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Resident murine alveolar and peritoneal macrophages differ in adhesion of apoptotic thymocytes

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Abstract

Apoptotic cells must be cleared efficiently by macrophages (Mø) to prevent autoimmunity, yet their ingestion impairs Mø microbicidal function. The principal murine resident lung phagocyte, the alveolar Mø (AMø), is specifically deficient at apoptotic cell ingestion, both in vitro and in vivo, compared to resident peritoneal Mø (PMø). To further characterize this deficiency, we assayed static adhesion in vitro using apoptotic thymocytes and resident AMø and PMø from normal C57BL/6 mice. Adhesion of apoptotic thymocytes by both types of Mø was rapid, specific, and cold-sensitive. Antibody against the receptor tyrosine kinase MerTK (Tyro12) blocked phagocytosis but not adhesion in both types of Mø. Surfactant protein A increased adhesion and phagocytosis by AMø, but not to the levels seen using PMø. Adhesion was largely cation-independent for PMø and calcium-dependent for AMø. Adhesion was not inhibited in either Mø type by mAbs against $\beta 1$ or $\beta 3$ integrins or scavenger receptor I/II (CD204), but AMø adhesion was inhibited by specific mAbs against CD11c/CD18. Thus, resident murine tissue Mø from different tissues depend on qualitatively disparate receptors systems to bind apoptotic cells. The decreased capacity of murine AMø to ingest apoptotic cells is only partially explained by reduced initial adhesion.

Keywords

Apoptosis; Adhesion Molecules; Mice; inbred strains; Lung

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INTRODUCTION

Efficient clearance of apoptotic cells by mononuclear phagocytes is essential to permit normal organogenesis, to maintain homeostasis of rapidly dividing tissues such as the bone marrow, and to terminate inflammatory responses gracefully (1). The rationale for efficiently clearing dying neutrophils or eosinophils is obvious, as their enzymes would be highly damaging if leaked as these cells progress to necrosis (2). Apoptotic T cells might seem less potentially damaging, but they are a potential source of nucleosomes, the macromolecular complexes that form the basic units of chromatin. Nucleosomes, which in vivo are formed exclusively by chromatin digestion during apoptosis, are a major immunogen for autoantibody production in systemic lupus erythematosus (3). Hence, impaired clearance of apoptotic T cells may break peripheral self-tolerance.

Nevertheless, this clearance process comes at a potential price to host defense against pathogens, because Mø that ingest apoptotic cells actively and specifically down-regulate their own capacity to produce inflammatory cytokines and mediators (4). This effect is mediated chiefly by TGF- β 1, with a lesser contribution by PAF and PGE₂ (4). The potential clinical relevance of this down-regulation has been shown in a murine model of trypanosomiasis, in which ingestion of apoptotic T cells decreased Mø inflammatory cytokine production and parasite killing (5). Whether such down-regulation is a problem during acute infections in most tissues is unclear. Although blood-derived monocytes appear to be unable to ingest apoptotic cells immediately upon recruitment (6, 7), contact with apoptotic cells alone may be sufficient in some circumstances to initiate an anti-inflammatory response (8). Hence, increased understanding of the process by which various cell types ingest their dying neighbors, and its consequences for host defense, is urgently needed. Clearance of apoptotic cells is particularly relevant to the lungs, where a significant fraction of lymphocytes recovered from the alveolar spaces of both normal mice and mice undergoing antigen-induced inflammation are apoptotic (9). Secretion of crucial pro-inflammatory cytokines could be suppressed if resident alveolar Mø (AMø) ingest dying T cells before or during encounters with pathogens. Thus, the lungs, a mucosal surface frequently exposed to pathogens, present a unique challenge in regulating clearance of apoptotic T cells.

In fact, decreased phagocytosis of apoptotic leukocytes by resident lung Mø has been demonstrated in three species. The phenomenon was first identified in phagocytosis of apoptotic neutrophils by resident rabbit AMø, relative to inflammatory Mø recovered from rabbit lungs (6). We reported markedly decreased phagocytosis of apoptotic thymocytes, which were used as a model of clearance of lymphocytes undergoing activation-induced cell death (AICD), by resident murine AMø relative to resident murine peritoneal Mø (PMø) (10). This defect was seen both in vitro in seven murine strains and in vivo, and was specific for apoptotic cells (as opposed to three other phagocytic targets), and extended to apoptotic neutrophils (10, 11). Importantly, the phagocytic defect in AMø could not be explained by differences in expression of several Mø receptors previously implicated in phagocytosis of apoptotic cells, including β 1 and β 3 integrins (10). Human AMø also show reduced apoptotic cell ingestion (12). Defining the basis of this deficit could provide key insights into the molecular mechanisms underlying Mø clearance of apoptotic cells.

