Supplemental Methods

Immunophenotyping

Thymus, spleens, and lymph nodes were dispersed into single cell suspension with frosted microscope slides in PBS with 2% FBS. For bone marrow isolation, femurs were wiped cleaned and flushed with PBS plus 2% FBS. The cells were blocked with anti-CD32/CD16 (Fc receptor) mAb (Becton Dickinson) and stained with the following antibody combinations in PBS plus 2mM EDTA and 0.5% FBS. T and NK cell subsets: pacific blue anti-CD4 (RM4-5; eBioscience), PE-Cy7 anti-CD8α (53-6.7; eBioscience), FITC anti-TCRβ (H57.597; BD Biosciences), PerCP-Cy5.5 anti-CD44 (1M7; eBioscience), APC anti-CD25 (PL61.5; eBioscience) and PE anti-TCRγδ (V65; eBioscience), anti-CD49b (DX5; eBioscience), or FoxP3 staining kit (eBioscience). Myeloid lineages: pacific blue anti-CD4 (RM4-5; eBioscience), PE anti-CD8α (53-6.7; eBioscience), APC anti-CD11c (N418, eBioscience), PE-Cy7 anti-CD11b (M1170; eBioscience), FITC anti-GR1 (RB6-8C5; eBioscience), and PerCP Cy5.5 anti-MHC class II (M5114.15-2; eBioscience). Bone marrow hardy fractions: stain 1, Lineage, (PE-Cy7 anti-CD3, CD8, Ter119, and Gr1), FITC anti-CD43, PE anti-CD24, APC anti-CD19, APC-Cy7 anti-B220, biotin anti-BP1 followed by streptavidin-PE-Texas Red (SA-PE-TR). Stain 2, Lineage, (PE-Cy7 anti-CD3, CD8, Ter119, and Gr1), FITC anti-CD43, PE anti-IgM, APC anti-CD19, APC-Cy7 anti-B220, biotin anti-IgD followed by streptavidin-PE-TR. Splenic Hardy Fractions: FITC anti-IgD, PE anti-CD21, APC anti-AA4.1, APC-Cy7 anti-B220, biotin anti-IgM followed by SA-PE-TR. Samples were analyzed by flow cytometry on a LSRII (Becton Dickinson).

Measurement of Cytokines and Chemokines

Cytokines and chemokines in the BAL fluid were measured with the Milliplex-32-plex cytokine assay kit (Millipore) according to manufacturers instructions. The plate was

read on a Luminex Bioplex System (Biorad). Interferon-β was measured using the Interferon-β ELISA kit (PBL Interferon Source) according to manufacturers instructions.

T cell and B cell Proliferation Assays

For proliferation assays, splenocytes were incubated with lipopolysaccharide (LPS; 0.1-10 ug/ml; Sigma, cat. no. L3012) or anti-TCRβ (H57; 0.1-10 ug/ml; purified from Hybridoma H57.597) for 48 hours. 3H-thymidine (2μCi/well) was added and cells were incubated for an additional 24 hours harvested and counted.

T cell differentiation and cytokine expression analysis

Naïve $CD4^+$ T cells from spleen and lymph node were purified by magnetic cell sorting (CD4 Purification Kit and CD62L Purification kit; Miltenyi). BALB/c splenic APCs were isolated by complement-mediated lysis with antibody to heat-stable antigen (Thy1; J1j) and irradiated with 3000 rads. T cells and APCs were co-cultured in the presence of IL-2 (10U/ml), anti-TCRβ (H57.597; 10 μg/ml), and anti-CD28 (20 μg/ml; 37N51.1) for four days. Cells were re-plated, rested for two days, and re-stimulated with plate bound anti-TCRβ (10μg/ml) and anti-CD28 (20μg/ml) in the presence of monensin (Golgi stop; BD Biosciences) for 5 hours. Cells were harvested and surfaced stained with FITC anti-CD4 (RM4-5; eBioscience). Intracellular cytokine staining was performed according manufacturer's instructions (Cytofix/Cytoperm Fixation/Permeabilization Kit; BD Biosciences) with PE-cy7 anti-IFNγ (XMG; eBioscience) and PE anti-IL4 (11B11; eBioscience) or PE anti-IL13 (eBioscience) and analyzed on a FACS Caliber (Becton Dickinson).

