

## Intranasal priming of newborn mice with microbial extracts increases opsonic factors and mature CD11c<sup>+</sup> cells in the airway

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**Kasahara K, Matsumura Y, Ui K, Kasahara K, Komatsu Y, Mikasa K, Kita E.** Intranasal priming of newborn mice with microbial extracts increases opsonic factors and mature CD11c<sup>+</sup> cells in the airway. *Am J Physiol Lung Cell Mol Physiol* 303: L834–L843, 2012. First published August 24, 2012; doi:10.1152/ajplung.00031.2012.—Nasal exposure to the mixture of microbial extracts (MME) after ab lactation enhanced airway resistance of newborn mice to *Streptococcus pneumoniae* (*J Physiol Lung Cell Mol Physiol* 298: L67, 2010). The present study was addressed to elucidate effective factors responsible for the enhanced innate resistance in the airway of MME-exposed newborn mice. MME exposure significantly increased the amount of pulmonary surfactants (SP-A and SP-D) in the airway. Bronchoalveolar lavage fluid of the exposed mice exhibited greater levels of opsonic activity, thereby enhancing the phagocytic and intracellular killing activities of alveolar macrophages (MØ) against *S. pneumoniae*. The exposure itself did not increase a complement component C3 and mannan-binding lectin-A (MBL-A) in the airway, whereas intratracheal infection with *S. pneumoniae* increased the quantity of SP-A, SP-D, C3, and MBL-A in the exposed mice to a significant extent compared with control mice. The exposure enhanced the expression of the class A scavenger MØ receptor with collagenous structure on alveolar MØ and also increased the frequency of major histocompatibility complex II+ CD11c<sup>+</sup> cells in the lung; the cells were able to produce IL-10 and transforming growth factor- $\beta$  in vitro. These results suggest that microbial exposure early in life increases the amounts of SP-A and SP-D and the number of scavenger MØ and also promotes maturation of CD11c<sup>+</sup> cells in the airway of newborn mice, which may be involved in airway resistance to *S. pneumoniae*.

commensal flora; regulation of acute inflammation; surfactants

THE NEWBORN IS EXPOSED to the variety of environmental microorganisms immediately after birth. The immunological competence in neonatal gut progresses in parallel to the increase in the density of microbial colonization (17, 45). Because host defense of newborns mainly depends on the components of innate immunity until immune system fully develops (27, 36), early establishment of microbial colonization is crucial to the defense against invading pathogens in neonatal gut (14, 39, 44). In the respiratory tract, the airways are continuously exposed to an enormous load of airborne microorganisms and environmental antigens during respiration. Although newborns are generally protected by the antibodies transferred across the placenta before birth and through their mother's breast milk after birth (23), their respiratory mucosal system allows some of the airborne and environmental microorganisms to colonize

the airways soon after birth. *Neisseria lactamica*, one of the human nasopharyngeal flora, expresses a product capable of lowering Toll-like receptor (TLR) 2-dependent local inflammation, thereby delaying or avoiding clearance by innate immunity (25). Thus commensal organisms appear to have their own specific strategies to reduce inflammatory response for colonization (reviewed in Ref. 28), possibly by exploiting the TLR pathway (38, 40).

Our previous study (48) showed that nasal exposure to the mixture of microbial extracts (MME) in weanling mice conferred a beneficial effect on local resistance to intratracheal infection with type 3 *Streptococcus pneumoniae*. MME exposure promoted the colonization of microbial flora including lactobacilli in the airway and also induced higher levels of TLR expression, compared with control exposure. Intratracheal infection with *S. pneumoniae* induced greater magnitude of macrophage (MØ) accumulation than polymorphonuclear leukocyte (PMN) response in the airway of MME-exposed mice. However, the infection did not upregulate TLR expression levels to a further extent in MME-exposed mice, contrasting with strong TLR expression in mice receiving control exposure. Despite such alterations of airway response, pneumococcal growth in the lung of MME-exposed mice was suppressed to a significant extent, leading to increase in average survival time of the exposed mice.

One major concern over the interpretation of our previous results is whether these effects are due to the rapid increase in microbial colonization after MME exposure or the direct stimulatory effect of MME. Importantly, the difference in elements composing innate immunity between the exposed and the control newborn mice was only the number and species of airway flora (48). Thus, we speculate that rapid colonization of bacteria including *Lactobacillus* spp. in the airway of newborn mice is related to the alteration of airway response, as demonstrated by Gabryszewski et al. (9). In addition, we thought that some effective factors might be involved in compensation for the suppressed PMN and TLR response after pneumococcal infection.

The present study was therefore addressed to elucidate the factors that augment antimicrobial activity in alveolar MØ or regulate inflammatory response in the airway of MME-exposed newborn mice. We showed here that MME exposure enhanced pulmonary surfactant production (SP-A, SP-D), MARCO (macrophage receptor with collagenous structure) expression, and also increased the number of major histocompatibility complex (MHC) II-positive CD11c<sup>+</sup> cells in the airway. Especially, the enhanced production of SP-A and SP-D was crucial to enhancement of the phagocytic and intracellular killing activities of alveolar MØ. We therefore propose that

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exposure to environmental microbes immediately after birth reinforces airway innate resistance by increasing cellular or soluble factors, which may be related to dense microbial colonization in the airway.

## MATERIALS AND METHODS

**Mice.** Weanling C57BL/6 mice (specific pathogen-free 3-wk-old male) were purchased from Charles River Laboratories Japan (Kanagawa, Japan); they were kept and handled under sterile conditions throughout experiments as described before (48). The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Nara Medical University School of Medicine.

**Preparation of microbial extracts.** Culture and cell extraction of *Aspergillus fumigatus* [American Type Culture Collection (ATCC) 14109], *Penicillium marneffeii* (ATCC 201013), *Cladosporium herbarum* (ATCC 201090), *Candida albicans* (ATCC 14053), *Staphylococcus epidermidis* (clinical isolate), and *Escherichia coli* (clinical isolate) were performed exactly following the method as described before (48). The 1-ml volume of each microbial suspension (300 µg/ml in PBS) was mixed together to make a preparation of the mixture of microbial extracts (defined as MME), which contained 50 µg/ml of each microbial extract.

**Exposure to MME.** Exposure to MME was performed according to the protocol as described previously (48). Mice were used within 24 h after ab lactation; the day of the first exposure was defined as *day 0*. A group of mice was intranasally inoculated with 50 µl of MME containing 2.5 µg of each microbial extract (MME group) or the same volume of sterile PBS (control group) once a day, starting from *day 0* to *day 28* every other day (15 exposures in total). These two groups were used for experiments at 10 days after the final exposure.

**Bronchoalveolar lavage and alveolar macrophage preparation.** Bronchoalveolar lavage (BAL) was performed as described previously (48) with a slight modification. The tracheae of euthanized mice were cannulated, and the lungs were washed three times with 0.8 ml of ice-cold Hanks' Balanced Salt Solutions (HBSS) containing 0.6 mM EDTA (Dojindo Laboratories, Kumamoto, Japan) to provide >2 ml of BAL fluid (BALF). All retrieved fluids from each mouse were combined as a BALF sample, and cellular components were sedimented by centrifugation at 400 *g* for 10 min at 4°C. The sedimented components were used for isolation of alveolar MØ. BALF samples were frozen at -80°C for subsequent assays immediately after the volume and total protein were measured. Protein content was determined using a BCA protein assay kit (Bio-Rad Laboratories, Hercules, CA).