We subsequently found that both AM ϕ and PM ϕ appear to share many downstream signaling pathways required for apoptotic cell ingestion (11), leaving the mechanism for the AM ϕ phagocytic defect undefined. More recently, we showed that both types of M ϕ express the stereospecific receptor for phosphatidylserine (PS-R) (13), and that mAb against PS-R profoundly inhibited phagocytosis but not adhesion of apoptotic cells by both AM ϕ and PM ϕ (14). We also found that at least a portion of the decreased ingestion of apoptotic cells by murine AM ϕ can be attributed to markedly decreased expression of protein kinase C isoform beta II, which we proved to be uniquely required for this process in both types of M ϕ (14). However, it remained uncertain whether this difference was the sole explanation of the AM ϕ functional deficiency.

The purpose of the current study was to investigate whether differences between AM ϕ and PM ϕ in adhesion of apoptotic thymocytes could also contribute to the difference in phagocytosis, and to characterize the binding interaction in vitro. Because we have previously shown that AM ϕ have a similar defect in phagocytosis of apoptotic thymocytes, lymphocytes, and neutrophils (10), we used apoptotic thymocytes as a convenient model of T cells dying by AICD. We found that both types of M ϕ rapidly and specifically bound apoptotic thymocytes, but that the pronounced differences between AM ϕ and PM ϕ in the kinetics of adhesion and the dependence on Ca⁺⁺ and β 2 integrins indicate that the two cell types use disparate receptor systems.

MATERIALS AND METHODS

Antibodies

The following mAbs were purchased from PharMingen (San Diego, CA): M17/4 (anti-murine CD11a; rat IgG_{2a}, κ); M1/70 (anti-murine CD11b; rat IgG_{2b}, κ); HL3 (anti-murine CD11c; Armenian hamster IgG₁, λ); C71/16 (anti-murine CD18; rat IgG_{2a}, κ); M18/2 (anti-murine CD18; rat IgG_{2a}, κ); GAME-46 (anti-murine CD18; rat IgG₁, κ); Ha2/5 (anti-murine CD29; Armenian hamster IgM, κ); R1-2 (anti-murine CD49d; rat IgG_{2b}, κ); 5H10-27 (anti-murine CD49e; rat IgG_{2b}, κ); H9.2B8 (anti-murine CD51; Armenian hamster IgG₁, λ); 2C9.G2 (anti-murine CD61; Armenian hamster IgG₁, κ); R3-34 (control rat IgG₁, κ); R35-95 (control rat IgG_{2a}, κ); A95-1 (control rat IgG_{2b}, κ); G235-2356 (control Armenian hamster IgG₁, λ); A19-4 (control hamster IgG3, λ); A19-3 (control hamster IgG₁, κ); G235-1 (control hamster IgM). mAb 2F8 (rat IgG_{2b}) against the murine scavenger receptor type I/II (CD204) was obtained from Serotec (Raleigh, NC).

Mice

Specific pathogen-free inbred female C57BL/6 and BALB/c mice were purchased from Charles River Laboratory Inc. (Wilmington, MA). C57BL/6 were used unless otherwise specified. Mice were obtained at 7–8 weeks of age and used at 8–14 weeks of age. Mice were housed in the Animal Care Facility at the Ann Arbor VA Medical Center, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care, where they 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.

Isolation and culture of Mø

Mice were euthanized by asphyxia in a high CO₂ environment, which we have previously shown does not impair the capacity of AMø to ingest apoptotic thymocytes compared to mice euthanized by exsanguination while anesthetized using pentobarbital (10). Resident AMø and PMø were harvested and cultured as previously described (10). PMø among the peritoneal lavage cells were first enriched by negative selection using a mixture of anti-CD19- and anti-CD90-conjugated paramagnetic beads (Miltenyi Biotec; Auburn, CA) according to the manufacturer's instructions. Mø were plated at 2×10^5 cells/well in sterile 8-well Lab-Tek slides (Nalge Nunc International; Naperville, IL) and, after 1 h incubation at 37° C, nonadherent cells were removed by gentle washing. Mø monolayers were cultured for 5 h in complete medium {RPMI 1640 containing 10% heat-inactivated FBS, 25 mM HEPES, 2 mM L-glutamine, 1 mM pyruvate, 100 units/ml penicillin/streptomycin (all obtained from GIBCO-BRL) and 55 µM 2-mercaptoethanol (Sigma; St. Louis, MO)} in a 5% CO₂ environment at 37° C before use in the adhesion assay. Selected experiments were performed using the same medium without FBS.

Isolation and apoptosis induction of thymocytes

Thymuses harvested from normal C57BL/6 mice were minced to yield a single cell solution. To induce apoptosis, thymocytes were suspended with RPMI 1640 containing 10% heat-inactivated FBS at the concentration of 1×10^6 /ml and incubated with a final concentration of 10^{-6} M dexamethasone (Sigma) for 6 h (10). 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). In selected experiments, thymocytes were induced to undergo apoptosis in the absence of FBS.

