

ORIGINAL ARTICLE

Characteristics of alveolar macrophages from murine models of OVA-induced allergic airway inflammation and LPS-induced acute airway inflammation

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ABSTRACT

Background: Macrophages include the classically activated pro-inflammatory M1 macrophages (M1s) and alternatively activated anti-inflammatory M2 macrophages (M2s). The M1s are activated by both interferon- γ and Toll-like receptor ligands, including lipopolysaccharide (LPS), and have potent pro-inflammatory activity. In contrast, Th2 cytokines activate the M2s, which are involved in the immune response to parasites, promotion of tissue remodeling, and immune regulatory functions. Although alveolar macrophages (AMs) play an essential role in the pulmonary immune system, little is known about their phenotypes. **Methods:** Quantitative reverse transcription polymerase chain reaction and flow cytometry were used to define the characteristics of alveolar macrophages derived from untreated naïve mice and from murine models of both ovalbumin (OVA)-induced allergic airway inflammation and LPS-induced acute airway inflammation. AMs were co-cultured with CD4⁺ T cells and were pulsed with tritiated thymidine to assess proliferative responses. **Results:** We characterized in detail murine AMs and found that these cells were not completely consistent with the current M1 versus M2-polarization model. OVA-induced allergic and LPS-induced acute airway inflammation promoted the polarization of AMs towards the current M2-skewed and M1-skewed phenotypes, respectively. Moreover, our data also show that CD11c⁺ CD11b⁺ AMs from the LPS-treated mice play a regulatory role in antigen-specific T-cell proliferation in vitro. **Conclusions:** These characteristics of AMs depend on the incoming pathogens they encounter and on the phase of inflammation and do not correspond to the current M1 versus M2-polarization model. These findings may facilitate an understanding of their contributions to the pulmonary immune system in airway inflammation.

KEYWORDS allergic airway inflammation, alveolar macrophages, M1 macrophages, M2 macrophages

INTRODUCTION

Macrophages are highly plastic cells that acquire a diversity of activation states in response to specific stimuli that depend on the local environment [1, 2]. Macrophages have been broadly categorized into subsets, including classically activated M1 macrophages (M1s) and alternatively activated M2 macrophages (M2s) [3, 4]. Macrophages are skewed to the M1

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phenotype by interferon- γ (IFN- γ) and/or after the engagement of Toll-like receptors (TLR) by their cognate pathogen-associated molecular patterns, such as lipopolysaccharide (LPS) [3, 5]. M1s have a high bactericidal and tumoricidal capacity and are characterized by the synthesis and upregulation of pro-inflammatory cytokines and chemokines, including interleukin (IL)-12, IL-6, IL-1 β , and tumor necrosis factor- α (TNF- α), as well as inducible nitric oxide synthase (iNOS; also denoted as nitric oxide synthase 2, NOS2). In contrast, macrophages also undergo an alternative activation by Th2 cytokines, including IL-4 and IL-13. M2s are involved in the immune response to parasites, promotion of tissue remodeling and tumor progression, and immune regulatory functions. This M1 versus M2-polarization model has been very helpful in describing immune responses [6], although this useful oversimplification has caused a barrier to a better understanding of macrophage activation [7, 8]. Mosser DM *et al.* suggested that there may be many different 'shades' of activation that have yet to be identified, resulting in a spectrum of macrophage populations based on their function [9]. Recently, transcriptome-based network analysis has revealed a spectrum of macrophage activation states extending the M1 versus M2-polarization model [7]. Moreover, nomenclature and experimental guidelines for macrophage activation and polarization propose that researchers should consider that there may be more than two types of macrophages and should adopt a nomenclature linked to the activation conditions based on stimulation scenarios, i.e., M(IL-4), M(IL-10), M(IFN- γ), M(LPS), and so forth [8].

In the pulmonary immune system, alveolar macrophages (AMs) act as the first line of defense against inhaled particulates and pathogens, and they play an essential role in both the initiation and orchestration of inflammatory responses. Thus, AMs are not only excellent phagocytes capable of removing particulates and pathogens from the airway, but they can also promote innate and adaptive immune responses. Recent reports have shown that human AMs from bronchoalveolar lavage (BAL) of patients with asthma and from lung tissue of patients with chronic obstructive pulmonary disease (COPD) have not corresponded to the current M1 versus M2-polarization model [10, 11]. Furthermore, macrophages might be skewed from one state to another, depending on either the incoming pathogens they encounter or the phase of inflammation [12, 13]. However, whether different AM phenotypes reflect the conditions of local microenvironment is poorly understood.

In the present study, we examined the characteristics of AMs from untreated naïve mice and from murine models of both ovalbumin (OVA)-induced allergic airway inflammation and LPS-induced acute airway inflammation. We identified the detailed characteristics of these AMs and determined that AMs in the steady state do not correspond to the current M1 versus M2-polarization model. OVA-induced allergic airway inflammation and LPS-induced acute airway inflammation promoted the polarization of AMs towards the current M2 [after exposure to either Th2 cytokines or M(IL-4)]-skewed and either the current M1 [after exposure to LPS with or without IFN- γ , M(LPS) or M(LPS+IFN- γ)]-skewed phenotypes, respectively. However, these AMs did not correspond to the M1 versus M2-polarization model. Moreover, our data show that CD11c⁺ CD11b⁺ AMs from the LPS-treated mice might be recruited to a site of inflammation and play a regulatory role in antigen-specific T-cell proliferative responses *in vitro*.

MATERIALS AND METHODS

Mice

Female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). Mice transgenic for the OVA_{323–339}-specific and I-A^d-restricted DO11.10 TCR- $\alpha\beta$ on a Rag-2^{-/-} BALB/c background (DO11.10/Rag-2^{-/-}) were generously supplied by Dr. S. Koyasu (Keio University School of Medicine, Tokyo, Japan) [14]. All mice were kept under specific pathogen-free conditions during the experiments. All animal experiments were approved by the Juntendo University Animal Experimental Ethics Committee and complied with the National Institutes of Health guidelines for animal care.

