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Obesity-Related Upregulation of Monocyte Chemotactic Factors in Adipocytes

Involvement of Nuclear Factor- κ B and c-Jun NH₂-Terminal Kinase Pathways

Ping Jiao,^{1,2} Qiu Chen,^{1,3} Suketu Shah,¹ Jing Du,¹ Bo Tao,¹ Iphigenia Tzamelis,⁴ Weiqun Yan,² and Haiyan Xu¹

OBJECTIVE—We sought to evaluate the entire picture of all monocyte chemotactic factors that potentially contribute to adipose tissue macrophage accumulation in obesity.

RESEARCH DESIGN AND METHODS—Expression and regulation of members in the entire chemokine superfamily were evaluated in adipose tissue and isolated adipocytes of obese versus lean mice. Kinetics of adipose tissue macrophage infiltration was characterized by fluorescence-activated cell sorting. The effects of fatty acids on stimulation of chemokine expression in adipocytes and underlying mechanisms were investigated.

RESULTS—Six monocyte chemotactic factors were found to be predominantly upregulated in isolated adipocytes versus stromal vascular cells in obese mice for the first time, although most of them were previously reported to be upregulated in whole adipose tissue. In diet-induced obese mice, adipose tissue enlargement, increase of adipocyte number, and elevation of multiple chemokine expression precede the initiation of macrophage infiltration. Free fatty acids (FFAs) are found to be inducers for upregulating these chemokines in 3T3-L1 adipocytes, and this effect can be partially blunted by reducing Toll-like receptor 4 expression. FFAs induce expression of monocyte chemotactic factors in adipocytes via both transcription-dependent and -independent mechanisms. In contrast to the reported role of JNK as the exclusive mediator of FFA-induced monocyte chemoattractant protein-1 (MCP-1) expression in macrophages, we show a novel role of inhibitor of κ B kinase- β (IKK β) in mediating FFA-induced upregulation of all six chemokines and a role of JNK in FFA-induced upregulation of MCP-1 and MCP-3.

CONCLUSIONS—Multiple chemokines derived from adipocytes might contribute to obesity-related WAT macrophage infiltration with FFAs as potential triggers and involvement of both IKK β and JNK pathways. *Diabetes* 58:104–115, 2009

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Obesity-related type 2 diabetes is associated with low-intensity inflammation (1,2). Human studies have demonstrated elevated circulating levels of inflammatory markers in obese diabetic patients (1,3). Furthermore, blood mononuclear cells (MNCs) in the obese state are also in a proinflammatory state (3). The discovery of macrophage accumulation in adipose tissue of obese rodents and humans revealed a potentially important source of inflammatory molecules in obesity (4,5). Activated macrophages are well known to secrete a variety of inflammatory cytokines and chemokines, which impair insulin signaling (6,7). Dysregulation of lipolysis by increased expression of adipose cytokines is an important factor for causing systemic insulin resistance through elevated circulating free fatty acid (FFA) levels. Elevation of circulating FFAs has also been reported to induce inflammation in MNCs (8). Decreased adipose macrophage infiltration in diet-induced obese (DIO) mice deficient in monocyte chemoattractant protein-1 (MCP-1) and its major receptor CCR2, accompanied by decreased adipose expression of cytokines and lowered circulating FFA levels, has been associated with improved systemic insulin sensitivity (9,10). Transgenic mice overexpressing MCP-1 in adipose tissue, with increased adipose macrophage content and elevated circulating FFA levels, are insulin resistant (10,11). However, the role of MCP-1 in adipose macrophage infiltration in obesity is controversial because a recent study showed that MCP-1-deficient mice have unchanged adipose macrophage content (12). Decreased macrophage infiltration and reduction of inflammatory gene expression in adipose tissue have also been associated with weight loss in obese subjects (13,14). Thiazolidinediones, a class of insulin-sensitizing drugs that mainly improve adipose insulin sensitivity of type 2 diabetic patients, also have potent anti-inflammatory effects, suppress adipose macrophage gene expression in vitro and in vivo, and inhibit proinflammatory MNCs (15–22). DIO mice treated with CCR2 antagonist have a 28% reduction in adipose macrophage content and have improved hyperglycemia (9). These results indicate that eliminating macrophage extravasation into fat in obesity may be beneficial for improving whole-body insulin sensitivity.

MCP-1 has been a controversial candidate chemokine for recruiting macrophages into WAT in obesity, indicating involvement of other chemokines. In the present study, we examined the entire murine chemokine superfamily for

TABLE 1
Metabolic parameters of *ob/ob* and DIO mice

	9 weeks		20 weeks	
	Lean	<i>ob/ob</i>	Chow	High-fat diet
<i>n</i>	10	10	8	5
Body weight (g)	27.5 ± 0.4	48.5 ± 1.1*	31.2 ± 0.73	51.6 ± 1.14*
Fed plasma glucose (mg/dl)	221 ± 18.84	477 ± 2.81*	282 ± 14.37	378 ± 18.89*
Fed plasma insulin (ng/ml)	0.76 ± 0.15	7.99 ± 0.1*	1.61 ± 0.67	7.47 ± 0.59*
Epididymal fat (g)	0.75 ± 0.04	4.1 ± 0.1*	1.12 ± 0.05	2.06 ± 0.20*
Total adipocytes per mouse × 10 ⁵	8.74 ± 0.70	21.2 ± 3.97	18.7 ± 0.68	29.7 ± 6.1
Total stromal-vascular cells per mouse × 10 ⁵	7.75 ± 0.59	58.5 ± 5.12*	6.99 ± 0.49	100.9 ± 20.5*
F4/80 CD11C ⁺ cells (%)	8.09 ± 0.32	28.9 ± 0.32*	11.99 ± 2.39	28.99 ± 2.98*

Data are means ± SE. Metabolic parameters of *ob/ob* and DIO mice and lean controls. *ob/ob* mice and controls were 9 weeks old. DIO mice and controls were 24 weeks old. High-fat diet was initiated at the age of 4 weeks and continued for 20 weeks. Body weight and blood glucose levels were measured in the fed state. Mice were then killed in the fed state for plasma collection to measure insulin. Epididymal fat pads were isolated, weighed, and digested for isolation of stromal vascular fraction. Percentage of macrophage in stromal vascular fraction was determined by fluorescence-activated cell sorting using anti-F4/80 and CD11C antibodies (detailed methods are in the supplementary materials available in the online appendix). **P* < 0.05, obese vs. lean mice.

their expression and regulation in adipose tissue of obese mice. Six chemokines were found to be significantly increased in obese adipose tissue. We revealed for the first time that elevation of chemokines in obese adipose tissue is predominantly in adipocytes and that FFAs are inducers for specific upregulation of the aforementioned six chemokines in cultured adipocytes. Although JNK has been demonstrated to mediate FFA-induced MCP-1 expression in macrophages (23), inhibitor of κ B (I κ B) kinase- β (IKK β) is the main mediator of FFA-induced upregulation of multiple chemokines in adipocytes.

RESEARCH DESIGN AND METHODS

Cells, reagents, and treatments. 3T3-L1 cells were obtained from American Type Tissue Culture Collection. 3T3-L1 cell sublines stably expressing short hairpin interfering RNA (shRNA) against Toll-like receptor 4 (shTLR4) or scramble shRNA were established as described previously (24). 3T3-L1 CAR cells, a 3T3-L1 subline stably expressing the truncated adenovirus receptor, were provided by Dr. David Orlicky (University of Colorado Health Sciences Center, Denver, CO). 3T3-L1 CAR cells have dramatically improved adenovirus infection efficiency compared with regular 3T3-L1 cells (25). For differentiation, preadipocytes were grown to confluency and induced with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% cosmic calf serum (CCS), 1 μ g/ml insulin, 0.5 nmol/l isobutylmethyl xanthine, and 1 μ mol/l dexamethasone for 3 days. After induction, cells were maintained in DMEM containing 10% CCS and 1 μ g/ml insulin for 7 more days. FFA mixture, dexamethasone, actinomycin D, and cycloheximide were purchased from Sigma. For FFA treatment, 3T3-L1 adipocytes were treated with 0.5 nmol/l FFA mixture in the presence of BSA at the molar ratio of 4:1. NADPH, nuclear factor- κ B (NF- κ B), and JNK inhibitors were purchased from Calbiochem. Rosiglitazone was purchased from BioMol International. MCP-1, MCP-2, MCP-3, monocyte inflammatory protein-1 α (MIP-1 α), and macrophage-related protein (MRP)-1 ELISA construction kits were purchased from Antigenix America. MRP-2 ELISA kit was purchased from R&D Systems. JNK1 small interfering RNA (siRNA) was purchased from Dharmacon. JNK2 and IKK β siRNAs were purchased from Santa Cruz Biotechnology. JNK1 and IKK β antibodies were purchased from Santa Cruz Biotechnology. Phospho-JNK antibody was purchased from Cell Signaling Technology.

