Critical Role for CCAAT/Enhancer-Binding Protein β in Immune Complex-Induced Acute Lung Injury

Chunguang Yan, Min Wu, Jay Cao, Huifang Tang, Mei Zhu, Peter F. Johnson and Hongwei Gao

J Immunol 2012; 189:1480-1490; Prepublished online 25 June 2012;
doi: 10.4049/jimmunol.1200877
http://www.jimmunol.org/content/189/3/1480

References
This article cites 43 articles, 26 of which you can access for free at:
http://www.jimmunol.org/content/189/3/1480.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Critical Role for CCAAT/Enhancer-Binding Protein β in Immune Complex-Induced Acute Lung Injury

Chunguang Yan,*† Min Wu,† Jay Cao,‡ Huifang Tang,* Mei Zhu,§ Peter F. Johnson,* and Hongwei Gao*

C/EBPs, particularly C/EBPβ and C/EBPδ, are known to participate in the regulation of many genes associated with inflammation. However, very little is known regarding the activation and functions of C/EBPβ and C/EBPδ in acute lung inflammation and injury. In this study, we show that both C/EBPβ and C/EBPδ activation are triggered in lungs and in alveolar macrophages following intrapulmonary deposition of IgG immune complexes. We further show that mice carrying a targeted deletion of the C/EBPβ gene displayed significant attenuation of the permeability index (lung vascular leak of albumin), lung neutrophil accumulation (myeloperoxidase activity), total number of WBCs, and neutrophils in bronchoalveolar lavage fluids compared with wild-type mice. Moreover, the mutant mice expressed considerably less TNF-α, IL-6, and CXC/CC chemokine and soluble ICAM-1 proteins in bronchoalveolar lavage fluids, and corresponding mRNAs in the IgG immune complex-injured lung, compared with wild-type mice. These phenotypes were associated with a significant reduction in morphological lung injury. In contrast, C/EBPδ deficiency had no effect on IgG immune complex-induced lung injury. IgG immune complex-stimulated C/EBPβ-deficient alveolar macrophages released significantly less TNF-α, IL-6, MIP-2, keratinocyte cell-derived chemokine, and MIP-1α compared with wild-type cells. Similar decreases in IgG immune complex-induced inflammatory mediator production were observed following small interfering RNA ablation of C/EBPβ in a murine alveolar macrophage cell line. These findings implicate C/EBPβ as a critical regulator of IgG immune complex-induced inflammatory responses and injury in the lung. The Journal of Immunology, 2012, 189: 1480–1490.

Abbreviations used in this article: BAL, bronchoalveolar lavage; Cl2MDP, dichloromethylene diphosphonate; IC, keratinocyte cell-derived chemokine; LIP, liver-enriched inhibitory protein; MPO, myeloperoxidase; sICAM-1, soluble ICAM-1; siRNA, small interfering RNA.
In this study, we demonstrate that both C/EBPβ and C/EBPδ are activated in IgG immune complex-stimulated alveolar macrophages and in lung during IgG immune complex-induced acute lung injury. Importantly, we provide direct evidence that C/EBPβ, but not C/EBPδ, is a critical transcription factor that regulates IgG immune complex-induced inflammatory responses in the lung and alveolar macrophages.

Materials and Methods

Cells and reagents

Mouse alveolar macrophage-derived cell line, MH-S, was purchased from American Type Culture Collection (Manassas, VA), and cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.01 M HEPES, and maintained in a humidified incubator at 37˚C with 5% CO2. BSA was purchased from Sigma-Aldrich (St. Louis, MO). Anti-BSA IgG was purchased from MP Biomedicals (Solon, OH). ELISA kits for mouse IL-6, TNF-α, MIP-2, keratinocyte cell-derived chemokine (KC), MIP-1α, MIP-1β, and soluble ICAM-1 (sICAM-1) were obtained from R&D Systems (Minneapolis, MN).