Antibodies

Phycoerythrin (PE)-conjugated anti-CD206 (C068C2) and anti-Ly-6C (HK1.4) monoclonal antibodies (mAbs) were purchased from Bio Legend (San Diego, CA, USA). PE-conjugated anti-CCR3 (83101), anti-SR-AI/MSR1/CD204 (268318), and anti-Dectin-1 (#218820) mAbs were purchased from R&D Systems (Minneapolis, MN, USA). PE-conjugated anti-CD11b (M1/70), anti-Gr-1 (RB6-8C5), anti-Ly-6G (1A8), anti-Siglec-F (E50-2440), anti-B7-2/CD86 (GL1), and fluorescein isothiocyanate (FITC)-conjugated anti-CD11b (M1/70) mAbs were purchased from BD Pharmingen (San Diego, CA, USA). Rat IgG isotype control,

purified anti-CD16/32 (2.4G2), FITC-conjugated, PE-conjugated, PerCP/Cy5.5-conjugated, and APC-conjugated anti-CD11c (N418), PE-conjugated anti-F4/80 (BM8), anti-DEC205/CD205 (205yekta), anti-DC-SIGN/CD209 (5H10), anti-Toll-like receptor (TLR) 2 (6C2), anti-B7-1/CD80 (RM80), anti-programmed death receptor-ligand 1 (PD-L1) (MIH6), anti-PD-L2 (TY25), and anti-I-A^d and I-E^d class II MHC molecules (M5/114.15.2) mAbs were purchased from eBioscience (San Diego, CA, USA).

Induction of Airway Inflammation

The murine model of OVA-induced allergic airway inflammation was induced in a manner similar to previously described protocols [15]. Briefly, BALB/c mice were immunized by i.p. injection of 100 μ g of OVA (Sigma-Aldrich, St Louis, MO, USA) with 2 mg of alum adjuvant (Thermo Fisher Scientific, Yokohama, Japan) (OVA/alum) on days 0 and 14. On days 21, 23, and 25, the mice were challenged with 30 mL of aerosolized 1% OVA in PBS (OVA/PBS). The aerosol was generated by a nebulizer (NE-U07; Omron, Tokyo, Japan). For the murine model of LPS-induced acute airway inflammation, BALB/c mice were anesthetized with isoflurane and given intranasal LPS (10 μ g/mouse in PBS) from *Escherichia coli* serotype 055:B5 (Sigma-Aldrich, St. Louis, MO, USA) on days 0 and 4. In these different murine models, BAL fluids were collected as described below on the day following the last inhalation.

Preparation of Alveolar Macrophages

BAL fluids were harvested as previously described [15]. Briefly, the trachea was cannulated with a polyethylene tube through which the lungs were gently lavaged with 0.5 mL of PBS containing 10% fetal calf serum (FCS) four times. The total number of cells was determined by Turk dye exclusion. BAL fluid-derived cells were incubated at a concentration of 2×10^6 cells/mL in tissue culture dishes for 2 hours at 37°C, 5% CO₂, followed by extensive washing with prewarmed RPMI 1640 to remove non-adherent cells. The adherent cells, defined as AMs, were collected by scraping with a cell scraper (Sumitomo Bakelite, Tokyo, Japan). This procedure yielded a purity of >95% AMs as confirmed by morphology in cytopspins with Diff-Quik (Sysmex International Reagents, Kobe, Japan) and by flow cytometry. Viability of the AMs was confirmed by trypan blue dye exclusion.

RNA Isolation and Quantitative RT-PCR

Total cellular RNA was isolated from AMs using the RNeasy plus mini kit (Qiagen, Valencia, CA, USA) with DNase treatment, followed by cDNA synthesis with the First-Strand cDNA Synthesis kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and the ABI 7500 Fast real-time PCR system (Applied Biosystems, Warrington, UK) were used for quantitative real-time reverse transcription-PCR (qRT-PCR) with the gene-specific primer pairs listed in the Table. For data analyses, the comparative threshold cycle (CT) value for GAPDH was used to normalize loading variations in the real-time PCRs. A $\Delta\Delta$ CT value was then obtained by subtracting control Δ CT values from the corresponding experimental Δ CT values. The $\Delta\Delta$ CT values were converted to the fold difference as compared to the control by raising two to the $\Delta\Delta$ CT power.

In vitro AM Culture and Cytokine Measurements

AMs were plated ($1.0\text{--}5.0 \times 10^4$ cells/well) in 96-well plates with RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/mL penicillin and streptomycin, and 50 μ M 2-mercaptoethanol and incubated for 24 hours. AM culture supernates were collected for cytokine measurement. The supernates of the AM cultures and BAL fluids were assayed by the multiplex bead array assays in accordance with the manufacturer's instructions (Bio-Plex, Bio-Rad Laboratories, Hercules, CA, USA). The assay working range was determined between the lower limit of quantification and the upper limit of quantification (Supplemental Table).

Flow Cytometric Analysis

BAL fluid-derived cells were immediately stained for flow cytometric analysis after collection of the BAL fluids. Cells ($0.5\text{--}1.0 \times 10^6$) were first preincubated with unlabeled anti-CD16/32 mAbs to avoid nonspecific binding of antibodies to the Fc receptor (FcR) and then incubated with biotinylated mAbs. The cells were washed with PBS twice and incubated with either PE-labeled streptavidin or mAbs. After washing with PBS twice, the stained cells (live gated on the basis of forward and side scatter profiles and propidium iodide (PI) exclusion) were analyzed with a FACSCalibur (BD Biosciences, Mountain View, CA, USA). AMs were identified by expression of

CD11c and a high background from autofluorescence. For AMs, fluorochrome compensation settings were adjusted by using either CD11c-FITC, CD11c-PE, CD11c-percp/Cy5.5, or CD11c-APC staining. A staining protocol used four-color approaches in all experiments. CD11c, CD11b or autofluorescence, PI, and each cell surface molecular analysis were performed in the same tubes. The data were processed using the CellQuest program (BD Biosciences).

Stimulation of Antigen-specific CD4⁺ T Cells *in vitro*

DO11.10/Rag-2^{-/-} mice were subcutaneously immunized with OVA/alum, and cervical lymph node cells were isolated on day 7 after immunization. CD4⁺ T cells derived from these lymph node cells from the OVA-treated DO11.10/Rag-2^{-/-} mice were purified by magnetic bead separation (Easy-Sep, StemCell Technologies Inc., Vancouver, BC, Canada), according to the manufacturer's instructions. CD4⁺ T cells (1×10^5) were co-cultured with AMs at the indicated density in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin, and 50 μ M 2-mercaptoethanol in the presence of the OVA_{323–339} peptide (2 μ M). To assess the proliferative responses, the cultures were pulsed with tritiated thymidine ($[^3\text{H}]\text{TdR}$; 0.5 μ Ci/well) for the last 6 hours of a 48-hour culture and harvested on a Micro 96 Harvester (Molecular Devices, Sunnyvale, CA, USA). Incorporated radioactivity was measured on a microplate beta counter (PerkinElmer, Waltham, MA, USA).

