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Comparison of Lung Dendritic Cells and B Cells in Stimulating Naive Antigen-Specific T Cells

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Dendritic cells (DCs) are specialized APCs that are important in priming naive T cells and can be manipulated in vitro and in vivo to enhance immunizations against microorganisms and tumors. A limitation in the development of suitable immunotherapeutic vaccines for the lung is incomplete information on the role of DCs and other potential APCs in the lung in priming naive T cells. In the current study, we analyzed the relative contributions of murine lung DCs and B cells to process and present OVA to naive CD4+ OVA\textsubscript{323–339} specific (DO11.10) T cells in vitro. We also examined their expression of MHC class II and accessory molecules before and after maturation in culture. Similar to DCs from other sites, freshly isolated lung DCs can process OVA, spontaneously up-regulate MHC class II and accessory molecules during overnight culture, and stimulate naive T cells in an Ag-specific manner. In contrast, freshly isolated lung B cells were unable to both process and present native OVA. Furthermore, under conditions of limited OVA\textsubscript{323–339} peptide exposure, B cells had a significantly diminished capacity to stimulate T cells, and this correlated with a decreased density of both MHC class II and important costimulatory molecules as compared with lung DCs.

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2 Address correspondence and reprint requests to Dr. Barbara J. Masten, Department of Pathology, University of New Mexico School of Medicine, 915 Stanford Dr. NE, BRF 323, Albuquerque, NM 87131-5301. E-mail address: bmasten@salud.unm.edu
3 Abbreviations used in this paper: DC, dendritic cell; CD62L, CD62 ligand; cRPMI, complete RPMI; FS/SSC, forward scatter and 90° side scatter; LAd, loosely adherent cells; LN, lymph node; MFI, mean fluorescence intensity; PE, phycoerythrin.

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as compared with cultured lung DCs. Finally, we compared the function and phenotype of lung B cells to lung DCs.

Materials and Methods

Mice

BALB/c (H-2b) and heterozygous mice transgenic for the DO11.10 αβ-TCR on a BALB/c background were bred in specific pathogen-free facilities in the University of New Mexico (UNM) Animal Resource Facility (Albuquerque, NM). Heterozygous DO11.10 male breeders, a kind gift of Dr. Dennis Loh (Hoffman LaRoche), were bred to BALB/c females to generate heterozygous offspring. Offspring were tested for expression of the transgene by obtaining tail vein blood, staining leukocytes with mAb KJ1-26, and analyzing by flow cytometry. All mice were kept in a specific pathogen-free room in a room maintained in a sterilized environment with a laminar flow and water. A sentinel animal kept in this facility was monitored each quarter for Ab conversion to Sendai and mouse hepatitis viruses and Mycoplasma pulmonis. Both males and females were used at 10–12 wk of age.

Reagents

Ags used in this study were chicken OVA (Sigma, St. Louis, MO) and synthesized OVA peptide, representing amino acids 323–339 of chicken OVA (American Chemistry Laboratory, Albuquerque, NM). Heterozygous DO11.10 male breeders, a kind gift of Dr. Dennis Loh (Hoffman LaRoche), were bred to BALB/c females to generate heterozygous offspring. Offspring were tested for expression of the transgene by obtaining tail vein blood, staining leukocytes with mAb KJ1-26, and analyzing by flow cytometry. All mice were kept in a specific pathogen-free room in a room maintained in a sterilized environment with a laminar flow and water. A sentinel animal kept in this facility was monitored each quarter for Ab conversion to Sendai and mouse hepatitis viruses and Mycoplasma pulmonis. Both males and females were used at 10–12 wk of age.

Lung cells were prepared as described (18, 19). Briefly, mice were injected i.p. with 150 U heparin (ICN Biomedicals, Aurora, OH) and 1 ml of the anesthetic, Avertin (2, 2, 2-tribromoethanol, 71 μM; Sigma). Digested lung tissue was tapped through a wire screen, particulate matter was removed by rapid filtration through a nylon wool plug, and the filtered cells were washed twice in HBSS (Life Technologies). Lung cells were resuspended in high density Percoll (Pharmacia, Piscataway, NJ), overlaid with equal volume of lower density Percoll (1.075 g/ml), and centrifuged at 500 × g for 20 min. Cells at the lymphoproliferation assays and Ag pulsing of APCs, cRPMI was additionally supplemented with 250 U/ml cathepsin D and 1 mg/ml indomethacin (Sigma).

