IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

***In Vivo* Depletion of CD206⁺ M2 Macrophages Exaggerates Lung Injury in Endotoxemic Mice**

Kenta Kambara,^{*,†} Wakana Ohashi,^{*} Kengo Tomita,^{*} Michinori Takashina,^{*} Shiho Fujisaka,[†] Ryuji Hayashi,[†] Hisashi Mori,[‡] Kazuyuki Tobe,[†] and Yuichi Hattori^{*}

From the Departments of Molecular and Medical Pharmacology^{*} and Neuroscience[†] and the First Department of Internal Medicine,[‡] Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

Accepted for publication
September 17, 2014.

Address correspondence to
Yuichi Hattori, M.D., Ph.D.,
Department of Molecular and
Medical Pharmacology, Grad-
uate School of Medicine and
Pharmaceutical Sciences, Uni-
versity of Toyama, Toyama
930-0194, Japan. E-mail:
yhattori@med.u-toyama.ac.jp.

Although phenotypically polarized macrophages are now generally classified into two major subtypes termed proinflammatory M1 and anti-inflammatory M2 macrophages, a contributory role of lung M2 macrophages in the pathophysiological features of acute lung injury is not fully understood. Herein, we show in an endotoxemic murine model that M2 macrophages serve as key anti-inflammatory cells that play a regulatory role in the severity of lung injury. To study whether M2 macrophages can modify inflammation, we depleted M2 macrophages from lungs of CD206-diphtheria toxin (DT) receptor transgenic (Tg) mice during challenge with lipopolysaccharide. The i.p. administration of DT depleted CD206-positive cells in bronchoalveolar lavage fluid. The use of M2 macrophage markers Ym1 and arginase-1 identified pulmonary CD206-positive cells as M2 macrophages. A striking increase in neutrophils in bronchoalveolar lavage fluid cell contents was found in DT-treated CD206-DT receptor Tg mice. In CD206-DT receptor Tg mice given DT, endotoxin challenge exaggerated lung inflammation, including up-regulation of proinflammatory cytokines and increased histological lung damage, but the endotoxemia-induced increase in NF- κ B activity was significantly reduced, suggesting that M2 phenotype-dependent counteraction of inflammatory insult cannot be attributed to the inhibition of the NF- κ B pathway. Our results indicate a critical role of CD206-positive pulmonary macrophages in triggering inflammatory cascade during endotoxemic lung inflammation. (*Am J Pathol* 2015, 185: 162–171; <http://dx.doi.org/10.1016/j.ajpath.2014.09.005>)

Macrophages are critical regulators of many organ systems, including innate and adaptive immunity, systemic metabolism, hematopoiesis, vasculogenesis, malignancy, and reproduction.¹ Macrophages differentiate into functionally distinct immunological populations depending on the microenvironments. On the basis of type 1/2 helper T-cell polarization concepts,² phenotypically polarized macrophages are now generally termed proinflammatory M1 and anti-inflammatory M2.^{3,4} Macrophages exposed to microbial products and interferon- γ become classically activated macrophages (M1), which produce copious amounts of proinflammatory cytokines and chemokines; key among these are tumor necrosis factor (TNF)- α , IL-1 β , IL-6, IL-12, and chemokine ligand 2. The same macrophages also produce reactive oxygen intermediates, and function predominantly in inflammation, tissue damage, killing of intracellular microbes, and increased tumoricidal activity.³ Recent studies

also suggest that the phenotypic switching of adipose tissue macrophages from M2 to M1 is generated by obesity, leading to systemic inflammation and insulin resistance.^{5–7} In contrast, alternative activation of macrophages is promoted by a variety of stimuli, such as IL-4, IL-10, IL-13, and glucocorticoids, that elicit different M2 forms. M2 macrophages up-regulate scavenger, mannose receptor, galactose receptor, and IL-1 receptor antagonist, and down-regulate IL-1 β and other proinflammatory cytokines.^{3,8,9} They are principally able to tune inflammatory responses and adaptive type 2 helper T-cell immunity, and to promote tissue remodeling, angiogenesis, and tumor development.^{3,10} Furthermore, M2

Supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan Grants-in-Aid for Scientific Research 18209033 and 21591126 (K.T.).

Disclosures: None declared.

macrophage activation can protect neurons and facilitate neuronal regeneration in Alzheimer disease.¹¹ M2-polarized macrophages are further subdivided into the wound-healing M2a (elicited by IL-4 and IL-13), the regulatory M2b (after stimulation by immune complexes in the presence of Toll-like receptor ligands), and the M2c (when exposed to anti-inflammatory stimuli, such as glucocorticoids, IL-10, and transforming growth factor- β),⁴ but this sub-classification may not fully represent the complexity of the transitional states of macrophage activation, which is finely tuned in response to different microenvironments.¹²

Acute lung injury (ALI) is a common and highly morbid clinical disease marked by respiratory failure attributable to increased pulmonary endothelial and epithelial permeability, leading to alveolar flooding, and neutrophil accumulation in alveolar spaces and in adjacent capillaries.^{13,14} Although neutrophil influx and activation within the lung play a crucial role in the pathogenesis of ALI,¹⁵ growing evidence indicates that macrophages also contribute to the modulation of lung inflammation and the resultant lung injury.^{16–18} Several studies have found that macrophage depletion with clodronate-liposomes results in attenuation of lung injury after endotoxin administration, ischemia-reperfusion, and mechanical ventilation.^{19–22} Conversely, other studies suggest that the recruitment of macrophages to the lung is associated with attenuated lung injury.^{23,24} One possible explanation for such disparate roles of macrophages in ALI may be related to different depletion or recruitment of heterogeneous populations of proinflammatory and anti-inflammatory macrophages in the lung. However, how M2 macrophages have a protective anti-inflammatory role under the pathological condition of ALI is not fully understood.

