Supplemental Information

Adipose natural killer cells regulate adipose tissue macrophages to promote insulin resistance in obesity

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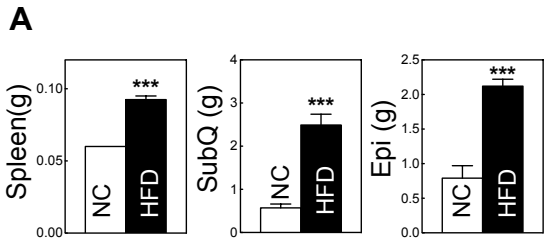
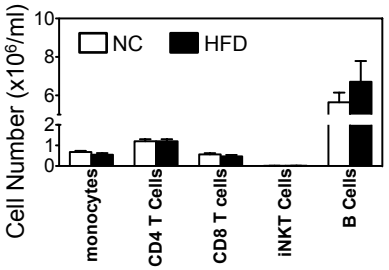
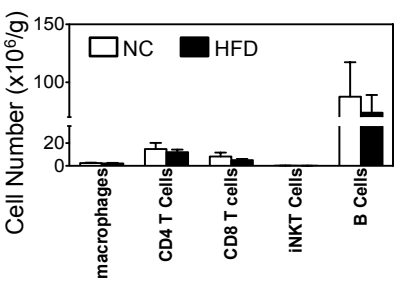
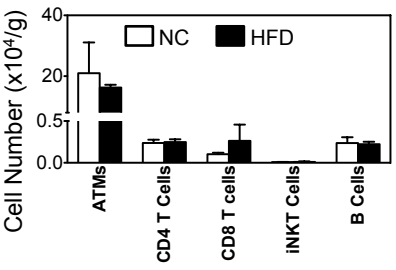
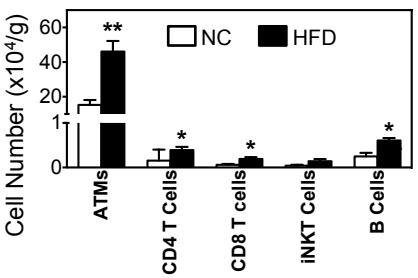
Figure S1 (Related to Figure 1). HFD increases organ weights and the numbers of epididymal fat immune cells.

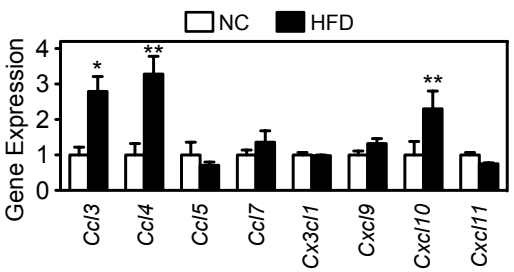


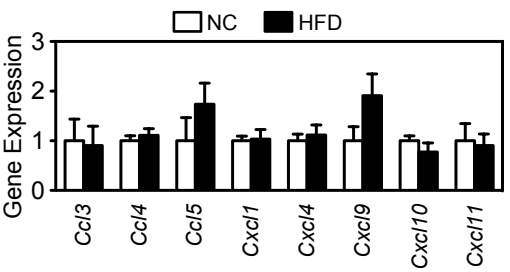


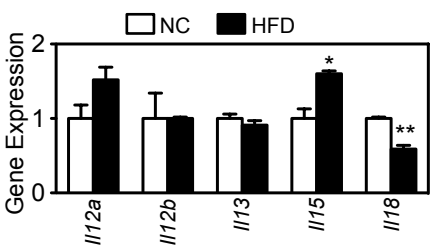












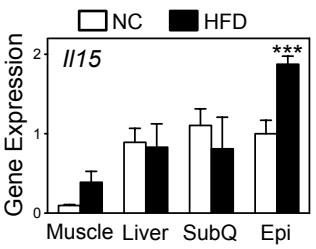


Figure S2 (Related to Figure 2). Tissue lymphocyte numbers and weights after anti-asialo GM1 antibody-mediated NK cell depletion and recovery of NK cells.

