Supplemental Information

Fig S1 The neonatal heart of NOD/SCID fails to regenerate. (A) Schematic diagram showing the experimental design. (B) Images of scar tissues at 4 weeks post CI to P3 or P8 hearts, scale bars: 2000 um. (C, E, G) Masson's trichrome staining showing representative serial cross sections of fibrotic tissues in blue at 4 weeks post CI, scale bars: 1000 um. (D, F, H) Quantification of fibrotic tissue coverage based on (C, E, G respectively). Data are presented as mean±S.E.M., n=6, *P<0.05, **P<0.01.

Figure S1

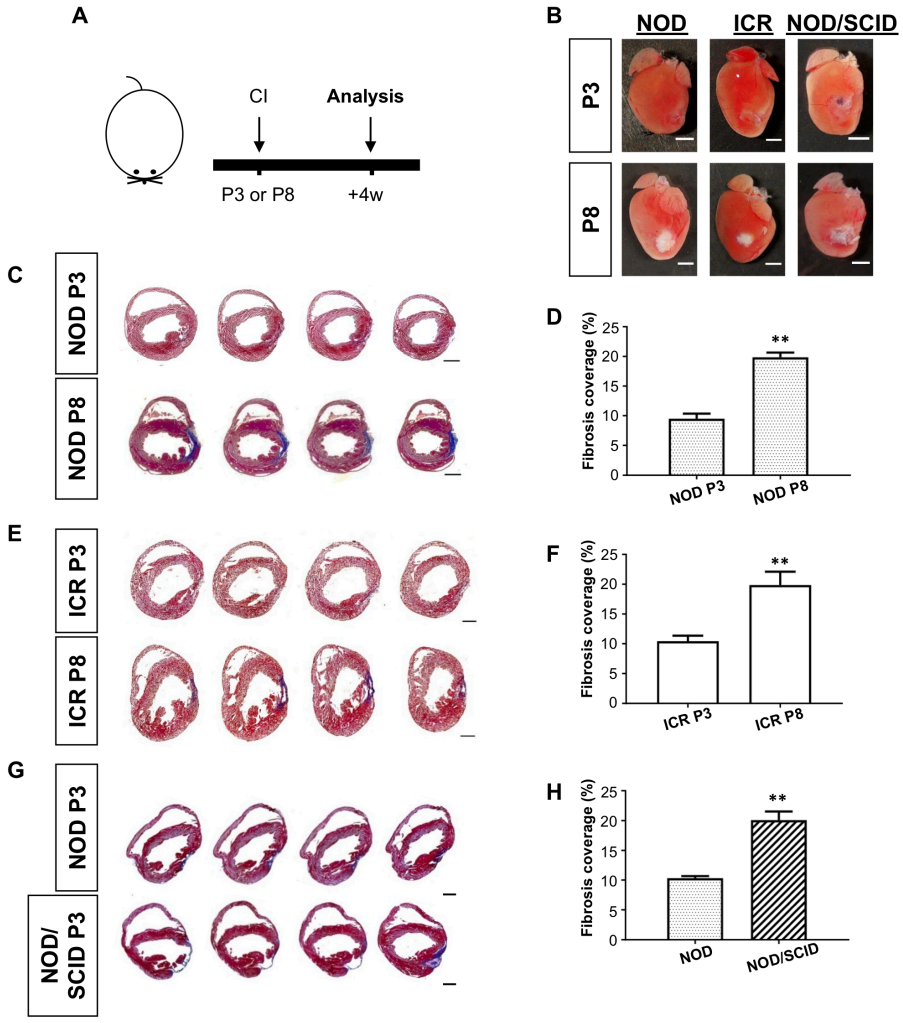

A
CI
Analysis
P3 or P8
+4w
B
NOD
ICR
NOD/SCID
P3
P8
C
NOD P3
NOD P8
D
Fibrosis coverage (%)
25
20
15
10
5
0
**
NOD P3
NOD P8
E
ICR P3
ICR P8
F
Fibrosis coverage (%)
ICR P3
ICR P8
G
NOD P3
NOD/ SCID P3
H
Fibrosis coverage (%)
NOD
NOD/SCID

Fig S2 Echocardiography showing functional and morphological change during neonatal heart regeneration. (A, G) Schematic diagrams showing the experimental design. (B, H) Echocardiographic analyses and quantification showing (C, I) %fractional shortening; (D, J) %ejection fraction; (E, K) end-diastolic diameter (LVID d) or (F, L) end-systolic diameter (LVID s) at (A-F) 4 days or (G-L) 4 weeks post CI, n=6. (C-F, I-L). Data are presented as mean±S.E.M., *P<0.05, **P<0.01, ***P<0.001.

