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Studies on IL-12 and IL-18-induced MCP-1 secretion in murine macrophages.

by

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ABSTRACT

IL-12 and IL-18 are important pro-inflammatory cytokines produced at the earlier stage of innate immunity. These cytokines are secreted mainly by activated macrophage and have been reported to be involved in the modulation and activation of adaptive immunity. However, the immuno-biologic function of these cytokine molecules in innate immunity has not yet been fully elucidated. Through this study, it was found that IL-12 and IL-18 independently stimulate macrophage to produce MCP-1 molecule, which plays an important role in the recruitment and activation of leukocytes at the inflammatory site, *via* an autocrine IFN-γ–dependent or independent mechanism, respectively. Moreover, it was also revealed that the MCP-1 inducing activity of IL-18 was mediated by direct IL-18R signaling such as the PI3K/Akt and MEK/Erk1/2 pathways. Finally, these results suggest that both IL-12 and IL-18 may actively regulate innate immunity by inducing the recruitment and activation of circulating leukocyte *via* MCP-1 production from macrophage.

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LIST OF ABBREVIATION

Ab: Antibody

Ag: Antigen

APC: Antigen Presenting Cell

CD: Cluster of Differentiation

ELISA: Enzyme-Linked Immunosorbent Assay

ERK: Extracellular signal-Regulated Kinase

FACS: Fluorescence Activating Cell Sorter

FasL: Fas-ligand

FBS: Fetal Bovine Serum

HPRT: Hypoxanthine Phosphoribosyl Transferase

HRP: Horseradish Peroxidase

IFN: Interferon

IGIF: IFN-γ Inducing Factor

IKK: Ikappa B kinase

IL: Interleukin

IRAK: IL-1 receptor associated kinase

JNK: *c*-Jun NH_2-terminal kinase

LPS: Lipopolysaccharide

MACS: Magnetic Activated Cell Sorting

MAPK: Mitogen Activated Protein Kinase

MCP: Monocyte Chemotactic Protein

MEK: Mitogen-activated Protein Kinase Kinase

Mϕ: Macrophage

MHC: Major Histocompatibility Complex

MIP: Macrophage Inflammatory Protein

MyD: Myeloid Differentiation

NF-κB: Nuclear Factor kappa-B

NK: Natural Killer

NO: Nitric Oxide

PAMP: Pathogen Associated Molecular Pattern

PBS: Phosphate-Buffered Saline

PI: Propidium Iodide

PI3K: Phosphatidly Inositol-3 Kinase

R: Receptor

RBC: Red Blood Cells

RT-PCR: Reverse transcriptase-polymerase chain reaction

TCR: T cells Receptor

TGF: Transforming Growth Factor

Th: T-helper

TNF: Tumor Necrosis Factor

VCAM: Vascular Cells Adhesion Molecules

CHAPTER 1. INTRODUCTION, HYPOTHESIS AND OBJECIVES

1. Introduction

1-1. Immune system

The immune system is self defense system that has evolved in organisms to protect themselves from invading pathogens. This self defense system is composed of a variety of cells and molecules which form a dynamic network to act together in the recognition and elimination of limitless variety of foreign invaders. The immune system is able to discriminate between self and non-self molecules. Self and non-self recognition is achieved by having every cell display a marker based on the major histo-compatibility complex (MHC). Any cell not displaying this marker is treated as non-self and attacked by immune cells. Once the immune system recognizes non-self molecules, a variety of cells and molecules of the system are activated and participate in mounting an appropriate response to eliminate the particular type of invaded foreign organisms (Goldsby, 2000; Janeway et al., 2001).

The immune system consists of two major types of immune components. One is innate immunity and the other is adaptive immunity. In terms of evolution, innate immunity is the more ancient form of host defense. This immunity shows antigen (Ag) non-specific immune responses, and is mediated mainly by granulocytes and macrophages (Mϕ). On the contrary, adaptive immunity, as an exclusively unique form of host defense system in vertebrates, can recognize invaded pathogens specifically, a feature which is mediated by lymphocytes such as B cells and T cells. Adaptive immunity efficiently eliminates invading microorganisms with two different kinds of immune components: cell-mediated immunity and humoral immunity. Adaptive immunity also confers lifelong protective immunity to re-infection with the same

pathogen, and this is called immunological memory (Janeway et al., 2001; Janeway et al., 2002).

1-1-1. Innate immunity

Innate immunity serves as a first line of defense, and this is mediated by white blood cells such as neutrophils, dendritic cells, and Mϕ. Innate immune cells recognize invaded pathogens through a limited number of germline-encoded receptors. These receptors have evolved to recognize pathogen-associated molecular patterns (PAMPs). Recognition of these molecular patterns is through their innate immune receptors, such as Fc-receptors, complement receptors or Toll-like receptors (TLRs) and allows the innate immune system to discriminate between non-self and self molecules. After recognition, these receptors trigger innate immune cells to produce multivalent inflammatory molecules such as cytokines and chemokines. These molecules in turn initiate the process of inflammation, which leads to the recruitment and activation of circulating leukocytes at the infected site. Immune recognition also induces the activation of innate immune cells to differentiate into short-lived effector cells whose main role is to eliminate the infected microorganisms through phagocytosis.

The innate immune response also contributes to the activation of adaptive immunity, and this is mediated by professional antigen presenting cells (APCs). APCs instruct adaptive immune cells, such as cluster of differentiation (CD) 4^+ and $CD8^+$ T lymphocytes, about the presence of non-self Ags through Ag presentation on their surface and the nature of the pathogenic challenge through the expression of pro-inflammatory cytokines or co-stimulatory molecules such as interleukin (IL)-4, IL-5, IL-12, IL-18, CD80, or CD86. Finally, innate immunity lies behind most inflammatory responses in the immune system (Janeway et al., 2001; Janeway et al., 2002).

1-2. The role of Mϕ in immunity

Mϕ are a heterogeneous group of cells derived from monocytes white blood cells. Blood monocytes migrate into various tissues of the body and there differentiate into macrophages. There are various subsets of Mϕ, such as synovial Mϕ in the joints, alveolar Mϕ in the lung, Kupffer cells in the liver and microglia in the central nervous system (CNS), with differing morphology and function depending upon their localization (Radzun et al. 1988). Originally, Mϕ have been known as scavenger-cells in organisms. Mϕ show phagocytic activity after recognition of foreign or damaged materials with surface receptors such as Fc-receptors, complement receptors, or TLRs. However, many studies revealed that Mϕ play a central role in the regulation of immune system at various loci in the body. Mϕ induce inflammation locally in the tissues by producing multivalent pro-inflammatory molecules such as chemokines, proteinases, prostaglandins, tumor necrosis factor (TNF), interleukin (IL)-1, oxygen free radicals, and nitric oxide ($NO^{\cdot}$), which subsequently results in the recruitment and activation of other immune cells (Hartung et.al, 1992; LeBlanc et al., 1990). Mϕ can also function as APCs to T lymphocytes to induce Ag specific adaptive immunity, and modulate the immune balance between T-helper (Th) 1 and Th2 through the secretion of innate cytokines such as IL-12 or IL-18 (Matsumoto et al., 1986).

There is increasing evidence that through their various immune functions, Mϕ are involved in inflammatory diseases such as insulin dependent type-I diabetes (IDDM), experimental allergic encephalomyelitis (EAE), and rheumatoid arthritis (RA) (Jun et al. 1999; Matsumoto et al, 1986). Mϕ present self-Ags to auto-reactive T-cells and produce variety of soluble mediators, such as IL-1β, TNF-α, IL-6, free radicals and

proteinases, which have a direct cytotoxic effect on target cells. Thus, Mϕ play a central role in regulation of immunity.

1-3. Innate cytokines in immunity

Innate cytokines are pro / anti-inflammatory cytokines produced mainly by innate immune cells such as Mϕ. These include interferon (IFN)-α/β, tumor growth factor (TGF)-β, TNF, IL-1α/β, IL-10, IL-12, IL-15, and interferon gamma inducing factor (IGIF: IL-18). This cytokine production is triggered by the recognition of PAMPs by innate immune cells. These cytokine molecules mediate multivalent immune responses such as chemotaxis of circulating leukocytes, activation of chemoattracted immune cells, and modulation of immune balance between Th1 and Th2. Moreover, through immune suppression, some of these cytokines inhibit suboptimal immune responses and protect against harmful effects resulted from toxic levels or combinations of cytokines (Biron et al., 1999). Therefore, innate cytokines appear to be crucial soluble factors in the regulation of immune responses.

1-3-1. Immunobiology of IL-12 and IL-18

1-3-1-1. Immunobiology of IL-12

IL-12 is a hetero-dimeric cytokine molecule that is composed of p35 and p40 subunits. This cytokine is produced primarily by APCs and exerts immunoregulatory functions on T and natural killer (NK) cells. Studies with IL-12-deficient mice and anti-IL-12 neutralizing antibodies showed that IL-12 is an important cytokine in the host defense against intracellular pathogens (Magram et al., 1996). Intracellular pathogens can be controlled by Th1 immunity and IL-12 appears to be essential factor for the generation of Th1 adaptive immunity. Through inducing the production of IFN-γ from

resting and activated T and NK cells, IL-12 also contribute to the clearance of intracellular pathogens. Besides IFN-γ, IL-12 induces the production of other inflammatory molecules, such as TNF-α, GM-CSF, IL-2 and IL-8, which play an important role in the initiation of inflammation (Gately et al., 1998; Kobayashi et al., 1989). Recently, it was reported that IL-12 induced cellular proliferation and inhibited passive cell death of T-lymphocytes through the phosphatidly inositol-3 kinase (PI3K)/Akt-depenent modulation of cell-cycle molecule and apoptotic molecules (Yoo et al., 2002). IL-12 plays a pivotal role in both regulating immune response against intracellular pathogens and homeostasis of immune system.

