SUPPLEMENTARY MATERIALS

Type I Interferon Responses Drive Intrahepatic T cells to Promote Metabolic Syndrome

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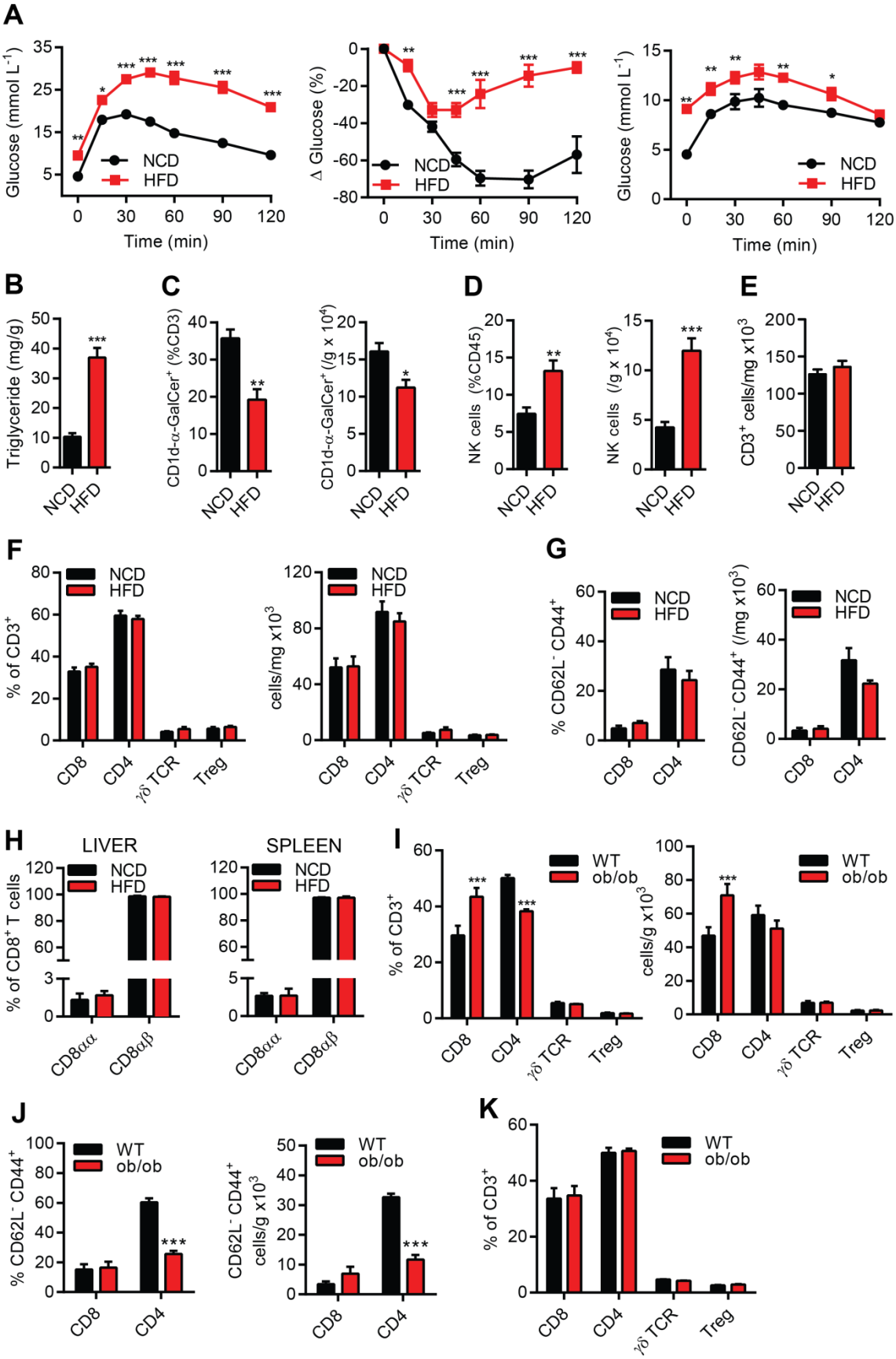
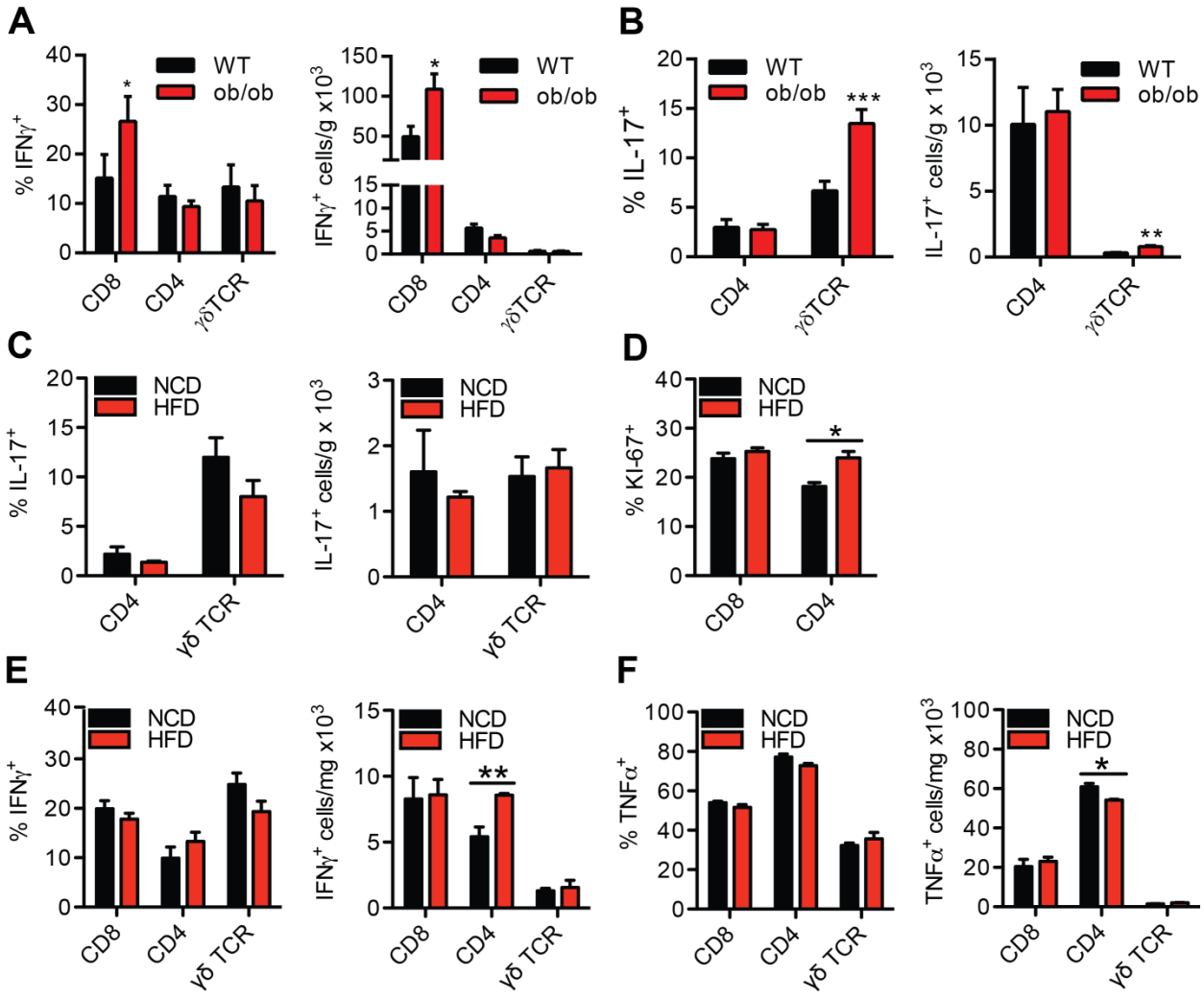
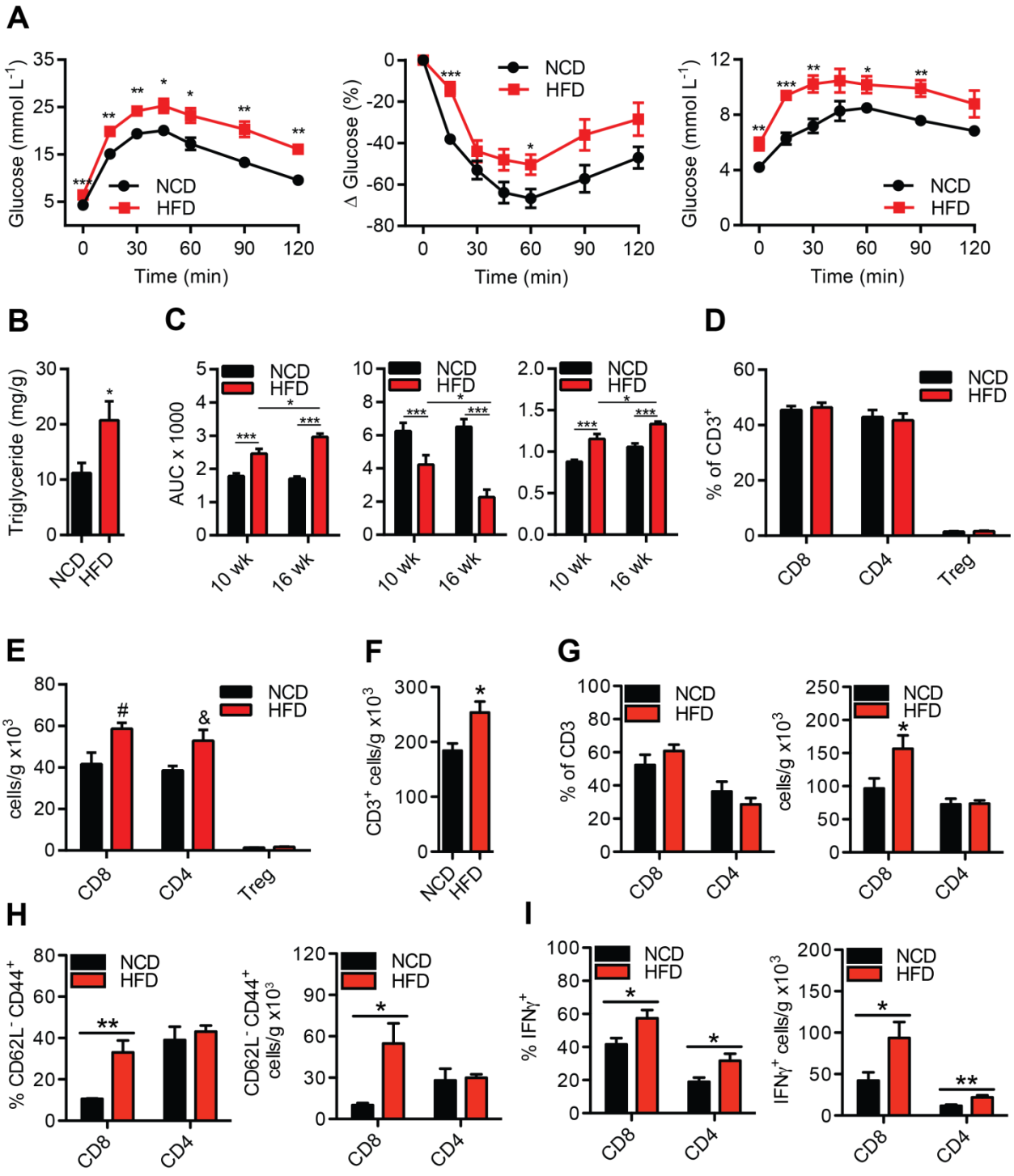

