**Supplemental Information**

# Macrophages Contribute to the Spermatogonial Niche in the Adult Testis

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# Figure S1

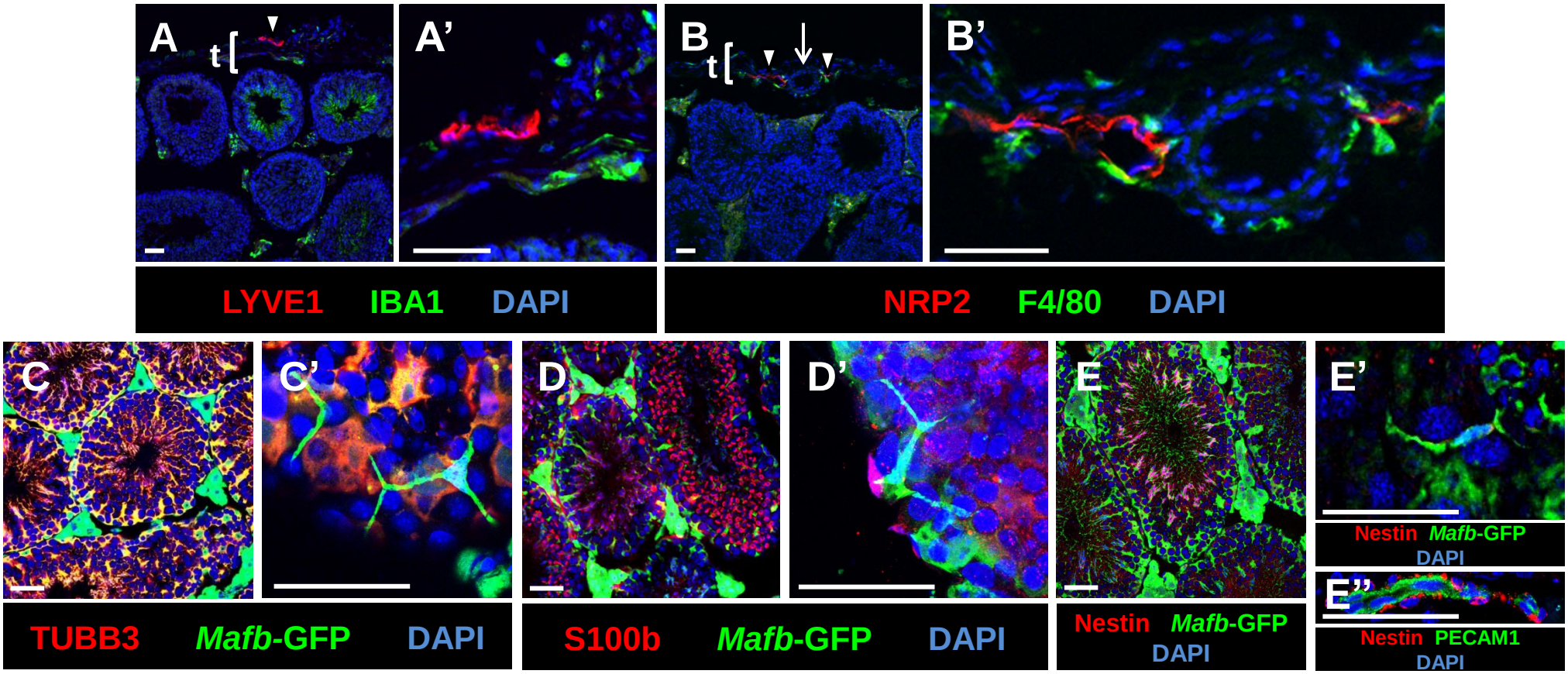
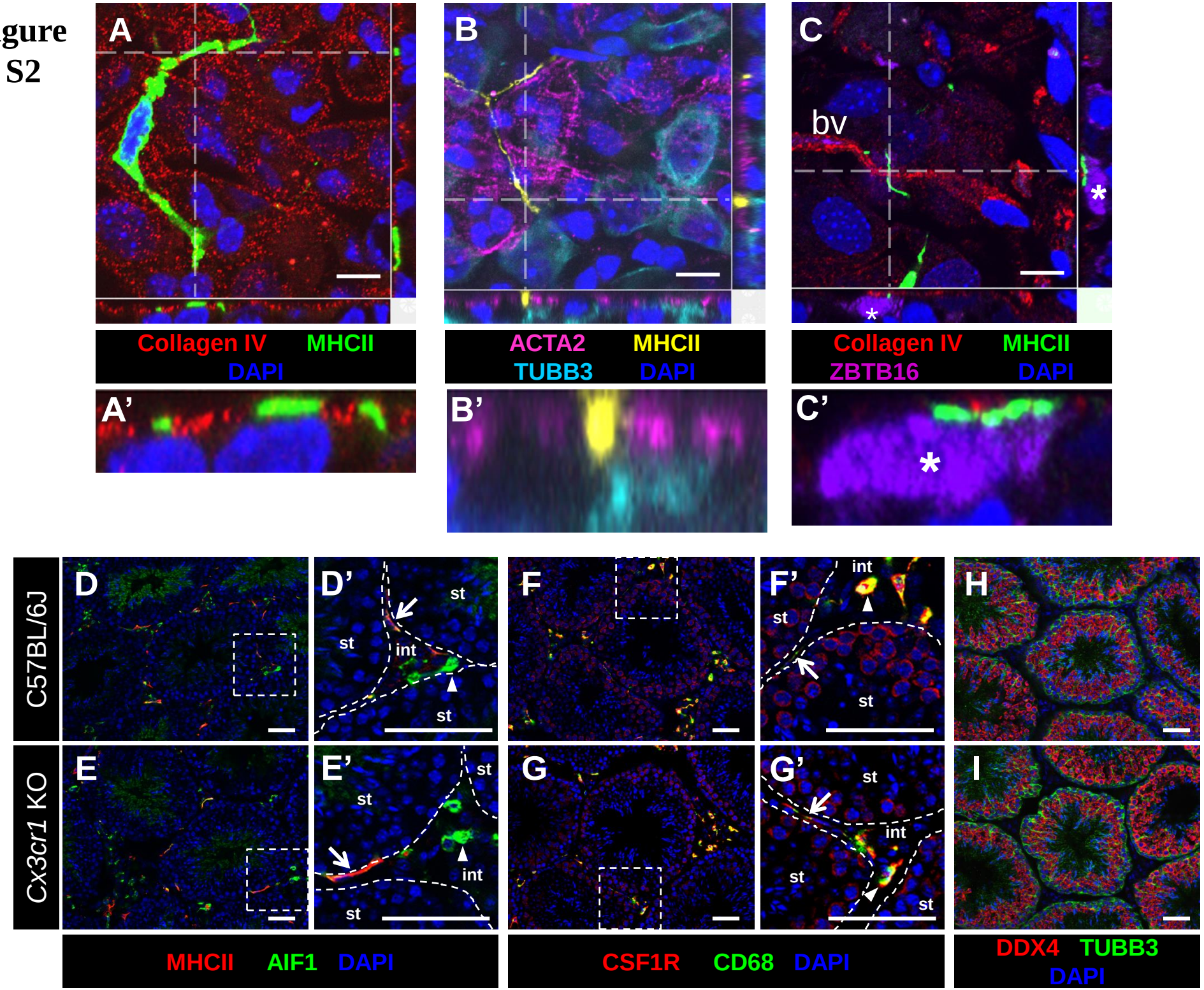
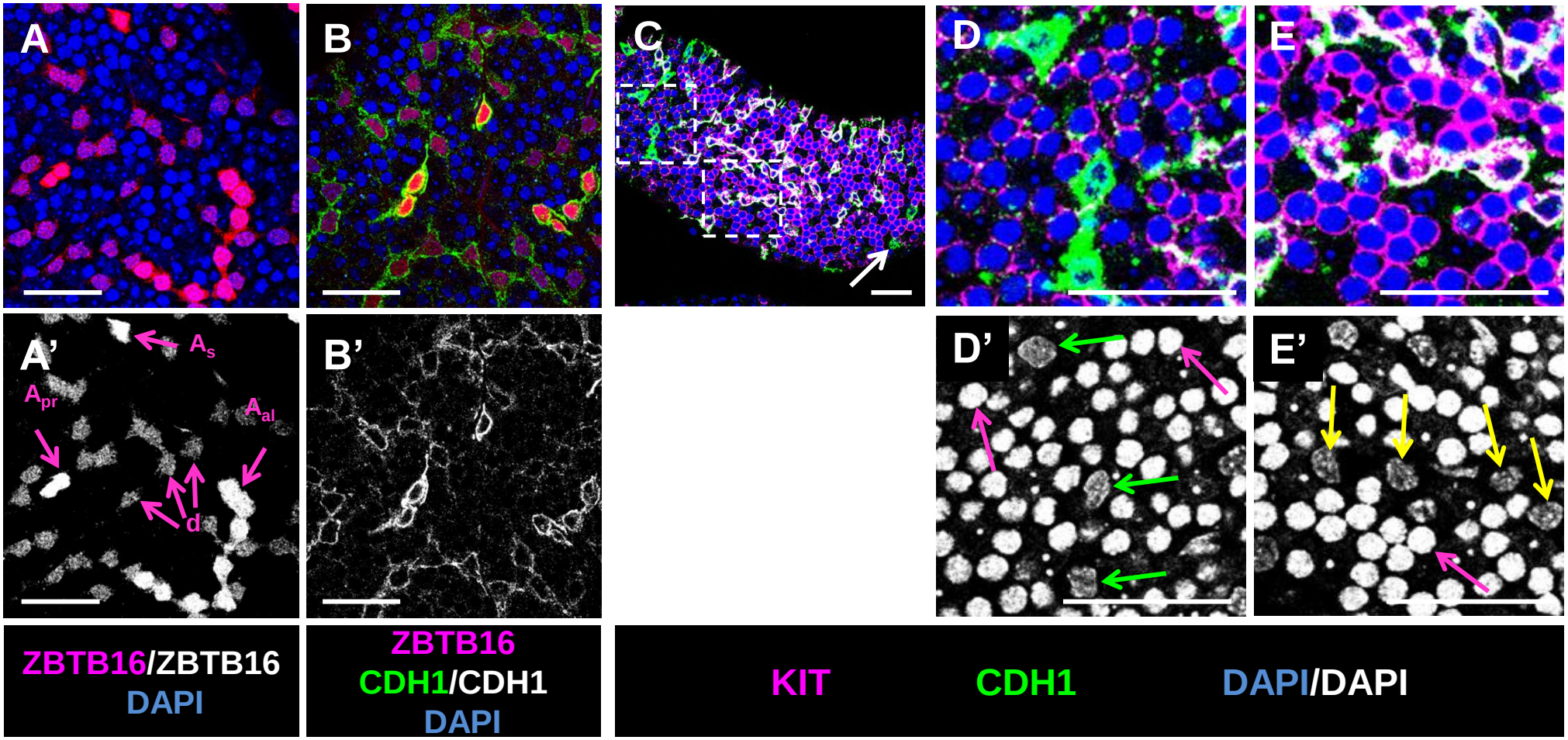