IL-12 also appears to be involved in the onset of immuno-pathology. In the inflammatory diseases associated with pathologic Th1 responses, such as multiple sclerosis, IL-12 produced by monocytes and dendritic cells is a crucial factor which influences the development and differentiation of pathogenic auto-reactive Th1 cells (Gately et al., 1998). Moreover, in diseases associated with pathologic Th2 responses, such as allergic asthma, administration of recombinant IL-12 can inhibit allergic airway inflammation and prevents the onset of allergic reaction (Gavett et al., 1995). Finally, IL-12 is one of the crucial cytokine molecules in the regulation of immune responses against both self and non-self Ags.

1-3-1-2. Immunobiology of IL-18

As a new member of IL-1 family, IL-18 is synthesized as a precursor protein (proIL-18) and is processed by the intracellular cysteine protease, caspase-1. Murine macrophages, such as Kupffer cells, splenic macrophages, alveolar macrophages, peritoneal exudate cells, and microglia, secrete functional IL-18 after activation with appropriate stimuli. Although the primary structure of IL-18 shows no homology with

that of IL-12, IL-18 shares some of its biologic activities with IL-12. In collaboration with IL-12, IL-18 stimulates Th1-mediated immune responses, which play a crucial role in the host defense against infection with intracellular pathogens through the induction of IFN-γ. However, it has been reported that IL-18, without help from IL-12, induces the production of Th2 cytokines from NK cells, basophils / mast cells, and T cells and stimulates NK cells and $CD8^+$ T cells to show cytotoxic activity, leading to killing virus-infected cells or tumor cells (Dinarello, 1999; Nakanishi et al., 2001). Studies using IL-18-deficient or IL-18Rα-deficient mice also showed that IL-18 is important for the functional development of NK cells (Hoshino et al., 1999; Takeda et al., 1998). In their reports, the number of NK cells of these IL-18 deficient mice was almost same as that of wild-type mice. However, the cytolytic activity against NK cell targets was significantly reduced in these mice. It was found that IL-18 regulates the cytolytic activity of NK cells through modulating the expression of cytocidal molecules such as perforin, Fas-ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) (Dao et al., 1996; Griffith et al., 1998; Kayagaki et al., 1999; Tsutsui et al., 1996; Wiley et al., 1995).

IL-18 also plays a role in the inflammatory cascade. Through inducing the secretion of pro-inflammatory molecules such as IL-1β, TNF-α, MIP-1α and IL-8 from human peripheral blood mononuclear cell (PBMC) (Puren et al., 1998), IL-18 seems to be involved in the chemoattraction and activation of multivalent immune cells at the inflammatory site.

1-3-1-2-1. IL-18 receptor (IL-18R)-mediated signaling pathway

IL-18R was recently identified and revealed to be expressed by many kinds of immune cells such as myeloid, monocytoid, erythroid and megakary-ocytic cells. IL-

18R shares a signal transduction pathway with IL-1R because the cytoplasmic regions of the receptors for IL-1 and IL-18 are homologous to each other and to members of the Toll-family. The engagement of IL-18R induces recruitment of myeloid differentiation (MyD88) molecules to the cytoplasmic domain of IL-18R, which provides a binding site for IL-1 receptor associated kinase (IRAK). Recruited IRAK autophosphorylates and dissociates from the receptor / MyD88 complex and subsequently interacts with TNFR-associated factor-6 (TRAF6). Activated TRAF6 molecules then interact with NF-kB-induced kinase (NIK) to activate two IkB kinases (IKK-1 and IKK-2) leading to the formation of activated transcription factor NF-kB (p65 homodimer or p65/p50 heterodimer). This signaling pathway is apparently major signaling pathway in IL-18R-mediated signaling (Nakanishi et al., 2001). However, it has been reported that different signaling pathways from this pathway are activated in some cell types.

c-Jun NH_2-terminal kinase (JNK) is activated by IRAK and lead to the activation of another transcription factor AP-1 in NK cells, and T cells (Kanakaraj et al., 1999). Moreover, Kalina et al. reported that mitogen-activated protein kinases (MAPKs) such as p38, p44 $^{erk\text{-}1}$, and p42 $^{erk\text{-}2}$ MAPK are activated and involved in IL-18-induced IFN-γ secretion (Kalina et al., 2000). IL-18 also seems to activate the PI3K/Akt pathway leading to the cell surface expression of vascular cells adhesion molecules (VCAM)-1 in fibroblasts (Neumann et al., 2002). These findings suggest that IL-18 might activate multiple signaling pathways for a variety of IL-18-induced cellular responses in different cell types.

1-4. Immunologic function of chemokine molecules

In the early phase of innate immunity, leukocyte extravasation is an important step for inflammation. Recruitment from the blood compartment involves

interaction of circulating leukocytes with vascular endothelium and response to tissue-derived chemotactic signals. Chemo-attractants induce the adhesion of leukocytes to endothelisal cells and directional locomotion.

As small (8- to 10-kDa) secreted proteins structurally related *via* a four-cysteine motif, chemokines (chemoattractant cytokines) are the molecules which are involved in the chemo-attraction and activation of circulating leukocytes during inflammation. Moreover, these molecules are involved in hemopoiesis, neutrophil degranulation, and T cell activation. In terms of the presence of an intervening amino acid between the first two of the four conserved cysteines, the chemokine superfamily can be divided into two major subfamilies such as α(C-X-C) and β (C-C) chemokines. There are also minor chemokine superfamilies, which lack the typical cysteine distribution. These are γ (C) and δ (CX_3C) chemokines represented by lymphotactin and fractalkine, respectivley. C chemokine lymphotactin, which lacks two of the four conserved cysteines, has chemotactic activity for lymphocytes. C-C chemokines, which consist of eotaxin, RANTES, monocyte chemotactic protein (MCP)-1/2/3/4, and MIP-1α/β, are monocyte, lymphocyte, basophil, eosinophil, and NK cell chemoattractants, and C-X-C chemokines, which consist of IL-8, GROα/β/γ, NAP-2, ENA78, GCP-2, IP10, Mig, and SDF-1, are known to be potent neutrophil chemoattractants. The CX_3C chemokine fractalkine is composed of two different forms: one is a membrane-bound form and the other is a soluble form. The expression of membrane-bound form of fractalkine is induced on activated primary endothelial cells, which promotes strong adhesion of leukocytes. In contrast, the soluble form of fractalkine is known to be a potent chemoattractant for T-cells and monocytes in immune system (Baggiolini et al., 1997; Rollins, 1997).

1-4-1. Immunobiologic role of MCP-1

MCP-1 (CCL2) was the first CC-chemokine to be characterized biologically and shown to attract monocytes. Until now, four related chemokine molecules (MCP-2, 3, 4 and 5) have been subsequently identified. These molecules share a pyroglutamate proline NH2-terminal motif and are structurally closely related to each other. However, MCP-1 has generally been observed to be the most potent and efficious chemo-attractant than other MCP-molecules. MCP-1 is produced by almost all cells such as Mϕ, smooth muscle cells, endothelial cells and fibroblasts, but its targets are limited to monocytes / macrophages, basophils, mast cells, T lymphocytes, NK cells, and dendritic cells that express CCR2 (Baggiolini et al., 1997; Rollins, 1997). MCP-1 was first designated monocyte chemotactic and activating factor because it stimulates chemotactic migration of human monocytes and activates monocytes to kill tumors *in vitro* (Valente et al., 1988). Although MCP-1 was first identified as a monocyte chemo-attractant, it was later identified as a major chemo-attractant for T cells of the activated memory phenotype ($CD45RA^-/CD45RO^+$/CD29/L-selectin and $CD26^+$). MCP-1 is also an activator of the adhesion of T lymphocytes to fibronectin through activation of β_1 integrins (Carr et al., 1994), and known as an inducer of degranulation and histamine-release from recruited leukocytes at the inflammatory site. Studies with MCP-1 knock-out mice also revealed that MCP-1 plays a pivotal role in the differentiation of Th2 adaptive immunity. MCP-1 knock-out mice showed decreased production of pro-inflammatory cytokine, such as IL-4, in draining lymph node cells and increased resistance to *Leishmania* infection, which is indicative of a shift from Th2 to Th1 immunity (Gu et al., 2000; Luther et al., 2001).

MCP-1 molecules are also involved in the immunopathogenesis. It was reported that MCP-1 expression was located in the injured tissue of allergic asthma, inflammatory bowl disease and allogeneic transplant rejection (Adams et al., 1993;

Brown et al., 1996; Grimm et al., 1996; Kondo et al., 1996; Pattison et al., 1994; Sousa et al., 1994; Teran et al., 1996). The presence of inflammatory cells in the joints of patient with RA can be explained in part by MCP-1 expression in the synovial fluid (Koch et al., 1992). Moreover, in experimental autoimmune encephalomyelitis, which is experimental animal model of human multiple sclerosis, the expression of MCP-1 was found immediately before the appearance of infiltrating cells in the central nerve system (CNS) (Godiska et al., 1995; Ransohoff et al., 1993; Ransohoff et al., 1994). Finally, these results suggest that MCP-1 is fundamental chemokine molecule in the regulation of the onset and progression of immune responses against both self and non-self Ag.

2. Hypothesis

As pro-inflammatory cytokines produced by Mϕ, IL-12 and IL-18 are known to be involved in the modulation and activation of adaptive immune response (Dinarello, 1999; Nakanishi et al., 2001). However, the immuno-biologic function of these cytokines in the innate immunity has not been clearly revealed yet. Recently, it was reported that these cytokine molecules can induce the activation of innate immune cell, Mϕ and that these activated Mϕ produce biologically active inflammatory molecules such as IFN-γ and $NO^{\cdot}$, which have an important role in the clearance of invaded pathogens at the early phase of infection (Munder et al., 1998). This result suggests that IL-12 and IL-18 may play a pivotal role in the regulation of innate immunity through autocrine activation of Mϕ.