Preparation of APCs

Lung cells were resuspended, washed with HBSS, and collected by centrifugation at 100 × g. A gate on I-Ad-positive, FITC-bead-negative cells; and a gate on B220-negative DCs. Therefore, in our phenotype analysis of DCs, we knowingly excluded all cells that could ingest latex beads. For DC phenotyping, analysis was performed on the I-A 4+ -positive, FITC-bead-negative, B220-negative population for expression of the PE-labeled marker. For B cell phenotyping, analysis was performed on the I-A 4+ -positive, FITC-bead-negative, B220-positive population for expression of the PE-labeled marker.

Isolation of DCs and B cells

To prepare DCs and B cells for sorting, whole lung cells, LAd cells, or enriched B cells were first incubated with FITC-latex beads, followed by peroxidase-digested LAd cells to block Fc receptors, and an additional gate was set using a log scale PE fluorescence histogram to exclude extremely bright FITC fluorescent cells with fluorescence signals that wrapped around from channel 1024 to channel 1 due to the electronics of the Elite, a second gate was set starting at channel 1 and encompassed the FITC-bright population within the FS/SSC gate. To exclude extremely bright FITC fluorescent cells with fluorescence signals that wrapped around from channel 1024 to channel 1 due to the electronics of the Elite, a second gate was set starting at channel 1 and encompassed the FITC-negative population using a linear scale FITC histogram. For DC sorting, an additional gate was set using a log scale PE fluorescence histogram to exclude CD45R/B220-positive cells (B cells) from the sort. Thus, four gates were used to sort I-A 4+ -positive, FITC-bead-negative, B220-negative cells, a gate on I-A 4+ -positive, FITC-bead-negative cells; and a gate on B220-negative cells. For sorting B cells, I-A 4+ -positive, FITC-bead-negative, B220-positive cells were collected as FITC-bead-negative, B220-positive population for expression of the PE-labeled marker.

Freshly isolated lung or spleen cells were resuspended (2–3 × 10 6 cells/ml) in cRPMI containing no Ag, intact OVA protein, or OVA 323–339 peptide and 250 U/ml catalse (Sigma), and 1 μg/ml indomethacin (Sigma) in 50 ml polypropylene conical centrifuge tubes at 37°C, 5% CO 2 for 18–24 h. Freshly isolated lung and spleen cells were washed with HBSS, resuspended, and incubated in cRPMI containing 20% FBS to identify phagocytic cells. Free latex beads were separated from the cells by centrifugation over FBS. Cells were then preincubated with purified anti-CD11c/CD32 (2.4G2, rat IgG2b, PharMingen, San Diego, CA) to block both FCR-mediated binding of staining Ab and cell aggregation by FcyRII binding by macrophages of Ab-stained DCs and B cells. Subsequently, cells were stained with biotinylated anti-I-A 4+ /I-E d (2G9, rat IgG2a, PharMingen), allopurinol-conjugated anti-CD45R/B220 (RA3-6B2, rat IgG2a, PharMingen), and a PE-conjugated mAb to the surface molecule of interest. I-A 4+ binding was identified by incubation with streptavidin-peridinin chlorophyll protein (PerCp) (Becton Dickinson, San Jose, CA), PE-conjugated anti-CD80 (1G10, rat IgG2a), anti-CD86 (GL1, rat IgG2a), anti-CD4 (30G12, rat IgG2b), anti-CD8 (53.6.7, rat IgG2b), anti-CD45R (3B2, hamster IgG), anti-CD25a (3C7, rat IgG2b), anti-CD24 (M1/69, rat IgG2b), anti-CD14 (1B1, rat IgG2b), anti-CD8a (53.6.7, rat IgG2a), anti-CD11b (M1/70, rat IgG2a), anti-CD2 (R35-95, rat IgG2b), and hamster IgG (G235-2356) were from PharMingen. Cells were always stained at 4°C and washed with PBS containing 2% FBS and 40 μg/ml EDTA. A Becton Dickinson flow cytometry cell sorter FACS Calibur was used for data acquisition and Cell Quest software (Becton Dickinson) for analysis. Presently, no markers are available that clearly distinguish lung DCs from lung macrophages. However, eliminating macrophage contamination was an important concern for us in our comparison of lung DCs and B cells. Therefore, in our phenotype analysis of DCs, we knowingly excluded all cells that could ingest latex beads. For DC phenotyping, analysis was performed on the I-A 4+ -positive, FITC-bead-negative, B220-negative population for expression of the PE-labeled marker. For B cell phenotyping, analysis was performed on the I-A 4+ -positive, FITC-bead-negative, B220-positive population for expression of the PE-labeled marker.
were examined for latex bead uptake, I-A^d expression, and elongated cell processes, using an epifluorescent photomicroscope. Sorted B cell populations (>97% purity) were examined for latex bead uptake, I-A^d expression, and lack of elongated cell processes. Sorted cell populations were 99% viable, determined by trypan blue dye exclusion. On average, this protocol yields 2.5 × 10^6 lung DCs/mouse. It was shown previously that this technique did not interfere with functional activity, as anti-I-A Abs must be continuously present in cultures to interfere with I-A-dependent lymphoproliferation (18). We always sorted for lung DCs after an overnight culture; a timepoint at which we believe the majority of DCs did not take up latex beads, and a timepoint at which DCs were still heterogeneous for the expression of I-A^d and accessory molecules. Thus, a significant portion of these nonphagocytic lung DCs studied was immature, i.e., still had a moderate density of I-A^d. A similar protocol was followed for staining and sorting spleen DCs.

