Blocking CCL8-CCR8-Mediated Early Allograft Inflammation Improves Kidney Transplant Function

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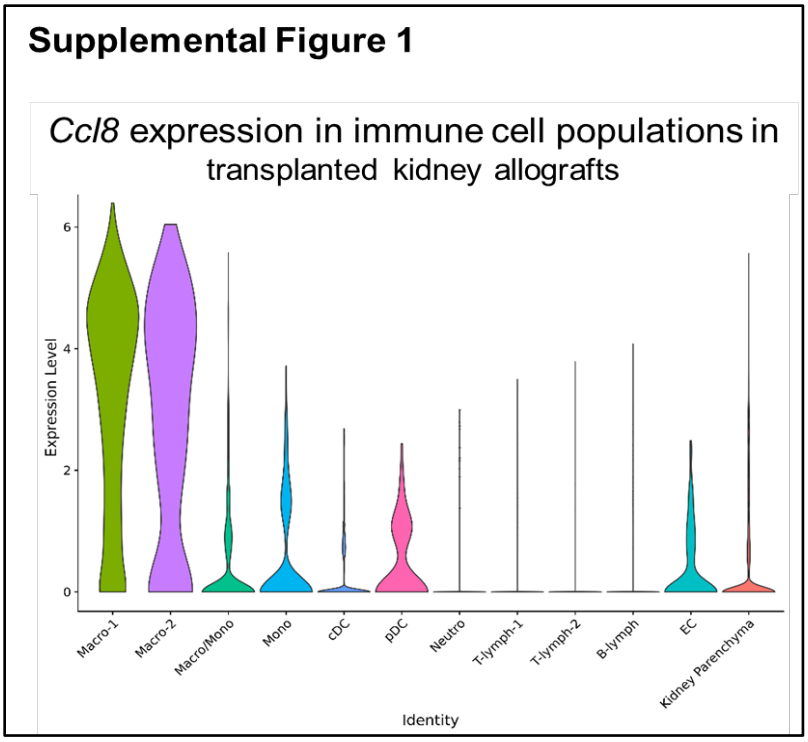
Supplemental Material

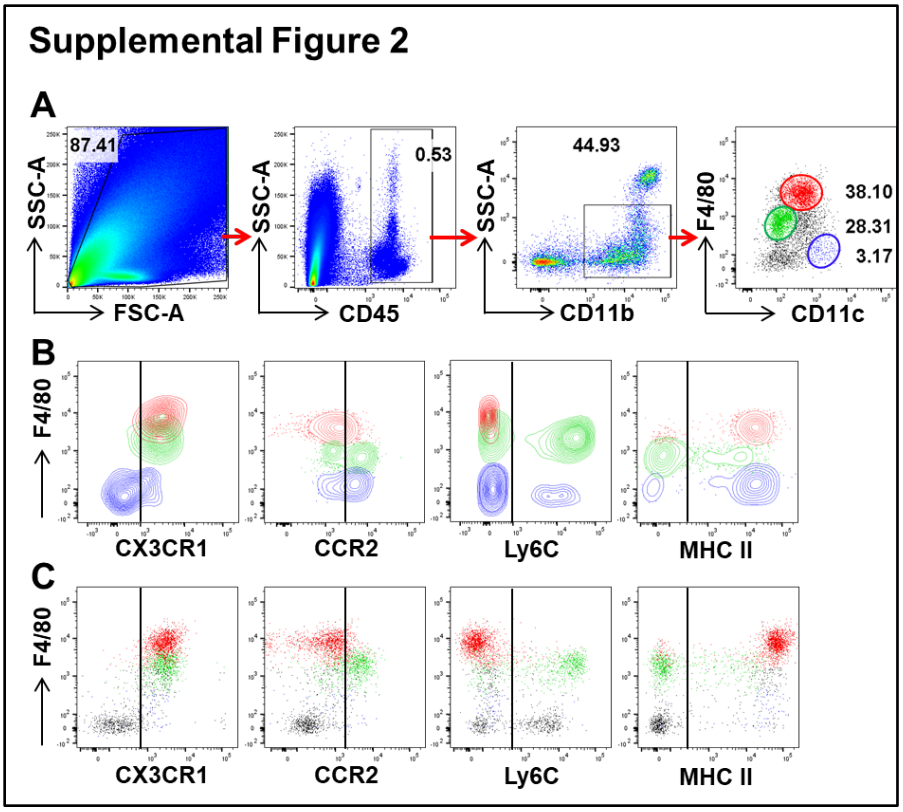
Supplemental Figures 1	Violin plot depicting *Ccl8* expression by various immune and non-immune cell clusters from rejection kidney allografts.
Supplemental Figures 2	Identification and phenotypic characterization of kidney resident macrophages.
Supplemental Figures 3	Donor kidney-resident and recipient graft-infiltrating macrophages co-exist in syngeneic kidney transplant grafts on day 15 post-transplantation.
Supplemental Figures 4	Up-regulation of F4/80 mean fluorescent intensity (MFI) on graft-infiltrating recipient $CD11b^+$ cells post allogeneic kidney transplantation.
Supplemental Figures 5	UMAPs of un-transplanted kidneys or transplanted kidneys on day 15 post-transplantation (in allogeneic recipients) showing *Ccr8* expression in distinct cell clusters.
Supplemental Figures 6	Recipient serum creatinine levels at various time points post-transplantation in CT-Ig and anti-CCL8 Ab treated recipients.
Supplemental Figures 7	Anti-CCL8 treatment permits longer survival of donor kidney resident macrophages post-transplantation.