Mouse models. Male *ob/ob* mice and littermate controls were purchased from The Jackson Laboratory. These mice were fed standard chow and killed at 9 weeks of age for tissue collection. For DIO mice, C57BL/6J mice were purchased from The Jackson Laboratory at 3 weeks of age, acclimated for a week, and fed on either a chow diet (5% kcal from fat) or a high-fat diet (60% kcal from fat; D12492; Research Diets) for 20 weeks. For rosiglitazone treatment, 9-week-old *ob/ob* male mice were orally gavaged once a day at the dose of 15 mg/kg for 28 consecutive days. At the end of the study, mice were killed by CO₂ inhalation, and epididymal fat pads were excised for RNA extraction. Animal experiments were approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital.

Isolation of primary adipocytes. Epididymal white fat pads from DIO mice were excised, weighed, and rinsed in isolation buffer. Fat pads were then cut

into small pieces in isolation buffer supplemented with 1 mg/ml type I collagenase and digested at 37°C in shaking water bath at 100 rpm for 45 min. Then, digested tissues were filtered through 400 μ mol/l mesh to get single cell suspension. Cells were rinsed twice with isolation buffer before RNA extraction.

Transcription profiling. Total RNA was extracted from epididymal adipose tissue of *ob/ob* and DIO mice. Twenty micrograms of RNA from each sample was further purified to remove contaminating organics and non-RNA species using a silica resin according to the manufacturer's instructions. Total RNA was converted to biotinylated, fragmented cRNA and hybridized to Murine U74Av2 chips using protocols recommended by the microarray manufacturer. The samples were stained and washed on Fluidics Station 400 and scanned on a GeneArray Scanner. Primary data extraction was performed with Microarray Suite 5.0 and signal normalization across samples was performed using all probe sets with a mean expression value of 500.

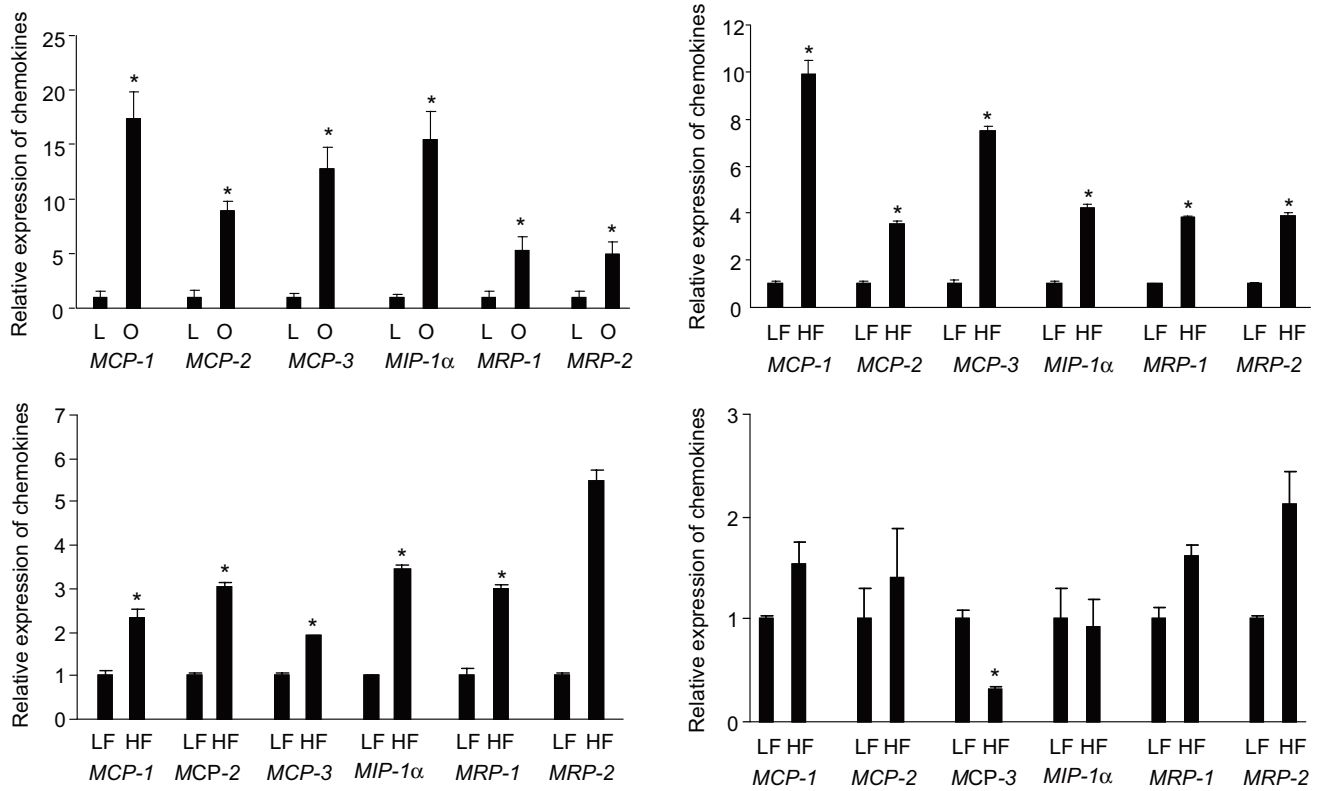
RNA isolation and real-time PCR. RNA samples were extracted using the TRIzol reagent. Chemokine expression and regulation profile were measured using real-time PCR analysis. Random hexamers were used for reverse transcription. Real-time PCR analysis was performed in a 15- μ l reaction on a 96-well clear plate using Power SYBR Green RT-PCR Reagents kit on ABI Prism thermal cycler model 7500. The relative mRNA expression levels were normalized to expression of 28S rRNA.

Electroporation of adipocytes and luciferase assay. 3T3-L1 adipocytes were transfected with NF- κ B-Luciferase (Firefly) construct by electroporation with a Nucleofector system (Amaxa Biosystems) according to the manufacturer's instructions. Briefly, 3T3-L1 adipocytes were trypsinized, resuspended in Nucleofector solution at 2.0×10^6 cells/100 μ l, and mixed with luciferase reporter vector. Cells were then electroporated by program A-033 using Nucleofector II (Amaxa Biosystems). Cells were immediately plated on 12-well plates (1.0×10^6 cells/well) after electroporation. Twenty-four hours after electroporation, cells were serum-free overnight. NF- κ B inhibitor was applied to adipocytes 1 h before FFA treatment and also added during FFA treatment. For luciferase assay, transfected cells were washed twice with PBS, lysed by two freeze-thaw cycles, and centrifuged at 10,000g for 5 min at 4°C to remove cellular debris. Firefly luciferase activity was measured by mixing 20 μ l cell extract with 100 μ l luciferase assay buffer containing firefly luciferase substrate. Light production was measured for 5 s on a Perkin Elmer luminometer.

RESULTS

Obesity is associated with upregulation of multiple monocyte chemotactic factors in adipose tissue and adipocytes. To date, 46 chemokines have been identified and 38 murine orthologs have been found, including a pseudogene. We analyzed the expression and regulation of murine chemokines in adipose tissue from both *ob/ob* and DIO mice by transcriptional profiling and real-time PCR analysis. We selected 9-week-old *ob/ob* mice and 24-week-old DIO mice fed on a high-fat diet for 20 weeks because of their comparable adiposity. The metabolic parameters