IgG immune complex-induced acute lung injury

All procedures involving mice were approved by the Animal Care and Use Committee of Harvard Medical School. Eight- to 12-wk-old specific pathogen-free male C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Generation of Cebpb−/− and Cebpd−/− mice by homologous recombination has been described previously (20, 21). Cebpb−/− mice and wild-type littermates were on a C57BL/6: Sv129 F1 hybrid background (to circumvent low mutant viability on each pure strain background), and Cebpd−/− animals and wild-type controls were on a C57BL/6 background. Mice were anesthetized i.p. with ketamine HCl (100 mg/kg), followed by intratracheal instillation of 40 μl rabbit anti-BSA IgG dissolved in PBS (6 mg/ml) during inspiration. Immediately after intratracheal injection of anti-BSA, mice received i.v. 200 μl BSA solution (5 mg/ml in PBS). Negative control mice received anti-BSA IgG or PBS intratracheally alone. Unless otherwise indicated, 4 h after IgG immune complex deposition, mice were exsanguinated and the pulmonary circulation was flushed with 1 ml PBS via the pulmonary artery. The lungs were surgically dissected and immediately frozen in liquid nitrogen.

Myeloperoxidase activity

Mice were scarified, and the lungs were perfused via the right ventricle with 3 ml PBS. To measure myeloperoxidase (MPO) activity, whole lungs were

![FIGURE 1. Lung C/EBP activation during IgG immune complex-induced alveolitis. C/EBP activation in the lung by IgG immune complex deposition.](http://www.jimmunol.org/Download)
homogenized in 50 mmol/l potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide and 5 mmol/L EDTA. The samples were sonicated for 1 min and centrifuged at 10,000 rpm for 10 min. A total of 10 μl recovered supernatants was added to 96-well plates, followed by addition of 100 mmol/l potassium phosphate buffer containing 1.5 mol/l H₂O₂ and 167 μg/ml o-dianisidine dihydrochloride. The enzyme activity was determined by measuring the change in OD at 450 nm over a period of 4.5 min using a 96-well plate reader.

**Histological assay**

Four hours after IgG immune complex deposition, 1 ml 10% buffered (pH 7.2) formalin was instilled into the lung via the trachea. The lungs were then surgically removed and further fixed in 10% buffered formalin solution for morphological assay by tissue sectioning and staining with H&E.

**Bronchoalveolar lavage fluid collection, total and differential WBC counts, albumin, and chemokine/cytokine ELISAs**

Four hours after initiation of the acute lung injury, the thorax was opened and 0.8 ml ice-cold, sterile PBS was instilled into the lung via a tracheal incision. The recovered lavage fluid (bronchoalveolar lavage [BAL]) was centrifuged at 450 × g for 6 min, and the cell-free supernatants were stored at −20°C. Cell pellets were resuspended in 1 ml HBSS containing 0.5% BSA, and differential cell analyses were performed by Diff-Quik–stained cytospin preparations (Dade, Duedingen, Switzerland) counting a total of 300 cells per slide in randomly selected high-powered fields (×1000). The supernatant was used for cytokine, chemokine, and sICAM-1 measurements by sandwich ELISA. Mouse albumin levels in BAL fluid were measured using a mouse albumin ELISA kit purchased from Bethyl Laboratories (Montgomery, TX). The permeability index was expressed as

**FIGURE 2.** Effects of C/EBPβ deficiency on IgG immune complex-induced acute lung injury. Four hours after IgG immune complex deposition, BAL fluids and whole lungs were harvested. (A) Mouse albumin content in BAL fluids was determined using ELISA as an index of lung microvascular permeability. (B) Changes in lung MPO activity was measured. (C and D), Total cell and neutrophil accumulation in BAL fluids was counted. (E) Lung sections were stained with H&E (original magnification ×40). Lung sections shown included the following: C/EBPβ+/+ + anti-BSA, C/EBPβ+/+ + IgG IC, C/EBPβ−/− + anti-BSA, C/EBPβ−/− + IgG IC, C/EBPβ−/− + anti-BSA, and C/EBPβ−/− + IgG IC. Results are means ± SEM for three (control group) or five (IgG immune complex-challenged group) mice for each group.
the ratio of the albumin in the IgG immune complex-injured lungs versus that in the control-treated lungs of same type of mice.

Alveolar macrophage isolation and in vitro IgG immune complex treatment

BAL fluids were collected using repetitive (three times) instillation and withdrawal of 1 ml saline via an intratracheal cannula. BAL samples were centrifuged at 1500 rpm for 10 min, and cell pellets were resuspended and plated in 96-well plate. Alveolar macrophages were allowed to adhere at 37°C for 1 h, and nonadhering cells were removed. The purity of the cell suspension was ~96%, which is determined by staining of BAL cells with HEMA 3 STAIN SET, obtained from Fisher Scientific. For IgG immune complex formation in vitro, 100 μg BSA in 100 μl PBS buffer was incubated with 25 μl 2.5 mg/ml anti-BSA IgG at 37°C for 30 min. Supernatant fluids were collected for assessment of cytokines/chemokines by ELISA.

