Leptin Induces CD40 Expression through the Activation of Akt in Murine Dendritic Cells*

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Increasing evidence suggests a regulatory role for leptin, an adipocyte-derived hormone, in immunity. Although recent studies indicated an essential role of leptin signaling in dendritic cell (DC) maturation, the molecular mechanisms by which leptin modulates DC functional maturation remained unclear. In this study, we showed that leptin induced CD40 expression in murine DC and significantly up-regulated their immunostimulatory function in driving T cell proliferation. Moreover, leptin markedly enhanced lipopolysaccharide-mediated DC activation. Using pharmacological inhibitors for Akt, STAT-1, and NF-κB and the dominant negative forms of Akt and IκB kinase α/β/γ, as well as small interfering RNA for STAT-1α, we showed that Akt, STAT-1α, and NF-κB were important for the leptin- or lipopolysaccharide-induced CD40 expression. Coimmunoprecipitation analysis revealed that leptin promoted immune complex formation between Akt and the IκB kinase subunits as well as STAT-1α. Blocking the activity of Akt demonstrated a crucial role for Akt in translocation of STAT-1α and NF-κB to the nucleus and activation of the CD40 promoter. Further analysis with chromatin immunoprecipitation assay confirmed that leptin recruited STAT-1α, NF-κBp65, and RNA polymerase II to the CD40 promoter and enhanced histone 4 acetylation in a time-dependent manner. Thus, our results have elucidated the molecular mechanisms underlying leptin-induced CD40 expression and DC maturation.

Leptin, encoded by the obes (ob) gene, is an adipocyte-derived hormone that has long been recognized as a satiety factor in regulating nutrient intake and energy homeostasis (1). Leptin is structurally related to members of the long chain helical cytokine family such as IL-6, IL-11, IL-12, and granulocyte colony-stimulating factor. The leptin receptor (Ob-R), a member of class I cytokine receptors, is encoded by the diabetes (db) gene and is expressed in peripheral B cells, T cells, NK cells, and monocyte macrophages (2–6). Leptin has been increasingly recognized as a cytokine-like hormone with pleiotropic actions in modulating immune responses (7). It has been shown that leptin can modulate the adaptive immunity via enhancing T cell survival and stimulating their production of pro-inflammatory cytokines such as interferon γ and IL-2 (8). Moreover, leptin activates NK cells and monocyte macrophages and enhances their immune functions and cytokine production (3, 9). Recent studies indicate that leptin promotes differentiation and survival of human DC (10). We also demonstrated the expression of functional leptin receptor on murine DC and revealed significantly impaired maturation and survival of bone marrow (BM)-derived DC in leptin-receptor deficient db/db mice (11), but the underlying molecular mechanisms by which leptin regulates DC maturation and function remained largely unclear.

DC are the most potent antigen-presenting cells and undergo maturation process by up-regulating the expression of MHC and costimulatory molecules and become phenotypically and functionally differentiated. DC maturation can be induced by multiple stimuli such as pathogen-related molecules and cytokines in the local environment and T cell-derived signaling molecules such as CD40L (12). CD40 functions as a receptor for a variety of ligands including CD40L and heat shock proteins and is crucial for the concerted actions of DC in both innate and adaptive immunity. aberrant CD40 expression has been found in a variety of autoimmune and inflammatory diseases (13, 14). Therefore, modulation of CD40 expression can have profound effects on the induction of immunity or tolerance by DC. The maturation of DC is also regulated by innate immune signals via TLR. LPS is known for its ability to induce CD40 expression on antigen-presenting cells including DC and acts as an immune adjuvant that induces a variety of genes involved in metabolic and inflammatory responses (15). We recently showed that DC from db/db mice displayed markedly reduced levels of CD40 expression (11). However, the underlying signal transduction pathways by which leptin affects CD40 expression in DC remained unknown. In this study, we revealed a previously unrecognized role for leptin in inducing CD40 expression in murine DC either alone or in cooperation with LPS, a process mediated through the activation of regulated kinase kinase; DN, dominant negative; IKK, IκB kinase; CA, constitutively active; PE, phycoerythin.
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of Akt and subsequently the downstream transcription activators, STAT-1α and NF-κBp65, to target the CD40 promoter. We also identified a crucial role for Akt in controlling leptin-induced recruitment of STAT-1α and NF-κBp65 to the CD40 promoter in DC. Thus, our findings provide new insights in understanding the molecular mechanisms by which leptin modulates the maturation and function of murine DC.

EXPERIMENTAL PROCEDURES

Animals—Male C57BL/6 and BALB/c wild type (WT) mice between 4 and 5 weeks old were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free animal facility at the University of Hong Kong under the institutional guidelines for the usage of experimental animals.

