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Monocytes Recruited to the Lungs of Mice during Immune Inflammation Ingest Apoptotic Cells Poorly

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Abstract

Apoptotic cells must be cleared to resolve inflammation, but few resident alveolar macrophages (AM ϕ) from normal lungs ingest apoptotic cells. We examined how M ϕ ingestion of apoptotic cells is altered during immune inflammation induced by intratracheal challenge of primed C57BL/6 mice using sheep red blood cells. Resident AM ϕ were labeled *in situ* before challenge using intravenous PKH26 to distinguish them from recruited monocytes. Using flow cytometry, we identified phagocytosis of fluorescently-labeled apoptotic thymocytes by alveolar mononuclear phagocytes *in vitro* and *in vivo*, and measured surface molecule expression. Intratracheal challenge induced rapid recruitment of monocytes, peaking at Day 3 and decreasing thereafter, whereas numbers of resident AM ϕ did not change significantly. At all times, the percentage of phagocytes ingesting apoptotic thymocytes *in vitro* was greater among resident AM ϕ (28–45%) than among recruited monocytes (9–19%), but was low in both cell types relative to ingestion of immunoglobulin-opsonized targets. There was also a nonsignificant trend toward lower ingestion by monocytes *in vivo*. MerTK, a receptor tyrosine kinase crucial for apoptotic cell phagocytosis, was expressed by resident AM ϕ , but not by recruited monocytes. Relative to resident AM ϕ , monocytes recruited to the alveolus ingest apoptotic cells meagerly, possibly due to absence of MerTK expression.

Keywords

apoptosis; adhesion molecules; mice; inbred strains; macrophage; receptor tyrosine kinase

Efficient clearance of apoptotic leukocytes is crucial for the resolution of inflammation (1). Defective clearance of apoptotic bodies is associated with autoimmunity in some mouse strains (2) and appears to be involved in the pathogenesis of systemic lupus erythematosus in humans (3). During resolving inflammation, apoptotic leukocytes are believed to be cleared predominantly by resident tissue macrophages (M ϕ), which are abundant in virtually all organs (4). M ϕ that ingest apoptotic cells accelerate the resolution of inflammation by producing anti-inflammatory mediators such as transforming growth factor- β and prostaglandin E₂ (5). Because these mediators also have the potential to induce fibrosis and to impair antimicrobial defenses, understanding the molecular mechanisms of apoptotic cell clearance by M ϕ is an important goal.

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How apoptotic cells are cleared from the lungs remains incompletely defined. Decreased phagocytosis of apoptotic cells by resident alveolar M ϕ (AM ϕ), relative to their own ingestion of other targets or to ingestion of apoptotic cells by other tissue M ϕ , has been demonstrated in rabbits, mice, and most recently in humans (6–8). In mice, the most thoroughly studied species, relatively deficient apoptotic cell phagocytosis was seen both *in vitro* in seven murine strains and *in vivo*, was specific for apoptotic cells (as opposed to three other phagocytic targets), and extended to ingestion of apoptotic neutrophils (7,9). This deficiency is explained in part by reduced adhesion of apoptotic cells (10), and in part by marked reduction in the expression of protein kinase C (PKC) β II, which is necessary for apoptotic cell ingestion (11). Nevertheless, AM ϕ share many features of apoptotic cell recognition with the avidly phagocytic resident peritoneal M ϕ (PM ϕ) (9), including reliance on the stereospecific phosphatidylserine receptor (PSR) (11,12) and the receptor tyrosine kinase MerTK (also known as Tyro12) (2,10). This combination of unique and shared features implies that the alveolar surface constitutes an ideal environment in which to investigate the mechanisms and consequences of altered apoptotic cell clearance.

The reduced capacity of resident murine AM ϕ to ingest apoptotic cells raises two questions. First, does phagocytosis of apoptotic cells by resident AM ϕ increase during resolving inflammation, when the burden of cells to be cleared rises? Second, might apoptotic cells actually be cleared under these circumstances primarily by recruited monocytes? *In vitro*, human monocyte-derived macrophages can clearly develop the capacity to ingest apoptotic cells, and the rate at which they develop this capacity is accelerated by cytokines that are likely to be expressed during resolving inflammation (13). However, it is unproven whether monocytes actually develop this capacity *in vivo*, and if so, over what time period.

To address these questions experimentally, we analyzed phagocytosis of apoptotic thymocytes by murine lung mononuclear cell phagocytes *in vitro* and *in vivo*, distinguishing resident AM ϕ from monocytes recruited to the alveolar spaces by a previously described vital staining technique (14). This technique, which relies on differential uptake of the vital dye PKH26 by phagocytic resident M ϕ versus the nonphagocytic peripheral blood monocyte, has been used extensively to analyze monocyte recruitment in murine models of lung inflammation (14–16). We employed a well characterized model of CD4 T cell-dependent lung inflammation, the secondary immune response of primed mice to intratracheal challenge with the particulate antigen sheep red blood cells (SRBC) (17). A salient feature of this experimental model system in addressing the current questions is that we have previously shown that a substantial fraction of alveolar lymphocytes are apoptotic throughout the course of this response (18). We found that the fraction of resident AM ϕ ingesting apoptotic cells remained low over the course of the pulmonary immune response, and at all times exceeded the fraction of recruited monocytes ingesting apoptotic cells.