A
t[
A'
B
t[
B'
LYVE1 IBA1 DAPI
NRP2 F4/80 DAPI
C
C'
D
D'
E
E'
Nestin Mafb-GFP
DAPI
E''
Nestin PECAM1
DAPI
TUBB3 Mafb-GFP DAPI
S100b Mafb-GFP DAPI
Nestin Mafb-GFP
DAPI

**Figure S2**


A
B
C
bv
*
Collagen IV MHCII
DAPI
ACTA2 MHCII
TUBB3 DAPI
Collagen IV MHCII
ZBTB16 DAPI
A'
B'
C'
C57BL/6J
Cx3cr1 KO
D
D'
E
E'
F
F'
G
G'
H
I
st
int
MHCII AIF1 DAPI
CSF1R CD68 DAPI
DDX4 TUBB3
DAPI

# Figure S3


A
B
C
D
E
A'
B'
D'
E'
$A_s$
$A_{pr}$
$A_{al}$
d
ZBTB16/ZBTB16
DAPI
ZBTB16
CDH1/CDH1
DAPI
KIT
CDH1
DAPI/DAPI

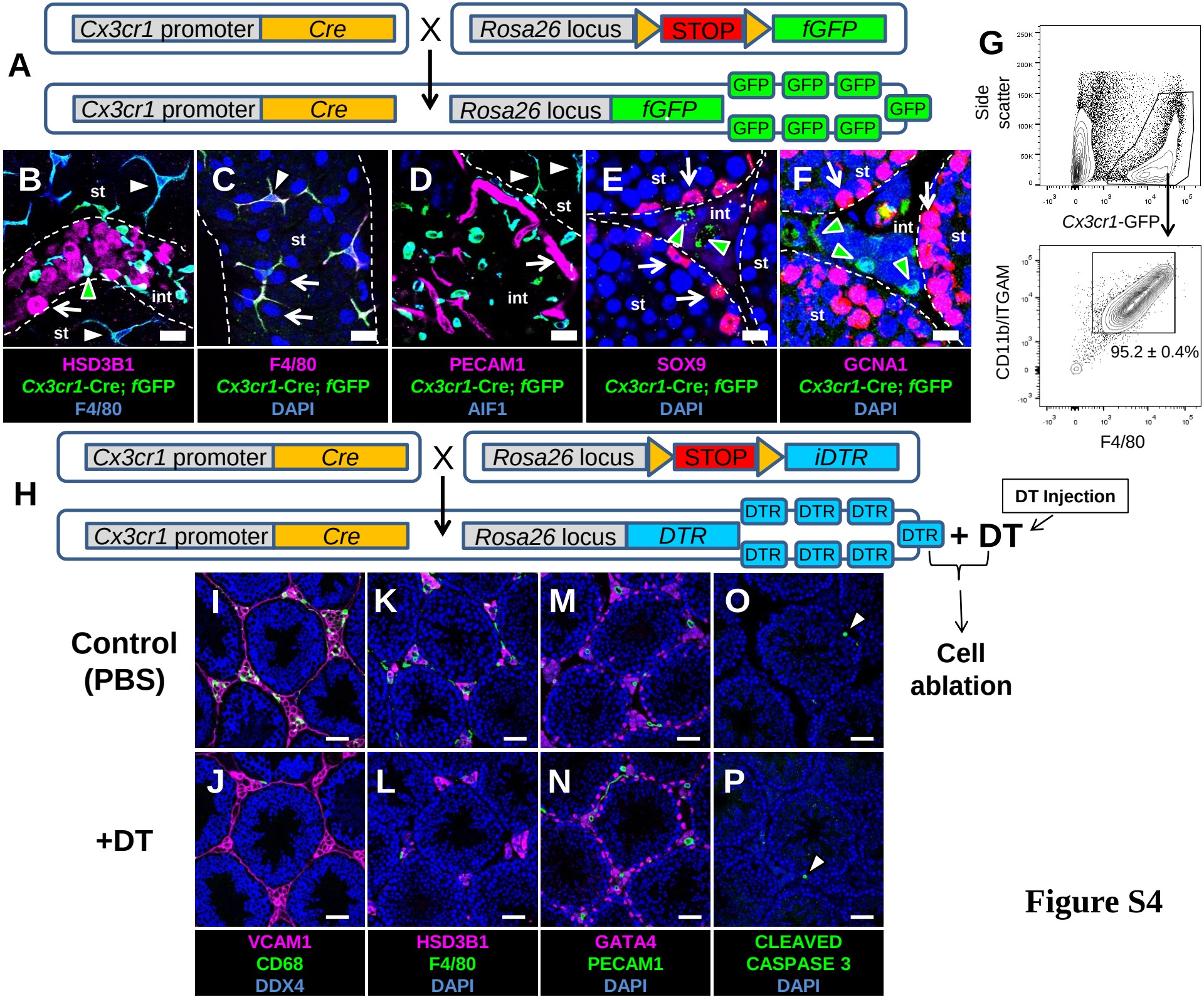

A
Cx3cr1 promoter
Cre
X
Rosa26 locus
STOP
fGFP
GFP
B
C
D
E
F
st
int
HSD3B1
Cx3cr1-Cre; fGFP
F4/80
DAPI
F4/80
Cx3cr1-Cre; fGFP
DAPI
PECAM1
Cx3cr1-Cre; fGFP
AIF1
SOX9
Cx3cr1-Cre; fGFP
DAPI
GCNA1
Cx3cr1-Cre; fGFP
DAPI
G
Side scatter
Cx3cr1-GFP
CD11b/ITGAM
95.2 ± 0.4%
F4/80
H
Cx3cr1 promoter
Cre
X
Rosa26 locus
STOP
iDTR
DTR
DT Injection
+ DT
Cell ablation
Control (PBS)
+DT
I
K
M
O
J
L
N
P
VCAM1
CD68
DDX4
HSD3B1
F4/80
DAPI
GATA4
PECAM1
DAPI
CLEAVED CASPASE 3
DAPI