**Enrichment of naive (CD62L^hi) CD4^+ DO11.10 T cells**

To prepare T cells for flow cytometry cell sorting on a Coulter Epics Elite (Coulter, Hialeah, FL), spleen cells from DO11.10 mice were passed on nylon wool, stained with FITC-conjugated anti-CD4 (RM4-5, rat IgG2a; PharMingen), PE-conjugated anti-CD62L (Mel-14, rat IgG2a; PharMingen), and biotinylated anti-I-A^d/I-E^d (2G9, rat IgG2a; PharMingen), followed by secondary staining with streptavidin-allophycocyanin (Becton Dickinson). To obtain naive CD4^+ T cells in high purity, cells expressing both CD4 and CD62L, but negative for I-A^d, were collected in a tube. The FS/SSC profile of stained cells was used to set a gate on the lymphocyte population. A second gate was set on cells expressing high levels of CD4 and CD62L. A third gate was set on cells expressing high levels of CD62L, but lacking expression of I-A^d to exclude APCs. All three gates were used together to sort naive (CD62L^hi) CD4^+ T cells. Sorted cells were 99% viable, as determined by trypan blue dye exclusion.

**Lymphoproliferation assay**

For whole lung cell experiments, the lung cells were washed and irradiated (2000 rad) before use in the lymphoproliferation assay. For experiments using sorted APCs, B cells and DCs were purified from nonirradiated whole lung cells, LAd, or enriched B cells by FACS before use in the lymphoproliferation assay. Sorted naive CD4^+ DO11.10 T cells (2.5 × 10^5/well) were cultured with APCs in triplicate, unless noted, in 96-well flat-bottom plates at 37°C, 5% CO_2 for 4 days. Culture conditions were 2000 µl RPMI containing no Ag, OVA, or OVA 323–339 peptide, and 250 U/ml catalase (Sigma) and 1 µg/ml indomethacin (Sigma). Catalase and indomethacin were added to inhibit macrophage-derived hydrogen peroxide and PGE_2 production, respectively, which was needed for whole lung cells and was also used for purified B cell and DC cultures to assure valid comparisons. Eighteen hours before harvesting, 0.5 µCi methyl [3H]thymidine (Amersham, Arlington Heights, IL) was added to each well. To determine whether APCs were a contaminant in the sorted T cell population, sorted T cells (2.5 × 10^5/well) were cultured with 10 µM OVA 323–339 peptide. Controls included each cell type with media only and media with OVA or OVA 323–339 peptide, where appropriate. Cultures were harvested with an automated cell harvester and analyzed in a Beckman LS 6300 liquid scintillation counter (Beckman Instruments, Fullerton, CA). Results were reported as cpm. For whole spleen cell experiments, the spleen cells were washed and irradiated (2000 rad) before use in the lymphoproliferation assay. For experiments using sorted spleen APCs, DCs were purified from nonirradiated spleen cells or LAd by FACS before use in the lymphoproliferation assay.

