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Interstitial lung macrophages interact with dendritic cells to present antigenic peptides derived from particulate antigens to T cells

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SUMMARY

When the protective structural and functional barriers of the lung are breached, immune responses must be generated in order to contain invading micro-organisms. This requires the presence of accessory cells capable of phagocytosing and presenting immunogenic peptides to either naive or sensitized T cells. In contrast to dendritic cells (DC) present in the airway epithelium, those within the lung parenchyma do not readily engulf particulates and, therefore, other mechanisms must account for their apparent ability to present immunogenic peptides derived from micro-organisms. The purpose of the present study was to determine the extent to which interstitial macrophages (IM) interact with lung DC to process and present antigenic peptides, derived from particulate, heat-killed *Listeria monocytogenes* (HKL), to HKL-immune T cells. Results show that highly purified Ia^- lung IM avidly phagocytose fluorescent-labelled HKL, but they do not present antigen to primed T cells. Their ability to present antigen is only modestly increased following interferon-γ (IFN-γ) stimulation. Conversely, mature DC isolated from the lung interstitium do not phagocytose fluorescent-labelled HKL. In antigen presentation assays, however, addition of 10% ($2{\cdot}5 \times 10^3$/ml) Ia^- IM to DC and HKL results in a two- to threefold increase in antigen presentation by DC to HKL-immune T cells. Conditioned medium (CM), generated by $2{\cdot}5 \times 10^4$/ml IM induced to phagocytose HKL, when administered to DC and HKL-sensitized T cells without added intact HKL, resulted in brisk mitogenesis, a response that did not occur in T cells sensitized to an irrelevant antigen. Conditioned medium derived from larger numbers of IM was inhibitory. When IM phagocytosed inert polystyrene beads, the resulting CM induced modest T-cell mitogenesis, suggesting that small amounts of cytokines were released. The results indicate that in small numbers, IM augment DC function, in part, by the release of antigenic peptides which are then presented by DC to T cells. When present in numbers greater than 50% of DC, however, they inhibit DC function, probably due to the release of soluble inhibitors.

INTRODUCTION

The epithelial surfaces of the lung are continuously exposed to potential pathogens in the external environment. A variety of protective mechanisms, including mucociliary clearance, phagocytosis by alveolar macrophages and secreted antibodies in alveolar fluid, guard against the penetration of inhaled pathogens into the lung. When these functional barriers are breached, for example during inflammation, an immune response to inhaled microbial antigens must be elicited, requiring the presence of accessory cells capable of endocytosing, processing and presenting antigen to appropriate subsets of T cells. Studies in a variety of species indicate that dendritic cells (DC), residing in airway epithelium,[1-3] in connective tissue cuffs surrounding major airways and blood vessels, and in alveolar walls,[4] are the principal accessory cells in the lung. Their phenotype and function vary depending on their anatomical location and degree of maturation.[3,5] DC in airway epithelium, like epidermal Langerhans' cells,[6] are capable of limited phagocytosis,[3] whereas mature DC isolated from alveolar walls endocytose small amounts of soluble antigen but are not phagocytic.[3,5] Interstitial macrophages (IM) may participate in the uptake and processing of antigen in the lung;[7] however, their ability to present immunogenic peptides to T cells remains to be established. Furthermore, their ability to regulate the degree of T-cell proliferation, either directly or via accessory cells,[8] is unclear.

Utilizing a recently developed micro-dissection technique,[3,9] data were obtained indicating that DC residing in alveolar walls of the lung are relatively mature, in that they express surface adhesins and co-stimulatory molecules necessary for the activation of non-sensitized T cells.[3] However, this subpopulation

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of interstitial DC is not phagocytic and is not as effective in processing and presenting native antigen as its more immature counterpart in airway epithelium.[3] Other phagocytic cells within the lung capable of uptake and processing of particulate pathogens include alveolar macrophages. These cells, however, even when stimulated by interferon-γ (IFN-γ) to express MHC class II antigen, are poor accessory cells in most species[10,11] and in fact suppress the activity of DC both *in vitro*[10] and *in vivo*.[8] Macrophages residing in the interstitium of the lung are intermediate in differentiation between that of alveolar macrophages and circulating monocytes.[12,13] They may be in close proximity to DC, which could facilitate the interaction of these two types of cells in the processing and presentation to T cells of immunogenic peptides derived from particulate antigens. The present study was undertaken to determine, *in vitro*, the extent to which IM interact with interstitial lung DC to process and present antigens derived from heat-killed *Listeria monocytogenes* (HKL) to HKL-immune T cells.

MATERIALS AND METHODS

Animals

Pathogen-free, 6–8-week-old female Lewis rats (160–180 g) were obtained from Charles River Breeding Laboratories (Kingston, NY). Animals, housed in restricted-access research animal care facilities at Massachusetts General Hospital, were permitted free access to food and water and underwent monthly monitoring for viral infections.