Figure S4

**Figure S5**

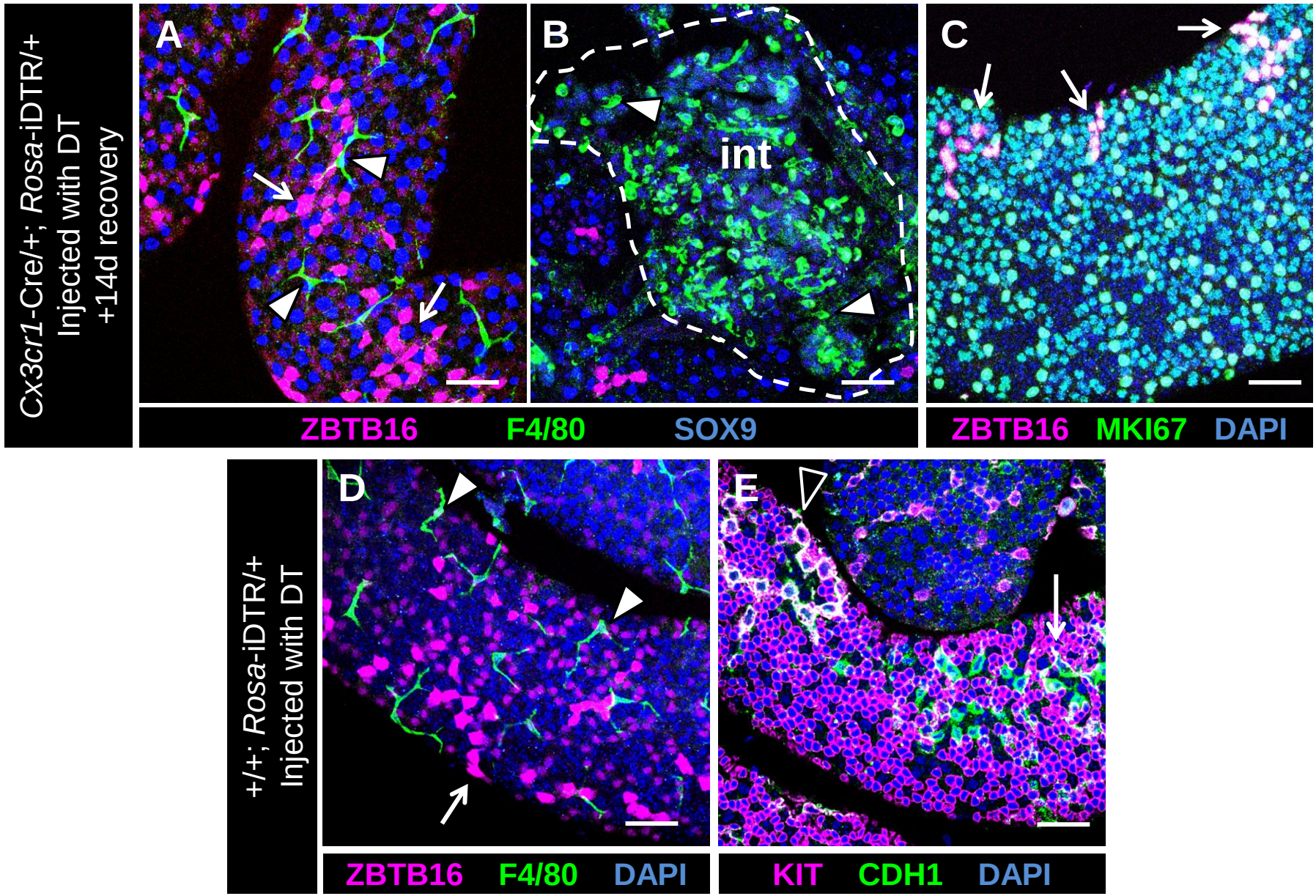

Cx3cr1-Cre/+; Rosa-iDTR/+
Injected with DT
+14d recovery
A
B
int
C
ZBTB16 F4/80 SOX9
ZBTB16 MKI67 DAPI
+/+; Rosa-iDTR/+
Injected with DT
D
E
ZBTB16 F4/80 DAPI
KIT CDH1 DAPI

