Supplemental Methods

Cloning and Viral Production. The MND promoter[1] and the WS1.6 promoter[2,3] have been previously described. The promoters and human WASp cDNA were cloned into the pRRL lentiviral vector[4] (obtained from Addgene, Cambridge MA) using standard molecular biology techniques. PCR primers are available upon request. Viral constructs have been deposited in Addgene. Lentiviral vectors were produced as previously described[5]. Briefly, the day before transfection, 293T cells were plated into 10cm TC plates at $4x10^6$ cells/plate. The next day, the cells were transfected with a VSV-G envelope plasmid, packaging plasmid and the pRRL transfer plasmid (1:2:4 ratio) using Polyethylenimine (PEI, Polysciences, Warrington PA, Cat # 23966-2). The supernatant was collected 48hr post-transfection, clarified through a 0.22um filter and concentrated via overnight centrifugation (8000g, 12-16hrs). The supernatant was discarded and the pellet resuspended in 200μl 1x PBS.

Several methods were used to determine viral titers. The GFP-containing vectors were titrated using the Nalm6 pre- B cell as previously described[5]. Cells were transduced with various dilutions of virus in the presence of polybrene (Sigma, 8μg/ml) and transduction efficiency (%GFP^+) was analyzed at 5 days post-transduction using flow cytometry. WASp expressing LVs were titrated using real-time PCR, with titration, viral integration and LAM-PCR analysis performed as previously described[5].

Stem cell isolation and transduction. Isolation, transduction and transplantation of human $CD34^+$ cells was performed as previously described[6]. For murine stem cell transplantation, we used a split-dose conditioning dose to deliver a total of 9Gy irradiation over a 24hr period unless noted otherwise. The first dose (4.5Gy) was given 12-24hr prior to transplant and 2^{nd} dose (4.5Gy) was given immediately prior to transplantation.

Murine stem cell isolation and transduction was performed essentially as described[5]. Crude BM suspension was isolated from the femurs, tibias, hips and spines of donor animals following crushing with mortar and pestle in isolation buffer (PBS + 5% FCS + 100U/ml Penicillin/Streptomycin). The cell suspension was filtered through a 40μm filter (BD Biosciences), washed with isolation buffer and resuspended in 10ml isolation buffer. Crude BM cell suspensions were underplayed with equal volume of room temperature Optiprep density media (*p*=1.09g/ml, 28% in HBSS; Sigma-Aldrich, St. Louis MO) and centrifuged for 20 min at 400g with low acceleration and no brake. The interface was collected, washed with isolation buffer and used for lineage depletion according to manufacturer's instructions (Stem Cell Technologies, Vancouver BC).

Lin^{neg} cells were cultured overnight in LV-containing StemSpan SFEM media (Stem Cell Technologies) supplemented with Penicillin/Streptomycin (100U/ml), 2mM L-Alanyl-L-Glutamine, mSCF (50ng/ml) and mTPO(20ng/ml, all cytokines from R&D Systems, Minneapolis MN). Polybrene was added at 4μg/ml and total transduction time varied from 9-12 hours. Multiplicity of infection (MOI) of 10 was used for *in vivo* competition experiments, MOI = 1-5 in MND alone transplants and MOI = 20 for MND vs. WS1.6 comparison studies. Transduced cells were washed 2-3 times in 1x PBS, counted and resuspended in 1x PBS for injection. A small number of cells were cultured for additional 4-5 days in cytokine-supplemented StemSpan SFEM media without polybrene for *in vitro* analysis of viral transduction. Following transduction, $1x10^6$ lin^{neg} cells in 300μl PBS were retro-orbitally injected into each recipient. The animals were maintained on antibiotics for 2wk following transplantation and then transferred to standard caging conditions.

Platelet isolation and counting. Platelet isolation and counting was performed as previously described[7]. Peripheral blood from retro-orbital or saphenous bleeds was collected using heparinized tubes into White's buffer (2.94% sodium citrate, 2.46% Glucose, pH 7.4) supplemented with 500μM Indomethacin (Sigma). Blood was spun at 300g for 5 min, the platelet-rich supernatant (PRP) was collected and the

cells were washed with staining buffer (PBS + 0.5% BSA). The cells were spun at 300g for another 5 min and the supernatant was combined with the PRP. The blood pellet was resuspended in 500μl staining buffer for FACS staining while the PRP was centrifuged at 3000g for 5 min and resuspended in 100μl of staining buffer.

For quantitative platelet counting, 5μl of whole blood was collected in 45μl of sodium citrate buffer (0.11M Sodium Citrate, pH 6.5). Ten microliters of diluted blood was mixed with 10μl of FACS antibodies for staining. The αCD41 and αCD45 antibodies were used to detect platelets and leukocytes respectively. Cells were incubated for 15min and combined with 965μl staining buffer and 15μl of Rainbow beads (Spherotech, Lake Forest, IL). Samples were analyzed with a FACS Calibur analyzer (BD Biosciences, San Jose, CA) and total platelet numbers were extrapolated based on the ratio between the number of beads per sample and the number of $CD41^+$ platelet and $CD45^+$ leukocytes.

Flow Cytometry. Studies were performed on single cell suspensions using either the FACSCalibur or LSR II instruments (BD Biosciences) and FACS data analyzed with FlowJo software (TreeStar, Ashland OR). WASp expression was detected was performed as previously described[8]. Briefly, cells were stained for extracellular antigens for 15 min in staining buffer, washed and fixed with 2% paraformaldehyde (PFA) for 12 min at room temperature. The cells were washed with 1x PermWash buffer (BD Biosciences) and stained with αWASp antibody (1:1000) for 20min at 4°C. Following WASp staining, cells were washed with 1x PermWash and incubated with 2^{nd} anti-rabbit Alexa568 antibody (1:2000, Invitrogen, Carlsbad CA) for 20 min at 4°C.