Herein, we decided to deplete M2 macrophages in mice during exposure to lipopolysaccharide (LPS). We generated a line of transgenic (Tg) mice expressing human diphtheria toxin (DT) receptor under the control of the *Mrc1* gene promoter. Murine cells are naturally resistant to DT because they lack functional cell surface receptors for the toxin.^{25–27} Thus, the technique with human DT receptor transduction allows us to achieve efficient inducible ablation of a specific type of murine cells by DT administration *in vivo*. Because *Mrc1* (alias CD206) is a cell surface receptor as an M2 phenotypic marker, systemic administration of DT was able to lead to a sharp decline in lung M2 macrophages in CD206-DT receptor Tg mice. Herein, we identify lung M2 macrophages as key anti-inflammatory cells that play a regulatory role in the severity of endotoxin-induced lung injury.

Materials and Methods

Generation and Screening of CD206-DT Receptor Tg Mice

We obtained the mouse BAC clone RP 24-377 D19 carrying a 152-kbp insert containing the exon coding translational

start Met and upstream 133-kbp sequence of *CD206* gene from the BACPAC Resources Center CHORI (Oakland, CA). The plasmid pTRECK6 that includes noncoding exon and intron from rabbit β -globulin gene, human HB-EGF (DT receptor) cDNA, and rabbit β -globulin and simian virus 40 polyadenylation (pA) signals was kindly provided by Dr. Kenji Kohno (Nara Institute of Science and Technology, Nara, Japan).^{28,29} By using a Counterselection BAC modification kit (Gene Bridges, Dresden, Germany), we inserted the noncoding exon and intron from rabbit β -globulin gene, human DT receptor cDNA, and rabbit β -globulin and simian virus 40 pA signals at the initiation site of translation in the *CD206* gene to yield the pTg-CD206-DT receptor (Figure 1A).

The purified pTg-CD206-DT receptor BAC DNA was microinjected into pronuclei of fertilized one-cell embryos from C57BL/6 mice. Founder mice were crossed with C57BL/6 mice to produce +/CD206^{DT receptor} mice. The wild-type (WT; +/+) and heterozygous CD206-DT receptor (+/CD206DTR) littermates were used for analysis. All mice were housed in a specific pathogen-free facility. Experiments were performed according to institutional guidelines.

For the genotyping of the Tg mouse lines by Southern blot analysis, genomic DNA prepared from tail biopsy specimens was digested with BamHI, separated by electrophoresis on a 0.6% agarose gel, and transferred to a nylon membrane (Hybond-N⁺; GE Healthcare Japan, Tokyo, Japan). Hybridization was conducted with a 900-bp ³²P-labeled DNA fragment derived from the exon 1 and intron 1 (Figure 1B). The detected band sizes of endogenous and Tg genes were 8.3 and 3.8 kb, respectively.

Experimental Procedures

In a first series of experiments to examine the effects of M2 macrophage depletion under normal noninflammatory conditions, CD206-DT receptor Tg mice and WT littermates received an i.p. injection of 15 μ g/kg DT. Mice were administered DT for 3 consecutive days and sacrificed 24 hours after the last injection (Figure 2A). Blood samples were collected, and various tissues and organs were removed. Some mice were given phosphate-buffered saline (PBS) instead of DT.

In another series of experiments to address the role of M2 macrophages in endotoxin-induced ALI, mice received an i.p. injection of 15 μ g/kg DT for 3 consecutive days and were subsequently challenged with i.v. LPS (0.1 mg/kg; List Biological Laboratories, Campbell, CA) 24 hours after the last injection. Mice were sacrificed 2 hours later, and blood collection and lung tissue isolation were performed (Figure 3A).

RNA Extraction and Quantitative Real-Time PCR

Total RNA was isolated from tissues with an RNeasy Mini Kit (Qiagen, Tokyo, Japan). RNA was reverse transcribed to cDNA, and real-time PCR analyses were performed as

Figure 1 CD206 expression in C57BL/6 mice. **A**: Schematic representation of WT and DT receptor (DTR)-inserted transgene in BAC DNA (pTg-CD206-DT receptor). Exons 1 through 3 of *CD206* gene are indicated by an open box. Met is the initiation site of translation in *CD206*. In the pTg-CD206-DT receptor BAC DNA, rabbit β -globin intron, human DT receptor cDNA, and rabbit β -globin/simian virus 40 pA signals are indicated by line and gray box. The probe used for Southern blot analysis is indicated as a solid line together with the predicted hybridizing fragments. After insertion of DT receptor, the CD206 of the transgene is not expressed. **B**: Southern blot analysis of BamHI-digested genomic DNA from CD206-DT receptor Tg mice. Genomic DNAs were hybridized with the CD206 probe, as shown in **A**. Location of WT (8.6 kbp) and CD206-transgene (Tg; 3.8 kbp)-derived bands are shown. The positions of DNA size markers (kbp) are indicated on the left. **C**: CD206 mRNA expression in different tissues of normal mice. The data are expressed as a fold increase above kidney value normalized to 18S ($n = 3$ mice). **D**: CD206 (D) and Ym1 (E) mRNA expression in lung tissues and in BALF from normal mice. The data are normalized to GAPDH ($n = 3$). The IHC detection of arginase-1-positive cells (F) and CD206-positive cells (G) in lung sections and in BALF (H) from normal mice. Lung sections and BALF were stained with hematoxylin. **Arrows** indicate CD206-positive cells (G). **I**: Immunofluorescence images in BALF. **Left panel**: CD206 (red). **Middle panel**: arginase-1 (Arg-1; green). **Right panel**: Overlap images with the two stains are presented. Nuclei were counterstained with DAPI (blue). Shown are representative micrographs from two independent experiments. * $P < 0.05$, ** $P < 0.01$.

described previously³⁰ using a Takara RNA PCR kit (Takara Bio, Ohtsu, Japan). Thermal cycler parameters were as follows: 1 cycle of 50°C for 2 minutes, 50 cycles of denaturation (95°C for 15 seconds), and combined annealing/extension (60°C for 30 seconds). Gene expression changes were calculated by the comparative C_T method, and 18S ribosomal RNA was used as the reference gene for normalization.

