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Obesity

TLR9 Regulates Adipose Tissue Inflammation and Obesity-Related Metabolic Disorders

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Objective: Recent studies have revealed a link between Toll-like receptor (TLR) signaling and the adipose tissue inflammation associated with obesity. Although TLR9 is known to play an important role in inflammation and innate immunity, its role in mediating adipose tissue inflammation has not yet been investigated. Thus, the objective of this study was to determine the role of TLR9 in regulating immune cells in visceral adipose tissue and maintaining the metabolic homeostasis.
Methods: Wild-type and TLR9-deficient mice were fed with a high-fat diet, and the body weight gain, glucose tolerance, insulin sensitivity, and adipose tissue inflammation were examined.
Results: TLR9-deficient mice gained significantly more weight and body fat under a high-fat diet than wild-type mice and exhibited more severe glucose intolerance and insulin resistance. We also found a dramatic increase of M1 macrophages as well as T_H1 cells in the adipose tissue of TLR9-deficient mice compared to wild-type mice. Furthermore, the levels of various proinflammatory cytokines and chemokines were higher in TLR9-deficient mice.
Conclusions: TLR9 signaling is involved in regulating adipose tissue inflammation and controlling obesity and the metabolic syndrome.

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Introduction

Obesity and obesity-related metabolic diseases are associated with excessive infiltration of immune cells and chronic inflammation in multiple metabolic tissues such as visceral adipose tissue (VAT), liver, and skeletal muscles (1). The concomitant increase in circulating cytokines, including IL-1β, TNF-α, and IL-6, leads to insulin resistance and disruption of metabolic homeostasis.

In obese mice, macrophages highly infiltrate into the VAT and secrete proinflammatory cytokines. In addition to the increase in numbers of macrophages, obesity induces the conversion of M2 (or *alternatively activated*) macrophages into M1 (or *classically activated*) macrophages, further promoting the tissue inflammation (2,3). T cells also accumulate in the VAT of obese mice and, in some cases, they seem to arrive even before the recruitment of macrophages (4). Recent studies show that other immune cells such as B cells, mast cells, eosinophils, neutrophils, and NKT cells also contribute to the insulin resistance and metabolic diseases in obese mice via low-grade chronic inflammation (5-10).

Mammalian Toll-like receptors (TLRs) belong to a major pattern recognition receptor family, and their activation typically leads to the production of proinflammatory cytokines and chemokines, thereby triggering innate immune responses (11). The role of TLRs is not limited to the host defense against infection, and they are also implicated in the regulation of metabolic health. For example, TLR5-deficient mice exhibit spontaneous obesity and metabolic diseases (12). On the other hand, mice lacking TLR2 or TLR4 are protected from diet-induced obesity and insulin resistance (13,14). However, little is known about the role of TLR9 signaling in the regulation of metabolic function and adipose tissue inflammation. TLR9 is originally identified as a receptor for unmethylated bacterial CpG DNA and synthetic CpG-containing oligodeoxynucleotides (15). Upon activation, it promotes secretion of proinflammatory cytokines and chemokines, as well as type I interferons, in a MyD88-dependent manner, and plays a major role in defense against many bacterial and viral infections (16-18). TLR9 can also be stimulated by mammalian DNA and is implicated in pathogenesis of several autoimmune diseases, such as systemic erythematosus lupus and psoriasis (19,20). In addition, TLR9 seems to mediate the beneficial

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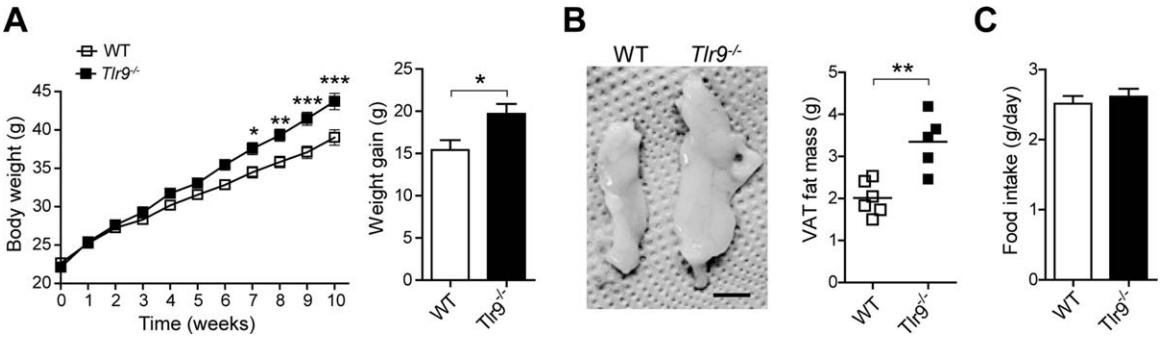

