

## ORIGINAL ARTICLE

# Oral alloantigen exposure promotes donor-specific tolerance in a mouse model of minor-mismatched skin transplantation

Peter Wang<sup>1,2</sup>  | Luqiu Chen<sup>1</sup> | Christine M. McIntosh<sup>1,3</sup> | Jorden I. Lane<sup>4</sup> |  
 Rena Li<sup>1,2</sup> | Stephen Z. Xie<sup>1,2</sup> | Husain Sattar<sup>4</sup> | Daria Esterhazy<sup>4</sup> | Anita S. Chong<sup>5</sup>  |  
 Maria-Luisa Alegre<sup>1</sup> 

<sup>1</sup>Department of Medicine, Section of Rheumatology, University of Chicago, Chicago, Illinois, USA

<sup>2</sup>The College, University of Chicago, Chicago, Illinois, USA

<sup>3</sup>Pritzker School of Medicine, University of Chicago, Chicago, Illinois, USA

<sup>4</sup>Department of Pathology, University of Chicago, Chicago, Illinois, USA

<sup>5</sup>Department of Surgery, Section of Transplantation, University of Chicago, Chicago, Illinois, USA

## Correspondence

Maria-Luisa Alegre, Department of Medicine, Section of Rheumatology, Chicago, IL, USA.  
 Email: [malegre@midway.uchicago.edu](mailto:malegre@midway.uchicago.edu)

## Funding information

NIAID, Grant/Award Number: P01AI-97113; Animal Resources Center, Grant/Award Number: 75N93020D00005; University of Chicago Human Tissue Resource Center, Grant/Award Number: RRID:SCR\_019199; Cancer Center Support Grant, Grant/Award Number: P30CA014599; University of Chicago Flow Cytometry Core Facility, Grant/Award Number: RRID: SCR\_017760

## Abstract

Oral antigen exposure is a powerful, non-invasive route to induce immune tolerance to dietary antigens, and has been modestly successful at prolonging graft survival in rodent models of transplantation. To harness the mechanisms of oral tolerance for promoting long-term graft acceptance, we developed a mouse model where the antigen ovalbumin (OVA) was introduced orally prior to transplantation with skin grafts expressing OVA. Oral OVA treatment pre-transplantation promoted permanent graft acceptance and linked tolerance to skin grafts expressing OVA fused to the additional antigen 2W. Tolerance was donor-specific, as secondary donor-matched, but not third-party allografts were spontaneously accepted. Oral OVA treatment promoted an anergic phenotype in OVA-reactive CD4<sup>+</sup> and CD8<sup>+</sup> conventional T cells (Tconvs) and expanded OVA-reactive Tregs pre-transplantation. However, skin graft acceptance following oral OVA resisted partial depletion of Tregs and blockade of PD-L1. Mechanistically, we revealed a role for the proximal gut draining lymph nodes (gdLNs) in mediating this effect, as an intestinal infection that drains to the proximal gdLNs prevented tolerance induction. Our study extends previous work applying oral antigen exposure to transplantation and serves as proof of concept that the systemic immune mechanisms supporting oral tolerance are sufficient to promote long-term graft acceptance.

## KEYWORDS

basic (laboratory) research/science, immunosuppression/immune modulation, T cell biology, tolerance: experimental

## 1 | INTRODUCTION

Oral tolerance, the phenomenon of local and systemic immune unresponsiveness to ingested and commensal microbe-derived antigens within the gastrointestinal tract, has evolved as a mechanism to

prevent food allergy or unwanted intestinal inflammation. Tolerance to the gut luminal contents is imparted through antigen uptake and presentation in the gdLNs and involves many peripheral T cell tolerance mechanisms, including deletion, anergy, and Treg induction.<sup>1-3</sup> Interestingly, oral tolerance is maintained by immune mechanisms

**Abbreviations:** B6, C57BL/6; CoB, costimulation blockade; gdLNs, gut draining lymph nodes; i.p., intraperitoneal; iTregs, induced Tregs; MFI, mean fluorescence intensity; mLN, mesenteric lymph node; OVA, ovalbumin; *S. venezuelensis*, *Strongyloides venezuelensis*; Tconvs, conventional T cells.

First Author: Peter Wan. Last Author: Maria-Luisa Alegre.

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that are known to also occur in costimulation blockade (CoB)-induced transplantation tolerance, in particular, antigen-specific Treg induction, T cell dysfunction, and T cell deletion.<sup>4-9</sup> In solid organ transplantation, achieving a state of donor-specific tolerance, where after a short treatment regimen, the host becomes tolerant to the graft while remaining immunocompetent against other antigens, is a major goal. As a therapeutic approach, acquiring transplantation tolerance via oral tolerance would be desirable as it is a non-invasive treatment expected to result in relatively few side effects. Several groups have attempted to induce oral tolerance to environmental antigens or autoantigens, with varying degrees of success in models of food allergy, autoimmunity, rheumatoid arthritis, and diabetes.<sup>10</sup>

In transplantation, oral tolerance has had modest success in reducing alloreactivity or prolonging graft survival, and there are hints that it might have some efficacy. For instance, recipients of maternal kidney transplants that had been breast-fed during infancy had better 1-year post-transplantation prognosis and graft survival than non-breast-fed controls, supporting the hypothesis that early oral antigen exposure to maternal alloantigens may have some tolerogenic effects for subsequent alloantigen challenge.<sup>11</sup> In addition, a small pilot study in humans found that feeding synthetic peptides derived from HLA-DR2 to transplant recipients of HLA-DR2<sup>+</sup> kidneys correlated with reduced panel-reactive antibody titers to those same peptides, though whether it improved transplant outcome was not reported.<sup>12</sup> Oral alloantigen administration has also been explored in rodent models of transplantation, albeit with very modest success. For instance, intra-portal injection or gavage of C57BL/6 (B6) splenocytes into BALB/c mice prolonged skin allograft survival by 2 days,<sup>13</sup> while gavage of donor splenocytes prevented hyperacute rejection of heart allografts in pre-sensitized rats but did not prolong allograft survival compared to unsensitized hosts.<sup>14</sup> Finally, in a rat model of lung transplantation, oral administration of collagen V reduced delayed-type hypersensitivity in recipient rats, correlating with systemic production of TGF- $\beta$ . Oral administration of collagen V did not decrease lung pathology but synergized with low-dose cyclosporin A to reduce pathology in MHC class I and II-mismatched lung allografts.<sup>15,16</sup> Although oral tolerance has been able to prolong graft survival in small animal models, it has not promoted long-term graft acceptance, hindering further translatability for use in the clinic.

One concern with prior methodologies is whether sufficient alloantigen survives the harsh enzymatic environment of the stomach to reach the gdLNs. Indeed, it has recently been shown that the gdLNs that primarily drain the upper small intestine/duodenum dictate the induction of pro-tolerogenic responses to oral antigens.<sup>17</sup> In this study, we revisited whether oral antigen exposure can promote tolerance to a subsequent graft expressing the same model antigen. To enrich antigen delivery to the upper intestinal gdLNs, we treated mice with omeprazole (to prevent proteolytic cleavage of antigen in the stomach) and olive oil plus antigen gavage orally, as previous work has shown that lymphatic lipid absorption enhances lymphatic antigen drainage to the upper small intestinal gdLNs.<sup>18</sup> Moreover, we and others have shown the importance of alloantigen

persistence for programming a state of exhaustion in alloreactive T cells, a peripheral mechanism of T cell tolerance associated with graft acceptance.<sup>6</sup> These observations prompted us to maintain oral donor antigen exposure beyond the initial antigen gavage, via provision in the animals' drinking water for 10 days until transplantation, to enable longer durations of antigen exposure, and investigate if this results in prolonged survival of skin grafts harboring expression of the same model antigen.

Our results provide proof of the principle that oral exposure to a model antigen can induce donor-specific tolerance and linked antigen tolerance in a mouse skin graft model, and that transplant tolerance can be prevented by immunological conflict in the duodenal lymph nodes following duodenal-draining parasitic infection at the time of oral tolerization.

