Ischemia augments alloimmune injury through IL-6-driven CD4$^+$ alloreactivity

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Supplementary Figure Legend:

Supplementary Figure 1. Allospecific $CD8^+$ T cells lead to prompt rejection, which is not augmented by IRI. Control or ischemic OVA hearts were transplanted into OTI recipients. (A) Heart grafts were rejected around 3 days post-transplant with no difference between control and ischemic groups (MST: 3 vs. 3 days, p=0.51, n=5/group). (B) Heart grafts were harvested at 4 days post-transplant for histological analysis. Both control and ischemic grafts showed severe allograft injury with dense lymphocyte infiltration, parenchymal necrosis and hemorrhage in both groups. (Scale bar 100μm for H&E. Inset scale bar 30μm).

Supplementary Figure 2. Analysis of graft infiltrating $CD4^+$ T cell populations shows a pro-inflammatory phenotype following IRI. Control or ischemic OVA hearts were transplanted into OT II recipients and harvested at day 6 post-transplant. Graft infiltrating lymphocytes were isolated and underwent intracellular staining to analyze intra-graft $CD4^+$ T cell populations. (A) No differences were observed in numbers of graft-infiltrating $CD4^+$ $Foxp3^+$ T cells between ischemic and control grafts (absolute cell count: 6498±844.7 vs. 5364±2925.1, p=0.6, n=3/group). (B) Absolute number of $CD4^+$ $IL17^+$ cells were higher in ischemic grafts compared to control (absolute cell count: 2615±461.5 vs. 816.1±328.9, *p<0.05, n=3/group). (C) A greater number of $CD4^+$ $IFN\gamma^+$ cells were found in ischemia group compared to control (absolute cell count: 8518±1503 vs. 2206±888.8, *p<0.05, n=3/group). Representative flow plots for all are shown.

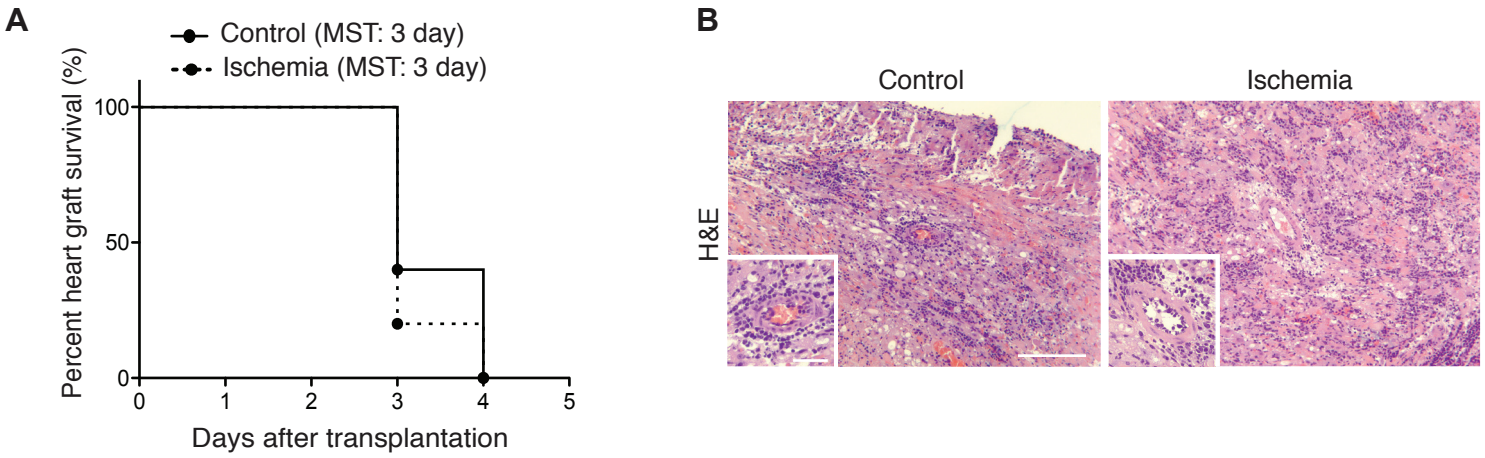
Supplementary Figure 3. IRI did not alter frequency of pro-inflammatory T cells in secondary lymphoid tissues. Control or ischemic OVA hearts were transplanted into OTII

recipients and spleen and graft draining lymph nodes were harvested at day 6 post-transplant. (A) No differences were seen in frequency of splenic $CD4^+$ $Foxp3^+$ or $CD8^+$ $IFN\gamma^+$ T cell between groups, as assessed by flow cytometry. (B) No differences were seen in frequency of $CD4^+$ $IL17^+$ T cell or $CD8^+$ $IFN\gamma^+$ T cell in DLN between both groups by flow cytometry.

