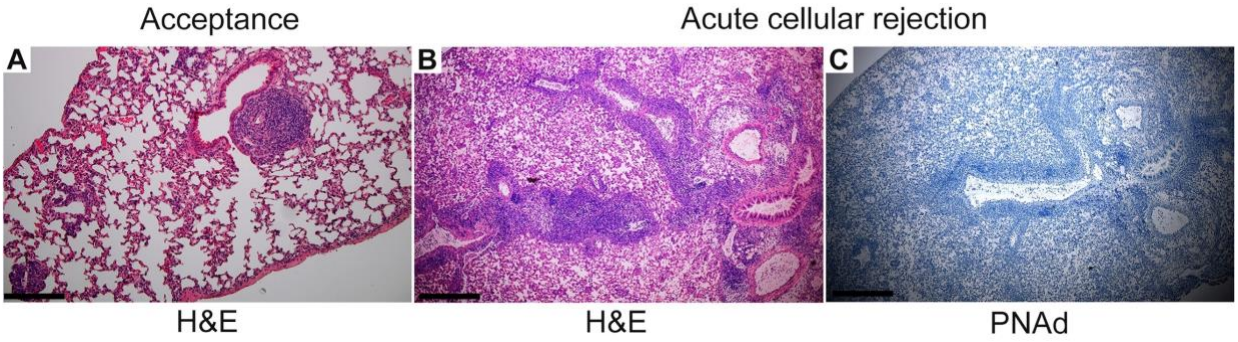
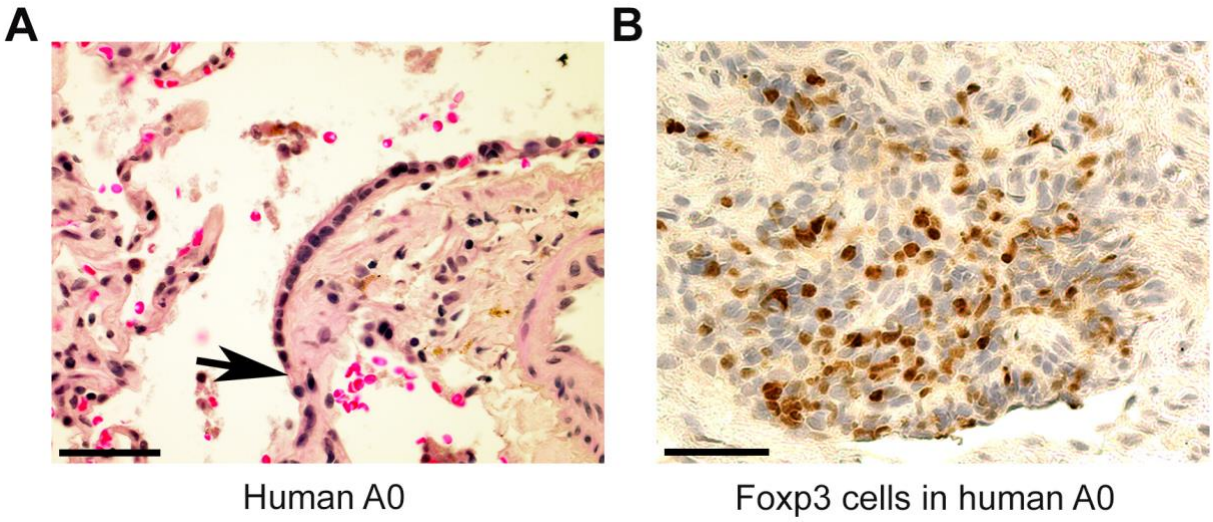
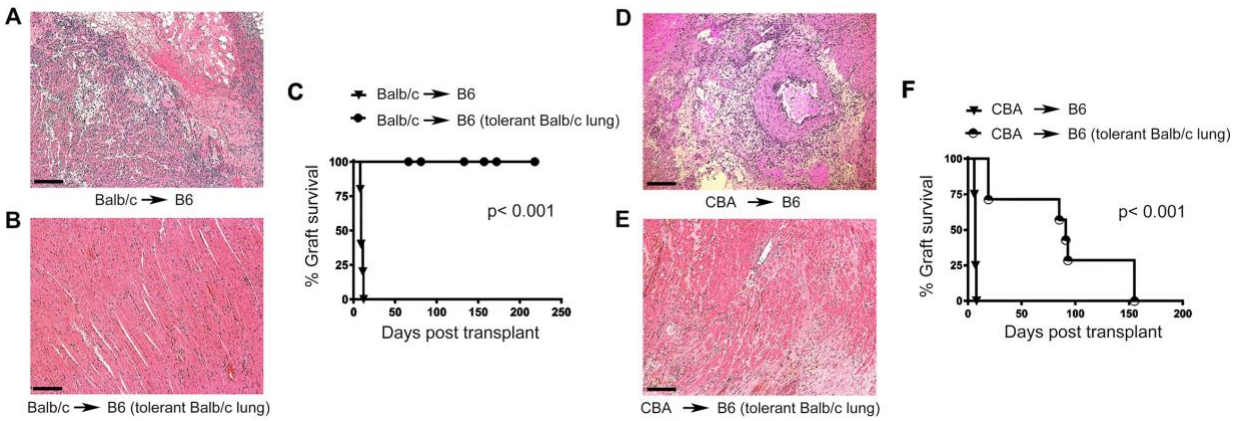
Supplemental data