Adhesion assays

Adherence of apoptotic thymocytes to Mø in vitro was assayed by adding 2×10^7 apoptotic thymocytes suspended in 400 µl of complete medium to each well of the Lab-Tek slides containing adherent Mø monolayers, yielding a 100:1 ratio of thymocytes to Mø. Thus, this assay used a 10-fold higher thymocyte:Mø ratio than we had previously used to assay phagocytosis (10); this change was made empirically due to the shorter duration of the current assay, to assure that Mø would have adequate exposure to apoptotic cells. The slides were incubated for various times at 37°C, and then washed in a standardized fashion, by dipping individual slides 5 times in each of two Wheaton jars filled with ice-cold PBS, stained using hematoxylin-eosin Y (H & E) (Richard-Allan; Kalamazoo, MI), and coverslipped. Adhesion was evaluated by counting 200 to 300 macrophages per well at 1000 × magnification under oil immersion, and scoring for bound thymocytes. Results were expressed as percentage of Mø binding at least one thymocyte (percent adhesion-positive Mø), and as adhesion index, which was calculated using the following formula:

$$\text{Adhesion index} = \text{adhesion-positive Mø (\%)} \times \text{mean number of adherent thymocytes per adhesion-positive Mø}$$

where the percent adhesion-positive M ϕ is expressed as a decimal (e.g., 75% = 0.75). In blocking experiments, mAbs were used at concentrations previously determined to be saturating by flow cytometry, and culture supernatant containing mAb 217 was concentrated using YM-50 Centricon tubes (Amicon; Bedford, MA).

Phagocytosis assays

Phagocytosis of apoptotic thymocytes in vitro was assayed by co-incubation with adherent M ϕ monolayers in complete medium as previously described (10). Results were expressed as percentage of M ϕ containing at least one ingested thymocyte (percent phagocytic M ϕ), and as phagocytic index, which was generated by multiplying the percent phagocytic M ϕ by the mean number of phagocytosed cells per M ϕ .

Flow cytometry

M ϕ were stained and analyzed as previously described (10).

Isolation of surfactant protein A (SP-A)

Human SP-A was purified from bronchoalveolar lavage of alveolar proteinosis patients using 1-butanol extraction followed by homogenization, dialysis, and centrifugation, as previously described in detail (15). Purified SP-A was confirmed to be >98% pure by two-dimensional gel electrophoresis followed by Western blotting and silver staining, and was found to contain <0.1 pg LPS/10 μ g SP-A by QCL-1000 *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD).

Purification of recombinant annexin V

For use in blocking experiments, recombinant human annexin was purified from TG1 strain *E. coli* containing a plasmid encoding human placental annexin V (clone pRK6; American Type Culture Collection; Rockville, MD) as previously described (10).

Statistical analysis

Data were expressed as mean $\pm$ SEM. Statistical calculations were performed using Statview v 5.0 and Super ANOVA v 1.11 (SAS Institute, Inc., Cray, NC) on a Macintosh PowerPC G4 computer. Continuous ratio scale data were evaluated by unpaired Student 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, which compares treatment groups to a specific control group (16). Significant differences were defined as $p < 0.05$.

RESULTS

AM ϕ and PM ϕ specifically bind apoptotic thymocytes in vitro, but differ in kinetics of adhesion

Both AM ϕ and PM ϕ bound apoptotic thymocytes, but the two types of M ϕ differed considerably, both in rate of adhesion and in the maximal percentage of M ϕ showing thymocyte binding (Fig. 1). By five minutes, a majority of PM ϕ had bound apoptotic thymocytes, and the number of apoptotic thymocytes bound per M ϕ continued to increase

slightly throughout the 30 minute assay. By contrast, the rate of binding by AM ϕ rose more slowly to a lower plateau at 15 minutes, and the number of bound apoptotic thymocytes per AM ϕ did not increase thereafter. The kinetic analysis was terminated at 30 minutes, a time we had previously shown corresponds to the beginning of phagocytosis (10). Based on these results, plus those of additional control experiments (Online supplemental data) all subsequent assays were performed at 15 minutes of co-incubation.

To exclude definitively a contribution of ingested cells, in control experiments we fluorescently-labeled apoptotic cells before co-incubating them with M ϕ , and quenched fluorescence at the end of co-incubation using methylene blue. Quenching totally abolished fluorescence (data not shown), implying that at the time-point studied, all apoptotic cells were extracellular. Because the method we use to induce apoptosis produces early apoptotic cells that are not entirely PS-positive, we also considered the possibility that binding of viable thymocytes by the two M ϕ types was a potential confounding factor in our analysis. However, results of control experiments (Online Supplemental Data) indicated that adhesion of viable thymocytes and apoptotic thymocytes is independent, and that under the conditions used in our assay any potential interference by viable thymocytes in the mixture would be negligible.