For isolation of alveolar MØ, the sedimented cells (~1.2–1.8 × 10<sup>6</sup> cells per mouse) were suspended in phenol red-free MEM (GIBCO, Life Technologies Japan, Tokyo, Japan) containing 10% FBS (GIBCO) with 1 mM HEPES (Wako Pure Chemical Industries, Osaka, Japan) (pH 7.4). After 2-h adherence to culture dishes (35 mm; BD Biosciences, San Diego, CA) (5 × 10<sup>5</sup> cells/dish), adherent cells were detached by incubation with accutase (PAA Laboratories, Cölbe, Germany), and the detached cells were used as MØ after washing. Higher than 98% of the cells were MØ, as confirmed by a modified Wright-Giemsa stain, by flow cytometry using FITC-conjugated rat anti-mouse F4/80 MAb (Cell Sciences, Canton, MA), and by enzyme cytochemistry for the absence of demonstrable myeloperoxidase activity (26). MØ were suspended at a density of 10<sup>6</sup>/ml in MEM for phagocytosis assay, 2.5 × 10<sup>6</sup>/ml in HBSS containing 0.5% FBS (HBSS/FBS) for chemiluminescence (CL) or 10<sup>8</sup>/ml in HBSS for Western blot assay. MØ from control mice and MME-exposed mice were defined as C-MØ and M-MØ, respectively.

**Isolation of lung parenchymal CD11c+ cells.** The tracheae of euthanized mice were cannulated, and the lungs were perfused with 5 ml of warm Ca<sup>2+</sup>-, Mg<sup>2+</sup>-free HBSS (CMFS) containing 5% FBS and antibiotics (100 U/ml penicillin + 100 µg/ml streptomycin) via the right ventricle of the heart. The lung from each mouse was

digested enzymatically for 40 min in 10 ml/lung of CMFS containing 0.5% FBS, antibiotics, 1 mg/ml collagenase (Roche Applied Science, Indianapolis, IN), and 30 µg/ml DNase (Sigma-Aldrich, St. Louis, MO). The action of the enzymes was stopped by adding 10 ml of CMFS supplemented with 1% glutamine, 0.1 mM nonessential amino acids (Life Technologies, Gaithersburg, MD), and 50 mM 2-mercaptoethanol (2-ME) (Wako Pure Chemical Industries). Single-cell suspensions were made by filtering digested tissues through a nylon screen, and cells were harvested by centrifugation. Red blood cells in the cell pellet were lysed with Gey's solution. After viable cell numbers of the cells from individual mice (defined as N<sub>1</sub>) were counted by Trypan blue exclusion, cells prepared from five control mice (on average, 2.2–2.5 × 10<sup>7</sup> cells/mouse) and from 4 MME-exposed mice (on average, 2.6–3.3 × 10<sup>7</sup> cells/mouse) were mixed, respectively. Each single-cell suspension was made in HBSS/FBS containing 2.5 mM EDTA. The number of viable cells in each suspension was adjusted to ~2 × 10<sup>8</sup>/ml for treatment with 20 µg of Fc block (rat anti-mouse CD16/CD32) (BD Pharmingen, San Diego, CA). After treatment, the cells were incubated for 20 min at 4°C with 100 µl of CD11c microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) for 10<sup>8</sup> cells/500 µl. After the incubation, the cells were washed, and CD11c+ cells were selected on an automated magnetic cell sorter (AutoMACS; Miltenyi Biotec) using the posselds program. Residual MØ were removed from the CD11c+ cell population by plastic adherence. Nonadherent cells were pooled and used as parenchymal CD11c+ cells. The viable number of the CD11c+ cells isolated from 10<sup>8</sup> pooled cells (N<sub>2</sub>) was determined by Trypan blue staining. The purity of the CD11c+ cells was determined by flow cytometry as described below. Viability was consistently ≥98%. The number of viable parenchymal CD11c+ cells in each mouse (N<sub>3</sub>) was calculated as follows: N<sub>3</sub> = (N<sub>1</sub> × N<sub>2</sub> × 1/4)/10<sup>8</sup> for an MME-exposed mouse and (N<sub>1</sub> × N<sub>2</sub> × 1/5)/10<sup>8</sup> for a control mouse, respectively. The cells were washed twice and suspended in RPMI 1640 (GIBCO) containing 10% FBS, 400 mM L-glutamine, antibiotics, 5 × 10<sup>-4</sup> M 2-ME with 1 mM HEPES (pH 7.4) (defined as complete medium) for further analyses.

**Flow cytometry.** Parenchymal CD11c+ cells were suspended at 10<sup>5</sup> per 100 µl in staining buffer (BD Pharmingen). After incubation with Fc block, the cells were stained on ice for 30 min with 1 µg of FITC-conjugated hamster anti-mouse CD11c MAb (Miltenyi Biotec) for identification of CD11c+ cells. For determination of MHC II expression by CD11c+ cells, cells were stained with 0.2 µg of PE-Cy5 rat anti-mouse MHC II (eBioscience, San Diego, CA). Stained cells were washed and resuspended in 0.4 ml PBS containing 1% FBS and 0.01% sodium azide and immediately analyzed by flow cytometry using a FACScan flow cytometer (BD Biosciences). Results were analyzed using CellQuest software (BD Biosciences), and fluorescence data were collected on a minimum of 10,000 cells. FITC-labeled hamster IgG (eBioscience) and rat IgG2b (BD Pharmingen) were used as isotype controls. Data were represented as histograms.

For the analysis of MARCO expression, MØ were suspended at 10<sup>5</sup> per 100 µl in staining buffer. After incubation with Fc block, cells were stained with phycoerythrin (PE)-conjugated rat anti-mouse MARCO (IgG1) (R & D Systems, Minneapolis, MN), or PE-labeled rat IgG1 antibody (Ab) (R & D Systems). All samples were analyzed on a FACScan analyzer using CellQuest. A minimum of 10,000 viable cells and cell-bound fluorescence intensity was expressed as the mean intensity of fluorescence (MIF).

**Assay for cytokine production by CD11c+ cells.** Parenchymal CD11c+ cells were incubated with formalin-killed pneumococci of strain NMU112 (44) for 24–48 h. The cells were plated in 500 µl of complete medium in wells (4 × 10<sup>5</sup> per well) of 24-well culture plates (BD Biosciences), and killed pneumococci were added (number of bacteria/cell = 20). Semi-quantitative RT-PCR at 24 h of incubation and ELISA at 48 h were used for characterization of the CD11c+ cells as follows.

**Semi-quantitative RT-PCR analysis.** Semi-quantitative RT-PCR was performed using total RNA extracted from CD11c+ cells as described before (13). The relative expression levels of each selected gene were determined by semi-quantitative RT-PCR. The primers used for PCR amplification were as follows: indoleamine 2,3-dioxygenase (IDO), forward 5'-GTA CATCACCATGGCGTATG-3', reverse 5'-GCCTTCGTC AAGTCTTCATTG-3'; transforming growth factor (TGF)- $\beta$ , forward 5'-CTACTGCTTCAGCTCCACAG-3', reverse 5'-TGCACCTGCAGG AGCGCAC-3'; IL-10, forward 5'-ATCGATTTCTCCCCTGTG-3', reverse 5'-AATGGGAAGTGGATATCAG-3'; IFN- $\alpha$  forward 5'-CTCTGTGCTTTCCTGATGGT-3', reverse: 5'-AGAGAGGGAGTCTCCTCATT-3'; and GAPDH, forward 5'-ACCA-CAGTCCATGCCATCAC-3', reverse: 5'-TCCACCACCCTGTTGCT-GTA-3'. PCR amplification was performed for 30 cycles consisting of the following steps: 5 min at 94°C for denaturing, 30 s at the optimal temperatures specific to the primers used for annealing, and 30 s at 72°C for extension. The products were resolved on a 2% agarose gel, followed by densitometric analysis using Gel analyst system (ICONIX, Paris, France) with NIH Image program (NIH, Bethesda, MD) (48). All genes were normalized to GAPDH.