Assessment of C/EBP activation by EMSA

Nuclear extracts of alveolar macrophages or MH-S were prepared as follows. Cells were washed in PBS and lysed in buffer A (15 mM KCl, 10 mM HEPES [pH 7.6], 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.1% [v/v] Nonidet P-40, 0.5 mM PMSF, 2.5 μg/ml leupeptin, 5 μg/ml antipain, and 5 μg/ml aprotinin). Proteins were extracted from nuclei by incubation at 4°C with vigorous vortexing in buffer C (420 mM NaCl, 20 mM HEPES [pH 7.9], 0.2 mM EDTA, 25% [v/v] glycerol, 1 mM DTT, 0.5 mM PMSF, 2.5 μg/ml leupeptin, 5 μg/ml antipain, and 5 μg/ml aprotinin). Nuclear extracts of whole-lung tissues were prepared, as described previously (17). C/EBP probes (5′- TGCGAGATggcGACaTgCTgCA-3′; Santa Cruz Biotechnology, Santa Cruz, CA) were labeled with [32P]ATP (3000 Ci/mmol at 10 mCi/ml; Amersham Biosciences, Sunnyvale, CA). EMSA was performed, as described previously (17). The following Abs were from Santa Cruz Biotechnology: C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, C/EBPγ, and normal rabbit IgG.

Small interfering RNA transfection

Transient small interfering RNA (siRNA) transfections were performed by transfecting 1–2 × 10⁶ MH-S cells with control siRNA or C/EBPβ siRNA (Santa Cruz Biotechnology) using Amaxa nucleofector kit V. Twelve hours later, MH-S cells were treated with or without 100 μg/ml IgG immune complexes for 6 h. RNAs were harvested for RT-PCR to analyze downregulation of C/EBPβ expression, or supernatants were collected for ELISAs.

Alveolar macrophage depletion

Mice were anesthetized with ketamine HCl (100 mg/kg, i.p.). A suspension of dichloromethylene diphosphonate (Cl₂MDP) liposomes in PBS (10 μl of

FIGURE 3. Effects of C/EBPβ deficiency on IgG immune complex-induced production of cytokines and chemokines in BAL fluids and lung. (A–F) ELISAs were performed to determine the levels of TNF-α (A), IL-6 (B), MIP-2 (C), KC (D), MIP-1α (E), and MIP-1β (F) in BAL fluids. (G–L) Four hours after IgG immune complex deposition, whole-lung RNAs were extracted from wild-type and C/EBPβ-deficient mice, respectively. Real-time PCR was conducted to determine the expression of TNF-α (G), IL-6 (H), MIP-2 (I), KC (J), MIP-1α (K), and MIP-1β (L). Results are means ± SEM for three (control group) or five (IgG immune complex-challenged group) mice for each group.
the liposome stock in a total volume of 50 μl was administered intratracheally during inspiration. As a control, PBS liposomes were administered in a similar fashion. All subsequent interventions were performed 24 h after liposome instillation. Liposome-encapsulated clodronate was prepared, as previously described (22). Mice receiving Cl2MDP liposomes showed >75% depletion of alveolar macrophages compared with mice receiving PBS liposomes. Administration of PBS liposomes did not reduce the number of alveolar macrophages.

Luciferase assay

MH-S cells were transfected with indicated reporter plasmids by using Fugene6 Transfection Reagent (Roche, Indianapolis, IN). Forty-eight hours after transfection, cells were treated with or without 100 μg/ml IgG immune complex. Four hours later, cells were lysed in Passive Lysis 5× Buffer (Promega, Madison, WI), and luciferase activity was measured. The mouse IL-6 promoter-reporter and TNF-α promoter-reporter as well as the C/EBPβ expression plasmid and C/EBP6 expression plasmid have been described in our previous publications (5, 23, 24).

