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Research Letter In-Press Preview Cardiology Inflammation

Type 1 Classical Dendritic Cells Govern Long-term Cardiac Allograft Acceptance

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Cardiac transplantation is a lifesaving procedure for patients with complex congenital heart diseases and end-stage heart failure. Unfortunately, rejection remains common due to limitations in current immunosuppressive strategies and alternative therapies are needed. Among proposed strategies, co-stimulation blockade (CSB) represents a promising approach, promoting tolerance rather than suppressing alloimmune responses. CSB with CTLA4-Ig and anti-CD40L antibodies is efficacious in experimental models and early clinical studies in islet and kidney transplantation (1,2). How CSB modulates recipient immune responses remains incompletely understood.

Co-stimulation pathways signal bidirectionally influencing both antigen presenting cells (APCs) and T-cells. While CSB's impact on T-cells is well studied, less is known about its effects on APCs. To investigate how CSB and a conventional immunosuppressant (cyclosporine, CSA) influence APCs in cardiac allografts, we performed single-cell RNA sequencing (scRNA-seq) on murine hearts 7 days post-transplant. BALB/c donor hearts were transplanted into B6 *Zbtb46*$^{gfp/+}$ recipients treated with either CSB (anti-CD40L and CTLA4-Ig) or CSA. Histologically, CSA-treated grafts exhibited increased cellular infiltration compared to CSB-treated counterparts (**Supplemental Fig.1A**). Flow cytometry-isolated mononuclear phagocytes were used for 10X Genomics scRNA-seq, yielding 14,524 high-quality cells (**Supplemental Fig.1B,C**) including monocyte, macrophage and classical dendritic cell (cDC) subsets (**Fig.1A, Supplemental Fig.1D,E**). CSB-treated grafts were enriched for recipient cDCs (GFP$^+$), whereas CSA-treated grafts had increased monocytes and macrophages (**Fig.1B**). Reference mapping of naïve hearts, syngeneic grafts, and a second model of allograft rejection (low-dose CTLA4-Ig) highlighted that cDC enrichment was CSB-specific (**Supplemental Fig.1F**). Differential gene expression analysis revealed upregulation of genes involved in cDC activation, antigen presentation, and immunoregulation in CSB samples (**Supplemental Fig.1G,H**). Flow cytometry and immunostaining confirmed increased frequencies of GFP$^+$ cDCs in CSB-treated allografts,

with a shift toward higher proportions of type 1 cDCs (cDC1s) (**Fig.1C-E, Supplemental Fig.2A**). Moreover, cDCs in CSB-treated allografts expressed PDL1 at a higher frequency than cDCs in CSA-treated allografts (**Fig.1D,E**).

We next set out to define the requirement for recipient cDC1s and cDC2s in CSB-mediated long-term cardiac allograft acceptance. BALB/c hearts were transplanted into wildtype (WT), Δ1+2+3 (cDC2-deficient), and *Irf8+32*$^{-/-}$ (cDC1-deficient) B6 CSB-treated recipients (**Supplemental Fig.2B**)(3, 4). While WT and cDC2-deficient recipients accepted cardiac allografts long-term, cDC1-deficient recipients rejected the transplanted hearts (**Fig. 1F,G**). *Irf8+32*$^{-/-}$ recipients exhibited intragraft infiltration of CD4^{+} and CD8^{+} T-cells at day 14 post-transplant and the time of rejection. WT and ∆1+2+3 recipients had significantly fewer T-cells at both time points (**Fig.1H**, **Supplemental Fig.2C**). We also observed increased Foxp3^{+} CD4 T-cells in allografts transplanted into WT versus *Irf8+32*$^{-/-}$ recipients, suggesting that cDC1s recruit regulatory T-cells (**Supplemental Fig.2D**).

To examine if cDC1 deficiency impacts the composition and transcriptional state of intragraft T-cells, BALB/c donor hearts were transplanted into CSB-treated B6 WT or *Irf8+32*$^{-/-}$ recipients. Extravascular immune cells were isolated from allografts 14 days post-transplant by flow cytometry and scRNA-seq was performed, yielding 12,580 high-quality cells (**Supplemental Fig.3A, B**). Allografts transplanted into *Irf8+32*$^{-/-}$ recipients exhibited shifts in CD4^{+} and CD8^{+} T-cell phenotype (**Fig.1I,J, Supplemental Fig.3C-E)**. We observed an increase in Rora^{+}CD4^{+} effector T-cells in allografts from *Irf8+32*$^{-/-}$ recipients. Rora, a key regulator of T_h17 cells, has been implicated in colitis where it drives T-cell infiltration, activation, and prevention of apoptosis (5). Moreover, we observed marked reduction in a CD8^{+} T-cell subset expressing *Tcf7*, *Xcl1* and immunoregulatory genes (*Cd200*, *Cd160, Lag3*) in allografts from *Irf8+32*$^{-/-}$ recipients. *Xcl1* is secreted by CD8^{+} T-cells and is a ligand for *Xcr1*, a cDC1-specific receptor that regulates antigen presentation, regulatory T-cell activation, and prevents intestinal