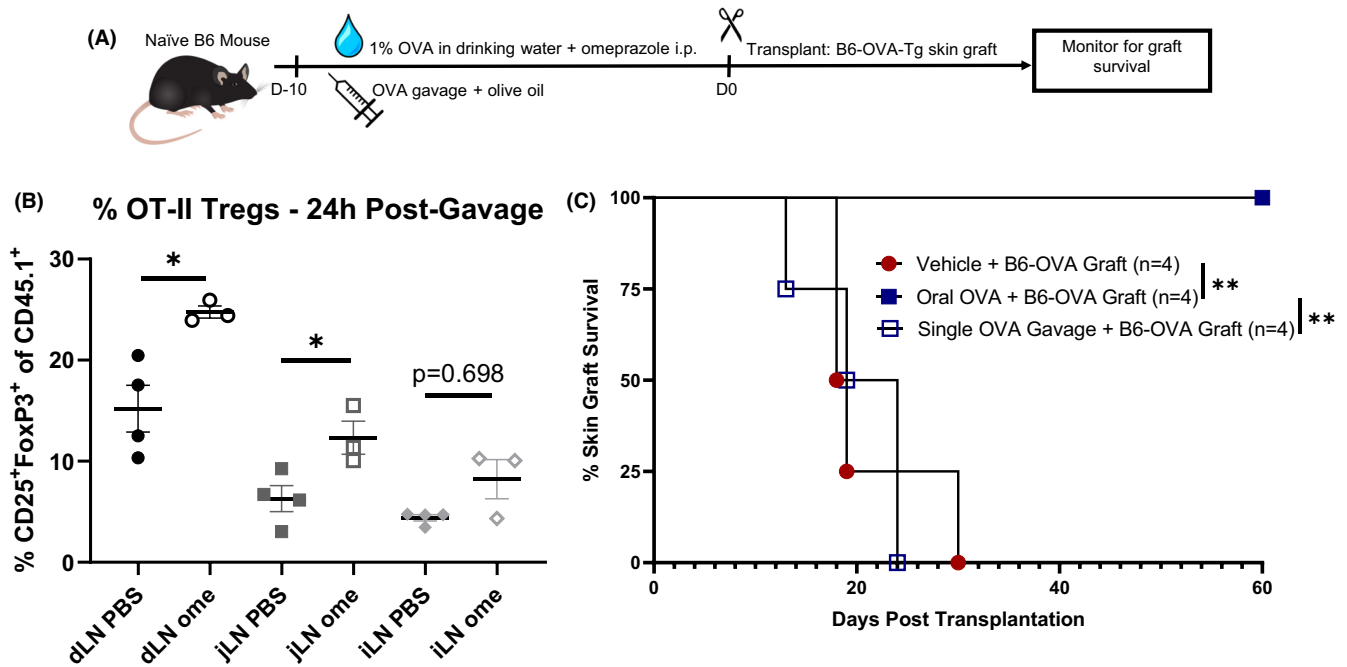
## 2 | RESULTS

### 2.1 | Oral OVA administration pre-transplantation promotes long-term acceptance of OVA-expressing skin allografts

To investigate if oral tolerance to donor antigen prior to transplantation can prolong skin graft survival, we gavaged mice with OVA protein once, on day minus 10 relative to transplantation, with or without the addition of OVA in the drinking water as a form of persistent antigen exposure until the time of transplantation. To increase the bioavailability of OVA in proximal gdLNs, we gavaged olive oil once prior to the OVA gavage, and also treated mice with omeprazole ip daily (Figure 1A), a proton pump inhibitor that raises the pH of the stomach and increased Treg induction in the proximal gdLNs early after OVA gavage (Figure 1B). Following 10 days of oral OVA, the mice were switched to regular water and transplanted with skin grafts where membrane-bound OVA was the only mismatched alloantigen. Remarkably, all mice pre-treated with the full oral OVA regimen, but not OVA gavage alone, accepted OVA-expressing skin grafts long term in the absence of immunosuppression (Figure 1C). Thus, prolonged oral exposure to donor antigen can induce long-term skin graft acceptance.

### 2.2 | Oral OVA administration promotes an anergic phenotype of OVA-reactive CD8<sup>+</sup> and CD4<sup>+</sup> conventional T cells (Tconvs)

To understand the immunological changes imparted by oral OVA pre-treatment to OVA-reactive T cells, we first used fluorescent OVA:K<sup>b</sup> peptide:MHC tetramers to identify endogenous polyclonal OVA-reactive CD8<sup>+</sup> T cells by flow cytometry prior to skin transplantation. The mesenteric lymph nodes (mLNs), and spleen were analyzed on day 10 of the oral OVA treatment, at the time when transplantation would be performed. As a positive control for activation, naive mice were immunized intravenously with splenocytes



**FIGURE 1** Oral OVA administration pre-transplantation promotes long-term acceptance of OVA-expressing skin grafts. (A) Model of oral OVA administration prior to skin transplantation. (B)  $10^6$  congenically labeled (CD45.1<sup>+</sup>) OT-II T cells were seeded into B6 mice prior to gavaging with 50  $\mu$ l olive oil 15 min prior to OVA gavage with or without omeprazole injections 48 h and 24 h prior to analysis of percent Tregs in each respective gdLN via flow cytometry. (C) B6-OVA skin grafts were transplanted onto vehicle-treated, oral OVA (OVA gavage + OVA in drinking water for 10 days)-treated, or single OVA gavage (day -10)-treated B6 recipients and graft survival was assessed over time. The experiment in 1C was pooled from two independent experiments. Statistical analysis via log-rank test. \*\* $p < .01$ . [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

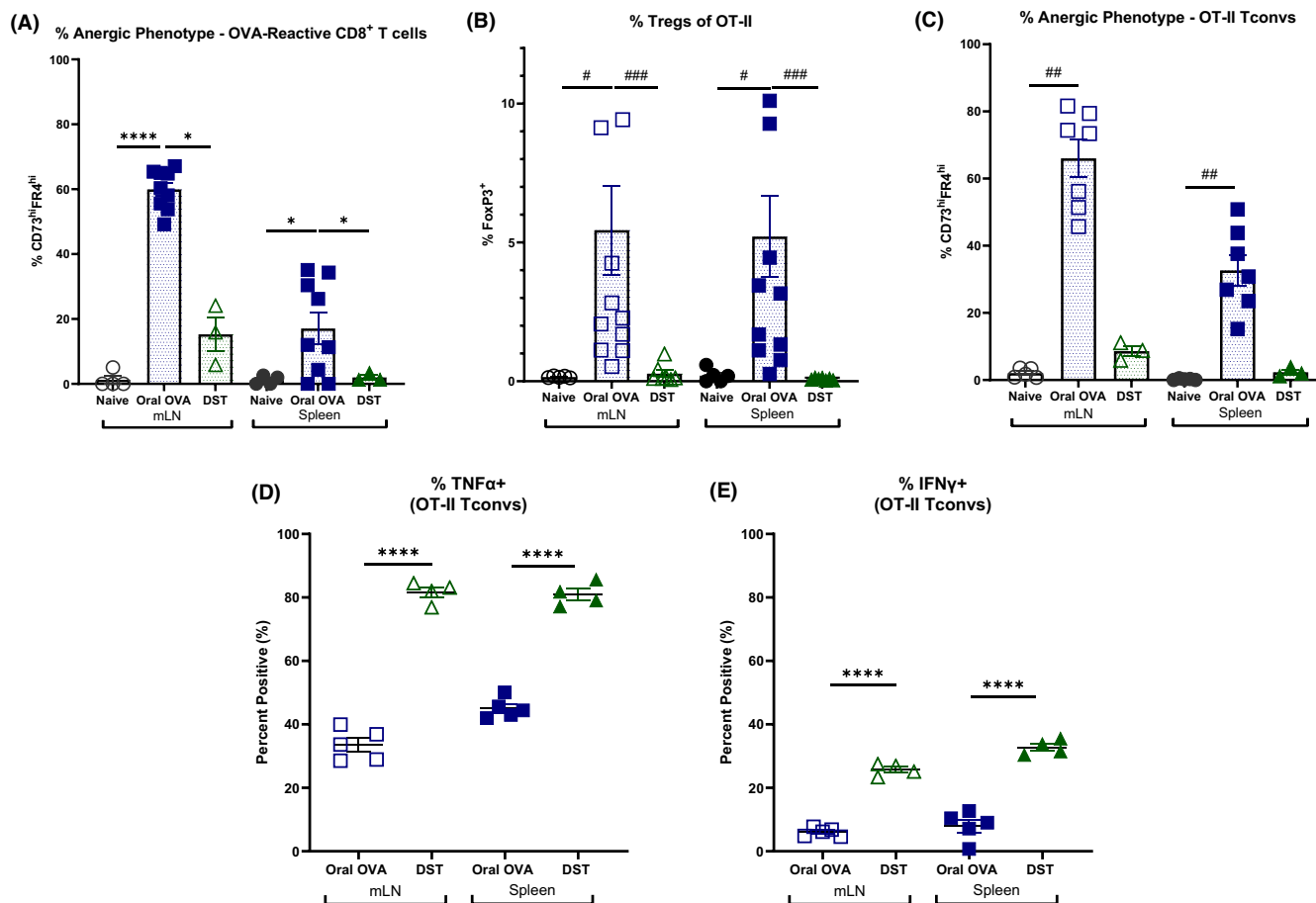
from OVA-Tg mice (donor-specific transfusion, DST) and analyzed 10 days later. The majority of OVA:K<sup>b</sup>-reactive CD8<sup>+</sup> T cells from both oral OVA-pre-treated and DST-immunized mice became antigen-experienced, as shown by upregulation of CD44 in both mLNs and spleen (Figure S1A). However, oral OVA, but not DST-immunization induced a large proportion of CD44<sup>hi</sup> OVA-reactive CD8<sup>+</sup> T cells to upregulate CD73 and FR4 - surface markers were reported to mark anergic CD4<sup>+</sup> T cells in the context of autoimmunity and pregnancy<sup>19</sup> (Figure 2A; Figure S1B-E). This anergic phenotype in CD8<sup>+</sup> T cells was unique to oral tolerance as it was not observed in OVA-reactive CD8<sup>+</sup> T cells recovered from heart transplant recipients where tolerance was induced with anti-CD154/DST (Figure S1F). Whereas oral OVA and DST-immunization resulted in a similar expansion of OVA-reactive CD8<sup>+</sup> T cells in the mLNs, oral OVA prevented the systemic splenic accumulation of OVA-reactive CD8<sup>+</sup> T cells (Figure S1G).