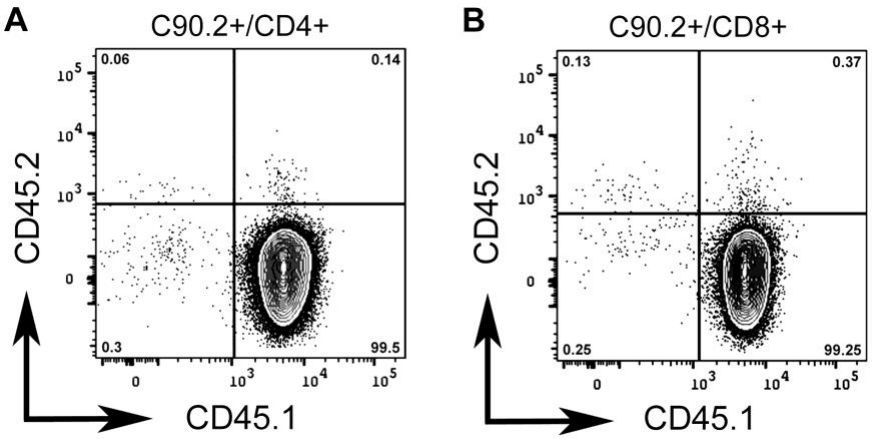
Supplemental Figure 1. Histology of accepting and rejecting lung allografts. (**A**) Histological appearance (H&E, scale bar 50μm) of Balb/c lung graft at least 30 days after transplantation into an immunosuppressed B6 host (n=8). (**B**) Histological appearance (H&E, scale bar 50μm) and (**C**) absent PNAd staining in Balb/c lung grafts that have acute cellular rejection 7 days after transplantation into non-immunosuppressed B6 recipients (n≥4).



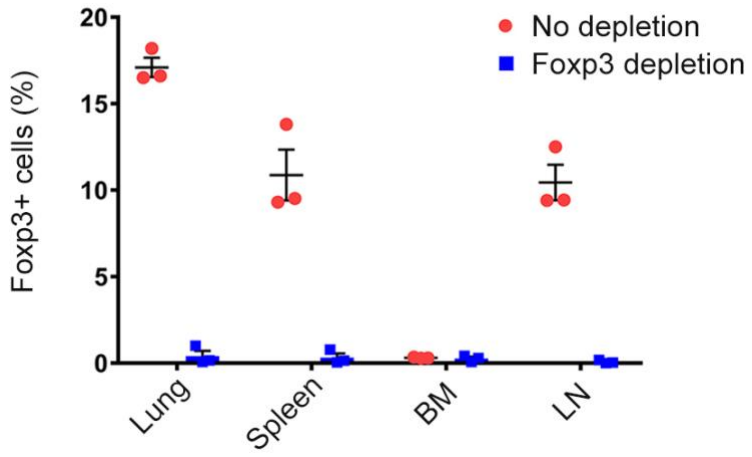
Supplemental Figure 2. Histology of human A0 lung rejection. (**A**) Histological appearance of airway epithelium (arrow) in a human lung graft with A0 rejection (H&E, scale bar 100μm). (**B**) Foxp3 immunostaining of tissue from transbronchial biopsy of human lung recipient with A0 rejection showing lymphoid aggregates with accumulation of $Foxp3^+$ cells (brown, scale bar 100μm) ($n \geq 3$).