Figure 1 TLR9 deficiency accelerates HFD-induced obesity. WT and TLR9-deficient ($Tlr9^{-/-}$) mice were monitored for 10 weeks while being fed a HFD. (**A**) Body weight gain ($n = 15$ mice per group). (**B**) Epididymal fat mass. Scale bar: 1 cm. (**C**) Food intake. Results are representative of two separate analyses. Means ± SEM are shown. $^{*}P < 0.05$, $^{**}P < 0.01$, *t* test or two-way ANOVA.

effect of certain probiotics, and recently the anti-inflammatory function of TLR9 started to be more appreciated (21,22). In this study, we investigated the role of TLR9 in induction of obesity and adipose tissue inflammation. We show that mice lacking TLR9, compared to wild-type (WT) mice, exhibit excessive weight gain with development of obesity-associated glucose intolerance and insulin resistance under a high-fat diet (HFD) condition. We also show that M1 macrophages and T_H1 cells accumulate significantly more in the VAT of TLR9-deficient mice, resulting in the increased levels of proinflammatory cytokines and chemokines.

Taken together, our findings suggest that TLR9 signaling is involved in regulating adipose tissue inflammation and protecting against obesity and the metabolic syndrome in mice.

Methods

Mice

WT (C57BL/6) and TLR9-deficient ($Tlr9^{-/-}$; B6.129P2-*Tlr9*^{tmAki}) mice were purchased from Oriental BioService, and housed in a specific pathogen-free facility at Pohang University of Science and Technology. TLR9-deficient mice were backcrossed more than 15 generations with C57BL/6 mice. All animal experiments were performed under protocols approved by the Ethics Review Committee for Animal Experimentation of Pohang University of Science and Technology. Only age-matched male mice were used for each experiment.

Diet-induced obesity and metabolic studies

For obesity induction, mice were fed with HFD (60 kcal% fat, Research Diets) starting at 6 weeks of age and maintained for 8-10 additional weeks. Body weight was measured every week. Fasting blood glucose and insulin concentrations were measured with a glucometer (Accu-Chek Performa kit, Roche) and by insulin ELISA (Mercodia), respectively. For glucose tolerance tests (GTT), glucose (1 g/kg body weight) was injected i.p., after a 16 h fast and blood glucose levels were measured at indicated time points. For insulin tolerance tests (ITT), human insulin (0.75 U/kg body weight) was administered by i.p., after a 4 h fast and blood glucose levels were measured at indicated time points.

Isolation of adipocytes and VAT-associated immune cells

Adipocytes were isolated from epididymal adipose tissues as previously described (3). For isolation of fat-associated leukocyte and mononuclear cells, epididymal adipose tissues without contaminating lymph nodes were minced, and digested in the enzyme media (RPMI 1640 media containing 400 U/ml of collagenase D, 10 μg/ml DNase I, 3% FCS, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 1 mM NEAA) for 45 min at 37°C. EDTA (final concentration of 10 mM) was added to the cell suspension, and the cells were incubated for an additional 5 min at 37°C. After filtering through a 40 μm cell strainer, the red blood cells were removed by lysis. For enrichment of lymphocytes, the cells were spun on a 40/75% Percoll gradient (GE Healthcare Life Sciences). For qRT-PCR analysis, macrophages (F4/80^{+}CD11b^{+}MHCII^{+}), T cells (SSC^{lo}TCRβ^{+}) and eosinophils (MHCII^{-}Siglec-F^{+}) from stromal vascular fraction (SVF) were further purified using MoFlo Astrios cell sorter (Beckman Coulter)

Antibodies and reagents

All fluorescence-conjugated antibodies used for flow cytometric analyses were purchased from BD Biosciences, eBioscience, or Biolegend. T cells were labeled with antibodies against TCRβ (H57-597), CD4 (RM4-5), and macrophages were labeled with MHCII (M5/114/15/2), F4/80 (BM8), CD11b (M1/70), CD11c (HL3), and CD206 (c068c2) antibodies after FcR blocking with anti-CD16/CD32 antibodies (2.4G2). Eosinophils were stained with siglec-F (E50-2440) antibody. For Treg cell labeling, cells were stained with TCRβ, CD4, and Foxp3 (FJK-16s) antibodies using the Foxp3 staining buffer solution (eBioscience). For T cell intracellular cytokine staining, T cells were surface-labeled with TCRβ and CD4 antibody and then stained with IFN-γ (XMG1.2), IL13 (eBio13A), and IL-17A (JES5-16E3) antibodies after permeabilization with the Cytofix/Cytoperm Kit (BD Biosciences). The stained cells were analyzed using LSRFortessa (BD Biosciences) and FlowJo software (Tree Star, San Carlos, CA).

qPCR

Total RNA was isolated from adipocytes, VAT-associated immune cells or whole-liver tissue with the TRIzol Reagent (Invitrogen) and

subjected to reverse transcription with Superscript II (Invitrogen) and oligo-dT primers. cDNA was amplified using Applied Biosystems ViiA7 using SYBR green (Takara) and specific primers. The results were analyzed by the $\Delta\Delta C_t$ (change in cycle threshold) method and normalized to *HPRT* expression.

