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Macrophages and Stromal Cells Phagocytose Apoptotic Bone Marrow-Derived B Lineage Cells

Zeynep Dogusan, Encarnacion Montecino-Rodriguez, and Kenneth Dorshkind

It has been hypothesized that B cell precursors that undergo programmed cell death due to nonproductive Ig gene rearrangements are cleared from the bone marrow by macrophages. However, a role for macrophages in this process is supported only by micrographs showing their association with apoptotic-appearing, B lineage cells. Functional data demonstrating phagocytosis of apoptotic, bone marrow lymphocytes by macrophages have not been presented, nor have receptors potentially involved in that process been identified. The data in this report demonstrate that macrophages isolated from murine bone marrow efficiently phagocytose apoptotic murine B lineage cells using multiple receptors that include CD14, integrins, class A scavenger receptor, and CD31 (PECAM-1). In addition, the results further reveal a new role for the hemopoietic microenvironment in B cell development in view of data demonstrating that murine bone marrow stromal cells are also capable of clearing apoptotic cells via an integrin-dependent mechanism. The Journal of Immunology, 2004, 172: 4717–4723.

B lymphopoiesis occurs in the bone marrow during postnatal life in association with a supporting population of stromal cells that are the source of various growth and differentiation factors (1–4). The seminal event in B lymphopoiesis is the expression of cell surface Ig, and this process is dependent upon rearrangement of the H and L chain genes that encode the Ig H and L chain proteins (5, 6). Ig gene recombination is an error-prone process. For example, it has been estimated that only about one-third of developing B lineage cells successfully recombine the genes encoding the H chain of the Ig molecule (7). The remaining B lineage cells in which rearrangements are nonproductive undergo apoptosis (8).

The rapid removal of apoptotic cells from tissues is a fundamental and critical process. Otherwise, escaping cytoplasmic and nuclear material from dying cells could trigger inflammatory responses or be a source of Ags that stimulate the formation of autoantibodies (9–13). It would appear that apoptotic B lineage cells are rapidly removed from the bone marrow under normal circumstances. Although B lineage cells comprise up to 40% of nucleated bone marrow cells, it is difficult to detect apoptotic B lineage cells in that tissue in vivo (14). This observation is analogous to the situation in the thymus, in which very few apoptotic cells can be detected even though numerous immature thymocytes undergo cell death (15, 16).

The potential of macrophages to clear apoptotic thymocytes has been demonstrated (16, 17), and it has generally been accepted that these phagocytes are the cells responsible for eliminating dying B lineage cells from the bone marrow (14, 18). However, this claim is supported only by micrographs showing B lymphoid cells with apoptotic morphology being ingested by macrophage-like cells. Functional data demonstrating the phagocytosis of apoptotic B lineage cells by macrophages have not been presented, and little is known about the cell surface receptors they may use to do so.

Multiple receptor systems are involved in the elimination of apoptotic cells by macrophages, thus ensuring functional redundancy (9, 19–24). This diverse array of receptors includes the CD14 LPS receptor (25), the class A scavenger receptor (SR-A) (16, 20), and various integrins (9). In addition, it has recently been reported that macrophages use CD31-mediated homotypic interactions with cells undergoing programmed cell death. When CD31 expressing macrophages encounter viable CD31+ cells, repulsion occurs. However, if the target cell is dead or dying, engulfment takes place (26, 27).

The data in this report provide evidence that bone marrow macrophages efficiently phagocytose apoptotic B lineage cells using all of the above receptor systems. In addition to these observations, the results further demonstrate an unappreciated role for bone marrow stromal cells in clearing apoptotic cells via an integrin-dependent mechanism, thus defining a new role for the hemopoietic microenvironment in B cell development.

Materials and Methods

Mice

BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the vivarium of Division of Laboratory Animal Medicine at University of California (Los Angeles, CA). Mice were 4–6 wk old at the time of use.

Preparation of cell suspensions

Bone marrow cells were flushed from femurs using a syringe fitted with a 23-gauge needle into Ca2+/Mg2+-free PBS. Cells were counted with a hemocytometer, and cell viability was determined by eosin dye exclusion.