Dendritic Cell Culture—DC were generated from BM cell suspensions as previously described (11). BM cells were grown in RPMI 1640 complete medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) in the presence of 20 ng/ml of murine granulocyte-macrophage colony-stimulating factor and 10 ng/ml of murine IL-4 (Alexis Biochemical, San Diego, CA). Purity and differentiation of the day 7 BM-derived DC were determined by flow cytometric analysis. 

Reagents—LPS was reconstituted in phosphate-buffered saline without additive at 100 μg/ml stock (Sigma-Aldrich). Murine leptin (Peprotech, Rocky Hill, NJ) and leptin R/Fc chimera (Ob-R:Fc) (R & D Systems, Minneapolis, MN) were used. Recombinant human leptin (Elabscience, Houston, TX) was used in the experiments. Antibodies used in this study have been previously described (11). Briefly, sorting-purified CD11c+MHC II+ C57BLKS/J DC after treatment were cocultured with sorted splenic CD4+ T cells from BALB/c mice at different stimulator:responder cell ratios for 3 days. The mixed cells were then pulsed with [3H]thymidine (25 μCi/well) for the final 16 h of culture and analyzed in a Top Count scintillation counter (Beckman LS5801; PerkinElmer Life Sciences).

Constructs—pGL3-Basic Luciferase reporter plasmids and Renilla luciferase plasmids were from Promega (Madison, WI). The CD40 promoter luciferase reporter plasmid was constructed by inserting a 707-bp-long fragment (–662 to +45) of the CD40 promoter into the KpnI and BglII sites of pGL3-Basic vector. Constitutively active and dominant negative plasmids of Akt contain a consensus Src myristoylation signal sequence and a point mutation at 179 from lysine to methionine, respectively. All of the Akt constructs including the full-length WT Akt are based in the pCR3.1 plasmids and have been previously described (17). The full-length IKK-α and -β and their dominant negative mutants with point mutation are based in pHA plasmids as previously described (20). All of the Akt and IKK proteins expressed by the constructs are HA-tagged. siRNA for STAT-1α and its scrambled control were from Ambion, Inc (Austin, TX). The sense sequences of the siRNA duplex are: STAT-1α siRNA 1, ACA CAG UUA AAG GAU UUC Gtt; control siRNA 1, GAA CAU AGC AAU AGU UUC Gtt; STAT-1α siRNA 2, UCU CUC CUU CUU CCU GAA Ctt; and control siRNA 1, ACCU CCU CUC UCU CUG AAC tt.

Transient Transfection and Luciferase Assays—Luciferase assays were performed as previously described (21). Briefly, DC2.4 cells in 6-well plates were transfected with 0.2 μg of CD40-luc reporter plasmid and 20 ng of Renilla-luc plasmid using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen) (22). For cotransfections with Akt and IKK expression constructs, the difference in amount of total DNA being transfected was normalized by the corresponding empty vectors. 4 h after transfection cells were trypsinized, divided into four 24-well plates and rested for 3 h before receiving treatment of LPS and leptin. After an additional 24 h, cell lysates were prepared for luciferase assays using a luciferase reporter assay system (Promega) according to the manufacturer’s manual. The relative luciferase activities were determined by quantitation of luminescent signal from the firefly luciferase normalized by Renilla luciferase.

Coimmunoprecipitation and Immunoblotting—For coimmunoprecipitation, equal amount of proteins from treated BMDC were immunoprecipitated with anti-Akt or anti-normal.
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medium; thin gray lines, leptin + Ob-R:Fc; black thin lines, leptin; black thick lines, LPS; black dotted lines, leptin + LPS. C, BMDC were treated with different amounts of leptin from 0 to 1600 ng/ml in the presence of LPS (400 ng/ml). Average MFI ± S.E for CD40 as analyzed by flow cytometry were summarized from three independent experiments. Open bars, medium; closed bars, LPS. D, RNA samples from BMDC were harvested 3 h after treatment, and cDNA were synthesized for quantitative PCRs. Average CD40 expression after normalization to corresponding β-actin values was presented as fold induction ± S.E. The data were summarized from three independent experiments. Open bars, medium; closed bars, LPS. E and F, DC2.4 cells were treated with 800 ng/ml leptin, 800 ng/ml leptin + 5 μg/ml Ob-R:Fc, 400 ng/ml LPS, or both leptin and LPS at 400 ng/ml for 24 h and analyzed by flow cytometry. The representative histograms show the surface CD40 expression on the gated CD11c+/MHC II+ live cells. Gray thick lines, leptin + Ob-R:Fc; gray thin lines, leptin; black thick lines, medium; black pointed lines, leptin + LPS. G and H, DC2.4 cells were treated with different amounts of leptin from 0 to 1600 ng/ml in the presence of LPS (400 ng/ml). Average MFI ± S.E. for CD40 were summarized from four independent experiments. I, RNA samples from DC2.4 cells were harvested 3 h after treatment and processed for quantitative RT-PCRs. The data are presented as the average CD40 fold inductions ± S.E. in a similar manner to BMDC and summarized from four independent experiments. L, leptin-treated DC show enhanced immunostimulatory capacity on the proliferation of allogeneic responder T cells. Immature BMDC were treated for 24 h with 800 ng/ml of leptin, 800 ng/ml leptin + 5 μg/ml Ob-R:Fc, 400 ng/ml of LPS, or a combination of 400 ng/ml leptin and 400 ng/ml LPS. CD11c+/MHC II+ DC were cocultured with allogeneic responder T cells for 3 days. T cell proliferation was determined by 3H incorporation assay. The data are the mean values of one representative set of triplicate samples for three experiments (means ± S.E., * p < 0.05; **, p < 0.01; *** p < 0.001).
tion, DNA was purified using spin columns and analyzed by PCR (34 cycles) with the primer pairs used to amplify a 250-bp region in the mouse CD40 promoter encompassing the NF-κB and IFN-γ activated site elements as described (23).

