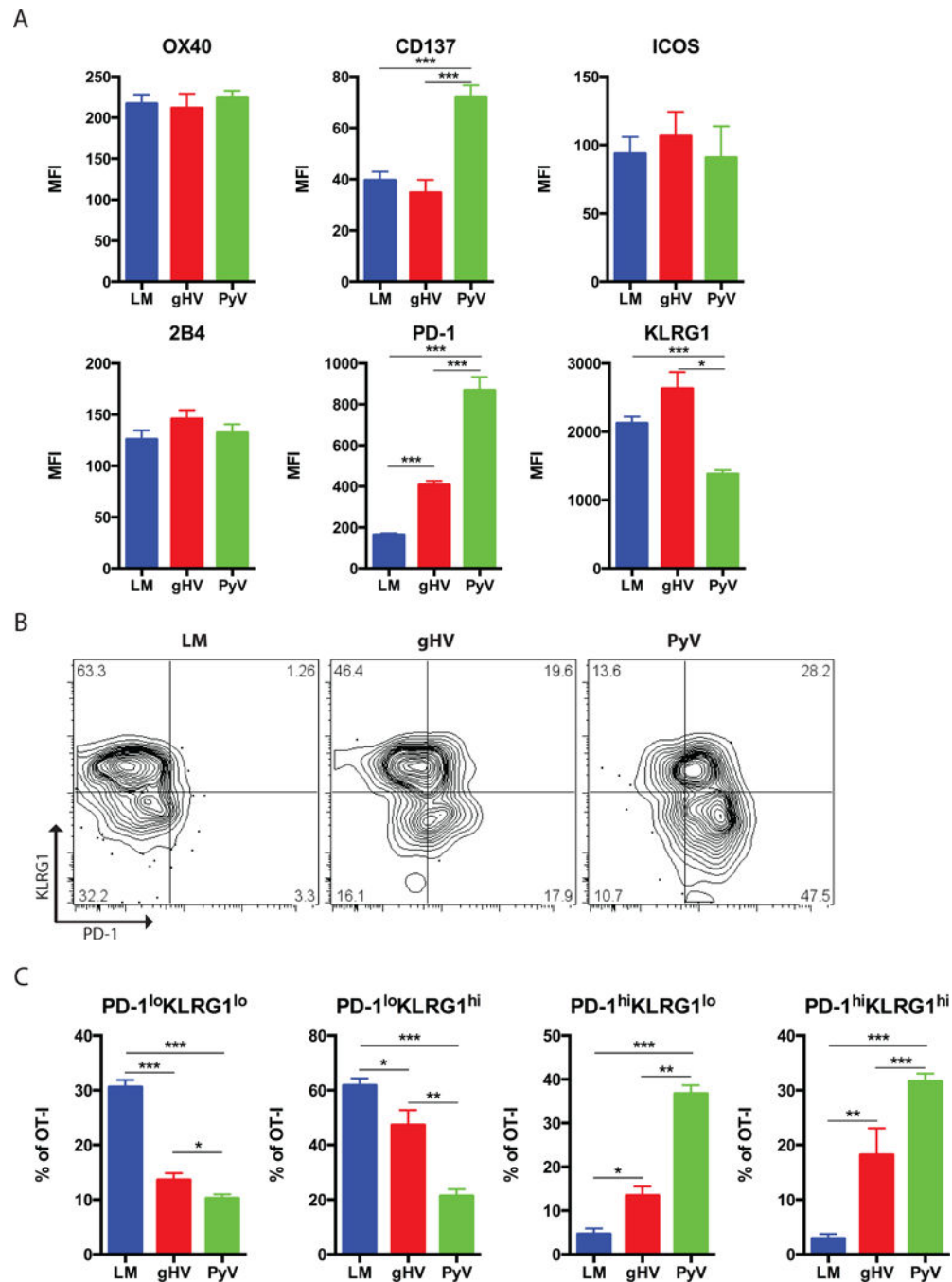
gated on CD8<sup>+</sup>Thy1.1<sup>+</sup> T cells. (E) Summary data of the frequency of CD43<sup>lo</sup>CD27<sup>hi</sup>, CD43<sup>hi</sup>CD27<sup>hi</sup>, and CD43<sup>lo</sup>CD27<sup>lo</sup> T cells (n = 8–10 per group). Summary data represent mean (SE). \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

