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Roles of CCAAT/enhancer-binding protein [beta] and [-delta] in immunoglobulin G immune complex-induced Inflammation

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ROLES OF CCAAT/ENHANCER-BINDING PROTEIN β AND -δ IN IMMUNOGLOBULIN G IMMUNE COMPLEX-INDUCED INFLAMMATION

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This thesis, submitted by Chunguang Yan in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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This dissertation meets the standards for appearance, conforms to the style and format requirements of the Graduate School of the University of North Dakota, and is hereby approved.

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ABSTRACT

CCAAT/enhancer-binding protein (C/EBP) β and C/EBPδ are known to participate in the regulation of many genes associated with inflammation. However, little is known about the activation and function of C/EBPβ and -δ in inflammatory responses elicited by Fcγ receptor (FcγR) activation. Here I showed that C/EBPβ and -δ activation were induced in immunoglobulin G immune complex (IgG IC)-treated macrophages by using gel shift assays. The increased expression of C/EBPβ and -δ occurred at both mRNA and protein levels. Furthermore, induction of C/EBPβ and -δ was mediated, to a large extent, by activating FcγRs. Using small interfering RNA (siRNA)-mediated knockdown as well as macrophages deficient for C/EBPβ and/or -δ, I demonstrated that C/EBPβ and -δ played a critical role in the production of tumor necrosis factor-α (TNF-α), macrophage inflammatory protein-2 (MIP-2), and macrophage inflammatory protein- 1α (MIP-1α) in IgG IC-stimulated macrophages. Moreover, both extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen activated protein kinase (MAPK) were involved in C/EBP induction and TNF-α, MIP-2, and MIP-1α production induced by IgG IC. I provided the evidence that complement component 5a (C5a) regulated IgG immune complex-induced inflammatory responses in macrophages by enhancing ERK1/2 and p38 MAPK activities as well as C/EBPβ and -δ activities. To further explore the roles of C/EBPβ and C/EBPδ in FcγR-mediated inflammatory responses *in vivo*, I used IgG IC-induced acute lung injury model. I showed that both C/EBPβ and C/EBPδ activation were triggered in lungs

challenged by IgG IC. I further demonstrated that C/EBPβ but not C/EBPδ deficient mice displayed significant attenuation of pulmonary vascular permeability and neutrophil accumulation when compared with wild type mice. Moreover, C/EBPβ deficient mice expressed considerable less inflammatory mediators compared with wild type littermates. Together, these data indicate that both C/EBPβ and C/EBPδ act as inflammatory stimulators *in vitro* during IgG IC-mediated inflammation. However, it is C/EBPβ but not C/EBPδ depletion attenuates IgG IC-induced lung inflammatory reactions *in vivo*.

CHAPTER Ⅰ

INTRODUCTION

IgG IC-induced Acute Pulmonary Inflammation

Acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) are important problems in human beings, leading to 75,000 deaths each year in the United States (1). To dissect the molecular mechanisms of ALI and ARDS, a number of animal models have been developed. By using animal models to mimic pulmonary inflammation, we can explore possible signaling cascades involved in inflammation-related lung damages. Such experimental models do not necessarily reproduce what happened during human pulmonary inflammation, but they allow us to investigate the molecular mechanisms that result in damages of pulmonary endothelial barrier and alveolar epithelial barrier. In addition, information obtained from experimental lung inflammatory models is beneficial to understating of inflammation-mediated diseases in other organs. In my dissertation, IgG IC-induced ALI in rodents is used. The model is applied to determine the roles of cytokines, chemokines and the complement system in inflammation, although IgG IC-induced tissue injury in human beings is mainly observed during autoimmune diseases, such as immune thrombocytopenia, systemic lupus erythematosus, extrinsic allergic alveolitis, and rheumatoid arthritis (2-5). IgG IC-stimulated ALI is initiated by intratracheal injection of IgG antibodies against bovine serum albumin (BSA), followed by intravenous administration of BSA. After IgG IC formation in the lung, damages of interstitial

capillary endothelial cells and alveolar epithelial cells are induced through a series of pathophysiological events.

Effects of FcγRs on IgG IC-mediated Immune Responses

IgG IC-induced immune responses is achieved by binding of IgG IC to FcγRs. FcγRs, which can specifically bind to the Fc region of IgG, are divided into four classes. Three classes of activating FcγRs are discovered: 1) FcγRⅠ with high affinity for Fc region of IgG; 2) FcγRⅣ with intermediate affinity; 3) FcγRⅢ with low affinity (6, 7). All these FcγRs share a common FcR γ-chain containing an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasm, which is indispensible for cell activation (8). Association of IgG IC with FcγRⅠ/FcγRⅢ/FcγRⅣ results in signal transduction from cytoplasm to the nucleus, which is achieved by interaction between the γ chain formed-homodimer and the receptor α chain (6, 9). IgG IC-induced clustering of FcγRⅠ/FcγRⅢ/FcγRⅣ stimulates tyrosine phosphorylation within ITAM and subsequent activation of downstream signaling cascades involved in functional responses (6). Because FcγRⅡ, with low affinity for Fc region of IgG, has an immunoreceptor tyrosine-based inhibition motif (ITIM), which can abrogate activation signaling induced by ITAM, it functions as an inhibitory FcγR (3, 8). FcγRⅠ is constitutively expressed by macrophages, monocytes and human airway smooth muscle cells while FcγRⅢ can be found on the surfaces of many types of cells, such as natural killer, eosinophils, macrophages, neutrophils, monocytes, human airway smooth muscle cells and platelets (10, 11). FcγRⅡ is expressed by almost all hematopoietic cells and human airway smooth muscle cells (10, 11). FcγRⅣ, a recently identified IgG IC receptor, is restricted to myeloid lineage cells (7).

The mechanisms by which IgG IC induces inflammation remain largely unknown, but the role of each FcγR in IC-stimulated immune responses is well established by using the gene knockout mice.

Contribution of each FcγR to IgG IC-induced inflammation has been extensively examined by using mice genetic deficiency in a variety of FcγRs. FcRγ$^{-/-}$ mice are completely protected from IgG IC-induced ALI when compared with wild type mice (12). In addition, it has been demonstrated that genetic depletion of FcγR Ⅰ leads to impaired cellular processes induced by IgG IC, including cytokine generation, cellular cytotoxicity, phagocytosis, and antigen presentation (13). Moreover, FcγR Ⅰ deficient mice are resistant to hypersensitivity responses, as reflected by decreased cartilage damages in an arthritis model (13). Recent studies show that microRNA-127 (miR-127) reduces IgG IC-induced ALI by downregulating FcγR Ⅰ expression (14). When compared with wild type mice, FcγRⅢ knockout mice show markedly attenuated inflammatory responses during IgG IC-induced alveolitis, with reduced expressions of TNF-α, interleukin(IL)-1β, and MIP-2 (15). Antibody-induced thrombocytopenia could be prevented by blocking binding of FcγR Ⅳ to pathogenic antibodies against platelets (7). Blocking FcγR Ⅳ results in decreased phagocytosis and cytokine expressions during IgG IC-induced ALI, though augmented expression of FcγRⅣ alone could not trigger the disease in FcγRⅢ knockout mice (16). FcγR Ⅱ deficiency lowers the threshold of IC stimulation of alveolar macrophages, as proved by analysis of FcγR Ⅱ knockout macrophages which display increased calcium and phagocytosis upon IC challenge (12). FcγR Ⅱ deficient mice are more sensitive to IC-induced pulmonary injury, as reflected by increased neutrophil

accumulation, edema, and hemorrhage (12). As mentioned above, both neutrophils and alveolar macrophages constitutively express FcγRs. Moreover, it has been demonstrated that airway epithelial cells can also express functional FcγRs (17). Thus, to understand the role of different FcγR in IgG IC-induced acute lung inflammation in more detail, mice selectively depleted of distinct FcγR in neutrophils, lung epithelial cells, and macrophages will be useful.

Roles of C5a Signaling in IgG IC-mediated Inflammation

Complement is discovered about a century ago as a heat-labile component of normal plasma. Activation of complement system plays pivotal roles in innate immune defense to eradicate invading microbial pathogens. However, overactivation of complement has been reported to lead to many inflammation-related diseases, such as IgG immune complex (IC)-induced acute lung injury (18, 19), caecal ligation and puncture (CLP)-induced sepsis (20-27), and asthma (28). By now, four pathways have been demonstrated to be involved in complement activation—classical pathway, mannan-binding lectin pathway, alternative pathway, and coagulation pathway. The classical pathway can be activated by direct association of C1q with the microbial pathogen surfaces. It can also be initiated by binding of C1q to antigen-antibody complexes during an adaptive immune response. Thus, it is a bridge linking innate immune responses and adaptive immune responses. The mannan-binding lectin pathway is trigged by binding of mannan-binding lectin to carbohydrate structures containing mannose that present on bacterium or virus surfaces. The alternative pathway is activated by binding of spontaneously activated complement peptide to pathogen surfaces. All three pathways described above result in a series of enzymatic cleavage reactions, leading to formation of

C3 convertase, at which the three pathways converge. C3 convertase has an important effect on complement activation, because it can lead to formation of C3a, C3b, C5a, C5b, C6, C7, C8, and C9, among which C5b, C6, C7, C8 and C9 form a membrane attack complex (C5b-9), which is used by host to lyse gram-negative bacteria. Coagulation pathway is recently identified at Peter A. Ward's lab at the University of Michigan, and they find that thrombin functions as C5 convertase in the absence of C3, leading to production of C5a and formation of C5b-9 (18). In addition, tissue factor (TF) and other factors may be responsible for thrombin production from prothrombin in C3 knockout mice (18). Moreover, most recent study demonstrates that in multiple trauma patients, factor Ⅶ-activating protease (FASP), which is activated by circulating nucleosomes released from necrotic cells, interacts with complement proteins in plasma, and cleaves C3 and C5 to produce C3a and C5a (29). Though complement initiation is necessary for appropriate immune reactions, excessive complement activation does harm to hosts. Thus, activation of complement system is considered as "double-edge sword". During IgG IC-induced acute lung injury, excessive production of C5a occurs, and its function has been extensively investigated.

Under inflammatory conditions, C5a can activate signaling cascades in both endothelial cells and phagocytic cells. For example, C5a treatment greatly augments TNF-α and MIP-2 expressions in alveolar macrophages challenged by IgG IC (30). In contrast, in the presence of C5a, lipopolysaccharide (LPS)-induced TNF-α expression in neutrophils is significantly reduced, which is mediated by increased levels of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) that can suppress nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation

(31). In human basophils, C5a is a potent stimulator of plasminogen activator inhibitor-1 expression (32). In addition, cluster of differentiation molecule 11b/18 (CD11b/CD18) expression on neutrophils and eosinophils is significantly increased by C5a, which leads to augmented adhesion of both granulocytes to human umbilical vein endothelial cells (HUVECs) or human bronchial epithelial cells (HBECs) (33). It has been demonstrated that binding of C5a to HUVECs significantly increases P-selectin expression, which is required for early adhesive interaction between endothelium and polymorphonuclear neutrophils (PMNs) (34). Moreover, intravenous injection of anti-rat C5a antibodies almost abrogates lung vascular P-selectin generation during cobra venom factor (CVF)-induced acute lung injury, which indicates that C5a is required for pulmonary vascular P-selectin expression (35). Recently, cDNA microarray analysis shows that C5a treatment markedly increases expressions of cell adhesion molecules in HUVECs, including E-selectin, vascular cell adhesion protein 1 (VCAM-1), and (intercellular adhesion molecule 1) ICAM-1, which are necessary for transendothelial trafficking of neutrophils (36). In the same study, it is observed that gene expression for cytokines and chemokines is also greatly elevated (36). Initial exposure of mouse dermal microvascular endothelial cells (MDMECs) to LPS, interferon-gamma (IFN-γ), or interleukin-6 (IL-6), followed by C5a challenge causes synergistic generation of monocyte chemotactic protein-1 (MCP-1) and MIP-2, which indicates that C5a in the co-presence of additional stimulators could induce strong immune reactions in endothelium (37).

During IgG IC-induced acute lung inflammation, growing evidence suggests that activation of complement system is indispensible for full pulmonary inflammatory responses (38). When compared with C5 sufficient mice, C5 deficient mice displays

reduced edema, hemorrhage, neutrophil accumulation, and wet/dry ratios of lung (a marker of lung vascular permeability) after pulmonary deposition of IgG IC, suggesting that C5a—a phlogistic fragment of C5, might be involved in IgG IC induced ALI (39). Intratracheal instillation of antibodies against C5a markedly inhibits IgG IC-induced pulmonary damages by decreasing TNF-α concentration in bronchoalveolar lavage (BAL) fluids, lung vascular ICAM-1 expression, pulmonary myeloperoxidase (MPO) activity, and lung vascular permeability (40). In contrast, intravenous instillation of anti-C5a antibodies has no protective effects against IgG IC-induced ALI, which suggests that IgG IC-induced C5a generation in the airway is indispensible for full development of pulmonary inflammation (40). Recent studies show that in the presence of activated neutrophils or alveolar macrophages, C5a is generated by enzymatic cleavage of C5, which means that C5a could be produced in the absence of plasma complement system (41). It is also found that treatment with antibodies to C5a protects mice from intravenous administration of CVF-induced lung injury, which indicates that systemic activation of complement-mediated lung injury is dependent on C5a (40). Both *in vitro* and *in vivo* experiments further prove the critical roles of C5a in IgG IC-induced inflammation. *In vitro* studies show that in the presence of anti-C5a antibodies, IgG IC-induced production of MIP-2, cytokine-induced neutrophil chemoattractant (CINC), MIP-1α, macrophage inflammatory protein 1 beta (MIP-1β), and MCP-1 is greatly reduced in rat alveolar macrophages (42). Moreover, treatment with blocking antibody to C5a also significantly inhibits generation of the chemokines in the rodent pulmonary model stimulated by IgG IC (42). It is further demonstrated that C5 and C5a positively regulate IgG IC-induced severe airway hyperreactvity by using mice genetically depleted of C5, and mice receiving

blocking antisera to C5a, respectively (43). However, recent data show that systemic activation of complement is not observed after airway injection of LPS, and immunopathogenesis of LPS-induced experimental ALI is independent of C3, C5, and C5a (44). Take together, these data show that during IgG IC-induced ALI, C5a generation through activation of complement system is an early event triggering inflammatory signaling cascades.

C5a exerts the biological activity through binding to its receptors—C5a receptor (C5aR) and C5a receptor-like 2 (C5L2). C5aR, which is also known as CD88 or complement component 5a receptor 1 (C5AR1), is a G-protein-coupled receptor with seven transmembrane segments. C5aR has a molecular weight of 45 kDa, and binds to C5a with high affinity, to a lesser extent, to C5a des Arg. Originally, it is thought that C5aR is exclusively expressed by myeloid cells, such as macrophages, monocytes, neutrophils, basophils, and eosinophils (45). Now there are growing evidences that C5aR expression could also be found to occur on a variety of nonmyeloid cells, such as bronchial and alveolar epithelial cells, smooth muscle cells, Kupffer cells, endothelial cells, astrocytes, kidney tubular epithelial cells, and other parenchymal cells of solid organs, including lung, kidney, liver, and heart (19). Treatment of alveolar epithelial cells (AECs) with LPS, TNF-α, IL-6, or C5a greatly increases binding of C5a, which is linked to elevated C5aR expression (46). Moreover, in the presence of C5a, LPS further augments inflammatory responses (46). Exposure of mouse dermal microvascular endothelial cells to LPS, IFN-γ, or IL-6 enhances binding of C5a, which is related to C5aR expression on surfaces of the cells (37). Also, IL-6-mediated immune reactions are further increased by C5a in the cells (37). Incubation of HUVECs with C5a significantly upregulates expressions of IL-1β,

interleukin-8 (IL-8), and regulated and normal T cell expressed and secreted (RANTES) at mRNA level (47). However, their expressions are greatly suppressed by treatment of the cells with antibodies against C5aR, indicating that interaction between C5a and C5aR is required for full production of the proinflammatory mediators (47). Interestingly, IL-6 generation is downregulated by C5a, which suggests that other signaling pathways activated by C5a are responsible for the inhibition of IL-6 expression (47). *In vivo* studies show that gene-targeted disruption of C5aR alleviates IgG IC-induced acute lung injury (48). IgG IC-mediated lung inflammation is also attenuated by using competitive C5aR antagonist that can inhibit the receptor phosphorylation and ensuing signaling transduction, indicating that C5aR is required for inflammatory signaling transduction induced by C5a in the model (49). The strict requirement of FcγRs for a variety of IgG IC-mediated immune reactions make us hypothesize that inflammatory signaling transduction triggered by complement activation is secondary to or independent of FcγRs activation (50, 51). However, recent studies show that signaling cascades induced by both FcγRs and complement system are necessary for full immune responses after intratracheal deposition of IgG IC in rodents, which suggests that FcγRs-induced signaling transduction might be integrated through C5a-meidated C5aR activation (10, 15, 30, 50, 51). *In vitro* data show that C5aR activation increases FcγRⅢ expression while decreases FcγRⅡ production, which augments IgG IC-induced TNF-α and MIP-2 generation in alveolar macrophages (30). *In vivo* studies demonstrate that C5aR depletion decreases FcγRⅢ/FcγRⅡ ratio as compared with wild type counterparts, causing reduced inflammatory mediators' generation, neutrophil accumulation, and ensuing lung injury induced by pulmonary

deposition of IgG IC (30). Therefore, binding of C5a to C5aR plays an essential role in promoting IgG IC-induced ALI by increasing FcγRⅢ/FcγRⅡ ratio.

C5L2 is encoded by the *GPR77* gene in humans, and has a molecular weight similar to C5aR. It belongs to a subfamily of C3a, C5a, and N-formyl-methionyl-leucyl-phenyl-alanine (fMLP) receptors, and its expression could be detected in different types of cells, such as granulocytes and immature dendritic cells (52). While C5L2 binds to C5a and C5a des Arg with high affinity, the interaction between C5L2 and other ligands, such as C3a and C3a des Arg, is still a matter of controversy (53-57). Unlike the previously described C5aR that is a G-protein-coupled receptor, C5L2 is uncoupled from G-proteins because of an amino acid alteration in the so-called DRY region of the third intracellular loop, and the association of C5L2 with C5a could not induced transient intracellular calcium influx due to the replacement of arginine by leucine in the DRY region (58). In addition, rat C5L2 first is cloned by Peter A. Ward group, and its cDNA sequence is 59.8%, and 86.4% identical to human C5L2, and mouse C5L2, respectively (59). Hydropathy analysis shows that rat C5L2 has seven transmembrane segments (59). C5a binding domain is located at the N-terminus of C5L2, and rat C5L2 has the DRY motif in the third intracellular loop, which is much shorter than that of C5aR (59). The bulk of C5L2 are located in cytosol in the "resting" PMN, which is in striking contrast to C5aR that mainly appears to be on cell surfaces (59).

First C5L2 knockout mouse is constructed by Craig Gerard group, Harvard Medical School, and C5L2 deficiency has no effect on C5aR function, because 1) C5L2 knockout mice has no influence on C5aR expression, as confirmed by Northern blot assay; 2) intraperitoneal injection of C5a into wild type and C5L2 deficient mice induced

indistinguishable neutrophilic infiltrates (53). However, C5L2 could act as a "decoy" receptor, and protect mice from IgG IC-induced acute lung injury. In IgG IC-mediated acute lung inflammation model, C5L2 knockout deteriorates inflammatory parameters: 1) pulmonary deposition of immune complex leads to greater influx of PMNs in C5L2 knockout mice when compared with wild type littermates; 2) C5L2 deficient mice express significantly more TNF-α and IL-6 in comparison with wild type counterparts; 3) histologic analysis of lung lavage and tissue sections show that enhanced inflammation occurred in C5L2 knockout mice compared with wild type rodents (53). Though C5L2 is reported as a "decoy" receptor, most recent data show that C5L2 could also be a functional receptor. In a mouse model of ovalbumin (OVA)- or house dust mite-induced experimental allergic asthma, C5L2 deficiency leads to attenuated asthmatic phenotype: 1) decreased airway hyperresponsiveness (AHR) and Th2 cytokine (IL-5, IL-13, IL-4, and IL-10) expressions are observed in C5L2 knockout mice upon pulmonary exposure to either OVA or house dust mite; 2) C5L2 knockout results in reduced lymphocytes accumulation, and eosinophils numbers as well as serum IgE level; 3) histologic analysis reveals reduced airway inflammatory cells and mucus production (60). Therefore, C5L2 may play different roles in distinct disease models.

Activation of Transcription Factors by IgG IC

IgG IC-induced FcγR aggregation initiates immune response-related signaling transduction, which lead to expressions of inflammatory genes encoding cytokines and chemokines, such as TNF-α, IL-1, IL-10, MIP-2, CINC, keratinocyte-activated cytokine (KC), MIP-1α, MIP-1β, eotaxin, and MCP-1 (10). The expressed genes include both proinflammatory and anti-inflammatory mediators, and their relative level determines the

inflammatory intensity. The precise mechanisms involved in signaling transduction from FcγR to cytoplasm and then to nucleus remains largely unknown, but it has been demonstrated that several transcription factors including NF-κB, Activator Protein-1 (AP-1), and Signal Transducer and Activator of Transcription 3 (STAT3) are activated during IgG IC-mediated inflammatory response (61-63).

As transcription factors, NF-κB family is composed of five members: p65 (RelA), RelB, c-Rel, p52/p100, and p50/p105, and they share a conserved Rel homology domain (RHD), which is consisted of 300 amino acids, and located toward the N-terminus of the protein. RHD is required for their DNA binding, activity regulation, and dimerization (64). The family members exist in cells by forming homodimers or heterodimers (64). There are two pathways involved in NF-κB activation—classical pathway and alternative pathway (64). In the classical pathway, inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) is activated by stimulators, leading to phosphorylation of IκB while in the alternative pathway, activated IKKα phosphorylates p100 (64). These two pathways converge at proteasomes, leading to release of mature NF-κB dimers that translocate to the nucleus and promote transcription of target genes (64, 65).

A variety of inflammation-related gene expressions, which include cytokines, adhesion molcules, and chemokines, are regulated by NF-κB. These inflammatory mediators play a critical role in inflammation-induced pulmonary damages. Thus, NF-κB activation is an upstream event in the inflammatory cascades, and may be involved in regulation of inflammation-induced lung injury. *In vivo* studies show that pulmonary transcription of ICAM-1, E-selectin, P-selectin, VCAM-1 genes follows NF-κB activation during endotoxin-induced acute lung injury (66). Furthermore, endotoxin-induced binding

of NF-κB to its consensus sequence in E-selectin promoter region, indicating that NF-κB directly mediates target genes' production at transcription level (66). During IgG IC-induced ALI, whole lung NF-κB is activated, followed by TNF-α and IL-1β expressions (61). In addition, NF-κB DNA binding activity is also enhanced in alveolar macrophages by intrapulmonary deposition of IgG IC (61). Now, it has been demonstrated that alveolar macrophages are required for intrapulmonary activation of NF-κB in IgG IC-induced alveolitis, because alveolar macrophage depletion leads to depressed lung NF-κB activity (67). Whole lung NF-κB activation could be restored in alveolar macrophage-depleted mice receiving intrapulmonary administration of TNF-α (67). In the model of IgG IC-triggered lung injury, TNF-α plays an essential role in full development of acute lung injury by increasing neutrophil accumulation, and is expressed mainly by alveolar macrophages as evidenced by immunohistochemical assay of cells recovered from BAL fluids and pulmonary sections (67). Therefore, TNF-α expression by IgG IC-activated alveolar macrophages is an upstream event in the inflammatory cascades, which is required for full activation of NF-κB in other types of cells in the lung, including pulmonary vascular endothelium and alveolar epithelial cells. NF-κB activation is also regulated by anti-inflammatory mediators, such as IL-10 and IL-13. Both cytokines increase IκBα expression at both mRNA and protein levels, which inhibits nuclear translocation of NF-κB after intrapulmonary deposition of IgG IC (68). Suppression of NF-κB activation in alveolar macrophages and whole lungs results in decreased TNF-α expression, leading to reduced neutrophil influx and ensuing lung injury (68). Thus, both proinflammatory and anti-inflammatory pathways converge at NF-κB, and their balance determines the intensity of lung damages. Interestingly, both *in vitro* and *in vivo* studies

shows that NF-κB activation is positively regulated by TNF-α and IL-1β, but C5a has no effect on its activity-induced by IgG IC, which suggests that C5a-mediated inflammation might be involved in other transcription factors (61).

It has been proved that reactive oxygen species (ROS) are produced by neutrophils and macrophages during stimulation by various agents or phagocytosis, and are promoters of NF-κB activation (69, 70). NF-κB activity is significantly reduced in p47phox deficient mice when compared with wild type mice during endotoxin-induced ALI (71). p47phox is the cytosolic subunit of the multi-protein complex known as NADPH (Nicotinamide Adenine Dinucleotide Phosphate, reduced) oxidase that is responsible for ROS generation. Therefore, NF-κB activation is positively regulated by ROS in the model. In IgG IC-induced ALI, NF-κB activation is greatly impaired in mice receiving N-acetylcysteine (NAC), but not catalase that could not penetrate cell membrane due to its large molecular weight, indicating that it is intracellular but not extracellular ROS play an important role in pulmonary NF-κB activation (61). In addition, IgG IC-induced NF-κB activation is independent of neutrophils, thus, intracellular ROS produced by alveolar macrophages are responsible for partial pulmonary NF-κB activation (61). Recent data show that NAC treatment downregulates NF-κB activation by impairing interaction of TNF-α with its receptor but not ROS generation (72). Thus, to further examine the role NF-κB activation mediated by ROS, p47phox knockout mice should be used in the IgG IC model of lung injury. Furthermore, in the endotoxin-induced ALI model, ROS has no effect on pulmonary inflammation and ensuing lung injury, thus, more effects should be made to investigate the role of ROS themselves in IgG IC-mediated pulmonary damages (71).

AP-1 are transcription factors, and exist in the cells as homodimers or heterodimers composed of proteins that belong to Fos, Jun, and activating transcription factor (ATF) families (73). AP-1 could be activated by a variety of stimuli, such as ROS, DNA damage, and proinflammatory cytokines (eg, TNF-α, and transforming growth factor-β) (10, 63, 74-76) . Activated AP-1 play an important role in cellular differentiation, proliferation, apoptosis, and transformation (10, 75). However, its effect on lung inflammation remains largely unknown. Transcription of many inflammation-related genes, especially those encoding chemokines and cytokines is dependent on binding of AP-1 to the consensus sequences in their promoter regions, which indicates that AP-1 activation might be involved in inflammatory diseases (77-79). AP-1 is activated during endotoxin-induced ALI (80, 81). Additionally, AP-1 overexpression is observed in lungs of smokers (82). In a rat model of lung fibrosis, AP-1 activation is induced in lungs after airway instillation of bleomycine due to augmented expression of c-Jun and c-Fos in alveolar macrophages and type Ⅱ lung epithelial cells (83). In asthmatic patients, c-Fos expression is increased in epithelial cells (84). Corticosteroid-treated patients rarely express c-Fos, which suggests that AP-1 play an essential role in inflammatory reactions in asthma (84). During IgG IC-induced ALI, AP-1 activation is observed in both alveolar macrophages and whole lungs, which is due to increased expressions of c-Fos, c-Jun, Jun-B, and Jun-D (63). Moreover, IgG IC-induced pulmonary AP-1 activation is dependent on alveolar macrophages, because AP-1 activity is sharply reduced in alveolar macrophage-depleted mice (63). Furthermore, TNF-α but not C5a is required for full activation of AP-1 in IgG IC-challenged lungs (63). More studies will be needed to determine the role of AP-1 in IgG IC-mediated pulmonary injury, and the contribution of neutrophils and IgG IC-induced

ROS to AP-1 activation. Also, extensive experiments should be performed to investigate whether there is cooperation between AP-1 and NF-κB during IgG IC-induced inflammation responses, because the cooperation is necessary for the maximal expressions of various inflammation-related genes (85).

Signal Transducer and Activator of Transcription 3 (STAT3) belongs to STAT protein family that is composed of seven members—STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. STAT proteins are activated in response to a variety stimuli, including cytokines, and growth factors. STAT3 is first described in hepatocytes as a transcription factor, and capable of binding to the acute-phase response element in the promoter regions, leading to transcription of acute-phase genes (86-90). STAT3 activation is initiated by ligand-induced receptor dimerization, followed by activation of receptor-associated Janus kinase (JAK), which leads to STAT3 phosphorylation. Phosphorylation induces dimerization, and subsequent nuclear translocation, resulting in transcription of target genes (91). STAT3 knockout mice die during embryonic development, but studies from tissue or cell-specific depletion of the STAT3 gene have indicated that STAT3 might be involved in inflammatory reactions (92, 93). When compared with wild type littermates, mice selectively depleted of STAT3 in respiratory epithelial cells are more susceptible to hyperoxia-induced acute lung injury, which is linked to lung microvascular permeability and acute respiratory distress (94). In STAT3 deficient mice, a more rapidly progressive epithelial cell injury and inflammatory reactions are observed during hyperoxia-mediated ALI (94). However, intratracheal administration of surfactant protein B could attenuate features of lung injury and improve survival rates in STAT3 knockout mice, indicating STAT3-regulated expression of surfactant protein B at

transcription level protects mice from hyperoxia-induced ALI (94, 95). Additionally, in LPS-induced ALI, conditional depletion of STAT3 in respiratory epithelial cells in the lung results in decreased surfactant lipid synthesis, leading to increased inflammatory mediators' production and vascular permeability (96). Human beings with dominant-negative mutations in the STAT3 gene develop recurrent pulmonary infections, indicating that STAT3 plays an essential role in host defense against the lung infection (97). During Escherichia coli-induced pneumonias, mice selectively depleted of STAT3 in lung epithelial cells are more sensitive to Escherichia coli infection, evidenced by fewer neutrophil accumulation and more viable bacterial load (97). Furthermore, IL-6 and leukemia inhibitory factor-activated STAT3 in lung epithelial cells is indispensible for recruitment of neutrophils into lungs (97). Taken together, the above data show that STAT3 activation in lung epithelial cells inhibits acute pulmonary inflammation. A recent study displays that overexpression of suppressor of cytokine signaling 3 (SOCS3) in lung epithelial cells leads to increased production of proinflammatory mediators, and recruitment of neutrophils into lung interstitial and alveolar compartments, and ensuing exacerbated lung damages, which indicates a negative feedback regulation of JAK-STAT3 signaling cascades by SOCS3 (98).

During IgG IC-induced ALI, STAT3 are activated in alveolar macrophages, as well as whole lungs (62). IgG IC-mediated whole lung STAT3 activation is dependent on neutrophils and alveolar macrophages, because depletion of either neutrophils or alveolar macrophages significantly impairs its activation (62). Unlike NF-κB and AP-1, C5a are required for full activation of STAT3 in the lung (62). Interestingly, both IL-6 and IL-10 are necessary for the maximal activation of STAT3 (62). IL-6 is a proinflammatory

cytokine while IL-10 is an anti-inflammatory cytokine, indicating that STAT3 may participate in both proinflammatory and anti-inflammatory reactions during IgG IC-mediated ALI. Recent data show that in the presence of LPS, IL-6-induced STAT3 activation is greatly impaired by upregulated SOCS3 expression whereas IL-10-mediated STAT3 activation is not affected by SOCS3, and in the absence of SOCS3, IL-6 participates in anti-inflammatory responses by increased STAT3 activation (99). Because intrapulmonary deposition of IgG IC significantly induces SOCS3 expression, leading to reduced STAT3 activation mediated by IL-6, IL-6-induced STAT3 activation promotes IgG IC-stimulated inflammation (98). Our *in vitro* data demonstrate that STAT3 plays an important role in IgG IC-induced inflammatory responses by promoting proinflammatory mediators' generation in alveolar macrophages (93). Moreover, our *in vivo* studies further prove that knock down of STAT3 leads to reduced production of inflammatory mediators, pulmonary microvascular permeability, neutrophil accumulation, and ensuing lung injury after pulmonary deposition of IgG IC (93). Therefore, the exact role of STAT3 in pulmonary inflammation is dependent on specific diseases. By now, four different isoforms of STAT3 have been defined: STAT3α, STAT3β, STAT3γ, and STAT3δ (100, 101). Thus, more studies should be performed to investigate the individual activity of those four STAT3 isoforms during IgG IC-mediated ALI, and examine their relative contribution to inflammation and ensuing lung injury.

Roles of CCAAT/Enhancer-binding Proteins in Inflammation

CCAAT/enhancer-binding protein (C/EBP) family consists of six members: C/EBPα, β, γ, δ, ε, and ξ, which belong to basic region-leucine zipper (bZIP) transcription factors (102). The bZIP domain is located in a conserved sequence in the C-terminal 55-56

amino acid residues (103). In contrast to the bZIP domain in the conserved C-termini, the sequence identity in the N-termini of C/EBP proteins is less than 20%, which determines their different roles in transcription (103). bZIP domain contains a DNA binding region that is rich of basic amino acids, which is followed by the"leucine zipper" that is responsible for dimerization (104-116). C/EBP proteins exist in cells as homodimers or heterodimers, and the formation of heterodimers between different C/EBP proteins is due to the highly conserved bZIP domain. The C/EBP proteins interact with a consensus DNA sequences: 5'-T(T/G)NNGNAA(T/G)-3', and play roles in transcriptional activation or repression *via* the N-terminal domains (105, 107, 109, 117, 118). However, C/EBPξ is an exception, because it cannot bind to the identical recognition sequence as other family members due to the two proline residues in the basic region which prevent the formation of α-helical conformation (116). Because of the intact leucine zipper, C/EBPξ can still form heterodimers with other family members, and inhibit the regulation of target gene transcription by other C/EBP proteins. However, the heterodimers formed by C/EBPξ and other C/EBP proteins could bind to the promoter regions of various cellular stress-regulated genes, and promote their transcription (119). Thus, C/EBPξ might function as both transcriptional promoter and inhibitor, which is dependent on the stimuli. The *in vitro* binding activities of these C/EBP family members are almost the same, however, the diversity of C/EBP family members and their potential for forming different heterodimers can provide a large pool of transcription factors with complicated regulatory functions *in vivo*. All C/EBP family members could participate in inflammation by exerting influence on myeloid development or inflammation-related cytokine production, and play important roles in pulmonary diseases.

