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Chunguang Yan

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ROLES OF CCAAT/ENHANCER-BINDING PROTEIN  $\beta$  AND  $\delta$  IN  
IMMUNOGLOBULIN G IMMUNE COMPLEX-INDUCED INFLAMMATION

by

Chunguang Yan  
Master of Science, Nanjing University, 2007

A dissertation

Submitted to the Graduate Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Grand Forks, North Dakota

December  
2012

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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## PERMISSION

Title            Roles of CCAAT/enhancer-binding Protein  $\beta$  and  $-\delta$  in Immunoglobulin G  
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## ABSTRACT

CCAAT/enhancer-binding protein (C/EBP)  $\beta$  and C/EBP $\delta$  are known to participate in the regulation of many genes associated with inflammation. However, little is known about the activation and function of C/EBP $\beta$  and  $\delta$  in inflammatory responses elicited by Fc $\gamma$  receptor (Fc $\gamma$ R) activation. Here I showed that C/EBP $\beta$  and  $\delta$  activation were induced in immunoglobulin G immune complex (IgG IC)-treated macrophages by using gel shift assays. The increased expression of C/EBP $\beta$  and  $\delta$  occurred at both mRNA and protein levels. Furthermore, induction of C/EBP $\beta$  and  $\delta$  was mediated, to a large extent, by activating Fc $\gamma$ Rs. Using small interfering RNA (siRNA)-mediated knockdown as well as macrophages deficient for C/EBP $\beta$  and/or  $\delta$ , I demonstrated that C/EBP $\beta$  and  $\delta$  played a critical role in the production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), macrophage inflammatory protein-2 (MIP-2), and macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) 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- $\alpha$ , MIP-2, and MIP-1 $\alpha$  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/EBP $\beta$  and  $\delta$  activities. To further explore the roles of C/EBP $\beta$  and C/EBP $\delta$  in Fc $\gamma$ R-mediated inflammatory responses *in vivo*, I used IgG IC-induced acute lung injury model. I showed that both C/EBP $\beta$  and C/EBP $\delta$  activation were triggered in lungs

challenged by IgG IC. I further demonstrated that C/EBP $\beta$  but not C/EBP $\delta$  deficient mice displayed significant attenuation of pulmonary vascular permeability and neutrophil accumulation when compared with wild type mice. Moreover, C/EBP $\beta$  deficient mice expressed considerable less inflammatory mediators compared with wild type littermates. Together, these data indicate that both C/EBP $\beta$  and C/EBP $\delta$  act as inflammatory stimulators *in vitro* during IgG IC-mediated inflammation. However, it is C/EBP $\beta$  but not C/EBP $\delta$  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 Fc $\gamma$ Rs on IgG IC-mediated Immune Responses

IgG IC-induced immune responses is achieved by binding of IgG IC to Fc $\gamma$ Rs. Fc $\gamma$ Rs, which can specifically bind to the Fc region of IgG, are divided into four classes. Three classes of activating Fc $\gamma$ Rs are discovered: 1) Fc $\gamma$ R I with high affinity for Fc region of IgG; 2) Fc $\gamma$ RIV with intermediate affinity; 3) Fc $\gamma$ RIII with low affinity (6, 7). All these Fc $\gamma$ Rs share a common FcR  $\gamma$ -chain containing an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasm, which is indispensable for cell activation (8). Association of IgG IC with Fc $\gamma$ R I /Fc $\gamma$ RIII/Fc $\gamma$ RIV results in signal transduction from cytoplasm to the nucleus, which is achieved by interaction between the  $\gamma$  chain formed-homodimer and the receptor  $\alpha$  chain (6, 9). IgG IC-induced clustering of Fc $\gamma$ R I /Fc $\gamma$ R III /Fc $\gamma$ R IV stimulates tyrosine phosphorylation within ITAM and subsequent activation of downstream signaling cascades involved in functional responses (6). Because Fc $\gamma$ R II, 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 Fc $\gamma$ R (3, 8). Fc $\gamma$ R I is constitutively expressed by macrophages, monocytes and human airway smooth muscle cells while Fc $\gamma$ RIII 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). Fc $\gamma$ R II is expressed by almost all hematopoietic cells and human airway smooth muscle cells (10, 11). Fc $\gamma$ RIV, 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 Fc $\gamma$ R in IC-stimulated immune responses is well established by using the gene knockout mice.

Contribution of each Fc $\gamma$ R to IgG IC-induced inflammation has been extensively examined by using mice genetic deficiency in a variety of Fc $\gamma$ Rs. FcR $\gamma^{-/-}$  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 Fc $\gamma$ R I leads to impaired cellular processes induced by IgG IC, including cytokine generation, cellular cytotoxicity, phagocytosis, and antigen presentation (13). Moreover, Fc $\gamma$ R 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 Fc $\gamma$ R I expression (14). When compared with wild type mice, Fc $\gamma$ RIII knockout mice show markedly attenuated inflammatory responses during IgG IC-induced alveolitis, with reduced expressions of TNF- $\alpha$ , interleukin( IL)-1 $\beta$ , and MIP-2 (15). Antibody-induced thrombocytopenia could be prevented by blocking binding of Fc $\gamma$ R IV to pathogenic antibodies against platelets (7). Blocking Fc $\gamma$ R IV results in decreased phagocytosis and cytokine expressions during IgG IC-induced ALI, though augmented expression of Fc $\gamma$ RIV alone could not trigger the disease in Fc $\gamma$ RIII knockout mice (16). Fc $\gamma$ R II deficiency lowers the threshold of IC stimulation of alveolar macrophages, as proved by analysis of Fc $\gamma$ R II knockout macrophages which display increased calcium and phagocytosis upon IC challenge (12). Fc $\gamma$ R 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 Fc $\gamma$ Rs. Moreover, it has been demonstrated that airway epithelial cells can also express functional Fc $\gamma$ Rs (17). Thus, to understand the role of different Fc $\gamma$ R in IgG IC-induced acute lung inflammation in more detail, mice selectively depleted of distinct Fc $\gamma$ R 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 triggered 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- $\alpha$  and MIP-2 expressions in alveolar macrophages challenged by IgG IC (30). In contrast, in the presence of C5a, lipopolysaccharide (LPS)-induced TNF- $\alpha$  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 ( $I\kappa B\alpha$ ) that can suppress nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa B$ ) 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- $\gamma$ ), or interleukin-6 (IL-6), followed by C5a challenge causes synergistic generation of monocyte chemoattractant 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 indispensable 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- $\alpha$  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 indispensable 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 C5F-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-1 $\alpha$ , macrophage inflammatory protein 1 beta (MIP-1 $\beta$ ), 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 hyperreactivity 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- $\alpha$ , 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- $\gamma$ , 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-1 $\beta$ ,

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 Fc $\gamma$ Rs 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 Fc $\gamma$ Rs activation (50, 51). However, recent studies show that signaling cascades induced by both Fc $\gamma$ Rs and complement system are necessary for full immune responses after intratracheal deposition of IgG IC in rodents, which suggests that Fc $\gamma$ Rs-induced signaling transduction might be integrated through C5a-mediated C5aR activation (10, 15, 30, 50, 51). *In vitro* data show that C5aR activation increases Fc $\gamma$ RIII expression while decreases Fc $\gamma$ R II production, which augments IgG IC-induced TNF- $\alpha$  and MIP-2 generation in alveolar macrophages (30). *In vivo* studies demonstrate that C5aR depletion decreases Fc $\gamma$ RIII/Fc $\gamma$ R II 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 Fc $\gamma$ RIII/Fc $\gamma$ R II 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- $\alpha$  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 Fc $\gamma$ R aggregation initiates immune response-related signaling transduction, which lead to expressions of inflammatory genes encoding cytokines and chemokines, such as TNF- $\alpha$ , IL-1, IL-10, MIP-2, CINC, keratinocyte-activated cytokine (KC), MIP-1 $\alpha$ , MIP-1 $\beta$ , 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 Fc $\gamma$ R to cytoplasm and then to nucleus remains largely unknown, but it has been demonstrated that several transcription factors including NF- $\kappa$ B, 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- $\kappa$ B 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- $\kappa$ B activation—classical pathway and alternative pathway (64). In the classical pathway, inhibitor of nuclear factor kappa-B kinase subunit beta (IKK $\beta$ ) is activated by stimulators, leading to phosphorylation of I $\kappa$ B while in the alternative pathway, activated IKK $\alpha$  phosphorylates p100 (64). These two pathways converge at proteasomes, leading to release of mature NF- $\kappa$ B 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 molecules, and chemokines, are regulated by NF- $\kappa$ B. These inflammatory mediators play a critical role in inflammation-induced pulmonary damages. Thus, NF- $\kappa$ B 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- $\kappa$ B activation during endotoxin-induced acute lung injury (66). Furthermore, endotoxin-induced binding

of NF- $\kappa$ B to its consensus sequence in E-selectin promoter region, indicating that NF- $\kappa$ B directly mediates target genes' production at transcription level (66). During IgG IC-induced ALI, whole lung NF- $\kappa$ B is activated, followed by TNF- $\alpha$  and IL-1 $\beta$  expressions (61). In addition, NF- $\kappa$ B 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- $\kappa$ B in IgG IC-induced alveolitis, because alveolar macrophage depletion leads to depressed lung NF- $\kappa$ B activity (67). Whole lung NF- $\kappa$ B activation could be restored in alveolar macrophage-depleted mice receiving intrapulmonary administration of TNF- $\alpha$  (67). In the model of IgG IC-triggered lung injury, TNF- $\alpha$  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- $\alpha$  expression by IgG IC-activated alveolar macrophages is an upstream event in the inflammatory cascades, which is required for full activation of NF- $\kappa$ B in other types of cells in the lung, including pulmonary vascular endothelium and alveolar epithelial cells. NF- $\kappa$ B activation is also regulated by anti-inflammatory mediators, such as IL-10 and IL-13. Both cytokines increase I $\kappa$ B $\alpha$  expression at both mRNA and protein levels, which inhibits nuclear translocation of NF- $\kappa$ B after intrapulmonary deposition of IgG IC (68). Suppression of NF- $\kappa$ B activation in alveolar macrophages and whole lungs results in decreased TNF- $\alpha$  expression, leading to reduced neutrophil influx and ensuing lung injury (68). Thus, both proinflammatory and anti-inflammatory pathways converge at NF- $\kappa$ B, and their balance determines the intensity of lung damages. Interestingly, both *in vitro* and *in vivo* studies

shows that NF- $\kappa$ B activation is positively regulated by TNF- $\alpha$  and IL-1 $\beta$ , 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- $\kappa$ B activation (69, 70). NF- $\kappa$ B 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- $\kappa$ B activation is positively regulated by ROS in the model. In IgG IC-induced ALI, NF- $\kappa$ B 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- $\kappa$ B activation (61). In addition, IgG IC-induced NF- $\kappa$ B activation is independent of neutrophils, thus, intracellular ROS produced by alveolar macrophages are responsible for partial pulmonary NF- $\kappa$ B activation (61). Recent data show that NAC treatment downregulates NF- $\kappa$ B activation by impairing interaction of TNF- $\alpha$  with its receptor but not ROS generation (72). Thus, to further examine the role NF- $\kappa$ B 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- $\alpha$ , and transforming growth factor- $\beta$ ) (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- $\alpha$  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- $\kappa$ B 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 of 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 indispensable 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- $\kappa$ B 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: STAT3 $\alpha$ , STAT3 $\beta$ , STAT3 $\gamma$ , and STAT3 $\delta$  (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/EBP $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\xi$ , 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/EBP $\xi$  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  $\alpha$ -helical conformation (116). Because of the intact leucine zipper, C/EBP $\xi$  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/EBP $\xi$  and other C/EBP proteins could bind to the promoter regions of various cellular stress-regulated genes, and promote their transcription (119). Thus, C/EBP $\xi$  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/EBP $\alpha$  isoforms—full-length C/EBP $\alpha$  with a molecular weight of 42 kDa (p42C/EBP $\alpha$ ) and p30C/EBP $\alpha$  (30 kDa) (120). p30C/EBP $\alpha$  is produced by alternative use of translation initiation codons (120). C/EBP $\alpha$  is an important regulator of myeloid lineage commitment. This is because C/EBP $\alpha$  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/EBP $\alpha$  (121). However, it is found that the phenotype in C/EBP $\alpha$  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/EBP $\alpha$ . It is demonstrated that expressions of IL-6 receptor and IL-6-responsive colony-forming units, which contribute to granulopoiesis, are abrogated in mice depleted of C/EBP $\alpha$  (122). Interestingly, blocking of granulocyte colony-stimulating factor signaling has no effect on differentiation of macrophages (121). In adult mice, deleted C/EBP $\alpha$  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/EBP $\alpha$  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/EBP $\alpha$ -induced PU.1 expression and interaction of C/EBP $\alpha$  with AP-1 and NF- $\kappa$ B (125).

C/EBP $\alpha$ , C/EBP $\beta$  and C/EBP $\delta$  expressions could be detected in bone marrow-derived macrophages (126). In the presence of LPS, DNA binding activity of both

C/EBP $\beta$  and C/EBP $\delta$  is increased while that of C/EBP $\alpha$  is reduced, indicating that C/EBP $\beta$ /C/EBP $\delta$ , and C/EBP $\alpha$  may have different role in inflammatory responses (126). Knockdown of C/EBP $\alpha$  significantly reduces IL-6 and TNF- $\alpha$  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/EBP $\alpha$ , C/EBP $\beta$  and C/EBP $\delta$  are redundant, indicating that lack of C/EBP $\beta$ /C/EBP $\delta$  could be compensated by C/EBP $\alpha$  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/EBP $\alpha$  production is significantly reduced while C/EBP $\beta$  and C/EBP $\delta$  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/EBP $\beta$  and C/EBP $\delta$  are thought to function as the major stimulators of acute phase response (129). Further studies show that C/EBP $\alpha$  is also involved in the inflammatory process (129). Neonatal mice lacking C/EBP $\alpha$  is completely resistant to LPS- or IL-1 $\beta$ -mediated acute phase response when compared with wild type littermates, though compensatory increase of both C/EBP $\beta$  and C/EBP $\delta$  DNA binding activities is obvious in C/EBP $\alpha$  null mice (129). Thus, C/EBP $\alpha$  is indispensable for acute phase response in neonatal mice. C/EBP family members exist in cells as homodimers or heterodimers, the absolute requirement of C/EBP $\alpha$  for acute phase response might be explained by the essential roles of C/EBP $\alpha$ :C/EBP $\beta$  heterodimer and C/EBP $\alpha$ :C/EBP $\delta$

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

Though *C/EBP $\alpha$*  null mice die shortly after birth, the role of *C/EBP $\alpha$*  during postnatal development has not been elucidated. Recent studies show that mice die about 30 days after total depletion of *C/EBP $\alpha$*  during postnatal development due to abnormal change in metabolic processes (130). *C/EBP $\alpha$*  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/EBP $\alpha$*  knockout mice demonstrates that type II lung epithelial cells undergo hyperproliferation and alveolar architecture is disturbed, which suggests an essential role for *C/EBP $\alpha$*  in lung development (132). To understand the role of *C/EBP $\alpha$*  in lungs, use of siRNA targeting *C/EBP $\alpha$*  is an acceptable method. Exposure of newborn mice to 95% oxygen leads to enhanced *C/EBP $\alpha$*  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/EBP $\alpha$* -specific siRNA treatment alleviates these hyperoxic features with an exception of lung morphologic change (133). Nevertheless, lung morphology is restored in *C/EBP $\alpha$*  siRNA-, but not control siRNA-treated mice after 2-week recovery in normal air, which suggests that *C/EBP $\alpha$*  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/EBP $\alpha$*  is

genetically depleted from Clara cells and type II lung epithelial cells after birth, indicating that C/EBP $\alpha$  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/EBP $\alpha$  plays different roles in newborn and adult mice during hyperoxia-induced ALI. Whereas newborn mice benefit from downregulation of C/EBP $\alpha$  expression, adult mice benefit from upregulation of C/EBP $\alpha$  expression, indicating that specific tissue levels of C/EBP $\alpha$  induce beneficial or detrimental effects (133). Immunohistochemistry staining shows that expression of C/EBP $\alpha$  could be found in alveolar macrophages and type II lung epithelial cells (134). During bleomycin-induced pulmonary fibrosis, C/EBP $\alpha$  expression is significantly induced in some of alveolar epithelial cells and fibroblasts in the fibrotic lesions (134). C/EBP $\alpha$  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/EBP $\alpha$  leads to increased generation of surfactant proteins and hyperproliferation of alveolar type II cells (135). Thus, bleomycin-induced upregulation of C/EBP $\alpha$  expression in alveolar epithelium and fibroblasts might be considered as an endogenous anti-fibrotic factor. In LPS-treated lungs, C/EBP $\alpha$  expression is also significantly increased, indicating that C/EBP $\alpha$  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/EBP $\alpha$  can function as a disease suppressor or stimulator, which is dependent on specific diseases.

C/EBP $\beta$  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/EBP $\beta$  isoforms found in the cells (137). The three different isoforms of C/EBP $\beta$  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/EBP $\beta$  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/EBP $\beta$  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/EBP $\beta$  links TLR4 activation to inflammatory mediator's expression (139). Many gene promoter regions contain C/EBP $\beta$  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/EBP $\beta$  (138). All functions of C/EBP $\beta$  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/EBP $\beta$  can mediate the IL-10 gene expression in mouse macrophage RAW264.7 challenged with LPS (140). C/EBP $\beta$  inhibits expressions of inflammatory mediators in

TNF tolerant monocytes by interacting with NF- $\kappa$ B 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/EBP $\beta$ , mice depleted of C/EBP $\beta$  are generated. When compared with wild type mice, C/EBP $\beta$  knockout mice are more sensitive to infection by *Listeria monocytogenes* due to defect in macrophage bactericidal activity (143). However, TNF and IFN- $\gamma$  expressions are not impaired in C/EBP $\beta$  deficient mice, indicating that the role of C/EBP $\beta$  in cytokine expression might be compensated by other C/EBP proteins, such as C/EBP $\alpha$  and C/EBP $\epsilon$  (143).

Higher C/EBP $\beta$  expression is found in the fibrotic lesions during bleomycin-induced pulmonary fibrosis (134). *In vitro* studies show that C/EBP $\beta$  plays an important role in myofibroblast differentiation, indicating that C/EBP $\beta$  might be a key regulator of fibrosis (144). *In vivo* data demonstrate that C/EBP $\beta$  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/EBP $\beta$  attenuates fibrosis suggest that when compared with C/EBP $\beta$  deficient mice, the wild type counterparts express more inflammatory mediators after intratracheal instillation of bleomycin (144). In addition, pulmonary fibroblasts lacking C/EBP $\beta$  generate less  $\alpha$ -smooth muscle actin, indicating that myofibroblast differentiation is positively regulated by C/EBP $\beta$  (144). Moreover, pulmonary fibroblasts isolated from wild type mice display less proliferative rates than those from C/EBP $\beta$ <sup>-/-</sup> mice. Overall, C/EBP $\beta$  plays multifactorial roles in lung fibrosis by regulating inflammatory mediators' production,

fibroblast proliferation, and differentiation (144). Although C/EBP $\beta$  knockout protects against bleomycin-induced pulmonary fibrosis, the molecular mechanisms by which C/EBP $\beta$  positively regulates fibrosis remain largely unknown. Recent studies demonstrate that C/EBP $\beta$  carrying a single point mutation (Thr217 to Ala217) can block phosphorylation of C/EBP $\beta$  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/EBP $\beta$  on Thr217 (145). Phosphorylated C/EBP $\beta$  is associated with procaspase 8, leading to inactivation of the apoptotic pathway in pulmonary fibroblasts (145). Moreover, in human patients, phosphorylation of C/EBP $\beta$  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/EBP $\beta$ , which can prevent fibroblast from apoptosis, might be involved in human pulmonary fibrosis (145). Interestingly, a block peptide, which can suppress phosphorylation of C/EBP $\beta$  on Thr217, alleviates bleomycin-mediated pulmonary fibrosis, indicating its possible clinical applications (145). *In vitro* data show that different C/EBP $\beta$  isoforms may have distinct roles in fibrosis. IL-1 $\beta$  suppresses  $\alpha$ -smooth muscle actin in rat lung fibroblasts by increasing the ratio of LIP to LAP (146). LIP-mediated inhibition of  $\alpha$ -smooth muscle actin expression is initiated at transcription level due to its ability to binding to the consensus sequence in the  $\alpha$ -smooth muscle actin promoter region (146). However, LAP is a stimulator of  $\alpha$ -smooth muscle actin expression, because  $\alpha$ -smooth muscle actin is elevated by ectopic expression of LAP while reduced by knockout of C/EBP $\beta$  (146). Further studies on mechanisms by which IL-1 $\beta$  augments LIP



expression suggest that IL-1 $\beta$  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/EBP $\beta$  could be found in alveolar macrophages and type II lung epithelial cells (134). In LPS-challenged lungs, C/EBP $\beta$  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/EBP $\beta$  express less KC in response to intratracheal administration of LPS, leading to reduced recruitment of neutrophils into lungs (147). Therefore, C/EBP $\beta$  expression in lung epithelial cells contributes to LPS-induced acute lung inflammation by increasing neutrophil influx. However, C/EBP $\beta$  is also necessary for anti-inflammatory responses induced by formoterol—a  $\beta_2$ -adrenergic agonist, because inhibition of LPS-mediated neutrophilia by formoterol is alleviated in mice lacking C/EBP $\beta$  in pulmonary epithelial cells by increasing KC production (147). The proinflammatory and anti-inflammatory effects of C/EBP $\beta$  might be explained by preferential expressions of different C/EBP $\beta$  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/EBP $\delta$  in inflammatory responses has also been investigated. Many gene promoter regions contain C/EBP $\delta$  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/EBP $\delta$  (148). In addition, LPS-induced binding of C/EBP $\delta$  to the consensus sequence is indispensable for cyclooxygenase-2 transcription in macrophages (149). However,

evidence has shown that C/EBP $\delta$  is also involved in anti-inflammatory mediators' generation. For example, C/EBP $\delta$  expression is significantly increased in  $\beta$ -cells and human islets incubated with IL-1 $\beta$  and IFN- $\gamma$ , leading to decreased production of chemokines in  $\beta$ -cells. In the lungs, immunohistochemistry staining shows that C/EBP $\delta$  expression is restricted to some of type II lung epithelial cells in a spatial pattern in the normal lung (134). Intensive expression of C/EBP $\delta$  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/EBP $\delta$  expression is found in proliferating alveolar and bronchiolar epithelial cells (134). C/EBP $\delta$  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/EBP $\beta$  and C/EBP $\delta$  to its promoter region (151). In addition, C/EBP $\delta$  induced-Clara cell secretory protein (CCSP) expression in lung epithelial cells is also involved in binding of C/EBP $\delta$  to its consensus sequence in the proximal CCSP promoter (151, 152). Furthermore, it is found that in pulmonary epithelium, C/EBP $\delta$  preferentially interacts with C/EBP $\alpha$  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/EBP $\alpha$  and C/EBP $\delta$  (153).

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

C/EBP $\epsilon$  expression is restricted to lymphocytes and myeloid cells including macrophages and granulocytes (155-157). C/EBP $\epsilon$  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/EBP $\epsilon$  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/EBP $\epsilon$  is an important regulator of myeloid lineage commitment. Morphologically, macrophages lacking C/EBP $\epsilon$  expression is immature (159). Additionally, C/EBP $\epsilon$  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/EBP $\epsilon$  knockout macrophages demonstrates that a subset of gene expressions related to inflammatory reaction and differentiation are significantly reduced in C/EBP $\epsilon$  deficient macrophages (159). Overall, as a transcription factor, C/EBP $\epsilon$  is necessary for macrophage development and subsequent normal functions in inflammation. C/EBP $\epsilon$  null mice develop normally but are susceptible to opportunistic infections by 3-5 months of age due to lack of functional neutrophils, indicating that C/EBP $\epsilon$  is required for terminal differentiation and functional maturation of granulocyte progenitor cells (160, 161). *In vivo* studies demonstrate that neutrophils deficient in C/EBP $\epsilon$  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/EBP $\epsilon$  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/EBP $\epsilon$  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- $\alpha$  (161). Interestingly, spontaneous expression of TNF- $\alpha$  is also elevated in neutrophils deleted of C/EBP $\epsilon$ , indicating the C/EBP $\epsilon$  might be involved in autoimmune diseases (161). Overall, the normal functions are severely impaired in C/EBP $\epsilon$  knockout neutrophils, leading to the abnormal immune reactions to inflammatory challenges. Therefore, C/EBP $\epsilon$  is necessary for generation of functional macrophages and neutrophils, which is important for host defense against infections.

To examine if C/EBP $\beta$  and C/EBP $\epsilon$  could be compensated for each other, double knockout mice are constructed. Though C/EBP $\beta$  and C/EBP $\epsilon$  double knockout mice are smaller than single deficient and wild type mice, morphological assay shows that lungs are normally developed in the double knockout mice (162). However, mice deficient in C/EBP $\beta$  and C/EBP $\epsilon$  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 IFN $\gamma$ -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/EBP $\beta$  and C/EBP $\epsilon$  are important for innate immune responses, and could be partially compensated for each other in the single knockout mice.

C/EBP $\gamma$  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/EBP $\alpha$  or C/EBP $\beta$  (164). For example, C/EBP $\gamma$  represses C/EBP $\beta$ -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/EBP $\beta$  to its consensus site in the promoter region, which is inhibited by C/EBP $\gamma$  (166). In reporter assays, it has been demonstrated that C/EBP $\delta$  cooperates with EKLF-a CACC-box binding protein, to enhance the beta-globin promoter-driven reporter gene expression, while C/EBP $\gamma$  inhibits the transcription activity of EKLF (167). In contrast to the notion of C/EBP $\gamma$  as an inhibitor,

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

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

C/EBP $\zeta$ , 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-1 $\beta$  level and neutrophil accumulation (173). Further studies demonstrate that CHOP is indispensable for LPS-induced caspase-11, which is necessary for activation of pro-IL-1 $\beta$  (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  $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\epsilon$ ,  $\gamma$ , and  $\zeta$  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/EBP $\beta$  and C/EBP $\delta$  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/EBP $\beta$  and C/EBP $\delta$  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/EBP $\beta$  and C/EBP $\delta$  in the lung (134, 188). However, the signals that mediate the activation of C/EBP $\beta$  and C/EBP $\delta$  and their functions in macrophages and lungs during inflammation remain largely unknown. A recent study demonstrates that C/EBP $\beta$  in the airway epithelium mediates inflammatory and innate immune responses in lung to cigarette smoke (189). C/EBP $\beta$  is also reported to play an essential role in bleomycin-induced pulmonary fibrosis (144). These observations suggest that C/EBP $\beta$  and/or C/EBP $\delta$  may play important roles during lung inflammation. However, no information exists about the role of C/EBP $\beta$  and C/EBP $\delta$  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- $\alpha$  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- $\kappa$ B 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/EBP $\beta$  and C/EBP $\delta$  have been demonstrated to be targets of STAT3 (191, 192).

The central hypothesis is that the activities of C/EBP $\beta$  and C/EBP $\delta$  are induced in immune complex-induced inflammation, and they may play an important regulatory role in Fc $\gamma$ R-mediated inflammatory responses.

#### *Problems*

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

functionality (6). Macrophages, the sentinels of innate immunity, play key roles in inflammation. Activation of macrophages by Fc $\gamma$ Rs 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 Fc $\gamma$ R 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 Fc $\gamma$ R signaling in macrophages remain unknown. Several transcription factors, such as NF- $\kappa$ B, AP-1, C/EBP $\beta$ , and STAT3, are activated in macrophages/monocytes following IgG IC stimulation (10, 194). Studies using pharmacological inhibitors suggest that NF- $\kappa$ B activation is involved in the expression of several chemokines, including MIP-1 $\alpha$  and MIP-1 $\beta$ , upon cross-linking of Fc $\gamma$ R (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 Fc $\gamma$ Rs.

C/EBP $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\epsilon$ ,  $\gamma$ , and  $\zeta$  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/EBP $\beta$  and  $\delta$  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/EBP $\beta$  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/EBP $\beta$  deficient

mice leads to normal induction of several inflammatory cytokines, including IL-6 and TNF- $\alpha$ , 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/EBP $\delta$  deficient macrophages show nominal defects in IL-6 and TNF- $\alpha$  production in response to several TLR ligands (199). In contrast, the absence of both C/EBP $\beta$  and - $\delta$  results in a significant decrease in the TLR ligand-induced production of both IL-6 and TNF- $\alpha$  (199). In another study, Gorgoni *et al.* find that LPS-induced expression of IL-1 $\beta$ , TNF- $\alpha$ , IL-6, and inducible nitric-oxide synthase is partially impaired in C/EBP $\beta$  deficient macrophages and that expression of IL-12 p35 is completely defective (183). However, IL-12 p40, RANTES, and MIP-1 $\beta$  are more efficiently induced in response to IFN- $\gamma$  and LPS in the absence of C/EBP $\beta$  (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/EBP $\beta$  and - $\delta$  deficiency. Thus, the role of C/EBP $\beta$  and - $\delta$  in regulating transcription of the different inflammation-regulated mediators warrants further investigation. Furthermore, whether and how C/EBP $\beta$  and - $\delta$  are involved in the Fc $\gamma$ R-induced inflammatory response remains unclear, and the signals that mediate the activation of C/EBP $\beta$  and - $\delta$  are unknown. Furthermore, the roles of C/EBP $\beta$  and C/EBP $\delta$  in IgG IC-induced acute lung injury are not known.

## CHAPTER II

### METHOD

#### Cells and Reagents

RAW264.7 cells (ATCC; TIB-71<sup>TM</sup>) and MH-S cells (ATCC; CRL-2019<sup>TM</sup>) 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<sub>2</sub>; 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<sub>2</sub>. 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/EBP $\beta$  and/or C/EBP $\delta$  as well as their wild type littermates (201, 202). Bone marrow was collected from the femurs and tibiae 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<sub>4</sub>Cl 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,  $1-5 \times 10^7$  BM cells were resuspended in  $\Psi$ CREJ2 cell supernatant supplemented with 8  $\mu$ g/ml Polybrene (Sigma) and 100 ng/ml macrophage colony-stimulating factor.  $\Psi$ CREJ2 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- $\alpha$ , MIP-2, KC, MIP-1 $\alpha$ , MIP-1 $\beta$ , 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- $\alpha$  promoter-reporter containing sequences extending to -1260 bp, C/EBP $\beta$  expression vector as well as the C/EBP $\delta$  expression vector are kindly provided by Richard C. Schwartz (Michigan State University). The reporter plasmid ( $2 \times$  C/EBP-Luc) containing two copies of a C/EBP binding site is kindly provided by Peter F. Johnson (NCI-Frederick). NF- $\kappa$ B 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  $\mu$ g of DNA and 1.5  $\mu$ l of Fugene6 transfection reagent (Roche

Applied Science) in 50  $\mu$ l of Opti-MEM I medium (Invitrogen). 24 h after transfection, the cells were treated either with or without 100  $\mu$ g/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  $1-2 \times 10^6$  cells with 600 nM control siRNA or C/EBP $\beta$ / $\delta$  siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) using the Amaxa<sup>®</sup> nucleofector kit V. 12 h later, the cells were treated with or without 100  $\mu$ g/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. Fc $\gamma$ R- and Fc $\gamma$ R II -deficient mice were obtained from Taconic. C5aR knock-out mice were kindly provided by Craig Gerard (Harvard Medical School). C/EBP $\beta$ <sup>-/-</sup> and C/EBP $\delta$ <sup>-/-</sup> 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<sub>2</sub>O. 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  $\mu$ g/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<sup>®</sup> 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 ( $2-2.5 \times 10^4$ /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<sup>®</sup> 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- $\alpha$ , MIP-2, KC, MIP-1 $\alpha$ , MIP-1 $\beta$ , 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/EBP $\beta$ , 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/EBP $\delta$ , 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- $\alpha$ : 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-1 $\alpha$ : 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  $\mu$ g total RNA was submitted to reverse transcription by using the Superscript II RNase H-Reverse Transcriptase. Following reverse transcription, the cDNA (2  $\mu$ l) 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- $\alpha$ , IL-6, MIP-2, ICAM-1, VCAM-1, KC, MIP-1 $\beta$ , and MIP-1 $\alpha$  were determined by quantitative real-time PCR. PCR was performed with primers for TNF- $\alpha$ , 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-1 $\alpha$ , 5' primer, 5'-ATG AAG GTC TCC ACC ACT GC-3' and 3' primer, 5'-CCC AGG TCT CTT TGG AGT CA-3'; MIP-1 $\beta$ , 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  $\mu$ g of protein were electrophoresed in a 12% polyacrylamide gel and then transferred to a PVDF membrane. Membranes were incubated with rabbit anti-C/EBP $\beta$  antibody (Santa Cruz Biotechnology, Inc.), rabbit anti-C/EBP $\delta$  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<sub>2</sub>, 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<sub>2</sub>, 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  $5000 \times g$  for 5 min at  $4^{\circ}\text{C}$ , and the supernatant extract was collected and stored at  $-80^{\circ}\text{C}$ . 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- $\kappa$ B consensus oligonucleotide (AGTTGAGGGGACTTTCCCAGGC, Promega, Madison, WI). C/EBP probes and NF- $\kappa$ B probes were labeled with  $\gamma$  [ $^{32}\text{P}$ ] ATP (3,000 Ci/mmol at 10 mCi/ml, GE Healthcare, Piscataway, NJ). DNA binding reactions were performed at room temperature in a 25  $\mu\text{l}$  reaction mixture containing 6  $\mu\text{l}$  of nuclear extract (1 mg/ml in buffer C or solution B) and 5  $\mu\text{l}$  of  $5 \times$  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  $\mu\text{g}$  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  $\mu\text{l}$ . Samples were electrophoresed through 5.5% polyacrylamide gels in  $1 \times$  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  $\mu\text{g}$ ) for 0.5 h at  $4^{\circ}\text{C}$  prior to the binding reaction. The following antibodies were purchased from Santa Cruz, CA: C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\delta$ , C/EBP $\epsilon$ , C/EBP $\gamma$ , 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/EBP $\beta$ - and C/EBP $\delta$ -deficient mice by homologous recombination has been described previously (201, 202). C/EBP $\beta$  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/EBP $\delta$  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  $\mu$ l 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  $\mu$ l 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  $\mu$ l recovered supernatants was added to 96-well plates, followed by addition of 100 mmol/l potassium phosphate buffer containing 1.5 mol/l H<sub>2</sub>O<sub>2</sub> and 167  $\mu$ g/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 ( $\times 1000$ ). 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<sub>2</sub>MDP) liposomes in PBS (10  $\mu$ l of the liposome stock in a total volume of 50  $\mu$ l) 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<sub>2</sub>MDP 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  $\pm$  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.