SP-A slightly increases adhesion of apoptotic thymocytes by murine AM ϕ

The pulmonary collectin SP-A has been shown to increase the in vitro phagocytosis of apoptotic neutrophils by rat AM ϕ (17) and, more recently, of apoptotic Jurkat cells by murine AM ϕ (18). In agreement with those studies, we found that purified human SP-A significantly increased the phagocytosis of thymocytes by murine AM ϕ in three separate experiments using different doses and preparations of SP-A (Figure 2). Nevertheless, the absolute increase in phagocytosis by AM ϕ was small, and did not bring phagocytosis by AM ϕ to the level seen in PM ϕ . By contrast, SP-A had an inconsistent effect on phagocytosis by murine PM ϕ , with a slight but statistically significant overall increase in the percentage of phagocytic PM ϕ , but a slight decrease in the phagocytic index (Figure 2).

Similarly, SP-A slightly but significantly increased adhesion of apoptotic thymocytes to AM ϕ (adhesion-positive AM ϕ : control $65.6 \pm 1.8\%$ versus SP-A $74.1 \pm 1.8\%$, $n = 9-12$; mean $\pm$ SEM, $p < 0.005$; adhesive index: control, 1.0 ± 0.1 versus SP-A, 1.5 ± 0.1 , $p < 0.001$, unpaired t test), but had no significant effect on adhesion to PM ϕ (adhesion-positive PM ϕ : control, $90.8 \pm 1.3\%$ versus SP-A, $89.3 \pm 1.0\%$, $n = 9-12$; mean $\pm$ SEM, $p = 0.34$; adhesive index, control, 3.0 ± 0.2 versus SP-A, 3.2 ± 0.1 , $p = 0.28$, unpaired t test).

To test the potential dependence of adhesion on serum proteins other than complement components, in control experiments we both induced thymocyte apoptosis and performed the adhesion assay in medium devoid of heat-inactivated FBS. Adhesion of apoptotic thymocytes in this assay was reduced in the absence of serum, but the effect was small (compared to assay in 10% serum, adhesion positive M ϕ in serum-free conditions = $87.3 \pm 1.3\%$ of control for PM ϕ and $80.8 \pm 7.6\%$ of control for AM ϕ ; mean $\pm$ SEM, $n = 5$, $p < 0.05$ for unpaired t test compared to assay in serum-containing medium). However, as shown by the adhesion index, the efficiency of adhesion to PM ϕ was more sensitive to the absence of serum (compared to assay in 10% serum, adhesion index = $46.3 \pm 7.6\%$ of control for PM ϕ

versus $84.8 \pm 7.8\%$ of control for AM ϕ versus; mean $\pm$ SEM, $n = 5$, $p < 0.05$ unpaired t test). Subsequent experiments were performed in medium containing heat-inactivated FBS unless otherwise specified.

MerTK is dispensable for apoptotic cell adhesion in vitro

We next considered the possible involvement of the two cell surface receptors shown to be necessary for M ϕ ingestion of apoptotic cells, the stereospecific receptor for phosphatidylserine (PS-R) (13), and the receptor tyrosine kinase MerTK (also known as Tyro12) (19). We have recently shown that both AM ϕ and PM ϕ express PS-R, and that mAb 217 against the PS-R has no effect on adhesion of apoptotic cells to either AM ϕ or PM ϕ , yet profoundly inhibits phagocytosis by both types of M ϕ (14). However, because receptors other than the PS-R can also bind PS in a stereo-nonspecific fashion, we next used annexin V to examine the importance of PS-binding in adhesion in this assay. Results showed that blocking PS on the thymocyte surface by annexin pre-treatment inhibited adhesion profoundly and to a similar degree in both AM ϕ and PM ϕ (Online Supplemental Data). These results imply that it is unlikely that differences in overall PS-binding underlies the observed disparity between the two types of tissue M ϕ in apoptotic cell adhesion.

Pre-treatment of M ϕ with anti-MerTK antibody (50 μ g/ml) had no effect on adhesion of apoptotic thymocytes to either type of tissue M ϕ (Fig 3A, B). By contrast, anti-MerTK inhibited phagocytosis of apoptotic thymocytes, in both AM ϕ and PM ϕ , with a greater relative effect in PM ϕ (Fig 3C, D), confirming the functional activity of the antibody. Thus, although these data provide novel confirmation of the importance of MerTK in M ϕ apoptotic cell phagocytosis, they do not explain the difference in adhesion between the two types of M ϕ .

Adhesion of apoptotic thymocytes by resident tissue M ϕ s is cold-inhibitable

Some adhesive interactions, notably those mediated by lectin-carbohydrate interactions, are impervious to chilling; consequently, we next performed the binding assay for apoptotic cells on ice. Chilling to 4 $^{\circ}$ C decreased adhesion substantially in both PM ϕ and AM ϕ (Fig. 4). The degrees of inhibition induced by cold was similar in the two types of M ϕ (percent adhesion was suppressed $77.6 \pm 5.8\%$ in PM ϕ , versus $85.1 \pm 3.6\%$ in AM ϕ ; $p = 0.2821$, unpaired t test comparing the two cell types; adhesive index was suppressed $91.9 \pm 2.4\%$ in PM ϕ , versus $87.6 \pm 3.3\%$ in AM ϕ ; $p = 0.2996$, unpaired t test comparing the two cell types). Thus, a major component of adhesion of apoptotic cells by resident tissue M ϕ appears to require a change in conformation or membrane location of the involved receptors.