The following antibodies were used for staining: CD45.1-PeCy7 (clone A20), CD45.2-APC (104), CD11b-FITC (M1/70), CD3-Apc/PeCy7/ApcCy7 (145-2C11), GR1-APC (RB6-8C5), IL2-PE (JES6-5H4), FoxP3-Fitc (FJK-16s), CD25-PE (PC61.5), CD62L-APC (MEL-14), B220-Fitc/PeCy7/PeCy5.5 (RA3-6B2), CD11c (N418) and Streptavidin-PeCy7 from Ebioscience (San Diego, CA); CD24-Biotin/APC (M1/69), CD4-Fitc/PerCP/Pe/PeCy5 (RM4-5), CD21-PE/APC (7G6), FAS-PE (Jo2), IgG1-Fitc(A85-1), IgG2b-Biotin (R12-3) from BD Biosciences; CD8-ApcCy5.5 (53-6.7), CD44-Biotin/ApcCy7 (IM7) and NK1.1-FITC or PE (PK136) from Biolegend (San Diego, CA); CD23-APC (B3B4) from Invitrogen; κLC-Fitc (187.1), λLC-PE (JC5-1), IgG3-Cy5 (Cat. #1100-15) from Southern Biotech (Birmingham, AL) and PNA-FITC from Vector Labs (Burlingame, CA).

Proliferation Assays. For T cell stimulation, plates were coated overnight at 4°C or for 2hr at 37°C with αCD3 (1μg/ml, UCSF Monoclonal Antibody core, San Francisco, CA) in 1x PBS. The plates were washed twice and the cells added in complete media (RPMI). Some cells were stimulated with PMA (2ng/ml)/Ionomycin (500ng/ml) (Sigma). Cells were incubated for 60hr with ^3H thymidine added during the last 12hr.

Detection of interleukin-2 (IL2) production. Culture plates were coated overnight at 4°C or for 2hr at 37°C with αCD3 (16μg/ml, UCSF Monoclonal Antibody core, San Francisco, CA) in 1x PBS. PMA (10ng/ml) and Ionomycin (500ng/ml) stimulation was used as a positive control. Cells were stimulated for 6hr, with Brefeldin-A (1μg/ml, Sigma) added during the last 5hr to block cytokine secretion. Intracellular IL2 staining was performed as described above for intracellular WASp detection.

Anti-DNA ELISA. To detect anti-dsDNA antibodies, NUNC ELISA Plates (Thermo, Rochester NY) were coated with poly-L-lysine (Sigma) followed by calf thymus dsDNA (50μl, 100μg/ml in TE buffer, Sigma). The plates were coated for 2hrs at 4°C and blocked with 1x PBS/0.5 % BSA for 1 hour. Serum was added in triplicate wells, at a 1:200 dilution in ELISA buffer (1x PBS, 0.5% BSA, 0.05% Tween 20). The plates were incubated for 24hrs at 4°C and washed 6x with wash buffer (1x PBS/0.05% Tween 20). Following

the wash, HRP-conjugated secondary antibodies were added at a 1:2000 dilution in 50μl/well secondary buffer (1x PBS, 0.5% BSA, 0.05 Tween20, 1% Goat Serum). The plates were incubated for 1hr at room temperature and washed 6x with wash buffer. Wells were developed with 80μl OptEIA HRP substrate Kit (BD Biosciences). The reaction was stopped by addition of 40μl 2N H_2SO_4 and the 450nm absorbance was determined with SpectraMax190 reader and SoftMax software (Molecular Devices, Sunnyvale, CA).

qPCR Analysis. Real-time PCR analysis of *Atf7ip* expression was performed as previously described[9]. Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia CA). The RNA was reverse transcribed using SuperScript II RT kit (Invitrogen) according to manufacturer's instruction. The mRNA was amplified using SYBR green real-time PCR kit (Bio-Rad labs, Hercules CA) and normalized to beta-2-microglobulin[10]. Primer sequences are available upon request.

B cell isolation. Splenic B220+ B cells were isolated using B220 conjugated-magnetic beads and an AutoMACS instrument from Miltenyi Biotec (Auburn CA). Briefly, 3-4x10^7 total splenocytes were labeled with B220-conjugated MACS beads according to manufacturer's instructions, the cells were washed and B220+ cells isolated using automatic positive selection setting on AutoMACS instrument. The resultant population was at least 90% $B220^+$.

In vitro isotype class switching. In vitro class switching was analyzed as previously described[11]. Briefly, isolated B220+ B cells were cultured in complete media at 0.5x10^6 cells/ml in 96-well U-bottom plates in conjunction with either LPS (10μg/ml) or αCD40 (10μg/ml) + IL-4 (100ng/ml). After 5 days, the cells were washed and stained with AlexaFluor 350 Succinimidyl Ester (Invitrogen, cat # A10168) to label dead cells[12]. After an additional wash, the cells were fixed and permeabilized using the BD cytofix/cytoperm kit and stained with isotype-specific antibodies for flow cytometry analysis.

Analysis of MZ turnover with Bromodeoxyuridine (BrdU) uptake. Analysis of MZ B cell turnover was performed as previously described[13]. BrdU was administered via drinking water provided ad libitum (1mg/ml brdu in 5% sucrose sugar water) and the mice were sacrificed 3 days after BrdU initiation. Total splenocytes were stained for B cell differentiation markers (B220, CD21, CD23, CD24) and BrdU incorporation was determined using FITC BrdU Flow Kit (BD Biosciences), based on manufacturer's protocol.

Quantification of antibody-secreting cells with ELISpot. Detection of antibody-secreting cells by ELISpot has been described[9]. Filter plates (MultiScreen HTS, Millipore, Billerica, MA) were coated overnight with anti-IgM or anti-IgG according to manufacturer's instructions. The following antibodies were used (all at 5μg/ml): IgM, cat. # 1020-01 and anti-IgG, cat #, 1030-01 (Southern Biotech). Plates were washed with PBS and blocked with complete media for 1-2 hours at 37° C. Isolated splenic B cells were added at defined dilutions (4x10^5; 2x10^5, 1x10^5 cells/ml) and cultured at 37° C for ~16 hours. Plates were washed six times with PBS and incubated with horseradish peroxidase-conjugated anti-IgM (1020-05) or anti-IgG (1030-05; 1:2,000 dilution; Southern Biotech). Plates were developed with the AEC developer kit (3-amino-9-ethyl carbazole; Vector Labs) and spots were counted with an ImmunoSpot counter and software (Cellular Technology).

