TLR4, TRIF, and MyD88 are essential for myelopoiesis and CD11c+ adipose tissue macrophage production in obese mice

Cameron Griffin, Leila Eter, Nico Lanzetta, Simin Abrishami, Mita Varghese, Kaitlin McKernan, Lindsey Muir, Jamie Lane, Carey N. Lumeng, and Kanakadurga Singer

From the Department of Pediatrics and Communicable Disease, University of Michigan Medical School, Ann Arbor, Michigan 48109

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Obesity-induced chronic inflammation is associated with metabolic disease. Results from mouse models utilizing a high-fat diet (HFD) have indicated that an increase in activated macrophages, including CD11c+ adipose tissue macrophages (ATMs), contributes to insulin resistance. Obesity primes myeloid cell production from hematopoietic stem cells (HSCs) and Toll-like receptor 4 (TLR4), and the downstream TIR domain–containing adapter protein–inducing interferon-β (TRIF)– and MyD88-mediated pathways regulate production of similar myeloid cells after lipopolysaccharide stimulation. However, the role of these pathways in HFD-induced myelopoiesis is unknown. We hypothesized that saturated fatty acids and HFD alter myelopoiesis by activating TLR4 pathways in HSCs, differentially producing pro-inflammatory CD11c+ myeloid cells that contribute to obesity-induced metabolic disease. Results from reciprocal bone marrow transplants (BMTs) with Tlr4−/− and WT mice indicated that TLR4 is required for HFD-induced myelopoiesis and production of CD11c+ ATMs. Experiments with homozygous knockouts of Irafkm (encoding a suppressor of MyD88 inactivation) and Trif in competitive BMTs revealed that MyD88 is required for HFD expansion of granulocyte macrophage progenitors and that Trif is required for pregranulocyte macrophage progenitor expansion. A comparison of WT, Tlr4−/−, Myd88−/−, and Trif−/− mice on HFD demonstrated that TLR4 plays a role in the production of CD11c+ ATMs, and both Myd88−/− and Trif−/− mice produced fewer ATMs than WT mice. Moreover, HFD-induced TLR4 activation inhibited macrophage proliferation, leading to greater accumulation of recruited CD11c+ ATMs. Our results indicate that HFD potentiates TLR4 and both its MyD88– and TRIF-mediated downstream pathways within progenitors and adipose tissue and leads to macrophage polarization.

Three decades of research have established the association among obesity, inflammation, and metabolic disease (1). Many of the associated co-morbidities of overnutrition in obese individuals (including insulin resistance, type 2 diabetes, atherosclerosis, cardiovascular disease, and some cancers) have been attributed to chronic low-grade inflammation, also known as meta-inflammation. More specifically, these obesity-induced diseases have been strongly correlated with an increase in myeloid leukocyte populations (macrophages and neutrophils) (2).

Obesity-induced alterations of tissue inflammatory macrophages, both in number and in activation state, have been directly associated with tissue dysfunction. Whereas local macrophage proliferation contributes to this accumulation (3), circulating pro-inflammatory Ly6ch+ monocyte populations are an important source of tissue macrophages (4). Ly6ch+ monocytes polarize and traffic into adipose tissue, giving rise to CD11c+ macrophages, specifically in the visceral white adipose tissue (WAT)2 of obese individuals (5). CD11c+ adipose tissue macrophages (ATMs) are distinguished by a CD64+/CD11c+ phenotype in murine models and a CD206+/CD11c+ phenotype in humans and are recruited through chemokines and adipoctye signals that promote chemotaxis of monocytes (6–11). CD11c+ ATMs accumulate disproportionately in visceral WAT and form ring structures known as crown-like structures (CLS) around damaged adipocytes in obese individuals. In contrast, CD11c− anti-inflammatory resident macrophages predominate in lean individuals (4). The presence of CLS is indicative of a pro-inflammatory state, as these macrophages have been found to secrete cytokines such as tumor necrosis factor-α and interleukin-1β (12) and are thus important contributors to

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This article contains Figs. S1–S4.

1 To whom correspondence should be addressed. Dept. of Pediatrics and Communicable Diseases, Division of Pediatric Endocrinology, D1205 MPB, 1500 E. Medical Center Dr., Ann Arbor, MI 48109. Tel.: 734-764-5175; Fax: 734-615-3353; E-mail: ksender@umich.edu.

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2 The abbreviations used are: WAT, white adipose tissue; ATM, adipose tissue macrophage; HSC, hematopoietic stem cells; TLR, Toll-like receptor; TRIF, TIR domain–containing adapter–inducing interferon-β; MyD88, myeloid differentiation primary response 88; IRAKM, interleukin-1 receptor–associated kinase-M; CFU, colony-forming unit(s); HFD, high-fat diet; ND, normal diet; PA, palmitic acid; GWAT, gonadal white adipose tissue; IWAT, inguinal white adipose tissue; GM, granulocyte macrophage; APC, allophycin; CLS, crown-like structures; LPS, lipopolysaccharide; BMT, bone marrow transplant; FFA, free fatty acid; DC, dendritic cell; BM, bone marrow; SVF, stromal vascular fraction.
metabolic syndrome, specifically in visceral adipose tissue of males (13).

