Molecules and Cells

Research Article

NK cell-activating receptor NKp46 does not participate in the development of obesity-induced inflammation and insulin resistance

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ABSTRACT

Recent evidence establishes a pivotal role for obesity-induced inflammation in precipitating insulin resistance and type-2 diabetes. Central to this process is the proinflammatory M1 adipose-tissue macrophages (ATMs) in epididymal white adipose tissue (eWAT). Notably, natural killer (NK) cells are a crucial regulator of ATMs since their cytokines induce ATM recruitment and M1 polarization. The importance of NK cells is shown by the strong increase in NK-cell numbers in eWAT, and by studies showing that removing and expanding NK cells respectively improve and worsen obesity-induced insulin resistance. It has been suggested that NK cells are activated by unknown ligands on obesity-stressed adipocytes that bind to NKp46 (encoded by *Ncr1*), which is an activating NK-cell receptor. This was supported by a study showing that NKp46-knockout mice have improved obesity-induced inflammation/insulin resistance. We therefore planned to use the NKp46-knockout mice to further elucidate the molecular mechanism by which NKp46 mediates eWAT NK-cell activation in obesity. We confirmed that obesity increased eWAT NKp46$^+$ NK-cell numbers and NKp46 expression in wild-type mice and that NKp46-knockout ablated these responses. Unexpectedly, however, NKp46-knockout mice demonstrated insulin resistance similar to wild-type mice, as shown by fasting blood glucose/insulin levels and glucose/insulin tolerance tests. Obesity-induced increases in eWAT ATM numbers and proinflammatory gene expression were also similar. Thus, contrary to previously published results, NKp46 does not regulate obesity-induced insulin resistance. It is therefore unclear whether NKp46 participates in the development of obesity-induced inflammation and insulin resistance. This should be considered when elucidating the obesity-mediated molecular mechanisms that activate NK cells.

Keywords: Inflammation, Insulin resistance, Natural killer cells, Obesity

INTRODUCTION

There is currently a growing worldwide epidemic of insulin resistance and type-2 diabetes, the main cause of which is the rising prevalence of obesity (Hotamisligil, 1999; Koska et al., 2008; Muoio and Newgard, 2008; Shin et al., 2015; Standl et al., 2019). It is widely thought that obesity induces insulin resistance by activating tissue-resident immune cells, particularly those in the epididymal (visceral) white adipose tissue (eWAT) (Li et al., 2023; Rohm et al., 2022). The immune cell that plays the most important role in this is the adipose-tissue macrophage (ATM): obesity converts the alternatively-activated M2 ATMs in eWAT into classically-activated M1 ATMs. These cells then proliferate and produce large amounts of proinflammatory cytokines that leach into the circulation and generate a low-grade but chronic systemic inflammation that leads to insulin resistance (Kolb, 2022; Lumeng et al., 2007; Reilly and Saltiel, 2017; Weisberg et al., 2003; Xu et al., 2003). Another important immune cell type is the natural killer (NK) cell. This innate immune cell has 2 major functions, namely, killing infected/foreign cells and regulating other immune cells with the cytokines it releases (Prager and Watzl, 2019). Numerous studies show that obesity increases NK-cell numbers, particularly in the eWAT. However, the cytotoxic activity of these cells is either suppressed or unaffected (Michelet et al., 2018). Rather, these cells release abundant amounts of cytokines such as Interferon Gamma (IFNγ) and Tumor Necrosis Factor Alpha (TNFα) (Wrann et al., 2012) that are key drivers of the diabetogenic M2 to M1 conversion of ATMs in the eWAT Lee et al., 2016; O'Rourke et al., 2014; Wensveen et al., 2015). This crucial role of NK cells in insulin resistance has been confirmed by experiments showing that antibody-mediated or genetic depletion of these cells in mice ameliorates obesity-induced insulin

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resistance (Lee et al., 2016; O'Rourke et al., 2014; Wensveen et al., 2015).

It remains unclear how obesity activates eWAT NK cells, but Wensveen et al. (2015) suggest that it is mediated by as yet-unidentified ligands that are expressed by obesity-stressed adipocytes and that bind to the NK-cell receptor NKp46 (encoded by *Ncr1*); this binding event activates NK cells and induces their release of M1 ATM-inducing cytokines. This notion was supported by their finding that $Ncr1^{gfp/gfp}$ mice are resistant to obesity-induced inflammation and insulin resistance (Wensveen et al., 2015): these mice lack functional NKp46 proteins because the *Ncr1* gene is knocked in by green fluorescent protein (GFP) (Gazit et al., 2006). However, the molecular mechanisms by which NKp46 regulates NK cells in obesity have not yet been explored.

To elucidate these molecular mechanisms, we aimed to feed wild-type (WT) and $Ncr1^{gfp/gfp}$ littermates with either a low-fat diet (LFD) or a high-fat diet (HFD) and then subject their tissues to next-generation sequencing, including single-cell RNA-sequencing (RNAseq) and Assay for Transposase-Accessible Chromatin using Sequencing (ATAC-seq). Unexpectedly, however, our initial experiments showed that the ablation of NKp46 did not alter obesity-induced inflammation and insulin resistance. The NKp46-knockout mouse strain is the same as that used by Wensveen et al. (2015). Thus, our experimental data suggest that NKp46 does not regulate obesity-induced inflammation and insulin resistance and that alternative mechanisms underpin the obesity-stimulated activities of NK cells. The discrepancy between our study and that of Wensveen et al. is discussed.

MATERIALS AND METHODS

Animals

All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Soonchunhyang University (South Korea) or the Joslin Diabetes Center (USA). The $Ncr1^{gfp/gfp}$ NKp46-knockout mice (B6:129-*Ncr1*^{tm1Oman}/J strain) were purchased from the Jackson Laboratory. All experiments were conducted with male mice, consistent with Wensveen et al. (2015). The WT controls in all experiments were littermates. All animals were maintained in specific pathogen-free facilities under a standard light cycle (12 hours light/dark) and allowed free access to water and food. To induce obesity and insulin resistance, mice were fed a HFD (60% fat, D12492; Research Diets, Inc) for the indicated time. Mice fed a LFD (10% fat, D12450B; Research Diets, Inc) served as controls.

Metabolic Phenotype Measurements

After overnight fasting, fasting blood glucose (FBG) levels were determined by using a glucometer (Accu-chek), and fasting insulin (FI) levels were assayed by using an Enzyme-linked Immunosorbent Assay (ELISA) kit (Crystal Chem). The insulin resistance index, namely, Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), was then calculated on the basis of the FBG and FI (Matthews et al., 1985). The glucose tolerance test (GTT) was performed by intraperitoneally injecting glucose (1.5 g/kg body weight) after overnight fasting. The insulin tolerance test (ITT) was performed by intraperitoneally injecting insulin (0.75 U/kg body weight, Sigma-Aldrich) after 6 hours of fasting. Blood samples were drawn from the tail vein before, and 15, 30, 60, and 120 minutes after the glucose or insulin injection, and glucose levels were measured by using an Accu-chek glucometer.