RNA isolation and detection of mRNA by quantitative real-time PCR

Total RNAs were extracted from lungs with TRIzol (Invitrogen, Carlsbad, CA), according to the manufacturer’s procedure. After isolation, total cellular RNA was incubated with RQ1 RNase-free DNase (Promega) to remove contaminating DNA. A quantity amounting to 2 μg total RNA was submitted to reverse transcription by using the Superscript II RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA). The cDNA (2 μl) was amplified and quantified using a Sequence Detection System (SDS 7300; Applied Biosystems, Foster City, CA) according to the manufacturer’s procedure. After isolation, total RNA was submitted to reverse transcription by using the Superscript II RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA). Following reverse transcription, the cDNA (2 μl) was amplified and quantified using a Sequence Detection System (SDS 7300; Applied Biosystems, Foster City, CA) and a PCR universal protocol as follows: AmpliTag Gold activation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The fluorescence of the double-stranded products accumulated was monitored in real time. The levels of mRNA of TNF-α, IL-6, MIP-2, and MIP-1α were determined by quantitative real-time PCR. PCR was performed with primers for TNF-α, 5'-primer, 5'-CGT CAG CCG ATT TGC TAT CT-3' and 3'-primer, 5'-AGT GAA GCC GGT AGG AGC TC-3'; 5'-primer, 5'-AGT GAA GCC GGT AGG AGC TC-3'; MIP-1α, 5'-primer, 5'-AGT AAG GTC TCC ACC ACT GC-3' and 3'-primer, 5'-CCC AGG TCT CTT TGAG AGT CA-3'; ICAM-1, 5'-primer, 5'-GTC TCA CTG AAT GCC GGC AC-3' and 3'-primer, 5'-GTC TCA CTG AAT GCC GGC AC-3'; VCAM-1, 5'-primer, 5'-ATT TTC TGG GGC AGG AAT TT-3' and 3'-primer, 5'-AGG TCT CTT CGG AAT TG-3'; the relative mRNA levels were normalized to levels of GAPDH mRNA in the same sample.

Statistical analysis

All values were expressed as the mean ± SEM. Data sets were analyzed using Student t test or one-way ANOVA, with individual group means being compared with the Student–Newman–Keuls multiple comparison test.

Results

Lung C/EBPs are activated during IgG immune complex-induced alveolitis

We evaluated C/EBP DNA-binding activity in IgG immune complex-injured lung by EMSA (Fig. 1A). C/EBP-binding species were detected in control-treated lung. Increased C/EBP binding was evident by 1 h after IgG immune complex deposition, and became strongest at 4 and 8 h. Subsequently, C/EBP-binding activity subsided, reaching basal levels by 24 h. To determine which C/EBP family members are induced by IgG immune complexes, we performed supershift assays. As shown in Fig. 1B, there are three major DNA-binding species in the nuclear proteins of control-treated lungs, as follows: low levels of C/EBPβ heterodimers; heterodimers between C/EBPβ (liver-enriched activating protein) and its short isoform, liver-enriched inhibitory protein (LIP), which is translated from an alternative start site in the same mRNA (25); and LIP/LIP homodimers. In IgG immune complex-injured lungs, the DNA-binding activities of both C/EBPβ (mainly β/LIP and LIP/LIP) and C/EBP6 were significantly induced (Fig. 1B). Using real-time RT-PCR analysis, we further show there is a time-dependent increase in the abundance of lung C/EBPβ and C/EBP6 transcripts after IgG immune complex deposition (Fig. 1C and data not shown). Thus, the increased mRNA expression of C/EBPβ and C/EBP6 is consistent with their increased DNA-binding activity in the lung.

Alveolar macrophages play a key role in the IgG immune complex-induced lung injury (16, 26, 27). Therefore, we determined whether alveolar macrophage dephlution would affect C/EBP activation in whole-lung tissues 4 h after onset of injury. Mice pretreated with PBS liposomes and challenged with IgG immune complexes showed the expected C/EBP activation (Fig. 1D, lanes 1 and 2). By contrast, depletion of alveolar macrophages with Cl2MDP liposomes markedly reduced the extent of lung C/EBP activation (Fig. 1D). These data suggest that alveolar macrophages play a critical role in IgG immune complex-induced activation of C/EBPs in lung.