Given that the associated cytokines produced by ATMs mimic the inflammation from lipopolysaccharide (LPS) stimulation (14, 15), the Toll-like receptor 4 (TLR4) pathway has been implicated in obesity-induced inflammation. TLR4 complexes recognize pathogen-associated molecular patterns and signal via either an MyD88-dependent or -independent pathway (12). Downstream of TLR4, the MyD88 pathway activates signal via either MyD88-dependent or MyD88-independent pathways to diet-induced inflammation. We demonstrate that these pathways contribute significantly to production of CD11c+ ATMs and myelopoiesis during HFD challenge.

**Results**

**Hematopoietic-specific TLR4 knockout animals have reduced meta-inflammation compared with WT animals after HFD challenge**

Whereas prior studies have demonstrated that hematopoietic Tlr4−/− mice have improved metabolism (18), it is unclear whether TLR4 deficiency has a direct impact on myeloid cell induction. Because it has been demonstrated that Tlr4−/− animals have fewer activated CD11c+ ATMs with HFD (19), we sought to assess whether the production of CD11c+ ATMs depended upon hematopoietic TLR4 expression by generating bone marrow transplant (BMT) chimeras. Young WT and Tlr4−/− mice have improved metabolism (18), it is unclear whether TLR4 deficiency in either BM or non-BM cells thereby exists as well.

**Whereas it has been shown that in obesity TLR4 deficiency significantly promotes alternative macrophage activation (19) and adipose tissue fibrosis (20), recent work in our laboratory demonstrated an additional role for TLR4 in the expansion of hematopoietic stem cells (HSCs) in obesity (2) and a role for MyD88 in the generation of myeloid progenitors in obesity. TLR4 activation in differentiated ATMs with fatty acids (17) in a high-fat diet environment is one possible mechanism explaining macrophage polarization, but a second mechanism of fatty acid activation within the hematopoietic progenitor cells probably exists as well.

**There is a paucity of information on the possible dual role for TLR4, TRIF, and MyD88 pathways in hematopoietic precursors and in mature tissue macrophages. Therefore, we chose to investigate these pathways together in the context of diet-induced obesity. This is critical to understand, as interventions on these inflammatory pathways probably play a role in both the generation of myeloid cells and the activation profile of macrophages in obesity. Based on the literature and prior studies, we sought to evaluate the hypothesis that TLR4, TRIF, and MyD88 are required for hematopoietic stem and progenitor cell responses and tissue macrophage activation with obesity. Studies were carried out using hematopoietic knockout models and in vivo and in vitro assays of progenitor and macrophage activation to determine the contribution of TLR4, TRIF, and MyD88 pathways to diet-induced inflammation. We demonstrate that these pathways contribute significantly to production of CD11c+ ATMs and myelopoiesis during HFD challenge.**

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10 weeks of HFD, there were no differences in fasting glucose or fasting insulin levels (Fig. 2, A and B), but fasting free fatty acids (FFAs) were higher in WT→Tlr4<sup>−/−</sup> compared with Tlr4<sup>−/−</sup>→WT animals (Fig. 2C). At 12 weeks of HFD, a glucose tolerance test demonstrated that Tlr4<sup>−/−</sup>→WT animals had overall improved glucose tolerance (Fig. 2E), and after 16 weeks of HFD, even with similar fed glucose levels, insulin levels were lower in Tlr4<sup>−/−</sup> recipient animals (Fig. 2, D and F). These results suggest that both hematopoietic Tlr4<sup>−/−</sup> and whole-body Tlr4<sup>−/−</sup> contribute to the regulation of fatty acid and insulin levels, whereas glucose tolerance is related to hematopoietic TLR4 deficiency.

Leukocytes were evaluated in blood 2 weeks after BMT, and animals with WT BM had more B220<sup>+</sup> cells in circulation, suggesting faster reconstitution (Fig. S2A). Two weeks after HFD exposure, there was a significant increase in circulating B220<sup>+</sup>, Ly6G, and CD115<sup>+</sup> myeloid cells in both WT recipient animals, but Ly6<sup>chi</sup> populations were similar (Fig. 3A). Adipose tissue evaluation at 16 weeks of HFD by flow cytometry (Fig. 3B) demonstrated equal CD45<sup>+</sup> leukocytes and ATMs in GWAT (Fig. 3D) but a larger CD11c<sup>+</sup>CD64<sup>+</sup> ATM population in WT→Tlr4<sup>−/−</sup>→WT compared with Tlr4<sup>−/−</sup>→WT (Fig. 3E). Further quantification showed a significant increase in CD11c<sup>+</sup> ATMs in GWAT of WT donor animals, whereas CD11c<sup>+</sup> ATMs and dendritic cells (DCs) were equal in both groups (Fig. 3F). Gene expression showed a slight but nonsignificant decrease in Tlr4 expression in Tlr4<sup>−/−</sup>→WT GWAT (Fig. S2B) due to nonhematopoietic TLR4-expressing cells in adipose tissue.

