Supplementary Information for:

Western diet regulates immune status and the response to LPS-driven sepsis independent of diet-associated microbiome

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Supplementary text
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Figs. S1 to S5

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SI MATERIALS & METHODS

Diet and temperature tracking in mice

Mice were fed either irradiated Standard Chow (2018; Envigo; Huntingdon, UK) or Western Diet (TD.88137; Envigo). In mice tracked for temperature, ID transponders were implanted subcutaneously, as previously described *(1).* For GF experiments, ID transponders and transponder injectors were sterilized via chemical sterilant (Clidox, Pharmacal Inc.) or via autoclaving, respectively, and transponders were injected under GF conditions.

CFU analysis

In experiments analyzing colony-forming units (CFUs) in lung and blood samples, all materials, homogenizations, and dilutions were done in pre-reduced environments and buffers. At specified time points, mice were sacrificed and lungs or 50μL of blood were harvested in pre-reduced 1x PBS. Lungs were homogenized and lungs and blood were then plated on Schaedler Agar with 5% sheep blood (defibrinated) and grown overnight at 37°C anaerobically or aerobically.

Real-time PCR

Mice treated with PBS or LPS were sacrificed at specified time points and 30μL of blood was collected from via cardiac puncture and transferred into 300μL of RNALater (Invitrogen) and frozen at -80°C. RNA was isolated using the RNeasy Mini Kit (QIAGEN). For kinetic studies, 10μL of blood was collected via tail snip and transferred into 150μL of RNALater (Invitrogen) frozen at -80°C. RNA was isolated using the RNeasy Plus Micro Kit (QIAGEN). Following RNA extraction, cDNA was synthesized from RNA samples using SuperScript III First-Strand synthesis system (Invitrogen). Gene specific primers were used to amplify transcripts using FastStart Universal SYBR Green Master (Rox; Roche). IL-6-F: 5’ TCCAGTTGCCTTCTTGGGAC 3’; IL-6-R: 3’ AGTCTCCTCTCCGGACTTGT 5’; TNF-F: 5’ GATCGGTCCCCAAAGGGATG 3’; TNF-R: 3’

TGGTTTGTGAGTGTGAGGGTC 5'; IL-10-F: 5' GTTGCCAAGCCTTATCGGA 3'; IL-10-R: 3' ACCTGCTCCACTGCCTTGCT 5'.

Flow cytometry

To analyze presence of white blood cells in 200μL blood samples, blood was processed on ice in PBS-EDTA. Cells were subject to ACK buffer for 1 minute at room temperature to insure red blood cell lysis. Then, cells were incubated with blocking mAb 2.4G2 anti-FcγRIII/I (BD Biosciences) and live/dead probe (Invitrogen) along with labeled CD11b (M1/70), CD19 (6D5), Ly6G (1A8), CD8 (GL-1), CD4 (L3T4, T4), CD335 (29A1.4), all from Biolegend, and Ly6C (HK1.4), CD115 (AFS98), TCRβ (H57-597), all from eBioscience and CD11c (HL3; BD Biosciences). Cells were then fixed for 15 minutes in 1.6% PFA and kept at 4°C until samples were acquired.

To isolate neutrophils for activation and migration assays, blood samples were processed on ice and in PBS-EDTA buffer to prevent activation of the cells. Then, cells were incubated with blocking mAb 2.4G2 anti-FcγRIII/I (BD Biosciences) and live/dead probe (Invitrogen) along with labeled Ter119 (TER119), CD3e (145-2C11), CD19 (1D3), CD49b (DX5), CD66b (G10F5), CXCR4 (2B11) all from eBioscience/Invitrogen, and CD11b (M1/70), Ly6G (1A8), CD62L (MEL-14), CD63 (NVG-2), all from Biolegend, and CXCR2 (Met1-Leu359; R&D Systems). Cells were then fixed for 15 minutes in 1.6% PFA and kept at 4°C until samples were acquired. Isotype control was run for CXCR2 by staining with Ig2a-APC. A sample treated with phorbol myristate acetate (PMA) for 30 minutes was used as a positive control. Neutrophil functional analysis and qRT-PCR analysis were previously described (*1, 2*). Neutrophil depletion assays were carried out one day prior to LPS treatment as described previously (*2, 3*).