There are two C/EBPα isoforms—full-length C/EBPα with a molecular weight of 42 kDa (p42C/EBPα) and p30C/EBPα (30 kDa) (120). p30C/EBPα is produced by alternative use of translation initiation codons (120). C/EBPα is an important regulator of myeloid lineage commitment. This is because C/EBPα knock out newborn mice show a defect in differentiation of neutrophils, which is due to loss of the granulocyte colony-stimulating factor receptor's expression positively regulated by C/EBPα (121). However, it is found that the phenotype in C/EBPα deficient mice is different from that of granulocyte colony-stimulating factor receptor knockout mice, which suggests that other gene expressions that are involved in granulopoiesis are regulated by C/EBPα. It is demonstrated that expressions of IL-6 receptor and IL-6-responsive colony-forming unites, which contribute to granulopoiesis, are abrogated in mice depleted of C/EBPα (122). Interestingly, blocking of granulocyte colony-stimulating factor signaling has no effect on differentiation of macrophages (121). In adult mice, deleted C/EBPα in bone marrow thwarts the transition of the common myeloid progenitor to granulocyte-monocyte progenitor, thus, bone marrow is filled with myeloblasts, which is similar to human acute leukemia (123). In addition, deletion of C/EBPα in hematopoietic progenitor cells demonstrates hyperproliferation of the cells and inability of multipotential progenitors to differentiate into granulocyte/macrophage progenitors, leading to markedly diminished macrophages and neutrophils (124). All the myeloid development processes as mentioned above are involved in C/EBPα-induced PU.1 expression and interaction of C/EBPα with AP-1 and NF-κB (125).

C/EBPα, C/EBPβ and C/EBPδ expressions could be detected in bone marrow-derived macrophages (126). In the presence of LPS, DNA binding activity of both

C/EBPβ and C/EBPδ is increased while that of C/EBPα is reduced, indicating that C/EBPβ/C/EBPδ, and C/EBPα may have different role in inflammatory responses (126). Knockdown of C/EBPα significantly reduces IL-6 and TNF-α expressions in macrophages treated with LPS (127). In P388 lymphoblasts, ectopic expression of any C/EBP protein is sufficient to confer LPS-stimulated production of MCP-1 and IL-6 to the lymphoblasts (126). Because these lymphoblasts do not express C/EBP proteins, and are unresponsive to LPS treatment, the activities of C/EBPα, C/EBPβ and C/EBPδ are redundant, indicating that lack of C/EBPβ/C/EBPδ could be compensated by C/EBPα expression under certain inflammatory conditions (126). Upon infection and/or tissue damage, an inflammatory reaction called acute phase response is induced. During the acute phase response induced by intraperitoneal injection of LPS, expression of C/EBP family members is dramatically altered (128). C/EBPα production is significantly reduced while C/EBPβ and C/EBPδ expressions are markedly increased in the lung, indicating that C/EBP family members might be involved in regulation of acute phase gene expressions in lungs (128). Among the C/EBP family members, C/EBPβ and C/EBPδ are thought to function as the major stimulators of acute phase response (129). Further studies show that C/EBPα is also involved in the inflammatory process (129). Neonatal mice lacking C/EBPα is completely resistant to LPS- or IL-1β-mediated acute phase response when compared with wild type littermates, though compensatory increase of both C/EBPβ and C/EBPδ DNA binding activities is obvious in C/EBPα null mice (129). Thus, C/EBPα is indispensible for acute phase response in neonatal mice. C/EBP family members exist in cells as homodimers or heterodimers, the absolute requirement of C/EBPα for acute phase response might be explained by the essential roles of C/EBPα:C/EBPβ heterodimer and C/EBPα:C/EBPδ

heterodimer in the inflammatory process (129). In addition, in C/EBPα null mice, STAT3 DNA binding are not activated in response to LPS, indicating that C/EBPα-mediated STAT3 activation might be important for acute phase response (129).

Though C/EBPα null mice die shortly after birth, the role of C/EBPα during postnatal development has not been elucidated. Recent studies show that mice die about 30 days after total depletion of C/EBPα during postnatal development due to abnormal change in metabolic processes (130). C/EBPα deficient in lung epithelial cells also leads to about 56% death within hours after birth because of respiratory distress (131). The survival mice develop a severe pathological pattern similar to chronic obstructive pulmonary disease (COPD) (131). Pulmonary histological assay of C/EBPα knockout mice demonstrates that type Ⅱ lung epithelial cells undergo hyperproliferaion and alveolar architecture is disturbed, which suggests an essential role for C/EBPα in lung development (132). To understand the role of C/EBPα in lungs, use of siRNA targeting C/EBPα is an acceptable method. Exposure of newborn mice to 95% oxygen leads to enhanced C/EBPα expression which is primarily derived from type Ⅱ lung epithelial cells (133). Although hyperoxia results in increased differentiation and expression of markers for type Ⅰ lung epithelial cells, C/EBPα-specific siRNA treatment alleviates these hyperoxic features with an exception of lung morphologic change (133). Nevertheless, lung morphology is restored in C/EBPα siRNA-, but not control siRNA-treated mice after 2-week recovery in normal air, which suggests that C/EBPα expression in type Ⅱ lung epithelial cells is responsible for cell proliferation and differentiation during pulmonary damage and repair (133). Pulmonary abnormalities are not observed in the transgenic mice, in which C/EBPα is

genetically depleted from Clara cells and type Ⅱ lung epithelial cells after birth, indicating that C/EBPα is critical for early pulmonary development but not required for maintenance of lung architecture after birth (96). When compared with wild type adult mice, the transgenic mice are more susceptible to hyperoxia-induced ALI, which is due to severe pulmonary inflammatory responses, abnormal pulmonary mechanics, reduced mature surfactant protein B and C, and decreased surfactant lipids (96). Overall, C/EBPα plays different roles in newborn and adult mice during hyperoxia-induced ALI. Whereas newborn mice benefit from downregulation of C/EBPα expression, adult mice benefit from upregulation of C/EBPα expression, indicating that specific tissue levels of C/EBPα induce beneficial or detrimental effects (133). Immunohistochemistry staining shows that expression of C/EBPα could be found in alveolar macrophages and type Ⅱ lung epithelial cells (134). During bleomycin-induced pulmonary fibrosis, C/EBPα expression is significantly induced in some of alveolar epithelial cells and fibroblasts in the fibrotic lesions (134). C/EBPα is involved in proliferation of type Ⅱ lung epithelial cells and expressions of surfactant proteins, including surfactant A, B, and C (135). Lung specific deletion of C/EBPα leads to increased generation of surfactant proteins and hyperproliferation of alveolar type Ⅱ cells (135). Thus, bleomycin-induced upregulation of C/EBPα expression in alveolar epithelium and fibroblasts might be considered as an endogenous anti-fibrotic factor. In LPS-treated lungs, C/EBPα expression is also significantly increased, indicating that C/EBPα may have a proinflammatory effect due to its inhibitory influence on generation of surfactant proteins B that is necessary for

suppression of LPS-mediated lung injury (134, 136). Overall, C/EBPα can function as a disease suppressor or stimulator, which is dependent on specific diseases.

C/EBPβ can give rise to three polypeptides, 38 kDa [liver-enriched transcriptional activator protein* (LAP*)], 35 kDa (LAP), and 20 kDa [liver-enriched inhibitory protein (LIP)], among which LAP and LIP are the major C/EBPβ isoforms found in the cells (137). The three different isoforms of C/EBPβ could be generated from the same mRNA by alternative use of translation initiation codons (137). LAP contains both transactivation domain and bZIP domain, thus, it can act as a transcription stimulator (137). bZIP domain but not activation domain is present in LIP, therefore, it could function as a transcription repressor by forming homodimers or heterodimers with other C/EBP family proteins (137). The function of C/EBPβ has been extensively investigated, and its main role is implicated in the regulation of proinflammatory mediators' production induced by ligands of Toll-like receptors (TLRs), IL-1, and IL-6 (109, 110, 138). C/EBPβ plays an essential role in heat shock protein 60-induced cyclooxygenase-2 expression in macrophages, as well as endothelial cells. Because heat shock protein 60 is an endogenous ligand of TLR4, C/EBPβ links TLR4 activation to inflammatory mediator's expression (139). Many gene promoter regions contain C/EBPβ binding site. For example, it has been shown that the activity of a reporter gene controlled by IL-6 promoter is greatly stimulated by C/EBPβ (138). All functions of C/EBPβ discussed above focus on its role in boosting proinflammatory responses; however, evidence has shown that the C/EBP family protein is also dedicated to suppression of inflammatory reactions. For example, cooperation between Sp1 and C/EBPβ can mediate the IL-10 gene expression in mouse macrophage RAW264.7 challenged with LPS (140). C/EBPβ inhibits expressions of inflammatory mediators in

TNF tolerant monocytes by interacting with NF-κB p65 and preventing its phosphorylation (141). In addition, the expression of Clara cell secretory protein (CCSP) that has anti-oxidant effects is inhibited by hyperoxia, which is mediated, at least in part, at transcription level by increased expression of LIP (142). To investigate the *in vivo* role of C/EBPβ, mice depleted of C/EBPβ are generated. When compared with wild type mice, C/EBPβ knockout mice are more sensitive to infection by Listeria monocytogenes due to defect in macrophage bactericidal activity (143). However, TNF and IFN-γ expressions are not impaired in C/EBPβ deficient mice, indicating that the role of C/EBPβ in cytokine expression might be compensated by other C/EBP proteins, such as C/EBPα and C/EBPε (143).

Higher C/EBPβ expression is found in the fibrotic lesions during bleomycin-induced pulmonary fibrosis (134). *In vitro* studies show that C/EBPβ plays an important role in myofibroblast differentiation, indicating that C/EBPβ might be a key regulator of fibrosis (144). *In vivo* data demonstrate that C/EBPβ knockout leads to decreased collagen deposition and fibrotic lesions in the lung during bleomycin-induced pulmonary fibrosis (144). Further studies on mechanisms by which knockout of C/EBPβ attenuates fibrosis suggest that when compared with C/EBPβ deficient mice, the wild type counterparts express more inflammatory mediators after intratracheal instillation of bleomycin (144). In addition, pulmonary fibroblasts lacking C/EBPβ generate less α-smooth muscle actin, indicating that myofibroblast differentiation is positively regulated by C/EBPβ (144). Moreover, pulmonary fibroblasts isolated from wild type mice display less proliferative rates than those from C/EBPβ$^{-/-}$ mice. Overall, C/EBPβ plays multifactorial roles in lung fibrosis by regulating inflammatory mediators' production,

fibroblast proliferation, and differentiation (144). Although C/EBPβ knockout protects against bleomycin-induced pulmonary fibrosis, the molecular mechanisms by which C/EBPβ positively regulates fibrosis remain largely unknown. Recent studies demonstrate that C/EBPβ carrying a single point mutation (Thr217 to Ala217) can block phosphorylation of C/EBPβ on Thr217 by ribosomal S-6 kinase (RSK), leading to attenuated pulmonary injury and fibrosis induced by intratracheal administration of bleomycin (145). In addition, *in vitro* studies find that ERK1/2 is an upstream stimulator of RSK, and is required for phosphorylation of C/EBPβ on Thr217 (145). Phosphorylated C/EBPβ is associated with procaspase 8, leading to inactivation of the apoptotic pathway in pulmonary fibroblasts (145). Moreover, in human patients, phosphorylation of C/EBPβ on Thr266 (identical to mouse phosphoacceptor Thr217) is observed in activated pulmonary fibroblasts, which could not be detected in control groups, indicating that the signaling pathway—ERK1/2-RSK-C/EBPβ, which can prevent fibroblast from apoptosis, might be involved in human pulmonary fibrosis (145). Interestingly, a block peptide, which can suppress phosphorylation of C/EBPβ on Thr217, alleviates bleomycin-mediated pulmonary fibrosis, indicating its possible clinical applications (145). *In vitro* data show that different C/EBPβ isoforms may have distinct roles in fibrosis. IL-1β suppresses α-smooth muscle actin in rat lung fibroblasts by increasing the ratio of LIP to LAP (146). LIP-mediated inhibition of α-smooth muscle actin expression is initiated at transcription level due to its ability to binding to the consensus sequence in the α-smooth muscle actin promoter region (146). However, LAP is a stimulator of α-smooth muscle actin expression, because α-smooth muscle actin is elevated by ectopic expression of LAP while reduced by knockout of C/EBPβ (146). Further studies on mechanisms by which IL-1β augments LIP

expression suggest that IL-1β treatment induces the generation of eukaryotic initiation factor 4E that plays a critical role in LIP expression (146). Immunohistochemistry staining shows that expression of C/EBPβ could be found in alveolar macrophages and type Ⅱ lung epithelial cells (134). In LPS-challenged lungs, C/EBPβ expression is found in alveolar macrophages, alveolar epithelium, and other inflammatory cells (134). When compared with wild type mice, mice with lung specific depletion of C/EBPβ express less KC in response to intratracheal administration of LPS, leading to reduced recruitment of neutrophils into lungs (147). Therefore, C/EBPβ expression in lung epithelial cells contributes to LPS-induced acute lung inflammation by increasing neutrophil influx. However, C/EBPβ is also necessary for anti-inflammatory responses induced by formoterol—a β_2-adrenergic agonist, because inhibition of LPS-mediated neutrophilia by formoterol is alleviated in mice lacking C/EBPβ in pulmonary epithelial cells by increasing KC production (147). The proinflammatory and anti-inflammatory effects of C/EBPβ might be explained by preferential expressions of different C/EBPβ isoforms induced by different stimulants. LPS treatment may induce expression of LAP—a transcription stimulator, while formoterol preferentially promotes expression of LIP, which is a repressor of transcription due to its lacking of transactivation domain located in N-terminus.

The role of C/EBPδ in inflammatory responses has also been investigated. Many gene promoter regions contain C/EBPδ binding sites. For example, it has been shown that the activity of a reporter gene controlled by IL-6 promoter is greatly stimulated by C/EBPδ (148). In addition, LPS-induced binding of C/EBPδ to the consensus sequence is indispensible for cyclooxygenase-2 transcription in macrophages (149). However,

evidence has shown that C/EBPδ is also involved in anti-inflammatory mediators' generation. For example, C/EBPδ expression is significantly increased in β-cells and human islets incubated with IL-1β and IFN-γ, leading to decreased production of chemokines in β-cells. In the lungs, immunohistochemistry staining shows that C/EBPδ expression is restricted to some of type Ⅱ lung epithelial cells in a spatial pattern in the normal lung (134). Intensive expression of C/EBPδ is observed in some of alveolar and bronchiolar epithelium in the fibrotic lesions during bleomycin-induced pulmonary fibrosis (134). In LPS-challenged lungs, higher C/EBPδ expression is found in proliferating alveolar and bronchiolar epithelial cells (134). C/EBPδ expression is significantly increased during differentiation of human feta lung in culture, and is mainly produced by alveolar epithelial cells (150). Moreover, its production can be induced by cyclic adenosine 3', 5'-monophosphate (cAMP) and glucocorticoids (150). Glucocorticoids play an important role in pulmonary development and maturation by regulating different gene expressions, among which P450-enzyme CYP2B1 synthesis is significantly induced by glucocorticoids in lung epithelial cells (151). Further studies show that glucocorticoids induce its expression by enhancing binding of C/EBPβ and C/EBPδ to its promoter region (151). In addition, C/EBPδ induced-Clara cell secretory protein (CCSP) expression in lung epithelial cells is also involved in binding of C/EBPδ to its consensus sequence in the proximal CCSP promoter (151, 152). Furthermore, it is found that in pulmonary epithelium, C/EBPδ preferentially interacts with C/EBPα to form heterodimers that can superinduce the gene expression driven by CCSP promoter (152). Secretoglobin 3A2, a secreted protein with small molecular weight, is primarily generated in lung airways, and its transcription is also dependent on C/EBPα and C/EBPδ (153).

C/EBPδ expression is significantly induced in lungs and kidneys at both mRNA and protein levels during endotoxin-induced disseminated intravascular coagulation (DIC), indicating that C/EBPδ might play a role in DIC (154). Further studies demonstrate that C/EBPδ knockout partially protects mice from DIC-mediated death by downregulation of systematic inflammation, as evidenced by decreased TNF-α and IL-6 levels in circulation (154). In addition, DIC-induced kidney injury is impaired in C/EBPδ deficient mice (154). Moreover, ischemia/reperfusion-induced acute kidney injury is also alleviated in C/EBPδ knockout mice, indicating that C/EBPδ plays an essential role in inflammation-related acute kidney injury (154). However, DIC-induced liver injury is exacerbated in C/EBPδ null mice, which suggests that C/EBPδ plays different roles in distinct organs, and explains the partially protective effect of C/EBPδ during LPS-induced DIC (154).

C/EBPε expression is restricted to lymphocytes and myeloid cells including macrophages and granulocytes (155-157). C/EBPε can give rise to four polypeptides with molecular weight of 32 kDa, 30 kDa, 27 kDa, and 14 kDa, respectively. The four different isoforms of C/EBPε could be produced by differential splicing and use of two alternative promoters (157, 158). 32 kDa and 30 kDa forms contain both transactivation domain and bZIP domain, thus, they can act as transcription stimulators (157, 158). bZIP domain but not activation domain is present in 14 kDa form, therefore, it could function as a transcription repressor (157, 158).

C/EBPε is an important regulator of myeloid lineage commitment. Morphologically, macrophages lacking C/EBPε expression is immature (159). Additionally, C/EBPε deficient macrophages show reduced expression of macrophage markers, and defect in phagocytic function due to decreased uptake of opsonized yeast

when compared with wild type macrophages (159). Analysis of different gene expression profiles between wild type and C/EBPε knockout macrophages demonstrates that a subset of gene expressions related to inflammatory reaction and differentiation are significantly reduced in C/EBPε deficient macrophages (159). Overall, as a transcription factor, C/EBPε is necessary for macrophage development and subsequent normal functions in inflammation. C/EBPε null mice develope normally but are susceptible to opportunistic infections by 3-5 months of age due to lack of functional neutrophils, indicating that C/EBPε is required for terminal differentiation and functional maturation of granulocyte progenitor cells (160, 161). *In vivo* studies demonstrate that neutrophils deficient in C/EBPε show reduced bactericidal activities due to defect in uptake of opsonized bacteria as well as generation of secondary granule proteins (161). In addition, decreased recruitment of C/EBPε deficient neutrophils into the local inflammatory site is observed, which is because of abnormal expressions of L-selectin and CD11b integrin on PMNs (161). Moreover, C/EBPε deficient neutrophils display different cytokine expression profile when compared with wild type neutrophils in response to inflammatory stimulation, as evidenced by reduced production of interleukin-1 receptor antagonist (IL-1Ra) but augmented expression of TNF-α (161). Interestingly, spontaneous expression of TNF-α is also elevated in neutrophils deleted of C/EBPε, indicating the C/EBPε might be involved in autoimmune diseases (161). Overall, the normal functions are severally impaired in C/EBPε knockout neutrophils, leading to the abnormal immune reactions to inflammatory challenges. Therefore, C/EBPε is necessary for generation of functional macrophages and neutrophils, which is important for host defense against infections.

To examine if C/EBPβ and C/EBPε could be compensated for each other, double knockout mice are constructed. Though C/EBPβ and C/EBPε double knockout mice are smaller than single deficient and wile type mice, morphological assay shows that lungs are normally developed in the double knockout mice (162). However, mice deficient in C/EBPβ and C/EBPε are sensitive to fatal infections and die with 2-3 months due to defective differentiation of neutrophils (162). Furthermore, cDNA microarray analysis demonstrates that LPS- and IFNγ-induced gene expressions involved in immune reactions are inhibited in bone marrow-derived macrophages from the double knockout mice when compared those from the single knockout mice (162). Taken together, both C/EBPβ and C/EBPε are important for innate immune responses, and could be partially compensated for each other in the single knockout mice.

C/EBPγ is an ubiquitously expressed member of the C/EBP family. It contains a C/EBP-like bZIP region; however, it lacks an amino terminal transactivation domain (163, 164). Consistent with the truncated structure, it has been considered as a "buffer" for C/EBP activator (164). Therefore, it can counteract transcriptional activation of other C/EBP family members such as C/EBPα or C/EBPβ (164). For example, C/EBPγ represses C/EBPβ-mediated human immunodeficiency virus (HIV)-1 long terminal repeat-driven transcription in human brain cells (165). Ethanol induction of class Ⅰ alcohol dehydrogenase expression in the rat requires binding of C/EBPβ to its consensus site in the promoter region, which is inhibited by C/EBPγ (166). In reporter assays, it has been demonstrated that C/EBPδ cooperates with EKLF-a CACC-box binding protein, to enhance the beta-globin promoter-driven reporter gene expression, while C/EBPγ inhibits the transcription activity of EKLF (167). In contrast to the notion of C/EBPγ as an inhibitor,

C/EBPγ is also characterized as an activator in recent studies. C/EBPγ is involved in the transcriptional upregulation of beta-globin gene in murine erythroleukemia cells (168). C/EBPγ is also a positive regulator of IFN-γ expression in splenocytes and natural killer (NK) cells (169), and gamma-globin in fetal liver (170). Furthermore, it has been reported that C/EBPγ inhibits reporter gene expression in L cell fibroblasts, but it is an ineffective repressor in HepG2 hepatoma cells, demonstrating that C/EBPγ modulates gene expression in a cell-specific manner (171). Taken together, these findings indicate that C/EBPγ has complex effects on gene transcription, and its role remains enigmatic.

The role of C/EBPγ in inflammation is largely unknown. Previous study suggests that C/EBPγ dramatically enhances the activity of C/EBPβ in LPS-mediated IL-6 promoter-driven reporter gene expression in a B lymphoblast cell line (102). Recently, we find that C/EBPγ expression is induced by IL-1β in alveolar type Ⅱ cells, and contributes to the inhibition of IL-1β-mediated IL-6 production (172). Further, we demonstrate that C/EBPγ inhibits IL-6 expression by forming homodimers that compete with C/EBPβ:C/EBPγ heterodimers to bind to the consensus sequence in the IL-6 promoter region (172). However, the binding of NF-κB to IL-6 promoter region is not impaired by C/EBPγ (172). The data indicate that C/EBPγ suppresses IL-1β-induced IL-6 expression in alveolar type Ⅱ cells by inhibiting C/EBPβ:C/EBPγ heterodimers but not NF-κB binding to the IL-6 promoter region (172).

C/EBPζ, also known as C/EBP homologous protein (CHOP), is induced in response to endoplasmic reticulum (ER) stress. In airway epithelial cells, LPS-induced CHOP expression is positively regulated by MUC5AC. Knockdown of MUC5AC significantly reduces CHOP production and protects against ER stress-induced apoptosis.

Mice receiving intratracheal injection of LPS express higher level of mRNAs for CHOP when compared with control mice, which suggests that ER stress-induced CHOP expression might play a critical role in LPS-mediated ALI (173). Indeed, *in vivo* studies show that LPS-induced acute pulmonary inflammation is attenuated in CHOP deficient mice, as evidenced by reduced mature IL-1β level and neutrophil accumulation (173). Further studies demonstrate that CHOP is indispensible for LPS-induced caspase-11, which is necessary for activation of pro-IL-1β (173). Overall, CHOP positively regulates LPS-induced pathogenesis of pulmonary inflammation by inducing caspase-11 expression. During LPS-induced acute endotoxemia, C/EBP activation is evident only in alveolar epithelial type Ⅱ cells (174). CHOP expression is significantly increased due to elevated activating transcription factor 3 and X-box binding protein 1 levels, suggesting that CHOP might play an essential role in endotoxin-induced septic shock (175). Furthermore, *in vitro* studies show that overexpression of CHOP induces apoptosis in lung cells, and *in vivo* data demonstrate that CHOP is required for apoptotic death of lung cells during LPS-induced septic shock, indicating that ER stress-induced CHOP expression plays an essential role in lung pathogenesis (175). The molecular mechanisms underlying idiopathic pulmonary fibrosis (IPF) are largely unknown. Recent data show that CHOP expression is significantly induced in the whole lung and alveolar epithelial type Ⅱ cells, leading to expression of proapoptotic, oligomeric forms of Bcl-2–associated X (Bax) protein that accelerates apoptosis of alveolar epithelial type Ⅱ cells and development of fibrosis in patients with sporadic IPF (176). Therefore, ER stress mediated-CHOP expression in alveolar epithelial type Ⅱ cells plays an essential role in fibrosis by inducing apoptosis of

this cell type. Hypersecretion of prostaglandin E2 induces IL-8 generation in cystic fibrosis bronchial epithelial cells, which is positively regulated by CHOP at transcription level (177). Because IL-8 is a powerful chemoattractant for neutrophils that play a critical role in inflammatory reactions in cystic fibrosis, CHOP enhances development of cystic fibrosis by increasing IL-8 transcription and subsequent neutrophil accumulation (177). Therefore, IPF is worsened by CHOP expression-mediated apoptosis of alveolar epithelial type Ⅱ cells and neutrophil accumulation.

However, CHOP expression can also have protective effects against acute lung injury. Both *in vitro* and *in vivo* studies show that CHOP expression is significantly increased by hyperoxia, which is dependent on double-stranded RNA-activated protein kinase pathway, but independent on p53 and ER stress-mediated signaling cascades (178, 179). CHOP expression is restricted to the bronchiolar epithelial cells, which is demonstrated by in site hybridization and immunohistochemistry (179). When compared with wild type mice, CHOP knockout mice are more sensitive to hyperoxia-induced pulmonary injury, as evidenced by increased lung edema and permeability (178). Therefore, increased CHOP expression induced by hyperoxia protects mice from hyperoxia-mediated acute pulmonary injury by boosting DNA repair (178-180).

Experimental Hypotheses

C/EBPs α, β, δ, ε, γ, and ζ comprise a family of basic region-leucine zipper transcription factors that dimerize through a leucine zipper and bind to DNA through an adjacent basic region (181). Among them, C/EBPβ and C/EBPδ appear to be effectors in the induction of genes responsive to LPS, IL-1, or IL-6 stimulation, and have been implicated in the regulation of inflammatory mediators as well as other gene products

associated with the activation of macrophages and the acute-phase inflammatory response (126, 182-187). Both C/EBPβ and C/EBPδ are expressed in the lung, and agents including classical acute-phase stimuli as well as those causing acute lung injury such as bleomycin and oxidative stress have been shown to increase the levels of C/EBPβ and C/EBPδ in the lung (134, 188). However, the signals that mediate the activation of C/EBPβ and C/EBPδ and their functions in macrophages and lungs during inflammation remain largely unknown. A recent study demonstrates that C/EBPβ in the airway epithelium mediates inflammatory and innate immune responses in lung to cigarette smoke (189). C/EBPβ is also reported to play an essential role in bleomycin-induced pulmonary fibrosis (144). These observations suggest that C/EBPβ and/or C/EBPδ may play important roles during lung inflammation. However, no information exists about the role of C/EBPβ and C/EBPδ in acute lung injury

The IgG immune complex model in the rodent lung has been employed to determine the molecular mechanisms of acute lung inflammatory injury (98, 190). In this model, intra-alveolar deposition of IgG immune complexes results in an acutely damaging process that affects capillary endothelial cells and alveolar epithelial cells, and also stimulates alveolar macrophages. Activation of alveolar macrophages results in formation of the early response cytokines such as TNF-α and IL-6, which induce expression of adhesion molecules on leukocytes and on endothelial cells/epithelial cells, all of which trigger a strong proinflammatory cascade. The induction of cytokine and chemokine gene expression involves the actions of specific transcription factors. For example, the activation of NF-κB and AP-1 has been observed in IgG immune complex-injured lung (61, 63). However, their functions in IgG immune complex-induced lung injury are only

speculative, and no definitive study demonstrates their pivotal roles in the development of lung inflammatory responses. Our recent study indicates that STAT3 may play an important role in IgG immune complex-induced inflammation in macrophages and lungs (93), but the molecular mechanisms responsible for this observation remain to be defined. In this regard, C/EBPβ and C/EBPδ have been demonstrated to be targets of STAT3 (191, 192).

The central hypothesis is that the activities of C/EBPβ and C/EBPδ are induced in immune complex-induced inflammation, and they may play an important regulatory role in FcγR-mdeiated inflammatory responses.

Problems

The FcγRs are a family of cell surface molecules that bind the Fc portion of IgG immunoglobulins. Different cell types bear different sets of FcγRs, and their specificities for various antibody classes thus determine which types of cells will be engaged in given responses. Four types of FcγRs are identified in mice: FcγRⅠ, FcγRⅡB, FcγRⅢ, and the newest member, FcγRⅣ. FcγRⅠ, FcγRⅢ, and FcγRⅣ are activating receptors, all of which associate with FcR common γ-chain, whereas FcγRⅡB is an inhibitory member. The corresponding human FcγRs are FcγRⅠA, FcγRⅡA, FcγRⅢA (activating), and FcγRⅡB (inhibitory) receptors. Human FcγRⅠA and mouse FcγRⅠ have high affinity for IgG. Based on the sequence similarity, human FcγRⅡA is most closely related to mouse FcγRⅢ, and human FcγRⅢA seems to be the orthologue of mouse FcγRⅣ (6). However, human FcγRⅡA has an immunoreceptor tyrosine-based activation motif in its cytosolic domain, which is not present in mouse FcγRⅢ and does not require γ-chain for

functionality (6). Macrophages, the sentinels of innate immunity, play key roles in inflammation. Activation of macrophages by FcγRs triggers a wide variety of cellular responses (6, 193). In addition to phagocytosis, one of the major cellular responses in macrophages initiated by IgG immune complex (IC)-mediated FcγR cross-linking is the activation of genes encoding chemokines and cytokines important in inflammation. Although the production of these mediators is mainly controlled at the transcriptional level in response to various inflammatory stimuli, the key nuclear molecules that mediate FcγR signaling in macrophages remain unknown. Several transcription factors, such as NF-κB, AP-1, C/EBPβ, and STAT3, are activated in macrophages/monocytes following IgG IC stimulation (10, 194). Studies using pharmacological inhibitors suggest that NF-κB activation is involved in the expression of several chemokines, including MIP-1α and MIP-1β, upon cross-linking of FcγR (194, 195). However, these lines of evidence are either indirect or correlative. Therefore, additional studies are necessary to elucidate the molecular mechanisms whereby the expression of inflammatory mediators is induced by FcγRs.

C/EBPα, β, δ, ε, γ, and ζ comprise a family of basic region-leucine zipper (bZIP) transcription factors that dimerize through a leucine zipper motif and bind to DNA through an adjacent basic region. C/EBPβ and -δ have been implicated in the regulation of inflammatory mediators as well as other gene products associated with the activation of macrophages and the acute phase inflammatory response (196). Two major forms of C/EBPβ are translated from the same messenger RNA, LAP and LIP (137). The roles of C/EBP family members in regulating inflammation have also been studied using knockout mice. Interestingly, the LPS stimulation of peritoneal macrophages from C/EBPβ deficient

mice leads to normal induction of several inflammatory cytokines, including IL-6 and TNF-α, with the exception of granulocyte colony-stimulating factor (G-CSF), Mincle, and microsomal prostaglandin E synthase-1 (mPGES-1) (143, 184, 197, 198), although macrophages from these mice demonstrate defective intracellular bacteria killing. Similarly, C/EBPδ deficient macrophages show nominal defects in IL-6 and TNF-α production in response to several TLR ligands (199). In contrast, the absence of both C/EBPβ and -δ results in a significant decrease in the TLR ligand-induced production of both IL-6 and TNF-α (199). In another study, Gorgoni *et al.* find that LPS-induced expression of IL-1β, TNF-α, IL-6, and inducible nitric-oxide synthase is partially impaired in C/EBPβ deficient macrophages and that expression of IL-12 p35 is completely defective (183). However, IL-12 p40, RANTES, and MIP-1β are more efficiently induced in response to IFN-γ and LPS in the absence of C/EBPβ (183). These results raise the possibility that the redundant expression of multiple C/EBP isoforms as well as differences in C/EBP homo-/heterodimer occupancy in specific gene promoters may account for the differential effects of C/EBPβ and -δ deficiency. Thus, the role of C/EBPβ and -δ in regulating transcription of the different inflammation-regulated mediators warrants further investigation. Furthermore, whether and how C/EBPβ and -δ are involved in the FcγR-induced inflammatory response remains unclear, and the signals that mediate the activation of C/EBPβ and –δ are unknown. Furthermore, the roles of C/EBPβ and C/EBPδ in IgG IC-induced acute lung injury are not known.

CHAPTER II

METHOD

Cells and Reagents

RAW264.7 cells (ATCC; TIB-71™) and MH-S cells (ATCC; CRL-2019™) were obtained from the American Type Culture Collection (Manassas, VA). RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% FBS, 2 mM L- glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and maintained in a humidified incubator at 37°C with 5% CO_2; MH-S cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.01 M HEPES, and maintained in a humidified incubator at 37°C with 5% CO_2. ΨCREJ2 cells (a producer cell line for the J2 retrovirus (200) kindly provided by H. Young) were maintained in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS; Hyclone, Rockford, IL), 200 mM L-glutamine, and 100 units/ml penicillin-streptomycin (Invitrogen). Transformed C/EBP deficient macrophage cell lines were generated from bone marrow of mice lacking C/EBPβ and/or C/EBPδ as well as their wild type littermates (201, 202). Bone marrow was collected from the femurs and tibias and placed into phosphate-buffered saline (PBS) containing 2% FBS. The bone marrow plugs were disaggregated and pelleted, and red blood cells were lysed using NH_4Cl lysis solution (Sigma). The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 ng/ml macrophage

colony-stimulating factor (Pepro- tech, Rocky Hill, NJ), 200 mM L-glutamine, 100 units/ml penicillin-streptomycin. After 24 h, macrophages suspended in the medium were collected by centrifugation. To generate immortalized macrophage cell lines, 1–5 × 10^7 BM cells were resuspended in ΨCREJ2 cell supernatant supplemented with 8 μg/ml Polybrene (Sigma) and 100 ng/ml macrophage colony-stimulating factor. ΨCREJ2 cell supernatant was removed after 24 h, and cell lines were maintained in DMEM supplemented with 10% heat-inactivated FBS, 100 ng/ml macrophage colony-stimulating factor, 200 mM L-glutamine, 100 units/ml penicillin-streptomycin. Rabbit anti-BSA IgG was purchased from ICN Biomedicals (Solon, OH). ELISA kits for mouse IL-6, TNF-α, MIP-2, KC, MIP-1α, MIP-1β, and soluble ICAM-1 (sICAM-1) were obtained from R&D Systems (Minneapolis, MN). p38 MAPK inhibitor Ⅷ and ERK1/2 inhibitor, U0126, were purchased from EMD Biosciences (Gibbstown, NJ). C5a and BSA were purchased from Sigma-Aldrich.