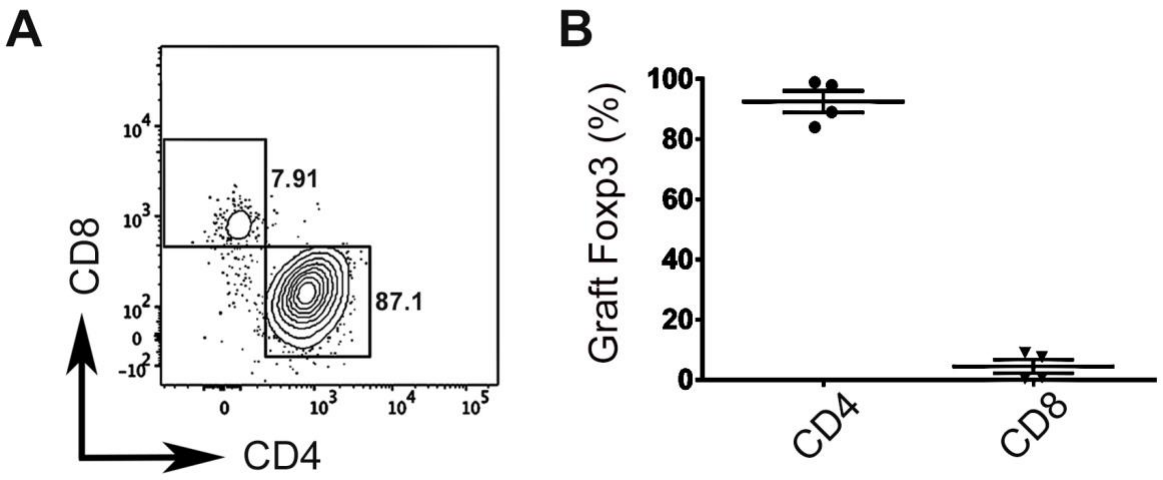
Supplemental Figure 3. Induction and maintenance of systemic tolerance after lung transplantation is dependent on the presence of the pulmonary allograft. Histological appearance of Balb/c heart grafts after transplantation into non-immunosuppressed (**A**) wild-type B6 mice (▼) (n=5) and (**B**) B6 hosts that have previously received and accepted Balb/c lungs (●)(n=8) (H&E, scale bars 100μm) as well as their (**C**) Kaplan-Meier survival curves. Histological appearance of CBA heart grafts after transplantation into non-immunosuppressed (**D**) wild-type B6 mice (▼)(n=4) and (**E**) B6 hosts that have previously received and accepted Balb/c lungs (◓)(n=7) (H&E, scale bars 100μm) as well as their (**F**) Kaplan-Meier survival curves.