Oil-red O staining

The liver was fixed in paraformaldehyde, embedded in Tissue-Tek O.C.T compound (SAKURA), and the frozen sections were stained with Oil-red O (Sigma Aldrich).

Statistical analyses

Results are presented as the mean ± SEM. Statistical significance was evaluated with an unpaired two-tailed Student's *t* test or two-way ANOVA. Significance was set at $P \leq 0.05$.

Results

TLR9 is highly expressed in macrophages and eosinophils in adipose tissues of mice (Supporting Information Figure S1A). TLR9 mRNA is also detected in adipocytes, albeit at a much lower level. To examine the role of TLR9 during the development of obesity, we fed 6-week-old WT and TLR9-deficient ($Tlr9^{-/-}$) mice with HFD for 10 weeks and measured body weight weekly. We found a significant difference in body weight changes with TLR9-deficient mice having approximately 20% higher body weight than WT mice after 10 weeks of HFD (Figure 1A). In addition, the epididymal fat mass was higher in TRL9-deficient mice (Figure 1B). Food consumption was not significantly different between the groups (Figure 1C). Compared to HFD-fed mice, mice fed with normal chow diet (NCD) did not show significant differences in weight gain (Supporting Information Figure S1B). Thus, TLR9 signaling regulates the development of diet-induced obesity.

To test if TLR9 deficiency also affects metabolic processes, we measured the fasting blood glucose levels of HFD-fed mice and found that it was significantly increased in mice lacking TLR9 compared to WT mice (Figure 2A). Moreover, TLR9-deficient mice exhibited an impaired ability to restore blood glucose to a baseline level in the GTT, a key indicator of altered metabolism (Figure 2B). Similarly, HFD-fed TLR9-deficient mice showed dramatically elevated blood insulin concentration compared with WT mice and decreased insulin sensitivity in the ITT, indicating more severe insulin resistance (Figure 2C,D). In contrast, mice fed with NCD showed no significant differences in serum glucose concentration, glucose tolerance and insulin sensitivity (Supporting Information Figures S2C and S2D).

Next, we examined the adipocyte function of TLR9-deficient obese mice. As shown in Figure 2E, TLR9 deficiency resulted in the significant decrease of adiponectin expression in adipose tissue. Adiponectin is a well-known adipokine that sensitizes insulin signaling,