A



B

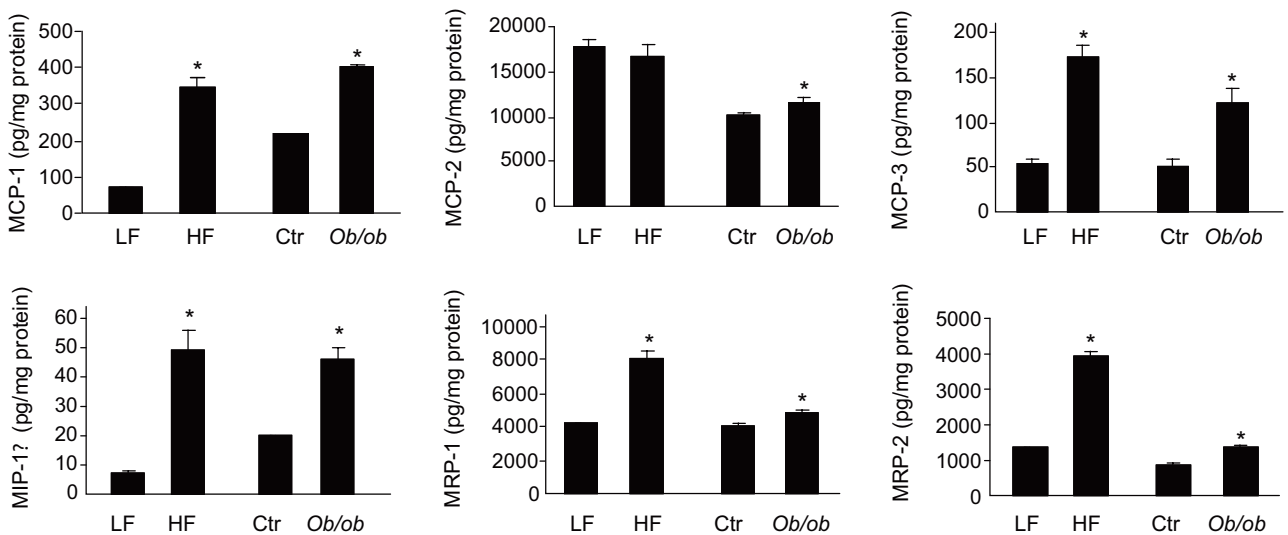


FIG. 1. Obesity is associated with upregulation of multiple monocyte chemotactic factors in adipose tissue and adipocytes. **A:** Significantly upregulated monocyte chemotactic factors in epididymal fat pads of *ob/ob* mice versus lean controls ($n = 5$, top left), in DIO mice versus lean controls ($n = 5$, top right), in isolated adipocytes of DIO mice versus lean controls ($n = 4$, bottom left), and in isolated stromal vascular cells in DIO mice versus controls ($n = 4$, bottom right). Expression of chemokines was measured by real-time PCR analysis. For comparison, the expression level of these genes in lean mice was arbitrarily set at 1. **B:** Protein levels of MCP-1, MCP-2, MCP-3, MIP-1 α , MRP-1, and MRP-2 in adipose tissue of *ob/ob* and DIO mice. **C:** Plasma levels of MCP-1, MCP-2, MCP-3, MRP-1, and MRP-2 in *ob/ob* and DIO mice. **D:** Protein levels of MCP-1, MCP-2, MCP-3, MIP-1 α , MRP-1, and MRP-2 in adipose tissue of lean mice on acute high-fat diet. Error bars represent \pm SE. * $P < 0.05$, *ob/ob* (O) vs. lean (L) or high fat (HF) vs. low fat (LF). HFD, high-fat diet; WK, week.

of these mice are listed in Table 1. Six CC and one CXC chemokines were found to be significantly upregulated in WAT of both *ob/ob* and DIO mice. These CC chemokines include *MCP-1*, *MCP-2*, *MCP-3*, *MIP-1 α* , *MRP-1*, and *MRP-2* (Fig. 1). CXC chemokine *MIP-2* was also significantly upregulated in WAT of both *ob/ob* and DIO mice;

MIP-1 β was significantly upregulated in WAT of *ob/ob* but not DIO mice; *MIP-2 γ* was significantly upregulated in WAT of DIO but not *ob/ob* mice. Interestingly, all six upregulated CC chemokines have monocyte chemotactic capabilities. Separation of adipocytes and stromal vascular cells indicate that these CC chemokines are mainly

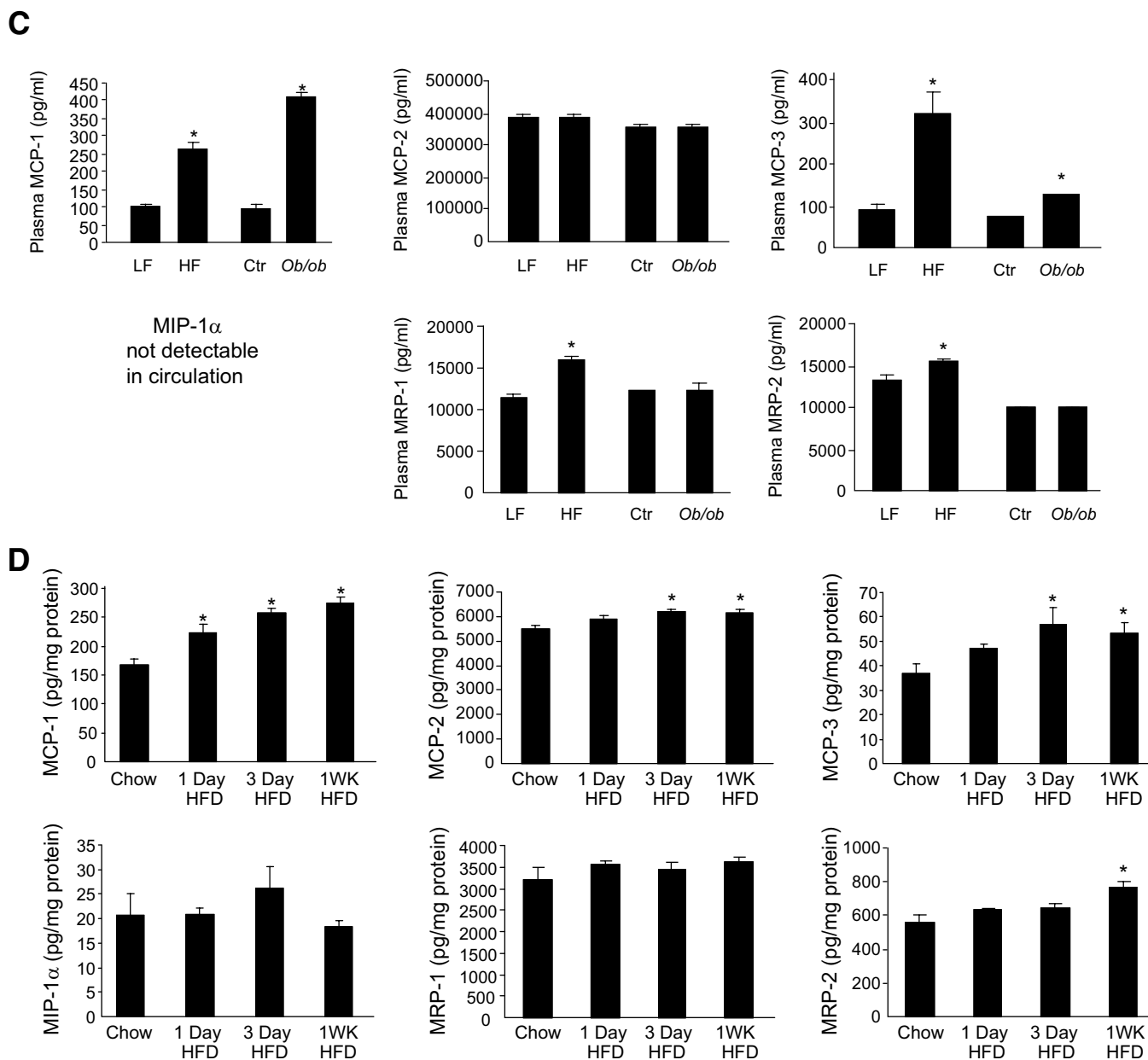


FIG. 1. Continued

increased in primary adipocytes (Fig. 1). MCP-1, MCP-2, MCP-3, MIP-1 α , and MRP-2 have been previously reported to increase at the mRNA level in whole adipose tissue (4,7,9,26). Our study not only adds MRP-1 to the list but also points out that elevation of chemokine expression mainly occurs in adipocytes. The protein levels of MCP-1, MCP-3, MIP-1 α , and MRP-2 were significantly increased by 402, 213, 604, 92, and 190%, respectively, in WAT of DIO mice with the protein level of MCP-2 unchanged (Fig. 1B). The protein levels of MCP-1, MCP-2, MCP-3, MIP-1 α , MRP-1, and MRP-2 were significantly increased by 85, 16, 134, 134, 21, and 60%, respectively, in WAT of *ob/ob* mice (Fig. 1B). The circulating levels of MCP-1, MCP-3, MRP-1, and MRP-2 were significantly increased by 160, 254, 41, and 17%, respectively, in plasma of DIO mice (Fig. 1C). The circulating levels of MCP-1 and MCP-3 were also significantly increased by 345 and 71%, respectively, in plasma of *ob/ob* mice (Fig. 1C). Circulating levels of MCP-2 did not change in either DIO mice or *ob/ob*

mice. Circulating levels of MRP-1 and MRP-2 only increased in DIO mice but remained unchanged in *ob/ob* mice. Circulating level of MIP-1 α was undetectable in either *ob/ob* or DIO mice. To understand when these chemokines start to increase, a high-fat diet was applied to 4-week-old C57BL/6J mice for a short period of time. The protein level of MCP-1 rapidly increased 1 day after high-fat diet and continued to increase at 3 and 7 days on high-fat diet (Fig. 1D). The protein levels of MCP-2 and MCP-3 trended up 1 day on high-fat diet and the elevation became significant at 3 and 7 days on high-fat diet. The protein level of MRP-2 significantly increased at 7 days on high-fat diet. However, MIP-1 α and MRP-1 protein levels did not significantly increase within 1 week of high-fat diet. These results indicate that MCP-1, MCP-2, MCP-3, and MRP-2 might be more important in the initial macrophage extravasation into adipose tissue compared with MIP-1 α and MRP-1, which probably play a role in attracting macrophage into adipose tissue in a later stage of obesity

