**Toll-like Receptor 4 Deficiency Promotes the Alternative Activation of Adipose Tissue Macrophages**

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Obesity is characterized by adipose tissue (AT) macrophage (ATM) accumulation, which promotes AT inflammation and dysfunction. Toll-like receptor 4 (TLR4) deficiency attenuates AT inflammation in obesity but does not impede the accumulation of ATMs. The purpose of the current study was to determine whether TLR4 deficiency alters ATM polarization. TLR4−/− and wild-type mice were fed a low-fat, high-monounsaturated fat (HFMUFA), or a high-saturated fat (HFSFA) diet for 16 weeks. Further, we used a bone marrow transplant model to determine the influence of hematopoietic cell TLR4 signaling. The metabolic and inflammatory responses to high-fat feeding and ATM phenotype were assessed. Global and hematopoietic cell TLR4 deficiency, irrespective of recipient genotype, produced a shift in ATM phenotype toward an alternatively activated state, which was accompanied by reduced AT inflammation. Despite the observed shift in ATM phenotype, neither global nor hematopoietic cell TLR4 deficiency influenced systemic insulin sensitivity after high-fat feeding. Results of the current study suggest that TLR4 directly influences ATM polarization but question the relevance of TLR4 signaling to systemic glucose homeostasis in obesity.

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In 1993, Hotamisligil et al. (1) first demonstrated that tumor necrosis factor-α (TNF-α) is expressed in adipose tissue (AT) and elevated in various rodent models of obesity. Subsequent research has led to the characterization of the obese state as one of chronic low-grade inflammation, which contributes to the development of systemic insulin resistance (IR) (2). Implicit in the idea that inflammatory status links obesity and IR is the notion that signaling pathways exist whereby the recognition of nutrient excess elicits an immune response. Confirmation of such a mechanism came with the discovery of the first human homolog of the Drosophila Toll receptor, Toll-like receptor 4 (TLR4) (3), and the subsequent finding that saturated fatty acids (SFAs) were capable of activating nuclear factor-κB (NF-κB) in a TLR4-dependent manner (4).

Numerous studies have explored the role of TLR4 signaling in the metabolic consequences of diet-induced obesity (DIO). Despite contradictory findings with respect to the influence of TLR4 on weight gain, the present literature consistently demonstrates that TLR4 deficiency reduces AT inflammation and hepatic steatosis after a high-fat (HF) diet (HFD) (5–7). Thus, the available evidence suggests that the beneficial metabolic effects of TLR4 deficiency may be due, in part, to the preservation of AT function during the development of DIO.

The capacity of AT to act as an effective buffer against lipid “spillover” into metabolic tissues (e.g., skeletal muscle, liver, kidneys) relies on its ability to expand, via hypertrophy and hyperplasia, in response to chronic overnutrition. This process involves the coordinated interaction of various cell populations comprising the AT stromal vascular fraction (SVF), including AT macrophages (ATMs), which have received a great deal of attention due to their progressive accumulation during AT expansion as well as their high inflammatory potential and influence on AT insulin sensitivity and angiogenesis (8,9). It is interesting to note that although hematopoietic or global TLR4 deficiency results in decreased AT inflammation, the prevalence of ATMs is not reliably reduced (10–13), thus raising questions about the influence of TLR4 signaling on AT phenotype.

Recently, the phenotypic diversity of ATMs has come to the forefront. In keeping with the T helper 1 and 2 (Th1/Th2) paradigm, the current literature relies on the M1/M2 nomenclature to refer to classically and alternatively activated macrophages, respectively. The prevailing model of ATM polarization holds that “resident” ATMs display an alternatively activated phenotype, whereas macrophages recruited to AT during the onset of obesity exhibit a predominantly M1 “classical activation” state (14). This obesity-associated shift in ATM polarization leads to a pronounced increase in the ratio of M1-to-M2 ATMs, thus promoting an inflammatory state within the AT.