To confirm appropriate reconstitution of these mice, hematopoietic progenitors and mature leukocytes were evaluated. CD3<sup>+</sup> and CD4<sup>+</sup> cells were decreased in BM of Tlr4<sup>−/−</sup>→WT mice compared with WT→Tlr4<sup>−/−</sup>→WT (Fig. S2C). Hematopoietic stem and progenitor cells were generally similar in both groups of reconstituted mice (Fig. S2D). Myeloid cells, however, were reduced in the spleen (CD11c<sup>+</sup> and CD115<sup>+</sup>) with an increase in CD19<sup>+</sup> cells (Fig. S2E). In the blood, CD115<sup>+</sup> and Ly6<sup>chi</sup> monocytes were also reduced, whereas circulating CD4<sup>+</sup> cells were increased in mice with TLR4<sup>−/−</sup>→BM (Fig. S2F). Both circulating and splenic monocyte profiles were consistent with a reduction in myeloid response to HFD in the absence of TLR4.

**TLR4<sup>−/−</sup> ATMs proliferate more robustly in response to HFD compared with WT ATMs**

Based on our findings of decreased recruited CD11c<sup>+</sup> ATMs in animals with Tlr4<sup>−/−</sup> marrow, we anticipated that resident Tlr4<sup>−/−</sup> ATMs may expand earlier in HFD challenge, reducing the requirement for recruitment of myeloid cells to the adipose tissue. TLR4 activation in nonhematopoietic cells has been shown to inhibit cell proliferation (21, 22). To understand the initial macrophage response to adipose tissue expansion in TLR4 knockout animals, a short-term diet model was used wherein mice at 10 weeks of age were challenged with HFD until 12 weeks of age. After 2 weeks of HFD, body weights remained consistent with normal diet (ND)-fed animals in both WT and Tlr4<sup>−/−</sup> groups (Fig. 4A). GWAT pads expanded in both WT and Tlr4<sup>−/−</sup> animals (Fig. 4B), and GWAT immunofluorescence demonstrated more proliferating (Ki67<sup>+</sup>) cells in the Tlr4<sup>−/−</sup> animals (Fig. 4C). Flow cytometry demonstrated that Tlr4<sup>−/−</sup> mice fed HFD had more ATMs (Fig. S3A and Fig. 4D). CD11c<sup>+</sup> ATMs were increased in both WT and Tlr4<sup>−/−</sup> mice after 2 weeks of HFD, but only Tlr4<sup>−/−</sup> mice showed...
expansion of CD11c– ATMs (Fig. 4D). DCs were unchanged in either group. When we assessed proliferation, HFD caused proliferation of both ATM types; however, there was enhanced proliferation in CD11c– ATMs of Tlr4−/− animals compared with WT CD11c– ATMs (Fig. 4E). This increase in ATM proliferation was not seen after chronic HFD feeding in GWAT and IWAT, where a large number of both WT and Tlr4−/− leukocytes were proliferating compared with ND-fed animals (Fig. S3, B and C). We further challenged 10-week-old Myd88−/− and Trif−/− mice to 2 weeks of HFD and found that Myd88−/− mice mimic the results seen with the Trif−/− animals (Fig. 4E). These data demonstrate that TLR4 and MyD88 inhibit the ability of resident ATMs to proliferate with adipose tissue expansion in short-term HFD exposure.

Hematopoietic TRIF and MyD88 pathways contribute to myeloid expansion in obesity

We have previously shown that TLR4 and MyD88 pathways both regulate the generation of obesity-induced inflammation. Although TLR4 pathways were mostly necessary for long-term HSC expansion, MyD88 was mostly required for myeloid progenitor expansion, monocyte production, and hence ATM accumulation in obesity (2). Two competitive BMTs were designed to further define the role of MyD88, as well as to determine the role of the noncanonical TRIF (Ticam1) pathway in inflammation. First, Trif−/− (CD45.2) and WT (CD45.1) BM was injected in a 1:1 ratio into irradiated CD45.1/CD45.2 heterozygous mice (Fig. 5A). These animals were started on HFD at 6 weeks post-BMT for 16 weeks. TRIF was required to expand pre-GMs, blood monocytes, and ATMs of both types, as seen from lower Trif−/− BM contribution to these cell types. To further delineate the role of MyD88 pathways, IRAKm, a negative regulator of MyD88 activation, was studied. Irakm−/− BM was competed with WT BM (Fig. 5B). Irakm−/− animal BM contributed to a greater number of pre-GMs, blood monocytes, and both types of ATMs, emphasizing a role for MyD88 pathways in generating bone marrow–derived myeloid cells during obesity (Fig. 5B). This same phenomenon for IRAKm pathways has been shown in LPS treatment of animals (23).

TLR4, TRIF, and MyD88 pathway knockouts respond with increased adiposity and glucose intolerance with HFD

WT, Tlr4−/−, Trif−/−, and Myd88−/− mice were placed on 60% HFD for 16 weeks. Animals in all groups gained weight (Fig. 6A), visceral fat (Fig. 6B), and subcutaneous fat (Fig. 6C) when challenged to HFD. Interestingly, liver mass did not increase in Trif−/− and was smaller overall in both Trif−/− and Myd88−/− mice with HFD compared with WT (Fig. 6D). Spleen weights were significantly larger after HFD in WT and Trif−/− mice (Fig. 6E). GWAT demonstrated increased adipocyte hypertrophy in all groups on HFD (Fig. 6F). Hematoxylin and eosin images of GWAT showed similar adipocyte hypertrophy but an increase in CLS and fibrosis in WT HFD-fed mice (Fig. 6G). Purely based on weight and adiposity, all groups were susceptible to obesity with HFD exposure.