A
Glucose (mmol L-1)
Time (min)
NCD
HFD
Δ Glucose (%)
B
Triglyceride (mg/g)
C
CD1d-α-GalCer+ (%CD3)
CD1d-α-GalCer+ (/g x 10^4)
D
NK cells (%CD45)
NK cells (/g x 10^4)
E
CD3+ cells/mg x10^3
F
% of CD3+
cells/mg x10^3
CD8
CD4
γδ TCR
Treg
G
% CD62L- CD44+
CD62L- CD44+ (/mg x10^3)
H
LIVER
SPLEEN
% of CD8+ T cells
CD8αα
CD8αβ
I
WT
ob/ob
cells/g x10^3
J
CD62L- CD44+ cells/g x10^3
K

Supplemental Figure 1. Metabolic parameters and hepatic T cell subsets of HFD-fed and ob/ob mice. Metabolic testing of wildtype (WT) mice fed either a NCD or HFD for 16 weeks including **(A)** a glucose tolerance test (GTT; Left, n=5), insulin tolerance test (ITT; Center, n=5), pyruvate tolerance test (PTT; Right, n=5), and **(B)** liver triglyceride content. Flow cytometry analysis of livers from 16 wk HFD- or NCD-fed WT mice showing **(C)** the frequency and number of CD1d-restricted invariant NKT cells, as indicated by αGC-loaded CD1d tetramer staining (n=5) and **(D)** the frequency and number of CD45+CD3-NK1.1+ gated NK cells (n=8). Flow cytometry analysis of splenocytes isolated from 16 wk HFD- or NCD-fed WT mice showing the **(E)** total number of splenic T cells (n=4), **(F)** frequency (Left) and total number (Right) of CD8+, CD4+, γδTCR+, or CD4+Foxp3+ (Treg) gated splenic T cell subsets (n=4-7), and **(G)** CD8+ or CD4+ CD62L- CD44+ gated effector memory T cell subsets (n=3). Flow cytometry analysis of **(H)** hepatic (left) or splenic (right) CD8αα+ or CD8αβ+ cells (n=3). Flow cytometry analysis of 12 week-old ob/ob and WT mice showing the **(I)** frequency and number of CD45+CD3+NK1.1- gated hepatic T cell subsets (n=4-8), **(J)** CD8+ or CD4+ CD62L- CD44+ hepatic effector memory T cell subsets (n = 4), and the **(K)** frequency of splenic T cell subsets (n=3-7). Statistical significance is denoted by *($p<0.05$), **($p<0.01$), and ***($p<0.001$).

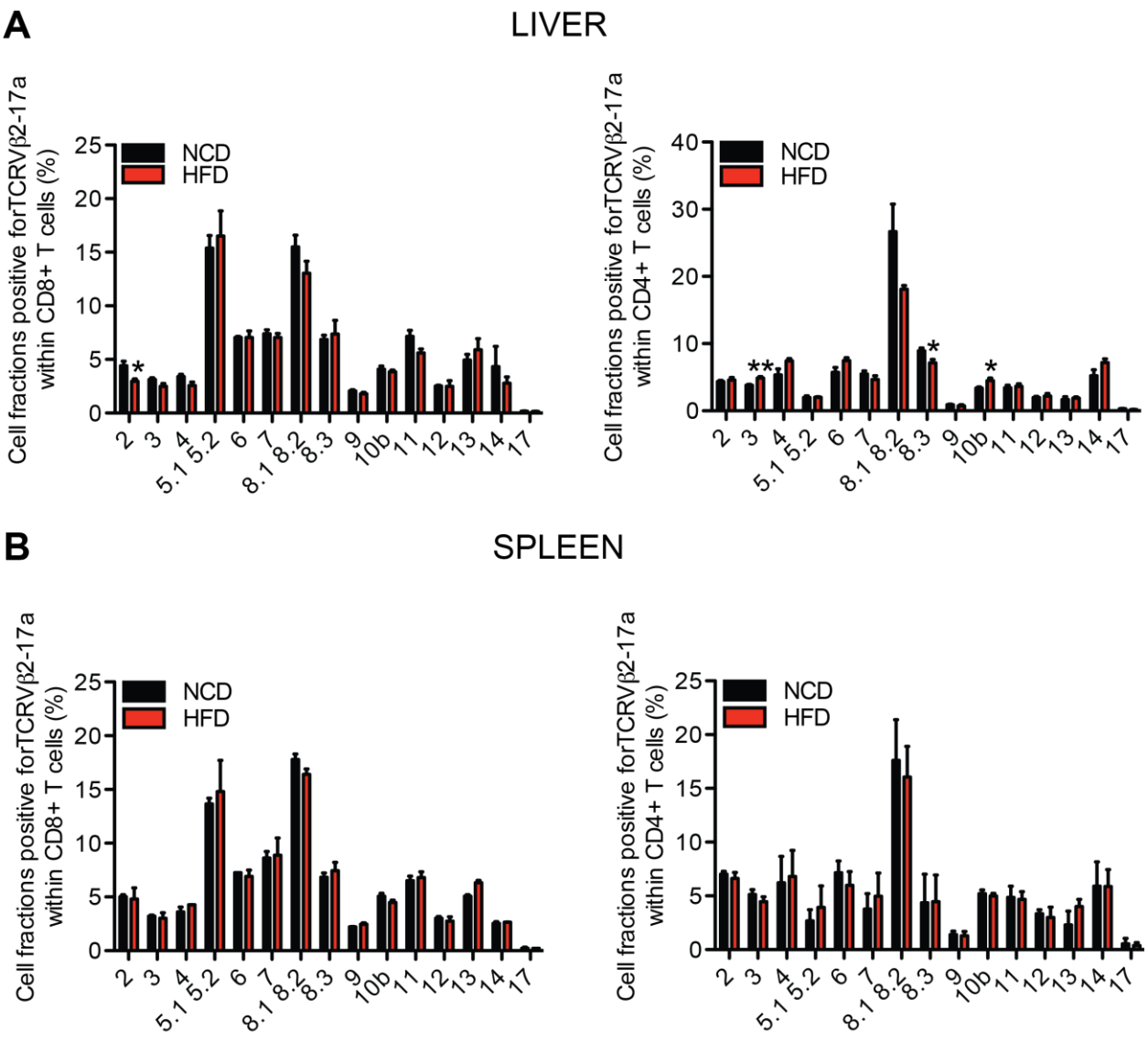


Supplemental Figure 2. Cytokine production by T cell subsets in the livers of ob/ob mice and WT HFD-fed mice, as well as spleens of 16 week HFD-fed mice. Intracellular stain for **(A)** IFNγ+ T cell subsets, **(B)** IL-17A+ CD4 and γδTCR T cells in the livers of 12-week old WT and ob/ob mice (n=4) and **(C)** IL-17A+ CD4 and γδTCR T cells in the livers of 16 week NCD- and HFD-fed WT mice (n=9). Intracellular stain for **(D)** KI-67+ (n = 3), **(E)** IFNγ+ (n=4), and **(F)** TNFα+ (n=3) in CD8+, CD4+ or γδTCR+ gated T cells in the spleen of NCD- or HFD-fed mice. Statistical significance is denoted by * ($p<0.05$), and ** ($p<0.01$).