## CHAPTER III

### RESULTS

#### Roles of C/EBP $\beta$ and $\delta$ in IgG IC-induced Inflammation in Macrophages

##### *IgG IC Induces Expression of C/EBP $\beta$ and $\delta$ 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/EBP $\beta$  LAP/C/EBP $\delta$  heterodimers, C/EBP $\beta$  LIP/C/EBP $\delta$  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/EBP $\beta$  (mainly LAP/LIP, LAP/LAP and LIP/LIP) and C/EBP $\delta$  were markedly induced (Fig. 1B). I further examined the IgG IC-induced C/EBP activation in transient transfections using 2  $\times$  C/EBP-Luc, a promoter-reporter that contains two copies of a canonical C/EBP binding site, and expression vectors for C/EBP $\beta$  and/or C/EBP $\delta$ . These transfections were carried out with and without IgG IC treatment. Consistent with the results from EMSA, IgG IC



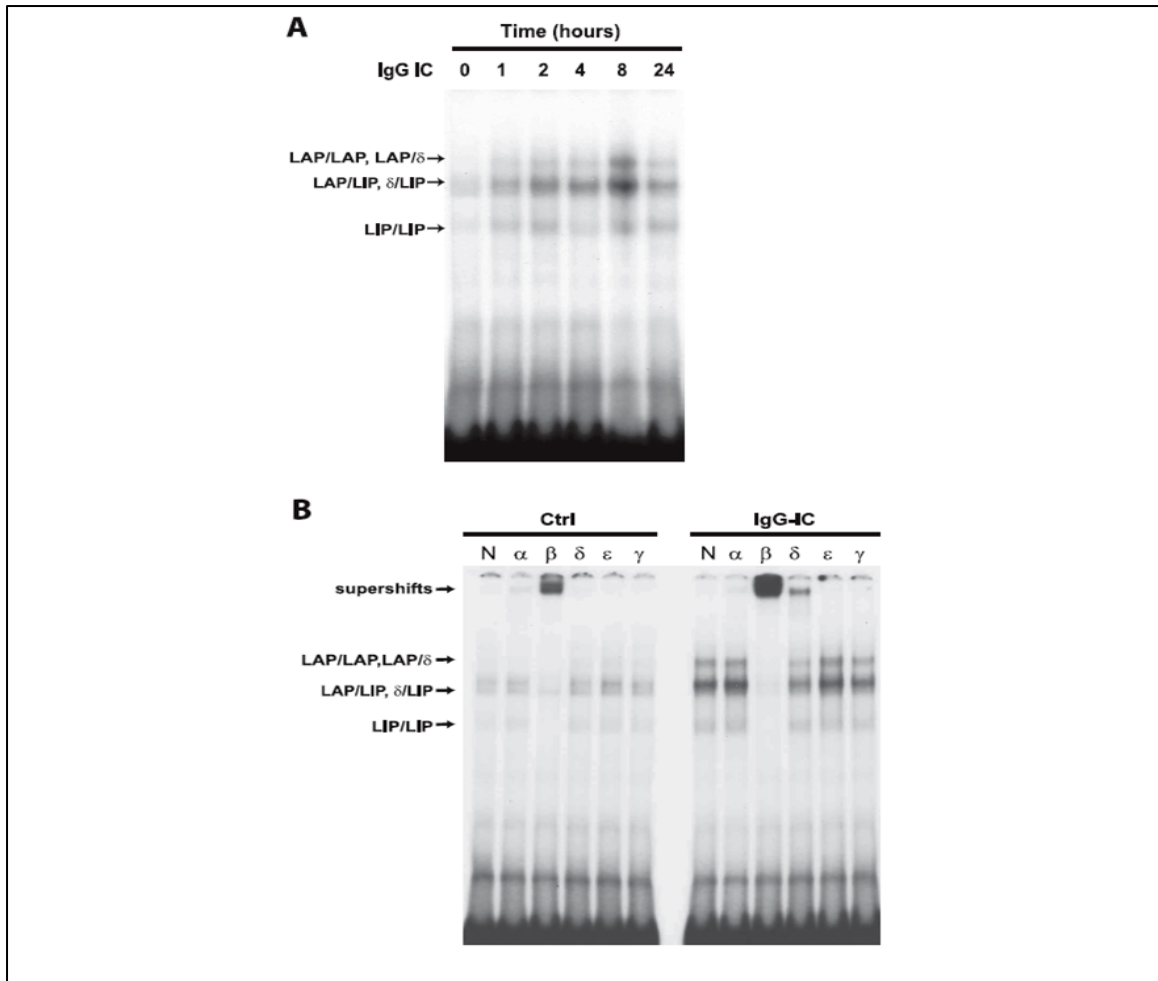


Figure 1. IgG Immune Complex Treatment Increases C/EBP $\beta$  and - $\delta$  DNA Binding Activities. A, RAW264.7 cells were stimulated with 100  $\mu$ g/ml IgG IC for the times indicated. Nuclear proteins were harvested and subjected to EMSA to measure C/EBP DNA binding activity. B, RAW264.7 cells were treated or left untreated with 100  $\mu$ g/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,  $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\epsilon$ , and  $\gamma$  indicate normal rabbit IgG, anti-C/EBP $\alpha$  antibody, anti-C/EBP $\beta$  antibody, anti-C/EBP $\delta$  antibody, anti-C/EBP $\epsilon$  antibody, and anti-C/EBP $\gamma$  antibody, respectively. The arrows indicate C/EBP complex and supershift species. The same experiment was repeated once.

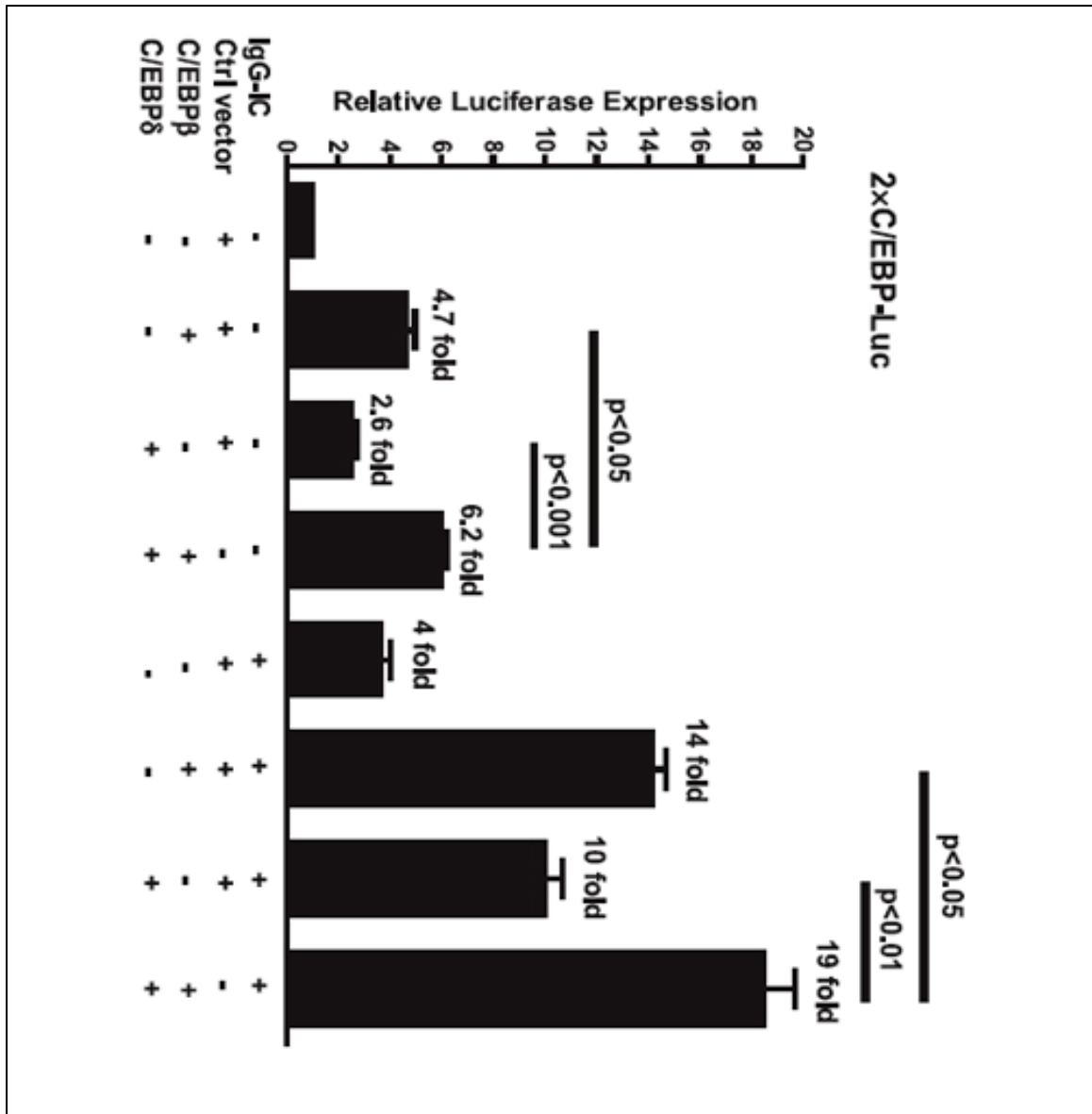


Figure 2. IgG Immune Complex Stimulation Augments C/EBP Transcriptional Activity. RAW264.7 cells were transiently transfected with a total of 0.5  $\mu$ g of the indicated DNA. 24 h after transfection, the cells were treated with 100  $\mu$ g/ml IgG immune complex for 5 h. Cell lysates were used to perform a luciferase activity assay. Luminometer values were normalized for expression from a co-transfected thymidine kinase reporter gene. The data were expressed as means  $\pm$  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/EBP $\beta$ , C/EBP $\delta$ , and C/EBP $\beta$  plus C/EBP $\delta$  transfectants induced luciferase expression 14-, 10-, and 19-fold, respectively, over the control value. Interestingly, overexpression of C/EBP $\beta$ , C/EBP $\delta$ , and C/EBP $\beta$  plus C/EBP $\delta$  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/EBP $\beta$  and  $\delta$  together were more potent activators than either protein alone, indicating that C/EBP $\beta$  and  $\delta$  are preferential dimerization partners, C/EBP $\beta/\delta$  heterodimers have stronger DNA binding activity than homodimers, or both.

I next examined whether IgG IC induced expression of C/EBP $\beta$  and  $\delta$  at the mRNA level. RT-PCR showed that C/EBP $\beta$  and  $\delta$  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/EBP $\beta$  and  $\delta$  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/EBP $\beta$  (mainly LAP) and C/EBP $\delta$  proteins (Fig. 3B). These data demonstrate that increased abundance of C/EBP $\beta$  and  $\delta$  is coincident with their increased DNA binding activity in RAW264.7 cells.

#### *C/EBP $\beta$ and $\delta$ are Required for IgG IC-mediated Cytokine and Chemokine Expression in Macrophages*

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

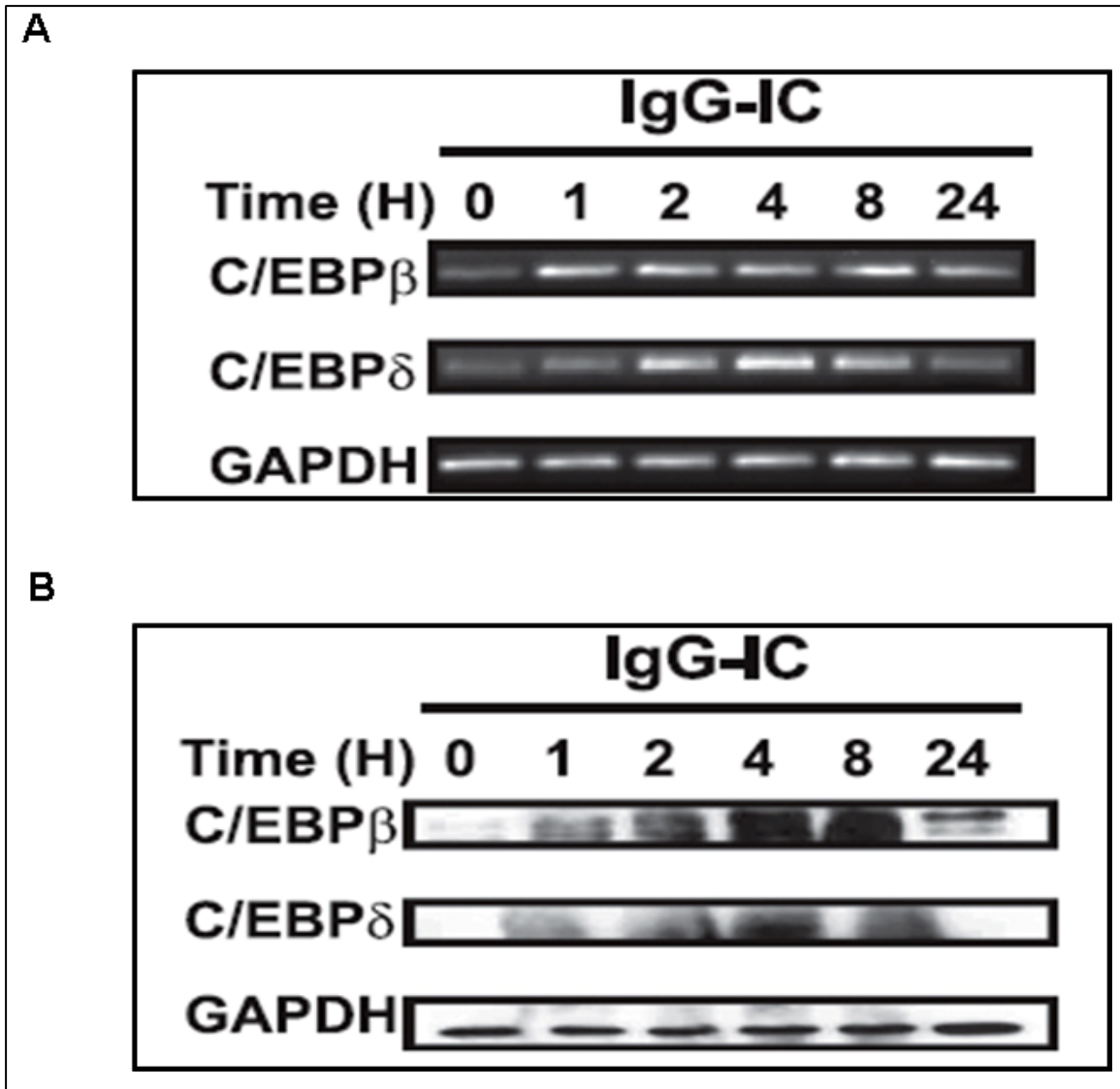


Figure 3. IgG Immune Complexes Induce C/EBP $\beta$  and - $\delta$  Expressions. A, RAW264.7 cells were stimulated with 100  $\mu$ g/ml IgG immune complexes for different time periods. Then total cellular RNA was isolated for RT-PCR with primers for C/EBP $\beta$ , C/EBP $\delta$ , and GAPDH, respectively. B, RAW264.7 cells were stimulated with 100  $\mu$ g/ml IgG immune complexes for different time periods. Then total cellular proteins were extracted to conduct Western blot using rabbit anti-C/EBP $\beta$  antibody, rabbit anti-C/EBP $\delta$  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 Fc $\gamma$ R cross-linking is unknown. TNF- $\alpha$  is an early proinflammatory cytokine secreted by macrophages during inflammation. Both MIP-2 and MIP-1 $\alpha$  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-1 $\alpha$ , 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/EBP $\beta$  and - $\delta$  deficiency on the expression of TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$  from IgG-IC-stimulated macrophages. To that end, I first showed that both C/EBP $\beta$  and - $\delta$  expression were efficiently depleted by their respective siRNAs in RAW264.7 cells (Figs. 4A and 6A). IgG immune complexes dramatically increased expressions of TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$  at transcriptional level in control cells, whereas all were markedly reduced when C/EBP $\beta$  or - $\delta$  was ablated, and the numbers indicated relative abundance of gene expression (Figs. 4B and 6B). To examine if knockdown of C/EBP $\beta$  or - $\delta$  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- $\alpha$ , MIP-2, and MIP-1 $\alpha$  from RAW264.7 cells were all markedly stimulated by IgG immune complexes treatment. However, when compared with control siRNA treatment, C/EBP $\beta$  siRNA suppressed TNF- $\alpha$  production by ~60%, MIP-2 by ~46%, and MIP-1 $\alpha$  by ~25%, respectively. Furthermore, C/EBP $\delta$  knockdown inhibited TNF- $\alpha$  generation by ~43%, MIP-2 by ~17%, and MIP-1 $\alpha$  by ~43%, respectively, as shown in Fig. 7.

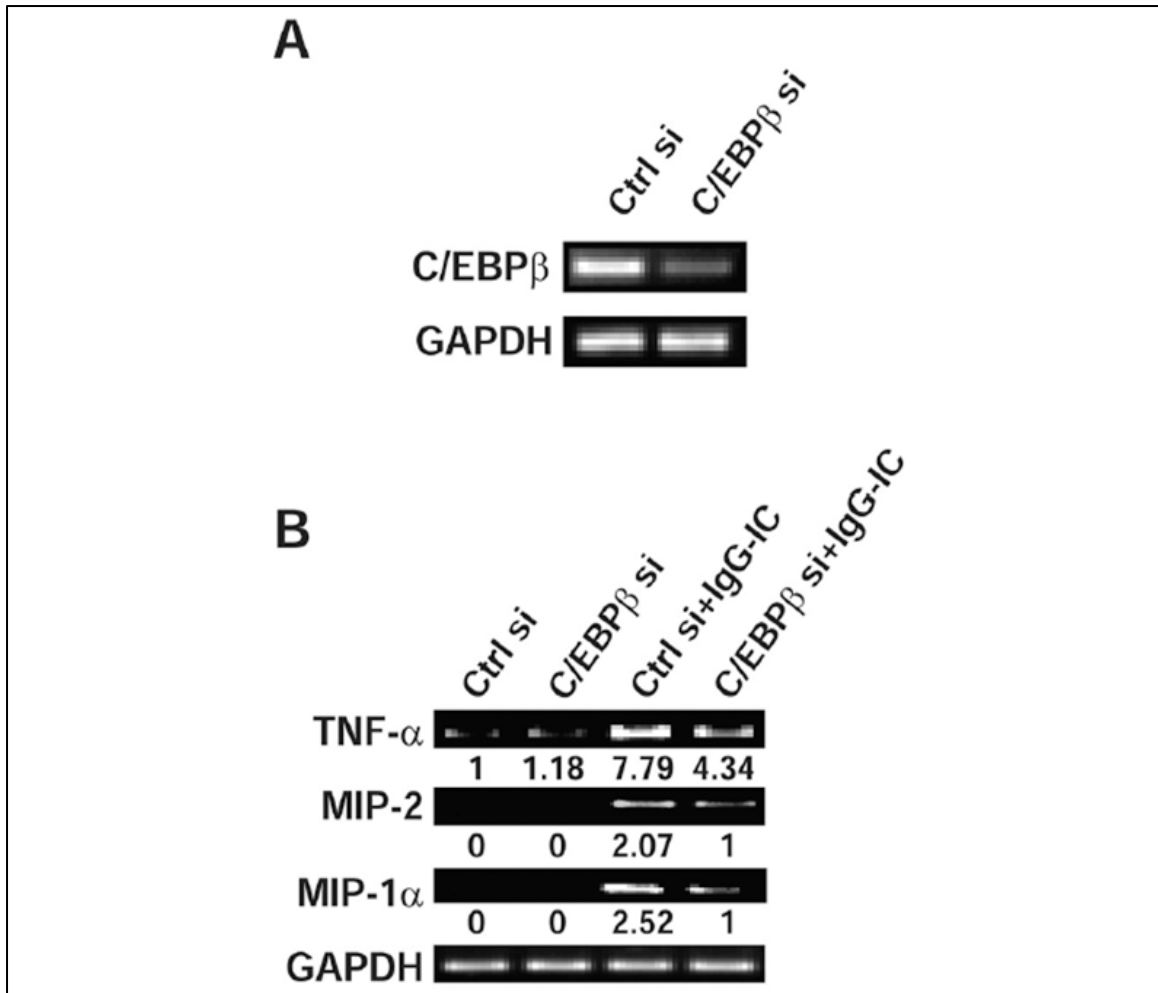


Figure 4. IgG IC-mediated Generation of Proinflammatory Mediators is Regulated by C/EBPβ at Transcription Level. A, RAW264.7 cells were transiently transfected with control siRNA, or C/EBPβ siRNA. 12 h later, RNAs were extracted and subjected to RT-PCR to examine expression of C/EBPβ, and GAPDH, respectively. 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.

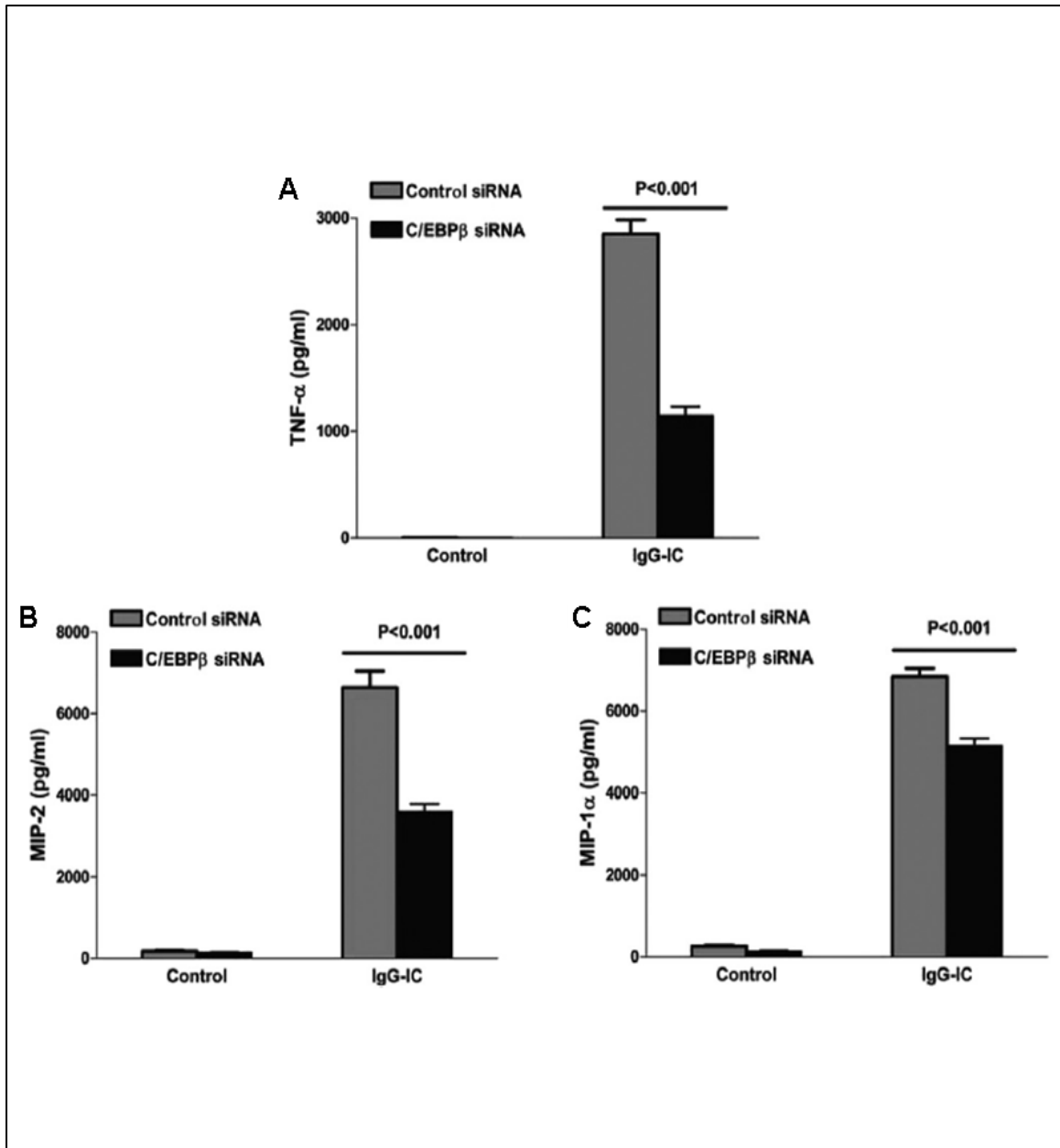


Figure 5. IgG IC-mediated TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$  Production is Regulated by C/EBP $\beta$  at Protein Level. RAW264.7 cells were transiently transfected with control siRNA or C/EBP $\beta$  siRNA. 12 h later, cells were incubated with or without 100  $\mu$ g/ml IgG IC for 5 h. The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

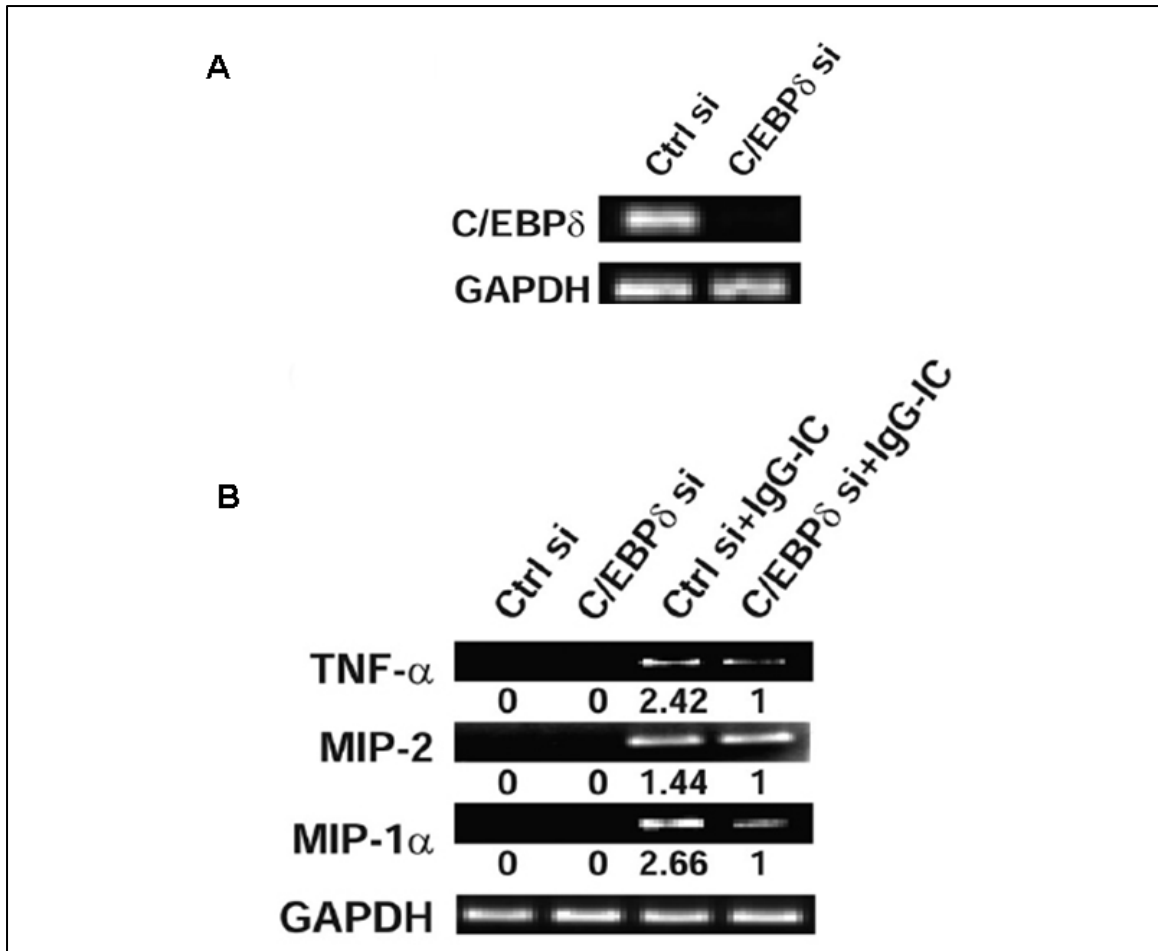


Figure 6. IgG IC-mediated Generation of Proinflammatory Mediators is Regulated by C/EBP $\delta$  at Transcription Level. A, RAW264.7 cells were transiently transfected with control siRNA, or C/EBP $\delta$  siRNA. 12 h later, RNAs were extracted and subjected to RT-PCR to examine expression of C/EBP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



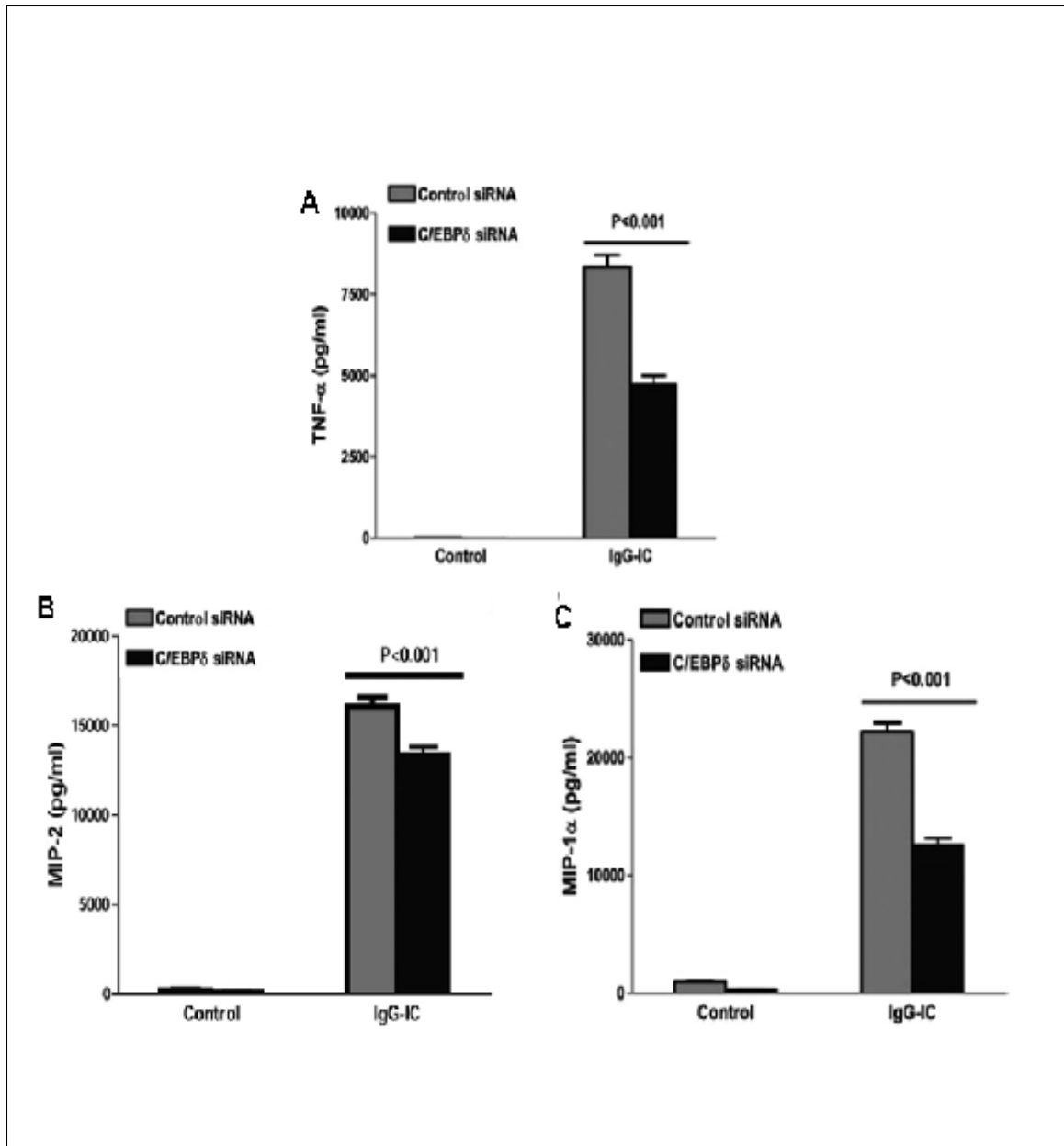


Figure 7. IgG IC-mediated TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$  Production is Regulated by C/EBP $\delta$  at Protein Level. RAW264.7 cells were transiently transfected with control siRNA or C/EBP $\delta$  siRNA. 12 h later, cells were incubated with or without 100  $\mu$ g/ml IgG IC for 5 h. The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

I further determined whether the observed regulatory role of C/EBP $\beta$  and - $\delta$  on the IgG IC-induced TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$  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/EBP $\beta$  LAP/C/EBP $\alpha$  heterodimers, C/EBP $\beta$  LIP/C/EBP $\delta$  heterodimers, LIP/C/EBP $\alpha$  heterodimers, heterodimers LAP and LIP, LAP/LAP homodimers, and LIP/LIP homodimers (Fig. 8). The DNA binding activities of both C/EBP $\beta$  and - $\delta$  (to a minor extent) were induced by IgG IC (Fig. 8). I next demonstrated that, upon IgG IC treatment, C/EBP $\beta$ - or C/EBP $\delta$ -deficient macrophages released less amounts of TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$  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/EBP $\beta$ <sup>-/-</sup>, C/EBP $\delta$ <sup>-/-</sup>, and C/EBP $\beta$ <sup>-/-</sup>/ $\delta$ <sup>-/-</sup> cells. When compared with WT macrophages, C/EBP $\beta$  knockout suppressed TNF- $\alpha$  induction by ~80%, MIP-1 $\alpha$  by ~58%, and MIP-2 by ~77.7%, and C/EBP $\delta$  knockout also significantly inhibited production of TNF- $\alpha$  (~24%), MIP-1 $\alpha$  (~49%), and MIP-2 (~34%) (Fig. 10). Moreover, C/EBP $\beta$ / $\delta$  double knockout further reduced secretion of TNF- $\alpha$  (~98%), MIP-1 $\alpha$  (~99%), and MIP-2 (~98%) (Fig. 10). Thus, there is a redundant role for C/EBP $\beta$  and - $\delta$  in IgG IC-induced TNF- $\alpha$ , MIP-1 $\alpha$ , and MIP-2 production in macrophages. Taken together, these findings show that C/EBP $\beta$  and - $\delta$  are critical regulators of cytokine and chemokine production in IgG IC-stimulated macrophages.