Histological analysis of kidney pathology. Formalin fixed, paraffin embedded tissue was sectioned and stained with periodic acid Schiff's (PAS) and silver methenamine. In a semi-quantitative fashion, slides were scored for overall percentages of affected glomeruli on a scale of 0 to 4 (none, less than 25%, 25 to 50%, 50 to 75% and greater than 75% respectively). The scoring system was an ad hoc qualitative assessment of global changes that included membranoproliferative and mesangial abnormalities with

variable features of matrix expansion with or without mesangiolysis and focal duplication of capillary basement membranes. This scoring system used does not communicate what the underlying pathologic processes might be and whether they are in evolution/progression.

Supplemental References

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13. Meyer-Bahlburg A, Becker-Herman S, Humblet-Baron S, et al. Wiskott-aldrich syndrome protein deficiency in b cells results in impaired peripheral homeostasis. *Blood*. 2008;112(10):4158-4169.

Supplemental Figures

Supplemental Figure 1: Lentiviral vector constructs and gating strategy used to identify immunologic subsets

(A) Schematic diagram of the LV vectors used for these studies. All LV vectors were based on the self-inactivated pRRL backbone. (B) Representative analysis of *in vitro* transduction efficiency. Mouse lin^{neg} stem cells were transduced with MND-WASp lentiviral vector and cultured for 5 days in StemSpan media supplemented with SCF and TPO. (C) Flow cytometry gating strategy for hematopoietic subsets. The same antibody panel was used for peripheral blood, BM and spleen. For the *in vivo* competition experiments, $CD45.1^+$ vs. $CD45.2^+$ subsets were identified as shown in (D). For T cell subset analysis, $CD3^+$ T cells were first sub-divided into $CD4^+$ or $CD8^+$ populations and then analyzed using FoxP3, CD25, CD62L and CD44 (E). Splenic $B220^+$ B cells were categorized using the CD24, CD21 and CD23 antibodies (F).

Supplemental Figure 2: The WS1.6 promoter is less active than MND in human leukocytes

Immunodeficient NSG mice were transplanted with human $CD34^+$ cord blood stem cells transduced with MND-GFP or WS1.6-GFP lentiviral vectors. (A) Percent GFP^+ in the splenic $hCD45^+$ compartment in the MND or WS1.6 recipients. (B) Relative GFP expression in engrafted human $CD33^+$ myeloid cells, $CD19^+$ B cells and $CD3^+$ T cells. Fold change was calculated by dividing the MFI of GFP^+ cells by the MFI of GFP^{neg} population. Representative FACS data for each hematopoietic subset is shown in (C). The fold change and percent GFP^+ for each sample is shown in the upper right corner. These data represent at least 7 mice for each vector from 3 unique experiments. Only animals with $>20\%^+$ $hCD45^+$ engraftment in the spleen were used for detailed analysis. * $p<0.05$; ** $p<0.01$, *** $p<0.001$.

Supplemental Figure 3: MND-huWASp LV treatment leads to selection of $WASp^+$ PB T cells and platelets

WASp expression in peripheral blood (PB) subsets was analyzed at various times post-transplant. (A) Representative PB FACS staining at 5 wk post-transplantation. (B) Summary of WASp expression in PB subsets at different time-points. (C) Analysis of $CD41^+$ platelet numbers at 38 wk post-transplant.

Similar numbers were obtained at all time-points. Error bars represent standard deviation from the mean derived from 4 unique experiments. Stars indicate significant differences in WASp expression compared to $CD11b^+$ monocytes during the same time-point. ** $p<0.01$, *** $p<0.001$.

Supplemental Figure 4: MND-huWASp LV treatment results in selection of $WASp^+$ $CD8^+$ T cells and normalized IL2 production on a per cell basis.

(A) Summarized data of WASp expression in $CD8^+$ splenic T cell subsets. (B) MFI of $WASp^+$ cells from different $CD8^+$ splenic T cell subsets. (C) Analysis of IL2 production on a per cell basis. Splenocytes from secondary recipients were stimulated with αCD3 or PMA/Ionomycin and stained with αWASp in conjunction with αIL2 and αCD4. In MND recipients, $CD4^+$ cells were sub-divided into $WASp^+$ or $WASp^-$ cells prior to IL2 quantification. Only the WAS^+ or WAS^- $CD4^+$ cells were analyzed in WT and KO recipients. These findings are indicative of 2 unique experiments, with at least 4 animals per treatment. ** $p<0.01$, *** $p<0.001$.

Supplemental Figure 5: The WS1.6 LV mediates low-level expression in T and B cells and limited selection of $WASp^+$ T cells.

WASp expression was analyzed at 24 wk post-transplant in recipients treated with MND- or WS1.6-huWASp containing lentiviral vectors. The proportion of $WASp^+$ cells within splenic $CD4^+$ (A) and $CD8^+$ (B) T cells is shown. The MFI values were obtained by gating only on $WASp^+$ cells and used to compare relative WASp expression in splenic monocyte vs. $CD4^+$ T cells subsets (C) or B cell subsets (D). WASp expression within splenic B cell compartments is shown in (E). Percent $WASp^+$ cells in each subset is shown in the upper right corner and the MFI of $WASp^+$ cells is shown in the upper left corner. These data represent at least two unique experiments, n= 5 for WTM, 5 for KOM and 8 each for MND and WS1.6. Error bars represent standard deviation. * $p<0.05$; ** $p<0.01$, *** $p<0.001$

Supplemental Figure 6: Normal IL-2 expression in T cells but B cell hyperactivity in WS1.6 recipients