Long term bone marrow cultures

Long term myeloid bone marrow cultures were initiated as described by Dexter et al. (28) Briefly, the contents of a femur were flushed into a 25-cm2 tissue culture flask in α-MEM (Life Technologies, Grand Island,
NY) supplemented with 20% horse serum and 10^{-6} M sodium hydrocortisone succinate. Cell cultures were incubated at 33°C in a 5% CO_2/air incubator. After 2 wk, cultures were recharged with 10^5 bone marrow cells/flask. B cell development was induced by switching cultures (29) to the B lymphoid conditions described by Whitlock and Witte (30) (RPMI 1640 supplemented with 5% FCS and 5 \times 10^{-5} M 2-ME) and transferring the flask to a 5% CO_2/air incubator at 37°C. Cultures were used 4–6 wk after transfer to the B lymphoid-permissive conditions. At this time, virtually all the cultured cells were small to medium-sized lymphocytes by morphologic analysis.

**Stromal cell cultures**

Primary stromal cell cultures were generated by treating established, non-reconstituted, long-term myeloid bone marrow cultures with mycophenolic acid as previously described (31). This procedure depletes hematopoietic cells and leaves a viable adherent layer composed of stromal cells and macrophages. The generation and maintenance of the S17 bone marrow stromal cell line, which is capable of supporting long term myelopoiesis and B lymphopoiesis have been described previously (32).

**Preparation of bone marrow macrophages**

Bone marrow-derived macrophages were established by culturing bone marrow cells in MEM supplemented with t-valine (Mediatech, Herndon, VA), 10% FCS, 5 \times 10^{-5} M 2-ME, and 50 ng/ml GM-CSF (BioSource, Camarillo, CA). The cells were cultured in wells of chamber slides or in 24-well tissue culture plates. Seven days later, wells or slides were gently washed, leaving a nonconfluent layer of adherent macrophages.

**Flow cytometry**

Fresh bone marrow cells from which RBC were lysed by treatment with Tris-ammonium chloride (pH 7.2) or nonadherent cells from long term bone marrow cultures were suspended at 10^6 cells/sample in Ca^{2+}- and Mg^{2+}-free PBS. After incubation with an anti-CD16/CD32 Ab (FcγRII/III, clone 2.4G2; BD PharMingen, San Diego, CA) to reduce nonspecific labeling, cells were incubated with one or more of the following anti-mouse Abs: CD45R (PE-conjugated, BD PharMingen), IgM (Southern Biotechnology Associates, Birmingham, AL), and CD11b (clone M1/70.15; Caltag Laboratories, Burlingame, CA). These Abs were conjugated to FITC, PE, TriColor, or biotin. Biotinylated Abs were revealed with TriColor-conjugated streptavidin (Southern Biotechnology Associates). Optimal working dilutions were determined for each Ab before use. All incubations were performed in Ca^{2+}- and Mg^{2+}-free PBS at 4°C for 30 min. After the last wash, 10^6–10^7 live cells/sample were analyzed by flow cytometry on a FACScan (BD Biosciences, San Jose, CA) with CellQuest software (BD Biosciences, Mountain View, CA).

**Preparation of apoptotic cells**

Nonadherent cells harvested from long term bone marrow cultures were resuspended at 5 \times 10^5 cells/ml in RPMI 1640, 1% FCS, 5 \times 10^{-5} M 2-ME, and 1 \times 10^{-5} M hydrocortisone sodium succinate for 5 h to induce apoptosis. After treatment, cells were labeled with 2 μM Cell Tracker Green (5-chloromethyllfluorescein diacetate; Molecular Probes, Eugene, OR) in phenol-free RPMI 1640 supplemented with 5% FCS and 5 \times 10^{-5} M 2-ME at 37°C for 30 min according to the manufacturer’s specifications. In some experiments cells were labeled with YO-PRO-1 dye (Molecular Probes, Eugene, OR) at 37°C. Cells were subsequently washed three times and resuspended in serum-free medium before incubation with macrophages or stromal cells.