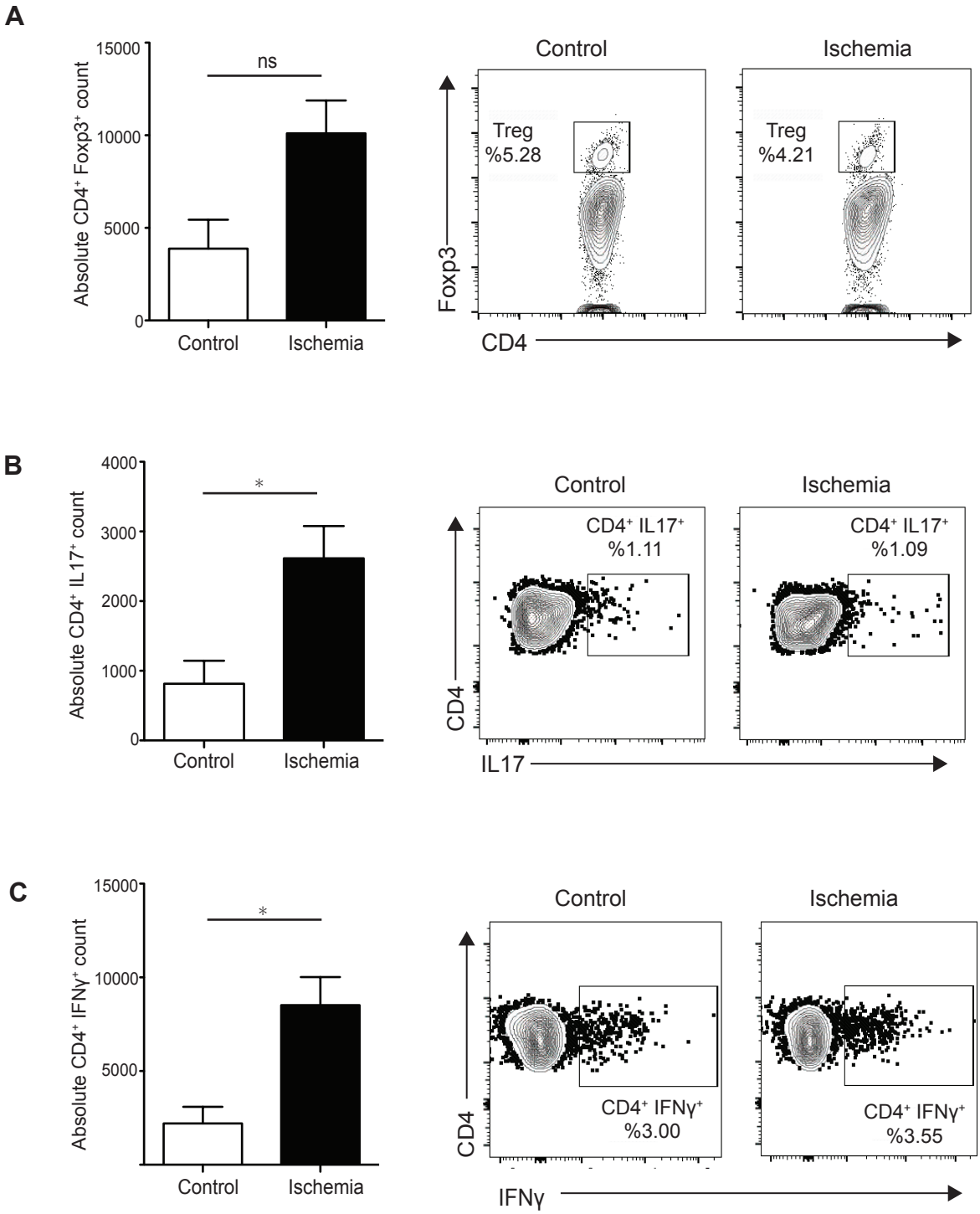
Supplementary Figure 4. IRI leads to increased allospecific IFNγ production by splenocytes; an effect abrogated by $CD4^+$ T cell depletion. (A) Control or ischemic OVA hearts were transplanted into OTII recipients, and spleens were harvested at day 6 post-transplant. Recipient whole splenocytes were incubated with irradiated OVA cells and allospecific IFNγ production was measured by ELISPOT analysis. Significantly greater splenocyte IFNγ production was seen in ischemia group (68.17±3.32 vs. 25.63±3.45 spots/10^6 cells, ***p<0.001, n=3/group). (B) OTII recipients were depleted of $CD4^+$ T cell before transplantation of control or ischemic OVA heart grafts. Spleens were harvested at 6 days post-transplant and allospecific IFNγ production was assessed by ELISPOT. No difference was seen in splenocyte IFNγ production in control or ischemia group following CD4 depletion (2.4±0.5 vs. 9.3±3.7 spots/10^6 cells, p=0.13, n=3/group).