Figure S2

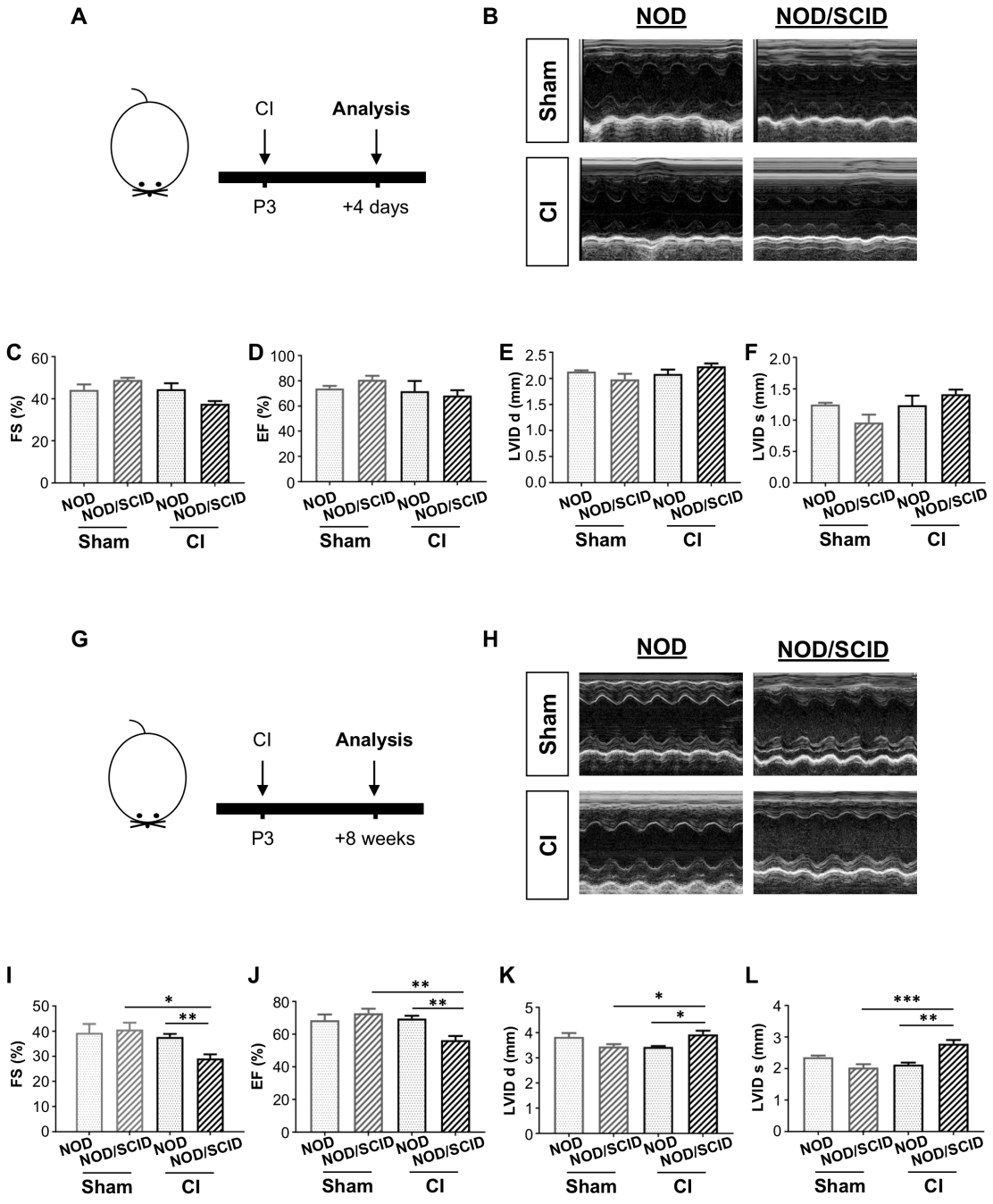

A
CI
Analysis
P3
+4 days
B
NOD
NOD/SCID
Sham
CI
C
FS (%)
D
EF (%)
E
LVID d (mm)
F
LVID s (mm)
NOD
NOD/SCID
Sham
CI
G
CI
Analysis
P3
+8 weeks
H
I
J
K
L
*
**
***

Figure S3 Quantification of Treg in the neonatal mouse heart after injury. (A) Flow cytometric analysis showing infiltration of $CD3^+CD4^+hCD2^+$ Treg into the injured myocardium at day 7 post CI to the P3 heart compared to the sham control. Quantification of (B) %Treg among total $CD4^+$ T-cells or (C) the absolute number of Treg per mg heart tissue within the first 2 weeks post CI based on (A). Data are presented as mean±S.E.M., n=3 per time point, *P<0.05, **P<0.01.