AM ϕ adhesion of apoptotic thymocytes is entirely calcium-dependent

Studies of cation chelation and repletion disclosed a fundamental difference between resident murine AM ϕ and PM ϕ in apoptotic cell adhesion. We used EDTA, which binds Mg^{++} more effectively than Ca^{++} , and EGTA, which potently chelates Ca^{++} . EDTA slightly inhibited adhesion by PM ϕ , and this effect was reversed by re-administration of Ca^{++} (Fig. 5A). EGTA more markedly inhibited adhesion by PM ϕ , and this effect was not reversed by re-administration of Mg^{++} . By contrast, adhesion of apoptotic thymocytes to AM ϕ was almost totally abolished by cation chelation using either EDTA or EGTA. Re-administration

of Ca^{++} restored binding in AM ϕ , whereas Mg^{++} did not (Fig. 5B). These findings indicated that PM ϕ were able to bind apoptotic thymocytes by both Ca^{++} -dependent and cation-independent mechanisms, whereas binding of apoptotic thymocytes to AM ϕ was entirely Ca^{++} -dependent.

One receptor that potentially could be involved in the Ca^{++} -independent component of M ϕ adhesion of apoptotic thymocytes is the murine scavenger receptor type I/II (CD204), which has been implicated in the phagocytosis of apoptotic cells (20). We tested the possible role of this receptor using the function-blocking mAb 2F8 (21), and M ϕ from BALB/c mice, because C57BL/6 mice have been reported to have a polymorphism in the gene for the scavenger receptor type I/II that eliminates the epitope recognized by mAb 2F8 (22). Incubation with mAb 2F8 at 30 $\mu\text{g}/\text{ml}$, which was a saturating concentration as confirmed by flow cytometry, had no significant effect on adhesion of apoptotic thymocytes to either type of M ϕ (adhesion-positive PM ϕ : mAb 2F8, $83.7 \pm 2.5\%$ versus control IgG, $83.7 \pm 2.6\%$; adhesion-positive AM ϕ , mAb 2F8, $37.7 \pm 4.8\%$ versus control IgG, $37.6 \pm 3.3\%$; adhesion index for PM ϕ , mAb 2F8, 3.1 ± 0.2 versus control IgG, 2.8 ± 0.2 ; adhesion index for AM ϕ , mAb 2F8, 0.5 ± 0.1 versus control IgG, 0.5 ± 0.1 ; mean $\pm$ SEM, $n = 10$, all comparisons $p > 0.05$, unpaired t test). Control experiments using the assay originally described by Fraser and colleagues (21) confirmed the ability of mAb 2F8 to totally inhibit Ca^{++} -independent adhesion of both types of M ϕ to tissue-culture plastic in the presence of serum (data not shown). These data do not support an essential role for scavenger receptor A in this assay.

Binding of apoptotic thymocytes by AM ϕ is inhibited by blocking CD11c/CD18

The cold-sensitivity and calcium dependence of binding, and the known roles of integrins in phagocytosis of apoptotic cells in some experimental systems (7) next led us to study the effect of integrin-blocking mAbs on adhesion. These experiments showed no necessary role for either $\beta 1$ or $\beta 3$ integrins for adhesion of apoptotic cells by murine AM ϕ or PM ϕ in this assay system (Online Supplemental Data), and, thus, also failed to explain the difference between the two types of M ϕ .

However, examination of $\beta 2$ integrins showed an additional difference between the two types of tissue M ϕ . Adhesion to PM ϕ was not inhibited by blocking the common $\beta 2$ integrin chain CD18 using mAb GAME-46 (Fig. 6) or mAbs C71/16 and M18/2 (data not shown). Neither was adhesion to PM ϕ inhibited by mAbs against the two alpha chains with which CD18 pairs in resident PM ϕ , CD11a and CD11b (Fig. 6), even though the mAbs we used have previously been shown to block adhesion in several other systems. These results are in accordance with the largely calcium-independent binding of apoptotic cells by PM ϕ (Fig. 5A). In PM ϕ , mAb HL3 against CD11c had no effect on adhesion (Fig. 6), as anticipated based on our previous finding that resident murine PM ϕ are virtually devoid of CD11c/CD18 (10). None of the isotype-treated groups were significantly different from untreated control groups for either M ϕ cell type (data not shown), excluding a non-specific effect of antibody treatment on apoptotic cell adhesion.