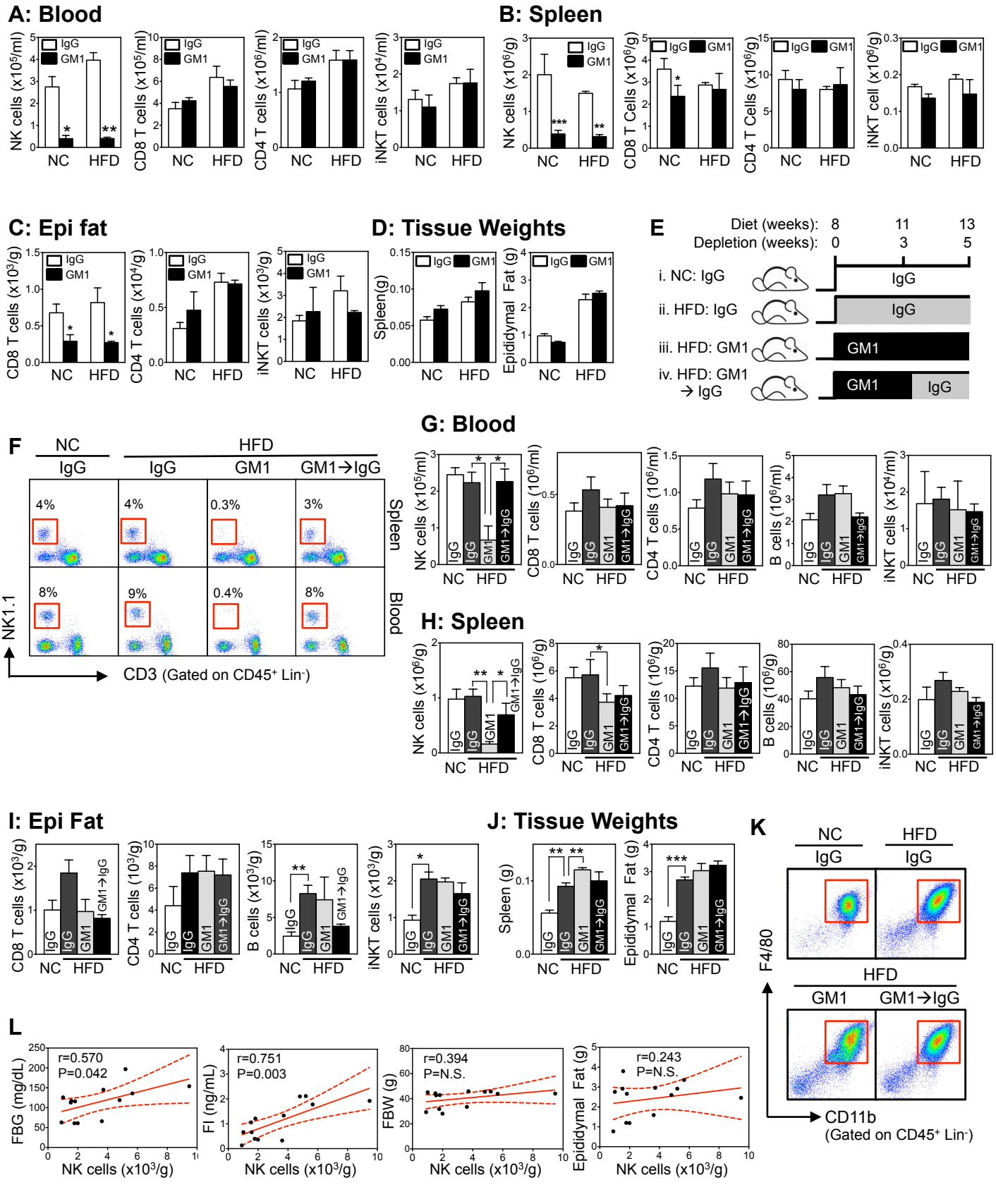


Figure S3 (Related to Figure 3). Tissue immune cell numbers and weights after anti-NK1.1 antibody-mediated NK cell depletion.

