Brief Cutting Edge Report
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

Systemic NK Cell Ablation Attenuates Intra-Abdominal Adipose Tissue Macrophage Infiltration in Murine Obesity

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Objective: Natural killer (NK) cells are understudied in the context of metabolic disease and obesity. The goal of this study was to define the effect of NK cell ablation on systemic inflammation and glucose homeostasis in murine obesity.

Methods: A transgenic murine model was used to study the effect of NK cell ablation on systemic inflammation and glucose homeostasis in the context of diet-induced obesity using flow cytometry, QRTPCR, and glucose tolerance and insulin sensitivity testing.

Results: NK cell ablation achieved a three to fourfold decrease in NK cells but had no effect on T-cell levels in adipose tissues and spleen. NK cell ablation was associated with decreased total macrophage infiltration in intra-abdominal adipose tissue, but macrophage infiltration in subcutaneous adipose tissue and spleen was unaffected. NK cell ablation was associated with modest improvement in insulin sensitivity but had no effect on tissue transcript levels of inflammatory cytokines.

Conclusions: NK cells play a role in promoting intra-abdominal adipose tissue macrophage infiltration and systemic insulin resistance in obesity.

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Introduction

NK cells are increased in murine and human obesity and implicated in obesity-related inflammation (1-8), suggesting a role in metabolic disease. These findings prompted the current study, the goal of which was to determine the effect of in vivo NK cell ablation on systemic inflammation and glucose homeostasis in murine obesity. We used mice containing a transgene encoding Cre recombinase under control of the NK-cell-specific NKp46 promoter along with a transgene that permits diphtheria toxin (DT)-induced ablation of Cre-expressing cells (9). We demonstrate that NK cell ablation attenuates intra-abdominal adipose tissue macrophage (ATM) infiltration and induces modest improvement in systemic insulin sensitivity. This is the first report to describe the effect of NK cell ablation on metabolic disease in an in vivo obesity model.

Methods

Animals

Research adhered to NIH, Oregon Health & Science University, and University of Michigan guidelines. C57Bl/6 NKp46-Cre transgenic mice (from Dr. Eric Vivier and INSERM) and C57Bl/6 mice with a 5′ loxP-stop codon-loxP huDTR transgene (Jackson Laboratory, Bar Harbor, ME) were crossed to generate mice heterozygous for the NKp46-Cre transgene and homozygous for the flox-stop codon-huDTR transgene (experimental Cre^{+} mice) (9). Mice homozygous for the flox-stop huDTR transgene but lacking the NKp46-Cre transgene were controls (Cre^{-} mice). Six-week-old littermate male mice were maintained on high-fat diet (HFD, 60% fat; Research Diets Inc., New Brunswick, NJ) for 18 weeks. Mice received a 3.5-week course of biweekly intraperitoneal (IP) injections (7 doses) of 500 ng of DT in 500 μL phosphate buffered saline (PBS) (Sigma-Aldrich Inc., St. Louis, MO) beginning at week 14 after initiation of HFD until sacrifice at the end of week 18 of HFD. Glucose tolerance testing (GTT) was performed at week 17, followed by insulin tolerance testing (ITT) then sacrifice at week 18 on the fourth day after final DT injection. For GTT and ITT, 12-h fasted mice received either glucose IP (2 g/kg) or recombinant human insulin IP (0.75 units/kg) and tail vein blood glucose was measured. Liver, spleen, intra-abdominal adipose tissue (IAT, epididymal fat pad), and subcutaneous adipose tissue (SAT, subcutaneous flank fat pad) were harvested, and splenocytes and SVF isolated (8). RNA from liver was