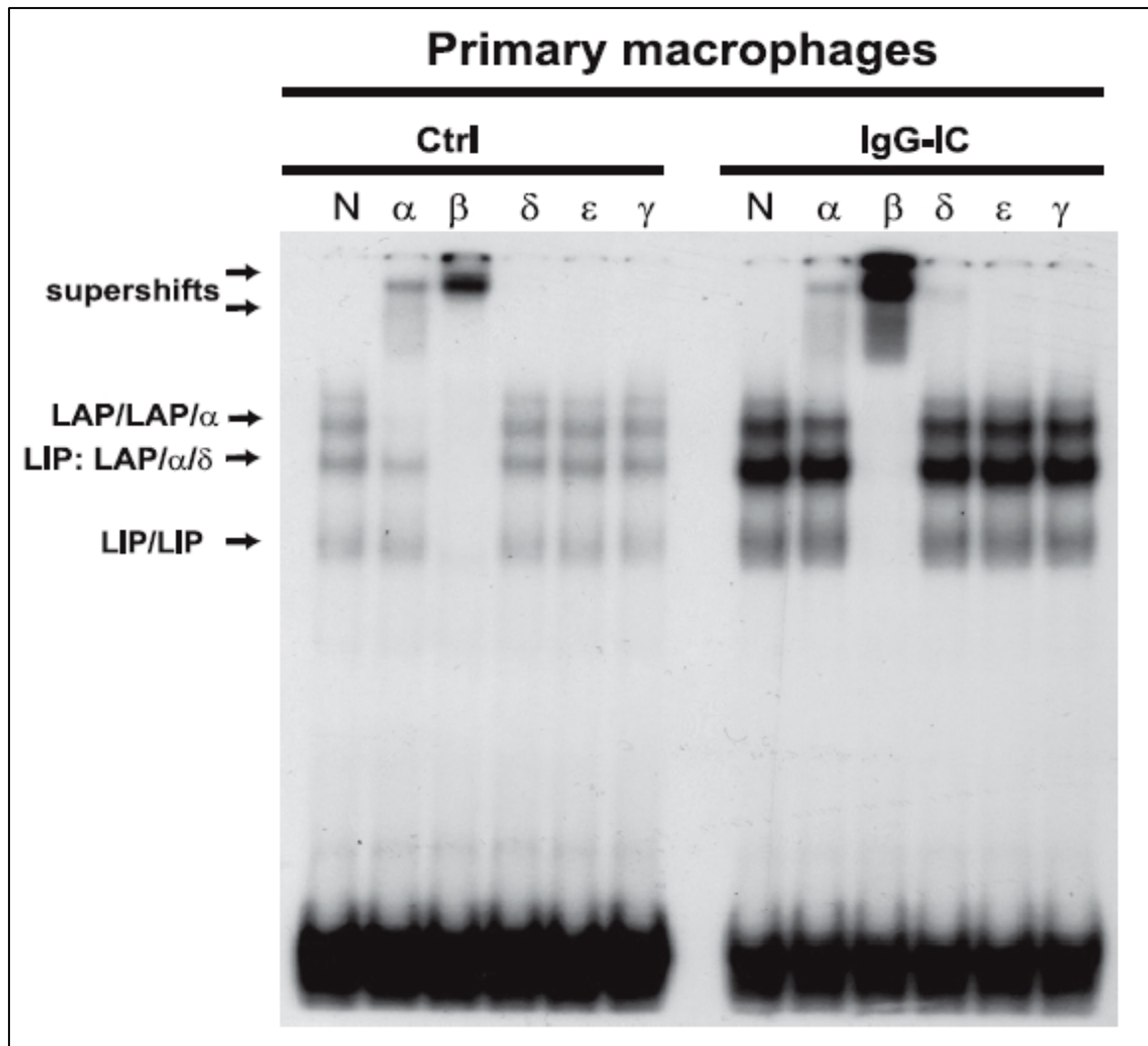


Figure 8. IgG Immune Complex Treatment Elevates C/EBP $\beta$  and - $\delta$  DNA Binding Activity in Primary Peritoneal Macrophages. Primary peritoneal macrophages were obtained from wild type mice, and treated or left untreated with 100  $\mu$ g/ml IgG immune complexes for 4 h. The nuclear extracts were harvested for gel supershift assay to identify which C/EBP family member DNA binding activity was regulated by IgG immune complex treatment. N,  $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\epsilon$ , and  $\gamma$ , normal rabbit IgG, anti-C/EBP $\alpha$  antibody, anti-C/EBP $\beta$  antibody, anti-C/EBP $\delta$  antibody, anti-C/EBP $\epsilon$  antibody, and anti-C/EBP $\gamma$  antibody, respectively. The arrows indicate C/EBP complex and supershift species. The same experiment was repeated three times.

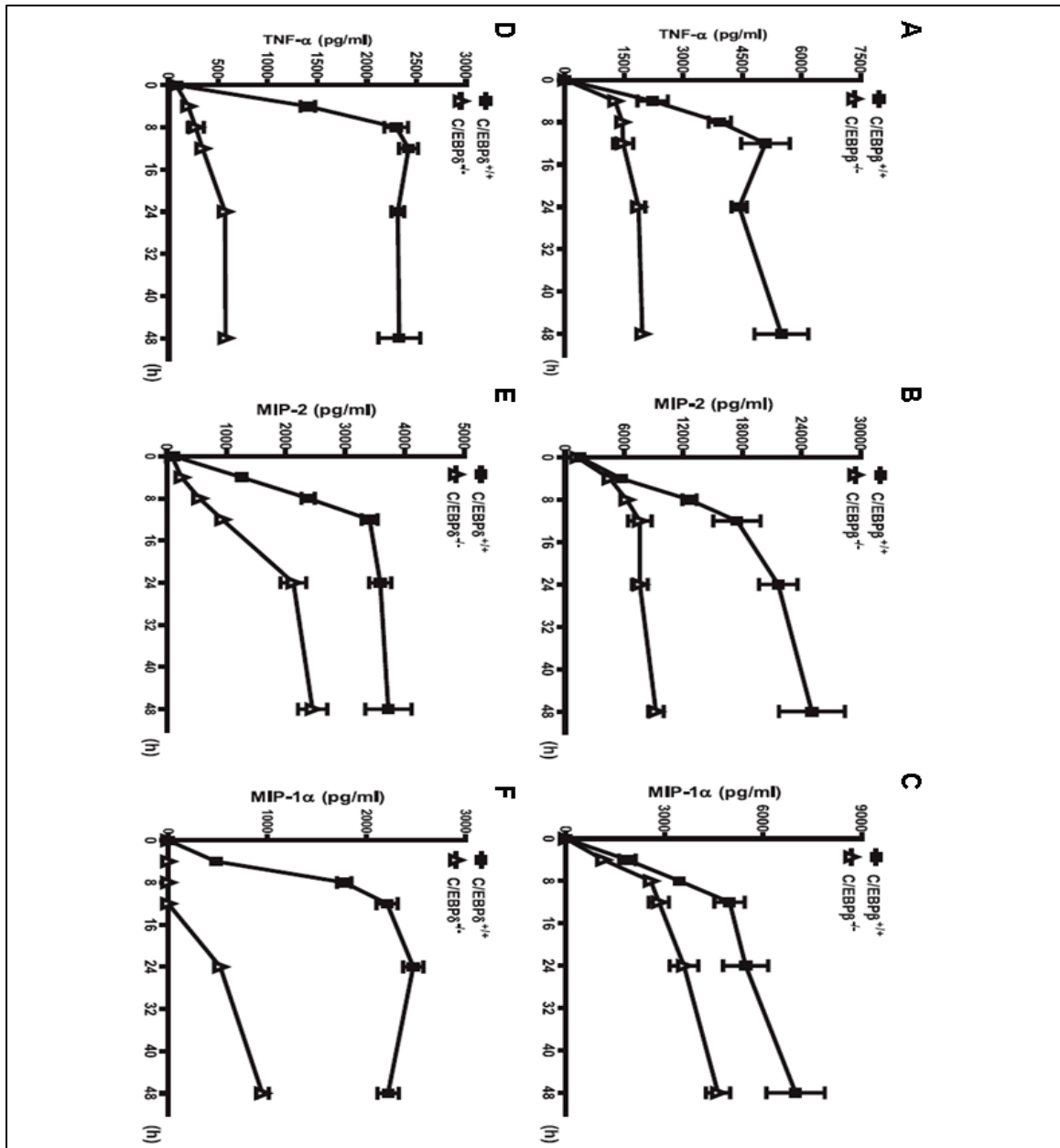


Figure 9. IgG IC-mediated Inflammation is Decreased in Macrophages Lacking C/EBPβ or C/EBPδ. Primary peritoneal macrophages obtained from corresponding wild type, and C/EBPβ or C/EBPδ knockout mice were treated with 100 μg/ml IgG immune complex for different time periods, and supernatants were subjected 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.

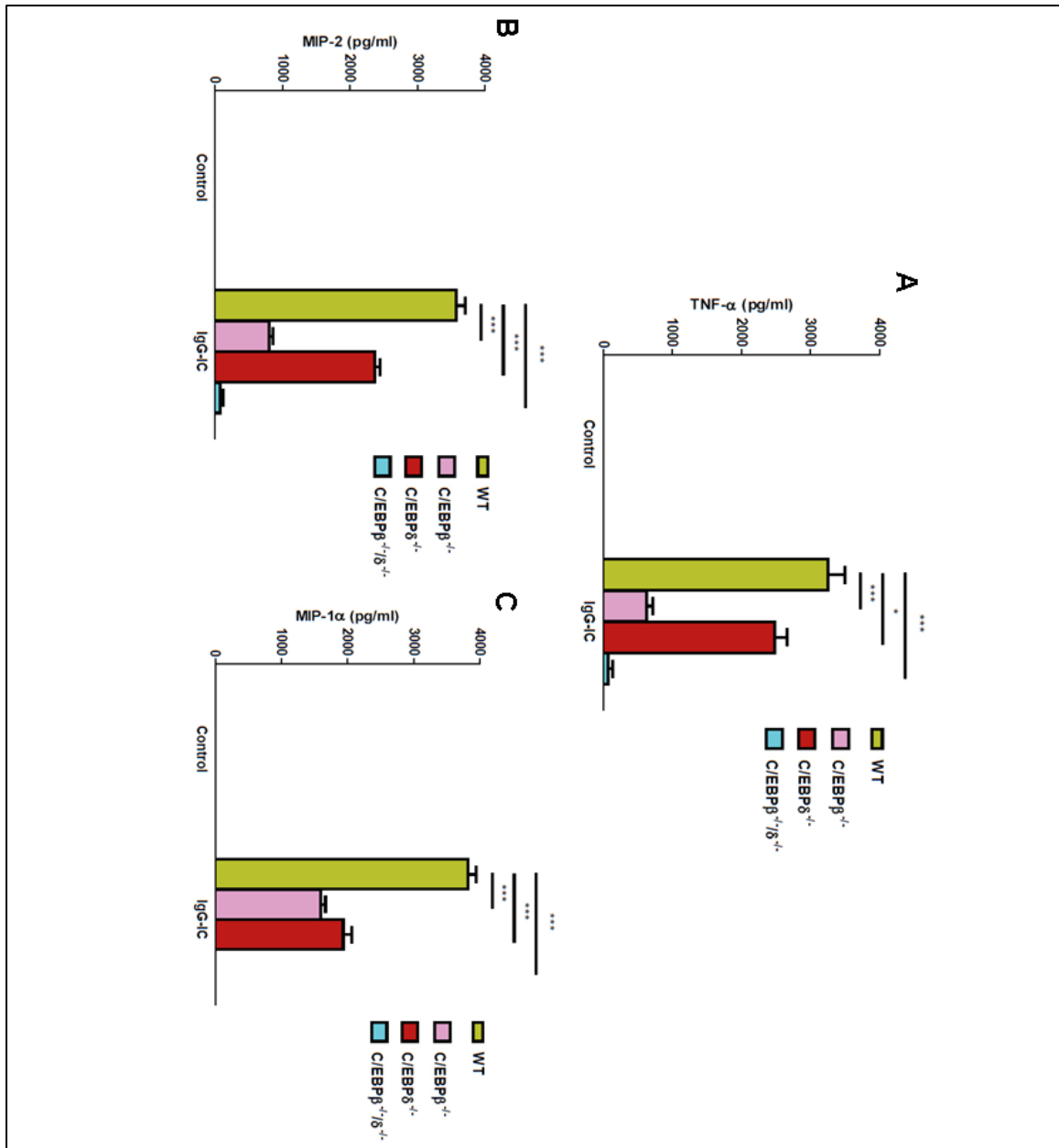


Figure 10. C/EBPβ and -δ are Critical Regulators of Inflammatory Reactions in IgG IC-stimulated Macrophages. Macrophages obtained from corresponding wild type, C/EBPβ knockout, C/EBPδ knockout, and C/EBPβ/δ double knockout mice were treated with 100 μg/ml IgG immune complex for 5 h, and supernatants 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- $\kappa$ B 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- $\kappa$ B 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- $\alpha$  and MIP-1 $\alpha$  production, suggesting that NF- $\kappa$ B also plays an important regulatory role in IgG IC-induced cytokine and chemokine production.

*Fc $\gamma$ Rs Trigger C/EBP-mediated Cytokine and Chemokine Production in IC-stimulated Macrophages*

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

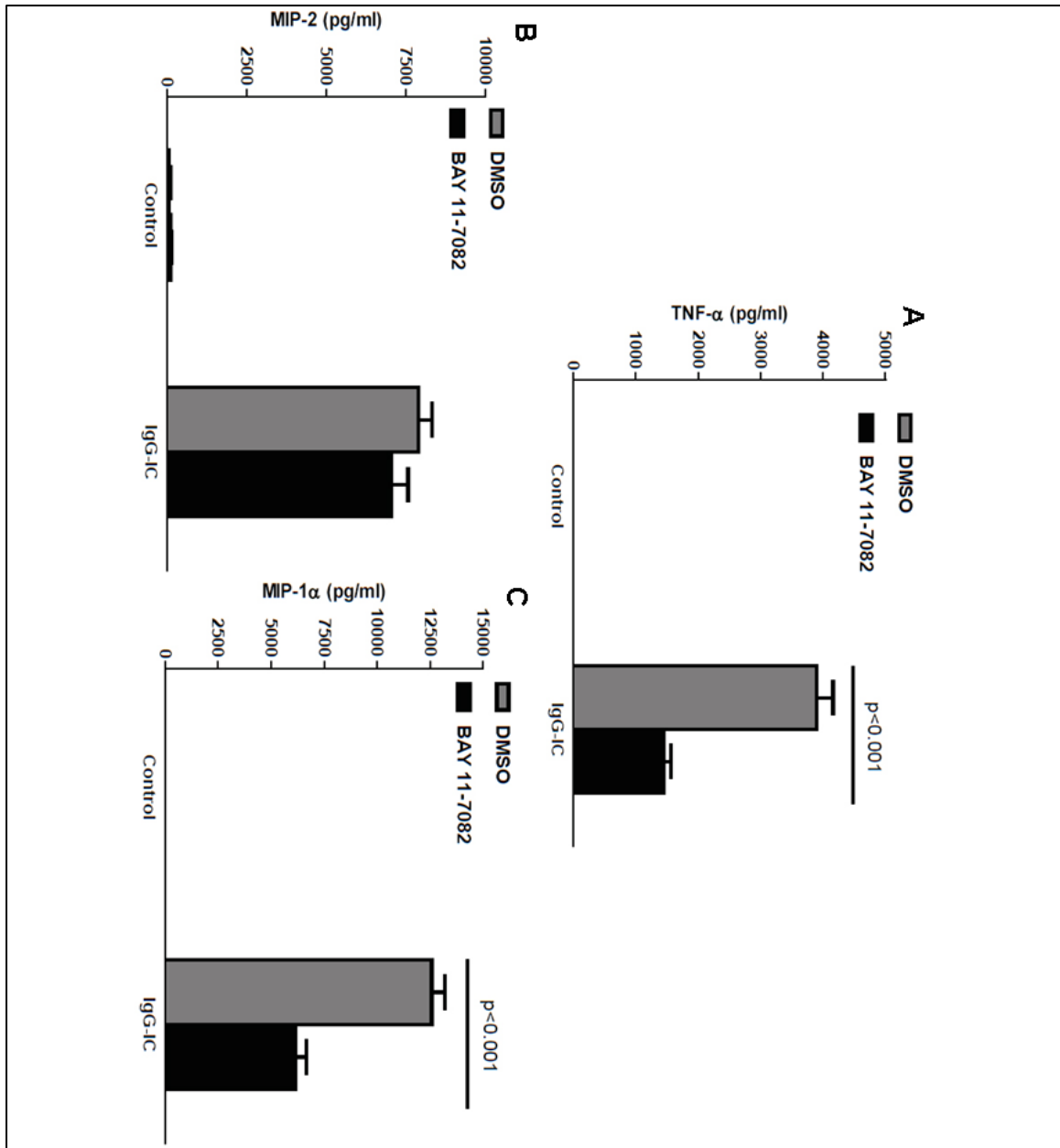


Figure 11. NF- $\kappa$ B Plays an Important Role in IgG Immune Complex-induced Inflammatory Mediators's Expressions. RAW264.7 cells were treated with 100  $\mu$ g/ml IgG immune complex, or IgG immune complex plus NF- $\kappa$ B inhibitor BAY 11-7082 (2  $\mu$ M) for 5 h. Supernatants were harvested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

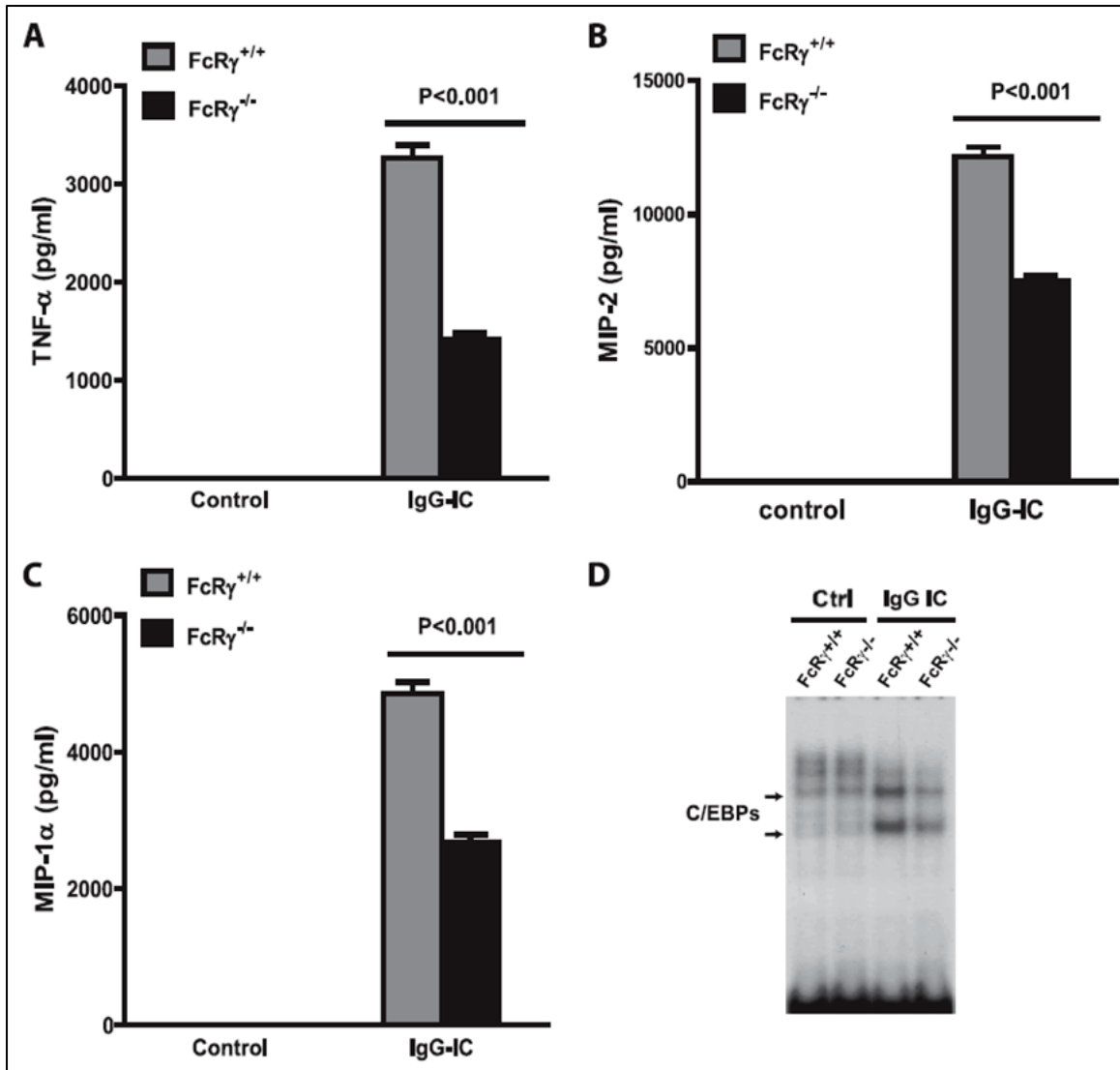


Figure 12. Effect of FcR  $\gamma$ -chain Deficiency on IgG IC-induced Inflammation. Primary peritoneal macrophages obtained from wild type and FcR  $\gamma$ -chain knockout mouse were treated with 100  $\mu$ g/ml IgG immune complex for 5 h, and supernatants were subjected to ELISA analysis for production of TNF- $\alpha$  (A), MIP-2 (B), and MIP-1 $\alpha$  (C). Data are presented as  $\pm$  S.E., n=8. D, Primary peritoneal macrophages obtained from corresponding wild type and FcR  $\gamma$ -chain deficient mice were treated or left untreated with 100  $\mu$ g/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.



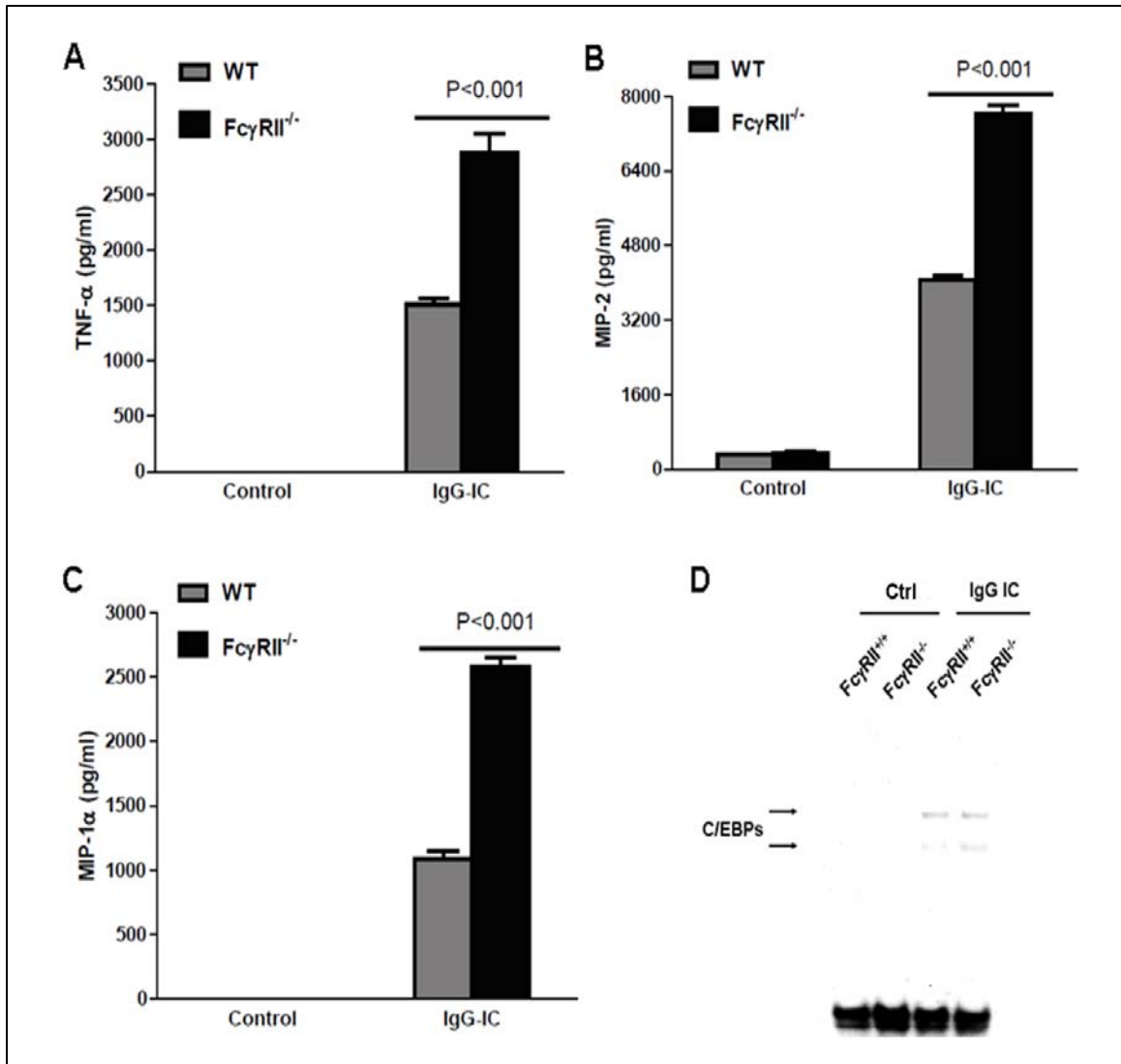


Figure 13. Effect of FcγR II Deficiency on IgG IC-induced Inflammation. Primary peritoneal macrophages obtained from wild type and FcγR II knockout mouse were treated with 100 μg/ml IgG immune complex for 5 h, and supernatants were subjected to ELISA analysis for production of TNF-α (A), MIP-2 (B), and 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/EBP $\beta$  and  $\delta$  may play important roles in cytokine and chemokine expression in IgG immune complex-stimulated macrophages, I next examined the effect of Fc $\gamma$ R deficiency on C/EBP $\beta$  and  $\delta$  activation. I found that knockout of FcR  $\gamma$ -chain suppressed induction of C/EBP DNA binding activity (Fig. 12D). Furthermore, inhibitory Fc $\gamma$ R II deficiency elevated C/EBP DNA binding activity (Fig. 13D). In summary, these findings suggest that activating Fc $\gamma$ R play an important role in IgG immune complex-induced C/EBP $\beta$  and  $\delta$  induction, leading to production of TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$ , whereas the inhibitory Fc $\gamma$ R has an opposite effect.

*ERK1/2 and p38 MAPK are Involved in IgG IC-induced C/EBP $\beta$  and  $\delta$  Activation and Subsequent Cytokine/Chemokine Production*

Previous studies have shown that Fc $\gamma$ R 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 Fc $\gamma$ R stimulation to C/EBP $\beta$  and  $\delta$  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/EBP $\beta$  and  $\delta$  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

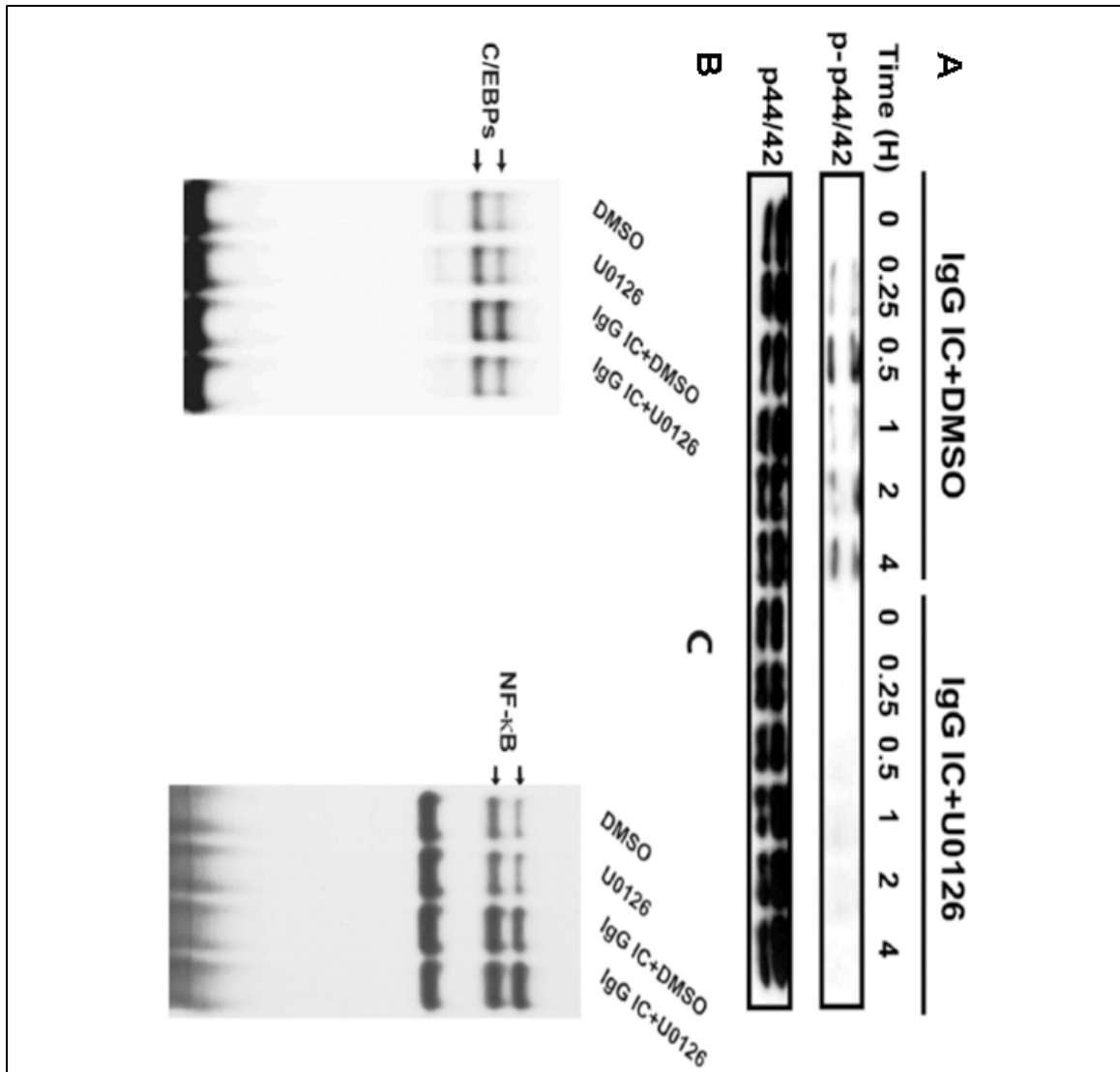


Figure 14. ERK1/2 is Involved in IgG IC-induced C/EBP $\beta$  and - $\delta$  Activation. A, RAW264.7 cells were treated with 100  $\mu$ g/ml IgG immune complexes in the presence or absence of ERK1/2 inhibitor, U0126 (10  $\mu$ M) for the indicated time periods. Total proteins were subjected to Western 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  $\mu$ g/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- $\kappa$ B 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- $\kappa$ B activation was also examined. EMSA showed that none of these inhibitors reduced NF- $\kappa$ B DNA binding activity (Figs. 14C and 16C). Interestingly, U0126 slightly elevated IgG IC-induced NF- $\kappa$ B activation (Fig. 14C). To determine whether MAPK activation is involved in IgG IC-induced cytokine and chemokine production (which are regulated by C/EBP $\beta$  and - $\delta$ ), I evaluated the effect of U0126 and p38 MAPK inhibitor VIII on TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$  secretion from RAW264.7 macrophages. As shown in Figs. 15 and 17, U0126 and p38 MAPK inhibitor VIII inhibited IgG IC-stimulated TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$  production. To determine whether ERK1/2 and p38 MAPK acted in concert or sequentially downstream of Fc $\gamma$ Rs, 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- $\alpha$  (~100%), MIP-2 (~100%), and MIP-1 $\alpha$  (~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- $\kappa$ B DNA Binding Activity*

C5a has been shown to synergistically enhance IgG immune complex-induced TNF- $\alpha$  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- $\alpha$ , MIP-2, and MIP-1 $\alpha$  production, whereas stimulation with C5a