**ELISA for cytokines.** The production of TGF- $\beta$ , INF- $\alpha$ , IL-10, and IDO in culture supernatants of pneumococci-treated CD11c+ cells was measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

**Phagocytosis and killing ability of alveolar M $\phi$ .** Type 3 *S. pneumoniae* strain NMU112 was grown and prepared for the assay of phagocytosis and intracellular killing by M $\phi$  as described before (47). The colony-forming unit (cfu) numbers of strain NMU112 were determined by plating serial dilutions of bacterial suspension on Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) agar. The procedure used for phagocytosis assay was similar to that described by Nishikawa et al. (33). M $\phi$  were suspended in complete medium devoid of antibiotics (assay medium) at a density of 10<sup>6</sup>/ml. A total of 100  $\mu$ l of the cell suspension was added to each well of a 16-well tissue culture chamber slide (Nunc, Naperville, IL). Following 2-h attachment, nonadherent cells were washed out with warm HBSS. This procedure made higher than 92.5% of the initial input attach to a well, regardless of M $\phi$  sources; an average number of M $\phi$  in a well was  $\sim 9 \times 10^4$  in this assay. Subsequently, 180  $\mu$ l of assay medium containing 1% gelatin and 20  $\mu$ l of opsonized pneumococcal suspension in HBSS containing 1% gelatin (GHBSS) (at a multiplicity of infection = 10) were added to each well; plates were centrifuged (200 g for 3 min) at 4°C and then incubated at 37°C for 30 min under weak horizontal agitation using a micromixer (Taiyokagakukogyo, Tokyo, Japan) for phagocytosis. After several washings with HBSS, gentamicin (Sigma-Aldrich) was added to each well (7.5  $\mu$ g/ml) for 45 min at 4°C. The cells were stained for 45 s with 0.1 mg/ml of acridine orange (Sigma-Aldrich) after washing. Bacteria that fluoresced green were scored as live (L), and those that fluoresced red-orange were scored as dead (D) (22). A total of 500 cells were examined for each well with a fluorescence microscope at  $\times 1,000$  magnification. Each assay was performed in triplicate. Phagocytic ability was evaluated by the mean number of M $\phi$  phagocytizing bacteria (either L or D) and also by the mean number of total bacteria (L + D) per cell. Data are expressed as means  $\pm$  SE from triplicate determinations of three different experiments.

To determine the intracellular killing ability by direct plate counting, M $\phi$  were washed several times with cold HBSS after a 30-min period of phagocytosis, and then 1 ml of HBSS containing 300  $\mu$ g/ml of gentamicin was added to each well. After 45-min incubation at 4°C, gentamicin was removed by extensive washing. Thereafter, half of the cells were lysed with 500  $\mu$ l of 1.5% saponin (Sigma-Aldrich) in HBSS for 1 min, and cfu numbers at *time 0* (CFU<sub>t=0</sub>) were determined by plating 50  $\mu$ l of the lysate on THY agar. The other half was incubated for additional 60 min at 37°C and then subjected to enumeration of intracellular CFU (CFU<sub>t=60</sub>). Each assay was per-

formed in triplicate. Intracellular killing ability was expressed as  $(1 - \text{CFU}_t - 60/\text{CFU}_t - 0) \times 100$  (%). Data are expressed as means  $\pm$  SE from triplicate determinations of three different experiments.

**Opsonization of *S. pneumoniae*.** The opsonin source was fresh mouse serum or cell-free BALF. Fresh serum was prepared from five mice, and pooled sera were used as a serum opsonin. Cell-free BALF samples from four mice were pooled, concentrated to 1 mg/ml by ultrafiltration, and used as a BALF opsonin. All opsonin preparations were membrane sterilized and immediately stored at -80°C until use. Serum and BALF prepared from control mice were defined as C-serum and C-BALF, respectively, and those from MME-exposed animals defined as M-serum and M-BALF, respectively.

To opsonize bacteria, pneumococcal suspension was spun at 2,000 g for 3 min, and the pellets were washed three times in HBSS. Aliquots then were resuspended in GHBSS with 10% (vol/vol) serum or BALF opsonin and incubated for 30 min at 37°C on a rotating rack. Opsonized bacteria were washed three times and resuspended in 1 ml of GHBSS at  $5 \times 10^7$  cfu/ml for phagocytosis assay and  $2 \times 10^8$  cfu/ml for CL assay, respectively.

**Luminol-dependent CL assay.** The activity of respiratory burst in alveolar M $\phi$  was evaluated using luminol-dependent CL as described previously (8). In brief, cell suspension (10<sup>6</sup> cells in 400  $\mu$ l GHBSS) was mixed with 50  $\mu$ l of luminol solution ( $1.0 \times 10^{-6}$  M; Laboscience, Tokyo, Japan) in a counting tube, which was kept at 37°C for 5 min with gentle agitation. To generate CL, opsonized pneumococci (10<sup>7</sup> cfu in 50  $\mu$ l of GHBSS) was added to the cell mixture. Light release was quantified for 30 s per sample at 5-min intervals for 30 min using the Lumiphotometer TD-4000 (Laboscience). Data output is relative light units per s (RLU/s) over a 30-min period. The CL intensity was presented as a slope [peak RLU / peak time (s)]  $\times 100$ . Data are expressed as means  $\pm$  SE from triplicate determinations of three different experiments.

**Western blot assay for surfactant proteins.** A 50- $\mu$ l aliquot of BALF samples was mixed with an equivalent volume of SDS sample buffer (50 mM Tris-HCl, pH 6.5, 10% glycerol, 2% SDS, 10% 2-ME, and 0.1% bromophenol blue) and incubated at 98°C for 5 min. Five micrograms of protein of each sample was loaded on to each lane of 12% SDS-PAGE gel, electrophoresed under reducing conditions, using a minigel apparatus (Bio-Rad). Western blot assay was performed as described before (47). Primary Abs were as follows: rabbit or goat Abs against SP-A (1: 500), SP-C (1: 400), and SP-D (1: 500), all of which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and SP-B (1:2,000) from Millipore (Billerica, MA). Proteins were visualized with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Santa Cruz) or HRP-conjugated rabbit anti-goat IgG (Santa Cruz) and ECL Western blotting detection system (GE Healthcare Japan, Tokyo) according to the manufacturer's instructions. Densitometry analysis was performed using gel analysis system with NIH Image program as described before (48). Results are expressed as the percentage of the density of each protein in C-BALF at 0 h (before infection). Data are expressed as mean  $\pm$  SE from triplicate determinations of three different experiments.

**Measurement of C3 and MBL-A in BALF.** C3 in BALF was determined by ELISA, using a kit of Mouse C3 ELISA (GenWay Biotech, San Diego, CA) according to the instructions provided. Mannan-binding lectin (MBL)-A in BALF was measured by sandwich ELISA kits (HyCult Biotechnology, Uden, The Netherlands). ELISA was performed according to manufacturer's instructions. For MBL-A ELISA, PBS supplemented with 10% FBS was used as a diluent for the detection antibody and the HRP-Streptavidin, and EDTA was added to a BALF sample at a final concentration of 0.6 mM. In both ELISA tests, BALF samples of individual mice were used without concentration after centrifugation to remove cellular components, and individual samples were measured in triplicate. Data are expressed as means  $\pm$  SE from triplicate determinations of three different experiments.