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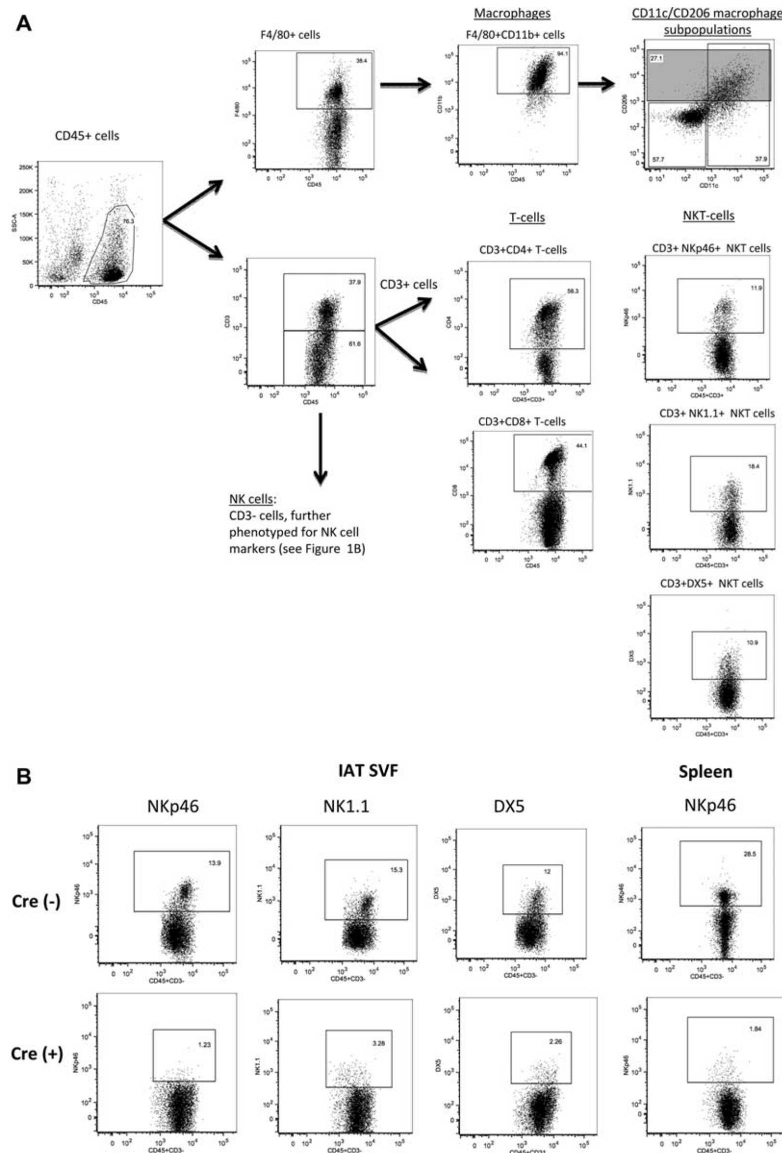

Figure 1 Effects of NK cell ablation on tissue leukocyte frequencies. (**A**) Flow cytometry gating strategy: representative scatterplots from IATSVF demonstrating flow cytometry gating strategy. After exclusion of doublets and nonviable cells, $CD45^+$ cells were gated. Macrophages ($F4/80^+CD11b^+$) were defined by sequential gating on $F4/80^+$ cells followed by $CD11b^+$ cells. $CD11c^+$ cells, $CD206^+$ cells, and $CD11c^-CD206^-$ (double-negative) cells within the F4/80+CD11b+ macrophage population were also studied (overlapping gates displayed). T cells ($CD3^+$) were defined by gating on $CD3^+$ cells, followed by gating on $CD4^+$ and $CD8^+$ T-cell subpopulations. NKT cells were defined by gating on $CD3^+$ cells followed by gating on NKp46, NK1.1, and DX5 separately. NK cells were defined by gating on $CD3^-$ cells, followed by gating on DX5, NKp46, and NK1.1 separately (see B). An identical gating strategy was used for SAT and spleen (not shown). Metric in each scatterplots is percent of parent gate for gated cell population. (**B**) NK cell ablation: representative scatterplots of NK cell markers (NKp46, NK1.1, DX5) in $CD45^+CD3^-$ cells after exclusion of doublets and nonviable cells in IATSVF and splenocytes (NKp46 only shown). Similar results were observed in SAT (scatterplots not shown). Flow cytometry analysis demonstrates ablation of NK cells in obese DT-treated Cre^+ experimental animals but not in obese DT-treated Cre^- control animals. Metric in each scatterplot is percent of parent gate for gated cell population. (**C**) The effect of NK cell ablation on tissue NK cell, T-cell, and NKT cell frequencies: Tissue frequencies of NK cells, T cells, and NKT cells in obese mice after treatment with DT. Ordinates: percent of designated cell population within all viable cells isolated from designated tissue. Single asterisk: $P < 0.050$, double asterisk: $P < 0.100$, independent *t*-test comparing Cre^- and Cre^+ cohorts. (**D**) The effect of NK cell ablation on tissue macrophage frequency: tissue frequencies of macrophages ($F4/80^+CD11b^+$) in Cre^+ and Cre^- obese mice after treatment with DT. Ordinate: percent of macrophages within all viable cells within each tissue. Asterisk: $P = 0.008$, independent *t*-test comparing Cre^- and Cre^+ cohorts. (**E**) The effect of NK cell ablation on CD11c/CD206 subpopulation frequencies in IAT: Frequencies of CD11c+, CD206+, and CD11c−CD206− macrophage subpopulations in IAT from Cre^+ and Cre^- obese mice after treatment with DT. Ordinate: percent of each cell subpopulation within all viable cells. Double asterisk: $P = 0.071$, independent *t*-test comparing Cre^- and Cre^+ cohorts. $n = 15$ Cre^+ and 17 Cre^- animals.