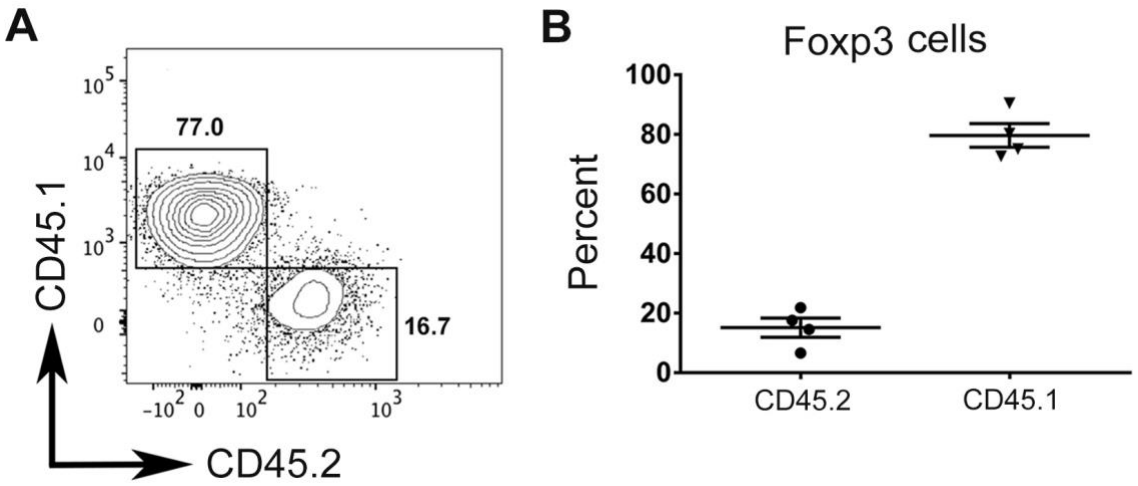
Supplemental Figure 4. T cells within accepted lung allografts are of recipient origin. Contour plots depicting expression of CD45.2 (donor) vs. CD45.1 (recipient) on (**A**) $CD4^+$ and (**B**) $CD8^+$ T cells within Balb/c lungs ($CD45.2^+$) at least 30 days after transplantation into immunosuppressed B6 ($CD45.1^+$) recipients. Plots are gated on live $CD90.2^+CD4^+$ and $CD90.2^+CD8^+$ cells, respectively. Plots are representative of two independent experiments with similar results.



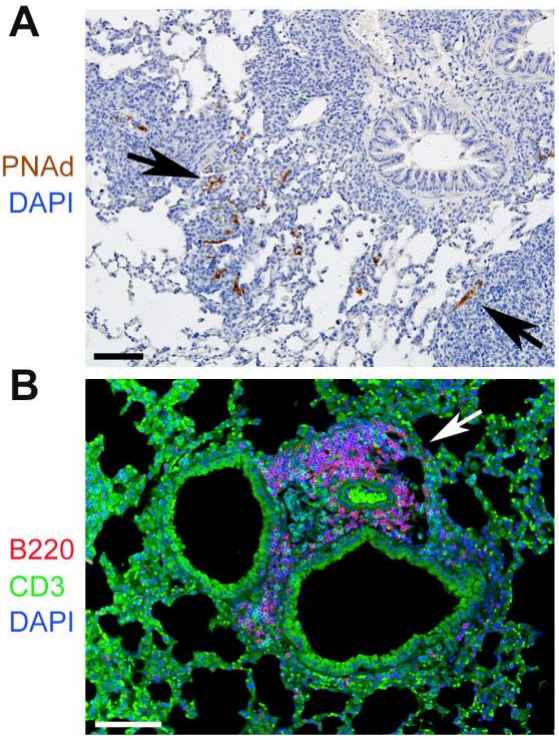
Supplemental Figure 5. Global elimination of Foxp3$^+$ cells after treatment of Foxp3-DTR mice with DT. Balb/c lungs were transplanted into costimulatory blockade-treated B6 wildtype (no depletion) or B6 Foxp3-DTR mice (Foxp3 depletion). Recipient mice were treated with DT (1 μg i.p.) on days 7 and 8 after transplantation. Frequencies of Foxp3-expressing live CD45$^+$CD90.2$^+$CD4$^+$CD8$^-$ cells were determined by flow cytometry in lung grafts, spleen, bone marrow and pooled peripheral lymph nodes 11 days after transplantation. Data are expressed as mean ± SEM (n=3).