Effects of C/EBPβ deficiency on IgG immune complex-induced lung injury

We sought to determine whether C/EBPβ contributed to IgG immune complex-induced lung injury by using C/EBPβ-deficient mice. As shown in Fig. 2A, C/EBPβ deficiency resulted in a significant decrease (p < 0.001) of permeability index (albumin leakage) when compared with wild-type mice after IgG immune complex deposition, whole-lung RNAs were extracted from wild-type and C/EBPβ-deficient mice, respectively. Real-time PCR was conducted to determine the expression of ICAM-1 (A) and VCAM-1 (B). Results are means ± SEM for three (control group) or five (IgG immune complex-challenged group) mice for each group.
complex deposition. We also examined MPO content to evaluate neutrophil accumulation in lungs (Fig. 2B). As with albumin leakage, MPO content in C/EBP\(^{\beta}\)-deficient mice was much lower (\(p, 0.01\)) when compared with values in control-treated wild-type mice during lung injury. We further found that C/EBP\(^{\beta}\)-deficient mice displayed significant attenuation of the total number of WBCs (by 59%, \(p, 0.001\); Fig. 2C) and neutrophils (by 61%, \(p, 0.001\); Fig. 2D) in BAL fluids from IgG immune complex-injured lung compared with wild-type mice. We examined whether C/EBP\(^{\beta}\)-deficient mice exhibited reduced lung injury by histological analyses. As shown in Fig. 2E, both wild-type and C/EBP\(^{\beta}\)-deficient mice receiving \(\alpha\)BSA alone exhibited normal lung architecture. As expected, lung hemorrhage and inflammatory cell influx were observed in wild-type mice after IgG immune complex deposition. In contrast, IgG immune complex-injured lungs from C/EBP\(^{\beta}\)-deficient mice showed significantly decreased neutrophil accumulation and reduced intra-alveolar hemorrhage compared with wild-type animals.

Production of cytokines and chemokines in lung after IgG immune complex deposition is impaired in C/EBP\(^{\beta}\)-deficient mice

We determined BAL levels of several inflammatory cytokines and chemokines that are involved in IgG immune complex-induced lung injury (26). As expected, wild-type mice undergoing IgG immune complex deposition showed increased production of TNF-\(\alpha\), IL-6, MIP-2, KC, MIP-1\(\alpha\), and MIP-1\(\beta\) compared with controls (Fig. 3A–F). The levels of all these inflammatory mediators were dramatically decreased in IgG immune complex-injured mutant mice when compared with wild-type mice. Moreover, in the presence of IgG immune complexes, lung from C/EBP\(^{\beta}\)-deficient mice expressed considerably lower amounts of mRNAs for TNF-\(\alpha\), IL-6, MIP-2, KC, MIP-1\(\alpha\), and MIP-1\(\beta\) (Fig. 3G–L), compared with the lung from wild-type mice. Adhesion molecules are also involved in lung inflammatory injury after intra-alveolar deposition of IgG immune complexes (28). Notably, IgG immune complex-injured lungs from C/EBP\(^{\beta}\)-deficient mice showed a marked reduction in ICAM-1 mRNA, whereas very little decrease in VCAM-1 expression was observed (Fig. 4). Because soluble ICAM-1 can directly bind and activate lung macrophages, and enhance lung injury after intrapulmonary disposition of IgG immune complexes (29), we measured soluble ICAM-1 level in BAL fluids of IgG immune complex-injured lungs. We found that C/EBP\(^{\beta}\) deficiency resulted in a significant decrease (\(p < 0.01\)) of
ICAM-1 change when compared with wild-type mice after IgG immune complex deposition in the lung (Fig. 5).

Effects of C/EBPβ deficiency on cytokine and chemokine protein production in alveolar macrophages stimulated by IgG immune complexes

We evaluated the effects of C/EBPβ deficiency on the expression of cytokines and chemokines in primary alveolar macrophages. Upon IgG immune complex treatment, C/EBPβ-deficient alveolar macrophages released significantly less TNF-α, IL-6, MIP-2, KC, and MIP-1α than wild-type macrophages at all time points analyzed (Fig. 6A–E). Interestingly, no significant change was found for MIP-1β, suggesting that other cells in the lung may contribute to the reduced levels of this chemokine in the BAL fluid and lung of C/EBPβ-deficient mice (Fig. 6F). We next sought to determine the effects of C/EBPβ on inflammatory mediator production following IgG immune complex stimulation of the alveolar macrophage cell line, MH-S. We first show that C/EBP-binding activity (mainly C/EBPβ) in IgG immune complex-treated MH-S cells increased with kinetics similar to that of the IgG immune complex-injured lung (Fig. 7A, 7B). We ablated C/EBPβ expression by siRNA-mediated silencing (Fig. 7C). Analysis of in-