Innate immunity initiates by chemoattraction and activation of circulating leukocytes at the inflammatory site, which is regulated by chemokines such as MCP-1. Produced by almost all cells, including Mϕ, MCP-1 molecules are known as a key regulator molecule in the initiation step of innate immunity through modulating

agranulocyte (monocyte/T cells)-chemoattraction, de-granulation and histamine-release from circulating leukocytes at the inflammatory site (Rollins, 1997).

Thus, the hypothesis of this study was that **both IL-12 and IL-18 might actively regulate the innate immunity by inducing the recruitment and activation of circulating leukocyte *via* MCP-1 production in Mϕ.**

3. Objectives

3-1. Overall objective

To examine whether or not IL-12 and IL-18 can induce MCP-1 secretion in murine Mϕ and, if so, to find molecular mechanisms involved in MCP-1 induction.

3-2. Specific objectives

3-2-1. Specific Objective 1: To optimize Mϕ-purification / *in vitro* -culture protocol to minimize non-specific Mϕ–activation.

3-2-2. Specific Objective 2: To determine whether or not IL-12 and IL-18 can induce MCP-1 secretion in murine Mϕ.

3-2-3. Specific Objective 3: To investigate the molecular mechanisms through which IL-12 and IL-18 induces MCP-1 secretion in murine Mϕ.

3-2-3-1. Specific Objective 3-1: To determine whether IL-12 or IL-18-induced MCP-1 secretion is mediated by autocrine expression of IFN-γ.

3-2-3-2. Specific Objective 3-2: To investigate the signaling mechanism through which IL-18 induces MCP-1 secretion in murine Mϕ.

3-2-3-2-1. Specific Objective 3-2-1: To elucidate the signaling components of the IL-18R in murine Mϕ.

3-2-3-2-2. Specific Objective 3-2-2: To determine which signaling pathway is involved in IL-18-induced MCP-1 secretion in murine Mϕ

3-2-3-2-3. Specific Objective 3-2-3: To determine the cytotoxicity of each pharmacological inhibitor to murine Mϕ.

CHAPTER 2. MATERIALS AND METHODS

1. Animals

Eight-week old male C57BL/6J and B6.129S7-*Ifng*^{tm1Ts}/J mice were purchased from Taconic Farms and the Jackson Laboratory, respectively. The animals were maintained under specific pathogen-free conditions and provided with sterile food and water at the Animal Resource Centre, Faculty of Medicine, University of Calgary.

2. Antibodies and chemicals

For MACS, biotinylated anti-mouse CD11b, CD11c, B220, CD3e and DX5 Abs were purchased from BD PharMingen (San Diego, CA) and anti-biotin microbeads were obtained from Miltenyi Biotec (Auburn, CA). For flow cytometric analysis, anti-mouse CD11b-FITC Ab was purchased from BD PharMingen. Recombinant mouse IL-12 and IL-18 were purchased from Peprotech (Ottawa, Ontario) and Medical & Biological Lab (Nagoya, Japan), respectively. General reagents used were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. Inhibitors such as LY294002 and PD98059 were purchased from Calbiochem (La Jolla, CA). RPMI 1640 and FBS were purchased from Life Technologies (Grand Island, NY) and HyClone Laboratories (Logan, UT), respectively. For in vitro chemotaxis assay, anti-mouse JE/CCL2 blocking Ab was purchased form R&D Systems (Minneapolis, MN), and 6.5 mm Transwells with 5 μm Pore Polycarbonate Membrane Inserts were purchased from Corning Costar (Acton, MA). For immunoblot assay, rabbit Abs against p65, p38 MAPK, Erks, Akt, SAPK / JNK, phosphorylated-p65 (Ser^{536}), phosphorylated-p38 MAPK (Thr^{180}/Tyr^{182}), phosphorylated-Erks (Thr^{202}/Tyr^{204}), phosphorylated-Akt (Ser^{473}/Thr^{308}) and phosphorylated-SAPK / JNK (Thr183/Tyr185) were all purchased from New England Biolabs (Beverly, MA). Nucleopore (0.8 μm) polycarbonate membranes were purchased from Fisher Scientific (Pittsburgh, PA). Mouse MCP-1 and

IFN-γ ELISA kits were purchased from R&D Systems. NE-PER Nuclear and Cytoplasmic extraction reagents was purchased from PIERCE (Rockford, IL).

3. Puification of peritoneal Mϕ

Peritoneal Mϕ were purified and used for this study. Briefly, 4 days after 4 % thioglycolate treatment (2 ml) through i.p-injection, whole peritoneal cells were harvested and then treated with ammonium chloride lysing buffer to clear RBC. For Mϕ-purification through the plate-attachment method, cells (1 x 10^6/well) were resuspended with RPMI-1640 media supplemented with 2 % heat-inactivated FBS (Gibco) and then seeded into 24-well cell-culture plates. Six hrs after incubation at 37°C in 5 % CO_2 humidified incubator, unattached cells were washed out using cold PBS, and the remaining cells were used as purified Mϕ (purity $\geq$ 96%). For Mϕ-purification through MACS, thioglycolate-elicited cells were treated with biotinylated anti-CD11c, CD3e, DX5 and B220 Abs and then incubated with anti-biotin microbeads. After that, Mϕ were purified by negative selection using the MACS Separation System. Cell-purity was assessed by flow cytometric analysis on a FACScan flow cytometer (BD Biosciences, Heidelberg, Germany) after CD11b and F4/80-staining (purity≥98%).

4. Enzyme-linked immuno-sorbent assay (ELISA) for mouse IFN-γ and MCP-1 measurement

After purification by MACS, peritoneal Mϕ (1 x 10^6 cells / well) were placed on 0.8 μm polycarbonate filters (Costar,Cambridge MA), floated on RPMI-1640 media supplemented with 2 % FBS, 2 mM glutamine, 5 x 10^{-5}M β-mercaptoethanol, 100 U / ml penicillin and 100 μg / ml streptomycin, and incubated for 24 hr at 37°C in

5 % CO_2 humidified incubator. Mϕ were treated with indicated concentrations of cytokines in the presence or absence of the inhibitors. At 48 hrs after stimulation, conditioned media were harvested and used for the quantification of both IFN-γ and MCP-1 production through sandwich ELISA as described in the manufacturer's manual. Briefly, conditioned media and standards were added to ELISA-plates coated with capture-Abs. At 2 hrs after incubation, the plates were washed several times with washing buffer and then treated with HRP-conjugated detection-Abs. The plates were incubated for additional 2 hrs at 37 °C. After extensive washings, color reagents were then added and incubated. The reaction was stopped after 20 min and the absorbance at 450 / 540 nm was measured in an ELISA reader. The optical density read at 540 nm was subtracted from that of 450 nm to correct for optical imperfections in the plate.

5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

At 6 hrs after stimulation of Mϕ with cytokines in the presence or absence of the inhibitors, total RNA was harvested using Trizol-buffer as described in the manufacturer's protocol. Harvested RNA (0.5 μg) was used to synthesize cDNA using Superscript II reverse transcriptase and oligo$(dT)_{12\text{-}18}$. PCR was then performed using specific primers for both murine MCP-1 and HPRT. The sense- and antisense-primers used were as follows: MCP-1: sense, 5'-AGAGAGCCAGACGGGAGGAA-3', antisense, 5'-GTCACACTGGTCACTCCTAC-3', HPRT: sense, 5'-GTAATGATCAGT CAACGGGGGAC-3', antisense, 5'-CCAGCAAGCTTGCAACCTTAACCA-3'.

The PCR-condition was optimized for each set of primers. The reaction was initially denatured at 95°C for 5 min, then subjected to each cycle (MCP-1: 30 cycles, HPRT: 33 cycles) of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min before a final extension at 72°C for 5 min. The products

were then subjected to electrophoresis on a 1 % agarose gel and detected by ethidium bromide staining.

6. Preparation of cell-extracts and immuno-blot analysis

Peritoneal Mϕ (1 x 10^6 cells / sample) were treated with medium alone or the indicated concentration of cytokines in the presence or absence of each inhibitor. Cells were then harvested at each time point, and whole cell-lysates were prepared as described previously (Baek et al., 2001; Yoo et al., 2002). To quantify the phosphorylation of Akt, Erks, JNK, p38 MAP kinases and p65, whole-cell lysates were subjected to electrophoresis in a 10 % polyacrylamide gel containing 0.1% SDS (SDS-PAGE). After that, western blots were immunoanalyzed as described previously using specific Abs against the phosphorylated form of each protein. To confirm that the same amount of cellular protein had been loaded in each lane, the primary / secondary-Ab complex was removed by incubating the blot in Mild Ab Stripping Solution (Chemicon, Temecula, CA) for 15 min at 37°C. The blots were then subjected to autoradiography to confirm that the Ab signal had been removed. After this procedure, the blots were reprobed with the specific Abs against total Akt, Erks, p38 MAP kinase, JNK and p65.

To detect nuclear translocation of p65 molecules, cells were harvested at indicated times and nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic extraction reagents as described in the manufacturer's manual. Briefly, after cytokine stimulation, cells were harvested by centrifugation at 500 x g. Pellets were then treated with ice-cold CER-I and CER-II buffers. At 10 min after incubation on ice, the resuspended pellets were subjected to centrifugation to discard the cytoplasmic fraction. After discarding supernatants, the remaining pellets were then treated with NER-buffer and incubated on ice. Forty minutes later, homogenized

samples were subjected to centrifugation at 16,000 x g, the supernatant was harvested and used as nuclear extract. The nuclear extract was subjected to electrophoresis in a 10 % polyacrylamide gel containing 0.1% SDS, and western blots were immuno-analyzed using specific Abs against NF-kB p65 molecule.