Table 1. Effect of MME exposure on CL generation by airway MØ

MØ	Opsonins	Peak CL	CL intensity
C-MØ	C-serum	19.73 ± 2.86	4.88 ± 0.36
	M-serum	26.27 ± 3.94	7.98 ± 0.84
	C-BALF	28.34 ± 2.37	7.89 ± 0.53
	M-BALF	48.16 ± 5.88*	16.00 ± 1.71*
M-MØ	C-serum	21.24 ± 1.52	6.47 ± 0.28
	M-serum	30.44 ± 4.28	10.67 ± 0.67
	C-BALF	36.52 ± 3.94	10.00 ± 1.33
	M-BALF	63.29 ± 6.34*	30.48 ± 1.02†

*S. pneumoniae* was opsonized with serum derived from control mice (C-serum), serum derived from mixture of microbial extracts (MME)-exposed mice (M-serum), C-bronchoalveolar lavage fluid (BALF), or M-BALF before incubation with macrophages (MØ). MØ suspension ( $10^6$  cells in 400 µl HBSS containing 1% gelatin, GHBS) was mixed with 50 µl of  $1.0 \times 10^{-6}$  M luminol solution in a counting tube, and opsonized pneumococci [ $10^7$  colony-forming units (cfu) in 50 µl of GHBS] were added to the suspension. Light release was quantified for 30 s per sample at 5-min intervals for 30 min. Data output is relative light units per s (RLU/s) over a 30-min period. Peak chemiluminescence (CL) is expressed as RLU/s  $\times 10^{-3}$ . The mean intensity of CL was calculated by [peak RLU/peak time (s)]  $\times 100$ . MØ were obtained from 3 mice in each group, and pooled cells were assayed using respective opsonins. Data are shown as means  $\pm$  SE from triplicate determinations of 3 different experiments. Both peak CL and CL intensity were significantly greater in M-MØ than in C-MØ only when M-BALF was used as an opsonin ( $P < 0.05$ ). \* $P < 0.05$  vs. C-BALF and M-serum,  $P < 0.025$  vs. C-serum, † $P < 0.025$  vs. C-BALF and M-serum,  $P < 0.01$  vs. C-serum.

**Absorption of opsonic factors in BALF by specific antibodies.** A BALF sample obtained before infection was dispensed in 250-µl aliquots (250 µg protein) into four sterile plastic tubes containing 50 µl of 5 µg of one of the following IgG Abs: rabbit Ab to SP-A, rabbit Ab to MBL-A, goat Ab to SP-D, or goat Ab to C3, all of which were purchased from Santa Cruz Biotechnology. All antibodies were coupled to protein G beads (Amersham Pharmacia Biotech, Piscataway, NJ) (47). For control absorption, the same volume of the BALF sample was added to a sterile plastic tube containing 50 µl of an isotype-matched Ab (5 µg) (normal rabbit IgG or goat IgG) coupled to protein G beads. All tubes were rotated at 4°C overnight and subsequently centrifuged at 4,000 g for 10 min at 4°C. Supernatants of individual test tubes were used as absorbed opsonins. Absorption procedure using each specific Ab was confirmed to reduce the amount of a corresponding target protein to the levels lower than the detection limit by Western blot. Deletion of individual opsonins was evaluated by the assay for the phagocytic and intracellular killing activities of M-MØ. Control absorption of either C-BALF or M-BALF at 0 h did not affect both activities of alveolar MØ to a significant extent; thus, both activities using respective BALFs receiving control absorption (control BALF) were defined as control activity. Results of these assays were expressed as a percentage of control activity. Data are expressed as means  $\pm$  SE from triplicate determinations of three different experiments.

**Statistics.** All data are presented as means  $\pm$  SE. Statistical analyses were performed using StatView 4.01 (Abacus Concepts, Berkeley, CA). A two-way ANOVA for repeated measures with Bonferroni post hoc analyses was used to determine whether the levels of surfactant proteins, C3, and MBL-A in BALF, and the expression levels of MARCO by MØ, were altered over time after pneumococcal infection. For the remainder of the data analysis, including the number of CD11c+ cells, the frequency of MHC II+ CD11c+ cells, CL intensity, levels of cytokine expression and production, and the phagocytosis and intracellular killing assays, the nonparametric Mann-Whitney test was utilized. Significance was set at  $P < 0.05$ .

## RESULTS

**Alveolar MØ assay.** The antimicrobial capacity of alveolar MØ was evaluated by the luminol-dependent CL and by the phagocytosis and intracellular killing assays. Opsonization with M-BALF elicited significantly greater levels of peak CL and CL intensity in both MØ during phagocytosis, compared with opsonization with other opsonins (in both MØ;  $P < 0.05$  vs. C-BALF and M-serum,  $P < 0.025$  vs. C-serum, in M-MØ;  $P < 0.01$  vs. C-serum) (Table 1). Especially, opsonization with M-BALF elicited the highest levels of both peak CL and CL intensity in M-MØ, and both parameters generated in M-MØ were significantly greater than in C-MØ ( $P < 0.05$ ). Compared with C-serum, opsonization with M-serum appeared to elicit relatively higher CL intensity in both MØ although not at a significant extent.

Next, we examined the phagocytic ability of MØ against *S. pneumoniae* opsonized with various opsonins. First, phagocytic ability of MØ was assessed by staining with acridine orange; the ability was evaluated by the mean number of phagocytizing MØ and also by that of pneumococci within a cell (Table 2). Especially, opsonization with M-BALF allowed greater numbers of both MØ to engulf pneumococci compared with that with C-BALF ( $P < 0.05$ ); additionally, the number of pneumococci opsonized with M-BALF in a cell was significantly greater than that of bacteria opsonized with C-BALF ( $P < 0.05$ ), independently of the source of MØ. Opsonization with M-BALF resulted in significantly lower ratio of L/D in both MØ, meaning greater degrees of intracellular killing, compared with other opsonins ( $P < 0.05$  vs. C-BALF, C-serum, and M-serum). These findings were consistent with those of intracellular killing assay by direct plate counting on THY agar (Table 3).

Table 2. Effect of MME exposure on phagocytosis by alveolar MØ

MØ	Opsonized With	No. of MØ-Engulfing bacteria	No. of bacteria per MØ	Average L/D
C-MØ	C-serum	53 ± 5	5.2 ± 2.3	84.5
	M-serum	64 ± 12	6.4 ± 3.3	80.3
	C-BALF	96 ± 19	8.2 ± 1.6	78.6
	M-BALF	213 ± 18*	16.5 ± 4.1*	53.4*
M-MØ	C-serum	62 ± 17	5.8 ± 3.7	80.6
	M-serum	76 ± 11	7.2 ± 4.2	78.2
	C-BALF	98 ± 23	9.6 ± 2.5	72.5
	M-BALF	242 ± 37*	21.8 ± 4.2*	50.2*

Opsonized pneumococcal suspension in 20 µl of GHBS was added to MØ monolayers ( $9 \times 10^4$  per well) in 180 µl of DMEM containing 1% gelatin (multiplicity of infection = 10); plates were centrifuged (200 g for 3 min) at 4°C and then incubated at 37°C for 30 min for phagocytosis. After 2 washings with HBSS, gentamicin was added to each well (final concentration: 7.5 µg/ml) for 45 min. The cells were stained for 45 s with acridine orange after washing. Bacteria that fluoresced green were scored as live (L), and those that fluoresced red-orange were scored as dead (D). Numbers of MØ-engulfing pneumococci, either L or D, were counted, and numbers of L and D pneumococci in a cell were counted. A total of 500 cells were examined for each well with a fluorescence microscope. Data are expressed as means  $\pm$  SE from triplicate determinations of 3 different experiments. The average ratio of L to D per cell (average L/D) was determined for a total of 300 MØ-ingesting organisms. Data are expressed as the average value from triplicate determinations of 3 different experiments, \* $P < 0.05$  vs. C-BALF.