Isolation of Adipose Stromal Vascular Cells and Splenocytes

Stromal vascular cells (SVCs) were isolated from adipose tissues by using a well-established collagenase-based method (Lee et al., 2016). Briefly, after epididymal and subcutaneous fat pads were harvested from mice, they were chopped in Krebs Ringer Phosphate buffer containing 2% (w/v) Bovine Serum Albumin (BSA) and collagenase type 2 (10 mg/mL, Sigma-Aldrich) and DNAse I (2 mg/mL, Sigma-Aldrich) were added. After incubating the mixtures for 25 minutes at 37℃, the cell suspensions were filtered through a 250-μm nylon mesh and centrifuged at 300× g for 5 minutes. This separated the floating adipocyte layer from the pelleted SVC fraction. The SVC fraction was kept. The floating adipocytes were transferred to a new tube, 5 mM EDTA was added, and the mixture was centrifuged again to collect the remaining SVCs. The 2 SVC pellets were combined and washed with KRB solution. To obtain splenocytes, spleens harvested from mice were weighed and gently mashed in 5 mL of Phospate-Buffered Saline (PBS) containing 2% (v/v) Fetal Bovine Serum (FBS). After being filtered through 70-μm mesh, the suspensions were centrifuged at 300× g for 5 minutes. Ammonium Chloride Potassium (ACK) lysis buffer (Thermo Fisher) was added to the pellet to remove the red blood cells. The splenocytes were then washed with 2% (v/v) FBS-PBS solution. The SVC and splenocytes were then subjected to flow cytometric analyses.

Flow Cytometric Analyses

The adipose SVCs and splenocytes were incubated for 10 minutes with CD16/32 antibody (FC block) (eBioscience) and then stained with other fluorochrome-conjugated antibodies for 20 minutes at 4℃. All cells were stained with antibodies against CD45-PE/Cy7 (eBioscience), CD4-BV500 (BD Biosciences), CD8-APC/Cy7 (Biolegend), CD19-PE (Biolegend), NK1.1-BV421 (Biolegend), NKp46-PE/Dazzle594 (Biolegend), CD11b-BV786 (Biolegend), CD11c-APC (Biolegend), CD3-BV711 (Biolegend), and F4/80-BV650 (Biolegend). 7AAD (Invitrogen) was used for live/dead staining. The stained cells were washed and analyzed with an LSRFortessa flow cytometer (BD Biosciences). NK cells were defined as $CD45^+$ $F4/80^-$ $CD19^-$ $CD3^-$ $NK1.1^+$ in lymphocyte gating. The $NKp46^+$ and GFP^+ cells were then gated. ATMs were defined as $CD45^+$ $CD19^-$ $CD3^-$ $NK1.1^-$ $F4/80^+$ $CD11b^+$. Total cell numbers were counted with a hemocytometer and normalized according to the tissue weight (cells/g). Flow cytometric data were analyzed by using the FlowJo software (Flow Jo).

Quantitative Real-time Reverse Transcription Polymerase Chain Reaction (qRRT-PCR) Analysis

Total RNA from fat tissues was prepared by using the RNEasy Lipid Tissue Mini Kit according to the manufacturer's protocol (Qiagen). RNAs from other tissue samples were isolated using the Trizol-based method (Life Technologies). complementary DNA (cDNA) was synthesized by using iScript cDNA Synthesis Kit (Bio-Rad). Gene expression levels were determined by real-time

RT-PCR by using SYBR green (Applied Biosystems) and the following primers: F4/80 (*Adgre1*, F: TGCATCTAGCAATGGAC AGC, R: GCCTTCTGGATCCATTGAA), CD68 (F: CCA ATT CAG GGT GGA AGA AA, R: CTC GGG CTC TGA TGT AGG TC), insulin receptor (*Insr,* F: CAG CCG GAT GGG CCA ATG GGA, R: CTCGTCCGGCACGTACACAGAA), IRS1 (*Irs1,* F: TGG GCC TTT GCC CGA TTA TG, R: TCT ACA CCC GAG ACG AAC ACT), IRS2 (*Irs2,* F: GAC TTC CTG TCC CAT CAC TTG, R: TTT CAA CAT GGC GGC GA), TNFα (*Tnf*, F: AGTCCGGGCAGGTCTAC TTT, R: GTCACTGTCCCAGCATCTTGT) and as a housekeeping control gene, GAPDH (F: GGATTTGGCCGTATTGGG, R: GTT GAGGTCAATGAAGGGG).

Statistical Analysis

Metabolic phenotype experiments were repeated 3 times at the Joslin Institute (USA), and all other experiments, including metabolic phenotype measurements, were repeated 3 times or more in Soonchunhyang Institute of Medi-bio Science (Korea). Representative data are shown in the figures. At least 3 mice were used per experiment. Statistical significance was examined by using 2-tailed unpaired Student's *t*-tests or 1-way Analysis of Variance (ANOVA). A Tukey's post-hoc test was used for multiple comparisons. Data are presented as mean ± SEM. GraphPad Prism 9 (GraphPad Software, Inc) was used to perform statistical analyses.

RESULTS

Obesity Increases NKp46 Expression in NK Cells

We first investigated the effect of obesity on insulin sensitivity and the concomitant expression of NKp46 by NK cells in various tissues from WT C57BL/6J mice. Thus, the WT mice were fed with a LFD or HFD for 16 weeks. Compared to the LFD-fed mice, the HFD-fed mice became insulin resistant, as shown by elevated fasting body weight (FBW), FBG, FI, and HOMA-IR values (Fig. 1A-D). Flow cytometric analysis of the eWAT immune cells then showed that the HFD dramatically increased the number of ATMs per gram of eWAT (Fig. 1E and F). This is consistent with previously published studies (Weisberg et al., 2003; Xu et al., 2003). By contrast, there were fewer ATMs in the inguinal white adipose tissue (iWAT), and their numbers were negligibly increased by a HFD (Fig. 1F). We also examined the frequencies of M1 ATMs by staining for CD11c. The HFD increased the frequency of $CD11c^+$ ATMs in both the eWAT and iWAT (Fig. 1G). This is consistent with previous studies (Lumeng et al., 2007). Thus, a HFD generated proinflammatory M1 ATMs in both WATs.

Wensveen et al., O'Rourke et al., and our previous study show that obesity increases the NK-cell numbers in eWAT (Lee et al., 2016; O'Rourke et al., 2014; Wensveen et al., 2015). Consistent with this, when we defined NK cells as $CD45^+$ F4/80^- $CD19^-$ $CD3^-$ $NK1.1^+$ cells, we found that HFD increased the NK-cell numbers per gram of eWAT. There was also a negligible increase in these cells in iWAT, but none in the spleen (Fig. 1H). Next, we gated NK cells using the markers described above and then selected the $NKp46^+$ cells. We found that a HFD greatly increased the numbers of $NKp46^+$ NK cells in eWAT, negligibly increased them in iWAT, and had no effect in the spleen (Fig. 1I). Moreover, the NKp46-expression intensity on NK cells, which is a marker of their activation (Boyiadzis et al., 2008; Hadad et al., 2015), rose with a HFD in the eWAT but dropped in the iWAT (Fig. 1J and K). These data support the hypothesis of Wensveen et al. (2015), namely, that NKp46 may participate in the mechanism by which obesity regulates NK-cell numbers in eWAT.