Figure S3

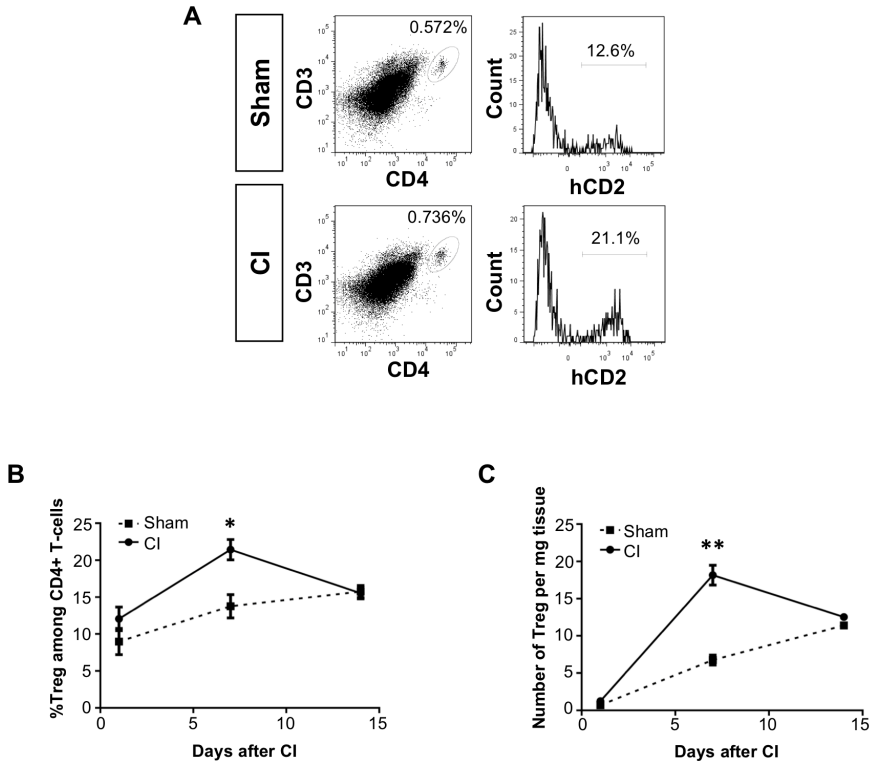
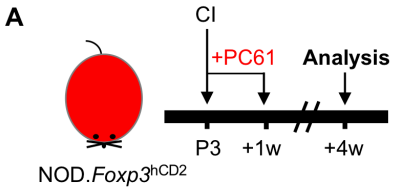
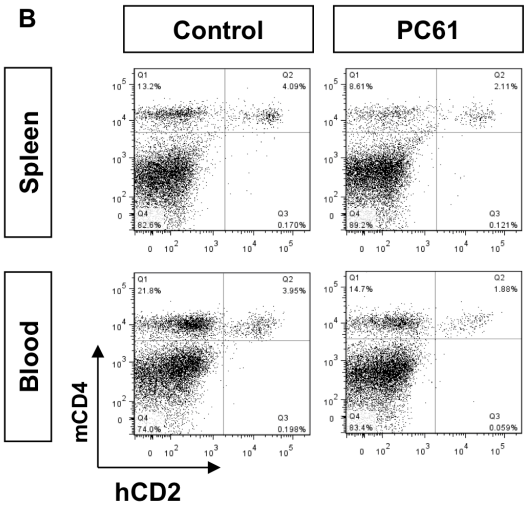


Figure S4 Depleting efficiency of PC61 targeting $CD4^+CD25^{hi}FOXP3^+$ Treg during neonatal heart regeneration. (A) Schematic diagram showing the experimental design. (B) Flow cytometric analyses and (C) quantification showing percentage of $CD4^+hCD2^+$ Treg among total splenocytes and peripheral blood cells, respectively, at 4 weeks after CI in control or anti-CD25 (PC61) monoclonal antibody-treated groups. Data are presented as mean±S.E.M., n=8, **P<0.01.