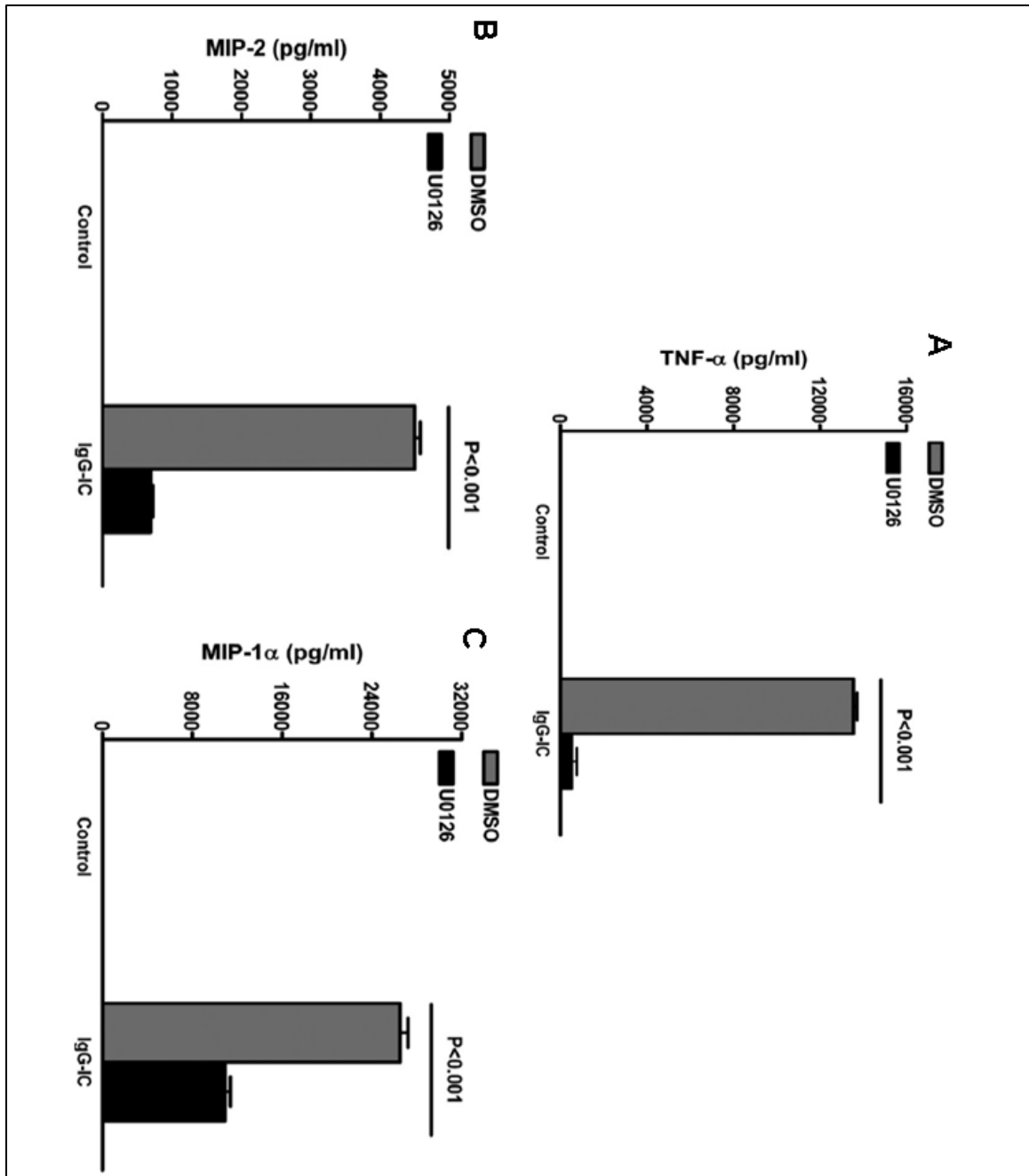


Figure 15. p44/42 MAPK is Involved in IgG IC-induced Inflammation. RAW264.7 cells were treated with 100  $\mu$ g/ml IgG immune complexes in the presence or absence of ERK1/2 inhibitor, U0126 (10  $\mu$ M) for 5 h, and supernatants were subjected to ELISA analysis for production of TNF- $\alpha$  (A),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

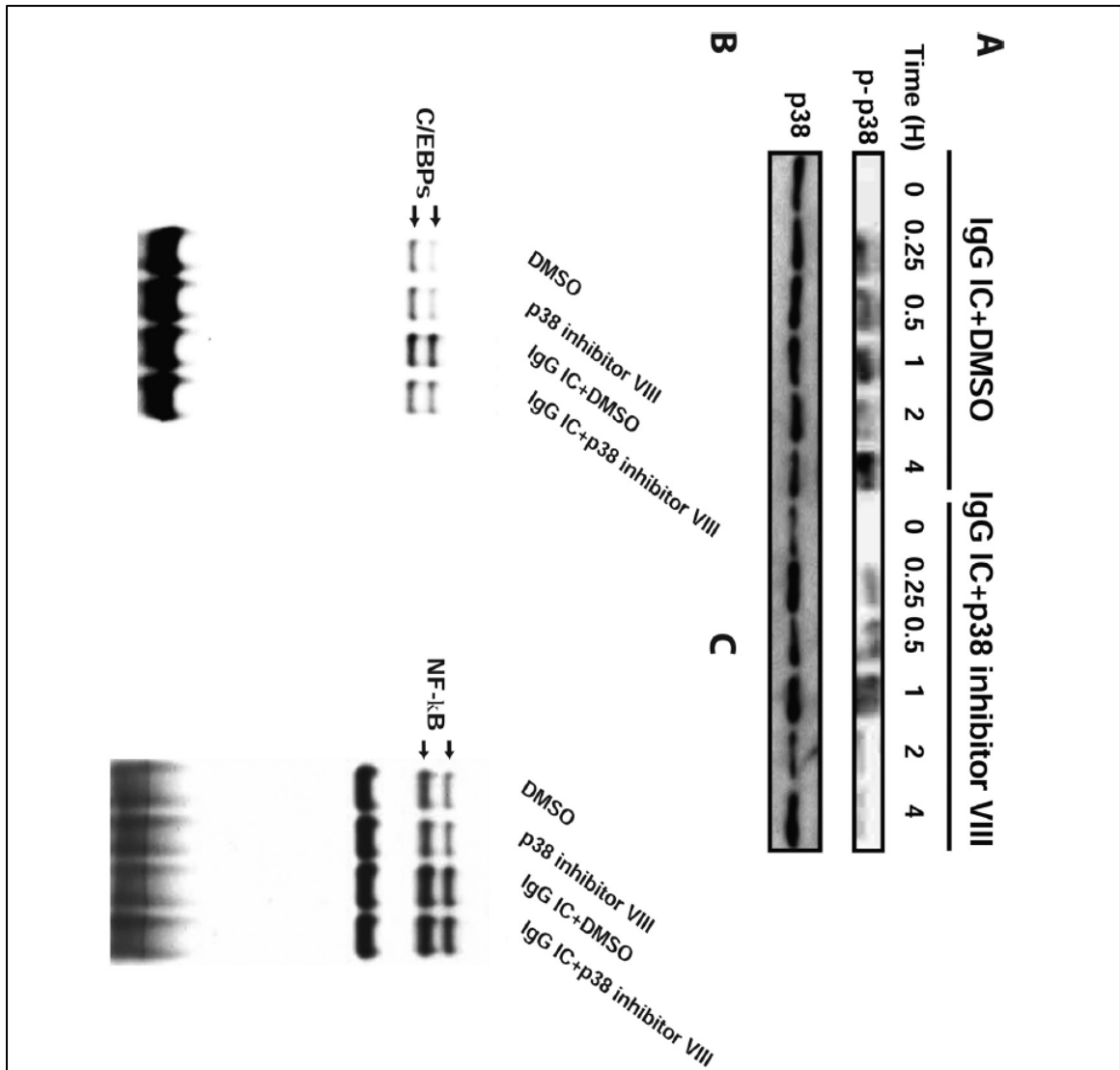


Figure 16. p38 MAPK is Involved in IgG IC-induced C/EBP $\beta$  and  $\delta$  Activation. A, RAW264.7 cells were treated with 100  $\mu$ g/ml IgG immune complexes in the presence or absence of p38 MAP Kinases Inhibitor VIII (10  $\mu$ M) for the indicated time periods. Total proteins were subjected 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  $\mu$ g/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- $\kappa$ B DNA binding (C), respectively. The same experiment was repeated once.

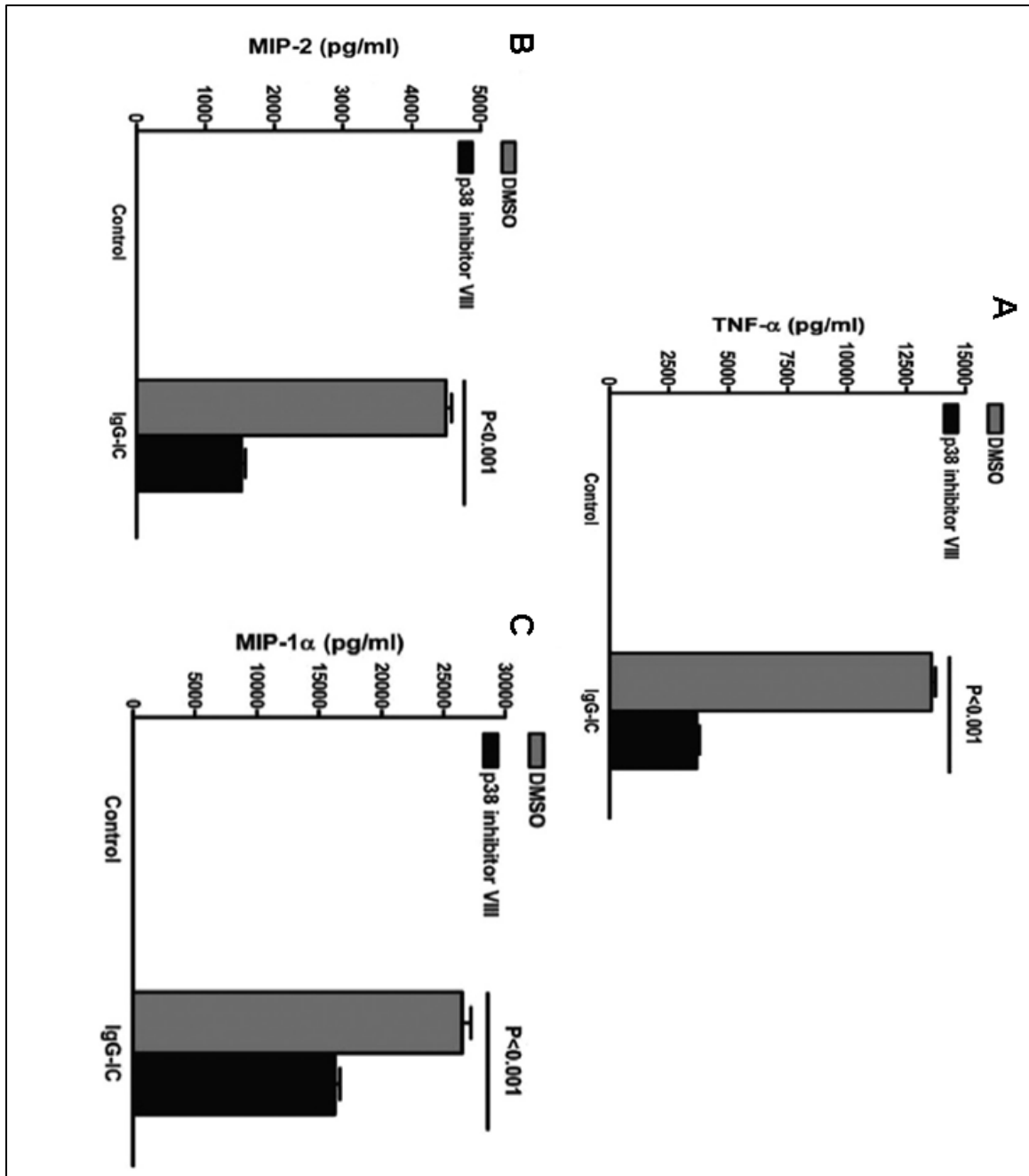


Figure 17. p38 MAPK is Involved in IgG IC-induced Inflammation. RAW264.7 cells were treated with 100  $\mu$ g/ml IgG immune complexes in the presence or absence of p38 MAPK inhibitor, p38 MAP Kinases Inhibitor VIII (10  $\mu$ M) for 5 h, and supernatants were subjected to ELISA analysis for production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

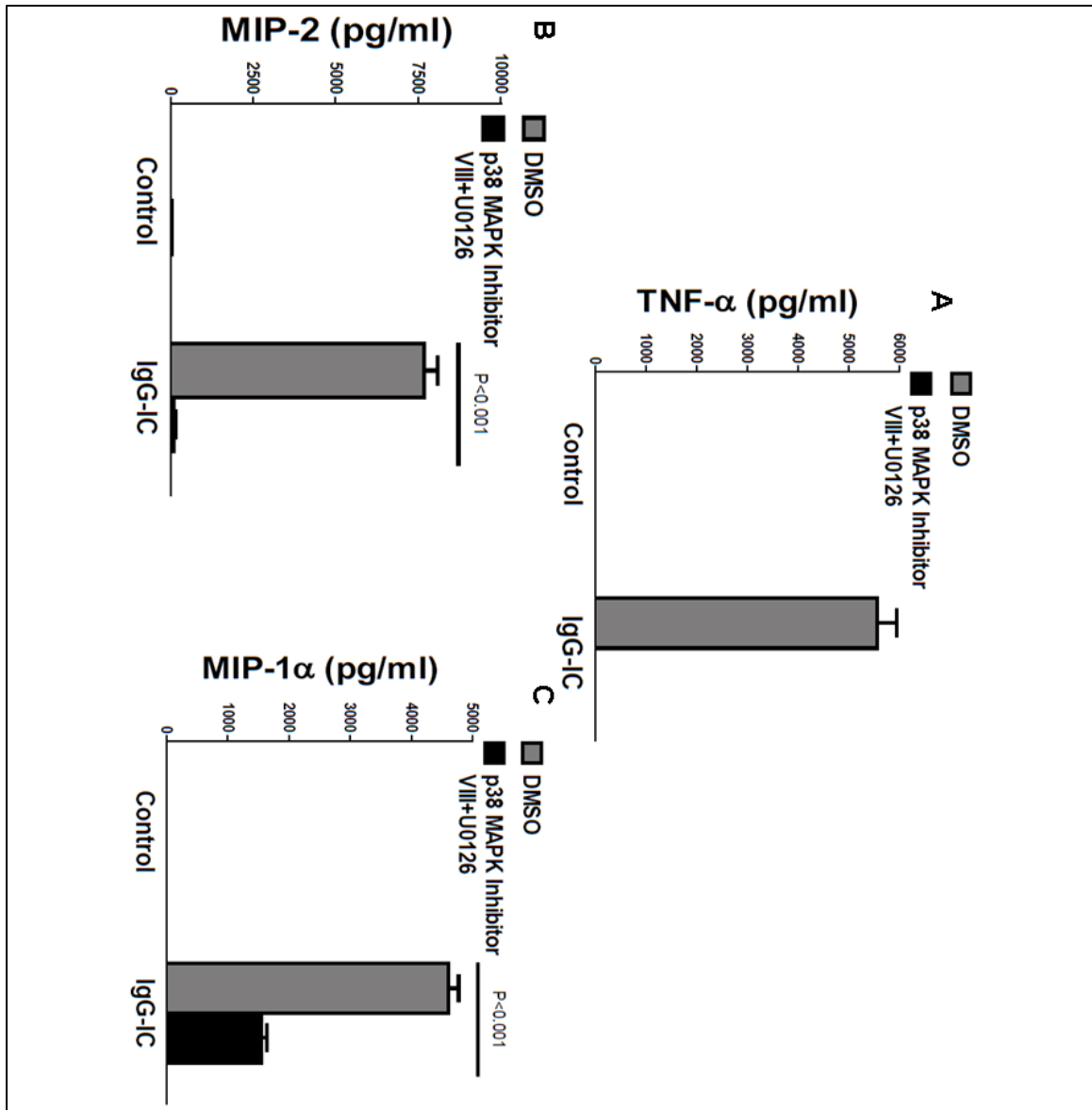


Figure 18. Both p38 and p44/42 MAPKs are Involved in IgG IC-induced Inflammation. RAW264.7 cells were treated with 100  $\mu$ g/ml IgG immune complex in the presence or absence of both p38 MAP kinase Inhibitor VIII(10  $\mu$ M) and p44/42 inhibitor U0126 (10  $\mu$ M) for 5 h. Supernatants were harvested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



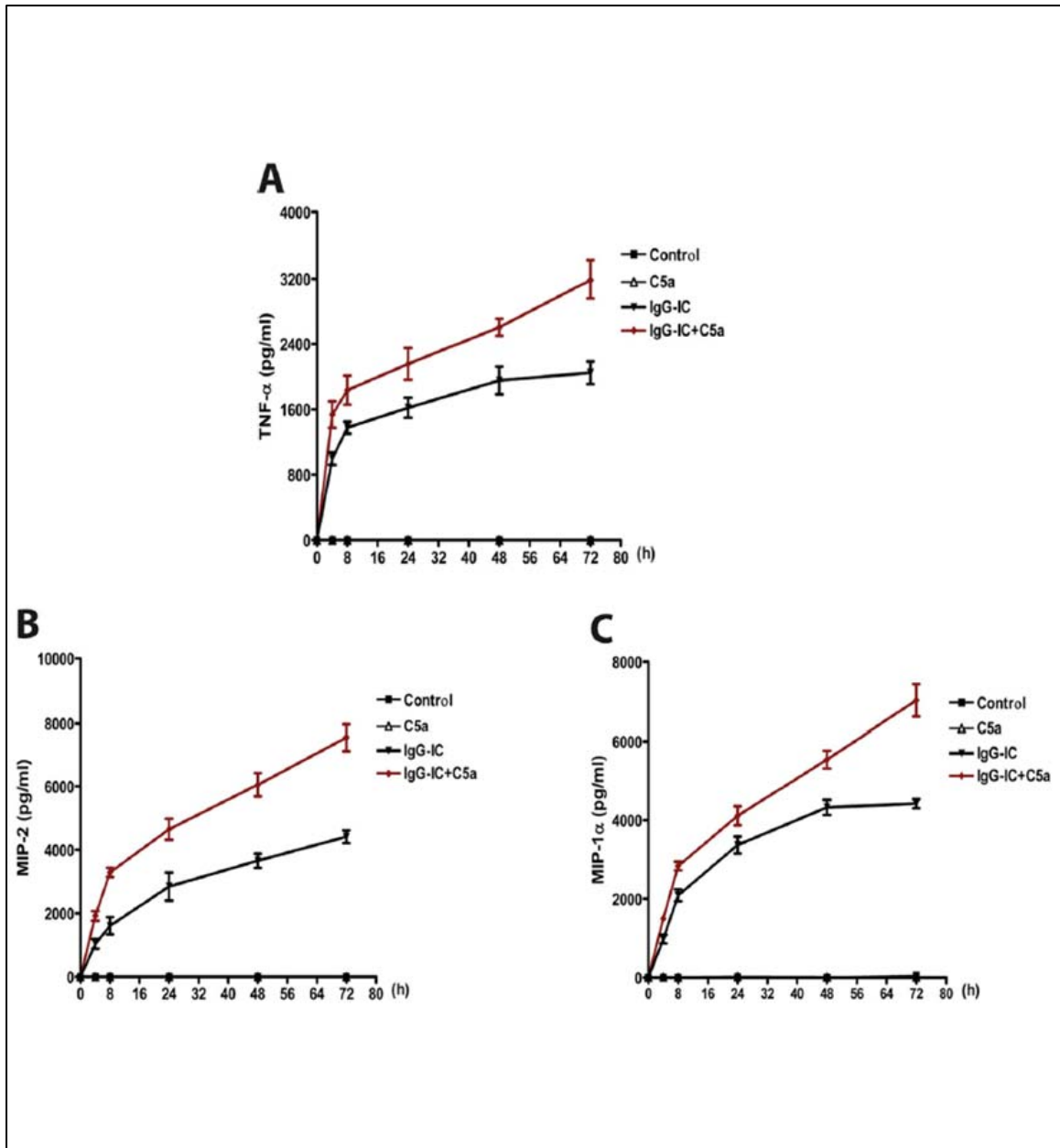


Figure 19. C5a Signaling Enhances IgG Immune Complex-induced Inflammation. Mouse peritoneal macrophages were treated with 10 nM recombinant human C5a, 100  $\mu$ g/ml IgG immune complexes, or a combination of both stimuli for different time points, and supernatants were subjected to ELISA analysis for production of TNF- $\alpha$  (A), MIP-2 (B), and MIP-1 $\alpha$  (C). Data are presented as mean  $\pm$  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 Fc $\gamma$ RIII/II pair to connect complement and Fc $\gamma$ R 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- $\kappa$ B, which may lead to the increased production of TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$ . Reporter assays demonstrated that IgG immune complex-induced C/EBP activity, but not NF- $\kappa$ B 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- $\kappa$ B 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/EBP $\beta$  and - $\delta$  activities.

#### Roles of C/EBP $\beta$ and - $\delta$ in IgG IC-induced ALI

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

Previous data have demonstrated the critical roles of C/EBP $\beta$  and - $\delta$  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-

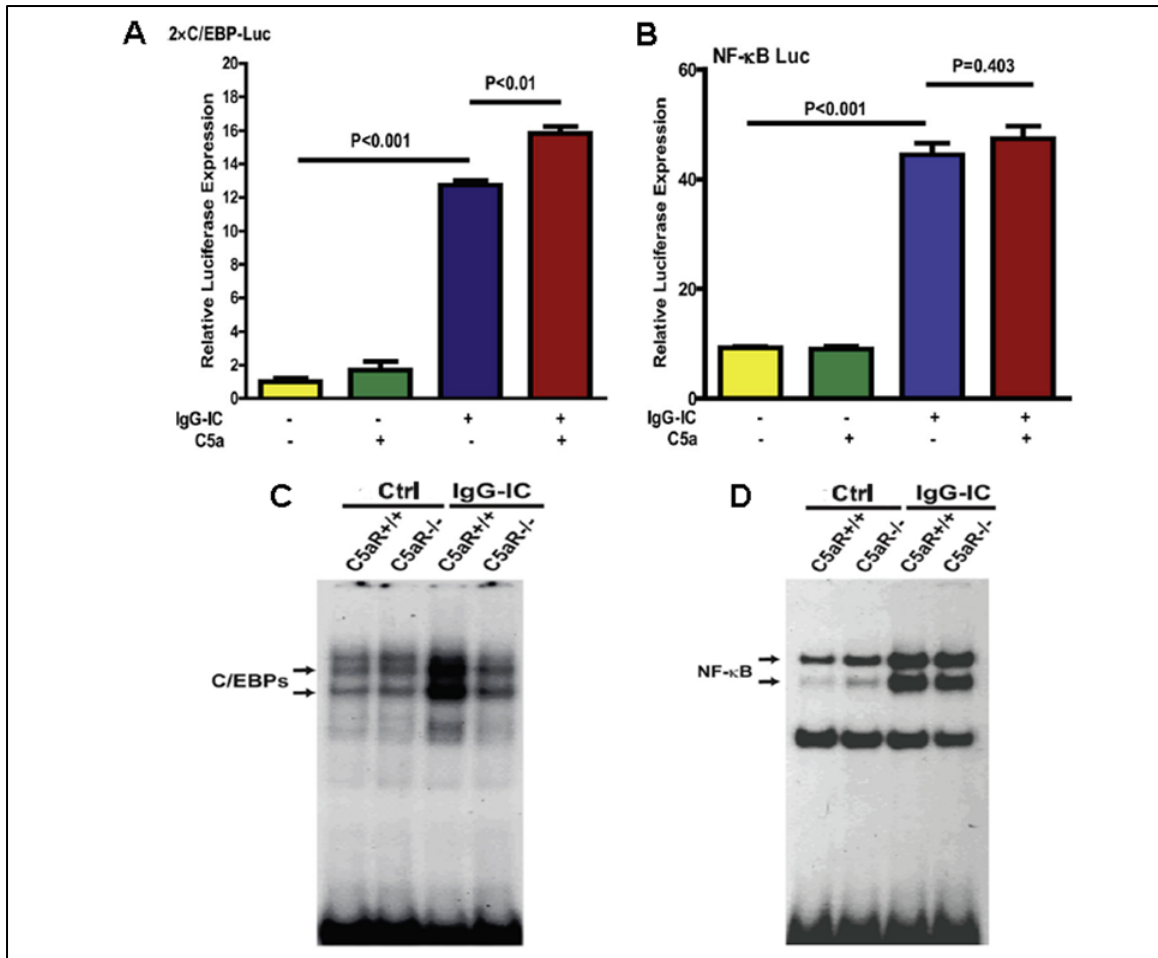


Figure 20. C5a Signaling Enhances IgG IC-induced C/EBP Activation. A and B, RAW264.7 cells were transiently transfected with a total of 0.5  $\mu$ g of DNA. 24 h after transfection, the cells were treated with 10 nM recombinant human C5a, 100  $\mu$ g/ml IgG immune complexes, or a combination of both 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  $\pm$  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  $\mu$ g/ml IgG IC for 4 h, and nuclear proteins were subjected to EMSA for C/EBP (C), and NF- $\kappa$ B (D), respectively. The same experiment was repeated once.

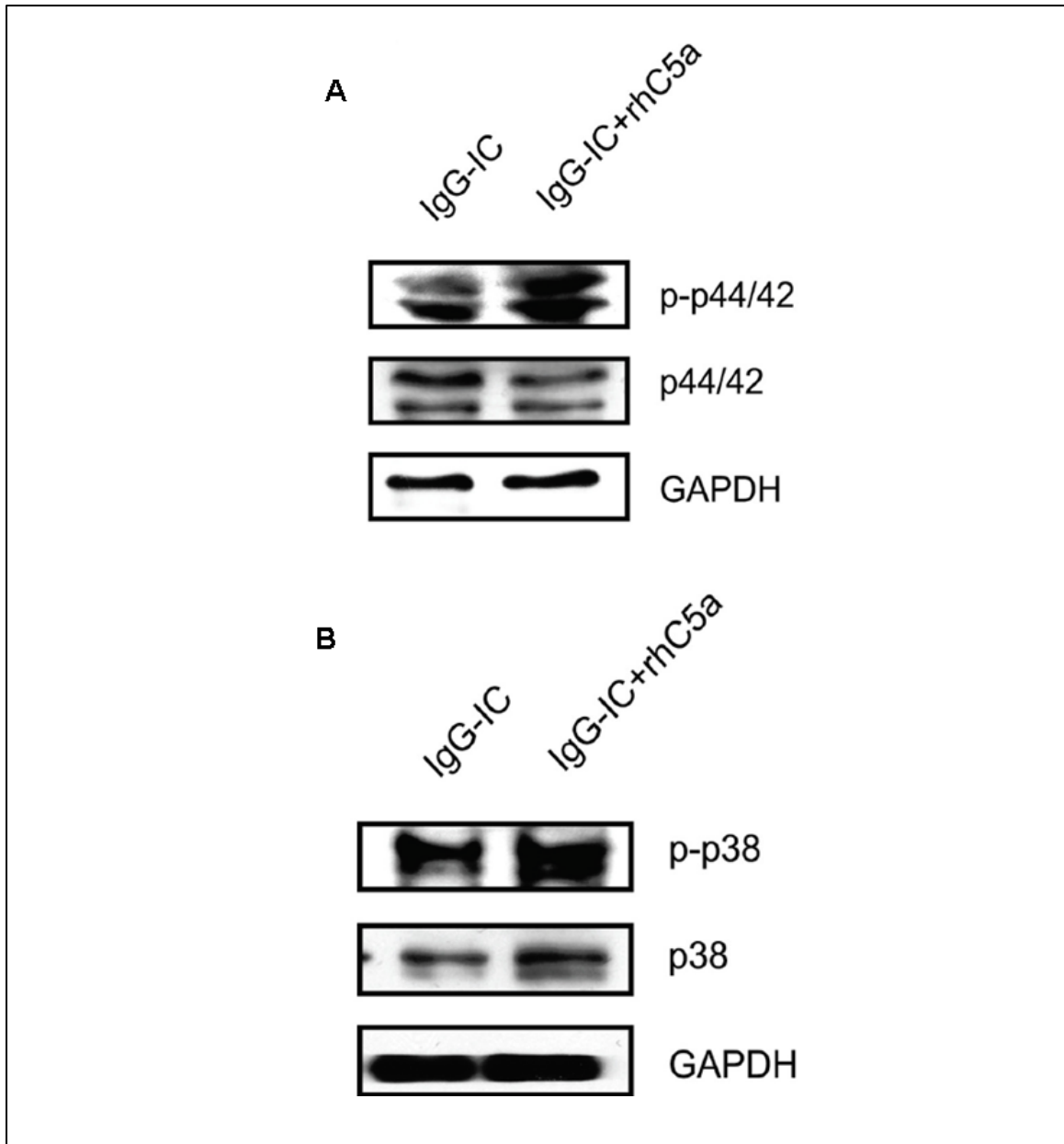


Figure 21. C5a Enhances IgG IC-induced p44/42 and p38 MAPKs Activation. RAW264.7 cells were treated with 100  $\mu$ g/ml IgG immune complex or IgG immune complex plus 10 nM rhC5a for 4 h. Total proteins were extracted and subjected to Western blot by using rabbit anti-phospho-p44/42 (p-p44/42) antibody (A), rabbit anti-p44/42 antibody (A), rabbit anti-phospho-p38 (p-p38) antibody (B), rabbit anti-p38 antibody (B), and rabbit anti-GAPDH, respectively. 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/EBP $\alpha$ / $\beta$  heterodimers; heterodimers between C/EBP $\beta$  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/EBP $\beta$  (mainly  $\beta$ /LIP and LIP/LIP) and C/EBP $\delta$  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/EBP $\beta$  mRNA after IgG immune complex deposition (Fig. 22C). Thus, the increased mRNA expression of C/EBP $\beta$  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<sub>2</sub>MDP 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.

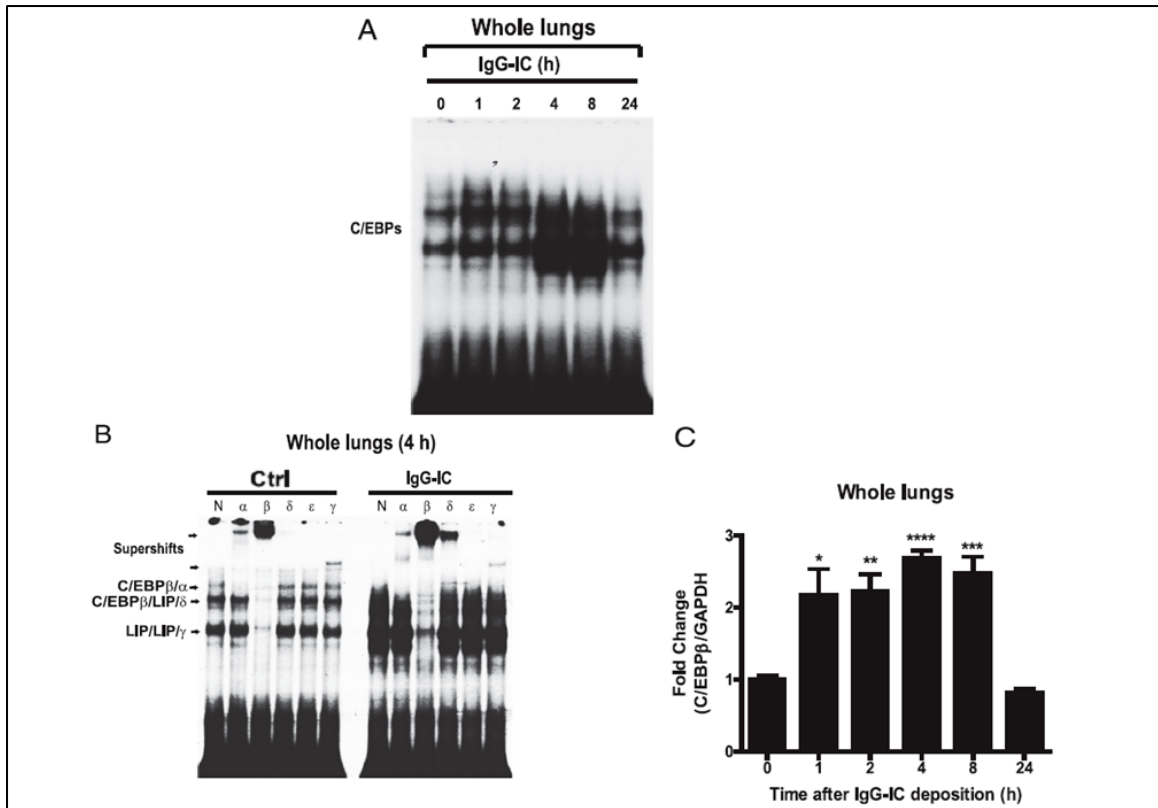


Figure 22. C/EBP $\beta$  and C/EBP $\delta$  DNA Binding Activities are Induced during IgG IC-induced Alveolitis. A, Mice lungs were challenged by IgG IC for indicated time periods. Then nuclear proteins were extracted from whole lung tissues and subjected to EMSA analysis. B, Nuclear proteins extracted 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/EBP $\alpha$  Ab ( $\alpha$ ), anti-C/EBP $\beta$  Ab ( $\beta$ ), anti-C/EBP $\delta$  Ab ( $\delta$ ), anti-C/EBP $\epsilon$  Ab ( $\epsilon$ ), and anti-C/EBP $\gamma$  Ab ( $\gamma$ ). 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/EBP $\beta$  during IgG immune complex-induced lung injury. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . Results are means  $\pm$  S.E.,  $n=4$ . The same experiment was repeated once.

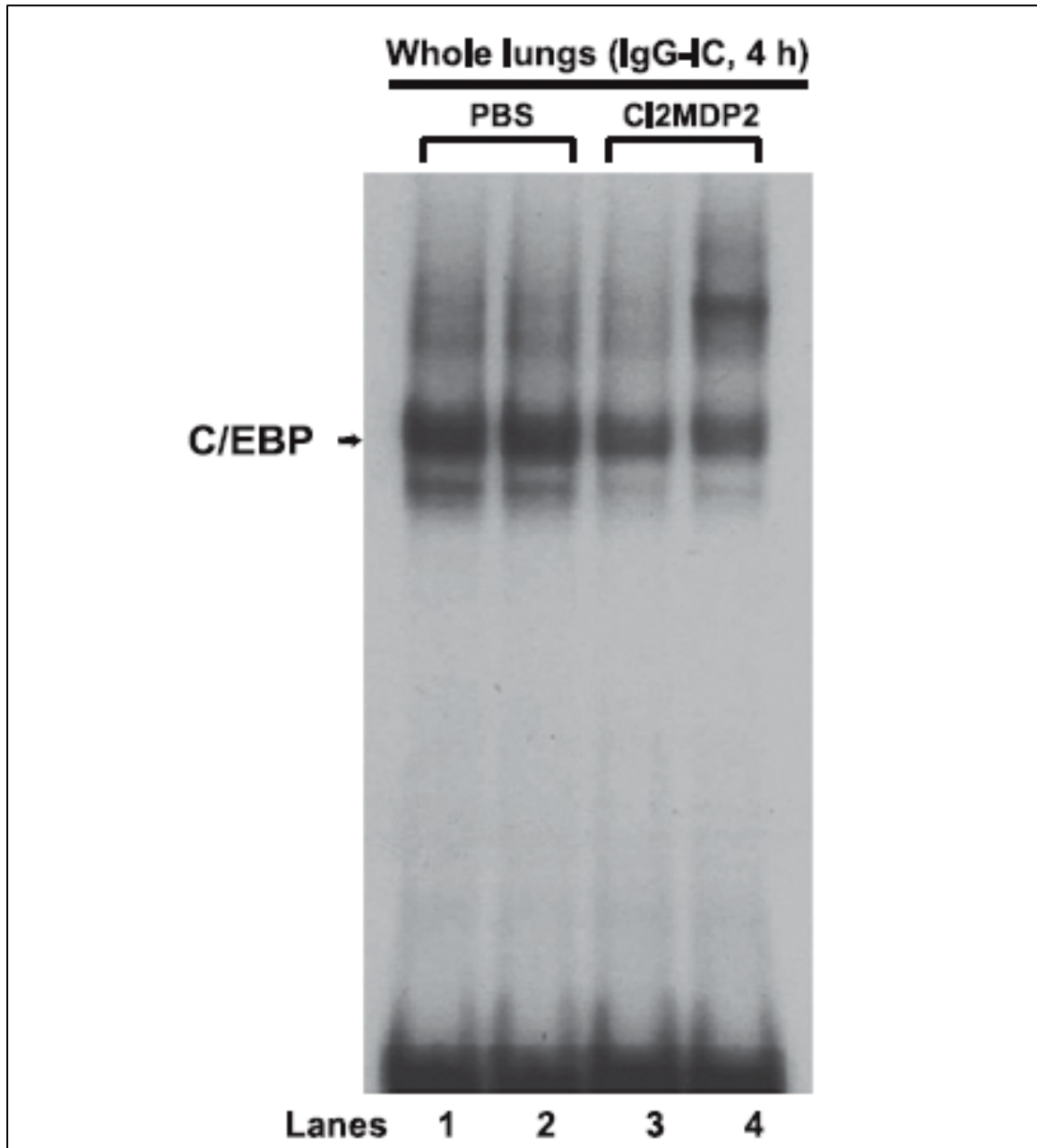


Figure 23. Effects of Alveolar Macrophage Depletion on C/EBP Activation during IgG Immune Complex-induced Lung Injury. Mice received PBS-liposome or Cl<sub>2</sub>MDP-liposomes 24 h before receiving intratracheal challenge with IgG immune complex. Four hours after immune complex challenge, lungs were harvested. C/EBP-binding activity of whole lung nuclear extracts was assessed by EMSA. The same experiment was repeated four times.