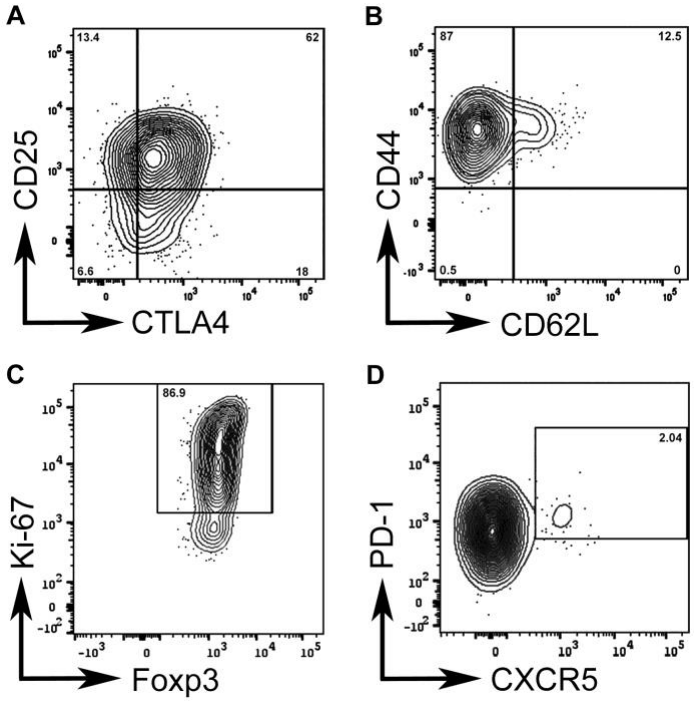
Supplemental Figure 6. Majority of graft-resident Foxp3^{+} cells in tolerant lungs express CD4. (**A**) Plot and (**B**) quantification of CD4 vs. CD8 expression on Foxp3^{+} cells from primary (CD45.2) recipient in Balb/c lungs, transplanted into immunosuppressed wildtype B6 (CD45.2) mice and re-transplanted into DT-treated B6 (CD45.1) hosts ≥ 30 days later. Grafts were analyzed by flow cytometry 7 days after re-transplantation. Plot is gated on live CD45.2^{+}CD45.1^{-}CD90.2^{+} cells (recipient). Data in (**B**) are expressed as mean ± SEM (n=4).



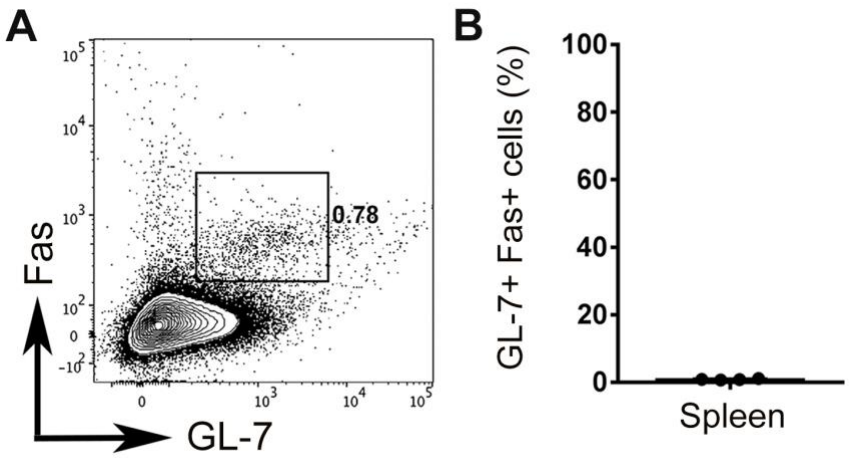
Supplemental Figure 7. Origin of Foxp3^{+} cells in lung grafts that are re-transplanted 72 hours after initial transplantation. (**A**) Plot and (**B**) quantification of distribution of CD45.1 vs. CD45.2 on live CD90.2^{+}CD4^{+}CD8^{-}Foxp3^{+} cells in Balb/c lungs that were initially transplanted into immunosuppressed wildtype B6 CD45.2 mice and 72 hours later re-transplanted into B6 CD45.1 hosts. Lung grafts were analyzed by flow cytometry 7 days after re-transplantation. Data in (**B**) are expressed as mean ± SEM (n=4).