Reagents and antibodies

The density gradients used included bovine serum albumin (BSA), fraction V, obtained from Intergen Co. (Purchase, NY), Percoll (Sigma Chemical Co., St Louis, MO), Metrizamide and Lymphoprep (Nycomed Pharmaceuticals AS, Oslo, Norway) and Histopaque-1077 (Sigma Chemical Co.). Enzymes used were collagenase (CLS 1; Worthington Biochemical Corp., Freehold, NJ) and DNAse I (Sigma Chemical Co.). Low melting temperature agarose Sea Plaque GTG was from FMC BioProducts (Rockland, ME). Cytokines used were murine recombinant granulocyte–macrophage colony-stimulating factor (rGM-CSF; a gift of Genetics Institute, Andover, MA), recombinant interleukin-2 (rIL-2; a gift of Hoffmann-LaRoche, Nutley, NJ) and IFN-γ (a gift of Genentech, S. San Francisco, CA). Cell culture reagents included RPMI-1640 medium (JRH Biosciences, Lenexa, KS), fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS) and penicillin/streptomycin (P/S; Gibco Laboratories, Grand Island, NY), and newborn calf serum (NCS; Hyclone Laboratories Inc., Logan, UT). Sheep red blood cells (SRBC; Whitaker Bioproducts, Walkersville, MD) were used. Monoclonal antibodies (mAb) included rabbit anti-SRBC IgG (Diamedix Corp., Miami, FL), OX6 (anti-Ia)[14] and OX41 (which recognizes an epitope on macrophages)[15] (Accurate Chem. & Sci. Corp., Westbury, NY). Goat anti-mouse $F(ab')_2$ IgG–fluorescein isothiocyanate (FITC) (Tago Inc., Burlingame, CA); rat gamma globulin (Pel-Freez Biologicals, Rogers, AK); tosylyl-activated Dynabeads (M-450; Dynal AS, Oslo, Norway); zynaxis dye PKH-26 (Cell Science, Inc., Malvern, PA); hen egg lysozyme (HEL; Sigma Chemical Co.); and polystyrene beads (1·16 μm in diameter; Seradyn, Indianapolis, IN) were purchased as indicated.

Surgery and enzyme digestion

Interstitial macrophages and DC were obtained from the peripheral, alveolar portions of the lung by micro-dissection, as previously described[3] (Fig. 1), except that enzyme was omitted from the instilled agarose. Briefly, female Lewis rats were anaesthetized with sodium pentobarbital, 50 mg/kg body weight. After exsanguination via the abdominal aorta, the diaphragm was cut and the lung lavaged with six 5-ml aliquots of 0·6 mM Na_2EDTA in phosphate-buffered saline (PBS). The thorax was opened, the left ventricle incised and the pulmonary vasculature perfused via the pulmonary artery with 1·5 mM Na_2EDTA and 0·075% sodium nitrite in PBS until the lung blanched completely.[16] The lungs were not ventilated. Ten millilitres of 1% agarose in HBSS at 37° was gently infused into the airways and lung. After tying off the trachea, the lung was immersed for 30 min in ice-cold PBS to solidify the agarose. Under sterile conditions, the lung parenchyma was dissected from the tracheobronchial tree and minced into 1-mm³ fragments. These were incubated in an enzyme solution, 4 ml/g tissue, containing collagenase (150 U/ml) and DNAase I (50 U/ml) in RPMI-1640 medium with 10% FBS, 1% P/S, 5 mM HEPES and 5×10^{-5} M 2-mercaptoethanol (complete medium) for 90 min at 37°.[3] After passing the fragments through an 80-mesh stainless steel screen and filtering through four layers of gauze, low-density cells were retrieved at the interface following equilibrium density centrifugation in a dense BSA solution.[17]

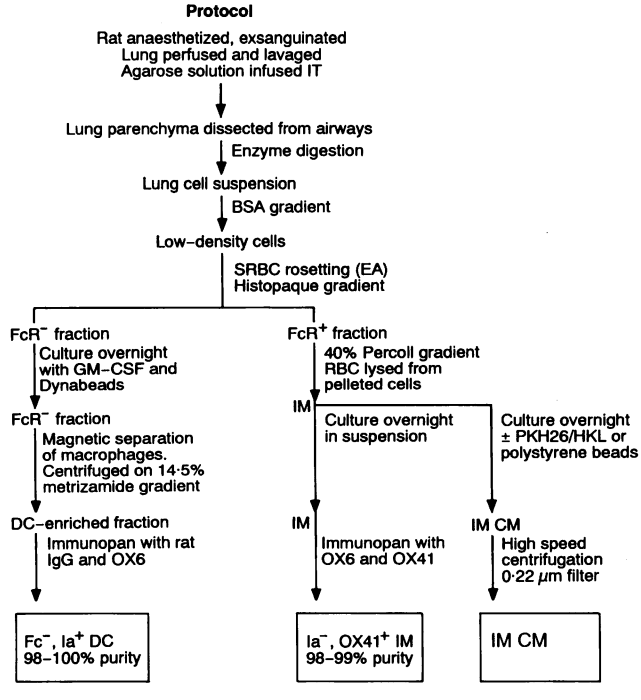

Figure 1. Flow diagram of procedure for the isolation of interstitial lung DC and IM and the production of CM. IT, intratracheal.