Statistical Analysis

Comparisons between multiple groups were made by one-way ANOVA with Tukey's multiple comparisons test. For Figure 3 and Supplemental Figure S2B, two-way ANOVA with Tukey's multiple comparisons test was used. All analyses were performed with the program GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA). Differences were considered to be statistically significant when *P*-values were 0.05 or less.

RESULTS

Bronchoalveolar Lavage-derived AMs and Cytokines in BAL Fluids

To explore the differences among AMs from untreated naïve mice and from either OVA-induced

allergic or LPS-induced acute airway inflammation murine models, we first defined the cellular composition of the BAL fluid that was collected from individual mice. As shown in Figure 1, mice in the OVA- and LPS-induced airway inflammation models had a higher number of AMs as compared to naïve mice. The BAL fluids collected from OVA- and LPS-induced airway inflammation murine models was characterized by large amounts of eosinophils and neutrophils, respectively. Next, we compared the secreted cytokines in the BAL fluids (Supplemental Figure S1). The concentrations of IL-1 α , IL-1 β , IL-6, IL-12p40, IL-12p70, Eotaxin-2/CC chemokine ligand (CCL) 24, granulocyte colony-stimulating factor (G-CSF), IFN- γ , monocyte chemotactic protein-1 (MCP-1)/CCL2, macrophage inflammatory protein (MIP)-1 α /CCL3, MIP-1 β /CCL4, regulated on activation, normal T cell expressed and secreted (RANTES)/CCL5, and TNF- α in the BAL fluids of the LPS-treated mice were significantly higher than that in the other groups. In contrast, the concentrations of Th2 cytokines, IL-4, IL-5, and IL-13 in the BAL fluids of the OVA-induced allergic airway inflammation model mice were significantly higher than that in the other groups. IL-2, IL-3, and IL-17A were not detectable in the BAL fluids.

AMs from Murine Models of OVA-induced Allergic and LPS-induced Acute Airway Inflammation Express Marker mRNA for M2s and M1s, Respectively

We examined M1- and M2-related gene expression in RNA isolated from AMs. The mRNA expression of the M2 markers arginase-1, Ym1 (also denoted as CHI3L3), found in the inflammatory zone 1 (FIZZ1; also denoted as resistin-like molecule- α , RELM- α), thymus and activation-regulated chemokine (TARC)/CCL17, macrophage-derived chemokine (MDC)/CCL22, Eotaxin-2/CCL24, and IL-4 were significantly increased in AMs from the murine model of OVA-induced allergic airway inflammation (OVA-AMs) as compared to that in the other groups (Figure 2). In contrast, the M1 markers iNOS, MCP-1/CCL2, RANTES/CCL5, and IL-12p40 mRNA were significantly increased in AMs from the murine model of LPS-induced acute airway inflammation (LPS-AMs) as compared to that in the other groups. IL-4 receptor α chain (IL-4R α) and TGF- β 1 mRNA were expressed in AMs from untreated naïve mice (Naïve-AMs) and OVA-AMs and were down-regulated in LPS-AMs. Although both OVA-AMs and LPS-AMs showed similar responses with respect to numbers in Figure 1, the mRNA profiles indicated that OVA-AMs skew towards the cur-

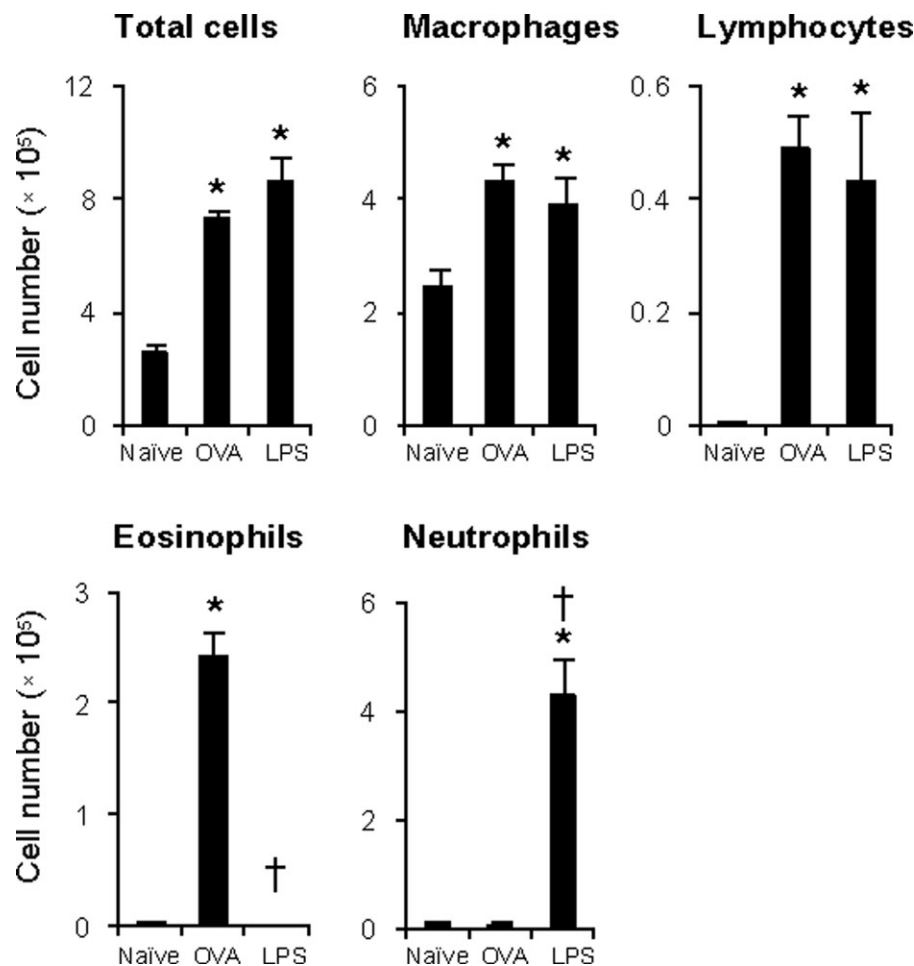


FIGURE 1. Comparison of cell number in bronchoalveolar lavage (BAL) fluid between untreated naïve mice (Naïve), mice in an OVA-induced allergic airway inflammation model (OVA), and mice in an LPS-induced acute airway inflammation model (LPS). BALB/c mice were immunized by injection of OVA with alum on days 0 and 14 and then challenged by inhalation of aerosolized OVA on days 21, 23, and 25. For the acute airway inflammation model, BALB/c mice received intranasal LPS on days 0 and 4. On the day following the last inhalation in both models, BAL fluids were collected from individual mice, and cellular composition of the airway infiltrates is shown. Results are expressed as the mean $\pm$ SEM of three independent experiments. Data are representative of 10 mice in each group analyzed. * $P < 0.05$ (Naïve vs OVA and Naïve vs LPS); † $P < 0.05$ (OVA vs LPS).

rent M2s or the M(IL-4) phenotype, and the LPS-AMs skew towards the current M1s, M(LPS) or M(LPS+IFN- γ) phenotype.