7. Fluorescence activating cell sorter (FACS)-analysis to detect apoptotic cell death of Mϕ

At 48 hrs after in vitro IL-18-stimulation, Mϕ were harvested to determine the apoptotic cell death of Mϕ. Cells were fixed in 50 % ethanol, washed in PBS and stained with PI (10 μg/ml: final concentration) in the presence of 50 μg/ml RNase A for 20 min at room temperature, followed by the analysis of a cell-cycle profile on FACScan (Becton Dickinson, San Jose, CA).

8. Statistical analysis

The statistical significance of differences between groups was analyzed by Student's t test. A level of $P < 0.05$ was accepted as significant. Data were expressed as means ± SD.

CHAPTER 3. RESULTS

1. Mϕ purification and *in vitro* culture protocol using magnetic activated cell sorting (MACS) and nucleopore polycarbonate membrane significantly decreased non-specific activation of murine Mϕ.

For studies on the role of IL-12 and IL-18 in activating Mϕ upon exposure to inflammatory signals, murine Mϕ were purified from the peritoneal cavity of 8-week-old C57BL/6 male mice through the commonly used plate-attachment method (Fig. 1). Briefly, 4 % thioglycolate was injected into 8-week old C57BL/6 male mice through i.p (intra-peritoneal) injection. At 4 days after injection, peritoneal cells were harvested through peritoneal cavity-washing with RPMI-1640 media. To purify Mϕ, these harvested peritoneal cells were seeded into cell-culture plates (Costar 24-well tissue culture plate) and incubated in RPMI-1640 media supplemented with 2 % FBS. At 6 hrs after plate-attachment, non-adherent cells were washed out using cold PBS, the remaining plate-attached Mϕ (purity $\geq$ 96 %) were incubated in RPMI-1640 media supplemented with 2 % FBS and used for further study.

To determine whether IL-12 and IL-18 can induce MCP-1 production from murine Mϕ, MCP-1 RT-PCR (Reverse Transcription-Polymerase Chain Reaction) was performed using RNA isolated from IL-12 and / or IL-18-stimulated Mϕ. However, it was difficult to determine differences in MCP-1 gene expression levels between cytokine-treated and non-treated Mϕ because the background expression levels of MCP-1 in Mϕ without treatment were so high (Fig. 2). It was also found that plate attachment itself induces the non-specific activation of the serine/threonine kinase Akt (Fig. 3). From these results, it was concluded that plate attachment induces non-specific activation of purified Mϕ and therefore, this Mϕ purification and *in vitro* culture protocol is not suitable for study of the role of

IL-12 and IL-18 in activating Mϕ.

Because plate attachment can activate Mϕ through the engagement of cell-adhesion molecules expressed on the cell surface, Mϕ purification and *in vitro* culture protocol using other methods were required. For this purpose, both MACS and nucleopore (0.8 μm) polycarbonate membranes were used for Mϕ purification and *in vitro* culture, respectively. Briefly, at 4 days after injection of 4 % thioglycolate, total peritoneal cells were harvested and then non-Mϕ cells (T cells, NK cells, B cells and dendritic cells) were depleted through positive selection using MACS. Purified Mϕ (purity ≥ 98 %) were then seeded on the nuclepore (0.8 μm) polycarbonate membrane and floated on the complete RPMI-1640 media supplemented with 2 % FBS for *in vitro* culture (Fig. 4). As shown in Figs. 5 and 6, almost no expression of MCP-1 or activation of Akt was observed in untreated Mϕ, suggesting that this modified protocol significantly decreased non-specific activation of purified Mϕ. In addition, it was found that from 12 hrs after incubation on polycarbonate membrane, the non-specific activation of MAPKs (p38, p44 $^{erk\text{-}1}$ and p42 $^{erk\text{-}2}$) was also significantly decreased (Fig. 7). Based on these results, it was decided to use this modified Mϕ purification and *in vitro* culture protocol for subsequent studies (Fig. 8).

Fig 1. Schematic diagram of Mϕ purification and *in vitro* culture protocol using the plate-attachment method. Four % thioglycolate was injected into 8-week-old C57BL/6 male-mice through i.p injection. At 4 days after injection, total peritoneal cells were harvested and seeded into 24-well culture-plates ($1x10^6$ cells / well). At 6 hrs after plate attachment, non-adherent cells were washed out using cold PBS and the remaining cells were used for further study as purified Mϕ (purity $\geq$ 96 %).

4 % thioglycolate
i.p injection (2 ml)

Eight-Week-old

Male C57BL/6 mice

↓

Four days after injection, total peritoneal cells are harvested.

↓

Cells are seeded ($1x10^6$ / well)

into 24-well cell-culture plates.

↓

Six hrs after attachment, unattached cells are washed out.

↓

Purified plate-bound Mϕ

(purity≥96%)

Fig 2. Mϕ purification and *in vitro* culture using the plate-attachment method showing high background expression level of MCP-1. Peritoneal Mϕ purified using the plate-attachment method were treated with media alone (-) or IL-12 and/or IL-18 (+) (10 ng / ml). At 6 hrs after cytokine-treatment, total RNAs were harvested and MCP-1 RT-PCR was performed as described in *Materials and Methods.* HPRT was amplified as an internal control. The expected size of PCR product for MCP-1 and HPRT was 530 bp and 176 bp, respectively. SM: 100 bp DNA ladder. Data are representative of three independent experiments.

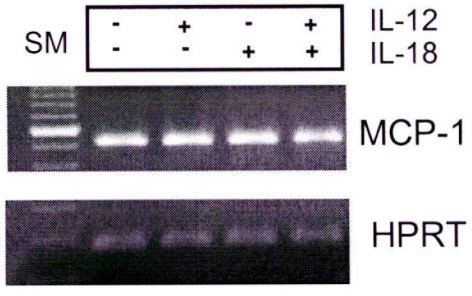

SM
- + - +
- - + +
IL-12
IL-18
MCP-1
HPRT

Fig 3. Mϕ purification and *in vitro* culture using the plate-attachment method showing high background activation level of Akt-kinase. Peritoneal Mϕ purified by the plate-attachment method were treated with media alone. At the indicated times, cells were harvested using cell-lysis buffer, and western blots were performed using anti-Akt (Akt) or anti-phosphorylated Akt (pAkt) Abs. Data are representative of three independent experiments.

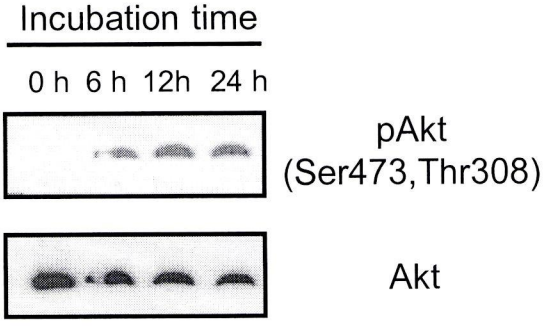

Incubation time
0 h 6 h 12h 24 h
pAkt
(Ser473,Thr308)
Akt

Fig 4. Schematic diagram of Mϕ purification and *in vitro* culture protocol using MACS and nucleopore polycarbonate membrane. Four % thioglycolate was injected into 8-week-old B6 male mice through i.p injection. At 4 days after injection, peritoneal cells were harvested and Mϕ were purified by negative selection using the MACS Separation System. Purified Mϕ (purity ≥ 98%) were seeded on polycarbonate filter membranes ($1x10^6$ cells / disc), floated on RPMI-1640 media supplemented with 2 % serum and used for further study.

4 % thioglycolate
i.p injection (2 ml)

Eight-Week-old
Male C57BL/6 mice

↓

Four days after injection, total peritoneal cells are harvested

↓

Mϕ-negative selection through MACS with anti-CD3e, B220, CD11c and DX5 Abs

↓

Purified Mϕ (purity≥98%)

↓

Purified Mϕ are seeded on 0.8 μm polycarbonate membrane ($1x10^6$ cells/disc).

↓

Membranes are floated on RPMI-1640 media supplemented with 2 % FBS.

Fig 5. Mϕ purification and *in vitro* culture using MACS and nucleopore poly-carbonate membrane showed significantly decreased background expression level of MCP-1. Purified Mϕ on the polycarbonate membranes were treated with media alone (-) or IL-12 and/or IL-18 (+) (10ng / ml). At 6 hrs after cytokine-treatment, total RNA was harvested and MCP-1 RT-PCR was performed as described in *Materials and Methods*. HPRT was amplified as an internal control. SM: 100 bp DNA ladder. Data are representative of three independent experiments.

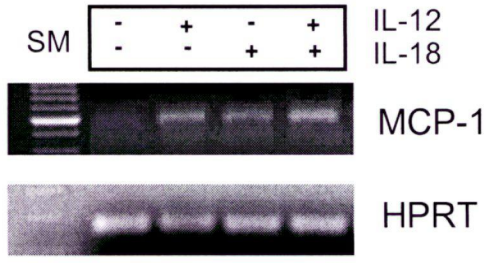

SM
- + - +
- - + +
IL-12
IL-18
MCP-1
HPRT

Fig 6. Mϕ purification and *in vitro* culture using MACS and nucleopore poly-carbonate membrane showed significantly decreased background activation level of Akt-kinase. Purified Mϕ on polycarbonate membranes were treated with media alone. At the indicated times, cells were harvested using cell-lysis buffer and western blots were performed using anti-Akt (Akt) or anti-phosphorylated Akt (pAkt) Abs. Data are representative of three independent experiments.


Incubation time
0 h 6 h 12 h 24 h
pAkt
(Ser473,Thr308)
Akt

Fig 7. Background activation level of MAPKs was significantly decreased after 12 hrs of incubation on the polycarbonate membrane. Purified Mϕ on polycarbonate membranes were treated with media alone. At the indicated times, cells were harvested using cell-lysis buffer and western blots were performed using: (A) anti-p38 MAPK (p38 MAPK) or anti-phosphorylated p38 MAPK (pp38MAPK); (B) anti-ERK1/2 (ERK1/2) or anti-phosphorylated ERK1/2 (pERK1/2) Abs. Data are representative of two independent experiments.