Enzyme Immunoassay for Cytokines

Blood levels of IL-1 β , TNF- α , monocyte chemotactic protein (MCP)-1, and IL-6 were measured by the use of a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The plate was read on a microplate reader (Nippon-InterMed, Tokyo, Japan). Assays were performed in duplicate.

Histological Examination

For routine histological examination, inflation-fixed lungs were harvested, fixed, dehydrated, paraffin embedded, and

sliced into sections (4 μ m thick).^{30,31} After deparaffinization, slides were stained with hematoxylin and eosin (H&E) using standard methods. A semiquantitative morphometric analysis of lung injury was performed by scoring from 0 to 4: 0 indicates none; 1 indicates light; 2 indicates moderate; 3 indicates severe; and 4 indicates very severe. The scores are for the following categories: neutrophil infiltration, pulmonary edema, and disorganization of lung parenchyma and hemorrhage.³⁰ A total lung injury score was calculated by adding the individual scores in every animal and averaging the total values in each group. All of the histological studies were performed in a blinded manner (K.K.).

IHC Data

Frozen lung sections (5 mm thick) were fixed in acetone for 10 minutes at -20°C and air dried. Bronchoalveolar lavage (BAL) was performed with five aliquots of 5 mL of PBS instilled into the lungs and gently withdrawn. Cells obtained from the total BAL fluid (BALF) were spun at 113 $\times g$ for 10 minutes onto glass slides by use of a Cytospin 4 (Thermo Fisher Scientific, Yokohama, Japan). Then, they were fixed with 4% buffered formalin solution for 10 minutes at room temperature.

Endogenous peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide for 15 minutes. After incubation overnight at 4°C with anti-CD206 antibody (AbD Serotec, Raleigh, NC) or anti-arginase-1 antibody (Sigma-Aldrich, St. Louis, MO), the samples were exposed to anti-rat or anti-rabbit IgG conjugated with horseradish peroxidase. Bound antibody was visualized by a light microscopy with diaminobenzidine. Omission of primary antibody and staining with nonimmune IgG served as negative control.

Immunofluorescence Staining

Cells obtained from the total BALF were adhered to a glass-bottom dish, fixed with 4% paraformaldehyde, and permeabilized in Perm Buffer (BD Biosciences, San Diego, CA). Cells were exposed to the fluorescent antibody Alexa 546-conjugated anti-rat IgG and Alexa 488-conjugated anti-rabbit IgG (Life Technologies, Carlsbad, CA) after overnight incubation with the primary antibody, anti-CD206 antibody and anti-arginase-1 antibody. The nucleus was counterstained with DAPI. Immunofluorescence images were observed under an Olympus (Tokyo, Japan) BX-51 fluorescence microscope and processed using Adobe Photoshop CS3 software (Adobe, San Jose, CA).

Cell Counting in BALF

The alterations in cells in BALF were examined as described previously.³¹ Cells in BALF obtained as mentioned above were counted with a hemacytometer. Slides were stained with Wright stain, and cell differentiation was tabulated using light microscopy.

NF-κB and AP-1 Binding Assays

Nuclear protein extracts from freshly isolated lungs were obtained with a commercially available nuclear extraction kit (Sigma-Aldrich), as described in the manufacturer's manual. TransAM NF-κB and TransAM AP-1 kits (Active Motif, Carlsbad, CA) were used to quantify the binding of p65 to the NF-κB site and c-Jun and c-Fos to the activator protein (AP)-1 site, according to the manufacturer's instructions. TransAM kits are DNA-binding ELISAs that quantify transcription factor activation in tissues.

Electrophoretic Mobility Shift Assay

NF-κB and AP-1 DNA binding reactions were performed according to the manufacturer's protocol using the Odyssey Infrared electrophoretic mobility shift assay kit (Li-Cor Biosciences, Lincoln, NE) and nuclei extracts from lung tissues. Double-stranded IRDye 700 infrared dye-labeled oligonucleotides with consensus sequences of NF-κB (forward, 5'-AGTT-GAGGGGACTTTCACAGGC-3' and reverse, 5'-GCCTGGG-AAHTCCCCCTCAACT-3') and AP-1 (forward, 5'-CGCTT-

GATGACTCAGCCGGAA-3' and reverse, 5'-TTCCGGCT-GAGTCATCAAGCG-3') were used.

Statistical Analysis

Data were analyzed by the use of Prism software version 6 (GraphPad Software, San Diego, CA). Statistical analysis was performed by Student's unpaired *t*-test or one-way analysis of variance, followed by Tukey's multiple comparison test. Differences were considered significant at $P \leq 0.05$. Data are presented as means $\pm$ SEM.