Comparison of Secreted Cytokines by AMs

Next, we examined the secretion of cytokines and chemokines by AMs. AMs from the BAL fluids were incubated in a culture dish for 24 h, and then the culture supernates were collected. The concentrations of IL-6 and IL-12p40, which are M1 secretory markers, and G-CSF and keratinocyte-derived cytokine (KC) in the culture supernates of LPS-AMs were significantly higher than that in cultures of Naïve- and OVA-

AMs in an AM density-dependent manner (Figure 3). Although the above results indicated that LPS-AMs skew towards the current M1 phenotype, the secretion of other M1 secretory markers, including IL-1 α , IL-1 β , MCP-1, MIP-1 α , MIP-1 β , TNF- α , and granulocyte-macrophage colony-stimulating factor (GM-CSF), was not significantly increased in the culture supernates of LPS-AMs as compared to that of Naïve-AMs. Similarly, whereas we hypothesized that the AMs derived from the allergic airway inflammation models would secrete M2 markers, the concentration of IL-13 in the culture supernates of OVA-AMs was lower than that in the other groups. Moreover, the concentrations of MIP-1 α , MIP-1 β ,

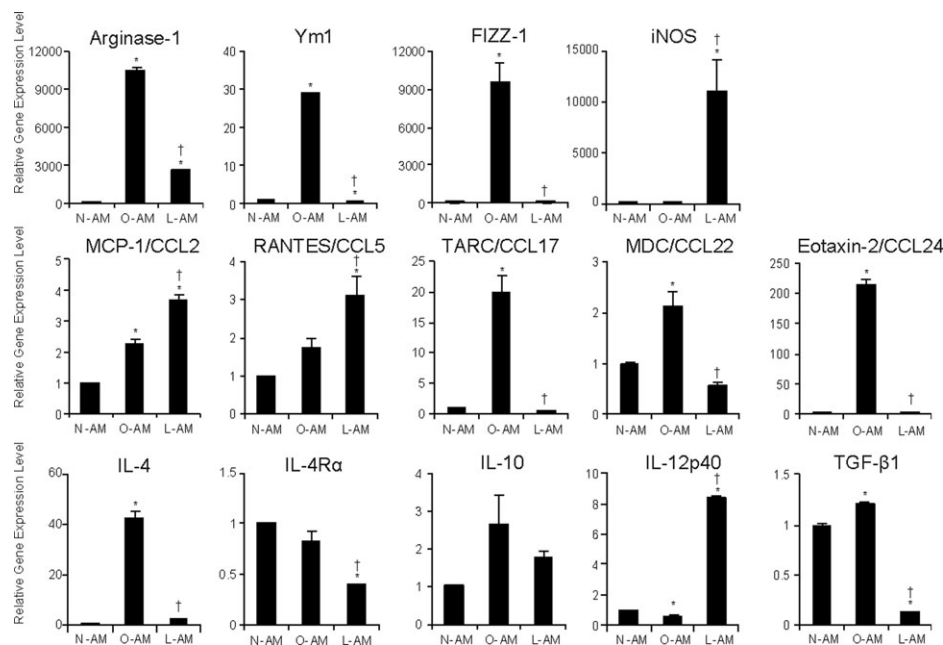


FIGURE 2. Expression of marker genes for M1 and M2 by AMs. Quantitative RT-PCR was used to measure mRNA levels of M1 and M2 related gene, arginase-1, Ym1, FIZZ1, iNOS, MCP-1, RANTES, TARC, MDC, Eotaxin-2, IL-4, IL-4R α , IL-10, IL-12p40, and TGF- β 1 in Naïve-AMs (N-AM), OVA-AMs (O-AM), and LPS-AMs (L-AM). These quantitative RT-PCR results were normalized to GAPDH in the same sample. Data represent the means $\pm$ SEM of three independent experiments. * $P < 0.05$ (N-AM vs. O-AM and N-AM vs. L-AM); † $P < 0.05$ (O-AM vs. L-AM).

and TNF- α in the culture supernates of the Naïve-AMs were significantly higher than that in the OVA- and LPS-AMs. Furthermore, the secretion of MCP-1 and RANTES into the culture supernates of AMs was different from the mRNA profile. The concentrations of IL-2, IL-3, IL-4, IL-5, IL-9, IL-17A, IFN- γ , and Eotaxin-2 were not detectable in the AMs culture supernates. These secretory marker profile results were not completely consistent with the current M1 versus M2-polarization model, however, at least LPS-AMs skewed towards the current M1, M(LPS) or M(LPS+IFN- γ) phenotype without TNF- α secretion. Furthermore, a comparison between these profiles and cytokines in BAL fluids suggested that AMs might have a minimal role in the secretion of cytokines in BAL fluids.

Surface Phenotypes of AMs

AMs differ from other tissue macrophages and dendritic cells (DCs) based on their autofluorescence and their high expression levels of CD11c and CD205 (also denoted as DEC205), which are commonly expressed on DCs [16–19]. AMs were identified by expression of CD11c and high background autofluorescence as examined in the FL1 channel (Figure

4A). CD11b expression, which is high in other tissue macrophage populations, is quite low in AMs from naïve mice [17, 18]. AMs from the murine model of LPS-induced acute airway inflammation showed increased expression of both CD11b and F4/80 as compared to that in the other groups (Figure 4B and Supplemental Figure S2). CD11c⁺ airway lavage cells were confirmed to be AMs by their expression profile of F4/80⁺, Siglec-F⁺, CD205⁺, CD209[−], and CC chemokine receptor 3 (CCR3)[−] (Figure 4B and Supplemental Figure S2). Although Siglec-F is a well-known and reliable distinguishing marker of eosinophils among granulocytes in the mouse, a recent report showed that Siglec-F was expressed on mouse CD11c⁺ AMs [20]. In Figure 4C and Supplemental Figure S2, Naïve-AMs expressed both M1 and M2 markers, including CD80 (also denoted as B7-1), PD-L1 (also denoted as B7-H1), and TLR2 as M1 markers and CD206 and Dectin-1 as M2 markers. OVA-AMs express a high level of PD-L2 (also denoted as B7-DC) and varying levels of CD206, scavenger receptor class A-1 (SRA-1), and Dectin-1, which are M2 markers, as compared to the expression profiles of the other groups. Moreover, the expression levels of Gr-1, Ly-6C, CD86 (also denoted as B7-2), PD-L1, TLR2, and also M2 mark-