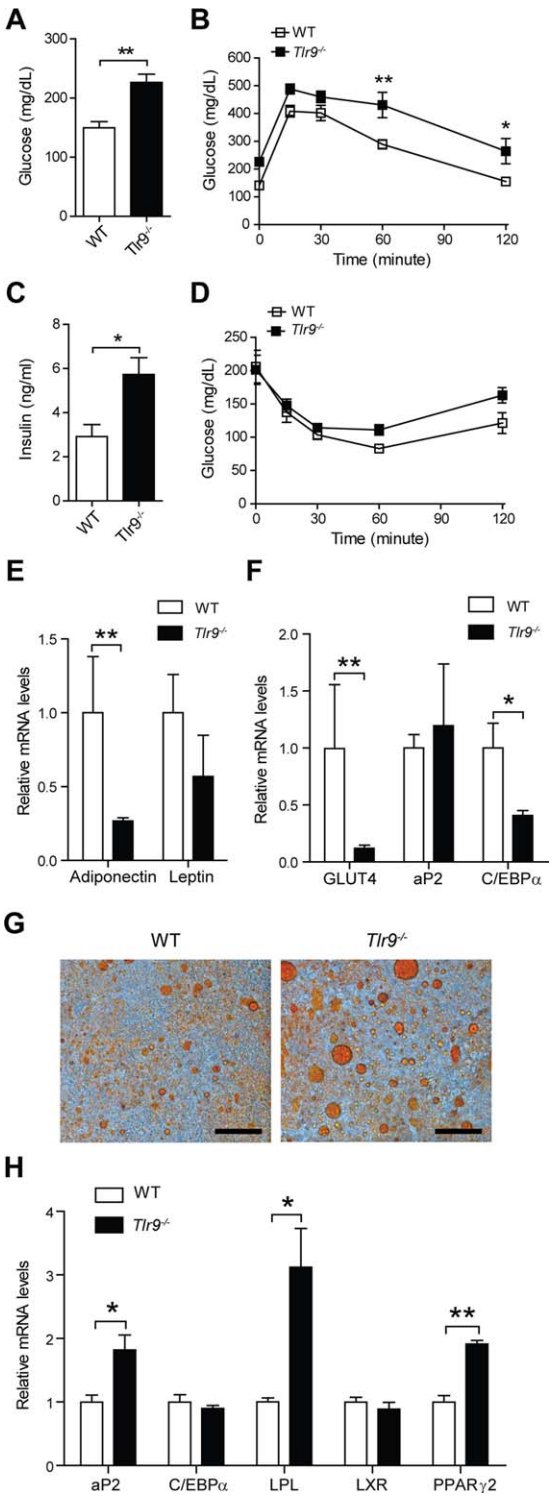

Figure 2 TLR9-deficient mice exhibit more severe metabolic disorders. After 10 weeks on a HFD, glucose homeostasis, adipocyte function, and fatty liver development were analyzed in WT and TLR9-deficient mice on a HFD. (**A**) Blood glucose level for 16 h fast. (**B**) Glucose tolerance test. Mice were fasted for 16 h and were injected i.p. with glucose (1 g/kg body weight). (**C**) Blood insulin level for 16 h fast. (**D**) Insulin tolerance test. Mice were fasted for 4 h and were injected i.p. with insulin (0.75 U/kg body weight) ($n = 6$). mRNA expression level of (**E**) adipokines and (**F**) adipogenic genes in epididymal fat pads. mRNA levels were normalized against *HPRT* mRNA, and the relative expression levels are shown ($n = 4$-5). (**G**) Oil-red O staining of the liver section. Scale bar: 200 μm. (**H**) mRNA expression level of hepatic lipogenic genes. Original magnification, 10×. Means ± SEM are shown. $^*P < 0.05$, $^{**}P < 0.01$, *t* test or two-way ANOVA.

