SUPPLEMENTAL METHODS

Analysis of regulatory T cell populations

$B6^{FoxP3GFP}$→$Balb/c^{FoxP3GFP}$ chimeric mice were generated as described in *Methods*. At 4 weeks of age, spleens were individually dissected and cut into several pieces with sterile surgical scissors and placed in 5mL polypropylene round bottom tubes (Evergreen Scientific, Rancho Dominguez, CA) with 300μL sterile phosphate-buffered saline (PBS). Using the plunger from a 1mL slip-tip tuberculin syringe (Becton Dickinson, Franklin Lakes, NJ), spleens were macerated vigorously for 1 minute, separating the splenocytes from the fibrous capsule. An additional 3mL cold PBS was added to the splenocyte suspension to further wash adherent cells from the capsule, and the suspension was then filtered through a 70μm nylon mesh filter into a 50mL conical tube. Mandibular, superficial parotid, proper and accessory axillary, and subiliac lymph nodes (LN) were dissected with sterile surgical scissors and placed on a 70μm nylon mesh filter moistened with 300μL cold PBS. LN were ground against the filter with a plunger from a 1mL slip-tip tuberculin syringe to separate cells from the fibrous capsule. Whole bone marrow (WBM) cells were isolated as described previously. Peripheral blood (PB) was harvested prior to euthanasia via retro-orbital venipuncture and diluted in heparinized PBS. Thus prepared, spleen, LN, WBM, and PB cells were then layered over sterile Ficoll. After centrifugation (850g × 20 minutes), the low density mononuclear cell (LDMC) layer was collected and washed with sterile PBS. Cells were resuspended in staining buffer for analysis by flow cytometry (FACSAria, BD Biosciences, San Jose, CA). Age-matched, uninjected $B6^{FoxP3GFP}$ and $Balb/c^{FoxP3GFP}$ mice served as controls.

Cells from each tissue were divided into two tubes for analysis. In the first tube, cells were stained first with CD49b-PE (BD Biosciences Cat# 553858) and CD223 (LAG-3)-APC (BD Biosciences Cat#562346) at a dilution of 1:33 in 200μL staining buffer at 37°C for 15 minutes and

then at room temperature for an additional 15 minutes as previously reported[1]. Cells were washed and then stained with $H2k^b$-PE/Cy7 (eBioscience Ref#25-5958-82), and CD4-APC/Cy7 (Biolegend Cat#100526) at a dilution of 1:100 in 200μL staining buffer at 4°C for 30 minutes. In the second tube, cells were stained with CD25-PE (BD Biosciences Cat#553075), $H2k^b$-PE/Cy7, and CD4-APC/Cy7 at a dilution of 1:100 in 200μL staining buffer at 4°C for 30 minutes. Tr1 cells were measured as $\%CD49b^+LAG\text{-}3^+$ / $CD4^+$ cells as previously described. Traditional Tregs were measured as $\%CD25^+FoxP3^+$ / $CD4^+$ cells. Donor-derived cells were isolated by gating on the $H2kb^+$ population, and host-derived cells were isolated by gating on the $H2kb^-$ ($H2kd^+$) population. Regulatory T cell populations were also analyzed in the spleens of chimeric animals at additional time points (2, 8, and 24 weeks of age) to evaluate the change in prevalence over time.

In addition to their co-expression of CD49b and LAG-3, Tr1 cells are characterized by robust production of the cytokine IL-10. In order to confirm the enrichment of donor-derived cells with this phenotype among $CD4^+$ T cells in the spleens and BM of chimeric animals, $B6^{IL\text{-}10GFP}$→Balb/c chimeric mice were generated as described previously. Of note, only donor-derived cells could be evaluated in this fashion because IL-10^{GFP}-labeled mice were only available in the B6 strain. Macrochimeric animals were sacrificed at 4 weeks of age, and cells were isolated from peripheral tissues as above. Cells were stained with $H2k^b$-PerCP/Cy5.5 (Biolegend Cat#116516), CD3-PE (BD Biosciences Cat#555275), and CD4-APC (eBioscience Ref#17-0042-82) at a dilution of 1:100 in 200μL staining buffer at 4°C for 30 minutes. The frequency of IL-10^{GFP+} cells was determined among $H2k^{b+}CD3^+CD4^+$ cells. Age-matched uninjected $B6^{IL\text{-}10GFP}$ males and females served as controls.