MATERIALS AND METHODS

Antibodies

The following monoclonal antibodies (mAbs) (clone; species isotype) were purchased from BD PharMingen (San Diego, CA): anti-CD11b (M1/70; rat IgG_{2b}, κ); anti-CD11c (HL3; Armenian hamster IgG₁, λ); anti-CD16/32 (2.4G2; rat IgG_{2b}, κ); anti-CD19 (1D3; rat IgG_{2a}, κ); anti-CD51 (RMV-7; rat IgG₁); anti-CD90.2 (30-H12; rat IgG_{2b}, κ); anti-H-2D^b (KH95; mouse IgG_{2b}, κ). Avidin-FITC and streptavidin-PerCP for use with biotinylated mAbs and the following controls (clone) were also purchased from BD PharMingen: control rat IgG_{2a}, κ (R35–95); control rat IgG_{2b}, κ (A95–1); control rat IgM (G155–228); anti-rat IgG_{2b} (G15–337; mouse IgG_{2b}, κ); control hamster IgG₁, κ (A19–3); control hamster IgG₃, λ (A19–4). mAb specific for the M ϕ isoform of Ly-6c (clone ER-MP20; rat IgG_{2a}, κ) was obtained from Accurate Chemical and Scientific Corp (Westbury, NY). Anti-F4/80 (clone CI:A3–1; rat

IgG_{2b}) was purchased from Caltag Laboratories (Burlingame, CA). mAb against PSR (clone 217; mouse IgM) was a generous gift of Drs. Valerie Fadok and Peter Henson (National Jewish Medical Center; Denver, CO). Goat anti-mouse IgM was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-murine MerTK monoclonal (clone 108921, rat IgG₁) and polyclonal (affinity-purified goat IgG) antibodies were obtained from R&D Systems (Minneapolis, MN). Normal goat IgG and rabbit anti-goat IgG (used as secondary antibody in MerTK staining) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Mice

Pathogen-free inbred female mice were used at 8–14 wk of age in all experiments. C57BL/6 (H-2^b) and BALB/c mice (H-2^d) were purchased from Charles River Laboratories (Wilmington, MA) and Jackson Laboratory (Bar Harbor, ME), respectively. Mice were housed in the Animal Care Facility at the Ann Arbor Veterans Affairs Medical Center, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were fed standard animal chow (rodent lab chow 5001; Purina, St. Louis, MO) and chlorinated tap water *ad libitum*. This study followed a protocol approved by the Animal Care Committee of the local Institutional Review Board, and complied with the latest version of the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences; www.nap.edu/readingroom/books/labrats/).

Experimental Design

Resident AM ϕ were labeled *in situ* by intravenous administration of the long-lasting orange fluorescent vital dye PKH26 PCL, as previously described (14). Briefly, C57BL/6 mice that had been antigen-primed by IP injection of 1×10^8 SRBC in 0.5 ml normal saline 2–3 wk previously received 100 μ l PKH26 PCL (Sigma, St. Louis, MO) (stock 1×10^{-3} M in ethanol, diluted 1:5 in Diluent B) by tail vein injection. This procedure labels mature phagocytic M ϕ throughout the body, with special efficiency in the lung, which receives the initial bolus of dye. Conversely, peripheral blood monocytes and their bone marrow precursors are not labeled, permitting their subsequent identification after recruitment to tissues (14–16).

In all experiments, alveolar mononuclear phagocytes were identified by light scatter characteristics using flow cytometry, distinguishing resident AM ϕ as PKH26-positive (PKH_{pos}) cells, and recruited monocytes as PKH26-negative (PKH_{neg}) cells with the same light scatter characteristics. Three types of experiments were performed. First, phagocytosis of fluorescently-labeled apoptotic cells was measured *in vitro*; second, labeled apoptotic cells were instilled directly into the lungs, and phagocytosis was measured after recovery of M ϕ by BAL; and third, M ϕ receptor expression was analyzed by two- and three-color flow cytometry. In most experiments, BAL cells from individual mice were analyzed, as it was important to verify that each mouse received an adequate aliquot of PKH26 by tail-vein injection. In some experiments of receptor expression, the adequacy of PKH26 labeling of each mouse was first confirmed by flow cytometric analysis of PM ϕ harvested from peritoneal lavage, and then BAL cells were pooled to obtain sufficient numbers of cells.

Induction of Pulmonary Immune Response

The particulate T cell-dependent antigen, sheep red blood cells (SRBC) (sheep 6115) (Colorado Serum, Denver, CO) were prepared and used as previously described (19). One day after intravenous injection of PKH26 PLC, SRBC-primed mice were antigen challenged by intratracheal injection of 5×10^8 SRBC in 50 μ l normal saline. IT SRBC-challenge induces highly reproducible but self-limited lung inflammation characterized by vigorous mononuclear cell influx (19,20). At various times after IT SRBC-challenge, mice were killed by asphyxia

in a high CO₂ environment, and bronchoalveolar lavage (BAL) and total cell counts were performed as previously described (19).

Thymocytes

Thymuses harvested from normal C57BL/6 mice were minced to yield a single cell suspension. Viable thymocytes were washed three times in Dulbecco's phosphate-buffered saline (PBS) (Invitrogen Life Sciences, Carlsbad, CA), suspended in PBS at a concentration of 2×10^7 /ml, and incubated with the green fluorescent dye 5-chloromethylfluorescein diacetate (CMFDA) (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. In some experiments, thymocytes from BALB/c mice (which express the MHC class I antigen H-2D^d) were used, so that target could be distinguished from phagocyte by staining for H-2D^b expressed by C57BL/6 mice.

To induce apoptosis, CMFDA-labeled thymocytes were suspended at a concentration of $1-3 \times 10^6$ /ml in RPMI 1640 medium containing 10% heat-inactivated FBS and a final concentration of 10^{-6} M dexamethasone (Sigma), and incubated for 6 h (7). This method results in a population of thymocytes in early apoptosis with little contamination by late apoptotic or necrotic cells (routinely 40–60 percent annexin-FITC positive, 12–18% annexin/PI double-positive, and < 2% trypan blue positive).

As a control target, CMFDA-labeled thymocytes were opsonized with 50 µl/ml of the rat monoclonal antibody CD90.2 for 30 min at 4°C, followed by mouse anti-rat mAb IgG for an additional 30 min. Opsonized thymocytes were washed with PBS and used immediately in the phagocytosis assay.