Although the direct influence of TLR4 signaling on ATM polarization remains unclear, several lines of evidence point to a potential role: 1) TLR4 deficiency attenuates obesity-associated AT inflammation and IR in the face of ATM accumulation (10–13); 2) recruited M1 ATMs express high levels of TLR4, whereas M2 ATMs highly express suppression of tumorigenicity 2 (ST2), a negative regulator of TLR4 signaling (15); 3) overexpression of activating transcription factor-3 (ATF3), a transcriptional repressor of TLR4 signaling, reduces M1 polarization of ATMs after 4 weeks of HF feeding (12). Taken together, the above evidence suggests that TLR4 deficiency may attenuate the inflammatory response to HF feeding, in part, by promoting the alternative activation of ATMs. Accordingly, the purpose of the current study was to test the hypothesis that TLR4 deficiency would promote the alternative activation of ATMs, which would be associated with a reduction in AT inflammation and systemic IR.

**RESEARCH DESIGN AND METHODS**

**Diets.** The current study used the following dietary treatments: low fat (LF; 10.5% kcal from fat), high-monounsaturated fat (HFMUFA; 45% kcal from fat, 2012 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See http://creativecommons.org/licenses/by-nc-nd/3.0/ for details.
which 68.5% were MUFA), and high-saturated fat (HFSFA; 45% kcal from fat, of which 91.5% were SFAs). Corn starch served as the predominant carbohydrate source to minimize the potential confounding influence of the increased sucrose content of commercial HFDs. All diets were purchased from Research Diets (New Brunswick, NJ). Mice had free access to water, and average daily food intake was assessed weekly in each cage of mice.

**Mice.** All animal care and experimental procedures were approved by the Vanderbilt University Institutional Animal Care and Usage Committee. Only male mice were included in the current study. TLR4<sup>−/−</sup> mice on a C57BL/6 background were provided by Satoshi Uematsu and Shizuo Akira (Department of Host Defense Research, Research Institute of Osaka University, Osaka, Japan) and subsequently bred with C57BL/6 mice from our colony. TLR4<sup>−/−</sup> offspring were interbred to obtain the TLR4<sup>−/−</sup> mice included in the current study (n = 13 LF, 13 HF<sub>MUFA</sub>, and 13 HF<sub>FSFA</sub>). C57BL/6, wild-type (WT) control mice (n = 15 LF, 14 HF<sub>MUFA</sub>, and 16 HF<sub>FSFA</sub>) were from our colony or purchased from Jackson Laboratory (Bar Harbor, ME). WT mice obtained from Jackson Laboratory were given a 1-week acclimation period before being included in the study. Similar results were obtained regardless of the source of WT mice.

**Bone marrow transplant.** After lethal irradiation, male WT and TLR4<sup>−/−</sup> mice were reconstituted with bone marrow (BM) from age-matched male WT or TLR4<sup>−/−</sup> donor mice, as previously described (13). WT recipients reconstituted with WT and TLR4<sup>−/−</sup> BM are denoted as WT<sub>WTBM</sub> and WT<sub>TLR4 BM</sub>, respectively. TLR4<sup>−/−</sup> recipients reconstituted with WT and TLR4<sup>−/−</sup> BM are denoted as TLR4<sup>−/−</sup> WT<sub>WTBM</sub> and TLR4<sup>−/−</sup> TLR4<sup>−/−</sup> BM, respectively.

**Body composition.** Total body fat, muscle, and free fluid were measured via nuclear magnetic resonance using a Bruker Minispec (Woodlands, TX) in the Vanderbilt University Mouse Metabolic Phenotyping Center (MMPC). Blood collection and plasma analyses. Fasting blood glucose, plasma insulin, adiponectin, total cholesterol, free fatty acids (FFA), and triglyceride (TG) concentrations were measured, as previously described (16). Plasma leptin was assessed via radioimmunoassay in the Vanderbilt Hormone Assay and Analytical Services Core.

**Adipocyte and SVF isolation.** Mice were killed, and blood was removed via central perfusion. Perigonadal AT depots were minced in Dulbecco’s PBS supplemented with 0.5% BSA. Subsequently, CaCl<sub>2</sub> and type II collagenase were added to a final concentration of 5 mM and 1 mg/mL, respectively. Tissue homogenates were incubated at 37°C for 20 min with shaking. After filtration and centrifugation, buoyant adipocytes were removed, washed twice, and collected as the adipocyte fraction. The remaining cell pellet was resuspended in ACK lysis buffer, washed, and collected as the SVF.

**Fluorescence-activated cell sorter analysis.** SVF cells were incubated with Fc block (BD Biosciences) on ice for 15 min. Subsequently, primary fluorophore-conjugated antibodies against F4/80 (eBioscience) and M<sub>1</sub>1 (Bio-Rad, Hercules, CA). Primer-probe sets were purchased from Applied Biosystem Assays-on-Demand (catalog numbers available upon request).