Supplemental Figure Legends

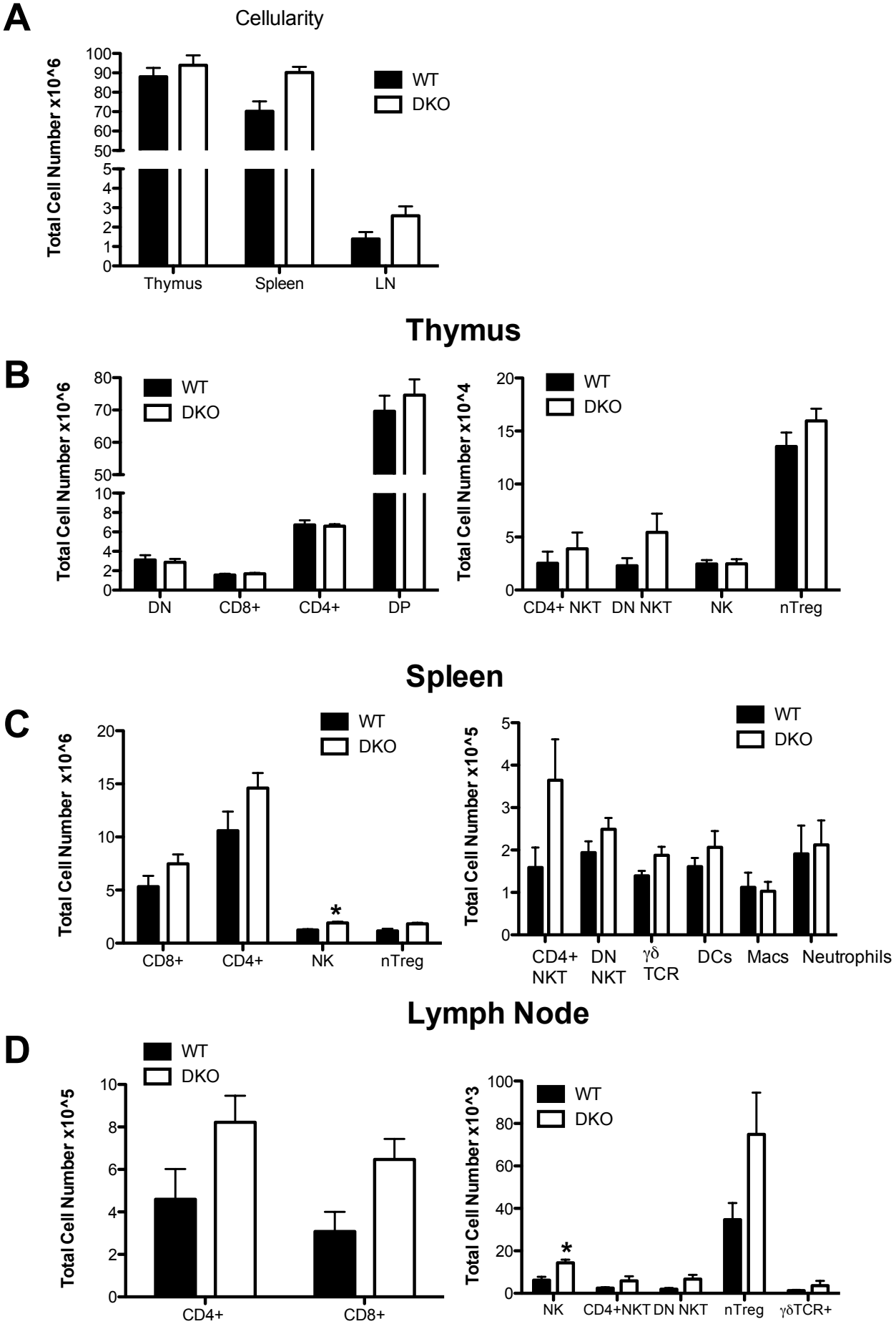
Supplemental Figure 1. Immune cell development in WT and DKO mice. (A) Depicted is the cellularity of the thymus, spleen, and lymph nodes in WT and DKO mice. (B) Depicted is the number of thymic DN ($CD4^-CD8^-$), DP ($CD4^+CD8^+$), $CD4^+$, $CD8^+$, CD4 NKT ($CD4^+CD8^-CD49b^+TCR\beta^+$), DN NKT ($CD4^-CD8^-CD49b^+TCR\beta^+$) and natural regulatory T (nTreg; $CD4^+FoxP3^+TCR\beta^+$) cells in WT and DKO mice. (C) Depicted is the number of splenic $CD8^+$, $CD4^+$, NK ($CD4^-CD8^-TCR\beta^-CD49b^+$), nTreg, $CD4^+$ NKT, DN NKT and γδT ($TCR\gamma\delta^+$) cells, macrophages ($CD11b^+Ly6G^+$MHC class II^+) and neutrophils ($CD11b^+Ly6G^+$MHC class II^-) in WT and DKO mice. (* p=.003, Student's T-test) (D) Depicted is the number of lymph node $CD4^+$, $CD8^+$, NK, CD4 NKT, DN NKT, nTreg and γδT cells in WT and DKO mice. (* p=.009, Student's T test) Data are from two independent experiments (mean and SEM).