Figure S4


A
CI
+PC61
Analysis
P3
+1w
+4w
NOD.Foxp3hCD2


B
Control
PC61
Spleen
Blood
mCD4
hCD2

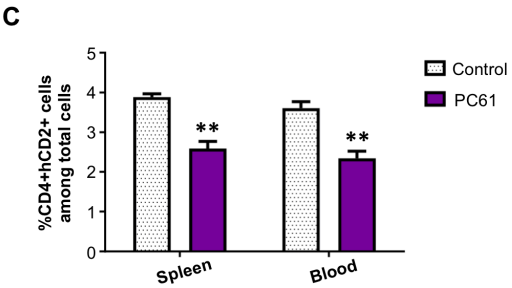

C
%CD4+hCD2+ cells among total cells
Control
PC61
**
**
Spleen
Blood

Figure S5 Loss-of-function of $CD4^+$ Treg leads to increased fibrosis and reduced cardiomyocyte proliferation after cryoinfarction. (A) Images of scar tissues, scale bars: 2000 um; and Masson's trichrome staining showing representative serial cross sections of fibrotic tissues in blue, scale bars: 1000 um. (B) Quantification of fibrotic tissue coverage based on (A), n=8. Immunostaining on frozen sections for (C) $COLA1^+$ (red) and $cTnT^+$ (green) cells within the infarct zone or (E) $Ki67^+$ (red) or $pH3^+$ (red) and $cTnT^+$ (green) cells within the border zone. Arrows indicate cardiomyocytes positive for Ki67 or pH3 and square denotes magnified images on the right. Quantification of (D) $\%cTnT^+$ coverage or the absolute number of (F) $Ki67^+cTnT^+$ or (G) $pH3^+cTnT^+$ cardiomyocytes per mm^2 area, n=4. (B, D, F, G) Data are presented as mean±S.E.M., *P<0.05, **P<0.01.