Phagocytosis Assays

Mø phagocytosis of apoptotic cells *in vitro* was analyzed as described by Jersmann and coworkers (21). BAL from individual mice were plated at 5×10^5 cells/well in sterile 48-well plates and allowed to adhere for 1–3 h at 37°C. Nonadherent cells were removed by gentle washing. Adherent Mø were incubated with 5×10^6 CMFDA-labeled apoptotic thymocytes or immunoglobulin-opsonized apoptotic thymocytes in 500 µl medium for 90 min (apoptotic) or 1 h (opsonized). Cells were extensively washed in ice-cold PBS to remove noningested thymocytes and were incubated in trypsin-EDTA at 4°C for 20 min and at 37°C for an additional 20 min. All aliquots of Mø released from the same plate were pooled and held on ice for analysis by flow cytometry immediately after the final wash. The adequacy of Mø release from the plate was confirmed in all experiments by direct inspection using an inverted microscope.

For *in vivo* experiments, 20×10^6 CMFDA-labeled apoptotic cells in 50 µl normal saline were administered intratracheally to SRBC-primed mice at various times after intratracheal SRBC challenge. Two hours later, mice were killed humanely by CO₂ asphyxia and BAL was performed with 0.5 M EDTA in PBS. Cells were washed three times with 0.5 M EDTA in ice-cold PBS and immediately analyzed by flow cytometry.

Flow Cytometry

Cells freshly isolated by BAL were used to analyze expression of receptors potentially involved in clearance of apoptotic cells. Cells were washed twice in staining buffer (DIFCO, Detroit, MI), resuspended in 100 µl staining buffer, and incubated for 30 min at 4°C in the dark with labeled Abs diluted in 100 µl staining buffer. Final antibody concentrations were 1–2 µg/10⁶ cells. FcγR was blocked using mAb 2.4G2. After incubation, cells were washed twice in PBS, resuspended in 0.5 ml marking buffer, and analyzed immediately.

Flow cytometry was performed as previously described in detail (7) using a FACScan cytometer (Becton Dickinson, Mountain View, CA). Data were collected using CellQuest software (version 3.3; Becton Dickinson) on a PowerPC microcomputer (Apple, Cupertino, CA) and subsequently analyzed using Flo-Jo software (version 4.4.1; Tree Star, Ashland, OR). A minimum of 10,000 viable cells was analyzed to determine cell-surface receptor expression. To compare fluorescence intensities, we calculated the change in mean channel fluorescence (ΔMCF) between cells stained with specific antibody versus isotype control antibody.

Statistics

Data were expressed as mean $\pm$ SEM. Continuous ratio scale data were evaluated by unpaired Student's *t* test (for two samples) or ANOVA (for multiple comparisons) with *post hoc* analysis by the Tukey-Kramer test or by the two-tailed Dunnett test (for comparison to a single control condition). Statistical calculations were performed using Statview v 5.0 and Super ANOVA v 1.11 (SAS Institute, Inc., Cary, NC) on a Macintosh PowerPC G4 computer. Significant differences were defined as $P < 0.05$.

RESULTS

Identification of Recruited Mononuclear Phagocytes in BAL

Resident AM ϕ of normal mice were labeled brightly and uniformly by intravenously administered PKH26. The FL2 (*orange*) fluorescence intensity of AM ϕ from mice receiving PKH26 did not overlap with that of resident AM ϕ from normal untreated mice (Figure 1; compare *Panel A* with *Panel B*). Moreover, PKH26-labeled AM ϕ of normal mice remained intensely stained for at least 13 d after a single intravenous administration, with virtually no loss of fluorescence intensity (data not shown). By contrast, prior treatment using PKH26 did not result in detectable staining of peripheral blood mononuclear cells during lung inflammation (Figures 1C and 1D). Flow cytometric analysis of BAL from normal mice that had been treated with PKH26 showed a distinct population of resident AM ϕ , identified as cells of elevated side scatter (SSC) (Figure 1E). As anticipated, cells within this gate showed uniformly and high FL2 fluorescence, i.e., were PKH26-positive (PKH_{pos}), but did not show high-level FL1 fluorescence (Figure 1F). This gate contained 92–97% of all BAL cells, the anticipated proportion of AM ϕ based on differential cell counts. Conversely, PKH_{pos} events were very uncommon outside these light scatter-defined gates (data not shown). Keeping instrument settings constant between experiments, we next applied identical light scatter gating to identify mononuclear phagocytes within BAL samples of intratracheally challenged mice (Figure 1G). At all times, this analysis disclosed a distinct population of PKH26-negative (PKH_{neg}) cells that did not overlap in FL2 fluorescence with the PKH_{pos} population (Figure 1H). Collectively, these data confirm that this technique permits clear distinction between resident AM ϕ and recruited monocytes during immune lung inflammation, in agreement with previous studies using this technique (14–16).

Antigen challenge induced a marked increase in absolute numbers of PKH_{neg} monocytes within BAL, from fewer than 100 cells/mouse at Day 0 to a peak at Day 3 of $0.5 \pm 0.2 \times 10^6$ cells/mouse (mean $\pm$ SEM, $n = 9$) (Figure 2). This marked increase on Day 3 ($> 8,000$ -fold above baseline) coincided with the peak of alveolar inflammation and of total M ϕ numbers previously described in this model system (19). Thereafter, numbers of PKH_{neg} monocytes decreased, but remained slightly above baseline through 12 d after intratracheal challenge. By contrast, absolute numbers of PKH_{pos} resident AM ϕ in BAL did not change significantly throughout the first 6 d of the response (Figure 2), although there was a nonsignificant trend toward higher numbers at Days 9 and 12 ($P > 0.5$ at all time points compared with Day 0, ANOVA with Dunnett *post hoc* testing).