NKp46 was Completely Ablated in NKp46 Knockout Mice

Wensveen et al. (2015) previously reported that the deletion of NKp46 improves obesity-induced inflammation and insulin resistance. For this, they used $Ncr1^{gfp/gfp}$ mice, where exons 5 to 7 of the NKp46-encoding gene *Ncr1* are replaced with a construct encoding GFP. Since NKp46 is a pan-NK cell marker (Narni-Mancinelli et al., 2011; Walzer et al., 2007), this means that all NK cells in the mutant mice cannot express NKp46 and instead express GFP when they receive a signal that would normally cause transcription of the *Ncr1* gene. It should be noted that apart from some reduction in cytotoxic activity against tumor or infectious cells, the NK cells in these mutant mice are otherwise generally functional. This likely reflects compensatory responses by other NK cell-activating receptors (Gazit et al., 2006; Imsirovic et al., 2023; Sheppard et al., 2013). Our flow cytometric analyses of LFD- and HFD-fed WT and homozygous $Ncr1^{gfp/gfp}$ (NKp46 Homo) littermates using NKp46 gating on $CD45^+$ F4/80^- $CD19^-$ $CD3^-$ $NK1.1^+$ NK cells confirmed that (1) the eWAT of WT mice bore $NKp46^+$ NK cells even in the LFD condition, (2) these cell numbers were hugely increased when a HFD was imposed, and (3) these cells were completely absent in NKp46 Homo mice regardless of diet (Fig. 2A and B). Moreover, when we examined NKp46 heterozygous knockout (Het) mice, their HFD-induced $NKp46^+$ NK-cell numbers in the eWAT were 10% of those in the WT mice (Fig. 2A and B). Conversely, there were no GFP^+ NK cells in the eWAT of WT mice regardless of diet but they were observed in the Het and especially Homo mice: specifically, GFP^+ NK cells were present in the LFD-fed Het mice and especially Homo mice, and these numbers were increased by a HFD (Fig. 2A and C). The same patterns were observed when examining the amount of GFP that was expressed per NK cell (Fig. 2D). Genotype had unremarkable effects on the $NKp46^+$ NK-cell numbers, GFP^+ NK-cell numbers, and GFP-expression intensity on NK cells in the iWAT and spleen (Fig. 2B-D).

NKp46 Deficiency did not Reduce $CD45^+$ F4/80^- $CD19^-$ $CD3^-$ $NK1.1^+$ NK-Cell Numbers

We found that when we defined NK cells as $CD45^+$ F4/80^- $CD19^-$ $CD3^-$ $NK1.1^+$ cells (ie, NKp46 expression was not included during gating), the Het and Homo mice did not differ from WT mice in terms of NK-cell numbers per gram of eWAT (or iWAT and spleen) in either the LFD or HFD condition (Fig. 2E). Thus, the general NK-cell population in the body, and its augmentation by a HFD, was unaffected by NKp46 deletion.

Genetic Deletion of NKp46 did not Improve Obesity-induced Insulin Resistance Phenotypes

We then compared the insulin-resistance phenotypes of the WT, Het, and Homo NKp46 knockout mice. Relative to a LFD, a HFD

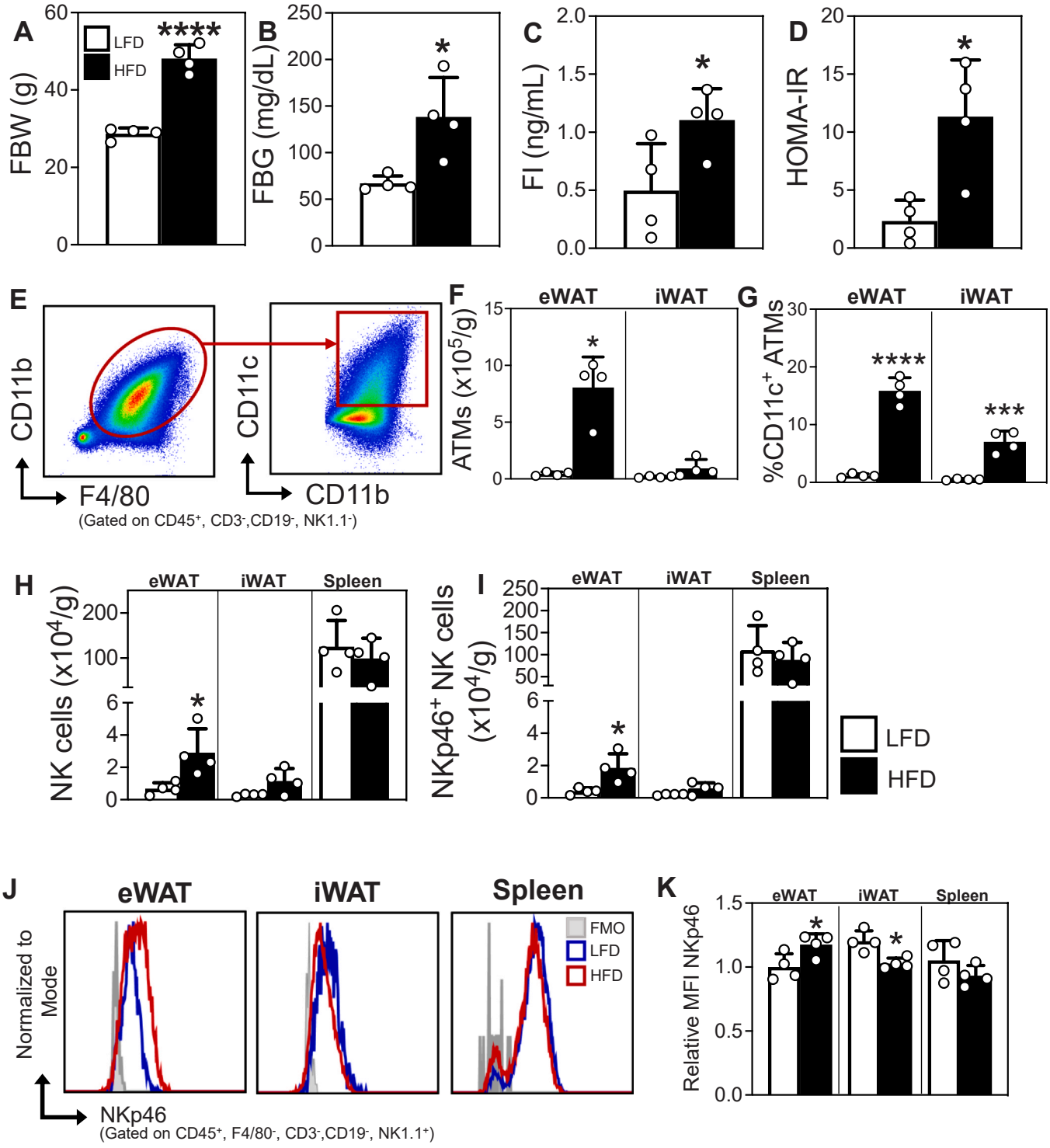

Fig. 1. Metabolic and inflammatory phenotypes of obese wild-type mice. Wild-type C57BL/6J were fed a LFD or HFD for 16 weeks, and their metabolic and inflammatory phenotypes were measured ($n = 4$). (A-D) Metabolic data. (A) Fasting body weight (FBW). (B) Fasting blood glucose (FBG). (C) Fasting insulin (FI). (D) Insulin resistance index (HOMA-IR). (E-K) Flow cytometric data of the indicated tissues. (E) Representative gating of ATMs. (F) ATM numbers per gram of tissue. (G) Frequency of $CD11c^+$ ATMs. (H) Numbers of NK cells per gram of tissue. NK cells were defined as $CD45^+$ $F4/80^-$ $CD19^-$ $CD3^-$ $NK1.1^+$ cells. (I) Numbers of $NKp46^+$ NK cells per gram of tissue. NK cells were gated as described in H, after which the $NKp46^+$ cells were selected. (J and K) Flow cytometric data showing the intensity of NKp46 expression on NK cells. The NK cells were defined as described in H, after which $NKp46^+$ cells were selected. (J) Representative flow cytometric histograms. (K) Relative mean fluorescence intensity (MFI) showing the NKp46 expression levels on NK cells. The data are presented as individual values ± SD. $^*P < .05$, $^{***}P < .001$, and $^{****}P < .0001$.