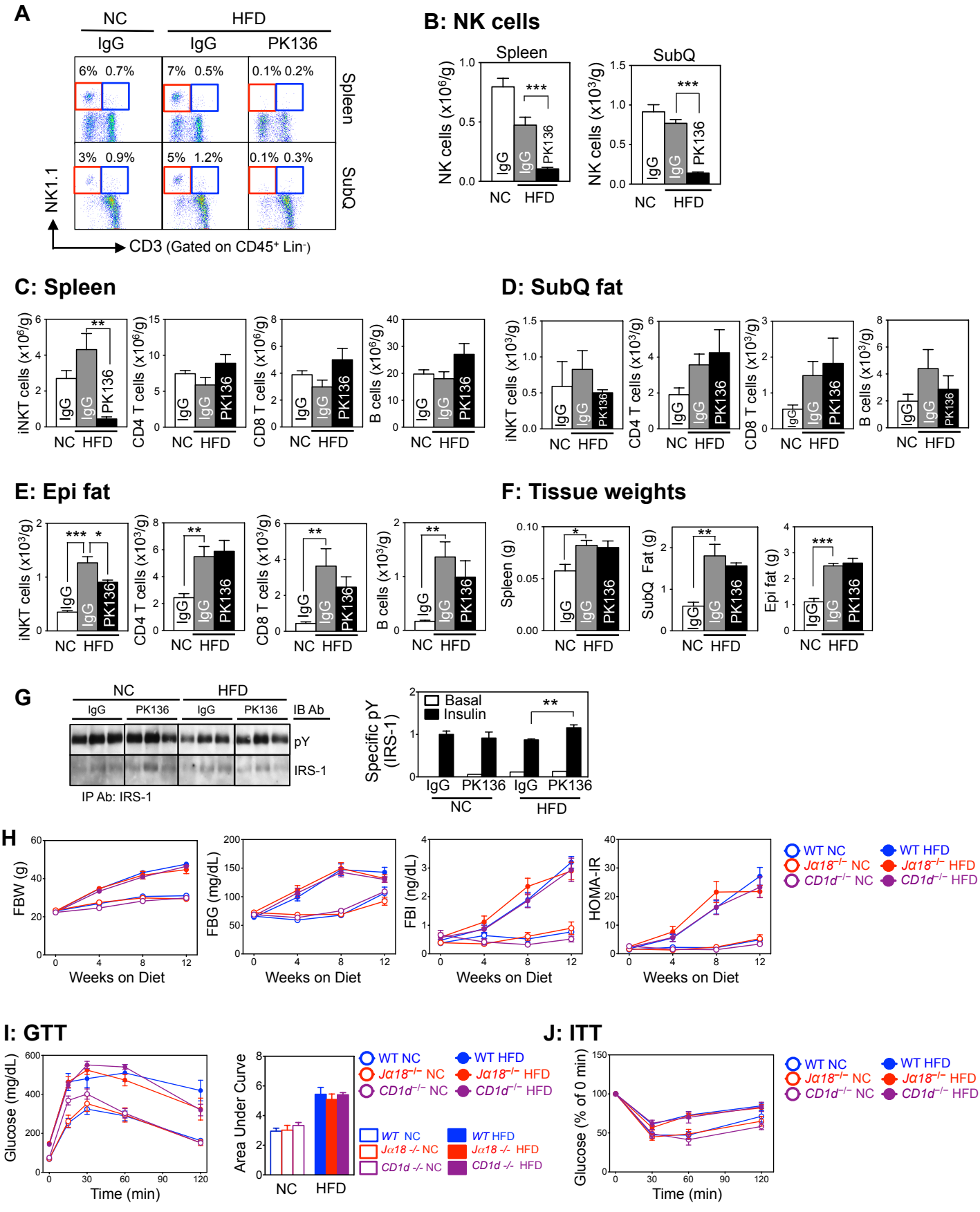


Figure S4 (Related to Figure 4). Tissue lymphocyte numbers and weights after IL-15-mediated NK cell expansion.