To characterize the two Mø populations further, BAL cells from primed mice were stained on various days after intratracheal challenge with mAbs against a variety of leukocyte antigens and analyzed by flow cytometry (Figure 3). PKH_{pos} Mø were only weakly positive for F4/80 (Figure 3A), in agreement with previous data on resident murine AMø (22), whereas PKH_{neg} Mø strongly expressed F4/80 (Figure 3B) (% fluorescence-positive: $2.5 \pm 0.9\%$ for PKH_{pos} Mø versus $28.2 \pm 2.6\%$ for PKH_{neg} Mø; ΔMCF : 41.6 ± 3.7 for PKH_{pos} Mø versus 121.1 ± 13.4 for PKH_{neg} Mø; mean $\pm$ SEM of eight mice assayed individually on Day 3 after intratracheal challenge; $P < 0.05$, unpaired t test). PKH_{pos} Mø stained weakly with mAb ER-MP20 (Figure 3C), whereas PKH_{neg} Mø were stained strongly (Figure 3D), as would be anticipated for relatively immature mononuclear phagocytes (23) (% fluorescence-positive: 1.1 ± 0.5 for PKH_{pos} Mø versus 77.2 ± 3.4 for PKH_{neg} Mø; ΔMCF : 52.0 ± 6.1 PKH_{pos} Mø versus 328.3 ± 15.2 PKH_{neg} Mø; mean $\pm$ SEM of eight mice assayed individually on Day 3 after intratracheal challenge; $P < 0.05$, unpaired t test). Negligible staining was seen within either the PKH_{pos} or PKH_{neg} Mø populations for the lymphocyte antigens TCR- β (Figures 3E and 3F) and CD19 (Figures 3G and 3H), which are pathognomonic of T cells and B-cells, respectively. As anticipated from our previous data on resident murine AMø (7), PKH_{pos} Mø were brightly positive for CD11c and showed minimal staining for CD11b, even late after intratracheal challenge (Figure 3G), whereas PKH_{neg} Mø remained mostly positive for CD11b alone (Figure 3H). Almost no cells within the Mø gate stained brightly for the granulocyte marker Gr-1 at any time (data not shown). To determine whether our gating strategy captured all BAL mononuclear phagocytes, we also examined cells of lower orthogonal light scatter ("lymphocyte gate"). Staining for F4/80 and ER-MP20 was essentially absent from this gate, whereas the majority of cells did stain for one or more typical lymphocyte markers such as CD3, CD19 and TCR- β (data not shown). These data collectively indicate that light scatter-gating accurately identifies a population of alveolar cells highly enriched for mononuclear phagocytes, with minimal contamination by other cell types, and that the population we analyzed contains the vast majority of alveolar mononuclear phagocytes.

***In Vitro* Phagocytosis by Alveolar Mononuclear Cells**

We next investigated the capacity of the two Mø populations to ingest fluorescently-labeled apoptotic cells *in vitro*. BAL was harvested from normal mice or from intratracheally challenged mice to permit isolation of Mø by adherence. In control experiments, we verified that this initial adherence step did not significantly change the ratio of PKH_{neg} Mø to PKH_{pos} ($12.2 \pm 4.1\%$ PKH_{neg} Mø pre-wash versus $8.8 \pm 1.5\%$ PKH_{neg} Mø post-wash; $P = 0.46$, Student's t test; mean $\pm$ SEM of triplicate samples in each of five individual experiments). Additional control experiments confirmed that *in vivo* labeling of AMø using intravenously administered PKH26 PCL had a minimal effect on phagocytic ability (PKH26 treatment $94.7 \pm 7.8\%$ of control; $P = 0.76$, Student's t test; mean $\pm$ SEM in three individual experiments).

Mø were next co-cultured with CMFDA-labeled apoptotic thymocytes. Uningested thymocytes were removed by standardized washing, and then Mø were released from the dishes using cold EDTA and analyzed by microscopy and two-color flow cytometry. The adequacy of Mø recovery from the dishes was confirmed in all experiments by direct inspection using an inverted microscope, with additional washes if necessary.

Using light and fluorescent microscopy, we first confirmed that fluorescent targets were inside Mø, and not merely adherent (Figures 4A and 4B). Flow cytometry showed that within both PKH_{pos} and PKH_{neg} populations, Mø that had ingested labeled thymocytes were easily distinguishable due to their very increased FL1 fluorescence (Figures 4C and 4D). Moreover, the percentage of FL1-positive Mø in the PKH_{pos} population in normal mice as determined by this method agreed with our previous data on resident murine AMø using direct inspection of microscopy slides (7). As a more definitive test that our assay measured only intracellular

thymocytes, we also performed a control phagocytosis experiment to quantify bound but uningested thymocytes, based on differences in MHC class I antigen expression between M ϕ and thymocytes. We used BAL cells from C57/BL/6 mice (H-2D^b) and apoptotic thymocytes from BALB/c mice (H-2D^d), and stained the M ϕ (without permeabilization) after the final wash step using biotinylated anti-H-2D^d plus streptavidin PerCP (an FL3 reagent). This reagent would stain any extracellular adherent thymocytes but not ingested thymocytes. Results confirmed minimal contribution of adherent uningested thymocytes ($2.8 \pm 1.1\%$ of CMFDA-positive M ϕ were H-2D^d positive; mean $\pm$ SEM of four mice assayed individually in two separate experiments). Thus, this assay system appears to measure ingested apoptotic cells accurately.