To understand the phenotypic and functional changes occurring in OVA-reactive CD4<sup>+</sup> T cells following oral OVA treatment, we adoptively transferred congenically marked (CD45.1<sup>+</sup>) OVA-reactive CD4<sup>+</sup> OT-II TCR-Tg T cells on a Rag<sup>-/-</sup> background 1 day prior to OVA gavage as a tracer population. On day 10 of the oral OVA treatment, we examined the phenotype and function of CD45.1<sup>+</sup> cells in the mLNs and spleen. While OT-II T cells were more likely to become FoxP3<sup>+</sup> in oral OVA-treated than DST-immunized mice, most of the recovered OT-II CD45.1<sup>+</sup> population remained FoxP3<sup>-</sup> (Figure 2B; Figure S2A,B). Similar to endogenous OVA-reactive CD8<sup>+</sup> T cells,

a high proportion of transferred OT-II CD4<sup>+</sup> T cells adopted the CD73<sup>hi</sup>FR4<sup>hi</sup> anergic phenotype in oral OVA-treated, but not DST-immunized mice (Figure 2C; Figure S2C,D). Whereas OT-II Tconvs accumulated similarly in mLNs and spleen of oral OVA-treated and DST immunized mice, OT-II Tconvs recovered from oral OVA-treated mice were less likely to produce IFN and TNF upon ex vivo restimulation than OT-II Tconvs recovered from DST-immunized mice (Figure 2D,E; Figure S2E), demonstrating donor-specific hyporesponsiveness. Together, these data indicate impaired expansion and functionality of OVA-reactive CD4<sup>+</sup> Tconvs and CD8<sup>+</sup> T cells following oral tolerance, correlating with the acceptance of a subsequent OVA-expressing skin graft.

### 2.3 | Oral OVA administration pre-transplantation promotes donor-specific tolerance

Because oral OVA-treated mice permanently accepted their subsequent OVA-expressing skin grafts, we investigated whether oral OVA treatment could extend to a linked antigen when skin grafts express OVA fused to the model antigen 2W (Figure 3A). Oral OVA-treated recipients accepted the majority of 2W:OVA-expressing skin grafts (Figure 3A) and exhibited a modest reduction in donor-specific alloantibody responses (Figure 3B). Surviving 2W:OVA skin grafts from OVA-pre-treated mice were characterized histologically by focal interstitial infiltrate and inflammation mainly localized to



**FIGURE 2** Oral OVA administration promotes an anergic phenotype on OVA-reactive CD8<sup>+</sup> and CD4<sup>+</sup> T cells prior to skin transplantation. Treatment groups and respective secondary lymphoid organs analyzed are displayed on the X-axis. mLN cells and splenocytes were harvested from untreated mice, or mice on day 10 of oral OVA treatment, or 10 days post-DST immunization and analyzed by flow cytometry. (A) Events were gated on OVA:K<sup>b</sup>-binding CD44<sup>hi</sup>CD8<sup>+</sup> T cells and analyzed for percent dual expression of CD73 and FR4. (B–E) OT-II CD45.1<sup>+</sup> T cells (10<sup>6</sup>) were seeded into mice day -1 from treatment initiation in the groups described above and analyzed 10 days later by flow cytometry. (B) Percent FoxP3<sup>+</sup> of OT-II CD45.1<sup>+</sup> T cells. (C) Percent of OT-II Tconvs (CD44<sup>hi</sup>FoxP3<sup>+</sup>) displaying dual upregulation of CD73 and FR4. (D, E) Percent of OT-II Tconvs producing TNFα (D) or IFNγ (E) after 24 h of ex vivo restimulation with plate-bound anti-CD3/anti-CD28. Data were pooled from 2 (A, D, E) or 3 (B, C) independent experiments. Statistical analysis via one-way ANOVA (\**p* < .05; \*\**p* < .01; \*\*\**p* < .001; \*\*\*\**p* < .0001), or via Kruskal Wallis non-parametric test (#*p* < .05; ##*p* < .001; ###*p* < .0001), or via *t*-test (D, E, \*\*\*\**p* < .0001). [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

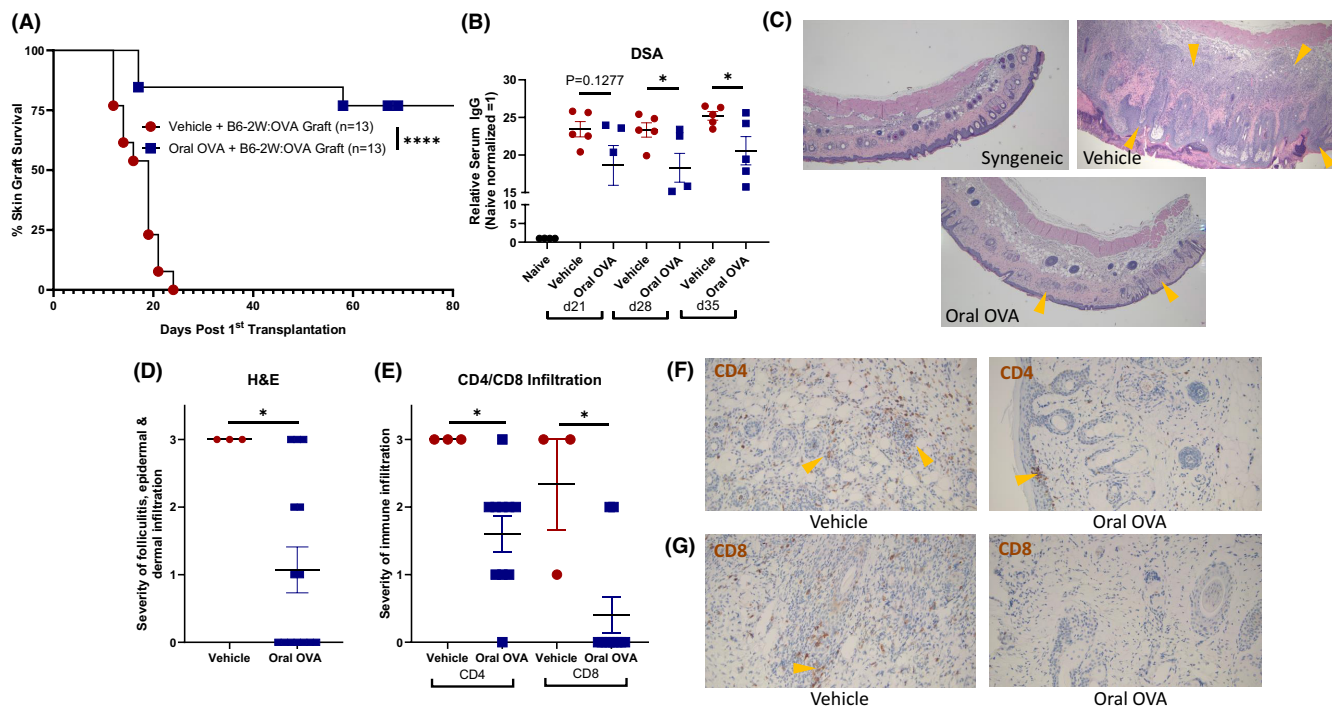
the dermal-epidermal junction and around hair follicles, and appeared similar to syngeneic grafts, whereas skin grafts undergoing active rejection in vehicle-pre-treated mice had diffuse inflammation, cellular infiltrates, tissue thickening and damage (Figure 3C,D). Immunohistochemical analysis of graft-infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> T cells revealed greater severity of immune infiltration in grafts from vehicle-treated mice compared to accepted grafts from oral OVA-treated recipients (Figure 3E–G).