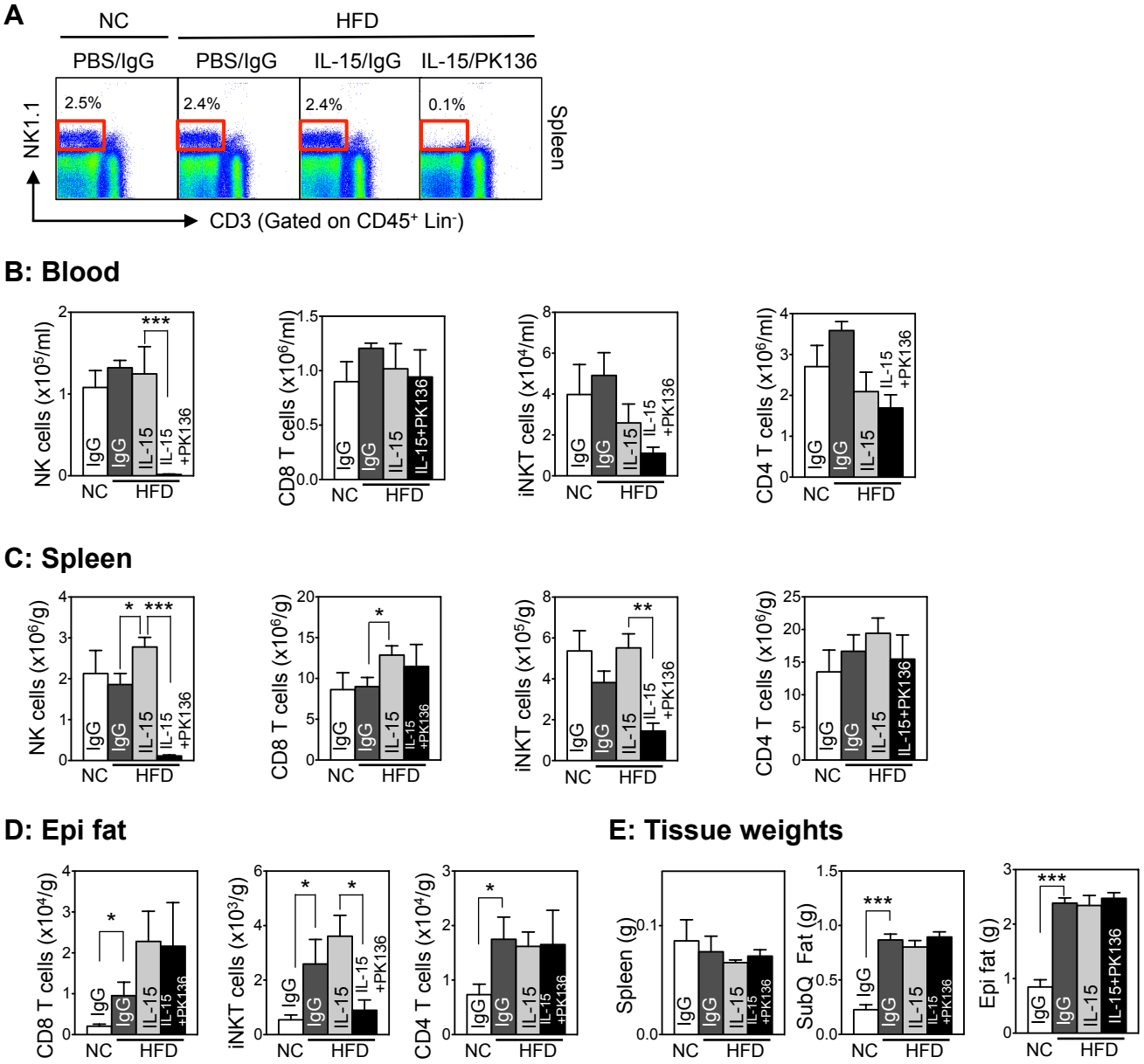


Figure S5 (Related to Figure 5). Genetic ablation of NK cells in *E4bp4* knockout mice associates with improved HFD-induced insulin resistance.