**Analysis of apoptosis and phagocytosis**

Cell Tracker Green- or YO-PRO-1-labeled cells in serum-free medium were added to cultures of adherent bone marrow-derived macrophages or S17 stromal cells. After a 90-min incubation at 37°C, wells or slides were gently washed four times with Ca^{2+}- and Mg^{2+}-free PBS to remove unbound cells. The cultures were then fixed with 1% formaldehyde for 10 min and washed with PBS, and the cultures were examined under epi-illumination with a fluorescent microscope (Leitz, Wetzlar, Germany).

Bone marrow-derived macrophages or stromal cells were incubated at 4°C for 30 min in the presence of the following reagents before addition of lymphocytes: poly(C) (50 μg/ml; Sigma-Aldrich, St. Louis, MO), chondroitin sulfate (100 μg/ml; Sigma-Aldrich), polyvinyl sulfate (50 μg/ml; Sigma-Aldrich), fucoidan (100 μg/ml; Sigma-Aldrich), RGDS (1 mM/ml; Sigma-Aldrich), RGES (1 mM/ml), purified anti-mouse CD14 (10 μg/ml, PharMingen), and purified anti-mouse CD31 (PECAM-1; 10 μg/ml, PharMingen). The studies using anti-CD14 and anti-CD31 Abs included appropriate isotype control Abs. In some experiments lymphoid cells were pretreated with anti-CD31 Abs or the appropriate isotype control. After incubation with the indicated blocking agent, cells were washed in medium before use.

**Lymphocytes**

Lymphocytes that were clearly bound to the surface of or internalized within a macrophage or stromal cell were enumerated as phagocytosed cells. The percent inhibition in cultures to which various specific inhibitors (RGDS, fucoidan, polyvinyl sulfate, and anti-CD14) were added was normalized to that in control cultures (untreated, isotype control Ab, poly C, and chondroitin sulfate) using the following formula: percentage phagocytosis = (number of lymphoid cells phagocytosed in inhibitor-containing cultures/number of lymphoid cells phagocytosed in control cultures) \times 100.

**Confocal microscopy**

In some experiments confocal microscopy was used to examine the association between apoptotic lymphocytes and stromal cells. After their culture in corticosteroids, lymphocytes were labeled with YO-PRO-1 and incubated with S17 stromal cells grown to subconfluence on microscope slides. The conditions for corticosteroid treatment of lymphocytes, labeling with YO-PRO-1, and incubation with stromal cells were identical with those described above for macrophages. Confocal microscopy was performed using a Fluoview laser scanning confocal microscope (Olympus, Melville, NY). Samples were excited at 488 nm with an argon laser, and light emitted between 525 and 540 nm was recorded in channel 1. Differential interference contrast images were obtained by collecting transmitted light in channel 2. Images were generated and analyzed using Fluoview software (version 2.139).

**Detection of DNA fragmentation**

Cells were lysed by incubation in extraction buffer (0.25 M NaCl, 0.1% SDS, 10 mM EDTA, and 1 mM Tris, pH 7.5) at room temperature for 30 min. The lysates were centrifuged and incubated with 20 μM RNase A (Promega, Madison, WI) for 30 min at 37°C. Proteinase K (100 μg/ml; Sigma-Aldrich) was added to the suspensions for 30 min at 37°C. After electrophoresis on 1.8% agarose gels, DNA fragmentation was visualized by staining with SYBR Green Gold nucleic dye (Molecular Probes, Leiden, The Netherlands).

**RT-PCR analysis**

Poly(A) mRNA was isolated using the MicroFastTrack 2.0 kit according to the manufacturer’s (Invitrogen, Carlsbad, CA) instructions. RT was performed using 200 U of SuperScript II reverse transcriptase (Invitrogen), 20 U of RNasin (Promega), 1 mM dNTPs, 0.5 μg oligo(dT) (Promega), 1 μg of hexanucleotide random primer (Promega), and 10 mM DTT in 20 μl of RT-DS, for 1 h at 37°C. PCR was performed using 1× PCR buffer; 2 mM MgCl_2; 0.2 μM each of dATP, dGTP, dCTP, and dTTP; and 2.5 μl of AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Foster City, CA) in a total volume of 50 μl. The primers used for PCR are shown in Table I along with the expected sizes of the products. Controls included template negative samples to test for amplification of contaminants. PCR conditions included the following: SR-A and GAPDH: 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min; αβ; 30 cycles of 95°C for 2 min, 60°C for 2 min, and 72°C for 3 min; β2; 30 cycles of 95°C for 30 s, 51°C 30 s, and 72°C 30 s; CD14: 30 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 90 s; CD31 (PECAM-1): 30 cycles of 95°C for 45 s, 58°C for 45 s, and 72°C for 1 min. The amplified products were separated on 1% agarose gels containing 0.25 mg/ml ethidium bromide, and bands were visualized and photographed using UV transillumination.