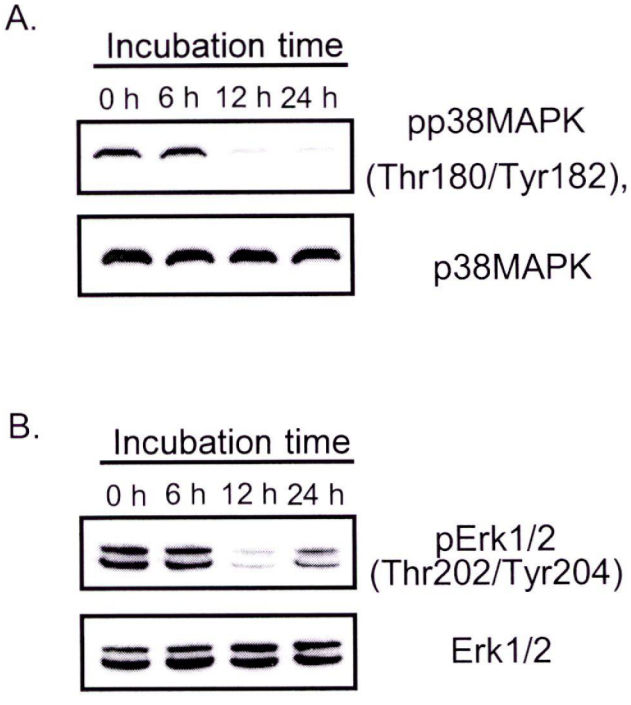
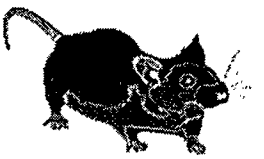

A.
Incubation time
0 h 6 h 12 h 24 h
pp38MAPK
(Thr180/Tyr182),
p38MAPK
B.
Incubation time
0 h 6 h 12 h 24 h
pErk1/2
(Thr202/Tyr204)
Erk1/2

Fig 8. Schematic diagram of optimized Mϕ purification and *in vitro* culture protocol using MACS and nucleopore polycarbonate membrane. Four % thioglycolate (2 ml) was intra-peritoneally injected into 8-weeks old B6 male-mice. At 4 days after injection, whole peritoneal cells were harvested and Mϕ were purified by negative selection using the MACS Separation System. Purified-Mϕ (purity≥98%) were seeded on polycarbonate membranes (1×10^6 cells / disc) and floated on RPMI-1640 media supplemented with 2 % FBS. Cells were rested for additional 24 hrs and used for further study.



4 % thioglycolate
i.p injection (2 ml)

Eight-Week-old
Male C57BL/6 mice

↓

Four days after injection, total peritoneal cells are harvested

↓

Mϕ-negative selection through MACS with anti-CD3e, B220, CD11c and DX5 Abs

↓

Purified Mϕ (purity≥98%)

↓

Purified Mϕ are seeded on 0.8 μm polycarbonate membrane ($1x10^6$ cells/disc).

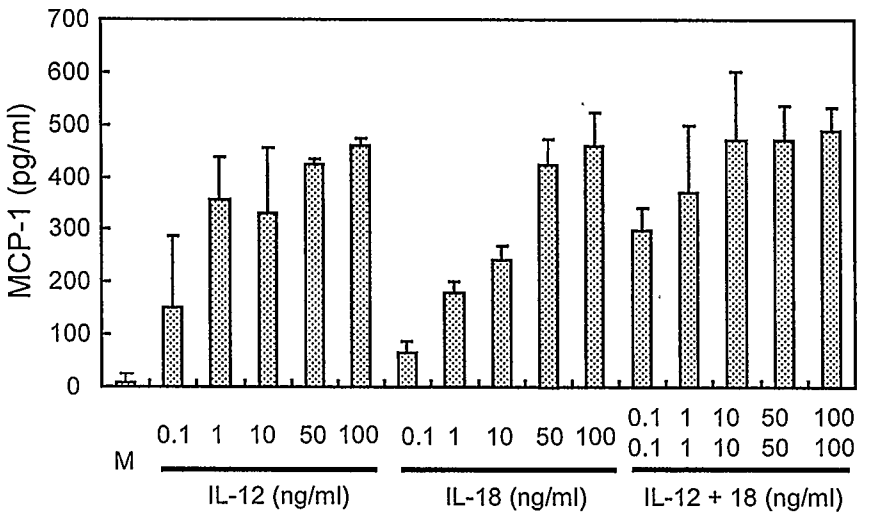
↓

Membranes are floated on RPMI-1640 media supplemented with 2 % FBS, and Mϕ are rested for additional twenty-four hrs.

2. IL-12 and IL-18 independently induce MCP-1 secretion in murine Mϕ.

With the optimized Mϕ purification and *in vitro* culture protocol, it was determined whether or not IL-12 and IL-18 can induce MCP-1 secretion in Mϕ. Purified peritoneal macrophages from C57BL/6J mice were treated with different concentrations of IL-12 and / or IL-18 (0.1, 1, 10, 50, or 100 ng/ml) for 48 hrs. Conditioned cell-culture media were then harvested and the secretion of MCP-1 was examined by MCP-1 ELISA. It was found that both of these cytokines induced the MCP-1 secretion into culture media in a dose-dependent manner (Fig. 9). However, there was no synergistic effect of these two cytokines on the MCP-1 secretion in Mϕ. Based on these results, it was concluded that IL-12 and IL-18 independently induce the MCP-1 secretion in murine Mϕ.

Fig. 9 IL-12 and IL-18 independently induce MCP-1 secretion in murine Mϕ. Mϕ were treated with media alone (M) or indicated concentrations of IL-12 and/or IL-18. At 48 hrs after cytokine treatment, conditioned media was harvested and used for MCP-1-ELISA as described in *Materials and Methods.* Data are shown as the mean ± SD of triplicate cultures and are representative of at least five independent experiments.


MCP-1 (pg/ml)
700
600
500
400
300
200
100
0
M
0.1 1 10 50 100
0.1 1 10 50 100
0.1 1 10 50 100
0.1 1 10 50 100
IL-12 (ng/ml)
IL-18 (ng/ml)
IL-12 + 18 (ng/ml)

3. IL-12 and IL-18 induce MCP-1 secretion in murine Mϕ *via* autocrine IFN-γ–dependent or -independent mechanisms, respectively.

Both IL-12 and IL-18 are known to be IFN-γ-inducing factors (Biron et al., 1999; Okamura et al., 1995), and the secretion of MCP-1 can be up-regulated by IFN-γ (Zhou et al., 1998). Thus, it was determined whether MCP-1 secretion induced by IL-12 or IL-18 is mediated by autocrine expression of IFN-γ. Initially, the IFN-γ production was verified by ELISA in culture supernatants of Mϕ treated with different doses of IL-12 or IL-18. It was found that small amounts of IFN-γ were secreted from IL-12-stimulated Mϕ. However, IFN-γ secretion was not induced in IL-18-stimulated Mϕ, regardless of the concentration of IL-18 (Fig. 10 A and B). These results suggest that IFN-γ may contribute to IL-12-induced MCP-1 secretion in Mϕ, but not to IL-18-induced MCP-1 secretion. To determine autocrine IFN-γ dependency of IL-12 or IL-18-induced MCP-1 secretion, MCP-1 production was examined by ELISA in the culture supernatant of IL-12 or IL-18-treated Mϕ derived from IFN-γ gene-deficient mice (B6.129S7-*Ifng*^{tm1Ts}/J). The IFN-γ gene-deficiency was confirmed by IFN-γ ELSIA with appropriate stimuli described previously (Munder et al., 1998) for IFN-γ secretion in Mϕ (Fig. 11). Through this knockout-mouse study, it was found that IL-12 could not induce MCP-1 secretion in Mϕ derived from the IFN-γ deficient mice. In contrast, there was no significant difference in the amount of MCP-1 secreted by Mϕ stimulated with IL-18 between wild-type and IFN-γ knockout mice (Fig. 12). Taken together, these results indicate that IL-12 induces MCP-1 secretion in murine Mϕ *via* an autocrine IFN-γ–dependent mechanism, whereas IL-18-induced MCP-1 secretion is independent of autocrine IFN-γ. The production of MCP-1 by IL-18 stimulation may be mediated by direct IL-18R signaling.

Fig 10. IL-12, but not IL-18, induces IFN-γ production in murine Mϕ. Mϕ were treated with media alone (M) or indicated concentration of (A) IL-12 or (B) IL-18. At 48 hrs after cytokine-treatment, conditioned media were harvested and used for IFN-γ ELISA as described in *Materials and Methods.* Data are shown as the mean ± SD of triplicate cultures and representative of three independent experiments.