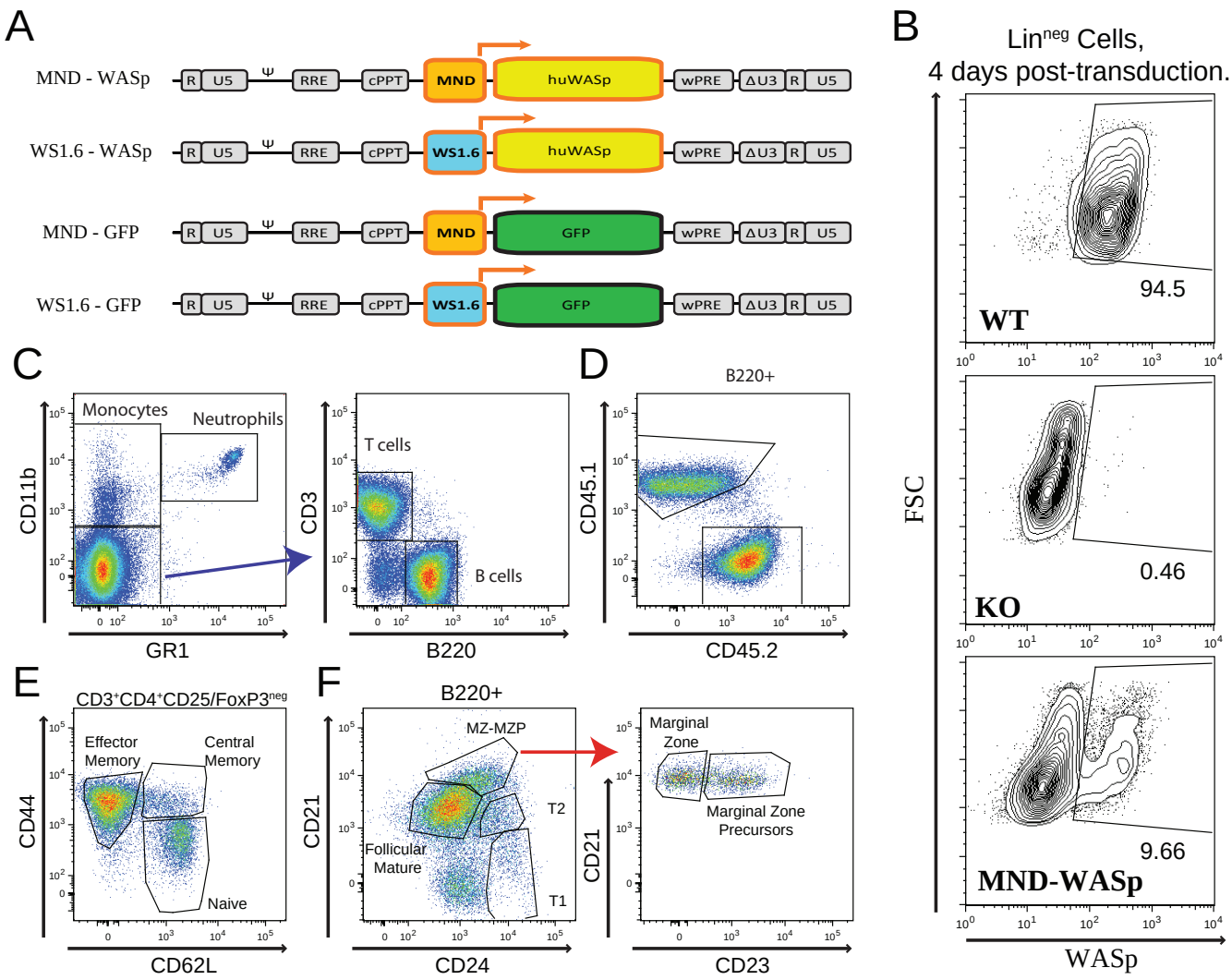
Splenocytes derived from LV treated mice or controls were stimulated with αCD3 and stained for CD4, WASp and IL2 six hr post-stimulation. Sample FACS plots are shown in (A), while pooled data showing IL2 production in $WASp^+$ cells from MND vs. WS1.6-derived $CD4^+$ T cells is shown in (B). Total splenic B

cells were isolated and cultured without stimulation overnight for ELISpot analysis. Number of IgM or IgG antibody-secreting cells (ASC) is shown in (C). For in vitro isotype switching, B cells were stimulated with αCD40 (D) or LPS (E) for 5 days and stained with αIgG1 or αIgG3 antibodies respectively. The numbers shown represent proportion of total live cells. With the exception of (D), these data represent at least two unique experiments, n= 5 for WTM, 5 for KOM and 8 each for MND and WS1.6. For (D), the data represents a single transplant with n= 2 for WTM, 2 for KOM, 4 for MND and 5 for WS1.6. Error bars represent standard deviation, * $p<0.05$; ** $p<0.01$, *** $p<0.001$.

Supplemental Figure 7: Enhanced glomerular injury in WS1.6 treated mice

Kidneys obtained from LV treated or control recipients were formalin fixed and paraffin embedded tissue was sectioned and stained using periodic acid Schiff's (PAS) and methenamine silver stain. (A) Representative images from each recipient group. Arrowheads indicate areas with mesangioproliferative changes and asterisk indicates areas of mesangiolysis. Bar is 50 microns. (B) Global mesangioproliferative and/or mesangial changes were scored by independent observers blinded to the identity of the experimental groups. The average score for each animal group is shown. These data represent five unique experiments, n= 11 for WTM, 11 for KOM, 20 for MND and 21 for WS1.6. Error bars represent standard deviation, * $p<0.05$; ** $p<0.01$, *** $p<0.001$.