**Statistical analysis**

All experiments were repeated at least four times. The significance of the data was evaluated by single-tailed Student’s t test.

**Results**

**Generation of apoptotic B lineage cells**

To identify the cells and molecules involved in the clearance of apoptotic lymphoid lineage cells from the bone marrow, it was necessary to develop a coculture system in which the interactions between these populations and phagocytes that potentially eliminate them could be dissected.
Purified macrophages were generated by culturing bone marrow cells in medium supplemented with GM-CSF. After a 7-day incubation, this protocol yielded a population in which >70% of the cells expressed CD11b (data not shown). Obtaining a reliable source of apoptotic B lineage cells was more problematic. As shown in Fig. 1A, CD45R<sup>+</sup> cells account for more than one-third of nucleated bone marrow cells, but only ~2.5% of the B lineage cells in freshly harvested marrow are apoptotic as indicated by annexin V staining. A comparable low frequency of apoptotic B lineage cells was present in long term B lineage cultures (Fig. 1B). Therefore, attempts were made to increase their frequency by exogenous steroid administration. The administration of corticosteroids to mice had little effect on the frequency of apoptotic B cells undergoing apoptosis (33), and studies confirmed that the YOPRO-1-labeled cells were also annexin V positive (data not shown). The increased frequency of apoptosis in cells harvested from the cultures was corroborated by the data in Fig. 1C showing increased DNA fragmentation. A feature of lymphoid long term bone marrow cultures is that not all cells in them express CD45R even though virtually all the cultured cells are small to medium-sized lymphocytes (29). Consistent with this fact is that those cells that are CD45R negative, annexin V positive displayed the light scatter characteristics of lymphocytes by FACS analysis (data not shown) and are also lymphoid lineage cells.

**Macrophages phagocytose bone marrow-derived lymphoid cells undergoing apoptosis**

To examine the potential of macrophages to clear apoptotic cells, bone marrow-derived macrophages were incubated with YOPRO-1-labeled steroid-treated lymphoid cells from lymphoid long term bone marrow cultures. This dye is preferentially incorporated into cells undergoing apoptosis (33), and studies confirmed that the YOPRO-1-labeled cells were also annexin V positive (data not shown). Fig. 2A shows one YOPRO-1 labeled cell within a macrophage and another that has bound to the macrophage cell membrane. The data in Fig. 2, B and C, show that >80% of the YOPRO-1-labeled lymphocytes in the cultures were primarily associated with macrophages, whereas the majority of the non-YOPRO-1-labeled lymphocytes were not macrophage associated. These findings indicate a preferential association of apoptotic lymphocytes with macrophages and are consistent with a report that viable lymphoid cells do not generally bind directly to macrophages in lymphoid long term bone marrow cultures (34).

Because of the preferential uptake of apoptotic lymphoid cells in this system and the instability of YOPRO-1 when exposed to UV light, steroid-treated, lymphoid cells from long term bone marrow cultures were labeled with Cell Tracker Green in the remaining experiments. Although this dye does not preferentially label dying cells, its fluorescence is not rapidly quenched, thereby allowing the hemopoietic cells to be easily visualized and counted under phase contrast fluorescence microscopy.