Supplemental Figures 8	Depletion of kidney resident macrophages in kidney donors.
Supplemental Figures 9	Alloantigen-specific CD4 T cell proliferation stimulated by donor kidney resident macrophages.
Supplemental Figures 10	Donor-specific alloantibodies on day 28 post-transplantation.

Supplemental Figure 1

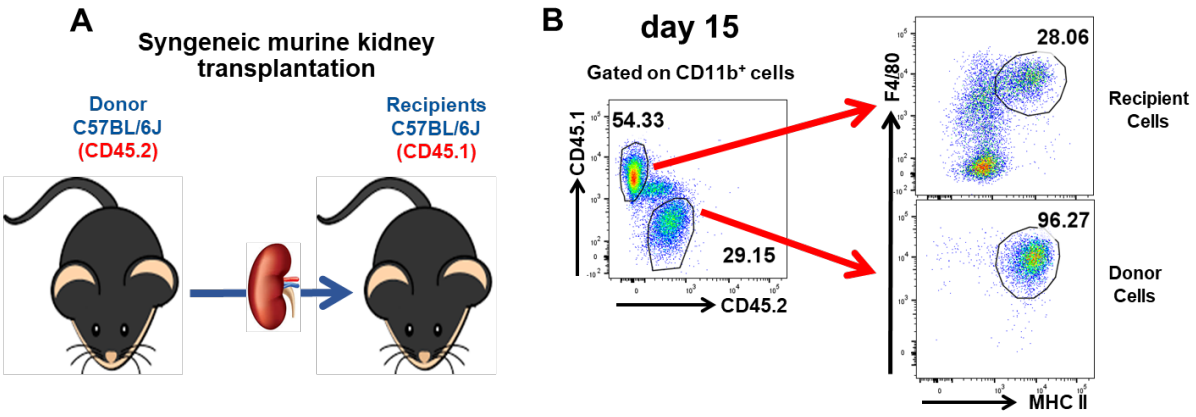


Supplemental Figure 1. Violin plot depicting *Ccl8* expression by various immune and non-immune cell clusters from rejection kidney allografts. Two macrophage clusters (Macro-1 and Macro-2) express the most prominent level of *Ccl8*. Samples were collected on day 15 post kidney transplantation for single cell RNA sequencing analysis. N=2.