In order to determine if donor-derived Tr1 cells arise from $CD4^+$ cells present in the allograft, $CD4^-$ progenitors, or a combination of both, $B6^{FoxP3}$ WBM was isolated as described

previously. CD4-depletion was performed using CD4 (L3T4) Microbeads, mouse (Miltenyi Biotec Cat#130-049-201) and LD columns per the manufacturer's instructions. $CD4^-$ purity was confirmed to be 99.9% by flow cytometry. 9.9×10^6 CD4-depleted BM cells were injected into Balb/c fetuses at 14 DPC, and the resulting chimeric animals were analyzed at 4 weeks of age as above.

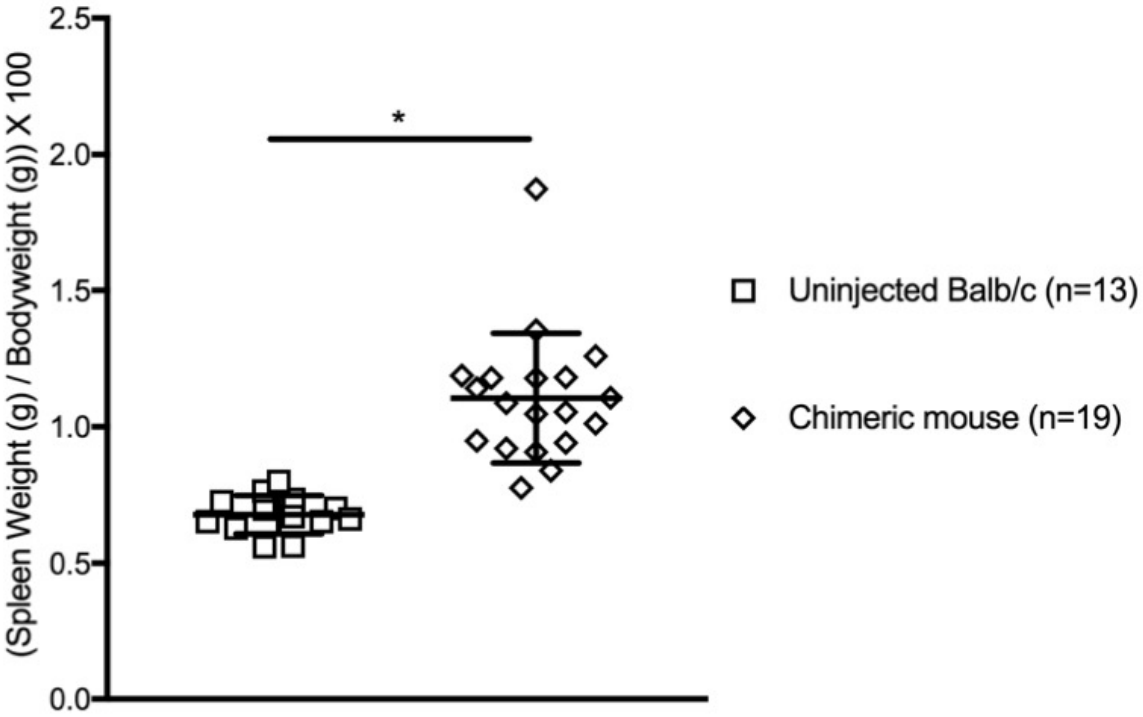
Separation of chimeric splenocytes into subpopulations