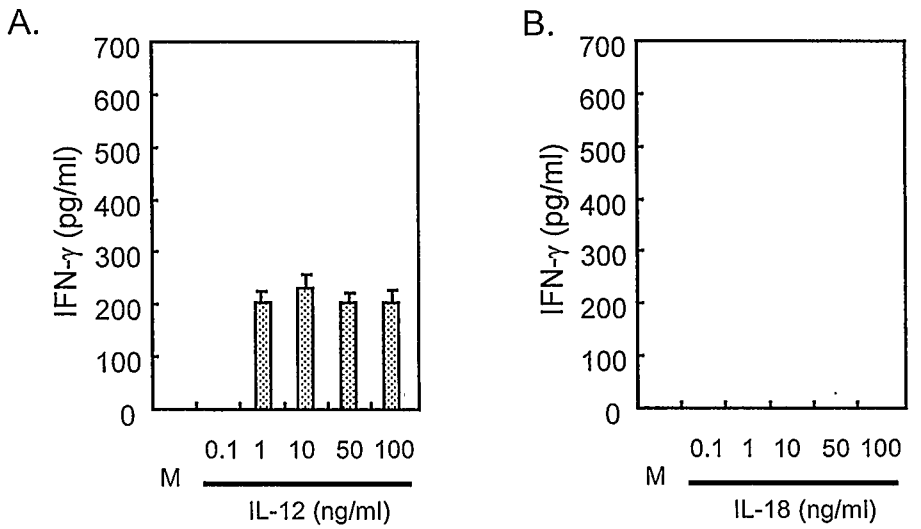

A.
IFN-γ (pg/ml)
700
600
500
400
300
200
100
0
0.1 1 10 50 100
M
IL-12 (ng/ml)
B.
IFN-γ (pg/ml)
700
600
500
400
300
200
100
0
0.1 1 10 50 100
M
IL-18 (ng/ml)

Fig 11. Mϕ from IFN-γ knockout mice do not produce IFN-γ upon appropriate stimuli. Peritoneal Mϕ from wild type (WT) or IFN-γ knockout mice (KO) were treated with media alone (M) or IL-12 and IL-18 (100 ng /ml for each). At 48 hrs after cytokine treatment, conditioned media was harvested and used for IFN-γ ELISA as described in *Materials and Methods.* Data are shown as the mean ± SD of triplicate cultures and representative of three independent experiments.

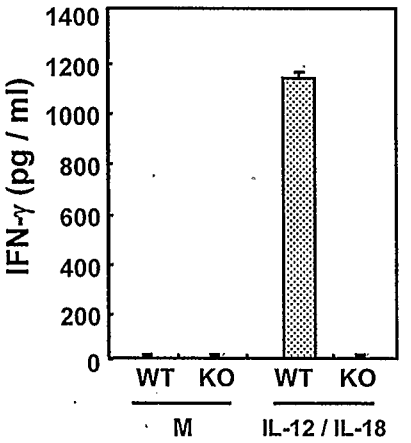
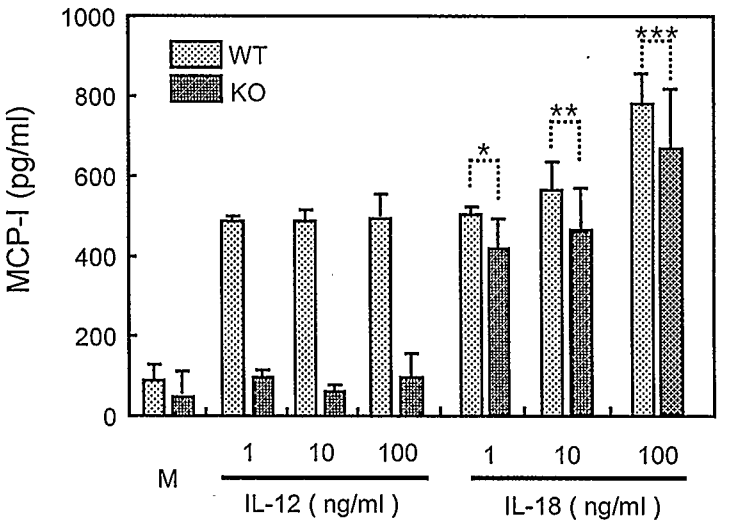

1400
1200
1000
800
600
400
200
0
IFN-γ (pg / ml)
WT
KO
WT
KO
M
IL-12 / IL-18

Fig 12. IL-12 and IL-18 induce MCP-1 secretion in murine Mϕ through IFN-γ–dependent or -independent mechanism, respectively. Peritoneal Mϕ from wild-type (WT) or IFN-γ knockout mice (KO) were treated with media alone (M) or indicated concentrations of IL-12 or IL-18. At 48 hrs after cytokine treatment, conditioned media was harvested and used for MCP-1 ELISA as described in *Materials and Methods.* Data are shown as the mean ± SD of triplicate cultures and representative of three independent experiments. (*: $p<0.12$, **:$p<0.15$, ***:$p<0.4$)


1000
800
600
400
200
0
MCP-I (pg/ml)
WT
KO
*
**
***
M
1
10
100
1
10
100
IL-12 (ng/ml)
IL-18 (ng/ml)

4. IL-18R engagement activates both the PI3K/Akt and MEK/ERK1/2 signaling pathways in murine Mϕ.

It is known that the IL-18 receptor is a member of the IL-1R family, which shares a signaling cascade of sequential recruitment of myeloid differentiation 88 (MyD88) and IL-1R-associated kinase (IRAK), followed by activation of IκB kinase (IKK), degradation of IκBα , and release of NF-κB p65 to translocate into the nucleus (Nakanishi et al., 2001). Therefore, it was examined whether the IKK/NF-κB pathway is activated by IL-18 in murine Mϕ. The treatment with lipopolysaccharide (LPS: 1 μg/ml), which is a well known stimulant of NF-κB molecule (Feng et al., 1999), clearly induced IκBα–degradation, nuclear-translocation and phosphorylation on Serine-536 of NF-κB p65 molecules (Fig. 13A - C). However, Mϕ stimulated with IL-18 (100 ng/ml) did not exhibit any detectable change in the amount of IκBα as compared to un-stimulated Mϕ (Fig. 13A), indicating that IκBα is not degraded. Moreover, the amount of NF-κB p65 in the nuclear extracts of IL-18-stimulated Mϕ was not different from that of unstimulated Mϕ (Fig. 13B), indicating that NF-κB p65 was not translocated into the nucleus. It was then determined whether or not NF-κB was activated by examining phosphorylated NF-κB p65 (Serine-536) through immuno-blot analysis. No phosphorylated NF-κB p65 was detected in the cytoplasm (Fig. 13C). All these results consistently show that the IKK/NF-κB signaling pathway is not activated by IL-18 in Mϕ.

IL-18 is also known to induce the activation of several signaling molecules such as the PI3K/Akt in synovial fibroblasts and MAPKs and JNK in a human natural killer cell line (Kalina et al., 2000; Kanakaraj et al., 1999; Morel et al., 2001; Morel et al., 2002). Therefore, it was determined whether or not the PI3K/Akt pathway is activated by IL-18 in Mϕ. Stimulation of Mϕ with IL-18 increased the

phosphorylation of Akt, and treatment with the PI3K-inhibitor, LY294002, completely inhibited this phosphorylation (Fig. 14A), indicating that IL-18 induces the activation of the PI3K/Akt pathway in Mϕ. Subsequently, the activation of p38 MAPK, ERK1/2, and JNK in IL-18-stimulated Mϕ was examined, and it was found that only the MEK/ERK1/2 pathway was activated, as evidenced by the increase in phosphorylated ERK1/2 after IL-18 treatment (Fig. 14B - D). Phosphorylation of ERK1/2 was blocked by treatment with the MEK inhibitor, PD98059 (Fig. 14D). As previously reported (Feng et al., 1999), LPS-stimulation clearly induced the activation of both p38MAPK and JNK kinases. However, neither phosphorylated p38 MAPK nor phosphorylated JNK was detected in IL-18-stimulated Mϕ (Fig. 14B and C), indicating that IL-18 does not activate these pathways in murine Mϕ.

Fig 13. IL-18 does not activate the IKK/NF-κB signaling pathway in murine Mϕ. Purified Mϕ from C57BL/6J mice were treated with media alone (M), LPS (1 μg/ml) or IL-18 (100 ng/ml) for the indicated times. (B) Nuclear extracts were prepared, and western blots were performed using anti-NF-κB p65 Ab (p65). (A) and (C) Total cell lysates were prepared, and western blots were performed using anti-IκBα (IκBα), anti-β-Actin (β-Actin), anti-phosphorylated NF-κB p65 (pp65) or anti-NF-κB p65 (p65) Ab. Data are representative of at least three independent experiments.

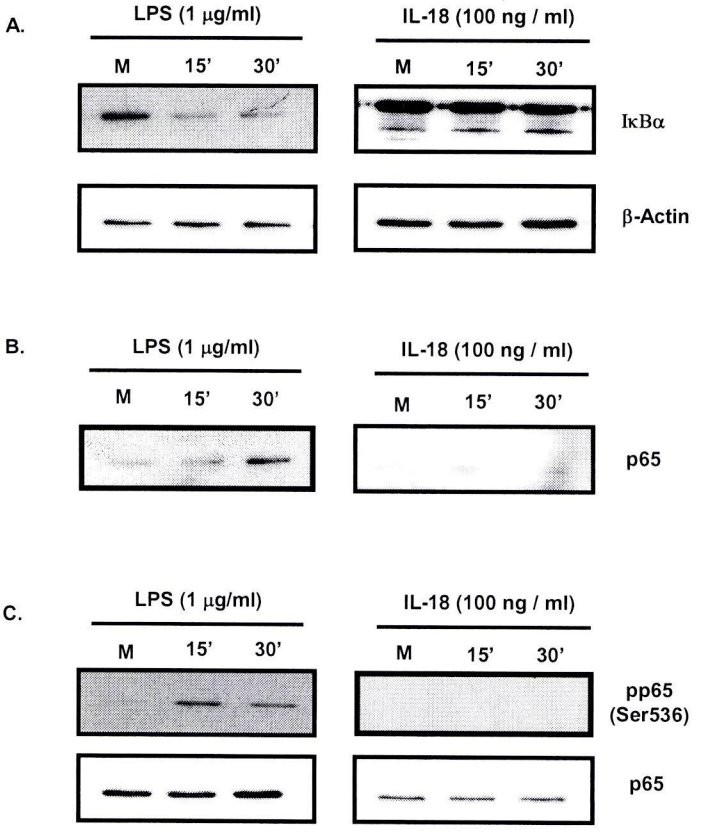
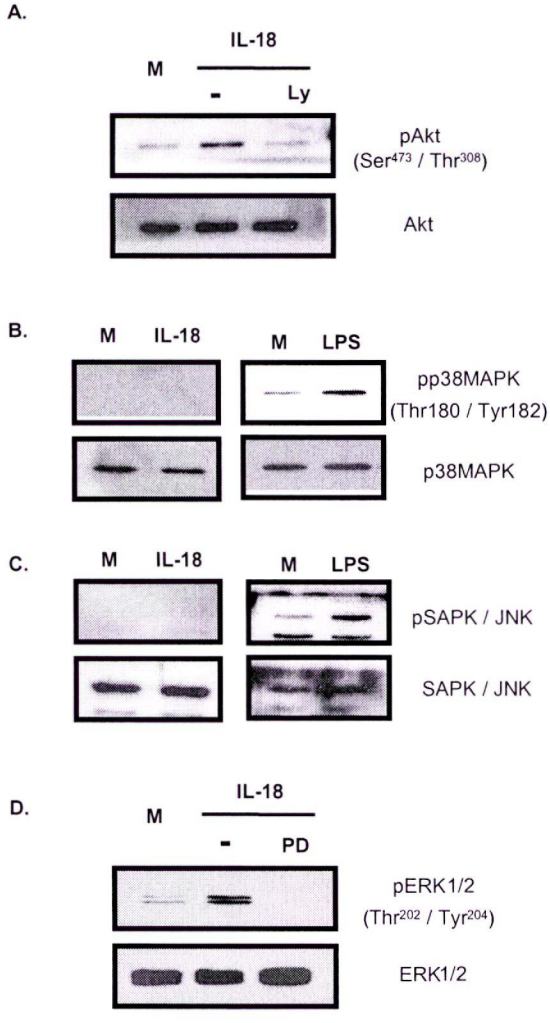