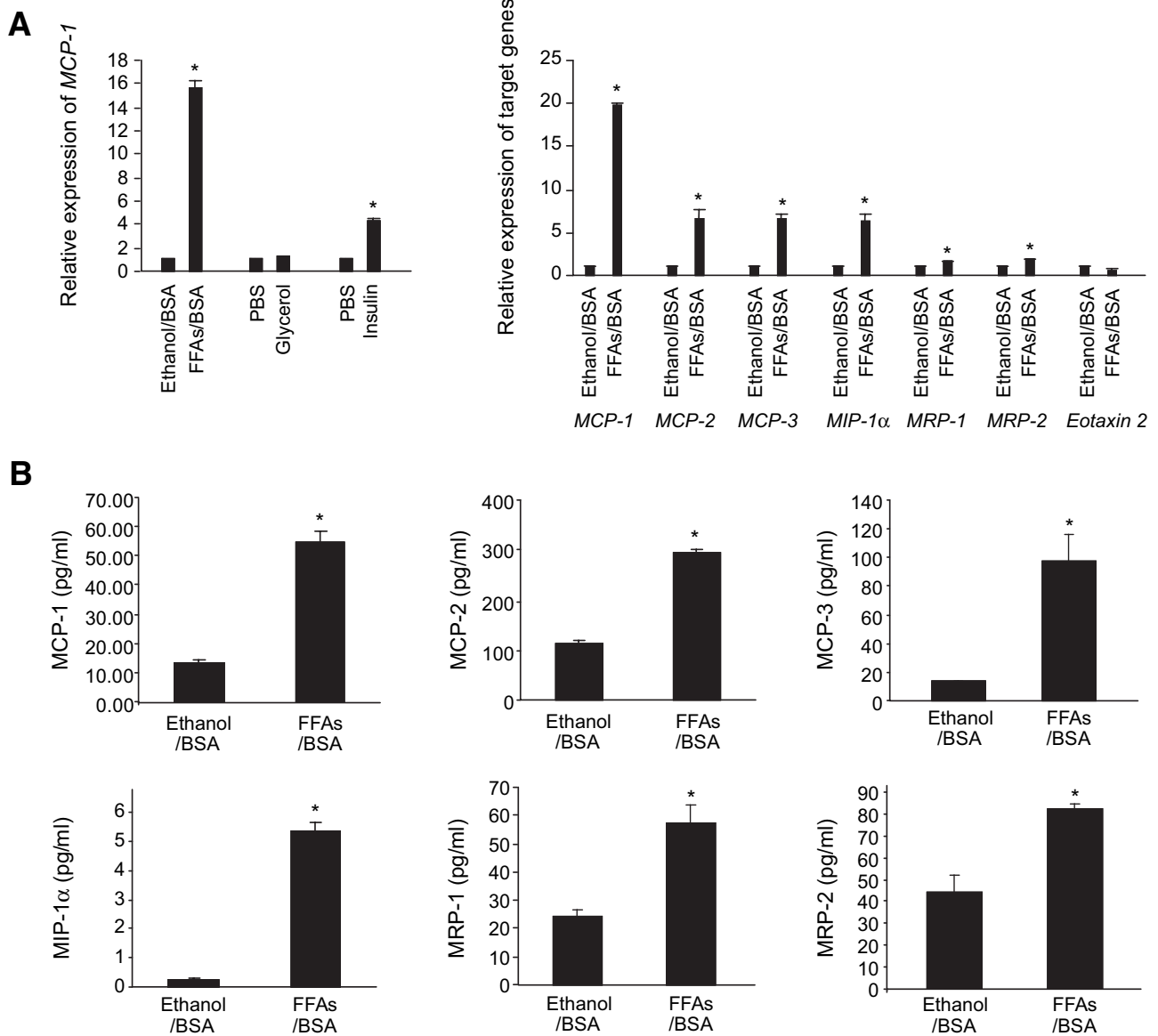


FIG. 2. FFAs stimulate chemokine production in 3T3-L1 adipocytes. **A:** FFAs upregulate monocyte chemotactic factors at mRNA level in 3T3-L1 adipocytes. **Left:** FFAs, not glycerol, are capable of inducing *MCP-1* expression in cultured 3T3-L1 adipocytes. RNAs were extracted from fully differentiated 3T3-L1 adipocytes treated with 0.5 mmol/l FFA/BSA, 0.5 mmol/l glycerol, 1 μ g/ml insulin, or respective vehicles (Veh) for 3 h after overnight incubation with serum-free medium. **Right:** FFAs are capable of inducing expression of multiple chemokines in cultured 3T3-L1 adipocytes. For comparison, the expression level of these genes in vehicle-treated 3T3-L1 adipocytes was arbitrarily set at 1. **B:** FFAs upregulate monocyte chemotactic factors in 3T3-L1 adipocytes at protein level. Conditioned medium was collected from adipocytes treated with ethanol/BSA or FFA/BSA for 3 h after overnight incubation with serum-free medium and used for enzyme-linked immunosorbent assay (ELISA) analysis. Error bars represent \pm SE. * $P < 0.05$, treated vs. vehicle. Results shown here are representative of three independent experiments.

development. To determine whether elevation of chemokine expression in adipose tissue occurs before macrophage infiltration, we evaluated the kinetics of macrophage infiltration by comparing adiposity and macrophage number in DIO mice versus lean controls 1, 4, 8, 12, 16, and 20 weeks on high-fat diet (supplementary Table 1, available in an online appendix at <http://dx.doi.org/10.2337/db07-1344>). Our results clearly show that fat pad enlargement, increase of adipocyte number, and elevation of expression of monocyte chemotactic factors occur as early as 1 week on high-fat diet, but adipose macrophage content did not significantly increase until 12 weeks on high-fat diet. A recent study reported that F4/80 and CD11c double positive cells are mainly responsible for macrophage-mediated inflammatory activities (23). We therefore analyzed the adipose macrophage content by counting F4/80 and CD11c double positive cells.

FFAs are potent inducers for chemokine expression.

Massive expansion of adipose tissue reflects the need for the body to store excessive amount of energy in the form of triglyceride, which is synthesized using FFAs and glycerol as substrates. The effects of FFAs and glycerol on chemokine production in adipocytes have not been documented. To determine whether a surplus of FFAs and/or glycerol could be the stimuli for increased chemokine production, 3T3-L1 adipocytes were stimulated with a mixture of either 0.5 mmol/l FFA/BSA or 0.5 mmol/l glycerol. We chose to use a mixture of 0.5 mmol/l saturated (lauric and myristic acid) and unsaturated (oleic, linoleic, and arachidonic acid) FFAs for stimulation. FFAs dramatically upregulated *MCP-1* expression, whereas glycerol had no effect (Fig. 2A). Further experiments indicated that FFAs are also able to upregulate *MCP-2*, *MCP-3*, *MIP-1 α* , *MRP-1*, and *MRP-2* in 3T3-L1 adipocytes