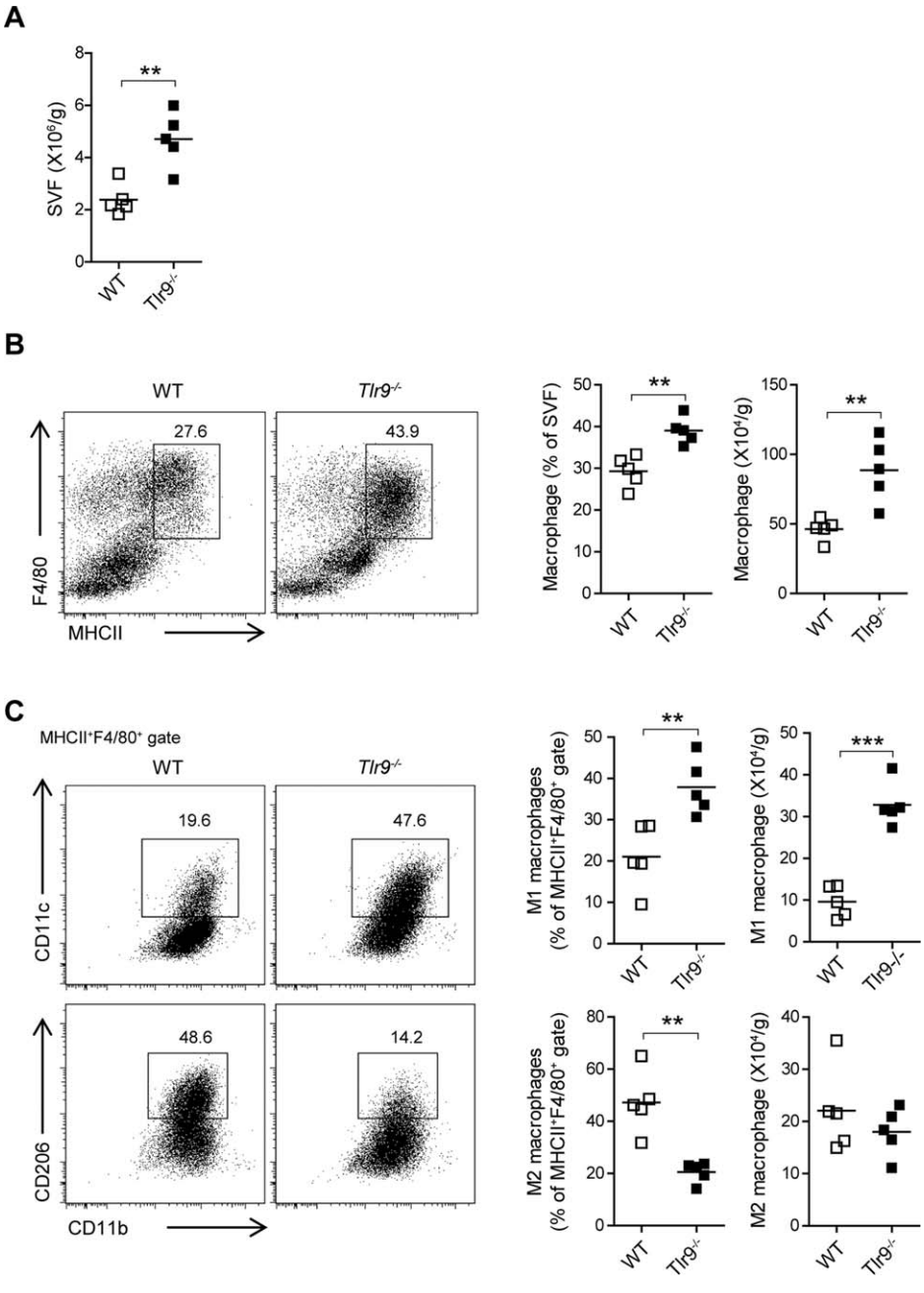

Figure 3 TLR9 deficiency increases macrophage accumulation in the VAT. Flow cytometric analysis of the SVF from the epididymal fat pads of WT and TLR9-deficient mice on a HFD. (**A**) Total SVF cell counts. (**B**) Proportion and number of macrophages in the VAT. (**C**) Proportion and number of M1 ($CD11c^+$) and M2 ($CD206^+$) macrophages in the VAT. Mean values and representative dot plots are shown. $^{**}P < 0.01$, $^{***}P < 0.001$, *t* test.

and its serum level is reduced in adults with obesity and patients with type 2 diabetes (23). Expression of adipogenic markers such as GLUT4 and CCAT/enhancer binding protein alpha (C/EBPα) in the adipose tissue was also significantly lower in TLR9-deficient mice compared to WT mice, suggesting that lack of TLR9 leads to more pronounced adipocyte dysfunction (Figure 2F). We also examined whether the TLR9 deficiency influences HFD-induced fatty liver development. HFD-induced lipid accumulation was dramatically enhanced in the liver of TLR9-deficient mice (Figure 2G). In addition, TLR9 deficiency significantly increased expression of the hepatic genes involved in lipogenesis, including fatty acid binding protein 4 (aP2), lipoprotein lipase (LPL), and PPARγ2 (Figure 2H). Collectively, these results imply that loss of TLR9 signaling amplifies the HFD-induced metabolic disorders.

The VAT plays an important role in controlling the systemic metabolism (1). In the obese state, macrophages infiltrate into the VAT and produce proinflammatory cytokines that decrease the insulin