Statistical Analysis—The data are presented as the means ± S.E. The means were compared with Student’s t test and analysis of variance where appropriate for statistical analysis. p values of <0.05 were considered statistically significant.
RESULTS

Leptin Induces CD40 Expression and Enhances DC Immunostimulatory Function—To investigate the role of leptin in modulating DC function, BMDC and DC2.4 cells were treated with recombinant murine leptin in the absence and in the presence of LPS before analysis by flow cytometry. Leptin significantly induced CD40 expression in a dose-dependent manner either alone or synergistically with LPS in BMDC (Fig. 1, A–C). Moreover, the presence of Ob-R:Fc, a soluble leptin blocker, abolished the effects of leptin on CD40 induction in DC. Quantitative RT-PCR analysis confirmed that leptin markedly increased CD40 gene expression up to ~10-fold, whereas in the presence of LPS, leptin up-regulated CD40 expression progressively with escalating concentrations (Fig. 1D). DC2.4 cells, a DC cell line, were also analyzed, and similar results were obtained (Fig. 1, E–H). These findings indicate that leptin can directly induce CD40 expression and synergizes with LPS to profoundly increase CD40 expression in DC.

To explore the functional implication of the leptin effect on CD40 induction, sorting-purified splenic CD11c<sup>+</sup> MHCI<sup>+</sup> DC were treated with leptin, LPS, or both for 24 h before incubation with naïve T cells for 3 days. As shown in Fig. 1I, leptin-treated DC significantly enhanced T cell proliferation compared with the untreated control DC. Moreover, DC treated with leptin plus LPS stimulated T cell proliferation more robustly than DC treated with either leptin or LPS alone. The addition of Ob-R:Fc abolished the effects of leptin on DC. Thus, these results clearly demonstrate that leptin can enhance the immunostimulatory function of DC.

Leptin Activates Multiple Intracellular Signal Transduction Pathways in DC—To investigate signal transduction pathways involved in leptin-induced CD40 expression in DC, we first assessed the phosphorylation of Janus kinase (JAK) 2, a key member of the JAK family that binds the leptin receptor. As shown in Fig. 2A, treatment of BMDC with leptin activated JAK2 in 7.5 min, and LPS triggered a marked increase in JAK2 phosphorylation more rapidly. DC pretreated with Ob-R:Fc before incubation with leptin abolished JAK2 phosphorylation. These results indicate that leptin can trigger intracellular signal transduction pathway(s) via its receptor in DC.

CD40 expression can be potentially regulated by STAT-1α and NF-κB in DC based on the presence of multiple STAT-1α-and NF-κB-binding elements in the CD40 promoter. In a variety of cell types including monocytes, both leptin and LPS have been shown to activate Akt (24). Moreover, leptin activates gene expression via MEK1/2 in monocytes and in the Ob-R-transfected BaF3 cells (24). To determine leptin-induced signaling pathways that can possibly stimulate CD40 expression in DC, we first evaluated whether leptin affected the activation of different transducers including Akt, NF-κB, STAT-1α, and MEK1/2 by Western blotting using antibodies against their phosphorylated forms. Leptin alone activated all these kinases (Fig. 2B). As such, leptin activated Akt at serine 473 rapidly (at 0.5 h), and the Akt activity was sustained throughout the time course. A greater activation of Akt occurred when DC were simultaneously stimulated with leptin and LPS. Moreover, mTOR, a downstream target of the phosphoinositide 3-kinase/Akt pathway, was markedly activated by either stimulant. Activation of the NF-κB pathway was evident by the enhanced phosphorylation of the negative regulator IκB-α in response to leptin treatment. Both leptin and LPS could stimulate nuclear translocation of NF-κBp65 at 0.5 h, and the peak levels occurred at 1 h after treatment. Leptin stimulation triggered nuclear translocation of phosphorylated STAT-1α as early as 0.5 h, whereas leptin plus LPS treatment showed more potent activity. Similarly, the MEK1/2 pathway was activated in a time-dependent manner in response to leptin treatment. These data show that leptin activates Akt, NF-κB, STAT-1α, and MEK1/2 pathways in BMDC.