A.
LPS (1 μg/ml)
IL-18 (100 ng / ml)
M
15'
30'
M
15'
30'
IκBα
β-Actin
B.
LPS (1 μg/ml)
IL-18 (100 ng / ml)
M
15'
30'
M
15'
30'
p65
C.
LPS (1 μg/ml)
IL-18 (100 ng / ml)
M
15'
30'
M
15'
30'
pp65
(Ser536)
p65

Fig 14. IL-18 activates the PI3K/Akt and MEK/ERK1/2 signaling pathways in murine Mϕ. Purified Mϕ from C57BL/6J mice were treated with media alone (M), LPS (1 μg/ml) or IL-18 (100 ng/ml) for 30 min. (A) A PI3K-inhibitor, LY294002 (Ly, 5 μM), or (D) a MEK- inhibitor, PD98059 (PD, 10 μM), was added at 20 min before IL-18 treatment. Cell lysates were prepared, and western blots were performed using: (A) anti-Akt (Akt) or anti-phosphorylated Akt (pAkt); (B) anti-p38 MAPK (p38 MAPK) or anti-phosphorylated p38 MAPK (pp38MAPK); (C) anti-JNK (SAPK/JNK) or anti-phosphorylated JNK (pSAPK/JNK); (D) anti-ERK1/2 (ERK1/2) or anti-phosphorylated ERK1/2 (pERK1/2) Abs. Data are representative of two independent experiments.


A.
IL-18
M
-
Ly
pAkt
(Ser473 / Thr308)
Akt
B.
M
IL-18
M
LPS
pp38MAPK
(Thr180 / Tyr182)
p38MAPK
C.
M
IL-18
M
LPS
pSAPK / JNK
SAPK / JNK
D.
IL-18
M
-
PD
pERK1/2
(Thr202 / Tyr204)
ERK1/2

5. IL-18-induced MCP-1 secretion in Mϕ is mediated by both the PI3K/Akt and MEK/ERK1/2 signaling pathways

Since it was determined that IL-18 induces the activation of the PI3K/Akt and the MEK/ERK1/2 pathways in Mϕ, I determined whether or not these signaling pathways are involved in IL-18-induced MCP-1 production. Mϕ were treated with LY294002 and / or PD98059 during stimulation with IL-18, and the secretion of MCP-1 into the culture supernatant was examined by ELISA. Inhibition of the PI3K/Akt pathway with LY294002 resulted in 51.6 % decreased of MCP-1 secretion and inhibition of the MEK/ERK1/2 with PD98059 resulted in 63.7 % decrease of MCP-1 secretion as compared with Mϕ stimulated with IL-18 without inhibitor treatment. Treatment with both inhibitors completely eliminated the secretion of MCP-1 to the level of Mϕ without IL-18 stimulation (Fig. 15). To confirm these results, gene expression of MCP-1 was examined by RT-PCR in Mϕ stimulated with IL-18 with or without the inhibitors. Consistent with the ELISA results, IL-18-treatment clearly induced MCP-1 gene expression in a dose-dependent manner (Fig. 16A), and this was partially inhibited by the treatment with either LY294002 or PD98059 and completely inhibited by a combination of the two inhibitors (Fig. 16B).

To determine whether or not the inhibition of MCP-1 secretion from Mϕ by the inhibitors was due to any toxic effects, apoptotic cell death of inhibitor-treated Mϕ was examined by the analysis of DNA content. Genomic DNA were stained with propidium iodide (PI) and FACS analysis was performed. No apoptotic cell death was found in Mϕ treated with inhibitors or their vehicle DMSO (Fig. 17). These results suggest that the inhibition of IL-18-induced MCP-1 secretion by these inhibitors is not due to any toxic effects, but rather to specific inhibition of the respective signaling pathways.

Fig 15. IL-18-induced MCP-1 secretion in Mϕ is mediated by the PI3K/Akt and MEK/ERK1/2 signaling pathways. Purified Mϕ from C57BL/6J mice were treated with media alone (M) or IL-18 (100 ng/ml) in the absence (-) or presence of LY294002 (Ly, 5 μM) and/or PD98059 (PD, 10 μM). Culture supernatant was harvested after 48 h for measurement of MCP-1 by ELISA. Data are shown as mean ± SD of triplicate cultures and are representative of at least three independent experiments. DMSO: vehicle control for LY and PD. [*: $p < 0.17$]

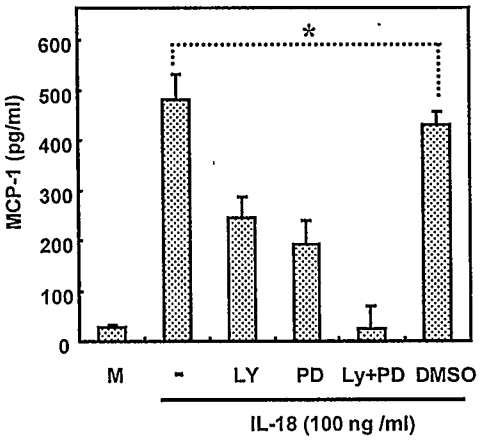
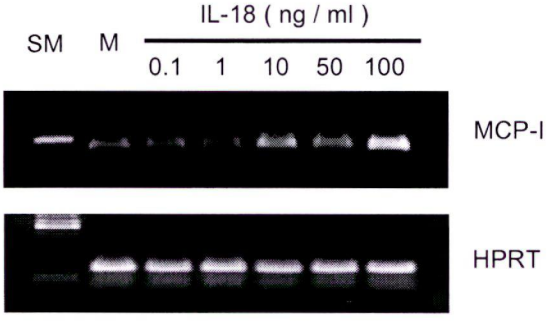

*
600
500
400
300
200
100
0
MCP-1 (pg/ml)
M
-
LY
PD
Ly+PD
DMSO
IL-18 (100 ng /ml)

Fig 16. IL-18 induces MCP-1 gene expression in Mϕ *via* the PI3K/Akt and MEK/ERK1/2 signaling pathways. Purified Mϕ from C57BL/6J mice were treated with media alone (M) or indicated concentrations of IL-18 in the absence (-) or presence of LY294002 (Ly, 5 μM) and/or PD98059 (PD, 10 μM). Total RNA was harvested after 6 h for RT-PCR analysis of MCP-1 expression. Hypoxanthine phosphoribosyltransferase (HPRT) was analyzed as an internal control. Data are representative of at least two independent experiments. SM: 100 bp DNA ladder.

A.


IL-18 (ng / ml)
SM
M
0.1
1
10
50
100
MCP-I
HPRT

B.

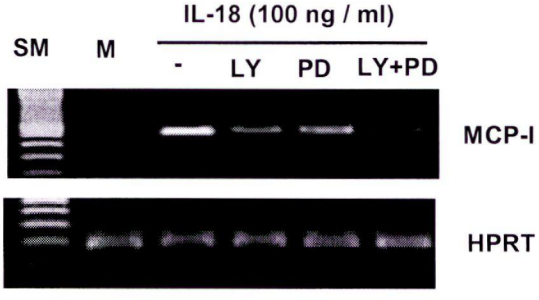
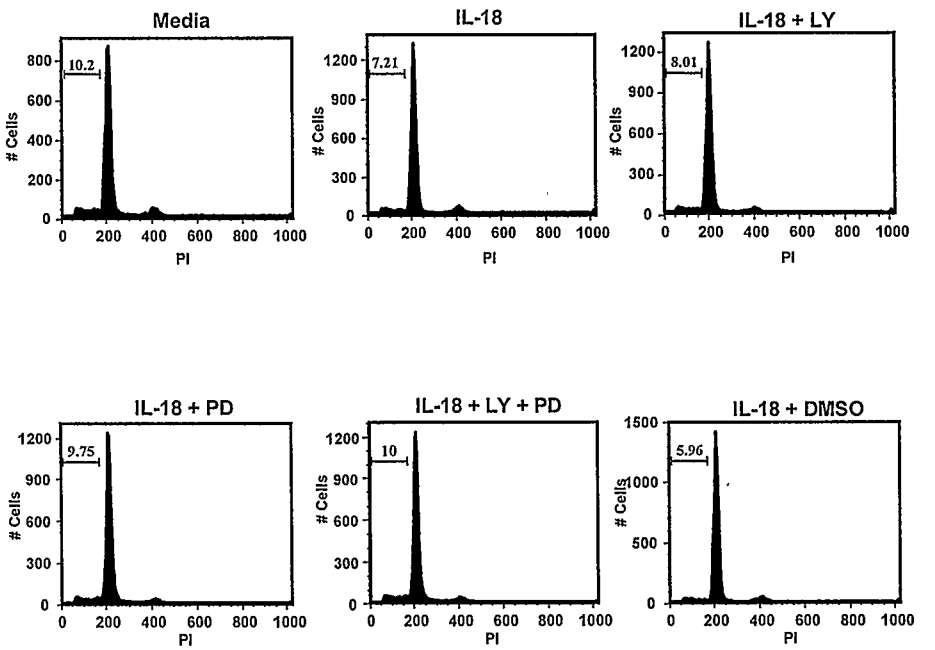

IL-18 (100 ng / ml)
SM
M
-
LY
PD
LY+PD
MCP-I
HPRT

Fig 17. The treatment of pharmacological inhibitors does not induce cytotoxicity in murine Mϕ. Purified Mϕ from C57BL/6J mice were treated with media alone (M) or IL-18 (100 ng / ml) in the absence (-) or presence of LY294002 (Ly, 5 μM) and/or PD98059 (PD, 10 μM). Cells were harvested after 48 hrs and stained with PI for apoptosis analysis by FACS. Data are representative of two independent experiments. DMSO: vehicle control for LY and PD.