Expression Vectors and Promoter Reporters

The mouse IL-6 promoter-reporter (-250 to +1), TNF-α promoter-reporter containing sequences extending to -1260 bp, C/EBPβ expression vector as well as the C/EBPδ expression vector are kindly provided by Richard C. Schwartz (Michigan State University). The reporter plasmid (2 × C/EBP-Luc) containing two copies of a C/EBP binding site is kindly provided by Peter F. Johnson (NCI-Frederick). NF-κB promoter-reporter was obtained from Promega, Madison, WI.

Luciferase Assay

Transient transfections were performed with RAW264.7 or MH-S cells plated in 12-well plates by using 0.5 μg of DNA and 1.5 μl of Fugene6 transfection reagent (Roche

Applied Science) in 50 μl of Opti-MEM I medium (Invitrogen). 24 h after transfection, the cells were treated either with or without 100 μg/ml IgG IC for the indicated time periods. Then cell lysates were subjected to luciferase activity analysis by using the Dual-Luciferase reporter assay system (Promega, Madison, WI). IgG IC were formed by the addition to anti-BSA of BSA at the point of antigen equivalence as described previously (203).

siRNA Transfection

Transient siRNA transfections were performed by transfecting 1-2 × 10^6 cells with 600 nM control siRNA or C/EBPβ/δ siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) using the Amaxa® nucleofector kit V. 12 h later, the cells were treated with or without 100 μg/ml IgG IC for the indicated time periods. Then supernatants were collected for ELISA analysis.

Peritoneal Macrophage Isolation and Culture

8–9-Weekold specific pathogen-free male C57BL/6 mice were obtained from Jackson Laboratories. FcRγ- and FcγR Ⅱ-deficient mice were obtained from Taconic. C5aR knock-out mice were kindly provided by Craig Gerard (Harvard Medical School). C/EBPβ$^{-/-}$ and C/EBPδ$^{-/-}$ mice have been described previously (201, 202). Mouse peritoneal macrophages were isolated by using the thioglycollate method. Briefly, mice were injected intraperitoneally with 1.5 ml of 2.4% sterile thioglycollate dissolved in double distilled H_2O. Peritoneal macrophages were obtained by instillation and aspiration of 10 ml of PBS 4 days after thioglycollate administration. The macrophages were maintained in DMEM supplemented with 5% FBS and allowed to adhere for 2 h, followed by 100 μg/ml IgG IC treatment for the indicated time periods. The supernatants were

harvested and subjected to ELISA analysis. The purity of the cell suspension was about 99%, which was determined by staining of the peritoneal cell suspension with the HEMA 3® Stain Set (Fisher).

Alveolar Macrophage Isolation and *in vitro* IgG IC 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 (2-2.5 × 10^4/well) were allowed to adhere at 37°C for 1 h, and nonadhering cells were removed, followed by 100 μg/ml IgG IC treatment for the indicated time periods. The supernatants were harvested and subjected to ELISA analysis. The purity of the cell suspension was about 96%, which is determined by staining of BAL cells with HEMA 3® Stain Set.

ELISA

The cell supernatants or BAL fluids were centrifuged at 3000 rpm for 5 min, and the cell-free supernatants or BAL fluids were harvested for IL-6, TNF-α, MIP-2, KC, MIP-1α, MIP-1β, and soluble ICAM-1 (sICAM-1) measurements according to the manufacturer's protocols.

RNA Isolation and Analysis by Semiquantitative RT-PCR

Total RNAs were extracted from cells with TRIzol (Invitrogen) according to the manufacturer's procedure. After isolation, total cellular RNA was incubated with RQ1 RNase-free DNase (Promega, Madison, WI) to remove contaminating DNA. 2 μg of total RNA was submitted to reverse transcription by using Superscript Ⅱ RNase H reverse transcriptase (Invitrogen). PCR was performed with the following primers: for C/EBPβ, 5'

primer (5’-CAA GCT GAG CGA CGA GTA CA-3’) and 3’ primer (5’- AGC TGC TCC ACC TTC TTC TG-3’); for C/EBPδ, 5’ primer (5’-CGC AGA CAG TGG TGA GCT T-3’) and 3’ primer (5’-CTT CTG CTG CAT CTC CTG GT-3’); for TNF-α: 5’ primer, 5’- CGT CAG CCG ATT TGC TAT CT -3’ and 3’ primer, 5’- CGG ACT CCG CAA AGT CTA AG -3’; for MIP-1α: 5’ primer, 5’- ATG AAG GTC TCC ACC ACT GC -3’ and 3’ primer, 5’- CCC AGG TCT CTT TGG AGT CA -3’; for MIP-2: 5’ primer, 5’- AGT GAA CTG CGC TGT CAA TG -3’ and 3’ primer, 5’- TTC AGG GTC AAG GCA AAC TT -3’for GAPDH, 5’ primer (5’-GCC TCG TCT CAT AGA CAA GAT G-3’) and 3’ primer (5’-CAG TAG ACT CCA CGA CAT AC-3’). After a “hot start” for 5 min at 94 °C, 28–33 cycles were used for amplification with a melting temperature of 94 °C, an annealing temperature of 60 °C, and an extending temperature of 72 °C, each for 1 min, followed by a final extension at 72 °C for 8 min. PCR was performed using different cycle numbers for all primers, to ensure that DNA was detected within the linear part of the amplifying curves for both primers.

RNA Isolation and Analysis by Real-time PCR

Total RNAs were extracted from lungs with TRIzol, according to the manufacturer’s procedure. After isolation, total cellular RNA was incubated with RQ1 RNase-free DNase 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. 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: AmpliTaq 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, ICAM-1, VCAM-1, KC, MIP-1β, 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'-CGG ACT CCG CAA AGT CTA AG-3'; KC, 5' primer, 5'-GCT GGG ATT CAC CTC AAG AA-3' and 3' primer, 5'-TGG GGA CAC CTT TTA GCA TC-3'; IL-6, 5' primer, 5'-AGT TGC CTT CTT GGG ACT GA-3' and 3' primer, 5'- TCC ACG ATT TCC CAG AGA AC-3'; MIP-2, 5' primer, 5'-AGT GAA CTG CGC TGT CAA TG-3' and 3' primer, 5'-TTC AGG GTC AAG GCA AAC TT-3'; MIP-1α, 5' primer, 5'-ATG AAG GTC TCC ACC ACT GC-3' and 3' primer, 5'-CCC AGG TCT CTT TGG AGT CA-3'; MIP-1β, 5' primer, 5'-GCC CTC TCT CTC CTC TTG CT-3' and 3' primer, 5'-GTC TGC CTC TTT TGG TCA GG-3'; ICAM-1, 5' primer, 5'-TTC ACA CTG AAT GCC AGC TC-3' and 3' primer, 5'-GTC TGC TGA GAC CCC TCT TG-3'; VCAM-1, 5' primer, 5'-ATT TTC TGG GGC AGG AAG TT-3' and 3' primer, 5'-ACG TCA GAA CAA CCG AAT CC-3'. The relative mRNA levels were normalized to levels of GAPDH mRNA in the same sample.

Western Blot Analysis

RAW264.7 cells were lysed in cold radioimmune precipitation assay buffer. Samples containing 80 μg of protein were electrophoresed in a 12% polyacrylamide gel and then transferred to a PVDF membrane. Membranes were incubated with rabbit anti-C/EBPβ antibody (Santa Cruz Biotechnology, Inc.), rabbit anti-C/EBPδ antibody (Santa Cruz Biotechnology, Inc.), rabbit anti-phospho-p38 MAPK antibody (Cell Signaling), rabbit anti-phospho-p44/42 MAPK antibody (Cell Signaling), rabbit anti-p38 MAPK antibody (Cell Signaling), rabbit anti-p44/42 antibody (Cell Signaling), and rabbit

anti-GAPDH antibody (Cell Signaling), respectively. After three washes in TBST, the membranes were incubated with a 1:5000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare). The membrane was developed by the enhanced chemiluminescence technique according to the manufacturer's protocol (Thermo Fisher Scientific, Rockford, IL).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts of RAW264.7 cells, primary peritoneal macrophages, or MH-S cells were prepared as follows. Cells were lysed in 15 mM KCl, 10 mM HEPES (pH 7.6), 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% (v/v) Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and complete protease inhibitors (Roche, Indianapolis, IN) for 10 min on ice. Nuclei were pelleted by centrifugation at 14,000 × g for 20 sec at 4°C. 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 dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and complete protease inhibitors (Roche, Indianapolis, IN). Nuclear extracts of whole lung tissues were prepared as described previously (62). Briefly, frozen lungs were homogenized in 0.6% (v/v) Nonidet P-40, 150 mM NaCl, 10 mM HEPES (pH 7.9), 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml antipain, 17 μg/ml calpain inhibitor, and 1×Halt™ Protease Inhibitor. The homogenate was incubated on ice for 5 min and then centrifuged for 5 min at 5000 x g at 4°C. Proteins were extracted from the pelleted nuclei by incubation at 4°C with solution B (420 mM NaCl, 20 mM HEPES (pH 7.9), 1.2 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml antipain, 17 μg/ml calpain inhibitor, and 1×Halt™ Protease Inhibitor. Nuclear

debris was pelleted by centrifugation at 5000 × g for 5 min at 4°C, and the supernatant extract was collected and stored at −80°C. Protein concentrations were determined by Bio-Rad protein assay kit (Thermo Fisher Scientific, Rockford, IL). The EMSA probes were C/EBP consensus oligonucleotide (TGCAGATTGCGCAATCTGCA, Santa Cruz Biotechnology, Inc.), or a NF-κB consensus oligonucleotide (AGTTGAGGGGACTTTCCCAGGC, Promega, Madison, WI). C/EBP probes and NF-κB probes were labeled with γ [^{32}P] ATP (3,000 Ci/mmol at 10 mCi/ml, GE Healthcare, Piscataway, NJ). DNA binding reactions were performed at room temperature in a 25 μl reaction mixture containing 6 μl of nuclear extract (1 mg/ml in buffer C or solution B) and 5 μl of 5 × binding buffer (20% (w/v) Ficoll, 50 mM HEPES pH 7.9, 5 mM EDTA, 5 mM dithiothreitol). The remainder of the reaction mixture contained KCl at a final concentration of 50 mM, Nonidet P-40 at a final concentration of 0.1%, 1 μg of poly (dI-dC), 200 pg of probe, bromphenol blue at a final concentration of 0.06% (w/v), and water to final volume of 25 μl. Samples were electrophoresed through 5.5% polyacrylamide gels in 1 × TBE at 190 V for approximately 3.5 h, dried under vacuum, and exposed to X-ray film. For supershifts, nuclear extracts were preincubated with antibodies (1 to 2 μg) for 0.5 h at 4°C prior to the binding reaction. The following antibodies were purchased from Santa Cruz, CA: C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, C/EBPγ, and normal rabbit immunoglobulin G.

IgG IC-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 C/EBPβ- and C/EBPδ-deficient mice by homologous recombination has been described previously (201, 202). C/EBPβ knockout mice and wild type littermates are on a C57BL/6:Sv129 F1 hybrid background (to circumvent low mutant viability on each pure strain background), and C/EBPδ knockout animals and wild type controls are on a C57BL/6 background. IgG immune complexes-mediated acute lung injury was induced as described previously (18, 62, 93, 98). Briefly, mice were anesthetized intraperitoneally (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 intravenous (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 sacrificed, and the lungs were perfused via the right ventricle with 3 ml PBS. Myeloperoxidase (MPO) is a peroxidase enzyme, which is abundantly generated in neutrophils, and its activity is the marker of neutrophil content. To measure MPO activity, whole lungs were 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 H_2O_2 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.

Cell Count and Albumin Concentration Assay in BAL Fluids

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 BAL fluid was centrifuged at 3000 rpm for 5 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). 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 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 Depletion

Mice were anesthetized with ketamine HCl (100 mg/kg, i.p.). A suspension of dichloromethylene diphosphonate (Cl_2MDP) liposomes in PBS (10 μl of 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 (204). Mice receiving Cl_2MDP liposomes showed more than 75% depletion of alveolar macrophages compared with mice receiving PBS liposomes. Administration of PBS liposomes did not reduce the number of alveolar macrophages.

Statistical Analysis

All values were expressed as the mean ± S. E.. Significance was assigned where $p < 0.05$. Data sets were analyzed using Student's t test or one-way ANOVA, with individual group means being compared with the Student-Newman-Keuls multiple comparison test.

CHAPTERIII

RESULTS

Roles of C/EBPβ and –δ in IgG IC-induced Inflammation in Macrophages

IgG IC Induces Expression of C/EBPβ and -δ in Macrophages

To determine if C/EBP activities were induced by IgG IC, I first examined the DNA binding activity of C/EBPs in RAW264.7 macrophages, which is the most popular peritoneal macrophage-derived cell line. As shown in Fig. 1A, RAW264.7 cells were challenged with IgG IC for different time periods, and extracted nuclear proteins were subjected to EMSA. The results showed that strong C/EBP DNA binding activity was induced after IgG IC stimulation (Fig. 1A). To determine which C/EBP family members were induced by IgG IC, supershift assays were performed. There are five major DNA-binding species in the nuclear proteins of control-treated macrophages, as follows: C/EBPβ LAP/C/EBPδ heterodimers, C/EBPβ LIP/C/EBPδ heterodimers, heterodimers LAP and LIP, LAP/LAP homodimers, and LIP/LIP homodimers (Fig. 1B). In IgG immune complex-treated macrophages, the DNA-binding activities of both C/EBPβ (mainly LAP/LIP, LAP/LAP and LIP/LIP) and C/EBPδ were markedly induced (Fig. 1B). I further examined the IgG IC-induced C/EBP activation in transient transfections using 2 × C/EBP-Luc, a promoter-reporter that contains two copies of a canonical C/EBP binding site, and expression vectors for C/EBPβ and/or C/EBPδ. These transfections were carried out with and without IgG IC treatment. Consistent with the results from EMSA, IgG IC

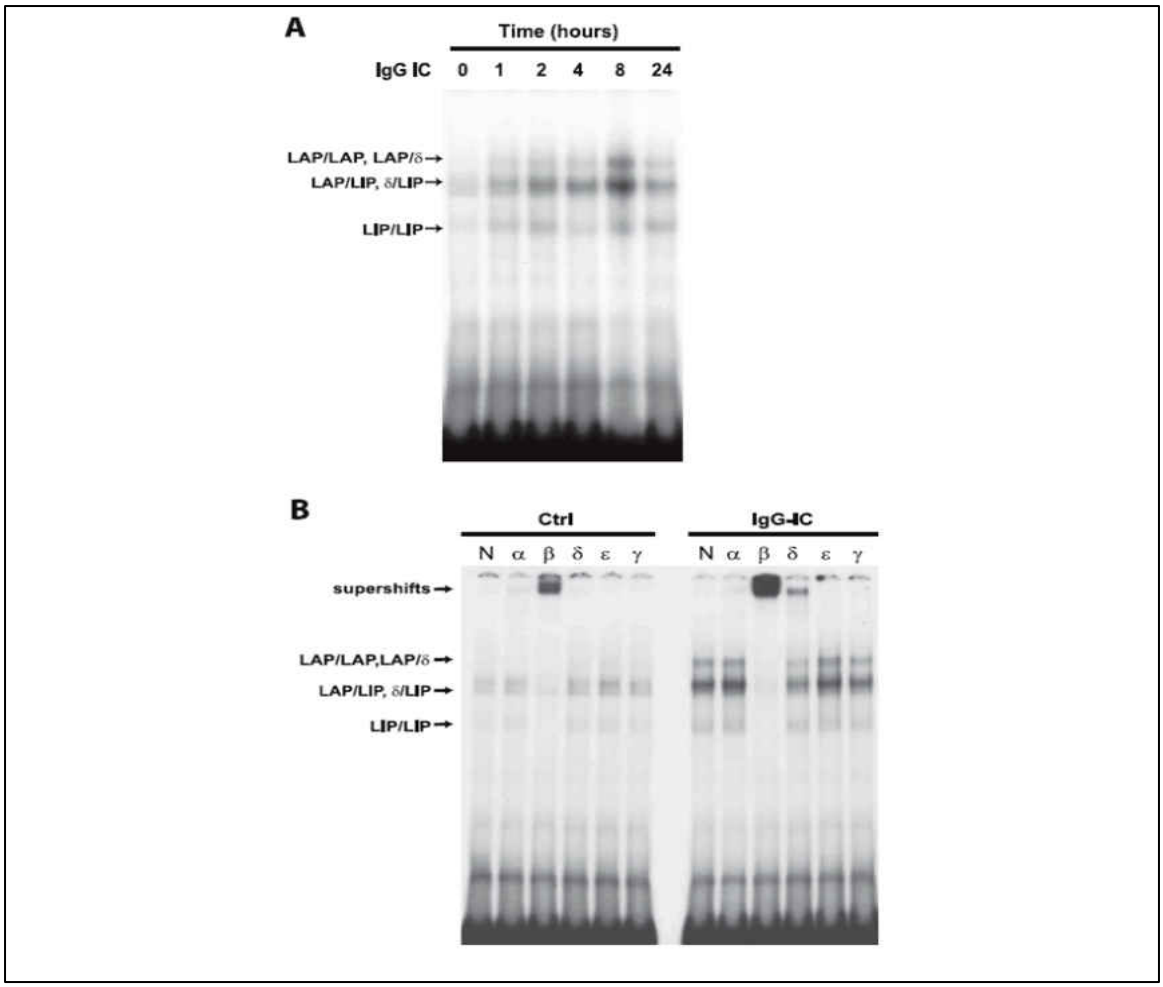

Figure 1. IgG Immune Complex Treatment Increases C/EBPβ and -δ DNA Binding Activities. A, RAW264.7 cells were stimulated with 100 μg/ml IgG IC for the times indicated. Nuclear proteins were harvested and subjected toEMSA to measure C/EBP DNA binding activity. B, RAW264.7 cells were treated or left untreated with 100 μg/ml IgG immune complexes for 4 h. The nuclear extracts were harvested for gel supershift assay to identify which C/EBP family member DNA binding activity was regulated by IgG immune complex treatment. N, α, β, δ, ε, and γ indicate normal rabbit IgG, anti-C/EBPα antibody, anti-C/EBPβ antibody, anti-C/EBPδ antibody, anti-C/EBPε antibody, and anti-C/EBPγ antibody, respectively. The arrows indicate C/EBP complex and supershift species. The same experiment was repeated once.

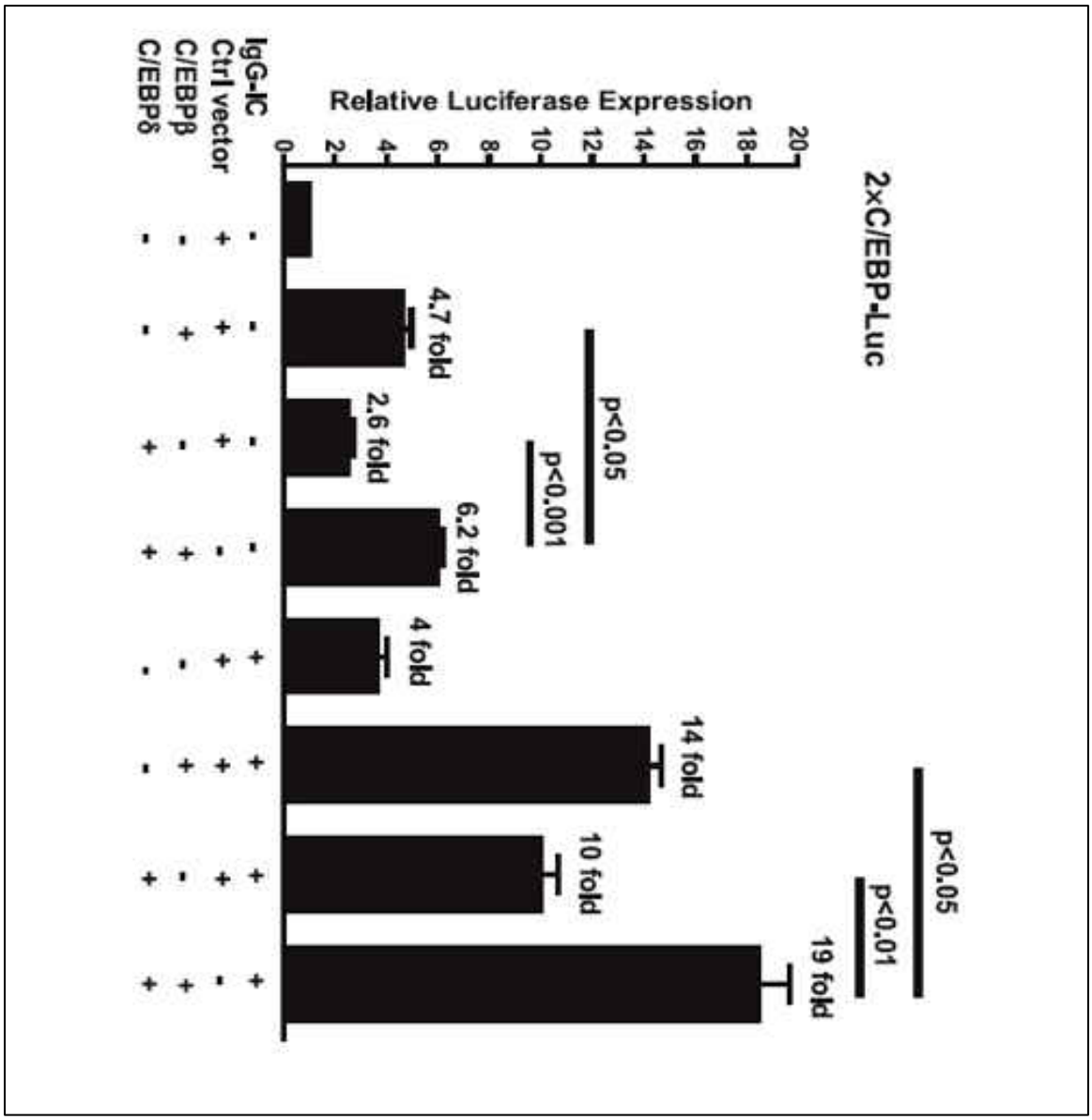

Figure 2. IgG Immune Complex Stimulation Augments C/EBP Transcriptional Activity. RAW264.7 cells were transiently transfected with a total of 0.5 μg of the indicated DNA. 24 h after transfection, the cells were treated with 100 μg/ml IgG immune complex for 5 h. Cell lysates were used to perform a luciferase activity assay. Luminometer values were normalized for expression from a co-transfected thymidine kinase reporter gene. The data were expressed as means ± S.E. (error bars), (n=3). The same experiment was repeated once.

stimulation alone increased luciferase activity 4-fold compared with the control group (Fig. 2). IgG IC treatment of C/EBPβ, C/EBPδ, and C/EBPβ plus C/EBPδ transfectants induced luciferase expression 14-, 10-, and 19-fold, respectively, over the control value. Interestingly, overexpression of C/EBPβ, C/EBPδ, and C/EBPβ plus C/EBPδ alone stimulated luciferase activity 4-, 2.6-, and 6.2-fold, respectively; this is less than the values observed in the presence of IgG IC. These data suggest that IgG IC signaling may stimulate C/EBP transcriptional activity in addition to inducing expression of the endogenous proteins. Moreover, C/EBPβ and -δ together were more potent activators than either protein alone, indicating that C/EBPβ and -δ are preferential dimerization partners, C/EBPβ/δ heterodimers have stronger DNA binding activity than homodimers, or both.

I next examined whether IgG IC induced expression of C/EBPβ and -δ at the mRNA level. RT-PCR showed that C/EBPβ and -δ mRNAs were highly induced in RAW264.7 cells following a 24-h treatment with IgG IC (Fig. 3A). I then sought to determine whether this induction resulted in increased abundance of C/EBPβ and -δ proteins in RAW264.7 cells. The Western blot analysis of proteins isolated over a time course of IgG IC treatment revealed a time-dependent increase in the abundance of both C/EBPβ (mainly LAP) and C/EBPδ proteins (Fig. 3B). These data demonstrate that increased abundance of C/EBPβ and -δ is coincident with their increased DNA binding activity in RAW264.7 cells.

C/EBPβ and -δ are Required for IgG IC-mediated Cytokine and Chemokine Expression in Macrophages

C/EBPβ and -δ play important roles in regulating inflammatory responses in many different cells, including macrophages (182, 183, 199, 205-207). However, whether they

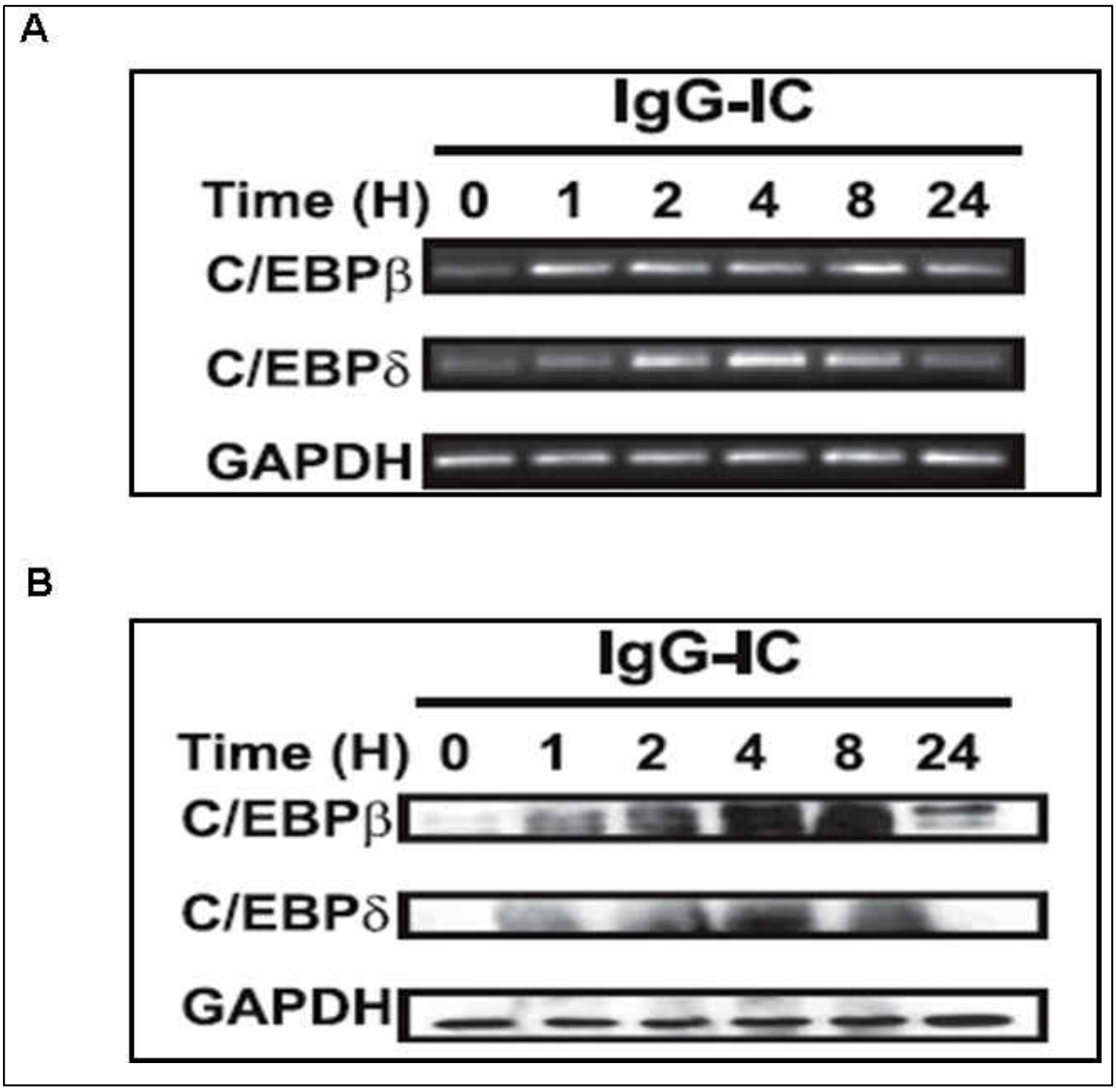

Figure 3. IgG Immune Complexes Induce C/EBPβ and -δ Expressions. A, RAW264.7 cells were stimulated with 100 μg/ml IgG immune complexes for different time periods. Then total cellular RNA was isolated for RT-PCR with primers for C/EBPβ, C/EBPδ, and GAPDH, respectively. B, RAW264.7 cells were stimulated with 100 μg/ml IgG immune complexes for different time periods. Then total cellular proteins were extracted to conduct Western blot using rabbit anti-C/EBPβ antibody, rabbit anti-C/EBPδ antibody, and rabbit anti-GAPDH antibody, respectively. The level of GAPDH is shown at the bottom as a loading control. The same experiment was repeated once.

are involved in macrophage inflammatory responses after FcγR cross-linking is unknown. TNF-α is an early proinflammatory cytokine secreted by macrophages during inflammation. Both MIP-2 and MIP-1α were initially identified as monokines secreted from LPS stimulated RAW264.7 cells (208-211). MIP-2 belongs to the C-X-C chemokine subfamily and has been shown to possess potent chemotactic activity for neutrophils, whereas MIP-1α, as a member of the C-C chemokine subfamily, has been shown to chemoattract leukocytes of the monocyte lineage (208, 209, 212, 213). Therefore, I sought to determine the effect of C/EBPβ and -δ deficiency on the expression of TNF-α, MIP-2, and MIP-1α from IgG-IC-stimulated macrophages. To that end, I first showed that both C/EBPβ and -δ expression were efficiently depleted by their respective siRNAs in RAW264.7 cells (Figs. 4A and 6A). IgG immune complexes dramatically increased expressions of TNF-α, MIP-2, and MIP-1α at transcriptional level in control cells, whereas all were markedly reduced when C/EBPβ or -δ was ablated, and the numbers indicated relative abundance of gene expression (Figs. 4B and 6B). To examine if knockdown of C/EBPβ or -δ could lead to a corresponding reduction in secretion of these proinflammatory proteins, I performed ELISA analysis. As shown in Figs. 5 and 7, the secretions of TNF-α, MIP-2, and MIP-1α from RAW264.7 cells were all markedly stimulated by IgG immune complexes treatment. However, when compared with control siRNA treatment, C/EBPβ siRNA suppressed TNF-α production by ~60%, MIP-2 by ~46%, and MIP-1α by ~25%, respectively. Furthermore, C/EBPδ knockdown inhibited TNF-α generation by ~43%, MIP-2 by ~17%, and MIP-1α by ~43%, respectively, as shown in Fig. 7.

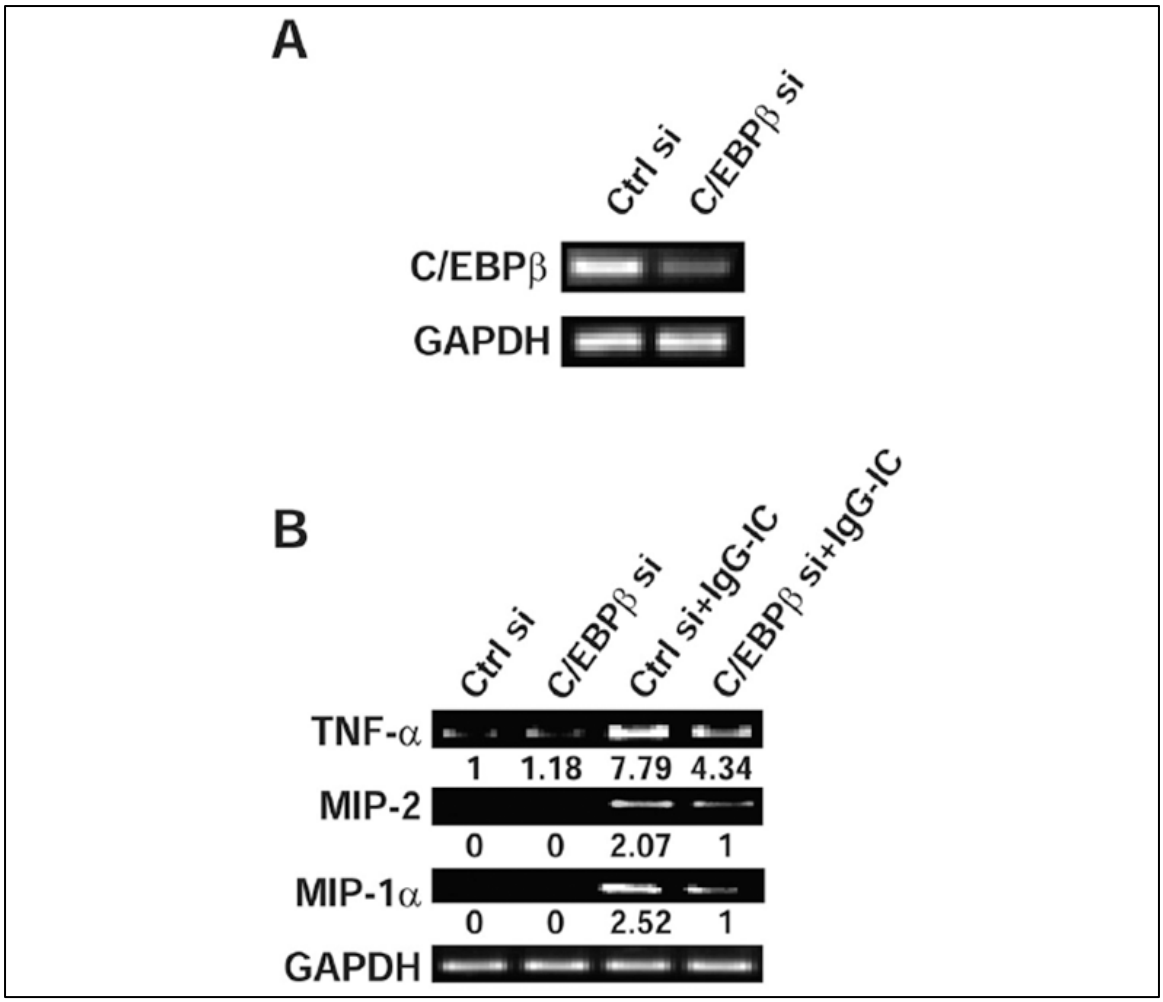

Figure 4. IgG IC-mediated Generation of Proinflammatory Mediators is Regulated by C/EBPβ at Transcription Level. A, RAW264.7 cells were transiently transfected with control siRNA, or C/EBPβ siRNA. 12 h later, RNAs were extracted and subjected to RT-PCR to examine expression of C/EBPβ, and GAPDH, respectively. B, RAW264.7 cells were transiently transfected with control siRNA or C/EBPβ siRNA. 12 h later, cells were incubated with or without 100 μg/ml IgG IC for 4 h. Total RNA was extracted, and semi-quantitative RT-PCR was performed to examine expression of TNF-α, MIP-2, and MIP-1α, respectively. The level of GAPDH is shown at the bottom as a loading control. The same experiment was repeated once.