**Identification of macrophage receptors that mediate phagocytosis of lymphoid cells**

Multiple classes of receptors expressed by macrophages that include CD14, integrins, and SR-A have been proposed to mediate

| Table I. Oligonucleotides used for detection of mRNA by RT-PCR |
|-----------------|-----------------|-----------------|
| PCR Product     | Sequence (5’ to 3’) | Expected Size (bp) |
| SR-A            | Sense CCA AGT CCT TGC AGA TGC TG | 236 |
|                 | Antisense GTC TGA GGT CGT TGG TGA TG | |
| CD14            | Sense GGA AGC CAG AGA ACA CCA TCG | 413 |
|                 | Antisense GCA GGG CTC CGA ATA GAA TCC | |
| α<sub>i</sub>   | Sense CCG CCG GTG CCA GCC CAT TGA G | 337 |
|                 | Antisense GCT ACC AGG ACC GAG AAG T | |
| β<sub>i</sub>   | Sense CTG GTG TTT ACC ACT GAT GCC AAG | 393 |
|                 | Antisense TGT TGA GCC AGG TGG CAT TGA AGG | |
| β<sub>3</sub>   | Sense GCG AAA AGA TGC TCT GCA | 598 |
|                 | Antisense GCC GCA TGT GCA ATT GTA | |
| CD31            | Sense AGG GGA CCA GCT GCA CAT TAG G | 452 |
|                 | Antisense AGG CCG CTT CTC TTG ACC ACT T | |

**FIGURE 1.** Detection of apoptotic cells in fresh and cultured bone marrow. A. Freshly harvested bone marrow cells were labeled with Abs to CD45R and annexin V. B. Cells from lymphoid long term bone marrow cultures were labeled with Abs to CD45R and annexin V. Left panel, Labeling of nonsteroid-treated cells from cultures; right panel, lymphoid cells harvested from cultures were treated with 1 × 10<sup>-5</sup> M hydrocortisone sodium succinate for 5 h. C. DNA was prepared from the cells described in B, and DNA fragmentation was detected by electrophoresis on 1.8% agarose gels.
phagocytosis of apoptotic cells (9, 19–24). Whether these receptors are involved in macrophage-mediated clearance of B lineage cells from the bone marrow has not been determined.

The data in Fig. 3A confirm that the bone marrow-derived macrophages generated in these studies express SR-A, CD14, and various integrin family members. Whether these receptors play a role in phagocytosis of apoptotic B lineage cells was tested by blocking them with specific inhibitors before their cocultures with Cell Tracker Green-labeled lymphocytes from lymphoid long term bone marrow cultures. Pretreatment of macrophages with the SR-A ligands fucoidan or polyvinyl sulfate inhibited phagocytosis by 40–50%, whereas the structurally related, non-SR-A ligands poly(C) and chondroitin sulfate had no effect (Fig. 3B). CD14 binding was inhibited by preincubation of macrophages with an anti-CD14 Ab, and this protocol resulted in a 25% inhibition of macrophage phagocytic activity (Fig. 3C). Finally, inhibition of binding to integrin receptors with the RGDS tetrapeptide inhibited phagocytosis by 35%, whereas the RGES peptide had no effect (Fig. 3D). Taken together, the data indicate that macrophages use multiple receptor systems to phagocytose apoptotic lymphoid cells.

FIGURE 2. Macrophages phagocytose apoptotic lymphoid cells. A, Interaction of YOPRO-1-labeled steroid-treated, lymphoid cells from long term bone marrow cultures in coculture with bone marrow macrophages. The fluorescence image is on the left, and the phase image is on the right. The macrophage on the left has internalized a YOPRO-1-labeled lymphocyte, whereas the macrophage on the right is associated with a YOPRO-1-labeled lymphocyte on its surface. B, Steroid-treated, lymphoid cells from long term bone marrow cultures were labeled with YOPRO-1 and cultured with bone marrow macrophages. The figure demonstrates that those lymphocytes that were bound to or internalized within macrophages were primarily those labeled with YOPRO-1. The frequencies were obtained based on counts of 300 total lymphocytes in multiple fields. C, The photomicrograph shows YOPRO-1-labeled lymphocytes within macrophages (arrows) and several non-YOPRO-1-labeled lymphocytes not associated with macrophages (*). D, Bone marrow stromal cells can internalize apoptotic lymphoid cells. Three confocal images are shown. From left to right these include a phase contrast view of stromal cell cultures, a fluorescent image of YOPRO-1-labeled lymphocytes, and a merged image. Note that the lymphocyte within the stromal cell at the upper left is relatively intact, but its nucleus is lobulated. The lymphocyte phagocytosed by the stromal cell at the lower right has been degraded, and the YOPRO-1 dye is beginning to diffuse throughout the stromal cell cytoplasm.