Kinetic analysis over the pulmonary immune response disclosed a marked difference in the efficiency of apoptotic cell phagocytosis between the two mononuclear phagocyte subpopulations. At all time points after intratracheal challenge, the percentage of phagocytic cells among PKH_{pos} M ϕ was greater than among PKH_{neg} M ϕ (Figure 4E). The fraction of phagocytic PKH_{pos} M ϕ remained statistically unchanged during the immune response, ranging between 27.6 and 44.9% ($P > 0.5$ at all time points compared with Day 0, ANOVA with Dunnett *post hoc* testing). Phagocytosis by PKH_{neg} M ϕ could not be measured at Day 0, due to the extreme paucity of that cell population, but thereafter ranged between 13 and 21%, also not changing statistically ($P > 0.5$, ANOVA with Tukey-Kramer *post hoc* testing). However, both PKH_{pos} and PKH_{neg} populations avidly ingested immunoglobulin-opsonized apoptotic cells (Figure 4F), confirming their capacity to ingest targets of identical size using an alternative recognition pathway (Fc γ R-mediated). These data demonstrate the ability of our assay system to detect high degrees of phagocytosis when present. The observed avid Fc γ R-mediated phagocytosis also argues strongly against the possibility that lung M ϕ were already stuffed with apoptotic cells they had ingested previously *in vivo*, an explanation we believed highly unlikely based on their morphologic appearance.

***In Vivo* Phagocytosis**

We next examined M ϕ clearance of apoptotic cells from the alveolar spaces. For this purpose, we administered CMFDA-labeled apoptotic thymocytes by the intratracheal route to SRBC-primed, PKH-treated mice that had been challenged at various earlier times with intratracheal SRBC, and 2 h later harvested BAL and analyzed M ϕ by flow cytometry. Phagocytosis of apoptotic thymocytes was seen in both M ϕ populations, and the percentage of phagocytic (FL1-positive) M ϕ in both populations agreed with results of the *in vitro* phagocytosis assay. PKH_{neg} M ϕ again ingested fewer than did PKH_{pos} M ϕ at all time points, although the difference between the two cell types was not significant (Figure 5) ($P > 0.05$, unpaired *t* test). When analyzed over time after intratracheal SRBC challenge, the percentage of phagocytic M ϕ did not change significantly in PKH_{pos} M ϕ ($P > 0.5$ at both time points compared with Day 0, ANOVA with Dunnett *post hoc* testing), or in PKH_{neg} M ϕ ($P > 0.5$, Day 5 versus Day 12, unpaired *t* test).

Because these *in vivo* experiments used large numbers of thymocytes per mouse, one potential confounding factor might be the presence of thymic M ϕ carried over in the thymocytes population. Such cells would have been CMFDA-labeled *ex vivo* before administration, and hence might be misidentified as phagocytosis-positive PKH_{neg} M ϕ . To verify that our assay measured only phagocytosis by recruited PKH_{neg} host monocytes, in a control experiment (Day 5 after intratracheal challenge) we again used thymocytes from mice that differed at the class I MHC locus. BAL M ϕ from C57BL/6 mice (which express H-2D^b) were incubated with CMFDA-labeled apoptotic thymocytes from BALB/c mice (which express H-2D^d). We performed the same *in vivo* phagocytosis assay, except that cells were also stained with biotinylated anti-H-2D^b and streptavidin-PerCP. Virtually no FL3-negative cells were

identified within the Mø gates, and identical percentages of phagocytosis were seen whether or not analysis was restricted to FL3-positive cells. These data exclude carryover of thymic Mø as a source of error in this assay.

We also considered the possibility that the intratracheal instillation might be inhomogeneous in individual mice, and that some Mø might be negative because they were not exposed to apoptotic cells *in vivo*. To address this possibility, we performed an additional experiment in which we mixed unstained apoptotic thymocytes with zymosan-FITC, instilled the mixture by the intratracheal route, and counted only FITC-containing Mø, using our previously described microscopic assay of phagocytosis (7). A preliminary experiment had shown that resident AMø from normal mice were able to ingest apoptotic thymocytes and FITC-labeled zymosan simultaneously at the same rate as they ingested each particle independently (*see* Table E1 in the online supplement), and that virtually all AMø avidly ingested zymosan. Therefore, we anticipated that ingestion of zymosan-FITC could be used as a surrogate for exposure to the intratracheal bolus. Indeed, 100% of Mø containing apoptotic thymocytes also were FITC-positive, and $22.8 \pm 4.6\%$ of FITC-positive Mø contained apoptotic thymocytes (mean $\pm$ SEM, $n = 3$), in close agreement with our previous results; note that because this experiment was counted using microscopy, we did not attempt to distinguish PKH positivity. Thus, inhomogeneity of apoptotic cell deposition within the lungs does not appear to explain the relatively low fraction of alveolar mononuclear phagocytes ingesting apoptotic cells *in vivo*.

Surface Molecule Expression

Finally, we analyzed expression of three Mø surface receptors previously implicated in the phagocytosis of apoptotic cells. In hematopoietic cells, the α_V integrin (CD51) forms a receptor for vitronectin together with either β_3 (CD61) or β_5 integrin chains. Through use of blocking mAb or inhibition with the tetrapeptide RGDS, experimental models utilizing human peripheral blood-derived monocytes or murine Mø cell lines have shown that binding of vitronectin to thrombospondin on the apoptotic cell surface is important for phagocytosis by these cell types (24,25). Nevertheless, we found that PKH_{pos} AMø did not express CD51 at any time during the pulmonary immune response (Figures 6A and 6C, *left-hand panels*) in agreement with our previous data on resident AMø from untreated mice (7). By contrast, PKH_{neg} Mø strongly expressed CD51 on Day 3 after IT SRBC-challenge (Figure 6B, *left-hand panel*), with a mean intensity of fluorescence that fell progressively thereafter (Figure 6C, *left-hand panel*). However, as we have previously shown for normal resident AMø and PMø from untreated mice (7), in separate functional experiments, the integrin-blocking tetrapeptide RGDS did not decrease phagocytosis by either PKH_{pos} Mø or PKH_{neg} Mø (data not shown). These results imply that clearance of apoptotic lung lymphocytes by murine lung Mø during immune inflammation does not depend on vitronectin binding.

The stereospecific PSR appears to be central to apoptotic cell recognition (12). We found that PKH_{pos} Mø and PKH_{neg} Mø had similar low PSR expression that changed little during the immune response (Figure 6, *center panels*).