Histopathology

The entirety of the left lung lobe was immersion-fixed in 10% neutral buffered formalin, routinely paraffin-embedded, sectioned at 5μm, and stained with hematoxylin and eosin. Lung samples were evaluated by a

board-certified veterinary pathologist for the following criteria: neutrophil presence within the alveolar septa (score 0-3), neutrophil margination within post-capillary venules and veins (score 0 or 1), and microvascular fibrin thrombi (score 0-2). Scores from each category were added to yield a cumulative lung pathology score for each mouse

Statistical analysis

For disease maps, to visualize the differences between the two experimental groups, confidence ellipses around mean group points were plotted. The *stat_ellipse* function in the ggplot2 package in R v3.2.0 was used to overlay 70% confidence ellipses for each of the diet groups. Two-factor multivariate analysis of variance (MANOVA) was used to test for differences in temperature and neutrophil level in blood between the two experimental groups. All statistics were calculated in R. All other statistical analysis not mentioned above was done using Prism 6.0 (GraphPad Software).

SI FIGURE LEGENDS

Figure S1. A) Aged-matched (6-8 weeks) female BALB/c mice were fed SC or WD for 16 days and weighed. Mice were fed SC and WD for 16 days and injected with 8 mg/kg of LPS i.p. and tracked for B) temperature loss and survival. Weight of mice was plotted against C) minimum temperature or D) survival time and correlation was calculated.

Figure S2. Aged-matched (6-8 weeks) female BALB/c mice were fed SC or WD for 16 day. Next they were sacrificed and 200μL of blood was analyzed by flow cytometry for the quantity of A) B cells, $CD4^+$ T cells, $CD8^+$ T cells, NK cells, and RBCs. B) Mice were treated with diets as indicated above and injected i.p. with 6 mg/kg of LPS and sacrificed at indicated time points and 200μL of blood was analyzed for B cells, $CD4^+$ T cells, $CD8^+$ T cells, and NK cells overtime.

Figure S3. A) Aged-matched (6-8 weeks) female BALB/c mice were injected with anti-Ly6G or PBS and 24h post-injection blood was harvested and analyzed for the presence of neutrophils. B) Aged-matched (6-8 weeks) female BALB/c mice were injected with anti-Ly6G or the isotype control and 24h p.t. these mice were injected i.p. with 6 mg/kg of LPS and tracked for B-C) temperature loss and D-E) weight loss.

Figure S4. Aged-matched (6-8 weeks) female BALB/c mice were fed SC or WD for 16 days and sacrificed. A-B) Lungs and C-D) 50µL of blood was collected and plated for microbial growth over 24h in aerobic or anaerobic conditions.

Figure S5. A) Aged-matched (6-8 weeks) female C57/B6 mice were injected i.p. with 6 mg/kg of LPS and monitored for survival. B-C) Germ-free mice were injected i.p. with varying levels of LPS and monitored for weight loss over time.

SI REFERENCES

1. B. A. Napier *et al.*, Complement pathway amplifies caspase-11-dependent cell death and endotoxin-induced sepsis severity. *J Exp Med*, (2016).
2. R. J. Napier *et al.*, Low doses of imatinib induce myelopoiesis and enhance host anti-microbial immunity. *PLoS Pathog* **11**, e1004770 (2015).
3. R. Blomgran, J. D. Ernst, Lung neutrophils facilitate activation of naive antigen-specific CD4+ T cells during Mycobacterium tuberculosis infection. *J Immunol* **186**, 7110-7119 (2011).

Figure S1.