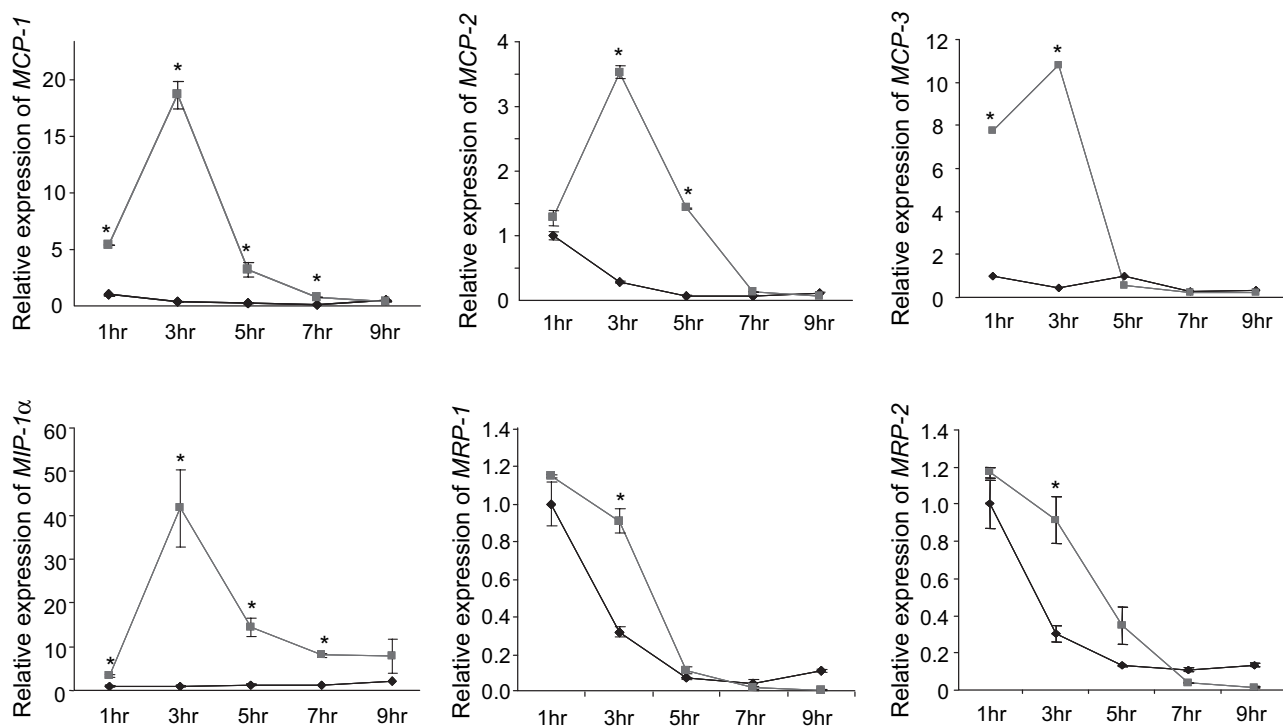


FIG. 3. Time course of FFA-induced upregulation of monocyte chemotactic factors in 3T3-L1 adipocytes. After overnight incubation with serum-free medium, 3T3-L1 adipocytes were treated with ethanol/BSA or FFA/BSA for 1, 3, 5, 7, or 9 h before RNA extraction. Expression of chemokines were evaluated by real-time PCR analysis. Error bars represent \pm SE. * $P < 0.05$, treated vs. vehicle. Results shown here are representative of three independent experiments.

(Fig. 2A). As a negative control, expression of *eotaxin 2*, which is not upregulated in obese adipose tissue, was examined in 3T3-L1 adipocytes and was found not to be increased by FFA treatment (Fig. 2A). The upregulation of mRNA level of the above chemokines by FFA treatment is accompanied by increased protein secretion into conditioned medium (Fig. 2B). FFA-stimulated chemokine production in 3T3-L1 adipocytes is dose (data not shown) and time dependent (Fig. 3). Detailed chemokine expression time course revealed that the peak expression of *MCP-1*, *MCP-2*, *MCP-3*, and *MIP-1 α* is \sim 3 h after treatment (Fig.

3). Interestingly, FFA-stimulated upregulation of *MRP-1* and *MRP-2* is due to delayed decrease rather than increase of the actual mRNA levels compared with vehicle-treated cells (Fig. 3). Experiments using individual FFAs indicate that the effect is most likely attributed to unsaturated FFAs (supplementary Fig. 1, available in the online appendix).

Role of TLR4 in FFA-induced chemokine expression. TLR4 plays an important role in innate immunity. Recently, TLR4 has been demonstrated to mediate fatty acid-induced activation of inflammatory pathways and

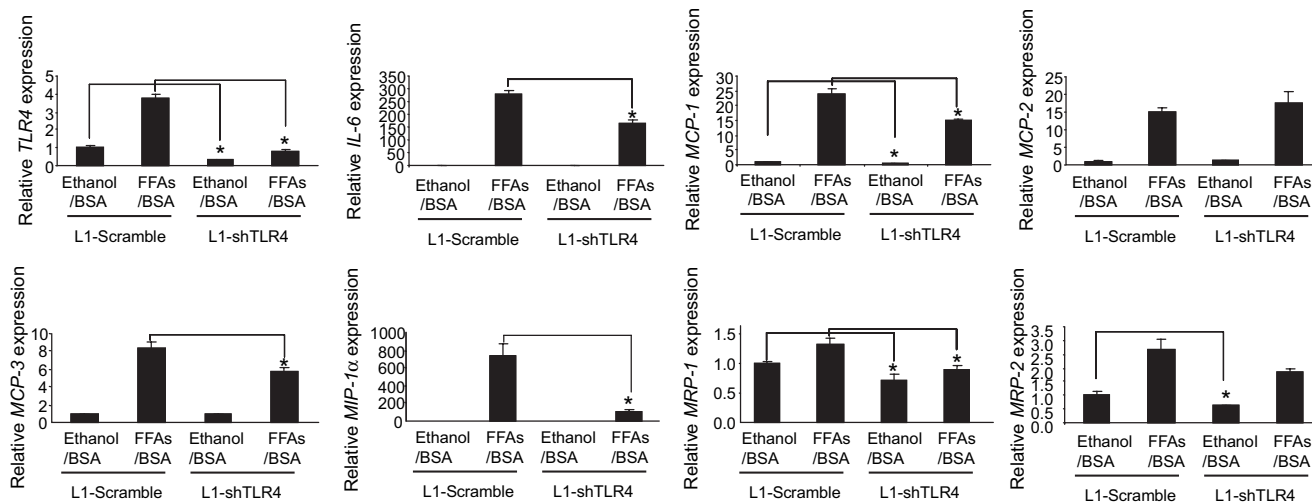


FIG. 4. TLR4 is involved in FFA-induced upregulation of chemokine expression. 3T3-L1 preadipocytes stably expressing shRNA against TLR4 (L1-shTLR4) or scramble shRNA (L1-scramble) were differentiated into adipocytes, treated with ethanol/BSA, or 0.5 mmol/l FFAs/BSA for 3 h after overnight incubation with serum-free medium before RNA extraction. For comparison, the expression level of chemokines in vehicle-treated 3T3-L1 adipocytes expressing scramble shRNA was arbitrarily set at 1. * $P < 0.05$, L1-shTLR4 vs. L1-scramble. Results shown here are representative of three independent experiments.

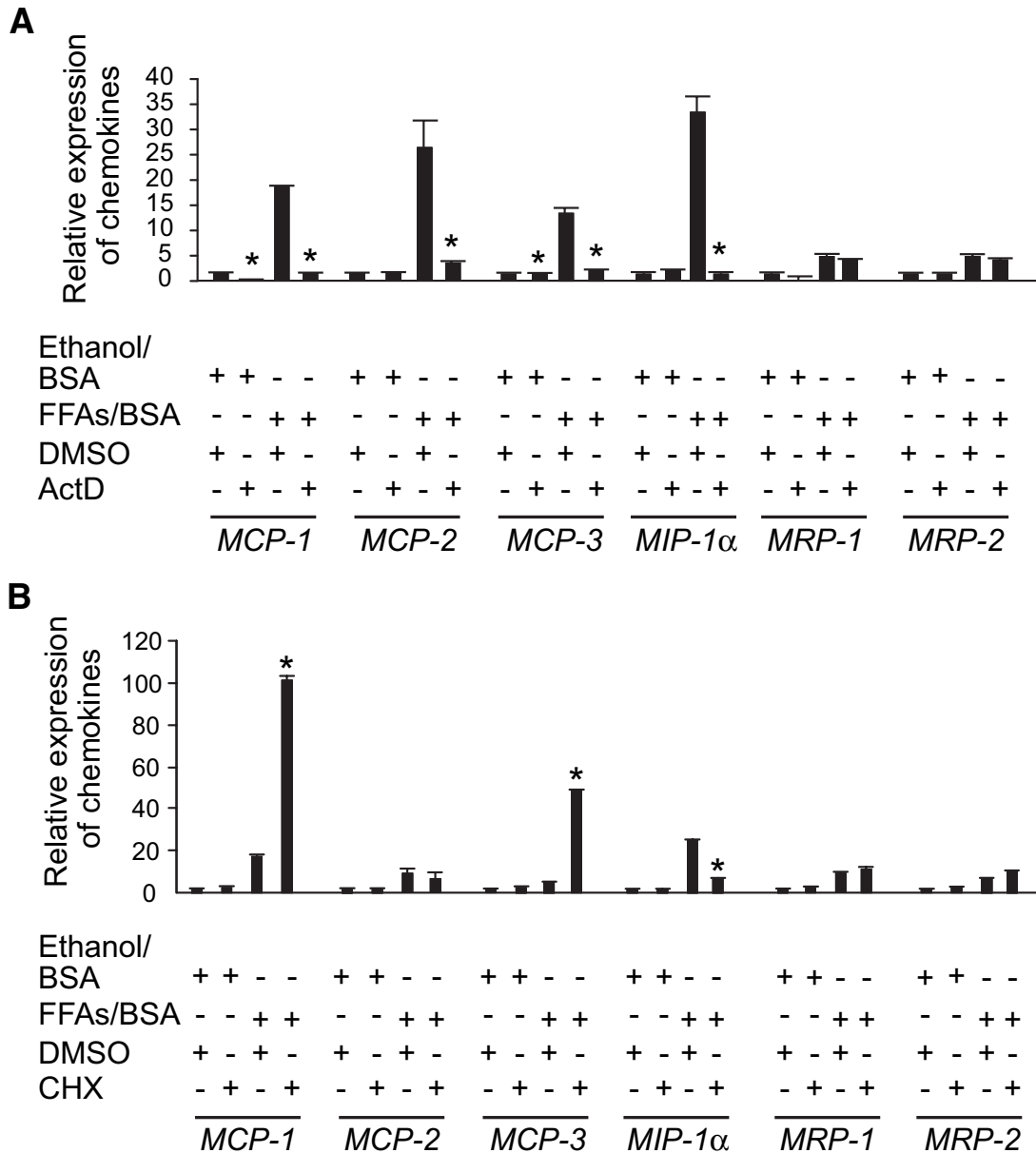


FIG. 5. Mechanism of FFA-induced upregulation of chemokines. **A:** FFA-induced upregulation of chemokine expression is both transcription dependent and independent. 3T3-L1 adipocytes were pretreated with 5 μ g/ml actinomycin D for 30 min before stimulation by 0.5 mmol/l FFAs for 3 h. **B:** Protein synthesis is only required for mediating FFA-induced MIP1 α expression. 3T3-L1 adipocytes were pretreated with 10 μ g/ml cycloheximide for 30 min before stimulation by 0.5 mmol/l FFAs for 3 h. For comparison, the expression level of chemokines in vehicle-treated 3T3-L1 adipocytes was arbitrarily set at 1. Veh, vehicle; ActD, actinomycin D; CHX, cycloheximide. Error bars represent \pm SE. * P < 0.05, treated vs. vehicle. Results shown here are representative of three independent experiments.