Results

CD206-Positive Cell Expression and Distribution in Mice

CD206 mRNAs were transcribed in a variety of tissues, including lung, liver, spleen, and kidney, of normal C57BL/6 mice, but they were most abundant in lung tissues (Figure 1C). CD206 mRNA expression levels were evidently higher in BALF than in lung tissues when normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Figure 1D). Likewise, high levels of mRNA expression of the classic M2 marker Ym1 were detected in BALF (Figure 1E). Immunohistochemical (IHC) studies showed that CD206-positive cells were present in the alveoli (Figure 1G) and constituted a great portion of the whole cells in BALF (Figure 1H). Arginase-1, a marker for the M2 subset, behaved in the same way as CD206 in terms of the alveolar localization (Figure 1F). Moreover, merging of CD206 and arginase-1 showed that CD206-positive cells in BALF were labeled with arginase-1 (Figure 1I).

DT Treatment of CD206-DT Receptor Tg Mice

DT treatment resulted in a sharp reduction in CD206 mRNA levels in lungs of CD206-DT receptor Tg mice without any change in those of WT mice (Figure 2B). Similarly, Ym1 mRNA expression in lungs was reduced when DT was given to CD206-DT receptor Tg mice (Figure 2C). Consistent with the effect on CD206 mRNA in lung tissues, DT treatment caused marked decreases in the CD206 and Ym1 mRNA expression levels (Figure 2, D and E) and the CD206-positive cell number (Figure 2, F and G) in BALF from the CD206-DT receptor Tg animal compared with WT. Furthermore, an immunofluorescence labeling study showed that no cells labeled with CD206 or arginase-1 were detectable in BALF from CD206-DT receptor Tg mice (data not shown).

DT treatment did not substantially affect mRNA levels of proinflammatory cytokines, IL-1 β , TNF- α , MCP-1, and IL-6, in lungs of WT mice. On the other hand, CD206-DT receptor Tg mice exhibited up-regulation of these proinflammatory cytokine mRNAs in response to DT treatment (Figure 2H). However, the effects of DT treatment on inflammatory gene expression were much less pronounced when compared with those of further challenge with LPS in

Figure 2 DT treatment on CD206 expression and proinflammatory cytokine production in CD206-DT receptor Tg mice. **A:** Experimental protocol. Mice were administered DT (or PBS) once daily for 3 days. The animals were then sacrificed 24 hours after the last DT injection. **B–E:** CD206 and Ym1 mRNA expression in lung tissues and BALF ($n = 2$ to 4). The data are normalized to GAPDH mRNA. IHC detection of CD206-positive cells in BALF from WT mice (**F**) and from DT-treated CD206-DT receptor Tg mice (**G**). Shown are representative micrographs from two independent experiments. **H:** mRNA levels of cytokines in lungs. The data are expressed as a fold increase above WT normalized 18S. **I:** Blood levels of cytokines. $n = 7$ to 8 (**H** and **I**). * $P < 0.05$.

DT-treated CD206-DT receptor Tg mice ([Supplemental Figure S1](#)).

When blood levels of proinflammatory cytokines were measured via an ELISA, the WT animals had low levels of the cytokines examined herein regardless of whether DT was given. In contrast, IL-1 β , MCP-1, and IL-6 showed an evident increase in CD206-DT receptor Tg mice when treated with DT, except that TNF- α was undetectable even though DT was administered ([Figure 2I](#)).

Effect of LPS in DT-Treated CD206-DT Receptor Tg Mice

DT treatment was without effect on lung and BALF CD206 mRNA levels in WT mice. When a low dose (0.1 mg/kg) of LPS was i.v. injected to DT-treated WT mice, CD206 mRNA levels were substantially unchanged in both lungs and BALF ([Figure 3, B and C](#)). Interestingly, challenge with the low dose of LPS did not alter the total number of BALF cells in WT mice ([Supplemental Figure S2](#)). This is due to the BALF cells being mainly composed of CD206-positive

macrophages. However, a notable increase in neutrophils in BALF cell contents was found in DT-treated CD206-DT receptor Tg mice ([Supplemental Figure S2](#)).

LPS challenge led to up-regulation of IL-1 β , TNF- α , MCP-1, and IL-6 mRNA levels in lungs of WT mice. This up-regulation was strikingly enhanced in DT-treated CD206-DT receptor Tg mice ([Figure 3D](#)). The ability of LPS at 0.1 mg/kg to up-regulate blood levels of TNF- α , MCP-1, and IL-6 was less pronounced in WT mice regardless of treatment with DT, although IL-1 β responded relatively well to LPS. However, LPS caused a significant and substantial impact on all proinflammatory cytokines examined herein in DT-treated CD206-DT receptor Tg mice ([Figure 3E](#)). LPS also led to an increase in the mRNA level of the anti-inflammatory cytokine IL-10 ([Supplemental Figure S3](#)). DT treatment enhanced the LPS-induced increase in IL-10 mRNA in CD206-DT receptor Tg mice.

Histological examination of H&E-stained sections of the lungs revealed that WT mice had alveolar septae that were normal in appearance, with no intra-alveolar inflammation

Figure 3 DT treatment on CD206 expression and proinflammatory cytokine production in CD206-DT receptor Tg mice exposed to LPS. **A:** Experimental protocol. Mice were administered DT once daily for 3 days. The animals then received an i.v. injection of LPS 24 hours after the last DT treatment and were sacrificed 2 hours later. CD206 mRNA expression in lung tissues (**B**) and BALF (**C**) from WT mice treated with PBS, DT, or DT plus LPS ($n = 4$). The data are normalized to GAPDH mRNA. **D:** Effect of LPS challenge on mRNA levels of cytokines in lungs. The data are expressed as a fold increase above control WT normalized to 18S. **E:** Effect of LPS challenge on blood levels of cytokines. $n = 5$ to 10 (**D** and **E**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

(Figure 4A). Lung histopathological analysis of WT mice given LPS showed modest inflammatory cell infiltrate (Figure 4B). DT-treated CD206-DT receptor Tg mice that received LPS had massive cell infiltration, hemorrhage resulting from ruptured capillary vessels, and diffuse septal edema (Figure 4C). Semiquantitative assessment using the lung injury score demonstrated that the score was significantly higher in CD206-DT receptor Tg mice treated with DT than in untreated Tg animals (Figure 4D). When DT-treated CD206-DT receptor Tg mice were challenged with LPS, the score increased strikingly.