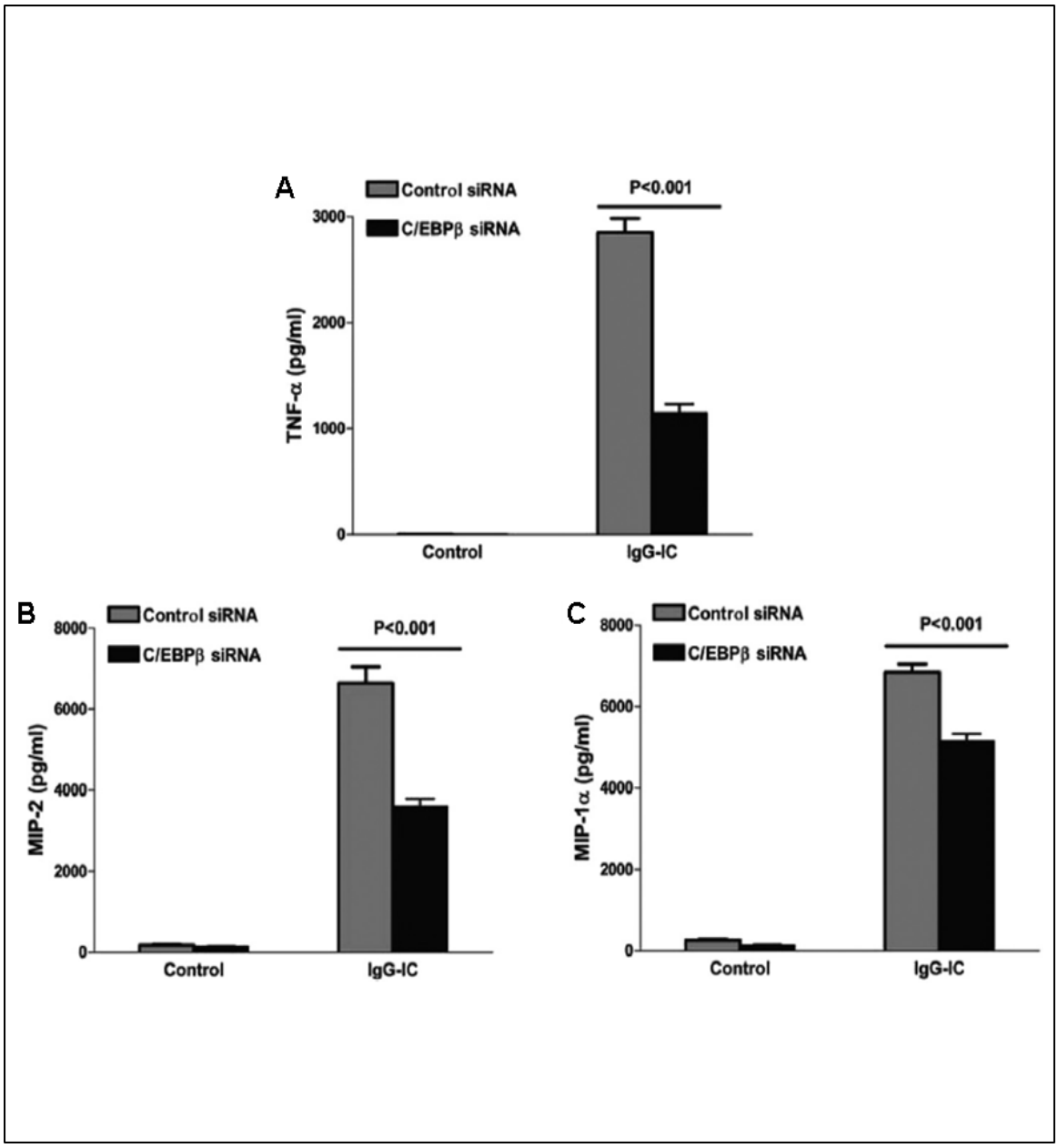

Figure 5. IgG IC-mediated TNF-α, MIP-2, and MIP-1α Production is Regulated by C/EBPβ at Protein Level. RAW264.7 cells were transiently transfected with control siRNA or C/EBPβ siRNA. 12 h later, cells were incubated with or without 100 μg/ml IgG IC for 5 h. The supernatants were harvested, and ELISA was performed to determine the production of TNF-α (A), MIP-2 (B), and MIP-1α (C). Data are presented as mean ± S.E. (n=7–12). The same experiment was repeated twice.

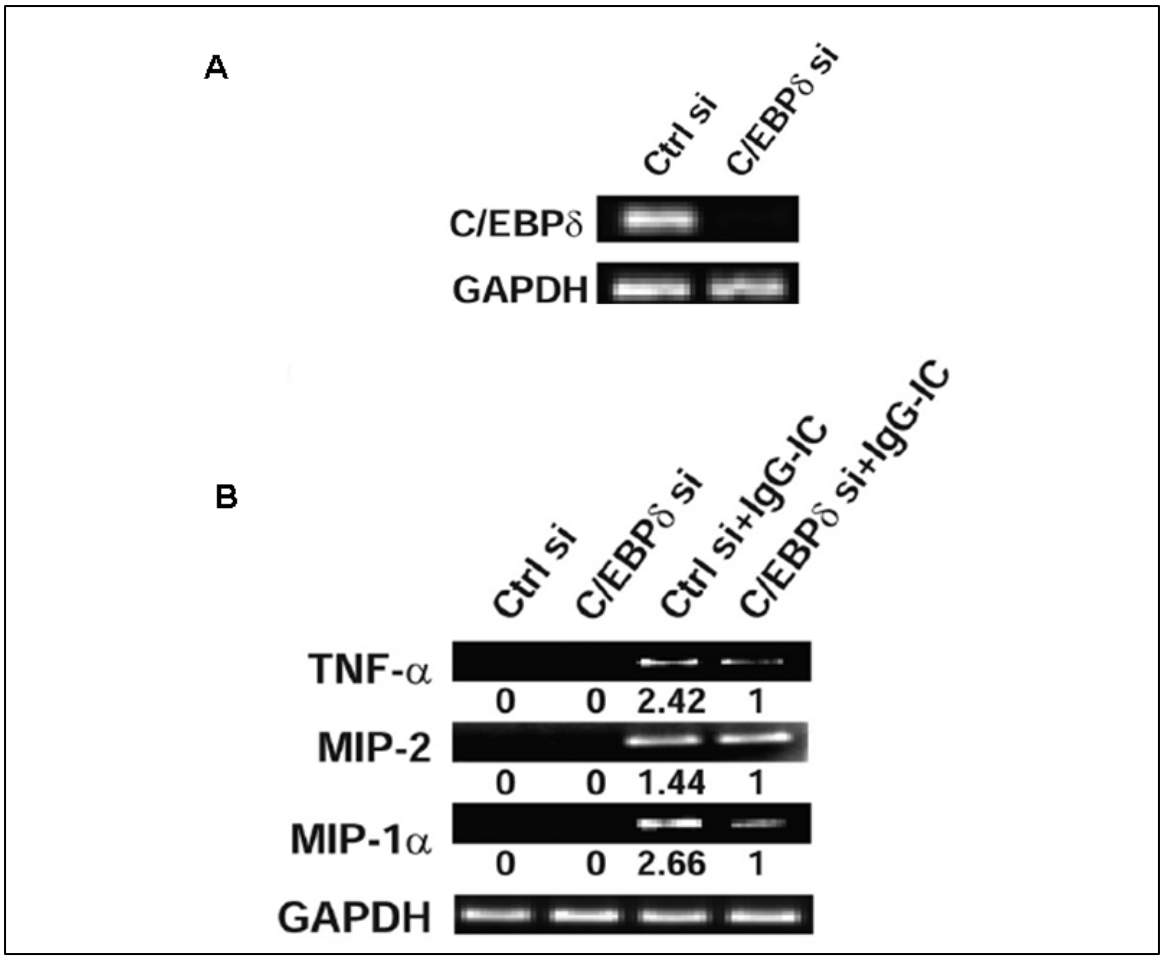

Figure 6. IgG IC-mediated Generation of Proinflammatory Mediators is Regulated by C/EBPδ at Transcription Level. A, RAW264.7 cells were transiently transfected with control siRNA, or C/EBPδ siRNA. 12 h later, RNAs were extracted and subjected to RT-PCR to examine expression of C/EBPδ, and GAPDH, respectively. B, RAW264.7 cells were transiently transfected with control siRNA or C/EBPδ siRNA. 12 h later, cells were incubated with or without 100 μg/ml IgG IC for 4 h. Total RNA was extracted, and semi-quantitative RT-PCR was performed to examine expression of TNF-α, MIP-2, and MIP-1α, respectively. The level of GAPDH is shown at the bottom as a loading control. The same experiment was repeated once.

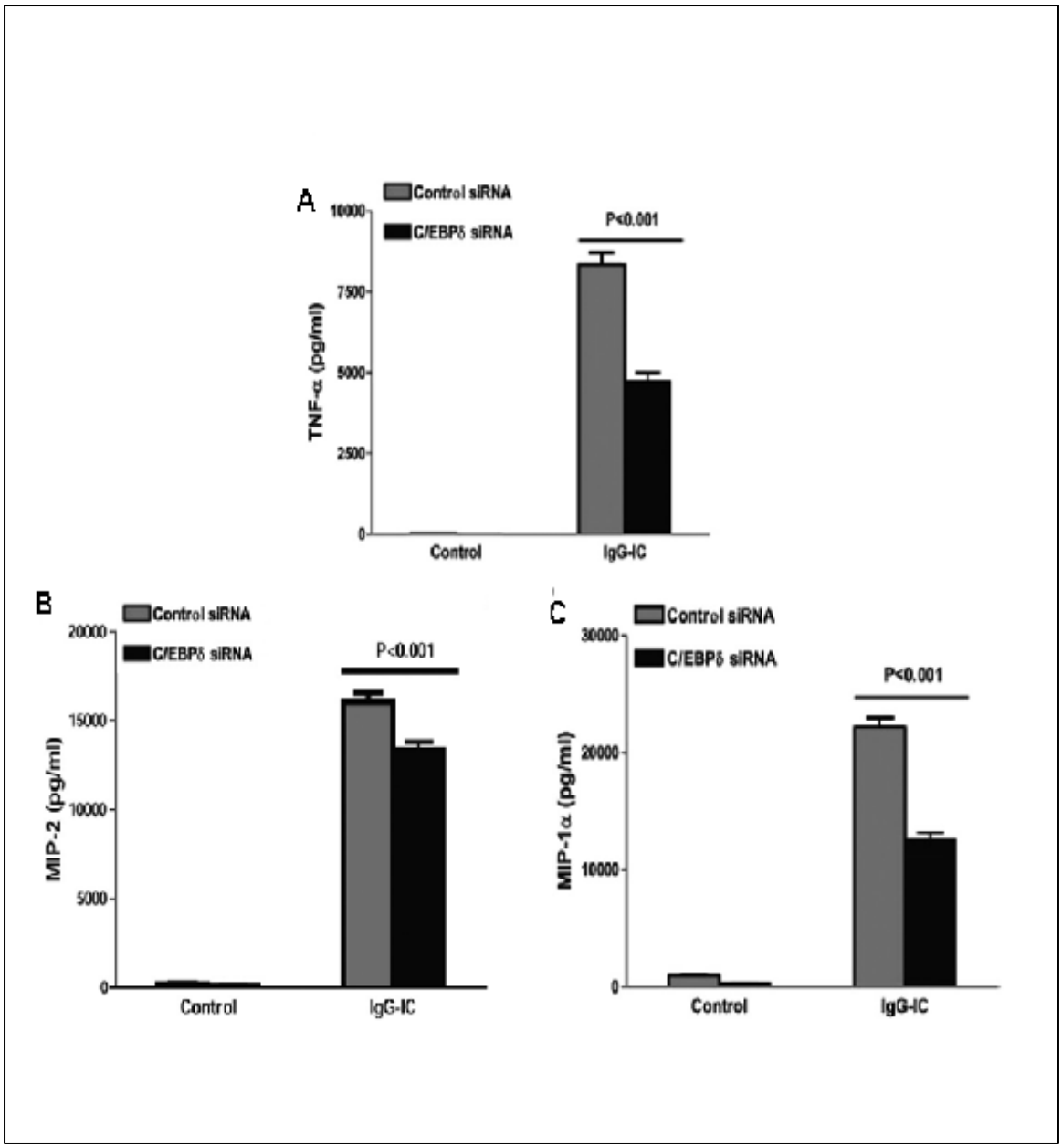

Figure 7. IgG IC-mediated TNF-α, MIP-2, and MIP-1α Production is Regulated by C/EBPδ at Protein Level. RAW264.7 cells were transiently transfected with control siRNA or C/EBPδ siRNA. 12 h later, cells were incubated with or without 100 μg/ml IgG IC for 5 h. The supernatants were harvested, and ELISA was performed to determine the production of TNF-α (A), MIP-2 (B), and MIP-1α (C). Data are presented as mean ± S.E. (n=7–12). The same experiment was repeated twice.

I further determined whether the observed regulatory role of C/EBPβ and -δ on the IgG IC-induced TNF-α, MIP-2, and MIP-1α production in RAW264.7 cells was also applicable to primary macrophages. I first performed gel supershift assay in control, and IgG IC-treated peritoneal macrophages, respectively. There are six major DNA-binding species in the nuclear proteins of control-treated macrophages, as follows: C/EBPβ LAP/C/EBPα heterodimers, C/EBPβ LIP/C/EBPδ heterodimers, LIP/C/EBPα heterodimers, heterodimers LAP and LIP, LAP/LAP homodimers, and LIP/LIP homodimers (Fig. 8). The DNA binding activities of both C/EBPβ and -δ (to a minor extent) were induced by IgG IC (Fig. 8). I next demonstrated that, upon IgG IC treatment, C/EBPβ- or C/EBPδ-deficient macrophages released less amounts of TNF-α, MIP-2, and MIP-1α at all time points when compared with wild type macrophages (Fig. 9), consistent with the results obtained from RAW264.7 cells. In addition, I compared the IgG IC responses of four different immortalized macrophage cell lines established from WT, C/EBPβ$^{-/-}$, C/EBPδ$^{-/-}$, and C/EBPβ$^{-/-}$/δ$^{-/-}$ cells. When compared with WT macrophages, C/EBPβ knockout suppressed TNF-α induction by ~80%, MIP-1α by ~58%, and MIP-2 by ~77.7%, and C/EBPδ knockout also significantly inhibited production of TNF-α (~24%), MIP-1α (~49%), and MIP-2 (~34%) (Fig. 10). Moreover, C/EBPβ/δ double knockout further reduced secretion of TNF-α (~98%), MIP-1α (~99%), and MIP-2 (~98%) (Fig. 10). Thus, there is a redundant role for C/EBPβ and -δ in IgG IC-induced TNF-α, MIP-1α, and MIP-2 production in macrophages. Taken together, these findings show that C/EBPβ and -δ are critical regulators of cytokine and chemokine production in IgG IC-stimulated macrophages.

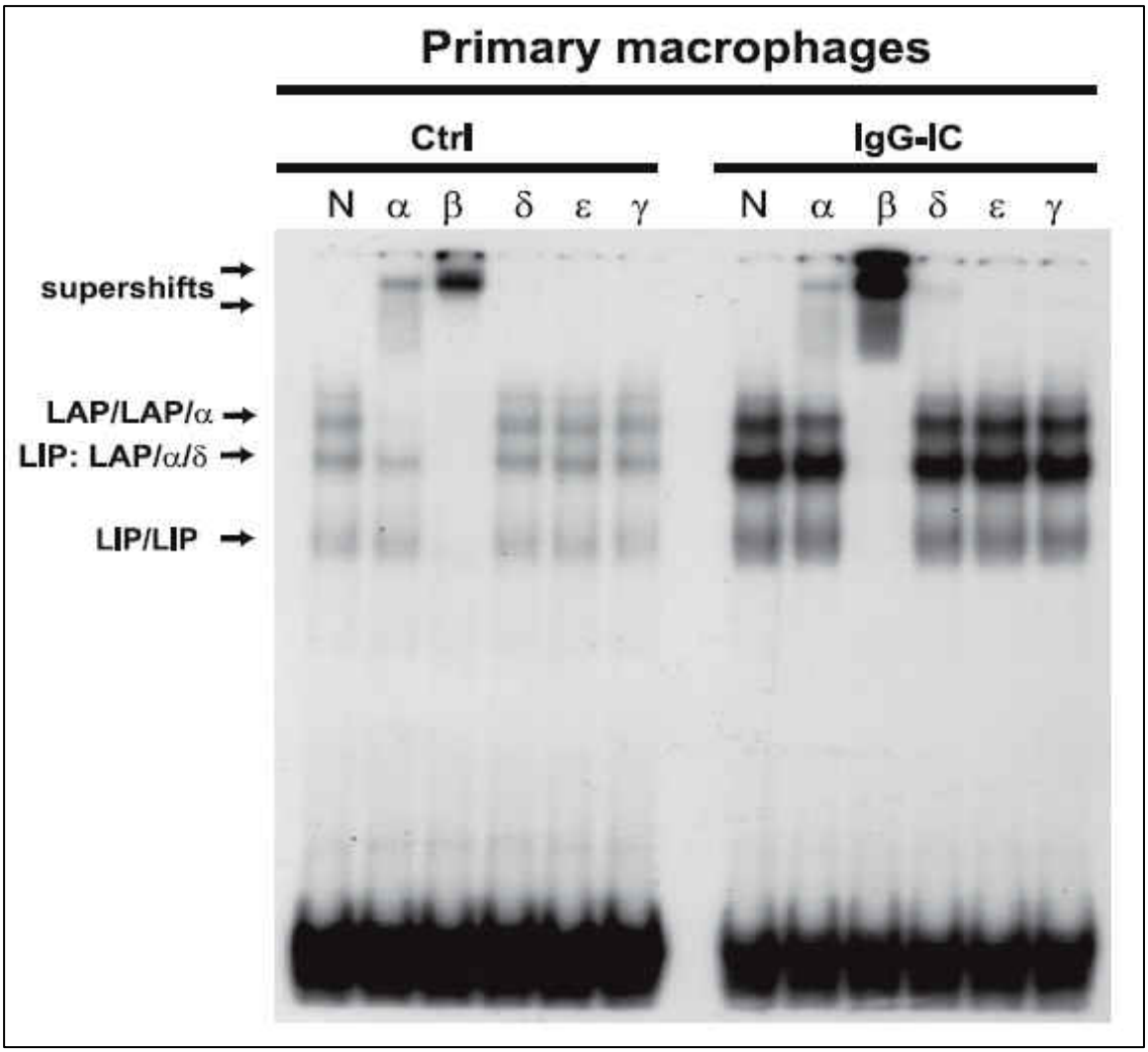

Figure 8. IgG Immune Complex Treatment Elevates C/EBPβ and -δ DNA Binding Activity in Primary Peritoneal Macrophages. Primary peritoneal macrophages were obtained from wild type mice, and treated or left untreated with 100 μg/ml IgG immune complexes for 4 h. The nuclear extracts were harvested for gel supershift assay to identify which C/EBP family member DNA binding activity was regulated by IgG immune complex treatment. N, α, β, δ, ε, and γ, normal rabbit IgG, anti-C/EBPα antibody, anti-C/EBPβ antibody, anti-C/EBPδ antibody, anti-C/EBPε antibody, and anti-C/EBPγ antibody, respectively. The arrows indicate C/EBP complex and supershift species. The same experiment was repeated three times.

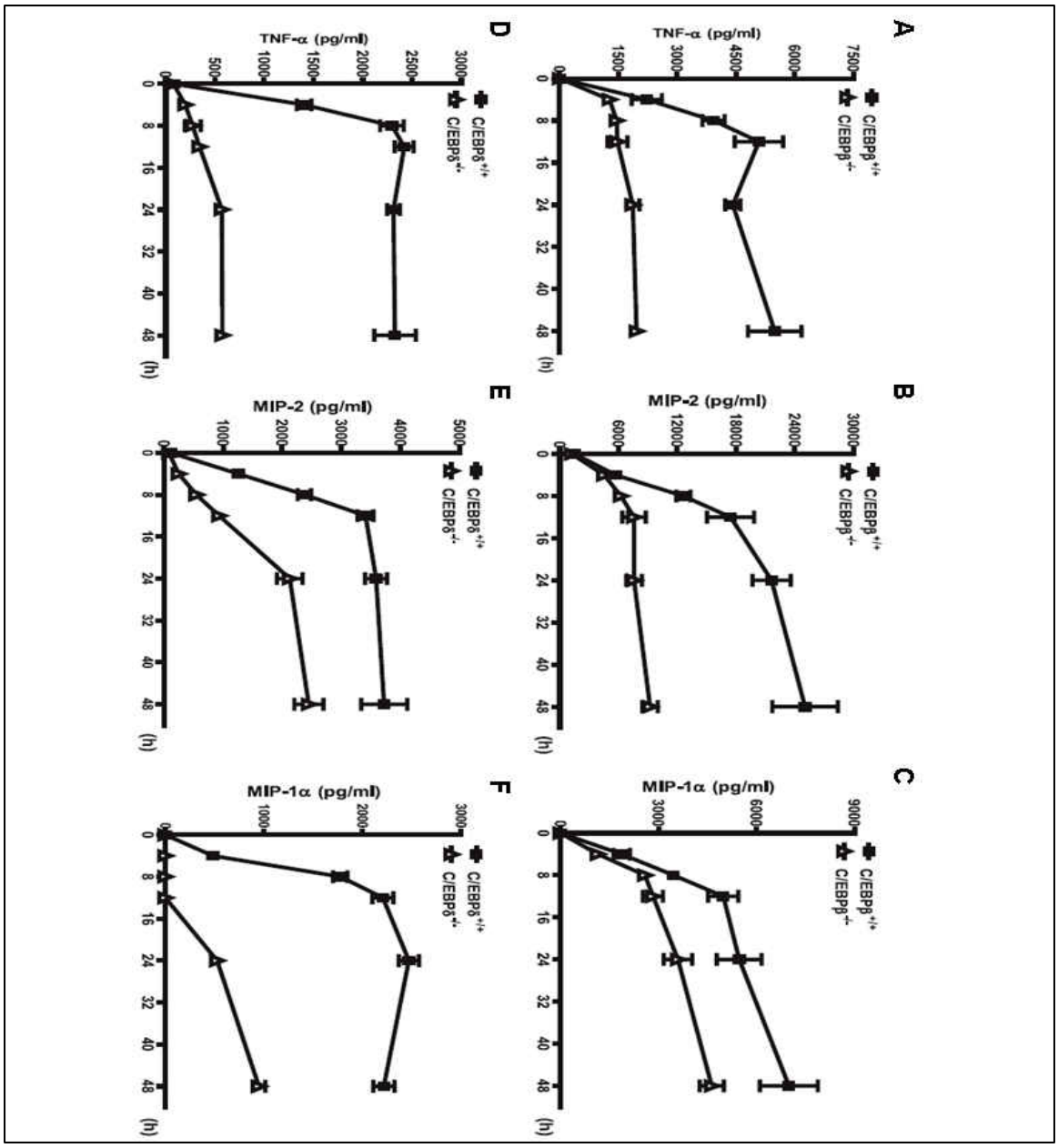

Figure 9. IgG IC-mediated Inflammation is Decreased in Macrophages Lacking C/EBPβ or C/EBPδ. Primary peritoneal macrophages obtained from corresponding wild type, and C/EBPβ or C/EBPδ knockout mice were treated with 100 μg/ml IgG immune complex for different time periods, and supernatants were subjected to ELISA analysis for TNF-α (A, D), MIP-2 (B, E), and MIP-1α (C, F) production. Data are presented as mean ± S.E. (n=6). The same experiment was repeated twice.

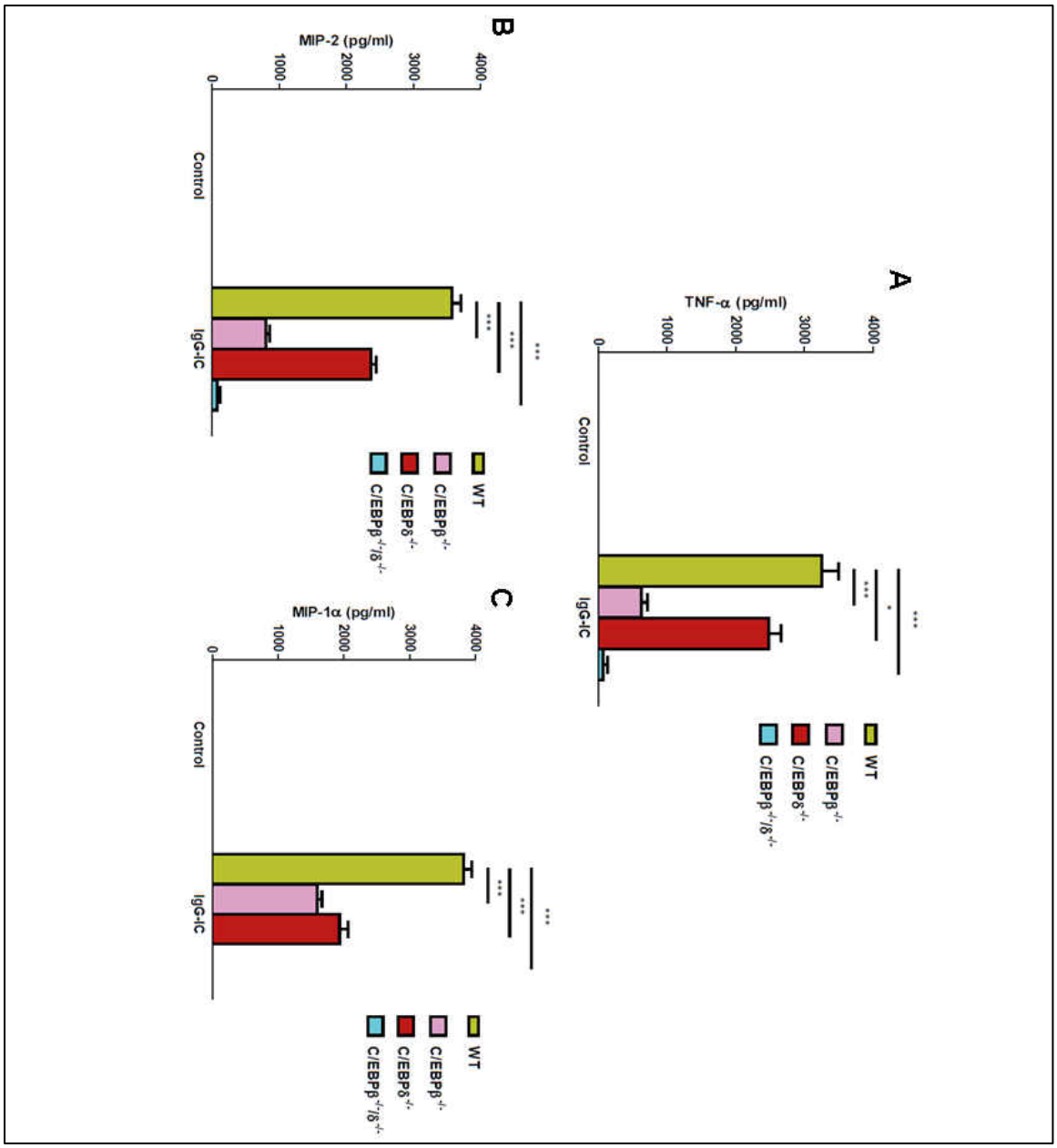

Figure 10. C/EBPβ and -δ are Critical Regulators of Inflammatory Reactions in IgG IC-stimulated Macrophages. Macrophages obtained from corresponding wild type, C/EBPβ knockout, C/EBPδ knockout, and C/EBPβ/δ double knockout mice were treated with 100 μg/ml IgG immune complex for 5 h, and supernatants were subjected to ELISA analysis for TNF-α (A), MIP-2 (B), and MIP-1α (C) production. Data are presented as mean ± S.E., n=7-8. The same experiment was repeated once.

NF-κB plays a central role in coordinating immune and inflammatory responses. To test its involvement in IgG immune complex-induced inflammation, RAW264.7 cells were treated with a NF-κB inhibitor, BAY 11-7082 (Fig. 11). The data showed that upon IgG immune complex stimulation, the levels of all three mediators were down-regulated by BAY 11-7082, with a significant inhibitory effect on TNF-α and MIP-1α production, suggesting that NF-κB also plays an important regulatory role in IgG IC-induced cytokine and chemokine production.

FcγRs Trigger C/EBP-mediated Cytokine and Chemokine Production in IC-stimulated Macrophages

FcγRs exert both activating and inhibitory effects on inflammatory responses, and the cellular threshold for inflammation is dependent on the ratio of the opposing signaling FcγRs. Because both activating and inhibitory FcγRs (Fcγ receptors) are expressed on macrophages (6, 214), I sought to determine the role of FcγRs in IgG immune complex-induced inflammatory reactions in macrophages. All the activating FcγRs that have been identified share a common γ-chain usually expressed as FcRγ, which is required for inflammatory signaling transduction. Thus, FcR γ-chain deficiency can lead to disability of the activating FcγRs. Using peritoneal macrophages from FcR γ-chain knockout mice, I first examined the influence of conventional activating FcγRs on IgG immune complex-induced cytokine and chemokine production. As shown in Fig. 12A-C, FcR γ-chain mutation resulted in a significantly decreased production of TNF-α (~57%), MIP-2 (~38%), and MIP-1α (~45%) in IgG immune complex-treated macrophages compared with their wild type counterparts. In contrast, FcγR Ⅱ deficiency resulted in an approximately 2-fold increase of TNF-α, MIP-2, and MIP-1α production (Fig. 13A-C).

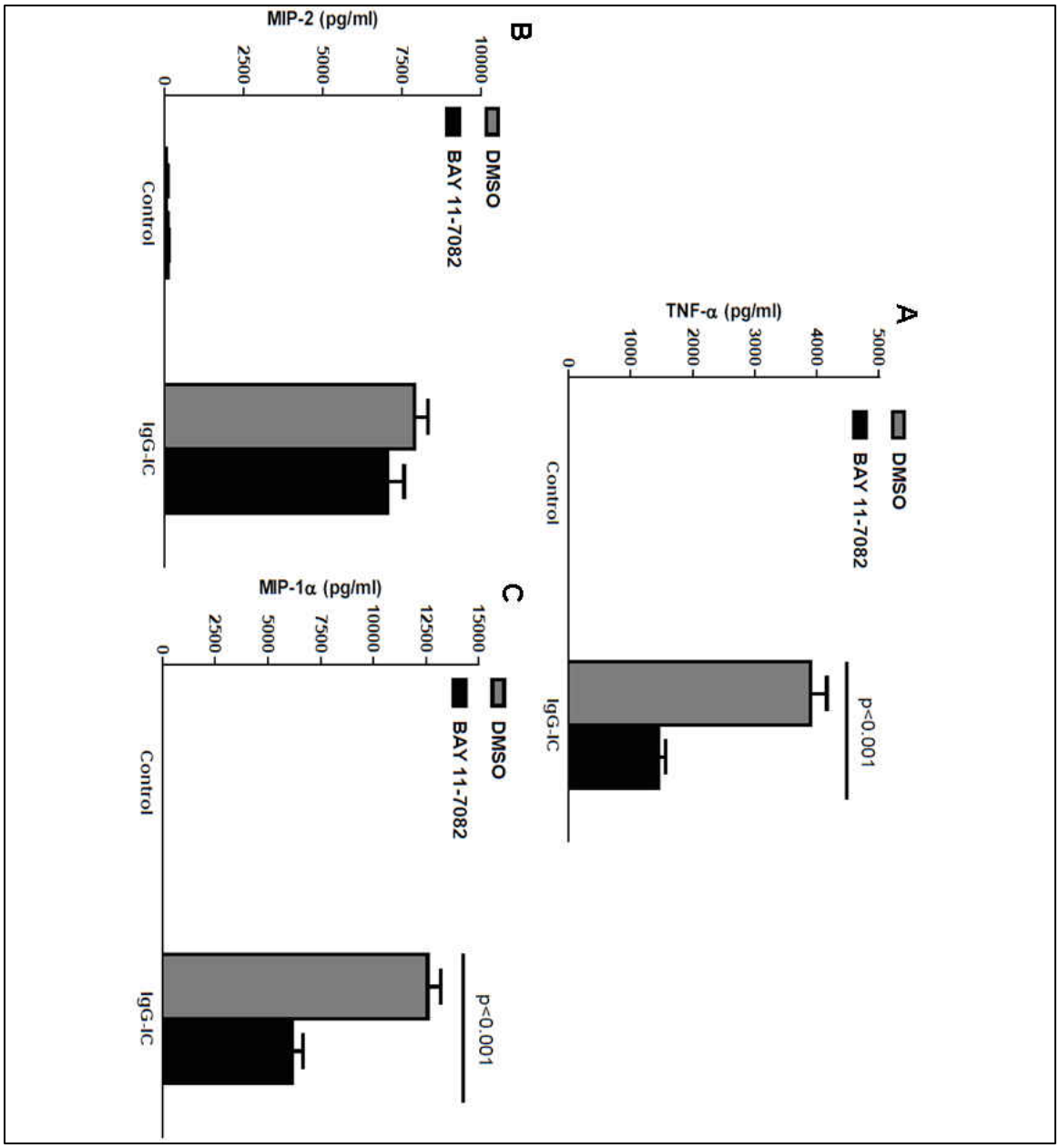

Figure 11. NF-κB Plays an Important Role in IgG Immune Complex-induced Inflammatory Mediators's Expressions. RAW264.7 cells were treated with 100 μg/ml IgG immune complex, or IgG immune complex plus NF-κB inhibitor BAY 11-7082 (2 μM) for 5 h. Supernatants were harvested and subjected to ELISA analysis for generation of TNF-α (A), MIP-2 (B), and MIP-1α (C). Data are presented as ± S.E., n=6. The same experiment was repeated twice.