Figure S1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WS1.6 - WASp
MND - GFP
WS1.6 - GFP
R
U5
Ψ
RRE
cPPT
MND
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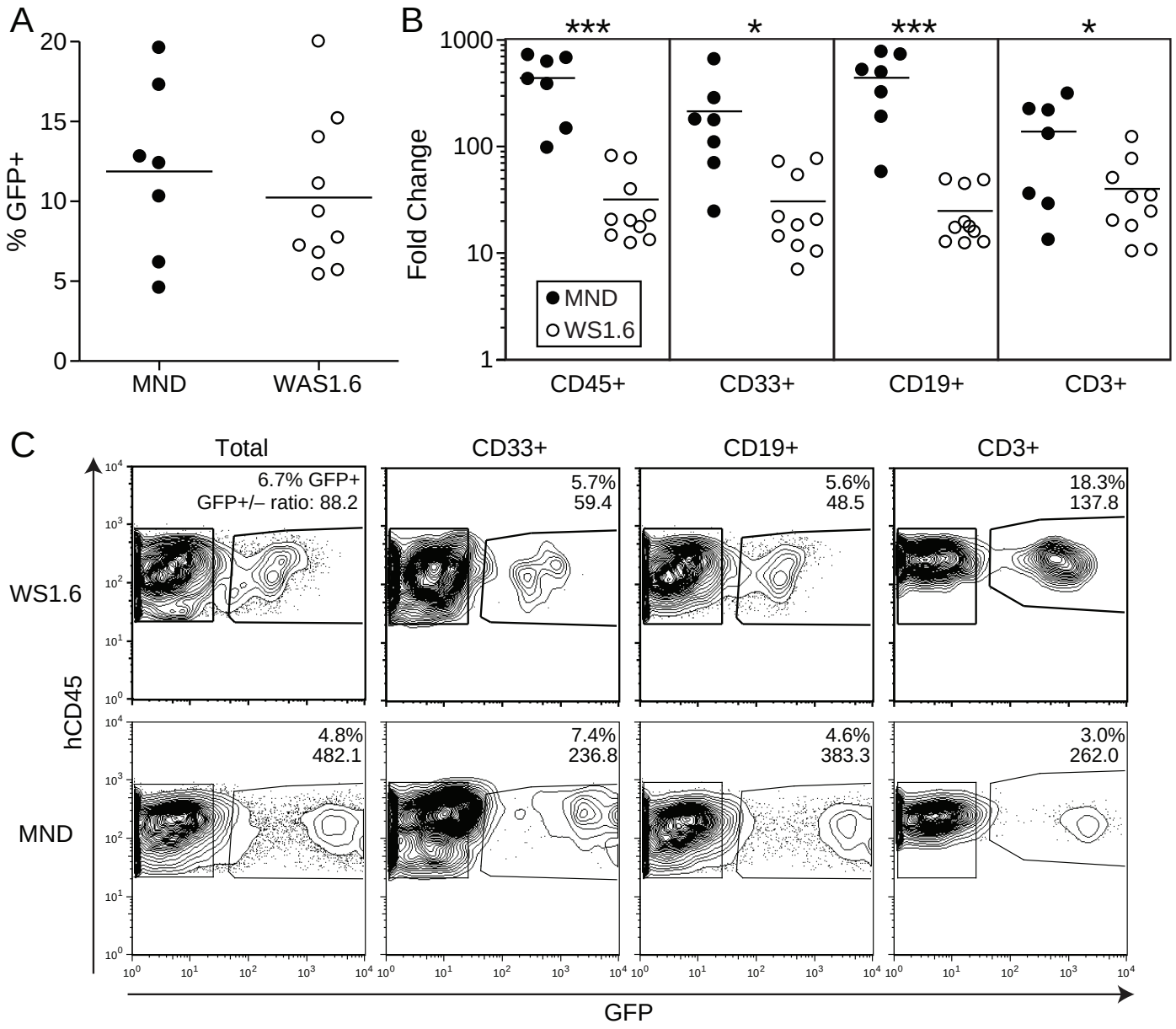
huWASp
GFP
wPRE
ΔU3
B
Lin^neg Cells,
4 days post-transduction.
94.5
WT
0.46
KO
9.66
MND-WASp
FSC
WASp
C
Monocytes
Neutrophils
CD11b
GR1
T cells
B cells
CD3
B220
D
B220+
CD45.1
CD45.2
E
CD3+CD4+CD25/FoxP3neg
Effector Memory
Central Memory
Naive
CD44
CD62L
F
B220+
MZ-MZP
T2
T1
Follicular Mature
CD21
CD24
Marginal Zone
Marginal Zone Precursors
CD23

Figure S2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% GFP+
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15
10
5
0
MND
WAS1.6
B
Fold Change
1000
100
10
1
***
*
***
*
MND
WS1.6
CD45+
CD33+
CD19+
CD3+
C
Total
CD33+
CD19+
CD3+
WS1.6
MND
hCD45
6.7% GFP+
GFP+/– ratio: 88.2
5.7%
59.4
5.6%
48.5
18.3%
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4.6%
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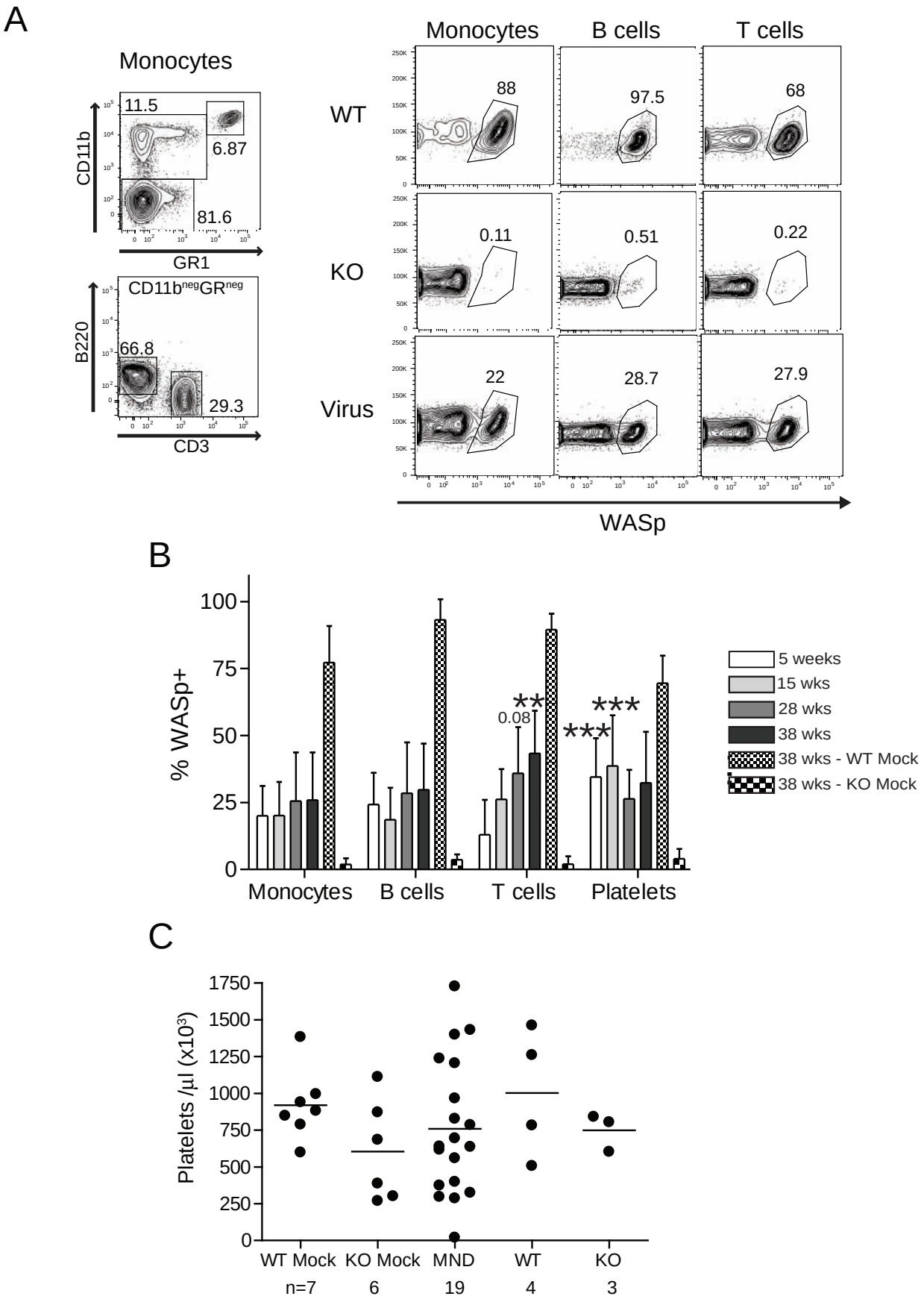
262.0
GFP