### *Effects of C/EBP $\beta$ Deficiency on IgG Immune Complex-induced Lung Injury*

I sought to determine whether C/EBP $\beta$  contributed to IgG IC-induced lung injury by using C/EBP $\beta$  deficient mice. As shown in Fig. 24A, C/EBP $\beta$  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/EBP $\beta$  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/EBP $\beta$  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/EBP $\beta$  deficient mice exhibited reduced lung injury by histological analyses. As shown in Fig. 25, both wild type and C/EBP $\beta$  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/EBP $\beta$  deficient mice showed significantly decreased neutrophil accumulation and reduced intra-alveolar hemorrhage compared with wild type animals.

### *Production of Cytokines and Chemokines in Lung after IgG Immune Complex Deposition is Impaired in C/EBP $\beta$ Deficient Mice*

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- $\alpha$ , IL-6, MIP-2, KC, MIP-1 $\alpha$ , and MIP-1 $\beta$  compared with controls (Fig. 26A-F). The levels of



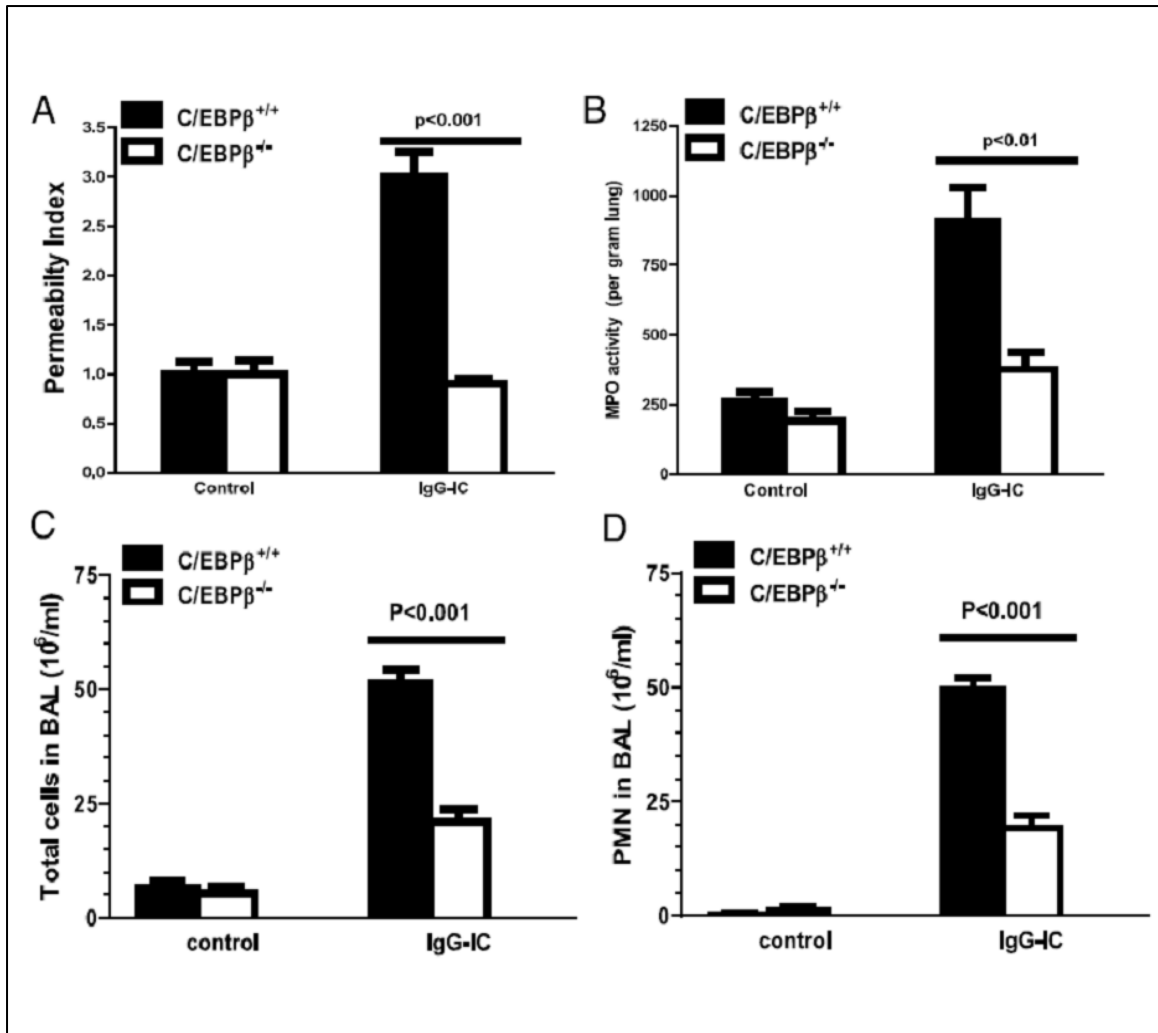


Figure 24. Effects of C/EBP $\beta$  Deficiency on IgG Immune Complex-induced Pulmonary Inflammation. Four hours after IgG immune complex deposition, BAL fluids and lungs were harvested. A, mouse albumin content in BAL fluids was determined using ELISA. The permeability index was expressed as the ratio 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  $\pm$  S.E. for three (control group) or five (IgG immune complex-challenged group) mice for each group. The same experiment was repeated once.

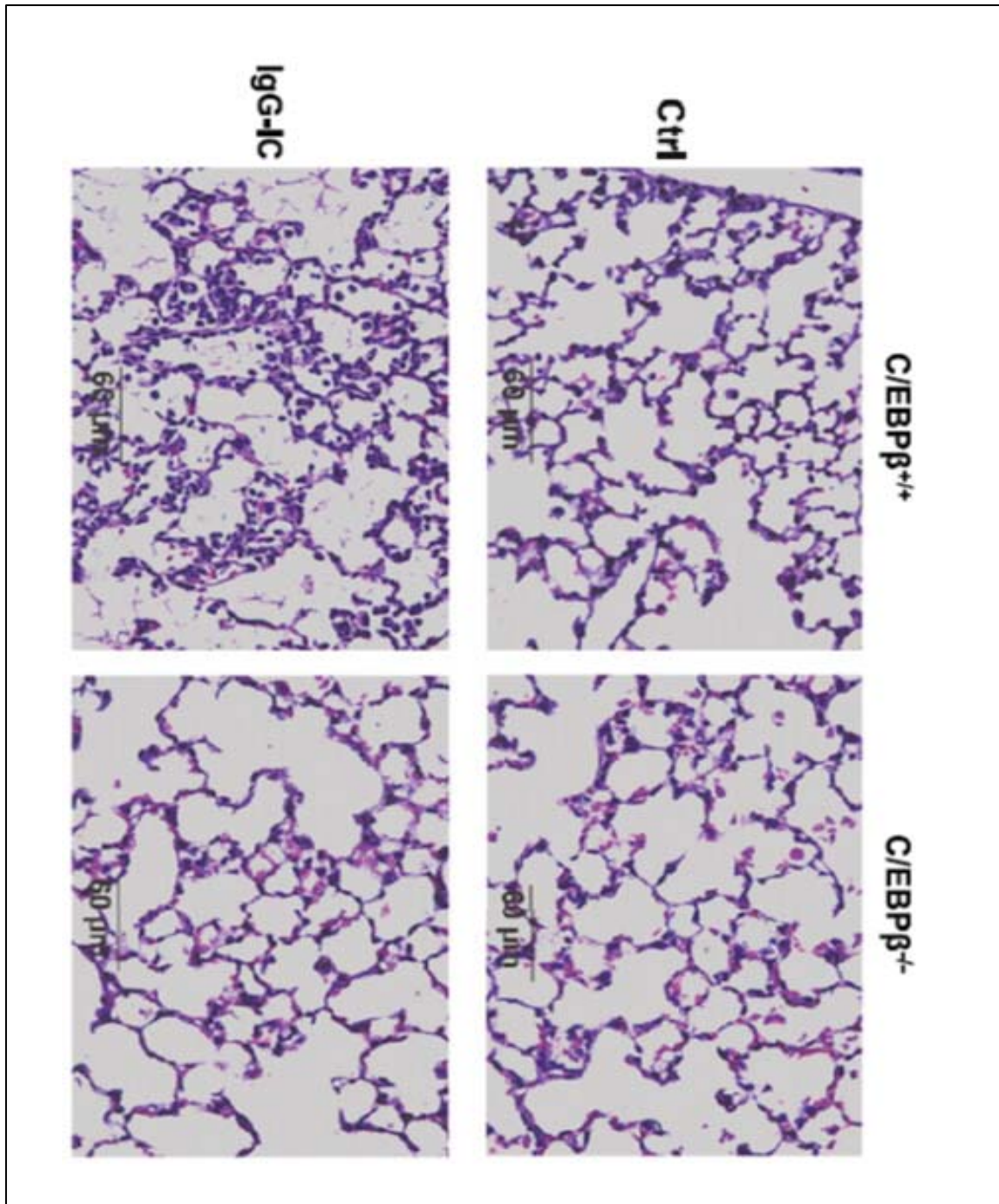


Figure 25. Effects of C/EBP $\beta$  Deficiency on IgG IC-induced Lung Injury Bases on Histological Assay. Lung sections were stained with H&E (original magnification  $\times 40$ ). Lung sections shown included the following: C/EBP $\beta^{+/+}$  + Ctrl, C/EBP $\beta^{+/+}$  + IgG IC, C/EBP $\beta^{-/-}$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

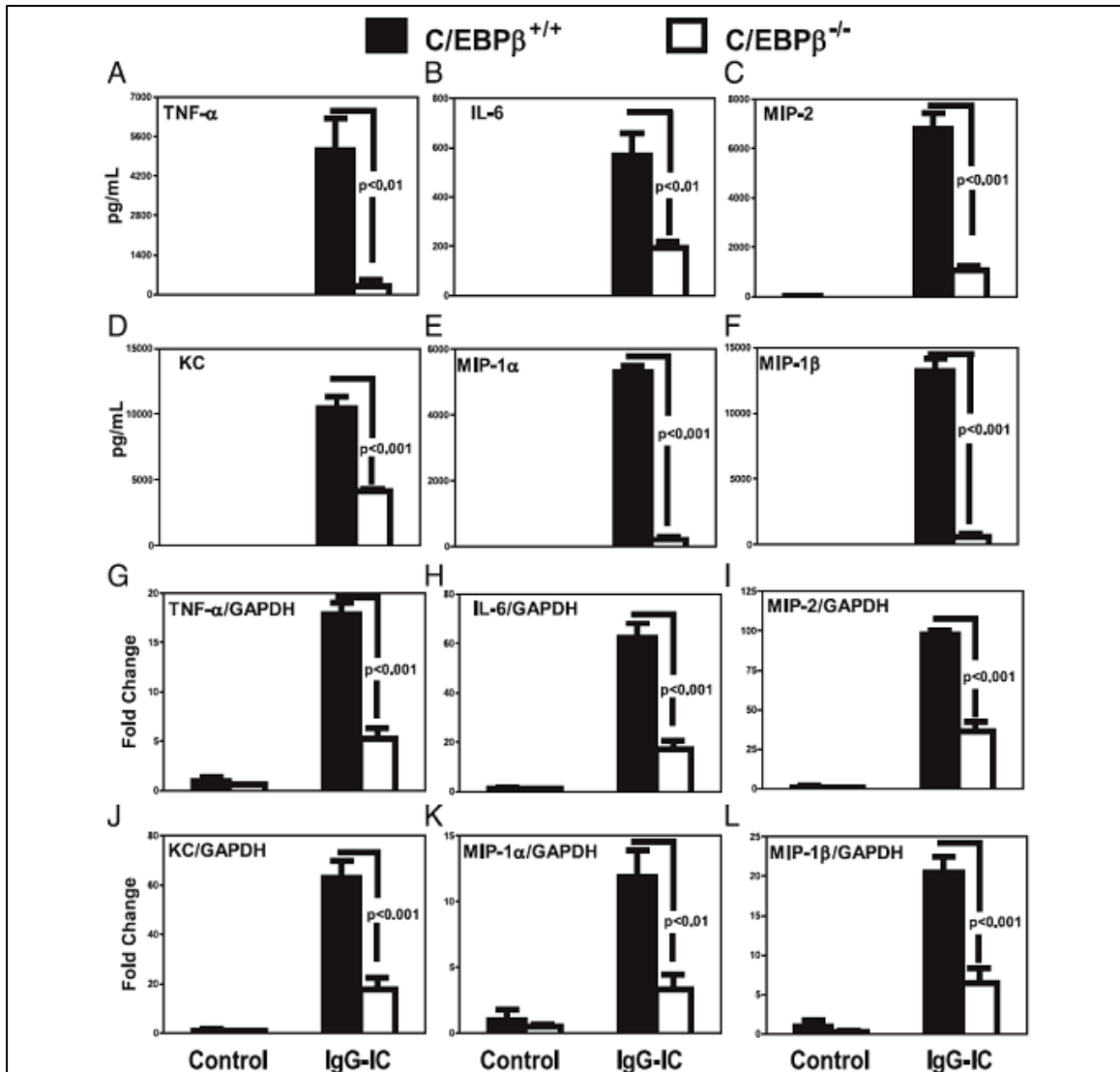


Figure 26. Effects of C/EBP $\beta$  Deficiency on Proinflammatory Mediators' Expressions during IgG IC-induced Acute Lung Injury. Four hours after IgG immune complex deposition, BAL fluids and lungs were harvested. Cell-free BAL fluids were subjected to ELISA to measure TNF- $\alpha$  (A), IL-6 (B), MIP-2 (C), KC (D), MIP-1 $\alpha$  (E), and MIP-1 $\beta$  (F) levels. RNAs were extracted, and real-time PCR was then used to analyze mRNA expression of TNF- $\alpha$  (G), IL-6 (H), MIP-2 (I), KC (J), MIP-1 $\alpha$  (K), and MIP-1 $\beta$  (L). Results are means  $\pm$  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/EBP $\beta$  deficient mice expressed considerably lower amounts of mRNAs for TNF- $\alpha$ , IL-6, MIP-2, KC, MIP-1 $\alpha$ , and MIP-1 $\beta$  (Fig. 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/EBP $\beta$  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/EBP $\beta$  deficiency resulted in a significant decrease ( $p < 0.01$ ) of ICAM-1 change when compared with wild type mice after IgG immune complex deposition in the lung (Fig. 27).

*Effects of C/EBP $\beta$  Deficiency on Cytokine and Chemokine Protein Production in Alveolar Macrophages Stimulated by IgG IC*

I evaluated the effects of C/EBP $\beta$  deficiency on the expression of cytokines and chemokines in primary alveolar macrophages. Upon IgG immune complex treatment, C/EBP $\beta$  deficient alveolar macrophages released significantly less TNF- $\alpha$ , IL-6, MIP-2, KC, and MIP-1 $\alpha$  than wild-type macrophages at all time points analyzed (Fig. 28). Interestingly, no significant change was found for MIP-1 $\beta$ , suggesting that other cells in the lung may contribute to the reduced levels of this chemokine in the BAL fluid and lung of C/EBP $\beta$  deficient mice (Fig. 28). To perform EMSA, about 2.5 million cells are required

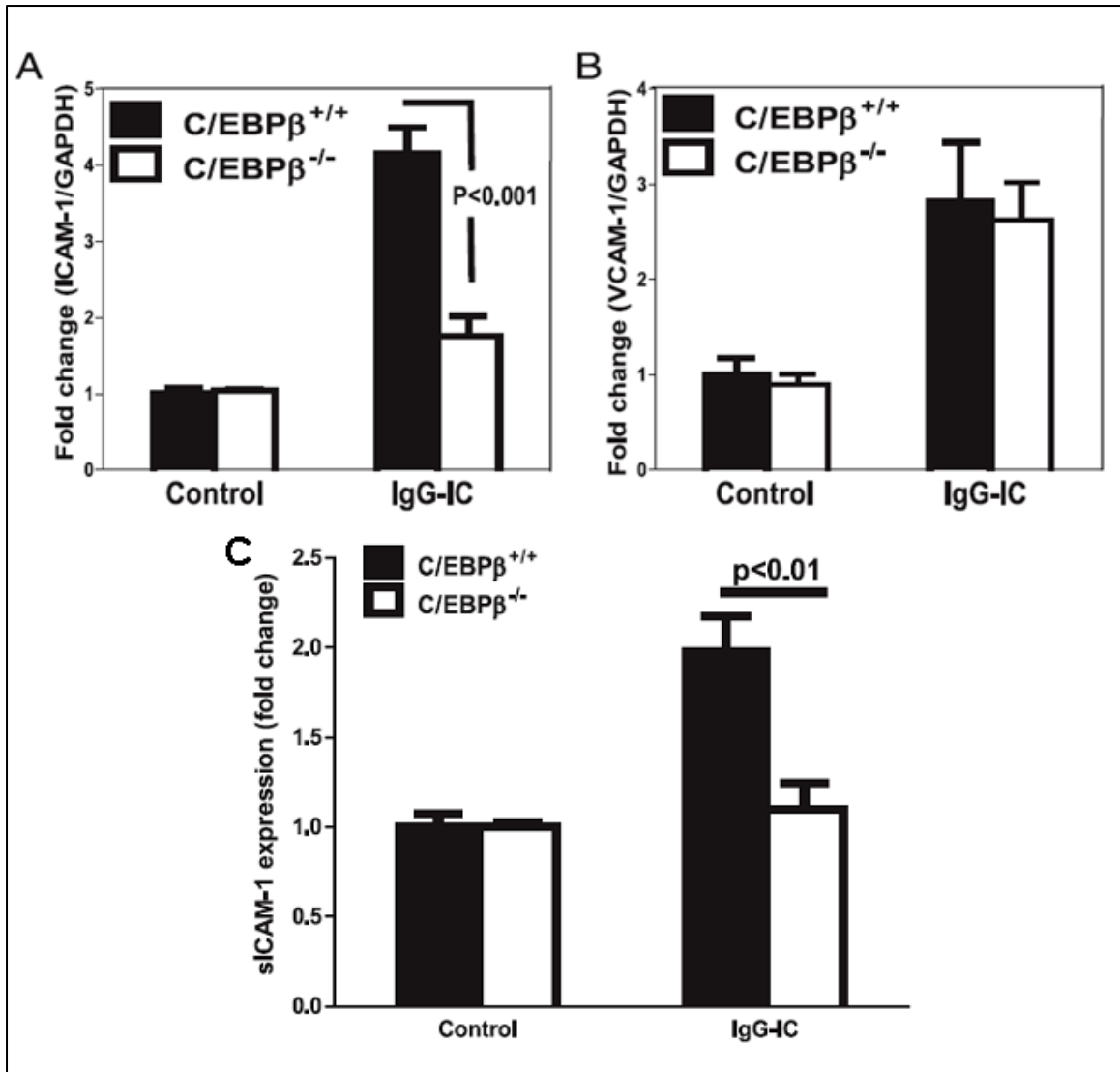


Figure 27. Effects of C/EBP $\beta$  Deficiency on IgG Immune Complex-induced ICAM-1 and VCAM-1 Expressions. Four hours after IgG immune complex deposition, BAL fluids and lungs were harvested. Real-time PCR was then used to analyze mRNA expression of ICAM-1 (A), and VCAM-1 (B). (C) Cell-free BAL fluids 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  $\pm$  S.E. for three (control group) or five (IgG IC-challenged group) mice for each group. The same experiment was repeated once.

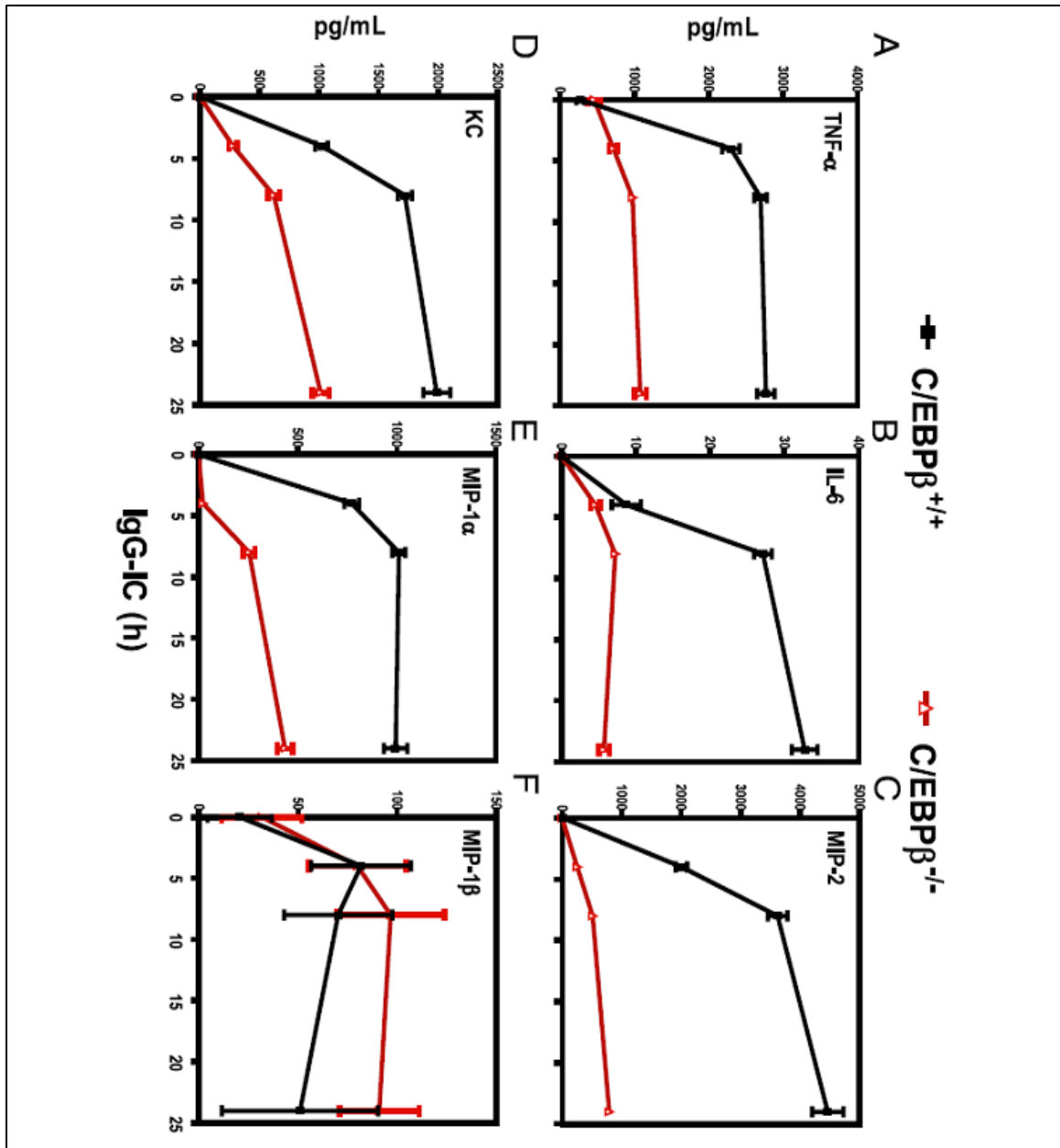


Figure 28. Effects of C/EBPβ Deficiency on IgG immune Complex-induced Inflammatory Reactions in Alveolar Macrophages. Alveolar macrophages obtained from wild-type and C/EBPβ knockout mice were treated with 100 μg/ml IgG immune complexes for the times indicated, and supernatants were subjected to ELISA 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/EBP $\beta$  LAP/C/EBP $\gamma$  heterodimers; heterodimers between C/EBP $\beta$  LAP and LIP; LAP/LAP homodimers, and LIP/LIP homodimers. In IgG immune complex-treated macrophages, the DNA-binding activity of C/EBP $\beta$  (mainly LAP/LIP, and LAP/LAP) was induced (Fig. 29B). To examine the role of C/EBP $\beta$  in IgG IC-induced inflammation in MH-S cells, I ablated C/EBP $\beta$  expression by siRNA-mediated silencing (Fig. 30A). Analysis of inflammatory mediator production showed impaired induction by IgG immune complexes following C/EBP $\beta$  knockdown in MH-S cells (TNF- $\alpha$  decreased by 62%, IL-6 by 77%, and MIP-2 by 48%, respectively; Fig. 30B-C).

*Effects of C/EBP $\beta$  Overexpression on IgG IC-induced TNF- $\alpha$  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  $\times$  C/EBP-Luc, a promoter-reporter that contains two copies of a C/EBP binding site, and an expression vector for C/EBP $\beta$  (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

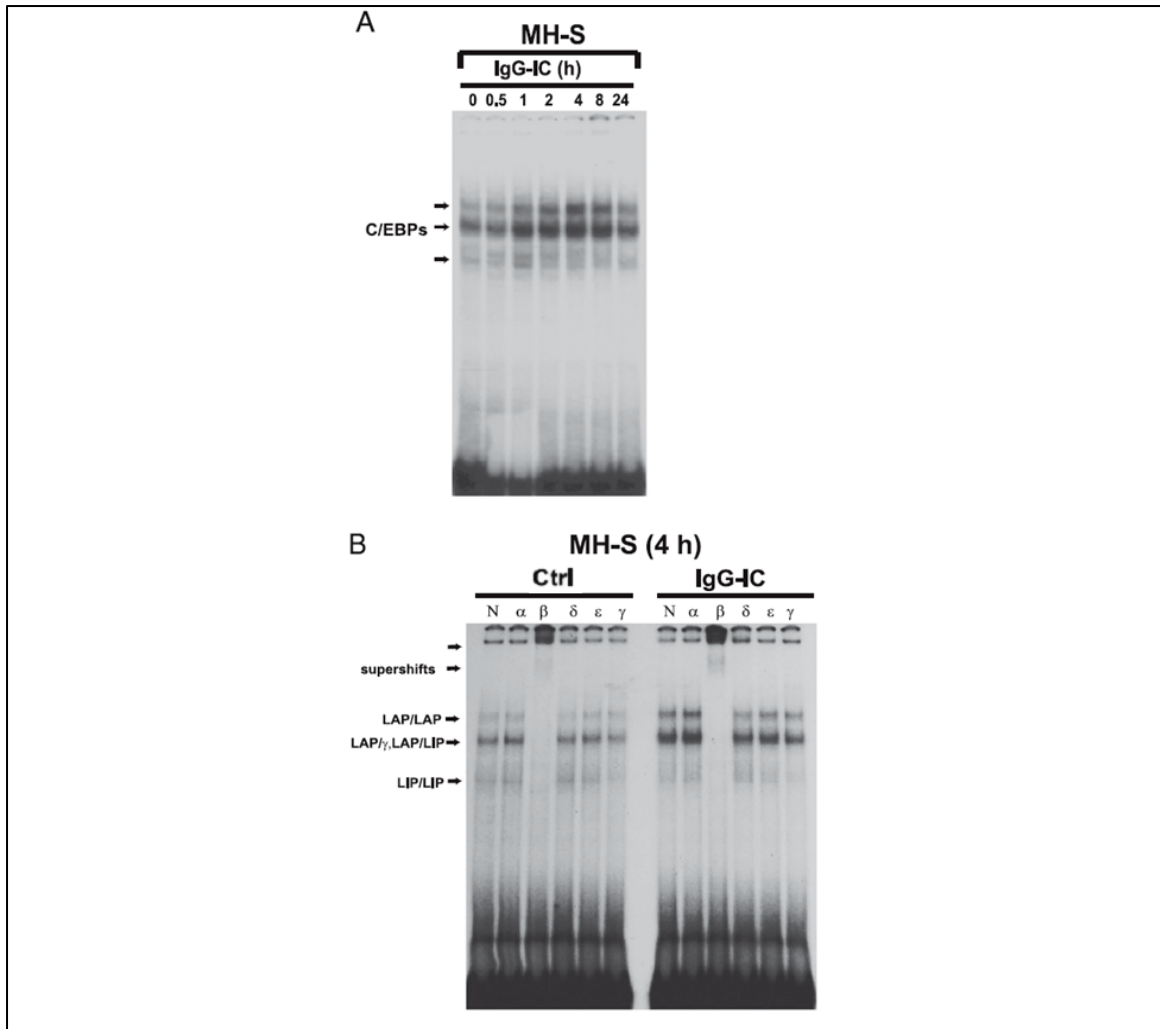


Figure 29. C/EBP $\beta$  and C/EBP $\delta$  DNA Binding Activities are Upregulated in MH-S Cells during IgG IC-induced Inflammatory Responses. A, MH-S cells were challenged by 100  $\mu$ g/ml IgG IC for different time periods as indicated. Nuclear extracts were then extracted, and subjected to EMSA 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/EBP $\alpha$  Ab ( $\alpha$ ), anti-C/EBP $\beta$  Ab ( $\beta$ ), anti-C/EBP $\delta$  Ab ( $\delta$ ), anti-C/EBP $\epsilon$  Ab ( $\epsilon$ ), and anti-C/EBP $\gamma$  Ab ( $\gamma$ ). Arrows indicated supershifts and C/EBP dimers, respectively. The same experiment was repeated three times.



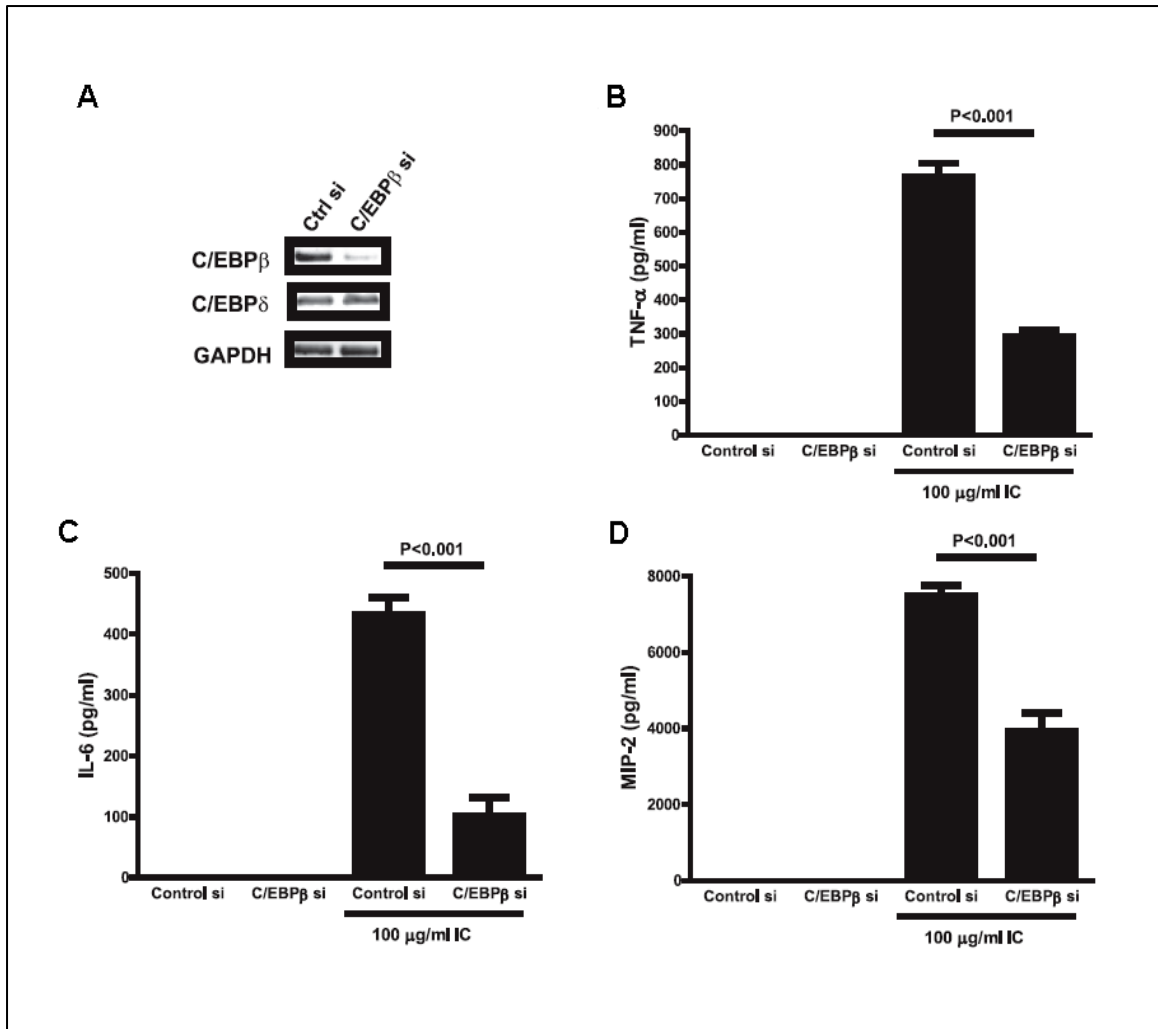


Figure 30. C/EBPβ is Required for IgG IC-induced TNF-α, IL-6 and MIP-2 Expressions in MH-S Cells. A, MH-S cells were transiently transfected with control siRNA or C/EBPβ siRNA. Twelve hours after transfection, RNAs were isolated. Then RT-PCR was performed by using primers for C/EBPβ, C/EBPδ, and GAPDH, 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.

