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Epithelial cells as phagocytes: apoptotic epithelial cells are engulfed by mammary alveolar epithelial cells and repress inflammatory mediator release

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Abstract

Clearance of apoptotic cells is critical to tissue homeostasis and resolution of inflammatory lesions. Macrophages are known to remove dying cells and release anti-inflammatory mediators in response; however, many cells traditionally thought of as poor phagocytes can mediate this function as well. In the lactating mammary gland following weaning, alveolar epithelial cell death is massive, yet the gland involutes rapidly, attaining its prepregnancy state in a matter of days. We found histologic evidence of apoptotic cell phagocytosis by viable mammary epithelial cells (MEC) in the involuting mouse mammary gland. Cultured MEC were able to engulf apoptotic cells *in vitro*, utilizing many of the same receptors used by macrophages, including the phosphatidylserine receptor (PSR), CD36, the vitronectin receptor $\alpha_v\beta_3$, and CD91. In addition, MEC, like macrophages, produced TGFβ in response to stimulation of the PSR by apoptotic cells or the anti-PSR ab 217G8E9, and downregulated endotoxin-stimulated proinflammatory cytokine production. These data support the hypothesis that amateur phagocytes play a significant role in apoptotic cell clearance and its regulation of inflammation.
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Abbreviations: PSR, phosphatidylserine receptor; MEC, mammary epithelial cell; PMEC, primary MEC; CD91/LRP, low-density lipoprotein-related receptor; MBP, mannose-binding protein

Introduction

The mechanisms for recognition and engulfment of apoptotic cells by macrophages are legion, and have been reviewed in some detail.[1–9] Many of these receptors are pattern recognition receptors also utilized for uptake of microbial organisms or their byproducts. The paradox is that the consequences for the macrophage following uptake of microbes compared to those observed after uptake of apoptotic cells are diametrically opposed. Phagocytosis of microbial organisms is followed by a proinflammatory response, which enables the activation of a productive immune response. By contrast, apoptotic cells fail to induce inflammation and, in many cases, they induce an anti-inflammatory reaction, thus presumably inhibiting an immune response. We have suggested that the difference lies in the fact that most pathogens fail to activate the receptor for phosphatidylserine (PSR), in contrast to apoptotic cells, which do.[6] In fact, certain pathogens (trypanosomes, *Leishmania* spp.) have evolved ways to evade immune destruction precisely by utilizing the same anti-inflammatory mechanisms utilized by apoptotic cells.[10–12]

Efficient removal of apoptotic cells is believed to be necessary for tissue development and homeostasis, as well as protection against neoplasia and chronic inflammation. The mammary gland provides a unique and ideal system in which to study the physiological removal of apoptotic cells, because it undergoes cycles of repetitive growth, death, and remodeling during the normal reproductive cycles of the adult female mammal. Given that macrophages have been shown to be dispensable with regard to apoptotic cell clearance, at least during development,[13] and given the reported role for mammary epithelial cells (MEC) in apoptotic cell engulfment during involution,[14] we chose to study MEC as a model 'amateur' phagocyte both *in vivo* and *in vitro*.

Little is known about receptors utilized for apoptotic cell uptake by epithelial cells or other 'amateur' phagocytes, yet it is clear that a variety of cells possess the ability to engulf apoptotic cells. These include epithelial cells, mesangial cells, vascular smooth muscle cells, hepatic cells, and endothelial cells.[15–28] It has been shown that integrins and the PSR are used by lung alveolar epithelial cells in the engulfment of apoptotic eosinophils,[15,16] but the consequences of apoptotic cell uptake on these amateur phagocytes are not known. Understanding how amateur phagocytes engulf and respond to apoptotic cells is important, because these cells can serve as important sources of cytokines (e.g. see Boudjellab *et al.*[29,30]), thus having the potential to contribute significantly to the inflammatory response. Epithelial cells, in particular, produce a variety of cytokines, and can initiate an inflammatory response themselves in response to noxious environmental stimuli.