In order to investigate which cell population(s) were important for preventing graft rejection in our model of late-gestation IUHCT, $CD4^+$ splenocytes harvested from 4-week-old B6→Balb/c chimeric mice were separated by MHC ($H2k^b$ and $H2k^d$) as well as by CD25. In order to maintain optimal cell viability, cells were sorted using rapid, sequential negative and positive selection by MACS. Briefly, cells were first stained with $H2k^b$-biotin (eBioscience Ref#13-5958-82) or $H2k^d$-biotin (eBioscience Ref#13-5957-82) at a dilution of 1:100 at a final concentration of 5×10^4 cells/μL for 25 minutes at 4°C. Cells were washed once with SB and pelleted by centrifugation at 350g × 10 minutes. When CD25-depletion was performed, cells were then stained with CD25-PE (Miltenyi Biotec Cat#130-102-593) at a dilution of 1:100 at a final concentration of 0.91×10^5 cells/μL for 15 minutes at 4°C. Cells were washed and then incubated with Anti-Biotin Microbeads (Miltenyi Biotec Cat#130-090-485) at a dilution of 1:50 at a final concentration of 1×10^5 cells/μL for 15 minutes at 4°C. When CD25-depletion was performed, cells were simultaneously incubated with Anti-PE Microbeads (Miltenyi Biotec Cat#130-048-801) at a dilution of 1:100. Depletion of labeled cells was performed using LS columns. Positive selection of $CD4^+$ cells was then performed as described previously using MS columns (Miltenyi Biotec Cat#130-042-201). Cells were confirmed to be >99.5% $H2k^{b-}$ or $H2k^{d-}$ by flow cytometry after

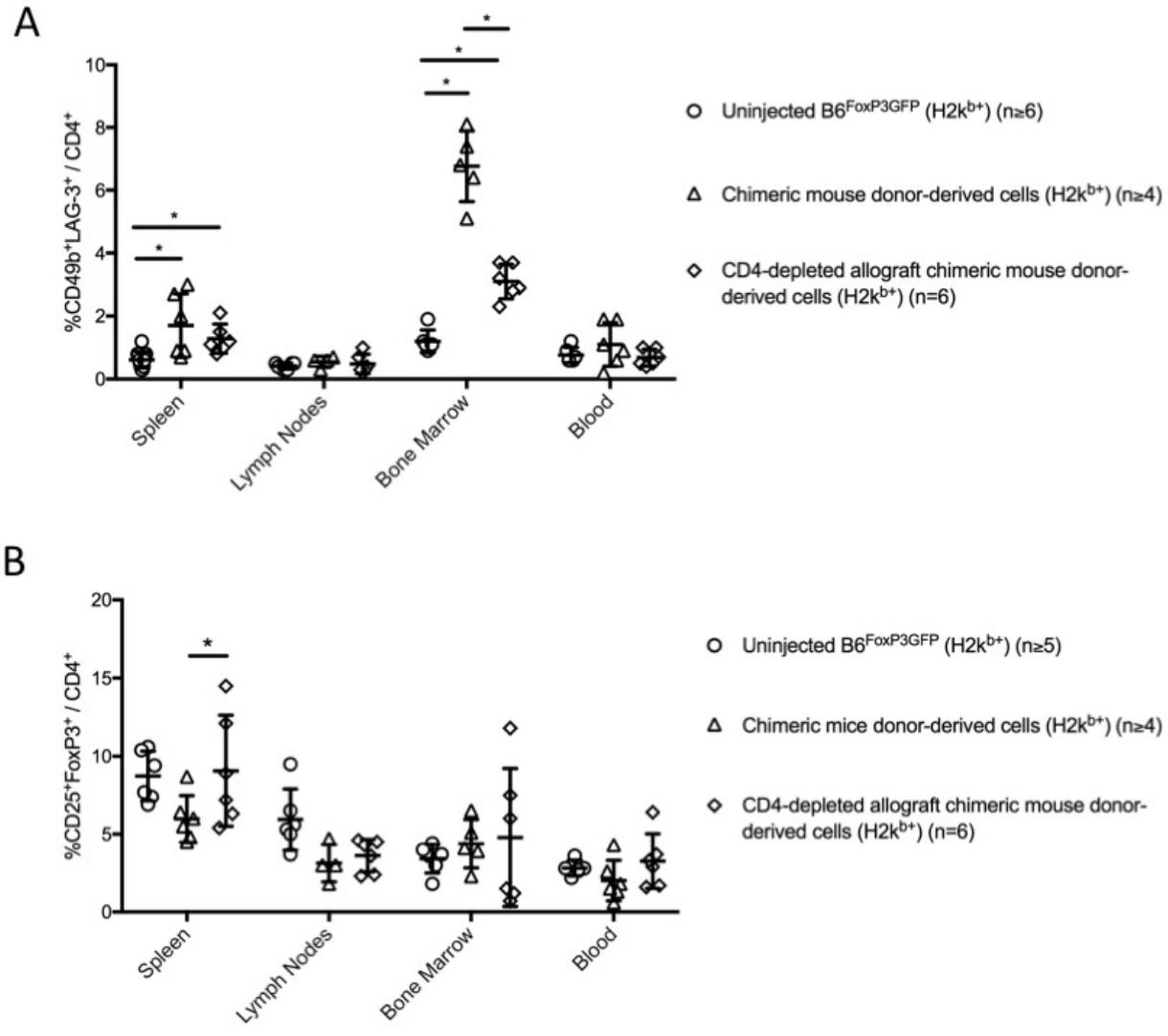
depletion. Strain-dependent differences in $CD4^+$ purity were detected, with Balb/c (host-derived, $H2k^{d+}$) cells being 80–95% $CD4^+$ after positive selection and B6 (donor-derived, $H2k^{b+}$) cells being 40–55% $CD4^+$. $CD25^-$ purity among $CD4^+$ cells was 97.0–99.9% for both strains. Donor-derived Tr1 cells were purified from 4-week-old $B6^{IL\text{-}10GFP}$→Balb/c chimeric mice using CD4-positive selection followed by flow cytometry cell sorting for $CD4^+IL\text{-}10^{GFP+}$ cells. Cell doses were chosen based on the relative frequency of the subpopulations in a 4-week-old chimeric mouse, which yields approximately 5×10^6 $CD4^+$ splenocytes and for which average splenic chimerism is 20–50%.