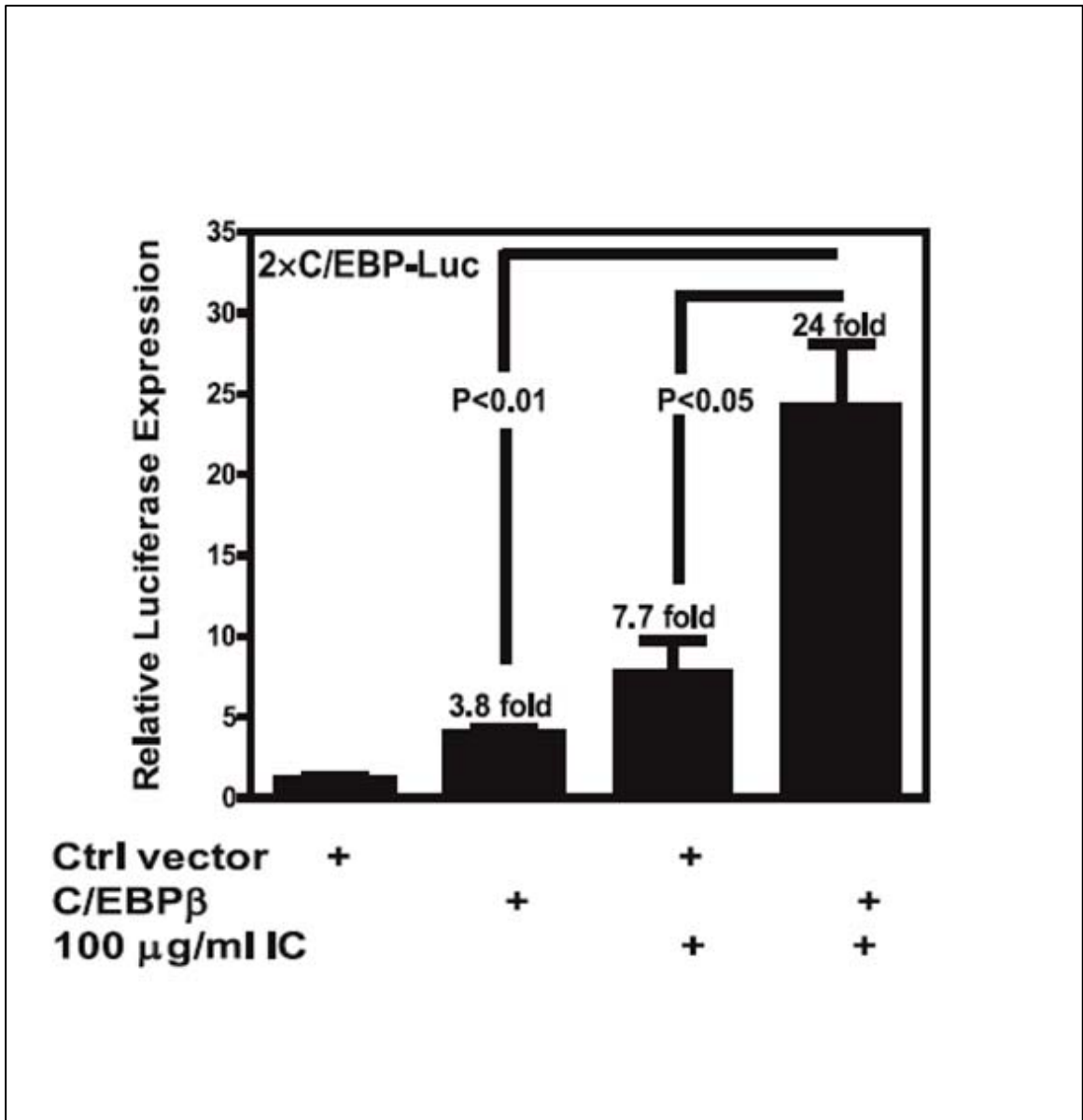


Figure 31. Effects of C/EBP $\beta$  on IgG Immune Complex-induced C/EBP DNA Binding in Alveolar Macrophage. MH-S cells were transiently transfected with total of 0.5  $\mu$ g indicated DNA. Twenty four hours after transfection, the cells were challenged with indicated stimulus for 4 h. Cell lysates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

luciferase activity compared with the untreated control (Fig. 31). C/EBP $\beta$  vector alone also elevated  $2 \times$  C/EBP-Luc transcription (7.7-fold). Importantly, IgG immune complex treatment of C/EBP $\beta$  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/EBP $\beta$  as well as its expression.

I next evaluated the role of C/EBP $\beta$  in IgG immune complex-induced transcription from the TNF- $\alpha$  and IL-6 promoters. As shown in Fig. 32, IgG immune complex stimulation alone markedly increased luciferase activity (2.2-fold for TNF- $\alpha$  and 4.56-fold for IL-6). C/EBP $\beta$  overexpression in the absence of IgG immune complex resulted in 4-fold (TNF- $\alpha$ ) and 1.56-fold (IL-6) induction of luciferase expression. Importantly, IgG immune complex treatment of C/EBP $\beta$  transfectants induced luciferase expression 8.6-fold and 9.65-fold for TNF- $\alpha$  and IL-6, respectively. These data are consistent with the ELISA results obtained from primary macrophages and MH-S cells.

#### *Effects of C/EBP $\delta$ Deficiency on IgG Immune Complex-induced Lung Inflammation*

Because C/EBP $\delta$  DNA-binding activity was induced by IgG immune complex deposition in the lung, I examined whether C/EBP $\delta$  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/EBP $\delta$  deficient mice 4 h after IgG immune complex deposition. Consistently, BAL fluids from lungs of C/EBP $\delta$  deficient mice showed similar levels of TNF- $\alpha$  and MIP-2 when compared with BAL fluids from injured wild-type mice (Fig. 34). I next determined the effects of C/EBP $\delta$  on the inflammatory mediator production from IgG immune complex-stimulated primary alveolar macrophages. As shown in Fig. 35, C/EBP $\delta$  deficiency in alveolar macrophages

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

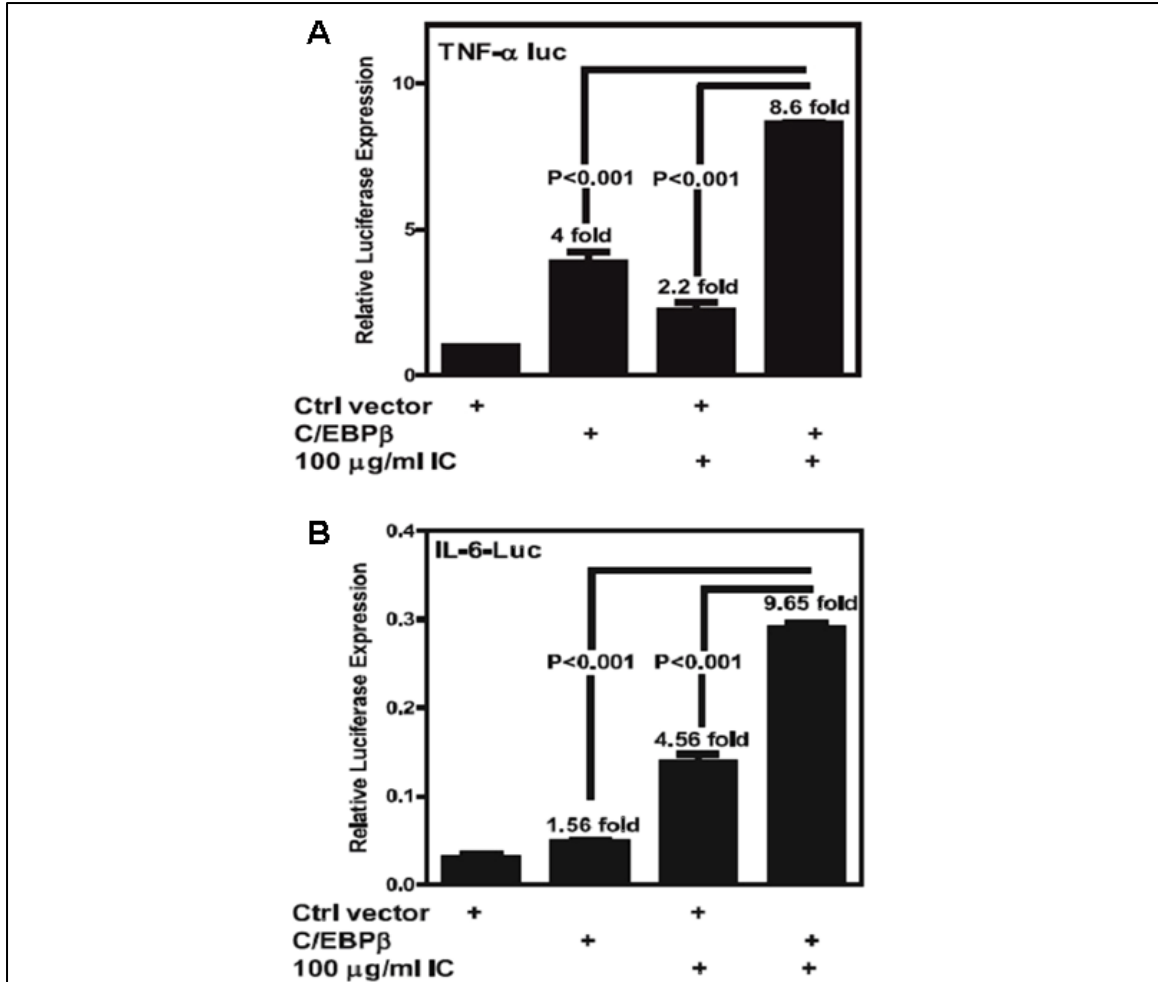


Figure 32. Effects of C/EBP $\beta$  Expression on IgG Immune Complex-induced TNF- $\alpha$  and IL-6 Production in MH-S Cells. MH-S cells were transiently transfected with total of 0.5  $\mu$ g indicated DNA. Twenty four hours after transfection, the cells were challenged with indicated stimulus for 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  $\pm$  S. E. (n=3). The same experiment was repeated three times.

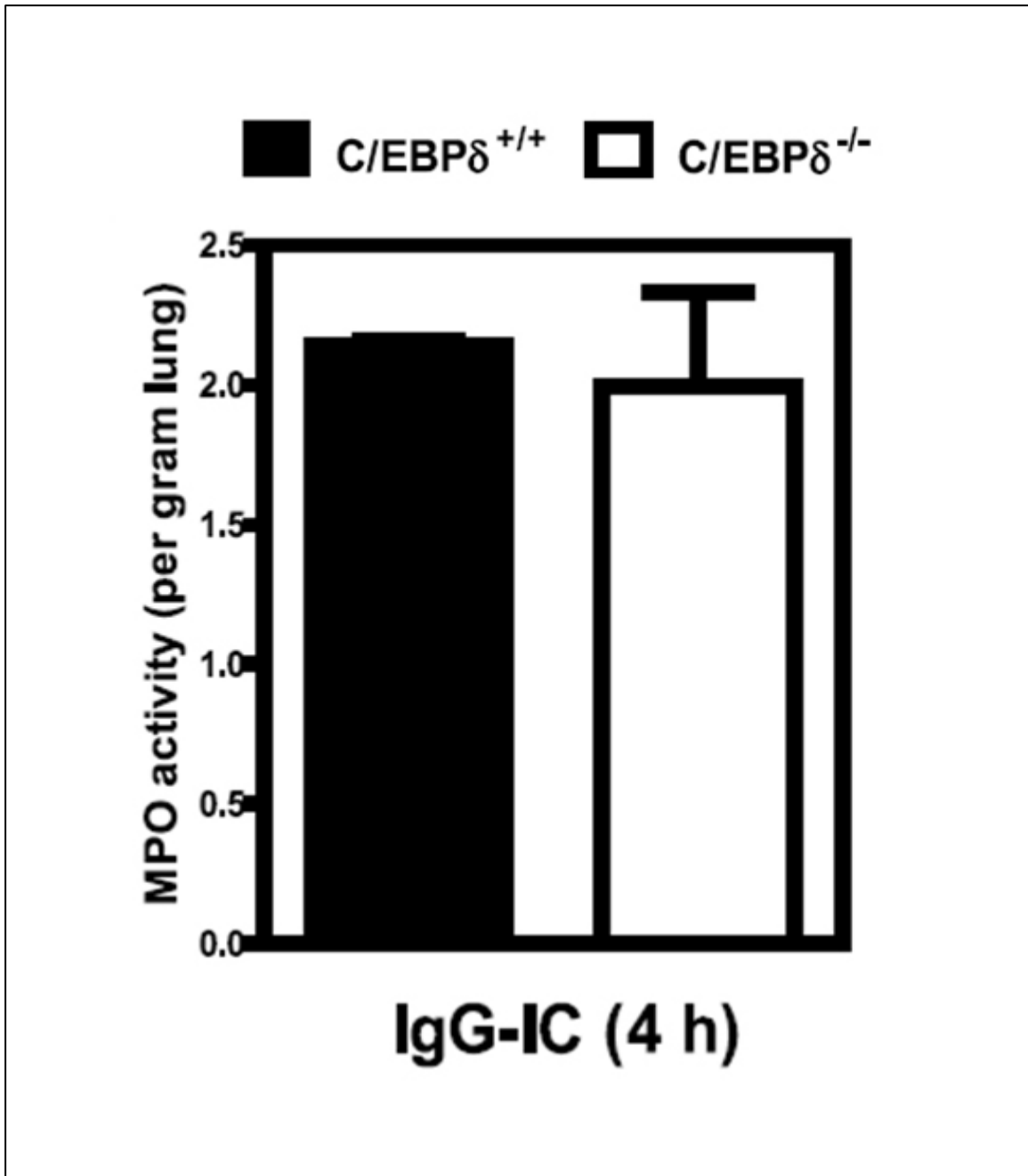


Figure 33. Effects of C/EBP $\delta$  Deficiency on Neutrophil Influx during IgG IC-induced Acute Lung Injury. Four hours after IgG IC deposition, mice lungs were harvested, and changes in lung MPO activity was measured as a marker for pulmonary neutrophil accumulation. Results are means  $\pm$  S.E.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

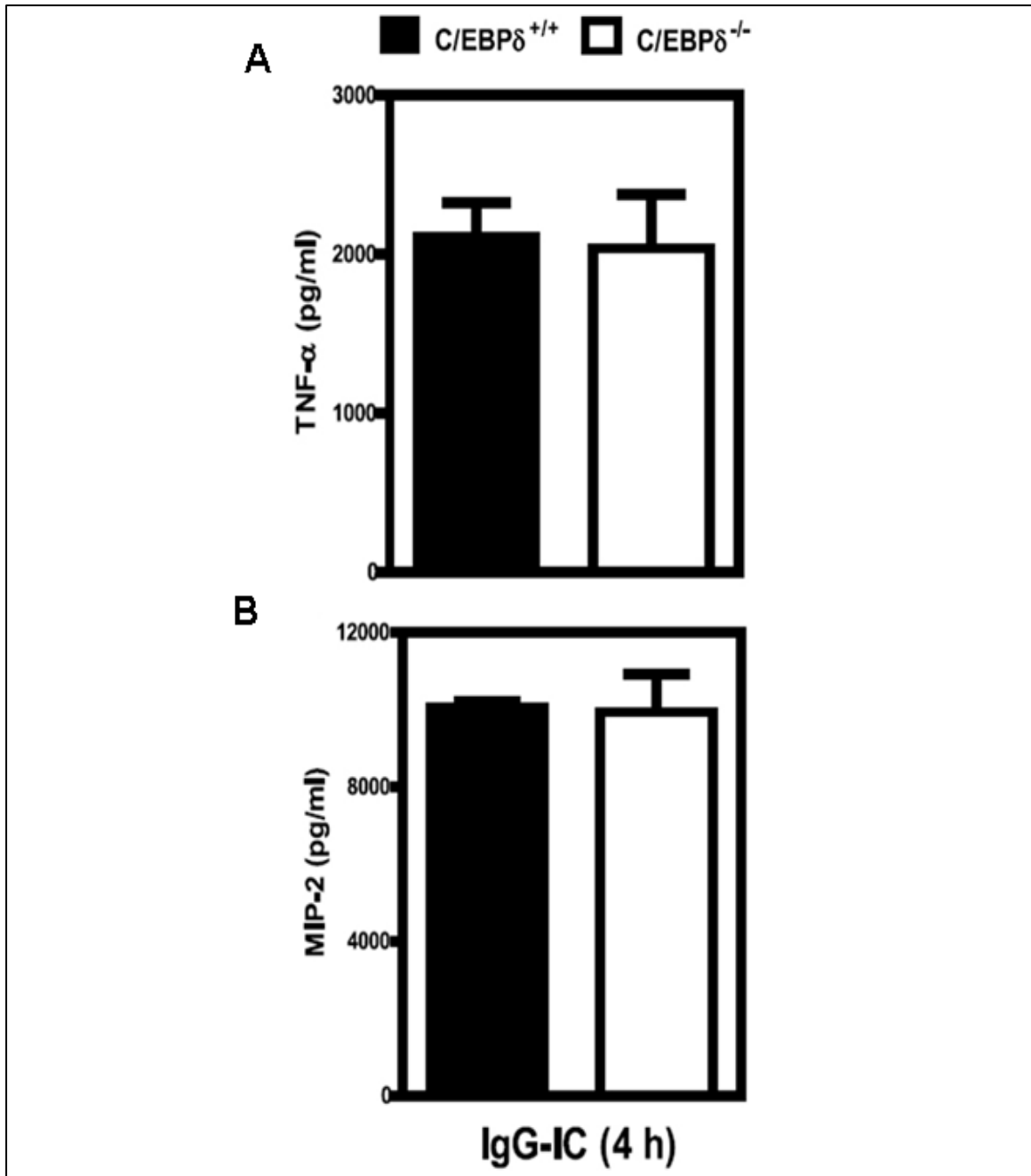


Figure 34. Effects of C/EBP $\delta$  Deficiency on TNF- $\alpha$  and MIP-2 Expressions during IgG Immune Complex-induced Acute Lung Injury. Four hours after IgG immune complex deposition, BAL fluids were harvested, and subjected to ELISA to measure TNF- $\alpha$  expression. Results are means  $\pm$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

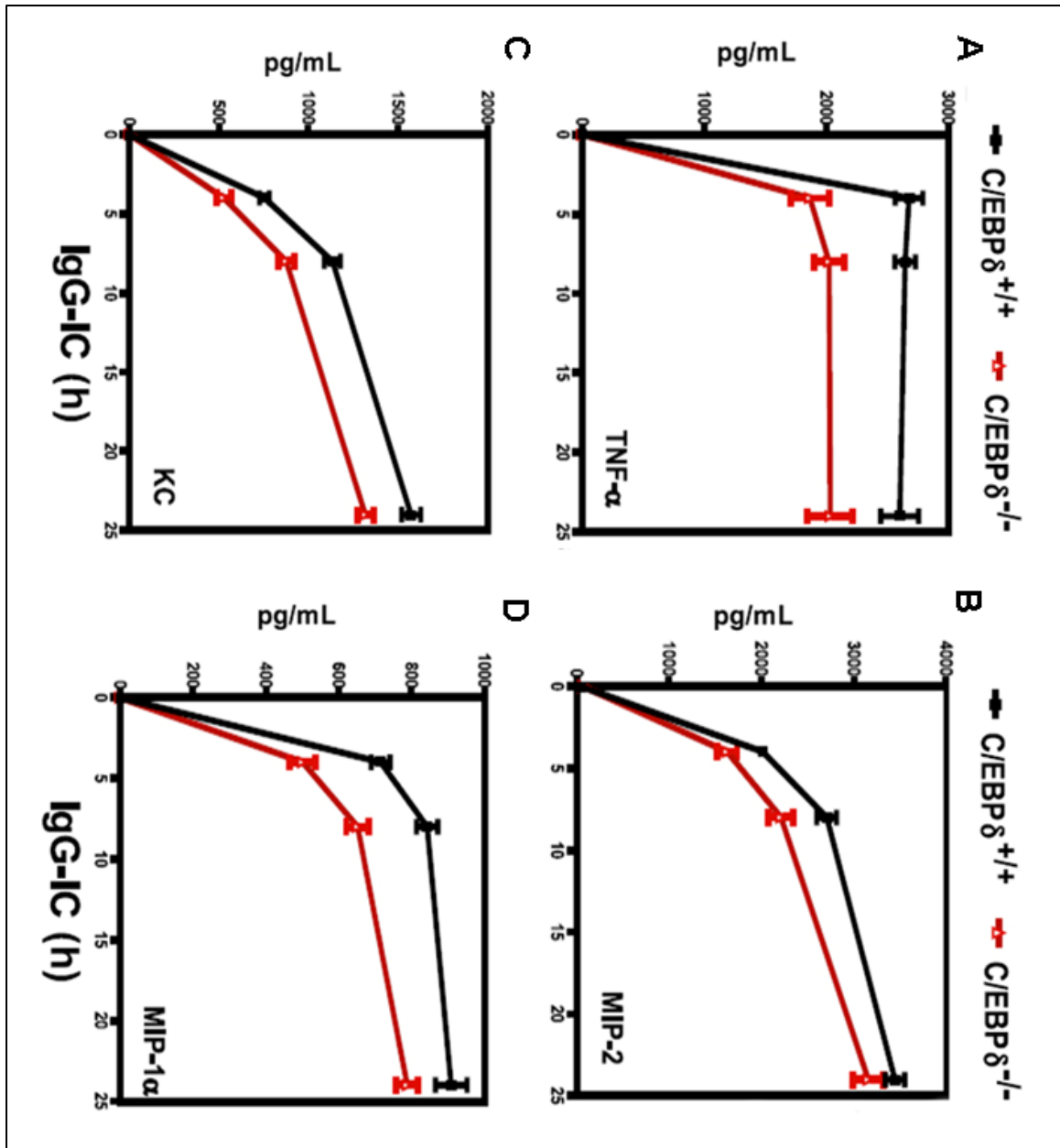


Figure 35. Effects of C/EBP $\delta$  Deficiency on IgG IC-induced Inflammation in Alveolar Macrophages. Alveolar macrophages obtained from wild-type and C/EBP $\beta$  knockout mice were treated with 100  $\mu$ g/ml IgG immune complexes for the times indicated, and supernatants were subjected to ELISA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

## CHAPTER IV

### DISCUSSION

#### Roles of C/EBP $\beta$ and $\delta$ in IgG IC-induced Inflammation in Macrophages

Macrophage Fc $\gamma$ R activation plays a central role in the immune defense system (6, 193). However, the signaling pathways from Fc $\gamma$ Rs to the nucleus remain largely unknown. In my study, I provide the evidence that C/EBP $\beta$  and  $\delta$  are key transcription factors that regulate Fc $\gamma$ R-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/EBP $\beta$  and  $\delta$ , in inflammation (199, 220, 221). For example, C/EBP $\beta$  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/EBP $\delta$  is relatively less well characterized than C/EBP $\beta$ . However, similarly to C/EBP $\beta$ , C/EBP $\delta$  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/EBP $\delta$  in a regulatory circuit that discriminates between transient and persistent TLR4 stimulation (220). In another study, Maitra *et al.* show that C/EBP $\delta$  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- $\alpha$ , MIP-2, and MIP-1 $\alpha$  (194, 223-225). For example, C/EBP $\beta$  has been shown to play an important role in



the regulation of the TNF- $\alpha$  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/EBP $\beta$  and NF- $\kappa$ B 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/EBP $\beta$  and NF- $\kappa$ B are involved in the upregulation of MIP-1 $\alpha$  expression in chondrocytes treated with IL-1 $\beta$  (225). Using monocytic cells, Fernández *et al.* recently show that cross-linking of Fc $\gamma$ R induces C/EBP $\beta$  DNA binding to its binding site in the MIP-1 $\alpha$  promoter, which suggests the possible involvement of C/EBP $\beta$  in IC-induced MIP-1 $\alpha$  expression (194). However, whether and to what extent C/EBP $\beta$  and - $\delta$  contribute to Fc $\gamma$ R-mediated inflammatory mediator production has been unclear. My study shows that C/EBP $\beta$  and - $\delta$  are expressed in untreated macrophages, and IgG IC stimulation upregulates C/EBP $\beta$  and - $\delta$  activities. Using a siRNA-mediated knockdown approach and mice deficient for C/EBP $\beta$  or/and - $\delta$ , my results clearly demonstrate that C/EBP $\beta$  and - $\delta$  play critical roles in the production of TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$  in IgG IC-stimulated macrophages. Interestingly, the functions of C/EBP $\beta$  and - $\delta$  seem to be partially redundant, although lack of either protein has a significant effect on TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$  production. This could also indicate the importance of C/EBP $\beta$  and - $\delta$  heterodimer occupancy in regulating these promoters. This hypothesis is further supported by data showing that co-expression of C/EBP $\beta$ - and C/EBP $\delta$ -expressing vectors stimulates more expression of a C/EBP-driven luciferase reporter than either C/EBP $\beta$  or C/EBP $\delta$  alone (Fig. 2). Further, because a relatively low level of DNA binding for C/EBP $\delta$  contributes a vigorous induction of TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$ , it is tempting to speculate that C/EBP $\delta$

may be more effective than C/EBP $\beta$  in supporting the IgG IC-induced transcription of TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$  genes.

Although the primary mechanism of C/EBP $\beta$  regulation within inflammatory responses appears to be post-transcriptional, C/EBP $\beta$  mRNA levels are also induced by inflammatory stimuli, including LPS (109, 226). On the other hand, unlike C/EBP $\beta$ , the primary mechanism of C/EBP $\delta$  regulation within the inflammatory responses is transcriptional (148, 226). For example, LPS, IL-1, and IL-6 all can induce C/EBP $\delta$  expression at the mRNA level (222). Here I show that IgG IC induces C/EBP $\beta$  and - $\delta$  expressions at both mRNA and protein levels. Further, the DNA binding activities of both C/EBP $\beta$  and - $\delta$  are increased upon Fc $\gamma$ R 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 Fc $\gamma$ R-dependent induction of TNF- $\alpha$  expression in monocytes (227). Furthermore, Fc $\gamma$ R 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-1 $\alpha$  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/EBP $\beta$  and - $\delta$  activation, thus stimulating cytokine and chemokine production in macrophages. Interestingly, neither ERK nor p38 MAPK affects NF- $\kappa$ B 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 Fc $\gamma$ R-dependent activation of NF- $\kappa$ B in monocytes (231-233). Collectively, however, these data do not exclude the requirement for NF- $\kappa$ B activation in Fc $\gamma$ R-mediated inflammation. In the current study, IgG IC-stimulated production of TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$  is inhibited by a NF- $\kappa$ B inhibitor with a significant effect observed on TNF- $\alpha$  and MIP-1 $\alpha$  levels (Fig. 11). These data suggest that NF- $\kappa$ B may play important but differential regulatory roles in Fc $\gamma$ R-mediated inflammatory mediators' production.

Products of macrophages play a major role in events leading to tissue injury. TNF- $\alpha$ , other cytokines, and chemokines, such as MIP-2 and MIP-1 $\alpha$ , 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 Fc $\gamma$ Rs on macrophages and other cells thus ensure the homeostasis of IC-induced inflammatory responses. For example, genetic deletion of FcR  $\gamma$ -chain, which leads to the loss of cell surface expression and functional inactivation of all three activating Fc $\gamma$ Rs, results in dramatically impaired inflammatory responses associated with IC formation (193, 214). In contrast, enhanced macrophage responses are observed in Fc $\gamma$ R II knockout mice (193, 214). Using macrophages from mice lacking FcR  $\gamma$ -chain (FcR $\gamma$ ), we demonstrate that activation of C/EBP $\beta$  and - $\delta$  in macrophages by IgG IC stimulation is mediated, to a large extent, by activating Fc $\gamma$ Rs. Furthermore, we show an enhanced C/EBP DNA binding activity in macrophages from Fc $\gamma$ R II deficient mice.

These data further suggest that activation of C/EBP $\beta$  and  $-\delta$  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- $\kappa$ B 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- $\gamma$ , and LPS have been shown to upregulate activating Fc $\gamma$ R production (193, 214, 234). Recent studies have shown that C5a causes induction of Fc $\gamma$ RIII and suppression of Fc $\gamma$ R II on both alveolar macrophages and RAW264.7 cells (216, 235, 236). Furthermore, genetic ablation of C5aR expression completely abolishes this regulation of Fc $\gamma$ Rs. These studies provide definite evidence that C5a plays a critical role in regulating the Fc $\gamma$ RIII/ II pair to connect complement and Fc $\gamma$ R 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 phospho-44/42 MAPK levels. Interestingly, my finding that the interaction between C5a and C5aR has no effect on IgG IC-induced NF- $\kappa$ B activation suggests that the C5a/C5aR pathway may have a specific role in C/EBP activation.

In summary, for this part of my study, Fc $\gamma$  receptors-mediated activation of C/EBP $\beta$  and  $-\delta$  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/EBP $\beta$  and  $-\delta$  in immune complex-related inflammation. Understanding the underlying roles of various transcription

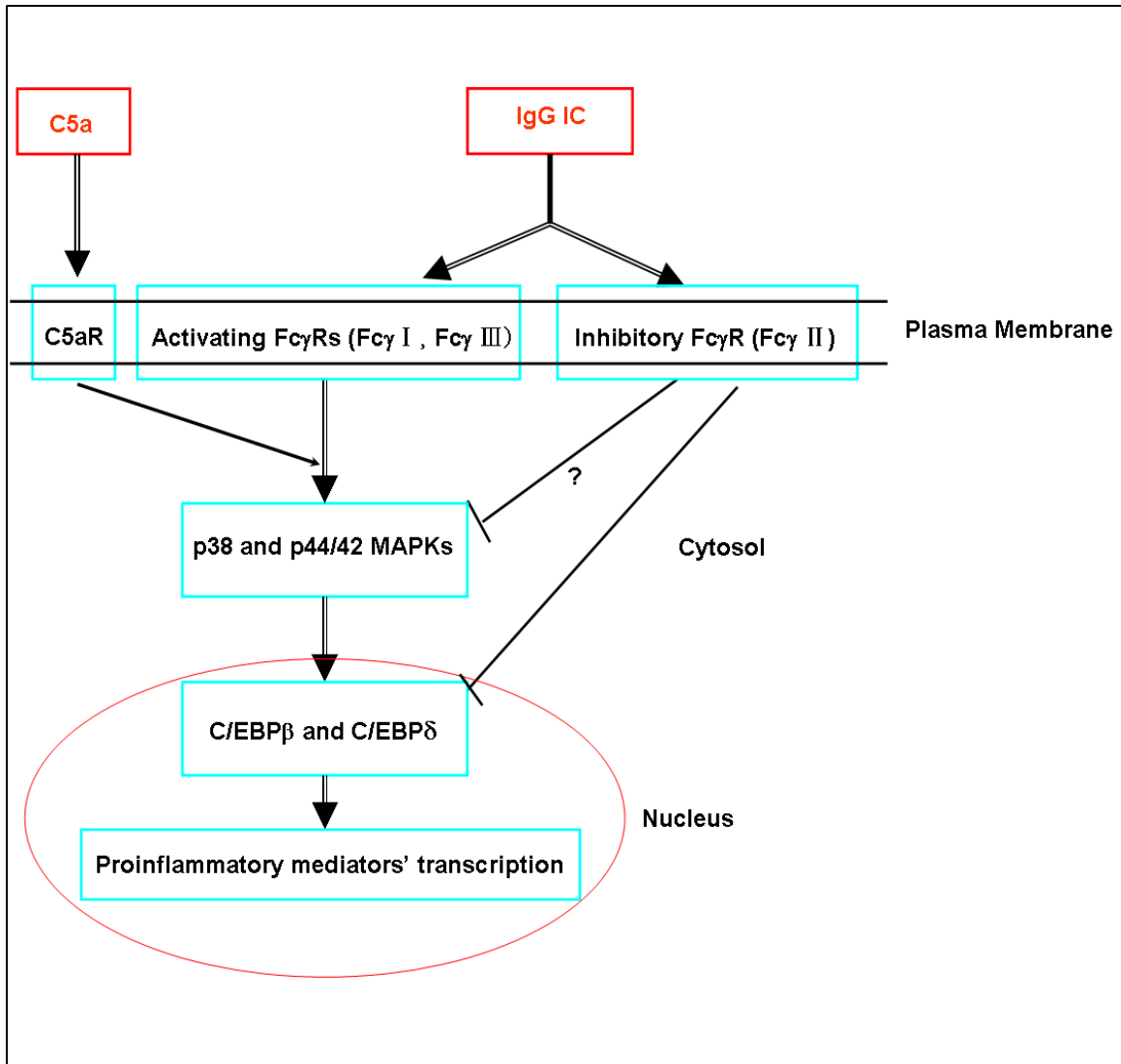


Figure 36. Cell Signaling Transduction Pathways from Membrane Receptors (FcγRs and C5aR) to Nucleus in Peritoneal Macrophages Challenged by IgG IC. Cross-linking of activating FcγRs leads to activation of p38 and p44/42 MAPKs, which can be further enhanced by interaction between C5a and C5aR. Phosphorylation 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/EBP $\beta$ and $\delta$ 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- $\kappa$ B 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- $\alpha$  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/EBP $\beta$  and C/EBP $\delta$ , 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/EBP $\beta$  as a critical mediator of IgG immune complex-induced acute lung injury and inflammatory response in alveolar macrophages.