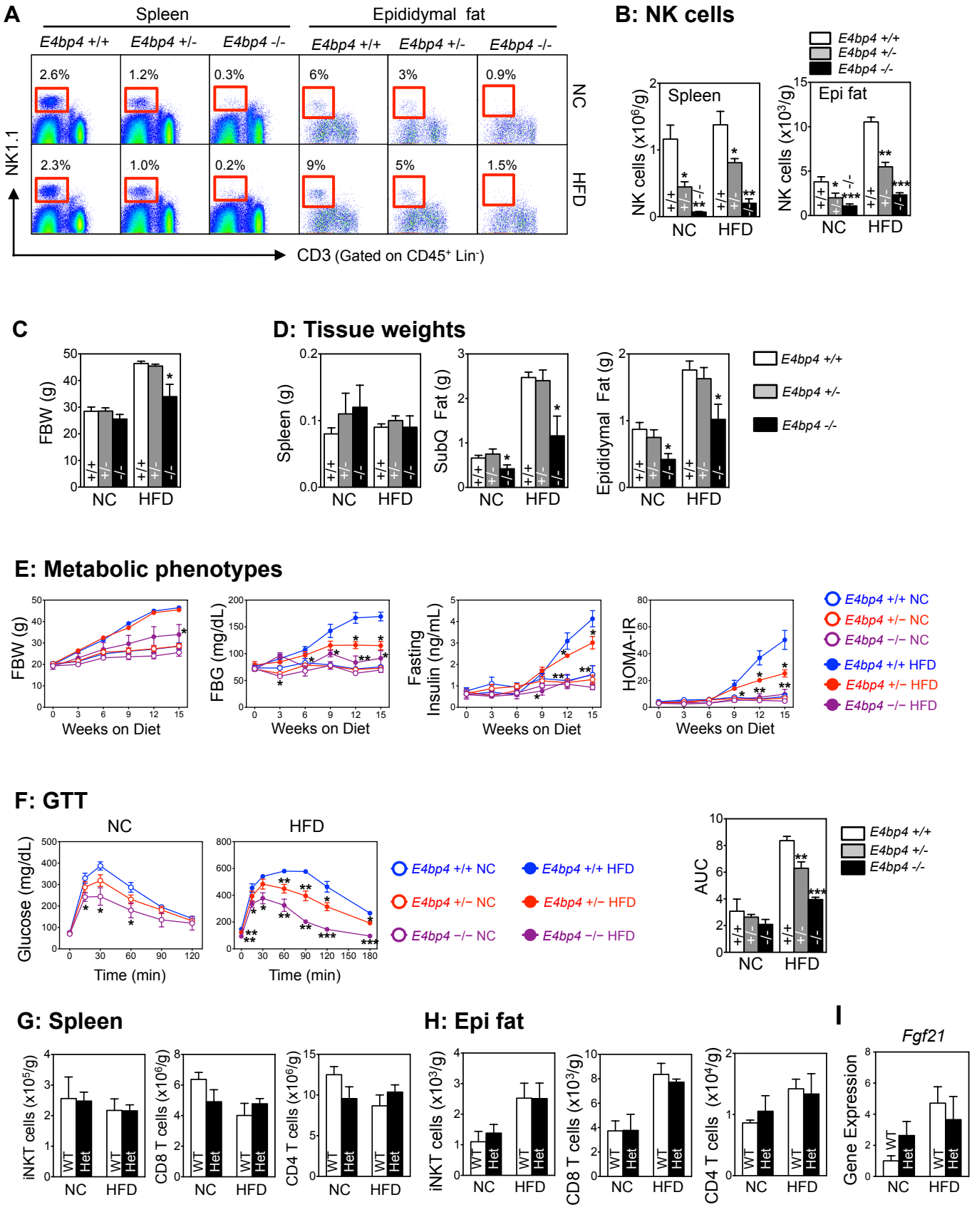
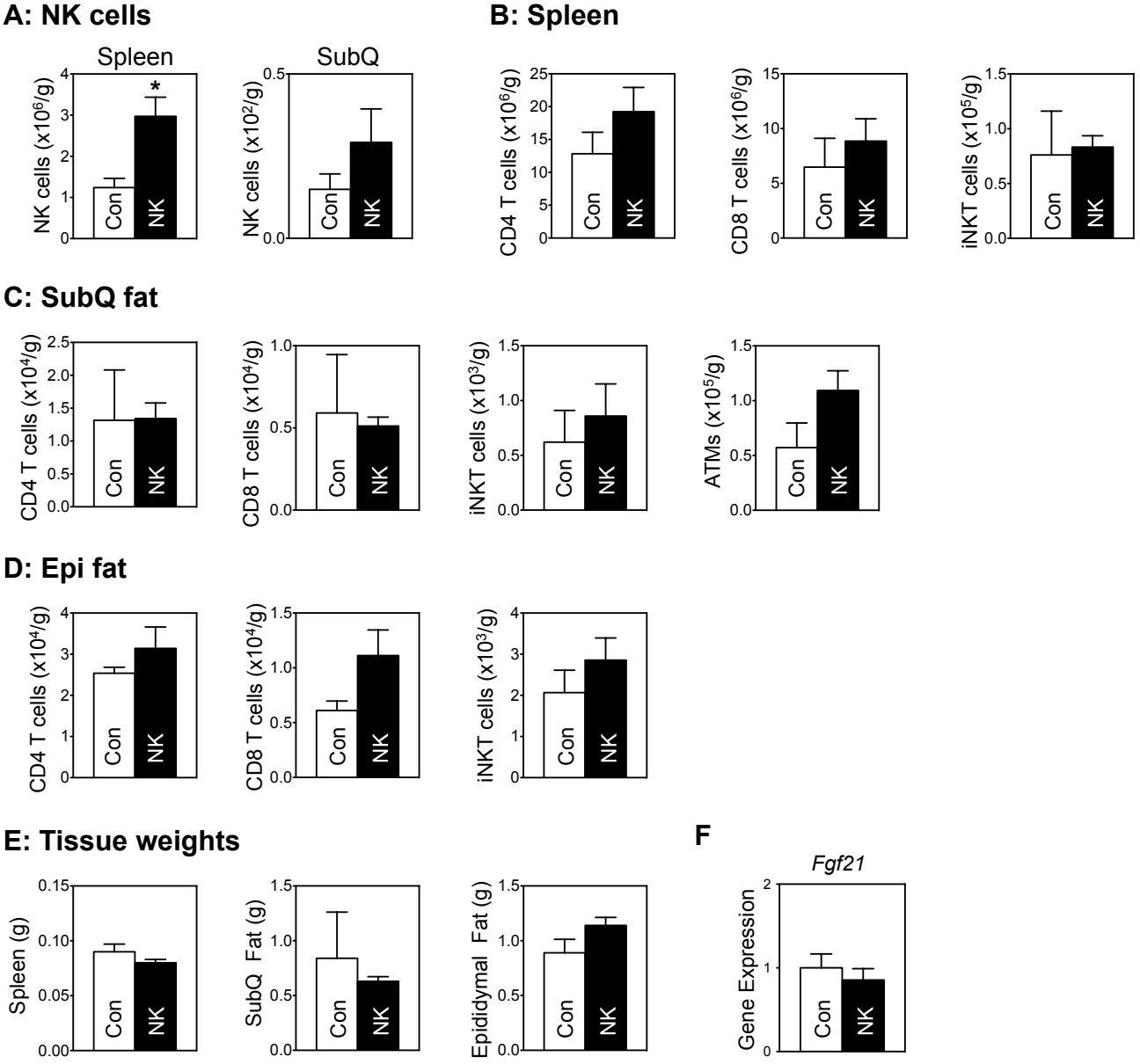