FIGURE 3. Identification of macrophage receptors involved in phagocytosis of apoptotic cells. A, RT-PCR analysis of receptor expression on bone marrow macrophages (BM), stromal cells, and macrophage-containing adherent layers from mycophenolic acid-treated, long-term bone marrow cultures (adherent layer) and the S17 bone marrow stromal cell line (stromal cells). H2O indicates control reactions containing water and no template. B–D, Macrophages were preincubated with specific inhibitors before addition of steroid-treated, lymphoid cells from long term bone marrow cultures. B, Addition of the SR-A-specific inhibitors fucoidan and polyvinyl sulfate and structurally related non-SR-A inhibitors poly(C) and chondroitin sulfate. C, Addition of anti-CD14 Abs or IgG1 isotype control. D, Addition of RGDS integrin binding peptide or a nonbinding RGES peptide. The percent phagocytosis determined as indicated in Materials and Methods. #, p < 0.025; *, p < 0.005; **, p < 0.0025 (relative to untreated control cultures). All experiments were repeated four times.
Cells undergoing apoptosis express cell surface determinants that allow their recognition by phagocytes. The recent description of CD31 (PECAM-1) on apoptotic leukocytes prompted an analysis of its role in the clearance of apoptotic B lineage cells.

As shown in Fig. 4A, corticosteroid-treated, lymphoid cells from long term bone marrow cultures express CD31. The data in Fig. 4B further suggest that CD31 is preferentially expressed by cells undergoing apoptosis, because >70% of the annexin V− cells were CD31+. Approximately one-fifth of the annexin V-negative cells also expressed CD31, but whether these cells were destined to undergo programmed cell death was not determined.

Functional studies were conducted to determine whether blocking of CD31 on corticosteroid steroid-treated, lymphoid cells from long term lymphoid cultures interfered with their clearance by macrophages. As shown in Fig. 4C, anti-CD31 treatment inhibited their phagocytosis by ~25%. CD31 is also expressed by macrophages (Fig. 4A), and their pretreatment with anti-CD31 Abs also inhibited phagocytosis. Pretreatment of both macrophages and lymphoid cells with anti-CD31 increased the inhibition of phagocytosis to ~35% (Fig. 4C).

The above data indicate that homophilic interactions between macrophages and apoptotic lymphocytes occur through CD31. In addition, heterophilic interactions between CD31 and αβ integrin receptors have been described (35). To determine whether these latter interactions were involved in the clearance of apoptotic bone marrow lymphocytes, anti-CD31-treated lymphoid cells were cocultured with RGDS-treated macrophages. As shown in Fig. 4C, this combined treatment did not increase the degree to which the anti-CD31-treated lymphoid cells were phagocyted, suggesting that heterophilic interactions between lymphocyte CD31 and integrins expressed by macrophages are not involved in clearance of the former cells.

Bone marrow stromal cells can phagocytose apoptotic B lineage cells

Although macrophages are present in the adherent layer of long term bone marrow cultures, stromal cells are the predominant population in that compartment. In view of this, further analyses were performed to determine whether stromal cells could also participate in the clearance of apoptotic lymphoid cells. Because the S17 stromal cell line can support both myelopoiesis and B lymphopoiesis (32), it was used as a model to test this hypothesis. As shown by the confocal images in Fig. 2D, stromal cells can internalize apoptotic B lineage cells.