In order to understand how epithelial cells recognize and respond to apoptotic cells, we studied phagocytosis during mammary gland involution *in vivo*, and modeled this process *in vitro* using the murine mammary epithelial cell lines HC-11[31] and EpH4,[32] as well as primary mammary epithelial cells (PMEC) isolated from mid-pregnant mice. MEC were able to act as phagocytes, in that they bound and engulfed apoptotic MEC, albeit much less efficiently than macrophages. They expressed

and utilized many of the receptors used by macrophages, including the PSR; its stimulation in macrophages has been shown to cause membrane ruffling, rapid cytoskeletal actin reorganization, and macropinocytosis.[33] In addition, following exposure to apoptotic cells, MEC-secreted TGFβ1 down-regulated LPS-induced proinflammatory cytokine release. The implications of these findings are that epithelial cells can contribute significantly to apoptotic cell clearance in the involuting mammary gland, and that apoptotic cell uptake by amateur phagocytes warrants serious study in other organs.

Results

Mammary epithelial cells engulf apoptotic bodies *in vivo* and *in vitro*

We began our studies by histologic analysis of mammary glands induced to involute by forced weaning at day 14. The third and fourth glands on each side were harvested at various time points and processed for light and electron microscopy. During early involution, apoptotic epithelial cells were seen to be released from the basement membrane zone and were subsequently engulfed by their viable epithelial neighbors. Shed cells were evident within the lumen of an alveolus in routine hematoxylin and eosin-stained paraffin sections. (Figure 1a, arrow) Additionally, rounded apoptotic cells were found within phagosomes in healthy cells within the epithelial monolayer. (Figure 1a, arrowheads) These cells were shown to be apoptotic both by morphology in electron micrographs (Figure 1c) and by immunostaining with an antibody to caspase-cleaved keratin-18 (Figure 1b).

In order to begin to describe the mechanisms used by MEC to clear apoptotic cells during gland remodeling, we used the murine mammary epithelial cell lines HC-11 and EpH4, as well as PMEC isolated from mid-pregnant mice. Figure 2 shows that both HC-11 MEC, which have a more mesenchymal phenotype, and EpH4 cells, which have a polarized epithelial phenotype, bound and engulfed apoptotic HC-11 cells. Figure 2a shows a fluorescent photomicrograph showing the engulfment of apoptotic HC-11 cells (stained red) by HC-11 and EpH-4 cells (stained green). In Figure 2b, an electron micrograph shows HC-11 cells, which have bound and engulfed apoptotic bodies showing condensed chromatin, as well as smaller membrane bound bodies derived from late-stage apoptotic cells.

Mammary epithelial cells express and utilize many receptors for phagocytosis of apoptotic cells

Flow cytometry analysis (Figure 3a) shows that the MEC expressed CD36 and the $\alpha_v\beta_3$ integrin (vitronectin receptor, VnR), implicated in apoptotic cell uptake by macrophages.[34,35] Additionally, MEC expressed surface calreticulin and CD91, thought to act together to promote the binding of macrophages to collectin-opsonized apoptotic cells.[36,37]