PKH_{pos} Mø abundantly expressed the receptor tyrosine kinase MerTK (Figures 6A and 6C, *right-hand panels*), which we have recently shown is involved in apoptotic cell phagocytosis by resident murine AMø (10). The intensity of MerTK expression by PKH_{pos} Mø increased markedly during resolution of lung inflammation, as demonstrated by Δ MCF (Figure 6C, *right-hand panel*). Importantly, PKH_{neg} Mø in the same samples barely expressed MerTK at any time-point (Figures 6B and 6C, *right-hand panels*). Antibody against MerTK significantly inhibited *in vitro* phagocytosis of apoptotic cells by PKH_{pos} Mø (anti-MerTK $21.4 \pm 2.6\%$ phagocytic Mø versus isotype control $45.3 \pm 2.7\%$ phagocytic Mø, $n = 9$, $P < 0.0001$), but had no significant effect on PKH_{neg} Mø (anti-MerTK $20.8 \pm 4.2\%$ phagocytic Mø versus isotype control $20.6 \pm 3.1\%$ phagocytic Mø, $P = 0.81$).

DISCUSSION

The results of this study define the clearance of apoptotic lymphocytes by murine alveolar mononuclear phagocytes during antigen-induced lung inflammation. The principal conclusion is that among both resident AM ϕ and recruited monocytes, the percentage of individual alveolar mononuclear phagocytes ingesting apoptotic lymphocytes remains low at all times relative to ingestion of immunoglobulin-opsonized targets. This conclusion is based on the following findings: (1) the fraction of PKH_{pos} AM ϕ ingesting apoptotic cells did not increase significantly from low baseline levels, *in vitro* or *in vivo*; and (2) despite a rapid influx of PKH_{neg} recruited monocytes that greatly yet transiently increased total numbers of mononuclear cells in the alveoli, recruited monocytes displayed an even lower rate of apoptotic cell ingestion *in vitro* than PKH_{pos} resident AM ϕ at all times. The relative deficiency in apoptotic cell ingestion by recruited monocytes was specific, as shown by the avid ingestion of immunoglobulin-opsonized particles, and was associated with low expression of the receptor tyrosine kinase MerTK. Collectively, these data explain our previous observation that apoptotic lymphocytes, which are easily detected in the alveoli in this experimental model system (18), are present due to inefficient clearance by phagocytes in the alveolar space. We also found that even though the vitronectin receptor chain CD51 was abundantly expressed by recruited monocytes (but not by resident AM ϕ throughout the immune response), phagocytosis of apoptotic cells by both populations of alveolar mononuclear phagocytes was not inhibited by the integrin-blocking tetrapeptide RGDS. These novel findings have important implications for the clearance of CD8 + T cells following viral pneumonias (26), and motivate additional investigation of how lung mononuclear cells contribute to resolution of inflammation.

Our results showing the accurate distinction of recruited monocytes from resident AM ϕ using PKH26 confirm and extend those of Maus and colleagues, who pioneered this technique to label lung M ϕ *in vivo* (14–16). Monocytes have been studied to a much lesser degree in murine models than in humans, due in part to the marked difficulty in obtaining pure populations from the peripheral blood of animals as small as mice. Moreover, there have been no clear-cut alternative methods to distinguish unambiguously between recently recruited monocytes and resident AM ϕ M ϕ , either morphologically or by surface markers (4). By permitting the unique contribution of recruited monocytes to immune and inflammatory responses to be defined, this technique is an important innovation that could be applied to other organs. We believe that analysis by flow cytometry is essential to provide reproducible results; preliminary experiments using immunofluorescence microscopy convinced us that the human eye is unable to distinguish reliably between the fluorescence intensity of PKH_{pos} M ϕ and PKH_{neg} M ϕ (unpublished observation). The lung appears to be a particularly favorable tissue for use of this technique. We noted that staining of AM ϕ was consistently of greater intensity than that of PM ϕ in the same mice (unpublished observation), likely due to the high first-pass effect of an intravenous injection on the pulmonary vasculature.

An important facet of our data is the insights they give into the differing fates of resident AM ϕ and recruited monocytes during immune lung inflammation. Particle-laden mature AM ϕ have been shown previously to migrate to regional lymph nodes (27), but the degree to which this migration depletes AM ϕ numbers within the lung has not been investigated. The finding that overall numbers of resident AM ϕ did not change significantly over the course of this immune response implies that the number of AM ϕ leaving the lungs during the pulmonary immune response is relatively small. Support for this conclusion comes from preliminary experiments which show very few PKH_{pos} M ϕ in mediastinal lymph nodes in this model system (unpublished observation). Emigration of resident AM ϕ may also have been balanced by transmigration into the alveoli of interstitial lung M ϕ , which should also have been stained by PKH26. This possibility is supported by the trend toward increased numbers of PKH_{pos} M ϕ seen at our later two time-points. Conversely, our kinetic data indicate that the vast majority

of monocytes recruited to the lungs during pulmonary immune responses do not become long-term residents of the alveolar spaces. Although we did not directly investigate the mechanism for the rapid decrease in numbers of monocytes after Day 3, it is likely to result primarily from emigration (Ref. 28, and unpublished data). Moreover, PKH_{neg} Mø remained relatively small in size, as shown by low forward light scatter, and continued to express surface antigens such as F4/80 and ER-MP20 (data not shown), consistent with an immature phenotype. Additional studies will be needed to explicate fully Mø population dynamics during lung inflammation.