Table 3. Effect of MME exposure on intracellular killing activity of alveolar MØ

MØ	Oponized With	Intracellular Killing, %
C-MØ	C-serum	19.7 ± 2.4
	M-serum	22.3 ± 3.3
	C-BALF	24.5 ± 4.6
	M-BALF	57.4 ± 3.8*
M-MØ	C-serum	20.8 ± 3.7
	M-serum	24.6 ± 5.2
	C-BALF	26.8 ± 2.9
	M-BALF	60.6 ± 4.3*

After a 30-min period of phagocytosis as described in Table 1, MØ were washed several times with cold HBSS, and then 1 ml of HBSS containing 7.5 µg/ml gentamicin was added to each well. After 45-min incubation at 4°C, the antibiotic was removed by serial washing. After the washing, half of the cells were treated with 500 µl of HBSS with 1.5% saponin for 1 min. CFU<sub>t-0</sub> in each lysate was determined by colony counting. The other half was incubated for additional 60 min at 37°C and then treated with 1.5% saponin for determination of CFU<sub>t-60</sub>. Intracellular killing ability was expressed as (CFU<sub>t-0</sub> - CFU<sub>t-60</sub>) / CFU<sub>t-0</sub> × 100 (%). \**P* < 0.05 vs. C-BALF.

**Assay for scavenger MØ in the lung.** Currently, alveolar MØ expressing a scavenger receptor, MARCO, are shown to have an important role for innate resistance against pneumococcal infection (2). Thus we examined the expression levels of MARCO on the surface of MØ by flow cytometry analysis (Fig. 1). The expression levels of MARCO by M-MØ, as determined by MIF, were significantly higher at 24 h (*P* < 0.05) and 36 h (*P* < 0.01) postinfection, compared with the preinfection levels (0 h). M-MØ expressed significantly higher levels of MARCO than did C-MØ at indicated time points (*P* < 0.025 at 0 h and 24 h, *P* < 0.05 at 36 h). In C-MØ, MARCO expression was increased to a significant extent only at 36 h postinfection compared with the level at 0 h (*P* < 0.05).

**Analysis of opsonic factors in BALF.** Next, we sought to elucidate the effective opsonin(s) in M-BALF. Because airway surfactants have been implicated in the regulation of pulmonary innate defense and inflammation (reviewed in Ref. 19), we quantified the amount of surfactants (SP-A, -B, -C, and -D) in BALF by Western blot and subsequent densitometry assay. Western blot assay showed that the protein levels of SP-A and SP-D in M-BALF increased in M-BALF at three time points, but in C-BALF only at 24 h (Fig. 2A). By contrast, the levels of SP-B and SP-C were not different between two groups of mice before and after infection although both surfactants slightly increased after infection (data not shown). Densitometry assays demonstrated that M-BALF at 0 h contained greater amounts of both SP-A and SP-D to a significant extent (*P* < 0.05), compared with C-BALF at 0 h (Fig. 2B). At 24 h and 36 h postinfection, these two surfactants in M-BALF increased to further higher levels (*P* < 0.025 at 24 h, *P* < 0.01 at 36 h vs. M-BALF at 0 h; *P* < 0.025 at 24 h, *P* < 0.01 at 36 h vs. C-BALF at the same time point).

In both mouse groups, pneumococcal infection increased C3 levels within 1 h after infection (a 2.2-fold increase in C-BALF, a 2.4-fold increase in M-BALF, *P* < 0.05 vs. before infection, respectively) (Fig. 3A). During the next 2 h, C3 levels rapidly decreased in both groups to the levels similar to those at 0 h. Between 6 h and 24 h of infection, C3 levels reincreased in M-BALF to a significant extent (*P* < 0.05 at 6 h,

*P* < 0.01 at 12 h and 24 h). Despite a rapid decline in C3 concentration during the next 12 h, C3 levels in M-BALF at 36 h remained higher compared with those at 0 h (*P* < 0.05). In C-BALF, C3 levels slowly reincreased after 6 h of infection; they remained at the same levels from 6 h to 36 h although at significantly greater levels compared with those at 0 h (*P* < 0.05). At 12 h and 24 h postinfection, C3 levels in C-BALF were significantly lower than in M-BALF (*P* < 0.05).

MME exposure itself did not increase MBL-A levels (at 0 h) compared with control exposure (Fig. 3B). After infection, MBL-A levels increased in M-BALF (*P* < 0.05 at 6 h and 36 h, *P* < 0.01 at 12 h and 24 h) and in C-BALF (*P* < 0.05 at 12 h, 24 h and 36 h), respectively, compared with those at 0 h of the same mouse group. However, MBL-A levels were statistically higher in M-BALF than in C-BALF at 12 h and 24 h postinfection (*P* < 0.05).

Absorption of either SP-A or SP-D most significantly reduced the phagocytic and intracellular killing activities of M-MØ (phagocytosis; *P* < 0.025, intracellular killing; *P* < 0.01) compared with control absorption (Fig. 4A). Absorption of MBL-A and C3 slightly, but not significantly, reduced both activities of M-MØ, compared with control absorption. Similar results were obtained when C-MØ were used instead of M-MØ (data not shown). Depletion of opsonins, except SP-D, from C-BALF decreased slightly, although not significantly, the phagocytic and intracellular killing activities of M-MØ. Only the intracellular killing activity of M-MØ was reduced by depletion of SP-D from C-BALF to a significant extent (*P* < 0.05) (Fig. 4B); however, its reduction level was significantly lower than that by SP-D depletion from M-BALF (*P* < 0.05).

**Properties of lung parenchymal CD11c+ cells.** Purity of the isolated CD11c+ cells was assessed by staining with FITC-conjugated CD11c MAb; ~97.1–98.2% of the cells obtained before infection were positive for CD11c (Fig. 5A), irrespective of mouse groups, validating the isolation procedure. The number of CD11c+ cells was not statistically different be-

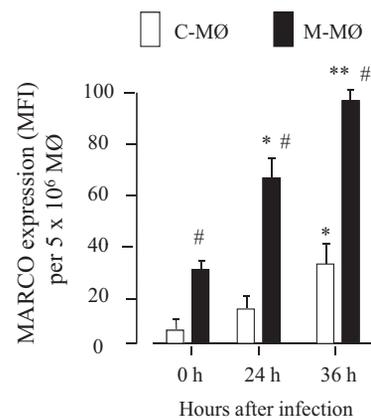


Fig. 1. Effect of mixture of microbial extracts (MME) exposure on the expression of MARCO by alveolar macrophages (MØ). MØ ( $10^5$  per 100 µl) were incubated with 2 µg/ml FITC rat anti-mouse MARCO antibodies (Abs) (IgG1), or FITC isotype-matched control Abs after incubation with Fc block (20 µg/ml). All samples were analyzed on a FACScan analyzer using Cell-Quest. A minimum of 10,000 viable cells was analyzed. Cell-bound fluorescence intensity was expressed as mean fluorescence intensity (MFI). Results are presented as means ± SE from triplicate determinations of 3 different experiments. \**P* < 0.05, \*\**P* < 0.01 vs. preinfection levels (0 h) of the same group, #*P* < 0.05 vs. control MØ (C-MØ) at the same time point. M-MØ, macrophages from MME-exposed mice.