**Statistics**

All statistical analyses were performed with the Statview software (SAS Institute, Cary, NJ). For data in Figs. 3, 4, and 5, an analysis of variance model and an unpaired t test were used for examining data. A p value <0.05 was considered significant.

**Results**

**Lung cells process and present Ag to naive T cells**

Previous studies (18, 19) showed that freshly isolated lung cells were highly efficient in stimulating allogeneic T cells in a mixed lymphocyte response. To determine the capacity of lung cells to process and present soluble protein to naive Ag-specific T cell, freshly isolated irradiated lung cells were immediately cultured with naive CD4^+ DO11.10 T cells and different concentrations of intact OVA protein or OVA 323–339 Peptide. This experiment was biased toward detecting the APC activity of lung cells that were resistant to 2000 rad, i.e., DCs. Freshly isolated lung cells were capable of stimulating naive T cells to proliferate in an Ag-specific manner when OVA 323–339 Peptide or OVA protein was included throughout the lymphoproliferation assay (Fig. 1a). However, the molar concentration of protein required to stimulate T cell proliferation equal to that observed with peptide was at least 1000-fold more. The concentration of protein that showed optimal T cell proliferation was 100 µM OVA protein for all lung cell concentrations and was used for all subsequent assays, including experiments in which overnight pulsing was done. In a separate experiment, freshly isolated spleen cells were used as a comparative APC population. Freshly isolated spleen cells showed similar Ag dose and cell number responses (Fig. 1b). However, on average, freshly isolated spleen cells were 40% more effective at stimulating naive DO11.10 T cells than freshly isolated lung cells.

**Lung DCs process and present Ag to naive T cells**

Our previous studies also showed that DCs sorted from cultured lung cells were potent stimulators of allogeneic T cell proliferative responses (18). To address whether isolated lung DCs have the capacity to stimulate naive Ag-specific T cells, FACS-purified lung DCs from nonirradiated lung cells were cultured with naive CD4^+ DO11.10 T cells and different concentrations of OVA 323–339 Peptide. Lung DCs were very efficient at presenting peptide to naive Ag-specific T cells when the peptide was included throughout the lymphoproliferation assay (Fig. 2). Optimal responses were obtained between 1 and 10 µM OVA 323–339 peptide;
Lung DCs (1×10³ cells/well) were cocultured with naive CD4⁺ DO11.10 T cells (2.5×10⁵ cells/well) and increasing concentrations of OVA323-339 peptide. After 5 days, T cell proliferation was assessed as described in Fig. 1. The data shown are the mean and SEM of triplicate wells and are representative of two independent experiments.

10 μM of peptide was used in all subsequent experiments. A direct comparison of lung DCs with spleen DCs showed similar Ag dose responses and equivalent capacities to stimulate naive CD4⁺ DO11.10 T cells (data not shown).

Naïve T cells are stimulated equally by lung DCs and B cells with continuous OVA peptide in culture, but not with continuous OVA protein

DCs and B cells are two cell types in the murine lung with the potential to capture and display Ag to CD4⁺ T cells. To determine whether these two MHC class II (I-A<sub>d</sub>)-expressing cell populations show a difference in Ag-specific APC activity, we compared the relative ability of lung B cells and DCs to process and present OVA protein to naive CD4⁺ DO11.10 T cells. DCs sorted from nonirradiated LAd and B cells sorted from nonirradiated nonadherent lung cells were cocultured with naive CD4⁺ DO11.10 T cells and OVA protein or OVA<sub>323-339</sub> peptide. On average, DCs were the only APCs able to efficiently stimulate T cell proliferation with intact protein (Fig. 3; DC+T+ova vs B+T+ova, overall p ≤ 0.001). However, lymphoproliferation cultures in which OVA peptide was continuously present showed that B cells were equally efficient to DCs in presenting peptide to Ag-specific naive CD4⁺ T cells (DC+T+pep vs B+T+pep, overall p ≤ 0.3493).