To confirm that leptin enhanced the LPS-induced phosphorylation of the kinases, we analyzed activation of Akt, STAT-1α, NF-κB, and MEK1/2 by combined treatment of leptin and LPS in BMDC. Levels of phosphorylated Akt at serine 473 progressively increased with escalating doses of leptin (Fig. 2C). In addition, the combination of a higher dose of leptin than that in Fig. 2B with LPS induced Akt activation more rapidly than LPS alone especially in the case of phosphorylated Akt at threonine 308, where leptin triggered its phosphorylation at 0.5 h, whereas LPS alone showed an activity at 2 h. Similarly, treatment with both leptin and LPS activated the NF-κB pathway, as indicated by increased phosphorylation of IκB-α and its degradation at 2 h. Leptin plus LPS also stimulated nuclear translocation of NF-κBp65 and phosphorylated STAT-1α more potently and rapidly than LPS alone. Finally, leptin showed a similar enhancing effect on LPS-stimulated MEK1/2 phosphorylation. Taken together, these results indicate that leptin can enhance LPS-induced activation of the Akt, STAT-1α, NF-κB, and MEK1/2 pathways in BMDC.

FIGURE 2. Leptin activates multiple intracellular signal transduction pathways in DC. A, BMDC were serum-starved for 3 h before incubation in the absence or presence of 400 ng/ml recombinant murine leptin, 400 ng/ml of LPS or 400 ng/ml of leptin plus 400 ng/ml of LPS at the indicated time intervals. 5 μg/ml of Ob-R:Fc was used to pretreat DC for 30 min before incubation with 400 ng/ml of leptin. Protein lysates subjected to immunoblotting with anti-phospho-JAK2 (Tyr<sup>1007/1008</sup>). The same blot was stripped and reprobed with anti-JAK2. GAPDH protein detection was used as a loading control. B, starved BMDC were treated in the same manner for the indicated time intervals. Total protein, nuclear, and cytoplasmic extracts were harvested and subjected to immunoblotting as indicated using the following antibodies: anti-phospho-Akt (Ser<sup>473</sup>), anti-Akt, anti-phospho-mTOR (Ser<sup>2448</sup>), anti-phospho-IκB-α (Ser<sup>32/36</sup>), anti-STAT-1α, anti-phospho-MEK1/2 (Ser<sup>27/28</sup>), anti-phospho-STAT-3 (Ser<sup>27/28</sup>), and anti-MEK1/2. The same blots were stripped and reprobed with anti-GAPDH for equal loading confirmation. Samples from DC of three different cultures were examined, and one set of representative results is shown. C, confirmation on the enhancing of leptin on LPS-induced phosphorylation. Starved BMDC were incubated with 400 ng/ml of LPS and treated with increasing doses of leptin as indicated for 0.5 h for the examination of Akt and 1 h for analysis of NF-κB. In a separate experiment, BMDC were treated with either 400 ng/ml of LPS or 400 ng/ml LPS together with a higher dose of leptin (1000 ng/ml) for the indicated time intervals for analysis of Akt, NF-κB, STAT-1α, and MEK1/2. Total, nuclear, and cytoplasmic extracts were harvested and subjected to immunoblotting as indicated with the following antibodies: anti-phospho-Akt (Ser<sup>473</sup>), anti-phospho-Akt (Thr<sup>308</sup>), anti-Akt, anti-phospho-mTOR (Ser<sup>2448</sup>), anti-mTOR, anti-phospho-IκB-α (Ser<sup>32/36</sup>), anti-IκB-α, anti-NF-κBp65, anti-phospho-STAT-1α (Ser<sup>72/110</sup>), anti-phospho-MEK1/2 (Ser<sup>27/28</sup>), anti-phospho-STAT-3 (Ser<sup>27/28</sup>), anti-STAT-1α, anti-phospho-MEK1/2 (Ser<sup>27/28</sup>), and anti-MEK1/2. The same blots were stripped and reprobed with anti-GAPDH for equal loading confirmation. Samples from DC of three different cultures were examined, and one set of representative results is shown.
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Akt, NF-κB, and STAT-1α but Not MEK1/2 Activity Are Required for Leptin-mediated CD40 Induction in DC—Next, we employed specific pharmacological inhibitors to identify the relevant kinase activation required for CD40 induction. Flow cytometric analysis of viable DC revealed that treatment with LY294002, pyrrolidine dithiocarbamate, and fludarabine abolished CD40 induction by leptin and/or LPS in a dose-dependent manner (Fig. 3A). In contrast, U0126, a MEK1/2 inhibitor, did not affect CD40 expression. The results were further confirmed at the transcriptional level by quantitative RT-PCR (Fig. 3B), suggesting that Akt, NF-κB, and STAT-1α but not MEK1/2 pathways are required for CD40 induction by leptin and/or LPS in DC.