Figure S3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GR1
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B220
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29.3
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Monocytes
B cells
T cells
WT
KO
Virus
88
97.5
68
0.11
0.51
0.22
22
28.7
27.9
WASp
B
% WASp+
100
75
50
25
0
0.08
**
***
***
Monocytes
B cells
T cells
Platelets
5 weeks
15 wks
28 wks
38 wks
38 wks - WT Mock
38 wks - KO Mock
C
Platelets /µl (x10³)
1750
1500
1250
1000
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KO Mock
MND
WT
KO
n=7
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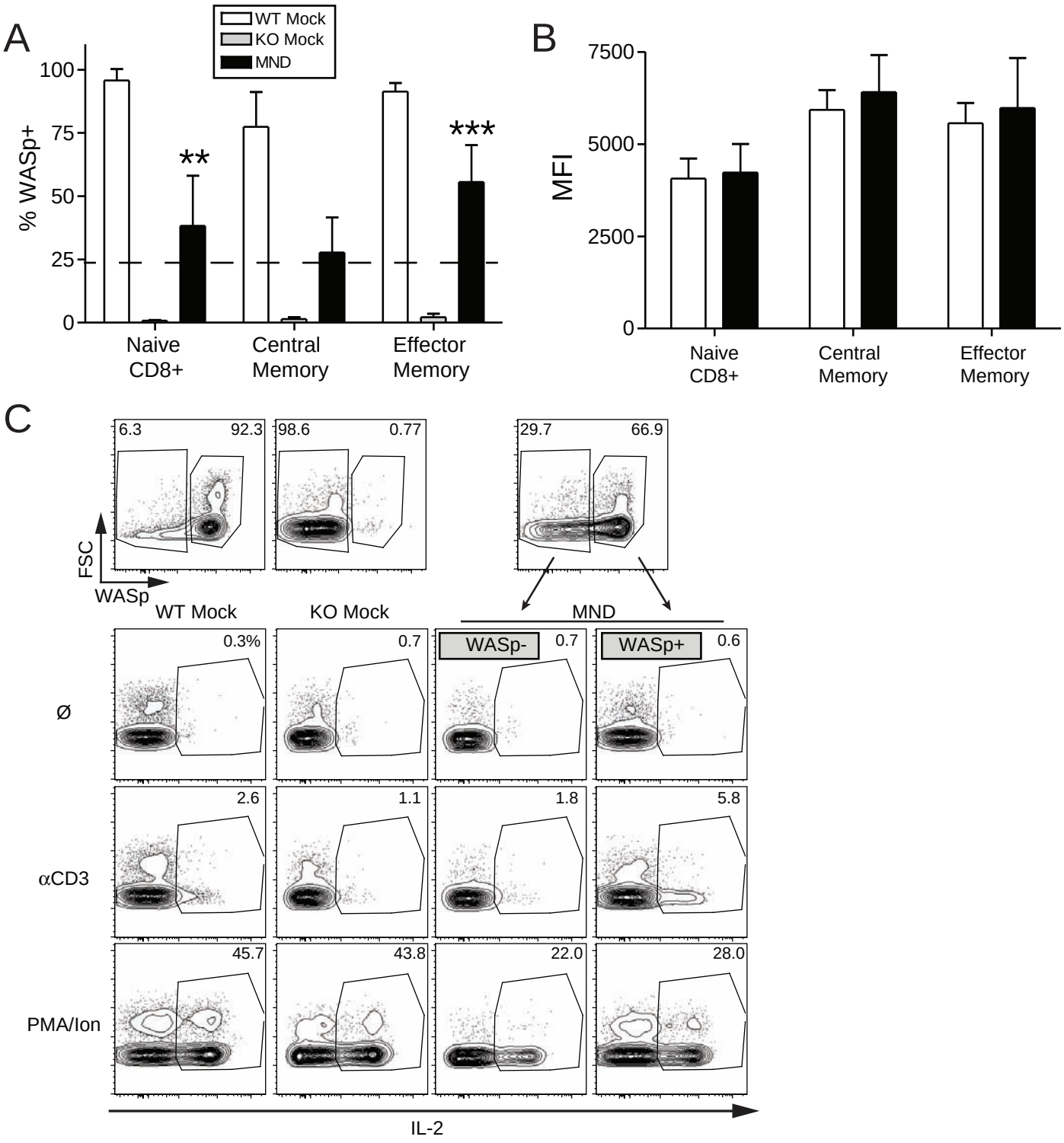
19
4
3