FIGURE 7. Expression and regulatory function of C/EBPβ in IgG immune complex-treated alveolar macrophage cells. (A) Nuclear extracts from MH-S cells stimulated with 100 μg/ml IgG immune complexes for different time points were subjected to EMSA. (B) Nuclear extracts were harvested from MH-S cells stimulated with 100 μg/ml IgG immune complexes for 0 and 4 h, respectively, and subjected to supershift. The following Abs were used: normal rabbit IgG (N), anti-C/EBPα Ab (α), anti-C/EBPβ Ab (β), anti-C/EBPδ Ab (δ), anti-C/EBPε Ab (ε), and anti-C/EBPg Ab (γ). Arrows indicated supershifts and C/EBP dimers, respectively. (C–F) MH-S cells were transiently transfected with 600 nM control siRNA or C/EBPβ siRNA. (C) Twelve hours after transfection, the cells were incubated with 100 μg/ml IgG immune complexes for 6 h. RNAs were isolated, and RT-PCR was performed by using primers for C/EBPβ and GAPDH, respectively. The level of GAPDH was shown at the bottom as a loading control. (D–F) Supernatants were harvested, and ELISA was performed to investigate the expressions of TNF-α (D), IL-6 (E), and MIP-2 (F), respectively. The data were expressed as means ± SEM (n = 12).
flamatory mediator production showed impaired induction by IgG immune complexes following C/EBPβ knockdown in MH-S cells (TNF-α decreased by 62%, IL-6 by 77% IL-6, and MIP-2 by 48%, respectively; Fig. 7D–F).

Effects of C/EBPβ overexpression on IgG immune complex-induced TNF-α and IL-6 expression in macrophages

We examined IgG immune complex-induced C/EBP transcriptional activity in transiently transfected MH-S cells using 2XC/EBP-Luc, a promoter-reporter that contains two copies of a C/EBP binding site, and an expression vector for C/EBPβ (liver-enriched activating protein). Consistent with the results from EMSA (Fig. 1), IgG immune complex stimulation alone induced a 3.8-fold increase in luciferase activity compared with the untreated control (Fig. 8A). C/EBPβ vector alone also elevated 2XC/EBP-Luc transcription (7.7-fold). Importantly, IgG immune complex treatment of C/EBPβ transfectants induced luciferase expression 24-fold over the control value. Because these effects are superadditive, IgG immune complexes may stimulate the intrinsic activity of C/EBPβ as well as its expression.

We next evaluated the role of C/EBPβ in IgG immune complex-induced transcription from the TNF-α and IL-6 promoters. As shown in Fig. 8B and 8C, IgG immune complex stimulation alone significantly increased luciferase activity (2.2-fold for TNF-α and 4.56-fold for IL-6). C/EBPβ overexpression in the absence of IgG immune complex resulted in 4-fold (TNF-α) and 1.56-fold (IL-6) induction of luciferase expression. Importantly, IgG immune complex treatment of C/EBPβ transfectants induced luciferase expression 8.6-fold and 9.65-fold for TNF-α and IL-6, respectively. These data are consistent with the ELISA results obtained from primary macrophages and MH-S cells.

Effects of C/EBPβ deficiency on IgG immune complex-induced lung inflammatory responses

Because C/EBPβ DNA-binding activity was induced by IgG immune complex deposition in the lung (Fig. 1B), we examined whether C/EBPβ contributes to IgG immune complex-induced lung inflammatory responses. As shown in Fig. 9A, there was no significant difference in lung MPO activity between wild-type and C/EBPβ-deficient mice 4 h after IgG immune complex deposition. Consistently, BAL fluids from lungs of C/EBPβ-deficient mice showed similar levels of TNF-α and MIP-2 when compared with BAL fluids from injured wild-type mice (Fig. 9B, 9C). We next determined the effects of C/EBPβ on the inflammatory mediator production from IgG immune complex-stimulated primary alveolar macrophages. As shown in Fig. 9D–G, C/EBPβ deficiency in alveolar macrophages caused only modest decreases in TNF-α, MIP-2, KC, and MIP-1β production compared with wild-type macrophages at all time points analyzed. Thus, C/EBPβ (but not C/EBPβ) is critical for IgG immune complex-induced inflammatory injury in the lung.