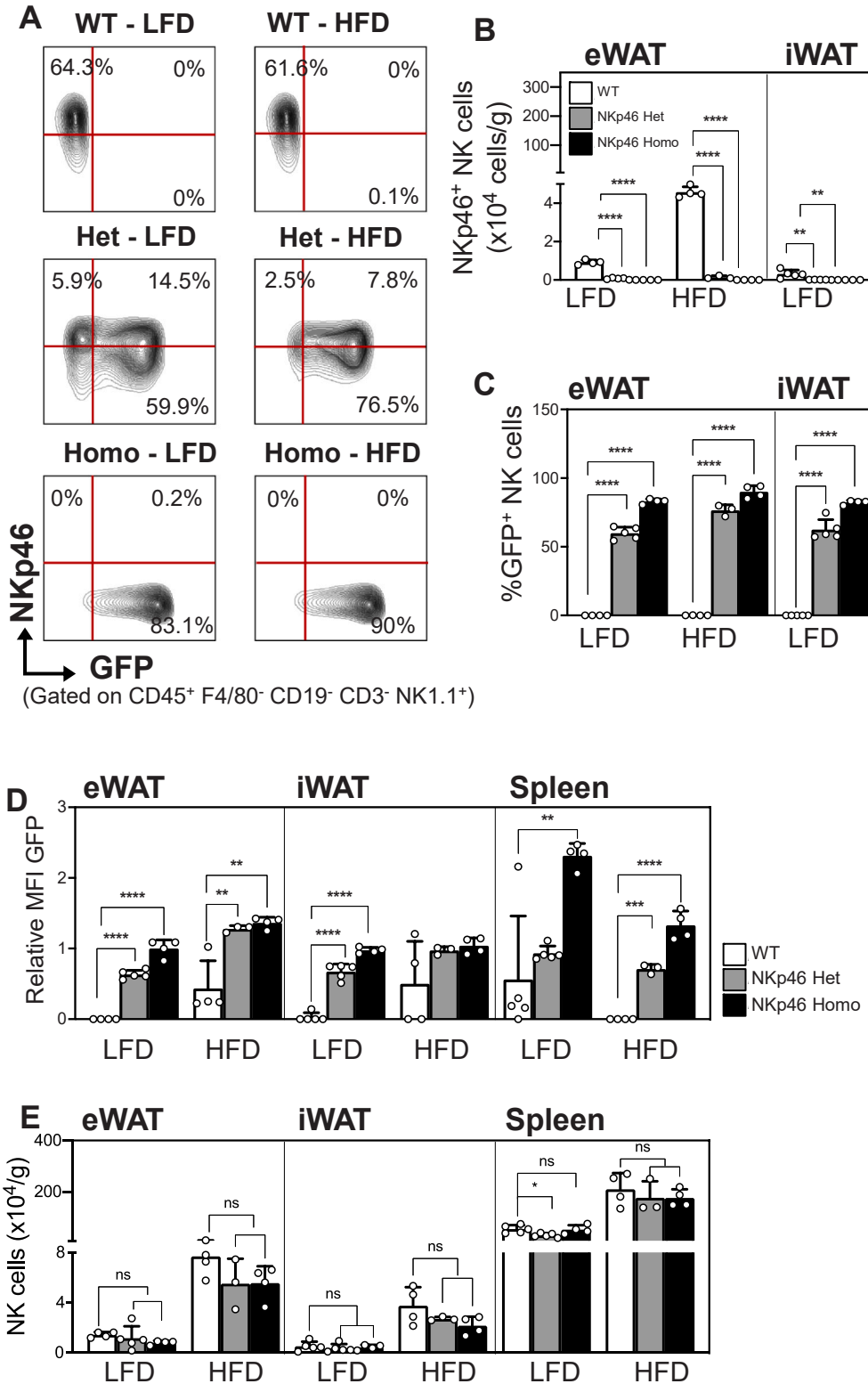

Fig. 2. Effects of NKp46 knockout on the obesity-induced increase in NK cells. Wild-type mice (WT), heterozygous NKp46-knockout mice (NKp46 Het), and homozygous NKp46-knockout mice (NKp46 Homo) were fed a LFD or HFD for 16 weeks ($n = 3$-5) and flow cytometric analyses were conducted on NK cells in the indicated tissues. NK cells were defined as $CD45^+$ $F4/80^-$ $CD19^-$ $CD3^-$ $NK1.1^+$ cells. (A) Representative flow cytometric data of the GFP and NKp46 expression of the NK cells. (B) Numbers of $NKp46^+$ NK cells per gram of tissue. (C) Frequencies of GFP^+ cells in the NK cell populations. (D) Relative mean fluorescence intensity (MFI) of GFP^+ cells. (E) Numbers of NK cells per gram of tissue. NK cells were defined as $CD45^+$ $F4/80^-$ $CD19^-$ $CD3^-$ $NK1.1^+$ cells. The data are presented as individual values ± SD. *P < .05, **P < .01, ***P < .001, and ****P < .0001. ns, not significant.