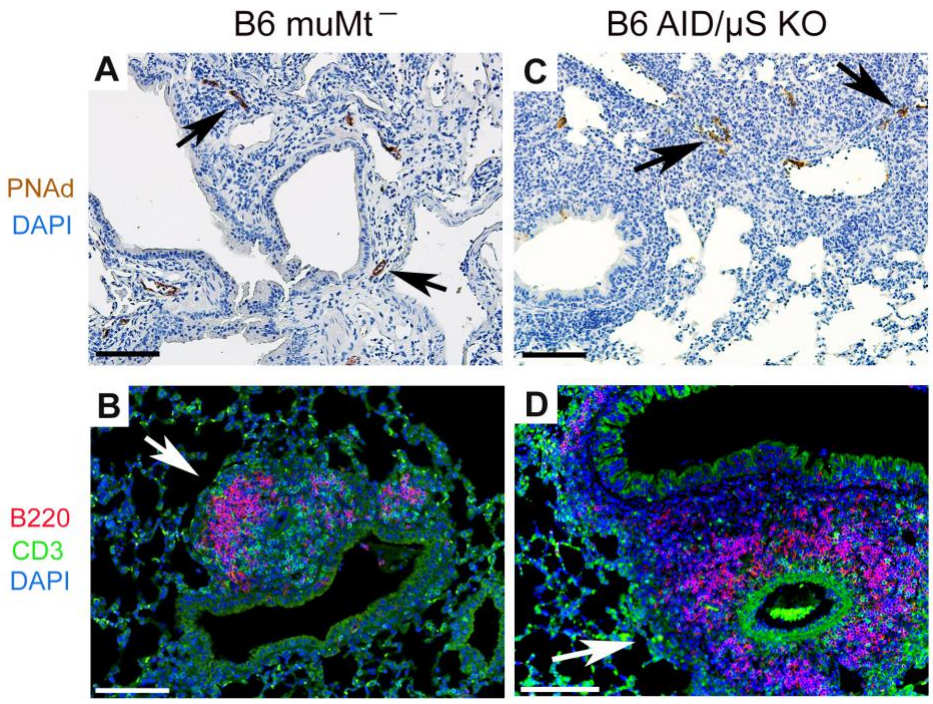
Supplemental Figure 8. BALT in re-transplanted tolerant lungs without depletion of graft-resident Foxp3^{+} cells. (**A**) PNAd (brown) (black arrow) and DAPI (blue) staining as well as (**B**) B220 (red), CD3 (green) and DAPI (blue) immunofluorescent staining in BALT (white arrow) in Balb/c lung grafts that were initially transplanted into immunosuppressed wildtype B6 mice and ≥ 30 days later re-transplanted into DT-treated B6 CD45.1^{+} hosts. Scale bars 100μm. Immunostaining was performed 7 days after re-transplantation (n=4).



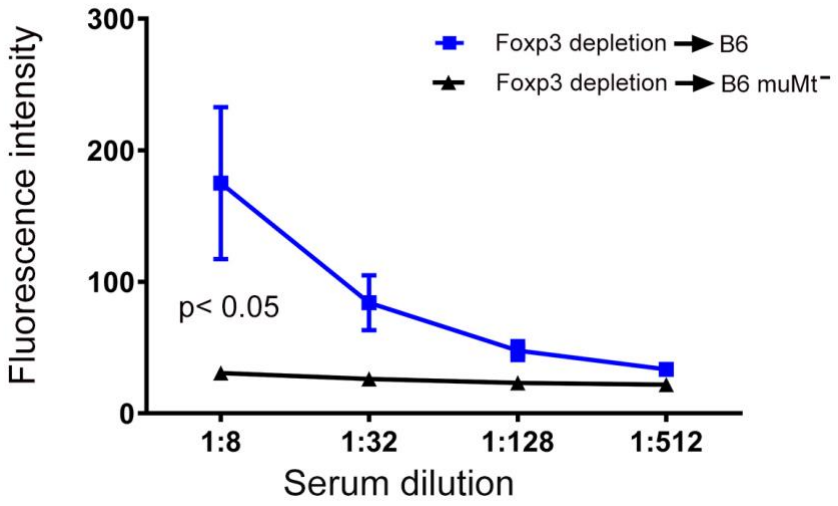
Supplemental Figure 9. Phenotype of graft-resident Foxp3$^+$ T cells. Contour plots depicting (**A**) expression of CD25 and intracellular CTLA4, (**B**) expression of CD44 and CD62Ligand, **(C)** Ki-67 expression and (**D**) expression of PD1 and CXCR5 in CD45.2$^+$CD45.1$^-$ Foxp3$^+$ T cells residing in Balb/c lung grafts that were initially transplanted into immunosuppressed B6 (CD45.2$^+$) mice and at least 30 days later re-transplanted into DT-treated B6 CD45.1$^+$ hosts. Pulmonary grafts were examined 7 days after re-transplantation. Plots are gated on live CD45.2$^+$CD45.1$^+$CD90.2$^+$CD4$^+$CD8$^-$ Foxp3$^+$ cells (n≥3 each).