Fasting glucose levels were not significantly different by genotype after 10 weeks on HFD but were lower in Myd88−/− mice on HFD compared with WT (Fig. 7A). Fasting insulin levels were substantially lower in all genotypes compared with WT (Fig. 7B). Glucose tolerance tests at 12 weeks on HFD were also unaffected by genotype (Fig. 7, C and D). Fed glucose levels were higher in mice on HFD, but insulin levels, although elevated in
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Figure 4. Increased CD11c⁺ ATM proliferation in Tlr4⁻/⁻ and Myd88⁻/⁻ animals after short-term high-fat diet. 10-Week-old WT and TLR4⁻⁻ animals were challenged to ND or HFD for 2 weeks. At 12 weeks of age, weight (A) and weight gain (B) were determined by flow cytometry. (C, red) and WT, responses in the knockout models, ATMs were measured in CD11c⁺ ATM subsets as determined by flow cytometry. E, Ki67 proliferation as determined by intracellular flow cytometry staining in GWAT ATMs (CD11c⁺ and CD11c⁻ ATMs). Significant differences between WT HFD-fed controls, TLR4⁻⁻, TRIF⁻⁻, and Myd88⁻⁻ mice, although Il1b was highest in WT HFD-fed mice (Fig. 8G). Genes generally activated in CD11c⁺ alternative resident macrophages, such as Arg1 and Tgfβ, showed increased expression in all groups. Hematopoietic compartment analysis demonstrated that granulocyte macrophage progenitors were only increased in WT animals on HFD (Fig. 8H), whereas pre-GMs were increased in all groups except the Myd88⁻⁻ HFD animals. Overall, these experiments demonstrate that the TLR4, TRIF, and Myd88 pathways are necessary for CD11c⁺ ATM accumulation and myeloid progenitor expansion with HFD.

TLR4, MyD88, and TRIF are required for hematopoietic responses to saturated fatty acid

We employed in vitro myeloid colony-forming unit (CFU) assays to assess stem cell capacity in the obese knockout mice. Compared with WT HFD-fed controls, TLR4⁻⁻, TRIF⁻⁻, and Myd88⁻⁻ knockouts generated fewer myeloid colonies (Fig. 9A). To understand the mechanism of this effect, we stimulated BM with fatty acids or LPS ex vivo and then performed myeloid CFU assays. For these assays, we used saturated fatty acids (palmitic and stearic acid, 10 μM) and unsaturated fatty acids (linoleic and oleic acid, 10 μM). Whereas WT marrow increased CFU generation in response to saturated fatty acids, TLR4⁻⁻, TRIF⁻⁻, and Myd88⁻⁻ marrow did not respond with a similar increase in CFU (Fig. 9B). Similar results were seen with LPS treatment. Overall, oleic acid (an unsaturated fatty acid) decreased the saturated fatty acid response (Fig. 9B).

To determine whether this fatty acid response in myeloid cells was due to stimulation of mature cells or progenitors, we isolated lineage-negative (Lin⁻) cells (immature noncommitted bone marrow cells), followed by treatment of isolated cells with palmitate. Palmitic acid stimulated WT lineage-negative BM (Fig. 9C), whereas TLR4⁻⁻ lineage-negative BM was resistant to this stimulation and yield lower colonies compared with WT lineage-negative PA treatment (p = 0.06).
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Figure 5. In competitive BMT models, TRIF and MyD88 pathways are necessary for expansion of myeloid progenitors and ATMs in response to HFD. Lethally irradiated CD45.1/CD45.2 heterozygote animals were injected with CD45.1 WT and CD45.2 Tlr4−/− BM in a 1:1 ratio (A) or CD45.1 WT and CD45.2 Irakm−/− BM in a 1:1 ratio (B). The ratio of quantitation of myeloid progenitors and leukocytes in animals reconstituted after 16 weeks of HFD (long-term hematopoietic stem cells (LT-HSC), granulocyte macrophage progenitor (GMP), pre-granulocyte macrophage (Pre-GM), blood monocytes, and ATMs and CD11c subsets) was calculated. ***, p < 0.005; ****, p < 0.001. n = 9 in TRIF competitive BMT and n = 6 in IRAKM competitive BMT. Error bars, S.E.

Figure 6. WT, Tlr4−/−, Trif−/−, and MyD88−/− animals respond to HFD challenge. Male animals were started on HFD at 6 weeks of age for 16 weeks. Shown are weight (A), GWAT weight (B), IWAT weight (C), liver weight (D), spleen weight (E), and average cross-sectional adipocyte size (F) of GWAT fat adipocytes. **, p < 0.01; ***, p < 0.005; ****, p < 0.001. n = 23–24 in WT groups, n = 14–21 in Tlr4−/− groups, n = 17–19 in Trif−/−, and n = 5–8 in MyD88−/− for spleen weight, n = 15 ND and 16 HFD in WT groups, n = 4 ND and 10 HFD in Tlr4−/− groups, n = 17 ND and HFD in Trif−/−, and n = 5 ND and 7 HFD for MyD88−/−; for adipocyte sizing, n = 8–9 in WT groups, n = 3–4 in Tlr4−/− groups, and n = 5 in Trif−/− and MyD88−/−). #, p < 0.05; ##, p < 0.01; ###, p < 0.005; ####, p < 0.001 when knockout groups were compared with WT ND or HFD control. Error bars, S.E.