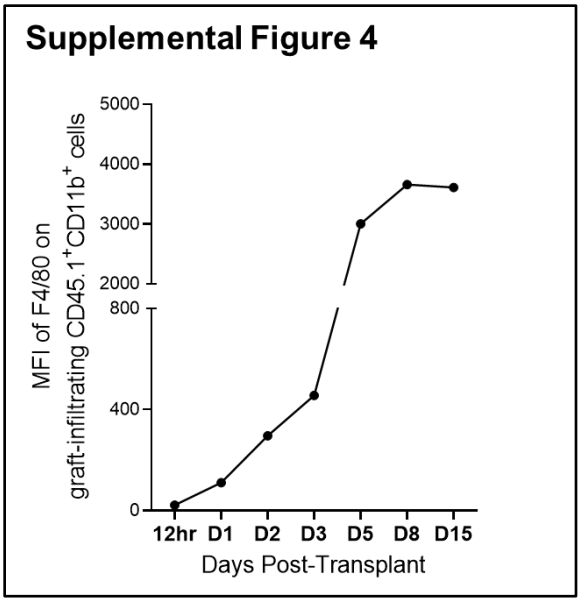


Supplemental Figure 2. Identification and phenotypic characterization of kidney resident macrophages. **(A)** Representative FACS plots demonstrating the gating strategy for various resident myeloid cells in naive BALB/c kidneys. Kidney resident macrophages of the yolk-sac origin are identified as F4/80^{HI}CD11c^{+} cells (red). Kidney macrophages of the bone marrow origin are identified as F4/80^{LO}CD11c^{-} cells (green). Kidney dendritic cells are identified as F4/80^{-} CD11c^{HI} cells (blue). **(B)** Contour plots showing the three kidney resident myeloid cells phenotypically compared for expressions of CX_3CR1, CCR2, Ly6C, and MHC-II. **(C)** Dot plots gated on all CD45^{+}CD11b^{+} cells as in (**A**), showing various sub-populations phenotypically compared for expressions of CX_3CR1, CCR2, Ly6C, and MHC-II. Data shown were representative of at least six naïve BALB/c kidneys.

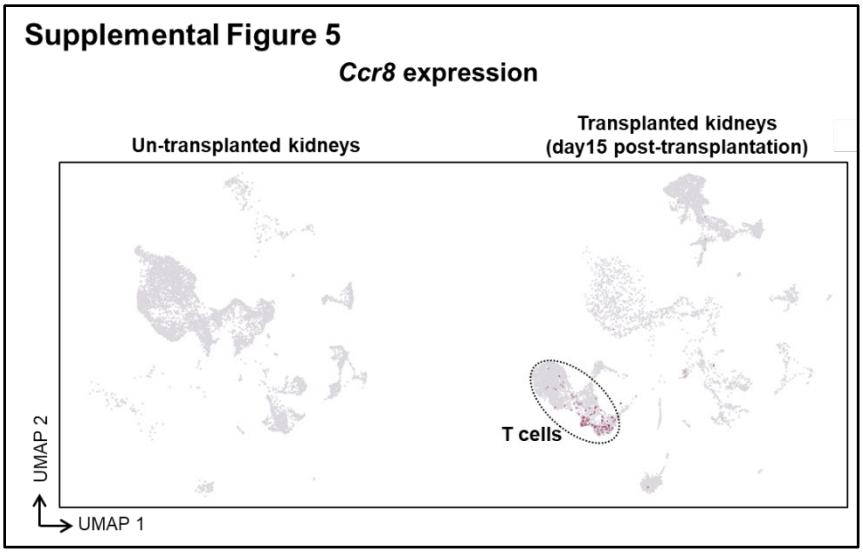
Supplemental Figure 3



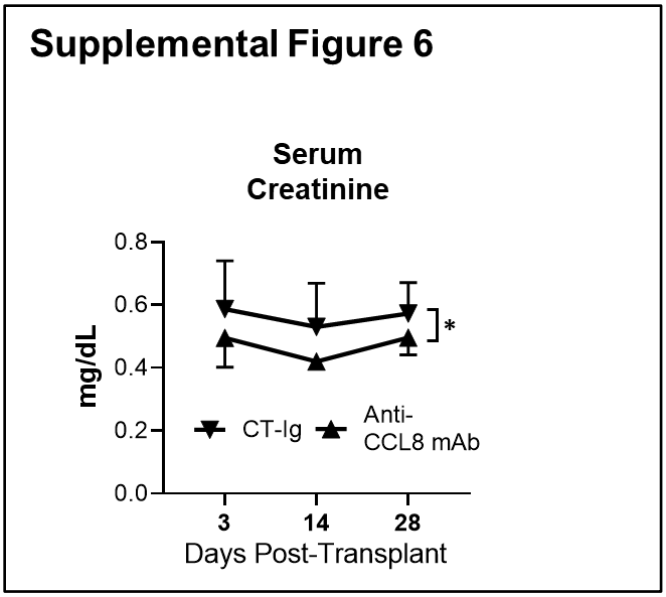
Supplemental Figure 3. Donor kidney-resident and recipient graft-infiltrating macrophages co-exist in syngeneic kidney transplant grafts on day 15 post-transplantation. **(A)** Cartoon of syngeneic kidney transplantation. Kidneys from CD45.2 B6 mice were transplanted into bilaterally nephrectomized CD45.1 B6 recipients. **(B)** FACS plot on left shows $CD45.2^+$ donor and $CD45.1^+$ recipient myeloid cells on day 15 post-transplantation. FACS plots on the right show $F4/80^{HI}MHC\text{-}II^{HI}$ macrophages of both donor and recipient origin in the syngeneic kidney grafts. Data were collected from N=2 mice.