SRBC rosetting for fractionation of FcR$^+$ and Fc'R$^-$ cell populations

After washing in PBS, the low density cells were fractionated into FcR$^+$ and FcR$^-$ cells by rosetting with antibody-coated sheep erythrocytes.[18] The two populations of cells were separated by centrifugation on a Histopaque density gradient at 800 *g* for 15 min at 4°. FcR$^-$ cells were retrieved at the interface, washed and incubated overnight in RPMI-1640 with rGM-CSF (30 U/ml) to improve DC survival,[19] as well as with non-coated, tosalyl-activated M-450 Dynabeads for uptake by and subsequent removal of phagocytic cells. The FcR$^+$ cells were gently resuspended from the pellet and layered onto 40% Percoll in HBSS, 5% FBS. Rosetted cells were retrieved by centrifugation at 2·5 *g* and the attached SRBC lysed in ACK-lysing buffer (0·15 M NH_4Cl, 1·0 mM $KHCO_3$, 0·1 mM EDTA).[20] After washing twice in PBS, the FcR$^+$ cells were cultured overnight in suspension in a 50-ml polypropylene tube.

The following day, phagocytic cells were removed from the FcR$^-$ population utilizing a magnetic particle concentrator (Dynal AS). The remaining non-phagocytic cells were then layered on top of 14·5% metrizamide and centrifuged at 600 *g* for 10 min at room temperature to remove B and natural killer (NK) cells.[21] The DC-enriched fraction at the interface was harvested, and washed twice in progressively less hypertonic medium until the cells were once again in isotonic complete medium.

Immunopanning

Interstitial macrophages. All immunopanning was conducted at 4°. To remove Ia$^+$ cells from the FcR$^+$ IM population, the cells were subjected to two rounds of immunopanning, utilizing bacteriological grade Petri dishes (8-757-13-A; Fisher Scientific Co., Pittsburgh, PA) coated with OX6 (1:100). The non-adherent Ia$^-$ IM were further enriched by panning three times on OX41 (1:200)-coated plates. The adherent IM were retrieved by gently scraping them from the Petri dish with a plastic cell scraper (NUNC, Naperville, IL). This resulted in an IM population that was >98% pure and >90% viable, as determined by immunofluorescence and by trypan blue dye exclusion, respectively.

Dendritic cells. To remove the residual FcR$^+$ cells (macrophages, monocytes, B cells) from the DC-enriched population, the latter was panned twice on rat IgG-coated Petri dishes. The non-adherent FcR$^-$, Ia$^+$ DC were retrieved by panning three times on OX6 (1:400 dilution)-coated Petri dishes which yielded a >98% pure population of cells. Utilizing such stringent isolation techniques, while resulting in highly purified interstitial lung DC, yielded, on average, 1×10^5 DC from 10 rats.

Labelling of HKL with zynaxis PKH-26

The PKH-26 fluorescent dye enables stable cell labelling by the incorporation of fluorochrome-labelled aliphatic reporter molecules into the lipid bilayer of cell membranes[22] without affecting cell function.[23] To label HKL, 1·0 ml of $1{\cdot}5 \times 10^{-6}$ M PKH-26 and 1·0 ml of 2×10^8 HKL, both in diluent (supplied by the manufacturer), were mixed together and incubated for 5 min at room temperature. Uniform staining was confirmed with a Zeiss Universal fluorescence microscope (Zeiss Inc., Oberkochen, Germany). The staining reaction was stopped by the addition of 2 ml of FBS. The labelled HKL were washed three times with PBS, utilizing a fresh tube each time to ensure the complete removal of free dye.

Interstitial macrophage-conditioned medium

Conditioned media (CM) were produced by utilizing either 'low' ($2{\cdot}5 \times 10^4$/ml) or 'high' ($5{\cdot}7 \times 10^5$/ml) numbers of IM with and without PKH-26-labelled HKL. Briefly, the CM were generated in 24-well plates by incubating 5×10^4 IM with $1{\cdot}8 \times 10^6$ HKL ('low') in 2 ml or $8{\cdot}5 \times 10^5$ IM with 4×10^7 HKL ('high') in 1·5 ml complete RPMI-1640 medium, for 15–16 hr in 5% CO_2 at 37°. The CM were aspirated, centrifuged at 27,000 *g* for 30 min at 4° and filtered through a 0·22 μm filter. The complete removal of PKH-26-labelled HKL was confirmed by inspection of aliquots of CM by fluorescence microscopy. As a further test, macrophages were incubated with the CM for 18 hr and then inspected for evidence of uptake of fluorescent lipid material. They were consistently negative. Control CM were generated by incubating a similar number of IM alone or by allowing IM ($2{\cdot}5 \times 10^4$/ml) to phagocytose polystyrene beads ($3{\cdot}5 \times 10^7$/ml) for 18 hr.

Proliferation assays

Addition of interstitial macrophages. IC and DC were irradiated (1000 rads) and washed. DC were plated (5×10^3/well) in triplicate in 96-well flat-bottomed culture plates (Becton Dickinson & Co., Lincoln Park, NJ) in complete medium. To these wells were added HKL-immune T lymphoblasts (5×10^4/well) and either 0%, 10% (5×10^2/well), 20% (1×10^3/well), or 50% ($2{\cdot}5 \times 10^3$/well) of purified IM, with and without PKH-26-stained HKL (4×10^6/well). Interstitial macrophages alone (5×10^3/well) incubated with HKL-immune T cells, with and without PKH-26-stained HKL, served as controls. To examine the ability of Ia$^+$ IM alone to present HKL to HKL-immune T cells, they were incubated either with or without IFN-γ (20 U/ml) for 18 hr prior to the assay, or the antigen presentation assay was conducted in the presence or absence of IFN-γ at the same dose.