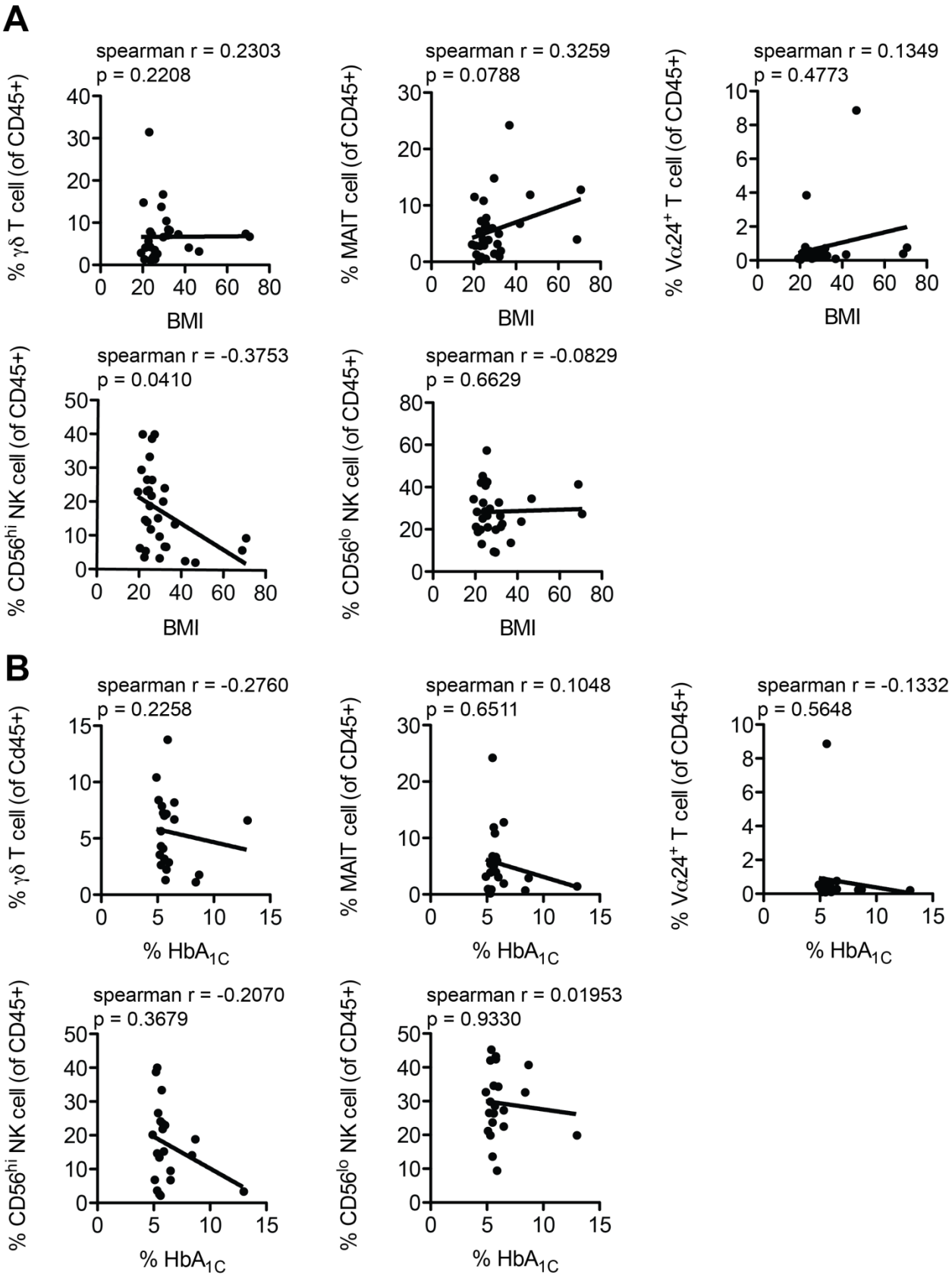


Supplemental Figure 3. Metabolic parameters and/or hepatic T cell populations of wildtype mice fed HFD for shorter (10 weeks) or longer (32 weeks) duration. Metabolic testing of mice fed either a NCD or HFD for 10 weeks including a **(A)** glucose tolerance test (GTT; Left, n=5), insulin tolerance test (ITT; Center, n=5), pyruvate

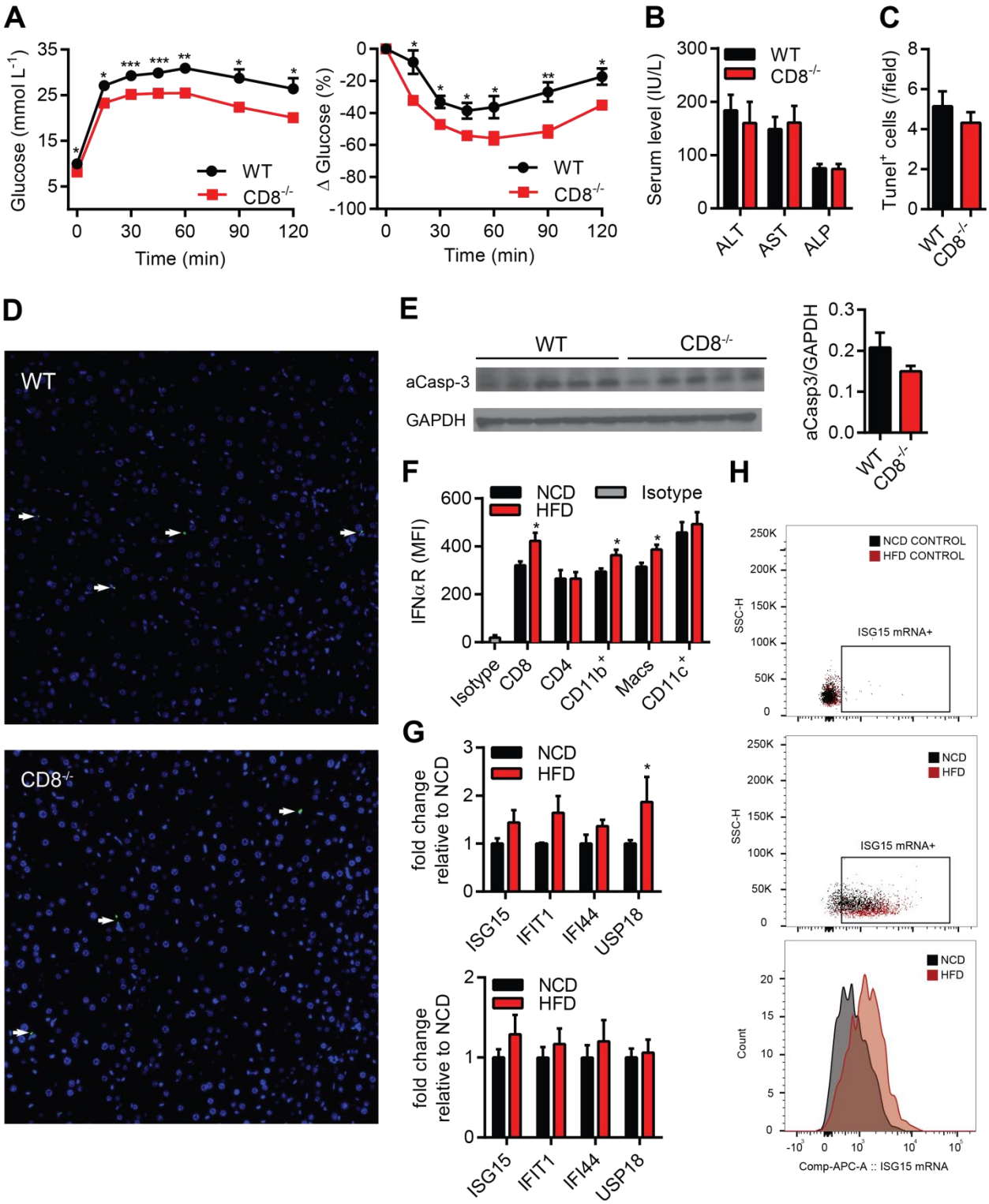
tolerance test (PTT; Right, n=5), and **(B)** liver triglyceride content. **(C)** Calculated areas under the curves (AUC) for the GTT (Left), ITT (Center), and PTT (Right) of mice fed either a NCD or HFD for 10 and 16 weeks (n=5). Flow cytometry analysis of liver immune cells isolated from 10 wk HFD- or NCD-fed WT mice representing the **(D)** frequency and **(E)** total number of hepatic T cells subset (n=3; $^{\#}p=0.051$, $^{\&}p=0.064$). Flow cytometry analysis of liver immune cells isolated from 32 wk HFD- or NCD-fed WT mice representing **(F)** total number of hepatic CD45+CD3+NK1.1- gated T cells per gram of tissue (n=5), **(G)** CD8+ or CD4+ gated hepatic T cell subsets (n=5), **(H)** CD8+ or CD4+ CD62L- CD44+ gated effector memory T cell subsets (n=5), and **(I)** intracellular T cell stains for IFNγ (n=5) in CD8+ or CD4+ gated T cells. Statistical significance is denoted by * ($p<0.05$), and ** ($p<0.01$).