Figure S6 (Related to Figure 6). Tissue lymphocyte numbers and weights in NK cell-reconstituted HFD-fed *E4bp4-/-* homozygous knockout mice.



Supplemental Information

All experiments were repeated at least twice except for the depletion/recovery experiment with anti-asialo GM1 antibody (Figure 2G-P and S2E-J) and the *in vivo* insulin signaling experiment (Figure 3I, 3J, and S3G). Those experiments were performed only once. The data are presented as mean ± S.E.M. P-values <0.05 were considered to indicate statistical significance.

Supplemental Figure Legends

Figure S1 (Related to Figure 1). HFD increases organ weights and the numbers of epididymal fat immune cells.

Starting at 7 weeks of age, C57BL/6 male mice were given a HFD or NC for 12 weeks (n=5–7/group). (A) Tissue weights after overnight fasting. (B–E) Numbers of specific immune cells in the blood (B), spleen (C), and subcutaneous (SubQ) (D) and epididymal (E) fat stromal vascular cells (SVCs), as measured by flow cytometry and normalized by blood volume or tissue weights. NK cell chemokine gene expression levels in sorted ATMs (F) and epididymal fat (G). Expression of genes that are important for NK cell proliferation and activation in sorted ATMs (H) and epididymal fat (I). Gene expression was determined by real-time RT-PCR. The data are presented as mean ± S.E.M. For all figures: *p<0.05, **p<0.01, and ***p<0.001.

Figure S2 (Related to Figure 2). Tissue lymphocyte numbers and weights after anti-asialo GM1 antibody-mediated NK cell depletion and recovery of NK cells.

(A–D) C57/B6 mice fed NC or HFD for 8 weeks were injected with anti-asialo GM1 or isotype-control (IgG) antibodies for another 4 weeks while continuing their diet (n=4–5/group). After overnight fasting, tissue immune cell numbers and tissue weights were measured. (A–C) Immune cell numbers in the blood (A), spleen (B), and epididymal fat SVCs (C), as measured by flow cytometry and normalized by blood volume or tissue weights. (D) Tissue weights. (E–J) C57/B6 mice fed NC or a HFD for 10 weeks were injected with GM1 or control (IgG) antibodies for another 3 weeks while continuing their diet. The GM1-injected HFD-fed mice were then divided into two groups: one group continued to be injected with GM1 while the other group was switched from GM1 to the control antibody (IgG) to allow the NK cell population to recover from the GM1-mediated NK cell depletion (GM1→IgG). After 2 weeks of NK cell recovery, tissue lymphocyte numbers and tissue weights were measured after an overnight fast (n=4–5/group). (E) Schematic depiction of the experiment. (F) Representative flow cytometric profiles of blood and splenic NK cells (red boxes) after depletion and recovery. The lineage markers (Lin) were TER-119, Gr-1, CD19, and CD19. (G–I) Lymphocyte numbers in the blood (G), spleen (H), and epididymal SVCs (I) after NK cell depletion and recovery, as measured by flow cytometry and normalized by blood volume or tissue weights. (J) Tissue weights. (K) Representative flow cytometric plots of epididymal ATMs (red box). The lineage markers were TER-119, CD3, CD19, and NK1.1. (L) Correlation between epididymal fat NK cell numbers after depletion and after recovery, and fasting blood glucose, fasting insulin, body weight, and fat weight. The data are presented as mean ± S.E.M. For all figures: *p<0.05, **p<0.01, and ***p<0.001.