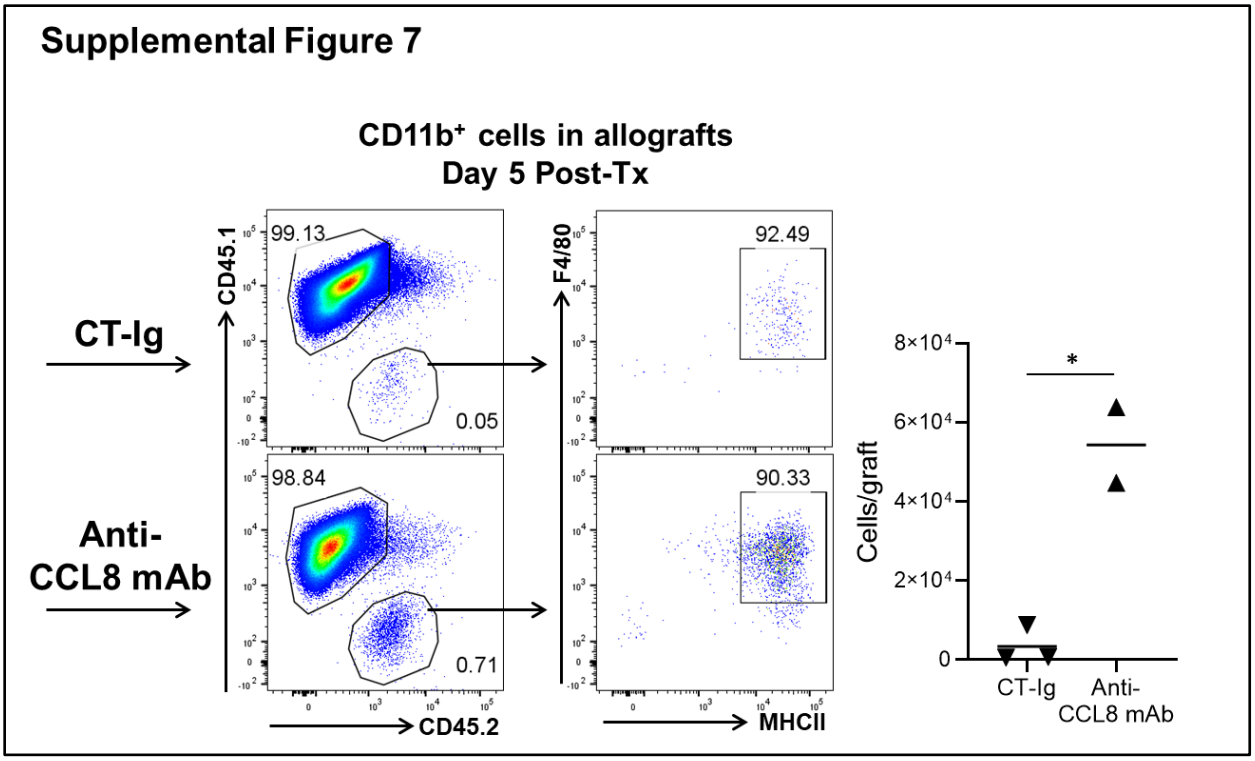
Supplemental Figure 4. Up-regulation of F4/80 mean fluorescent intensity (MFI) on graft-infiltrating recipient $CD11b^+$ cells post allogeneic kidney transplantation. Kidney allografts were harvested at the indicated time points and single cell preparations were stained for F4/80 for FACS analysis. F4/80 expression on total graft-infiltrating recipient $CD45.1^+CD11b^+$ myeloid cells was analyzed. The MFI of F4/80 was normalized to that of the isotype control. N=2-5 per time point.