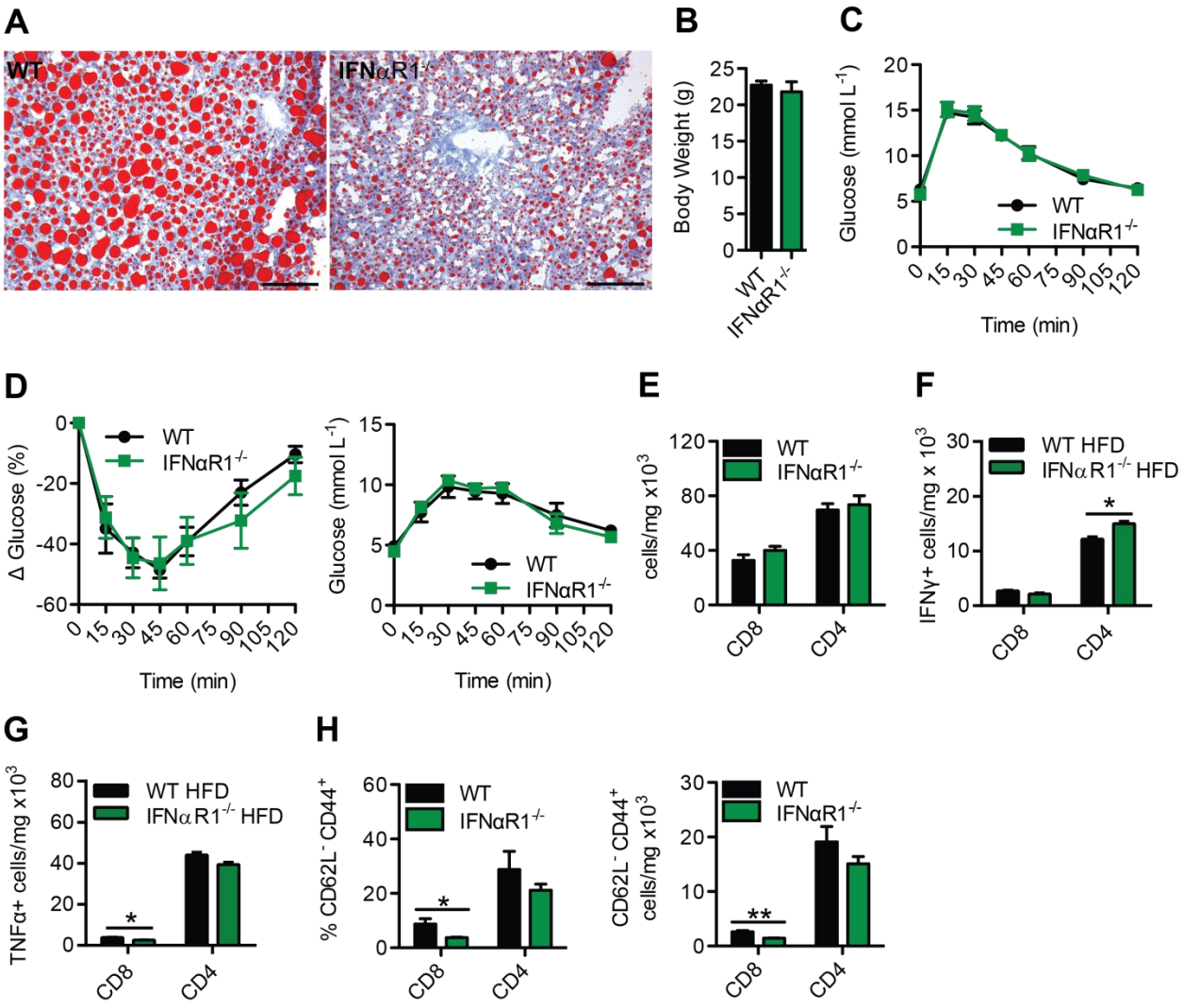
Supplemental Figure 4. Surface TCRvβ expression in hepatic and splenic T cells in HFD- or NCD-fed mice. Flow cytometry analysis of surface TCRvβ2-17a expression in 16 wk HFD- or NCD-fed WT mice representing **(A)** hepatic (n=4, 2 mice pooled per sample), and **(B)** splenic (n=2, 2 mice pooled per sample) CD45+CD3+NK1.1- CD8+ or CD4+ gated T cells. Statistical significance is denoted by * ($p<0.05$), and ** ($p<0.01$).