Discussion

The intrapulmonary deposition of IgG immune complexes in mice results in a complex cascade of inflammatory mediators that control an ordered sequence of events, including the activation of residential macrophages and recruitment of neutrophils to the site of injury (26). Previous studies in this lung model suggest that activation of NF-κB appears to play a central role in the pulmonary inflammatory response to IgG immune complexes (30). However, no direct evidence supports this hypothesis. Moreover, growing evidence indicates that the regulation of gene expression in the lung is mediated by a highly intricate network of transcription factors (31, 32). Our recent studies show that Stat3 is activated in both alveolar macrophages and whole-lung extracts following IgG immune complex deposition (17, 33). Using an adenoviral vector expressing a dominant-negative Stat3 isoform, we further show that Stat3 plays an important regulatory role in the pathogenesis of IgG immune complex-induced acute lung injury (17). Because the promoter regions of several important inflammatory mediators such as IL-6 and TNF-α do not contain functional Stat3 binding sites, the molecular mechanism whereby Stat3 regulates lung inflammation remains unknown. Interestingly, it has been demonstrated that Stat3 can bind the promoter regions of both C/EBPβ and C/EBPα, which are involved in IL-6 signaling in hepatoma cells (19). Thus, we hypothesized that Stat3 may regulate lung inflammation by affecting C/EBP activity. In the current study, we have identified C/EBPβ as a critical mediator of IgG immune complex-induced acute lung injury and inflammatory response in alveolar macrophages.

FIGURE 8. Effects of C/EBPβ expression on IgG immune complex-induced C/EBP DNA binding (A) and expression of TNF-α (B) and IL-6 (C) in alveolar macrophage cells. MH-S cells were transiently transfected with total of 0.5 μg indicated DNA. Twenty-four hours after transfection, the cells were challenged with indicated stimulus for 4 h. Cell lysates were used for luciferase activity assay. Luminometer values were normalized for expression from a cotransfected thymidine kinase reporter gene. The data were expressed as means of three experiments ± SEM.
C/EBPβ and C/EBPδ are expressed in many tissues, including lung. However, the expression and function of C/EBPβ and C/EBPδ during acute lung inflammatory response are still largely unknown. We now provide evidence that both C/EBPβ and C/EBPδ are activated in lung during IgG immune complex-induced acute lung injury. We further show the IgG immune complexes regulate the lung expression of C/EBPβ and C/EBPδ at mRNA level (Fig. 1C and data not shown). However, the molecular mechanisms by which IgG immune complexes induce C/EBPβ and C/EBPδ gene expression in the lung remain unclear. We recently found that C/EBP activation in peritoneal macrophages is mediated, to a large extent, by Fcγ receptors (FcγRI and FcγRIII) (34). Therefore, it would be interesting to investigate in the future study whether FcγR signaling is involved in the C/EBP activation after IgG immune complex deposition in the lung.

C/EBPβ is a known regulator of several genes that are involved in the inflammatory responses, including those coding for cytokines, chemokines, and their receptors and acute-phase proteins (2). In the current study, we have used C/EBPβ-deficient mice to clearly demonstrate that C/EBPβ play a critical role in acute lung inflammation and injury. The current data indicate that several mechanisms are involved in C/EBPβ regulation of acute immunological pulmonary alveolitis. Our results that C/EBPβ deficiency significantly mitigates IgG immune complex-induced lung injury as defined by decreased albumin leakage into lung and reduced MPO content as well as less BAL cells indicate a critical role for C/EBPβ in neutrophil accumulation in lung. Neutrophil transmigration into the alveolar compartment and lung interstitium plays a key role in the development of acute lung injury. Using an Ab-mediated blocking approach, both the CXC chemokines (MIP-2 and KC) and CC chemokines (MIP-1α and MIP-1β) have been shown to play an important role in intrapulmonary recruitment of neutrophils and development of lung injury induced by the IgG immune complex deposition (35–37). Our observation that C/EBPβ deficiency resulted in a significant decrease of these chemokines in BAL fluids from IgG immune complex-injured lungs supports this hypothesis. Another possible mechanism is the C/EBPβ regulation of TNF-α and IL-6, both of which play an important role in the development of acute lung injury by inducing the expression of molecules mediating adhesive interactions between endothelial cells and leukocytes (38). Indeed, we show that C/EBPβ deficiency caused a reduced expression of TNF-α and IL-6 as well as ICAM-1 in the lung. Together, our data suggest that upon IgG immune complex deposition, C/EBPβ affects neutrophil migration into lung and alveolar space by regulating the expression of chemokines, cytokines, and adhesion molecule.