C/EBP $\beta$  and C/EBP $\delta$  are expressed in many tissues, including lung. However, the expression and function of C/EBP $\beta$  and C/EBP $\delta$  during acute lung inflammatory response are still largely unknown. My current study provides evidence that both C/EBP $\beta$  and C/EBP $\delta$  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/EBP $\beta$  at mRNA level (Fig. 22C). However, the molecular mechanisms by which IgG immune complexes induce C/EBP $\beta$  gene expression in the lung remain unclear. I have found that C/EBP activation in peritoneal macrophages is mediated, to a large extent, by Fc $\gamma$  receptors (Fc $\gamma$ R I and Fc $\gamma$ RIII). Therefore, it would be interesting to investigate in the future study whether Fc $\gamma$ R signaling cascade is involved in the C/EBP activation after IgG immune complex deposition in the lung.

C/EBP $\beta$  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/EBP $\beta$  deficient mice to clearly demonstrate that C/EBP $\beta$  plays a critical role in acute lung inflammation and injury. The current data indicate that several mechanisms are involved in C/EBP $\beta$  regulation of acute immunological pulmonary alveolitis. The results that C/EBP $\beta$  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/EBP $\beta$  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-1 $\alpha$  and MIP-1 $\beta$ ) 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/EBP $\beta$  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/EBP $\beta$  regulation of TNF- $\alpha$  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/EBP $\beta$  deficiency causes a reduced expression of TNF- $\alpha$  and IL-6 as well as ICAM-1 in the lung. Together, the data suggest that upon IgG immune complex deposition, C/EBP $\beta$  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- $\alpha$  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/EBP $\beta$ , siRNA-mediated knockdown in cell lines, and luciferase reporter assays, I show that C/EBP $\beta$  plays a critical role in the production of cytokines and chemokines in IgG immune complex-stimulated macrophages. These data together indicate that C/EBP $\beta$  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/EBP $\beta$  and C/EBP $\delta$ , in supporting the induction of inflammatory cytokines and chemokines. For example, LPS stimulation of peritoneal macrophages from C/EBP $\beta$  deficient mice leads to normal induction of several inflammatory cytokines, including IL-6 and TNF- $\alpha$ , 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/EBP $\alpha$ , C/EBP $\beta$ , and C/EBP $\delta$  are redundant in regard to the expression of IL-6 and MCP-1 (126). In addition, a recent study shows that C/EBP $\delta$  deficient macrophages have no significant defects in IL-6 and TNF- $\alpha$  production in response to several TLR ligands, whereas the absence of both C/EBP $\beta$  and C/EBP $\delta$  results in a significant decrease in the TLR ligand-induced production of IL-6 and TNF- $\alpha$  (206). Interestingly, our *in vitro* study shows that lack of either C/EBP $\beta$  or C/EBP $\delta$  has a significant effect on the production of TNF- $\alpha$ , MIP-2, and MIP-1 $\alpha$  in IgG immune complex-stimulated peritoneal macrophages, indicating the possible importance of C/EBP $\beta$  and C/EBP $\delta$  heterodimer occupancy in regulating activation of these promoters. In addition, I have directly compared the effect of C/EBP $\beta$  and C/EBP $\delta$  deficiency on lung inflammatory responses. I clearly show that C/EBP $\delta$  could not compensate for C/EBP $\beta$  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/EBP $\beta$  and C/EBP $\delta$  in inflammation are cell and tissue specific.

Together, I present evidence that mutant mice defective in C/EBP $\beta$  but not C/EBP $\delta$  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

Fc $\gamma$ R, Fc $\gamma$  receptor

IgG IC, immunoglobulin G immune complex

siRNA, small interfering RNA

TNF- $\alpha$ , tumor necrosis factor- $\alpha$

MIP-2, macrophage inflammatory protein-2

MIP-1 $\alpha$ , macrophage inflammatory protein-1 $\alpha$

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, factor VII-activating protease

LPS, lipopolysaccharide

I $\kappa$ B $\alpha$ , nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

NF- $\kappa$ B, 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- $\gamma$ , interferon-gamma

MCP-1, monocyte chemotactic protein-1

BAL, bronchoalveolar lavage

MPO, myeloperoxidase

CINC, cytokine-induced neutrophil chemoattractant

MIP-1 $\beta$ , 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

IKK $\beta$ , 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<sub>2</sub>MDP, dichloromethylene diphosphonate

MEK, mitogen-activated protein kinase kinase

## REFERENCES

1. Rubenfeld, G. D., Caldwell, E., Peabody, E., Weaver, J., Martin, D. P., Neff, M., Stern, E. J., and Hudson, L. D. (2005) Incidence and outcomes of acute lung injury. *N Engl J Med* 353, 1685-1693.
2. Robles-Carrillo, L., Meyer, T., Hatfield, M., Desai, H., Davila, M., Langer, F., Amaya, M., Garber, E., Francis, J. L., Hsu, Y. M., and Amirkhosravi, A. Anti-CD40L immune complexes potently activate platelets in vitro and cause thrombosis in FCGR2A transgenic mice. *J Immunol* 185, 1577-1583.
3. Smith, K. G., and Clatworthy, M. R. FcγRIIB in autoimmunity and infection: evolutionary and therapeutic implications. *Nat Rev Immunol* 10, 328-343.
4. Schuyler, M., Gott, K., and French, V. (2004) The role of MIP-1α in experimental hypersensitivity pneumonitis. *Lung* 182, 135-149.
5. Schubert, D., Schmidt, M., Zaiss, D., Jungblut, P. R., and Kamradt, T. (2002) Autoantibodies to GPI and creatine kinase in RA. *Nat Immunol* 3, 411; author reply 412-413.
6. Nimmerjahn, F., and Ravetch, J. V. (2008) Fcγ receptors as regulators of immune responses. *Nat Rev Immunol* 8, 34-47.
7. Nimmerjahn, F., Bruhns, P., Horiuchi, K., and Ravetch, J. V. (2005) FcγRIIV: a novel FcR with distinct IgG subclass specificity. *Immunity* 23, 41-51.
8. Ravetch, J. V., and Bolland, S. (2001) IgG Fc receptors. *Annu Rev Immunol* 19, 275-290.
9. Gerber, J. S., and Mosser, D. M. (2001) Stimulatory and inhibitory signals originating from the macrophage Fcγ receptors. *Microbes Infect* 3, 131-139.
10. Gao, H., Neff, T., and Ward, P. A. (2006) Regulation of lung inflammation in the model of IgG immune-complex injury. *Annu Rev Pathol* 1, 215-242.
11. Xia, Y. C., Schuliga, M., Shepherd, M., Powell, M., Harris, T., Langenbach, S. Y., Tan, P. S., Gerthoffer, W. T., Hogarth, P. M., Stewart, A. G., and Mackay, G. A. Functional expression of IgG-Fc receptors in human airway smooth muscle cells. *Am J Respir Cell Mol Biol* 44, 665-672.
12. Clynes, R., Maizes, J. S., Guinamard, R., Ono, M., Takai, T., and Ravetch, J. V. (1999) Modulation of immune complex-induced inflammation in vivo by the coordinate expression of activation and inhibitory Fc receptors. *J Exp Med* 189, 179-185.



13. Ioan-Facsinay, A., de Kimpe, S. J., Hellwig, S. M., van Lent, P. L., Hofhuis, F. M., van Ojik, H. H., Sedlik, C., da Silveira, S. A., Gerber, J., de Jong, Y. F., Roozendaal, R., Aarden, L. A., van den Berg, W. B., Saito, T., Mosser, D., Amigorena, S., Izui, S., van Ommen, G. J., van Vugt, M., van de Winkel, J. G., and Verbeek, J. S. (2002) FcγRI (CD64) contributes substantially to severity of arthritis, hypersensitivity responses, and protection from bacterial infection. *Immunity* 16, 391-402.
14. Xie, T., J. Liang, N. Liu, Q. Wang, Y. Li, P. W. Noble, and D. Jiang. MicroRNA-127 inhibits lung inflammation by targeting IgG Fcγ receptor I. *J Immunol* 188:2437-2444.
15. Chouchakova, N., J. Skokowa, U. Baumann, T. Tschernig, K. M. Philippens, B. Nieswandt, R. E. Schmidt, and J. E. Gessner. 2001. Fc γ RIII-mediated production of TNF-α induces immune complex alveolitis independently of CXC chemokine generation. *J Immunol* 166:5193-5200.
16. Syed, S. N., S. Konrad, K. Wiege, B. Nieswandt, F. Nimmerjahn, R. E. Schmidt, and J. E. Gessner. 2009. Both FcγRIV and FcγRIII are essential receptors mediating type II and type III autoimmune responses via FcRγ-LAT-dependent generation of C5a. *Eur J Immunol* 39:3343-3356.
17. Salik, E., M. Tyorkin, S. Mohan, I. George, K. Becker, E. Oei, T. Kalb, and K. Sperber. 1999. Antigen trafficking and accessory cell function in respiratory epithelial cells. *Am J Respir Cell Mol Biol* 21:365-379.
18. Huber-Lang, M., J. V. Sarma, F. S. Zetoune, D. Rittirsch, T. A. Neff, S. R. McGuire, J. D. Lambris, R. L. Warner, M. A. Flierl, L. M. Hoesel, F. Gebhard, J. G. Younger, S. M. Drouin, R. A. Wetsel, and P. A. Ward. 2006. Generation of C5a in the absence of C3: a new complement activation pathway. *Nat Med* 12:682-687.
19. Sun, L., R. F. Guo, H. Gao, J. V. Sarma, F. S. Zetoune, and P. A. Ward. 2009. Attenuation of IgG immune complex-induced acute lung injury by silencing C5aR in lung epithelial cells. *Faseb J* 23:3808-3818.
20. Huber-Lang, M., V. J. Sarma, K. T. Lu, S. R. McGuire, V. A. Padgaonkar, R. F. Guo, E. M. Younkin, R. G. Kunkel, J. Ding, R. Erickson, J. T. Curnutte, and P. A. Ward. 2001. Role of C5a in multiorgan failure during sepsis. *J Immunol* 166:1193-1199.
21. Huber-Lang, M. S., J. V. Sarma, S. R. McGuire, K. T. Lu, R. F. Guo, V. A. Padgaonkar, E. M. Younkin, I. J. Laudes, N. C. Riedemann, J. G. Younger, and P. A. Ward. 2001. Protective effects of anti-C5a peptide antibodies in experimental sepsis. *Faseb J* 15:568-570.
22. Laudes, I. J., J. C. Chu, S. Sikranth, M. Huber-Lang, R. F. Guo, N. Riedemann, J. V. Sarma, A. H. Schmaier, and P. A. Ward. 2002. Anti-c5a ameliorates coagulation/fibrinolytic protein changes in a rat model of sepsis. *Am J Pathol* 160:1867-1875.
23. Huber-Lang, M. S., N. C. Riedeman, J. V. Sarma, E. M. Younkin, S. R. McGuire, I. J. Laudes, K. T. Lu, R. F. Guo, T. A. Neff, V. A. Padgaonkar, J. D. Lambris, L. Spruce, D. Mastellos, F. S. Zetoune, and P. A. Ward. 2002. Protection of innate immunity by C5aR antagonist in septic mice. *Faseb J* 16:1567-1574.

24. Guo, R. F., N. C. Riedemann, and P. A. Ward. 2004. Role of C5a-C5aR interaction in sepsis. *Shock* 21:1-7.
25. Rittirsch, D., M. A. Flierl, B. A. Nadeau, D. E. Day, M. Huber-Lang, C. R. Mackay, F. S. Zetoune, N. P. Gerard, K. Cianflone, J. Kohl, C. Gerard, J. V. Sarma, and P. A. Ward. 2008. Functional roles for C5a receptors in sepsis. *Nat Med* 14:551-557.
26. Guo, R. F., and P. A. Ward. 2005. Role of C5a in inflammatory responses. *Annu Rev Immunol* 23:821-852.
27. Huber-Lang, M. S., E. M. Younkin, J. V. Sarma, S. R. McGuire, K. T. Lu, R. F. Guo, V. A. Padgaonkar, J. T. Curnutte, R. Erickson, and P. A. Ward. 2002. Complement-induced impairment of innate immunity during sepsis. In *J Immunol*. 3223-3231.
28. Lajoie, S., I. P. Lewkowich, Y. Suzuki, J. R. Clark, A. A. Sproles, K. Dienger, A. L. Budelsky, and M. Wills-Karp. Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. *Nat Immunol* 11:928-935.
29. Kanse, S. M., A. Gallenmueller, S. Zeerleder, F. Stephan, O. Rannou, S. Denk, M. Etscheid, G. Lochnit, M. Krueger, and M. Huber-Lang. Factor VII-Activating Protease Is Activated in Multiple Trauma Patients and Generates Anaphylatoxin C5a. *J Immunol* 188:2858-2865.
30. Shushakova, N., J. Skokowa, J. Schulman, U. Baumann, J. Zwirner, R. E. Schmidt, and J. E. Gessner. 2002. C5a anaphylatoxin is a major regulator of activating versus inhibitory FcγR3s in immune complex-induced lung disease. *J Clin Invest* 110:1823-1830.
31. Riedemann, N. C., R. F. Guo, K. D. Bernacki, J. S. Reuben, I. J. Laudes, T. A. Neff, H. Gao, C. Speyer, V. J. Sarma, F. S. Zetoune, and P. A. Ward. 2003. Regulation by C5a of neutrophil activation during sepsis. *Immunity* 19:193-202.
32. Wojta, J., C. Kaun, G. Zorn, M. Ghannadan, A. W. Hauswirth, W. R. Sperr, G. Fritsch, D. Printz, B. R. Binder, G. Schatzl, J. Zwirner, G. Maurer, K. Huber, and P. Valent. 2002. C5a stimulates production of plasminogen activator inhibitor-1 in human mast cells and basophils. *Blood* 100:517-523.
33. Jagels, M. A., P. J. Daffern, and T. E. Hugli. 2000. C3a and C5a enhance granulocyte adhesion to endothelial and epithelial cell monolayers: epithelial and endothelial priming is required for C3a-induced eosinophil adhesion. *Immunopharmacology* 46:209-222.
34. Foreman, K. E., A. A. Vaporciyan, B. K. Bonish, M. L. Jones, K. J. Johnson, M. M. Glovsky, S. M. Eddy, and P. A. Ward. 1994. C5a-induced expression of P-selectin in endothelial cells. *J Clin Invest* 94:1147-1155.
35. Mulligan, M. S., E. Schmid, G. O. Till, T. E. Hugli, H. P. Friedl, R. A. Roth, and P. A. Ward. 1997. C5a-dependent up-regulation in vivo of lung vascular P-selectin. *J Immunol* 158:1857-1861.
36. Albrecht, E. A., A. M. Chinnaiyan, S. Varambally, C. Kumar-Sinha, T. R. Barrette, J. V. Sarma, and P. A. Ward. 2004. C5a-induced gene expression in human umbilical vein endothelial cells. *Am J Pathol* 164:849-859.

37. Laudes, I. J., J. C. Chu, M. Huber-Lang, R. F. Guo, N. C. Riedemann, J. V. Sarma, F. Mahdi, H. S. Murphy, C. Speyer, K. T. Lu, J. D. Lambris, F. S. Zetoune, and P. A. Ward. 2002. Expression and function of C5a receptor in mouse microvascular endothelial cells. *J Immunol* 169:5962-5970.
38. Johnson, K. J., and P. A. Ward. 1974. Acute immunologic pulmonary alveolitis. *J Clin Invest* 54:349-357.
39. Larsen, G. L., B. C. Mitchell, and P. M. Henson. 1981. The pulmonary response of C5 sufficient and deficient mice to immune complexes. *Am Rev Respir Dis* 123:434-439.
40. Mulligan, M. S., E. Schmid, B. Beck-Schimmer, G. O. Till, H. P. Friedl, R. B. Brauer, T. E. Hugli, M. Miyasaka, R. L. Warner, K. J. Johnson, and P. A. Ward. 1996. Requirement and role of C5a in acute lung inflammatory injury in rats. *J Clin Invest* 98:503-512.
41. Huber-Lang, M., E. M. Younkin, J. V. Sarma, N. Riedemann, S. R. McGuire, K. T. Lu, R. Kunkel, J. G. Younger, F. S. Zetoune, and P. A. Ward. 2002. Generation of C5a by phagocytic cells. *Am J Pathol* 161:1849-1859.
42. Czermak, B. J., V. Sarma, N. M. Bless, H. Schmal, H. P. Friedl, and P. A. Ward. 1999. In vitro and in vivo dependency of chemokine generation on C5a and TNF-alpha. *J Immunol* 162:2321-2325.
43. Lukacs, N. W., M. M. Glovsky, and P. A. Ward. 2001. Complement-dependent immune complex-induced bronchial inflammation and hyperreactivity. *Am J Physiol Lung Cell Mol Physiol* 280:L512-518.
44. Rittirsch, D., M. A. Flierl, D. E. Day, B. A. Nadeau, S. R. McGuire, L. M. Hoesel, K. Ipaktchi, F. S. Zetoune, J. V. Sarma, L. Leng, M. S. Huber-Lang, T. A. Neff, R. Bucala, and P. A. Ward. 2008. Acute lung injury induced by lipopolysaccharide is independent of complement activation. *J Immunol* 180:7664-7672.
45. Fayyazi, A., R. Sandau, L. Q. Duong, O. Gotze, H. J. Radzun, S. Schweyer, A. Soruri, and J. Zwirner. 1999. C5a receptor and interleukin-6 are expressed in tissue macrophages and stimulated keratinocytes but not in pulmonary and intestinal epithelial cells. *Am J Pathol* 154:495-501.
46. Riedemann, N. C., R. F. Guo, V. J. Sarma, I. J. Laudes, M. Huber-Lang, R. L. Warner, E. A. Albrecht, C. L. Speyer, and P. A. Ward. 2002. Expression and function of the C5a receptor in rat alveolar epithelial cells. *J Immunol* 168:1919-1925.
47. Monsinjon, T., P. Gasque, P. Chan, A. Ischenko, J. J. Brady, and M. C. Fontaine. 2003. Regulation by complement C3a and C5a anaphylatoxins of cytokine production in human umbilical vein endothelial cells. *Faseb J* 17:1003-1014.
48. Bozic, C. R., B. Lu, U. E. Hopken, C. Gerard, and N. P. Gerard. 1996. Neurogenic amplification of immune complex inflammation. *Science* 273:1722-1725.
49. Heller, T., M. Hennecke, U. Baumann, J. E. Gessner, A. M. zu Vilsendorf, M. Baensch, F. Boulay, A. Kola, A. Klos, W. Bautsch, and J. Kohl. 1999. Selection of a C5a receptor antagonist from phage libraries attenuating the inflammatory response in immune complex disease and ischemia/reperfusion injury. *J Immunol* 163:985-994.

50. Ravetch, J. V., and R. A. Clynes. 1998. Divergent roles for Fc receptors and complement in vivo. *Annu Rev Immunol* 16:421-432.
51. Trcka, J., Y. Moroi, R. A. Clynes, S. M. Goldberg, A. Bergtold, M. A. Perales, M. Ma, C. R. Ferrone, M. C. Carroll, J. V. Ravetch, and A. N. Houghton. 2002. Redundant and alternative roles for activating Fc receptors and complement in an antibody-dependent model of autoimmune vitiligo. *Immunity* 16:861-868.
52. Ohno, M., T. Hirata, M. Enomoto, T. Araki, H. Ishimaru, and T. A. Takahashi. 2000. A putative chemoattractant receptor, C5L2, is expressed in granulocyte and immature dendritic cells, but not in mature dendritic cells. *Mol Immunol* 37:407-412.
53. Gerard, N. P., B. Lu, P. Liu, S. Craig, Y. Fujiwara, S. Okinaga, and C. Gerard. 2005. An anti-inflammatory function for the complement anaphylatoxin C5a-binding protein, C5L2. *J Biol Chem* 280:39677-39680.
54. Kalant, D., R. MacLaren, W. Cui, R. Samanta, P. N. Monk, S. A. Laporte, and K. Cianflone. 2005. C5L2 is a functional receptor for acylation-stimulating protein. *J Biol Chem* 280:23936-23944.
55. Chen, N. J., C. Mirtsos, D. Suh, Y. C. Lu, W. J. Lin, C. McKerlie, T. Lee, H. Baribault, H. Tian, and W. C. Yeh. 2007. C5L2 is critical for the biological activities of the anaphylatoxins C5a and C3a. *Nature* 446:203-207.
56. Johswich, K., M. Martin, J. Thalmann, C. Rheinheimer, P. N. Monk, and A. Klos. 2006. Ligand specificity of the anaphylatoxin C5L2 receptor and its regulation on myeloid and epithelial cell lines. *J Biol Chem* 281:39088-39095.
57. Scola, A. M., K. O. Johswich, B. P. Morgan, A. Klos, and P. N. Monk. 2009. The human complement fragment receptor, C5L2, is a recycling decoy receptor. *Mol Immunol* 46:1149-1162.
58. Okinaga, S., D. Slattery, A. Humbles, Z. Zsengeller, O. Morteau, M. B. Kinrade, R. M. Brodbeck, J. E. Krause, H. R. Choe, N. P. Gerard, and C. Gerard. 2003. C5L2, a nonsignaling C5A binding protein. *Biochemistry* 42:9406-9415.
59. Gao, H., T. A. Neff, R. F. Guo, C. L. Speyer, J. V. Sarma, S. Tomlins, Y. Man, N. C. Riedemann, L. M. Hoesel, E. Younkin, F. S. Zetoune, and P. A. Ward. 2005. Evidence for a functional role of the second C5a receptor C5L2. *Faseb J* 19:1003-1005.
60. Zhang, X., I. Schmutde, Y. Laumonier, M. K. Pandey, J. R. Clark, P. Konig, N. P. Gerard, C. Gerard, M. Wills-Karp, and J. Kohl. A critical role for C5L2 in the pathogenesis of experimental allergic asthma. *J Immunol* 185:6741-6752.
61. Lentsch, A. B., B. J. Czermak, N. M. Bless, and P. A. Ward. 1998. NF-kappaB activation during IgG immune complex-induced lung injury: requirements for TNF-alpha and IL-1beta but not complement. *Am J Pathol* 152:1327-1336.
62. Gao, H., R. F. Guo, C. L. Speyer, J. Reuben, T. A. Neff, L. M. Hoesel, N. C. Riedemann, S. D. McClintock, J. V. Sarma, N. Van Rooijen, F. S. Zetoune, and P. A. Ward. 2004. Stat3 activation in acute lung injury. *J Immunol* 172:7703-7712.
63. Guo, R. F., A. B. Lentsch, J. V. Sarma, L. Sun, N. C. Riedemann, S. D. McClintock, S. R. McGuire, N. Van Rooijen, and P. A. Ward. 2002. Activator protein-1 activation in acute lung injury. *Am J Pathol* 161:275-282.

64. Hayden, M. S., and S. Ghosh. 2004. Signaling to NF-kappaB. *Genes Dev* 18:2195-2224.
65. Karin, M., Y. Yamamoto, and Q. M. Wang. 2004. The IKK NF-kappa B system: a treasure trove for drug development. *Nat Rev Drug Discov* 3:17-26.
66. Manning, A. M., F. P. Bell, C. L. Rosenbloom, J. G. Chosay, C. A. Simmons, J. L. Northrup, R. J. Shebuski, C. J. Dunn, and D. C. Anderson. 1995. NF-kappa B is activated during acute inflammation in vivo in association with elevated endothelial cell adhesion molecule gene expression and leukocyte recruitment. *J Inflamm* 45:283-296.
67. Lentsch, A. B., B. J. Czermak, N. M. Bless, N. Van Rooijen, and P. A. Ward. 1999. Essential role of alveolar macrophages in intrapulmonary activation of NF-kappaB. *Am J Respir Cell Mol Biol* 20:692-698.
68. Lentsch, A. B., T. P. Shanley, V. Sarma, and P. A. Ward. 1997. In vivo suppression of NF-kappa B and preservation of I kappa B alpha by interleukin-10 and interleukin-13. *J Clin Invest* 100:2443-2448.
69. Forman, H. J., and M. Torres. 2002. Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. *Am J Respir Crit Care Med* 166:S4-8.
70. Iles, K. E., and H. J. Forman. 2002. Macrophage signaling and respiratory burst. *Immunol Res* 26:95-105.
71. Koay, M. A., J. W. Christman, B. H. Segal, A. Venkatakrisnan, T. R. Blackwell, S. M. Holland, and T. S. Blackwell. 2001. Impaired pulmonary NF-kappaB activation in response to lipopolysaccharide in NADPH oxidase-deficient mice. *Infect Immun* 69:5991-5996.
72. Hayakawa, M., H. Miyashita, I. Sakamoto, M. Kitagawa, H. Tanaka, H. Yasuda, M. Karin, and K. Kikugawa. 2003. Evidence that reactive oxygen species do not mediate NF-kappaB activation. *Embo J* 22:3356-3366.
73. Liu, W., X. Ouyang, J. Yang, J. Liu, Q. Li, Y. Gu, M. Fukata, T. Lin, J. C. He, M. Abreu, J. C. Unkeless, L. Mayer, and H. Xiong. 2009. AP-1 activated by toll-like receptors regulates expression of IL-23 p19. *J Biol Chem* 284:24006-24016.
74. Wisdom, R. 1999. AP-1: one switch for many signals. *Exp Cell Res* 253:180-185.
75. Karin, M., Z. Liu, and E. Zandi. 1997. AP-1 function and regulation. *Curr Opin Cell Biol* 9:240-246.
76. Moriguchi, T., F. Toyoshima, N. Masuyama, H. Hanafusa, Y. Gotoh, and E. Nishida. 1997. A novel SAPK/JNK kinase, MKK7, stimulated by TNFalpha and cellular stresses. *Embo J* 16:7045-7053.
77. Rahman, I., and W. MacNee. 1998. Role of transcription factors in inflammatory lung diseases. *Thorax* 53:601-612.
78. Roebuck, K. A., L. R. Carpenter, V. Lakshminarayanan, S. M. Page, J. N. Moy, and L. L. Thomas. 1999. Stimulus-specific regulation of chemokine expression involves differential activation of the redox-responsive transcription factors AP-1 and NF-kappaB. *J Leukoc Biol* 65:291-298.
79. Zagariya, A., S. Mungre, R. Lovis, M. Birrer, S. Ness, B. Thimmapaya, and R. Pope. 1998. Tumor necrosis factor alpha gene regulation: enhancement of C/EBPbeta-induced activation by c-Jun. *Mol Cell Biol* 18:2815-2824.

80. Rocksén, D., B. Ekstrand-Hammarström, L. Johansson, and A. Bücht. 2003. Vitamin E reduces transendothelial migration of neutrophils and prevents lung injury in endotoxin-induced airway inflammation. *Am J Respir Cell Mol Biol* 28:199-207.
81. Bozinovski, S., J. E. Jones, R. Vlahos, J. A. Hamilton, and G. P. Anderson. 2002. Granulocyte/macrophage-colony-stimulating factor (GM-CSF) regulates lung innate immunity to lipopolysaccharide through Akt/Erk activation of NF- $\kappa$ B and AP-1 in vivo. *J Biol Chem* 277:42808-42814.
82. Wodrich, W., and M. Volm. 1993. Overexpression of oncoproteins in non-small cell lung carcinomas of smokers. *Carcinogenesis* 14:1121-1124.
83. Haase, M., R. Koslowski, A. Lengnick, R. Hahn, K. W. Wenzel, D. Schuh, M. Kasper, and M. Müller. 1997. Cellular distribution of c-Jun and c-Fos in rat lung before and after bleomycin induced injury. *Virchows Arch* 431:441-448.
84. Demoly, P., P. Chanez, J. L. Pujol, C. Gauthier-Rouvière, F. B. Michel, P. Godard, and J. Bousquet. 1995. Fos immunoreactivity assessment on human normal and pathological bronchial biopsies. *Respir Med* 89:329-335.
85. Stein, B., A. S. Baldwin, Jr., D. W. Ballard, W. C. Greene, P. Angel, and P. Herrlich. 1993. Cross-coupling of the NF- $\kappa$ B p65 and Fos/Jun transcription factors produces potentiated biological function. *Embo J* 12:3879-3891.
86. Akira, S., Y. Nishio, M. Inoue, X. J. Wang, S. Wei, T. Matsusaka, K. Yoshida, T. Sudo, M. Naruto, and T. Kishimoto. 1994. Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. *Cell* 77:63-71.
87. Luticken, C., U. M. Wegenka, J. Yuan, J. Buschmann, C. Schindler, A. Ziemiecki, A. G. Harpur, A. F. Wilks, K. Yasukawa, T. Taga, and et al. 1994. Association of transcription factor APRF and protein kinase Jak1 with the interleukin-6 signal transducer gp130. *Science* 263:89-92.
88. Zhong, Z., Z. Wen, and J. E. Darnell, Jr. 1994. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* 264:95-98.
89. Raz, R., J. E. Durbin, and D. E. Levy. 1994. Acute phase response factor and additional members of the interferon-stimulated gene factor 3 family integrate diverse signals from cytokines, interferons, and growth factors. *J Biol Chem* 269:24391-24395.
90. Levy, D. E., and C. K. Lee. 2002. What does Stat3 do? *J Clin Invest* 109:1143-1148.
91. Gao, B. 2005. Cytokines, STATs and liver disease. *Cell Mol Immunol* 2:92-100.
92. Fu, X. Y. 2006. STAT3 in immune responses and inflammatory bowel diseases. *Cell Res* 16:214-219.
93. Tang, H., C. Yan, J. Cao, J. V. Sarma, E. B. Haura, M. Wu, and H. Gao. An essential role for Stat3 in regulating IgG immune complex-induced pulmonary inflammation. *Faseb J* 25:4292-4300.
94. Hokuto, I., M. Ikegami, M. Yoshida, K. Takeda, S. Akira, A. K. Perl, W. M. Hull, S. E. Wert, and J. A. Whitsett. 2004. Stat-3 is required for pulmonary homeostasis during hyperoxia. *J Clin Invest* 113:28-37.

95. Yan, C., A. Naltner, M. Martin, M. Naltner, J. M. Fangman, and O. Gurel. 2002. Transcriptional stimulation of the surfactant protein B gene by STAT3 in respiratory epithelial cells. *J Biol Chem* 277:10967-10972.
96. Xu, Y., C. Saegusa, A. Schehr, S. Grant, J. A. Whitsett, and M. Ikegami. 2009. C/EBP $\alpha$  is required for pulmonary cytoprotection during hyperoxia. *Am J Physiol Lung Cell Mol Physiol* 297:L286-298.
97. Quinton, L. J., M. R. Jones, B. E. Robson, B. T. Simms, J. A. Whitsett, and J. P. Mizgerd. 2008. Alveolar epithelial STAT3, IL-6 family cytokines, and host defense during Escherichia coli pneumonia. *Am J Respir Cell Mol Biol* 38:699-706.
98. Gao, H., L. M. Hoesel, R. F. Guo, N. J. Rancilio, J. V. Sarma, and P. A. Ward. 2006. Adenoviral-mediated overexpression of SOCS3 enhances IgG immune complex-induced acute lung injury. *J Immunol* 177:612-620.
99. Yasukawa, H., M. Ohishi, H. Mori, M. Murakami, T. Chinen, D. Aki, T. Hanada, K. Takeda, S. Akira, M. Hoshijima, T. Hirano, K. R. Chien, and A. Yoshimura. 2003. IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. *Nat Immunol* 4:551-556.
100. Kato, T., E. Sakamoto, H. Kutsuna, A. Kimura-Eto, F. Hato, and S. Kitagawa. 2004. Proteolytic conversion of STAT3 $\alpha$  to STAT3 $\gamma$  in human neutrophils: role of granule-derived serine proteases. *J Biol Chem* 279:31076-31080.
101. Yoo, J. Y., D. L. Huso, D. Nathans, and S. Desiderio. 2002. Specific ablation of Stat3 $\beta$  distorts the pattern of Stat3-responsive gene expression and impairs recovery from endotoxic shock. *Cell* 108:331-344.
102. Gao, H., S. Parkin, P. F. Johnson, and R. C. Schwartz. 2002. C/EBP gamma has a stimulatory role on the IL-6 and IL-8 promoters. *J Biol Chem* 277:38827-38837.
103. Ramji, D. P., and P. Foka. 2002. CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem J* 365:561-575.
104. Landschulz, W. H., P. F. Johnson, E. Y. Adashi, B. J. Graves, and S. L. McKnight. 1988. Isolation of a recombinant copy of the gene encoding C/EBP. *Genes Dev* 2:786-800.
105. Landschulz, W. H., P. F. Johnson, and S. L. McKnight. 1989. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. *Science* 243:1681-1688.
106. Vinson, C. R., P. B. Sigler, and S. L. McKnight. 1989. Scissors-grip model for DNA recognition by a family of leucine zipper proteins. *Science* 246:911-916.
107. Agre, P., P. F. Johnson, and S. L. McKnight. 1989. Cognate DNA binding specificity retained after leucine zipper exchange between GCN4 and C/EBP. *Science* 246:922-926.
108. Hurst, H. C. 1995. Transcription factors 1: bZIP proteins. *Protein Profile* 2:101-168.
109. Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto. 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *Embo J* 9:1897-1906.
110. Poli, V., F. P. Mancini, and R. Cortese. 1990. IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. *Cell* 63:643-653.

111. Descombes, P., M. Chojkier, S. Lichtsteiner, E. Falvey, and U. Schibler. 1990. LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. *Genes Dev* 4:1541-1551.
112. Chang, C. J., T. T. Chen, H. Y. Lei, D. S. Chen, and S. C. Lee. 1990. Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family. *Mol Cell Biol* 10:6642-6653.
113. Roman, C., J. S. Platero, J. Shuman, and K. Calame. 1990. Ig/EBP-1: a ubiquitously expressed immunoglobulin enhancer binding protein that is similar to C/EBP and heterodimerizes with C/EBP. *Genes Dev* 4:1404-1415.
114. Cao, Z., R. M. Umek, and S. L. McKnight. 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev* 5:1538-1552.
115. Williams, S. C., C. A. Cantwell, and P. F. Johnson. 1991. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. *Genes Dev* 5:1553-1567.
116. Ron, D., and J. F. Habener. 1992. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev* 6:439-453.
117. Sawata, M., H. Takeuchi, and T. Kubo. 2004. Identification and analysis of the minimal promoter activity of a novel noncoding nuclear RNA gene, AncR-1, from the honeybee (*Apis mellifera* L.). *Rna* 10:1047-1058.
118. Landschulz, W. H., P. F. Johnson, and S. L. McKnight. 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* 240:1759-1764.
119. Ubeda, M., X. Z. Wang, H. Zinszner, I. Wu, J. F. Habener, and D. Ron. 1996. Stress-induced binding of the transcriptional factor CHOP to a novel DNA control element. *Mol Cell Biol* 16:1479-1489.
120. Lin, F. T., O. A. MacDougald, A. M. Diehl, and M. D. Lane. 1993. A 30-kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: transcriptional activator lacking antimitotic activity. *Proc Natl Acad Sci U S A* 90:9606-9610.
121. Zhang, D. E., P. Zhang, N. D. Wang, C. J. Hetherington, G. J. Darlington, and D. G. Tenen. 1997. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. *Proc Natl Acad Sci U S A* 94:569-574.
122. Zhang, P., A. Iwama, M. W. Datta, G. J. Darlington, D. C. Link, and D. G. Tenen. 1998. Upregulation of interleukin 6 and granulocyte colony-stimulating factor receptors by transcription factor CCAAT enhancer binding protein alpha (C/EBP alpha) is critical for granulopoiesis. *J Exp Med* 188:1173-1184.
123. Zhang, P., J. Iwasaki-Arai, H. Iwasaki, M. L. Fenyus, T. Dayaram, B. M. Owens, H. Shigematsu, E. Levantini, C. S. Huettner, J. A. Lekstrom-Himes, K. Akashi, and D. G. Tenen. 2004. Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. *Immunity* 21:853-863.