A
spearman r = 0.2303
p = 0.2208
% γδ T cell (of CD45+)
BMI
spearman r = 0.3259
p = 0.0788
% MAIT cell (of CD45+)
BMI
spearman r = 0.1349
p = 0.4773
% Vα24+ T cell (of CD45+)
BMI
spearman r = -0.3753
p = 0.0410
% CD56hi NK cell (of CD45+)
BMI
spearman r = -0.0829
p = 0.6629
% CD56lo NK cell (of CD45+)
BMI
B
spearman r = -0.2760
p = 0.2258
% γδ T cell (of Cd45+)
% HbA1C
spearman r = 0.1048
p = 0.6511
% MAIT cell (of CD45+)
% HbA1C
spearman r = -0.1332
p = 0.5648
% Vα24+ T cell (of CD45+)
% HbA1C
spearman r = -0.2070
p = 0.3679
% CD56hi NK cell (of CD45+)
% HbA1C
spearman r = 0.01953
p = 0.9330
% CD56lo NK cell (of CD45+)
% HbA1C

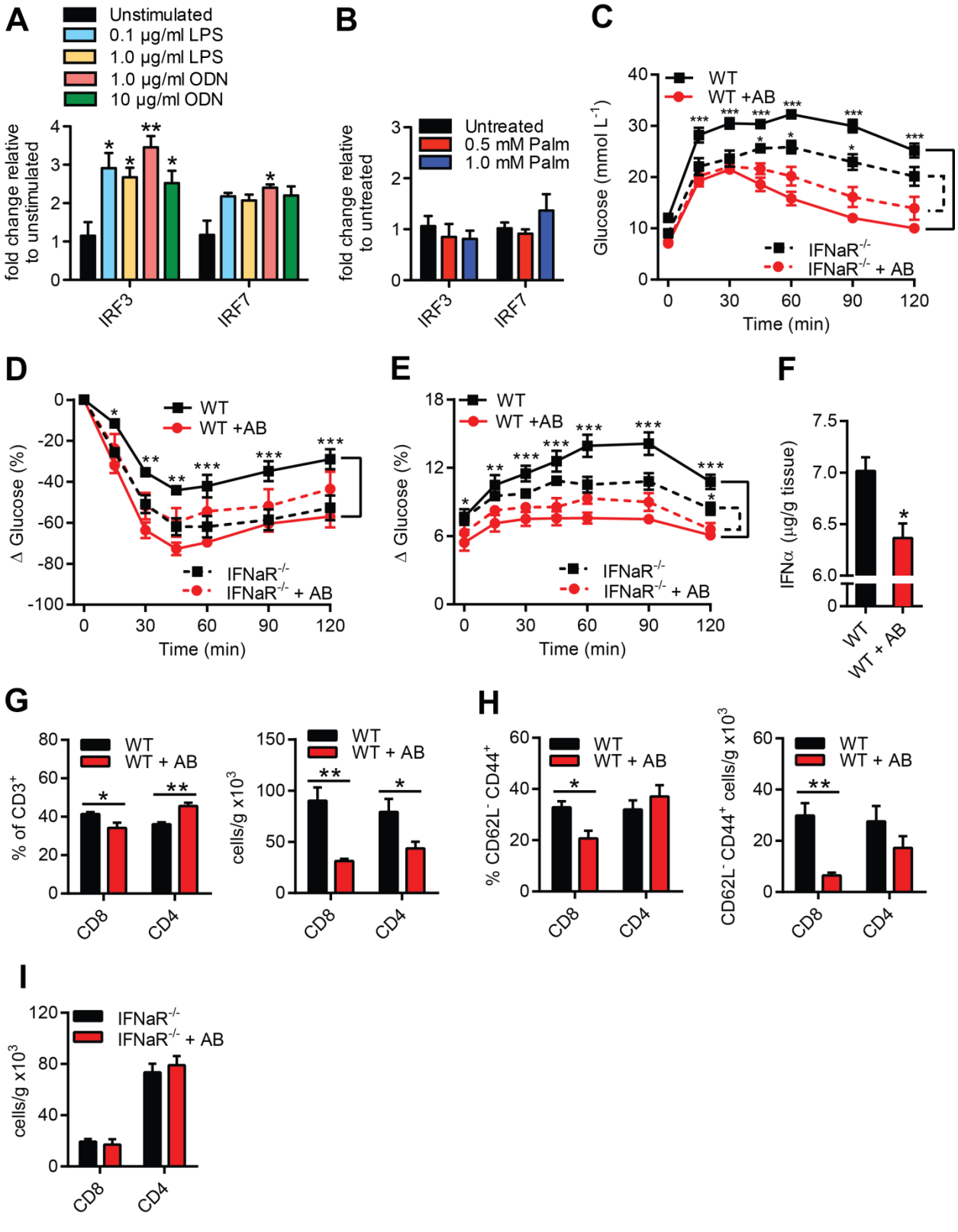
Supplemental Figure 5. Correlative analyses of human intrahepatic mononuclear cell subsets to BMI or % HbA1c. Correlations between the percentage of various intrahepatic mononuclear cell subsets of total CD45+ gated cells by flow cytometry to **(A)** BMI and **(B)** % HbA1c. Spearman r values and p values are indicated in figure graphs.


A
Glucose (mmol L-1)
Time (min)
WT
CD8-/-
Δ Glucose (%)
B
Serum level (IU/L)
ALT
AST
ALP
C
Tunel+ cells (/field)
D
WT
CD8-/-
E
aCasp-3
GAPDH
aCasp3/GAPDH
F
IFNαR (MFI)
NCD
HFD
Isotype
CD8
CD4
CD11b+
Macs
CD11c+
G
fold change relative to NCD
ISG15
IFIT1
IFI44
USP18
H
NCD CONTROL
HFD CONTROL
ISG15 mRNA+
SSC-H
Count
Comp-APC-A :: ISG15 mRNA

Supplemental Figure 6. Metabolic and liver function parameters of HFD-fed CD8$^{-/-}$ mice and expression of IFN-responsive genes in immune cells. Metabolic and liver function testing of WT and CD8$^{-/-}$ mice fed a HFD for 16 weeks including a **(A)** GTT (Left, n=5), ITT (Right, n=5), and **(B)** serum levels of alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP, n=5). TUNEL assay of liver sections from HFD-fed WT and CD8$^{-/-}$ mice (n=7) showing the **(C)** average number of TUNEL positive cells (4 fields, 100X magnification) and **(D)** representative images. **(E)** Representative western blot and analysis of liver lysates immunoblotted for active caspase-3 (aCasp-3) and GAPDH proteins in HFD-fed WT and CD8$^{-/-}$ mice (n=5). Flow cytometry analysis of liver immune cells isolated from 16 wk HFD- or NCD-fed WT mice representing the **(F)** mean fluorescent intensity (MFI) expression of IFNαR1 in CD8+ and CD4+ T cells, CD11b+ monocytes, F4/80+ macrophages, and CD11c+ cells (n=4-8). **(G)** Gene expression analysis of ISGs in CD11b+ monocytes (top, n=8) and CD4+ T cells (bottom, n=5) from the livers of 16 wk HFD- or NCD-fed WT mice. **(H)** Representative flow cytometry dot plots (Top, negative control; Middle) and histogram (Bottom) showing the expression of ISG15, in CD8+ T cells from NCD (black)- and HFD (red)-fed mice using RNA fluorescent in situ hybridization (FISH) followed by branched amplification and flow cytometry analysis (PrimeFlow®). Statistical significance is denoted by * ($p<0.05$), and ** ($p<0.01$).