Long-term graft acceptance does not necessarily equate to donor-specific tolerance. To determine whether oral OVA pre-treatment could achieve donor-specific tolerance, we challenged mice with surviving 2W:OVA-expressing skin grafts with secondary donor-matched 2W:OVA-expressing skin grafts (Figure 4A). Remarkably, mice initially tolerized with oral OVA and having accepted a 2W:OVA-expressing primary skin graft spontaneously accepted a donor-matched secondary graft but rejected third-party

male grafts expressing H-Y-encoded minor antigens (Figure 4B). Thus, oral OVA can induce bona fide donor-specific transplantation tolerance.

## 2.4 | Oral OVA administration pre-transplantation promotes linked suppression to minor mismatched antigens

The 2W:OVA system allowed us to track endogenous 2W-reactive CD4<sup>+</sup> T cells in addition to endogenous OVA-reactive CD8<sup>+</sup> T cells. We thus investigated whether oral OVA could drive linked suppression and reduce immune responses to the linked antigen 2W expressed in the subsequent graft. Oral OVA-pre-treated and vehicle-pre-treated mice were sacrificed on day >60 post-primary 2W:OVA skin transplantation and splenocytes were stained with



**FIGURE 3** Oral OVA administration pre-transplantation promotes linked suppression to minor-mismatched antigens. (A) Skin graft survival of mice transplanted with primary B6-2W:OVA skin grafts in oral OVA or vehicle-treated B6 recipients. (B) Serum was collected at d21, d28, and d35+ post-transplantation and analyzed for relative DSA via flow cytometry. (C) Representative hematoxylin and eosin (H&E) staining of B6 syngeneic skin grafts in untreated mice, of actively rejecting 2W:OVA-expressing skin grafts (d11–15) from vehicle-treated mice, and of tolerant 2W:OVA-expressing skin grafts (day 40+) from oral OVA-pre-treated mice. Arrows indicate infiltrates. (D) H&E slides were scored according to the scoring system described in the methods section and the severity of dermal/epidermal inflammation and folliculitis was quantified. (E) Slides were stained with immunohistochemistry antibodies against CD4 and CD8 and the degree of immune infiltration was quantified by an independent pathologist blinded to the experimental conditions as described in the methods. (F) Representative IHC staining of graft infiltrating CD4<sup>+</sup> T cells. (G) Representative IHC staining of graft infiltrating CD8<sup>+</sup> T cells. Data were pooled from three independent experiments. Statistical analysis via log-rank test (A), *t*-test (B), or Mann–Whitney non-parametric *t*-test for categorical variables (D, E). \**p* < .05. \*\*\*\**p* < .0001. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

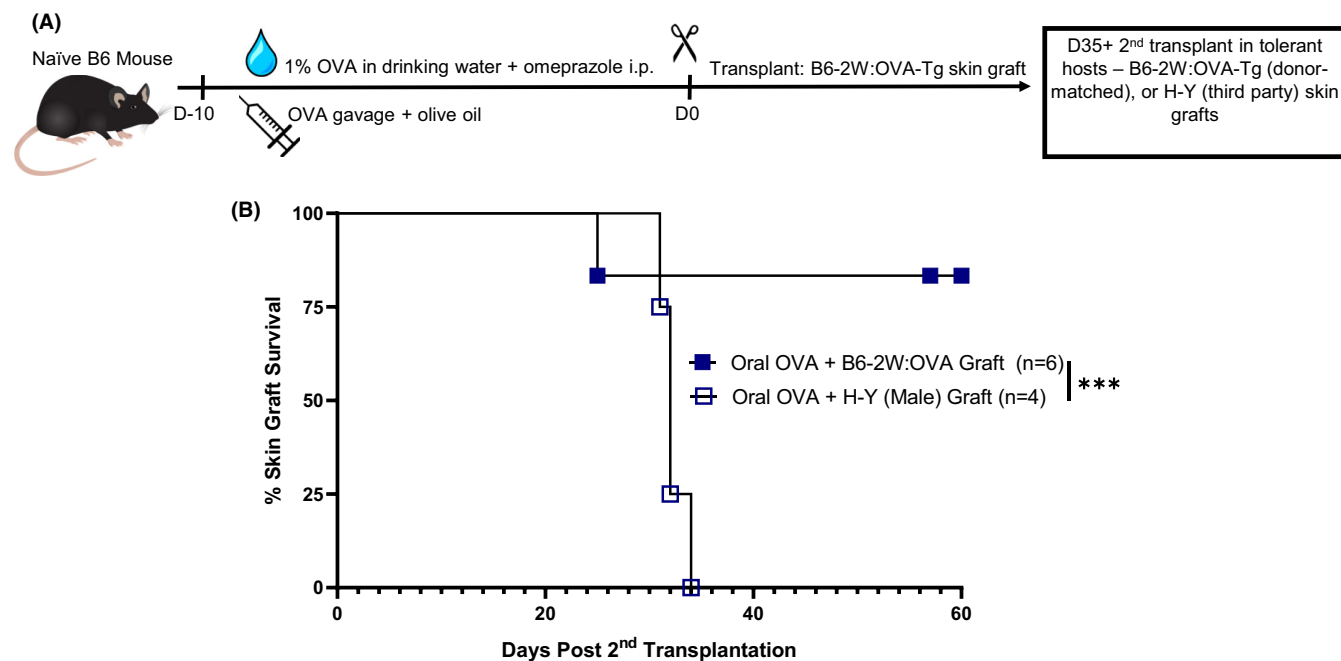
2W:I-A<sup>b</sup> and OVA:K<sup>b</sup> pMHC tetramers (Figure S3A). Oral OVA pre-treatment resulted in reduced accumulation of not only the OVA-reactive CD8<sup>+</sup> T cells that had been pre-tolerized with oral OVA but also of 2W-reactive CD4<sup>+</sup> T cells when compared to in vehicle-treated mice that had rejected their grafts (Figure 5A,B).

In CoB-induced transplantation tolerance, using p:MHC multimer mean fluorescence intensity (MFI) as a readout for the relative avidity for a cognate antigen of a T cell population, we have previously reported that tolerant mice display reduced expansion of high-affinity alloreactive T cell clones compared to rejecting mice, resulting in a low avidity state at the population level.<sup>20</sup> Similar to CoB-mediated tolerance, reduced expansion of graft-reactive T cells in oral OVA-pre-treated mice correlated with lower MFI of 2W:I-A<sup>b</sup> and OVA:K<sup>b</sup> pMHC tetramer binding compared to vehicle-pre-treated recipients and was indistinguishable from the MFI in T cells from naive, untransplanted mice (Figure S3B,C). Whereas tolerant and rejecting mice displayed similar numbers of 2W-reactive Tregs, the limited accumulation of 2W-reactive Tconvs in oral OVA-pre-treated mice resulted in high allospecific Treg:Tconv ratios (Figure 5C,D), a feature that correlates with graft acceptance in other settings.<sup>4,5,9</sup> Thus, oral OVA tolerization resulted in control of not only OVA-reactive T cells

but also of CD4<sup>+</sup> T cells reactive to the 2W antigen fused to OVA in the skin graft, with several parameters associated with transplantation tolerance, indicating linked suppression to the 2W antigen.