To examine the activation of the proinflammatory transcription factor NF- κ B in lungs after LPS challenge, NF- κ B DNA binding activity in nuclear protein extracts from lungs was quantitatively measured with its ELISA. The DNA binding activity of NF- κ B was greatly increased in WT mice after LPS injection. This increase was significantly attenuated in DT-treated CD206-DT receptor Tg mice (Figure 5A). Another proinflammatory transcription factor, AP-1, consists of Jun and Fos family proteins. Thus, c-Jun and c-Fos proto-oncogenes encode proteins that form a complex that regulates transcription from promoters containing AP-1 activation elements.³² Both c-Jun and c-Fos binding activities were significantly increased when WT mice were challenged with LPS. Further increases in these binding activities in response to LPS were observed in DT-treated CD206-DT receptor Tg mice (Figure 5A). These findings obtained using the ELISA method were confirmed by gel mobility shift assay. The

LPS-induced great increase in the NF- κ B binding was blunted when DT was given to CD206-DT receptor Tg mice (Figure 5B). In contrast, DT treatment markedly heightened the LPS-induced increase in the AP-1 binding in CD206-DT receptor Tg mice.

Discussion

The concept of macrophages existing in functionally distinct phenotypes from proinflammatory to anti-inflammatory states has been fully established by detailed studies on M1 and M2 macrophages.^{3,4,8,9} A growing body of evidence is accumulating that macrophages are key regulators of the induction and resolution phases of ALI, suggesting distinct roles of macrophage subpopulations.³³ Understanding this heterogeneity of pulmonary macrophages may be important in developing strategies to modulate ALI. However, there is no direct evidence that pulmonary M2 macrophages make a significant contribution to the pathophysiological features of ALI. In this work, we addressed this issue by studying how depletion of M2 macrophages can modify lung inflammation in endotoxemic mice.

CD206 encodes the mannose receptor C-type lectin, a cell surface protein that belongs to a family of C-type lectin receptors.³⁴ This endocytic receptor is known to be expressed on macrophages and probably other types of cells, including airway smooth muscle cells.^{35–38} The expression of the

Figure 4 Lung sections stained with H&E. **A:** Control WT mice. **B:** DT-treated WT mice who received LPS. **C:** DT-treated CD206-DT receptor Tg mice who received LPS. **D:** Semiquantitative analysis of lung tissues by lung injury score. $n = 5$ to 7 (**D**). * $P < 0.05$, *** $P < 0.001$.

receptor is highly regulated and correlates with the functional state of macrophages. Thus, fully mature activated M2 macrophages express CD206 as a phenotypic hallmark.³⁹ Therefore, CD206 can be widely used to identify the M2 phenotype.³ The use of M2 macrophage markers Ym1 and arginase-1 identified pulmonary CD206-positive cells as M2 macrophages. Furthermore, this was confirmed by our immunofluorescence labeling study showing that CD206-positive cells in BALF were labeled with arginase-1. Herein, DT administration on CD206-DT receptor Tg mice led to conditional CD206-positive cell ablation, which implies that pulmonary macrophages, which are M2 polarized under homeostatic conditions, can be effectively depleted *in vivo*.

Many M2 macrophages were found in the BALF of C57BL/6 mice under normal conditions. Lung sections from normal C57BL/6 mice showed that M2 macrophages were also detected in the alveoli. DT-mediated M2 macrophage depletion in CD206-DT receptor Tg mice demonstrated that mRNA levels of proinflammatory cytokines, IL-1 β , TNF- α , MCP-1, and IL-6, were up-regulated in lungs. We interpret these observations to indicate that M2 macrophages lead to a tonic inhibition of pulmonary production of proinflammatory cytokines. Blood levels of IL-1 β , MCP-1, and IL-6 were apparently increased when abolition of M2 macrophages was made by administration of DT to CD206-DT receptor Tg mice. This suggests that M2 macrophage-mediated tonic inhibition of the genesis of proinflammatory

cytokines under basal subinflammatory conditions may be found systemically.

Administration of LPS at a low dose did not substantially alter expression of CD206 mRNA in mouse lungs, suggesting that M2 macrophages may be unchanged by a low dose of LPS. In contrast, a recent report has shown that low doses of LPS can effectively suppress expression of the M2 marker, arginase-1, when bone marrow-derived macrophages were treated with M2-skewing mediators.⁴⁰ This report suggests that M2 macrophages may be transformed into M1 macrophages after LPS stimulation to promote inflammation. However, our present study indicates that the presence of M2 macrophages is important in the regulation of the inflammatory state during endotoxemia. DT-mediated depletion of M2 macrophages in CD206-DT receptor Tg mice led to a discernible enhancement of the LPS-induced increases in pulmonary mRNA levels of proinflammatory cytokines. Furthermore, this manipulation significantly aggravated LPS-induced lung injury. These results strongly suggest that M2 macrophages serve as a protective phenotype and can lessen lung inflammation during endotoxemia. DT treatment accelerated the deleterious effect of LPS on blood proinflammatory cytokine levels in CD206-DT receptor Tg mice, implying that M2 macrophages appear essential for appropriate moderation of systemic endotoxemic inflammation.