**RESULTS**

**Body weight and body fat gain was slightly reduced by TLR4 deficiency.** In light of previous studies, suggesting that SFAs act as a ligand for TLR4 (4,24,25), we chose an experimental design whereby the diet-treatment groups differed in fat content (i.e., 10 vs. 45% kcal from fat), as well as fatty acid profile (i.e., HF<sub>MUFA</sub> vs. HF<sub>FSFA</sub>). Although HF<sub>MUFA</sub> and HF<sub>FSFA</sub> feeding induced dramatic weight gain in WT and TLR4<sup>−/−</sup> mice, TLR4 deficiency blunted weight gain in response to both HFDs (Fig. 1A). Consistent with the observed differences in weight gain, food intake was significantly reduced in TLR4<sup>−/−</sup> compared with WT counterparts (Supplementary Fig. 1A). Because TLR4 signaling has previously been implicated in the development of hypothalamic inflammation in response to HF feeding (26), we assessed hypothalamic inflammatory gene expression to determine whether alterations in hypothalamic inflammation are associated with the observed reduction in food intake by TLR4-deficient mice. TLR4<sup>−/−</sup> mice exhibited significantly greater hypothalamic Il10 expression after HF<sub>FSFA</sub> feeding; however, hypothalamic expression of Tnfα, Il6, chemokine (C-C motif) ligand (CCL) 2 (Ccl2), and Il1b was unchanged after HF feeding and was not influenced by genotype (Supplementary Fig. 1B–F). Despite producing similar weight gain, significant diet effects were observed with respect to total adiposity, as HF<sub>FSFA</sub> feeding induced the greatest increases in body fat (Supplementary Table 1). In contrast to total body fat, TLR4 deficiency reduced visceral fat accumulation, as significant genotype effects were present for perirenal and perirenal fat pad weight (Supplementary Table 1). As expected, HF feeding produced significant elevations in plasma leptin concentrations; however, TLR4 deficiency attenuated this increase in HF<sub>FSFA</sub>-fed mice (Supplementary Table 2). There was a significant diet × genotype interaction for plasma adiponectin, as concentrations were reduced in TLR4<sup>−/−</sup> compared with WT mice fed the LF diet. In addition, the HF<sub>FSFA</sub> diet reduced adiponectin concentrations regardless of genotype (Supplementary Table 2).

**Oil Red O staining.** Neutral lipid accumulation in liver sections was visualized by Oil Red O (ORO) staining, as previously described (19).

**Statistics.** Statistical analyses were performed using GraphPad Prism 4.03 (GraphPad, La Jolla, CA) and JMP 8.0.2 (SAS Institute, Inc., Cary, NC) software. Non-normally distributed data were log-transformed. Changes in dependent variables over time were assessed using repeated-measures ANOVA with the Bonferroni post hoc test. Diet and genotype effects, as well as diet × genotype interactions were determined by two-way ANOVA with the Tukey honestly significant difference post hoc test. Comparisons within BM transplant (BMT) recipient groups were performed using unpaired t tests. The Grubbs test was used to detect significant outliers, which were removed before statistical analysis. Data are expressed as mean ± SEM, and significance was set at P < 0.05.
to the LF diet, HF -SFA feeding increased Ccl2 expression regardless of genotype. Interestingly, expression of the chemokine Ccl2 was significantly greater in HF-SFA-fed TLR4−/− mice than in WT mice. Il6 and Il10 expression were not influenced by diet or genotype.

**TLR4 deficiency promotes M2 polarization of ATMs and PMs.** ATM accumulation, as quantified by the macrophage markers F4/80 (Emr1; Fig. 3A) and cluster of differentiation (CD) 68 (Cd68; Fig. 3B), was significantly increased by both HFDs in WT and TLR4−/− mice. Likewise, WT and TLR4−/− mice displayed similar increases in the prevalence of Mac-2-positive crown-like structures in the AT after HF feeding (data not shown). To assess ATM polarization, several markers of classical (M1) and alternative (M2) activation were quantified by real-time RT-PCR (Fig. 3C–H). Significant genotype effects were observed for mannose receptor C type 1 (Mrc1), C-type lectin domain family 10 member A (Clec10a), and macrophage galactose N-acetyl-galactosamine specific lectin 2 (Mgl2) as shown by these M2 markers being upregulated in the absence of TLR4 expression. In the case of Clec10a, there was a significant diet × genotype interaction, such that expression was significantly greater in HF-SFA-fed TLR4−/− mice than in WT mice. Ccl2 and Emr1 expression were not influenced by diet or genotype.