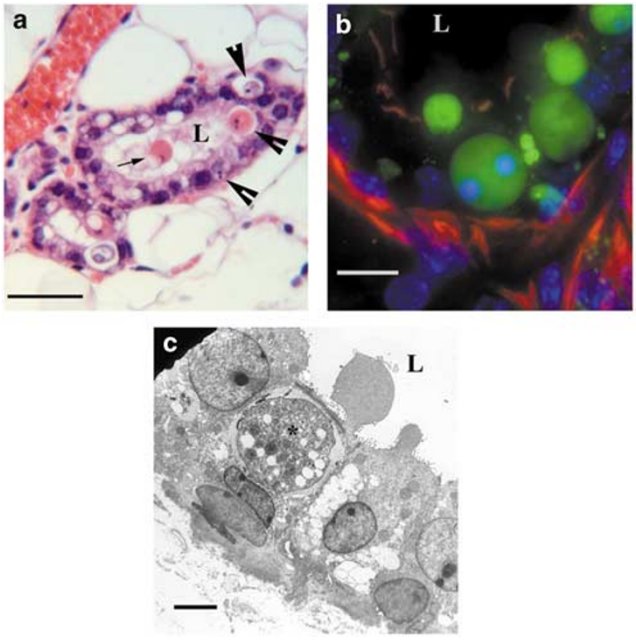

Figure 1 MEC engulf apoptotic cells *in vivo*. (**a**) Hematoxylin and eosin staining of paraffin-embedded tissue taken 3 days after weaning. Arrow shows an apoptotic cell shed into the lumen of the alveolus. Arrowheads point to apoptotic bodies, which appear to be contained within viable neighbors. Bar is 50 μm. (**b**) Frozen sections (8 μm thick) of mammary gland tissue harvested 3 days after weaning were stained with Cytodeath, an antibody recognizing caspase-cleaved keratin-18, and counterstained with rhodamine–phalloidin and DAPI. Optical slices through the tissue are shown as the maximum intensity projection of the data. Intact and fragmented apoptotic bodies within phagosomes in viable epithelial cells are shown. Bar is 10 μm (**c**). Electron micrograph of an engulfed apoptotic cell (asterisk) within a mammary epithelial cell, 1.5 days postwean. The lumen of the alveolus is marked with an L. Bar is 5 μm

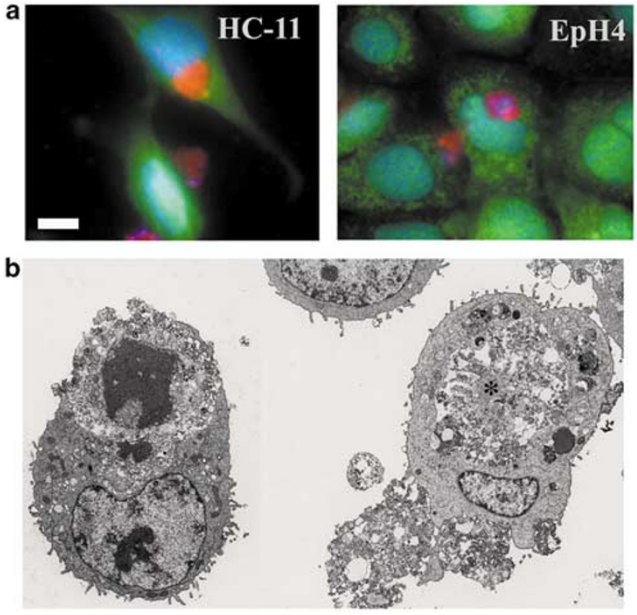

Figure 2 MEC bind and engulf apoptotic cells *in vitro*. (**a**) HC-11 and EpH4 cells, labeled with Cell Tracker Green, were incubated with Texas red-labeled apoptotic HC-11 cells. The complexes were counterstained with Hoechst. Bar is 5 μm. Large vacuoles within the green phagocytes are evident with red-stained apoptotic bodies within. (**b**) HC-11 cells were incubated with apoptotic HC-11 cells and the complexes were processed for electron microscopy. Two phagocytic complexes, one with dead material inside a large phagosome (asterisk) are shown