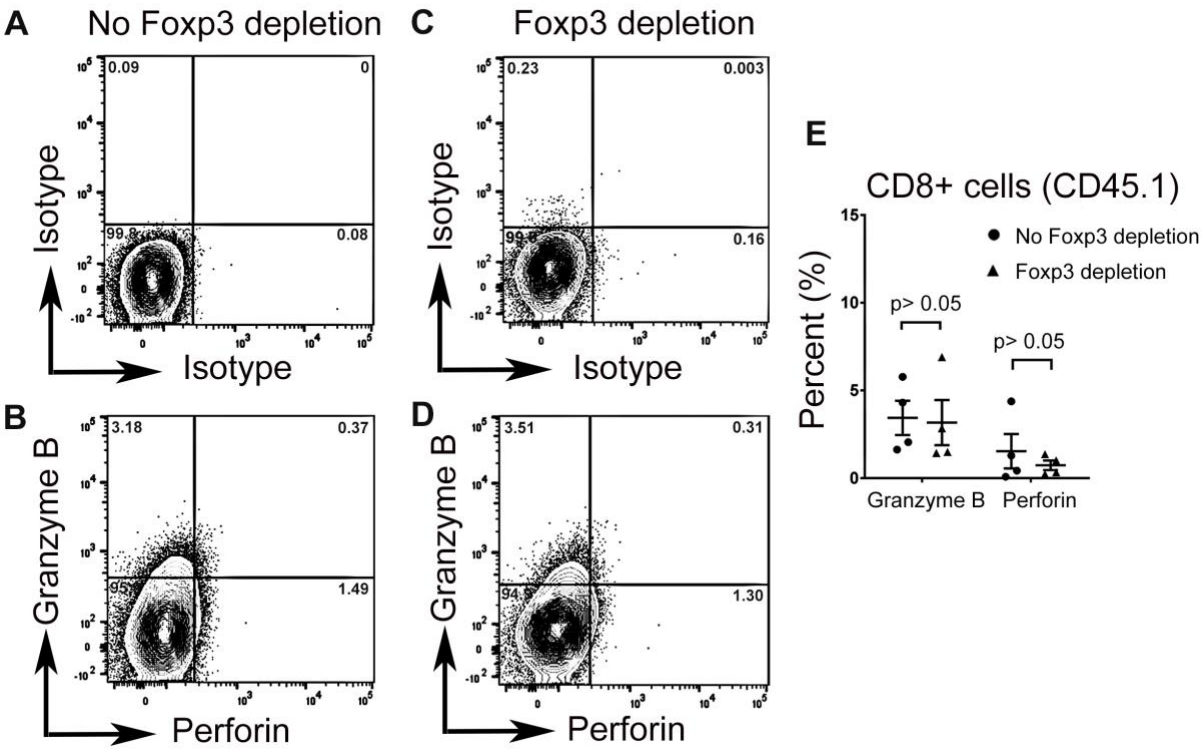
Supplemental Figure 10. Activated B cells in spleens of resting mice. (**A**) Plot and (**B**) quantification of $GL7^{+}Fas^{+}$ B cells in spleens of resting wildtype B6 mice. Plot is gated on live $CD45^{+}B220^{+}$ cells. Data in (**B**) are expressed as mean ± SEM. (n=4).



Supplemental Figure 11. BALT in tolerant lungs after re-transplantation into muMt$^-$ or AID/μS knockout mice after depletion of graft-resident Foxp3$^+$ cells. (**A**) PNAd (brown) (black arrow) and DAPI staining as well as (**B**) B220 (red), CD3 (green) and DAPI (blue) immunofluorescent staining in BALT (white arrow) in Balb/c lung grafts that were initially transplanted into immunosuppressed wildtype B6 Foxp3-DTR mice and ≥ 30 days later re-transplanted into DT-treated B6 muMt$^-$ hosts. (**C**) PNAd (brown) (black arrow) and DAPI staining as well as (**D**) B220 (red), CD3 (green) and DAPI (blue) immunofluorescent staining in BALT (white arrow) in Balb/c lung grafts that were initially transplanted into immunosuppressed wildtype B6 Foxp3-DTR mice and ≥ 30 days later re-transplanted into DT-treated B6 AID/μS knockout hosts. Scale bars 100μm. Immunostaining was performed 7 days after the re-transplantation procedures (n=4).