Supplemental Figure 7. Additional metabolic assessment and T cell subset analysis of IFNαR1$^{-/-}$ mice. (A) Lipid staining by oil red O of liver sections from WT and IFNαR1$^{-/-}$ (n=3) mice fed a HFD for 16 weeks (200X magnification, Scale bar: 100μm). Metabolic testing of IFNαR1$^{-/-}$ mice fed a NCD including **(B)** body weight, **(C)** GTT (n=5), and **(D)** ITT (Left, n=5) and PTT (Right, n=5). Flow cytometry analysis of splenocytes isolated from 16 wk HFD-fed WT and IFNαR1$^{-/-}$ mice representing **(E)** splenic CD8+ and CD4+ T cells (n=4), intracellular T cell stains for **(F)** IFNγ and **(G)** TNFα (n=2), and **(H)** effector memory T cell subsets (n=4), and using previously stated gating strategies. Statistical significance is denoted by * ($p<0.05$), and ** ($p<0.01$).


A
Unstimulated
0.1 µg/ml LPS
1.0 µg/ml LPS
1.0 µg/ml ODN
10 µg/ml ODN
fold change relative to unstimulated
IRF3
IRF7
B
Untreated
0.5 mM Palm
1.0 mM Palm
fold change relative to untreated
IRF3
IRF7
C
WT
WT +AB
IFNaR-/-
IFNaR-/- + AB
Glucose (mmol L-1)
Time (min)
D
WT
WT +AB
IFNaR-/-
IFNaR-/- + AB
Δ Glucose (%)
Time (min)
E
WT
WT +AB
IFNaR-/-
IFNaR-/- + AB
Δ Glucose (%)
Time (min)
F
IFNα (µg/g tissue)
WT
WT + AB
G
WT
WT + AB
% of CD3+
cells/g x10^3
CD8
CD4
H
WT
WT + AB
% CD62L- CD44+
CD62L- CD44+ cells/g x10^3
CD8
CD4
I
IFNaR-/-
IFNaR-/- + AB
cells/g x10^3
CD8
CD4

Supplemental Figure 8. Bacterial products drive hepatic type I interferon production and T cell inflammation. RT-PCR gene expression analysis of IRF3 and IRF7 from primary hepatocyte cell lysates that were treated with **(A)** 0.1 or 1.0 μg/ml LPS and 1.0 or 10 μg/ml unmethylated CpG (ODN2395) (n=3), and **(B)** 0.5 or 1.0 mM sodium palmitate for 12 hrs at 37°C (n=3-6). Metabolic testing of HFD-fed WT and IFNαR1$^{-/-}$ mice including a **(C)** GTT (n=5), **(D)** ITT (n=5), and **(E)** PTT (n=5) after 4 weeks of treatment with either antibiotics (AB) or control water. **(F)** Quantification of IFNα protein, **(G)** flow cytometry analysis of total CD8+ and CD4+ T cells, **(H)** CD8+ and CD4+ T effector memory cells of antibiotic-treated HFD-fed WT mice (n=5). **(I)** Flow cytometry analysis of total CD8+ and CD4+ T cells in antibiotic-treated HFD-fed IFNαR1$^{-/-}$mice (n=5). Statistical significance is denoted by * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$).

Supplemental Table 1. Fluorophore-conjugated antibodies used for flow cytometry

Antibody	**Clone, Supplier**
AlexaFluor 700 anti-mouse CD45.2	104, BioLegend
FITC anti-mouse CD45.1	A20, BioLegend
AlexaFluor 647 anti-mouse CD3ε	145-2C11, BioLegend
PE-Cy7 anti-mouse CD4	RM4-5, BioLegend
PE-Cy5 anti-mouse CD8a	53-6.7, BioLegend
APC-Cy7 anti-mouse NK1.1	PK136, BioLegend
PerCP-Cy5.5 anti-mouse γδ TCR	GL3, BioLegend
FITC anti-mouse CD62L	MEL-14, BioLegend
PerCP anti-mouse CD44	IM7, BioLegend
BV421 anti-mouse IFN-γ	XMG1.2, BioLegend
PE-Cy7 anti-mouse TNF-α	MP6-XT22, BioLegend
PE anti-mouse Ki-67	16A8, BioLegend
PE anti-mouse IFNAR1	MAR1-5A3, BioLegend
AlexaFluor 488 anti-mouse FOXP3	150D, BioLegend
FITC anti-mouse CD4	GK1.5, BioLegend
PE anti-mouse IL-17A	TC11-18H10.1, BioLegend

Supplemental Table 2. Primer sets used for real-time-PCR

Gene	**Primer Sequences**
G6Pc	F: 5'-CGACTCGCTATCTCCAAGTGA-3' R: 5'-GTTGAACCAGTCTCCGACCA-3'
PEPCK1	F: 5'-CTGCATAACGGTCTGGACTTC-3' R: 5'-CAGCAACTGCCCGTACTCC-3'
IRF3	F: 5'-GAGAGCCGAACGAGGTTCAG-3' R: 5'-CTTCCAGGTTGACACGTCCG-3'
IRF7	F: 5'-GAGACTGGCTATTGGGGGAG-3' R: 5'-GACCGAAATGCTTCCAGGG-3'
ISG15	F: 5'-GGTGTCCGTGACTAACTCCAT-3' R: 5'-TGGAAAGGGTAAGACCGTCCT -3'
IFIT1	F: 5'-CTGAGATGTCACTTCACATGGAA-3' R: 5'-GTGCATCCCCAATGGGTTCT-3'
IFIT2	F: 5'-AGTACAACGAGTAAGGAGTCACT-3' R: 5'-AGGCCAGTATGTTGCACATGG-3'
IFI44	F: 5'-AACTGACTGCTCGCAATAATGT-3' R: 5'-GTAACACAGCAATGCCTCTTGT-3'
USP18	F: 5'-TTGGGCTCCTGAGGAAACC-3' R: 5'-CGATGTTGTGTAAACCAACCAGA-3'
GAPDH	F: 5'-TCACCACCATGGAGAAGGC-3' R: 5'-GCTAAGCAGTTGGTGGTGCA-3'

Supplemental Experimental Procedures

Mice. Knockout or transgenic Mice had been backcrossed at least 10 generations on C57BL/6J background and maintained in a pathogen-free, temperature-controlled environment on a 12 hr light and dark cycle at the Toronto Medical Discovery Tower facility.