By contrast, adhesion to AM ϕ was significantly inhibited by the anti-CD18 mAb, GAME-46, and to a somewhat lesser degree by mAb against one of the two alpha chains

with which CD18 pairs in resident AM ϕ , CD11c (Fig. 6). Flow cytometric analysis showed that the level of staining by GAME-46 was uniform among AM ϕ , identical to expression by PM ϕ , and identical to staining using two non-blocking anti-CD18 mAbs, C71/16 and M18/2 (data not show), excluding the possibility that GAME-46 recognized an antigen found only on a subset of AM ϕ . In separate experiments, phagocytosis of apoptotic cells by AM ϕ was also inhibited by GAME-46 (compared to untreated control, the percent phagocytosis was suppressed $58.8 \pm 1.4\%$, and the phagocytic index was suppressed $64.7 \pm 2.4\%$).

DISCUSSION

The results of these studies indicate that resident murine M ϕ from two different tissues rapidly and specifically bind apoptotic thymocytes using mechanisms distinct from adhesion of viable thymocytes, but that AM ϕ differ both qualitatively and quantitatively from PM ϕ in this function. Similarities between the two types of M ϕ included inhibition of adhesion by chilling the cells to 4°C or by masking PS on the apoptotic cell using annexin V, and insensitivity to blocking $\beta 1$ or $\beta 3$ integrins. However, there were also notable differences between PM ϕ and AM ϕ in adhesion of apoptotic thymocytes. Compared to adhesion of apoptotic thymocytes to PM ϕ , adhesion to AM ϕ was much less rapid, somewhat less sensitive to the absence of serum, and characterized by markedly lower maximal binding. Adhesion was largely cation-independent in PM ϕ , but almost totally calcium-dependent in AM ϕ . Moreover, a major component of adhesion by AM ϕ depended on CD11c/CD18, a $\beta 2$ integrin not expressed by resident PM ϕ , whereas adhesion by PM ϕ was independent of $\beta 2$ integrins. These novel findings provide further support for the possibility that the disparity between resident AM ϕ and PM ϕ in recognition of apoptotic cells may be a evolutionary adaptation of teleological value because it preserves the crucial surveillance and microbicidal functions of AM ϕ against autocrine down-regulation (1, 4, 5, 23). This study also showed that the receptor tyrosine kinase MerTK did not mediate M ϕ adhesion of apoptotic cells, and that addition of SP-A did not correct the relative deficiency of murine AM ϕ in adhesion and phagocytosis of apoptotic thymocytes to the levels seen in resident murine PM ϕ .

Results of our chelation and $\beta 2$ integrin-blocking experiments indicate the existence of at least two distinct pathways of apoptotic cell adhesion to resident tissue M ϕ . PM ϕ exhibited both a major cation-independent pathway and a minor calcium-dependent pathway, whereas AM ϕ essentially showed only a calcium-dependent pathway that is partially explained by binding via CD11c/CD18. Absence of cation-independent adhesion is a partial explanation for the relative defect in phagocytosis by AM ϕ , since 30–50% of AM ϕ do not interact with apoptotic thymocytes at all. Importantly, the performance of PM ϕ in this short-term adhesion assay very closely paralleled the phagocytosis data we have previously reported (10), indicating a close functional linkage between adhesion and ingestion in this cell type. By contrast, a much greater percentage of AM ϕ bound at least one apoptotic thymocyte (maximum adhesion = 50–70%, current study) than ingested any apoptotic thymocytes (maximum phagocytosis = $19.1 \pm 1.0\%$ positive at 90 minutes) (10). Thus, although initial adhesion must constitute an obligatory step in phagocytosis, adhesion itself did not appear to be the rate-limiting step in the process for the majority of AM ϕ that do bind apoptotic thymocytes. Why many of the remaining AM ϕ that bind apoptotic cells do not go on to

ingest any is uncertain, although post-receptor defects in signal transduction, such as the markedly reduced expression of PKC β II in murine AM ϕ (14), probably contribute.

The current results using purified human SP-A agree with the previous findings of Schagat et al (17) and Vandivier et al (18) with regard to the effect on AM ϕ phagocytosis, and extend them by analysis of adhesion and by comparison with resident murine M ϕ from another anatomic site. Like those investigators, we found 160–300% increases from control values of either percent of phagocytic M ϕ or phagocytic index (although the absolute value of the phagocytic index we report is lower by a factor of 100 due to a difference in the method of calculation). Importantly, however, even on addition of SP-A, phagocytosis by AM ϕ remained markedly reduced relative to that of PM ϕ . These persistent differences between the two types of M ϕ argue that the selective defect we and others reported in resident AM ϕ phagocytosis of apoptotic cells (6, 11) is not due simply to a unique requirement for bridging surfactant proteins absent from the in vitro assay, as has been suggested (24).