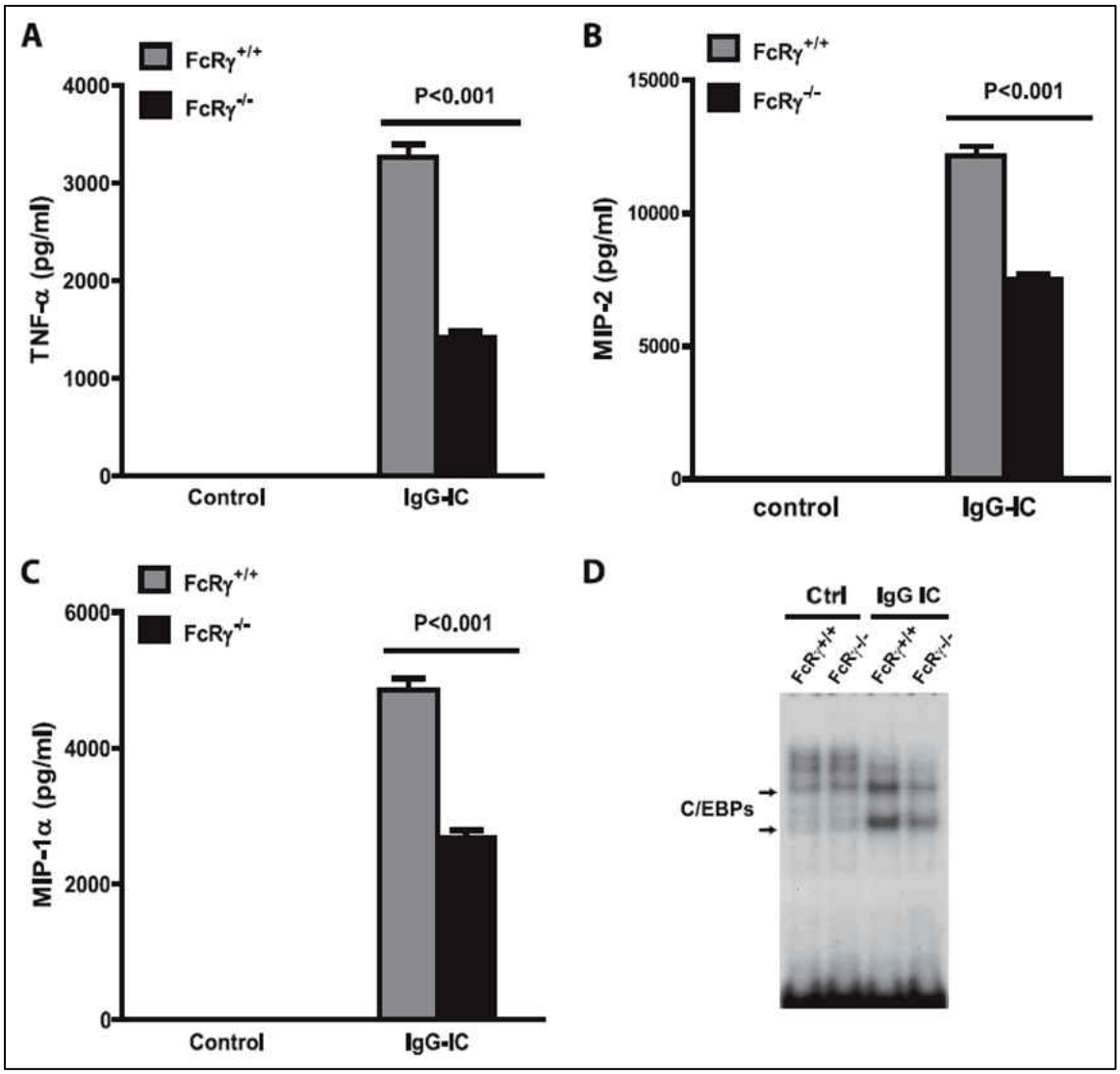

Figure 12. Effect of FcR γ-chain Deficiency on IgG IC-induced Inflammation. Primary peritoneal macrophages obtained from wild type and FcR γ-chain knockout mouse were treated with 100 μg/ml IgG immune complex for 5 h, and supernatants were subjected to ELISA analysis for production of TNF-α (A), MIP-2 (B), and MIP-1α (C). Data are presented as ± S.E., n=8. D, Primary peritoneal macrophages obtained from corresponding wild type and FcR γ-chain deficient mice were treated or left untreated with 100 μg/ml IgG immune complexes for 4 h. The nuclear proteins were harvested and subjected to EMSA to measure C/EBP DNA binding activity. The same experiment was repeated once.

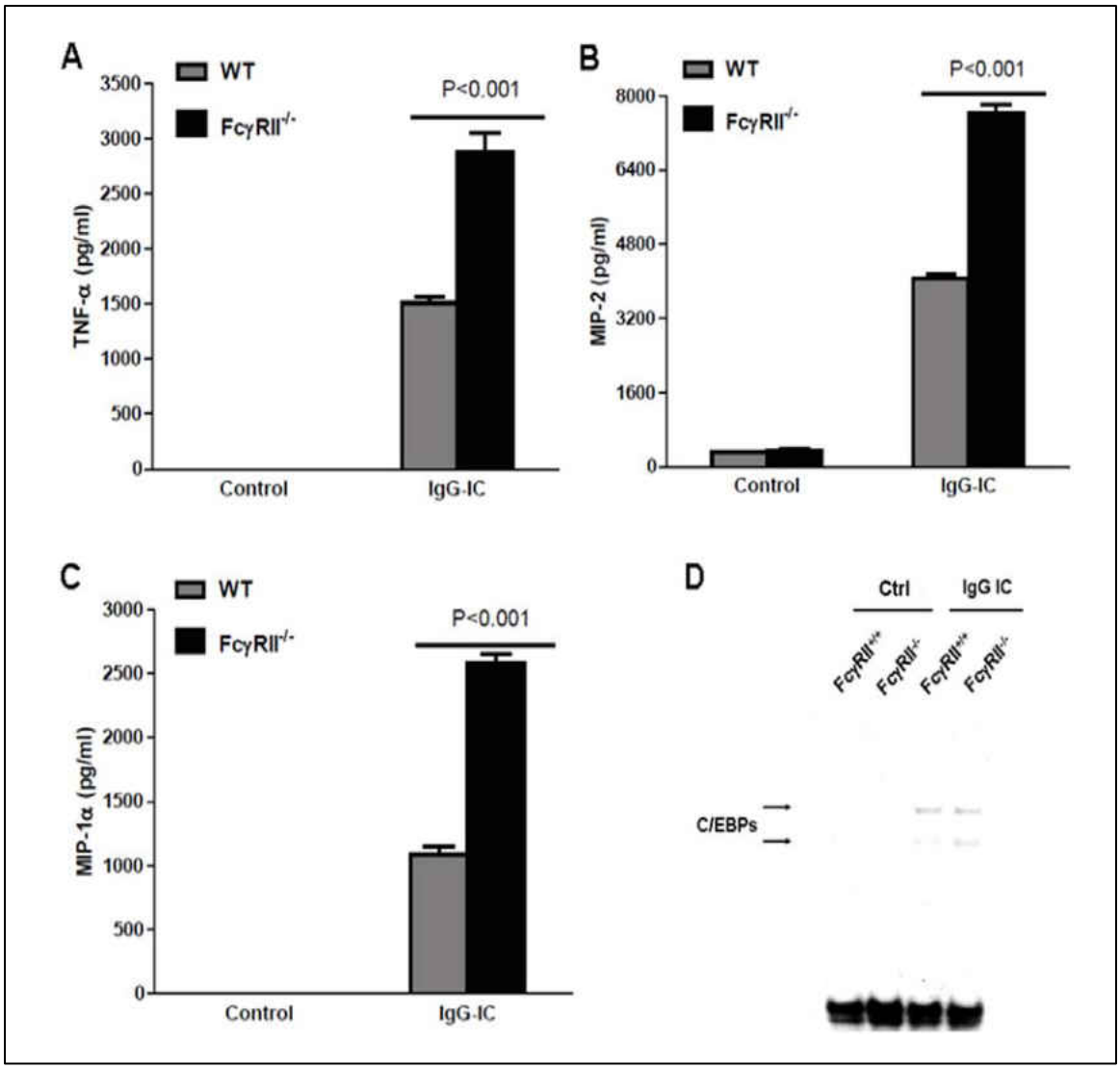

Figure 13. Effect of FcγR Ⅱ Deficiency on IgG IC-induced Inflammation. Primary peritoneal macrophages obtained from wild type and FcγR Ⅱ knockout mouse were treated with 100 μg/ml IgG immune complex for 5 h, and supernatants were subjected to ELISA analysis for production of TNF-α (A), MIP-2 (B), and MIP-1α (C). Data are presented as ± S.E., n=8. D, Primary peritoneal macrophages obtained from corresponding wild type and FcγR Ⅱ deficient mice were treated or left untreated with 100 μg/ml IgG immune complexes for 4 h. The nuclear proteins were harvested and subjected to EMSA to measure C/EBP DNA binding activity. The same experiment was repeated once.

Because the current data suggest that C/EBPβ and -δ may play important roles in cytokine and chemokine expression in IgG immune complex-stimulated macrophages, I next examined the effect of FcγRs deficiency on C/EBPβ and -δ activation. I found that knockout of FcR γ-chain suppressed induction of C/EBP DNA binding activity (Fig. 12D). Furthermore, inhibitory FcγRⅡ deficiency elevated C/EBP DNA binding activity (Fig. 13D). In summary, these findings suggest that activating FcγRs play an important role in IgG immune complex-induced C/EBPβ and -δ induction, leading to production of TNF-α, MIP-2, and MIP-1α, whereas the inhibitory FcγR has an opposite effect.

ERK1/2 and p38 MAPK are Involved in IgG IC-induced C/EBPβ and -δ Activation and Subsequent Cytokine/Chemokine Production

Previous studies have shown that FcγR cross-linking on macrophages/monocytes activates the extracellular signal-regulated kinases (ERK1/2, p44/42) and p38 MAPK signaling pathways (215, 216). However, whether ERK and p38 signaling pathways function as transducers connecting FcγR stimulation to C/EBPβ and -δ activation remains unclear. To investigate this possibility, I first evaluated the MAPK pathways in IgG IC-stimulated macrophages. As shown in Figs. 14A and 16A, IgG IC treatment led to the phosphorylation of both ERK1/2 and p38 MAPK in a time-dependent manner. I next evaluated the influence of these phosphorylated MAPK on C/EBPβ and –δ activation by using specific pharmacological inhibitors for ERK1/2 and p38 MAPK. I observed that phosphorylation of ERK1/2 was greatly suppressed by U0126 at all time points (Fig. 14A). Though p38 MAPK activation was only markedly inhibited by p38 MAPK inhibitor Ⅷ at 4-h time point, it had no influence on my subsequent experiments—EMSA and ELISA were conducted 4 hrs, and 5 hrs, respectively, after IgG immune complex treatment in the

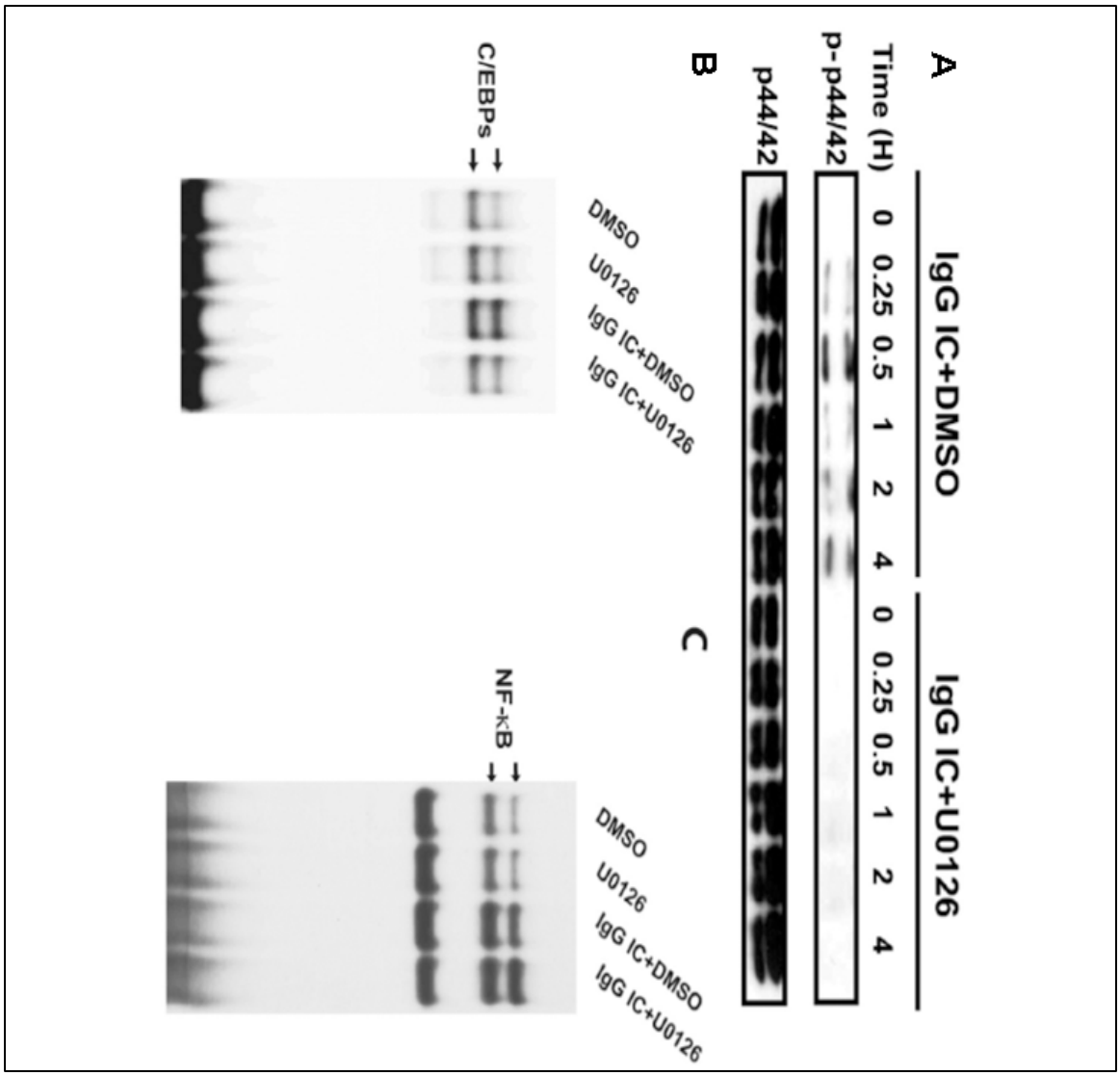

Figure 14. ERK1/2 is Involved in IgG IC-induced C/EBPβ and -δ Activation. A, RAW264.7 cells were treated with 100 μg/ml IgG immune complexes in the presence or absence of ERK1/2 inhibitor, U0126 (10 μM) for the indicated time periods. Total proteins were subjected to Western blot by using rabbit anti-phospho-p44/42 (p-p44/42) antibody and rabbit anti-p44/42 antibody, respectively. RAW264.7 cells were treated with 100 μg/ml IgG IC in the presence or absence of U0126 for 4 h. The nuclear proteins were subjected to EMSA for C/EBP DNA binding (B), and NF-κB DNA binding (C), respectively. The same experiment was repeated once.

presence of the inhibitor (Figs. 16 and 17). Further, U0126 and p38 MAPK inhibitor Ⅷ suppressed C/EBP DNA binding activity induced by IgG IC (Figs. 14B and 16B). The effect of MAPK inhibitors on IgG IC-induced NF-κB activation was also examined. EMSA showed that none of these inhibitors reduced NF-κB DNA binding activity (Figs. 14C and 16C). Interestingly, U0126 slightly elevated IgG IC-induced NF-κB activation (Fig. 14C). To determine whether MAPK activation is involved in IgG IC-induced cytokine and chemokine production (which are regulated by C/EBPβ and -δ), I evaluated the effect of U0126 and p38 MAPK inhibitor Ⅷ on TNF-α, MIP-2, and MIP-1α secretion from RAW264.7 macrophages. As shown in Figs. 15 and 17, U0126 and p38 MAPK inhibitor VIII inhibited IgG IC-stimulated TNF-α, MIP-2, and MIP-1α production. To determine whether ERK1/2 and p38 MAPK acted in concert or sequentially downstream of FcγRs, RAW264.7 cells were treated with both ERK1/2 inhibitor and p38 inhibitor. As shown in Fig. 18, when compared with p38 MAPK inhibitor Ⅷ or U0126 treatment alone, co-treatment resulted in a decreased production of TNF-α (~100%), MIP-2 (~100%), and MIP-1α (~66%). These data suggest that ERK1/2 and p38 MAPK act in concert to mediate IgG IC-induced inflammatory responses.

C5a Enhances IgG IC-stimulated Cytokine and Chemokine Production by Elevating C/EBPs but Not NF-κB DNA Binding Activity

C5a has been shown to synergistically enhance IgG immune complex-induced TNF-α and MIP-2 production in alveolar macrophages (30, 217). Thus, I examined the role of C5a in the activation of peritoneal macrophages treated with IgG immune complex. As shown in Fig. 19, the addition of C5a significantly increased IgG immune complex-induced TNF-α, MIP-2, and MIP-1α production, whereas stimulation with C5a

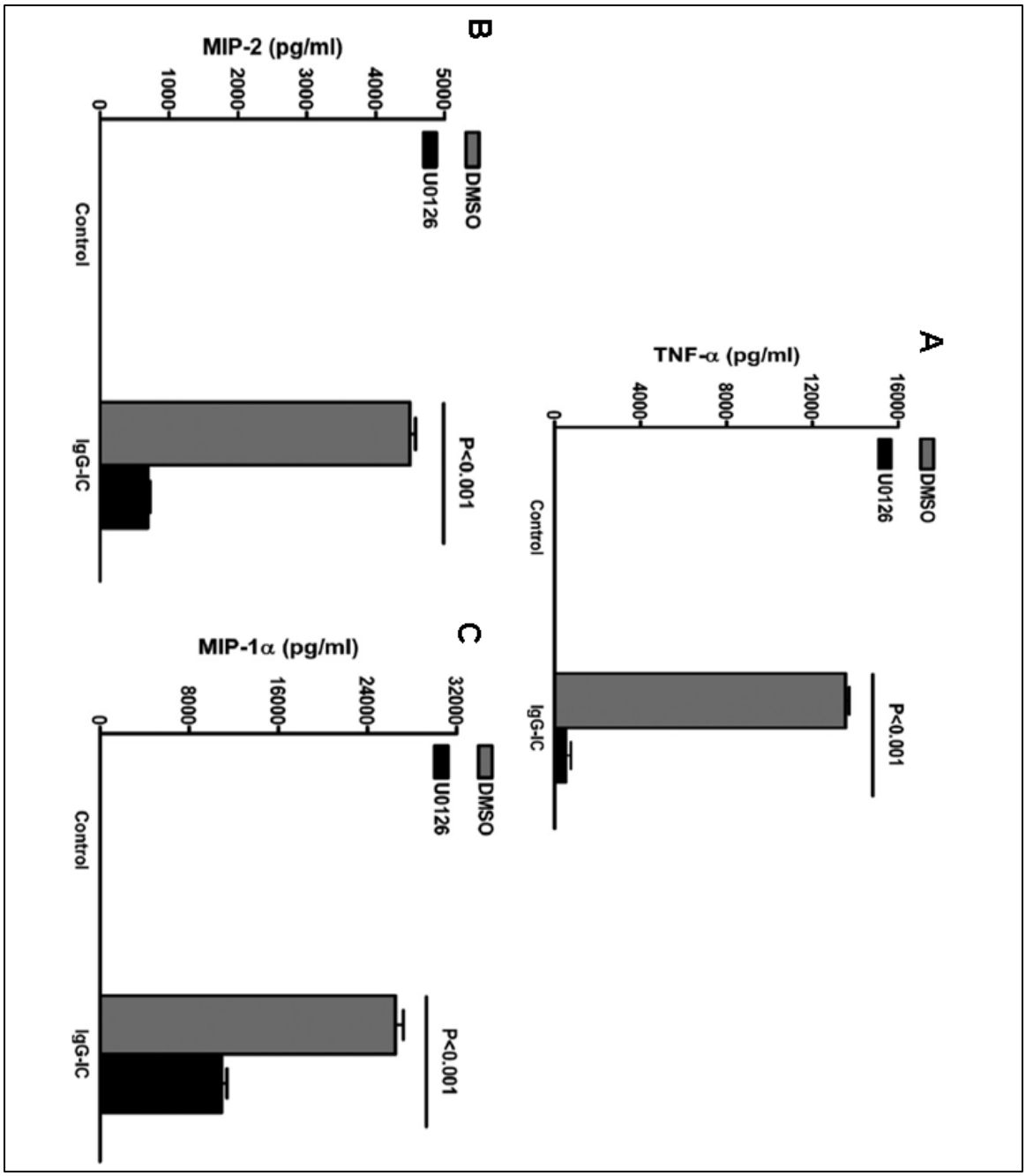

Figure 15. p44/42 MAPK is Involved in IgG IC-induced Inflammation. RAW264.7 cells were treated with 100 μg/ml IgG immune complexes in the presence or absence of ERK1/2 inhibitor, U0126 (10 μM) for 5 h, and supernatants were subjected to ELISA analysis for production of TNF-α (A), MIP-2 (B), and MIP-1α (C). Data are presented as mean ± S.E., n =10. The same experiment was repeated once.

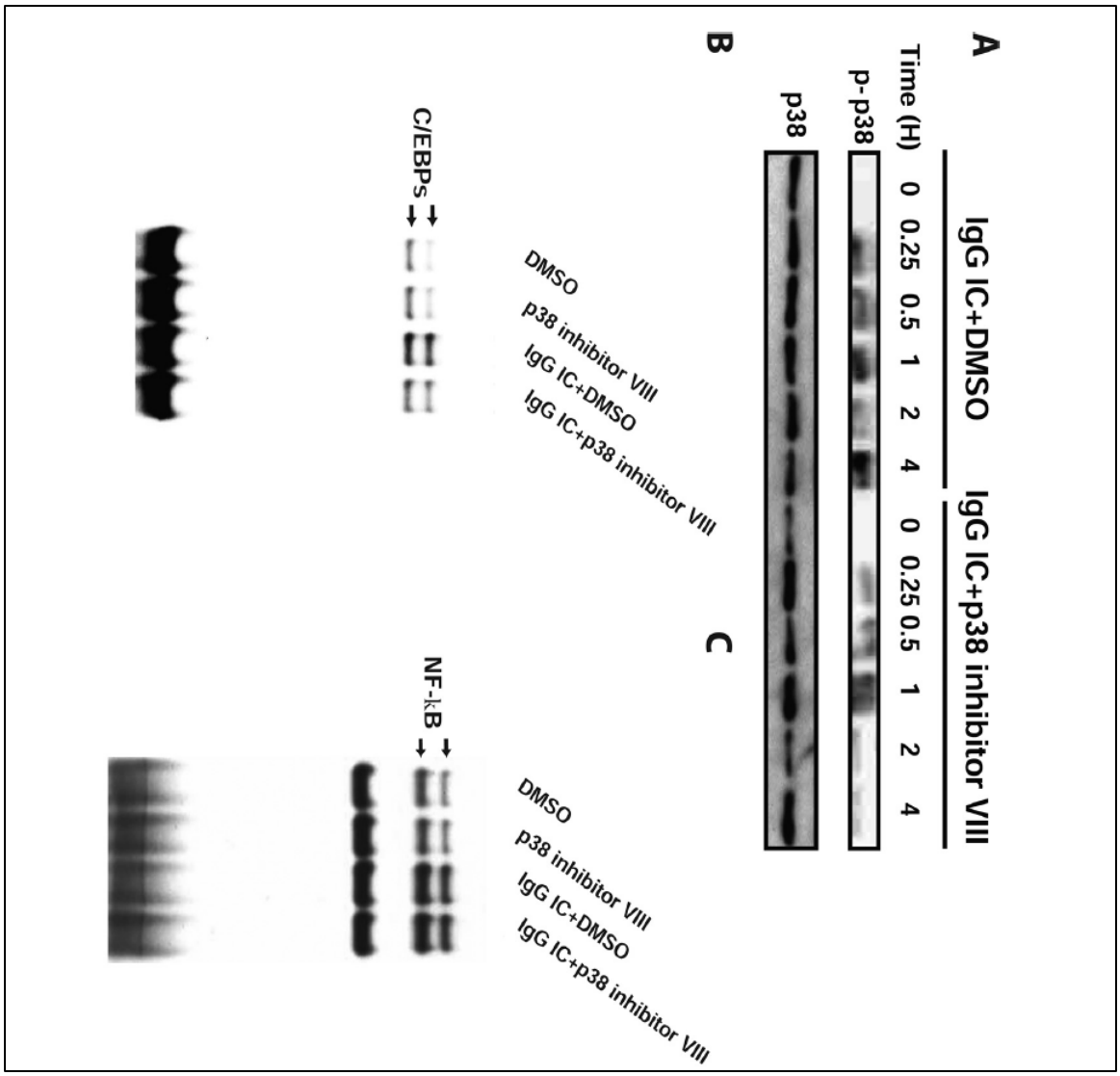

Figure 16. p38 MAPK is Involved in IgG IC-induced C/EBPβ and -δ Activation. A, RAW264.7 cells were treated with 100 μg/ml IgG immune complexes in the presence or absence of p38 MAP Kinases Inhibitor Ⅷ (10 μM) for the indicated time periods. Total proteins were subjected to Western blot by using rabbit anti-phospho-p38 (p-p38) antibody and rabbit anti-p38 antibody, respectively. RAW264.7 cells were treated with 100 μg/ml IgG IC in the presence or absence of p38 MAP Kinases Inhibitor Ⅷ for 4 h. The nuclear proteins were subjected to EMSA for C/EBP DNA binding (B), and NF-κB DNA binding (C), respectively. The same experiment was repeated once.

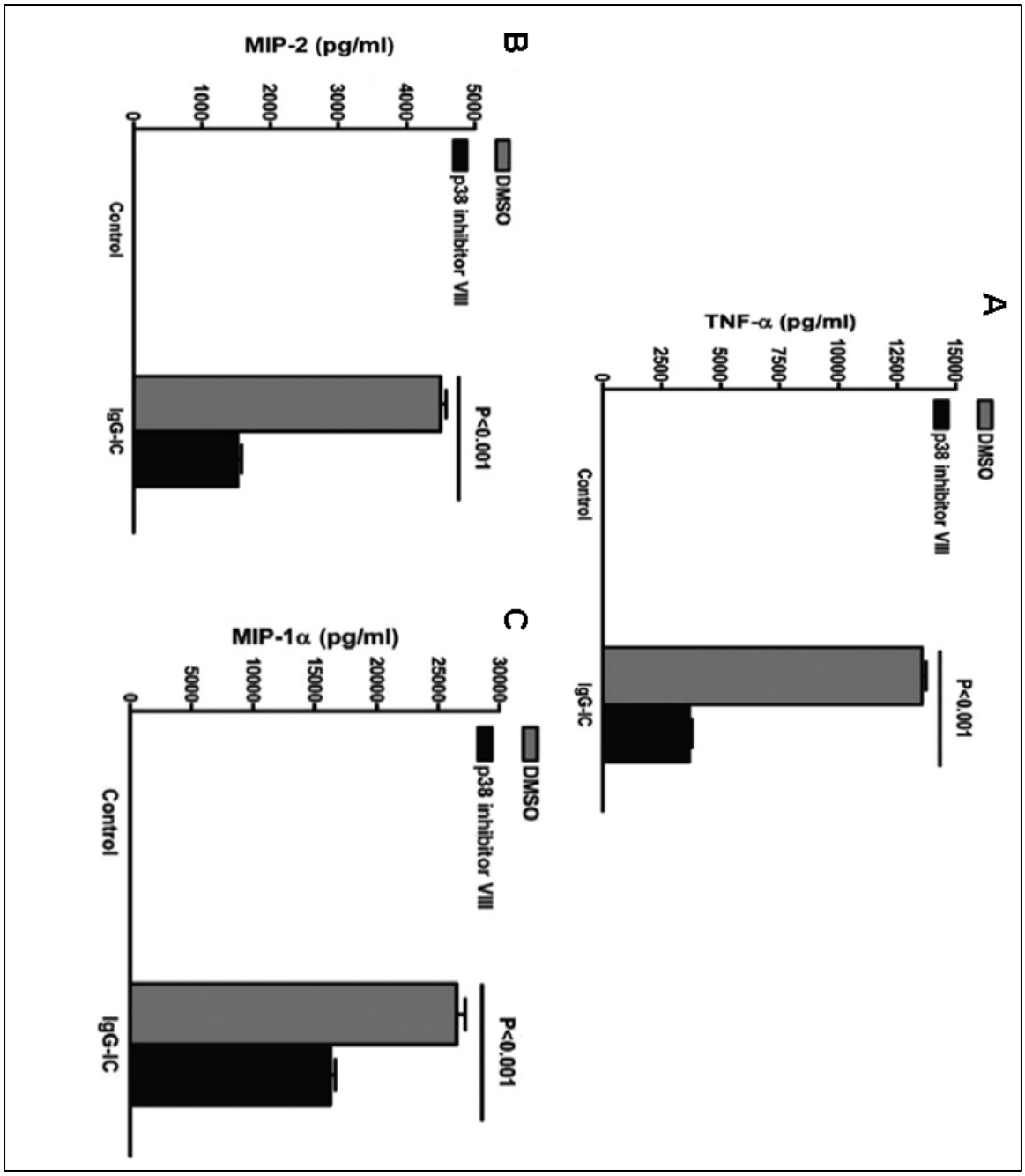

Figure 17. p38 MAPK is Involved in IgG IC-induced Inflammation. RAW264.7 cells were treated with 100 μg/ml IgG immune complexes in the presence or absence of p38 MAPK inhibitor, p38 MAP Kinases Inhibitor Ⅷ (10 μM) for 5 h, and supernatants were subjected to ELISA analysis for production of TNF-α (A), MIP-2 (B), and MIP-1α (C). Data are presented as mean ± S.E., (n =10). The same experiment was repeated once.