To further verify the involvement of Akt, NF-κB, and STAT-1α during leptin-induced CD40 expression, we individually expressed dominant negative (DN) versions of Akt and IKK subunits, IKK-α, IKK-β, and IKK-γ, as well as two siRNAs for STAT-1α in DC2.4 cell line, and the expressions were confirmed by immunoblotting (Fig. 3C). The DN forms of Akt, IKK-α, and IKK-β contain a point mutation in the catalytic ATP-binding domain, whereas the IKK-γ DN construct is a truncated mutant (17–19). Transfected cells were treated with leptin and/or LPS, and CD40 surface expression was analyzed by flow cytometry. Transfection with the empty vector pCR3.1 or expression construct for WT Akt did not influence surface expression of CD40 by leptin and/or LPS (Fig. 3D). However, expression of DN Akt almost completely abolished leptin- and MEK1/2 pathways were assessed in BMDC using increasing doses of inhibitors for the respective pathways: LY294002, pyrrolidine dithiocarbamate, fludarabine, and U0126. BMDC were pretreated with the inhibitors for 3 h before stimulation with 800 ng/ml of leptin, 400 ng/ml of LPS, or a combination of 400 ng/ml leptin and 400 ng/ml LPS. After 24 h, CD40 expression was analyzed on the CD11c+ MHC II+ gated live cells by flow cytometry. The representative line graphs show the average relative MFI ± S.E. of CD40 expression in treated DC normalized to MFI of control untreated DC. Black lines, leptin; dotted lines, LPS; gray lines, leptin + LPS. BMDC pretreated with inhibitors for 3 h were stimulated for 3 h as shown before RNA preparation for quantitative RT-PCRs. CD40 expression relative to untreated DC after normalization to corresponding β-actin values was presented as the averages ± S.E. The data were summarized from three independent experiments. Open bars, leptin; black bars, LPS; gray bars, leptin and LPS. C, the expression of DN proteins (500 ng) and the knockdown effect of STAT-1α siRNAs were assessed by Western blotting. The knockdown efficiency of STAT-1α siRNA 1 and 2 transfected at 20 nM was assessed by levels of detectable STAT-1α proteins. Control siRNA 1 and 2 were scrambled control oligonucleotides for STAT-1α siRNA 1 and 2, respectively. D, DC2.4 cells seeded into six-well plates were transfected with 500 ng of either the empty vector pCR3.1 or the expression vector for WT Akt or DN Akt. At 24 h post-transfection, 800 ng/ml of leptin, 400 ng/ml of LPS, or a combination of 400 ng/ml leptin and 400 ng/ml LPS was added for 24 h before cytometric analysis. Representative histograms are shown. Gray lines, medium; thin black lines, leptin; thick black lines, LPS; dotted lines, leptin + LPS. E, DC2.4 cells were transfected with either STAT-1α siRNAs or their control siRNAs for 24 h, treated, and analyzed in the same manner as described for D. F, DC2.4 cells were transfected with the indicated WT or DN IKK-α, IKK-β, or IKK-γ constructs (500 ng), as well as different combinations of DN IKK plasmids. The respective empty vectors (pRC-β-actin for WT and DN IKK-α and IKK-β, and pHA for WT and DN IKK-γ) were used as controls. Differences in the amount of DNA were adjusted with the corresponding empty vectors. The transfected DC were treated and analyzed in the same manner as described for D and E. G, DC2.4 cells were transfected and treated in the same manner as described for E. H, DC2.4 cells were transfected and treated as described for F. The data were summarized from three independent experiments (means ± S.E.; *, p < 0.05; **, p < 0.01; ***, p < 0.001).