Supplementary Figure 5. Liposomal clodronate depletes intra-graft CD11c and F4/80 cells. (A) Flow cytometric analysis of cardiac APCs in donor heart after clodronate administration showed significant reduction in $CD11c^+$ DCs (control vs. clodronate treatment; $109.9x10^3$±$13.0x10^3$ vs. $29.2x10^3$±$8.4x10^3$, *p<0.05, n=3/group). (B) A non-significant decrease in the absolute number of $F4/80^+$ macrophages was seen after clodronate administration (control vs. clodronate treatment; $545.1x10^3$±$223.5x10^3$ vs. $318.1x10^3$±$74.7x10^3$, p=0.39, n=3/group).

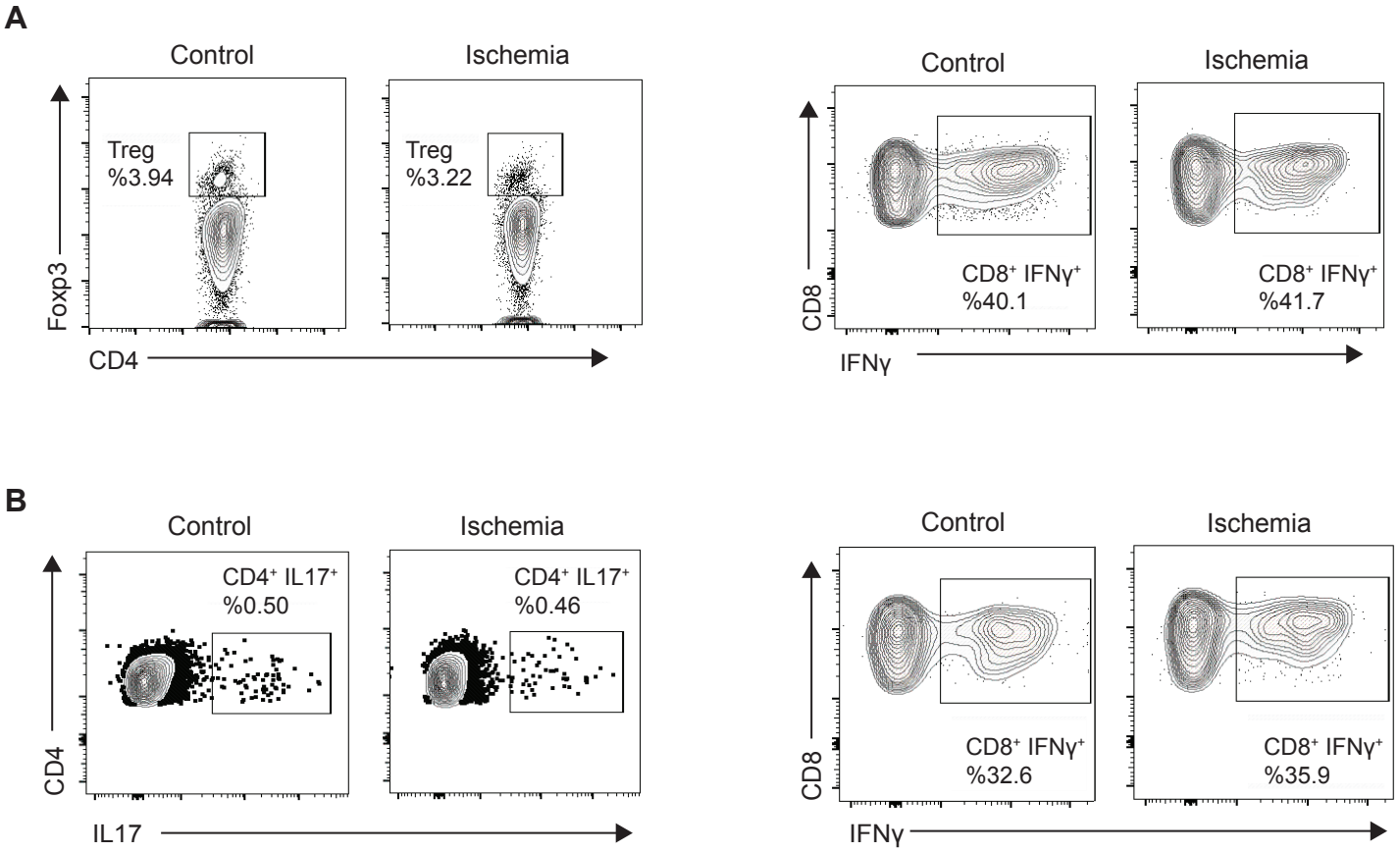
Supplementary Figure 6. Allograft resident APCs are the main source of increased IL-6 within ischemic allografts. (A) Ischemic and control OVA hearts were transplanted into OTII recipients, harvested on day 1, 3 and 6 post-transplant and RNA was extracted for PCR analysis at each time point. IL-6 gene expression was 8 times higher in ischemic grafts than control at 1 day post-transplant, and remained significantly higher up to day 3. (*p<0.05 for day 1, n=4/group, and *p<0.05 for day 3, n=3/group, respectively). (B) Control and ischemic grafts were harvested at day 1 and stained by immunohistochemistry for analysis of graft infiltrating cells. At day 1, no infiltrating cells, $CD4^+$ and $CD8^+$ T cells were detected in either control or ischemic grafts. (Scale bar 100μm for H&E, CD4 and CD8 stain. Inset scale bar 30μm)**.** (C) Control, ischemic or APC depleted ischemic OVA hearts were harvested at day 1 post-transplant from OTII recipients. Following RNA extraction, PCR analysis demonstrated a significant reduction of IL-6 expression, with undetectable IL-6 expression in APC depleted ischemic grafts as compared to non-depleted ischemic grafts.