attenuation of insulin signaling (24,27). To investigate whether TLR4 plays a role in FFA-induced chemokine expression in adipocytes, we treated 3T3-L1 adipocytes stably expressing scramble shRNA or shTLR4 with FFAs. Consistent with a previous report, reduction of *TLR4* expression in adipocytes significantly reduced FFA-induced *IL-6* expression (Fig. 4). FFA-induced expression of *MCP-1*, *MCP-3*, *MIP-1 α* , *MRP-1*, and *MRP-2* was also reduced (Fig. 4). In addition, the basal levels of *MCP-1*, *MRP-1*, and *MRP-2* were also decreased (Fig. 4). However, TLR4 knockdown did not have any effect on *MCP-2* expression in either basal or FFA-stimulated condition. These results indicate that TLR4 is partially responsible for FFA-induced chemokine expression and that an alternative pathway(s) also exists.

Mechanism of FFA-induced chemokine expression.

To further explore the mechanism of FFA-induced chemokine expression in 3T3-L1 adipocytes, we tested whether the regulation is dependent on transcription and/or protein synthesis. The upregulation of *MCP-1*, *MCP-2*, *MCP-3*, and *MIP-1 α* mRNA in response to FFAs is transcription dependent because the effect can be blocked by actinomycin D treatment (Fig. 5A). In contrast, the upregulation of *MRP-1* and *MRP-2* mRNA is posttranscriptional (Fig. 5A). Only FFA-stimulated *MIP-1 α* expression requires protein synthesis because it is blocked by cycloheximide treatment (Fig. 5B). In contrast, blockage of protein synthesis significantly further increased expression of *MCP-1* and *MCP-3* (Fig. 5B).

FFAs have been reported to induce production of hydrogen peroxide in 3T3-L1 adipocytes (28). Despite the

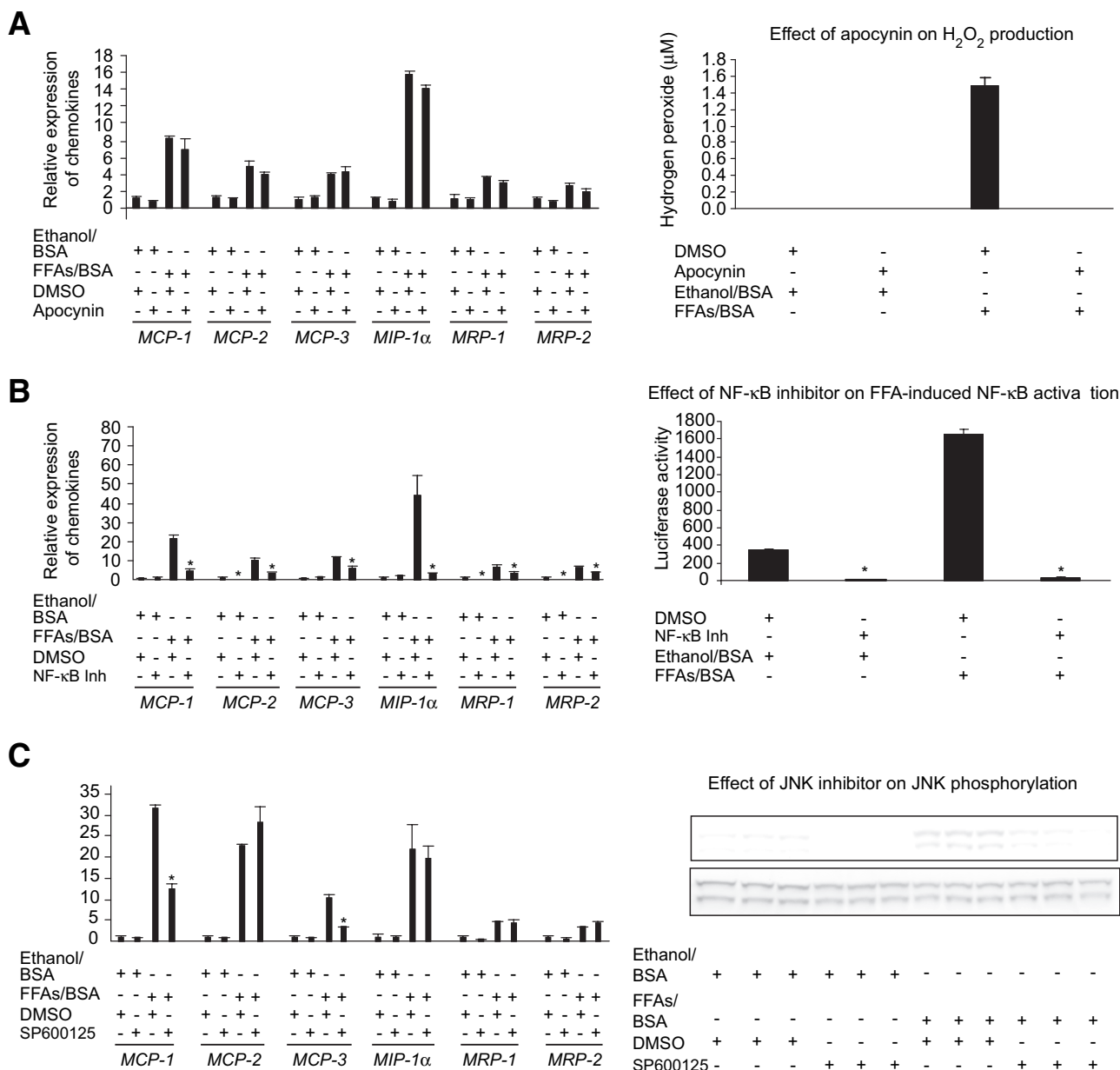


FIG. 6. Inflammatory pathways involved in FFA-induced chemokine expression. *A*: Oxidative stress is not required for mediating FFA-induced chemokine expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes were pretreated with 500 μmol/l apocynin (NADPH inhibitor) for 1 h before stimulation by 0.5 mmol/l FFAs for 3 h. *B*: NF-κB pathway is involved in FFA-mediated upregulation of chemokine expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes were pretreated with 200 μmol/l NF-κB inhibitor for 1 h before stimulation by 0.5 mmol/l FFAs for 3 h. *C*: JNK is necessary for mediating FFA-induced expression of *MCP-1* and *MCP-3*, but not other chemokines, in 3T3-L1 adipocytes. 3T3-L1 adipocytes were pretreated with 250 μmol/l JNK inhibitor for 1 h before stimulation by 0.5 mmol/l FFAs for 3 h. For comparison, the expression level of chemokines in vehicle-treated 3T3-L1 adipocytes was arbitrarily set at 1. **P* < 0.05, treated vs. vehicle. NF-κB Inh, NF-κB inhibitor; SP600125, JNK inhibitor. Results shown here are representative of three independent experiments.

fact that high concentration of hydrogen peroxide (0.5 mmol/l) can mildly induce MCP-1 mRNA in 3T3-L1 adipocytes 24 h after treatment, we found that 0.5 mmol/l hydrogen peroxide did not significantly upregulate these six chemokines in 3T3-L1 adipocytes 3 h after treatment (data not shown). In addition, treatment with 500 μmol/l NADPH oxidase inhibitor apocynin could not repress FFA-induced chemokine production but did effectively abolish production of hydrogen peroxide caused by FFA treatment (Fig. 6A). FFAs are also known to activate both NF-κB and JNK pathways (29,30). Therefore, we tested the possibility of whether FFA-induced chemokine expression is due to activation of NF-κB and/or JNK pathways. NF-κB transcriptional activation inhibitor was used to treat