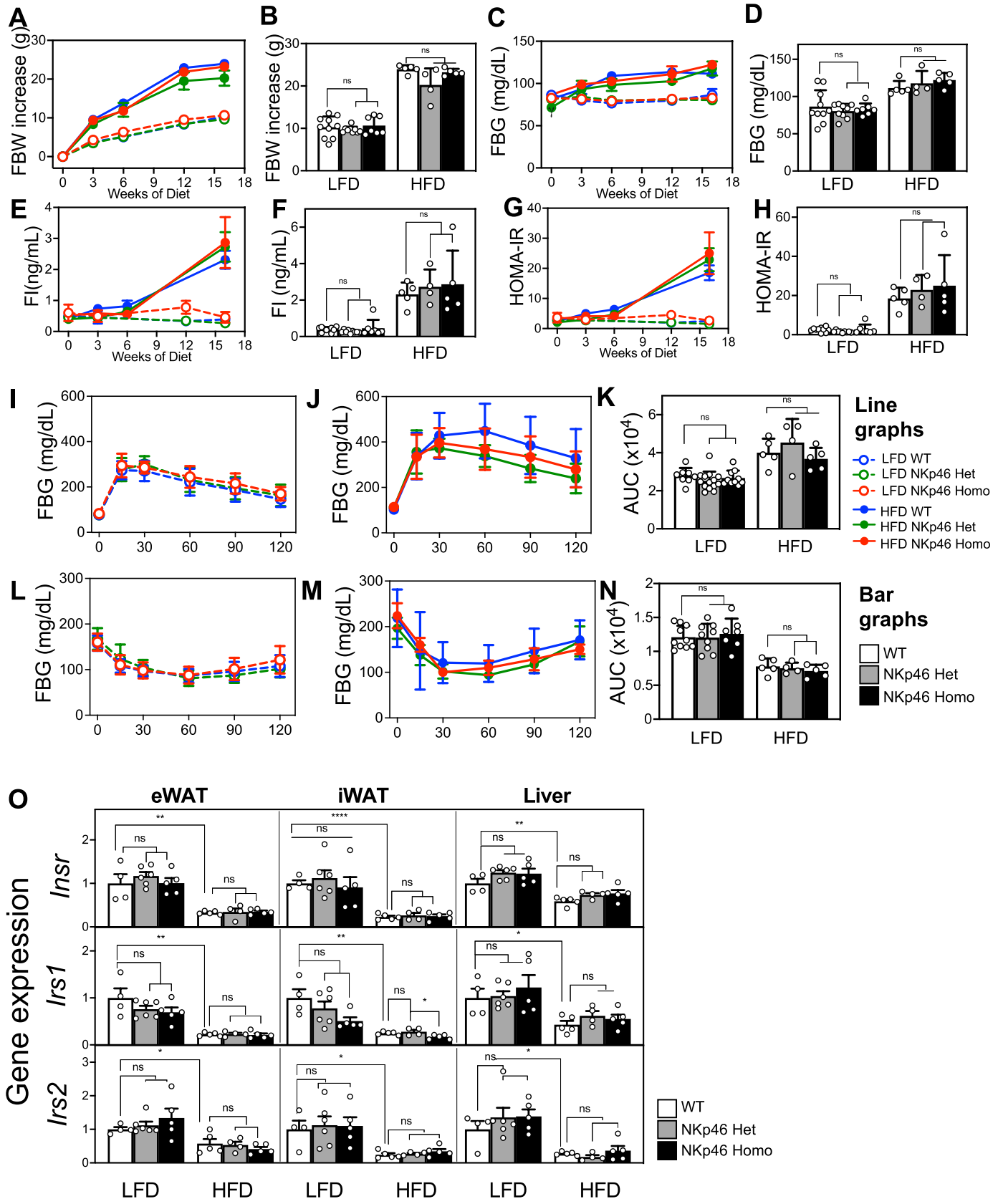

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Fig. 3. Genetic deletion of NKp46 did not improve obesity-induced insulin resistance. Wild-type mice (WT), heterozygous NKp46-knockout mice (NKp46 Het), and homozygous NKp46-knockout mice (NKp46 Homo) were fed a LFD or HFD and their metabolic phenotypes were determined (*n* LFD = 7-14, *n* HFD = 4-5). (A and B) Fasting body weight (FBW) over time (A) and at 16 weeks (B). (C and D) Fasting blood glucose (FBG) over time (C) and at 16 weeks (D). (E and F) Fasting insulin (FI) over time (E) and at 16 weeks (F). (G and H) HOMA-IR over time (G) and at 16 weeks (H). (I-K) GTT of LFD-fed (I) and HFD-fed (J) mice, and area under the curve (AUC) (K) at 12 weeks. (L-N) ITT of LFD-fed (L) and HFD-fed (M) mice, and AUC (N) at 9 weeks. (O) qRT-PCR analysis of *Insr, Irs1*, and *Irs2* in the eWAT, iWAT, and liver was conducted at 16 weeks on diets. The data are presented as individual values ± SD. **P* < .05, ***P* < .01, and *****P* < .0001. ns, not significant.

for 16 weeks increased the FBW of all 3 genotypes equally well (Fig. 3A and B). Moreover, the 3 genotypes did not differ in FBG levels, FI levels, or HOMA-IR at any time point, namely, at 3, 6, 12, and 16 weeks, regardless of whether they received a LFD or a HFD (Fig. 3C-H). Thus, deletion of NKp46 did not affect insulin resistance. The GTT (Fig. 3I-K) and ITT (Fig. 3L-N) confirmed that the Het and Homo mice did not show any improvements in glucose tolerance and insulin sensitivity.

We and others have shown that while a HFD impairs the insulin signaling pathway in eWAT, iWAT, and the liver, as shown by reduced expression of insulin receptor (*Insr*), IRS-1 (*Irs1*), and IRS-2 (*Irs2*), deleting NK cells reverses this (Lee et al., 2016; Wensveen et al., 2015). However, this reversal was not seen in the Het and Homo mice (Fig. 3O).

Thus, deleting NKp46 in NK cells did not affect the development of obesity-induced insulin resistance at any timepoint after starting a HFD. This suggests that NKp46 per se does not participate in obesity-induced insulin resistance.

Genetic Deletion of NKp46 did not Change Obesity-induced Inflammation in eWAT

We next examined the changes in obesity-induced inflammation in adipose tissues. We first assessed the expression of ATM marker genes. Consistent with the literature (Weisberg et al., 2003; Xu et al., 2003), a HFD increased eWAT expression of *Adgre1* (F4/80) and *Cd68* in WT mice (Fig. 4A). It also slightly increased iWAT expression of these genes. However, the Het and Homo NKp46-deficient mice showed similar changes in the eWAT and iWAT. This suggested that NKp46 deletion did not alter the ATM numbers (Fig. 4A). Indeed, flow cytometric analyses showed that a HFD increased ATM numbers in the eWAT in all 3 genotypes equally well. A HFD also negligibly increased the ATM numbers in the iWAT, and this was also seen in all 3 genotypes (Fig. 4B and C). A HFD also increased the eWAT (and iWAT) frequencies of $CD11c^+$ M1 ATMs equally well in the 3 genotypes (Fig. 4D and E).

We previously observed that in WT mice, NK cells at least partially regulate the recruitment of ATMs into eWAT by controlling eWAT expression of *Ccl2* (Lee et al., 2016). Our current analyses showed that a HFD upregulated *Ccl2* in the eWAT and iWAT regardless of the genotype (Fig. 4F). Moreover, we showed previously that in WT mice, a HFD increased eWAT expression of the proinflammatory cytokine *Tnf* (Lee et al., 2016). However, this was also observed in the Het and Homo mice. Moreover, a HFD negligibly increased the *Tnf* expression in the iWAT, with no difference between the 3 genotypes (Fig. 4F). These data show clearly that NKp46 in NK cells does not play a role in the development of obesity-induced inflammation in adipose tissues.

Thus, NKp46 does not participate in the development of obesity-induced inflammation and insulin resistance in our laboratory.