It is unclear if the expression of the 2W peptide alone is sufficient to trigger graft rejection. To address if oral OVA treatment-induced linked suppression could prolong the survival of skin grafts expressing antigens that can drive transplant rejection, we transplanted oral OVA-pre-treated female recipients with male 2W:OVA-expressing skin grafts, or male skin grafts devoid of 2W:OVA, as the Y-chromosome encodes several minor antigens recognized as foreign by female hosts. Whereas rejection kinetics of male skin grafts were similar in oral OVA-pre-treated and vehicle-pre-treated recipients, survival of male skin grafts expressing 2W:OVA was prolonged in hosts initially tolerized with oral OVA (Figure S4). Tolerization to OVA was not sufficient to induce long-term graft acceptance of 2W:OVA-expressing male skin grafts, most likely because of the many antigens encoded by the Y chromosome. Together with the reduced responses to the 2W antigen, prolongation of survival of OVA-expressing male skin grafts in oral OVA-pre-treated female mice supports linked suppression, with initial oral tolerance to OVA subsequently extending to immune responses against 2W and





**FIGURE 4** Oral OVA pre-transplantation promotes donor-specific transplantation tolerance. (A) Model for oral OVA administration and subsequent transplantation with primary 2W:OVA-expressing skin grafts, and secondary donor-matched or third party (H-Y<sup>+</sup>) skin grafts in the absence of any additional immunosuppression. (B) Survival of secondary donor-matched (2W:OVA-expressing) or third party (H-Y<sup>+</sup>) skin grafts following transplantation onto oral OVA-pre-treated mice that had accepted primary 2W:OVA-expressing skin grafts for >35 days. Data were pooled from two independent experiments. Statistical analysis via log-rank test. \*\*\* $p < .001$ . [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

promoting prolonged survival of grafts containing H-Y-encoded antigens, only if the grafts co-express OVA.

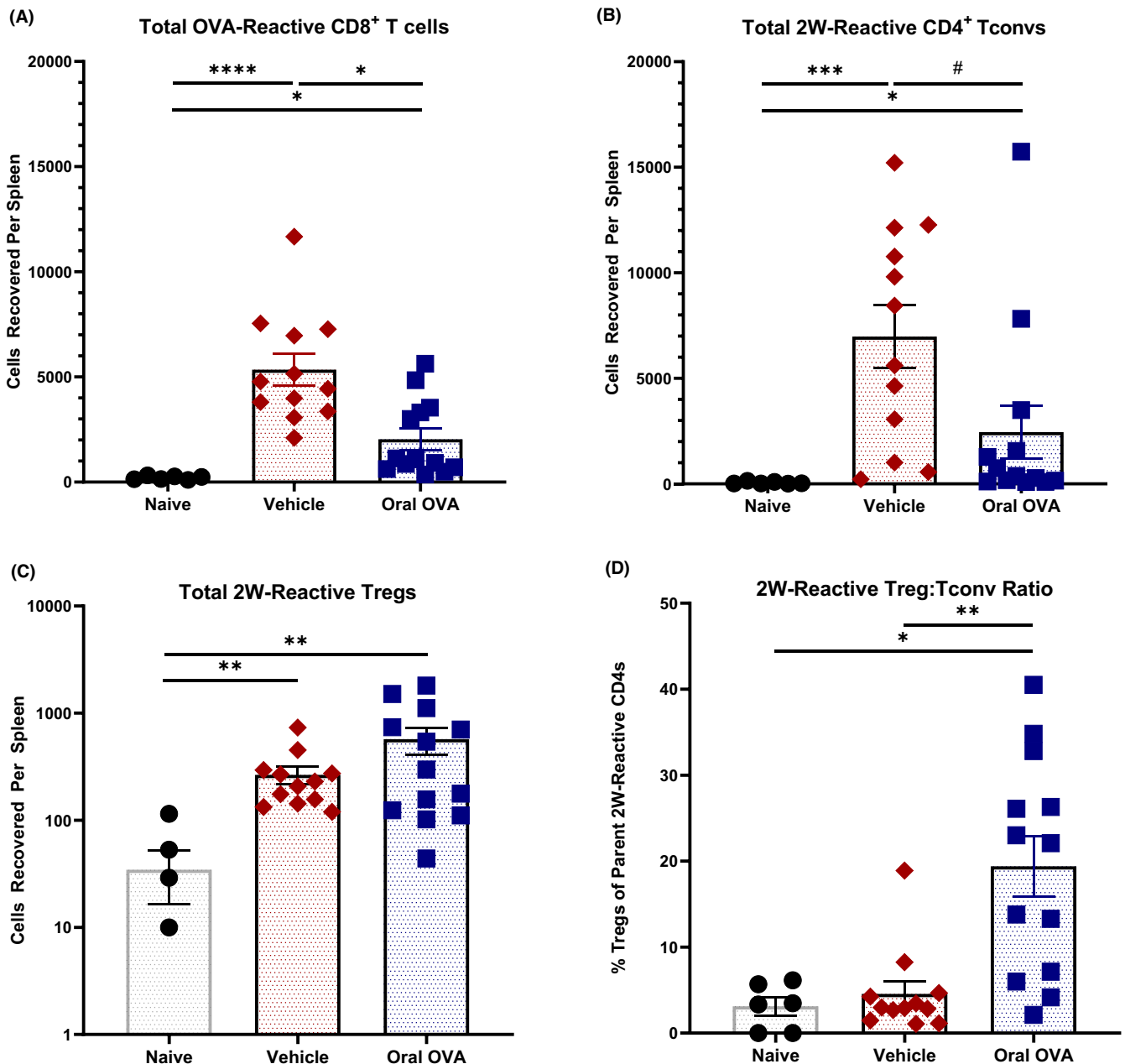
To investigate if the oral tolerance regimen could be applied to major mismatched alloantigens, we gavaged mice 10 days prior to skin transplantation with freeze-thawed K<sup>d</sup>-expressing splenocytes, prior to supplementation of lysed K<sup>d</sup>-expressing splenocytes in drinking water. Mice were subsequently transplanted with B6.K<sup>b/d</sup>-expressing skin grafts. Mice that were orally exposed to K<sup>d</sup> pre-transplantation displayed slower kinetics of graft rejection compared with vehicle-treated mice but were still able to generate donor-specific IgG antibodies. (Figure S5A,B). The addition of a low dose of CTLA4-Ig did not further prolong skin graft survival over that enabled by oral K<sup>d</sup>, but it abrogated alloantibody production (Figure S5A,B). Thus, oral tolerance significantly, though more moderately, extended the survival of a single MHC-mismatched skin allograft.

## 2.5 | Inflammation in the proximal gdLNs prevents the induction of oral antigen-mediated graft acceptance

Because we observed an increase in iTregs and high ratios of Treg:Tconv in oral OVA-treated mice, we investigated whether Tregs are required for subsequent acceptance of 2W-OVA skin grafts. Unlike in models of CoB-induced transplantation tolerance,<sup>8,21,22</sup> partial depletion of Tregs with anti-CD25 at the time of transplantation (Figure S6) failed to prevent the induction of oral OVA-mediated graft acceptance (Figure 6A). The maintenance of CoB-induced transplantation

tolerance depends both on Tregs and on co-inhibitory signals via the PD-1/PD-L1 pathway, as we have previously shown that multiple mechanisms can cooperate to maintain transplantation tolerance.<sup>7,23</sup> However, co-administration of anti-CD25 and anti-PD-L1 at the time of transplantation also failed to prevent graft acceptance by the oral OVA treatment protocol in most animals (Figure 6A), suggesting at least partial independence from these inhibitory mechanisms in contrast to CoB-induced cardiac transplantation tolerance.

Individual gdLNs are known to drain different parts of the gastrointestinal tract and harbor distinct niches that dictate either pro-tolerogenic or pro-inflammatory responses.<sup>17</sup> The proximal gdLNs have been shown to primarily mediate pro-tolerogenic responses, which can be abrogated during infections that drain to the same proximal LNs. To investigate mechanistically the role of the proximal gdLNs in transplant tolerance induction following oral donor antigen exposure, we infected mice with the helminth *Strongyloides venezuelensis* (*S. venezuelensis*), a parasitic worm with a duodenal tropism that has been shown to prevent oral tolerance.<sup>17</sup> *S. venezuelensis* infection is known to be controlled in immunocompetent hosts and cleared 10–12 days after infection.<sup>24</sup> Oral OVA treatment was initiated at the peak of infection to induce immune conflict during the time of first antigen exposure and 2W:OVA-expressing skin grafts were transplanted on day 10 of OVA treatment (Figure 6B). Oral OVA pre-treatment during *S. venezuelensis* infection was much less effective at inducing subsequent acceptance of 2W:OVA-expressing grafts. In previously infected mice, 46.67% of the skin grafts were rejected, and 100% of grafts demonstrated macroscopic damage as determined by their visual inspection scores at day 40 post-transplantation (Figure 6C,D).



