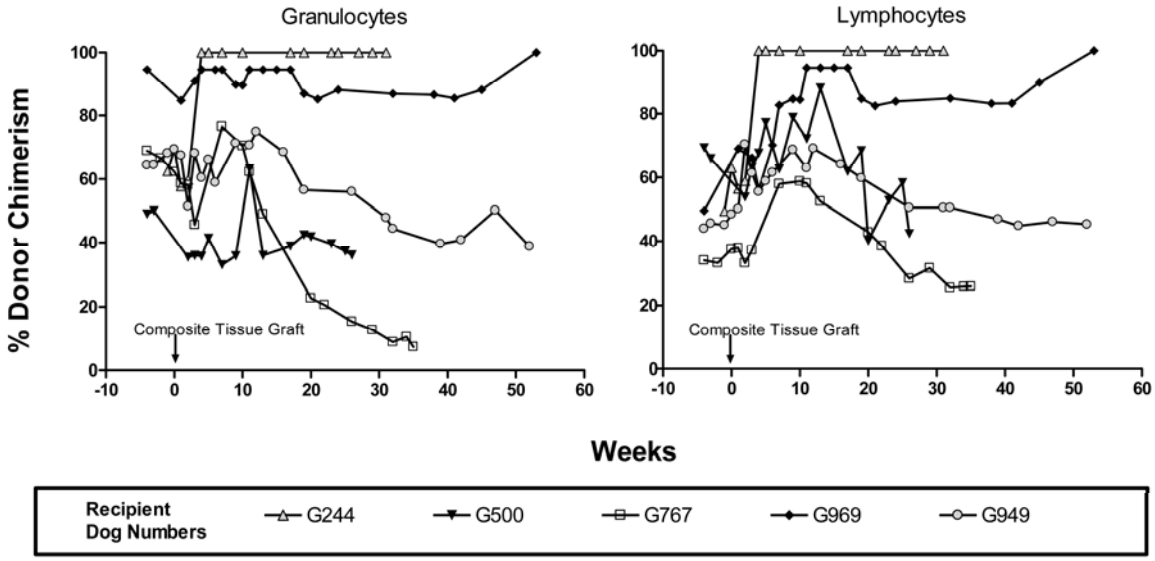
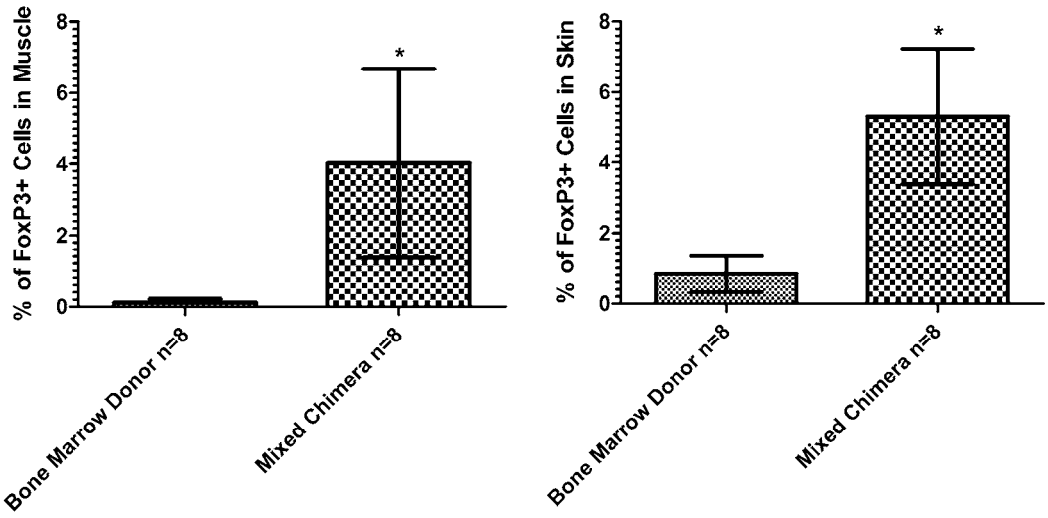
SDC 1 – Combined Supplemental Digital Content

SDC 2



Supplemental Figure 1. Marrow donor granulocyte (left panel) and lymphocyte (right panel) chimerism in 5 recipients before and after composite tissue grafts from their marrow donors.