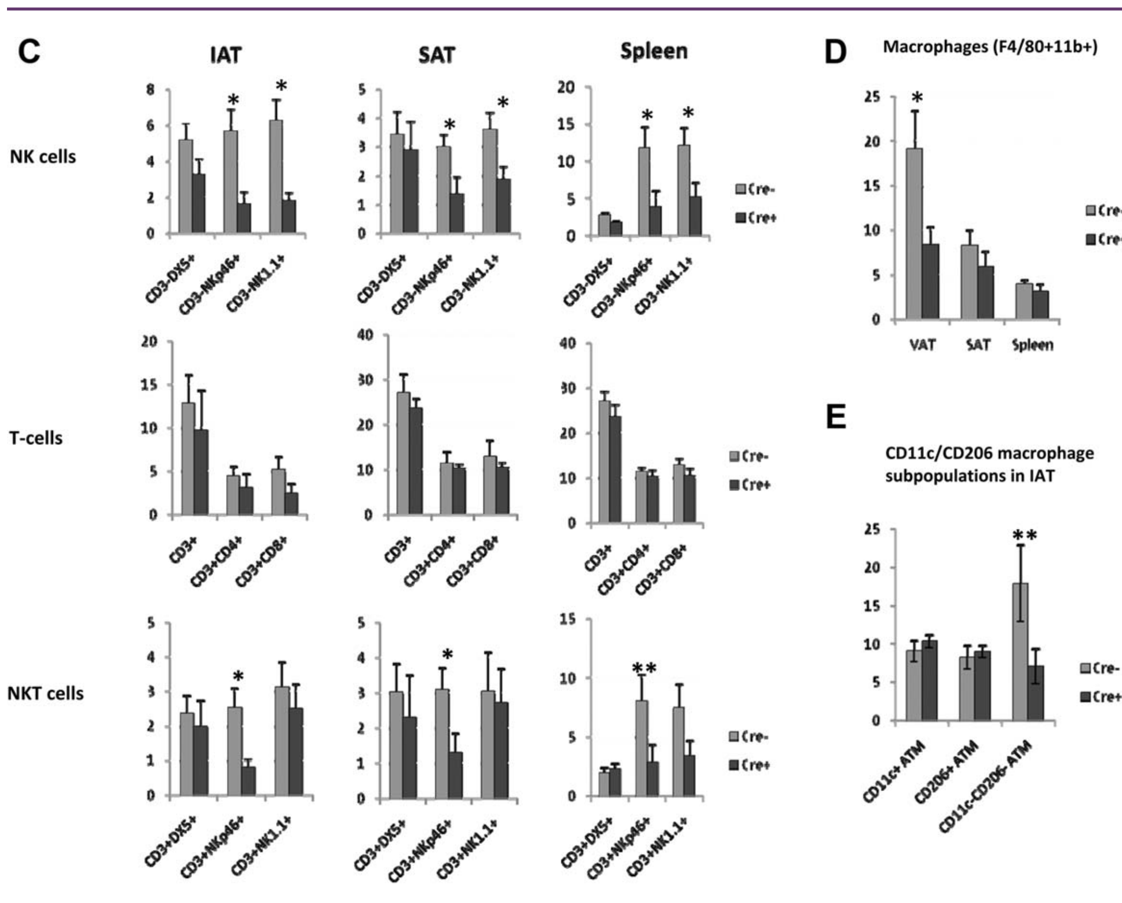

Figure 1 (Continued)

isolated for QRTPCR. Fasting serum insulin levels were measured with ELISA.