Addition of interstitial macrophage conditioned medium. To triplicate wells containing DC (5×10^3/well) and HKL-immune T cells (5×10^4/well) was added 0%, 10%, 20% or 30% (v/v) of either 'low' or 'high' IM-generated CM. No HKL were added to these wells. Controls included either DC or IM alone incubated with HKL-immune T cells, without HKL. In a second set of experiments, 0%, 10%, 20% or 30% (v/v) of 'low' IM-generated CM (produced by IM phagocytosing PKH-26-labelled HKL) was added to triplicate wells containing DC (1×10^4/well) and either HKL-immune T cells or HEL-immune T cells. CM generated by IM phagocytosing polystyrene beads served as control CM. Incubation in all the assays was for 72 hr in 5% CO_2 at 37°. Cells were pulsed with [^{3}H]thymidine ([^{3}H]TdR; 1 μCi/well; specific activity 50–80 Ci/mmol; New England Nuclear, Boston, MA) for the final 6 hr of the assay, harvested using a cell harvester (Skatron AS, Lierbyen, Norway), and counted in a Tri-Carb liquid β-scintillation spectrometer (Packard Instrument Co. Inc., United Technologies, Downers Grove, IL).

Immunofluorescence

To determine whether there is an association of IM and DC within heterotypic cell clusters with HKL-immune T cells, 1×10^4 DC and 1×10^3 IM were incubated with 5×10^5 HKL-immune T cells and 1×10^7 HKL stained with PKH-26 in 2 ml complete medium at 37° in 5% CO_2 for 18 hr. The cells were then

layered onto 5 ml of Lymphoprep and centrifuged at room temperature for 20 min at 500 *g* to remove free T cells. The cell clusters were harvested at the interface and slide preparations made using a Cytospin cytocentrifuge (Shandon Inc., Pittsburgh, PA). The cells were stained, utilizing the indirect immunofluorescence technique, with either OX6 (DC) or OX41 (IM) followed by goat anti-mouse $F(ab')_2$ IgG–FITC. The FITC (green)-labelled cells and the engulfed PKH-26-labelled HKL (red) were visualized by fluorescence microscopy using 3RS vertical illumination and a dichroic mirror system providing the appropriate wavelengths for fluorescein and rhodamine, respectively. The location of the clustered T cells was documented by phase microscopy.

Confocal microscopy

To determine the ability of DC to phagocytose PKH-26-labelled HKL, cells were prepared for confocal microscopy as follows. DC (1×10^5/well), isolated and purified as described above, were incubated with 4×10^6/well of PKH-26-labelled HKL in 200 μl of complete medium in a 96-well plate (Becton Dickinson) for 18 hr in 5% CO_2 at 37°. Slides of DC were prepared by cytocentrifugation and stained for Ia (OX6) as described above. Purified IM (1×10^5/well), isolated as described above, were incubated with 4×10^6/well of PKH-26-labelled HKL in Lab-Tek chambers (NUNC Inc.) in 200 μl complete medium for 18 hr in 5% CO_2 at 37°. Since the IM adhered to the glass surface, they were immunolabelled directly in the Lab-Tek chambers with OX41 mAb, as described above. To diminish fluorescence quenching, the slides were mounted in 50% glycerol containing 2% N-propyl gallate.[24]

Cells were examined at 100× (1·4 numeric aperture) using a Sarastro 2000 Confocal Laser Scanning Microscope (CLSM; Molecular Dynamics, Sunnyvale, CA) fitted with a 25 mW Argon-Ion laser. Fields were selected at random and the cells were brought into focus under bright field conditions. Dual-scan fluorescent CLSM images were then collected under the following operating parameters for both IM and DC preparations. Laser power 10 mW with excitation wavelengths of 488 nm for FITC and 514 for PKH-26; laser output was attenuated to 10% transmittance. The emission spectra were filtered with a high-pass 535 nm primary dichroic beam splitter. A dichroic secondary beam splitter of 565 nm separated the emission output to two photomultiplier tubes (PMT), with detector filters of 600 nm for PMT-1 (red, PKH-26) and 540 detector filters of 30 nm for PMT-2 (FITC).

RESULTS

Characteristics of dendritic cells and interstitial macrophages isolated from the lung interstitium

The multiple-step purification protocol utilized to isolate interstitial lung DC and IM resulted in preparations of DC that were >98% pure with <1–2% contamination of IM, as judged by immunofluorescence and confocal microscopy (Figs 2 and 3). Despite multiple panning and density gradient steps, this trace fraction of IM remained. The mean diameter ± SD of isolated IM was 12·3 ± 1·2 μm; they were Ia^- and stained positively with OX41 mAB (Figs 2 and 3). By contrast, the mean diameter of