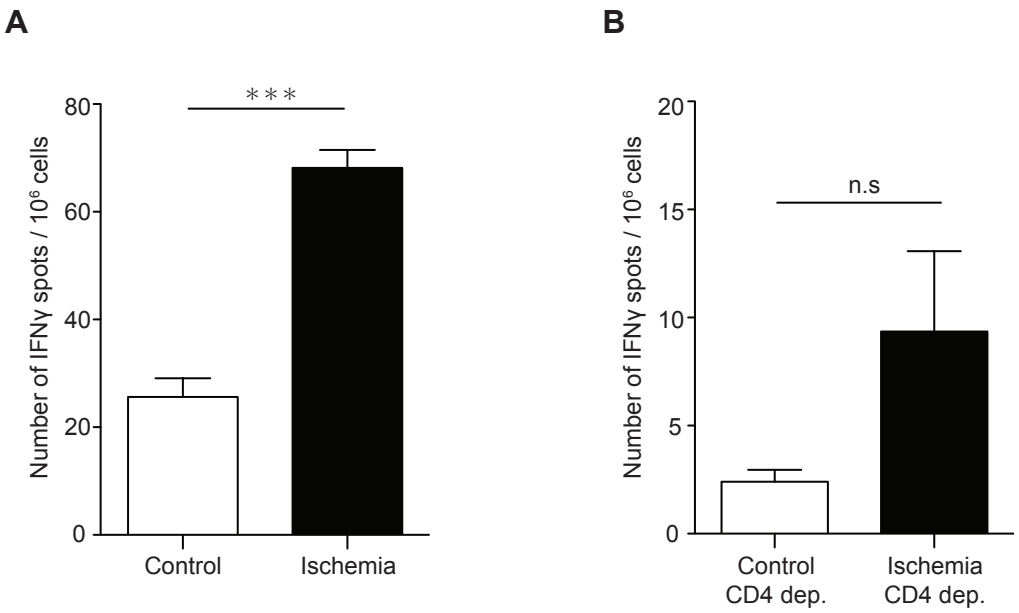
Uehara et al. Suppementary Figure 1



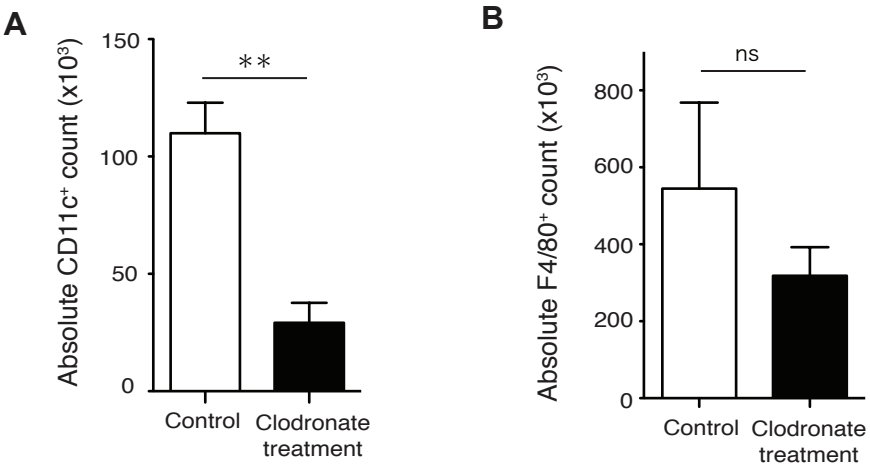
Uehara et al. Supplementary Figure 2

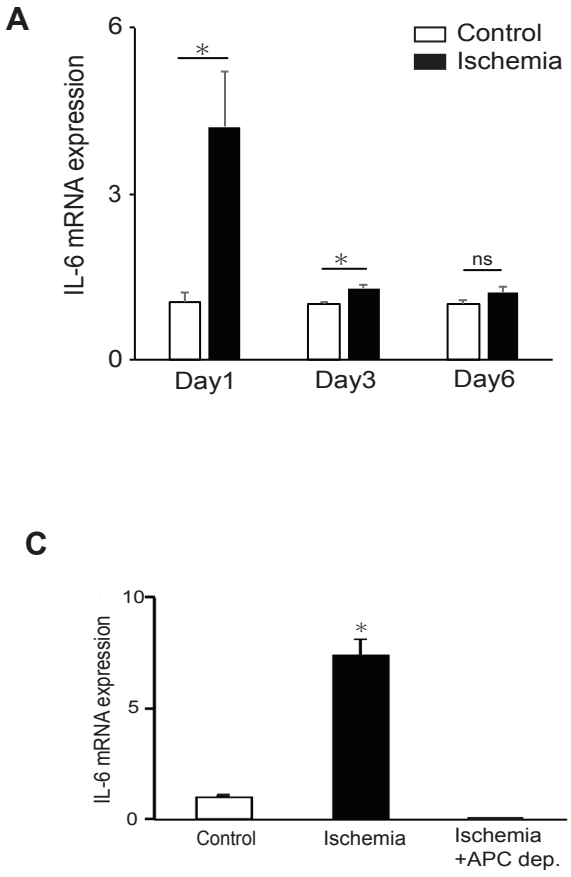


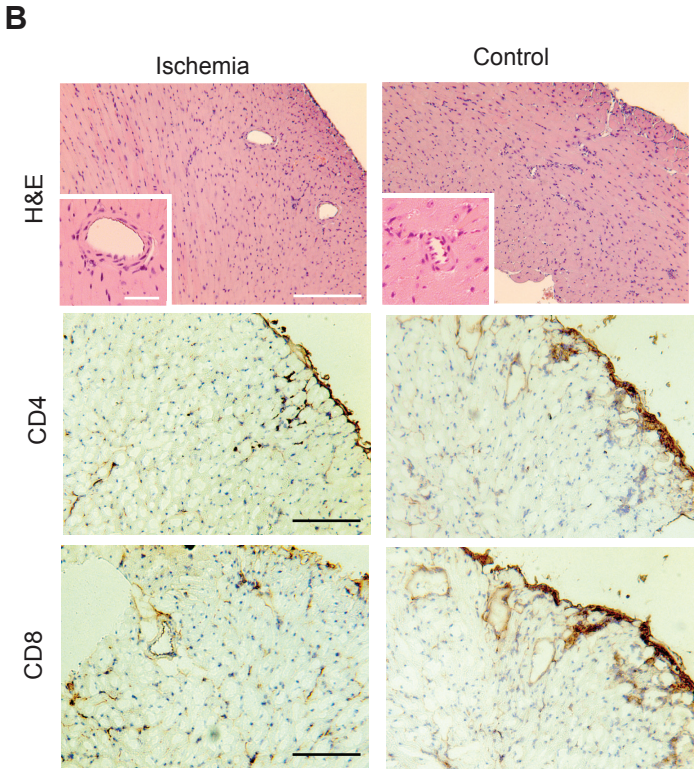
Uehara et al. Supplementary Figure 3



Uehara et al. Supplementary Figure 4


A
Absolute CD11c+ count (x10³)
150
100
50
0
**
Control
Clodronate treatment
B
Absolute F4/80+ count (x10³)
800
600
400
200
0
ns
Control
Clodronate treatment





Uehara et at. Supplementary Figure 6