SDC 3



Supplemental Figure 2. Analysis of overall FoxP3+ staining of muscle and skin biopsies from composite tissue allografts in bone marrow donor dogs and mixed chimeric recipients. Assessing T-regulatory cells in the transplanted allograft by quantifying the overall percentage of FoxP3 staining in the muscle (left) and skin (right). In both muscle and skin, there is a greater number of FoxP3 stained cells in the composite tissue allograft of a mixed chimera dog as compared to non-mixed chimera dog (statistically significant, * designates P≤ 0.05, two tailed Student's T-test).

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SDC 4 - Supplemental Materials and Methods

Quantitative Reverse Transcribed-PCR of Intracellular Cytokines and FoxP3 from CD3+ T-cells and FoxP3 Immunohistochemistry

T-cells were selected with canine CD3-specific monoclonal antibody CA17.6B3 (gift from P.F. Moore, University of California, Davis, CA) and Miltenyi magnetic bead technology (Miltenyi Biotec, Auburn, CA). Purity of the cell sorts was determined by flow cytometry. CD3+ T-cells were obtained from peripheral blood, lymph node, muscle and skin from marrow donors and mixed chimeras. In addition, samples were collected from normal dogs not irradiated, transplanted, or immunosuppressed. From the sorted CD3+ T-cells, mRNA was extracted, isolated, and transcribed to cDNA using μMACS One Step cDNA kits (Miltenyi Biotec, Auburn, CA). FoxP3 expression was measured by quantitative reverse transcribed (RT)-PCR using primers and Taqman probes designed by Primer Express (Applied Biosystems, Foster City, CA). The sequences of the primers used for the amplification and detection of canine FoxP3 were: forward primer, 5'-AGGATTTCCTCAAGCACTGC-3'; reverse primer, 5'-TGGAAGAACTCTGGGAATGTG-3' and probe 6FAM 5'-TGGTGCAGTCTCTGGAACAG- 3' TAMARA. The sequences of the primers used for quantitative RT-PCR of canine interleukin-10 (IL-10), canine transforming growth factor beta (TGFβ), and the housekeeping gene, canine glycerol-3-phosphate dehydrogenase (G3PDH), have been previously published (*49*). Absolute copy numbers were calculated based on FoxP3, IL-10 or TGFβ standard curves, and samples were normalized to a second standard curve for the housekeeping gene G3PDH (*49,50*).

FoxP3 Immunohistochemistry

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Immunohistochemistry and histology were performed on paraffin-embedded tissues. Four-micron sections were cut, deparaffinized and rehydrated in Dako Wash Buffer (Dako Corp., Carpinteria, CA). Slides were antigen retrieved in a Black & Decker® steamer for 20 minutes in preheated Trilogy buffer (Cell Marque, Hot Springs AZ). Slides were rinsed three times in wash buffer and stained using the Dako Autostainer. Endogenous peroxide activity was blocked using 3% H_2O_2 followed by blocking with15% goat serum and 5% canine serum in PBS containing 1% bovine serum albumin BSA. Anti-FoxP3 antibody (14-5773-82, eBioscience) was used at 10 µg/ml and detected with biotinylated goat anti-rat (112-065-167, Jackson ImmunoResearch) at 1:200 followed by Vector Elite ABC. The staining for all slides was visualized with 3,3'-diaminobenzidine (DAB, Dako) for 8 minutes, and the sections were counter-stained with hematoxylin (Dako) for 2 minutes. Concentration-matched isotype control slides were run for each tissue sample (Jackson ImmunoResearch Laboratories).

Analysis for percentage of cells stained for FoxP3 was performed using ImageJ software (NIH, Bethesda, MD). After staining for FoxP3, each slide was visualized at 200X and 400X magnification. Four representative pictures were taken from each slide at 200X. Each of these pictures were analyzed using ImageJ after color separation was performed using the ImageJ plugin color deconvolution (designed by Gabriel Landini). Noise was filtered at a median of 2 pixels, regions of FoxP3 staining and high cellularity were selected and thresholds were run. The final value was total volume of FoxP3 stained cells compared to the total volume of all cells. This value was defined as "% of FoxP3+ cells." Mean and standard deviation were run for all values.