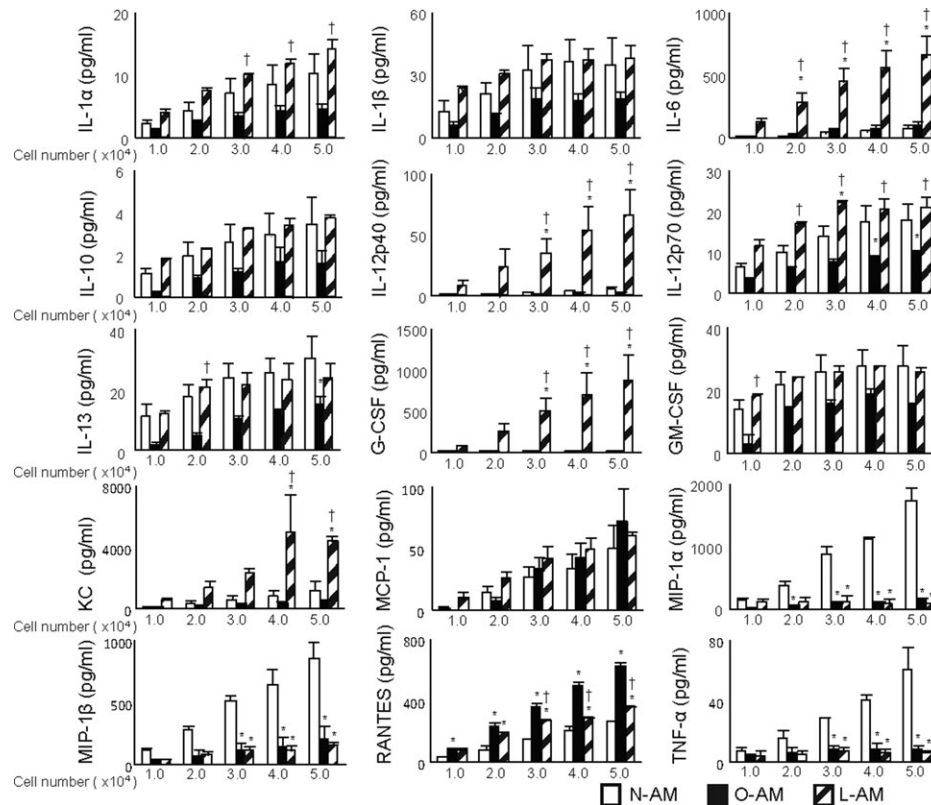


FIGURE 3. Comparison of secreted cytokines by Naïve-AMs (N-AM), OVA-AMs (O-AM), and LPS-AMs (L-AM) in an AM density-dependent manner. AMs were plated ($1.0\text{--}5.0 \times 10^4$ cells/well) in 96-well plates and incubated for 24 hours. The concentrations of IL-1 α , IL-1 β , IL-6, IL-10, IL-12p40, IL-12p70, IL-13, G-CSF, GM-CSF, KC, MCP-1, MIP-1 α , MIP-1 β , RANTES, and TNF- α in the culture supernatants of AMs were measured by using multiplex bead array assays. Data represent the means $\pm$ SEM of three independent experiments. * $P < 0.05$ (N-AM vs. O-AM and N-AM vs. L-AM); † $P < 0.05$ (O-AM vs. L-AM).

ers, including SRA-1, were increased in LPS-AMs as compared to that in the other groups. Furthermore, we followed the expression levels of PD-L2⁺ and CD206⁺ OVA-AMs in a murine model of OVA-induced allergic airway inflammation on days 3, 5, and 7 after the last inhalation. As shown in Figure 4D, we demonstrated that PD-L2, but not CD206, was gradually decreased after day 3 and almost resolved on day 7. The cell surface marker profile of the CD11c⁺ AMs was not completely consistent with the current M1 versus M2-polarization model, however at least OVA-AMs skewed toward the current M2 phenotype.

Differences between CD11b[−] and CD11b⁺ AMs

There was little difference in the cell-surface phenotypes among the three AM phenotypes excluding the expression of CD11b and PD-L2. To explore

the difference among the three AM phenotypes, we focused on CD11b expression. AMs were characterized as highly fluorescent cells with a CD11c⁺, F4/80⁺, Siglec-F⁺, and CD205⁺ phenotype. The use of CD11c and CD11b expression in combination with the autofluorescence further subdivided the AMs into either CD11b⁺ or CD11b[−] cells [21, 22]. CD11c⁺ AMs from LPS-treated mice had increased expression of CD11b. Thus, CD11c⁺ AMs from LPS-treated mice were gated as indicated in Figure 5. The AMs that were gated as CD11c⁺ CD11b[−] AMs appeared to be almost the same as the undivided AMs shown in Figure 4 without the expression of Gr-1, Ly-6C, and Ly-6G (Figure 5 and Supplemental Figure S2). However, CD11c⁺ CD11b⁺ AMs were different from the undivided AMs and from the CD11c⁺ CD11b[−] AMs (Figure 6 and Supplemental Figure S2). The CD11c⁺ CD11b⁺ AMs, but not the CD11c⁺ CD11b[−] AMs, from LPS-treated mice contained Gr-1⁺, Ly-6C⁺, and Ly-6G⁺ cells, naïve mice contained Gr-1⁺ and Ly-6C⁺ cells, and OVA-