Supplemental Figure 2. B cell development in WT and DKO mice. Depicted is the number of cells in Hardy Fractions A-F in (A) the bone marrow and in T1-T3, Fo, B1 and Mz in (B) the spleen of WT (n=4), Het (n=4), and DKO (n=4) mice. Fraction A ($B220^+CD43^+$, $CD24^-$, $BP1^-$), Faction B ($B220^+CD43^+$, $CD24^+$, $BP1^-$), Fraction C ($B220^+CD43^+$, $CD24^{med}$, $BP1^+$), Fraction C' ($B220^+CD43^+$, $CD24^{high}$, BP1+), Fraction D ($B220^+$, $CD43^-$, IgM^-, IgD^-), Fraction E ($B220^+$, $CD43^-$, IgM^+, IgD^-), Fraction F ($B220^+$, $CD43^-$, IgM^+, IgD^+), T1 ($CD23^-$, $AA4.1^+$, IgM^+, $CD21^-$), T2 ($CD23^+$, $AA4.1^+$, $CD21^-$, IgM^{high}), T3 ($CD23^+$, $AA4.1^+$, $CD21^-$, IgM^{high}), Fo ($CD21^+$, $CD23^+$, $AA4.1^-$, IgM^+), B1 ($CD23^-$, $AA4.1^-$, $CD21^{low}$, IgM^+), Mz ($CD23^-$, $AA4.1^-$, $CD21^{low}$, IgM^+). Data are from three independent experiments (mean and SEM).

Supplemental Figure 3. T and B cell proliferation and CD4 T cell cytokine production in WT and DKO mice. (A and B) Splenocytes were stimulated with anti-TCRβ (H57; 0.1 to 10 μg/ml) or LPS (0-1 μg/ml). 3H-thymidine was added after 48 hours

and cells were harvested and counted after 24 hours. (C and D) CD4 T cells were activated under neutral conditions (anti-TCRβ, anti-CD28, IL2) with BALB/c APCs for four days and rested for two days. Cells were re-stimulated with plate bound anti-TCRβ, anti-CD28 for five hours and IFNγ, IL-4, and IL-13 production were determined by intracellular cytokine staining. Data in (A) are from two independent experiments (mean and SEM). Data in (B) are representative of 3 independent experiments.