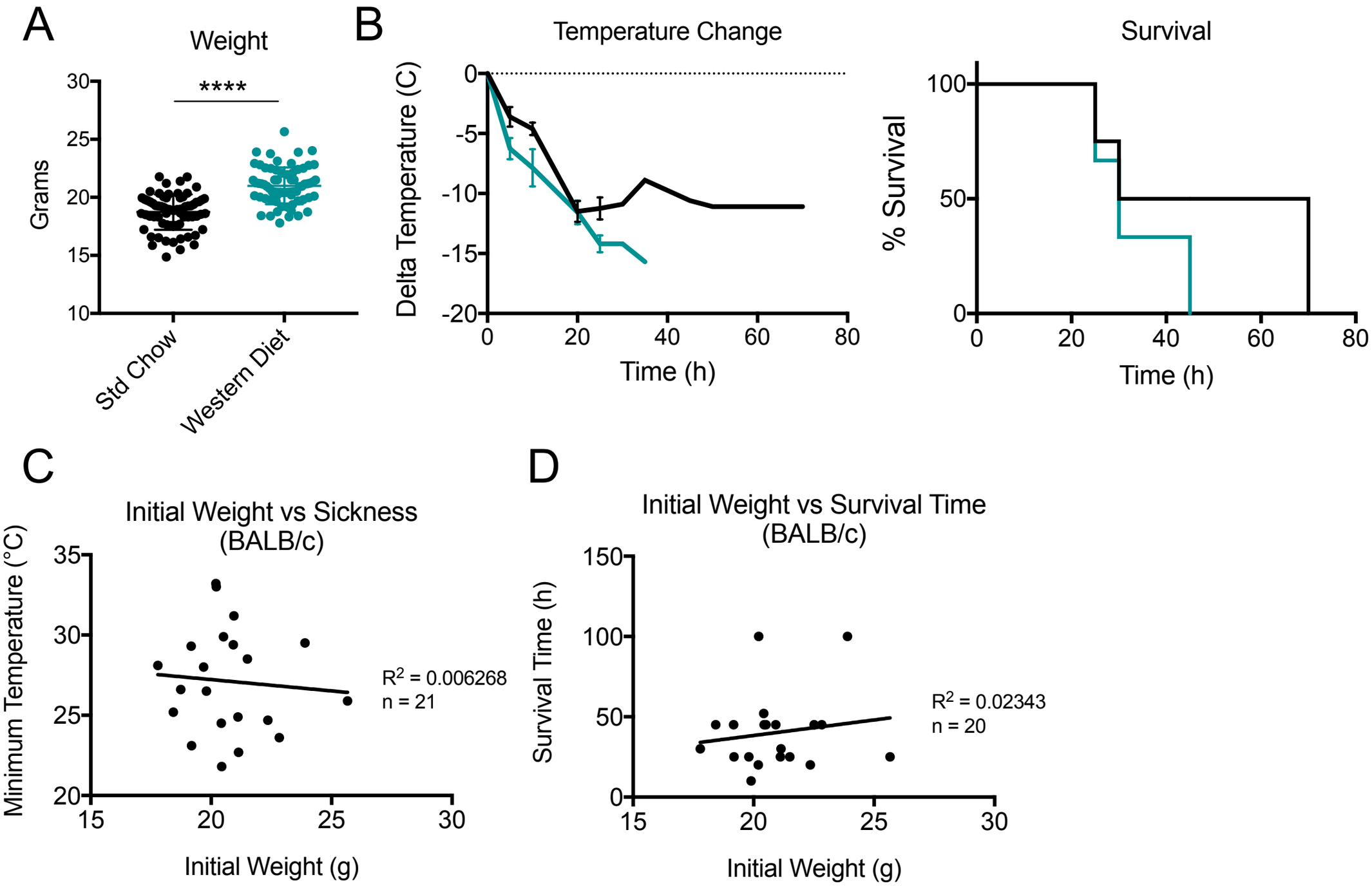
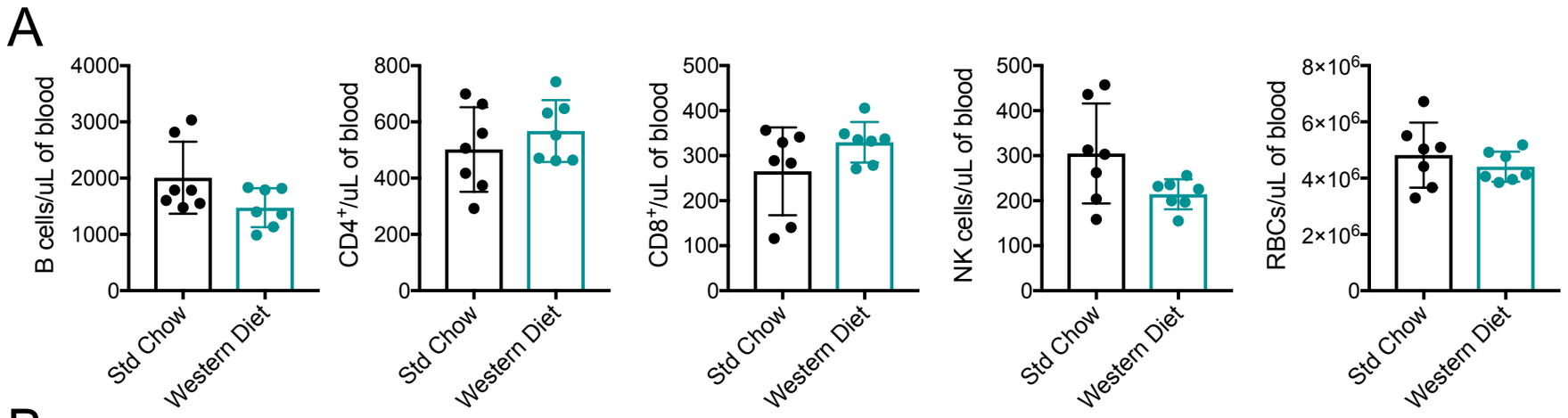