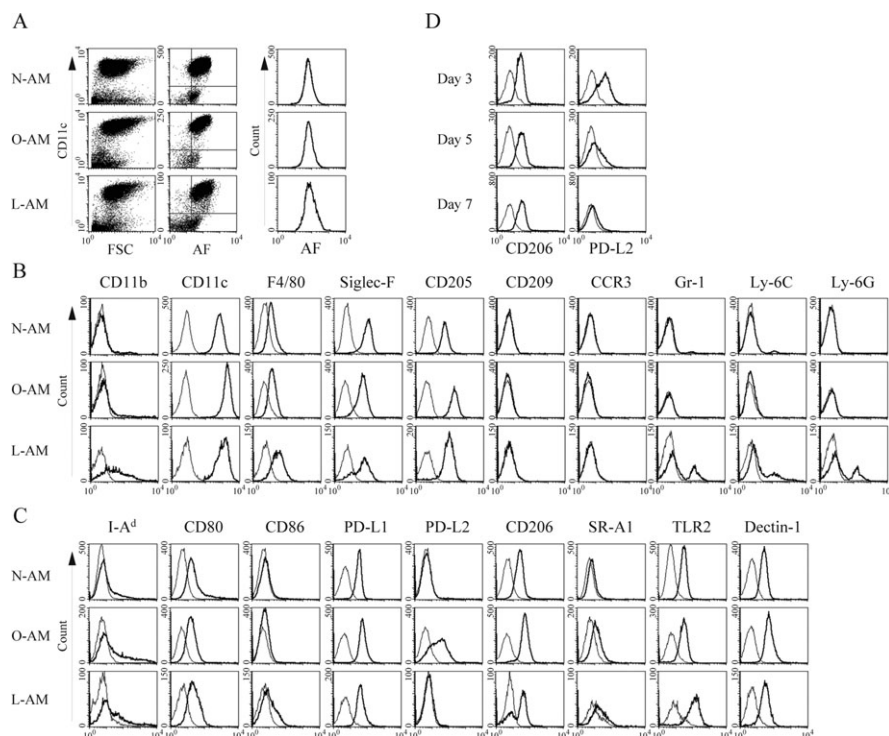


FIGURE 4. Surface phenotype of Naïve-AMs (N-AM), OVA-AMs (O-AM) and LPS-AMs (L-AM). (A) AMs were identified by high cellular autofluorescence and expression of CD11c marker. (B and C) Each CD11c⁺ AM was analyzed for CD11b, F4/80, Siglec-F, CD205, CD209, CCR3, Gr-1, Ly-6C, and Ly-6G, respectively, and was analyzed for the expression of M1/M2 markers. (D) PD-L2⁺ and CD206⁺ O-AMs were analyzed on day 3, 5, and 7 after the last OVA inhalation. Black and gray lines indicate marker expression and the isotype control staining, respectively. Similar results were obtained in four independent experiments.

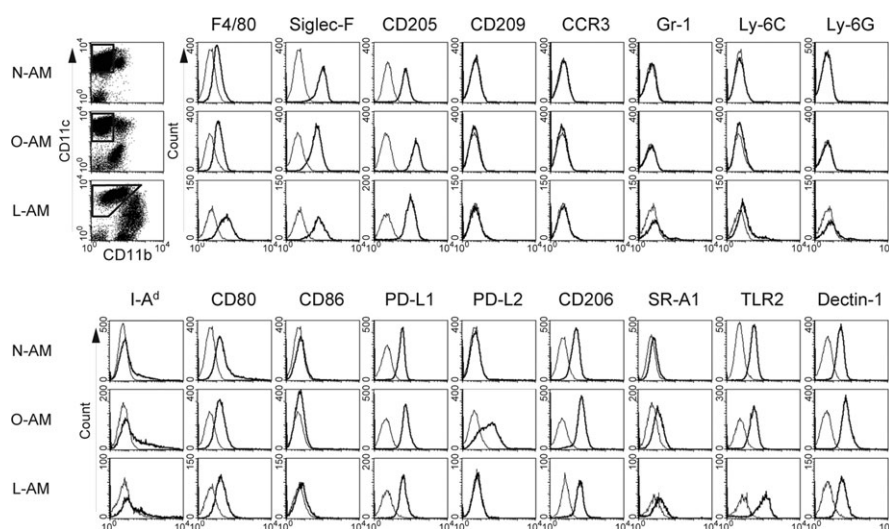


FIGURE 5. Surface phenotype of CD11c⁺ CD11b⁻ AMs from naïve mice (N-AM) and murine models of OVA-induced allergic inflammation (O-AM) and LPS-induced acute airway inflammation (L-AM). CD11c⁺ CD11b⁻ AMs were gated as indicated. Each CD11c⁺ CD11b⁻ AM was analyzed for F4/80, Siglec-F, CD205, CD209, CCR3, Gr-1, Ly-6C, Ly-6G, and M1/M2 markers, respectively. Black and gray lines indicate marker expression and the isotype control staining, respectively. Similar results were obtained in four independent experiments.

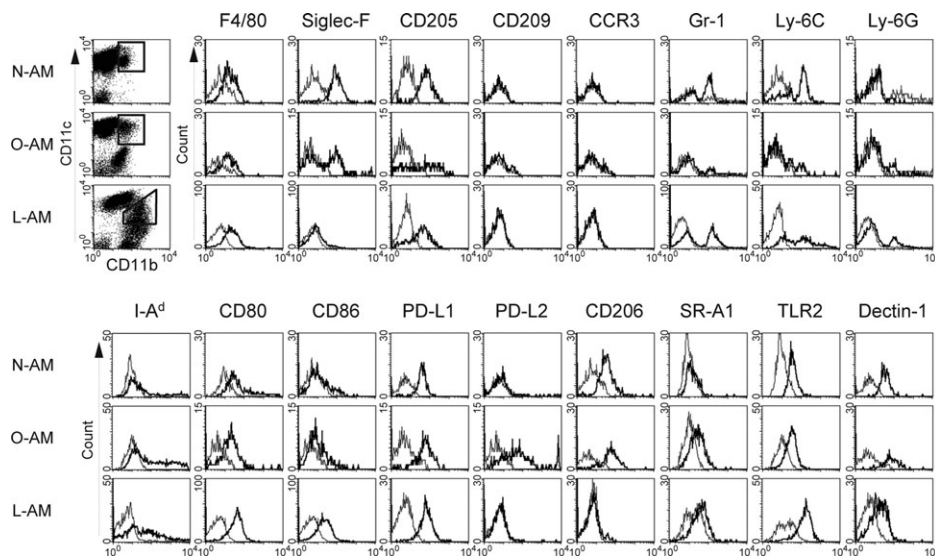


FIGURE 6. Surface phenotype of CD11c⁺ CD11b⁺ AMs from naïve mice (N-AM) and murine models of OVA-induced allergic inflammation (O-AM) and LPS-induced acute airway inflammation (L-AM). CD11c⁺ CD11b⁺ AMs were gated as indicated. Each CD11c⁺ CD11b⁺ AM was analyzed for F4/80, Siglec-F, CD205, CD209, CCR3, Gr-1, Ly-6C, Ly-6G, and M1/M2 markers, respectively. Black and gray lines indicate marker expression and the isotype control staining, respectively. Similar results were obtained in four independent experiments.