M2 macrophages may produce some anti-inflammatory cytokines, as typified by IL-10, and maximize the IL-10

Figure 5 Activation of proinflammatory transcriptional factors in lungs of DT-treated WT and CD206-DT receptor Tg mice who were challenged with LPS. **A:** NF-κB p65, AP-1 c-Jun, and AP-1 c-Fos DNA binding activities assessed using TransAM NF-κB and TransAM AP-1 kits. **B:** Gel mobility shift assays for NF-κB and AP-1-binding activity. The induced NF-κB and AP-1 shift bands are indicated. Complete competition for binding of NF-κB and AP-1 is shown in the presence of excess unlabeled NF-κB and AP-1 oligodeoxynucleotides, respectively. Values are means $\pm$ SEM (**A**). $n = 5$ to 7 mice (**A**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. NC, negative control.

signal to dampen the immune response. However, we observed that when LPS was challenged, the up-regulation of IL-10 mRNA expression was increased, rather than decreased, in CD206-depleted mice compared with WT. Thus, CD206-positive M2 macrophages are unlikely to be the principle source of production of IL-10 in lungs.

We found a notable increase in neutrophils in BALF cell contents in DT-treated CD206-DT receptor Tg mice. This suggests that the depletion of M2 macrophages altered inflammatory cell recruitment to the lung. This may account for the enhanced response to LPS in lungs of DT-treated CD206-DT receptor Tg mice.

In quiescent cells, NF-κB is maintained in inactive form by IκB.⁴¹ LPS stimulates IκB-kinase that specifically phosphorylates IκB, resulting in IκB polyubiquitination and subsequent degradation, followed by liberation of NF-κB.⁴² Many M1 genes have κB sites in their promoter region, including inducible nitric oxide synthase and cyclooxygenase 2.⁴³ Thus, in M1 macrophages, NF-κB orchestrates the expression of many proinflammatory genes in response to LPS. Herein, we showed that M2 macrophage depletion resulted in a significant reduction in LPS-induced NF-κB activity, suggesting that M2 phenotype-dependent counteraction of inflammatory insult cannot be attributed to the inhibition of the NF-κB pathway. At the present time, however, we do not have a clear understanding of whether the reduced NF-κB activity was the result of the critical regulation of the transcription factor by M2 macrophages. Reports from several laboratories suggest that macrophages play a role in inducing the activation of NF-κB in epithelial cells in lungs.^{44–46} M2 macrophage depletion

performed in the present study may have caused an alteration in NF-κB activation in alveolar epithelial cells. Such a regulation may be mediated by soluble mediators, such as TNF- α and IL-1 β , because they have been shown to be involved in the macrophage-induced modulation of NF-κB activity in alveolar epithelial cells.^{45,46}

Another major proinflammatory transcription pathway within macrophages involves the AP-1 pathway, whose proinflammatory targets overlap those of the NF-κB pathway.⁴⁷ AP-1 is a group of basic leucine zipper transcription factors, including the Fos and Jun families of transcription factors.⁴⁷ We found that AP-1 c-Jun and c-Fos activities were greatly enhanced when DT was given to CD206-DT receptor Tg mice with endotoxemia, which implies that M2 macrophages negatively regulate the AP-1 pathway. We, thus, suggest that AP-1 signaling is a key transcriptional regulator involved in the inhibitory modulation of lung inflammation by M2 macrophages. There were slight differences in the extents of the enhancing effect of DT treatment on an array of proinflammatory cytokine levels in CD206-DT receptor Tg mice. This may be associated with some differences in the dependence of the transcription of cytokines on the NF-κB and AP-1 pathways. In accord with the importance of AP-1 in regulating IL-10 promoter activity,⁴⁸ a striking up-regulation of IL-10 mRNA was found when CD206-depleted mice were challenged with LPS. However, the increased AP-1 activity may be secondary to the significant result of CD206 depletion leading to altered LPS responses. Further investigations are required to delineate a mechanistic role of AP-1 in the

increased pulmonary inflammatory response in CD206-depleted mice.

We have previously demonstrated that short-term treatment with high doses of statin can increase the number of alveolar macrophages in mouse lungs.⁴⁹ These alveolar macrophages display an unusual phenotype compared with typical tissue macrophages. Thus, we have found that alveolar macrophages express high levels of CD11c,⁴⁹ a molecule that is not expressed by their counterparts in other body sites and is generally expressed by dendritic cells.⁵⁰ However, contrary to dendritic cells, alveolar macrophages are unlikely to emigrate from the tissue and seem to have distinct roles in the initiation and maintenance of immune response.⁵¹ These alveolar macrophages may be now identified as M2 macrophages. Our statin treatment has been able to mitigate ALI and improve the survival of mice with cecal ligation and puncture-induced sepsis.⁴⁹

In conclusion, our findings identify lung CD206-positive M2 macrophages as key anti-inflammatory cells during endotoxemic lung injury. We clearly demonstrate that the depletion of M2 macrophages can aggravate lung inflammation that is linked with neutrophil recruitment to the lung. Our finding may become part of a therapeutic strategy for pulmonary inflammatory disease.

Acknowledgments

We thank Prof. Kenji Kohno (Nara Institute of Science and Technology, Nara, Japan) for providing the pTRECK6 vector and Toshio Fujimori for technical assistance.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.09.005>.