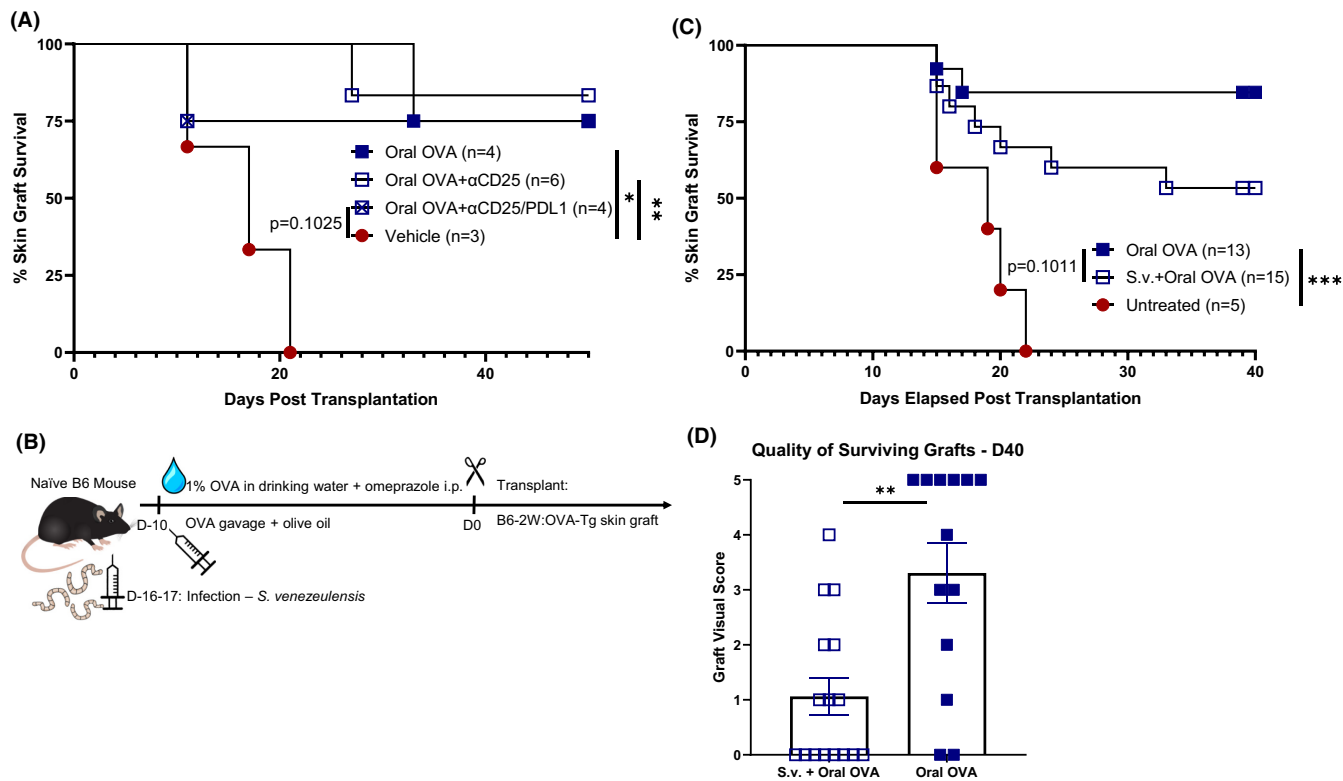
**FIGURE 5** Oral OVA pre-transplantation results in linked suppression of 2W-reactive CD4<sup>+</sup> T cells in mice tolerant to 2W:OVA-expressing skin grafts. Splenocytes were isolated from untransplanted naive, oral OVA-pre-treated, or vehicle-pre-treated mice on day 60+ post-transplantation of 2W:OVA-expressing skin grafts. Cells were stained with 2W:I-A<sup>b</sup> and OVA:K<sup>b</sup> tetramers and analyzed by flow cytometry. The gating strategy is outlined in Figure S3. (A) Total numbers of endogenous OVA:K<sup>b</sup>-reactive CD8<sup>+</sup> T cells recovered. (B) Total numbers of endogenous 2W:I-A<sup>b</sup>-reactive CD4<sup>+</sup> Tconvs (FoxP3<sup>-</sup>) recovered. (C) Total numbers of 2W-reactive (FoxP3<sup>+</sup>) Tregs recovered. (D) Treg:Tconv ratio of 2W-reactive CD4<sup>+</sup> T cells recovered. Data were pooled from 3 independent experiments. Statistical analysis via Kruskal Wallis non-parametric test (\* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$ ; \*\*\*\* $p < .0001$ ) and via Mann-Whitney non-parametric  $t$ -test (# $p < .05$ ). [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

Thus, oral tolerance induction pre-transplantation was precluded by immune conflict in the proximal gdLNs.

### 3 | DISCUSSION

In this study, we show that an improved oral antigen exposure regimen to the model antigen OVA pre-transplantation promoted

donor-specific tolerance and linked suppression to 2W and H-Y-expressing skin grafts. Oral OVA-induced expansion of OVA-reactive Tregs and acquisition of an anergic phenotype by OVA-reactive CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Mechanistically, we uncovered a role for the proximal intestinal gdLNs in mediating tolerance to distal skin grafts, as duodenal infection during oral OVA treatment prevented oral OVA-induced acceptance of subsequent 2W:OVA-expressing grafts in the majority of the animals.



**FIGURE 6** Inflammation in the proximal gdLNs prevents the induction of oral antigen-induced graft acceptance. (A) Survival curve of oral OVA-pre-treated mice that were challenged with either anti-CD25 or anti-CD25/anti-PDL1 at the time of transplantation with 2W:OVA-expressing skin grafts. (B) Model of subcutaneous *S. venezuelensis* infection to induce immune conflict in the proximal gdLNs at the time of oral OVA treatment. (C) Survival curve of 2W:OVA-expressing skin grafts transplanted onto oral OVA-pre-treated recipients where tolerance was induced 6–7 days after subcutaneous *S. venezuelensis* infection. (D) Quality of skin grafts upon visual inspection at day 40 post-transplantation as per (C). 1 point each was given for the graft being present, retaining its initial size (~1 cm<sup>2</sup>), containing hair, displaying pigmentation, and lacking red inflammatory spots for a total of 5 points for a perfect graft. Data were pooled from 2 (A) or 3 (C, D) independent experiments. Statistical analysis via log-rank test (A, B), or Mann-Whitney one-tailed *t*-test (C). \**p* < .05. \*\**p* < .01. \*\*\**p* < .0001. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

This new protocol was more successful at using oral antigen exposure to prolong graft survival due to the extended duration of oral donor antigen administration compared to previous studies,<sup>12,14–16</sup> as a single gavage of OVA was insufficient in our model to induce acceptance of subsequent OVA-expressing skin grafts. Indeed, in transplantation, the persistence of antigen exposure is a key determinant of alloreactive T cell dysfunction.<sup>6,25</sup> This is similar to the persistence of antigen being necessary for the induction of T cell exhaustion/dysfunction in settings of chronic viral infection and solid tumors.<sup>26</sup> Moreover, the addition of omeprazole, a selective proton pump inhibitor that raises the pH of the stomach, and gavage with olive oil that promotes lymphatic drainage,<sup>18</sup> likely helped improve antigen bioavailability in the upper intestinal gdLNs and may have enabled a more pro-tolerogenic environment given the increased number of Tregs in duodenal and jejunal lymph nodes when omeprazole was added.

Oral OVA administration not only resulted in iTreg generation and expansion, but also in an anergic phenotype of CD4<sup>+</sup> Tconvs. High expression of CD73 and FR4 has previously been shown to mark anergic CD4<sup>+</sup> Tconvs and Treg precursors in the context of autoimmunity and pregnancy,<sup>19</sup> consistent with our observation that OT-II Tconvs lose function after oral OVA administration. Oral

OVA administration also resulted in dual expression of CD73 and FR4 by CD44<sup>hi</sup>OVA:k<sup>b</sup>-reactive CD8<sup>+</sup> T cells. High CD73 expression by tumor-infiltrating CD8<sup>+</sup> T cells has been shown to be associated with resistance to checkpoint blockade immunotherapy,<sup>27,28</sup> and with metabolic deficiencies.<sup>29</sup> However, in the setting of acute myeloid leukemia, CD73<sup>+</sup> CD8<sup>+</sup> T cells were more functional than their CD73<sup>-</sup> counterparts.<sup>30</sup> Thus, the role of high CD73 expression in CD8<sup>+</sup> T cells following oral tolerance warrants further investigation. Similarly, the role of high FR4 expression in CD8<sup>+</sup> T cells that have encountered persistent antigen stimulation, or a tolerogenic environment, remains uncertain.