Metabolic Studies. After 16 weeks on diet, fasting blood glucose and insulin levels were measured (Crystal Chem Inc ELISA). For glucose and pyruvate tolerance tests, mice were fasted for 16 hours and then injected intraperitoneally (i.p.) with 1.5 g/kg (of body weight) D-glucose or sodium pyruvate (Sigma) in PBS. For insulin tolerance tests, mice were fasted for 6 hours and then injected i.p. with 0.75 U/kg (of body weight) insulin (Eli Lilly) in PBS. Blood glucose concentrations were determined at indicated time points using a Contour Next EZ (Bayer) glucometer and Contour Next strips (Bayer). Serum alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) were measured by the Centre for Phenogenomics (Mount Sinai Hospital).

Processing of immune cells from tissues. Perfused livers were disrupted using a gentleMACS tissue dissociator and resuspended in RPMI-1640 media containing 10% FBS. This suspension was centrifuged at 60 g for 1 min and the supernatant containing hepatic immune cells was collected and centrifuged again at 480 g for 8 min. The pellet was resuspended in 10 mL of 37.5% Percoll (Sigma) in PBS and centrifuged at 850 g for 30 min. The resulting pellet was washed followed by red blood cell lysis in ammonium-chloride-potassium (ACK) lysis buffer for 2 min at room temperature. VAT cells were isolated from epididymal VAT pads that were mashed and digested in collagenase (0.2 mg/ml) DMEM at 37°C for 45 min. Cells were then washed, pelleted

and filtered through a 70-μm nylon cell strainer, followed by red blood cell lysis and washed to obtain stromal vascular cells (SVC) containing immune cells. Splenocytes were isolated by mechanically dissociating spleen tissue on 70-μm nylon cell strainers, followed by red blood cell lysis and washing.

Flow Cytometry. TCRVβ studies were performed using the Mouse TCRVβ Screening Panel (BD Pharmingen). For intracellular staining of T cells, cells were incubated with Cell Stimulating Cocktail and Golgistop (eBioscience) for 5 hours at 37°C in lymphocyte culture media as described. Cells were then treated with fixation/permeabilization buffer (eBioscience) for 1 hour at 4°C and stained. For detection of NKT cells, we stained intrahepatic cells with CD1d tetramers loaded with the marine sponge glycosphingolipid α-galactosylceramide (αGalCer, NIH Tetramer Core Facility) for 30 min at 4°C followed by cell surface staining. Expression of ISG15 mRNA was analyzed in CD8+ surface-stained cells by flow cytometry using the PrimeFlow Assay kit (Affymetrix eBioscience) according to the manufacturer's protocols. All data were acquired on a Fortessa flow cytometer (BD Bioscience) and analyzed using FlowJo software (Tree Star).

Mixed Bone Marrow Chimeras. Six week old WT CD45.1 mice were irradiated with 1100 cGy divided in two cycles of 550 cGy on the same day using a Cesium-137 irradiator, and reconstituted with intravenous (i.v.) injections of $5x10^6$ BM mixtures (in 100μl PBS) of either (1) 85% $CD8^{-/-}$ and 15% $IFN\alpha R1^{-/-}$ BM cells, or (2) 85% $CD8^{-/-}$ and 15% WT BM cells from 6 week old $CD8^{-/-}$, $IFN\alpha R1^{-/-}$, or WT CD45.2 mice. BM cells were obtained by flushing mouse femurs and tibias with PBS using a 26-guage needle, dissociated into single-cell suspension, hemolyzed, washed in PBS and counted. BM chimeric mice were treated with Baytril drinking water for 2 weeks after BM transfer,

then fed a HFD for 16 weeks. Chimerism (>95%) and IFNαR1 expression was evaluated by flow cytometry.

Treatment of Primary Hepatocytes. Livers were perfused with digestion media containing collagenase (1 mg/ml) in DMEM then PBS. Livers were mechanically dissociated and single cell suspensions were centrifuged three times for 2 min at 60 g to eliminate small cells residing in supernatants. From the final pellet containing hepatocytes, $1x10^6$ cells were seeded onto 6 well plates and left over night in culture medium.

Western Blotting. Mice were fasted overnight and injected i.p. with 1.5 U/kg of insulin in PBS. Livers were harvested 15 min after acute insulin injection and snap frozen in liquid nitrogen. Tissue was mechanically homogenized in ice-cold lysis buffer (Santa Cruz) and protein concentration was determined using the Pierce BCA Protein Assay (Thermo Scientific). Protein lysates were separated by SDS-PAGE and immunoblotted with antibodies (Cell Signaling Technology) against Akt (Cell Signaling Technology) and Ser473-phosphorylated Akt (Signal Way Antibody), GAPDH or β-actin (Santa Cruz Biotechnology). The results are shown as fold changes relative to the pAkt/Akt ratio of the control group.

Histology and Human Liver Biopsies. Mouse liver tissue was fixed for 48 hrs in 10% buffered formalin before sectioning onto slides for hematoxylin-eosin staining and TUNEL (Roche). For Oil-red-O staining, mouse liver tissues were snap frozen before staining with Oil-red-O to visualize neutral lipids. The patient cohort consisted of 13 males and 6 females with an average age of 44.26. When selecting the patient cohort, cirrhosis was an exclusion criterion. Each human sample was given a NAFLD Activity Score (NAS), quantified for CD8+ cells per high power field (HPF = 0.237 mm^2) and

given an Expression Score for the degree of IRF3 or ISG15 staining. Expression scores for IRF3 and ISG15 were based on the intensity of staining (scored with range of 0-3, with 3 being most intense) and percentage of cells staining (0, 1 being 0-30%, 2 being 30-60%, 3 being 60-100%). The intensity and percentage scores were added to form the expression score out of 6 for both hepatocytes and mononuclear cells, and these scores were added to form a total expression score out of 12, which is plotted.

Isolation of Human Intrahepatic Mononuclear Cells. Peripheral blood was removed by aortic flush and the liver-associated mononuclear cells were collected by liver-specific perfusion with either HTK or University of Wisconsin solution. Mononuclear cells were isolated by density gradient centrifugation.