**FIG. 1.** TLR4 deficiency attenuates weight gain and hepatic TG accumulation after HF feeding. **A:** WT and TLR4-deficient mice display similar growth curves on a LF diet, whereas weight gain is attenuated in TLR4−/− mice after HF-SFA and HF-MUFA feeding (n = 13–15 for all diet conditions). **B:** TLR4 deficiency reduces HF-feeding–induced hepatic TG accumulation (n = 13–16). **C:** Representative images of ORO-stained liver sections. Data are presented as mean ± SEM. **P < 0.01 and ***P < 0.001 for WT HF-SFA vs. TLR4−/− HF-SFA; #P < 0.05 for WT HF-MUFA vs. TLR4−/− HF-MUFA; **P < 0.01 for genotype effect; and P < 0.05 for groups not connected by the same letter. (A high-quality digital representation of this figure is available in the online issue.)
expression data (Fig. 3I–K). HF<sup>SFA</sup>-fed WT and TLR4<sup>−/−</sup> mice displayed similar numbers of ATMs as a percentage of SVF cells. However, consistent with the previously described elevation in Clec10a gene expression, the proportion of F4/80<sup>+</sup>Mgl1<sup>+</sup> ATMs was significantly increased in TLR4-deficient mice. Finally, TLR4-deficient PMs appeared skewed toward an alternatively activated gene expression profile as TLR4<sup>−/−</sup> PMs exhibited significantly greater Il10 and Arg1 expression compared with WT (Supplementary Fig. 3A and B) as well as significantly greater Clec10a expression and reduced Nos2 expression after Th2 stimulation (Supplementary Fig. 3A and F).

**Systemic insulin sensitivity is unaffected by TLR4 deficiency.** Fasting plasma insulin and blood glucose concentrations were increased after HF feeding and were not influenced by genotype (Supplementary Table 2). Systemic insulin sensitivity was not influenced by genotype, because the glucose infusion rate required to maintain blood glucose between 120 and 130 mg/dL (Fig. 4A) during a hyperinsulinemic euglycemic clamp did not differ between HF<sup>SFA</sup>-fed WT (29.9 ± 2.6 mg/kg/min) and TLR4<sup>−/−</sup> (26.5 ± 3.4 mg/kg/min) mice (Fig. 4B). Likewise, WT and TLR4<sup>−/−</sup> mice did not differ with respect to the rate of endogenous glucose appearance or glucose disappearance, suggesting comparable hepatic and peripheral insulin sensitivity, respectively (Fig. 4C and D). However, we observed significant reductions in insulin-stimulated glucose uptake at the gastrocnemius, superficial vastus lateralis, and AT of TLR4<sup>−/−</sup> mice compared with WT mice (Fig. 4E).

**Hematopoietic cell TLR4 deficiency does not influence body weight, adiposity, or steatosis.** In light of our data suggesting that, after HF<sup>SFA</sup> feeding, TLR4 deficiency attenuates hepatic steatosis and AT inflammation comcomitant to a shift in ATM polarization toward an M2 phenotype, we next sought to determine whether hematopoietic cell TLR4 deficiency recapitulates the global TLR4<sup>−/−</sup> phenotype in response to HF<sup>SFA</sup> feeding. Toward this aim, we conducted both the forward and reverse BMT, whereby lethally irradiated groups of WT and TLR4<sup>−/−</sup> recipients were reconstituted with WT or TLR4<sup>−/−</sup>BM (i.e., WT<sub>WTBM</sub>, WT<sub>TLR4−/−BM</sub>, and TLR4<sup>−/−</sup>BM<sub>TLR4−/−BM</sub>). Subsequently, mice were fed the HF<sup>SFA</sup> diet for 20 weeks. Throughout the study, TLR4<sup>−/−</sup> recipient groups weighed less than their WT counterparts, irrespective of hematopoietic cell TLR4 expression (Supplementary Fig. 4A). Likewise, food consumption was significantly reduced in TLR4<sup>−/−</sup> compared with WT recipient groups and was not influenced by hematopoietic cell TLR4 expression (Supplementary Fig. 4B). Body fat and lean body mass were significantly reduced in TLR4<sup>−/−</sup> recipient groups (Supplementary Fig. 4C and D). Adiposity was similar between recipient groups and unaffected by hematopoietic cell TLR4 expression (Supplementary Fig. 4E).