REFERENCES

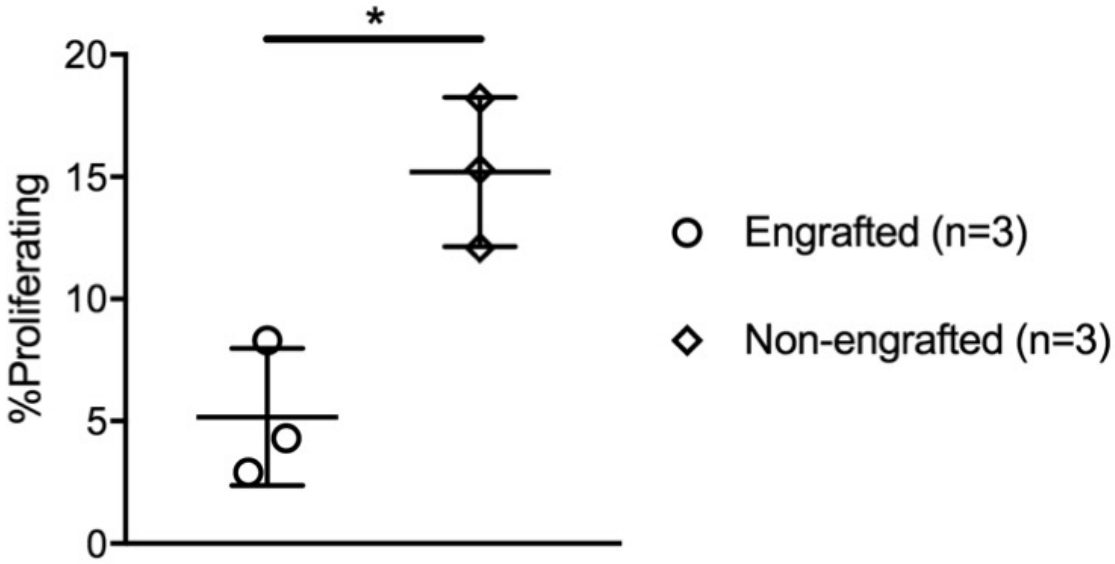
1. Gagliani N, Magnani CF, Huber S, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat. Med.* 2013;19(6):739–746.