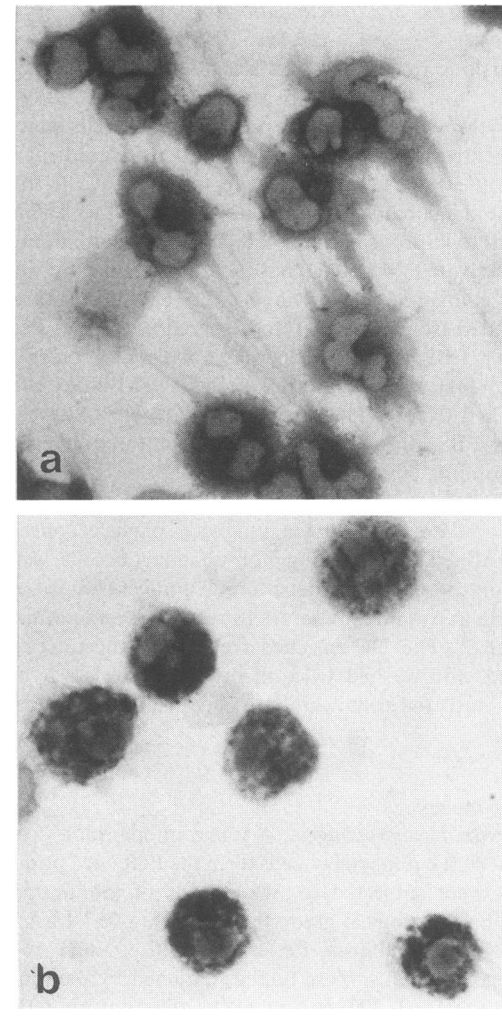

Figure 2. Cytospin preparations of (a) isolated, rat interstitial lung DC immunolabelled for Ia; (b) isolated Ia^- rat IC immunolabelled with OX41 mAb. Magnification (a) and (b) ×660.

DC, exclusive of cell processes, was 15·36 ± 3·01 μm; cell processes extended up to 40 μm from the cell body. They were $OX41^-$ and strongly Ia^+. To increase their viability during overnight incubations, DC were maintained in the presence of 30–60 U/ml of rGM-CSF, a cytokine that not only increases viability[19] but also promotes the differentiation of DC.[25] Thus the final population of interstitial lung DC examined was one of mature cells that were incapable of phagocytosing particulate HKL (Figs 3 and 4). The IM preparation was >98% pure, as determined by immunofluorescence utilizing the OX41 mAb and by examining their ability to phagocytose PKH-26-labelled HKL. Moreover, because residual DC were removed from the IM fraction by immunopanning with OX6 (anti-Ia) mAb, the final preparation of IM was Ia^-, thereby abrogating their ability to present antigen directly to primed T cells (Fig. 5). However, even when stimulated to express Ia by an 18-hr incubation with IFN-γ, IM presented antigen poorly to HKL-immune T cells relative to a DC preparation containing 5–10% added IM (Fig. 5).

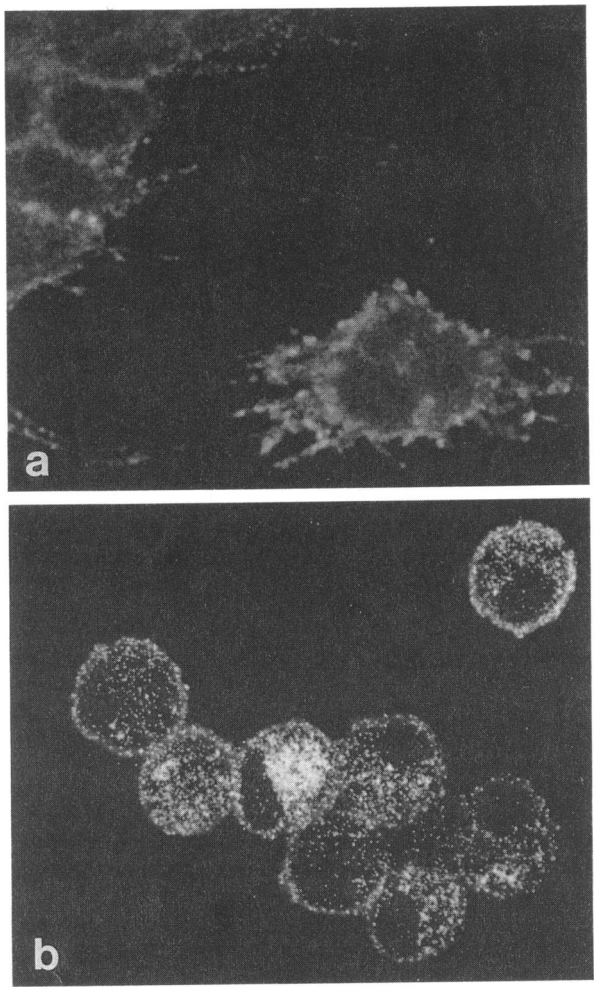

Figure 3. (a) Dual channel image of rat interstitial lung DC co-cultured for 18 hr with PKH-26-stained HKL (red). Cells were cytocentrifuged prior to immunolabelling for Ia (green). All DC were strongly Ia^+ and no HKL were found attached to the surface of, or internalized by, these cells. (b) Dual channel image of rat IC co-cultured with PKH-26-labelled HKL under the same conditions as those described for DC. The IM were immunolabelled with OX41 directly in Lab-Tek chambers and were not subjected to cytocentrifugation. All IM (green) contained PKH-26-stained HKL (red). At this time following ingestion, intact bacteria are no longer discernible. Magnification (a) and (b): × 1125.