Pulsed lung cells can prime Ag-specific naïve T cells

We next examined whether lung cells exposed to (pulsed) intact OVA protein before addition to the lymphoproliferation assay could stimulate naïve T cells in an Ag-specific manner. Freshly isolated lung cells cultured with OVA protein for 18–24 h were compared with lung cells that were not pulsed for their capacity to stimulate naïve CD4⁺ DO11.10 T cells. Both the pulsed and not pulsed lung cell populations were irradiated before use in the lymphoproliferation assay. Overnight pulsed lung cells showed specific priming in vitro, whereas not pulsed lung cells showed no APC activity (Fig. 4a: Not pulsed vs Pulsed, p ≤ 0.001). In a separate experiment, not pulsed and overnight pulsed spleen cells showed similar results to not pulsed and overnight pulsed lung cells, respectively (Fig. 4b).

Pulsed lung DCs are more efficient than pulsed lung B cells at priming Ag-specific naïve T cells

With APC activity evident in protein-pulsed populations of irradiated lung cells that contained 7.5 ± 2.4% B cells and 1.2 ± 0.6% DC, it was important to verify, under nonirradiating conditions, that DCs were the only APCs capable of inducing naïve T cells to proliferate and confirm the lack of APC activity by B cells. B cells and DCs sorted from nonirradiated lung cells that were cultured overnight with intact protein or OVA<sub>323-339</sub> peptide were compared for their capacity to stimulate naïve OVA-specific CD4⁺ T cells. In contrast to the similar levels of APC activities of lung B cells and DCs when peptide was present throughout the lymphoproliferation assay (refer to Fig. 3), peptide-pulsed lung DCs were more efficient than peptide-pulsed B cells at stimulating naïve T cells to proliferate (Fig. 5a; Pulsed lung B cells vs Peptide-pulsed DCs, overall p ≤ 0.0001). Significant differences between peptide-pulsed B cells and DCs were observed at each APC concentration: 1.25×10⁵ (p ≤ 0.0246); 2.5×10⁵ (p ≤ 0.0299); and 5×10⁵ (p ≤ 0.0173). As expected from the results of the experiments with OVA protein continuously present during the lymphoproliferation assay (refer to Fig. 4), only OVA protein pulsed DCs demonstrated any APC activity for naïve T cells (Fig. 5b; Protein-pulsed B cells vs Protein-pulsed DCs, overall p ≤ 0.0001). Significant differences between protein-pulsed B cells and DCs were observed at 2.5×10⁵ (p ≤ 0.0049) and 5×10⁵ (p ≤ 0.0005) concentrations of APCs.

MHC class II expression on freshly isolated and cultured lung DCs and lung B cells

Activation of CD4⁺ T cells is initiated when their MHC-restricted, peptide-specific TCRs bind MHC class II-peptide complexes expressed on APCs. The observation that peptide-pulsed B cells and DCs differed in their ability to stimulate Ag-specific naïve T cells prompted us to examine the levels of I-A<sub>d</sub> expression on these two APC types to determine whether lung DCs have the capacity to present more peptide at their surface than B. In addition, the expression of I-A<sub>d</sub> on lung APCs from both freshly isolated lung cells and lung cells cultured overnight in plastic culture dishes was compared to determine whether murine lung B cells and DCs upregulate MHC class II expression during culture in a manner similar to that described for murine skin and splenic DCs (5–7, 21–24). CD45R/B220 staining in combination with FITC-head uptake and I-A<sub>d</sub> staining was used to distinguish B cells from DCs so that the level of expression of I-A<sub>d</sub> on both of these nonphagocytic, MHC class II-positive populations could be determined.
Lung DCs in freshly isolated lung cell suspensions (I-A<sup>d</sup>; 100%, 451 MFI) were comprised of DCs expressing moderate levels of I-A<sup>d</sup> (83%, 286 MFI) and a smaller percentage expressing high levels of I-A<sup>d</sup> (17%, 1325 MFI) (Fig. 6). In contrast, DCs in lung cells cultured overnight (I-A<sup>d</sup>; 100%, 1057 MFI) were comprised mainly of high I-A<sup>d</sup> (54%, 1692 MFI) expressing DCs. The intensity of I-A<sup>d</sup> expressed on B cells in freshly isolated lung cell suspensions was uniform and at moderate levels (100%, 271 MFI) and changed little after culture (100%, 340 MFI). When lung B cells and lung DCs were compared under freshly isolated and cultured conditions, it was apparent that lung DCs expressed higher intensities of I-A<sup>d</sup> than lung B cells under either set of condition.