Figure S4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25
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***
Naive CD8+
Central Memory
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MFI
7500
5000
2500
0
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Central Memory
Effector Memory
C
6.3
92.3
98.6
0.77
29.7
66.9
FSC
WASp
WT Mock
KO Mock
MND
WASp-
WASp+
0.3%
0.7
0.7
0.6
Ø
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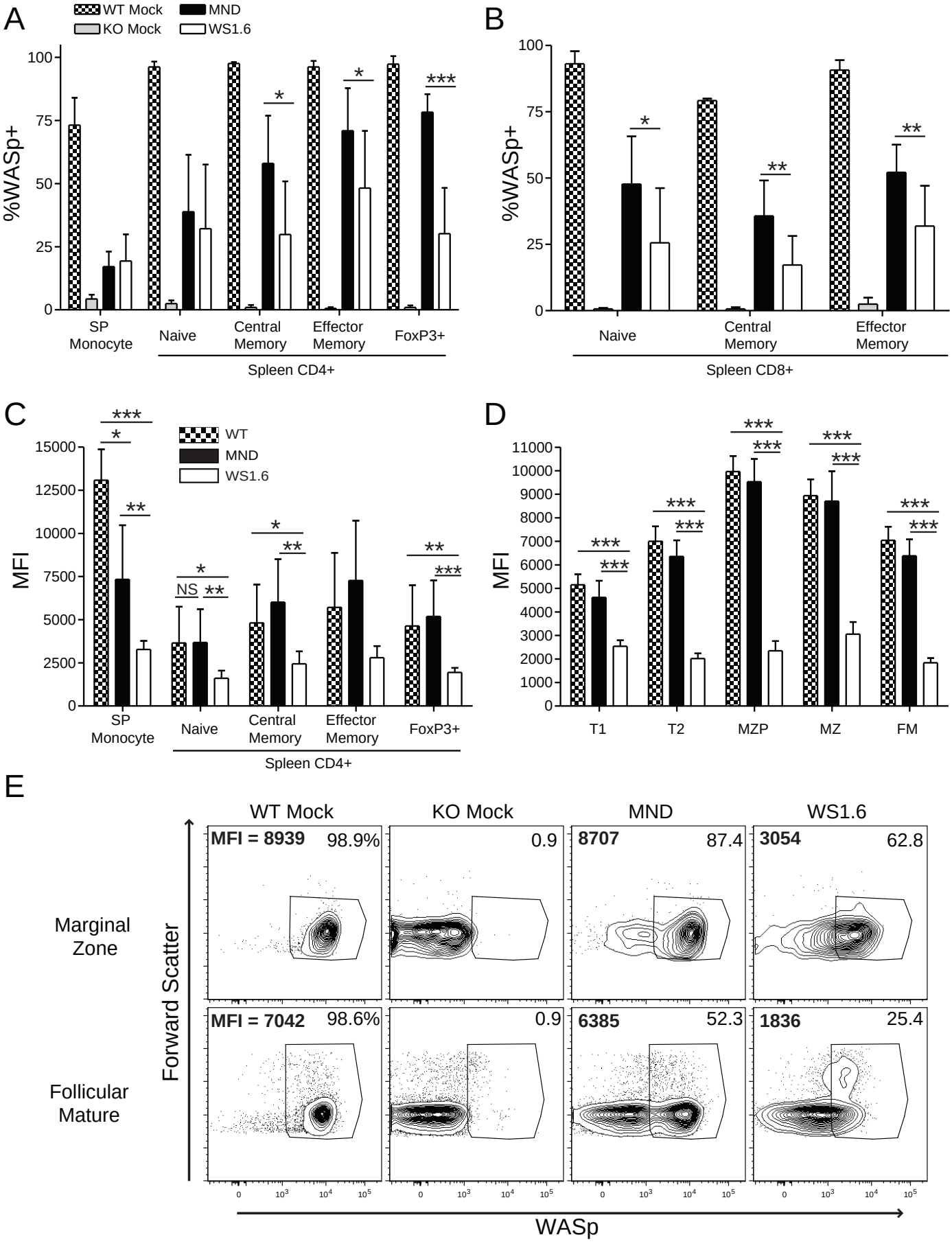
28.0
PMA/Ion
IL-2