**Figure S6**

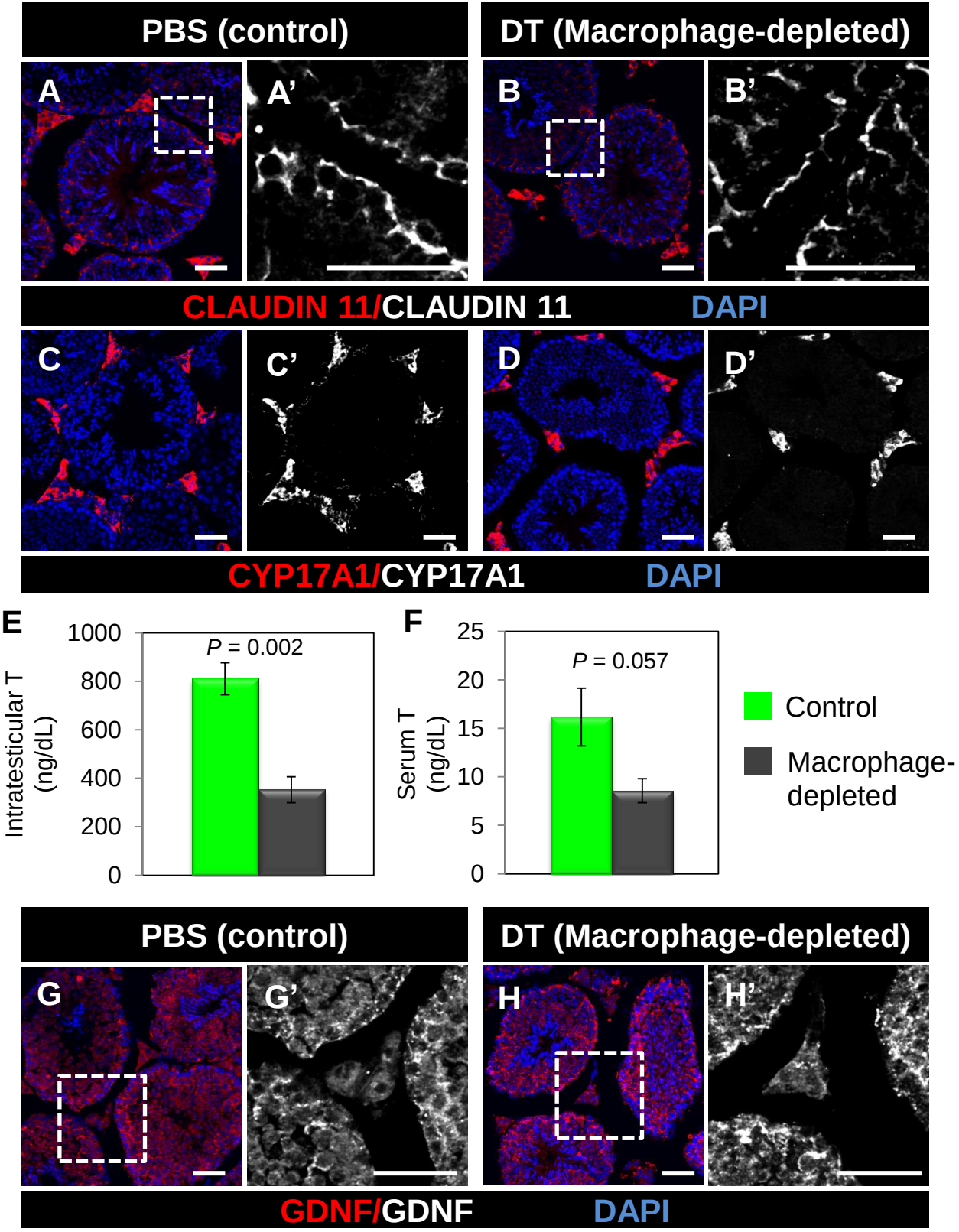
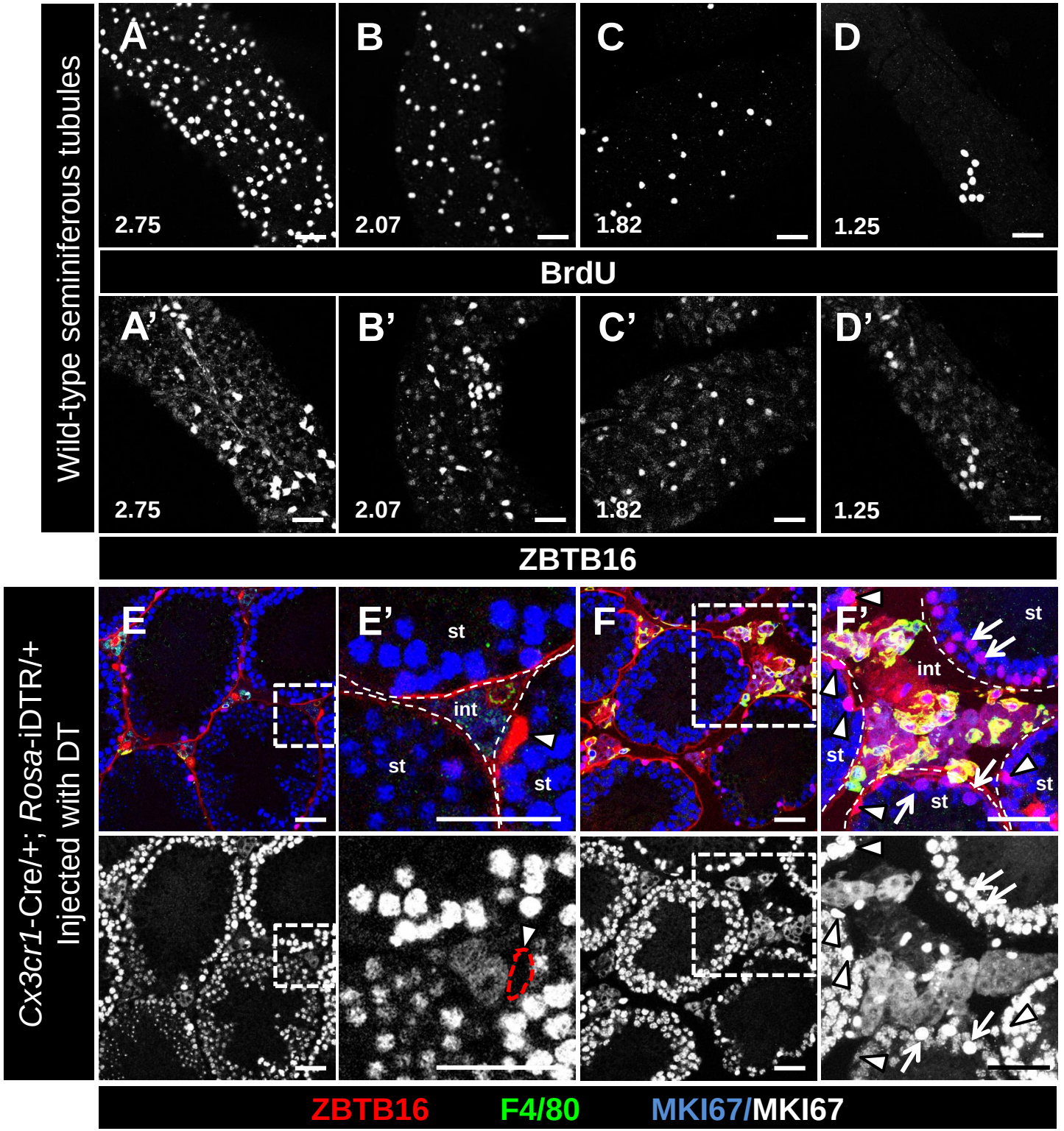