A
Weight
****
Grams
30
25
20
15
10
Std Chow
Western Diet
B
Temperature Change
Delta Temperature (C)
0
-5
-10
-15
-20
0
20
40
60
80
Time (h)
Survival
% Survival
100
50
0
0
20
40
60
80
Time (h)
C
Initial Weight vs Sickness
(BALB/c)
Minimum Temperature (°C)
35
30
25
20
15
20
25
30
Initial Weight (g)
R2 = 0.006268
n = 21
D
Initial Weight vs Survival Time
(BALB/c)
Survival Time (h)
150
100
50
0
15
20
25
30
Initial Weight (g)
R2 = 0.02343
n = 20

Figure S2


A
B cells/uL of blood
4000
3000
2000
1000
0
Std Chow
Western Diet
CD4+/uL of blood
800
600
400
200
0
CD8+/uL of blood
500
400
300
200
100
0
NK cells/uL of blood
RBCs/uL of blood
8×10^6
6×10^6
4×10^6
2×10^6

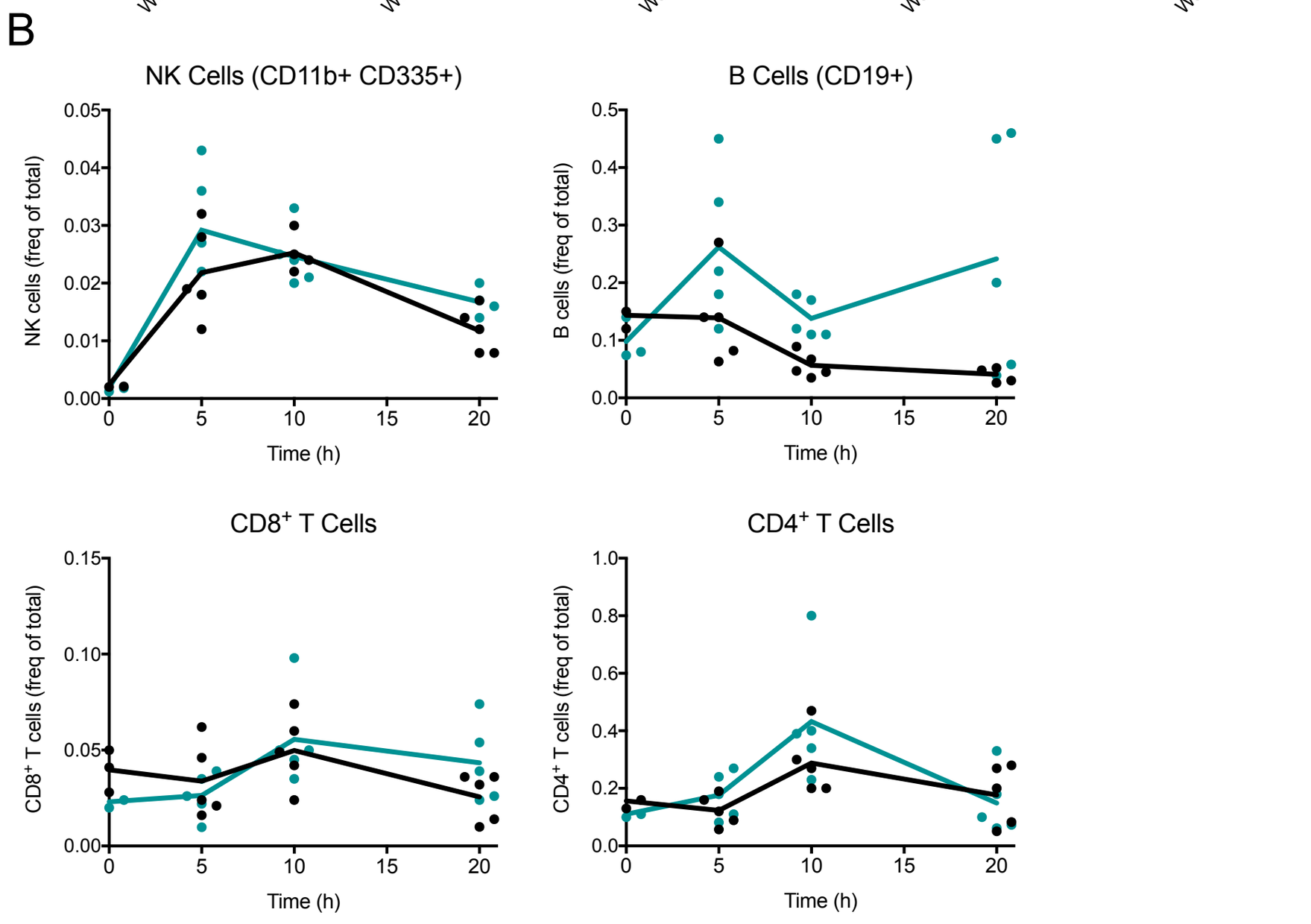
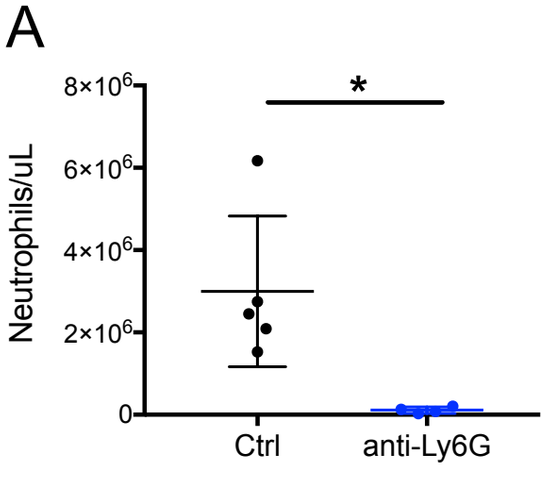
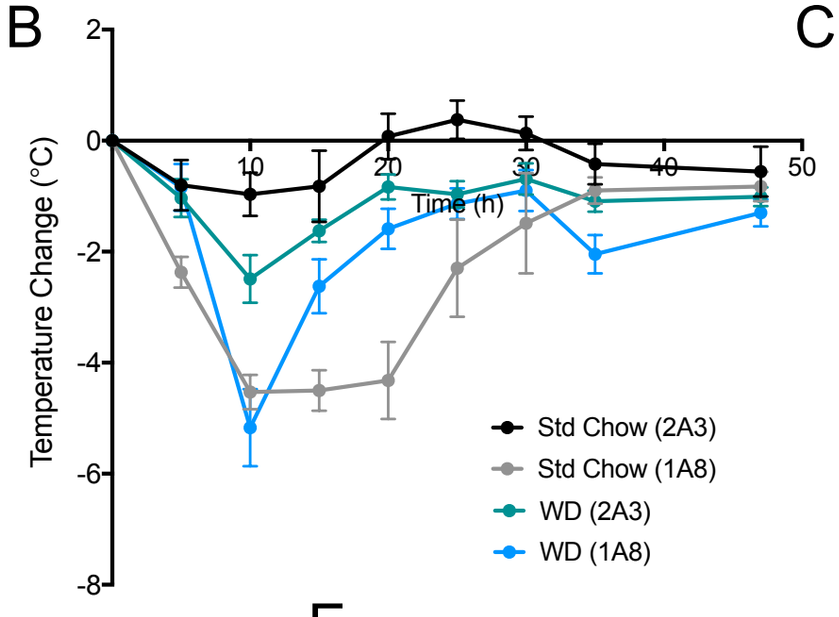
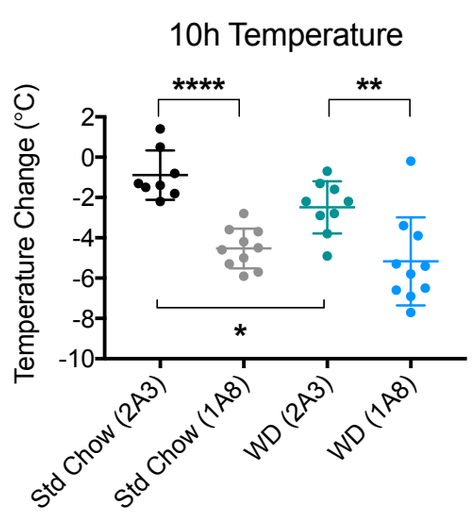
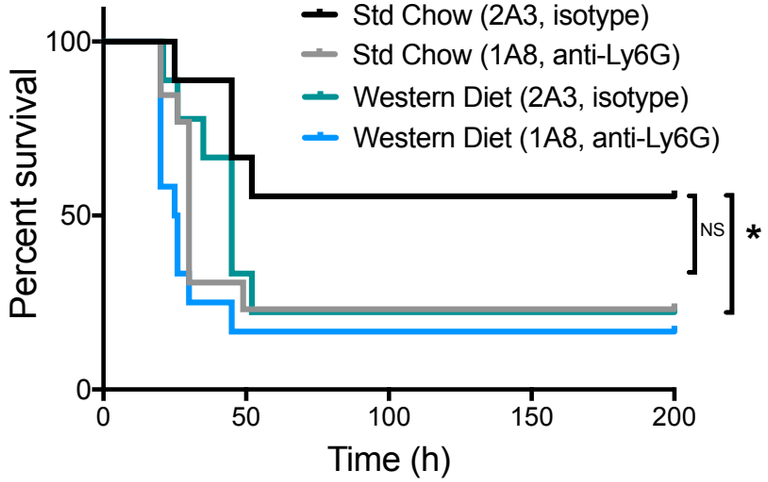