Figure S5

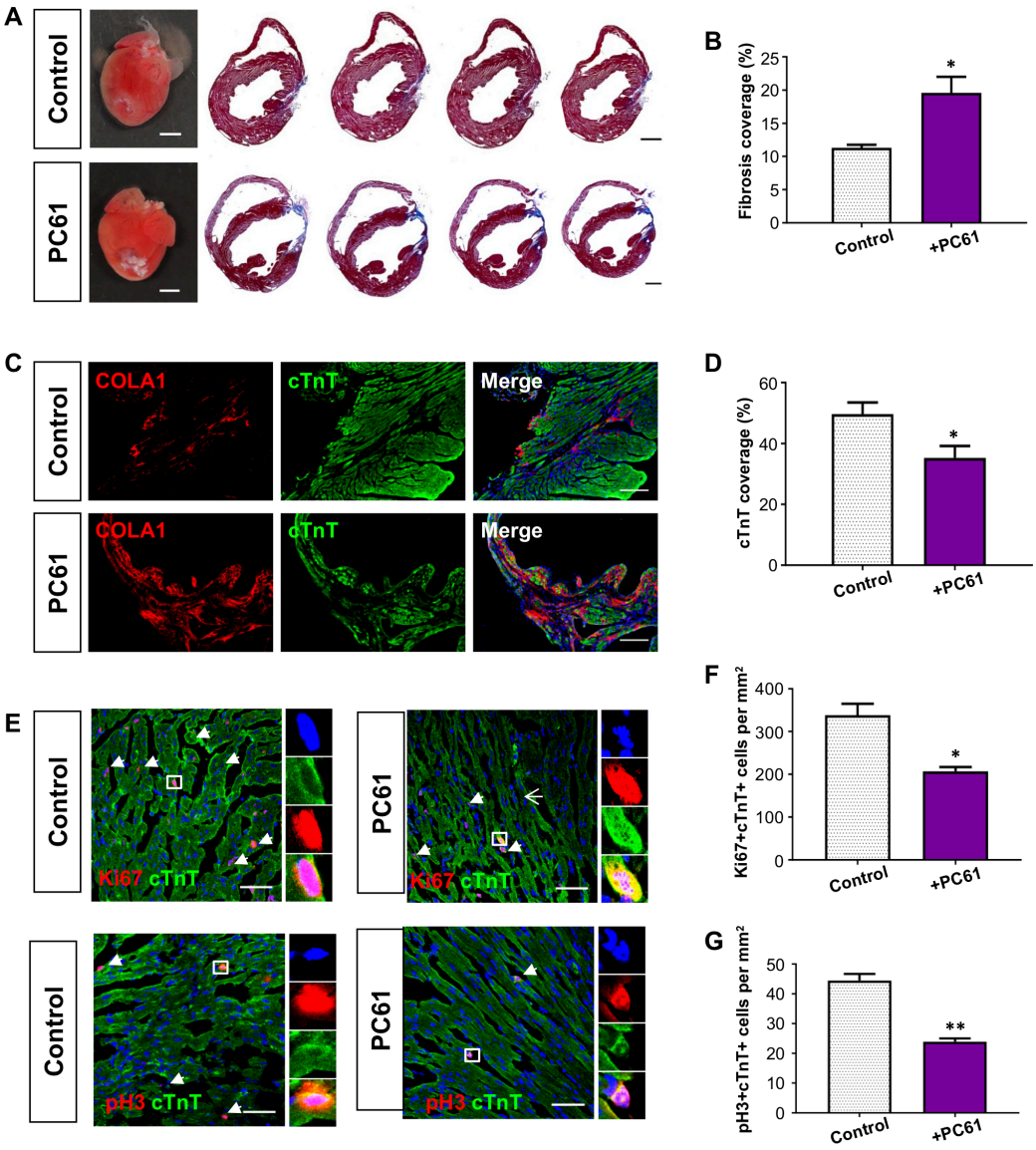

A
Control
PC61
B
Fibrosis coverage (%)
25
20
15
10
5
0
*
Control
+PC61
C
Control
COLA1
cTnT
Merge
PC61
COLA1
cTnT
Merge
D
cTnT coverage (%)
60
40
20
0
*
Control
+PC61
E
Control
Ki67 cTnT
PC61
Ki67 cTnT
Control
pH3 cTnT
PC61
pH3 cTnT
F
Ki67+cTnT+ cells per mm²
400
300
200
100
0
*
Control
+PC61
G
pH3+cTnT+ cells per mm²
50
40
30
20
10
0
**
Control
+PC61

Figure S6 Cardiomyocyte characterization during neonatal heart regeneration. (A) qRT-PCR showing expression levels of the respective CDK inhibitor genes in neonatal cardiomyocytes isolated from P1 ICR mice with or without cultured in Treg SN for 1 day; gene expression levels in Treg SN were compared to control (without Treg SN). Data are presented as mean±S.E.M., n=3, *P<0.05, **P<0.01. (B) Immunostaining on frozen heart sections of the P1 heart of ICR mice for cTnT (green) and CCR3 (red). (C) Flow cytometric analysis showing expression of CCR3 on neonatal cardiomyocytes isolated from the P1 heart of ICR mice.