Supplemental Figure 12. IgG titers after re-transplantation into B cell-deficient mice. Donor-specific IgG titers 7 days after re-transplantation of Balb/c lungs into DT-treated wildtype (blue squares) or muMt$^-$ (black triangles) B6 hosts ≥ 30 days after initial engraftment into immunosuppressed B6 Foxp3-DTR mice (Foxp3 depletion). Data are expressed as mean ± SEM. Mann-Whitney U test was used to compare the means. (n=4).



Supplemental Figure 13. Expression of perforin and granzyme B in graft-infiltrating $CD8^+$ T cells. (**A-D**) Plots and (**E**) quantification of granzyme B and perforin expression in graft-infiltrating $CD8^+$ T cells from secondary recipients (CD45.1) in Balb/c lungs, transplanted into immunosuppressed wildtype B6 (CD45.2) (no Foxp3 depletion) ((**A**) isotype control staining; (**B**) perforin / granzyme B staining)) or Foxp3-DTR B6 (CD45.2) (Foxp3 depletion) ((**C**) isotype control staining; (**D**) perforin / granzyme B staining) mice and re-transplanted into DT-treated B6 (CD45.1) hosts ≥ 30 days later. Data in (**E**) are expressed as mean ± SEM (n=4). Mann-Whitney U test was used to compare the means.

Supplemental Video 1. Foxp3$^+$ cells form aggregates in tolerant lung grafts. Time-lapse intravital two-photon imaging of Foxp3$^+$ cells (green) in Balb/c lung grafts 7 weeks after transplantation into B6 Foxp3 IRES-GFP recipient that was treated with peri-operative costimulatory blockade. Pulmonary vessels appear red after intravenous injection of non-targeted 655-nm quantum dots. Scale bar: 30 μm. Relative time is displayed in hrs:min:sec.

Supplemental Video 2. Graft-infiltrating CD4$^+$ T cells and B cells interact with graft-resident Foxp3$^+$ cells in tolerant lungs. Balb/c lungs were transplanted into immunosuppressed B6 Foxp3 IRES-GFP (green) recipients. Six weeks after transplantation, 10^7 CMTMR-labeled (red) CD4$^+$ T cells and 10^7 Cell Trace violet-labeled (blue) B cells, isolated from spleens of B6 mice, were injected into the recipient mice and lungs were imaged within 24 hours of cell injection. Time lapse imaging demonstrates interactions between Foxp3$^+$ cells, CD4$^+$ T cells and B cells. Scale bar: 10 μm. Relative time is displayed in hrs:min:sec.

Supplemental Video 3. Graft-infiltrating CD4$^+$ T cells and B cells form stable interactions in Foxp3$^+$ T cell-depleted lung grafts. Time-lapse intravital two-photon imaging of adoptively transferred CD4$^+$ T (CFSE-labeled; green) and B (Cell Trace violet-labeled; blue) cells in Balb/c lung grafts which were initially transplanted into immunosuppressed B6 Foxp3-DTR recipients and 5 weeks later re-transplanted into secondary B6 hosts that were treated with DT and control-Ig. Pulmonary vessels appear red after intravenous

injection of non-targeted 655-nm quantum dots. Scale bar: 10 μm. Relative time is displayed in hrs:min:sec.

Supplemental Video 4. CXCL13 inhibition prevents graft-infiltrating $CD4^+$ T cells and B cells from forming stable interactions in $Foxp3^+$ T cell-depleted lung grafts. Time-lapse intravital two-photon imaging of adoptively transferred $CD4^+$ T (CFSE-labeled; green) and B (Cell Trace violet-labeled; blue) cells in Balb/c lung grafts which were initially transplanted into immunosuppressed B6 Foxp3-DTR recipients and 5 weeks later re-transplanted into secondary B6 hosts that were treated with DT and anti-CXCL13 antibodies. Pulmonary vessels appear red after intravenous injection of non-targeted 655-nm quantum dots. Scale bar: 10 μm. Relative time is displayed in hrs:min:sec.