QRTPCR

RNA was reverse-transcribed using random hexamers and QRTPCR performed using SYBR Green (Applied Biosystems, Inc., Foster City, CA), transcript-specific primers using actin as an endogenous control, and 2^{-ddCT} quantification method. Primer sequences are previously published (8).

Flow cytometry

Cells were stained with viable dye and antibodies (CD45-FITC, F4/80-APC, CD11b-PE-CY7, CD11c-PE, CD206-PerCP-Cy5.5, CD3-PE-Cy7, CD4-PE, CD8-APC, DX5-APC, NKp46-PerCP-efluor710, and NK1.1-APC-Cy7; eBiosciences Inc., San Diego, CA) and analyzed on an LSRII flow cytometer (Becton, Dickinson, Inc., Franklin Lakes, NJ). Data were analyzed after exclusion of doublets and nonviable cells, using unstained and isotype controls, restricting analysis to $CD45^+$ cells (Figure 1A).

Results

NKp46 transcripts are stable from 6 to 18 weeks of HFD in wild-type mice IAT

To determine the kinetics of HFD's effects on IAT NK cell frequency, we compared NKp46 (NK cell) and CD11c (M1 macrophage) transcripts in IAT from wild-type C57Bl/6 mice maintained on HFD for 6 or 18 weeks. NKp46 transcript levels were similar (fold difference 1.06, $P = 0.914$) and CD11c transcripts were elevated (fold difference 25.61, $P = 0.000$) in IAT at 18 weeks compared to 6 weeks of HFD. Subsequent experiments in the transgenic model studied 18 week HFD.

Systemic NK cell ablation reduces tissue NK cell frequencies with no effect on T cells in murine obesity

DT induced a three to fourfold reduction in the frequency of CD3-NKp46+ and CD3-NK1.1+ but not CD3-DX5+ NK cells in IAT, SAT, and spleens of Cre^+ mice relative to Cre^- mice. NK cell ablation had no effect on the frequency of T cells ($CD3^+$), or NKT cells ($CD3^+DX5^+$, $CD3^+NK1.1^+$) in any tissue, but reduced the frequency of $CD3^+NKp46^+$ NKT cells (Figures 1B and 1C, Table 1).

NK cell ablation reduces IAT macrophages but has no effect on inflammatory cytokine transcript levels in murine obesity

NK cell ablation induced a marked reduction in macrophages ($F4/80^+CD11b^+$) in IAT but not SAT or spleen in Cre^+ mice relative to Cre^- mice. NK cell ablation had no effect on $CD11c^+$ or $CD206^+$ macrophage frequencies or numbers but was associated with

TABLE 1 Cells per gram of adipose tissue based on flow cytometry data, calculated by multiplying frequency of each cell subpopulation by each parent gate (including CD45+ cell gate, as shown in Figure 1) and by total SVF cell yield per gram of tissue; *P*-values derived from independent *t*-test comparing Cre$^-$ and Cre$^+$ animal cohorts

Cells/gram adipose tissue		IAT			SAT		
Cell subset	Cell subpopulation	Cre$^-$	Cre$^+$	*P*-value	Cre$^-$	Cre$^+$	*P*-value
NK cells	CD3$^-$DX5$^+$	97,336	44,100	0.162	14,507	9,979	0.239
	CD3$^-$NKp46$^+$	96,246	20,153	**0.014**	16,093	4,677	**0.039**
	CD3$^-$NK1.1$^+$	110,589	29,004	**0.007**	18,238	7,800	**0.050**
NKT cells	CD3$^+$DX5$^+$	41,636	22,171	0.328	11,503	11,395	0.927
	CD3$^+$NKp46$^+$	24,211	8,014	**0.038**	13,786	5,600	**0.022**
	CD3$^+$NK1.1$^+$	28,627	25,074	0.738	10,454	12,290	0.491
T cells	CD3$^+$	417,651	312,506	0.492	140,653	35,288	0.304
	CD3$^+$CD4$^+$	143,817	142,029	0.977	56,674	12,976	0.303
	CD3$^+$CD8$^+$	171,556	90,104	0.247	70,855	21,217	0.160
ATM	F4/80$^+$CD11b$^+$	487,951	211,011	**0.008**	47,028	52,950	0.731
	F4/80$^+$CD11b+CD11c$^+$	291,038	225,072	0.621	19,809	25,280	0.656
	F4/80$^+$CD11b$^+$CD206$^+$	278,054	200,535	0.573	51,141	44,935	0.592
	F4/80$^+$CD11b$^+$CD11$^-$CD206$^-$	373,274	197,938	**0.078**	41,843	27,035	0.496

These data parallel the frequency data presented in Figure 1. Bolded *P*-values are <0.100.

decreased frequency and number of CD11c$^-$CD206$^-$ (double-negative) macrophages in IAT (Figures 1D and 1E, Table 1). Expression of TNF-α, IL-6, IL-10, CCL2, and IFN-γ were similar between Cre$^+$ and Cre$^-$ mice in IAT, SAT, spleen, and liver (data not shown).