In parallel experiments, we determined whether culture on plastic might affect the expression of I-A<sup>d</sup> on lung APCs by comparing cells held overnight in suspension with lung APCs cultured overnight on plastic tissue culture dishes. The expression of I-A<sup>d</sup> on B cells and DCs cultured in suspension was similar to that seen on B cells and DCs cultured on plastic dishes, respectively (data not shown). In addition, the expression of I-A<sup>d</sup> on DCs and B cells pulsed with OVA protein or OVA peptide was similar to that seen on nonpulsed DCs and B cells, respectively (data not shown).

Accessory molecule expression on freshly isolated and cultured lung DCs and B cells

Previous studies showed that murine lung DCs utilized multiple accessory molecules in their role as initiators of allogeneic T cell responses, including CD2 ligand, CD11a, CD40, CD54, CD80, and CD86 (18). To further address possible mechanisms for the functional APC differences seen between peptide-pulsed lung B cells and DCs, the expression of accessory molecules was compared for these two I-A<sup>d</sup>-expressing populations. In addition, the expression of accessory molecules on B cells and DCs from freshly isolated and cultured lung cells was compared to determine whether their accessory molecule expression increased in a manner similar to that described for murine skin and splenic DCs (5–7, 1314).

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FIGURE 4. OVA-pulsed lung cells stimulate naive CD4<sup>+</sup> DO11.10 T cells to proliferate. Freshly isolated lung cells (a) and spleen cells (b) were either pulsed (within 3 h after the lung tissue harvest) or not pulsed overnight with 100 μM OVA and irradiated before coculture with naive CD4<sup>+</sup> DO11.10 T cells (2.5 × 10<sup>4</sup> cells/well). After 5 days, T cell proliferation was assessed as described in Fig. 1. The data shown are the mean and SEM of quadruplicate wells. T only, 338 cpm; T + 100 μM OVA, 800 cpm; T + 10 μM peptide, 5851 cpm.

FIGURE 5. Pulsed lung DCs are superior to pulsed lung B cells in stimulating naive CD4<sup>+</sup> DO11.10 T cells to proliferate. Freshly isolated lung cells were pulsed overnight with 10 μM OVA<sub>323-339</sub> peptide (a) or 100 μM OVA (b). Lung DCs and B cells were sorted from these nonirradiated lung cell populations and cocultured with naive CD4<sup>+</sup> DO11.10 T cells (2.5 × 10<sup>4</sup> cells/well). After 5 days, T cell proliferation was assessed as described in Fig. 1. T + 5K mock-pulsed DC, 311 cpm; T + 5K mock-pulsed B, 128 cpm. For a, the data shown are the mean and SEM of three independent experiments. For b, the data shown are the mean and SEM of triplicate wells and are representative of two independent experiments.

FIGURE 6. Lung DCs express higher levels of I-A<sup>d</sup> than lung B cells. Lung cells were stained as described in Materials and Methods. The FS/SSC profile of stained cells was used to set gate A to exclude debris, free latex beads, some macrophages, and possibly some NK cells. To optimize analysis, this gate was further refined by back-gating from gate A to set gate B on I-Ad<sup>-</sup> cells to ensure that DCs and B cells were the predominate populations within gate A. For DC analysis, gate C was set on CD45R/B220-negative cells within gate A and analyzed for the expression of I-Ad<sup>d</sup>. Note the majority of DCs in gate C are difficult to see because they fall into the lower B220 fluorescence channels that are not resolved from the baseline. However, the I-Ad<sup>d</sup> staining on DCs in gate C is clearly shown in the I-Ad<sup>d</sup> histogram for DCs. For B cell analysis, gate D was set on CD45R/B220-positive cells within gate A and analyzed for the expression of I-Ad<sup>d</sup>.
DCs expressing moderate levels of I-Ad might have also included a marker to clearly identify B cells. For B cell phenotyping, gates A, B, and D shown in Fig. 6 were used to perform analysis on the I-A\(^d\)-positive, FITC-head-negative, B220-negative population for the expression of a series of PE-labeled accessory molecule markers. Previous three-color phenotyping studies in our lab demonstrated that nonphagocytic, high I-A\(^d\)-expressing DCs in LAd generated DC subsets in the lung DCs.