Figure S3 (Related to Figure 3). Tissue immune cell numbers and weights after anti-NK1.1 antibody-mediated NK cell depletion.

(A–F) C57/B6 mice fed a HFD or NC for 8 weeks were injected with PK-136 antibody for another 4 weeks while continuing their diet. After overnight fasting, their immunological and metabolic phenotypes were measured (n=4–5/group). (A) Representative flow cytometric profiles of the NK cells in the spleen and subcutaneous fat SVCs after NK cell depletion. Red boxes, the NK cell population; blue boxes, the iNKT cell population. The lineage markers (Lin) were TER-119, Gr-1, CD19, and F4/80. (B) NK cell numbers in the spleen and subcutaneous fat SVCs (SubQ), as measured by flow cytometry and normalized by tissue weights. (C–E) NKT, CD4 T, CD8 T, and B cell numbers in the spleen (C) and subcutaneous (D) and epididymal (E) fat SVCs after NK cell depletion, as measured by flow cytometry and normalized by tissue weights. (F) Tissue weights. (G) Representative Western blot of IRS-1 in muscle (left) and quantitation

(right). (H-J) The constitutive depletion of iNKT cells in *Jα18*−/− and *CD1d*−/− homozygous knockout mice does not alter insulin sensitivity under either NC or HFD conditions. Six-week-old iNKT cell-deficient homozygous knockout mice (*Jα18*-/- and *CD1d*-/-) were fed with NC or a HFD for 12 weeks. (H) After overnight fasting, the metabolic phenotypes (fasting body weights, fasting blood glucose, fasting insulin, and HOMA-IR) over time were assessed (n=8–11/group). (I) Glucose tolerance test (GTT) and the AUC after overnight fasting. (J) Insulin tolerance tests (ITT) after 6 hours of fasting. The HFD-fed *Jα18*-/- and *CD1d*-/- mice did not differ from the HFD-fed wild-type mice. Similarly, the NC-fed *Jα18*-/- and *CD1d*-/- did not differ from the NC-fed wild-type mice. The data are presented as mean ± S.E.M. For all figures: *p<0.05, **p<0.01, and ***p<0.001.

Figure S4 (Related to Figure 4). Tissue lymphocyte numbers and weights after IL-15-mediated NK cell expansion.

C57BL/6 mice fed a HFD for 6 weeks were injected with PBS or IL-15 for another 4 weeks to expand the NK cells. To confirm that NK cells were responsible for the observed changes in metabolic phenotypes, the IL-15-injected HFD-fed mice were co-injected with PK-136 or the control antibody (IL-15/PK-136 and IL-15/IgG, respectively). Mice fed NC served as a control (n=5–7/group). (A) Representative flow cytometric profiles of the splenic NK cells (red boxes) after NK cell expansion with and without concomitant depletion. The lineage markers (Lin) were TER-119, Gr-1, CD19, and F4/80. (B-D) Numbers of lymphocytes in the blood (B), spleen (C), and epididymal fat SVCs (D) after NK cell expansion with and without concomitant depletion, as measured by flow cytometry and normalized by blood volume or tissue weights. (E) Tissue weights. The data are presented as mean ± S.E.M. For all figures: *p<0.05, **p<0.01, and ***p<0.001.

Figure S5 (Related to Figure 5). Genetic ablation of NK cells in *E4bp4* knockout mice associates with improved HFD-induced insulin resistance.

(A–F) *E4bp4* -/- homozygous knockout mice, *E4bp4*+/- heterozygous knockout mice, and littermate control *E4bp4*+/+ wild-type mice (WT) were fed NC or a HFD for 15 weeks. (A) Representative flow cytometric profiles of the splenic and epididymal NK cell populations (red boxes) at 15 weeks. The lineage markers (Lin) were TER-119, Gr-1, CD19, and F4/80. (B) Splenic and epididymal adipose NK cell numbers at 15 weeks, as measured by flow cytometry and normalized by tissue weights. (C and D) Fasting body weights (C) and tissue weights (D) at 15 weeks. (E) Fasting body weights, fasting blood glucose, fasting insulin, and HOMA-IR were measured every 3 weeks. (F) glucose tolerance test (GTT) and the AUC of homozygous, heterozygous, and wild-type control mice fed NC or a HFD for 13 weeks. (G and H) T-cell subpopulation numbers in the spleen and epididymal fat from NC- and HFD-fed *E4bp4*+/- heterozygous knockout mice. The splenocytes (G) and epididymal adipose SVCs (H) from the *E4bp4*+/- heterozygous mice and littermate control *E4bp4*+/+ wild-type mice (WT). (I) Expression levels of *Fgf21* mRNA in the liver, as determined by qRT-PCR. The data are presented as mean ± S.E.M. For all figures: *p<0.05, **p<0.01, and ***p<0.001.