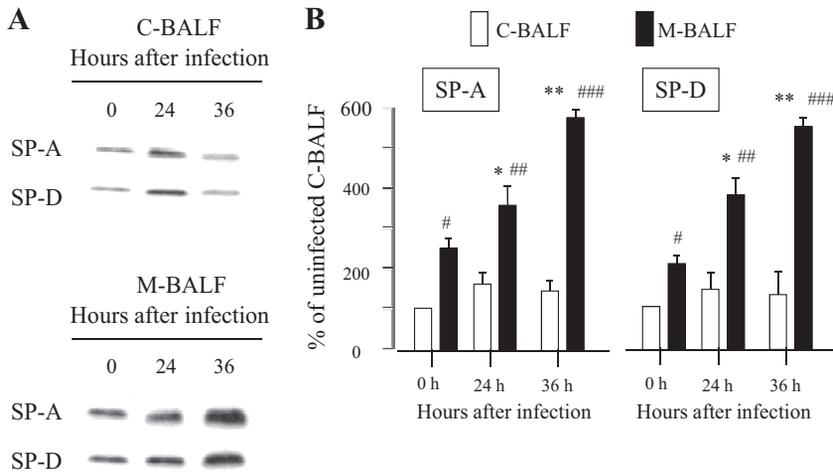


Fig. 2. Effect of MME exposure on the induction of surfactants in the airway. *A*: Western blot analysis using anti-pulmonary surfactant (SP)-A and anti-SP-D antibodies was performed to compare the expression levels of SP-A and SP-D in 5  $\mu$ g protein of C-bronchoalveolar lavage fluid (BALF) and M-BALF, respectively, obtained before (0 h) and at 24 h and 36 h of infection with pneumococci. The data are shown from 1 representative experiment of 3 different experiments with similar results ( $n = 3$  at each time point per experiment). *B*: densitometric analysis of Western blot assays (*A*) for SP-A and SP-D was performed. Density of the Ab-bound protein bands in individual samples collected at preinfection (0 h), at 24 h, and at 36 h postinfection was expressed as a percentage of the density of each corresponding Ab-bound protein band in C-BALF obtained at preinfection (0 h). Results are expressed as means  $\pm$  SE from triplicate determinations of 3 different experiments. \* $P < 0.05$ , \*\* $P < 0.025$  vs. M-BALF at 0 h, # $P < 0.05$ , ## $P < 0.025$ , ### $P < 0.01$  vs. C-BALF at the same time point.

tween two groups of mice before infection (Fig. 5B). However, MME-exposed mice had a 3.7-fold increase in numbers of CD11c+ cells during 24 h of infection ( $P < 0.025$ ), and a twofold increase between 12 h and 24 h of infection, respectively ( $P < 0.05$ ). By contrast, control mice had only a

1.25-fold increase during 24 h of infection. Although the proportion and absolute number of CD11c+ cells were not statistically different between two groups of mice before infection, the frequency of the cells expressing MHC II was

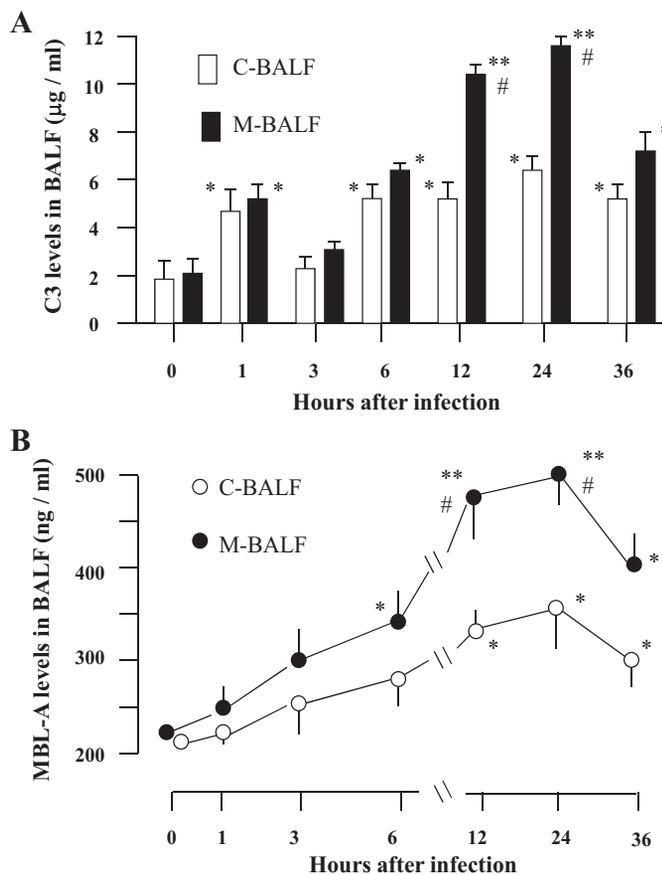


Fig. 3. Kinetics of C3 and mannan-binding lectin (MBL)-A production in the airway. Levels of C3 (*A*) and MBL-A (*B*) in BALF were measured using ELISA kits. At each time point, BALF samples were obtained from 3 mice, and measurement of individual samples was performed in triplicate. Results are presented as means  $\pm$  SE from 3 different experiments. \* $P < 0.05$ , \*\* $P < 0.01$  vs. BALF of the same group at 0 h, # $P < 0.05$  vs. C-BALF at the same time point.

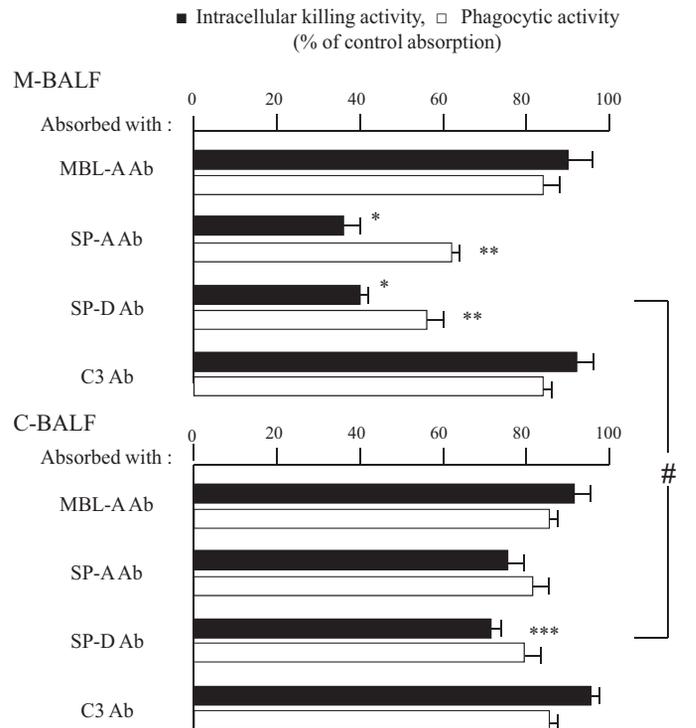


Fig. 4. Effect of opsonin absorption from M-BALF on the phagocytic and killing activities of alveolar M $\phi$ . SP-A, SP-D, MBL-A, and C3 were absorbed with corresponding specific antibodies coupled to protein G beads, respectively, from C-BALF and M-BALF obtained before infection (0 h). The phagocytosis and intracellular killing were assessed following the same methods described in Tables 2 and 3, respectively, using M-M $\phi$  or C-M $\phi$ , and pneumococci opsonized with 1 of absorbed BALF samples. For preparation of control BALF, BALF samples obtained at 0 h were treated with isotype-matched Abs coupled to protein G beads. Both activities of M $\phi$  determined using control BALF were defined as the control activity. At each time point, 3 different samples were used, and each sample was assayed in triplicate. Results are expressed as percentages of respective control activity and presented as means  $\pm$  SE from 3 different experiments. \* $P < 0.01$ , \*\* $P < 0.025$ , \*\*\* $P < 0.05$  vs. respective control absorption. # $P < 0.05$ , between C-BALF and M-BALF.