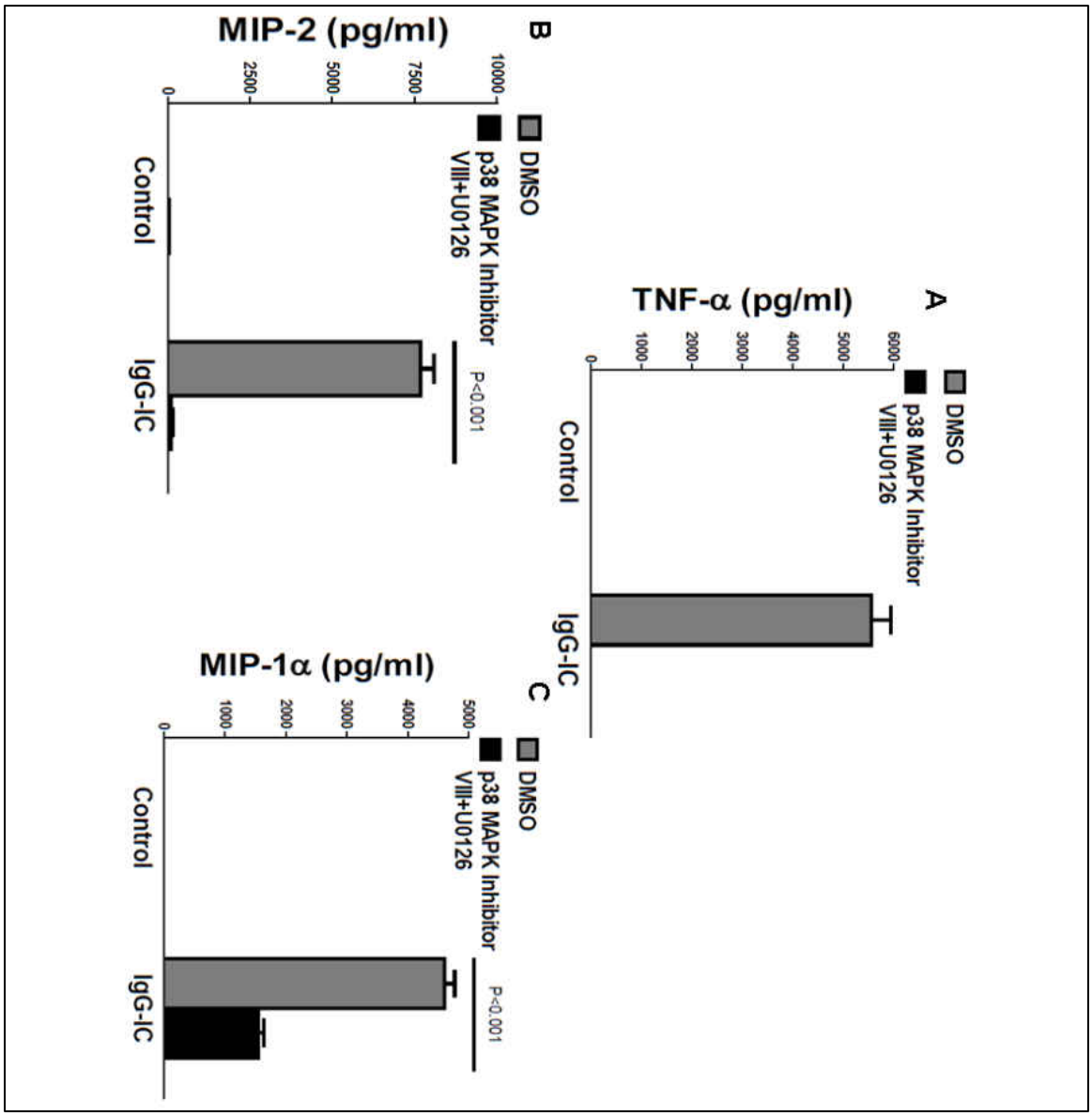

Figure 18. Both p38 and p44/42 MAPKs are Involved in IgG IC-induced Inflammation. RAW264.7 cells were treated with 100 μg/ml IgG immune complex in the presence or absence of both p38 MAP kinase Inhibitor VIII(10 μM) and p44/42 inhibitor U0126 (10 μM) for 5 h. Supernatants were harvested and subjected to ELISA analysis for TNF-α (A), MIP-2 (B), and MIP-1α (C). Data are presented as mean ± S.E., (n =6). The same experiment was repeated once.

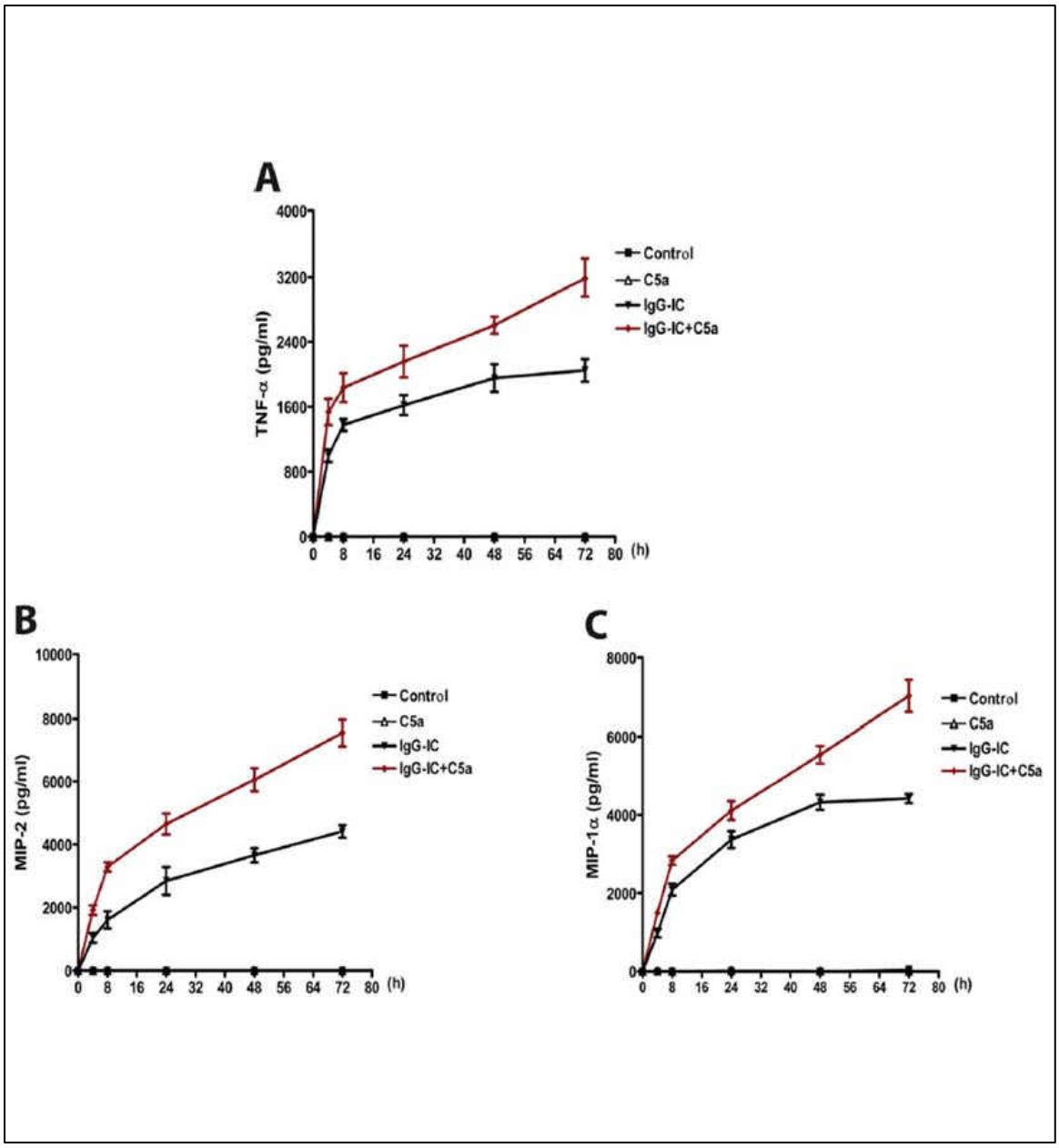

Figure 19. C5a Signaling Enhances IgG Immune Complex-induced Inflammation. Mouse peritoneal macrophages were treated with 10 nM recombinant human C5a, 100 μg/ml IgG immune complexes, or a combination of both stimuli for different time points, and supernatants were subjected to ELISA analysis for production of TNF-α (A), MIP-2 (B), and MIP-1α (C). Data are presented as mean ± S.E, (n =7). The same experiment was repeated twice.

alone had no effect on these inflammatory mediators' production. C5a plays a critical role in regulating the FcγRⅢ/Ⅱ pair to connect complement and FcγR pathways during immune complex-associated inflammatory responses (30). Furthermore, C5a can be generated by IC-stimulated macrophages (218). Therefore, I determined if C5a signaling plays a role in activation of C/EBPs and NF-κB, which may lead to the increased production of TNF-α, MIP-2, and MIP-1α. Reporter assays demonstrated that IgG immune complex-induced C/EBP activity, but not NF-κB activity, was further increased by the addition of C5a (Fig. 20A and B). Moreover, IgG immune complex-stimulated C/EBP DNA binding was significantly reduced in peritoneal macrophages from C5aR-deficient mice compared with wild type controls (Fig. 20C). In contrast, C5aR deficiency had no effect on NF-κB DNA-binding activity (Fig. 20D), consistent with the reporter data. Thus, C5a appears to have a specific role in activating C/EBPs.

To further address the underlying mechanisms whereby C5a enhances IC-induced cytokine and chemokine production, I explored the influence of C5a on MAPK pathways. As shown in Fig. 21, C5a treatment further increased both phospho-p38 and phospho-p44/42 levels induced by IC in RAW264.7 cells. Thus, these data indicate that C5a enhances IC-induced cytokine and chemokine production by elevating phospho-p38 and phospho-p44/42 levels, which lead to increased C/EBPβ and -δ activities.

Roles of C/EBPβ and –δ in IgG IC-induced ALI

Lung C/EBPs are Activated during IgG IC-induced Alveolitis

Previous data have demonstrated the critical roles of C/EBPβ and –δ in IgG immune complex-induced inflammation *in vitro*, I then sought to examine their effects on inflammatory reactions *in vivo*. I first evaluated C/EBP DNA-binding activity in IgG IC-

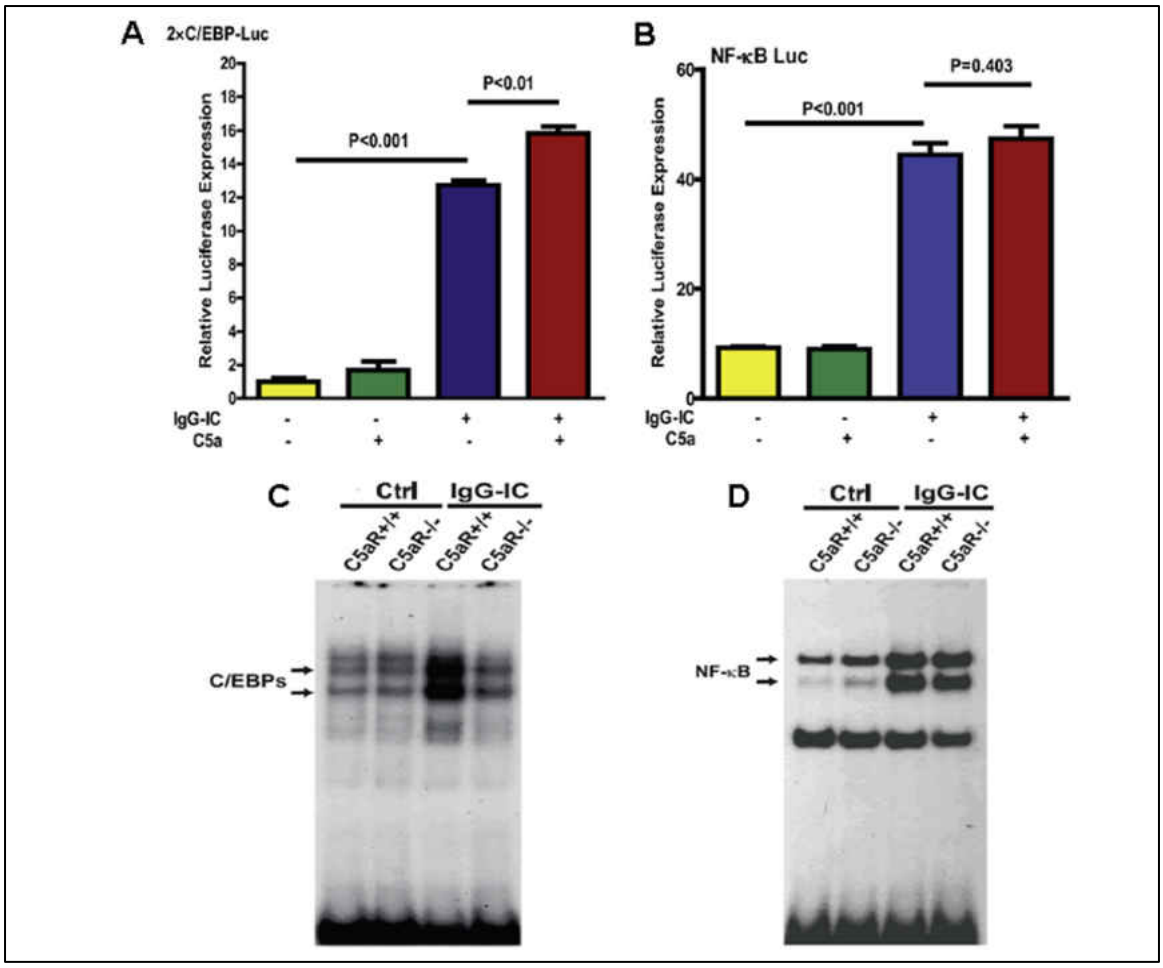

Figure 20. C5a Signaling Enhances IgG IC-induced C/EBP Activation. A and B, RAW264.7 cells were transiently transfected with a total of 0.5 μg of DNA. 24 h after transfection, the cells were treated with 10 nM recombinant human C5a, 100 μg/ml IgG immune complexes, or a combination of both stimuli for 4 h. Cell lysates were used to perform the luciferase activity assay. Luminometer values were normalized for expression from a co-transfected thymidine kinase reporter gene. Data are presented as mean ± S.E., (n =3). The same experiment was repeated three times. Peritoneal macrophages obtained from wild type and C5aR deficient mice were treated with or without 100 μg/ml IgG IC for 4 h, and nuclear proteins were subjected to EMSA for C/EBP (C), and NF-κB (D), respectively. The same experiment was repeated once.

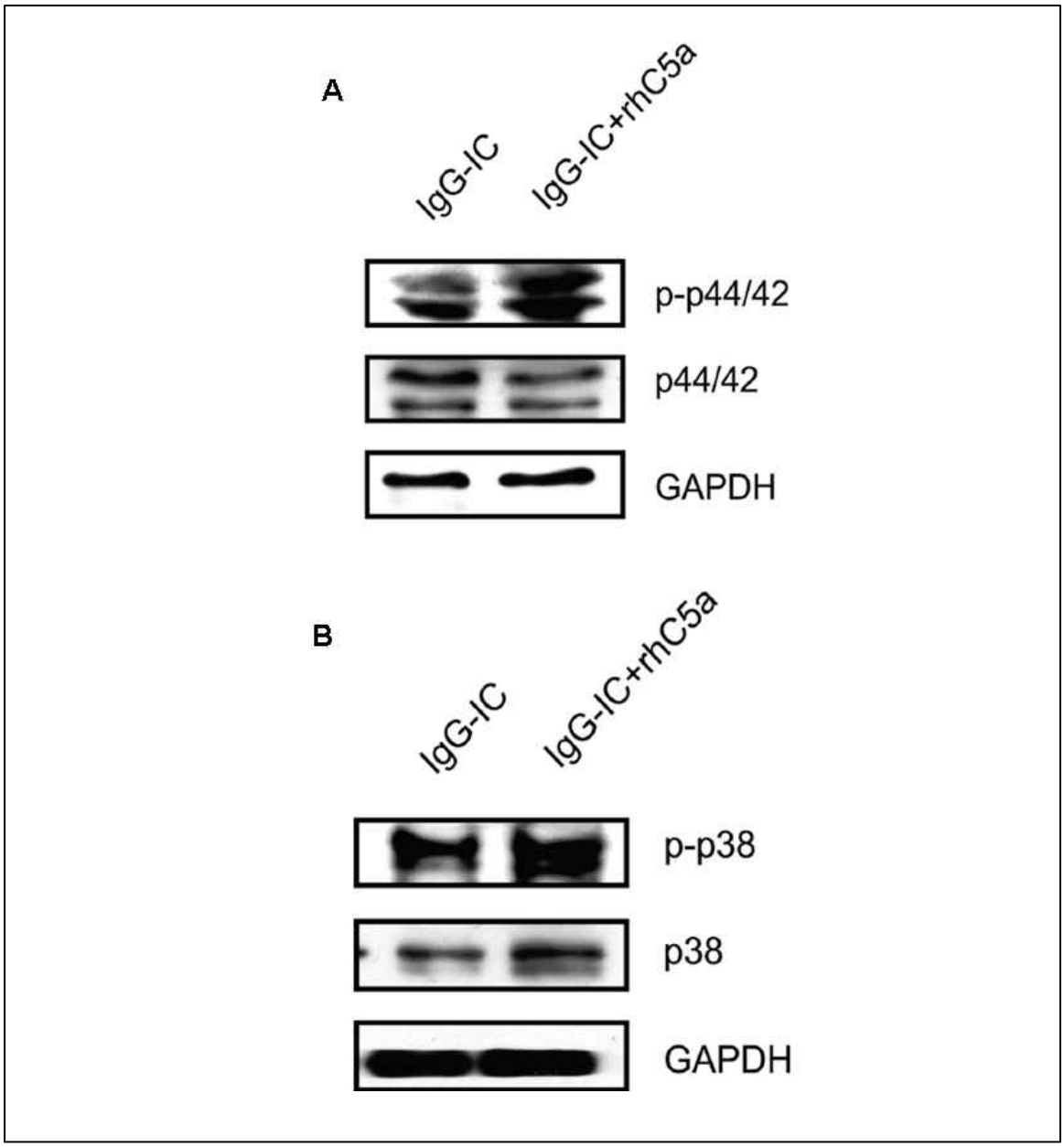

Figure 21. C5a Enhances IgG IC-induced p44/42 and p38 MAPKs Activation. RAW264.7 cells were treated with 100 μg/ml IgG immune complex or IgG immune complex plus 10 nM rhC5a for 4 h. Total proteins were extracted and subjected to Western blot by using rabbit anti-phospho-p44/42 (p-p44/42) antibody (A), rabbit anti-p44/42 antibody (A), rabbit anti-phospho-p38 (p-p38) antibody (B), rabbit anti-p38 antibody (B), and rabbit anti-GAPDH, repectively. The same experiment was repeated once.

injured lung by EMSA (Fig. 22A). 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, I performed supershift assays. As shown in Fig. 22B, there are three major DNA-binding species in the nuclear proteins of control-treated lungs, as follows: low levels of C/EBPα/β heterodimers; heterodimers between C/EBPβ LAP and its short isoform, LIP, which is translated from an alternative start site in the same mRNA (137); 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/EBPδ were significantly induced (Fig. 22B). Using real-time RT-PCR analysis, I further show there is a time-dependent increase in the abundance of lung C/EBPβ mRNA after IgG immune complex deposition (Fig. 22C). Thus, the increased mRNA expression of C/EBPβ is consistent with the increased DNA binding activity in the lung.

Alveolar macrophages play a key role in the IgG immune complex-induced lung injury (10, 61, 67). Therefore, I determined whether alveolar macrophage depletion 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. 23, lanes 1 and 2). By contrast, depletion of alveolar macrophages with Cl_2MDP liposomes markedly reduced the extent of lung C/EBP activation (Fig. 23). These data suggest that alveolar macrophages play a critical role in IgG immune complex-induced activation of C/EBPs in lung.

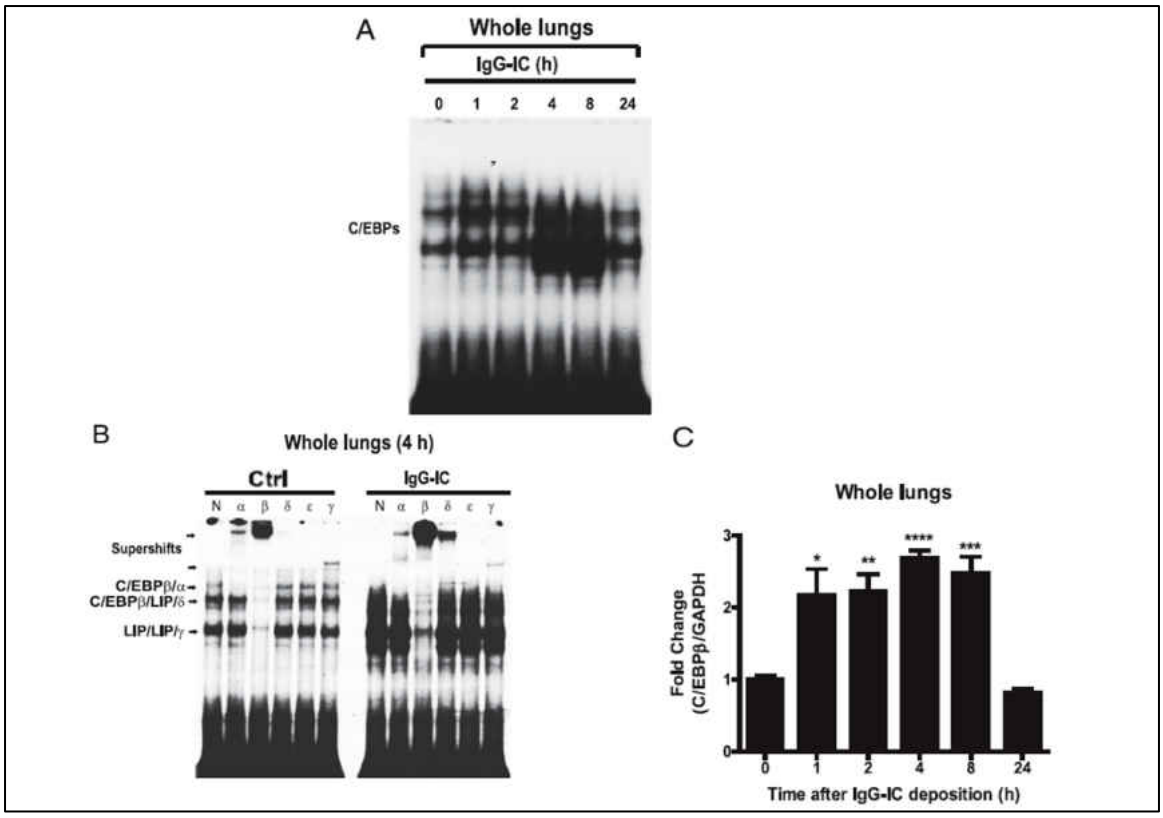

Figure 22. C/EBPβ and C/EBPδ DNA Binding Activities are Induced during IgG IC-induced Alveolitis. A, Mice lungs were challenged by IgG IC for indicated time periods. Then nuclear proteins were extracted from whole lung tissues and subjected to EMSA analysis. B, Nuclear proteins extracted from whole lung 0 and 4 h, respectively, after IgG immune complex deposition were subjected to supershift. The following antibodies (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/EBPγ Ab (γ). Arrows indicated supershifts and C/EBP dimers, respectively. The same experiment was repeated 3 times. C, RNAs were extracted from lung homogenates 0, 1, 2, 4, 8, and 24 h after initiation of IgG immune complex reactions. Real-time PCR was then used to analyze mRNA expression of C/EBPβ during IgG immune complex-induced lung injury. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Results are means ± S.E., n=4. The same experiment was repeated once.

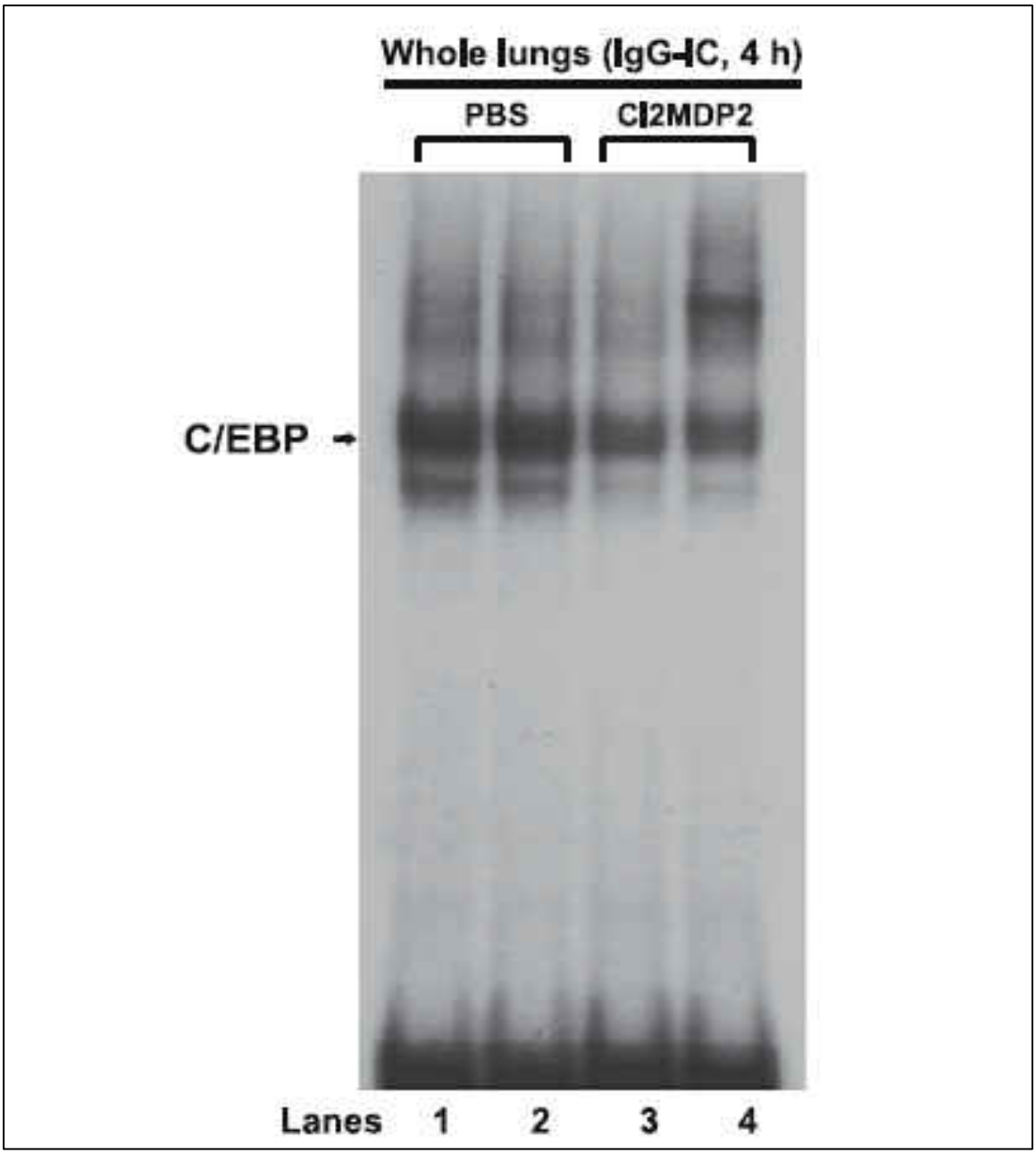

Figure 23. Effects of Alveolar Macrophage Depletion on C/EBP Activation during IgG Immune Complex-induced Lung Injury. Mice received PBS-liposome or Cl_2MDP-liposomes 24 h before receiving intratracheal challenge with IgG immune complex. Four hours after immune complex challenge, lungs were harvested. C/EBP-binding activity of whole lung nuclear extracts was assessed by EMSA. The same experiment was repeated four times.

Effects of C/EBPβ Deficiency on IgG Immune Complex-induced Lung Injury

I sought to determine whether C/EBPβ contributed to IgG IC-induced lung injury by using C/EBPβ deficient mice. As shown in Fig. 24A, C/EBPβ deficiency resulted in a significant decrease ($p < 0.001$) of permeability index (albumin leakage) when compared with wild-type mice after IgG IC deposition. I also examined MPO content to evaluate neutrophil accumulation in lungs (Fig. 24B). As with albumin leakage, MPO content in C/EBPβ deficient mice was much lower ($p < 0.01$) when compared with values in control-treated wild type mice during lung injury. I further found that C/EBPβ deficient mice displayed significant attenuation of the total number of white blood cells (WBCs) (by 59%, $p < 0.001$; Fig. 24C) and neutrophils (by 61%, $p < 0.001$; Fig. 24D) in BAL fluids from IgG IC-injured lung compared with wild-type mice. I examined whether C/EBPβ deficient mice exhibited reduced lung injury by histological analyses. As shown in Fig. 25, both wild type and C/EBPβ deficient control mice 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/EBPβ 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/EBPβ Deficient Mice

I determined BAL levels of several inflammatory cytokines and chemokines that are involved in IgG immune complex-induced lung injury (10). As expected, wild type mice undergoing IgG immune complex deposition showed increased production of TNF-α, IL-6, MIP-2, KC, MIP-1α, and MIP-1β compared with controls (Fig. 26A-F). The levels of

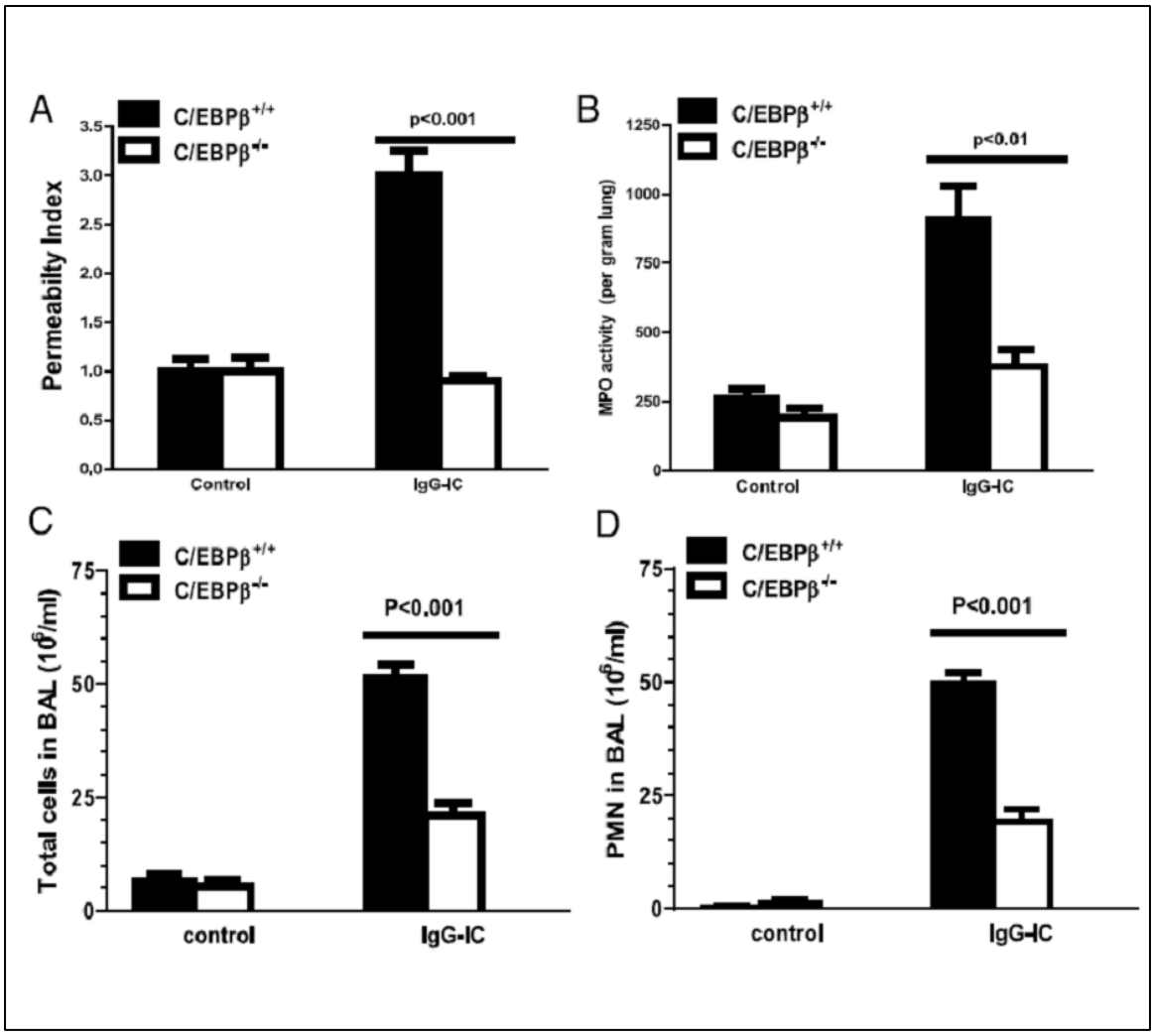

Figure 24. Effects of C/EBPβ Deficiency on IgG Immune Complex-induced Pulmonary Inflammation. Four hours after IgG immune complex deposition, BAL fluids and lungs were harvested. A, mouse albumin content in BAL fluids was determined using ELISA. The permeability index was expressed as the ratio of the albumin in the IgG immune complex-injured lungs versus that in the control-treated lungs of same type of mice. B, changes in lung MPO activity was measured as a marker for pulmonary neutrophil accumulation. Total cell accumulation (C), and neutrophil influx (D) in BAL fluids were counted. Results are means ± S.E. for three (control group) or five (IgG immune complex-challenged group) mice for each group. The same experiment was repeated once.