Alveolar macrophages function as regulatory cells that secrete TNF-α and other cytokines and chemokines to modulate the cell signaling cascade for the production of other inflammatory mediators during lung inflammation (2, 50). We previously showed that

![Figure 9](http://www.jimmunol.org/)

**FIGURE 9.** Effect of C/EBPδ deficiency on IgG immune complex-induced lung inflammation in vitro and in vivo. (A–C) Four hours after IgG immune complex deposition in the lungs of wild-type and C/EBPδ-deficient mice, BAL fluids and whole lungs were harvested. (A) Changes in lung MPO activity were measured. (B and C) ELISAs were performed to determine the levels of TNF-α (B) and IL-6 in BAL fluids. (D–G) Alveolar macrophages obtained from wild-type and C/EBPδ-deficient mice were treated with 100 μg/ml IgG immune complexes for the times indicated, and supernatants were subjected to ELISA to assess production of TNF-α (D), MIP-2 (E), KC (F), and MIP-1α (G), respectively. The data were expressed as means ± SEM (n = 6).
depletion of alveolar macrophages resulted in significantly reduced expression of inflammatory cytokines and chemokine genes in IgG immune complex-injured rat lungs (33). Data in the current study show that C/EBP activation induced by IgG immune complexes is suppressed by depletion of alveolar macrophages in whole lung tissues (Fig. 1D). Moreover, employing mice deficient for C/EBPβ, siRNA-mediated knockdown in cell lines, and luciferase reporter assays, we show that C/EBPβ plays a critical role in the production of cytokines and chemokines in IgG immune complex-stimulated macrophages. These data together indicate that C/EBPβ activation in alveolar macrophages is a key event in IgG immune complex-induced lung injury.

All C/EBP members can form homo- and heterodimers with other family members. It has been noted that, depending on the composition, C/EBP complexes may be associated with function differences in cell growth, cell activation, and apoptosis (39). However, several studies have suggested compensatory or redundant roles for C/EBPs, including C/EBPα and C/EBPβ, in supporting the induction of inflammatory cytokines and chemokines. For example, LPS stimulation of peritoneal macrophages from C/EBPβ-deficient mice led to normal induction of several inflammatory cytokines, including IL-6 and TNF-α, with the exception of G-CSF, Mincle, and mPGES-1 (4, 40–42). Using a B lymphoblast system, Hu et al. (5) reported that the activities of C/EBPα, C/EBPβ, and C/EBPα and C/EBPβ are redundant in regard to the expression of IL-6 and MCP-1. In addition, a recent study shows that C/EBPβ-deficient macrophages have no significant defects in IL-6 and TNF-α production in response to several TLR ligands, whereas the absence of both C/EBPβ and C/EBPβ results in a significant decrease in the TLR ligand-induced production of IL-6 and TNF-α (43). Interestingly, our recent study shows that lack of either C/EBPβ or C/EBPβ has a significant effect on the production of TNF-α, MIP-2, and MIP-1α in IgG immune complex-stimulated peritoneal macrophages, indicating the possible importance of C/EBPβ and C/EBPβ heterodimer occupancy in regulating these promoters (34). In this report, we have directly compared the effect of C/EBPβ and C/EBPβ deficiency on lung inflammatory responses. We clearly show that C/EBPβ could not compensate for C/EBPβ deficiency in the IgG immune complex lung injury model and alveolar macrophage responses. These data further support the idea that the functional roles of C/EBPβ and C/EBPβ in inflammation are cell and tissue specific.

In summary, we present evidence that mutant mice defective in C/EBPβ were significantly protected from acute lung inflammation and injury following intrapulmonary deposition of IgG immune complexes. Our study indicates that understanding the underlying roles of various transcriptional factors in regulating the pulmonary inflammation may be a crucial step for devising new therapeutic strategies for treatment of acute lung injury.

Acknowledgments
We thank Karen Saylor and Nancy Martin for assistance with mouse breeding and genotyping.

Disclosures
The authors have no financial conflicts of interest.

References