Distinct mature lymphoid- and myeloid-derived subpopulations of DCs have been described in the murine spleen. We evaluated by four-color flow cytometry the expression of the lymphoid-related markers, CD1d and CD80, and the myeloid-related marker, CD11b, to determine whether lung DCs could be divided into lymphoid and myeloid subpopulations, respectively. Lineage analysis revealed that the majority of freshly isolated lung B cells and DCs expressed low levels of CD1d, and a very small subpopulation expressed low levels of CD80. CD1d expression on lung B cells did not change after culture. However, CD1d was up-regulated on cultured lung DCs, specifically lung DCs expressing high levels of I-A\(^d\) (DC\(^{high\ I-Ad}\)) showed higher levels of accessory molecules than cultured DCs expressing moderate I-A\(^d\). Freshly isolated and lung cells cultured overnight were stained as described in Materials and Methods, and gates A, B, and C shown in Fig. 6 were used to identify the DC population. Two additional gates (not shown) were established to perform analysis on DCs expressing moderate levels of I-A\(^d\) (Fluorescence intensity, 40–750) and DCs expressing high levels of I-A\(^d\) (CDC\(^{high\ I-Ad}\)) (Fluorescence intensity, 751–10,000) for the expression of a series of PE-labeled accessory molecule markers.
freshly isolated and cultured lung B cells showed low levels of CD11b. The majority of freshly isolated lung DCs expressed moderate levels of CD11b that did not change after culture. However, the highest levels of I-A^d also expressed the highest level of CD11b.

Discussion

The present study resulted in four important observations. First, both murine lung DCs and lung B cells presented OVA_{323–339} peptide to Ag-specific naive CD4^+ T cells when the peptide was continuously present during the lymphoproliferation assay. However, when APCs were pulsed with peptide, DCs were clearly superior to B cells at stimulating T cell proliferation. Second, with optimal OVA protein continuously present in culture, lung DCs, but not lung B cells, stimulated naive T cells. Furthermore, as expected, protein-pulsed lung DCs were also efficient in presenting Ag to naive T cells, whereas protein-pulsed lung B cells showed no APC activity. Third, phenotypic characterization of lung DCs and B cells revealed important differences in the expression of MHC class II and accessory molecules that help explain why peptide-pulsed DCs were superior to peptide-pulsed B cells at stimulating naive T cell proliferation. Finally, the level of expression of I-A^d on DCs predicted the level of expression of accessory molecules and suggested heterogeneity of maturation of lung DCs even after overnight culture.

The principal aim of this work was to examine the ability of lung B cells and lung DCs to stimulate naive CD4^+ T cells to determine which APC was more important in priming the host to inhaled Ags. To test the accessory cell capacities of these two cell types, two assay systems, which make different demands on APCs, were used. One measured the response of naive CD4^+ DO11.10 T cells to OVA peptide. By using peptide, processing was bypassed, allowing us to test directly Ag presentation to naive CD4^+ T cells. Both B cells and DCs were capable of activating naive CD4^+ T cells when the peptide was present in nonlimiting concentrations, i.e., throughout the lymphoproliferation assay. However, when peptide was limited by pulsing freshly isolated lung cells overnight before purifying lung DCs and B cells, a relative deficiency of lung B cells became evident. The second assay used the response of naive CD4^+ DO11.10 T cells to intact OVA protein presented on APCs. Although B cells are known to take up and present soluble proteins independently of their surface Ig specificity, our results showed that lung B cells were deficient in stimulating naive CD4^+ T cells to proliferate even when the OVA was nonlimiting (16, 17). The results with OVA suggest that only lung DCs are capable of processing intact protein, and together with their increased MHC class II and accessory molecules are the most potent APC in the lung. It appears unlikely that lung B cells, even if they possessed sufficient accessory molecules, might not be capable of priming a host to inspired intact Ags.