The results presented here also extend our previous observation that resident murine AM ϕ and PM ϕ do not require RGDS-inhibitable integrins for phagocytosis (10). By contrast, previous studies have shown that human monocyte-derived M ϕ (HMDM) and MBMDM do use these systems (7, 25, 26). An obvious experimental difference between our system and those studies is the duration of culture before analysis: 6 h in our studies versus 5–7 d in the latter. This longer culture period is necessary because the precursors of HMDM and MBMDM lack the capacity to ingest apoptotic cells (6, 7), which can, however, be induced by cytokine stimulation (27). Because the receptor systems identified in studies of HMDM and MBMDM are also likely to be used by activated mononuclear phagocytes at sites of inflammation, results of those studies are nonetheless highly clinically relevant. The current analysis of constitutive clearance by resident mucosal M ϕ complements those studies by defining the mechanisms of normal physiologic clearance in the absence of inflammation. Our results also emphasize the importance of studying resident M ϕ from specific tissues. It is conceivable that still different physiologic behavior will be discovered in mononuclear phagocytes facing different burdens of apoptotic cells and different exposure to environmental challenges, as it may be, for example, the case with microglial or retinal pigment epithelial cells, which reside behind the blood-brain barrier, versus Kupffer cells, which are continuously exposed to bacterial products.

Using a direct antibody blocking approach to examine the role of MerTK in apoptotic cell adhesion and phagocytosis, our results complement and extend the results of gene deletion of MerTK (19), which verified an essential role for MerTK in apoptotic cell clearance in vitro and in vivo. MerTK possesses dual extracellular immunoglobulin and fibronectin-type III motifs that suggest potential adhesive capacities (28). Hence, our finding that function-blocking Ab against MerTK did not block apoptotic cell adhesion may seem surprising. Nevertheless, it is likely that any strictly adhesive function of MerTK is dispensable due to the redundancy of M ϕ receptors that can mediate apoptotic cell adhesion. It is unlikely, but conceivable, that the polyclonal Ab we used failed to block epitopes essential for apoptotic cell adhesion although it could block phagocytosis.

The apparent disparity between results of blocking PS expression on the apoptotic cells using annexin V in the current study (Online Supplemental Data), versus our previous finding that mAb 217 does not inhibit adhesion of apoptotic thymocytes (14), is explicable because PS can be bound in a stereo-nonspecific fashion by multiple receptors, including class B scavenger receptors, CD14, macrofialin (CD68) and thrombospondin-dependent vitronectin receptors {reviewed in (24)}. By coating the surface of the apoptotic cell densely, annexin V might antagonize adhesion via other receptors through steric hindrance, and hence be a highly effective inhibitor of both adhesion, and as we have previously shown, phagocytosis (10). It is formally possible from our collective data that the residual level of apoptotic cell phagocytosis by PM ϕ that is not inhibited by mAb 217 (14) results from the calcium-independent adhesive activity possessed by PM ϕ but absent from AM ϕ (Fig. 4), and that the receptor(s) mediating this binding can trigger phagocytosis without involvement of surface PS recognition. Unfortunately, this possibility cannot be tested directly by assaying phagocytosis in the presence of combined blockade using annexin V and mAb 217 without or with calcium chelation, because binding of annexin V to PS is also calcium-dependent. However, because mAb 217 also incompletely inhibits phagocytosis by AM ϕ , which clearly lack calcium-independent adhesion, we favor the interpretation that this mAb is simply an imperfect functional antagonist of phagocytosis.

A role for CD11c/CD18 in apoptotic cell recognition via C3bi in HMDM was previously shown by Mevorach et al (29), who employed an assay in the presence of fresh autologous serum to provide functional complement components. The difference in our assay system, which uses heat-inactivated serum, implies that we are detecting a complement-independent function of CD11c/CD18, as does the absence of effect of blocking CD11b/CD18 in PM ϕ . Therefore, our results do not disagree with those of Mevorach et al. At face value, our results using viable thymocytes (Online Supplemental Data) appear to differ from those of Duvall et al, who found that 64% of PM ϕ bound viable thymocytes (30), whereas we found virtually no binding of viable thymocytes by either type of M ϕ . This difference undoubtedly results from the difference in the assay conditions, which in their case involved incubation for 2h at 4°C. Because our experiments were not performed in a manner that would facilitate detection of lectin-mediated interactions, the current results should not be construed to call into question the substantial body of data indicating a role for carbohydrate recognition in apoptotic cell clearance (30–32).