Supplemental Figure 4. Cytokine expression in BAL from flu-infected WT and DKO mice. Mice were infected with 2000 EID50 of PR8 intranasally. BAL fluid was isolated at day 3 (A; WT N=12, DKO N=12) and day 7 (B; WT N=12, DKO N=12) post-infection. (A) IFNβ concentration in BAL was determined by ELISA. (B) Cytokine concentrations in BAL at day 7 post-infection were determined by multiplex analysis. Data are from 2 independent experiments (mean and SEM).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DKO
Thymus
Spleen
LN
Thymus
B
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DN
CD8+
CD4+
DP
Total Cell Number x10^4
CD4+ NKT
DN NKT
NK
nTreg
Spleen
C
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CD8+
CD4+
NK
nTreg
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CD4+ NKT
DN NKT
γδ TCR
DCs
Macs
Neutrophils
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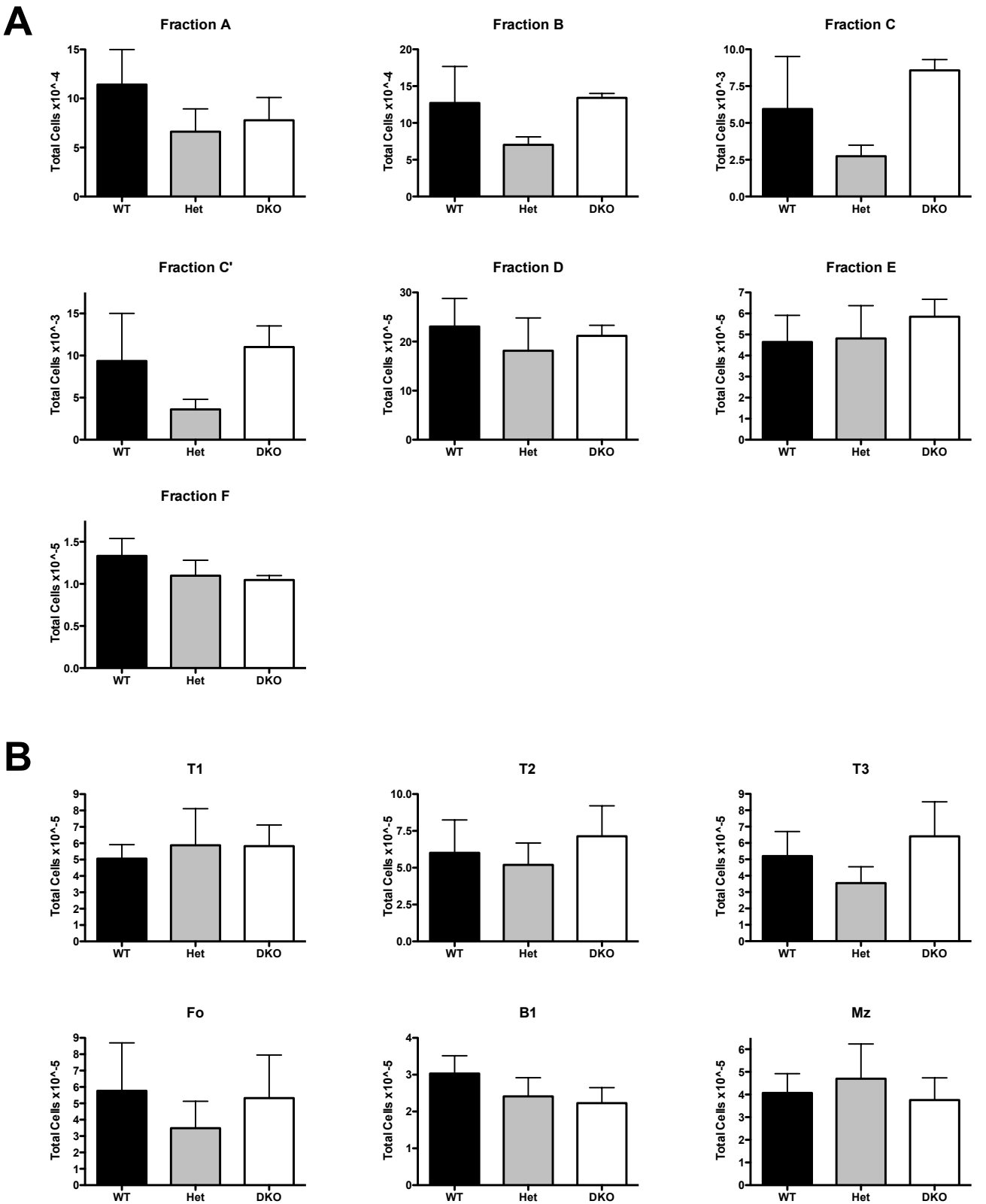
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10