Figure S6

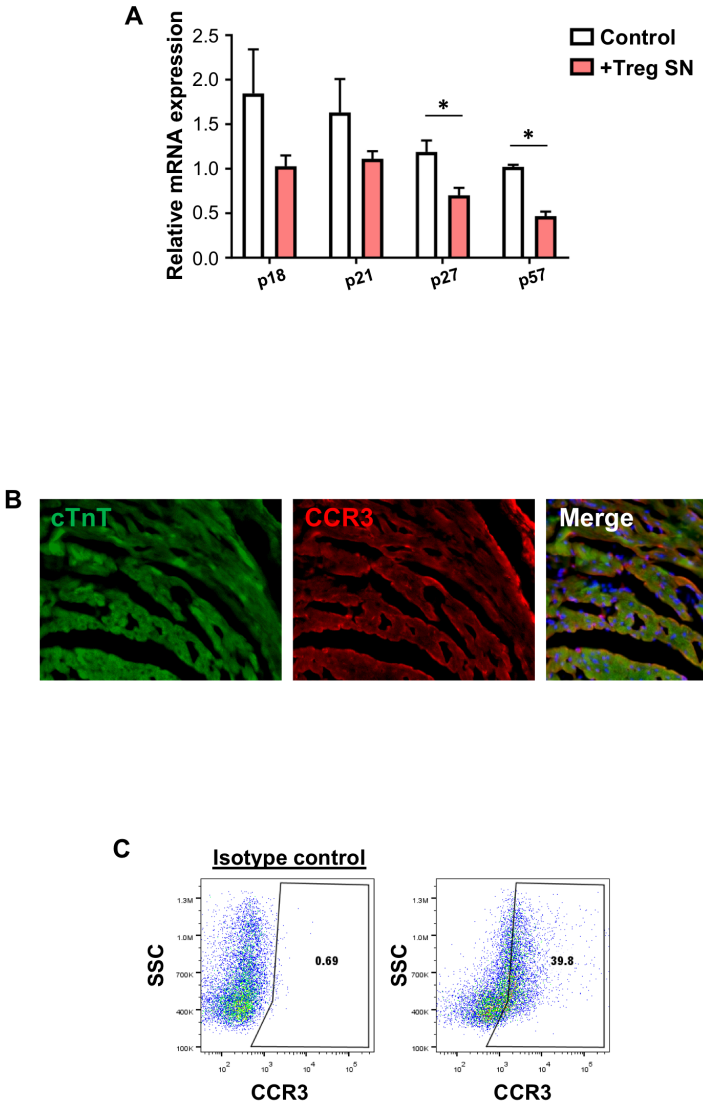

A
Relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
Control
+Treg SN
*
*
p18
p21
p27
p57
B
cTnT
CCR3
Merge
C
Isotype control
SSC
CCR3
0.69
39.8

Figure S7 Treg regulate accumulation of M2-like macrophages during neonatal heart regeneration. (A, D) Schematic diagrams showing the experimental design. (B, E) Flow cytometric analysis and (C, F) quantification showing %F4/80^{+}Ly6C^{high} M1-like or %F4/80^{+}CD206^{+}Ly6C^{low} M2-like macrophages among total F4/80^{+} macrophages after CI. Data are presented as mean±S.E.M., n=4, *P<0.05, **P<0.01.