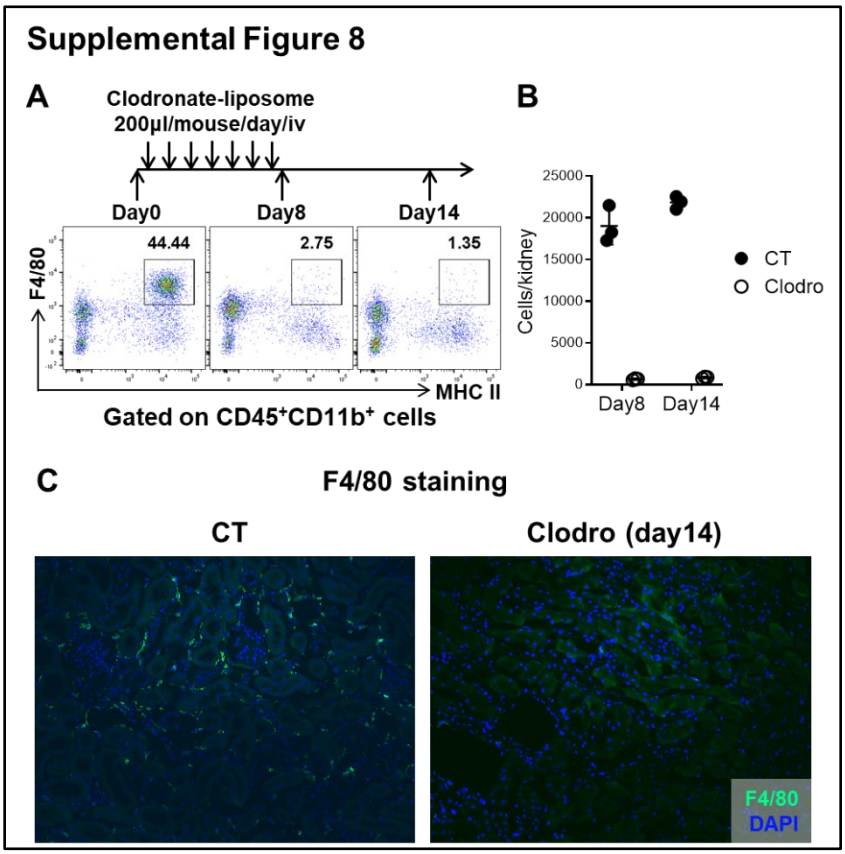
Supplemental Figure 5. UMAPs of un-transplanted kidneys or transplanted kidneys on day 15 post-transplantation (in allogeneic recipients) showing *Ccr8* expression in distinct cell clusters. *Ccr8* was primarily expressed by T cells in transplanted kidneys (circled). The UMAP of un-transplanted kidneys contained 8,552 cells and the UMAP of transplanted kidneys contained 9,434 cells. N=2 per group.