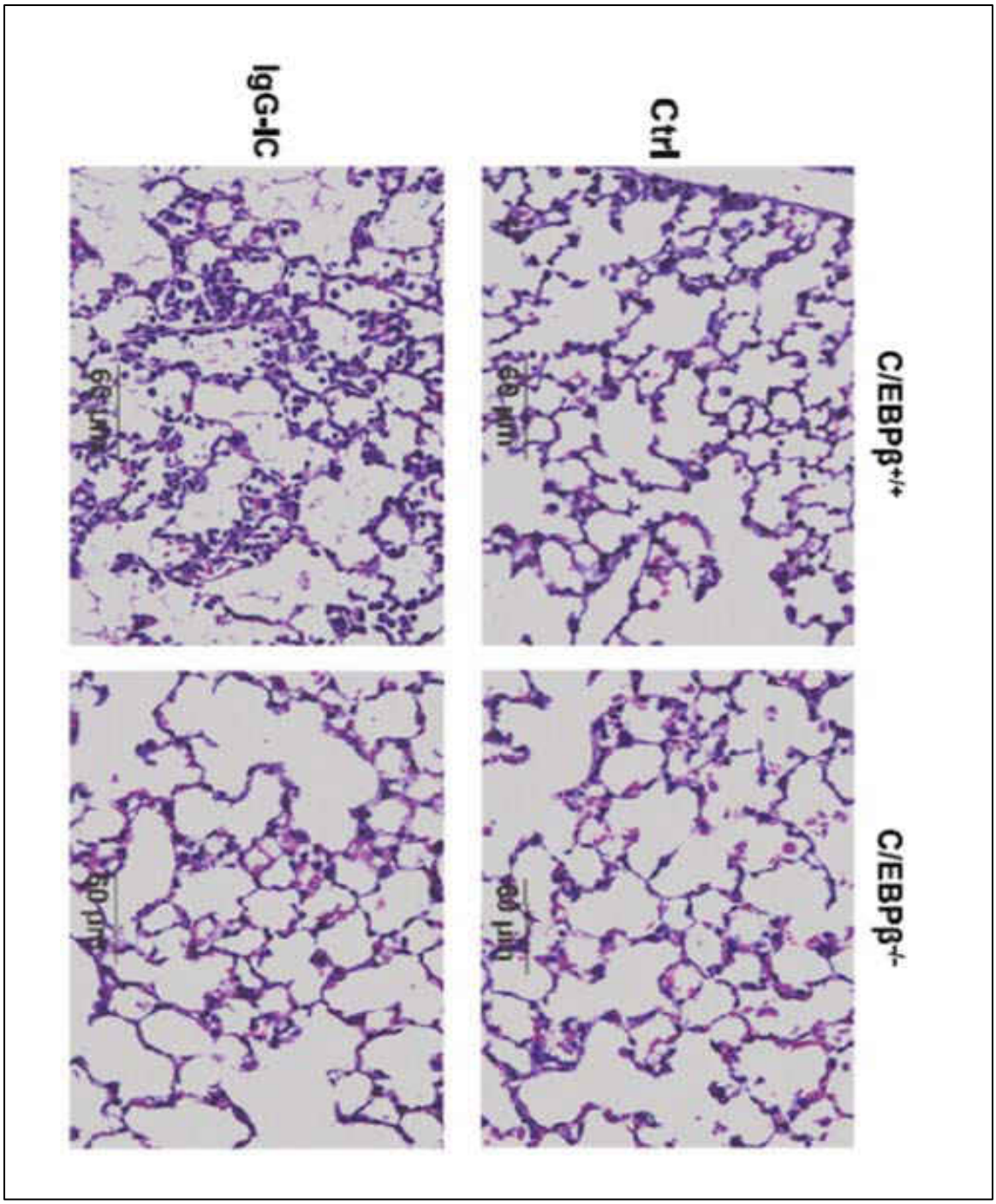

Figure 25. Effects of C/EBPβ Deficiency on IgG IC-induced Lung Injury Bases on Histological Assay. Lung sections were stained with H&E (original magnification × 40). Lung sections shown included the following: C/EBPβ$^{+/+}$ + Ctrl, C/EBPβ$^{+/+}$ + IgG IC, C/EBPβ$^{-/-}$ + Ctrl, and C/EBPβ$^{-/-}$ + IgG IC. The same experiment was repeated three times.

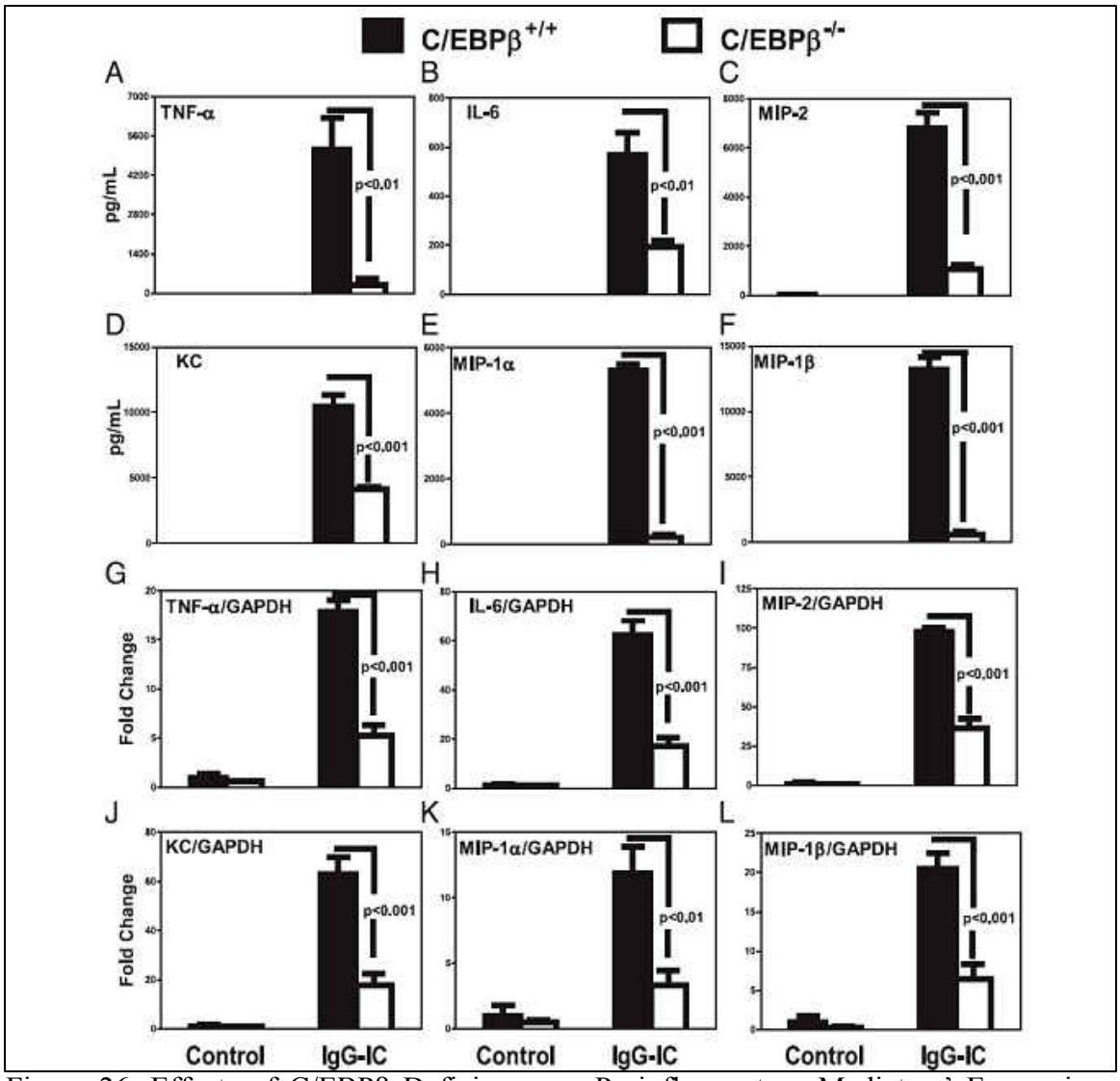

Figure 26. Effects of C/EBPβ Deficiency on Proinflammatory Mediators' Expressions during IgG IC-induced Acute Lung Injury. Four hours after IgG immune complex deposition, BAL fluids and lungs were harvested. Cell-free BAL fluids were subjected to ELISA to measure TNF-α (A), IL-6 (B), MIP-2 (C), KC (D), MIP-1α (E), and MIP-1β (F) levels. RNAs were extracted, and real-time PCR was then used to analyze mRNA expression of TNF-α (G), IL-6 (H), MIP-2 (I), KC (J), MIP-1α (K), and MIP-1β (L). Results are means ± S.E. for three (control group) or five (IgG immune complex-challenged group) mice for each group. The same experiment was repeated once.

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/EBPβ deficient mice expressed considerably lower amounts of mRNAs for TNF-α, IL-6, MIP-2, KC, MIP-1α, and MIP-1β (Fig. 26G-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 (219). Notably, IgG immune complex-injured lungs from C/EBPβ deficient mice showed a marked reduction in ICAM-1 mRNA, whereas very little decrease in VCAM-1 expression was observed (Fig. 27). Because soluble ICAM-1 can directly bind and activate lung macrophages, and enhance lung injury after intrapulmonary disposition of IgG immune complexes (196), I measured soluble ICAM-1 level in BAL fluids of IgG immune complex-injured lungs. I found that C/EBPβ 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. 27).

Effects of C/EBPβ Deficiency on Cytokine and Chemokine Protein Production in Alveolar Macrophages Stimulated by IgG IC

I 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. 28). 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. 28). To perform EMSA, about 2.5 million cells are required

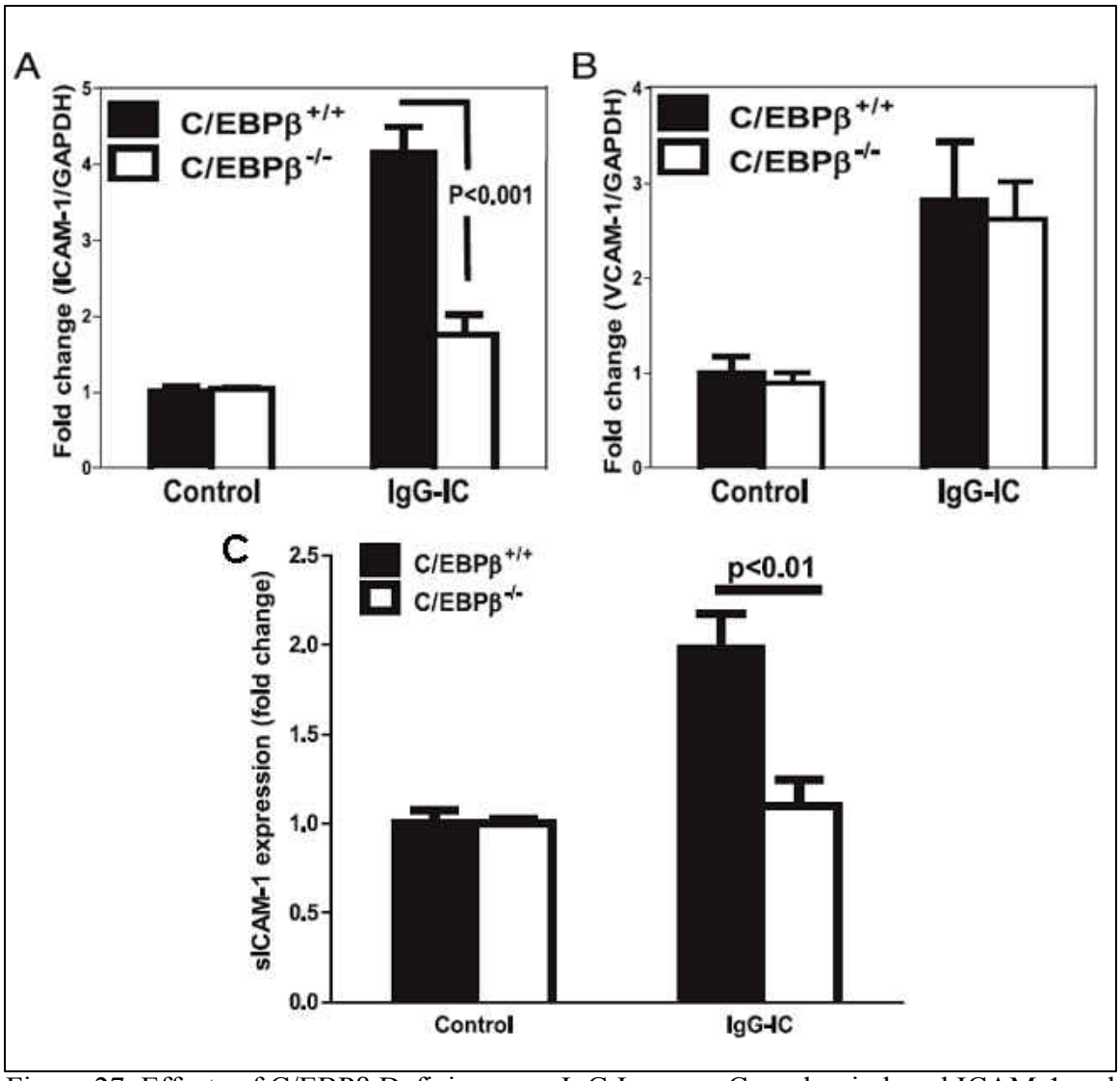

Figure 27. Effects of C/EBPβ Deficiency on IgG Immune Complex-induced ICAM-1 and VCAM-1 Expressions. Four hours after IgG immune complex deposition, BAL fluids and lungs were harvested. Real-time PCR was then used to analyze mRNA expression of ICAM-1 (A), and VCAM-1 (B). (C) Cell-free BAL fluids were subjected to ELISA to measure sICAM-1 level. The relative sICAM-1 level was expressed as the ratio of the sICAM-1 in the IgG immune complex-injured lungs versus that in the control-treated lungs of same type of mice. Results are means ± S.E. for three (control group) or five (IgG IC-challenged group) mice for each group. The same experiment was repeated once.

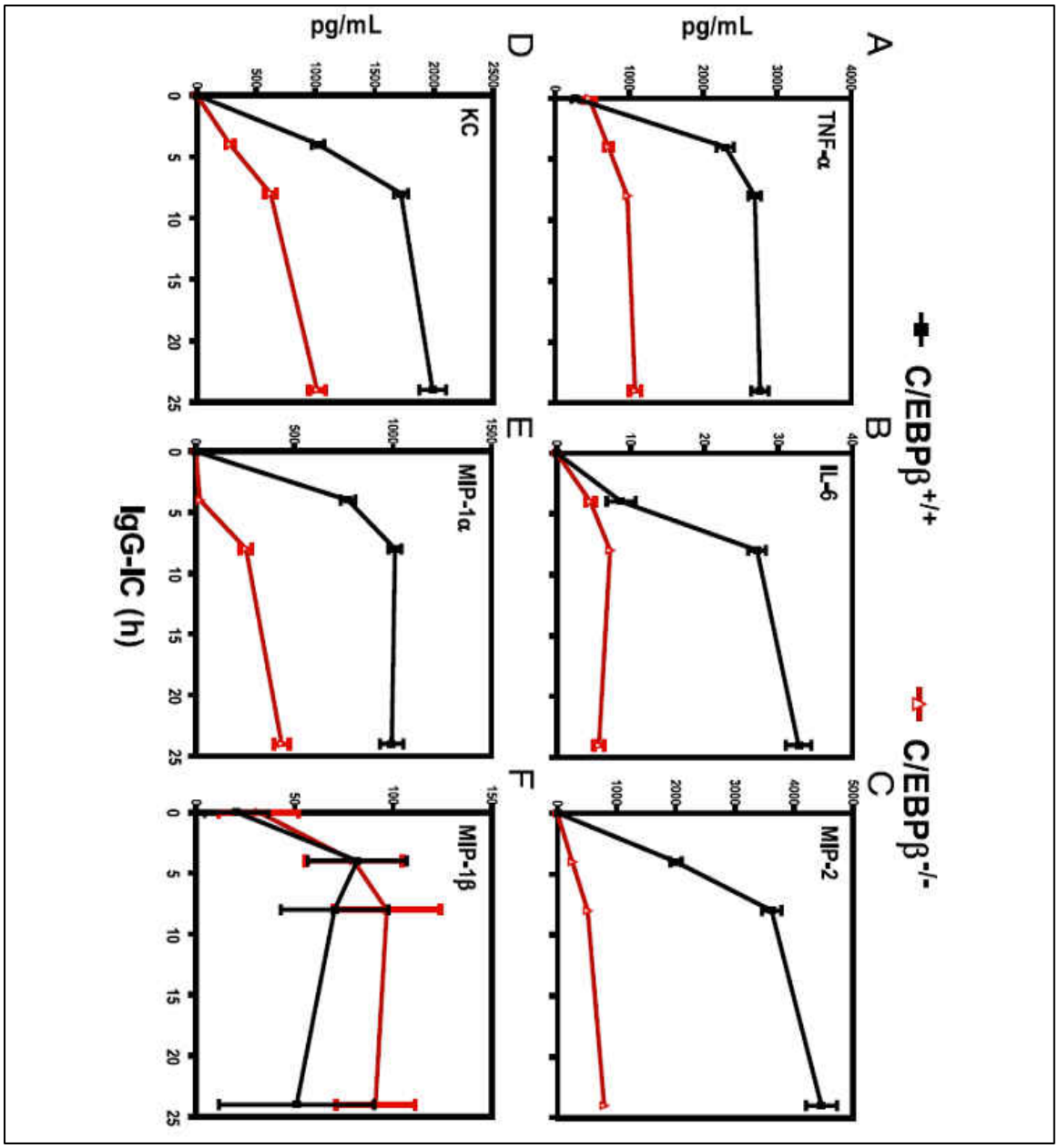

Figure 28. Effects of C/EBPβ Deficiency on IgG immune Complex-induced Inflammatory Reactions in Alveolar Macrophages. Alveolar macrophages obtained from wild-type and C/EBPβ knockout mice were treated with 100 μg/ml IgG immune complexes for the times indicated, and supernatants were subjected to ELISA to assess production of TNF-α (A), IL-6 (B), MIP-2 (C), KC (D), MIP-1α (E), and MIP-1β (F). The data were expressed as means ± S. E. (n = 6). The same experiment was repeated once.

for one time point, however, only 0.25 million primary alveolar macrophages could be harvested from each mouse. Thus, MH-S cells—alveolar macrophage-derived cell line, were used to determine the effects of IgG IC on C/EBP DNA binding activities in alveolar macrophage. As shown in Fig. 29A, C/EBP DNA binding activities was induced by IgG IC. To further determine which C/EBP family members were induced by IgG IC, supershift assays were performed. There are four major DNA-binding species in the nuclear proteins of control-treated macrophages, as follows: C/EBPβ LAP/C/EBPγ heterodimers; heterodimers between C/EBPβ LAP and LIP; LAP/LAP homodimers, and LIP/LIP homodimers. In IgG immune complex-treated macrophages, the DNA-binding activity of C/EBPβ (mainly LAP/LIP, and LAP/LAP) was induced (Fig. 29B). To examine the role of C/EBPβ in IgG IC-induced inflammation in MH-S cells, I ablated C/EBPβ expression by siRNA-mediated silencing (Fig. 30A). Analysis of inflammatory 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%, and MIP-2 by 48%, respectively; Fig. 30B-C).

Effects of C/EBPβ Overexpression on IgG IC-induced TNF-α and IL-6 Expression in Alveolar Macrophages

I examined IgG immune complex-induced C/EBP transcriptional activity in transiently transfected MH-S cells using 2 × C/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). Because the transfection efficiency is very low in primary alveolar macrophages, MH-S cells were used to conduct transfection. Consistent with the results from EMSA, IgG immune complex stimulation alone induced a 3.8-fold increase in

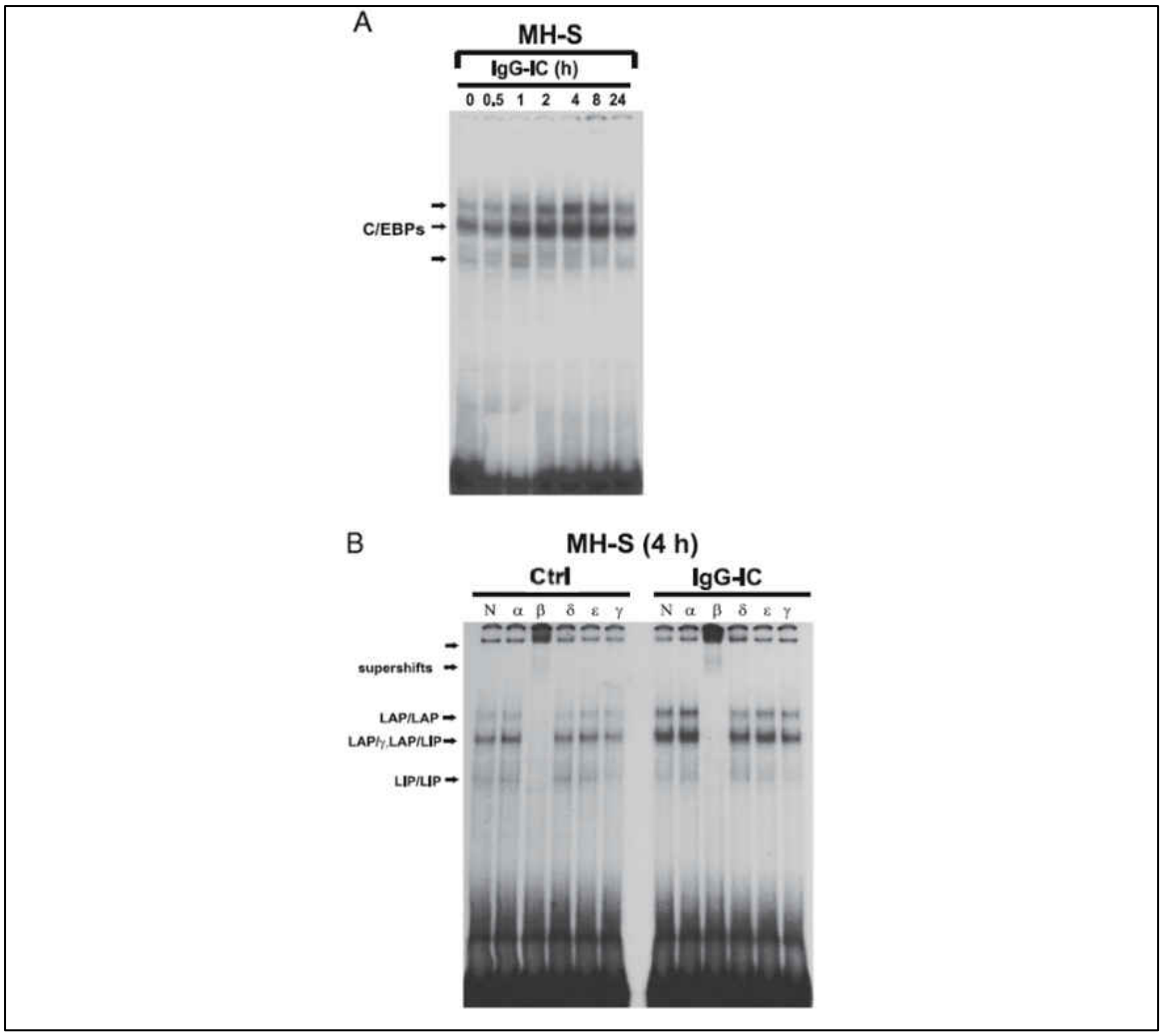

Figure 29. C/EBPβ and C/EBPδ DNA Binding Activities are Upregulated in MH-S Cells during IgG IC-induced Inflammatory Responses. A, MH-S cells were challenged by 100 μg/ml IgG IC for different time periods as indicated. Nuclear extracts were then extracted, and subjected to EMSA to detect C/EBP activity. B, Nuclear proteins extracted from MH-S cells, 0 and 4 h, respectively, after IgG immune complex challenge were subjected to supershift. The following antibodies (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/EBPγ Ab (γ). Arrows indicated supershifts and C/EBP dimers, respectively. The same experiment was repeated three times.

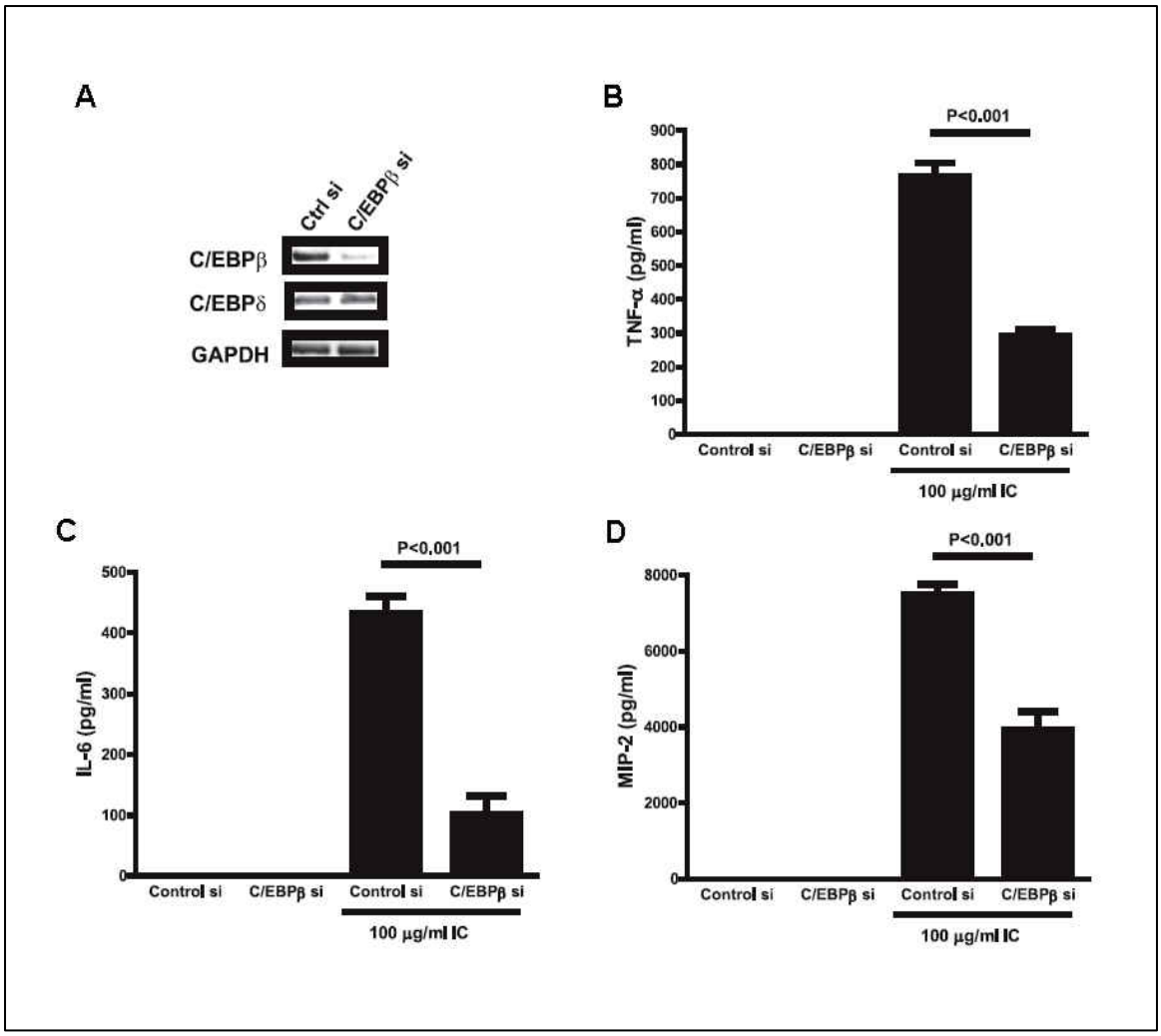

Figure 30. C/EBPβ is Required for IgG IC-induced TNF-α, IL-6 and MIP-2 Expressions in MH-S Cells. A, MH-S cells were transiently transfected with control siRNA or C/EBPβ siRNA. Twelve hours after transfection, RNAs were isolated. Then RT-PCR was performed by using primers for C/EBPβ, C/EBPδ, and GAPDH, respectively. The level of GAPDH was shown at the bottom as a loading control. Twelve hours after transfection, the cells were incubated with 100 μg/ml IgG immune complexes for 6 h. Supernatants were harvested, and ELISA was performed to investigate the expressions of TNF-α (B), IL-6 (C), and MIP-2 (D). The data were expressed as means ± S. E. (n = 12). The same experiment was repeated three times.

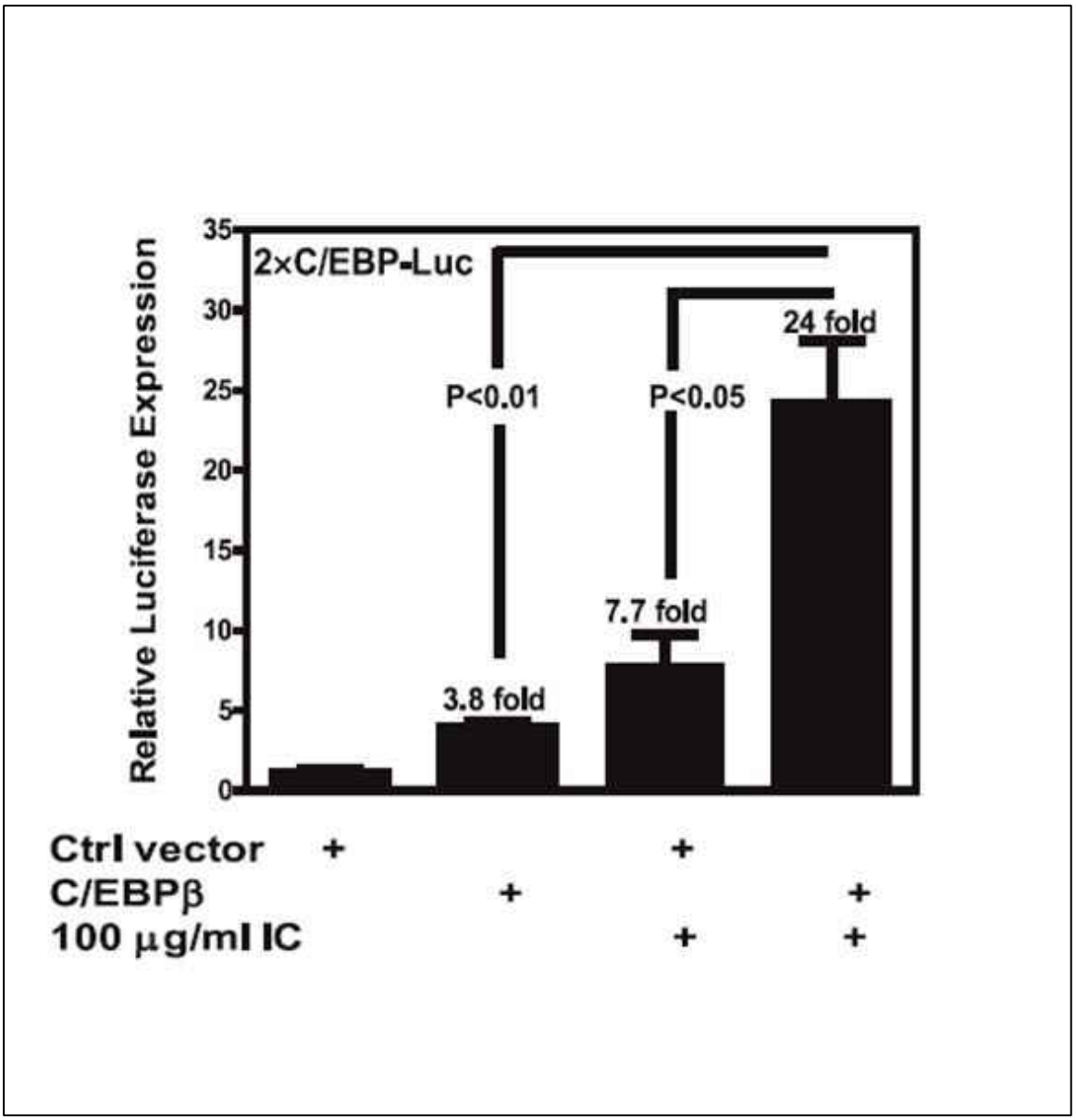

Figure 31. Effects of C/EBPβ on IgG Immune Complex-induced C/EBP DNA Binding in Alveolar Macrophage. MH-S cells were transiently transfected with total of 0.5 μg indicated DNA. Twenty four hours after transfection, the cells were challenged with indicated stimulus for 4 h. Cell lysates were used for 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 ± S. E. (n=3). The same experiment was reapted once.

luciferase activity compared with the untreated control (Fig. 31). C/EBPβ vector alone also elevated 2 × C/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 super additive, IgG immune complexes may stimulate the intrinsic activity of C/EBPβ as well as its expression.

I 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. 32, IgG immune complex stimulation alone markedly 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/EBPδ Deficiency on IgG Immune Complex-induced Lung Inflammation

Because C/EBPδ DNA-binding activity was induced by IgG immune complex deposition in the lung, I examined whether C/EBPδ contributes to IgG immune complex-induced lung inflammatory responses. As shown in Fig. 33, there was no significant difference in lung MPO activity between wild-type and C/EBPδ deficient mice 4 h after IgG immune complex deposition. Consistently, BAL fluids from lungs of C/EBPδ deficient mice showed similar levels of TNF-α and MIP-2 when compared with BAL fluids from injured wild-type mice (Fig. 34). I next determined the effects of C/EBPδ on the inflammatory mediator production from IgG immune complex-stimulated primary alveolar macrophages. As shown in Fig. 35, C/EBPδ deficiency in alveolar macrophages

caused only modest decreases in TNF-α, MIP-2, KC, and MIP-1α production compared with wild type macrophages at all time points analyzed. Thus, C/EBPβ (but not C/EBPδ) is critical for IgG immune complex-induced inflammatory injury in the lung.