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**Figure 7. Donor-specific CD8<sup>+</sup> memory T cells exhibit a less differentiated PD-1/KLRG1 phenotype**

Naive C57BL/6 mice were adoptively transferred  $10^4$  OT-I T cells and then infected with OVA-expressing LM, gHV or PyV. Peripheral blood lymphocytes were then characterized 30 days after infection. (A) Summary data of the MFI on antigen-specific CD8<sup>+</sup>Thy1.1<sup>+</sup> memory T cells for the designated surface markers (n = 8–10 per group). (B) Analysis of PD-1 and KLRG1 expression on antigen-specific memory T cells. Flow cytometry plots are representative and gated on CD8<sup>+</sup>Thy1.1<sup>+</sup> T cells. (C) Summary data of the frequency of

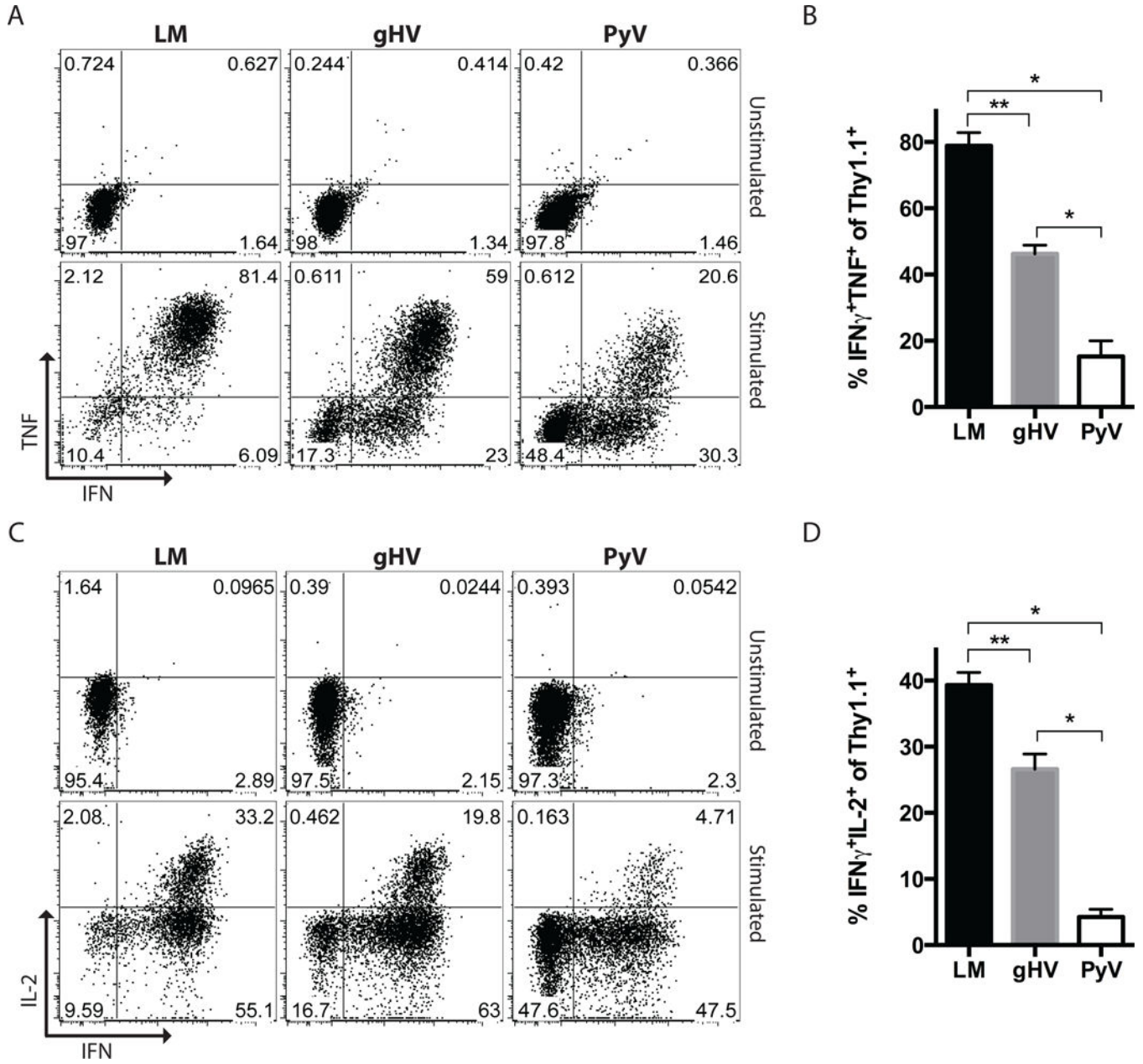
PD1<sup>lo</sup>KLRG1<sup>lo</sup>, PD1<sup>lo</sup>KLRG1<sup>hi</sup>, PD1<sup>hi</sup>KLRG1<sup>lo</sup>, and PD1<sup>hi</sup>KLRG1<sup>hi</sup> T cells (n = 8–10 per group). Summary data represent mean (SE). \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