Reflecting the differences in body weight between recipient groups, liver weight was significantly greater in WT recipient groups (Supplementary Fig. 5A). Given our observation of attenuated hepatic steatosis in TLR4<sup>−/−</sup> mice, we were surprised that TLR4 deficiency had no influence on hepatic TG accumulation in our BMT model (Supplementary Fig. 5B and C). Neither liver weight nor hepatic TG concentrations were influenced by hematopoietic cell TLR4 expression.

**Hematopoietic cell TLR4 deficiency attenuates AT inflammation in WT recipients.** To ascertain the influence of hematopoietic and parenchymal TLR4 deficiency on AT inflammation, the expression of various chemokines and cytokines were determined separately in isolated SVF and adipocyte fractions from the perigonadal AT. In the adipocyte fraction of WT<sub>TLR4−/−BM</sub>, Tnfa and Clec10a gene
expression was significantly reduced compared with WTWTBM (Fig. 5A and B). Likewise, hematopoietic cell TLR4 deficiency reduced SVF Ccl3 expression in WT recipients (Fig. 5F). Thus, reconstitution of WT recipients with TLR4−/− BM reduced AT inflammation. In contrast to WT recipients, hematopoietic cell TLR4 expression did not influence inflammatory gene expression in the AT of TLR4−/− recipients (Fig. 5A–H), despite exhibiting differential SVF expression of Tlr2 (Fig. 5I).

**Hematopoietic cell TLR4 deficiency promotes ATM alternative activation.** AT SVF expression of Emr1 and Cd68 were used as surrogate markers of AT accumulation. Whereas Emr1 expression was not influenced by recipient or hematopoietic cell TLR4 expression, Cd68 was significantly upregulated in the WTTLR4−/−BM group compared with the WTWTBM group (Fig. 6B). Similar to our findings of AT alternative activation in globally deficient TLR4−/− mice, hematopoietic cell TLR4 deficiency resulted in significantly greater Clec10a, Mgl2, and Mrc1 expression in WT recipients (Fig. 6C–E). Likewise, TLR4−/−TLR4−/−BM mice displayed elevated expression of Clec10a and Mrc1 as well as reduced expression of Nos2 compared with TLR4−/−WTBM (Fig. 6C, E, and F). SVF expression of Il10 was significantly reduced in WTTLR4−/−BM mice compared with WTWTBM mice (Fig. 6H).

**Hematopoietic cell TLR4 deficiency does not influence systemic insulin sensitivity.** To determine the contribution of parenchymal and hematopoietic cell TLR4 expression to the metabolic response to a HFSFA diet challenge, as well as to account for any significant baseline differences, mice from each BMT group underwent a glucose tolerance test (GTT) before the BMT procedure, and a GTT and insulin tolerance test (ITT) after 20 weeks of HFSFA feeding. Before BMT, all groups exhibited similar glucose excursion curves during the GTT (Fig. 7A). In contrast, TLR4−/− recipient groups displayed impaired glucose tolerance compared with WT recipients, regardless of hematopoietic cell TLR4 expression (Fig. 7B). In addition, IR, as assessed by ITT, was significantly greater in the TLR4−/− recipient groups than in WT groups; again, hematopoietic TLR4 deficiency had no effect on insulin sensitivity (Fig. 7C). A significant recipient × BM genotype interaction was observed for fasting blood glucose as shown by significantly greater concentrations in WTTLR4−/−BM than in WTWTBM (Fig. 7D). Plasma insulin was significantly elevated in WT recipients and was not influenced by hematopoietic cell TLR4 expression (Fig. 7E).

**DISCUSSION**

The primary findings of the current study are that global TLR4 deficiency reduces AT inflammation concomitant...
with a shift in ATM polarization toward an alternatively activated state. To our knowledge, this is the first direct experimental evidence for a role of TLR4 in ATM polarization, which is further supported by the finding that hematopoietic cell TLR4 deficiency is sufficient to elicit the alternative activation of ATMs irrespective of recipient genotype.