124. Heath, V., H. C. Suh, M. Holman, K. Renn, J. M. Gooya, S. Parkin, K. D. Klarmann, M. Ortiz, P. Johnson, and J. Keller. 2004. C/EBPalpha deficiency results in hyperproliferation of hematopoietic progenitor cells and disrupts macrophage development in vitro and in vivo. *Blood* 104:1639-1647.
125. Friedman, A. D. 2007. C/EBPalpha induces PU.1 and interacts with AP-1 and NF-kappaB to regulate myeloid development. *Blood Cells Mol Dis* 39:340-343.
126. Hu, H. M., M. Baer, S. C. Williams, P. F. Johnson, and R. C. Schwartz. 1998. Redundancy of C/EBP alpha, -beta, and -delta in supporting the lipopolysaccharide-induced transcription of IL-6 and monocyte chemoattractant protein-1. *J Immunol* 160:2334-2342.
127. Yang, I. V., S. Alper, B. Lackford, H. Rutledge, L. A. Warg, L. H. Burch, and D. A. Schwartz. Novel regulators of the systemic response to lipopolysaccharide. *Am J Respir Cell Mol Biol* 45:393-402.
128. Alam, T., M. R. An, and J. Papaconstantinou. 1992. Differential expression of three C/EBP isoforms in multiple tissues during the acute phase response. *J Biol Chem* 267:5021-5024.
129. Burgess-Beusse, B. L., and G. J. Darlington. 1998. C/EBPalpha is critical for the neonatal acute-phase response to inflammation. *Mol Cell Biol* 18:7269-7277.
130. Yang, J., C. M. Croniger, J. Lekstrom-Himes, P. Zhang, M. Fenyus, D. G. Tenen, G. J. Darlington, and R. W. Hanson. 2005. Metabolic response of mice to a postnatal ablation of CCAAT/enhancer-binding protein alpha. *J Biol Chem* 280:38689-38699.
131. Didon, L., A. B. Roos, G. P. Elmerger, F. J. Gonzalez, and M. Nord. Lung-specific inactivation of CCAAT/enhancer binding protein alpha causes a pathological pattern characteristic of COPD. *Eur Respir J* 35:186-197.
132. Flodby, P., C. Barlow, H. Kylefjord, L. Ahrlund-Richter, and K. G. Xanthopoulos. 1996. Increased hepatic cell proliferation and lung abnormalities in mice deficient in CCAAT/enhancer binding protein alpha. *J Biol Chem* 271:24753-24760.
133. Yang, G., M. D. Hinson, J. E. Bordner, Q. S. Lin, A. P. Fernando, P. La, C. J. Wright, and P. A. Dennerly. Silencing hyperoxia-induced C/EBPalpha in neonatal mice improves lung architecture via enhanced proliferation of alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 301:L187-196.
134. Sugahara, K., T. Sadohara, M. Sugita, K. Iyama, and M. Takiguchi. 1999. Differential expression of CCAAT enhancer binding protein family in rat alveolar epithelial cell proliferation and in acute lung injury. *Cell Tissue Res* 297:261-270.
135. Sugahara, K., K. I. Iyama, T. Kimura, K. Sano, G. J. Darlington, T. Akiba, and M. Takiguchi. 2001. Mice lacking CCAAT/enhancer-binding protein-alpha show hyperproliferation of alveolar type II cells and increased surfactant protein mRNAs. *Cell Tissue Res* 306:57-63.
136. Baron, R. M., I. M. Carvajal, L. E. Fredenburgh, X. Liu, Y. Porrata, M. L. Cullivan, K. J. Haley, L. A. Sonna, G. T. De Sanctis, E. P. Ingenito, and M. A. Perrella. 2004. Nitric oxide synthase-2 down-regulates surfactant protein-B expression and enhances endotoxin-induced lung injury in mice. *Faseb J* 18:1276-1278.

137. Descombes, P., and U. Schibler. 1991. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell* 67:569-579.
138. Yan, C., J. Cao, M. Wu, W. Zhang, T. Jiang, A. Yoshimura, and H. Gao. Suppressor of cytokine signaling 3 inhibits LPS-induced IL-6 expression in osteoblasts by suppressing CCAAT/enhancer-binding protein {beta} activity. *J Biol Chem* 285:37227-37239.
139. Billack, B., D. E. Heck, T. M. Mariano, C. R. Gardner, R. Sur, D. L. Laskin, and J. D. Laskin. 2002. Induction of cyclooxygenase-2 by heat shock protein 60 in macrophages and endothelial cells. *Am J Physiol Cell Physiol* 283:C1267-1277.
140. Liu, Y. W., H. P. Tseng, L. C. Chen, B. K. Chen, and W. C. Chang. 2003. Functional cooperation of simian virus 40 promoter factor 1 and CCAAT/enhancer-binding protein beta and delta in lipopolysaccharide-induced gene activation of IL-10 in mouse macrophages. *J Immunol* 171:821-828.
141. Zwergal, A., M. Quirling, B. Saugel, K. C. Huth, C. Sydlik, V. Poli, D. Neumeier, H. W. Ziegler-Heitbrock, and K. Brand. 2006. C/EBP beta blocks p65 phosphorylation and thereby NF-kappa B-mediated transcription in TNF-tolerant cells. *J Immunol* 177:665-672.
142. Ramsay, P. L., Z. Luo, A. Major, M. S. Park, M. Finegold, S. E. Welty, I. Kwak, G. Darlington, and F. J. Demayo. 2003. Multiple mechanisms for oxygen-induced regulation of the Clara cell secretory protein gene. *Faseb J* 17:2142-2144.
143. Tanaka, T., S. Akira, K. Yoshida, M. Umemoto, Y. Yoneda, N. Shirafuji, H. Fujiwara, S. Suematsu, N. Yoshida, and T. Kishimoto. 1995. Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. *Cell* 80:353-361.
144. Hu, B., M. R. Ullenbruch, H. Jin, M. Gharaee-Kermani, and S. H. Phan. 2007. An essential role for CCAAT/enhancer binding protein beta in bleomycin-induced pulmonary fibrosis. *J Pathol* 211:455-462.
145. Buck, M., and M. Chojkier. C/EBPbeta-Thr217 phosphorylation signaling contributes to the development of lung injury and fibrosis in mice. *PLoS One* 6:e25497.
146. Hu, B., Z. Wu, H. Jin, N. Hashimoto, T. Liu, and S. H. Phan. 2004. CCAAT/enhancer-binding protein beta isoforms and the regulation of alpha-smooth muscle actin gene expression by IL-1 beta. *J Immunol* 173:4661-4668.
147. Roos, A. B., J. L. Barton, A. Miller-Larsson, B. Dahlberg, T. Berg, L. Didon, and M. Nord. Lung epithelial-C/EBPbeta contributes to LPS-induced inflammation and its suppression by formoterol. *Biochem Biophys Res Commun* 423:134-139.
148. Kinoshita, S., S. Akira, and T. Kishimoto. 1992. A member of the C/EBP family, NF-IL6 beta, forms a heterodimer and transcriptionally synergizes with NF-IL6. *Proc Natl Acad Sci U S A* 89:1473-1476.
149. Uto, T., M. Fujii, and D. X. Hou. 2005. Inhibition of lipopolysaccharide-induced cyclooxygenase-2 transcription by 6-(methylsulfinyl) hexyl isothiocyanate, a chemopreventive compound from *Wasabia japonica* (Miq.) Matsumura, in mouse macrophages. *Biochem Pharmacol* 70:1772-1784.

150. Breed, D. R., L. R. Margraf, J. L. Alcorn, and C. R. Mendelson. 1997. Transcription factor C/EBPdelta in fetal lung: developmental regulation and effects of cyclic adenosine 3',5'-monophosphate and glucocorticoids. *Endocrinology* 138:5527-5534.
151. Berg, T., T. N. Cassel, P. E. Schwarze, and M. Nord. 2002. Glucocorticoids regulate the CCSP and CYP2B1 promoters via C/EBPbeta and delta in lung cells. *Biochem Biophys Res Commun* 293:907-912.
152. Cassel, T. N., L. Nordlund-Moller, O. Andersson, J. A. Gustafsson, and M. Nord. 2000. C/EBPalpha and C/EBPdelta activate the clara cell secretory protein gene through interaction with two adjacent C/EBP-binding sites. *Am J Respir Cell Mol Biol* 22:469-480.
153. Tomita, T., T. Kido, R. Kurotani, S. Iemura, E. Sterneck, T. Natsume, C. Vinson, and S. Kimura. 2008. CAATT/enhancer-binding proteins alpha and delta interact with NKX2-1 to synergistically activate mouse secretoglobin 3A2 gene expression. *J Biol Chem* 283:25617-25627.
154. Slofstra, S. H., A. P. Groot, M. H. Obdeijn, P. H. Reitsma, H. ten Cate, and C. A. Spek. 2007. Gene expression profiling identifies C/EBPdelta as a candidate regulator of endotoxin-induced disseminated intravascular coagulation. *Am J Respir Crit Care Med* 176:602-609.
155. Antonson, P., B. Stellan, R. Yamanaka, and K. G. Xanthopoulos. 1996. A novel human CCAAT/enhancer binding protein gene, C/EBPepsilon, is expressed in cells of lymphoid and myeloid lineages and is localized on chromosome 14q11.2 close to the T-cell receptor alpha/delta locus. *Genomics* 35:30-38.
156. Chumakov, A. M., I. Grillier, E. Chumakova, D. Chih, J. Slater, and H. P. Koeffler. 1997. Cloning of the novel human myeloid-cell-specific C/EBP-epsilon transcription factor. *Mol Cell Biol* 17:1375-1386.
157. Yamanaka, R., G. D. Kim, H. S. Radomska, J. Lekstrom-Himes, L. T. Smith, P. Antonson, D. G. Tenen, and K. G. Xanthopoulos. 1997. CCAAT/enhancer binding protein epsilon is preferentially up-regulated during granulocytic differentiation and its functional versatility is determined by alternative use of promoters and differential splicing. *Proc Natl Acad Sci U S A* 94:6462-6467.
158. Lekstrom-Himes, J. A. 2001. The role of C/EBP(epsilon) in the terminal stages of granulocyte differentiation. *Stem Cells* 19:125-133.
159. Tavor, S., P. T. Vuong, D. J. Park, A. F. Gombart, A. H. Cohen, and H. P. Koeffler. 2002. Macrophage functional maturation and cytokine production are impaired in C/EBP epsilon-deficient mice. *Blood* 99:1794-1801.
160. Yamanaka, R., C. Barlow, J. Lekstrom-Himes, L. H. Castilla, P. P. Liu, M. Eckhaus, T. Decker, A. Wynshaw-Boris, and K. G. Xanthopoulos. 1997. Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. *Proc Natl Acad Sci U S A* 94:13187-13192.
161. Lekstrom-Himes, J., and K. G. Xanthopoulos. 1999. CCAAT/enhancer binding protein epsilon is critical for effective neutrophil-mediated response to inflammatory challenge. *Blood* 93:3096-3105.

162. Akagi, T., N. H. Thoennissen, A. George, G. Crooks, J. H. Song, R. Okamoto, D. Nowak, A. F. Gombart, and H. P. Koeffler. In vivo deficiency of both C/EBPbeta and C/EBPepsilon results in highly defective myeloid differentiation and lack of cytokine response. *PLoS One* 5:e15419.
163. Thomassin, H., D. Hamel, D. Bernier, M. Guertin, and L. Belanger. 1992. Molecular cloning of two C/EBP-related proteins that bind to the promoter and the enhancer of the alpha 1-fetoprotein gene. Further analysis of C/EBP beta and C/EBP gamma. *Nucleic Acids Res* 20:3091-3098.
164. Cooper, C., A. Henderson, S. Artandi, N. Avitahl, and K. Calame. 1995. Ig/EBP (C/EBP gamma) is a transdominant negative inhibitor of C/EBP family transcriptional activators. *Nucleic Acids Res* 23:4371-4377.
165. Schwartz, C., P. Catez, O. Rohr, D. Lecestre, D. Aunis, and E. Schaeffer. 2000. Functional interactions between C/EBP, Sp1, and COUP-TF regulate human immunodeficiency virus type 1 gene transcription in human brain cells. *J Virol* 74:65-73.
166. He, L., M. J. Ronis, and T. M. Badger. 2002. Ethanol induction of class I alcohol dehydrogenase expression in the rat occurs through alterations in CCAAT/enhancer binding proteins beta and gamma. *J Biol Chem* 277:43572-43577.
167. Gordon, C. T., V. J. Fox, S. Najdovska, and A. C. Perkins. 2005. C/EBPdelta and C/EBPgamma bind the CCAAT-box in the human beta-globin promoter and modulate the activity of the CACC-box binding protein, EKLF. *Biochim Biophys Acta* 1729:74-80.
168. Wall, L., N. Destroismaisons, N. Delvoye, and L. G. Guy. 1996. CAAT/enhancer-binding proteins are involved in beta-globin gene expression and are differentially expressed in murine erythroleukemia and K562 cells. *J Biol Chem* 271:16477-16484.
169. Kaisho, T., H. Tsutsui, T. Tanaka, T. Tsujimura, K. Takeda, T. Kawai, N. Yoshida, K. Nakanishi, and S. Akira. 1999. Impairment of natural killer cytotoxic activity and interferon gamma production in CCAAT/enhancer binding protein gamma-deficient mice. *J Exp Med* 190:1573-1582.
170. Zafarana, G., R. Rottier, F. Grosveld, and S. Philipsen. 2000. Erythroid overexpression of C/EBPgamma in transgenic mice affects gamma-globin expression and fetal liver erythropoiesis. *Embo J* 19:5856-5863.
171. Parkin, S. E., M. Baer, T. D. Copeland, R. C. Schwartz, and P. F. Johnson. 2002. Regulation of CCAAT/enhancer-binding protein (C/EBP) activator proteins by heterodimerization with C/EBPgamma (Ig/EBP). *J Biol Chem* 277:23563-23572.
172. Yan, C., X. Wang, J. Cao, M. Wu, and H. Gao. CCAAT/enhancer-binding protein gamma is a critical regulator of IL-1beta-induced IL-6 production in alveolar epithelial cells. *PLoS One* 7:e35492.
173. Endo, M., M. Mori, S. Akira, and T. Gotoh. 2006. C/EBP homologous protein (CHOP) is crucial for the induction of caspase-11 and the pathogenesis of lipopolysaccharide-induced inflammation. *J Immunol* 176:6245-6253.

174. Sunil, V. R., K. J. Patel, M. Nilsen-Hamilton, D. E. Heck, J. D. Laskin, and D. L. Laskin. 2007. Acute endotoxemia is associated with upregulation of lipocalin 24p3/Lcn2 in lung and liver. *Exp Mol Pathol* 83:177-187.
175. Endo, M., S. Oyadomari, M. Suga, M. Mori, and T. Gotoh. 2005. The ER stress pathway involving CHOP is activated in the lungs of LPS-treated mice. *J Biochem* 138:501-507.
176. Korfei, M., C. Ruppert, P. Mahavadi, I. Henneke, P. Markart, M. Koch, G. Lang, L. Fink, R. M. Bohle, W. Seeger, T. E. Weaver, and A. Guenther. 2008. Epithelial endoplasmic reticulum stress and apoptosis in sporadic idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 178:838-846.
177. Vij, N., M. O. Amoako, S. Mazur, and P. L. Zeitlin. 2008. CHOP transcription factor mediates IL-8 signaling in cystic fibrosis bronchial epithelial cells. *Am J Respir Cell Mol Biol* 38:176-184.
178. Lozon, T. I., A. J. Eastman, G. Matute-Bello, P. Chen, T. S. Hallstrand, and W. A. Altemeier. PKR-dependent CHOP induction limits hyperoxia-induced lung injury. *Am J Physiol Lung Cell Mol Physiol* 300:L422-429.
179. O'Reilly, M. A., R. J. Staversky, R. H. Watkins, W. M. Maniscalco, and P. C. Keng. 2000. p53-independent induction of GADD45 and GADD153 in mouse lungs exposed to hyperoxia. *Am J Physiol Lung Cell Mol Physiol* 278:L552-559.
180. Lee, J., R. Reddy, L. Barsky, K. Weinberg, and B. Driscoll. 2006. Contribution of proliferation and DNA damage repair to alveolar epithelial type 2 cell recovery from hyperoxia. *Am J Physiol Lung Cell Mol Physiol* 290:L685-L694.
181. Lekstrom-Himes, J., and K. G. Xanthopoulos. 1998. Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *J Biol Chem* 273:28545-28548.
182. Poli, V. 1998. The role of C/EBP isoforms in the control of inflammatory and native immunity functions. *J Biol Chem* 273:29279-29282.
183. Gorgoni, B., D. Maritano, P. Marthyn, M. Righi, and V. Poli. 2002. C/EBP beta gene inactivation causes both impaired and enhanced gene expression and inverse regulation of IL-12 p40 and p35 mRNAs in macrophages. *J Immunol* 168:4055-4062.
184. Uematsu, S., M. Matsumoto, K. Takeda, and S. Akira. 2002. Lipopolysaccharide-dependent prostaglandin E(2) production is regulated by the glutathione-dependent prostaglandin E(2) synthase gene induced by the Toll-like receptor 4/MyD88/NF-IL6 pathway. *J Immunol* 168:5811-5816.
185. Caivano, M., B. Gorgoni, P. Cohen, and V. Poli. 2001. The induction of cyclooxygenase-2 mRNA in macrophages is biphasic and requires both CCAAT enhancer-binding protein beta (C/EBP beta) and C/EBP delta transcription factors. *J Biol Chem* 276:48693-48701.
186. Albina, J. E., E. J. Mahoney, J. M. Daley, D. E. Wesche, S. M. Morris, Jr., and J. S. Reichner. 2005. Macrophage arginase regulation by CCAAT/enhancer-binding protein beta. *Shock* 23:168-172.
187. Serio, K. J., K. V. Reddy, and T. D. Bigby. 2005. Lipopolysaccharide induces 5-lipoxygenase-activating protein gene expression in THP-1 cells via a NF-kappaB and C/EBP-mediated mechanism. *Am J Physiol Cell Physiol* 288:C1125-1133.

188. Cassel, T. N., and M. Nord. 2003. C/EBP transcription factors in the lung epithelium. *Am J Physiol Lung Cell Mol Physiol* 285:L773-781.
189. Didon, L., J. L. Barton, A. B. Roos, G. J. Gaschler, C. M. Bauer, T. Berg, M. R. Stampfli, and M. Nord. Lung epithelial CCAAT/enhancer-binding protein-beta is necessary for the integrity of inflammatory responses to cigarette smoke. *Am J Respir Crit Care Med* 184:233-242.
190. Ward, P. A. 1996. Role of complement, chemokines, and regulatory cytokines in acute lung injury. *Ann N Y Acad Sci* 796:104-112.
191. Zhang, K., W. Guo, Y. Yang, and J. Wu. JAK2/STAT3 pathway is involved in the early stage of adipogenesis through regulating C/EBPbeta transcription. *J Cell Biochem* 112:488-497.
192. Cantwell, C. A., E. Sterneck, and P. F. Johnson. 1998. Interleukin-6-specific activation of the C/EBPdelta gene in hepatocytes is mediated by Stat3 and Sp1. *Mol Cell Biol* 18:2108-2117.
193. Halstead, S. B., S. Mahalingam, M. A. Marovich, S. Ubol, and D. M. Mosser. Intrinsic antibody-dependent enhancement of microbial infection in macrophages: disease regulation by immune complexes. *Lancet Infect Dis* 10:712-722.
194. Fernandez, N., M. Renedo, C. Garcia-Rodriguez, and M. Sanchez Crespo. 2002. Activation of monocytic cells through Fc gamma receptors induces the expression of macrophage-inflammatory protein (MIP)-1 alpha, MIP-1 beta, and RANTES. *J Immunol* 169:3321-3328.
195. Bayon, Y., A. Alonso, and M. Sanchez Crespo. 1997. Stimulation of Fc gamma receptors in rat peritoneal macrophages induces the expression of nitric oxide synthase and chemokines by mechanisms showing different sensitivities to antioxidants and nitric oxide donors. *J Immunol* 159:887-894.
196. Schmal, H., B. J. Czermak, A. B. Lentsch, N. M. Bless, B. Beck-Schimmer, H. P. Friedl, and P. A. Ward. 1998. Soluble ICAM-1 activates lung macrophages and enhances lung injury. *J Immunol* 161:3685-3693.
197. Uematsu, S., T. Kaisho, T. Tanaka, M. Matsumoto, M. Yamakami, H. Omori, M. Yamamoto, T. Yoshimori, and S. Akira. 2007. The C/EBP beta isoform 34-kDa LAP is responsible for NF-IL-6-mediated gene induction in activated macrophages, but is not essential for intracellular bacteria killing. *J Immunol* 179:5378-5386.
198. Matsumoto, M., T. Tanaka, T. Kaisho, H. Sanjo, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, and S. Akira. 1999. A novel LPS-inducible C-type lectin is a transcriptional target of NF-IL6 in macrophages. *J Immunol* 163:5039-5048.
199. Cloutier, A., C. Guindi, P. Larivee, C. M. Dubois, A. Amrani, and P. P. McDonald. 2009. Inflammatory cytokine production by human neutrophils involves C/EBP transcription factors. *J Immunol* 182:563-571.
200. Cox, G. W., B. J. Mathieson, L. Gandino, E. Blasi, D. Radzioch, and L. Varesio. 1989. Heterogeneity of hematopoietic cells immortalized by v-myc/v-raf recombinant retrovirus infection of bone marrow or fetal liver. *J Natl Cancer Inst* 81:1492-1496.

201. Sterneck, E., R. Paylor, V. Jackson-Lewis, M. Libbey, S. Przedborski, L. Tessarollo, J. N. Crawley, and P. F. Johnson. 1998. Selectively enhanced contextual fear conditioning in mice lacking the transcriptional regulator CCAAT/enhancer binding protein delta. *Proc Natl Acad Sci U S A* 95:10908-10913.
202. Sterneck, E., L. Tessarollo, and P. F. Johnson. 1997. An essential role for C/EBPbeta in female reproduction. *Genes Dev* 11:2153-2162.
203. Rittirsch, D., M. A. Flierl, D. E. Day, B. A. Nadeau, F. S. Zetoune, J. V. Sarma, C. M. Werner, G. A. Wanner, H. P. Simmen, M. S. Huber-Lang, and P. A. Ward. 2009. Cross-talk between TLR4 and FcgammaReceptorIII (CD16) pathways. *PLoS Pathog* 5:e1000464.
204. Van Rooijen, N., and A. Sanders. 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* 174:83-93.
205. Anastasov, N., I. Bonzheim, M. Rudelius, M. Klier, T. Dau, D. Angermeier, J. Duyster, S. Pittaluga, F. Fend, M. Raffeld, and L. Quintanilla-Martinez. C/EBPbeta expression in ALK-positive anaplastic large cell lymphomas is required for cell proliferation and is induced by the STAT3 signaling pathway. *Haematologica* 95:760-767.
206. Lu, Y. C., I. Kim, E. Lye, F. Shen, N. Suzuki, S. Suzuki, S. Gerondakis, S. Akira, S. L. Gaffen, W. C. Yeh, and P. S. Ohashi. 2009. Differential role for c-Rel and C/EBPbeta/delta in TLR-mediated induction of proinflammatory cytokines. *J Immunol* 182:7212-7221.
207. Maitra, U., L. Gan, S. Chang, and L. Li. Low-dose endotoxin induces inflammation by selectively removing nuclear receptors and activating CCAAT/enhancer-binding protein delta. *J Immunol* 186:4467-4473.
208. Widmer, U., K. R. Manogue, A. Cerami, and B. Sherry. 1993. Genomic cloning and promoter analysis of macrophage inflammatory protein (MIP)-2, MIP-1 alpha, and MIP-1 beta, members of the chemokine superfamily of proinflammatory cytokines. *J Immunol* 150:4996-5012.
209. Wolpe, S. D., B. Sherry, D. Juers, G. Davatellis, R. W. Yurt, and A. Cerami. 1989. Identification and characterization of macrophage inflammatory protein 2. *Proc Natl Acad Sci U S A* 86:612-616.
210. Wolpe, S. D., G. Davatellis, B. Sherry, B. Beutler, D. G. Hesse, H. T. Nguyen, L. L. Moldawer, C. F. Nathan, S. F. Lowry, and A. Cerami. 1988. Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. *J Exp Med* 167:570-581.
211. Davatellis, G., P. Tekamp-Olson, S. D. Wolpe, K. Hermsen, C. Luedke, C. Gallegos, D. Coit, J. Merryweather, and A. Cerami. 1988. Cloning and characterization of a cDNA for murine macrophage inflammatory protein (MIP), a novel monokine with inflammatory and chemokinetic properties. *J Exp Med* 167:1939-1944.
212. Wolpe, S. D., and A. Cerami. 1989. Macrophage inflammatory proteins 1 and 2: members of a novel superfamily of cytokines. *Faseb J* 3:2565-2573.

213. Oppenheim, J. J., C. O. Zachariae, N. Mukaida, and K. Matsushima. 1991. Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu Rev Immunol* 9:617-648.
214. Nimmerjahn, F., and J. V. Ravetch. 2007. Fc-receptors as regulators of immunity. *Adv Immunol* 96:179-204.
215. Akagi, T., Q. T. Luong, D. Gui, J. Said, J. Selektar, A. Yung, C. M. Bunce, G. D. Braunstein, and H. P. Koeffler. 2008. Induction of sodium iodide symporter gene and molecular characterisation of HNF3 beta/FoxA2, TTF-1 and C/EBP beta in thyroid carcinoma cells. *Br J Cancer* 99:781-788.
216. Bianchi, G., F. Montecucco, M. Bertolotto, F. Dallegri, and L. Ottonello. 2007. Immune complexes induce monocyte survival through defined intracellular pathways. *Ann N Y Acad Sci* 1095:209-219.
217. Bless, N. M., R. L. Warner, V. A. Padgaonkar, A. B. Lentsch, B. J. Czermak, H. Schmal, H. P. Friedl, and P. A. Ward. 1999. Roles for C-X-C chemokines and C5a in lung injury after hindlimb ischemia-reperfusion. *Am J Physiol* 276:L57-63.
218. Kumar, V., S. R. Ali, S. Konrad, J. Zwirner, J. S. Verbeek, R. E. Schmidt, and J. E. Gessner. 2006. Cell-derived anaphylatoxins as key mediators of antibody-dependent type II autoimmunity in mice. *J Clin Invest* 116:512-520.
219. Mulligan, M. S., G. P. Wilson, R. F. Todd, C. W. Smith, D. C. Anderson, J. Varani, T. B. Issekutz, M. Miyasaka, T. Tamatani, and et al. 1993. Role of beta 1, beta 2 integrins and ICAM-1 in lung injury after deposition of IgG and IgA immune complexes. *J Immunol* 150:2407-2417.
220. Litvak, V., S. A. Ramsey, A. G. Rust, D. E. Zak, K. A. Kennedy, A. E. Lampano, M. Nykter, I. Shmulevich, and A. Aderem. 2009. Function of C/EBPdelta in a regulatory circuit that discriminates between transient and persistent TLR4-induced signals. *Nat Immunol* 10:437-443.
221. Adelmant, G., J. D. Gilbert, and S. O. Freytag. 1998. Human translocation liposarcoma-CCAAT/enhancer binding protein (C/EBP) homologous protein (TLS-CHOP) oncoprotein prevents adipocyte differentiation by directly interfering with C/EBPbeta function. *J Biol Chem* 273:15574-15581.
222. Zhang, Y., and W. N. Rom. 1993. Regulation of the interleukin-1 beta (IL-1 beta) gene by mycobacterial components and lipopolysaccharide is mediated by two nuclear factor-IL6 motifs. *Mol Cell Biol* 13:3831-3837.
223. Pope, R. M., A. Leutz, and S. A. Ness. 1994. C/EBP beta regulation of the tumor necrosis factor alpha gene. *J Clin Invest* 94:1449-1455.
224. Walpen, S., K. F. Beck, L. Schaefer, I. Raslik, W. Eberhardt, R. M. Schaefer, and J. Pfeilschifter. 2001. Nitric oxide induces MIP-2 transcription in rat renal mesangial cells and in a rat model of glomerulonephritis. *Faseb J* 15:571-573.
225. Arnett, B., P. Soisson, B. S. Ducatman, and P. Zhang. 2003. Expression of CAAT enhancer binding protein beta (C/EBP beta) in cervix and endometrium. *Mol Cancer* 2:21.
226. Ramji, D. P., A. Vitelli, F. Tronche, R. Cortese, and G. Ciliberto. 1993. The two C/EBP isoforms, IL-6DBP/NF-IL6 and C/EBP delta/NF-IL6 beta, are induced by IL-6 to promote acute phase gene transcription via different mechanisms. *Nucleic Acids Res* 21:289-294.



227. Trotta, R., P. Kanakaraj, and B. Perussia. 1996. Fc gamma R-dependent mitogen-activated protein kinase activation in leukocytes: a common signal transduction event necessary for expression of TNF-alpha and early activation genes. *J Exp Med* 184:1027-1035.
228. Hillyard, D. Z., A. G. Jardine, K. J. McDonald, and A. J. Cameron. 2004. Fluvastatin inhibits raft dependent Fc gamma receptor signalling in human monocytes. *Atherosclerosis* 172:219-228.
229. Lucas, M., X. Zhang, V. Prasanna, and D. M. Mosser. 2005. ERK activation following macrophage Fc gamma R ligation leads to chromatin modifications at the IL-10 locus. *J Immunol* 175:469-477.
230. Song, X., S. Tanaka, D. Cox, and S. C. Lee. 2004. Fc gamma receptor signaling in primary human microglia: differential roles of PI-3K and Ras/ERK MAPK pathways in phagocytosis and chemokine induction. *J Leukoc Biol* 75:1147-1155.
231. Sanchez-Mejorada, G., and C. Rosales. 1998. Fc gamma receptor-mediated mitogen-activated protein kinase activation in monocytes is independent of Ras. *J Biol Chem* 273:27610-27619.
232. Garcia-Garcia, E., G. Sanchez-Mejorada, and C. Rosales. 2001. Phosphatidylinositol 3-kinase and ERK are required for NF-kappaB activation but not for phagocytosis. *J Leukoc Biol* 70:649-658.
233. Nakano, H., M. Shindo, S. Sakon, S. Nishinaka, M. Mihara, H. Yagita, and K. Okumura. 1998. Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. *Proc Natl Acad Sci U S A* 95:3537-3542.
234. Gerbitz, A., P. Ewing, K. Olkiewicz, N. E. Willmarth, D. Williams, G. Hildebrandt, A. Wilke, C. Liu, G. Eissner, R. Andreesen, E. Holler, R. Guo, P. A. Ward, and K. R. Cooke. 2005. A role for CD54 (intercellular adhesion molecule-1) in leukocyte recruitment to the lung during the development of experimental idiopathic pneumonia syndrome. *Transplantation* 79:536-542.
235. Konrad, S., L. Engling, R. E. Schmidt, and J. E. Gessner. 2007. Characterization of the murine IgG Fc receptor III and IIB gene promoters: a single two-nucleotide difference determines their inverse responsiveness to C5a. *J Biol Chem* 282:37906-37912.
236. Konrad, S., S. R. Ali, K. Wiege, S. N. Syed, L. Engling, R. P. Piekorz, E. Hirsch, B. Nurnberg, R. E. Schmidt, and J. E. Gessner. 2008. Phosphoinositide 3-kinases gamma and delta, linkers of coordinate C5a receptor-Fc gamma receptor activation and immune complex-induced inflammation. *J Biol Chem* 283:33296-33303.
237. Fan, J., R. D. Ye, and A. B. Malik. 2001. Transcriptional mechanisms of acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 281:L1037-1050.
238. Shanley, T. P., H. Schmal, R. L. Warner, E. Schmid, H. P. Friedl, and P. A. Ward. 1997. Requirement for C-X-C chemokines (macrophage inflammatory protein-2 and cytokine-induced neutrophil chemoattractant) in IgG immune complex-induced lung injury. *J Immunol* 158:3439-3448.
239. Shanley, T. P., H. Schmal, H. P. Friedl, M. L. Jones, and P. A. Ward. 1995. Role of macrophage inflammatory protein-1 alpha (MIP-1 alpha) in acute lung injury in rats. *J Immunol* 154:4793-4802.

240. Bless, N. M., M. Huber-Lang, R. F. Guo, R. L. Warner, H. Schmal, B. J. Czermak, T. P. Shanley, L. D. Crouch, A. B. Lentsch, V. Sarma, M. S. Mulligan, H. P. Friedl, and P. A. Ward. 2000. Role of CC chemokines (macrophage inflammatory protein-1 beta, monocyte chemoattractant protein-1, RANTES) in acute lung injury in rats. *J Immunol* 164:2650-2659.
241. Mulligan, M. S., A. A. Vaporciyan, M. Miyasaka, T. Tamatani, and P. A. Ward. 1993. Tumor necrosis factor alpha regulates in vivo intrapulmonary expression of ICAM-1. *Am J Pathol* 142:1739-1749.
242. Kaminska, B., B. Pyrzynska, I. Ciechomska, and M. Wisniewska. 2000. Modulation of the composition of AP-1 complex and its impact on transcriptional activity. *Acta Neurobiol Exp (Wars)* 60:395-402.