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**Figure 8. Pathogen stimulation history impacts cytokine production amongst donor-specific CD8<sup>+</sup> T cells**  
 Naive C57BL/6 mice were adoptively transferred 10<sup>4</sup> OT-I T cells, infected with LM, gHV or PyV, and transplanted skin from mOVA mice. 7 days after transplant, lymphocytes from graft-draining axillary lymph nodes were isolated and restimulated ex-vivo with SIINFEKL peptide. (A) IFN-γ and TNF production by donor-reactive CD8<sup>+</sup>Thy1.1<sup>+</sup> T cells after antigen stimulation. Flow cytometry plots are representative and gated on CD8<sup>+</sup>Thy1.1<sup>+</sup> lymphocytes. (B) Summary data of the frequencies of IFN-γ<sup>+</sup>TNF<sup>+</sup> donor-reactive CD8<sup>+</sup>Thy1.1<sup>+</sup> T cells (n = 3–5 per group). (C) IFN-γ and IL-2 production by donor-reactive CD8<sup>+</sup>Thy1.1<sup>+</sup> T cells after antigen stimulation. Flow cytometry plots are representative and gated on CD8<sup>+</sup>Thy1.1<sup>+</sup> lymphocytes. (D) Summary data of the frequencies of IFN-γ<sup>+</sup>IL-2<sup>+</sup>

donor-reactive CD8<sup>+</sup>Thy1.1<sup>+</sup> T cells (n = 3–5 per group). Summary data represent mean (SE). \* p < 0.05, \*\* p < 0.01.

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**Table 1**

Treatment groups and skin allograft survival

Treatment	Mice per Group (n)	Median allograft Survival (days)		
		LM	gHV	PyV
No treatment	5–15	11	12	11
CoB (CTLA4-Ig + anti-CD154)	4–24	12	13	12
Anti-LFA-1	5–15	12	13	12
Anti-VLA-4	5	14	14	11
CoB + anti-LFA-1	5–22	101 <sup>**</sup>	13.5	22 <sup>*</sup>
CoB + anti-VLA-4	5–21	100 <sup>**</sup>	20.5 <sup>**</sup>	14

\*  
p < 0.01,\*\*  
p < 0.001