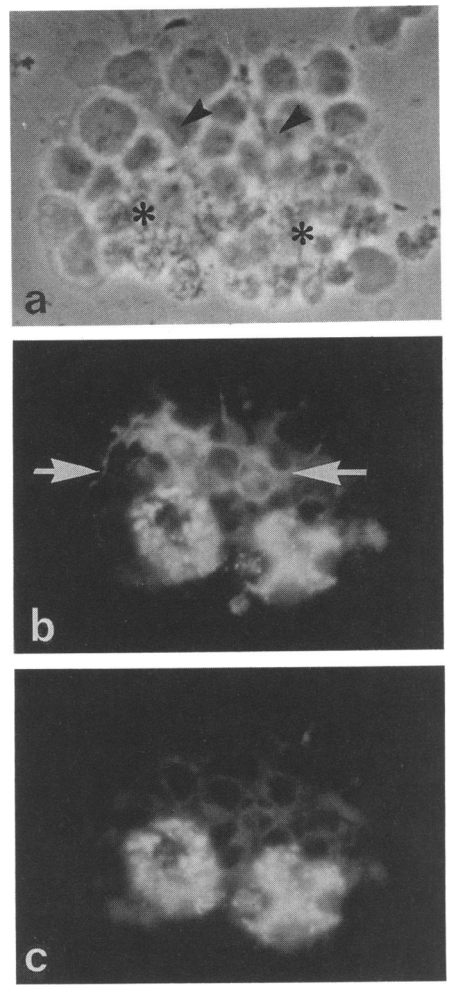

Figure 4. (a) Phase micrograph of an heterotypic cluster containing two IM (asterisks) and two DC (arrowheads). Note that the enlarged HKL-immune T lymphoblasts are clustered with the DC and not with the IM. (b) Immunofluorescence of the same cluster as in (a) viewed at an emission wavelength of 510 nm to visualize FITC staining. The long cell processes of the two Ia^+ DC are clearly visible (white arrows). (c) Immunofluorescence of the cluster shown in (a) viewed at an emission wavelength of 580 nm (RITC). The two IM contain large numbers of PKH-26-stained HKL. Magnification (a), (b), (c): × 340.

Clustering of interstitial macrophages and dendritic cells with T cells

Examination by immunofluorescence microscopy of cell clusters formed after addition of 10% IM to DC, together with HKL-immune T cells and PKH-26-labelled HKL, revealed that IM were frequently adherent to DC within the heterotypic DC, T-cell clusters (Fig. 4a–c). Moreover, T cells preferentially clustered with DC and not with the attached IM, even though labelled HKL were detected only in the latter. The selective adhesion of T cells to DC is probably due to the presence of a greater array of adhesions on mature DC than on Ia^- IM, as well as the interaction of the T-cell receptor with Ia and antigen on the DC surface. It is possible that immunogenic peptides released during phagocytosis of HKL by IM may interact directly with Ia present on the surface of DC. Alternatively, since DC are capable of a modest degree of endocytosis,[26] released peptides larger than 10–14 amino acids in length may be endocytosed, processed and presented by the DC. It should be noted that since the fluorescent dye PKH-26 partitions into the lipid bilayer of cell membranes, peptides released during phagocytosis by IM are not labelled and, therefore, are not visualized.

Effect of increasing numbers of interstitial macrophages on antigen presentation by dendritic cells

Addition of 10% IM to a constant number of interstitial lung DC in an antigen presentation assay consistently resulted in a two- to threefold increase in T-cell mitogenesis (Fig. 6). The inter-assay variation of the observed increase over the baseline value obtained with DC alone was probably the result of varying numbers of residual IM in the DC preparation. Increasing the number of added IM above 10% did not further augment T-cell

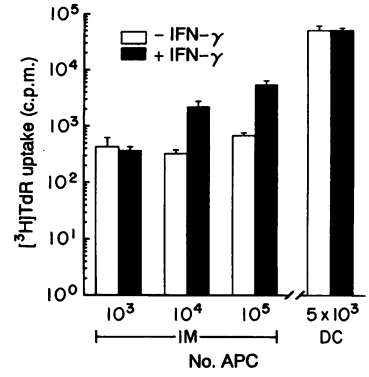

Figure 5. Graph indicating the effect of incubating Ia⁻ IM or DC for 18 hr with IFN-γ (20 U/ml) on antigen presentation. It should be noted that while IM were used as the sole antigen-presenting cells (APC), 10% IM were added to the DC preparations that had either been treated with IFN-γ or not. Although IFN-γ modestly augments antigen presentation by IM relative to DC plus 10% IM, they present HKL poorly to HKL-immune T cells. The data represent the mean ± SE of triplicate wells.

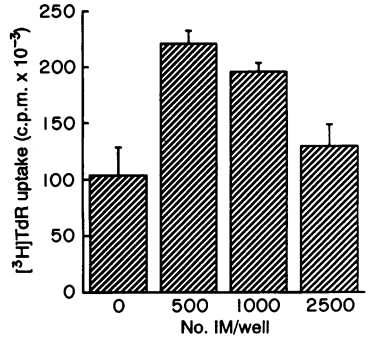

Figure 6. Graph showing the effect of increasing numbers of IM/well on the presentation of HKL by lung interstitial DC (5×10^3/well) to HKL-immune T cells (5×10^4/well). The data represent the mean ± SE of three separate experiments.