Despite using a dietary strategy aimed at maximizing our ability to detect TLR4-dependent effects of dietary SFA content, we failed to observe a robust difference in the metabolic or inflammatory response of TLR4−/− mice to the HFSFA and HP SFA diets. Results of previous studies suggesting that SFAs directly activate TLR4 (4,24,25) have been questioned.

FIG. 4. TLR4 deficiency does not attenuate systemic IR after HFSFA feeding. A: Time course of blood glucose throughout the hyperinsulinemic-euglycemic clamp is presented to illustrate clamp quality. B: The glucose infusion rate required to maintain blood glucose between 120 and 130 mg/dL did not differ between HP SFA-fed TLR4−/− and WT mice. WT and TLR4−/− mice displayed similar rates of endogenous glucose production (EndoRg) (C) and glucose disappearance (Rd) (D). E: Glucose uptake (Rg) was significantly reduced in the gastrocnemius (Gastroc), superficial vastus lateralis (SVL), and AT of TLR4−/− mice compared with WT. Data are presented as mean ± SEM (n = 4–7). **P < 0.01 and ***P < 0.0001 for TLR4−/− compared with WT.
recent report by Erridge and Samani (27) demonstrates that lipopolysaccharide and lipopeptide contamination of BSA accounts for the TLR4 activation commonly attributed to SFA treatment. We recognize that the current study does not directly address this issue; however, our findings do not generally support the notion that dietary SFAs directly induce TLR4 signaling. Irrespective of whether SFAs act as a TLR4 ligand, several lines of evidence suggest a much more complex and multifaceted immunomodulatory role for dietary fatty acids, such as inducing a shift to gram-negative intestinal microbiota and increasing gut permeability (28).

The finding of reduced weight gain in TLR4−/− mice after HF feeding is in line with some (5,7,26,29)—but not all—previous investigations (6,10–12,30,31). Careful examination of the available literature highlights the difficulty in drawing direct comparisons between previous studies because important differences exist with respect to 1) sex and age of the mice used; 2) percentage of dietary fat (ranging from 42–60% kcal from fat); 3) carbohydrate source (e.g., sucrose or corn starch); 4) TLR4-deficient model (i.e., C57BL/10ScN, C3H/HeJ, or TLR4−/− mice on a BL/6 or BL/10 background); and 5) study duration. Unfortunately, no discernible patterns exist that might explain the various weight gain results. Taken in context with the current literature, our study suggests that any protection TLR4 deficiency may confer with respect to weight gain is likely very modest and of questionable physiologic relevance.

Our results are in agreement with the general consensus of previous studies demonstrating a reduction in hepatic lipid accumulation in TLR4−/− mice after HF feeding (5–7). Perhaps surprising was the lack of effect noted in our BMT model with respect to the influence of parenchymal or hematopoietic cell TLR4 deficiency on hepatic steatosis. Whereas we observed similar levels of hepatic TG accumulation in WT and TLR4−/− recipients, regardless of hematopoietic cell TLR4 expression, Saberi et al. (32) recently reported a dramatic reduction in hepatic TG concentrations after

FIG. 5. Hematopoietic cell TLR4 deficiency attenuates AT inflammation in WT recipient mice. Real time RT-PCR was used to assess adipocyte fraction mRNA expression of Tnfa (A), Ccl3 (B), Ccl2 (C), and Il6 (D) as well as SVF mRNA expression of Tnfa (E), Ccl3 (F), Ccl2 (G), Il6 (H), and Tlr2 (I). Data are presented as mean ± SEM (n = 7–16). *P < 0.05 for WTTLR4−/−−BM compared with WTWTBM, and #P < 0.01 for TLR4−/−TLR4−/−BM compared with TLR4−/−−WTBM.
HF feeding in WT mice reconstituted with TLR4−/− BM. The reasons for this inconsistency are unclear but likely include differences in diet and the duration of HF feeding. Similar to previous investigations (10–13), we found that global as well as hematopoietic cell TLR4 deficiency produces modest reductions in AT inflammation despite comparable increases in ATM accumulation after HF feeding, suggesting that the absence of TLR4 signaling may influence ATM phenotype. Support for this possibility comes from the finding that the expression of various

### FIG. 6. Hematopoietic cell TLR4 deficiency promotes the alternative activation of ATMs. Real-time RT-PCR was used to assess SVF mRNA expression of Emr1 (A), Cd68 (B), Clec10a (C), Mgl2 (D), Mrcl (E), Nos2 (F), Arg1 (G), and Il10 (H). Data are presented as mean ± SEM (n = 7–16). *P < 0.05 for WTTLR4−/−BM compared with WTWTBM, and #P < 0.05 for TLR4−/−TLR4−/−BM compared with TLR4−/−WTBM.