FIGURE 3. Akt, NF-κB and STAT-1α but not MEK1/2 activity are required for CD40 induction by leptin in BMDC and DC2.4 cells. A, the effects on leptin-induced CD40 expression by blocking each of the Akt, STAT-1α, NF-κB,
and/or LPS-induced CD40 expression. Although siRNAs for STAT-1α markedly suppressed the CD40 expression induced by leptin and/or LPS, transfection with the scrambled control siRNAs had no effects (Fig. 3E). Moreover, expression of WT IKK-α, -β, and -γ did not affect leptin- and/or LPS-induced CD40 expression on the surface (Fig. 3F). In contrast, expression of any of DN IKK-α, DN IKK-β, or DN IKK-γ suppressed leptin- and/or LPS-induced CD40 expression down to the levels of the untreated control. Furthermore, concurrent expression of two or three of the DN IKK proteins completely abolished the surface expression of CD40. To further verify the effects of transfection with the various DN constructs, CD40 gene expression was analyzed by quantitative RT-PCR. Similar to the flow cytometric results, transfection with the empty vector pCR3.1 or expression construct for WT Akt did not influence CD40 induction by leptin and/or LPS, whereas the DN Akt expression construct completely abolished CD40 expression (Fig. 3G). Transfection with siRNAs for STAT-1α markedly suppressed the CD40 expression induced by leptin and/or LPS (Fig. 3H). Moreover, expression of WT IKK-α and β did not affect leptin- and/or LPS-induced CD40 expression (Fig. 3I). In contrast, DN IKK-α suppressed leptin- and/or LPS-induced CD40 expression down to the levels of the untreated control, whereas DN IKK-β displayed even greater suppressive activity. When DN IKK-γ was expressed, both basal levels of CD40 expression in untreated cells and the induced CD40 expression in the treated samples were severely suppressed. Furthermore, concurrent expression of two or three of the DN IKK proteins completely abolished the expression of CD40. These results demonstrate that activation of Akt, NF-κB, and STAT-1α is critically involved in leptin- and/or LPS-induced CD40 expression in DC.

**Akt Is Required for Activation of STAT-1α and NF-κB in Response to Leptin and LPS in DC**—We next performed coimmunoprecipitation to test whether Akt interacts with the IKK complex and STAT-1α in BMDC stimulated with leptin and/or LPS. As shown in Fig. 4A, Akt formed a complex with mTOR in the untreated DC, but leptin significantly enhanced the amount of coprecipitate. Importantly, when DC were stimulated with both leptin and LPS, Akt coprecipitated with the greatest amount of mTOR. In addition, leptin induced complex formation between Akt and STAT-1α. To determine whether treatment with both leptin and LPS could increase the active form of STAT-1α in the complex associated with Akt, we stripped and reprobed the blots with antibodies against the phosphorylated STAT-1α at serine 727. As shown in Fig. 4A, there was an enhanced level of activated STAT-1α that had been coprecipitated with Akt in leptin-treated DC. Notably, the addition of leptin to LPS-treated DC further up-regulated the complex formation. Furthermore, leptin up-regulated the levels of complex formation between Akt and each of the IKK subunits, and substantially increased complex formation was detected in DC treated with both leptin and LPS. These findings that Akt interacts with IKK as well as STAT-1α suggest a functional regulation between these molecules in DC in response to leptin stimulation.

To ascertain the role of Akt in leptin signaling, we used constitutively active (CA) and DN constructs of Akt and transfect them separately into DC2.4 cells prior to treatment with leptin, LPS, or a combination of the two stimulants. As shown in Fig. 4B, Akt-CA transfection enhanced phosphorylated Akt levels in either the untreated or treated DC compared with empty vector controls, whereas Akt-DN transfection down-regulated the phosphorylated Akt levels. Transfection of the Akt DN construct completely inhibited the leptin- and/or LPS-induced phosphorylation of mTOR at serine 2448, the activation site that was previously shown to be critical for STAT-1α activation (25). Moreover, we found that expression of Akt-CA enhanced nuclear translocation of phosphorylated STAT-1α, whereas

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**FIGURE 4.** Akt is required for activation of STAT-1α and NF-κB pathways by leptin in DC. A, a coimmunoprecipitation assay was performed to test whether Akt forms a complex with the IKK complex and STAT-1α in BMDC. Three hours after treatment with 5 μg/ml of leptin, 0.5 μg/ml of LPS, and 0.5 μg/ml leptin plus 0.5 μg/ml LPS for 1 h before immunoprecipitation, total protein lysates were used for immunoblotting. Western blotting was performed on the anti-Akt immunoprecipitates using anti-mTOR, anti-STAT-1α, and anti-IKK-α antibodies. Total protein lysates were probed with anti-phospho-Akt (Ser473), anti-phospho-STAT-1α (Ser727) and anti-NF-κBp65 (nuclear), and anti-phospho-IKK-α and anti-IKK-β and anti-IKK-γ. For detection of phosphorylated STAT-1α, 1A-α-probed blot was stripped and reprobed with anti-phospho-STAT-1α. As controls, anti-Akt antibodies were used to detect Akt protein in the immunoprecipitates. A representative blot of three similar sets of results is shown. B, DC2.4 cells were transfected with 0.5 μg of each of the empty vector pCR3.1, CA Akt construct, and DN Akt construct for 24 h prior to treatment with 0.5 μg/ml of leptin, 0.5 μg/ml of LPS, and 0.5 μg/ml LPS plus 0.5 μg/ml LPS. Total and nuclear protein extracts were collected after 1 h and subjected to Western blot analysis. The blots transferred with total proteins were probed with anti-phospho-Akt (Ser473), anti-phospho-mTOR (Ser2448), anti-CD40, and anti-HA. The blots transferred with nuclear extracts were probed with phospho-STAT-1α (Ser727) and anti-NF-κBp65. Blots probed with anti-GAPDH were used as loading control. A representative blot of two similar experiments is shown. IP, immunoprecipitation; IB, immunoblot.
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A