Evolutionarily, breaking of oral tolerance would be deleterious for survival, such that resistance to tolerance reversal may be a key feature of oral tolerance induction. Indeed, partial depletion of Tregs with anti-CD25 and the combination of anti-CD25 and anti-PD-L1 at the time of transplantation both failed to prevent oral OVA-induced transplantation tolerance. This is in contrast to the ability of anti-CD25 to prevent CD154/DST-mediated graft acceptance in mouse models of allogeneic skin, heart, and islet transplantation,<sup>19,31,32</sup> although these are models of full allogeneic mismatches where anti-donor responses may be more aggressive and more difficult to control by Tregs. Nevertheless, our results support the



hypothesis that the Tconv energy induced by oral tolerance plays a role in subsequent graft acceptance and suggests partial independence from Tregs and PD-1/PD-L1 pathways.

While the tolerogenic regimen was particularly robust with the model antigen OVA and imparted linked suppression to 2W-reactive T cells in the case of 2W:OVA-expressing skin grafts, graft survival was not permanent in the case of oral exposure to an MHC alloantigen prior to transplantation with an MHC-mismatched skin graft. This might be due to an insufficient load of oral MHC antigen or due to the membrane-bound nature of MHC when feeding freeze-thawed splenocytes in comparison to feeding soluble OVA protein. During tolerance induction, the gavage of a quarter donor spleen may not result in an MHC amount comparable to the 50mg of OVA administered in the initial gavage. This is in agreement with previous studies showing that the quality of oral tolerance achieved depends on the dose of antigen administered.<sup>33,34</sup> Future studies should focus on engineering allogeneic MHC such that higher doses of alloantigen can be administered orally and for sustained periods.

Our study showed that immune conflict in the proximal gdLNs induced by a pathogen at the time of oral OVA-exposure prevented acceptance of roughly half of the skin grafts, demonstrating that the “immune context” of proximal gdLNs is important for transplant tolerance induced by oral donor antigen exposure. The helminth *S. venezuelensis* is a parasitic worm whose pathogenic effects are restricted to the duodenum.<sup>24</sup> The fact that not all skin grafts were rejected in infected mice might be due to downstream gdLNs playing a compensatory effect to induce/maintain oral tolerance. Our results are consistent with the notion that the duodenum is the critical site for tolerance induction to dietary antigens, and that the overall “duodenal state,” is a critical determinant in the quality of tolerance, as previous studies have demonstrated that immune conflict induced in the proximal gdLNs, but not distal gdLNs, compromises the quality of oral tolerance.<sup>17</sup> Together, these results are consistent with the notion that the induction and maintenance of oral tolerance must be robust, but also tightly regulated to support host survival.

Peripheral LNs have been shown to play a critical role in the induction of CoB-mediated cardiac transplantation tolerance in mice,<sup>35,36</sup> despite lymphatic vessels of the donor being sectioned for organ harvest and not being re-anastomosed in the recipient, such that alloantigen and alloreactive T cells reach the LNs via systemic blood circulation. Our study highlights the importance of proximal gdLNs for the induction of oral donor antigen-induced transplantation tolerance, where the transcriptome of dendritic cells is thought to be more tolerogenic than that of dendritic cells in more distal gdLNs.<sup>17</sup> Enhancing the targeting of donor antigens to lymph nodes, and perhaps especially to gdLNs, may be desirable for improved graft outcome, as exemplified by decreased mucosal damage observed in a rat model of intestinal transplantation when donor lymphatic vessels were anastomosed to the host's lymphatics in animals treated with cyclosporin A.<sup>37</sup> Our study supports the hypothesis that pre-inducing tolerance ahead of transplantation by harnessing proximal gdLNs may be a worthwhile

consideration. As oral tolerance can achieve donor-specific tolerance and linked suppression in a mouse model of minor-mismatched skin transplantation, the concept of oral alloantigen administration to improve graft outcome may be worth re-examining.

## 4 | MATERIALS AND METHODS

### 4.1 | Mice

Both male and female mice were used throughout the duration of the study and age-matched whenever possible. Six-to eight-week-old C57BL/6 (B6) or BALB/c mice were purchased from Envigo TMS. B6.K<sup>d/d</sup>-Tg mice were obtained from Alexander Chervonsky (University of Chicago) and crossed to B6 mice to generate B6.K<sup>b/d</sup> mice. 2W-mOVA-Tg mice on the B6 background (B6-2W:OVA) which express the fused transmembrane model antigens 2W and OVA under the actin promoter were obtained from James Moon (Harvard Medical School). For DST immunizations, B6-2W:OVA males were crossed to BALB/c females to generate BALB/c x B6 F1-2W-mOVA-Tg mice (F1-2W:OVA). mOVA-Tg mice on the B6 background (B6-OVA) which express the transmembrane model antigen OVA under the actin promoter were obtained from Anita Chong (University of Chicago). OT-II-Tg mice, comprising CD4<sup>+</sup> cells recognizing the OVA<sub>323-339</sub> peptide presented on I-A<sup>b</sup> were obtained from Jackson Laboratories (JAX Stock #004194) and crossed to CD45<sup>1/1</sup>Rag1<sup>-/-</sup> mice to generate congenically marked CD45<sup>1+</sup> OT-II Rag<sup>-/-</sup> mice (OT-II). NSG mice were obtained from Jackson Laboratories (JAX Stock #005557). All animals were housed under specific pathogen-free conditions and used according to the University of Chicago Institutional Animal Care and Use Committee guidelines in accordance with National Institutes of Health guidelines for animal use (ACUP protocol #71095).

### 4.2 | Oral tolerance induction

Ten days prior to skin transplantation (d-10), mice were gavaged with 50 $\mu$ l olive oil 15min prior to gavage with 50mg OVA (grade III, Sigma) suspended in 200 $\mu$ l PBS. 1% OVA by weight was supplemented in drinking water. For oral tolerance to K<sup>d</sup>, mice were gavaged with 50 $\mu$ l olive oil 15min prior to gavage of 1/4 freeze-thawed donor splenocytes in 200 $\mu$ l PBS followed by supplementation of ACK-lysed K<sup>d</sup>-expressing splenocytes (1 spleen/30ml water) for 10 days. Mice were allowed to consume supplemented drinking water ad libitum. Mice received omeprazole (Sigma) injections (i.p.) at a dose of 50mg/kg body weight in PBS-1%Tween80 at least every other day, in most cases for the entire duration of tolerance induction. All reagents were prepared sterile in a tissue culture hood. Untreated control mice were gavaged with olive oil and received omeprazole injections for at least one replicate of all experiments. For simplicity, untreated or vehicle-treated mice are referred to as “vehicle” in the figures.

### 4.3 | Treatment with CTLA4-Ig, in vivo depletion and blockade with monoclonal antibodies

In the indicated experiments, mice received 500 µg CTLA-4Ig (abatacept, BMS) in 100 µl PBS i.p. on the day of transplantation (d0) and days 2, 4, 6 post-transplantation. CD25<sup>+</sup> T cells were depleted with anti-CD25 (clone PC.61, BioXcell) at a dose of 0.4 mg/200 µl PBS i.v. on the day of transplantation. Depletion was confirmed by the loss of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells in peripheral blood mononuclear cells (PBMCs) 7 days later. Anti-PD-L1 blocking antibody (clone B7-H1, BioXcell) was administered on the day of transplantation (d0) at a dose of 0.5 mg/200 µl PBS and 0.25 mg/100 µl PBS i.p. days 2, 4, and 6 post-transplantation.