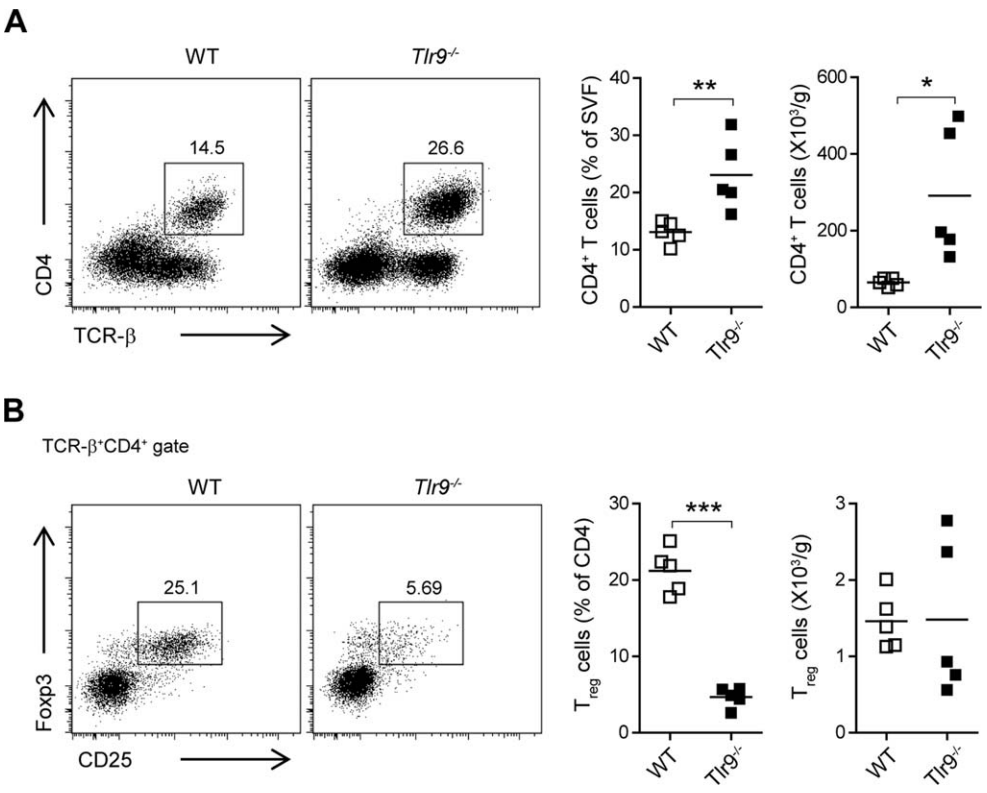

Figure 4 TLR9 deficiency increases CD4 T cell accumulation in the VAT. Flow cytometric analysis of the SVF from the epididymal fat pads of WT and TLR9-deficient mice on a HFD. (**A**) Proportion and cell numbers of $CD4^+$ T cells in the VAT. (**B**) Proportion and cell number of Treg ($Foxp3^+CD25^+$) cells in the VAT. Mean values and representative dot plots are shown. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, *t* test.

sensitivity (2). Additionally, obesity induces the phenotypic shift of VAT macrophages from the M2-polarized state to the proinflammatory M1 state (3). To analyze if TLR9 signaling affects VAT macrophages, we isolated SVF cells of VAT pads excised from obesity-induced WT and TLR9-deficient mice and analyzed the cells by flow cytometry. The total SVF cell count was higher in TLR9-deficient mice than WT mice (Figure 3A). Moreover, VAT macrophages ($MHCII^+F4/80^+$) were more enriched in TLR9-deficient mice (Figure 3B) (24). To assess the phenotypic polarization of macrophages, we measured the surface expression of CD11c and CD206, a marker of M1 and M2 macrophages, respectively. In a previous report, $CD206^{hi}$ macrophages were shown to play a dominant anti-inflammatory role through their expression of IL-10 (25), whereas $CD11c^+$ macrophages exert proinflammatory functions (3). We found that both the percentage and the number of proinflammatory M1 macrophages were higher in TLR9-deficient mice, whereas the proportion of anti-inflammatory M2 macrophages was lower in TLR9-deficient mice than in WT mice (Figure 3B). The numbers of M2 macrophages were similar in WT and TLR9-deficient mice, due to the higher total number of macrophages in TLR9-deficient mice.

Macrophage polarization can be influenced by T_H cells through their secretion of various cytokines (26). Therefore, we also analyzed $CD4^+$ T cells in the VAT and found that the percentage and the number of $CD4^+$ T cells were higher in TLR9-deficient mice (Figure 4A). We further examined $CD4^+$ T cell subsets, namely regulatory T cells ($CD25^+Foxp3^+$), T_H1 ($IFN\text{-}\gamma^+$), T_H2 ($IL\text{-}13^+$), and T_H17 ($IL\text{-}17A^+$) cells. Regulatory T cells (Treg) in the VAT are shown to exert antiobesity effects (27). Also, IL-10 secreted by Treg cells can alleviate the insulin resistance by inducing M2 macrophages (26). We found that the frequency of Treg cells was lower in TLR9-deficient mice. However, the numbers were similar in both WT and TLR9-deficient mice, because the total number of $CD4^+$ T cells was higher in the VAT of TLR9-deficient mice (Figure 4B).

VAT T_H1 cells can activate M1 macrophages via secretion of cytokines such as IFN-γ which is linked to metabolic syndrome (28). We found higher numbers of T_H1 cells in the VAT from obesity-induced TLR9-deficient mice than obesity-induced WT mice, which likely contribute to the proinflammatory environment of the VAT (Figure 5). In adoptive transfer experiments, T_H2 cells were shown to sustain VAT M2 macrophages and enhance insulin sensitivity (25). In comparison with WT mice, TLR9-deficent mice have a lower percentage of T_H2 cells in the VAT, but show similar overall numbers. We also found the presence of T_H17 cells in the VAT of both WT and TLR9-deficient mice. There was no significant alteration of T_H17 cells in TLR9-deficient mice. In summary, these results demonstrate that the lack of TLR9 signaling potentiates the increase of proinflammatory cells in the VAT of obese mice, which likely leads to a chronic inflammation of the VAT.