Figure S5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Central Memory
Effector Memory
FoxP3+
Spleen CD4+
B
%WASp+
Naive
Central Memory
Effector Memory
Spleen CD8+
C
WT
MND
WS1.6
MFI
NS
SP Monocyte
Naive
Central Memory
Effector Memory
FoxP3+
Spleen CD4+
D
MFI
T1
T2
MZP
MZ
FM
E
WT Mock
KO Mock
MND
WS1.6
MFI = 8939
98.9%
0.9
8707
87.4
3054
62.8
MFI = 7042
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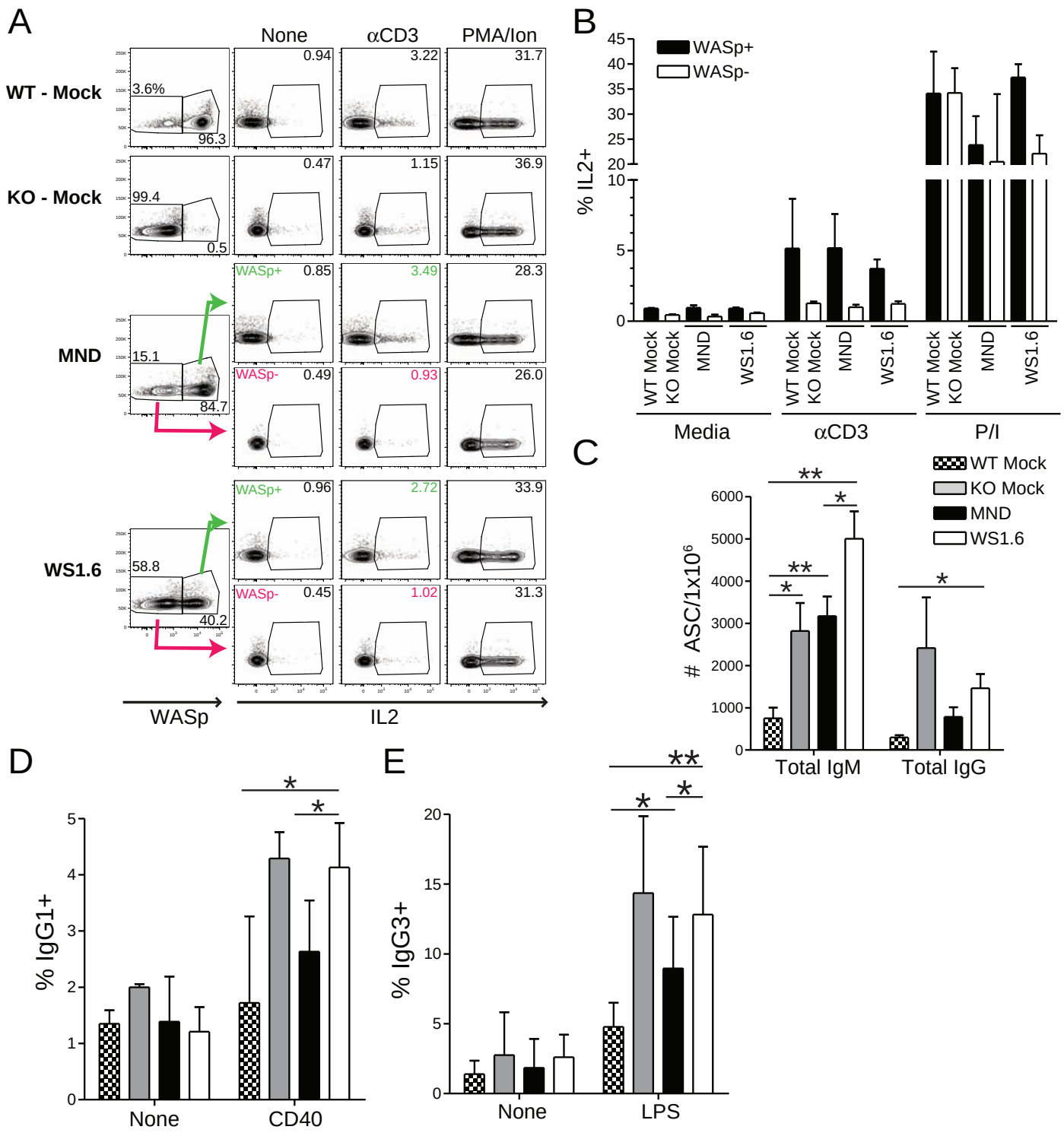
1836
25.4
Marginal Zone
Follicular Mature
Forward Scatter
WASp

Figure S6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MND
WS1.6
WASp+
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WASp+
WASp-
% IL2+
WT Mock
KO Mock
MND
WS1.6
Media
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P/I
C
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WS1.6
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Total IgG
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E
% IgG3+
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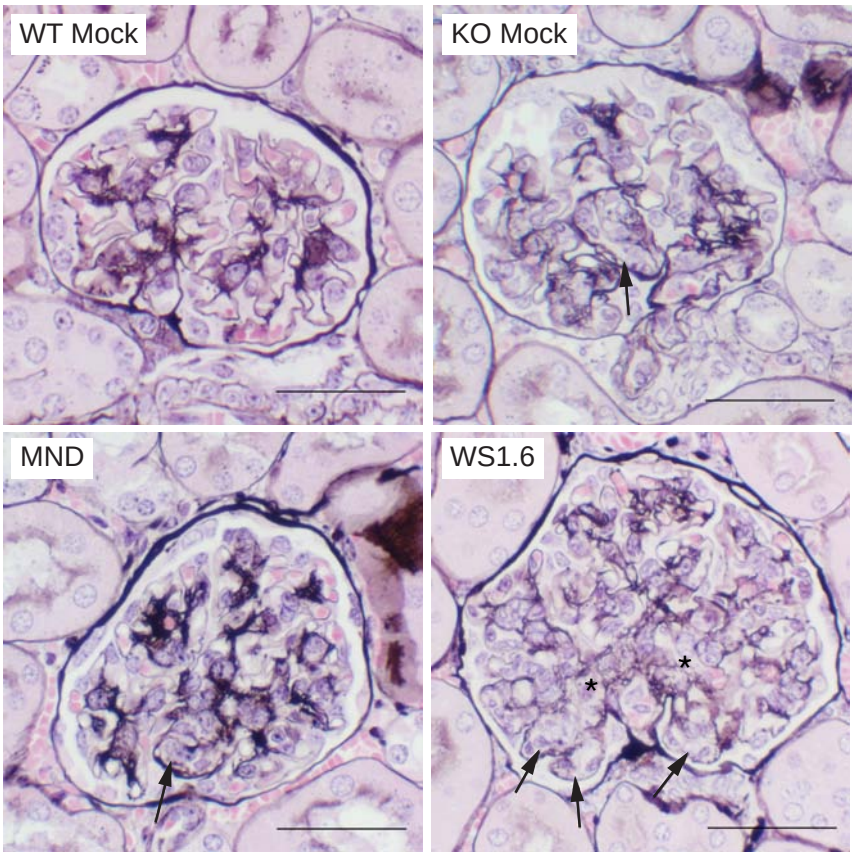
LPS

Figure S7

A.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KO Mock
MND
WS1.6
*
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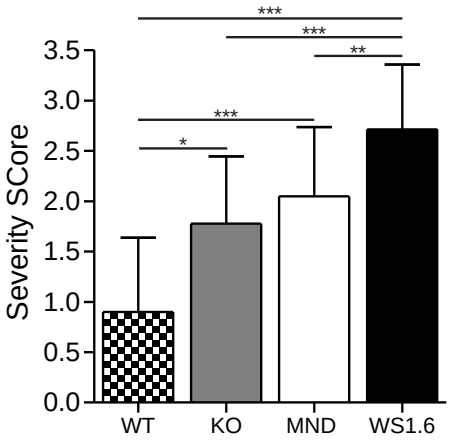
B.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Severity SCore
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
WT
KO
MND
WS1.6