inflammation (6). Pathway analysis revealed upregulation of IL-1, IL-5, TNF and CD40L signaling in T-cells from allografts transplanted into *Irf8+32*$^{-/-}$ recipients, and enhanced immunoregulatory responses and T-cell apoptosis in WT recipients (**Fig.1K**).

Collectively, we demonstrate that cDC1s expand in response to CSB and are essential for long-term allograft acceptance. CSB facilitates recruitment of immunoregulatory cDC1s which modulate T-cell phenotypes. CSB represents a tractable approach to achieve organ transplant tolerance in the clinical setting. Unlike other tolerance protocols, CSB does not necessitate exposure of the recipient to donor cells or tissues and instead only involves perioperative treatment with CTLA4-Ig and anti-CD40L antibodies. Identification of cDC1s as a key cell involved in cardiac allograft acceptance provides a critical clue regarding underlying mechanisms. Future studies dissecting tolerogenic cDC1 effector mechanisms may lead to improved CSB regimens, methodologies to measure CSB efficacy, and platforms to predict post-transplant outcomes.

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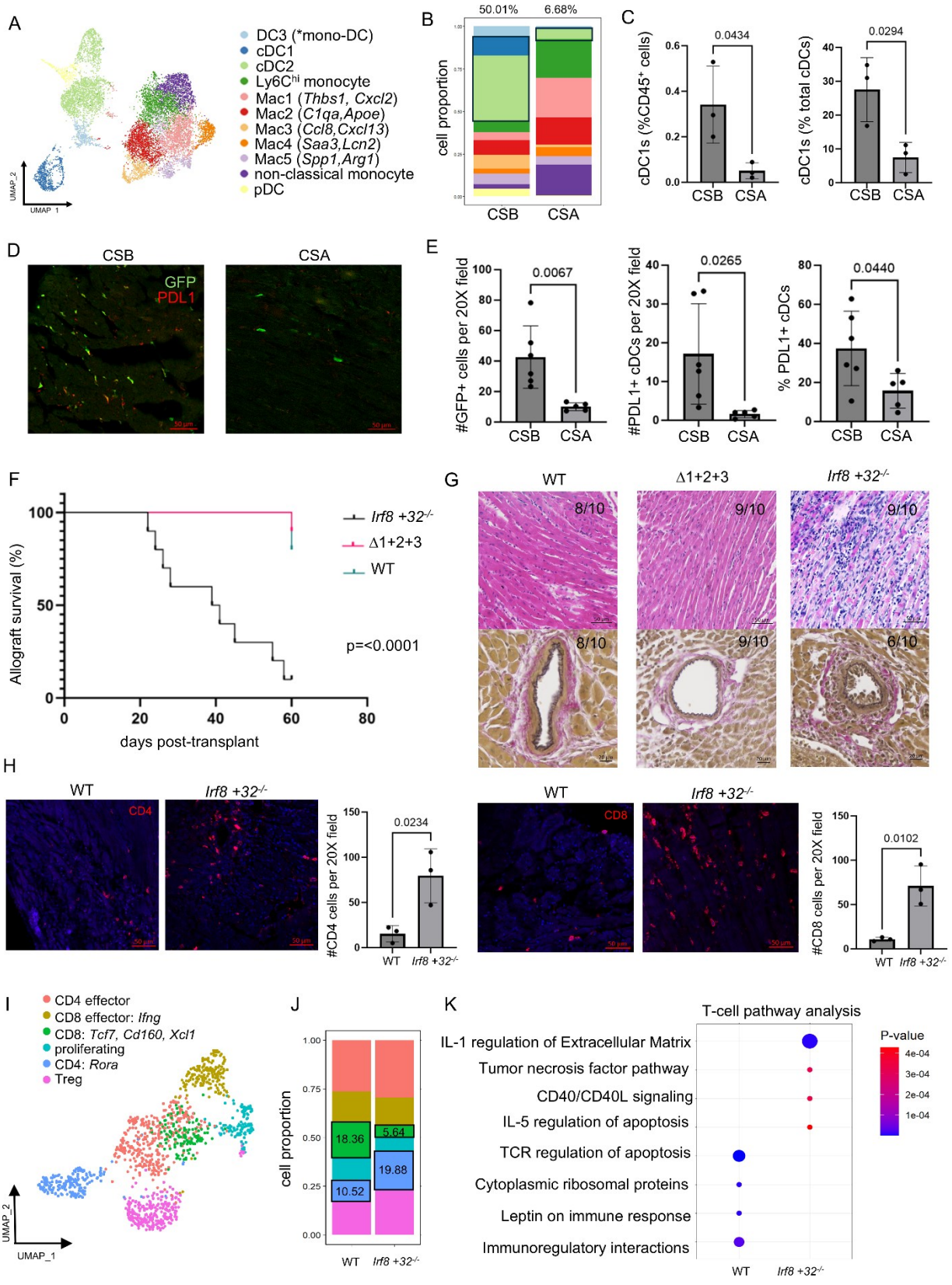
A
DC3 (*mono-DC)
cDC1
cDC2
Ly6C^hi monocyte
Mac1 (Thbs1, Cxcl2)
Mac2 (C1qa,Apoe)
Mac3 (Ccl8,Cxcl13)
Mac4 (Saa3,Lcn2)
Mac5 (Spp1,Arg1)
non-classical monocyte
pDC
UMAP_2
UMAP_1
B
50.01%
6.68%
cell proportion
CSB
CSA
C
cDC1s (%CD45+ cells)
0.0434
cDC1s (% total cDCs)
0.0294
CSB
CSA
D
CSB
CSA
GFP
PDL1
E
#GFP+ cells per 20X field
0.0067
#PDL1+ cDCs per 20X field
0.0265
% PDL1+ cDCs
0.0440
CSB
CSA
F
Allograft survival (%)
days post-transplant
Irf8 +32-/-
Δ1+2+3
WT
p=<0.0001
G
WT
Δ1+2+3
Irf8 +32-/-
8/10
9/10
9/10
8/10
9/10
6/10
H
WT
Irf8 +32-/-
CD4
#CD4 cells per 20X field
0.0234
CD8
#CD8 cells per 20X field
0.0102
I
CD4 effector
CD8 effector: Ifng
CD8: Tcf7, Cd160, Xcl1
proliferating
CD4: Rora
Treg
UMAP_2
UMAP_1
J
cell proportion
18.36
5.64
19.88
10.52
K
T-cell pathway analysis
IL-1 regulation of Extracellular Matrix
Tumor necrosis factor pathway
CD40/CD40L signaling
IL-5 regulation of apoptosis
TCR regulation of apoptosis
Cytoplasmic ribosomal proteins
Leptin on immune response
Immunoregulatory interactions
P-value