CD40 promoter

Luciferase

RLA

Control siRNA #1
STAT-1α siRNA #1
Control siRNA #2
STAT-1α siRNA #2
Control siRNA #1
STAT-1α siRNA #1
Control siRNA #2
STAT-1α siRNA #2
Control siRNA #1
STAT-1α siRNA #1
Control siRNA #2
STAT-1α siRNA #2
Control siRNA #1
STAT-1α siRNA #1
Control siRNA #2
STAT-1α siRNA #2

pCR3.1
Akt-CA
Akt-DN

B

RLA

actin
IKK-α
IKK-β
IKK-γ
IKK-α
IKK-β
IKK-γ
IKK-α
IKK-β
IKK-γ
IKK-α
IKK-β
IKK-γ
IKK-α
IKK-β
IKK-γ

pCR3.1
Akt-CA

C

Leptin - + + + - - - - + + + +
LPS - - - - + + + + + + + +

0 1 2 3 1 2 3 1 2 3 (h)

STAT-1α
NF-κBp65
RNA Pol II
AcH4
Normal IgG
Input
Akt-DN transfection suppressed it down to the basal level. Treatment with leptin or LPS could not rescue the inhibiting effect of Akt-DN. Remarkably, the nuclear translocation for NF-κBp65 was completely abolished when Akt-DN was expressed, whereas Akt-CA could up-regulate it in both the absence and presence of stimulation. The critical involvement of Akt was further confirmed by the up-regulation of CD40 protein expression in the presence of Akt-CA construct and by the abolishment of CD40 expression when Akt-DN was present. Thus, these results demonstrate that Akt is critically required for nuclear translocation of activated STAT-1α and NF-κBp65, as well as CD40 induction in DC upon stimulation by leptin, LPS, or their combination and lend further support to the notion that Akt is a major controlling factor in the signaling cascade triggered by leptin.

Akt Mediates Leptin-induced Recruitment of STAT-1α and NF-κBp65 to the CD40 Promoter in DC—To determine whether leptin can activate the CD40 promoter, we cloned the CD40 promoter region, which encompasses multiple STAT-1α- and NF-κB-binding elements (23, 26), into the pGL3 Basic vector upstream of the luciferase (Luc) gene, yielding a CD40-luc reporter construct. As shown in Fig. 5 (A and B), leptin and LPS can activate the CD40 promoter both individually and synergistically. Although cotransfection with the CD40-luc reporter construct and the Akt-CA construct slightly up-regulated the luciferase activity, the Akt-DN construct completely abolished the luciferase activity induced by leptin and/or LPS. On the other hand, siRNA for STAT-1α suppressed CD40 promoter activation by any of the stimulants, which was not rescued by the expression of Akt-CA construct. As shown in Fig. 5B, each of the IKK-DN constructs inhibited the luciferase activity even in the presence of leptin and LPS. When each IKK-DN construct was cotransfected with Akt-CA expression vector, the luciferase activity remained inhibited. Taken together, these results demonstrate that leptin and LPS can activate the CD40 promoter either individually or synergistically and that each Akt, STAT-1α, and NF-κB is absolutely required for the transactivating capacity of leptin and/or LPS. To assess in vivo binding of the transcriptional activators, BMDC were incubated in the absence or presence of leptin and/or LPS for ChIP assay. After the addition of leptin and/or LPS, STAT-1α and NF-κBp65 were rapidly recruited to the CD40 promoter in a time-dependent manner (Fig. 5C). The combined treatment with leptin and LPS enhanced the binding of both STAT-1α and NF-κBp65 to the CD40 promoter. Moreover, we detected the recruitment of RNA Pol II to the CD40 promoter as early as 1 h, which peaked at 3 h after treatment with leptin or LPS alone. Similarly, leptin was able to enhance histone 4 acetylation in a time-dependent manner. These data show that leptin can recruit STAT-1α, NF-κBp65, and RNA Pol II to the CD40 promoter with epigenetic modification.