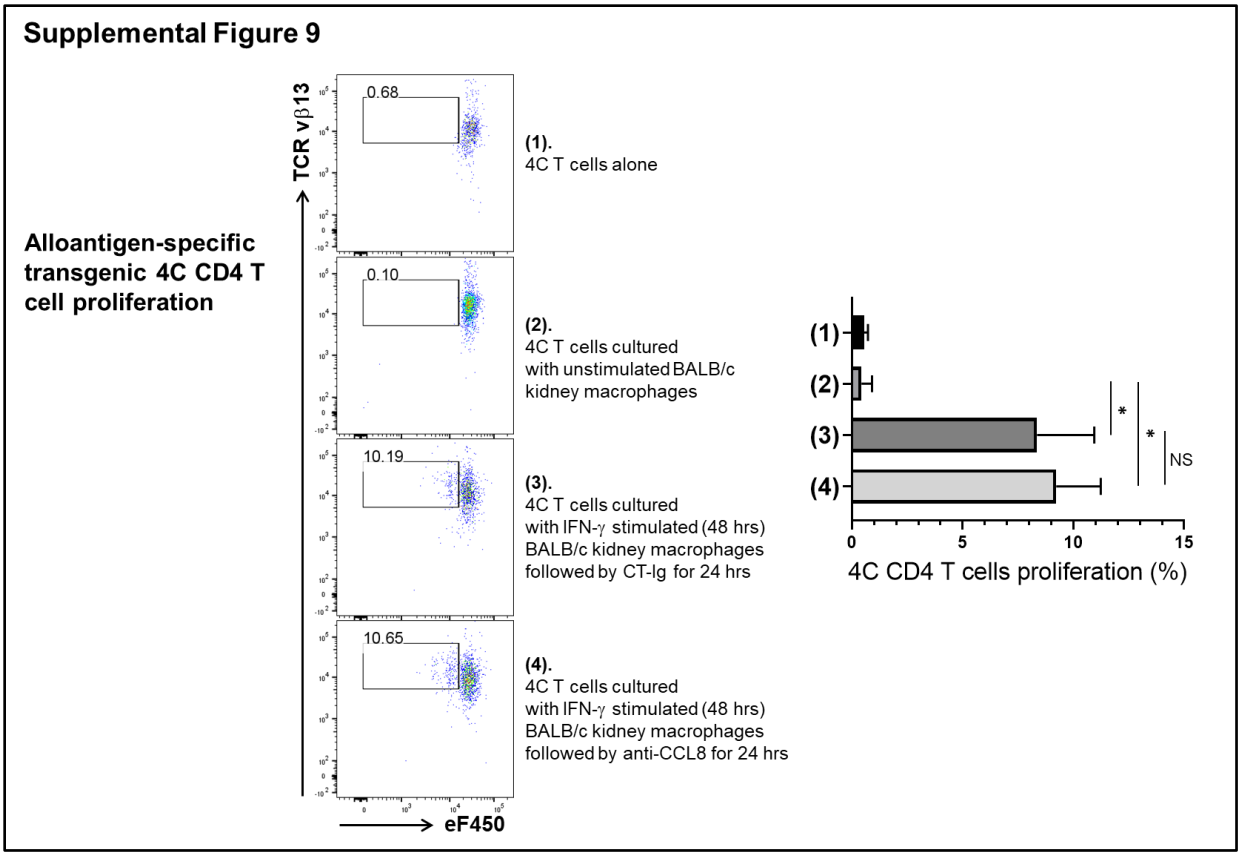
Supplemental Figure 6. Recipient serum creatinine levels at various time points post-transplantation in CT-Ig and anti-CCL8 Ab treated recipients. Bilateral nephrectomized B6 mice were transplanted with BALB/c kidneys. Recipients were either treated with CT-Ig or anti-CCL8 as shown in Figure 5A. Blood samples were collected on indicated days for serum creatinine measurements. N=3 for the day 3 time point; N=2-3 for the day 14 time point; N=5-9 for the day 28 time point. *$p \leq 0.05$, calculated using unpaired *t*-test.



Supplemental Figure 7. Anti-CCL8 treatment permits longer survival of donor kidney resident macrophages post-transplantation. CD45.1 B6 mice were transplanted with CD45.2 BALB/c kidneys. Recipients were treated with either control Ig (CT-Ig) or anti-CCL8 from day -1 to +4, and sacrificed on day 5 post-transplantation. Kidney allografts were harvested and cells were analyzed by FACS. Representative FACS plots on the left show the relative proportion of $CD45.1^+$ recipient and $CD45.2^+$ donor myeloid cells in CT-Ig or anti-CCL8 treated recipients. Cells were gated on total $CD11b^+$ myeloid cells. Representative FACS plots on the right confirm the $F4/80^{HI}MHCII^{HI}$ macrophage phenotype of the gated $CD45.2^+$ donor myeloid cells. Scatter plot shows the absolute number of surviving donor macrophages per kidney allograft in either CT-Ig or anti-CCL8 treated recipients on day 5 post-transplantation. N=2-3 per group. *$p \leq 0.05$, calculated using unpaired *t*-test.