Figure 7. WT, Tlr4−/−, Trif−/−, and MyD88−/− animals respond metabolically to HFD challenge. Shown are 10-week fasting glucose (n = 3–8) (A) and insulin (n = 3–7) (B). Shown are 12-week glucose tolerance tests (WT ND, n = 12; WT HFD, n = 21; Tlr4−/− HFD, n = 14; Trif−/− HFD, n = 4; MyD88−/− HFD, n = 6) (C), with calculated area under curve (AUC) (D). *, p < 0.05. Error bars, S.E.
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Figure 8. Tlr4<sup>−/−</sup>, Trif<sup>−/−</sup>, and Myd88<sup>−/−</sup> animals are protected from adipose tissue inflammation. After 16 weeks of HFD challenge, flow cytometry assessments were performed for CD45<sup>+</sup> cells (A), ATMs (B), CD11c<sup>+</sup> ATMs (C), CD11c<sup>+</sup> ATMs (D), and DCs (E) (n = 12–21 in WT groups, n = 10–17 in Tlr4<sup>−/−</sup> groups, n = 7–17 in Trif<sup>−/−</sup>, and n = 5–8 in Myd88<sup>−/−</sup>). F, Immunofluorescence in GWAT (caveolin (green) and Mac2 (magenta)). G, Quantitative real-time PCR (n = 11 WT ND, n = 20 WT HFD, n = 10 Trif<sup>−/−</sup> HFD, n = 5 Tlr4<sup>−/−</sup> HFD, and n = 6 MyD88<sup>−/−</sup> HFD). H, Flow cytometry evaluation of hematopoietic progenitors (n = 8 WT ND and HFD, n = 11 Tlr4<sup>−/−</sup>, n = 5 Trif<sup>−/−</sup>, and n = 8 Myd88<sup>−/−</sup>). *, p < 0.05; **, p < 0.01; ***, p < 0.005; ****, p < 0.001; #, p < 0.05; ##, p < 0.01; ###, p < 0.005; ####, p < 0.001. Error bars, S.E.
Comparison of WT and Tlr4−/− ND and HFD animals showed a brisk response to PA in ND WT mice that was blunted in HFD WT mice. Tlr4−/− mice on ND or HFD failed to induce colony formation in response to PA in Lin− cells (Fig. 9D). Lin− TRIF and MyD88 knockout mice demonstrated that Myd88−, but not Trif, was required for myeloid expansion in response to PA (Fig. 9E); however, both pathways yielded lower myeloid colonies compared with WT mice. This was consistent with our prior research (2) that determined that MyD88 pathways are critical for myeloid inflammation in obesity. Gene expression studies in harvested cells treated with PA (Fig. 9F) demonstrated that palmitate stimulation in WT mice, but not Tlr4−/− animals, induced Mcp1 expression (analysis of variance, p < 0.05).

Discussion

Our findings show that there are distinct roles for TLR4, TRIF, and MyD88 in the inflammatory response to obesity. Prior studies have identified a role for TLR4 and NF-κB (24) in obesity-induced inflammation and insulin resistance (25). Our studies demonstrate that TLR4 reduces local (CD11c−) macrophage proliferation in response to adipose tissue expansion, promotes recruitment of CD11c− ATM accumulation, and is also necessary for the generation of bone marrow–derived macrophages in response to saturated fatty acid.

Spontaneous mutants lacking TLR4 function are protected from insulin resistance (26), but studies have shown mixed effects of TLR4 knockouts in the metabolic response to HFD. Our reciprocal BMTs suggest that this may be because of opposing TLR4 function in bone marrow–derived and nonhematopoietic compartments, such as adipocytes. Whereas animals reconstituted with Tlr4−/− BM had improved metabolism, glucose tolerance in nonirradiated Tlr4−/− mice on HFD showed similar impairment as WT HFD-fed animals (Fig. 7). Tlr4−/− mice gained significantly more weight when nonirradiated, suggesting that the protection conferred by hematopoietic...
ETIC TLR4 DEFICIENCY MAY BE OVERRIDDEN BY EXCESS ADIPOSITY. TLR4 HAS A KNOWN ROLE IN ADIPOCYTES (27), ESPECIALLY IN SHORT-TERM HFD, AND IN RECRUITMENT OF INFLAMMATORY LEUKOCYTES (28). ONCE CHRONIC OBESITY HAS LED TO EXPANSION OF ADIPOSE TISSUE, THERE IS PROBABLY A SECONDARY ROLE FOR TLR4 IN THE GENERATION OF FIBROSIS, PROMOTING FURTHER ADIPOSE TISSUE DYSFUNCTION (20).