These results also provide a novel *in vivo* perspective on the important literature investigating phagocytic capacity of human monocyte-derived Mø during *in vitro* maturation. We showed that at least some murine monocytes develop phagocytic capacity by Day 3 after intratracheal challenge, which we believe from more detailed kinetic analyses of leukocyte trafficking in this model system (Refs. 17 and 19, and unpublished observations) indicates ~24–48 h of residence in the lungs. By contrast, freshly isolated human monocytes did not ingest apoptotic cells at all, and can require up to a week in culture to gain that function (13). For ethical reasons, it would be difficult to use this staining technique in humans to determine whether these disparate results reflect species differences or simply more rapid development of at least some phagocytic capacity *in vivo*; we favor the latter interpretation. Our finding that recruited murine monocytes did not further upregulate their phagocytic capacity over 12 d of lung inflammation could be interpreted to indicate that that individual monocytes may have stayed in the lungs for only a brief period. Our results in mice also differ somewhat from those of Newman and colleagues, who showed a more pronounced increase from baseline phagocytosis of neutrophils by alveolar mononuclear phagocytes of rabbits treated with intratracheal acid (6). This apparent disparity could be due to a greater degree of tissue injury in their model system or the difference in apoptotic target, as Mø have unique mechanisms restricted to recognition of apoptotic neutrophils (29).

The finding that recruited monocytes express extremely low levels of the Tyro3 receptor kinase family member MerTK provides one potential mechanism for their low rate of apoptotic cell phagocytosis. Several pieces of evidence indicate a central role for MerTK in apoptotic cell clearance (30). Transgenic mice exclusively expressing a kinase-deficient form of Mer (Mer^{kd}), which interferes with MerTK surface expression, clear apoptotic cells poorly and develop auto-immunity as they age (2,31,32). Genetic deficiency or mutation of MerTK leads to retinitis pigmentosa in rats and humans (30) due to defective clearance of apoptotic bodies by retinal pigment epithelial cells. We have shown that polyclonal antibody against MerTK blocks phagocytosis (but not adhesion) of apoptotic cells by murine Mø, including AMø (10); as anticipated, the current data extend that finding to mAb against MerTK and this flow cytometry-based assay. The other members of this receptor kinase family, Axl and Tyro3, have also been shown to be involved in apoptotic cell clearance *in vivo* (30,33), but little is known about their contribution to ingestion by resident Mø from specific tissues. Whether murine monocytes also lack expression of other Tyro3 family members is under current investigation.

We carefully considered a variety of technical limitations that could detract from this analysis. First, it is unlikely that our assay conditions missed very rapid uptake and ingestion of apoptotic cells, as we have previously performed extensive kinetic analysis (15 min to 8 h of co-incubation) of Mø adhesion and phagocytosis using a microscopic assay system (7,10). Moreover, CMFDA should be retained within Mø even if the ingested cells were digested rapidly. Second, we think it highly unlikely that some unique feature of SRBC challenge specifically reduces Mø ingestion of apoptotic cells, as our current data during inflammation agree closely with our current and previous results in normal mice. Third, because we have shown that a substantial fraction of murine alveolar lymphocytes are apoptotic throughout the course of this response (18), we cannot exclude the possibility that the relatively low capacity of resident PKH_{pos} AMø to ingest apoptotic cells reflects the effect of chronic low level

ingestion. Previous ingestion of apoptotic neutrophils has been shown to decrease the subsequent capacity of rat bone marrow-derived M ϕ to ingest apoptotic neutrophils *in vitro* (34). However, this critique would obviously not apply to PKH_{neg} monocytes. Overall, we are confident that our results accurately mimic the behavior of murine lung mononuclear phagocytes in both the normal state and in a variety of infectious and inflammatory diseases.

If both resident AM ϕ and especially recruited monocytes have limited capacity to ingest apoptotic leukocytes, how then are they cleared during resolving inflammation? The simple answer in this model system (18) is that they are cleared slowly and inefficiently, which is why they are easily detected. We suspect that similar delayed clearance occurs following viral pneumonias (26). It is also possible that apoptotic leukocytes are cleared in part by pulmonary airway epithelial cells, which can ingest apoptotic eosinophils *in vitro* (35). Epithelial cells appear to have an even lower efficiency per individual cell than do AM ϕ , but if type II alveolar epithelial cells show the same phagocytic behavior as the A549 cell line (36), their massive total numbers argue that they might make a sizeable net contribution. We did not specifically measure recovery of uningested thymocytes in our *in vivo* experiments, but it was clear from hemocytometer counts and inspection of the ungated flow cytometric results that the vast majority of apoptotic cells were not ingested during that short assay.

In summary, we have shown that both resident AM ϕ and, to a lesser degree, recruited monocytes, can ingest apoptotic lymphocytes during antigen-induced immune lung inflammation, but that the phagocytic fraction of both cell types is low, and does not increase markedly over time. When the overall phagocytic capacity of resident AM ϕ versus recruited monocytes for apoptotic cells is estimated as the product of the observed absolute numbers per mouse and the percent phagocytic cells of each subset, it becomes clear that resident AM ϕ contribute the overwhelming amount of ingestion at all times except Day 3. These results emphasize the continued need to study how the interaction of apoptotic cells with AM ϕ modulates both ongoing inflammation and host defense of the lungs.

Supplemental Table 1. AM ϕ independently ingest disparate particles simultaneously*

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

PKH26 treatment permits distinction between resident AMØ and recruited monocytes during immune lung inflammation. (A and B) Baseline BAL data. AMØ from unimmunized C57BL/6 mice were analyzed by flow cytometry using identical cytometer instrument settings, either without PKH treatment (A) or 24 h after injection of PKH26 by tail vein (B). Note the near absence of overlap in the FL2 fluorescence intensity (*vertical axis*) between the two populations. (C and D) Peripheral blood data. Mononuclear cells purified from peripheral blood of primed C57BL/6 mice 3 d after intratracheal SRBC challenge were analyzed by flow cytometry. Note minimal difference in FL2 fluorescence (*vertical axis*) between control mice not receiving PKH26 (C) and PKH26-treated primed mice (D). (E–H) BAL data during inflammation. SRBC-primed C57BL/6 mice received PKH26 by tail vein-injection, and 24 h later were either killed as a Day 0 control (E and F) or were challenged with 5×10^8 SRBC by the intratracheal route and assayed on various later days by flow cytometric analysis of BAL. Representative data are shown from a mouse 12 d after intratracheal challenge (G and H). (E and G) Light scatter data, as forward angle scatter (FSC) on the *horizontal axis* and side scatter

(SSC) on the *vertical axis*. (*F* and *H*) Fluorescence data, as FL1 on the *horizontal axis* and FL2 (PKH26 staining) on the *vertical axis*. Note the appearance of PKH_{neg} cells in the *lower left-hand quadrant* following immunization (*H*), and the absence of FL1 + cells (*F* and *H*).