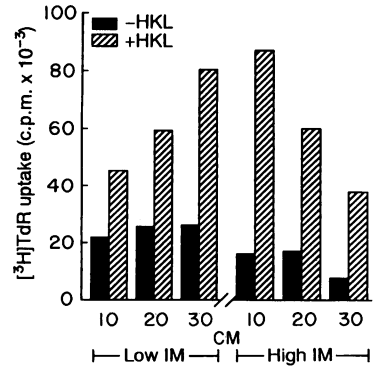

Figure 7. Graph showing the effect of addition of increasing amounts of IM CM produced by either $2{\cdot}5 \times 10^4$/ml IM ('low' CM) or $5{\cdot}7 \times 10^5$/ml IM ('high'CM) with or without HKL, on antigen presentation by interstitial lung DC to HKL-immune T cells. When no CM was added, a mean of $1{\cdot}3 \times 10^3 \pm 576$ c.p.m. was measured. The data are derived from three separate experiments.

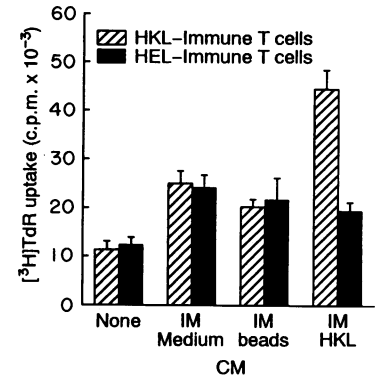

Figure 8. CM were generated by $2{\cdot}5 \times 10^4$/ml IM alone or IM induced to phagocytose HKL or polystyrene beads. Significant mitogenesis was observed only when HKL-immune T cells were incubated with HKL-generated CM. HEL-immune T cells did not respond. CM generated by IM that phagocytosed polystyrene beads had similar low stimulating activity as CM obtained from IM cultured in medium alone. [³H]TdR uptake by HEL- and HKL-immune T cells incubated with 5×10^4 spleen cells and their respective antigen was $2{\cdot}1 \times 10^5$ and $3{\cdot}0 \times 10^5$ c.p.m., respectively. The data are derived from two separate experiments.

mitogenesis. In fact, when wells were supplemented with 50% IM, mitogenesis was reduced to the level observed in the absence of added IM, suggesting that inhibitors generated by this number of IM abrogated any enhancement in antigen presentation gained from the capture of released immunogenic peptides by DC.

Effect of interstitial macrophage-conditioned medium on dendritic cell antigen presentation

To determine whether antigenic fragments released during phagocytosis by IM of PKH-26-labelled HKL are immunogenic and are presented by DC to HKL-immune T cells, CM was generated by using either $2{\cdot}5 \times 10^4$/ml ('low' CM) or $5{\cdot}7 \times 10^5$/ml ('high' CM) IM, with PKH-26-labelled HKL. Addition of as little as 10% (v/v) of the 'low' CM to wells containing DC and HKL-immune T cells resulted in a doubling of [³H] TdR uptake by the T cells as compared to CM generated without HKL (Fig. 7). A greater than fourfold increase in [³H]TdR uptake was observed when 30% of the 'low' CM or 10% of the 'high' CM was added. However, addition of amounts greater than 10% (v/v) of the 'high' CM resulted in progressive inhibition of T-cell mitogenesis. Conditioned media generated by IM in the absence of HKL or by their phagocytosis of polystyrene beads, did not significantly stimulate T-cell mitogenesis (Fig. 8). That immunogenic peptides are indeed released during the phagocytosis of HKL by IM is indicated by the fact that addition of such CM failed to induce mitogenesis in HEL-immune T cells while causing a twofold increase in mitogenesis by HKL-immune T cells (Fig. 8). That the amount of [³H]TdR taken up was always less than when intact HKL was added directly to the antigen presentation assay (Fig. 6) is probably due to the fact that the concentration of immunogenic peptides generated during phagocytosis was low.

DISCUSSION

Protocols developed to isolate DC from non-lymphoid tissues, such as the lung, have resulted in preparations of varying purity.[8,27] When these preparations were utilized in antigen presentation assays with particulate antigens, a brisk response by primed T cells was observed;[27] however, immuno-cytochemical examination revealed that macrophages were among the contaminating cells. This observation prompted the present study in which a concerted effort was made to (1) obtain pure cell preparations; (2) obtain DC and IM from the same interstitial compartment of the lung; and (3) utilize fluorescent-labelled bacteria to determine directly by confocal microscopy the phagocytic capacity of these two cell populations. The isolation protocol employed resulted in >98% pure IM which, because of a last panning step utilizing an anti-Ia mAb, were Ia^-, thereby abrogating their ability, at least initially, to present antigen directly to T cells. The lengthy isolation procedure for DC required an overnight incubation with rGM-CSF in order to maintain the viability of the partially purified DC.[19] Because rGM-CSF also promotes DC maturation,[25] the resulting preparation consisted of mature, differentiated, non-phagocytic DC that were >98% pure. While such highly purified cell populations are necessary to determine unequivocally whether there is a co-operative interaction between IM and mature DC, it is recognized that direct extrapolation to conditions prevailing *in vivo* requires caution. As shown previously, there are subsets of DC, particularly in airway epithelium, that express Fc receptors and are modestly phagocytic,[3] and IM can be stimulated to express Ia by exposing them to IFN-γ.[4]