5
0
WT
Het
DKO
Fraction B
Total Cells x10^-4
20
15
10
5
0
WT
Het
DKO
Fraction C
Total Cells x10^-3
10.0
7.5
5.0
2.5
0.0
WT
Het
DKO
Fraction C'
Total Cells x10^-3
15
10
5
0
WT
Het
DKO
Fraction D
Total Cells x10^-5
30
20
10
0
WT
Het
DKO
Fraction E
Total Cells x10^-5
7
6
5
4
3
2
1
0
WT
Het
DKO
Fraction F
Total Cells x10^-5
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1.0
0.5
0.0
WT
Het
DKO
B
T1
Total Cells x10^-5
9
8
7
6
5
4
3
2
1
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Het
DKO
T2
Total Cells x10^-5
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7.5
5.0
2.5
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WT
Het
DKO
T3
Total Cells x10^-5
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7
6
5
4
3
2
1
0
WT
Het
DKO
Fo
Total Cells x10^-5
9
8
7
6
5
4
3
2
1
0
WT
Het
DKO
B1
Total Cells x10^-5
4
3
2
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Mz
Total Cells x10^-5
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3
2
1
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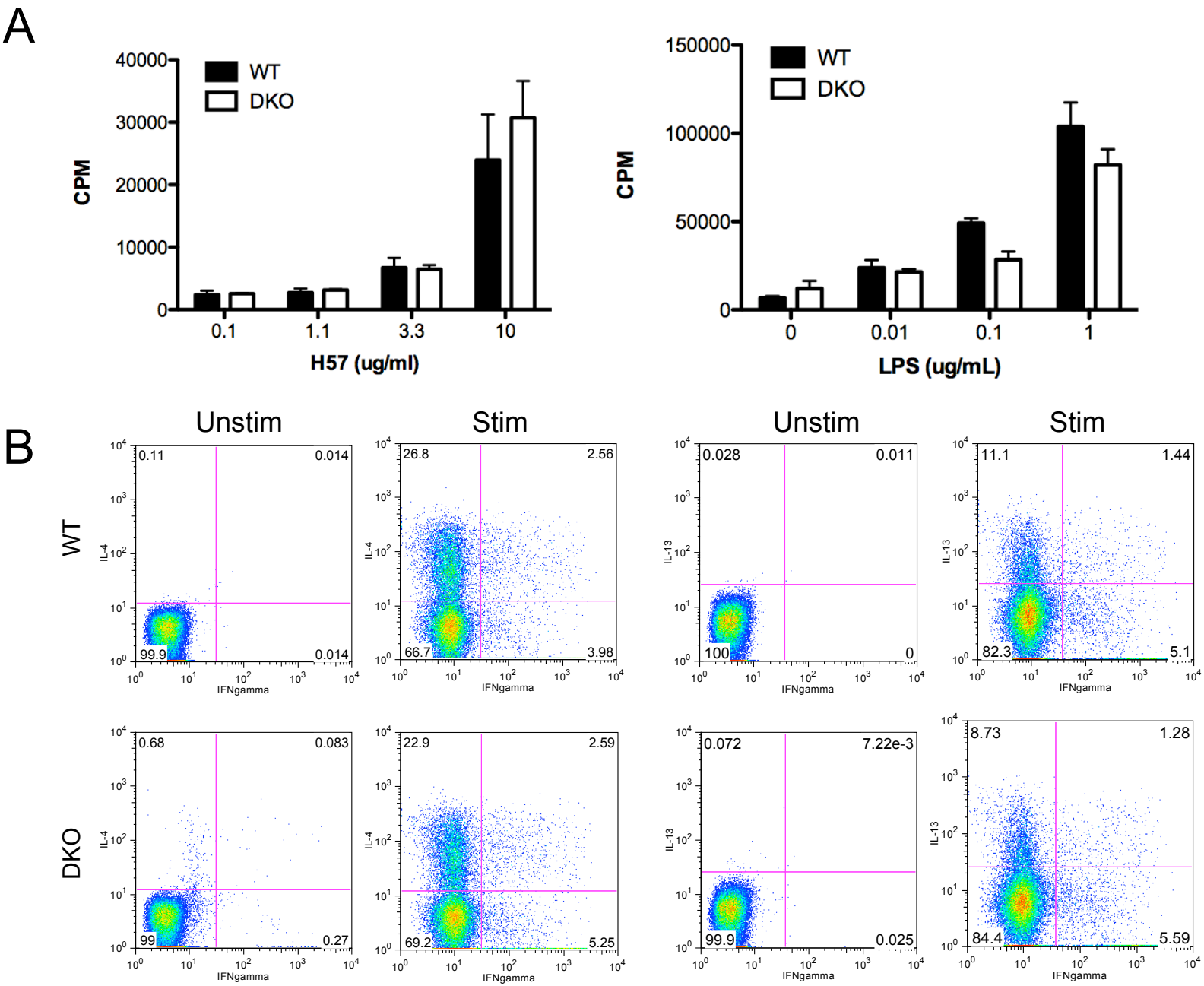
WT
Het
DKO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30000
20000
10000
0
CPM
WT
DKO
0.1
1.1
3.3
10
H57 (ug/ml)
150000
100000
50000
0
CPM
WT
DKO
0
0.01
0.1
1
LPS (ug/mL)
B
Unstim
Stim
Unstim
Stim
WT
DKO
IL-4
IL-13
IFNgamma
0.11
0.014
99.9
0.014
26.8
2.56
66.7
3.98
0.028
0.011
100
0
11.1
1.44
82.3
5.1
0.68
0.083
99
0.27
22.9
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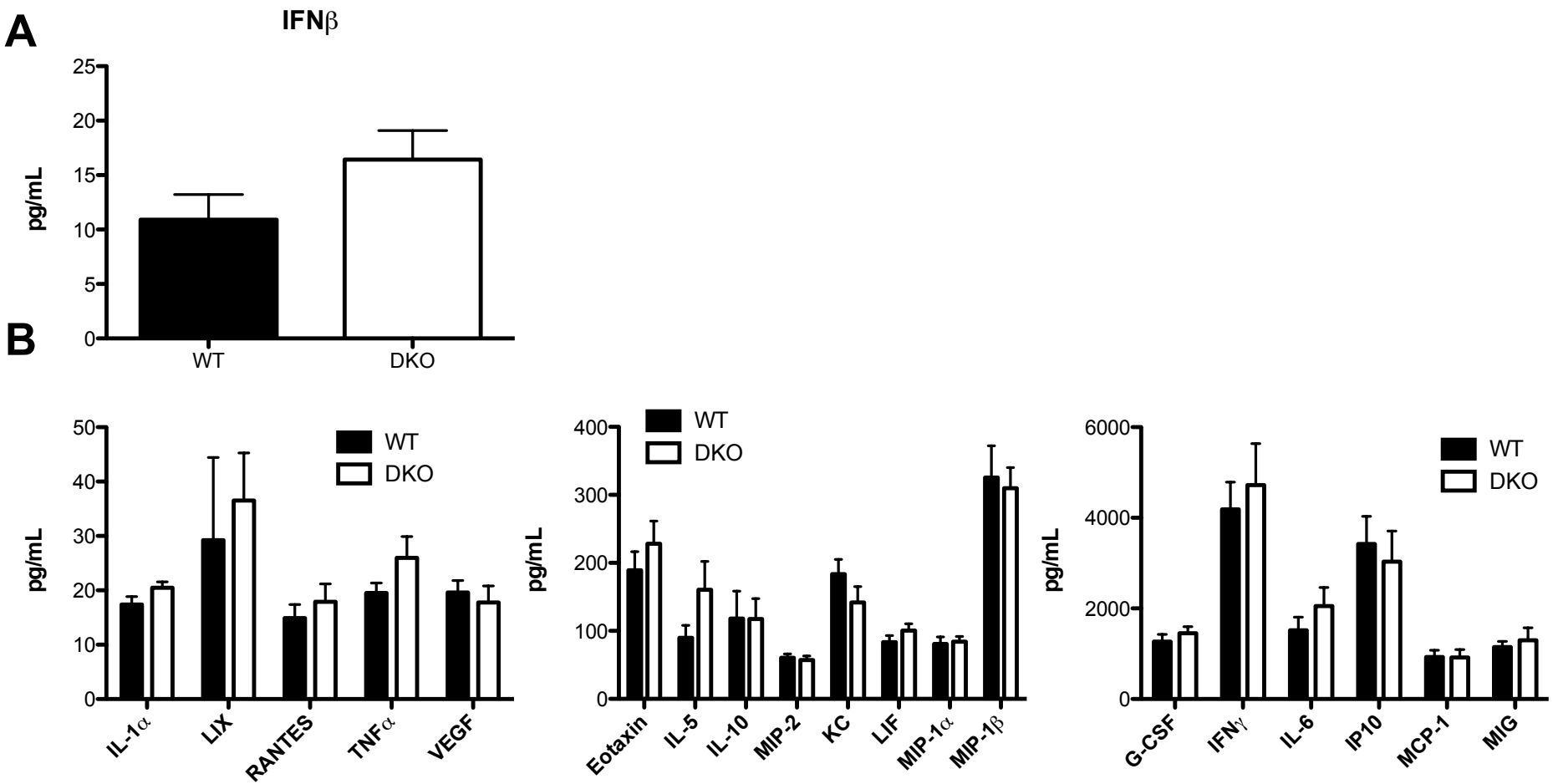
84.4
5.59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pg/mL
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20
15
10
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WT
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B
pg/mL
50
40
30
20
10
0
WT
DKO
IL-1α
LIX
RANTES
TNFα
VEGF
pg/mL
400
300
200
100
0
WT
DKO
Eotaxin
IL-5
IL-10
MIP-2
KC
LIF
MIP-1α
MIP-1β
pg/mL
6000
4000
2000
0
WT
DKO
G-CSF
IFNγ
IL-6
IP10
MCP-1
MIG