Figure 2.

Kinetics of mononuclear cell recruitment during immune lung inflammation. SRBC-primed mice received PKH26 by tail vein-injection and were challenged by the intratracheal route 24 h later with 5×10^8 SRBC. At the indicated days after intratracheal SRBC-challenge, mice underwent BAL, and absolute cell counts of PKH_{pos} Mφ (*black squares*) and PKH_{neg} Mφ (*open circles*) cells were calculated. Results are mean $\pm$ SEM of 3–10 mice per time point assayed individually in at least three separate experiments per time-point.

Figure 3.

Characterization of mononuclear phagocyte subpopulations during immune lung inflammation. SRBC-primed mice received PKH26 by tail vein-injection and were challenged by the intratracheal route 24 h later with 5×10^8 SRBC. At various later times, alveolar cells were harvested by BAL and cells within light scatter-defined gates were analyzed for the indicated antigens by flow cytometry. (A, C, E, G, and I) PKH_{pos} M ϕ . (B, D, F, H, and J) PKH_{neg} M ϕ . (A–H) Representative histograms from individual mice, 3 d after intratracheal challenge. *Horizontal axis*, log FL1 fluorescence; *dotted line*, isotype control; *solid line*, specific staining. (I and J) Representative two-color fluorescence plots from a single mouse, 12 d after intratracheal challenge. *Horizontal axis*, log FL1 fluorescence (CD11b); *vertical axis*, log FL3 fluorescence (CD11c). *Quadrants* indicate specific staining for each cell type.

Figure 4.

Phagocytosis of apoptotic thymocytes by recruited and resident mononuclear phagocytes *in vitro*. At various days after intratracheal SRBC challenge of primed PKH-labeled mice, cells were recovered by BAL, washed, and allowed to adhere to plastic plates. CMFDA-labeled apoptotic thymocytes (A–E) or CMFDA-labeled apoptotic thymocytes opsonized by IgG (F) were added to the adherent Mφ at a ratio of 10:1 and incubated for 90 min. Slides were vigorously washed to remove external thymocytes and Mφ were released into suspension using trypsin/EDTA, and analyzed by fluorescence microscopy (A and B) or flow cytometry (C–F). (A and B) Photomicrographs of Mφ containing internalized apoptotic thymocytes; epifluorescence illumination (A); phase contrast (B). Note the distinction between the uningested green thymocyte not associated with Mφ (white arrow), and a PKH_{pos} Mφ containing two ingested yellow thymocytes (yellow arrowheads). (C and D) Representative dot plots from individual mice incubated either without (C) or with (D) CMFDA+ apoptotic thymocytes. Note appearance of FL1+ cells in both *right-hand quadrants* of D, indicating Mφ that have ingested apoptotic thymocytes. (E) Kinetics of Mφ phagocytosis of apoptotic thymocytes *in vitro*. Black squares, PKH_{pos} Mφ; open circles, PKH_{neg} Mφ. (F) Kinetics of phagocytosis of opsonized apoptotic thymocytes *in vitro*. Dark bars, PKH_{pos} Mφ; light bars, PKH_{neg} Mφ; ND = not done. Results in E and F are mean ± SEM of 3–9 mice assayed individually in at least three independent experiments; **P* < 0.05, unpaired *t* test compared with PKH_{neg} Mφ at the same time-point.

Figure 5.

Kinetics of phagocytosis of apoptotic thymocytes *in vivo*. SRBC-primed mice received PKH26 by tail vein-injection and were challenged by the intratracheal route 24 h later with 5×10^8 SRBC to induce lung inflammation. At the indicated days after intratracheal challenge, mice each received 20×10^6 CMFDA-labeled apoptotic thymocytes by the intratracheal route, and were allowed to recover from anesthesia. Two hours later, mice were killed, alveolar cells were recovered by BAL, and the percentage of phagocytic Mø in the PKH_{pos} and PKH_{neg} subsets was determined by two-color flow cytometry. *Black bars*, PKH_{pos} Mø; *white bars*, PKH_{neg} Mø. Results are mean $\pm$ SEM of 3–8 mice in at least two independent experiments per time-point. Day 0 indicates SRBC-primed C57BL/6 mice that had received PKH26 and labeled apoptotic thymocytes, but no intratracheal SRBC. n.d., not determined.

Figure 6.

Expression of receptors important for apoptotic cell clearance. SRBC-primed mice received PKH26 by tail vein-injection, were challenged by the intratracheal route 24 h later with 5×10^8 SRBC, and were assayed on various later days for expression of CD51 (*left-hand column*), PSR (*middle column*), and MerTK (*right-hand column*) by flow cytometric analysis of BAL. (A and B) Representative flow cytometry data from individual mice 6 d after intratracheal challenge, gated on (A) PKH_{pos} Mφ and (B) PKH_{neg} Mφ. (C) Kinetics of receptor expression. *Black bars*, PKH_{pos} Mφ; *white bars*, PKH_{neg} Mφ. Results are expressed as change in mean fluorescence channel number (ΔMCF) from isotype control, as mean $\pm$ SEM of 3–5 mice per time-point, assayed individually. *Significantly different from other Mφ population, $P < 0.05$, unpaired t test. Note difference in scale of ΔMCF between different receptors.