In summary, we show rapid and specific adhesion of apoptotic thymocytes by resident murine tissue M ϕ , provide evidence that resident M ϕ in different organs use disparate adhesion systems, and have identified a receptor, CD11c/CD18, that mediates apoptotic cell adhesion in murine AM ϕ . The collective findings of this study and our previous data (10, 14) in this system support a model of apoptotic cell recognition by mononuclear phagocytes that distinguishes between receptors that mediate initial adhesion and those that trigger phagocytosis (33, 34). Recognition of surface PS expression on the apoptotic cell appears to be central to this process, yet we have shown that neither the stereo-specific PS-R itself nor MerTK, which also binds PS via the soluble intermediaries Gas6 (35) or protein S (36, 37), are required for adhesion. It is plausible that some of the M ϕ molecules involved in apoptotic clearance serve both adhesive and informational functions, much in the manner of co-stimulatory activity by a variety of receptors during T cell activation. Because selective

modulation of apoptotic cell clearance may be of therapeutic value in a wide variety of diseases, it will be important to define the unique aspects of this process used by Mø in different organs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

AICD	activation-induced cell death
AMø	alveolar macrophage
BAL	bronchoalveolar lavage
IT	intratracheal
HMDM	human monocyte-derived macrophage
mAb	monoclonal antibody
MBMDM	murine bone marrow-derived macrophage
PMø	peritoneal macrophage
PS	phosphatidylserine
PS-R	PS receptor

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FIGURE 1.

Kinetics of adhesion of apoptotic thymocytes to resident tissue Mφ. Resident PMφ (open circles) and AMφ (closed squares) from normal C57BL/6 mice (2×10^5 Mφ in a final volume of 400 μ l) were co-incubated at 37°C in 8-well chamber slides with apoptotic thymocytes (2×10^7 per well) without rocking. After various times, slides were washed in a standardized fashion using PBS, fixed in 10% neutral buffered formalin, and stained using H & E. Adhesion was evaluated by counting 200–300 Mφ per well at 1000 $\times$ magnification under oil immersion, and scoring for bound thymocytes. A. Percentage of adhesion-positive Mφ. B. Adhesion index, which was calculated by multiplying the percentage of adhesion-positive Mφ by the mean number of adherent thymocytes per adhesion-positive Mφ. Data are mean $\pm$ SEM of 3–5 replicates in each of four independent experiments. All time points differ significantly between the two Mφ types; *, $p < 0.05$; **, $p < 0.0001$, unpaired t test.

FIGURE 2.

SP-A has a small but significant effect on phagocytosis of apoptotic cells by murine AMØ. Resident murine PMØ or AMØ were untreated (light gray bars), or pre-incubated with human SP-A at 25 µg/ml (white bars) or 50 µg/ml (black bars), and then assayed for phagocytosis of apoptotic thymocytes. A. Percentage of phagocytic MØ. B. Phagocytic index. Data are mean $\pm$ SEM of 3 wells per condition in each of three independent experiments. *, $p < 0.05$ compared to untreated control for same cell type, ANOVA with Dunnett's post-hoc testing.

FIGURE 3.

The receptor tyrosine kinase MerTK is essential for apoptotic cell phagocytosis but not adhesion. Resident murine PMØ or AMØ were untreated (light gray bars), or pre-incubated with control IgG (white bars) or anti-MerTK (black bars), and then assayed for either adhesion (A, B) or phagocytosis (C, D) of apoptotic thymocytes. Data are mean $\pm$ SEM of 3 wells per condition in each of three independent experiments. *, $p < 0.05$ compared to other two conditions for same cell type, ANOVA with Tukey-Kramer post-hoc testing. There are no significant differences between untreated and isotype control groups.

FIGURE 4.

Resident tissue MØ adhesion of apoptotic thymocytes is sensitive to cold. Adhesion of apoptotic thymocytes to resident murine AMØ and PMØ was assayed as described in the legend to Figure 1, with the incubation either at 37°C (dark gray bars) or on melting ice (light gray bars). Data are mean $\pm$ SEM of at least 3 replicates in each of three independent experiments; **, $p < 0.0001$, unpaired t test.

FIGURE 5.

Cation sensitivity of apoptotic thymocyte adhesion. Adherent Mø were pre-incubated in complete medium (white bars) or medium containing 2 mM EDTA (dark gray bars), EDTA plus 2 mM CaCl₂ (light gray bars), 2 mM EGTA (black bars), or 2 mM EGTA plus 2 mM MgCl₂ (medium gray bars). A. PMø. B. AMø. Data are mean $\pm$ SEM of 3 replicates in each of three independent experiments. *, $p < 0.001$ compared to untreated group, ANOVA with Dunnett's post-hoc testing. Only data for the percentage of adhesion-positive Mø are shown, although for both PMø and AMø, the same treatment groups were also statistically different from control in adhesion index values.

FIGURE 6.

CD11c/CD18 mediates binding of apoptotic thymocytes to AMØ. MØ were pre-incubated with complete medium containing saturating concentration of mAb for 30 minutes, and then thymocytes were added in medium containing the same mAb concentration, and adhesion was assayed after an additional 15 minutes incubation. mAbs are M17/4 (anti-CD11a) (dark gray bars), M1/70 (anti-CD11b) (white bars), HL3 (anti-CD11c) (light gray bars) and GAME-46 (anti-CD18) (black bars). Data are expressed as a percentage of the result for the appropriate isotype control in the same experiment, and represent mean $\pm$ SEM of 6–9 wells in at least two experiments; *, $p < 0.05$, compared to other mAbs in same panel, ANOVA with Tukey-Kramer post-hoc testing.