3T3-L1 adipocytes at 200 μmol/l, which inhibited FFA-induced NF-κB activation by 99.8% (Fig. 6B). Significant repression on FFA-induced chemokine expression by NF-κB inhibitor was observed for all six chemokines (Fig. 6B). JNK inhibitor II (SP600125) was applied to assess the role of JNK in FFA-induced expression of chemokines in 3T3-L1 adipocytes. FFA-induced expression of *MCP-1* and *MCP-3* was significantly reduced in SP600125-treated 3T3-L1 adipocytes (Fig. 6C). The efficacy of SP600125 in 3T3-L1 adipocytes was confirmed by reduction of JNK phosphorylation (Fig. 6C). To confirm the role of NF-κB in FFA-induced chemokine expression by a biological approach, we reduced the expression of IKKβ by RNA interference (Fig. 7A). Consistent with our results with

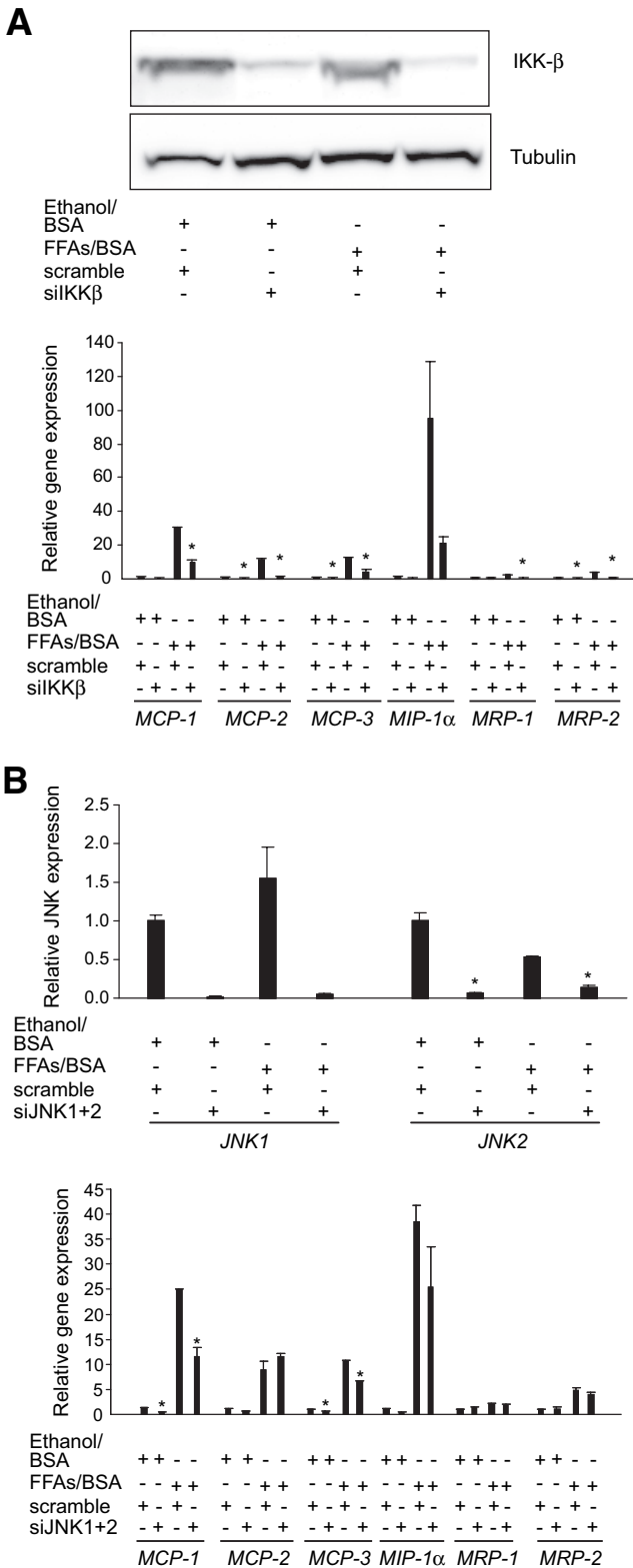


FIG. 7. Effect of IKKβ and JNK knockdown on FFA-induced upregulation of chemokines. *A: Top*, reduced IKKβ expression in 3T3-L1 adipocytes by RNA interference. 3T3-L1 adipocytes were electroporated with either scrambled siRNA or siRNA against IKKβ. Twenty-four hours after electroporation, adipocytes were incubated in serum-free DMEM overnight. Forty-eight hours after electroporation, adipocytes were treated with 0.5 mmol/l FFA/BSA or ethanol/BSA for 2 h and then harvested for examination of IKKβ expression. *Bottom*, expression of chemokines in 3T3-L1 adipocytes with IKKβ knockdown. Adipocytes treated as described in *A* were harvested for examination of chemokine expression by real-time PCR analysis. **P* < 0.05, scrambled vs. siIKKβ. *B: Top*, reduced JNK expression in 3T3-L1 adipocytes

NF-κB inhibitor, reduction of IKKβ expression in 3T3-L1 adipocytes by RNA interference also reduced FFA-induced upregulation of all six CC chemokines (Fig. 7A). The inhibitory effect of JNK inhibitor on FFA-induced MCP-1 and MCP-3 expression was also confirmed by RNA interference (Fig. 7B). In addition, overexpression of both wild type and the constitutively active form of IKKβ in 3T3-L1 CAR adipocytes via adenovirus-mediated gene transfer can significantly increase expression of MCP-1, MCP-2, MCP-3, MIP-1α, and MRP-2 (Fig. 8) but, surprisingly, not MRP-1 (data not shown).

Effects of rosiglitazone on expression of monocyte chemotactic factors in vitro and in vivo. Thiazolidinediones have been reported to have potent anti-inflammatory activity and suppress NF-κB activity in MNCs and lower plasma MCP-1 level (15,20). To address whether thiazolidinediones can repress FFA-induced expression of chemokines in adipocytes, 3T3-L1 adipocytes were treated with rosiglitazone for 24 h before stimulation with FFAs. Pretreatment with rosiglitazone significantly and dose dependently reduced expression of *MCP-1*, *MCP-3*, *MIP-1α*, *MRP-1*, and *MRP-2*, but not *MCP-2*, in 3T3-L1 adipocytes (supplementary Fig. 2, available in the online appendix). Obese mice treated with thiazolidinediones have been reported to have reduced expression of MCP-1, MCP-3, and MIP-1α but unchanged MCP-2 expression in adipose tissue; and obese humans treated with pioglitazone have reduced expression of MCP-1 in adipose tissue (4,9,31). However, potential regulation of MRP-1 and MRP-2 in adipose tissue by thiazolidinediones has never been examined. We treated *ob/ob* mice with rosiglitazone for 4 weeks and evaluated the expression of all six chemokines upregulated in obese fat. Consistent with previous reports, expression of *MCP-1*, *MCP-3*, and *MIP-1α* was repressed but *MCP-2* remains unchanged (Fig. 8B). Surprisingly, rosiglitazone can only reduce expression of *MRP-1* and *MRP-2* in vitro but not in vivo, suggesting that these two chemokines might be regulated by an additional mechanism in vivo compared with in vitro.

DISCUSSION

Obesity-related adipose tissue macrophage accumulation has been demonstrated to contribute to systemic insulin resistance (9–11). To understand the responsible chemotactic factors in addition to MCP-1, we systematically analyzed the expression and regulation of murine chemokines in adipose tissue of male *ob/ob* and DIO mice. Totally, five additional monocyte chemokines were found to be upregulated at both mRNA and protein level in WAT of *ob/ob* and DIO mice, indicating that macrophage extravasation into adipose tissue in the obese state might be very complicated. A recent publication showed that another monocyte chemokine MIP-2γ is upregulated at the mRNA level in WAT of both *ob/ob* and DIO mice, whereas we only detected mild upregulation in WAT of DIO but not *ob/ob* mice (32). The discrepancy is likely due to gender difference. In that report, MIP-2γ has been shown to play an important role in WAT macrophage infiltration in female but not male DIO mice (32). In our study, we only followed

by RNA interference. *Bottom*, expression of chemokines in 3T3-L1 adipocytes with JNK knockdown. For comparison, the expression level of chemokines in scrambled siRNA-electroporated and ethanol/BSA-treated 3T3-L1 adipocytes was arbitrarily set at 1. Results shown here are representative of three independent experiments.