References

1. Tugal D, Liao X, Jain MK: Transcriptional control of macrophage polarization. *Arterioscler Thromb Vasc Biol* 2013, 33:1135–1144
2. Romagnani S: T-cell subsets (Th1 versus Th2). *Ann Allergy Asthma Immunol* 2000, 85:9–18
3. Gordon S: Alternative activation of macrophages. *Nat Rev Immunol* 2003, 3:23–35
4. Martinez FO, Sica A, Mantovani A, Locati M: Macrophage activation and polarization. *Front Biosci* 2008, 13:453–461
5. Lumeng CN, Bodzin JL, Saltiel AR: Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* 2007, 117:175–184
6. Fujisaka S, Usui I, Burkhar A, Ikutani M, Oya T, Kanatani Y, Tsuneyama K, Nagai Y, Takatsu K, Urakaze M, Kobayashi M, Tobe K: Regulatory mechanisms for adipose tissue M1 and M2 macrophages in diet-induced obese mice. *Diabetes* 2009, 58:2574–2582
7. Fujisaka S, Usui I, Kanatani Y, Ikutani M, Takasaki I, Tsuneyama K, Tabuchi Y, Burkhar A, Yamazaki Y, Suzuki H, Senda S, Aminuddin A, Nagai Y, Takatsu K, Kobayashi M, Tobe K: Telmisartan improves insulin resistance and modulates adipose tissue macrophage polarization in high-fat-fed mice. *Endocrinology* 2011, 152:1789–1799
8. Scotton CJ, Martinez FO, Smelt MJ, Sironi M, Locati M, Mantovani A, Sozzani S: Transcriptional profiling reveals complex regulation of the monocyte IL-1 beta system by IL-13. *J Immunol* 2005, 174:834–845
9. Martinez FO, Gordon S, Locati M, Mantovani A: Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol* 2006, 177:7303–7311
10. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A: Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 2002, 23:549–555
11. Varnum MM, Ikezu T: The classification of microglial activation phenotypes on neurodegeneration and regeneration in Alzheimer's disease in brain. *Arch Immunol Ther Exp* 2012, 60:251–266
12. Cassetta L, Cassol E, Poli G: Macrophage polarization in health and disease. *ScientificWorldJournal* 2011, 11:2391–2402
13. Ware LB, Matthay MA: The acute respiratory distress syndrome. *N Engl J Med* 2000, 342:1334–1349
14. Manicone A: Role of the pulmonary epithelium and inflammatory signals in acute lung injury. *Expert Rev Clin Immunol* 2009, 5:63–75
15. Grommes J, Soehnlein O: Contribution of neutrophils to acute lung injury. *Mol Med* 2011, 17:293–307
16. Lomas-Neira J, Chung CS, Perl M, Gregory S, Biffl W, Ayala A: Role of alveolar macrophage and migrating neutrophils in hemorrhage-induced priming for ALI subsequent to septic challenge. *Am J Physiol Lung Cell Mol Physiol* 2006, 290:L51–L58
17. Kooguchi K, Hashimoto S, Kobayashi A, Kitamura Y, Kudoh I, Wiener-Kronish J, Sawa T: Role of alveolar macrophages in initiation and regulation of inflammation in *Pseudomonas aeruginosa* pneumonia. *Infect Immun* 1998, 66:3164–3169
18. Marriott HM, Dockrell DH: The role of the macrophage in lung disease mediated by bacteria. *Exp Lung Res* 2007, 33:493–505
19. Koay MA, Gao X, Washington MK, Parman KS, Sadikot RT, Blackwell TS, Christman JW: Macrophages are necessary for maximal nuclear factor-kappa B activation in response to endotoxin. *Am J Respir Cell Mol Biol* 2002, 26:572–578
20. Naidu BV, Krishnadasan B, Farivar AS, Woolley SM, Thomas R, Van Rooijen N, Verrier ED, Mulligan MS: Early activation of the alveolar macrophage is critical to the development of lung ischemia-reperfusion injury. *J Thorac Cardiovasc Surg* 2003, 126:200–207
21. Zhao M, Fernandez LG, Doctor A, Sharma AK, Zarbock A, Tribble CG, Kron IL, Laubach VE: Alveolar macrophage activation is a key initiation signal for acute lung ischemia-reperfusion injury. *Am J Physiol Lung Cell Mol Physiol* 2006, 291:L1018–L1026
22. Frank JA, Wray CM, McAuley DF, Schwendener R, Matthay MA: Alveolar macrophages contribute to alveolar barrier dysfunction in ventilator-induced lung injury. *Am J Physiol Lung Cell Mol Physiol* 2006, 291:L1191–L1198
23. Knapp S, Leemans JC, Florquin S, Branger J, Maris NA, Pater J, van Rooijen N, van der Poll T: Alveolar macrophages have a protective anti-inflammatory role during murine pneumococcal pneumonia. *Am J Respir Crit Care Med* 2003, 167:171–179
24. Amano H, Morimoto K, Senba M, Wang H, Ishida Y, Kumatori A, Yoshimine H, Oishi K, Mukaida N, Nagatake T: Essential contribution of monocyte chemoattractant protein1/C-C chemokine ligand-2 to resolution and repair processes in acute bacterial pneumonia. *J Immunol* 2004, 172:398–409
25. Pappenheimer AM, Harper AA, Moynihan M, Brockers JP: Diphtheria toxin and related proteins: effect of route of injection on toxicity and the determination of cytotoxicity for various cultured cells. *J Inf Dis* 1982, 145:94–102
26. Naglich JG, Metherall JE, Russell DM, Eidels L: Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell* 1992, 69:1051–1061
27. Mitamura T, Higashiyama S, Taniguchi N, Klagsbrun M, Mekada E: Diphtheria toxin binds to the epidermal growth factor (EGF)-like