These results prompted attempts to identify receptors involved in this process. PCR analysis demonstrated that stromal cells expressed CD14 and integrins, but not SR-A or CD-31 (Figs. 3A and 4A). Consistent with these results was the observation that preincubation of S17 stromal cells with the SR-A inhibitors fucoidan and polyvinyl sulfate did not inhibit their ability to phagocytose lymphoid cells (Fig. 5A). In addition, even though stromal cells express CD14, this cell surface determinant did not appear to play a role in phagocytosis, because pretreatment with anti-CD14 Abs was not inhibitory (Fig. 5B). However, pretreatment of stromal cells with RGDS significantly inhibited the uptake of apoptotic lymphoid cells (Fig. 5A).

Discussion

The efficient clearance of apoptotic cells from tissues is critical to prevent inflammation mediated by the release of toxic intracellular components. In addition, apoptotic cells have been suggested to be a source of autoantigens, so their rapid removal may be important in minimizing the development of autoimmune disease (8–12). It seems evident that the elimination of apoptotic B lineage cells from the bone marrow is a highly efficient process, because few annexin V-labeled B lineage cells can be detected in vivo. The aim of this study was to test the prevailing view that the clearance of apoptotic B lineage cells in the bone marrow is mediated by macrophages and, if so, to identify the cell surface receptors used by those scavengers to do so.

Initial efforts focused on developing a system to investigate the fate of apoptotic B lineage cells. Macrophage cell lines or activated peritoneal macrophages have been used in some studies (36). However, to approximate the in vivo situation more closely, bone marrow macrophages were obtained by growing bone marrow cells in recombinant...
GM-CSF, a process that yielded a relatively pure macrophage population. More challenging was the generation of significant numbers of apoptotic B lineage cells. Few apoptotic B lineage cells were detected in freshly harvested bone marrow. Although treatment of mice with steroids significantly reduced the frequency of B lineage cells, few CD45R− cells labeled with annexin V were observed, indicating that the clearance of apoptotic cells in vivo is an extremely efficient process. Few apoptotic cells were present in lymphoid long term bone marrow cultures as well, indicating that the mechanism(s) for their removal is also operative in this in vitro system. Therefore, subsequent studies used a coculture system in which CD45R+ cells harvested from lymphoid long term bone marrow cultures were treated with steroids and mixed with primary cultures of bone marrow-derived macrophages. A high proportion of these lymphoid cells were annexin V+. It has been reported that annexin V may label some bone marrow B lineage cells that are viable (37). This would not seem to be an issue in this study, because after steroid treatment, the annexin V+ cells from lymphoid long term bone marrow cultures exhibited considerable DNA fragmentation and were labeled with YOPRO-1 dye. The macrophages preferentially bound and phagocytosed apoptotic, YOPRO-1-labeled lymphoid cells in these cultures, indicating the appropriateness of the coculture system for dissecting the mechanisms involved in interactions between these two cell populations.

The results demonstrated that bone marrow macrophages identify apoptotic lymphoid cells via CD14, integrin receptors, and SR-A. It is important to stress that although the bone marrow-derived macrophages that were analyzed had been harvested from GM-CSF-stimulated cultures of fresh bone marrow, the expression of these receptors was not an artifact of cytokine activation in vitro. In situ studies of both murine and human bone marrow sections demonstrate that resident macrophages that express SR-A, CD14, and CD31 are present in normal bone marrow (38–40). That multiple macrophage receptors are involved in clearing apoptotic bone marrow-derived lymphoid cells is consistent with the results of studies in which the elimination of apoptotic thymocytes was examined in the SR-A knockout mouse (41). Thymic macrophages use SR-A in clearing apoptotic thymocytes (16). However, even when the rate of thymocyte apoptosis was increased by treatment of SR-A knockout mice with gamma irradiation, dying cells were still efficiently eliminated in vivo, thereby indicating the involvement of multiple receptor systems in this process.

Similar to what was reported in studies in which macrophages were treated with Abs to SR-A (16), it was difficult to completely inhibit the clearance of apoptotic lymphoid cells in the experiments reported in this study. For example, treatment of macrophages in this study with inhibitors of SR-A or with anti-SR-A Abs in the aforementioned studies of thymocyte apoptosis (16) resulted in only 50% inhibition of phagocytosis. One reason for this partial inhibition of phagocytosis is undoubtedly due to the involvement of multiple receptor systems. In addition, it is important to note that the present studies were performed under serum-free conditions. This is relevant because serum factors such as complement, which would not be present in the coculture system used in this study, have been shown to be involved in the clearance of dying cells (24, 42).