Figure 1: A) scRNA-seq UMAP and **B)** composition plot of mononuclear phagocytes sorted from allografts 7 days after transplantation of BALB/c hearts into CSB (n=3)- or CSA (n=3)-treated B6 WT mice. **C)** Flow cytometry quantification of cDC1s within allografts of CSB (n=3)- and CSA (n=3)-treated B6 WT recipients at 7 days post-transplant. **D)** Immunostaining of graft-infiltrating cDCs (GFP^+) and PDL1 in CSB (n=6)- and CSA (n=5)-treated B6 *Zbtb46*$^{gfp/+}$ recipients of BALB/c hearts at 7 days post-transplant. **E)** Quantification of GFP^+ and $PDL1^+$ cells in CSB (n=6)- and CSA (n=5)-treated allografts at 7 days post-transplant into B6 *Zbtb46*$^{gfp/+}$ mice. **F)** Kaplan-Meier survival curves of BALB/c hearts after transplantation into CSB-treated B6 WT, Δ1+2+3, and *Irf8+32*$^{-/-}$ mice (n=10 per condition). **G)** Histology (H&E, Verhoeff-van Gieson Elastin stain (VVG)) of allografts from B6 WT, Δ1+2+3, and *Irf8+32*$^{-/-}$ recipients at 60 days post-transplant (WT, Δ1+2+3) or time of rejection (*Irf8+32*$^{-/-}$). **H)** Immunostaining of $CD4^+$ and $CD8^+$ T-cells in allografts of B6 *Irf8+32*$^{-/-}$ (n=3) and WT (n=3) recipients at 14 days post-transplant. **I)** scRNA-seq UMAP and **J)** composition plot of subclustered T-cells B6 WT (n=3) and *Irf8+32*$^{-/-}$ (n=3) recipients. **K)** Pathway analysis of genes differentially expressed in T-cells.