- domain of human heparin-binding EGF-like growth factor/diphtheria toxin receptor and inhibits specifically its mitogenic activity. *J Biol Chem* 1995, 270:1015–1019
28. Saito M, Iwakaki T, Taya C, Yonekawa H, Noda M, Inui Y, Mekada E, Kimata Y, Tsuru A, Kohno K: Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat Biotechnol* 2001, 19:746–750
 29. Furukawa N, Saito M, Kimata Y, Kohno K: A diphtheria toxin receptor deficient in epidermal growth factor-like biological activity. *J Biochem* 2006, 140:831–841
 30. Oishi H, Takano K, Tomita K, Takebe M, Yokoo H, Yamazaki M, Hattori Y: Olprinone and colforsin daropate alleviate septic lung inflammation and apoptosis through CREB-independent activation of the Akt pathway. *Am J Physiol Lung Cell Mol Physiol* 2012, 303: L130–L140
 31. Matsuda N, Nishihira J, Takahashi Y, Kemmotsu O, Hattori Y: Role of macrophage migration inhibitory factor in acute lung injury in mice with acute pancreatitis complicated by endotoxemia. *Am J Respir Cell Mol Biol* 2006, 35:198–205
 32. Halazonetis TD, Georgopoulos K, Greenberg ME, Leder P: c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell* 1988, 55:917–924
 33. Herold S, Mayer K, Lohmeyer J: Acute lung injury: how macrophages orchestrate resolution of inflammation and tissue repair. *Front Immunol* 2011, 2:65
 34. Ezekowitz RA, Sastry K, Bailly P, Warner A: Molecular characterization of the human macrophage mannose receptor: demonstration of multiple carbohydrate recognition-like domains and phagocytosis of yeasts and Cos-1 cells. *J Exp Med* 1990, 172:1785–1794
 35. Haltiwanger H, Hill RL: The ligand specificity and tissue localization of a rat alveolar macrophage lectin. *J Biol Chem* 1986, 261: 15696–15702
 36. Magnusson S, Berg T: Endocytosis of ricin by rat liver cells in vivo and in vitro is mainly mediated by mannose receptors on sinusoidal endothelial cells. *Biochem J* 1993, 291:749–755
 37. Lew BD, Songu-Mize E, Pontow SE, Stahl PD, Rattazzi MC: A mannose receptor mediates mannose-rich glycoprotein-induced mitogenesis in bovine airway smooth muscle cells. *J Clin Invest* 1994, 94:1855–1863
 38. Norrman F, Braat WAM, Barrett-Bergshoeff M, Barbé E, van Leeuwen A, Lindeman J, Rijken DC: Monoclonal antibodies against the human mannose receptor as a specific marker in flow cytometry and immunohistochemistry for macrophages. *J Leukoc Biol* 1997, 61: 63–72
 39. Montovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M: The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* 2004, 25:677–686
 40. Surace MJ, Li L: Potent suppression of arginase 1 expression in murine macrophages by low dose endotoxin. *Am J Clin Exp Immunol* 2013, 2:117–123
 41. Baldwin AS: The NF- κ B and I κ B proteins: new discoveries and insights. *Annu Rev Immunol* 1996, 14:649–683
 42. Bährle PA, Henkel T: Function and activation of NF- κ B in the immune system. *Annu Rev Immunol* 1994, 12:141–179
 43. Huang W, Ghisletti S, Perissi V, Rosenfeld MG, Glass CK: Transcriptional integration of TLR2 and TLR4 signaling at the NCoR derepression check-point. *Mol Cell* 2009, 35:48–57
 44. Knapp S, Wieland CW, van't Veer C, Takeuchi O, Akira S, Florquin S, van der Poll T: Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defence. *J Immunol* 2004, 172:3132–3138
 45. Quinton LJ, Jones MR, Simms BT, Kogan MS, Robson BE, Skerrett SJ, Mizgerd JP: Functions and regulation of NF-kappaB RelA during pneumococcal pneumonia. *J Immunol* 2007, 178:1896–1903
 46. Hess C, Herr C, Beisswenger C, Zakharkina T, Schmid RM, Bals R: Myeloid RelA regulates pulmonary host defense networks. *Eur Respir J* 2010, 35:343–352
 47. Fujioka S, Niu J, Schmidt C, Sclabas GM, Peng B, Uwagawa T, Li Z, Evans DB, Abbruzzese JL, Chiao PJ: NF-kappaB and AP-1 connection: mechanism of NF-kappaB-dependent regulation of AP-1 activity. *Mol Cell Biol* 2004, 24:7806–7819
 48. Saraiva M, O'Garra A: The regulation of IL-10 production by immune cells. *Nat Rev Immunol* 2010, 10:170–181
 49. Takano K, Yamamoto S, Tomita K, Takashina M, Yokoo H, Matsuda N, Takano Y, Hattori Y: Successful treatment of acute lung injury with pitavastatin in septic mice: potential role of glucocorticoid receptor expression in alveolar macrophages. *J Pharmacol Exp Ther* 2011, 336:381–390
 50. Gonzalez-Juarrero M, Shin TS, Kipnis A, Junqueira-Kipnis AP, Orme IM: Dynamics of macrophage cell populations during murine pulmonary tuberculosis. *J Immunol* 2003, 171:3128–3135
 51. Jakubzick C, Tacke F, Llodra J, van Rooijen N, Randolph GJ: Modulation of dendritic cell trafficking to and from the airways. *J Immunol* 2006, 176:3578–3584