In addition to receptors expressed on macrophages, attempts were made to identify molecules expressed on apoptotic B lineage cells that would allow their recognition by phagocytes. Recent reports have identified CD31, also referred to as PECAM-1, as mediating homotypic interactions between macrophages and apoptotic leukocytes. Homophilic ligation of CD31 on viable cells results in their mutual repulsion, whereas CD31-mediated detachment was disabled in apoptotic neutrophils (27). Less is known about the involvement of CD31 in the clearance of apoptotic B lineage cells. Although there is evidence that CD31 plays a role in B cell development and B cell receptor-mediated activation (43), the data in this study indicate that the majority of CD45R+ annexin V+ cells express CD31. Conversely, <20% of the CD45R− annexin V+ cells are CD31+. The involvement of CD31 in homotypic interactions between apoptotic B lineage cells and macrophages was suggested by data demonstrating that pretreatment of either population alone or in combination inhibited the clearance of the latter population by ~20%. In contrast, no role for heterophilic interactions between lymphocyte CD31 and macrophage integrins was found.

The data also revealed a role for bone marrow stromal cells in the clearance of apoptotic lymphoid cells. This conclusion is based on the observation that stromal cells were able to internalize YOPRO-1-labeled B lineage cells, as demonstrated by confocal microscopy. Unlike macrophages, bone marrow stromal cells did not mediate their phagocytic effects via the SR-A or CD14 pathways, but instead used an integrin-dependent mechanism. That nonprofessional phagocytes, such as stromal cells, can clear cells undergoing apoptosis is not unprecedented, as other cell types, such as epithelial cells, have been implicated in this process (44, 45). Indeed, a role for thymic nurse cells in the phagocytosis of apoptotic thymocytes has been reported (46, 47), but a similar function for bone marrow stromal cells has not been described. This finding ascribes a new functional role to bone marrow stromal cells in addition to supporting hematopoietic cell development. This observation also places the findings of Lu and Osmond (14) in a new context. Those investigators reported that coculture of CD45R− IgM− bone marrow cells with stromal cells reduced the apoptotic rate compared with that in cells cultured in medium alone. Stromal cell-derived signals may provide survival signals for the B lineage cells, as suggested in that report. However, the data in this study suggest that the increased survival rate could also
be due to the removal of the dying B lineage cells by the stroma, thereby decreasing the relative proportion of viable cells.

The uptake of apoptotic lymphoid cells by bone marrow stromal cells is not a rare event. For example, confocal microscopy studies revealed that whereas individual stromal cells that had not phagocytosed apoptotic lymphocytes were observed, it was rare to observe YOPRO-1-labeled lymphocytes that had not been internalized within stromal cells. Further evidence that stromal cells clear apoptotic B lineage cells efficiently is that the frequency of annexin V+ cells in long term lymphoid bone marrow cultures from BALB/c mouse bone marrow (Fig. 1A) is similar to that observed in vivo (Fig. 1B). This observation is significant, because CD11b+ macrophages are a majority of cells in the long term cultures. The remaining hematopoietic cells are lymphoid, and the majority of nonhemopoietic cells in the adherent layer are stromal cells. Thus, if stromal cells did not efficiently eliminate apoptotic cells, a higher frequency of apoptotic B lineage cells should have been observed in the cultures than in vivo. However, additional studies will be needed to determine how the efficiency with which stromal cells clear apoptotic B lineage cells compares to that of macrophages.

In summary, only a proportion of developing B lineage cells successfully rearrange their Ig genes, and those that do not have no functional relevance to the organism and are thought to undergo programmed cell death and rapid removal from the bone marrow. Because few apoptotic B lineage cells can be detected in the bone marrow at any given time, they must be rapidly removed. The present results have confirmed that macrophages are involved in this process and have described mechanisms by which these cells may eliminate apoptotic B lineage cells from the bone marrow. In addition, the data have revealed a heretofore unappreciated role for bone marrow stromal cells in this process.

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