AMs contained a few Gr-1⁺ and Ly-6G⁺ cells. As previously described, the CD11c⁺ CD11b⁺ airway lavage-derived cells might contain Gr-1⁺ monocytes recruited from the peripheral blood [23]. Interestingly, LPS-induced inflammatory CD11c⁺ CD11b⁺ AMs were characterized as CD206⁻ and Siglec-F⁻, which is not the AM phenotype, indicating that the CD11c⁺ CD11b⁺ AMs from the murine model of LPS-induced acute airway inflammation might be monocytes that were recruited to the site of inflammation. In contrast, OVA-induced inflammatory CD11c⁺ CD11b⁺ AMs also included Siglec-F⁻ cells, but these cells were CD206⁺. Furthermore, the expression levels of TLR2 were increased on the cell surface of CD11c⁺ CD11b⁺ AMs from the LPS-treated mice, when compared to that of the other groups.

OVA-AMs and LPS-AMs Differentially Influence the Antigen-specific T-cell Proliferative Responses in vitro

The pathogenic and regulatory roles of AMs remain unidentified, but data increasingly suggest that macrophages can actively participate in asthma [24]. We next examined whether AMs could increase the proliferation of antigen-specific CD4⁺ T cells using

lymph node cells from OVA-treated DO11.10/Rag-2^{-/-} mice, which express an I-A^d-restricted TCR specific for an OVA₃₂₃₋₃₃₉ peptide, but do not express endogenous TCR. DO11.10/Rag-2^{-/-} mice were subcutaneously immunized with OVA/alum, and cervical lymph node cells were harvested 7 days after immunization. As shown in Figure 7, AMs were co-cultured with CD4⁺ T cells from cervical lymph nodes of the DO11.10/Rag-2^{-/-} mice with the OVA₃₂₃₋₃₃₉ peptide, and induced an OVA-specific T-cell proliferative response. Interestingly, T-cell proliferation was significantly reduced in co-culture with LPS-AMs in an AM density-dependent manner. These T-cell proliferative responses were opposite to the secretory marker profiles that were increased in an AM density-dependent manner in Figure 3.

Finally, to further explore the regulatory role of CD11b⁺ and CD11b⁻ AMs in antigen-specific T-cell proliferative responses, CD11b⁻ AMs were purified using magnetic beads containing anti-CD11b antibodies, and whole AMs were prepared as CD11b⁺ plus CD11b⁻ AMs. CD4⁺ T cells from the OVA-treated DO11.10/Rag-2^{-/-} mice and CD11b⁻ AMs or whole AMs were co-cultured with the OVA₃₂₃₋₃₃₉ peptide. Interestingly, only the CD11b⁻ AMs from LPS-treated mice showed inhibition of the suppressive effects on OVA-specific T-cell proliferative responses (Figure 7).

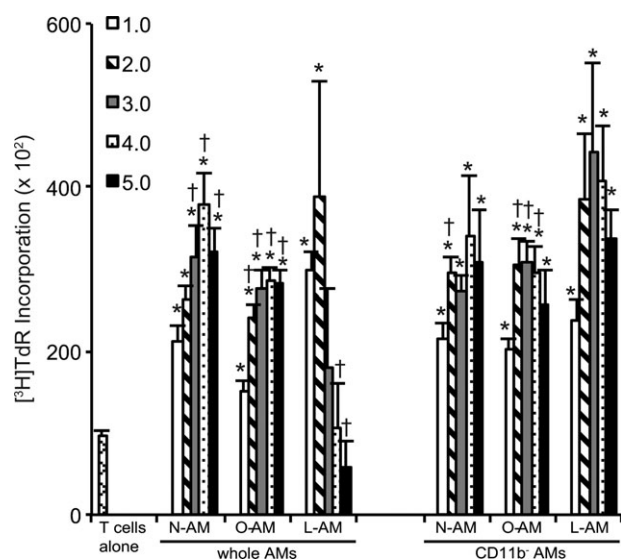


FIGURE 7. AMs differentially affect the antigen-specific T-cell proliferative responses in vitro. CD4⁺ T cells in cervical lymph node cells from OVA-immunized DO11.10/Rag-2^{-/-} mice and CD11b⁻ AMs were purified by magnetic bead separation, and whole AMs were prepared as CD11b⁺ plus CD11b⁻ AMs. CD4⁺ T cells (1.0×10^5 cells/well) were co-cultured with naïve-AMs (N-AM), OVA-AMs (O-AM), and LPS-AMs (L-AM) at the indicated density (1.0 – 5.0×10^4 cells/well) in the presence of an OVA_{323–339} peptide. [³H]thymidine (3H-TdR) uptake was measured at 48 hours. Data represent the means $\pm$ SEM of three independent experiments. * $P < 0.05$ compared with T cells alone. † $P < 0.05$ compared with a cell density of 1.0×10^4 cells/well.

DISCUSSION

Originally, it was apparent that the cell-surface markers used to define AMs may lead to inherent confusion, because AMs constitutively express DC markers, such as CD11c and CD205, and the eosinophilic marker Siglec-F with a reduction in CD11b [21, 25, 26]. Moreover, CD206, an important marker for M2, was constitutively expressed on AMs. Taken together, our findings indicate that several cell-surface markers for AMs, such as CD11c, F4/80, Siglec-F, and CD205, should be used in combination with autofluorescence to define AMs. Similarly, the current M1 versus M2-polarization model has led to inherent confusion. In our system, the mRNA profile, secretory marker profile, and cell surface marker profile of mouse AMs did not correspond to the current M1 versus M2-polarization model. However LPS-induced acute airway inflammation AMs skewed either toward the current M1, M(LPS), or M(LPS+IFN- γ) phenotype, and OVA-induced allergic airway inflammation AMs skewed toward either the current M2 or M(IL-4) phenotype. Moreover, the cell surface profile without PD-L2 indicated little dif-

ference to discriminate the phenotypes among AMs from untreated naïve mice and from murine models of an OVA-induced allergic airway inflammation or LPS-induced acute airway inflammation, suggesting that these cell surface markers without PD-L2 do not discriminate AM phenotypes.