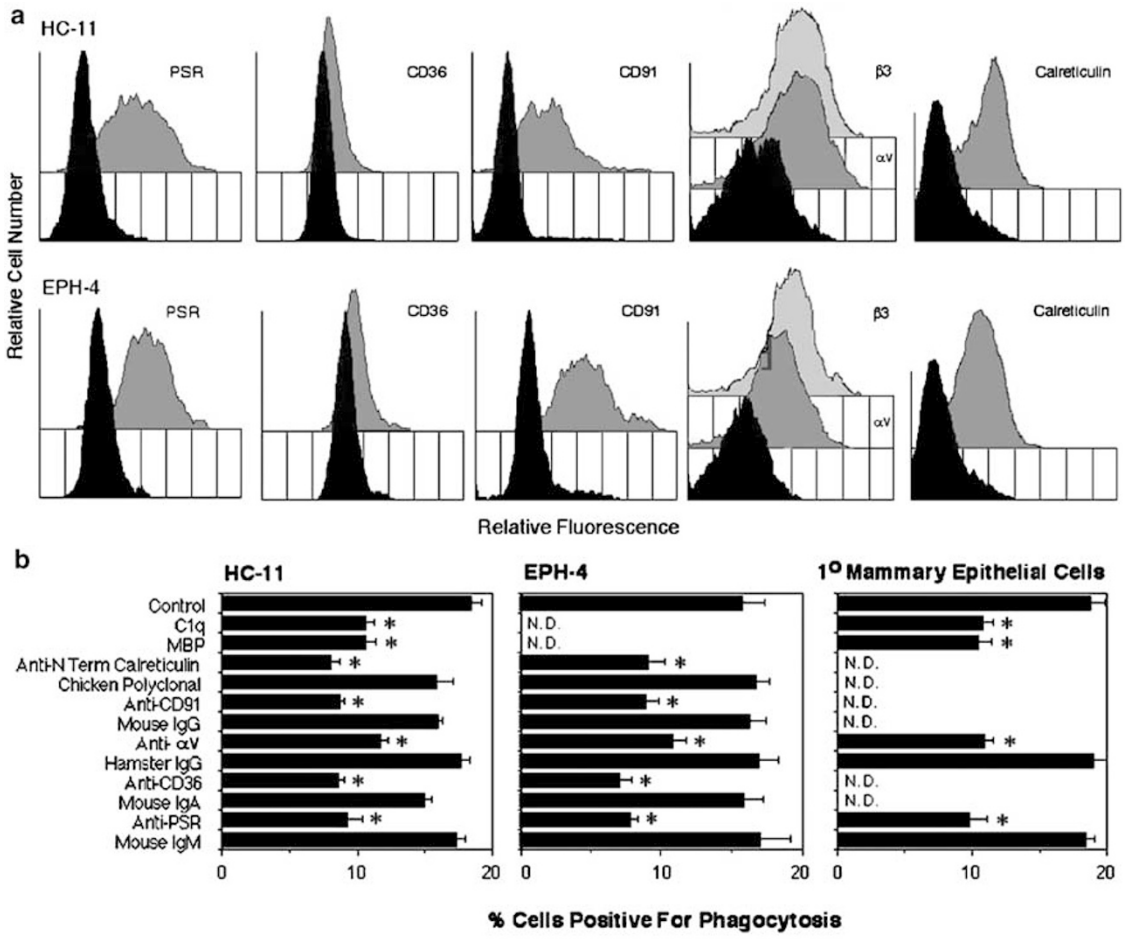

Figure 3 MEC express and utilize several receptors to bind and engulf apoptotic cells. (**a**) Analysis of receptor expression on HC-11 and EpH4 cells by flow cytometry. The isotype control for each antibody is shown in black with specific staining shown in gray. (**b**) HC-11, EpH-4, or primary mouse mammary epithelial cells (PMEC) were cultured in 24-well plates. Potential inhibitors of uptake were added 30 min prior to addition of apoptotic HC-11 cells as the target. The cells were incubated for 3 h, then washed and fixed. Phagocytosis is expressed as % epithelial cells positive for apoptotic bodies. $N = 12 \pm$ S.E.M. for HC-11 and PMEC; $N = 3 \pm$ for EpH 4 cells. *Represents those numbers significantly different from the control phagocytosis ($P < 0.0001$). N.D. indicates those inhibitors that are not determined. C1q is the first component of complement; MBL is mannose-binding lectin, Anti-N term calreticulin is the antibody directed against the N terminus of calreticulin and the chicken polyclonal is the isotype control, anti-PSR is the monoclonal antibody 217G8E9. For each antibody, the isotype control is shown directly beneath

Lastly MEC expressed PSR.[38] These cells were tested for surface expression of scavenger receptor A, CD14, and CD11b/CD18 (Mac-1) implicated in uptake of apoptotic cells by macrophages,[39–43] but were found to be negative (not shown). These cells also do not express classical F_c receptors and preliminary experiments suggested tht they do not engulf cells opsonized with mouse IgG (data not shown).

In Figure 3b, we show that antibodies to calreticulin, CD91, PSR, CD36, and the $\alpha_v\beta_3$ VnR reduced phagocytosis of apoptotic HC-11 cells by MEC. In addition, preincubation of the epithelial phagocytes with C1q or mannose-binding lectin also blocked uptake of apoptotic cells by HC-11 and PMEC, as was observed for macrophages.[36]

Mammary epithelial cells express PSR on their cell surfaces in a punctate pattern

We had demonstrated expression of PSR on the cell surface of epithelial cells initially by flow cytometry. We then undertook to demonstrate its presence by immunohistochemistry utilizing monoclonal antibody 217G8E9 (mAb217). The receptor appeared to be localized in small, undefined membrane microdomains on the surface. Background binding of non-specific mouse IgM to these cells was minimal (Figure 4).