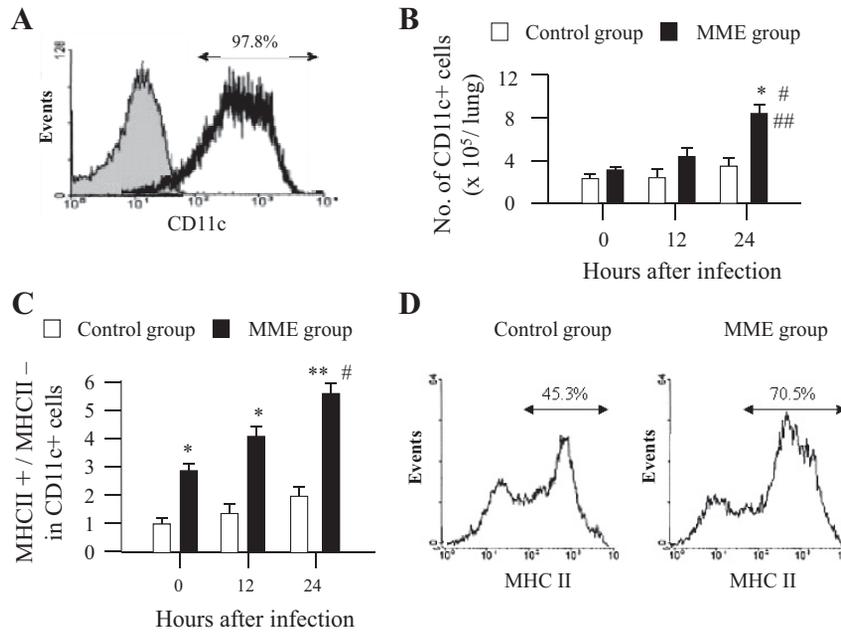


Fig. 5. Effect of MME exposure on the induction of major histocompatibility complex (MHC) II+ CD11c+ cells in the lung. CD11c+ cells enriched from respective cell suspensions using AutoMACS and CD11c microbeads and the enriched cells were depleted of M $\phi$  by plastic adherence for further purification. **A:** purity of the isolated parenchymal CD11c+ cells was assessed by staining with FITC anti-CD11c MAb (no shading) or isotype control (gray shading). The histogram was representative of 3 separate analyses using the cells from uninfected control group with similar results; the number indicated the mean proportion (%) of CD11c+ cells in the examined cells. The proportion of CD11c+ cells was not significantly different between 2 groups before infection. **B:** number of viable CD11c+ cells per lung was calculated as described in MATERIALS AND METHODS. At each time point, 3 sets of purified CD11c+ cells were analyzed in an experiment. Results are expressed as means  $\pm$  SE of triplicate determinations of 3 different experiments. \* $P$  < 0.05 compared with control mouse at 24 h, # $P$  < 0.025, ## $P$  < 0.05 vs. mice of the same group at 0 h and 12 h, respectively. **C:** MHC II expression by parenchymal CD11c+ cells isolated at indicated time points was determined by flow cytometry using PE-Cy5 rat anti-MHC II MAb. The ratio of MHC II+ cells to MHC II- cells was determined on at least 10,000 cells. At each time point, three sets of purified CD11c+ cells were analyzed in an experiment. Results are expressed as means  $\pm$  SE of triplicate determinations of 2 different experiments. \* $P$  < 0.05, \*\* $P$  < 0.025 vs. mice of control group at the same time point, respectively. # $P$  < 0.05 vs. mice of the same group at 0 h and 12 h, respectively. **D:** histograms showed expression of MHC II on CD11c+ cells isolated from mice of both groups at 24 h postinfection. The numbers in the histograms give the mean proportion (%) of MHC II+ cells in CD11c+ cells. The results are representative of 3 individual experiments with similar results.

significantly greater in MME-exposed mice compared with control mice ( $P$  < 0.05 at 0 h and 12 h) (Fig. 5C). Furthermore, greater frequencies of MHC II+ CD11c+ cells were observed in the lung of MME-exposed mice at 24 h postinfection ( $P$  < 0.05 compared with 0 h and 12 h). The mean proportion (%) of MHC II+ CD11c+ cells from MME-exposed mice at 24 h was  $\sim$ 1.6-fold greater than that from control mice at the same time point ( $P$  < 0.05) (Fig. 5D).

When CD11c+ cells isolated from mice before infection were incubated with killed pneumococci for 24 h, RT-PCR showed that mRNAs for IL-10 and TGF- $\beta$  were expressed at significantly greater levels in the cells of MME-exposed mice than in those of control mice ( $P$  < 0.05) (Fig. 6, A and B). Accordingly, protein levels of these two cytokines were increased in culture supernatants obtained at 48 h of stimulation to a significant extent in the cells of MME-exposed mice than in those of control mice ( $P$  < 0.05) (Fig. 6C). By contrast, neither mRNA expression nor protein production of IDO and IFN- $\alpha$  was significantly increased, regardless of mouse groups.

## DISCUSSION

In the present investigation, we sought to elucidate the helper factor(s) involved in local resistance in the airway of MME-exposed mice. Nasal exposure to MME induced greater amounts of SP-A and SP-D in the airway, compared with

control exposure. Opsonization with M-BALF significantly enhanced the phagocytic and killing activities of M-M $\phi$  against type 3 *S. pneumoniae*, compared with opsonization with C-BALF. Such enhancement was associated with greater amounts of SP-A and SP-D in M-BALF; depletion of both SP-A and SP-D from M-BALF significantly reduced the phagocytic and killing activities of M-M $\phi$ . Several reports have shown that both surfactants contribute to airway resistance and modulation of inflammatory response (5, 10, 19, 35). In addition, SP-A directly interacts with TLR4 and MD-2, resulting in alteration of TLR2-mediated signaling (31), as well as inhibition of LPS-elicited inflammation (46). SP-D also binds to TLR2 by a mechanism different from its binding to LPS (34). Moreover, both surfactants are shown to bind to several serotypes of *S. pneumoniae*, enhancing uptake of pneumococci by phagocytes (12). Interestingly, SP-D is shown to delay the pneumococcal blood invasion (15, 16), which is consistent with our previous observation (48). Taken together, these findings suggest that both of SP-A and SP-D serve to be effective helper factors in the airway for enhancing the phagocytic and intracellular killing activities of alveolar M $\phi$  and for regulating inflammatory response. Therefore, increased production of these surfactants must be crucial to enhancement of local resistance to pneumococci in the airway of MME-exposed newborn mice, as suggested by Haagsman et al. (11).