We also demonstrated that CD11c⁺ CD11b⁺ AMs from LPS-induced acute airway inflammation, which were characterized as lacking the markers for AM including CD206 and Siglec-F and having increased TLR2 expression, were different from CD11c⁺ CD11b⁺ AMs from both naïve and OVA-treated mice. These CD11c⁺ CD11b⁺ LPS-AMs lacking the markers for the AM phenotype might be recruited monocytes or macrophages. Further interventional studies are necessary to elucidate their origin. Furthermore, this subset could suppress the antigen-specific T-cell proliferative responses, in contrast to other subsets. AMs were reported to be negative regulators, which could modulate immune responses by inhibiting antigen presentation by DCs, T-cell activation, and antibody production [24, 27, 28]. In addition, although M2s expressing arginase 1, Ym1, and FIZZ1 can inhibit Th2 responses, macrophages, including M2s, participate in the pathogenesis of asthma and promote Th2 immunity [3, 12, 24, 29–33]. Moreover, using methodologies to deplete AMs and adoptive transfer experiments, some groups have also shown that M1- and M2-skewed macrophages can be pathogenic cells and suppress allergic lung inflammation *in vivo* [24, 34–38]. In this regard, the pathogenic or regulatory roles of AMs remain controversial. As found in the present study, the antigen-specific T-cell proliferative responses were suppressed at high ratios of CD11c⁺ CD11b⁺ LPS-AMs to CD4⁺ T cells and were induced by naïve AMs, OVA-AMs, and CD11c⁺ CD11b⁻ LPS-AMs. These results suggested that CD11c⁺ CD11b⁺ LPS-AMs might play a regulatory role in antigen-specific T-cell proliferative responses. The suppressive effect of CD11c⁺ CD11b⁺ LPS-AMs might reflect the findings in Figure 2 showing increasing iNOS expression, and therefore might be expected to express suppressor macrophage activity unlike Naïve and OVA-AMs [39–41]. Conversely, characterization of LPS-AMs might be influenced by recruited CD11c⁺ CD11b⁺ LPS-AMs. On the other hand, these lymphocyte responses by AMs might be explained by the high expression of PD-L1 on the three AM phenotypes and PD-L2 on OVA-AMs, because PD-L1 and PD-L2 are inhibitory costimulatory molecules. However, these OVA-specific T-cell proliferative responses were not inhibited by neutralizing anti-PD-L1 and PD-L2 mAbs (data not shown).

Table 1 Primer Sequences for Real-time PCR

cDNA	Forward	Reverse
iNOS	ACATCGACCCGTCCACAGTAT	CAGAGGGGTAGGCTTGTCTC
Arginase-1	CTCCAAGCCAAAGTCCTTAGAG	GGAGCTGTCATTAGGGACATCA
Ym1	TCACTTACACACATGAGCAAGAC	CGGTTCTGAGGAGTAGAGACCA
FIZZ-1	CCAATCCAGCTAACTATCCCTCC	CCAGTCAACGAGTAAGCACAG
MCP-1/CCL2	TAAAAACCTGGATCGGAACCAAA	GCATTAGCTTCAGATTTACGGGT
RANTES/CCL5	TGGGCCTGCTGTTTACAGTTGC	AGCAGGTGAGTGGGGCGTTA
TARC/CCL17	TACCATGAGGTCACTTCAGATGC	GCACTCTCGGCCTACATTGG
MDC/CCL22	AAGCCTGGCGTTGTTTTGATA	CCTGGGATCGGCACAGATA
Eotaxin-2/CCL24	ACGGCAGCATCTGTCCCAAGGC	GTGCCTCTGAACCCACAGCAGC
IL-4	GGGACGCCATGCACGGAGATG	TGCGAAGCACCTTGGAAGCCC
IL-4R α	ACACTACAGGCTGATGTTCTTCG	TGGACCGGCCTATTTCATTTC
IL-10	ATGCTCCTAGAGCTGCGGACTG	AGGCTTGGCAACCCCAAGTAACC
IL-12p40	CTCAGAAGCTAACCATCTCTCTG	CACAGGTGAGGTTCACTGTTTC
TGF- β 1	CCGCAACAACGCCATCTATG	CCCGAATGTCTGACGTATTGAAG
GAPDH	AACTTTGGCATTGTGGAAGG	GGATGCAGGGATGATGTTCT

Macrophages secrete pro-inflammatory cytokines, including IL-1 β and TNF- α , in response to LPS [42–44]. Our findings demonstrate the discrepant findings of secreted molecules between high concentrations of IL-1 α , IL-1 β , IL-6, IL-12, MCP-1, MIP-1 α , MIP-1 β , RANTES, and TNF- α in the BAL fluids of LPS-treated mice and high concentrations of MIP-1 α , MIP-1 β , TNF- α in the culture supernates of AMs from untreated naïve mice. Therefore, LPS-AMs do not align with the current M1 secretory markers, including IL-1 β and TNF- α . Production of IL-1 β , MIP-1 α , MIP-1 β , and TNF- α by AMs might be suppressed by apoptotic cells in the OVA-induced allergic and LPS-induced acute airway inflammation models, because phosphatidylserine-mediated efferocytosis reduces TNF- α production by macrophages [42–44]. Furthermore, AMs from the OVA-induced allergic airway inflammation model had lower production of IL-13 as compared to that of other AM phenotypes. A different production profile between this IL-13 secretion of AMs and the concentration of IL-13 in the BAL fluids suggest that OVA-AMs might have little role in the pathogenic process during allergic airway inflammation. Altogether, further studies are needed to elucidate our discrepant findings between the secretory marker profile in AMs culture supernates and in BAL fluids.

CONCLUSION

We have characterized in detail mouse AMs to show that AMs in the steady state and in OVA-induced allergic and LPS-induced acute airway inflammation do not correspond to the current M1 versus M2-polarization model. However, OVA-induced allergic and LPS-induced acute airway inflammation

promotes the polarization of AMs towards the current M2-skewed and current M1-skewed phenotypes, respectively. Furthermore, we have suggested that CD11c⁺ CD11b⁺ LPS-AMs might play a regulatory role in antigen-specific CD4⁺ T-cell proliferation. Although it could not be concluded from the present results that Naïve-AMs, OVA-AMs or LPS-AMs are clonally separable cells like macrophages, the different characterizations of AMs depending on the incoming pathogens they encounter or on the phase of inflammation may facilitate their contributions to host defense to both the innate and adaptive immune responses and emphasize their importance as cells of the pulmonary immune system in conditions of airway inflammation. However, our use of murine models is the most important limitation of this study. These phenotypes and functions of AMs in airway inflammation still require further investigation.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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