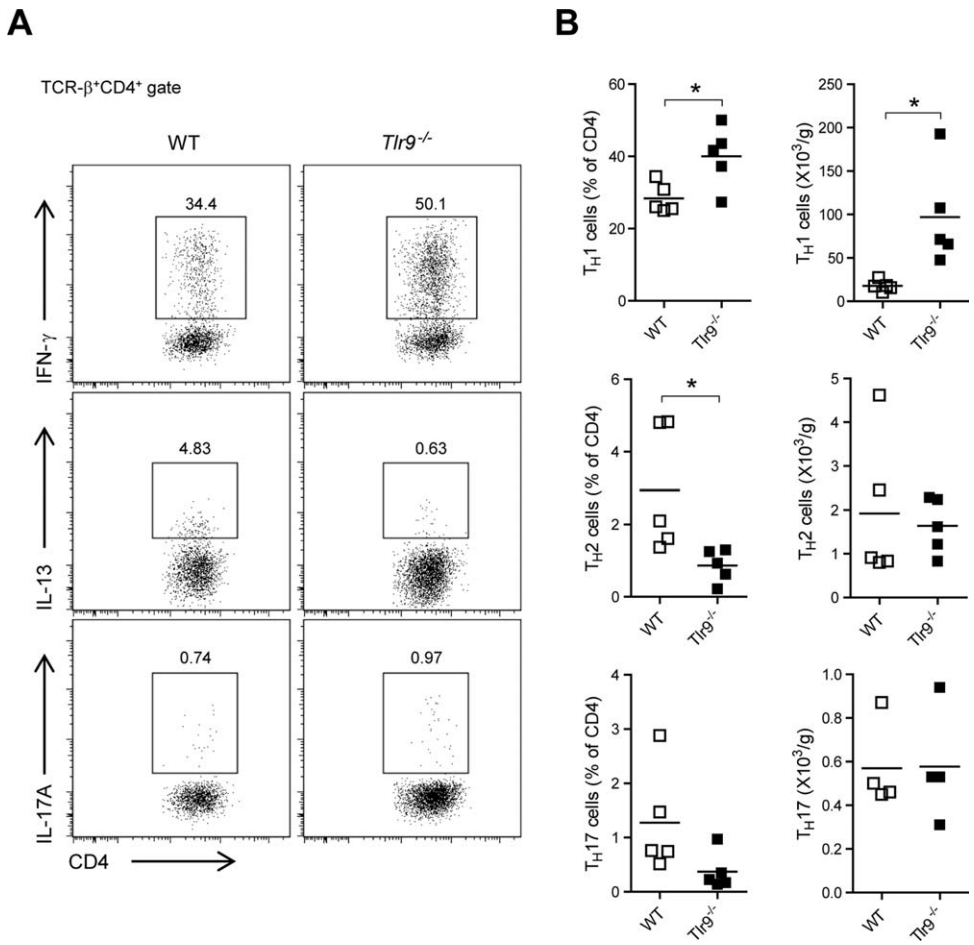

Figure 5 TLR9 deficiency results in increase of T_H1 cells in the VAT. Flow cytometric analysis of the SVF from the epididymal fat pads of WT and TLR9-deficient mice on a HFD. Isolated VAT T cells were stimulated with PMA and ionomycin in the presence of monensin for 4 h before intracellular cytokine staining. (**A**) Analysis of IFN-γ, IL-13, and IL-17A expressing $CD4^+$ T cells. (**B**) Proportion and cell number of T_H1, T_H2, and T_H17 cells in the VAT. Mean values and representative dot plots are shown. $^*P < 0.05$, *t* test.

To determine whether the VAT of TLR9-deficient obese mice suffers more severe inflammation, we measured cytokine mRNA levels in the VAT extracts. As shown in Figure 6A, TLR9 deficiency indeed caused a significantly higher expression of various proinflammatory cytokines (TNF-α, IL-6, and IFN-γ). In contrast, the levels of T_H2 cytokines, IL-4 and IL-13, were similar in both WT and TLR9-deficient mice. The lack of TLR9 also resulted in the increased expression of multiple chemokines, such as MCP-1, MIP-1α, and RANTES (Figure 6B). These findings clearly support a role of TLR9 in regulating inflammatory responses in the VAT of obese mice.

Discussion

Obesity is associated with low-grade inflammation caused by abnormal inflammatory cytokine production in adipose tissue (29). Although many studies have shown an important role of adipose tissue inflammation in metabolic syndrome (30), the underlying mechanisms are not fully understood.

TLRs, which become upregulated in affected tissues of most inflammatory disorders, can mediate crosstalk between the immune system and body metabolism (31). Recent findings have shed light on the role of several TLRs as important regulators of metabolic disorders such as obesity and insulin resistance (32). Especially, in the inflammatory environment of the VAT in obese mice, activation of TLRs by a variety of endogenous and exogenous ligands may contribute to adipocyte dysfunction and further promote insulin resistance (33). For example, it was reported that TLR4, the receptor for bacterial lipopolysaccharides, can sense free fatty acids and mediate insulin resistance in the adipose tissue (34). Despite the increased TLR9 expression in the adipose tissue of obese mice, however, the role of TLR9 in maintaining metabolic homeostasis has remained unknown (35).

In this study, we found that TLR9 deficiency accelerates HFD-induced weight gain, insulin resistance, and visceral fat accumulation. In addition, severe adipocyte dysfunction and fatty liver development were observed in HFD-fed TLR9-deficient mice.