NK cell ablation is associated with improved systemic insulin sensitivity in murine obesity

Cre$^+$ and Cre$^-$ animals gained similar weight on HFD. NK cell ablation induced modest improvement in insulin sensitivity but had no effect on glucose tolerance (Figure 2). No difference in fasting serum insulin levels were observed (0.66 vs. 0.80 ng/mL, Cre+ vs. Cre− mice, $P = 0.499$, independent *t*-test).

Discussion

Lack of specificity of in vivo ablation methods complicates study of NK cells (10), a challenge addressed with recent development of a transgenic model using an NKp46 promoter-driven Cre gene (8,11), which formed the basis for this manuscript. We observed an increased macrophage infiltrate in IAT relative to SAT in control Cre$^-$ animals that did not undergo NK cell ablation, consistent with prior reports in murine and human obesity. The decrease in macrophages in IAT but not SAT or spleen with NK cell ablation may be due to the fact that IAT macrophages are more susceptible to downregulation due to pre-existing elevated levels, while macrophages in other tissues are at a biologic "floor" not susceptible to further reduction. Qualitative differences may also exist between macrophages in IAT and other tissues with respect to NK cell interactions, such as different susceptibilities to NK cell-derived mediators such as IFN-γ, which promotes macrophage inflammation (6,12,13). Further experiments will be necessary to identify NK cell-derived factors that regulate macrophages in obesity.

NK cell ablation was associated with a decrease in IAT of CD11c−CD206− (double-negative) ATM but did not affect the proportions of CD11c$^+$ ATM, a diabetogenic subpopulation enriched in obesity (14), or CD206+ ATM, an M2 subpopulation, suggesting that NK cells do not regulate ATM phenotype with respect to these markers. NK cell ablation did not affect T cells, which are implicated in obesity-related inflammation and insulin resistance (15,16). The lack of effect of NK cell ablation on CD11c, ATM, CD206 ATM, and/or T cells may explain the only modest improvement in systemic insulin sensitivity and the lack of effect on inflammatory transcripts. The improvement in insulin sensitivity but not glucose tolerance suggests that NK cells may selectively regulate peripheral insulin sensitivity rather than insulin secretion. Studies with euglycemic clamps will be necessary to resolve this issue. Finally, in the absence of glucose homeostasis data from mice fed normal chow, and given the apparent disconnect between inflammation and improved insulin action in ablated mice, we cannot exclude the possibility that NK cells may attenuate peripheral insulin action in nonobese mice as well.

Despite specificity for NK cells, NKp46 is expressed by other cell types (17,18). DT induced decreased the frequency of CD3$^+$NKp46$^+$ NKT cells, a limitation of this model. In addition, while DT induced ablation of CD3$^-$NKp46$^+$ and CD3$^-$NK1.1$^+$ cells, CD3$^-$DX5$^+$ cells were preserved. DX5 is expressed on T cells, NKT cells, fibroblasts, and platelets (8,19). Persistent CD3$^-$DX5$^+$ cells likely represent DT-resistant non-NK cells.

Ablation accomplished a three to fourfold decrease in NK cells. Higher doses and/or longer courses of DT may accomplish more profound ablation with different metabolic effects, and represent avenues for future research. As tools develop, study of tissue-specific ablation will further elucidate the role of NK cells in obesity. We observed no difference in IAT NKp46 transcript levels in wild-type mice fed HFD for 6 weeks compared to 18 weeks, suggesting that IAT NK cell infiltration does not change substantially from 6 to 18 weeks of HFD; CD11c transcripts in contrast were markedly elevated at 18 compared to 6 weeks. We elected to study 18 week HFD in this manuscript to determine if NK cell