PBS (control)
DT (Macrophage-depleted)
A
A'
B
B'
CLAUDIN 11/CLAUDIN 11
DAPI
C
C'
D
D'
CYP17A1/CYP17A1
DAPI
E
Intratesticular T (ng/dL)
1000
800
600
400
200
0
P = 0.002
F
Serum T (ng/dL)
25
20
15
10
5
0
P = 0.057
Control
Macrophage-depleted
PBS (control)
DT (Macrophage-depleted)
G
G'
H
H'
GDNF/GDNF
DAPI

**Figure S7**


Wild-type seminiferous tubules
A
B
C
D
2.75
2.07
1.82
1.25
BrdU
A'
B'
C'
D'
2.75
2.07
1.82
1.25
ZBTB16
Cx3cr1-Cre/+; Rosa-iDTR/+
Injected with DT
E
E'
F
F'
st
int
st
st
st
int
st
st
st
ZBTB16
F4/80
MKI67/MKI67

**Supplemental Figure Legends**

**Figure S1. Testis macrophages do not associate with lymphatic vessels and do not express neural/CNS-associated markers, Related to Figure 1.** Immunofluorescent staining of 3-month-old cryosectioned adult testes. (A, B) Lymphatic vessels (arrowheads, marked by LYVE-1 or NRP2) are only detected in the tunica layer ("t") of the testis; macrophages are found throughout the tunica and are often associated with lymphatic vessels and blood vessels (arrow in B). A' and B' are higher-magnification images of the tunica layer in A and B, respectively. Note that AIF1 is also expressed in elongating spermatids in the center of a subset of tubules in A. C-E are low-magnification images, while C'-E' are higher-magnification images of superficial cryosections of the tubule surface that contains *Mafb*-GFP-expressing peritubular macrophages. *Mafb*-GFP is expressed in the adult testis in Sertoli cells, Leydig cells, and macrophages. While none of the neural markers TUBB3 (C, C'), S100b (D, D'), or Nestin (E, E') are expressed in peritubular macrophages, they are expressed in other testis cell types, such as Sertoli cells (TUBB3), round spermatids and a subset of PMCs (S100b), or elongated spermatids and endothelial cell (PECAM1)-proximal perivascular cells (Nestin, E"). Scale bar, 50 μm.

**Figure S2. Peritubular macrophages are located in the peritubular myoid layer atop the basement membrane overlying spermatogonia, and do not require *Cx3cr1* function for their presence in the testis, Related to Figure 1.** Immunofluorescent images of whole-mount 3 month-old adult seminiferous tubules (A-C) and cryosectioned testes (D-I). (A-C) Three-dimensional reconstruction of tubules was performed, and orthogonal views are shown on the right and bottom sides of each panel. A'-C' are higher-magnification images of orthogonal views focusing on regions containing macrophages. Dashed lines indicate planes of section for orthogonal cross-sections. (A) An MHCII-positive peritubular macrophage is contained within or

just above the Collagen-IV-containing peritubular basement membrane. (B) A peritubular macrophage is embedded between ACTA2-positive peritubular myoid cells, overlying Sertoli cells (Sertoli cells are labeled with TUBB3). (C) A macrophage arm is associated with a blood vessel (bright Collagen-IV-staining structure is blood vessel basement membrane, labeled as "bv"), directly above a ZBTB16-bright undifferentiated spermatogonium (marked by asterisk). (D-I) Control C57BL/6J (D, F, H) and *Cx3cr1*-GFP homozygous mutant ("*Cx3cr1* KO"; E, G, I) adult testes both contain similar populations of peritubular (MHCII-bright/CSF1R-dim, arrows in D'-G') and interstitial (MHCII-dim/CSF1R-bright, arrowheads in D'-G') macrophages, and exhibit normal testicular Sertoli cell (TUBB3-positive, H and I) and spermatocyte/spermatid (DDX4-positive, H and I) differentiation. D'-G' are higher-magnification images of the boxed regions in D-G. In D'-G', dashed lines denote boundaries between tubular and interstitial compartments; "st" refers to seminiferous tubule regions and "int" refers to interstitial regions. Scale bar, 10 μm in A-C and 50 μm in D-I.

**Figure S3. Distinct spermatogonial populations are labeled by KIT and CDH1, Related to Figure 2.** Immunofluorescent images of 3 month-old whole-mount adult seminiferous tubules. (A) Anti-ZBTB16 antibody stained brightly in $A_{single}$ ($A_s$), $A_{paired}$ ($A_{pr}$), or $A_{aligned\text{-}8}$ ($A_{al}$) cells and was dim (d) in longer chains of cells. (B) ZBTB16 co-stained with CDH1 in ZBTB16-positive cells. While KIT was not observed in $A_{single}$ cells (C, arrow) or shorter chains of CDH1-positive cells (D), KIT was found in other, longer chains of CDH1-expressing cells (E). CDH1-positive-only cells (D, green arrows in D') and CDH1/KIT double-positive cells (E, yellow arrows in E') had lightly staining nuclei indicative of an undifferentiated or less differentiated status. KIT-positive, CDH1-negative cells showed a more densely stained nucleus indicative of a more differentiated state (D and E, pink arrows in D' and E'). A', B', and D'- E' are ZBTB16-only,

CDH1-only, and DAPI-only channels of A, B, and D-E, respectively. D and E are higher-magnification images of the boxed regions in the left of C and center of C, respectively. White staining in multi-color images is indicative of co-localization of magenta and green stains (i.e., KIT/CDH1 double-positive cells). Scale bar, 50 μm.