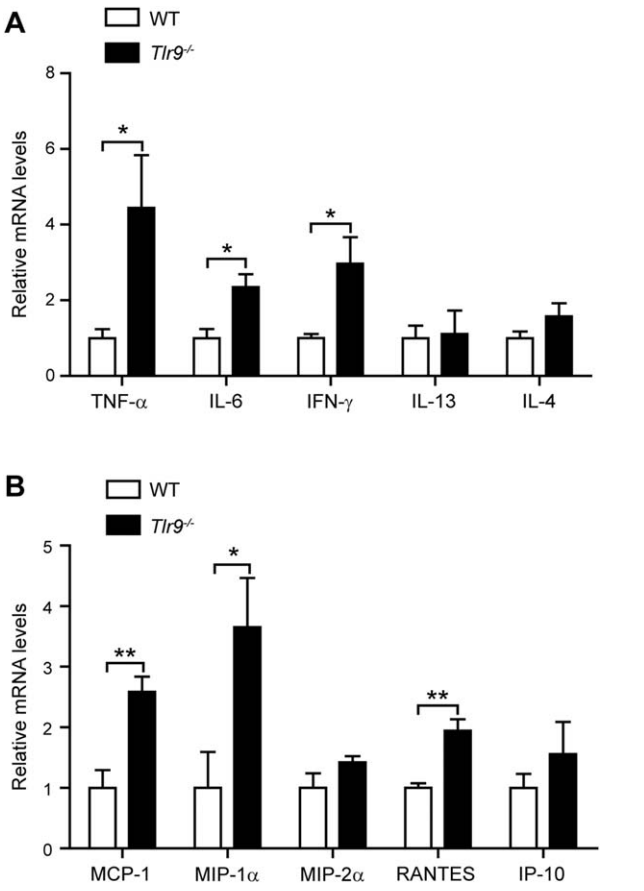

Figure 6 TLR9 deficiency results in adipose tissue inflammation. qRT-PCR analysis of (**A**) inflammatory cytokines and (**B**) chemokines in epididymal fat pads of WT (white bars) and TLR9-deficient (black bars) mice on a HFD. mRNA levels were normalized against *HPRT* mRNA, and the relative expression levels are shown. Means ± SEM are shown ($n = 4$–5). $^{*}P < 0.05$, $^{**}P < 0.01$, *t* test.

Furthermore, infiltration of immune cells, including macrophages and T_H1 cells, into the VAT was dramatically increased in TLR9-deficient mice. The crosstalk between VAT-infiltrating T cells and macrophages is important for altering the inflammatory phenotype of the macrophages and for regulating adipose tissue inflammation (4,36). Consistent with the increase of immune cell infiltration within the adipose tissue of TLR9-deficient mice, various proinflammatory cytokines, and chemokines were significantly increased in TLR9-deficient mice. Thus, our study implicates that TLR9 is required for regulating adipose tissue inflammation and obesity-related metabolic disorders.

How does TLR9 limit HFD-induced adipose tissue inflammation and metabolic syndrome? A majority of evidence suggests that the main role of TLR9 signaling in the innate immune responses is to exert proinflammatory actions (31,37). However, the anti-inflammatory function of TLR9 has recently been implicated in several inflammatory diseases including colitis and pneumonia (17,38). Moreover, TLR9-mediated signaling was linked to the anti-inflammatory cytokine production by macrophages. Stimulation of macrophages with CpG DNA resulted in IL-10 production through a TLR9-MyD88 dependent pathway (39). Further study will be required to determine if TLR9 signaling indeed directly suppresses the adipose tissue inflammation by exerting anti-inflammatory responses and controls obesity development.

Similar to our findings with TLR9-deficient mice, mice lacking TLR5 exhibited insulin resistance and obese phenotype and these defects were attributed to the altered intestinal microbiota community in TLR5-deficient mice (12). However, a recent study showed that TLR9 deficiency did not alter the composition of gut microbiota, suggesting that the exacerbated metabolic syndrome seen in TLR9-deficient mice is unlikely to be explained by changes in intestinal microbiota (40).

In conclusion, we found that TLR9 deficiency increases HFD-induced adiposity, VAT inflammatory responses, and insulin resistance in mice. Therefore, TLR9 is likely to play a key role in regulating adipose tissue inflammation and obesity-related metabolic disorders, although additional investigation is required to elucidate the underlying mechanisms. Probing the site of action and cell type-specific mechanism of TLR9-mediated signaling may provide further insight into the role of TLR9 in maintaining metabolic homeostasis and preventing adipose tissue inflammation. Our study also suggests that manipulation of TLR9 signaling using specific agonists might provide a useful therapeutic approach for treatment of inflammation-related metabolic syndrome. O

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