TLR4 AND MYD88 HAVE SPECIFICALLY BEEN REPORTED TO BE CRITICAL FOR ATMS THAT ARE “METABOLICALLY ACTIVATED” AND PLAY AN IMPORTANT ROLE IN ADIPOSE TISSUE HOMEOSTASIS (30). OUR RESULTS SUGGEST THAT MYD88 DEFICIENCY PROTECTS FROM META-INFLAMMATION BECAUSE MYD88 IS REQUIRED FOR ACCUMULATION OF ACTIVATED ATMs AND ENHANCED MELOPOIESIS. OUR RESULTS ARE CONSISTENT WITH PREVIOUS STUDIES (31, 32) THAT HAVE IMPLICATED MYD88 IN ITS CONTRIBUTION TO OBESITY-INDUCED ADIPOSE TISSUE INFLAMMATION (31), ATHEROSCLEROSIS (31, 33), AND IMPROVED GLUCOSE SENSITIVITY (34). STUDIES HAVE PREVIOUSLY DEMONSTRATED THAT MYD88 IS NECESSARY FOR TLR2 AND TLR4 SATURATED FATTY ACID–INDUCED INFLAMMATORY ACTIVATION OF MATURE MACROPHAGES (35). ADDITIONALLY, OUR RESULTS DEMONSTRATE THAT MYD88 PATHWAYS ARE ALSO RESPONSIBLE FOR THE HSC RESPONSES TO SATURATED FATTY ACIDS IN OBESITY.

WHEREAS MYD88-INDEPENDENT TRIF SIGNALING HAS BEEN IMPLICATED IN MELOPOIESIS (36) AND ATHEROSCLEROSIS (37), THERE IS A PAUCITY OF DATA ON THE ROLE OF TRIF IN META-INFLAMMATION. WE FOUND THAT LIN\(^{-}\) TRIF\(^{−/−}\) PROGENITORS PRODUCED MELOPOIESIS IN RESPONSE TO PALMITATE STIMULATION IN VITRO. IN VIVO, HOWEVER, TRIF\(^{−/−}\) ANIMALS HAD FEWER MELOPOIESIS PROGENITORS AND ATMs WITH HFD CHALLENGE WHEN COMPARED WITH WT ANIMALS. IT IS INTERESTING TO NOTE THAT ALTHOUGH THESE ANIMALS GAINED WEIGHT AND ADIPOSION, TRIF\(^{−/−}\) MICE WERE PROTECTED FROM HEPATICSTEATOSIS AND TRIGLYCERIDE ACCUMULATION DURING HFD. THIS FINDING IS CONTRARY TO WHAT HAS BEEN OBSERVED IN MODELS OF NONALCOHOLIC STEATOHEPATITIS, WHERE TRIF\(^{−/−}\) ANIMALS GENERALLY HAVE INCREASED INFLAMMATION AND FIBROSIS DUE TO ENHANCED STEATONE AND CYTOKINE ACTIVATION OF THE MYD88 PATHWAY, DEMONSTRATING THAT TRIF IS PROBABLY IMPORTANT FOR INHIBITING INFLAMMATION IN THE LIVER (38, 39). HOWEVER, INFLAMMATION IS PROVOKED IN THOSE STUDIES THROUGH AMINO ACID DEFICIENCY AS OPPOSED TO HFD-INDUCED OBESITY STIMULATION. THIS HIGHLIGHTS THE IMPORTANCE OF SOURCE AND DEGREE OF STIMULATION WHEN EVALUATING STEATOSIS AND HEPATIC INFLAMMATION (40, 41). THIS ALSO EMPHASIZES THAT THERE MAY BE A POTENTIAL DIFFERENT ROLE FOR TRIF PATHWAYS IN THE ADIPOSE TISSUE PROTECTING HFD-FED TRIF\(^{−/−}\) MICE FROM ADIPOSE TISSUE INFLAMMATION AND DYSFUNCTION, LEADING TO PROTECTION FROM FATTY ACID RELEASE AND STEATOSIS.

THE ROLE OF TLR4 PATHWAYS IN BIASING HEMATOPOIETIC PROGENITOR EXPANSION (42) TOWARD MELOPOIESIS (43) WITH LPS STIMULATION HAS BEEN DEMONSTRATED IN ACUTE SETTINGS BUT REMAINS UNRESOLVED IN THE CHRONIC INFLAMMATORY STATE OF OBESITY. THE STUDIES HERE REVEAL THAT TLR4 THROUGH TRIF AND MYD88 SIGNALING CASCADES ARE IMPORTANT FOR HEMATOPOIETIC CONTRIBUTION TO MELOPOIESIS CELLS AND MACROPHAGE POLARIZATION IN OBESITY-INDUCED INFLAMMATION. THE IMPORTANCE OF TLR4 IN BONE MARROW–DERIVED MELOPOIESIS CELLS FOR INSULIN RESISTANCE DURING OBESITY HAS ONLY RECENTLY BEEN CHARACTERIZED (2, 44, 45), BUT WITH A LIMITED UNDERSTANDING OF ITS TRiggers. OUR BM STUDIES INDICATE THAT SATURATED FATTY ACIDS HAVE A DIRECT ROLE IN ENHANCING MELOPOIESIS PROGENITORS VIA STIMULATION OF TLR4, TRIF, AND MYD88 PATHWAYS.

WHEREAS THIS DIRECT EFFECT OF FATTY ACIDS ON HEMATOPOIETIC PROGENITORS IS NOVEL, IT IS PROBABLY ONE OF MANY FACTORS ALTERED WITH HFD, GIVEN THAT INCREASED ENDOXOTINS (46) AND MICROBIOTA CHANGES HAVE ALSO BEEN DEMONSTRATED TO ENHANCE ATM RECRUITMENT VIA TLR4 (47).