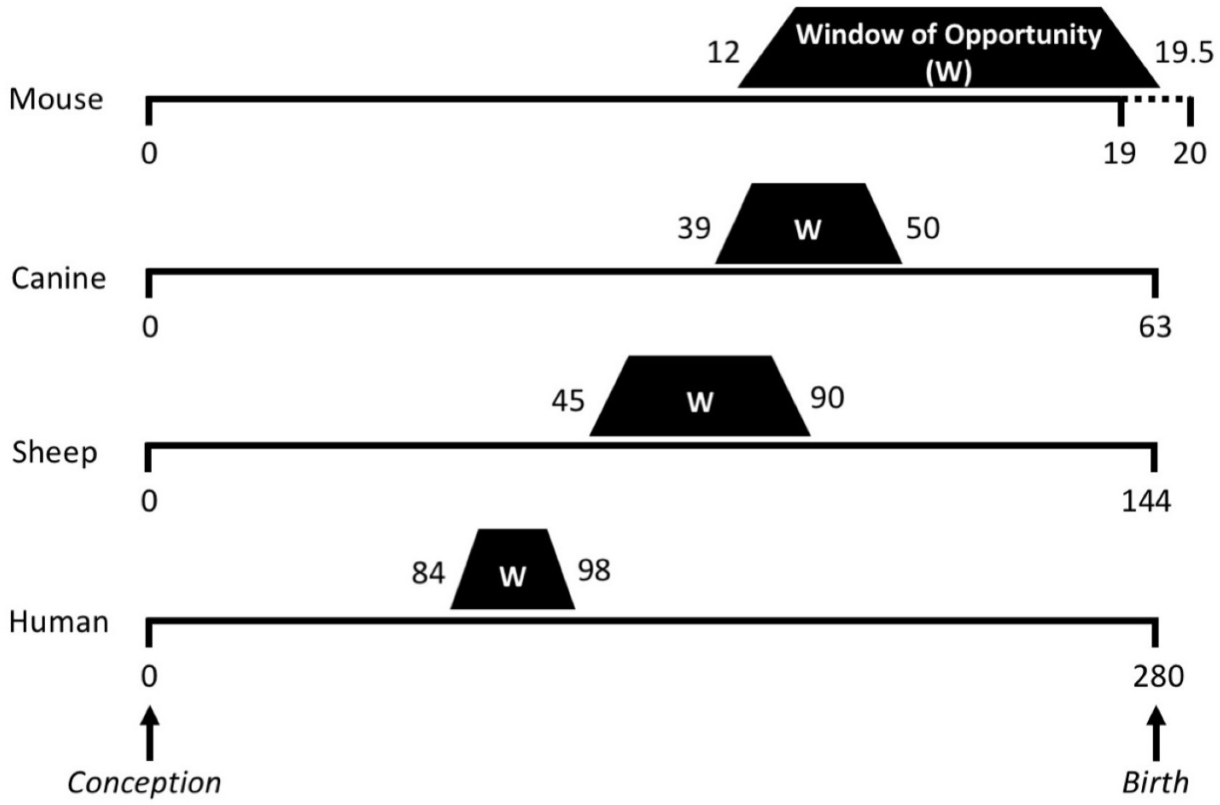
Supplemental Figure 1: Spleen weight as a percentage of total bodyweight among chimeric and naïve mice at 4 weeks of age. Mean and standard deviation are included in addition to individual data points. Data were analyzed using Student's t test assuming unequal variance with statistically significant differences ($P < 0.05$) indicated by *.



Supplemental Figure 2: Impact of CD4-depletion of the allograft upon regulatory T cell frequency. A) Tr1 cells and B) Traditional Tregs measured as a percentage of donor-derived $CD4^+$ cells at 4 weeks of age in the spleen, lymph nodes, bone marrow, and peripheral blood following IUHCT at 14 DPC with CD4-depleted allografts. Mean and standard deviation are included in addition to individual data points. Data were analyzed using ANOVA with Bonferroni multiple comparisons with statistically significant differences ($P < 0.05$) indicated by *.



Supplemental Figure 3: *In vivo* mixed lymphocyte reaction performed at 6 months of age comparing donor-reactivity among engrafted vs. non-engrafted animals following injection at 19 DPC with TCD BM. Lymphocytes were harvested from the spleen. Percent proliferating was defined as the percentage of $H2k^{b-}GFP^{-}CD3^{+}$ cells with APC fluorescence intensity ≤50% of undivided cells. Data were analyzed using Student's t test assuming unequal variance with statistically significant differences ($P < 0.05$) indicated by *.



Supplemental Figure 4: Interspecies variation in the window of opportunity for allograft tolerance relative to conception and birth. Compared to the mouse, canine, and sheep models of IUHCT, the window of opportunity (W) closes much earlier in gestation. The unit for all values is days.