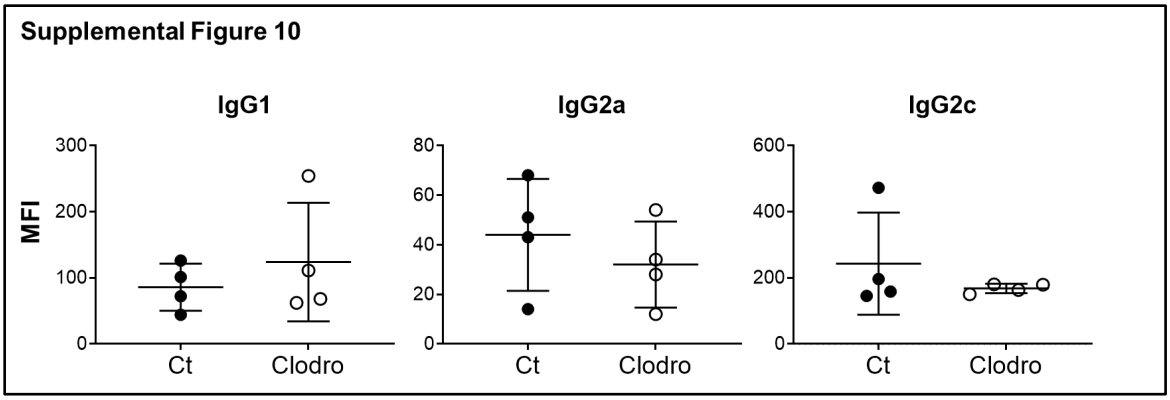


Supplemental Figure 8. Depletion of kidney resident macrophages in kidney donors. **(A)** Scheme of clodronate-liposome injections in BALB/c donor mice and FACS analysis of kidney resident myeloid cells on the indicated days. $CD45^{+}CD11b^{+}$ myeloid cells were analyzed to identify the $F4/80^{HI}MHC\text{-}II^{HI}$ kidney resident macrophage sub-population. **(B)** Scatter plot showing the absolute number of $F4/80^{HI}MHC\text{-}II^{HI}$ macrophages in control PBS (CT) and clodronate (Clodro) liposome treated mice at the indicated time points. N=3 mice per group per time-point. **(C)** Immunofluorescence staining of F4/80 of kidneys from clodronate-liposome treated donor mice. Frozen sections of kidneys collected on day14 as shown in (*A*) were used. F4/80-specific primary and AlexaFluor-488 conjugated secondary antibodies were used to identify kidney macrophages (green). DAPI was used to stain nuclei (blue).



Supplemental Figure 9. Alloantigen-specific CD4 T cell proliferation stimulated by donor kidney resident macrophages. 4C T cells are transgenic CD4 T cells (congenically marked by CD90.1) on the B6 background that recognize the I-A^d alloantigen expressed by BALB/c cells. 4C cells were labeled with the proliferation dye eF450 and cultured alone or with sorted BALB/c kidney macrophages. Co-culture conditions were: (1) 4C T cells alone; (2) 4C T cells + un-stimulated BALB/c kidney macrophages; (3) 4C T cells + IFN-γ stimulated (48 hrs) BALB/c kidney macrophages followed by CT-Ig for 24 hrs; (4) 4C T cells + IFN-γ stimulated (48 hrs) BALB/c kidney macrophages followed by anti-CCL8 for 24 hrs (see Methods for details). On day 7 of co-cultures, cells were harvested, stained for CD3, CD4, CD90.1, TCR vβ13$^+$ and analyzed by FACS for eF450 dilution. Representative FACS plots were gated on CD3$^+$CD4$^+$CD90.1$^+$ cells showing proliferation of TCR vβ13$^+$ 4C cells by eF450 dilution in the indicated groups. Bar graph shows

percentages of proliferating 4C T cells in the indicated groups. Data were presented as mead±SD. N=2. *$p \leq 0.05$, calculated using unpaired *t*-test. NS=Not Significant.



Supplemental Figure 10. Donor-specific alloantibodies on day 28 post-transplantation. Specific anti-donor antibodies of three IgG sub-classes (IgG1, IgG2a and IgG2c) were measured in sera from “CT” or “Clodro” recipients by flow cytometry. The mean fluorescence intensity (MFI) of each specific anti-donor antibody subclass in transplant recipients was normalized to those in naive un-transplanted mice (N=4 per group). Data are presented as mean±SD.