### FIG. 7. A: Hematopoietic cell TLR4 deficiency does not influence systemic insulin sensitivity. WT and TLR4−/− mice displayed similar glucose excursion curves before BMT (n = 5–9). After 20 weeks of an HFSFA diet, TLR4−/− recipient mice exhibited impaired glucose (n = 8–15) (B) and insulin (n = 6–8) (C) tolerance compared with WT recipients, regardless of hematopoietic cell TLR4 expression. D: Fasting blood glucose (n = 8–18). E: Fasting plasma insulin (n = 8–18). Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 for recipient genotype effect, and P < 0.05 for groups not connected by the same letter.
markers of M2 macrophage polarization are elevated in the AT of TLR4−/− mice. Likewise, the proportion of M2
ATMs (i.e., F4/80+M11+) is significantly greater in HF–fed TLR4−/− mice than in WT counterparts. Interestingly,
our BMT model points to a direct influence of TLR4 signaling on ATM polarization, as shown by the elevation of
M2 marker expression in the SVF of mice reconstituted with TLR4−/− BM, regardless of recipient genotype (i.e.,
WTTLR4−/− BM and TLR4−/− TLR4−/− BM). Additional support for a direct role of TLR4 in macrophage polarization comes
from the finding that TLR4−/− PMs are skewed toward an alternatively activated expression profile. In addition to
the potential for TLR4 deficiency to directly modulate ATM polarization, the possibility that the alternative activation
observed in TLR4-deficient ATMs may actually represent a relative resistance to the shift in ATM polarization toward
an M1 phenotype in response to elevated endotoxin concentration remains unclear. Regarding these inconsistencies, it is
interesting to note that those studies reporting an attenuation of IR after an HFD used diets providing 55–60% kcal
from fat (7,10,11), whereas studies (including the present) using diets consisting of 42–45% kcal from fat consistently
fail to show an effect of TLR4 deficiency on IR (5,6). This raises the possibility that our ability to detect the influence
of TLR4 on systemic insulin sensitivity was compromised by the use of 45% fat diets.

Similar to models of global TLR4 deficiency, the role of hematopoietic cell TLR4 signaling in HFD-induced IR is
uncertain. Olefsky and colleagues (32) recently reported that hematopoietic cell TLR4 deficiency, as well as lenthiviral
mediated knockdown of TLR4 in hematopoietic cells, attenuates diet-induced insulin resistance. In contrast, the current study
and a previous report by our laboratory (13) both failed to detect an improvement in systemic insulin sensitivity in mice
lacking hematopoietic TLR4 signaling. Notably, TLR4−/− recipients (i.e., TLR4−/− WTM and TLR4−/− WTM–TLR4−/− BM) were
significantly more glucose- and insulin-intolerant than WT recipients (i.e., WTM and WTM–TLR4−/− BM), regardless of
hematopoietic cell TLR4 expression. Because previous studies have only used WT recipients, we are unable to reconcile this unexpected finding. Nonetheless, this does not impact our finding that hematopoietic cell TLR4 deficiency does not influence glucose or insulin tolerance.

In summary, the current study demonstrates that TLR4 deficiency promotes the alternative activation of ATMs. Furthermore, the alternative activation of ATMs in hematopoietic cell TLR4−/− chimeras suggests that TLR4 signaling plays a direct role in mediating ATM phenotype in DIO. Lastly, it should be emphasized that, despite the observed influence of ATM phenotype on AT inflammation, in our hands, global and hematopoietic TLR4 deficiency produces a modest phenotype that does not manifest in improved systemic glucose homeostasis.

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J.S.O. and M.J.P. collected and analyzed the data, and wrote the manuscript. K.L.J.E. and C.N.L. helped collect the
data. D.H.W. and A.H.H. aided with data analysis and with writing and editing the manuscript. A.H.H. is the
 guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for
the integrity of the data and the accuracy of the data analysis.

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