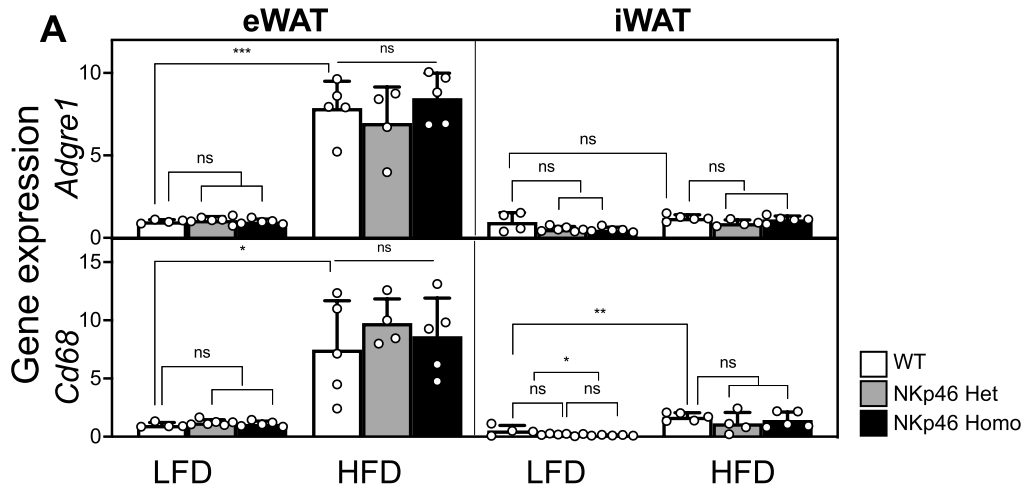
DISCUSSION

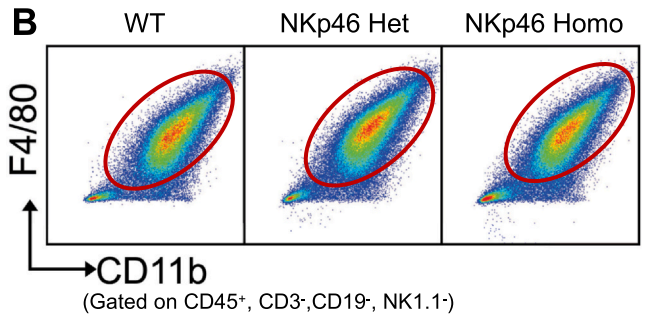
Several studies, including our own, show that NK cells play important roles in the development of obesity-induced inflammation and insulin resistance (Lee et al., 2016; O'Rourke et al., 2014; Wensveen et al., 2015). In particular, these studies show that obesity activates NK cells in eWAT only and that these cells then promote ATM recruitment and activation, again only in eWAT. This raises an important question: how does obesity activate NK cells in the eWAT only? Wensveen et al. proposed the following mechanism: (1) obesity induces epididymal adipocytes (but not subcutaneous adipocytes) to express as yet-identified ligands for NKp46 on their cell surface, and (2) when these ligands bind to NKp46 on epididymal NK cells, these cells become activated and release diabetogenic ATM-provoking cytokines (Wensveen et al., 2015). The key finding that supported this hypothesis was that $Ncr1^{gfp/gfp}$ NKp46-knockout mice did not develop insulin resistance after 12 weeks of a HFD (Wensveen et al., 2015).

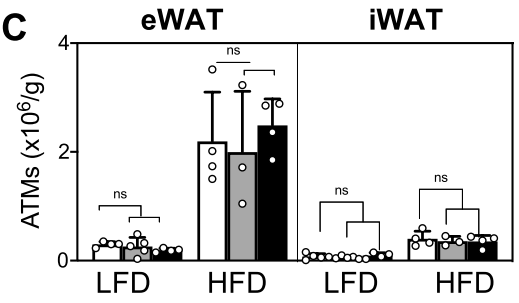
However, neither the NKp46 ligands nor the underlying molecular mechanism were determined. To address these issues, we commenced a study based on the $Ncr1^{gfp/gfp}$ NKp46-knockout mice. Like Wensveen et al. (2015), we found that obesity in WT mice increased eWAT NK-cell numbers and $NKp46^+$ NK-cell numbers. Obesity also elevated the expression intensity of NKp46 on eWAT NK cells, indicating their activation (Boyiadzis et al., 2008). Unexpectedly, however, we were not able to confirm the metabolic and inflammatory phenotypes of the $Ncr1^{gfp/gfp}$ NKp46-knockout mice: when these mice were fed a HFD, they, like WT mice, demonstrated increased insulin resistance, eWAT ATMs and $CD11c^+$ M1 ATMs, and eWAT inflammatory-gene expression, and decreased insulin signaling-gene expression in the fat and liver.

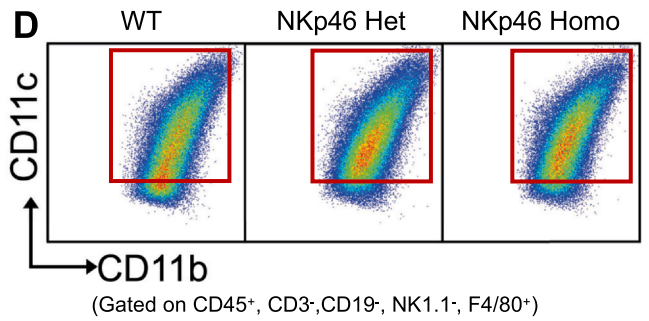
It should be noted that we measured the metabolic phenotypes with FBW, FBG, FI, GTT, and ITT, all of which showed that the WT and NKp46 knockout mice were largely identical in terms of all these variables (Figs. 2-4). By contrast, Wensveen et al. (2015) only measured the metabolic phenotypes with GTT and ITT, and the effects of NKp46 deletion were mild. In addition, the NKp46 deletion did not alter FBG, as shown by the identical FBG at 0 minutes of GTT. Thus, NKp46 deletion in the Wensveen et al. study had only mild metabolic effects.

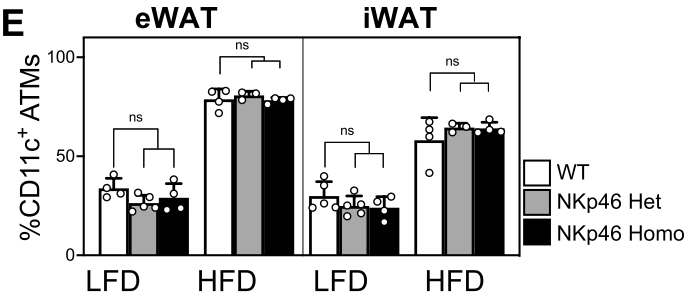
Nonetheless, Wensveen et al. (2015) showed that disrupting NKp46-gene expression can mildly improve insulin resistance while we found that it had no effect at all on insulin











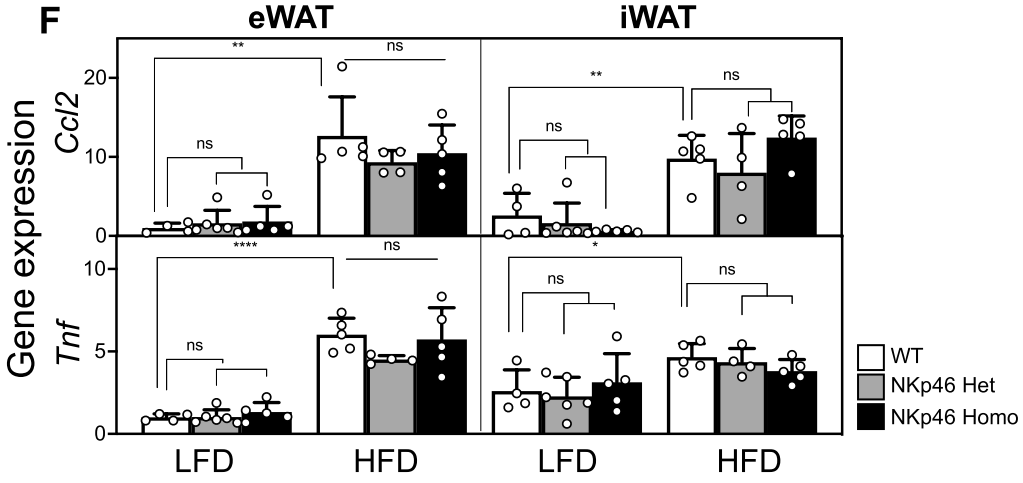


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Fig. 4. Genetic deletion of NKp46 did not improve obesity-induced inflammation. Wild-type mice (WT), heterozygous NKp46-knockout mice (NKp46 Het), and homozygous NKp46-knockout mice (NKp46 Homo) were fed a LFD or HFD for 16 weeks, and their immunological phenotypes in the indicated tissues were measured (n = 3-5). (A) Gene expression of the macrophage markers *Adgre1* (F4/80) and *Cd68* in whole tissue, as determined by qRT-PCR. (B-E) Flow cytometric data. (B) Representative flow cytometric data of ATMs in eWAT. (C) Numbers of ATMs per gram of tissue. (D) Representative flow cytometric data of $CD11c^+$ ATMs in eWAT. (E) Frequency of $CD11c^+$ ATMs. (F) Expression of *Ccl2* and *Tnf* in whole tissue. The data are presented as individual values ± SD. *P < .05, **P < .01, and ***P < .001. ns, not significant.

resistance. What could explain this discrepancy? There are several possibilities.