B
NK Cells (CD11b+ CD335+)
NK cells (freq of total)
0.05
0.04
0.03
0.02
0.01
0.00
0
5
10
15
20
Time (h)
B Cells (CD19+)
B cells (freq of total)
0.5
0.4
0.3
0.2
0.1
0.0
CD8+ T Cells
CD8+ T cells (freq of total)
0.15
0.10
0.05
0.00
CD4+ T Cells
CD4+ T cells (freq of total)
1.0
0.8
0.6
0.4
0.2
0.0

Figure S3


A
Neutrophils/uL
8×10^6
6×10^6
4×10^6
2×10^6
0
*
Ctrl
anti-Ly6G


B
Temperature Change (°C)
2
0
-2
-4
-6
-8
10
20
30
40
50
Time (h)
Std Chow (2A3)
Std Chow (1A8)
WD (2A3)
WD (1A8)


C
10h Temperature
Temperature Change (°C)
****
**
*
2
0
-2
-4
-6
-8
-10
Std Chow (2A3)
Std Chow (1A8)
WD (2A3)
WD (1A8)


D
Std Chow (2A3, isotype)
Std Chow (1A8, anti-Ly6G)
Western Diet (2A3, isotype)
Western Diet (1A8, anti-Ly6G)
Percent survival
100
50
0
0
50
100
150
200
Time (h)
NS
*

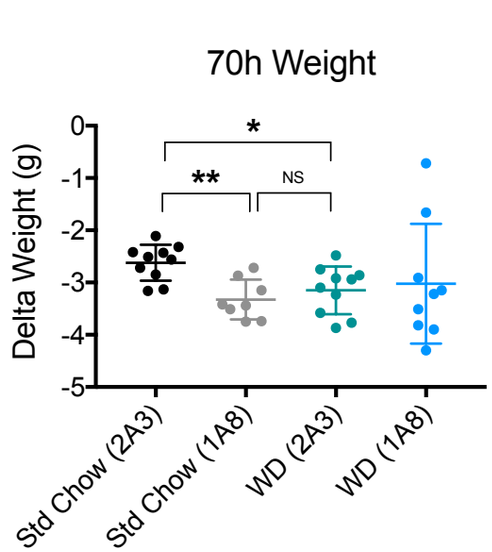

E
70h Weight
Delta Weight (g)
0
-1
-2
-3
-4
-5
*
**
NS
Std Chow (2A3)
Std Chow (1A8)
WD (2A3)
WD (1A8)