In the current study, we asked whether the APC potency differences between peptide-pulsed lung DCs and peptide-pulsed lung B cells might be due to differences in the expression of the required combination of peptide-MHC complexes and accessory molecules necessary to engage the TCR and initiate T cell proliferation. The concept that T cells count the number of triggered TCRs and respond when this number reaches an appropriate threshold was presented by Viola et al. (27). Based on our phenotyping data, lung DCs expressed higher levels of MHC class II molecules than lung B cells. Lung DCs also expressed higher levels of accessory molecules such as CD80, CD86, CD11a, and CD54 that are known to interact with counter-receptors on T cells to enhance costimulation and adhesion. However, lung DCs and lung B cells expressed similar levels of CD40 and CD24. In addition, MHC class II expression and the expression of accessory molecules were up-regulated on lung DCs within 1 day of culture, whereas little change was seen for lung B cells. These findings suggest that when peptide was not limiting, the low level of accessory molecules expressed by lung B cells was sufficient to augment TCR signaling and provide the overall signal necessary for T cell proliferation. However, under conditions of limited peptide, an overall activation threshold for cell proliferation was restricted and the number of naive CD4^+ T cells capable of responding to the peptide was low.

We noted heterogeneity of phenotype among our lung DC populations both in fresh and cultured lung cells. Recently, Koch et al. (8) demonstrated that skin and splenic DCs do not mature uniformly in culture, and the processing activity observed in these populations was most likely due to a subset of immature DCs retaining expression of the MHC class II-associated invariant chain. Whether murine lung DCs spontaneously mature in culture had not yet been reported. We found lung DCs up-regulated their expression of MHC class II, CD80, CD86, CD40, CD11a, and CD54 during culture in a manner similar to that described by others for skin and spleen DCs. In addition, a subpopulation of cultured lung DCs expressed MHC class II and accessory molecules at the same level as found on freshly isolated DCs. This finding is important, because it suggests that MHC class II expression might coincide with accessory molecule expression by lung DCs, and that lung DCs share a similar maturation scheme with skin and spleen DCs. Although not specifically tested, we hypothesize that the subpopulation of cultured lung DCs coexpressing lower MHC class II and accessory molecules comparable with freshly isolated DCs represents an immature population of DCs that retain the ability to process OVA. Indeed, the ability of lung DCs, even after overnight culture, to process and present OVA (as shown in Fig. 3) supports this hypothesis.

Finally, other markers for DCs were studied to further characterize lung DCs. It was established previously by three-color FACS analysis that the majority of cultured murine lung DCs expressed the IL-2R alpha chain (IL-2R, CD25a) (19). It has been suggested that CD25a is important for the formation of high affinity binding sites responding to IL-2, and that IL-2 is a specific chemotactic factor for DCs in lung and skin (28–30). Others also suggested CD25a expression might indicate DC maturation, and be important in the activation and migration of DCs (31, 32). We found that the percentage of CD25a-positive DCs increased after overnight culture and the density of this marker correlated with I-A^d expression. These results support the concept that CD25a might be a marker of DC maturation.

We failed to find lung DCs that correspond to either a strict lymphoid or myeloid lineage DC as was described in the spleen (25, 26). Pulendran et al. postulated that CD11b is a stable marker for myeloid-derived DCs, whereas Vremec et al. suggested CD11b is a marker of DC maturation. Our lineage phenotyping analysis supports the view of Vremec et al. because the level of CD11b and I-A^d expression on lung DCs correlated with one another. Pulendran et al. also postulated that CD11d is a stable marker for lymphoid-derived DCs. In contrast, our phenotyping data showed that CD11d was up-regulated on cultured lung DCs, again specifically on lung DCs expressing high levels of I-A^d, suggesting that CD11d is also a maturation marker. In the spleen, two populations of DCs have been described, one CD8alpha^- DEC205^- CD24^- CD11b^- and the other CD8alpha^- DEC205^ CD24^- CD11b^, representing lymphoid- and myeloid-derived DCs, respectively (32). Although we did not directly examine coexpression of CD8alpha and CD11b, we found that CD8alpha was only on a small subpopulation of lung DCs. Because both CD24 (a lymphoid-related marker) and CD11b (a
myeloid-related marker) were present on virtually 100% of lung DCs, it appears that the lung DC phenotype fits neither the splenic lymphoid nor myeloid designations described by Vremec et al.

In conclusion, when a host responds to new protein Ags introduced directly into the lungs, lung DCs most likely play the most important role in the generation of primary immune responses.

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