**Figure S4. *Cx3cr1-Cre*-targeted iDTR ablation is specific to macrophages and shows no gross effects on other testis-specific somatic cell types or structures, Related to Figure 3.** Immunofluorescent images of 3-month-old adult testes. (A) Diagram depicting strategy used for *Cx3cr1*-Cre lineage tracing using a farnesylated-GFP reporter (*Rosa26R-CAG-fGFP*). Orange triangles represent loxP sites which flank a STOP cassette; presence of Cre will excise the STOP cassette and allow GFP expression. GFP driven by *Cx3cr1*-Cre was observed in F4/80- and AIF1-positive interstitial (B, E, F, green arrowheads) and peritubular macrophages (B-D, white arrowheads), but not in HSD3B1-positive Leydig cells (B, arrow), peritubular myoid cells (determined by DAPI staining for large round nuclei on the surface of tubules; C, arrows), vasculature (PECAM1-positive cells; D, arrow), Sertoli cells (SOX9-positive cells; E, arrows), or germ cells (GCNA1-positive cells, F, arrows). Yellow staining in interstitial region of F is due to a blood vessel with autofluorescence in the GCNA1 channel. (G) Representative flow cytometric analysis of a *Cx3cr1*-GFP adult testis. Number in lower panel indicates the average percentage (± standard deviation) of macrophages (gated below as F4/80 and CD11b/ITGAM double-positive) among GFP-positive cells (top gating); average is from two separate experiments on pooled testes from two adult males. (H) Diagram depicting strategy used for DT-mediated adult macrophage ablation. Orange triangles represent loxP sites; in the presence of Cre, the STOP cassette is removed and *DTR* is expressed. Upon administration of DT, *Cx3cr1*-Cre-expressing cells (i.e., macrophages) will be susceptible to DT and will be ablated. (I, K, M, O)

Control PBS-injected *Cx3cr1*-Cre/+;*Rosa*-iDTR/+ testes. (J, L, N, P) DT-injected *Cx3cr1*-Cre/+;*Rosa*-DTR/+ macrophage-ablated testes. VCAM1 labels interstitial and vascular associated cells (I, J); DDX4 labels differentiated spermatocytes and spermatids (I, J); HSD3B1 labels Leydig cells (K, L); GATA4 labels Sertoli cells (M, N); and PECAM1 labels vascular endothelial cells (M, N). Anti-cleaved Caspase 3 staining revealed no significant increase in apoptotic cells (O, P, arrowheads) after transient macrophage depletion. Note that there are many fewer macrophages (labeled with CD68 in I-J or F4/80 in K-L) in DT-treated samples in J and L. Thick scale bar, 12.5 μm; thin scale bar, 50 μm. Panels B-D are whole-mount samples; all other samples are cryosections.

**Figure S5. Full recovery of macrophages and spermatogenesis occur in the testis 14 days after final DT injection, and DT administration alone does not affect spermatogenesis, Related to Figure 3.** Immunofluorescent images of whole-mount 3-month-old adult seminiferous tubules and interstitium. (A-C) After 14 days of recovery following final DT injection, *Cx3cr1*-Cre/+;*Rosa*-iDTR/+ males exhibited a full recovery of both peritubular (A, arrowheads) and interstitial (B, arrowheads) macrophages, in addition to recovery of long $A_{aligned}$ ZBTB16-bright spermatogonial chains (A, arrows) which were MKI67-positive (C, arrows). Dashed line in B indicates region of interstitium (int). (D, E) Cre-negative;*Rosa*-iDTR/+ males 72 hours after final injection of DT showed normal numbers of peritubular macrophages (D, arrowheads), ZBTB16-bright $A_{aligned}$ spermatogonia (D, arrow), CDH1-positive $A_{aligned}$ spermatogonia (E, arrow), and KIT/CDH1 double-positive A1-A3 spermatogonia (E, black arrowhead). White staining in E is indicative of co-localization of magenta and green stains (i.e., KIT/CDH1 double-positive cells). The DT treatment regimen is outlined in Figure 3A, and strategy used for macrophage ablation is depicted in Figure S4H. Scale bar, 50 μm.

**Figure S6. Effects of macrophage depletion on testosterone levels or functions and Sertoli cell GDNF expression, Related to Figure 3.** Immunofluorescent images of cryosectioned 3-month-old adult testes (A-D and G-H). A', B', G' and H' are higher-magnification images of the boxed regions in A, B, G, and H, respectively. Black-and-white images are CLAUDIN 11-only (A' and B'), CYP17A1-only (C' and D'), and GDNF-only (G' and H') channels. *Cx3cr1*-Cre/+;*Rosa*-iDTR/+ males injected with PBS (A, C, G) and with DT to deplete macrophages (B, D, H) possessed an intact blood-testis barrier (marked by CLAUDIN 11 in A and B), indicative of proper testosterone function, and showed similar expression of CYP17A1 (C, D) and the Sertoli-derived SSC maintenance factor GDNF (G, H). Graphs in E and F show intratesticular (E) and serum (F) testosterone levels in 3-month old *Cx3cr1*-Cre/+;*Rosa*-DTR/+ males injected with PBS (green, n=4) or with DT to deplete macrophages (dark gray, n=4). Samples for T measurements were taken 72 hours after final injection of DT or PBS; all images were from samples harvested 72 hours after final injection of DT or PBS. Data are represented as means ± SEM. *P* values for each comparison are noted above each graph. Scale bar, 50 μm.

**Figure S7. There is a positive correlation between presence of testicular macrophages and actively cycling spermatogonia, Related to Figure 4.** Immunofluorescent images of wild-type (C57BL/6J) whole-mount seminiferous tubules (A-D) and cryosectioned testes from 3-month-old adult *Cx3cr1*-Cre/+;*Rosa*-iDTR/+ mice injected with DT (E-F). A-D are the BrdU-only channels for the images in Figure 4A-D, and A'-D' are the ZBTB16-only channels for the images in Figure 4A-D. Numbers indicate macrophages per 10,000 $\mu m^2$ of seminiferous tubule surface area within frame of image (see Figure 4A-D). E' and F' are higher-magnification images of the boxed regions in E and F, respectively. Black-and-white panels below E and F are MKI67-only channels. DT-treated tubules with macrophage-depleted areas (E, E') had more

MKI67-negative ZBTB16-positive cells (E', arrowhead and red outline) with few MKI67-positive, ZBTB16-expressing spermatogonia, while DT-treated tubules with many remaining macrophages (F, F') were associated with areas of MKI67-positive ZBTB16-bright (F', arrowheads) and MKI67-positive ZBTB16-dim spermatogonia (F', arrows). Scale bar, 50 μm.