### 4.4 | Skin transplantation and histology

Donor mice were sacrificed while under ketamine anesthetic and the tail was wiped with an isopropyl wipe. Tail skin from donor mice was removed with a razor blade, cut into roughly 1 cm<sup>2</sup> pieces, and transplanted onto the prepared graft bed on the flank of shaved recipient mice under ketamine anesthetic. A vaseline-coated gauze was placed on top of the graft and bandages were removed on day 7 post-transplantation. Grafts were monitored two to three times per week and scored according to size, presence of hair, pigmentation, and the appearance of inflammatory red spots. Rejection was determined when <20% of viable skin tissue remained. For histology, skin grafts were removed, cut in half laterally, placed into Tissue-Tek cassettes (Sakura Finetek USA), and fixed for 36–48 h in 10% neutral buffered formalin. Cassettes containing fixed tissue were stored in 70% ethanol prior to processing and staining with hematoxylin/eosin (H&E) and immunohistochemistry (IHC) using antibodies against CD4 and CD8. Slides were imaged with an infinity HD camera mounted on an Olympus microscope (model #BX45TF), and analyzed by a blinded pathologist using the following criteria for H&E: 1 point—folliculitis—>25% of all hair follicles have moderate or severe lymphocytic infiltrates; 1 point—dermal inflammation—dense aggregates of lymphocytes in a band-like distribution or multiple patchy distributions; 1 point—epidermal infiltration—basilar and epidermal damage or necrosis for a total of 3 points. Some slides were analyzed and scored for the degree of CD4/CD8 infiltration according to the following criteria: 1 point—scattered, increased dermal positive staining; 2 points—mild, scattered, increased dermal staining with focal infiltration into the dermis and hair follicles; 3 points—marked, scattered positive staining, and dermal and hair follicle infiltration.

### 4.5 | Heterotopic heart transplantation and tolerance induction with anti-CD154/DST

Heterotopic heart transplantation was performed as described previously.<sup>5-7</sup> In brief, donor F1-2W:OVA donor hearts were

harvested and placed into cold saline. Recipient B6 mice were anesthetized with ketamine/xylazine, underwent a laparotomy, and the donor aorta and vena cava were anastomosed to the recipient abdominal aorta and vena cava via end-to-side anastomosis. Mice were allowed to recover under a heat lamp until they regained sternal recumbency and grafts were monitored twice a week via palpation of the abdomen. For tolerance induction, mice were treated with 500 µg anti-CD154 (clone MR1, BioXcell) i.v. on d0 and i.p. d7 and d14 post-transplantation and donor splenocyte transfusion (DST) on d0 (preparation described below) i.v.

### 4.6 | Adoptive cell transfer and donor splenocyte transfusion (DST)

Spleen and mLNs (pooled from the central mesenteric lobes that predominantly drain the jejunum, ileum, and colon) were isolated from OT-II CD45.1<sup>+</sup> Rag<sup>-/-</sup> mice, ACK-lysed, and resuspended cells were counted with a C6 Accuri (BD Biosciences), or Fortessa flow cytometer. Cells were washed with sterile PBS, filtered and 10<sup>6</sup> cells were injected i.v. in 200 µl of PBS. For DST, spleens from F1-2W:OVA mice were harvested and homogenized in a 40 µm filter cup with a syringe plunger. Cells were resuspended in 900 µl PBS and filtered through nylon mesh prior to i.v. injection. Mice receiving DST treatment were injected with roughly ¼–½ donor spleen in 200 µl PBS i.v.

### 4.7 | Cell harvest and cell staining for flow cytometry

All monoclonal antibodies were purchased from BD Biosciences, Invitrogen, or eBioscience. Spleens and mesenteric LNs were isolated from mice and homogenized with frosted glass slides. Spleens were ACK lysed prior to staining. Cells were counted with a C6 Accuri, Fortessa flow cytometer, or Moxi V cell counter (MXV102) prior to staining 1:1000 with fixable Live Aqua live/dead stain (Invitrogen) for 20–30 min at RT in the dark. For tetramer staining, 5 × 10<sup>6</sup> to 10<sup>7</sup> unenriched cells were stained with phycoerythrin (PE) or allophycoerythrin (APC)-coupled 2W(EAWGALANWAVDSA):I-A<sup>b</sup> for 60 min at RT in a dark water bath, washed and stained with PE or APC-coupled OVA(SIINFELK):K<sup>b</sup> tetramers (NIH tetramer core facility) for 20 min in a RT dark water bath. Cells were then surface stained with fluorophore-conjugated anti-CD4 (GK1.5), anti-CD8 (53–6.7), anti-TCRβ (H57-597), anti-B220 (RA3-6B2), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD127 (A7R34), anti-PD1 (29F.1A12), anti-CD73 (TY/11.8), and anti-FR4 (12A5) for 10 min at RT in the dark. Surface-stained cells were then fixed with a FoxP3 fixation-permeabilization kit (eBioscience) for 20–30 min at RT and washed with a 1× perm buffer. Some samples were intracellularly stained with anti-FoxP3 (FJK-16s) for 30 min at RT or overnight at 4°C. Samples were run on a LSR Fortessa 4–12, 4–15, 4–15HTS, or X20 flow cytometer (BD Biosciences).

#### 4.8 | Ex vivo stimulation for cytokine production

U-bottom tissue culture plates were coated for 90–120 min or overnight with 5 µg/ml anti-CD3 (2C11) and 1 µg/ml anti-CD28 (PV.1) (Fitch Monoclonal Antibody Facility) in PBS.  $1 \times 10^6$  lymphocytes from spleens or mLN were plated in duplicate or triplicate and incubated at 37°C, 5%–10% CO<sub>2</sub> for 16–24 h. Unstimulated controls for each individual sample were plated in uncoated wells with 1 ng/ml IL-7 to ensure cell viability (Peprotech). 60–120 min after plating, brefeldin A (Biolegend) was added to all wells. After stimulation, cells were stained with fixable viability dye (Invitrogen) and surface stained with anti-CD4, anti-CD8, anti-CD44, and anti-CD45.1. Cells were then fixed and permeabilized with the FoxP3 fixation-permeabilization kit (eBioscience) for 30 min at RT and washed twice with 1× perm buffer. Cells were subsequently stained with anti-IFN $\gamma$  (XMG1.2) and anti-TNF $\alpha$  (MP6-XT22) for 30 min at RT or 4°C overnight before washing and flow cytometry analysis. Data points are presented as the average of 2–3 replicate wells per mouse.

#### 4.9 | Alloantibody determination

Serum was collected from mice and stored at –20°C.  $5 \times 10^5$  2W:OVA or K<sup>d</sup>-expressing splenocytes in PBS 2%FBS were incubated with 5 µl serum for 20 min at RT. Cells were washed, then stained with a fixable viability dye (Invitrogen), anti-CD19 (6D5), and goat anti-mouse IgG (H+L) (catalog 1031-02, Southern Biotech) for 15 min at RT. Relative IgG was determined by the MFI of live CD19<sup>+</sup> cells.

#### 4.10 | *S. venezuelensis* Infection

*Strongyloides venezuelensis* (*S. venezuelensis*) was maintained in NSG mice via subcutaneous infection with 10000 larvae. Feces of NSG mice were collected, and spread on Whatman paper in a beaker containing water and incubated at 28°C. Hatching larvae were collected, mounted onto a glass slide with PBS, and counted manually with a fluorescent microscope. About 800–1200 larvae were resuspended in PBS and injected subcutaneously into mice (upper-flank, between shoulder blades). Oral OVA treatment commenced at the peak of worm load (6–7 days post-infection).

#### 4.11 | Data analysis

Flow cytometry data were analyzed using FlowJo (Tree Star). Flow cytometry samples were gated on live cells prior to analysis. Statistical analyses were performed using GraphPad Prism, with each statistical test, statistical significance, and the number of independent replicates noted in figure legends.

#### ACKNOWLEDGMENTS

We thank the University of Chicago Flow Cytometry Core Facility (RRID:SCR\_017760), Cancer Center Support Grant (P30CA014599) for their assistance in sample acquisition and analysis, and the University of Chicago Human Tissue Resource Center (RRID:SCR\_019199) for their assistance with processing, embedding, and staining of histology slides. We would also like to recognize the staff of the Animal Resources Center for their technical assistance and the NIH Tetramer Core Facility (contract number 75N93020D00005) for providing pMHC tetramer reagents. This work was supported by NIAID grants to ASC and MLA (P01AI-97113).

#### DATA AVAILABILITY STATEMENT

The data that support the results of this study, experimental protocols, and additional details regarding methods employed in this study will be made available through request with the corresponding author (MLA).

#### DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

#### ORCID

Peter Wang  <https://orcid.org/0000-0001-7790-2596>

Anita S. Chong  <https://orcid.org/0000-0003-0460-0196>

Maria-Luisa Alegre  <https://orcid.org/0000-0001-5707-6194>

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

**How to cite this article:** Wang P, Chen L, McIntosh CM, et al. Oral alloantigen exposure promotes donor-specific tolerance in a mouse model of minor-mismatched skin transplantation. *Am J Transplant.* 2022;22:2348-2359. doi: [10.1111/ajt.17107](https://doi.org/10.1111/ajt.17107)