Figure S7

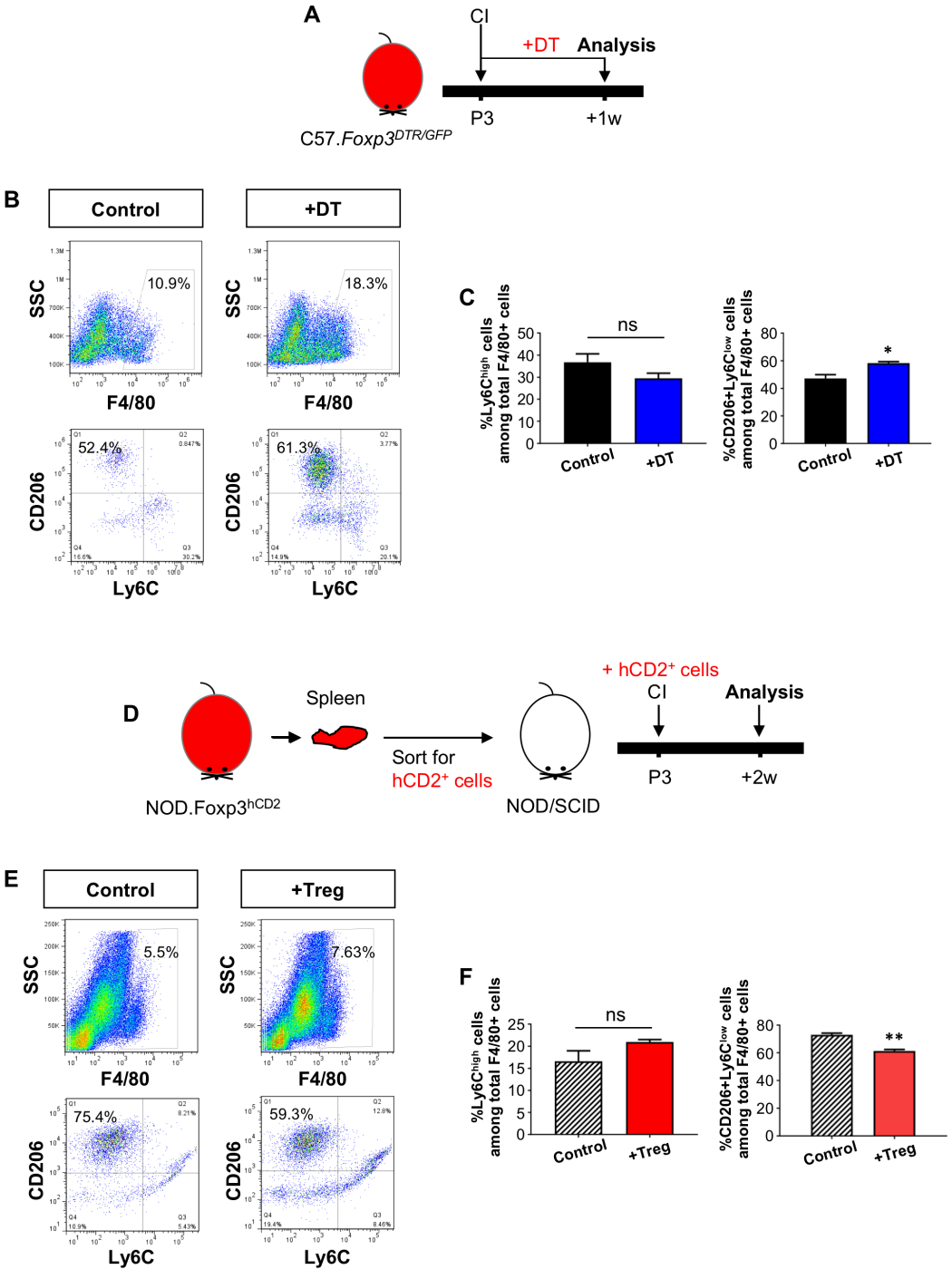

A
CI
+DT
Analysis
P3
+1w
C57.Foxp3DTR/GFP
B
Control
+DT
SSC
F4/80
10.9%
18.3%
CD206
Ly6C
52.4%
61.3%
C
%Ly6Chigh cells among total F4/80+ cells
ns
Control
+DT
%CD206+Ly6Clow cells among total F4/80+ cells
*
D
Spleen
Sort for hCD2+ cells
NOD.Foxp3hCD2
NOD/SCID
+ hCD2+ cells
CI
Analysis
P3
+2w
E
Control
+Treg
5.5%
7.63%
75.4%
59.3%
F
ns
**

Table S1 GO functional annotations showing a list of genes expressed by C1 Treg purified from the spleen of ICR mice at day 7 after CI to a P3 heart. The selected most significantly upregulated pathways in terms of biological processes are determined by scRNA-seq.

Upregulated Pathway	p-value	Molecules
GO:0007067~mitotic nuclear division	5.78E-10	CCNB1, CDK1, CDCA8, CCNB2, NUSAP1, BIRC5, CDC20, PTTG1, UBE2C, SMC2, CDC25B
GO:0051301~cell division	1.04E-08	CCNB1, CDK1, CDCA8, CCNB2, NUSAP1, BIRC5, CDC20, PTTG1, UBE2C, SMC2, CDC25B
GO:0007049~cell cycle	1.04E-06	CCNB1, CDK1, CDCA8, CCNB2, NUSAP1, BIRC5, CDC20, PTTG1, UBE2C, SMC2, CDC25B
GO:0032496~response to lipopolysaccharide	5.86E-06	HMGB2, S100A8, WFDC21, ELANE, MPO, CD6, TNFRSF4
GO:0002376~immune system process	2.58E-05	LCN2, HMGB2, S100A8, TAP2, S100A9, FCNB, PGLYRP1, LTF

Table S2 GO functional annotations showing a list of genes expressed by C2 Treg purified from the myocardium of ICR mice at day 7 after CI to a P3 heart. The selected most significantly upregulated pathways in terms of biological processes are determined by scRNA-seq.

Upregulated Pathway	p-value	Molecules
GO:0030593~neutrophil chemotaxis	2.12E-16	CCL24, CCL12, CCL3, CCL2, C5AR1, CXCL2, FCER1G, CCL4, CCL7, FCGR3, SYK, SPP1
GO:0006935~chemotaxis	1.00E-13	CCL24, CCL12, C3AR1, CCL3, CCL2, C5AR1, CCR1, CXCL16, CXCL2, CX3CR1, CCL4, CCL7
GO:0070374~positive regulation of ERK1 and ERK2 cascade	3.93E-10	CCL24, CCL12, CCL3, CCL2, C5AR1, CCR1, TREM2, CCL4, CCL7, GAS6, CSF1R
GO:0002548~monocyte chemotaxis	1.72E-07	CCL24, CCL12, CCL3, CCL2, CCL4, CCL7
GO:0048246~macrophage chemotaxis	1.11E-05	CCL12, CCL3, CCL2, CX3CR1