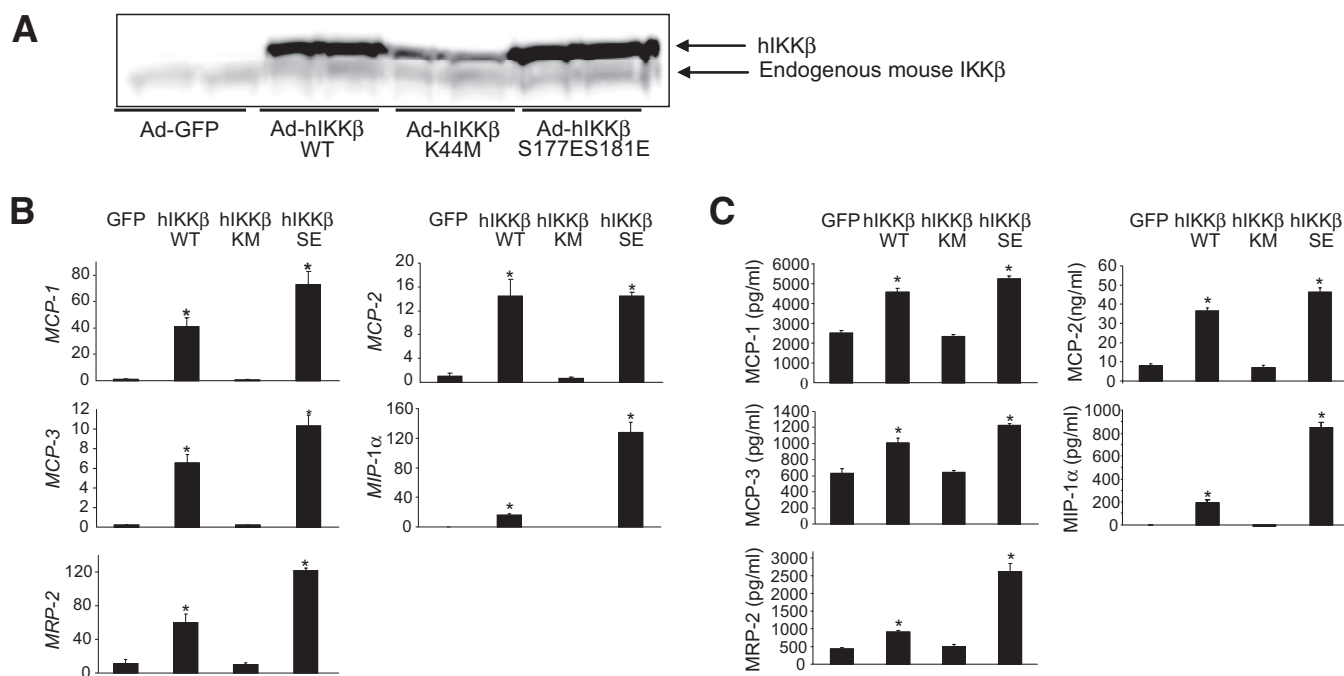


FIG. 8. Effect of IKK β overexpression on chemokine production. Wild-type, inactive, and constitutively active human IKK β (named hIKK WT, hIKK KM, and hIKK SE, respectively) were overexpressed in 3T3-L1 CAR cells. **A:** Overexpression of hIKK β in 3T3-L1 CAR cells at protein level. **B:** Expression of chemokine genes in 3T3-L1 CAR adipocytes overexpressing hIKK β . Fully differentiated 3T3-L1 CAR adipocytes were infected with adenoviruses overexpressing green fluorescent protein (GFP), hIKK β WT, hIKK β KM, or hIKK β SE at the dose of 1×10^9 plaque-forming units/ml for 6 h. Cells were then incubated in fresh medium for 48 h. Forty-eight hours after infection, cells were incubated in serum-free medium overnight. Seventy-two hours after infection, cells were harvested for RNA extraction to examine expression of chemokine genes by real-time PCR analysis. **C:** Expression of chemokine protein in conditioned supernatant. Conditioned supernatant collected from the experiment described in **B** was used to measure secreted chemokine protein by ELISA. Error bars represent means \pm SE. Methods for construction of adenovirus constructs are in supplementary materials available in the online appendix. * $P < 0.05$, IKK β -overexpressing cells vs. GFP-overexpressing cells. Results shown here are representative of three independent experiments.

up on the six CC chemokines that are upregulated in WAT of both *ob/ob* and DIO male mice. Isolation of primary adipocytes proves that aforementioned chemokines are predominantly increased in adipocytes, supporting the hypothesis that adipocyte-derived chemokines might play important roles in initial macrophage infiltration. Acute high-fat diet study showed rapid increase of MCP-1, MCP-2, MCP-3, and MRP-2 in fat within a week, at which time point circulating levels of these chemokines did not change. Our kinetic study with DIO mice indicates that fat mass enlargement precedes increase of macrophage content and further supports that adipocytes may play an important role in initiating macrophage infiltration by secreting chemokines.

Elevated level of FFAs, a well-known factor contributing to systemic insulin resistance and inflammation in MNCs in obesity (8), was found to potently induce expression of monocyte chemotactic factors in cultured 3T3-L1 adipocytes, accompanied by increased protein secretion into conditioned medium. FFA-stimulated expression of monocyte chemotactic factors is dose and time dependent, and unsaturated fatty acids are mainly responsible for this effect. The extent and duration of chemokine induction by FFAs in 3T3-L1 adipocytes vary among chemokines. Because these chemokines start to elevate in adipose tissue at different time points on high-fat diet, it is likely that they play unequal roles in attracting monocytes. Why so many monocyte chemotactic factors increase in the obese state is not clear. Genetic models deficient in these chemokines individually or in combination will provide more information regarding their relative importance for adipose tissue macrophage attraction. In addition to potential macrophage recruiting capability,

whether these chemokines have other biological functions, such as impairing insulin sensitivity in insulin target cells, like MCP-1 (7), remains to be investigated.

Interestingly, not all of the upregulated chemokines are due to the actual increase of mRNA. In the cases of MRP-1 and MRP-2, the mRNA levels decreased rapidly in vehicle-treated 3T3-L1 adipocytes, whereas delayed reduction of mRNA was observed in FFA-treated cells, suggesting that FFAs might protect mRNA stability of MRP-1 and MRP-2. For MCP-1, MCP-2, MCP-3, and MIP-1 α , mRNA levels were induced by FFA treatment via a transcription-dependent mechanism. TLR4 has been recently demonstrated to be an important receptor for mediating the effects of FFAs on activation of inflammatory pathways. We found that TLR4 is also partially responsible for FFA-induced upregulation of MCP-1, MCP-3, MIP-1 α , MRP-1, and MRP-2. FFAs are known to activate both NF- κ B and JNK pathways, the important intracellular pathways that are activated by inflammatory stimuli. The IKK β /NF- κ B axis has been demonstrated to be the molecular target for the hypoglycemic actions of salicylates (33,34). IKK β selectively phosphorylates the I κ B protein inhibitor of NF- κ B, which triggers degradation of I κ B and releases NF- κ B for translocation into the nucleus to transcribe many target genes that are related to insulin resistance (35). By application of the NF- κ B activation inhibitor, reduction of IKK β expression via RNA interference, and IKK β overexpression, we showed that the IKK/NF- κ B pathway has a broad effect on FFA-induced chemokine expression. JNK is a stress kinase that is involved in insulin resistance (36–38). Animals deficient in JNK-1 are protected from developing insulin resistance on a high-fat diet (39). In addition, JNK has also been demonstrated to be a major contributor to FFA-

induced cellular insulin resistance using 3T3-L1 adipocytes as a model system (29). In our study, inhibition of the JNK pathway partially reduced FFA-induced upregulation of MCP-1 and MCP-3. Because upregulation of MCP-1 and MCP-3 by FFA treatment is due to increased transcription, JNK-involved chemokine expression is most likely through activation of c-Jun. AP-1 sites have been reported to exist in the promoters of MCP-1 and MCP-3. Our data provide further evidence to demonstrate that JNK is not only important for mediating FFA-induced insulin resistance but also involved in FFA-induced expression of monocyte chemotactic factors in adipocytes.

Among the current therapeutic agents for treating type 2 diabetes, thiazolidinediones mainly improve adipose insulin sensitivity. Existing evidence indicates that thiazolidinediones have a potent anti-inflammation effect through repressing NF- κ B activity at least in MNCs, suggesting that improved insulin sensitivity might be partially through its anti-inflammatory effect (15,20,22). Rosiglitazone not only significantly reduced MCP-1, MCP-3, and MIP-1 α expression in adipose tissue, it can also repress FFA-induced expression of the aforementioned chemokines around the peak expression time in adipocytes. These results suggest that the anti-inflammatory effect of thiazolidinediones is likely through both macrophages and adipocytes. Surprisingly, rosiglitazone only represses FFA-induced expression of MRP-1 and MRP-2 in vitro but not in vivo. Whether expression of MRP-1 and MRP-2 in vivo is under a different control mechanism compared with in vitro remains to be studied. Upregulation of multiple monocyte chemotactic factors via different mechanisms in obesity may render it difficult to completely deplete adipose macrophages through targeting an individual chemokine or receptor. It is noteworthy to point out that improvement of systemic insulin resistance has been observed in MCP-1, MIP-2 γ , and CCR2-deficient mice and in mice treated with a CCR2 antagonist, although adipose tissue macrophage content was only partially reduced. These results not only demonstrate that adipose tissue-infiltrated macrophages play an important role for the development of obesity-related insulin resistance but also imply that reduction of adipose macrophage to a higher extent might be more beneficial.

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