ANOTHER LIMITATION OF OUR STUDIES IS THE TRIF AND MYD88 KNOCKOUT ANIMAL RESULTS ARE POSSIBLY CONFOUNDED BY SIGNALING PATHWAYS FROM OTHER TLRs THAT ARE ACTIVATED DURING AN OBESITY STATE, INCLUDING TLR2 (48, 49). ALSO, IT IS NOT CLEAR WHETHER THIS INCREASED TLR4 ACTIVATION IN HEMATOPOIETIC PROGENITORS THROUGH TRIF PATHWAYS LEADS TO TOLERANCE OR EXHAUSTION IN CHRONIC HFD, AS HAS BEEN DEMONSTRATED IN LPS STIMULATION (36, 50). TIMING AND DOSE OF STIMULATION ARE CRITICAL IN LPS STUDIES (51) AND HENCE MAY NOT MIMIC FINDINGS IN OBESITY. FURTHER, WHEREAS WE WERE ABLE TO CHARACTERIZE THE ROLE OF TLR4 VIA MYD88 FOR INHIBITION OF LOCAL CD11c\(^{−}\) ATM PROLIFERATION AND THE ROLE OF BOTH TRIF AND MYD88 FOR MELOPOIESIS EXPANSION, THE DOWNSTREAM PATHWAYS FROM THIS INITIAL SIGNALING ARE YET TO BE DETERMINED.

OVERALL, OUR STUDIES DEMONSTRATE THAT TLR4, TRIF, AND MYD88 PLAY CRITICAL RULES IN THE PROFILE OF ATMs IN VISCERAL FAT AFTER ACUTE AND CHRONIC HFD EXPOSURE. IN ADDITION, DEFICIENCY OF ANY OF THESE PATHWAYS REDUCES MELOPOIESIS STIMULATION OF BONE MARROW WITH SATURATED FATTY ACIDS. THESE RESULTS EMPHASIZE THAT WHEN EVALUATING OBESITY-INDUCED INFLAMMATION, THE TISSUE-SPECIFIC MACROPHAGE RESPONSE MAY ONLY BE PART OF THE UNDERLYING EFFECT AND THAT THERE IS AN ADDITIONAL MELOPOIESIS PROGENITOR ALTERATION THAT OCCURS WITH OBESITY.

EXPERIMENTAL PROCEDURES

ANIMAL MODELS AND TREATMENTS

C57Bl/6J (WT), TLR4\(^{−/−}\) (B6.B10ScN-Tlr4\(^{lps−delf/Jth};\) 007227), and MYD88\(^{−/−}\) (B6.129P2 (SJL)-Myd88\(^{m1.1Dfcf/J};\) 090888) MICE ON C57Bl/6J BACKGROUND WERE PURCHASED FROM JACKSON LABORATORIES. TRIF\(^{−/−}\) MICE WERE DONATED FROM DR. GABRIEL NUÑEZ’S LABORATORY, ORIGINALLY FROM THE AKIRA LABORATORY (52). IRAK4\(^{−/−}\) MICE WERE DONATED FROM A COLONY BREED ON A B6 BACKGROUND THAT WAS ESTABLISHED AT THE UNIVERSITY OF MICHIGAN (53). ALL MICE WERE MALE AND WERE FED AD LIBITUM EITHER A CONTROL ND CONSISTING OF 4.5% FAT (5001; LabDiet) OR AN HFD CONSISTING OF 60% OF CALORIES FROM FAT (Research Diets, Inc., D12492) STARTING AT 6 WEEKS OF AGE FOR 16 WEEKS DURATION. GLUCOSE TOLERANCE (WITH 0.7 g/kg) AND INSULIN TOLERANCE (WITH 1 UNIT/ KG) TESTING WERE CONDUCTED AFTER 6 HOURS OF FASTING. ANIMALS WERE HOUSED IN A SPECIFIC PATHOGEN-FREE FACILITY WITH A 12-H LIGHT/12-H DARK CYCLE AND GIVEN FREE ACCESS TO FOOD AND WATER. ANIMAL PROTOCOLS WERE IN COMPLIANCE WITH THE INSTITUTE OF LABORATORY ANIMAL RESEARCH GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS AND APPROVED BY THE UNIVERSITY COMMITTEE ON CARE AND USE OF ANIMALS AT THE UNIVERSITY OF MICHIGAN (ANIMAL WELFARE ASSURANCE NUMBER A3114-01).

QUANTITATIVE REAL-TIME PCR

RNA EXTRACTION WAS PERFORMED WITH AN RNEASY KIT (QIAGEN) FOLLOWED BY REVERSE TRANSCRIPTION (APPLIED BIOSYSTEMS) AND REAL-TIME PCR ANALYSIS USING GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE TO NORMALIZE (SYBR GREEN, ABI PRISM 7200 SEQUENCE DETECTION SYSTEM; APPLIED BIOSYSTEMS). RELATIVE
expression was assessed by the comparative CT method correcting for amplification efficiency of the primers and performed in duplicate as described previously (2). PCR primers used are reported in Table 1.