First, there were a number of environmental and experimental differences between the Wensveen et al. (2015) and our studies, including the fact that different animal facilities were used: it is well-known that animal facilities have disparate environmental microbiomes that can shape metabolic phenotypes (Musso et al., 2010; Turnbaugh et al., 2006). However, we repeated our studies in 2 separate facilities, namely, the Joslin Diabetes Center in the USA and the Soonchunhyang Institute of Medi-bio Science in Korea, where the environmental microbiomes are likely to be quite disparate. Thus, while the different microbiomes between our facility and that of Wensveen et al. could contribute to the different results, their effects may be limited.

Another important experimental difference is that we used a 60% fat diet (D12492, Research Diets, Inc) while Wensveen et al. (2015) used a diet where 50% of calories were from animal fat (Bregi). Different types and quantities of fat could potentially lead to quantitative metabolic/immunological differences, which may also produce different outcomes between 2 laboratories.

There is also an important immunological discrepancy between 2 studies. Our study showed that while NKp46 deletion did ablate $NKp46^+$ NK-cell numbers, it did not significantly change the numbers of total NK cells (ie, NK cells gated without NKp46) in any of the tissues. This is consistent with other studies on the same mutant mice (Pallmer et al., 2019; Sheppard et al., 2018). By contrast, Wensveen et al. (2015) showed that the total eWAT NK-cell numbers were greatly decreased in HFD-fed NKp46 knockout mice, although contradictorily, the description in the text of these data stated that there were no changes in total NK-cell numbers. If the total eWAT NK-cell numbers were in fact reduced by NKp46 knockout, this would mimic the effects of NK-cell depletion (eg, by anti-NK1.1 antibody), which reverses HFD-induced ATM activation and insulin resistance (Lee et al., 2016; Wensveen et al., 2015).

We initially speculated that the different total NK-cell numbers in the NKp46-knockout mice in our study and that of Wensveen et al. could reflect the fact that they examined the immunological phenotypes early in obesity (4-6 weeks on a HFD) whereas we examined it at 16 weeks. This would suggest that total NK-cell numbers in NKp46-knockout mice are low early in obesity but then recover in later stages of obesity. However, this does not explain why the NKp46-knockout mice had divergent metabolic outcomes in our study and that of Wensveen et al. (2015), because both studies examined metabolic phenotypes in late obesity (12-16 weeks). Moreover, NKp46 is not known to regulate NK-cell proliferation or development (Gazit et al., 2006), so it is unclear how NKp46 knockout would suppress NK-cell numbers early in obesity.

Thus, it is not clear why the NKp46-knockout mice had disparate immunological and metabolic outcomes in our study and that of Wensveen et al.

It should be noted that NKp46 is also expressed by other cells, including type-1 and -3 innate lymphoid cells (ILC1s and ILC3s) and NKT cells, all of which lack NKp46 expression in NKp46-knockout mice (Bonamichi and Lee, 2017). We showed previously with $Ja18^{-/-}$ and $CD1d^{-/-}$ models that NKT cells do not participate in obesity-induced insulin resistance (Lee et al., 2016). Hence, we do not expect that the loss of NKp46 expression by NKT cells will shape the development of obesity-induced inflammation/insulin resistance in NKp46-knockout mice. However, it should be noted that there is some controversy regarding the roles of NKT cells in obesity-induced insulin resistance: some laboratories show that NKT cells help resist it while others show that NKT cells promote it or play no role (Bonamichi and Lee, 2017; Huh et al., 2013; Satoh et al., 2016). Moreover, there is evidence that ILC1s may promote the development of obesity-induced inflammation and insulin resistance while ILC3s may either resist or promote it (O'Sullivan et al., 2016; Sasaki et al., 2019; Wu and Ballantyne, 2020). Thus, the role that NKp46 on NKT cells, ILC1s, and ILC3s plays in obesity-induced insulin resistance remains to be determined.

It should also be noted that although NKp46 is an important activating receptor for NK-cell cytotoxicity against cancer and infected cells, other NK-cell functions appear to be unaffected. This may reflect compensatory effects of other NK cell-activating receptors (Gazit et al., 2006; Imsirovic et al., 2023; Satoh-Takayama et al., 2009; Sheppard et al., 2013). This dispensability of NKp46 also casts some doubt on the notion that it plays a central role in obesity-induced NK-cell activation (Wensveen et al., 2015).

These observations together suggest that while NKp46 could drive obesity-induced insulin resistance, its role may be relatively weak and sensitive to environmental conditions and/or experimental approaches. Further studies are warranted, but nonetheless, it seems that obesity largely activates NK cells in epididymal fat via molecular mechanisms that do not involve NKp46.

DECLARATION OF COMPETING INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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AUTHOR CONTRIBUTIONS

G.N., B.D.S.F.B., E.R.H., and J.L. designed the studies. G.N., B.D.S.F.B., J.K., J.J, H.K., E.R.H., and E.E. performed the experiments and analyzed the data. G.N. and J.L. wrote the manuscript.

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REFERENCES

Bonamichi, B., and Lee, J. (2017). Unusual suspects in the development of obesity-induced inflammation and insulin resistance: NK cells, iNKT cells, and ILCs. Diabetes Metab. J. *41*, 229-250.

Boyiadzis, M., Memon, S., Carson, J., Allen, K., Szczepanski, M.J., Vance, B.A., Dean, R., Bishop, M.R., Gress, R.E., and Hakim, F.T. (2008). Up-regulation of NK cell activating receptors following allogeneic hematopoietic stem cell transplantation under a lymphodepleting reduced intensity regimen is associated with elevated IL-15 levels. Biol. Blood Marrow Transplant. *14*, 290-300.

Gazit, R., Gruda, R., Elboim, M., Arnon, T.I., Katz, G., Achdout, H., Hanna, J., Qimron, U., Landau, G., Greenbaum, E., et al. (2006). Lethal influenza infection in the absence of the natural killer cell receptor gene Ncr1. Nat. Immunol. *7*, 517-523.

Hadad, U., Thauland, T.J., Martinez, O.M., Butte, M.J., Porgador, A., and Krams, S.M. (2015). NKp46 clusters at the immune synapse and regulates NK cell polarization. Front. Immunol. *6*, 495.

Hotamisligil, G.S. (1999). The role of TNFalpha and TNF receptors in obesity and insulin resistance. J. Intern. Med. *245*, 621-625.

Huh, J.Y., Kim, J.I., Park, Y.J., Hwang, I.J., Lee, Y.S., Sohn, J.H., Lee, S.K., Alfadda, A.A., Kim, S.S., Choi, S.H., et al. (2013). A novel function of adipocytes in lipid antigen presentation to iNKT cells. Mol. Cell Biol. *33*, 328-339.

Imsirovic, V., Lenartic, M., Wensveen, F.M., Polic, B., and Jelencic, V. (2023). Largely preserved functionality after the combined loss of NKG2D, NCR1 and CD16 demonstrates the remarkable plasticity of NK cell responsiveness. Front. Immunol. *14*, 1191884.

Kolb, H. (2022). Obese visceral fat tissue inflammation: from protective to detrimental? BMC Med. *20*, Article 494.