In antigen presentation assays with PKH-26-labelled HKL, these highly purified DC were capable of a modest level of antigen presentation to primed T cells. The apparent ability of DC, in general, to present antigen derived either from whole bacteria[28,29] or from soluble antigens covalently bound to beads[30,31] has led to the suggestion that there may be proteases present on the DC plasma membrane.[28,30] In the present study, at the times examined, PKH-26-stained HKL were not observed to be attached to the surface of DC, either by immunofluorescence or confocal microscopy, a requirement for surface proteolysis. On the other hand, the possibility that intact, soluble antigens are spontaneously shed from the micro-organisms or the beads and subsequently internalized and processed by DC, cannot be excluded.

An alternative explanation for the low level of antigen presentation by highly purified lung DC is that the presence of 1–2% contaminating IM may have released sufficient quantities of antigenic peptides that either directly bound to Ia on the surface of DC, or were first endocytosed by the DC for subsequent presentation to primed T cells. Of interest is the fact that IM tended to cluster with DC (Fig. 4), a mechanism which could facilitate an efficient transfer of peptides when IM are present in concentrations as low as 1–2%. Such physical proximity between IC and DC in the lymph node and spleen could facilitate the transfer of peptides derived from particulate antigens, transported to these lymphoid organs by macrophages[32,33] from the latter cells to DC. When CM, generated by IM induced to phagocytose PKH-26-labelled HKL, was added to DC in the absence of added antigen, there was a four- to fivefold increase in [^{3}H]TdR uptake by HKL-immune T cells. By contrast, the same CM added to HEL-immune T cells did not stimulate mitogenesis (Fig. 8), suggesting that immunogenic peptides derived from HKL were released by the macrophages during phagocytosis. Although the release of cytokines, including IL-1β and GM-CSF, factors known to up-regulate DC function and DC differentiation, respectively,[25,34] may have contributed to this result, the fact that HEL-immune T cells did not respond indicates that released antigenic peptides were indeed present. The observations made in the present study are similar to those reported by Miyazaki *et al.*[35] who showed that addition of Ia^- splenic macrophages to splenic DC enhanced the presentation of keyhole limpet haemocyanin (KLH) to KLH-immune T cells. Utilizing murine peritoneal macrophages, Allen *et al.*[36] observed that the administration of isotopically labelled HKL to these cells resulted in the release of weakly immunogenic peptides into the supernatant. These peptides were detected in assays in which macrophages were the sole accessory cells; it is possible that utilization of DC might have enhanced the sensitivity of this assay as well as eliminate the suppressive effects of soluble inhibitors released by macrophages.[8]

Indirect evidence for the release of immunogenic peptides by macrophages was reported in a recent study in which the intratracheal administration of Ia^- alveolar macrophages, primed with a soluble antigen, resulted in T-cell priming in hilar lymph nodes.[37] That the priming occurred whether live or dead macrophages were administered, suggested that antigenic peptides released from these cells were captured by DC in the lung and presented to T cells in the hilar lymph nodes. In contrast to antigen-primed alveolar macrophages, the intratracheal administration of live, but not dead, antigen-primed DC stimulated T cells in hilar lymph nodes. Furthermore, *in vitro* assays indicated that whereas the addition of live, antigen-primed alveolar macrophages suppressed T cell proliferation in the presence of DC, addition of these cells, killed by freeze-thawing, resulted in brisk T-cell proliferation. It is likely that soluble inhibitors released from the live alveolar macrophages suppressed T-cell proliferation, whereas antigens released from the dead macrophages were presented by DC to T cells. A co-operative interaction between lung DC and macrophages *in vitro* has also been observed in the mixed leucocyte reaction.[38,39]

The release of antigenic peptides by viable macrophages and their capture by DC as a possible mechanism for the processing of proteins derived from particulates such as micro-organisms, however, remains controversial. Utilizing *in vivo* antigen-primed macrophages or DC, Crowley *et al.*[40] observed that only primed DC and not primed macrophages were capable of presenting protein in an immunogenic form to antigen-specific T cells. Furthermore, addition of cultured DC to a mixture of *in vivo*-pulsed macrophages and DC did not result in augmented antigen presentation to antigen-specific T cells, even in the continuous presence of exogenously added protein. Although these observations differ from those reported here, it is significant that in the present study the augmented presentation of HKL antigen by DC was observed only when macrophages comprised no more than 20% of the accessory cell population. Higher concentrations of macrophages resulted in suppression. Although the spontaneous release of HKL antigen or the presence of proteolytic enzymes on the surface of DC cannot be ruled out, results from the present study support the notion that macrophages are critical to the processing and presentation of peptides derived from particulates such as micro-organisms. In

small numbers, macrophages augment antigen presentation by DC, possibly through the release of immunogenic peptides, when present in larger numbers, however, antigen presentation is down-regulated by soluble inhibitors secreted by macrophages.

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