Adipose tissue stromal vascular fraction (SVF) isolation and flow cytometry

Adipose tissue fractionation and flow cytometry analyses were performed as described previously after Fc blocking of samples (54). SVF cells were stained with CD64 PE, CD45.2 e450, and CD11c-APC-Cy7 or APC (eBioscience) (55) for ATMs.

Flow cytometry assessment of HSC and myeloid progenitors

Bone marrow from one femur was flushed with PBS and made into single-cell suspension using a syringe and then centrifuged. Thereafter, cell pellets were treated with RBC lysis solution for 5 min. After resuspension in PBS, cells were stained with lineage markers on APC (CD4, CD5, CD8, CD11b, B220 (CD45R), Gr1, Ter119), CD117-APC-Cy7, Sca1-PECy7, with lineage markers on APC (CD4, CD5, CD8, CD11b, B220 (CD45R), Gr1, Ter119), CD117-APC-Cy7, Sca1-PECy7, and gating as described by Pronk et al. (56) and Oguro et al. (57).

cfu assays

Bone marrow from a femur was flushed with Iscove’s modified Dulbecco’s medium. This marrow was then treated with fatty acid–free BSA, with fatty acids (palmitic acid, linoleic acid, oleic acid, stearic acid) (10 μM, purchased from Sigma) in fatty acid–free BSA, or with 10 μg/ml LPS for 1 h at 37 °C. Cells were then resuspended in MethoCult medium and plated at a density of 10,000 cells/plate/protocol (Stem Cell Technology). After 7 days, colonies were counted. For sorted cfu assays, bone marrow was isolated from animals, and lineage-negative cells were separated using a stem-cell magnet column isolation kit (EasySep™ hematopoietic progenitor cell enrichment kit). After magnetic separation, 1000 cells were used per plate.

Bone marrow transplantation

Bone marrow cells were isolated from donor groups (58) and injected retro-orbitally into lethally irradiated (900 rads) 6-week-old recipient mice (10 million cells/mouse). Animals were treated with antibiotics (polymyxin and neomycin) for 4 weeks after BMt. Following 2 weeks of normal Chow diet, they were started on ND or HFD Chow. Glucose tolerance testing was performed as described previously (29).

Statistics

Results are presented as mean ± S.E. One-way or two-way analysis of variance was performed with factors of genotype and diet. If there was a main effect for either factor, then t tests were performed for WT versus knockout differences within each diet or for diet groups within each group, respectively.

Author contributions—C. G., L. E., N. L., S. A., M. V., K. M., L. M., J. L., and K. S. data curation; C. G., L. E., N. L., S. A., M. V., K. M., L. M., and K. S. formal analysis; C. G., S. A., and K. S. supervision; C. G. and K. S. methodology; C. G., S. A., and K. S. writing-original draft; L. E., N. L., M. V., C. N. L., and K. S. writing-review and editing; S. A. and K. S. investigation; L. M., C. N. L., and K. S. conceptualization; C. N. L. resources; C. N. L. and K. S. funding acquisition; K. S. validation; K. S. visualization; K. S. project administration.

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Table 1

| Primer sequences | Forward primer | Reverse primer |
|------------------|---------------|---------------|
| Gapdh            | TGAACGCAAGCACCACCTGAGG | CGAAGGCTGAGAGTGAGGGG |
| Il6              | TACGCTTCTCTACTCACCATAAT | AAAGGACCTCTCAGCTGGAGATC |
| Mcp1             | TAAATACCTGGAATTGCCCTGGGG | GCTATGCTGTCTGAGTTTACCTT |
| Il1b             | ATCTCCGGACAGCTTTAAAAGG | AGGAGGTCACCTAGTGGAAATCC |
| Arg1             | CTCCACCGCAAGGCTTTAGAG | GAAGCTGCTCAGCTAGTGGAAATCC |
| Tgfb             | GAGCCTCTACCCCTGACAGAC | GACGCGACCAAGCTATTGGT |
| Tic1             | AGGGCACTGCTTTACCACACC | GAGGACCCGAGGCTAAAAAG |
| Myd88            | CCACACTCCAGAGCCCTCAAGC | CAAAGGCTCTGAAGTTGCA |
| Irf4             | TTGGTCAGGATGAGAGGCTTA | CTCACATTGGGGTCTGAGT |
| Glut4            | GTGACCTCGGACACCTGGCCCTA | CGAAGGACCTCAGTGGAGG |

Statistics

Results are presented as mean ± S.E. One-way or two-way analysis of variance was performed with factors of genotype and diet. If there was a main effect for either factor, then t tests were performed for WT versus knockout differences within each diet or for diet groups within each group, respectively.

Author contributions—C. G., L. E., N. L., S. A., M. V., K. M., L. M., J. L., and K. S. data curation; C. G., L. E., N. L., S. A., M. V., K. M., L. M., and K. S. formal analysis; C. G., S. A., and K. S. supervision; C. G. and K. S. methodology; C. G., S. A., and K. S. writing-original draft; L. E., N. L., M. V., C. N. L., and K. S. writing-review and editing; S. A. and K. S. investigation; L. M., C. N. L., and K. S. conceptualization; C. N. L. resources; C. N. L. and K. S. funding acquisition; K. S. validation; K. S. visualization; K. S. project administration.

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