Figure S6. (Related to Figure 6) Tissue lymphocyte numbers and weights in NK cell-reconstituted HFD-fed *E4bp4-/-* homozygous knockout mice.

E4bp4-/- homozygous knockout mice fed a HFD for 8 weeks were reconstituted with NK cells isolated from the spleen of WT mice fed a HFD for 8 weeks. PBS was injected as a control (n=4–5/group). (A) NK cell numbers in the spleen and subcutaneous fat SVCs (SubQ) after reconstitution, as measured by flow cytometry and normalized by tissue weights. (B-D) Lymphocyte numbers in the spleen (B) and subcutaneous (C) and epididymal (D) fat SVCs after reconstitution, as measured by flow cytometry and normalized by tissue weights. (E) Tissue weights. (F) Expression levels of *Fgf21* mRNA in liver, as determined by qRT-PCR. The data are presented as mean ± S.E.M. For all figures: *p<0.05, **p<0.01, and ***p<0.001.

Table S1 (Related to Figure 1-6). Cell-surface markers for murine immune cells

Cell types	Markers	
	Positive selection	Negative selection
NK Cells	CD45 NK1.1+	Gr-1, F4/80, CD3, CD19, TER 119
iNKT Cells	CD45, CD3, NK1.1	Gr-1, F4/80, CD19, TER 119
B Cells	CD45, CD19	Gr-1, CD3, NK1.1, TER 119
CD4 T Cells	CD45, CD3, CD4	Gr-1, CD8, CD19, NK1.1, TER 119
CD8 T Cells	CD45, CD3, CD8	Gr-1, CD4, CD19, NK1.1, TER 119
Monocytes	CD45, CD11b	$Ly6G^{hi}$, CD3, CD19, NK1.1, TER 119
ATMs	CD45, CD11b, F4/80	TER-119, CD3, CD19, NK1.1
CD11c+ ATMs	CD45, CD11b, F4/80, CD11c	TER-119, CD3, CD19, NK1.1

Table S2 (Related to Figure 1-6). Cell-surface antibodies for flow cytometric analyses of mouse immune cells

Cell markers	Clone number	Fluorophore	Manufacturer
NK1.1	PK-136	PerCP Cy5.5	eBioscience 45-5941-82
		PE-Cy7	BD Pharmingen 550627
		APC	BioLegend 550627
CD49b	DX5	APC	eBioscience 17-5971-82
		PE	BioLegend 108908
CD3	145-2C11	PerCP Cy5.5	BD Pharmingen 551163
		PB	BioLegend 100214
CD19	1D3	PerCP Cy5.5	BioLegend 115534
		APC	BioLegend 115512
		PE	BioLegend 115508
TER119	TER-119	APC	eBioscience 17-5921-83
		PerCP Cy5.5	BioLegend 116228
CD45	30-F11	APC-Cy7	BD Pharmingen 557659
			BioLegend 103116
CD11c	N418	PE	eBioscience 2-0114-83
		PB	BioLegend 117322
F4/80	BM8	PE-Cy7	eBioscience 25-4801-82
			BioLegend 123114
CD11b	M1/70.15	PerCP Cy5.5	BD Pharmingen 550993
		PETR	Invitrogen RM2817
Gr1	RB6-8C5	PB	eBioscience57-5931-82
		APC	BioLegend 108412
CD4	RM4-5	PETR	Caltag Lab MCD0417
		PB	BioLegend 100531
CD8	53-6.7	PerCPCy5.5	BD Pharmingen 551162,
		PE Cy7	BioLegend 100722
CD25	3C7	FITC	BD Pharmingen 558689
IgG	R3-34	PE	BioLegend 400608
IFNγ	XMG1.2	PE	BioLegend 505808
TNFα	MP6-XT22	PE	BioLegend 506306
IL-10	JES5-16E3	PE	BioLegend 505008
IL-6	MP5-20F3	PE	BioLegend 504504