DISCUSSION

In this study, we demonstrate that leptin enhances CD40 expression and DC maturation synergistically with LPS. Moreover, we have elucidated the molecular mechanisms underlying leptin signaling and its synergistic action with LPS in enhancing CD40 expression and DC immunostimulatory function. We show that leptin-induced CD40 up-regulation is mediated by the activation of Akt, which controls the downstream transcription activators STAT-1α and NF-κBp65. Thus, our findings provide strong evidence of the molecular mechanism by which leptin enhances CD40 gene expression and signaling transductions in DC.

We previously showed that the microenvironment in lymphoid organs can modulate the functional differentiation of mature DC (12). As a protein hormone produced by the adipocytes, leptin has been increasingly recognized as a growth factor that modulates both immune and inflammatory responses (27, 28), but it was not until recently that the regulatory function of leptin in DC maturation and function was reported (10, 11). Recent studies have shown that leptin promotes DC differentiation and survival with polarized Th1 cytokine production (10). Our previous work also revealed an essential role of leptin signaling in DC maturation and costimulatory molecule expression, but the underlying molecular mechanisms remained largely unclear (11). In the present study, we show that leptin potently activates JAK2 in DC, which is specifically blocked by a soluble leptin blocker (Figs. 1 and 2A), suggesting that leptin functions via the leptin receptor expressed by DC (11). Leptin signaling involves the JAK-STAT pathway with the phosphorylated JAK2, and the cytoplasmic tyrosine residues serve as the binding sites for different transcription factors including the STAT proteins (24, 29). Recently, leptin has been shown to activate NF-κB in human DC, but the upstream signaling pathways and the promoter target remained unclear (10). Our current data demonstrate that both STAT-1α and NF-κB are critically involved in CD40 expression induced by either leptin or in combination with LPS in DC, consistent with previous findings that LPS treatment induces activation of STAT-1α and NF-κB in macrophages and microglia (23) (Fig. 6). Inter...
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Interestingly, the early findings that the nuclear translocation of STAT-1α exhibited a delayed kinetics compared with NF-κBp65 suggest that LPS-triggered IRF3 expression may induce interferon β production, which in turn activates STAT-1α in an autocrine manner (23). However, our present findings show highly similar kinetics of the nuclear translocation of STAT-1α and NF-κBp65 concurrently within the first 0.5–1 h, whereas JAK2 is rapidly activated within 7.5 min in DC in response to either leptin or LPS stimulation, indicating that both leptin and LPS can activate STAT-1α directly in DC (Fig. 2). Moreover, recent evidence of physical association of JAK2 with the TLR4-MyD88 complex further supports a direct activation of STAT-1α by LPS (30). Akt has been shown to regulate DC survival in innate and adaptive immune responses by modulating Bcl-2 levels (31–33). We previously revealed defective Akt activation and Bcl-2 expression along with increased apoptosis in DC from db/db mice (11). A recent study shows that Akt is required for LPS-mediated DC maturation (33). Here, we demonstrate that Akt is critical for CD40 induction by leptin and links both leptin receptor and TLR4 activation to the downstream transcription factors STAT-1α and NF-κBp65. Consistent with our data, previous studies have demonstrated that phosphoinositide 3-kinase/Akt and mTOR pathways converge to stimulate STAT-1α phosphorylation at serine 727 in response to LPS or interferon γ (25, 34). Because mTOR is a downstream target of Akt, and we detected complex formation between Akt and mTOR in DC treated with leptin, it remains possible that Akt activates STAT-1α via mTOR in response to leptin.

Levels of leptin rapidly increase during acute infection and sepsis because leptin expression is stimulated by LPS and inflammatory cytokines such as tumor necrosis factor-α, IL-6, and IL-1 (35). Moreover, leptin is implicated in autoimmune diseases such as type I diabetes and experimental arthritis (4, 36). Thus, our results may provide new insight in understanding the immunoregulatory role of leptin via Akt activation in DC maturation and function during infections and other inflammatory conditions. We have also identified a novel function for leptin in amplifying TLR4 signaling effects on DC maturation and function. CD40 is a key determinant for the activity of DC in immunity and tolerance. As a costimulatory molecule, CD40 is up-regulated during DC maturation to engage in the DC-T cell interaction. Thus, the up-regulation of CD40 expression and other costimulatory molecules on DC induced by leptin either alone or in combination with LPS may contribute to significantly enhanced DC stimulatory function in driving T cell proliferation. Aberrant expression of CD40 is implicated in a variety of autoimmune and inflammatory diseases such as rheumatoid arthritis and multiple sclerosis (13, 14). Blockade of the CD40-transduced signals have been shown to enhance the tolerogenic potential of antigen-specific DC for suppressing autoimmune conditions (37–40). Because leptin exhibits pro-inflammatory functions in immunity, our findings on the molecular basis for leptin-stimulated CD40 expression and DC function may benefit the design of immune therapy with genetically modified DC for treating autoimmune diseases.
and to validate leptin as an effective adjuvant in the development of DC vaccines for cancer.

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