Figure S4

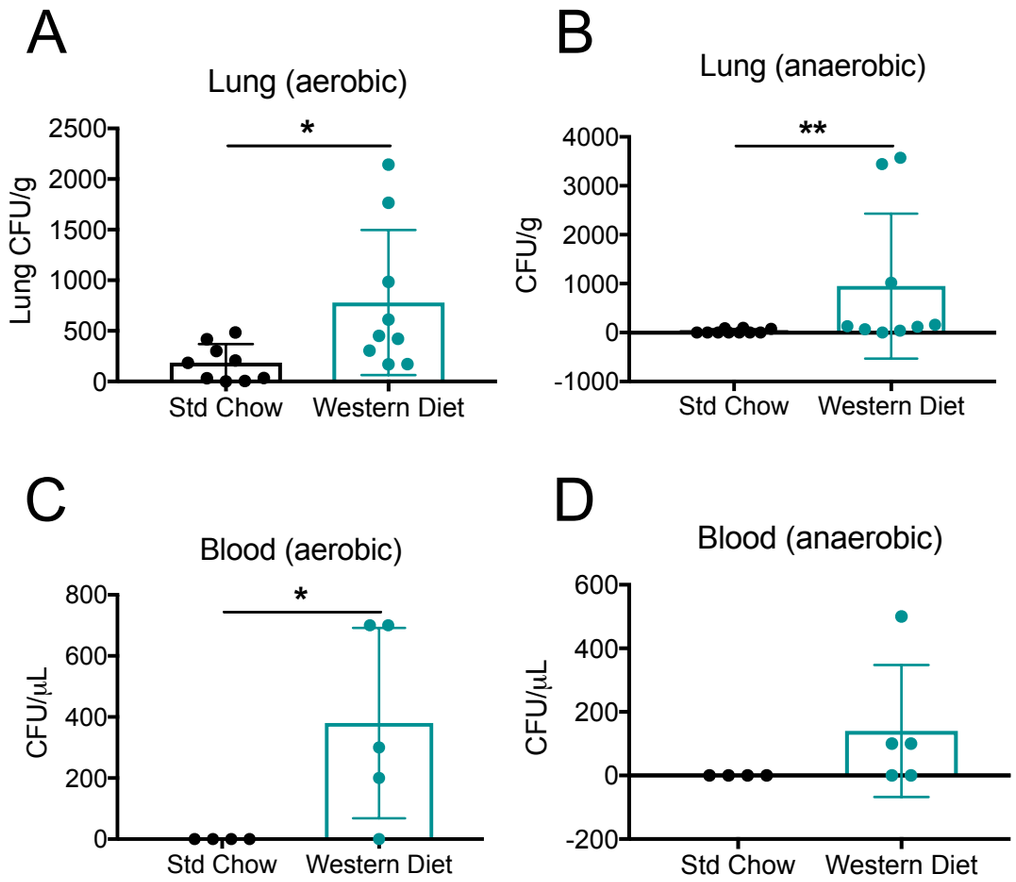
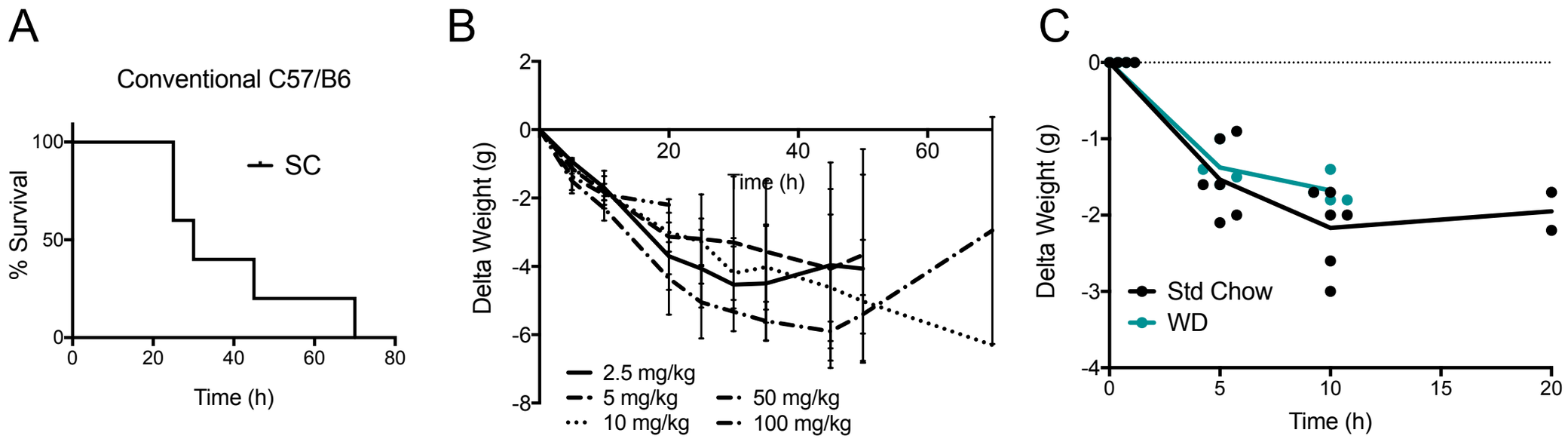

A
Lung (aerobic)
Lung CFU/g
2500
2000
1500
1000
500
0
*
Std Chow
Western Diet
B
Lung (anaerobic)
CFU/g
4000
3000
2000
1000
0
-1000
**
Std Chow
Western Diet
C
Blood (aerobic)
CFU/µL
800
600
400
200
0
*
Std Chow
Western Diet
D
Blood (anaerobic)
CFU/µL
600
400
200
0
-200
Std Chow
Western Diet

Figure S5


A
Conventional C57/B6
% Survival
100
50
0
SC
0
20
40
60
80
Time (h)
B
Delta Weight (g)
2
0
-2
-4
-6
-8
20
40
60
Time (h)
2.5 mg/kg
5 mg/kg
10 mg/kg
50 mg/kg
100 mg/kg
C
Delta Weight (g)
0
-1
-2
-3
-4
Std Chow
WD
0
5
10
15
20
Time (h)