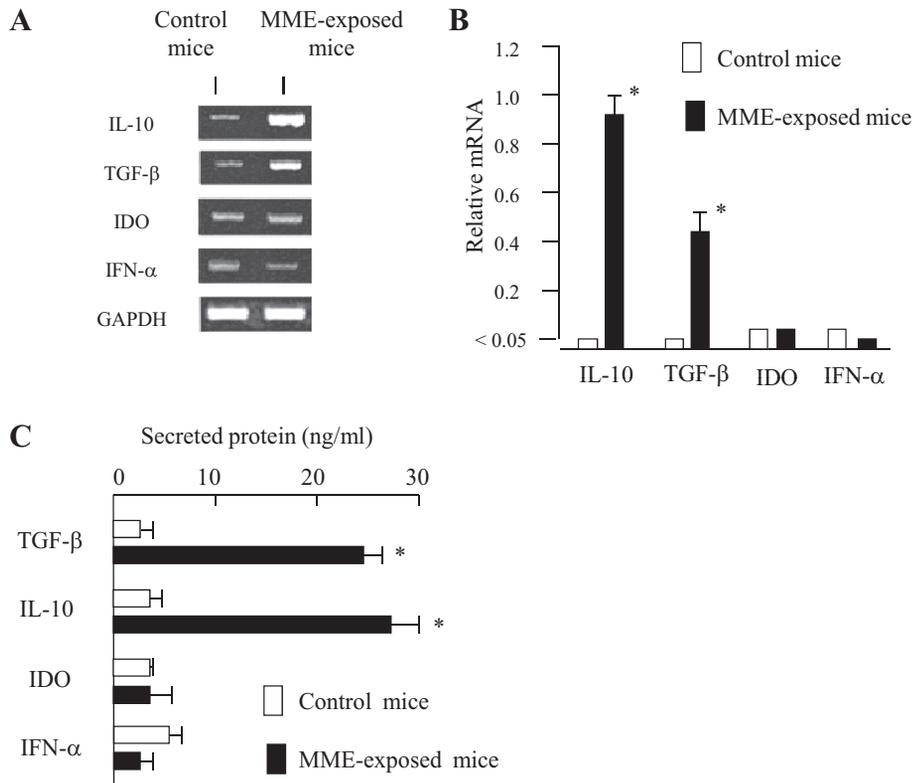


Fig. 6. Analysis of cytokine production by CD11c+ cells. CD11c+ cells ( $4 \times 10^5$  per well) were stimulated with formalin-killed pneumococci for 24–48 h. **A**: expression of mRNAs for individual soluble factors in cultured CD11c+ cells was examined by RT-PCR after 24-h stimulation. The representative results obtained from 3 separate experiments with similar results are shown. **B**: expression level of mRNA for each factor was normalized relative to GAPDH mRNA of the same sample. Results are presented as means  $\pm$  SE from 3 different experiments with similar results (4–6 mice/group in an experiment). \* $P < 0.05$  vs. control mouse cells at the same time point. **C**: concentrations of IL-10, IFN- $\alpha$ , TGF- $\beta$ , and indoleamine 2,3-dioxygenase (IDO) in cell-free culture supernatants collected at 48 h of stimulation were determined by ELISA. Data are expressed as means  $\pm$  SE of 3 separate experiments with 5 different measurements from each mouse sample. \* $P < 0.05$  vs. control mouse cells.

MME exposure increased the number of M $\phi$  expressing MARCO to a significant extent, compared with control exposure, and subsequent pneumococcal infection further upregulated MARCO expression. MARCO-deficient (MARCO<sup>-/-</sup>) mice are very susceptible to intranasal inoculation with *S. pneumoniae*, accompanied with severe tissue damage in the lung (2). Furthermore, SP-A enhances uptake of *S. pneumoniae* by alveolar M $\phi$  resulting from increased expression of scavenger receptor class A (20). These facts suggest the increased expression of MARCO by alveolar M $\phi$ , in association with increment of SP-A production, is able to enhance phagocytosis as well as regulate inflammatory response (6) in the lung of MME-exposed newborn mice.

Although mature CD11c+ dendritic cells (DCs) are known to regulate immune responses via production of TGF- $\beta$ , IL-10, IFN- $\alpha$ , or IDO (29, 30, 41), it is unclear about whether mature DCs could be involved in the regulation of acute inflammatory response. MME exposure itself did not increase the number of CD11c+ cells in the lung, but the frequency of MHC II+ CD11c+ cells was significantly higher in the exposed mice compared with control mice. Subsequent pneumococcal infection increased their numbers and promoted maturation to a further extent in the exposed mice. The CD11c+ cells of MME-exposed mice produced higher levels of IL-10 and TGF- $\beta$  in vitro upon stimulation with pneumococci, compared with the cells of control mice. However, the amount of IL-10 in the lungs of MME-exposed mice was not significantly greater at 24 h postinfection than in control mouse lungs (48), indicating that their capacity for producing IL-10 in vitro is not sufficient to increase this cytokine in the lung after infection, or that their numbers are small relative to other cells in lungs. Nevertheless, there is an apparent difference in the ability of

CD11c+ cells to produce in vitro IL-10 and TGF- $\beta$  between two groups of mice. Thus it is speculated that the cells may take part in the regulation of inflammatory response to *S. pneumoniae* in MME-exposed newborn mice. Clearly, more studies are required to elucidate the actual role of CD11c+ cells and the meaning of maturation of the cells in the airway of MME-exposed newborn mice.

There is strong evidence that the complement system is important for innate immunity to *S. pneumoniae* (16, 18); especially, the classical complement pathway is the dominant pathway for opsonization of *S. pneumoniae* at least in mice (4, 18). Although C3 is shown to be essential in the lung for an optimal immune response against *S. pneumoniae* (18), the pneumococcal surface proteins (PspA and PspC) act synergistically to inhibit C3 activation in combination with pneumolysin (1, 24, 37). Thus it appears that C3 may not be activated enough to enhance phagocytosis of *S. pneumoniae* by M $\phi$ , despite the fact that C3 increased in M-BALF during late infection. MBL-A also increased in M-BALF during late infection. However, this factor seems to exhibit low activity in opsonization against *S. pneumoniae* because pneumococci have a low binding capacity to MBL-A (32). Importantly, absorption of these two factors from M-BALF reduced the phagocytic and intracellular killing activities of M-M $\phi$  only to a lesser extent, compared with the absorption of SP-A and SP-D. Previous reports by others indicate that both C3 (18, 42) and MBL-A (43) play an anti-inflammatory role during bacterial infection by reducing production of inflammatory cytokines. Taken together, it is assumed that the elevated levels of both C3 and MBL-A in M-BALF during late infection (after 12 h of infection) might be more important for the regulation of inflammatory response than for opsonophagocytosis. Kerr

et al. (18) have proposed that the relevation of C3 levels during the late phase of pneumococcal infection is due to serum proteins leaking into the lungs rather than enhanced local production although we did not explore their proposal.

Colonizing bacteria are recognized by TLRs, which subsequently trigger innate immune responses; the responses may result in either eliminating unfavorable bacteria or shaping the airway microbial flora (38). Currently, Duggan et al. (7) have reported that simultaneous treatment with ligands for TLR2/6 and TLR9 induced a high level of resistance to microbial pathogens including *S. pneumoniae* in the lung of adult mice. One would predict that MME exposure in newborn mice may serve the same function as TLR agonists in adult mice. Although MME exposure enhanced the expression of TLR-2, -4 and -9 in the lung of newborn mice, their expression was not increased to a further extent after infection; nevertheless, the exposed mice survived significantly longer than control mice after infection (48). There are two explanations for the discrepancy in TLR responses between before infection and after infection in MME-exposed mice. The first one is that the first stimulation of TLRs with MME after birth may modify their responsiveness to subsequent stimulation with invading pneumococci as a second stimulus. This notion is based on our preliminary experimental findings that such different behavior of TLR response did not occur in newborn mice, when MME exposure started at 6 wk after birth (unpublished data). In this setting, mice are continuously stimulated with environmental microbial antigens before MME exposure; that is to say MME exposure is a second stimulus. The alternative explanation is that the dense colonization of microbes in the airway, especially *Lactobacillus* spp., in MME-exposed newborn mice may regulate the responsiveness of TLRs, as demonstrated by Gabryszewski et al. (9). They showed that priming with live lactobacilli protected mice independently of TLR pathways from a subsequent lethal respiratory microbial challenge because the effect was apparent in *MyD88* gene-deleted mice (9). Indeed, MME does not contain *Lactobacillus* spp., but MME exposure is likely to give the similar effect in the airway of newborn mice by inducing dense colonization of *Lactobacillus* spp. (48). Furthermore, greater levels of TLR expression by MME exposure might be related to early shaping of commensal flora in the airway of newborn mice because recent work emphasized the crucial role of TLRs in microbial colonization (3, 21, 40).

Overall, we have shown that the repeated exposure to microbial extracts (MME) in newborn mice increased helper factors, including production of SP-A and SP-D, MØ expressing MARCO, and mature CD11c+ cells. These factors are possibly to contribute to airway resistance as well as the regulation of acute inflammatory response in the lung after pneumococcal infection. Furthermore, we propose that such alterations for innate immunity after MME exposure might be related to a dense colonization of microbes including *Lactobacillus* spp. in the airway of newborn mice.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

#### AUTHOR CONTRIBUTIONS

Author contributions: Kazuki Kasahara, Y.M., K.U., Kei Kasahara, and Y.K. performed experiments; Kazuki Kasahara, K.U., and K.M. analyzed data; Kazuki Kasahara, Y.M., Kei Kasahara, Y.K., K.M., and E.K. interpreted results of experiments; Kazuki Kasahara and Y.M. prepared figures; K.M. and E.K. drafted manuscript; E.K. conception and design of research; E.K. edited and revised manuscript; E.K. approved final version of manuscript.

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