**Table S1. Primary antibodies used for immunofluorescence and flow cytometry, Related to Figure 1.** List of primary antibodies used for immunofluorescent stainings and flow cytometry in this study. Dilutions used and source of antibody are listed. Reagents used for flow cytometry are labeled with "(FC)" after the antibody name.

| Primary Antibody | Dilution | Company/Source |
|---|---|---|
| Rat anti-F4/80 | 1:2,000 | AbD Serotec #MCA497RT |
| Rabbit anti-GFP | 1:1,000 | Molecular Probes #A11122 |
| Chicken anti-GFP | 1:1,000 | Aves #GFP-1020 |
| Rabbit anti-HSD3B1 | 1:2,000 | K. Morohashi |
| Rabbit anti-HSD3B1 | 1:500 | TransGenic Inc. #KO607 |
| Rat anti-PECAM1 | 1:250 | BD Biosciences #553370 |
| Rabbit anti-AIF1 | 1:1,000 | Wako #019-19741 |
| Rat anti-ITGAM | 1:250 | BD Biosciences #557395 |
| Rat anti-CD68 | 1:1,000 | AbD Serotec #MCA1957T |
| Rat anti-MHCII | 1:1,000 | eBioscience #14-5321-81 |
| Rabbit anti-CSF1R | 1:1,000 | Santa Cruz #sc-692 |
| Mouse anti-ZBTB16 | 1:250 | Millipore #OP128-100UG |
| Rabbit anti-SOX9 | 1:4,000 | Millipore #AB5535 |
| Rat anti-CDH1 | 1:500 | Life Technologies #13-1900 |
| Goat anti-VCAM1 | 1:2,000 | R&D #AF643 |
| Rabbit anti-DDX4 | 1:1,000 | Abcam #ab13840 |
| Rat anti-BrdU | 1:200 | Accurate Chemical #OBT0030G |
| Rabbit anti-MKI67 | 1:500 | Thermo Scientific #RM-9106-S |
| Rabbit anti-MKI67 | 1:500 | Thermo Scientific #RB-1510-P1 |
| Rabbit anti-CDKN1B | 1:500 | Santa Cruz #sc-528 |
| Goat anti-CSF1 | 1:200 | Santa Cruz #sc-1324 |
| Rabbit anti-ACTA2 | 1:500 | Abcam #ab5694 |

| | | |
|---|---|---|
| Rabbit anti-ALDH1A2 | 1:200 | Sigma #HPA010022 |
| Goat anti-CYP17A1 | 1:500 | Santa Cruz #sc-46081 |
| Rabbit anti-RDH10 | 1:400 | ProteinTech #14644-1-AP |
| Mouse anti-TUBB3 | 1:1,000 | Covance #MMS-435P |
| Rabbit anti-S100b | 1:500 | Abcam #ab41548 |
| Rabbit anti-Nestin | 1:1,000 | Covance #PRB-314C |
| Rabbit anti-Collagen IV | 1:2,000 | AbD Serotec #2150-1470 |
| Goat anti-KIT | 1:400 | R&D #AF1356 |
| Mouse anti-GCNA1 | 1:10 | G. Enders |
| Goat anti-GATA4 | 1:100 | Santa Cruz #sc-1237 |
| Rabbit anti-Cleaved Caspase 3 (Asp175) | 1:250 | Cell Signaling #9661S |
| Rabbit anti-Claudin 11 | 1:1,000 | Life Technologies #36-4500 |
| Rabbit anti-GDNF | 1:800 | Santa Cruz #sc-328 |
| Rat anti-LYVE1 | 1:200 | eBioscience #14-0443-80 |
| Goat anti-NRP2 | 1:400 | R&D #AF567 |
| Anti-Mouse CD11b PE-Cyanine7 (FC) | 1:100 | eBioscience #25-0112-82 |
| Anti-mouse I-A/I-E (MHCII) PerCP (FC) | 1:100 | BioLegend #107624 |
| Anti-mouse F4/80 APC/Cy7 (FC) | 1:100 | BioLegend #123118 |
| Anti-Mouse CD115 (CSF1R) PE (FC) | 1:100 | BD Biosciences #565249 |
| Anti-Mouse CD45 eFluor® 650NC (FC) | 1:100 | eBioscience #95-0451-42 |

**Movie S1. 3D reconstruction of seminiferous tubule with macrophages, peritubular myoid cells, and Sertoli cells labeled, Related to Figure 1.** Movie shows 3D reconstruction of adult seminiferous tubule surface cell layers, with the following cell types labeled: peritubular macrophages marked in yellow with MHCII; peritubular myoid cells marked in magenta with ACTA2; and Sertoli cells marked in cyan with TUBB3. Nuclei are stained in blue (with Hoechst 33342 dye). The movie shows that macrophage cellular processes lie within the plane of the ACTA2-positive cells, above the Sertoli cell layer.

**Movie S2. 3D reconstruction of seminiferous tubule with macrophages, undifferentiated spermatogonia, and blood vessels labeled, Related to Figure 1.** Movie shows 3D reconstruction of adult seminiferous tubule surface cell layers, with the following cell types labeled: peritubular macrophages marked in green with MHCII; undifferentiated spermatogonia marked in purple with ZBTB16; and blood vessel basement membrane marked in red with Collagen IV. Nuclei are stained in blue (with Hoechst 33342 dye). The movie shows that macrophage cellular processes lie adjacent to and beneath the blood vessel, immediately overlying an undifferentiated spermatogonium.