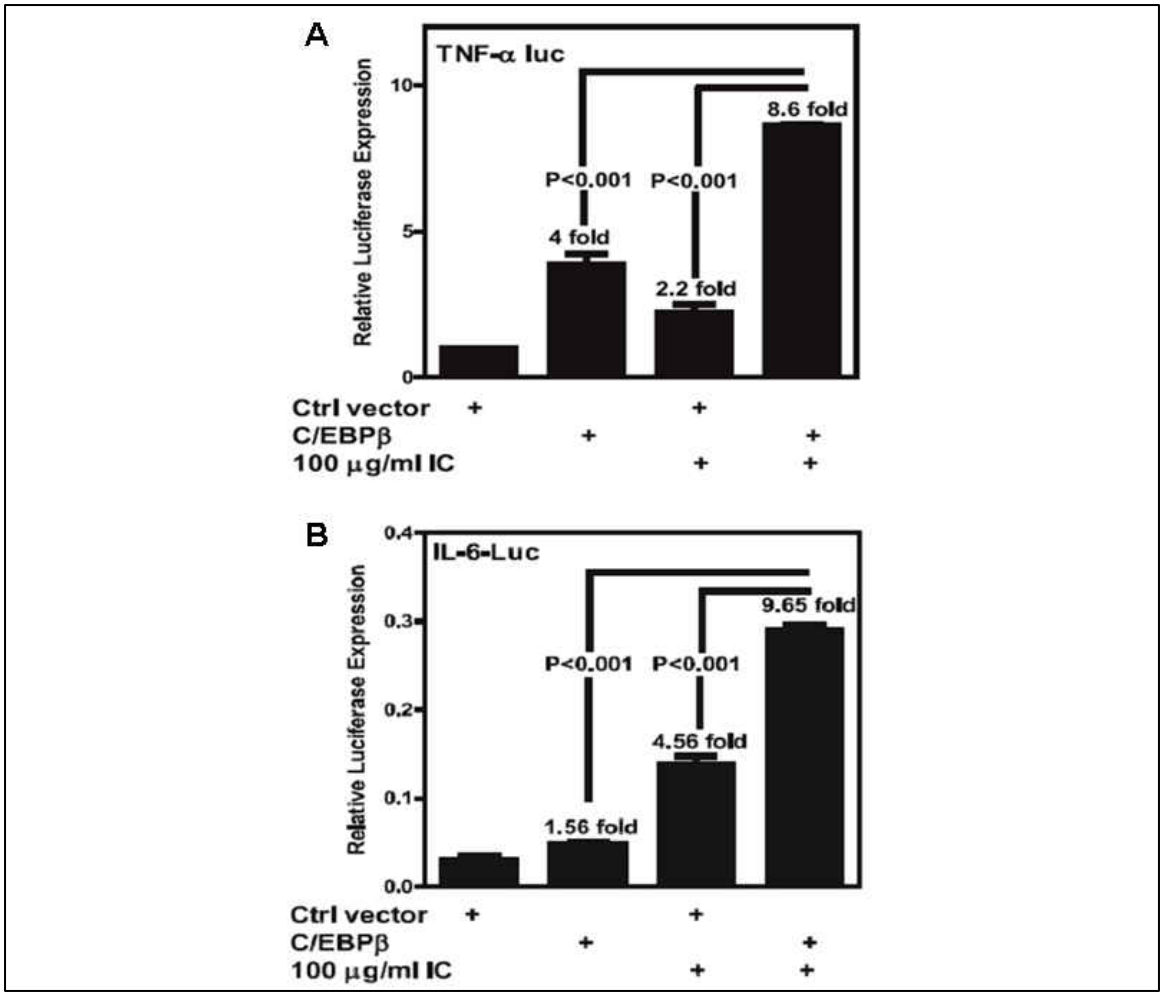

Figure 32. Effects of C/EBPβ Expression on IgG Immune Complex-induced TNF-α and IL-6 Production in MH-S Cells. MH-S cells were transiently transfected with total of 0.5 μg indicated DNA. Twenty four hours after transfection, the cells were challenged with indicated stimulus for 4 h. Cell lysates were 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 ± S. E. (n=3). The same experiment was repeated three times.

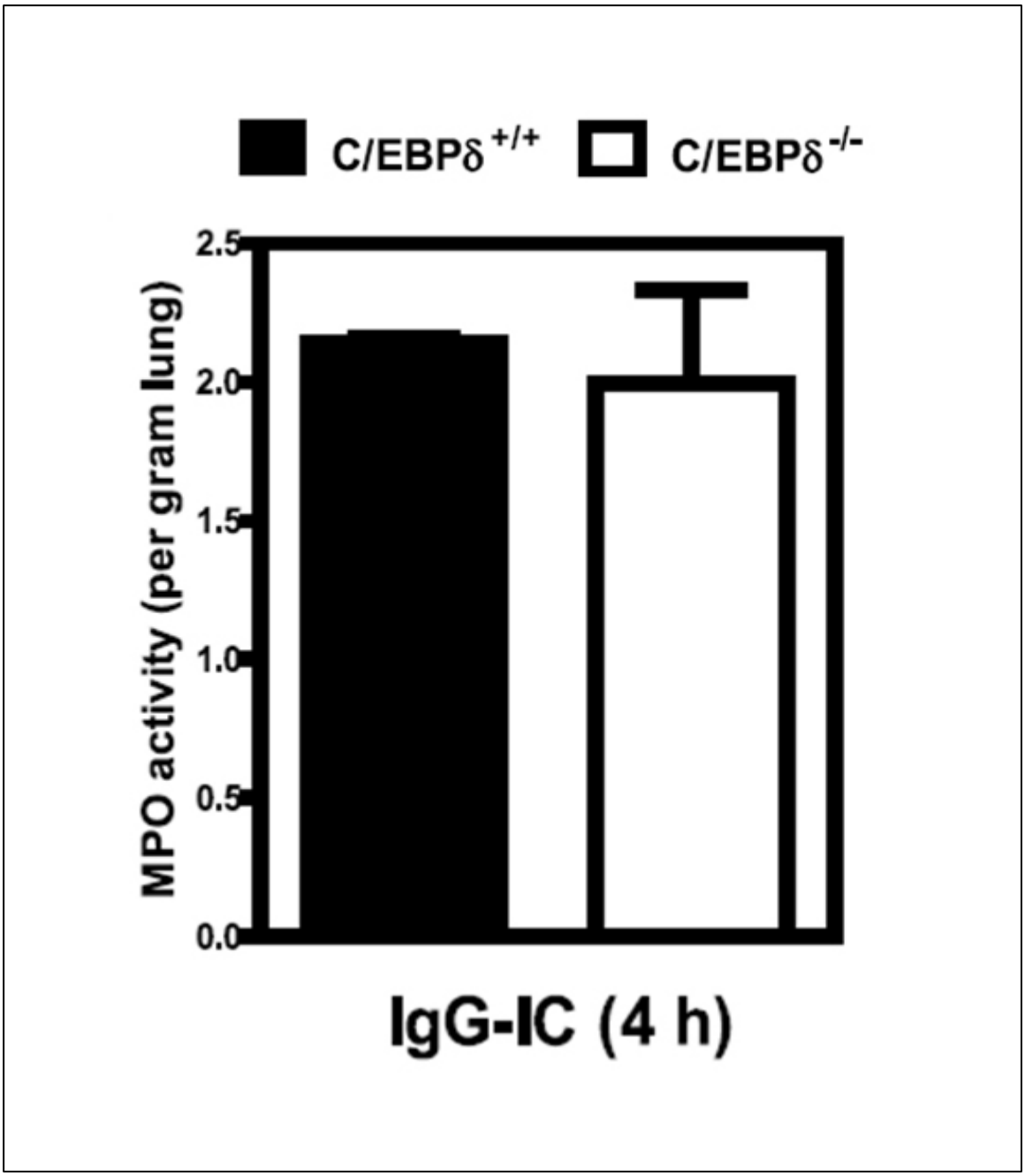

Figure 33. Effects of C/EBPδ Deficiency on Neutrophil Influx during IgG IC-induced Acute Lung Injury. Four hours after IgG IC deposition, mice lungs were harvested, and changes in lung MPO activity was measured as a marker for pulmonary neutrophil accumulation. Results are means ± S.E. for six mice for each group. The same experiment was repeated once.

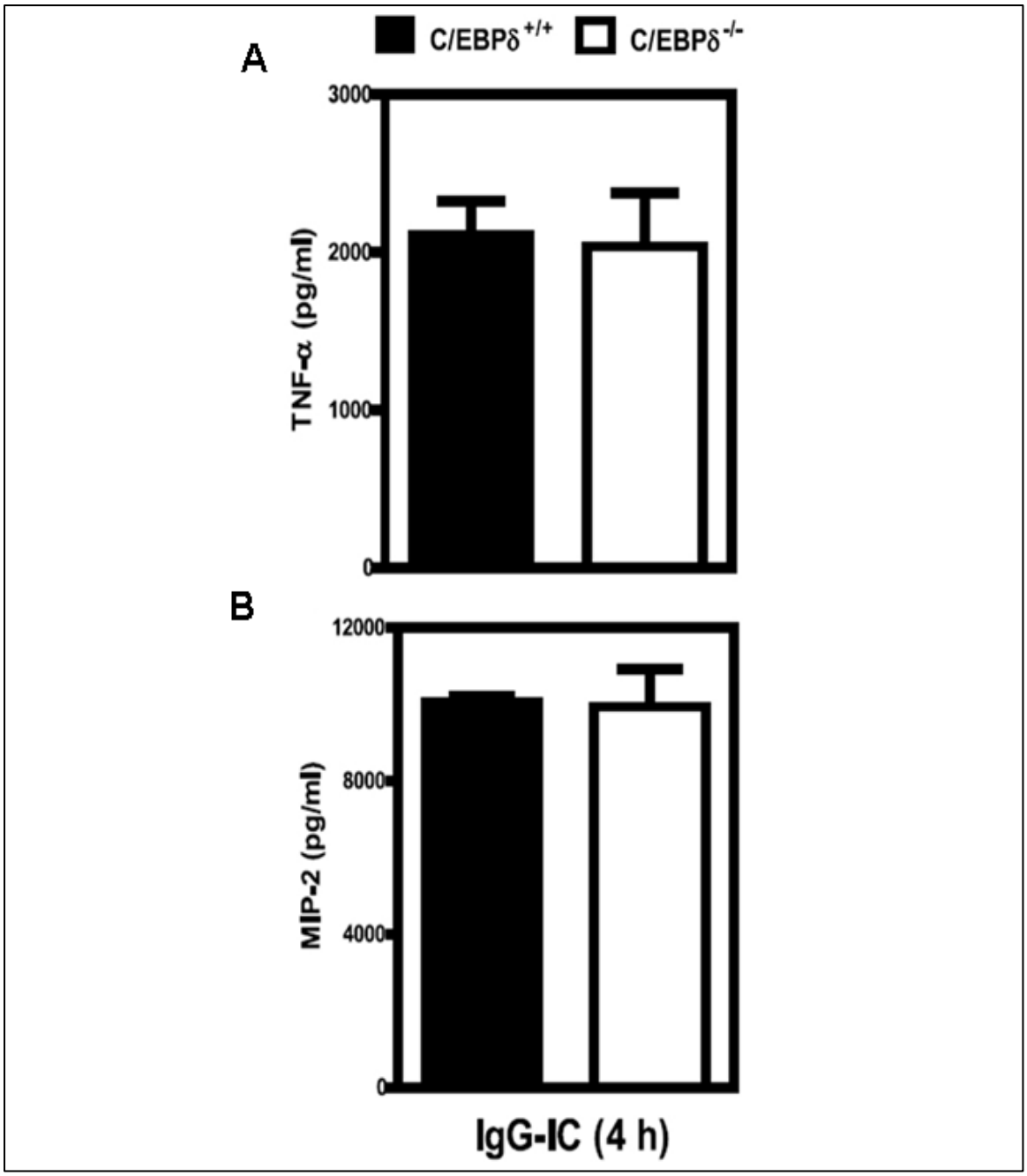

Figure 34. Effects of C/EBPδ Deficiency on TNF-α and MIP-2 Expressions during IgG Immune Complex-induced Acute Lung Injury. Four hours after IgG immune complex deposition, BAL fluids were harvested, and subjected to ELISA to measure TNF-α expression. Results are means ± S.E. for six mice for each group. The same experiment was repeated once.

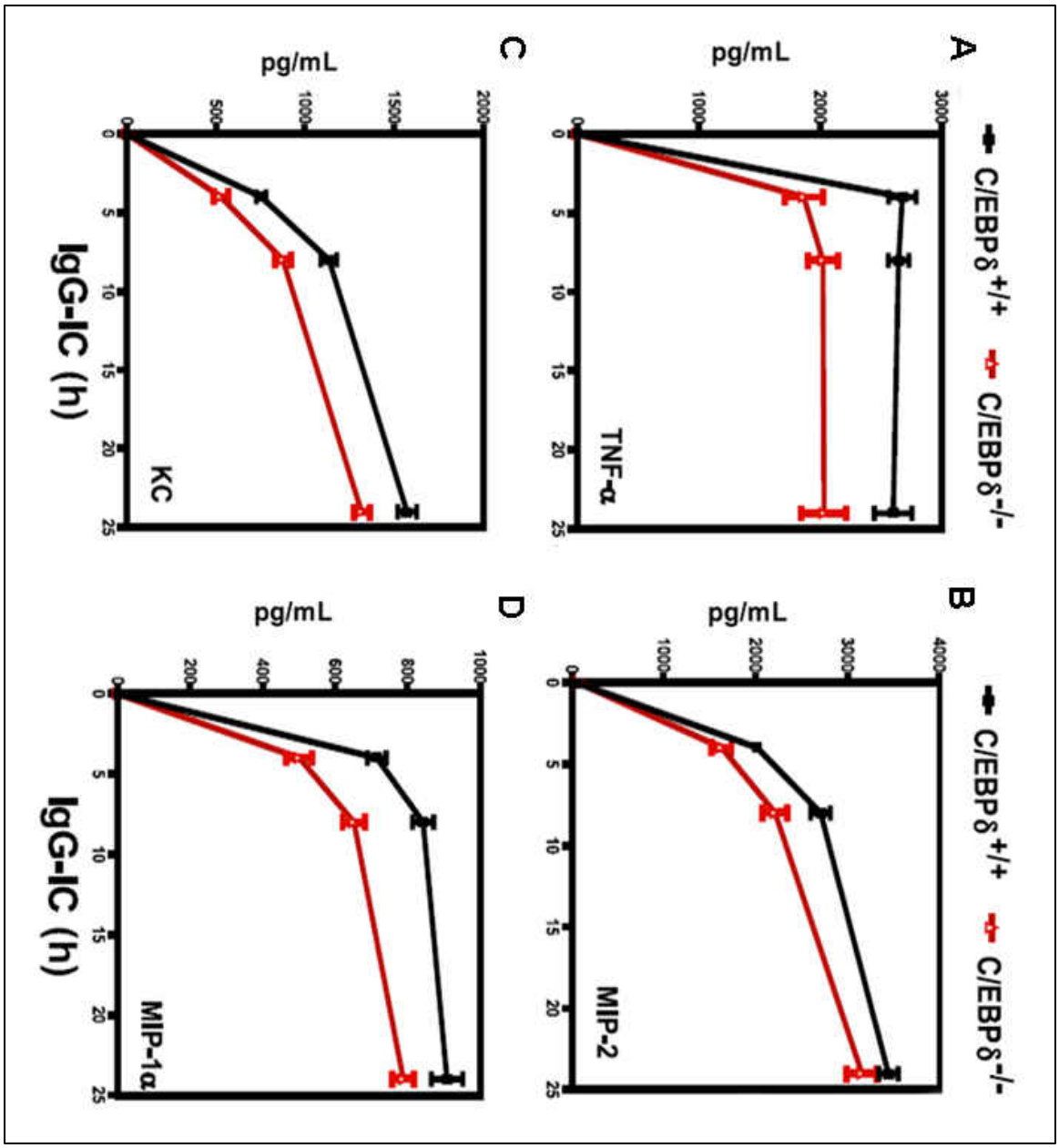

Figure 35. Effects of C/EBPδ Deficiency on IgG IC-induced Inflammation in Alveolar Macrophages. Alveolar macrophages obtained from wild-type and C/EBPβ knockout mice were treated with 100 μg/ml IgG immune complexes for the times indicated, and supernatants were subjected to ELISA to assess production of TNF-α (A), MIP-2 (B), KC (C), and MIP-1α (D). The data were expressed as means ± S. E. (n = 6). The same experiment was repeated once.

CHAPTER Ⅳ

DISCUSSION

Roles of C/EBPβ and –δ in IgG IC-induced Inflammation in Macrophages

Macrophage FcγR activation plays a central role in the immune defense system (6, 193). However, the signaling pathways from FcγRs to the nucleus remain largely unknown. In my study, I provide the evidence that C/EBPβ and -δ are key transcription factors that regulate FcγR-mediated generation of inflammatory cytokines and chemokines in macrophages. To our knowledge, this is the first study indicating that C/EBPs are key regulators of immune complex-induced inflammatory responses.

Increasing evidence suggests an important role for C/EBPs, such as C/EBPβ and -δ, in inflammation (199, 220, 221). For example, C/EBPβ has been shown to be an effector in the induction of acute phase and inflammatory genes responsive to LPS, IL-1, or IL-6 (109, 222). C/EBPδ is relatively less well characterized than C/EBPβ. However, similarly to C/EBPβ, C/EBPδ has also been implicated in regulation of the acute phase and inflammatory responses (128, 148). Interestingly, a recent study demonstrates a critical role for C/EBPδ in a regulatory circuit that discriminates between transient and persistent TLR4 stimulation (220). In another study, Maitra *et al*. show that C/EBPδ is the key mediator to initiate low dose endotoxin-induced inflammation (207). Functional C/EBP binding sites have been identified in the promoter regions of the TNF-α, MIP-2, and MIP-1α (194, 223-225). For example, C/EBPβ has been shown to play an important role in

the regulation of the TNF-α gene in myelomonocytic cells (223). Furthermore, serial and site-directed deletion mutants of MIP-2 luciferase reporter genes demonstrate that the binding sites for both C/EBPβ and NF-κB are essential for the activation of the MIP-2 promoter in response to nitric oxide (224). In addition, a recent study suggests that both C/EBPβ and NF-κB are involved in the upregulation of MIP-1α expression in chondrocytes treated with IL-1β (225). Using monocytic cells, Fernández *et al*. recently show that cross-linking of FcγR induces C/EBPβ DNA binding to its binding site in the MIP-1α promoter, which suggests the possible involvement of C/EBPβ in IC-induced MIP-1α expression (194). However, whether and to what extent C/EBPβ and -δ contribute to FcγR-mediated inflammatory mediator production has been unclear. My study shows that C/EBPβ and -δ are expressed in untreated macrophages, and IgG IC stimulation upregulates C/EBPβ and -δ activities. Using a siRNA-mediated knockdown approach and mice deficient for C/EBPβ or/and -δ, my results clearly demonstrate that C/EBPβ and -δ play critical roles in the production of TNF-α, MIP-2, and MIP-1α in IgG IC-stimulated macrophages. Interestingly, the functions of C/EBPβ and -δ seem to be partially redundant, although lack of either protein has a significant effect on TNF-α, MIP-2, and MIP-1α production. This could also indicate the importance of C/EBPβ and-δ heterodimer occupancy in regulating these promoters. This hypothesis is further supported by data showing that co-expression of C/EBPβ- and C/EBPδ-expressing vectors stimulates more expression of a C/EBP-driven luciferase reporter than either C/EBPβ or C/EBPδ alone (Fig. 2). Further, because a relatively low level of DNA binding for C/EBPδ contributes a vigorous induction of TNF-α, MIP-2, and MIP-1α, it is tempting to speculate that C/EBPδ

may be more effective than C/EBPβ in supporting the IgG IC-induced transcription of TNF-α, MIP-2, and MIP-1α genes.

Although the primary mechanism of C/EBPβ regulation within inflammatory responses appears to be post-transcriptional, C/EBPβ mRNA levels are also induced by inflammatory stimuli, including LPS (109, 226). On the other hand, unlike C/EBPβ, the primary mechanism of C/EBPδ regulation within the inflammatory responses is transcriptional (148, 226). For example, LPS, IL-1, and IL-6 all can induce C/EBPδ expression at the mRNA level (222). Here I show that IgG IC induces C/EBPβ and -δ expressions at both mRNA and protein levels. Further, the DNA binding activities of both C/EBPβ and -δ are increased upon FcγR activation, although whether this is due solely to increased expressions of the proteins *versus* post-translational regulation of C/EBP protein activity is unknown and requires further investigation.

Using MAPK inhibitors, previous studies have shown that activation of MAPK is necessary for the FcγR-dependent induction of TNF-α expression in monocytes (227). Furthermore, FcγR ligation of monocytes/macrophages leads to both ERK1/2 and p38 activation (228, 229). In addition, Song *et al*. demonstrate that in microglia, the Ras/MEK/ERK pathway is necessary and sufficient for IC-induced MIP-1α expression (230). Thus, my finding that IgG IC treatment leads to the phosphorylation of ERK1/2 and p38 MAPK is consistent with these previous reports. Importantly, my results suggest that both ERK and p38 MAPK pathways are involved in IgG IC-induced C/EBPβ and -δ activation, thus stimulating cytokine and chemokine production in macrophages. Interestingly, neither ERK nor p38 MAPK affects NF-κB DNA binding activity, suggesting that other signaling pathways are involved in its activation by IgG IC. This is

consistent with a previous report showing that a mitogen-activated protein kinase kinase (MEK) inhibitor fails to affect IC-induced p65 nuclear translocation in microglia (230). In contrast, other studies have suggested that MAPK activation is necessary for FcγR-dependent activation of NF-κB in monocytes (231-233). Collectively, however, these data do not exclude the requirement for NF-κB activation in FcγR-mediated inflammation. In the current study, IgG IC-stimulated production of TNF-α, MIP-2, and MIP-1α is inhibited by a NF-κB inhibitor with a significant effect observed on TNF-α and MIP-1α levels (Fig. 11). These data suggest that NF-κB may play important but differential regulatory roles in FcγR-mediated inflammatory mediators' production.

Products of macrophages play a major role in events leading to tissue injury. TNF-α, other cytokines, and chemokines, such as MIP-2 and MIP-1α, secreted by macrophages have been shown to modulate the cell signaling cascades for the production of other proinflammatory and anti-inflammatory mediators during inflammation. The coordinate expressions of activating and inhibitory FcγRs on macrophages and other cells thus ensure the homeostasis of IC-induced inflammatory responses. For example, genetic deletion of FcR γ-chain, which leads to the loss of cell surface expression and functional inactivation of all three activating FcγRs, results in dramatically impaired inflammatory responses associated with IC formation (193, 214). In contrast, enhanced macrophage responses are observed in FcγRⅡ knockout mice (193, 214). Using macrophages from mice lacking FcR γ-chain (FcRγ), we demonstrate that activation of C/EBPβ and -δ in macrophages by IgG IC stimulation is mediated, to a large extent, by activating FcγRs. Furthermore, we show an enhanced C/EBP DNA binding activity in macrophages from FcγRⅡ deficient mice.

These data further suggest that activation of C/EBPβ and –δ may function as a pivotal regulatory mechanism of IgG IC-associated immune responses.

Another interesting result in my study is that C5a enhances IgG IC-induced cytokine and chemokine production by elevating C/EBPs but not NF-κB DNA binding activities. However, the exact mechanism whereby C5a signals control C/EBP activation remains an important open question. Several inflammatory stimulators, such as TNF, IFN-γ, and LPS have been shown to upregulate activating FcγR production (193, 214, 234). Recent studies have shown that C5a causes induction of FcγRⅢ and suppression of FcγR Ⅱ on both alveolar macrophages and RAW264.7 cells (216, 235, 236). Furthermore, genetic ablation of C5aR expression completely abolishes this regulation of FcγRs. These studies provide definite evidence that C5a plays a critical role in regulating the FcγRⅢ/Ⅱ pair to connect complement and FcγR pathways during IC-associated inflammation. Moreover, the current data suggest that the enhancement by C5a of C/EBP activity may be mediated by increased phospho-p38 MAPK and phosphop-44/42 MAPK levels. Interestingly, my finding that the interaction between C5a and C5aR has no effect on IgG IC-induced NF-κB activation suggests that the C5a/C5aR pathway may have a specific role in C/EBP activation.

In summary, for this part of my study, Fcγ receptors-mediated activation of C/EBPβ and -δ leads to cytokine and chemokine production from IgG IC-stimulated macrophages, and both MAPKs and C5a signal pathways are involved in C/EBP activation (Fig. 36). These data support an important role of C/EBPβ and -δ in immune complex-related inflammation. Understanding the underlying roles of various transcription

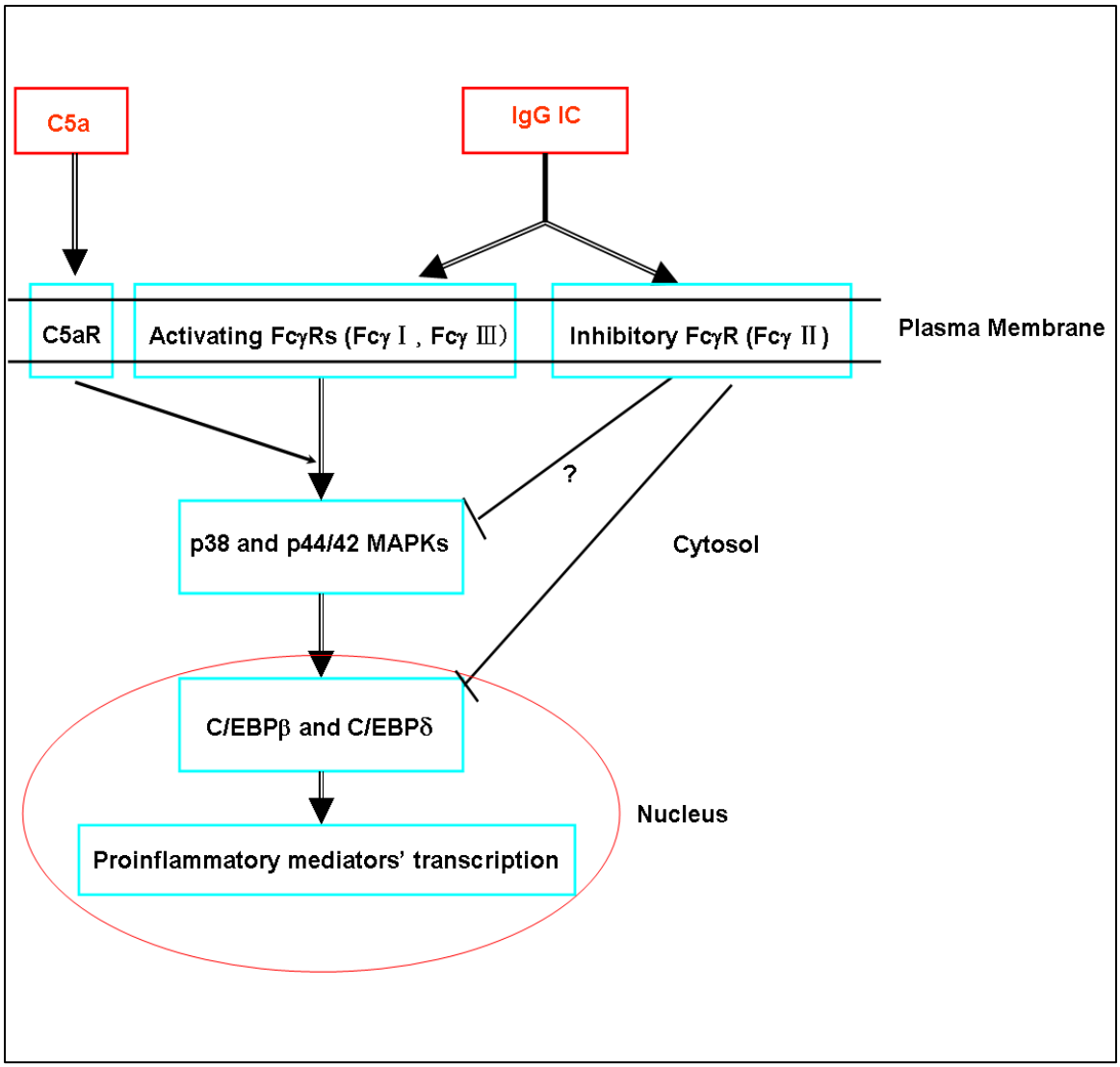

Figure 36. Cell Signaling Transduction Pathways from Membrane Receptors (FcγRs and C5aR) to Nucleus in Peritoneal Macrophages Challenged by IgG IC. Cross-linking of activating FcγRs leads to activation of p38 and p44/42 MAPKs, which can be further enhanced by interaction between C5a and C5aR. Phosphorylation of MAPKs results in elevated C/EBPβ and -δ DNA binding activities, which can finally promote inflammation-related gene transcription. FcγR Ⅱ as an inhibitory receptor could suppress proinflammatory mediator's transcription by downregulation of C/EBP DNA binding activities.

factors in regulating the network of inflammatory system may be a crucial step for the development of new therapeutic drugs for treatment of immune complex diseases.

Roles of C/EBPβ and –δ in IgG IC-induced ALI

The intrapulmonary deposition of IgG immune complexes in mice results in a complex cascade of inflammatory responses that control an ordered sequence of events, including the activation of residential macrophages and recruitment of neutrophils to the site of injury (10). 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 (68). 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 (77, 237). Our recent studies show that STAT3 is activated in both alveolar macrophages and whole lung extracts following IgG immune complex deposition (62, 93). 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 (93). 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/EBPδ, which are involved in IL-6 signaling in hepatoma cells (192). Thus, we hypothesize that STAT3 may regulate lung inflammation by affecting C/EBP activity. In the current study, I have identified C/EBPβ as a critical mediator of IgG immune complex-induced acute lung injury and inflammatory response in alveolar macrophages.

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. My current study provides evidence that both C/EBPβ and C/EBPδ are activated in lung during IgG immune complex-induced acute lung injury. I further show the IgG immune complexes regulate the lung expression of C/EBPβ at mRNA level (Fig. 22C). However, the molecular mechanisms by which IgG immune complexes induce C/EBPβ gene expression in the lung remain unclear. I have found that C/EBP activation in peritoneal macrophages is mediated, to a large extent, by Fcγ receptors (FcγR Ⅰ and FcγRⅢ). Therefore, it would be interesting to investigate in the future study whether FcγR signaling cascade 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 (182). In the current study, I have used C/EBPβ deficient mice to clearly demonstrate that C/EBPβ plays 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. The 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 lungs. Neutrophil transmigration into the alveolar compartment and lung interstitium plays a key role in the development of acute lung injury. Using an antibody-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 (238-240). My 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 (241). Indeed, I show that C/EBPβ deficiency causes a reduced expression of TNF-α and IL-6 as well as ICAM-1 in the lung. Together, the 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 (182). I previously showed that depletion of alveolar macrophages results in significantly reduced expression of inflammatory cytokines and chemokine genes in IgG immune complex-injured rat lungs (62). 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. 23). Moreover, employing mice deficient for C/EBPβ, siRNA-mediated knockdown in cell lines, and luciferase reporter assays, I 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 functional differences in cell growth, cell activation, and apoptosis (242). However, several studies have suggested compensatory or redundant roles for C/EBPs, including C/EBPβ and C/EBPδ, in supporting the induction of inflammatory cytokines and chemokines. For example, LPS stimulation of peritoneal macrophages from C/EBPβ deficient mice leads to normal induction of several inflammatory cytokines, including IL-6 and TNF-α, with the exception of G-CSF, Mincle, and mPGES-1 (143, 184, 197, 198). Using a B lymphoblast system, Hu *et al*. reports that the activities of C/EBPα, C/EBPβ, and C/EBPδ are redundant in regard to the expression of IL-6 and MCP-1 (126). In addition, a recent study shows that C/EBPδ 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/EBPδ results in a significant decrease in the TLR ligand-induced production of IL-6 and TNF-α (206). Interestingly, our *in vitro* study shows that lack of either C/EBPβ or C/EBPδ 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/EBPδ heterodimer occupancy in regulating activation of these promoters. In addition, I have directly compared the effect of C/EBPβ and C/EBPδ deficiency on lung inflammatory responses. I clearly show that C/EBPδ 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/EBPδ in inflammation are cell and tissue specific.

Together, I present evidence that mutant mice defective in C/EBPβ but not C/EBPδ are significantly protected from acute lung inflammation and injury following intrapulmonary deposition of IgG immune complexes. My 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.

APPENDICES

APPENDIX

Abbreviations

C/EBP, CCAAT/enhancer-binding protein

FcγR, Fcγ receptor

IgG IC, immunoglobulin G immune complex

siRNA, small interfering RNA

TNF-α, tumor necrosis factor-α

MIP-2, macrophage inflammatory protein-2

MIP-1α, macrophage inflammatory protein-1α

ERK, extracellular signal-regulated kinase

MAPK, mitogen activated protein kinase

C5a, complement component 5a

ALI, acute lung injury

ARDS, acute respiratory distress syndrome

BSA, bovine serum albumin

ITAM, immunoreceptor tyrosine-based activation motif

ITIM, immunoreceptor tyrosine-based inhibition motif

miR, microRNA

IL, interleukin

CLP, caecal ligation and puncture

TF, tissue factor

FASP, factorⅦ-activating protease

LPS, lipopolysaccharide

IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

CD, cluster of differentiation molecule

HUVECs, human umbilical vein endothelial cells

HBECs, human bronchial epithelial cells

PMNs, polymorphonuclear neutrophils

CVF, cobra venom factor

VCAM-1, vascular cell adhesion protein 1

ICAM-1, intercellular adhesion molecule 1

MDMEC, mouse dermal microvascular endothelial cell

IFN-γ, interferon-gamma

MCP-1, monocyte chemotactic protein-1

BAL, bronchoalveolar lavage

MPO, myeloperoxidase

CINC, cytokine-induced neutrophil chemoattractant

MIP-1β, macrophage inflammatory protein 1 beta

C5aR, C5a receptor

C5L2, C5a receptor-like 2

C5AR1, complement component 5a receptor 1

AECs, alveolar epithelial cells

RANTES, regulated and normal T cell expressed and secreted

fMLP, N-formyl-methionyl-leucyl-phenylalanine

OVA, ovalbumin

AHR, airway hyperresponsiveness

KC, keratinocyte-activated cytokine

AP-1, Activator Protein-1

STAT3, Signal Transducer and Activator of Transcription 3

RHD, Rel homology domain

IKKβ, inhibitor of nuclear factor kappa-B kinase subunit beta

ROS, reactive oxygen species

NADPH, Nicotinamide Adenine Dinucleotide Phosphate, reduced

NAC, N-acetylcysteine

ATF, activating transcription factor

JAK, Janus kinase

SOCS3, suppressor of cytokine signaling 3

bZIP, basic region-leucine zipper

COPD, chronic obstructive pulmonary disease

LAP*, liver-enriched transcriptional activator protein*

LAP, liver-enriched transcriptional activator protein

LIP, liver-enriched inhibitory protein

TLRs, Toll-like receptors

CCSP, Clara cell secretory protein

RSK, ribosomal S-6 kinase

cAMP, cyclic adenosine 3', 5'-monophosphate

DIC, disseminated intravascular coagulation

IL-1Ra, interleukin-1 receptor antagonist

HIV, human immunodeficiency virus

NK cell, natural killer cell

CHOP, C/EBP homologous protein

ER, endoplasmic reticulum

IPF, idiopathic pulmonary fibrosis

Bax, Bcl-2–associated X

G-CSF, granulocyte colony-stimulating factor

mPGES-1, microsomal prostaglandin E synthase-1

EMSA, Electrophoretic Mobility Shift Assay

i.p., intraperitoneal

i.v., intravenous

Cl_2MDP, dichloromethylene diphosphonate

MEK, mitogen-activated protein kinase kinase

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