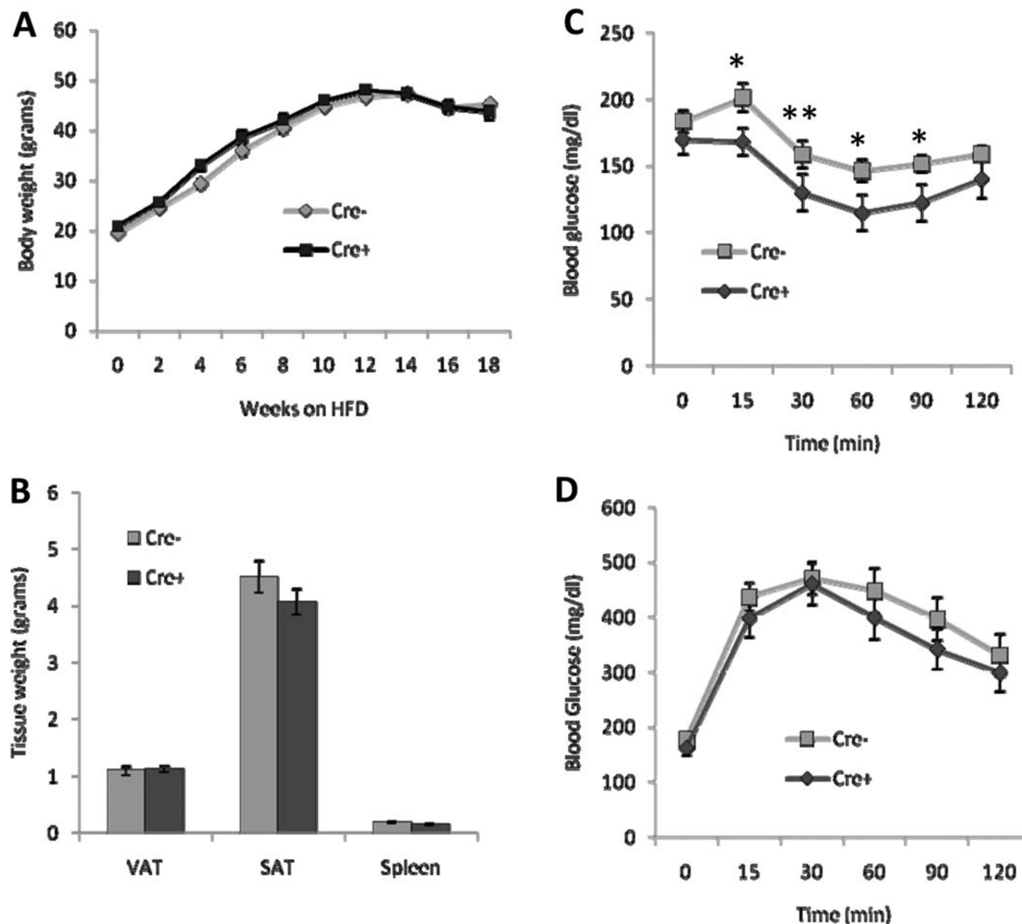

Figure 2 Effects of NK cell ablation on glucose homeostasis: body weights: ordinate: body weights (g) over the course of high-fat diet. (**A**) Tissue weights: ordinate: weights (grams) at sacrifice; no differences observed between Cre$^+$ and Cre$^-$ mice. (**B**) Insulin tolerance test: ordinate: blood glucose; single asterisk: $P < 0.050$, double asterisk: $P < 0.100$, independent *t*-test comparing Cre$^-$ and Cre$^+$ cohorts at each time point. Areas under curve for Cre− and Cre+ cohorts were 19,293 and 15,964 mg/dL/min, respectively, $P = 0.109$, independent *t*-test. (**C**) Glucose tolerance test: ordinate: blood glucose; no differences observed at any time point between Cre$^+$ and Cre$^-$ mice, independent *t*-test. $n = 17$ Cre$^+$ and 19 Cre$^-$ animals.

ablation regulates metabolism in the face of an established obesity-induced macrophage infiltrate. Future research will study the kinetics of the effects of HFD on NK cells over shorter durations of HFD.

Our findings support a role for NK cells in regulating IATATM infiltration and contributing to systemic insulin resistance in obesity. Despite these effects, NK cell ablation is not sufficient to completely resolve inflammation or metabolic dysfunction, suggesting that targeting NK cells alone will not be sufficient to treat obesity-related metabolic disease. O

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