Koska, J., Stefan, N., Permana, P.A., Weyer, C., Sonoda, M., Bogardus, C., Smith, S.R., Joanisse, D.R., Funahashi, T., Krakoff, J., et al. (2008). Increased fat accumulation in liver may link insulin resistance with subcutaneous abdominal adipocyte enlargement, visceral adiposity, and hypoadiponectinemia in obese individuals. Am. J. Clin. Nutr. *87*, 295-302.

Lee, B.C., Kim, M.S., Pae, M., Yamamoto, Y., Eberle, D., Shimada, T., Kamei, N., Park, H.S., Sasorith, S., Woo, J.R., et al. (2016). Adipose natural killer cells regulate adipose tissue macrophages to promote insulin resistance in obesity. Cell Metab. *23*, 685-698.

Li, J.H., Hepworth, M.R., and O'Sullivan, T.E. (2023). Regulation of systemic metabolism by tissue-resident immune cell circuits. Immunity, *56*, 1168-1186.

Lumeng, C.N., Bodzin, J.L., and Saltiel, A.R. (2007). Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J. Clin. Invest. *117*, 175-184.

Matthews, D.R., Hosker, J.P., Rudenski, A.S., Naylor, B.A., Treacher, D.F., and Turner, R.C. (1985). Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia, *28*, 412-419.

Michelet, X., Dyck, L., Hogan, A., Loftus, R.M., Duquette, D., Wei, K., Beyaz, S., Tavakkoli, A., Foley, C., Donnelly, R., et al. (2018). Metabolic reprogramming of natural killer cells in obesity limits antitumor responses. Nat. Immunol. *19*, 1330-1340.

Muoio, D.M., and Newgard, C.B. (2008). Mechanisms of disease: Molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. Nat. Rev. Mol. Cell Biol. *9*, 193-205.

Musso, G., Gambino, R., and Cassader, M. (2010). Obesity, diabetes, and gut microbiota: the hygiene hypothesis expanded? Diabetes Care, *33*, 2277-2284.

Narni-Mancinelli, E., Chaix, J., Fenis, A., Kerdiles, Y.M., Yessaad, N., Reynders, A., Gregoire, C., Luche, H., Ugolini, S., Tomasello, E., et al. (2011). Fate mapping analysis of lymphoid cells expressing the NKp46 cell surface receptor. Proc. Natl. Acad. Sci. U.S.A. *108*, 18324-18329.

O'Rourke, R.W., Meyer, K.A., Neeley, C.K., Gaston, G.D., Sekhri, P., Szumowski, M., Zamarron, B., Lumeng, C.N., and Marks, D.L. (2014). Systemic NK cell ablation attenuates intra-abdominal adipose tissue macrophage infiltration in murine obesity. Obesity, *22*, 2109-2114.

O'Sullivan, T.E., Rapp, M., Fan, X., Weizman, O.E., Bhardwaj, P., Adams, N.M., Walzer, T., Dannenberg, A.J., and Sun, J.C. (2016). Adipose-resident group 1 innate lymphoid cells promote obesity-associated insulin resistance. Immunity, *45*, 428-441.

Pallmer, K., Barnstorf, I., Baumann, N.S., Borsa, M., Jonjic, S., and Oxenius, A. (2019). NK cells negatively regulate CD8 T cells via natural cytotoxicity receptor (NCR) 1 during LCMV infection. PLoS Pathog. *15*, Article e1007725.

Prager, I., and Watzl, C. (2019). Mechanisms of natural killer cell-mediated cellular cytotoxicity. J. Leukoc. Biol. *105*, 1319-1329.

Reilly, S.M., and Saltiel, A.R. (2017). Adapting to obesity with adipose tissue inflammation. Nat. Rev. Endocrinol. *13*, 633-643.

Rohm, T.V., Meier, D.T., Olefsky, J.M., and Donath, M.Y. (2022). Inflammation in obesity, diabetes, and related disorders. Immunity, *55*, 31-55.

Sasaki, T., Moro, K., Kubota, T., Kubota, N., Kato, T., Ohno, H., Nakae, S., Saito, H., and Koyasu, S. (2019). Innate lymphoid cells in the induction of obesity. Cell Rep. *28*, 202-217 e207.

Satoh, M., Hoshino, M., Fujita, K., Iizuka, M., Fujii, S., Clingan, C.S., Van Kaer, L., and Iwabuchi, K. (2016). Adipocyte-specific CD1d-deficiency mitigates diet-induced obesity and insulin resistance in mice. Sci. Rep. *6*, Article 28473.

Satoh-Takayama, N., Dumoutier, L., Lesjean-Pottier, S., Ribeiro, V.S., Mandelboim, O., Renauld, J.C., Vosshenrich, C.A., and Di Santo, J.P.

(2009). The natural cytotoxicity receptor NKp46 is dispensable for IL-22-mediated innate intestinal immune defense against *Citrobacter rodentium*. J. Immunol. *183*, 6579-6587.

Sheppard, S., Schuster, I.S., Andoniou, C.E., Cocita, C., Adejumo, T., Kung, S.K.P., Sun, J.C., Degli-Esposti, M.A., and Guerra, N. (2018). The murine natural cytotoxic receptor NKp46/NCR1 controls TRAIL protein expression in NK cells and ILC1s. Cell Rep. *22*, 3385-3392.

Sheppard, S., Triulzi, C., Ardolino, M., Serna, D., Zhang, L., Raulet, D.H., and Guerra, N. (2013). Characterization of a novel NKG2D and NKp46 double-mutant mouse reveals subtle variations in the NK cell repertoire. Blood, *121*, 5025-5033.

Shin, K.O., Bae, J.Y., Woo, J., Jang, K.S., Kim, K.S., Park, J.S., Kim, I.K., and Kang, S. (2015). The effect of exercise on expression of myokine and angiogenesis mRNA in skeletal muscle of high fat diet induced obese rat. J. Exerc. Nutr. Biochem. *19*, 91-98.

Standl, E., Khunti, K., Hansen, T.B., and Schnell, O. (2019). The global epidemics of diabetes in the 21st century: Current situation and perspectives. Eur. J. Prev. Cardiol. *26*, 7-14.

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., and Gordon, J.I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. Nature, *444*, 1027-1031.

Walzer, T., Blery, M., Chaix, J., Fuseri, N., Chasson, L., Robbins, S.H., Jaeger, S., Andre, P., Gauthier, L., Daniel, L., et al. (2007). Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. Proc. Natl. Acad. Sci. U.S.A. *104*, 3384-3389.

Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L., and Ferrante, A.W., Jr. (2003). Obesity is associated with macrophage accumulation in adipose tissue. J. Clin. Invest. *112*, 1796-1808.

Wensveen, F.M., Jelencic, V., Valentic, S., Sestan, M., Wensveen, T.T., Theurich, S., Glasner, A., Mendrila, D., Stimac, D., Wunderlich, F.T., et al. (2015). NK cells link obesity-induced adipose stress to inflammation and insulin resistance. Nat. Immunol. *16*, 376-385.

Wrann, C.D., Laue, T., Hubner, L., Kuhlmann, S., Jacobs, R., Goudeva, L., and Nave, H. (2012). Short-term and long-term leptin exposure differentially affect human natural killer cell immune functions. Am. J. Physiol. Endocrinol. Metab. *302*, E108-E116.

Wu, H., and Ballantyne, C.M. (2020). Metabolic inflammation and insulin resistance in obesity. Circ. Res. *126*, 1549-1564.

Xu, H., Barnes, G.T., Yang, Q., Tan, G., Yang, D., Chou, C.J., Sole, J., Nichols, A., Ross, J.S., Tartaglia, L.A., et al. (2003). Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J. Clin. Invest. *112*, 1821-1830.