Media
IL-18
IL-18 + LY
IL-18 + PD
IL-18 + LY + PD
IL-18 + DMSO
Cells
PI
10.2
7.21
8.01
9.75
10
5.96

CHAPTER 4. DISCUSSION

The cytokines IL-12 and IL-18 are mainly induced in macrophages and involved in the regulation of both innate and adaptive immunity. These innate cytokines play a critical role in the pathophysiology of autoimmune, inflammatory, and infectious diseases (Nakanishi et al., 2001; Trinchieri, 1995). IL-12 is composed of p35 and p40 subunits, exerts immuno-regulatory effects on leukocytes, and regulates the immune-balance between Th1 and Th2 (Trinchieri, 1995). In contrast, IL-18, which is synthesized as a precursor protein and processed by the intracellular cysteine protease caspase-1, contributes to host defense and inflammatory responses through synergy with other inflammatory cytokines, particularly IL-12 (Nakanishi et al., 2001). It is known that these innate cytokines stimulate T and NK cells to produce IFN-γ (Biron et al., 1999; Okamura et al., 1995), which subsequently activates macrophages and other immune cells to secrete proinflammatory cytokines, chemokines, and nitric oxide . An earlier report suggests that IL-12 and IL-18 may directly regulate innate immunity through autocrine Mϕ activation (Munder et al., 1998).

In the early phase of innate immunity, leukocyte recruitment, including Mϕ, NK cells, and T cells, is an important step for inflammation, which is mediated by chemokine molecules. As a CC-chemokine, MCP-1 is a potent chemoattractant and de-granulation factor of circulating leukocytes, resulting in an accelerated induction of the pathogen-specific immune response at the inflammatory site (Baggiolini et al., 1997; Carr et al., 1994; Rollins, 1997). Thus, using murine peritoneal Mϕ, it was determined whether or not IL-12 and IL-18 can induce the production of MCP-1, contributing to the recruitment of mononuclear cells. Because plate-attachment can activate Mϕ non-specifically, MACS system and Nucleopore (0.8 μm) polycarbonate membranes have been used for purification and *in vitro* culture of Mϕ, respectively. This method prevents non-specific Mϕ activation and significantly reduces the background activation level of

signaling molecules.

First, it was examined whether or not IL-12 and IL-18 can induce the production of MCP-1 in Mϕ. I found that these innate cytokines independently activated Mϕ to produce MCP-1 in a dose-dependent manner. The production of MCP-1 by IL-12 was mediated by autocrine IFN-γ. On the contrary, IL-18 induced the MCP-1 production *via* an IFN-γ-independent mechanism. This difference might be due to the cytokine receptor signaling, since IL-12 and IL-18 stimulate distinct signaling pathways in immune cells (Nakanishi et al., 2001; Trinchieri, 1995).

Second, the signaling pathways involved in the production of MCP-1 were examined. IL-18R engagement is known to induce the activation of several signaling molecules such as IKK/NF-κB, PI3K/Akt, MAPK (p38, p42 and p44 MAPK), and JNK in several different cell types (Kalina et al., 2000; Kanakaraj et al., 1999; Morel et al., 2001; Morel et al., 2002; Nakanishi et al., 2001). IL-18R is a member of the IL-1R family, which shares a signaling cascade of sequential recruitment of MyD88 and IRAK, followed by activation of NF-κB. The activation of NF-κB was shown to be required for IL-18-induced IFN-γ expression in a human myelomonocyte cell line (Kojima et al., 1999). In addition, IL-18 was also shown to activate NF-κB in murine Th1 cells (Matsumoto et al., 1997). Furthermore, IRAK-deficient mice showed impairment of IL-18-induced NF-κB activation and of IL-18-mediated NK cells and Th1 responses (Kanakaraj et al., 1999; Suzuki et al., 2003). On the basis of these available information, it was expected that NF-κB can be activated in Mϕ when they are stimulated with IL-18. I examined the activation of NF-κB in Mϕ by IL-18. In contrast to the expectations, there was no activation of NF-κB by the stimulation with IL-18. Neither the degradation of IκB nor the translocation of NF-κB p65 was observed in IL-18-stimulated Mϕ. These results indicate that IL-18 does not induce activation of NF-κB in Mϕ. A recent study

showed that NF-κB was not activated by IL-18 but was activated by IL-1β in human epithelial cells (Lee et al., 2004). Taken together, it appears that the activation of NF-κB may be different in different cell types.

The PI3K/Akt pathway was shown to be involved in IL-18-induced expression of cell adhesion molecules such as vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in synovial fibroblasts (Morel et al., 2001; Morel et al., 2002). It has also been found that IL-18 induced cardiomyocyte hypertrophy by the PI3K/Akt-dependent signaling cascade (Chandrasekar et al., 2004). Third, the activation of the PI3K/Akt pathway in macrophages stimulated with IL-18 was examined. It was found that IL-18 induced the activation of the PI3K/Akt signaling pathway. In addition to the PI3K/Akt pathway, evidence suggests that MAPKs play a role in IL-18 signaling. The activation of p38 MAPK by IL-18 was detected in an epithelial cell line, and the activation of p38 MAPK and ERK1/2 by IL-18 was observed in a human NK cell line (Kalina et al., 2000). In the present study, only the MEK/ERK1/2 pathway was activated in IL-18-stimulated Mϕ, but not the p38 MAPK or JNK pathways. The expression and secretion of MCP-1 was attenuated by inhibiting either the PI3K/Akt or the MEK/ERK1/2 pathway and was almost completely abolished by inhibiting both pathways. On the basis of these observations, I would suggest that both the PI3K/Akt and MEK/ERK1/2 pathways are required for IL-18-induced production of MCP-1 in murine Mϕ.

Through this study, it was demonstrated that IL-12 and IL-18 can independently induce the expression of MCP-1 mRNA and the release of MCP-1 protein in Mϕ. This likely plays an important role in the recruitment of leukocytes at the inflammatory site and subsequently the development of Ag-specific immunity. MCP-1 secretion induced by IL-12 and IL-18 in murine Mϕ was mediated *via* autocrine IFN-

γ dependent or independent mechanism, respectively. IL-18 did not activate IKK/NF-κB pathways, but activated PI3K/Akt and MEK/ERK1/2 signaling pathways, contributing to the production of the chemokine MCP-1. These results suggest that both IL-12 and IL-18 may actively regulate innate immunity by inducing the recruitment and activation of circulating leukocyte *via* MCP-1 production from Mϕ.

MCP-1 produced by IL-12 or IL-18-stimulated Mϕ can also modulate adaptive immunity. MCP-1 has a biological activity to induce chemo-attraction of memory phenotype T cells ($CD45RA^-/CD45RO^+$/CD29/L-selectin and $CD26^+$) (Carr et al., 1994). Therefore, IL-12 or IL-18-stimulated Mϕ can induce the chemo-attraction of circulating memory T-cells to Ag-presenting cells (APC), which results in the induction of pathogen-specific T-cell immunity at the inflammatory site. Taken together, it can be suggested IL-12 and IL-18 may actively regulate both innate and adaptive immunity through multifunctional MCP-1 molecules.

Innate cytokine molecules contribute to host defense. However, overproduction of IL-12 or IL-18 is potentially harmful because these cytokines are powerful pro-inflammatory molecules with a wide-spectrum of influence within the immune system (Nakanishi et al., 2001; Trinchieri, 1995). A high level of IL-18 has been found in the synovial fluid from patients with rheumatoid arthritis (RA). In addition, IL-18 molecules were produced in synovial Mϕ from DBA/1 mice with collagen (type 2) induced arthritis, which is the animal model of human RA (Gracie et al., 1999; Nakanishi et al., 2001). In the present study, it was also found that IL-18 molecules can autocrinally activate Mϕ to produce MCP-1 molecules with monocyte-chemotaxis inducing biological activity. Taken together, these results strongly suggest that autocrine macrophage activation by IL-18 may play a pivotal role in the onset of inflammatory diseases such as RA. This is supported by a previous report that the

synovial production of MCP-1 plays an important role in the recruitment of mononuclear phagocytes during inflammation associated with RA and that the synovial tissue macrophages are the dominant source of this chemokine molecule (Koch et al., 1992). In conclusion, it has been determined that both IL-12 and IL-18 appear to regulate innate immunity by inducing the recruitment and activation of circulating leukocytes through MCP-1 production from Mϕ. This finding may unravel new biological functions of IL-12 and IL-18 molecules in the immune system and open up new avenues for the study of inflammatory diseases such as rheumatoid arthritis.

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