Mammary epithelial cells respond to apoptotic cells by releasing TGFβ and downregulating endotoxin-induced TNFα

We and others[44–47] have shown that macrophages exposed to apoptotic cells release anti-inflammatory mediators. These results can be mimicked by exposure to phosphatidylserine-containing liposomes or by direct stimulation of the PSR by the mAb217. Figure 5 shows that mammary epithelial cells have the same response. HC-11 cells stimulated with 2 μg/ml lipopolysaccharide (*Escherichia coli*) for 24 h secreted TNFα (Figure 5a) and MIP2 (Figure 5b). This response was abrogated by concurrent stimulation of the PtdSerR with

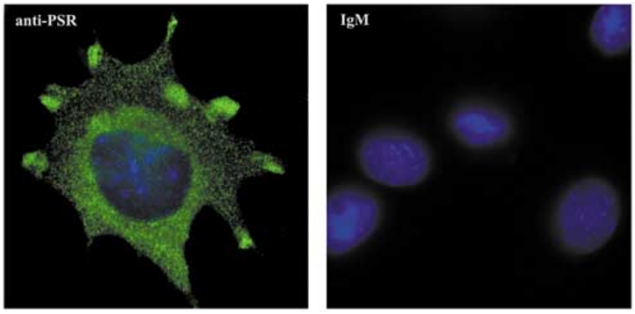

Figure 4 The PSR is expressed on the surface of cultured MEC. (**a**) Immunostaining of HC-11 cells with mAb 217 (shown in green) shows a punctate, surface localization of the PtdSerR. Cells are counterstained with DAPI. 'IgM' denotes the isotype control for anti-PtdSerR antibody

either mAb217 or phosphatidylserine-expressing apoptotic cells (Jurkat T cells). The inhibition appeared to be specifically mediated by an interaction between phosphatidylserine on the dying cell and PSR, as apoptotic PLB 985 cells, which fail to expose phosphatidyserine,[48] had no effect. As was observed for macrophages,[45] incubation of endotoxin-stimulated HC-11 cells with anti-TGFβ antibody partially blocked ($59 \pm 5.2\%$) the anti-inflammatory effects of the apoptotic cells, suggesting that TGFβ plays a role in the anti-inflammatory effects of apoptotic cells on epithelial proinflammatory cytokine production. In fact, HC-11 cells (Figure 5c) and PMEC (Figure 5d) isolated from a mid-pregnant mouse mammary gland were found to secrete TGFβ in response to apoptotic cells.

Discussion

We have shown that MEC, like macrophages, will bind and engulf apoptotic cells and that the consequences of such engulfment include the release of anti-inflammatory mediators and the repression of proinflammatory mediators. Further, we have shown that these cells utilize several receptors during the binding and phagocytic processes, and that mammary epithelial cells, like macrophages, respond to triggering of the PSR by membrane ruffling, suggesting that the mechanisms utilized for engulfment and many of the receptors are conserved among mammalian phagocytes, whether professional or not.

We have found that uptake of apoptotic cells by MEC *in vitro* is relatively inefficient when compared with that for macrophages. Our previous work has suggested that 40–60% of macrophages contain apoptotic cells after 30 min, whereas the maximal percentage of epithelial cells was only 15–20% even after 4 h of incubation with apoptotic cells. The reasons for these differences and whether they apply *in vivo* are not known. Parnaik *et al*.[49] compared the uptake of apoptotic cells by brain macrophages and microglia with uptake by lens epithelial cells and BHK cells. They found that while the nonprofessional phagocytes readily bound apoptotic cells, they did not ingest them for several hours, and concluded that apoptotic cells must develop additional surface changes that trigger engulfment. An alternate interpretation is that amateur phagocytes require induction of competency to phagocytose, which is acquired during the long binding period observed by Parnaik. Wood *et al*.[13] found that while apoptotic cell

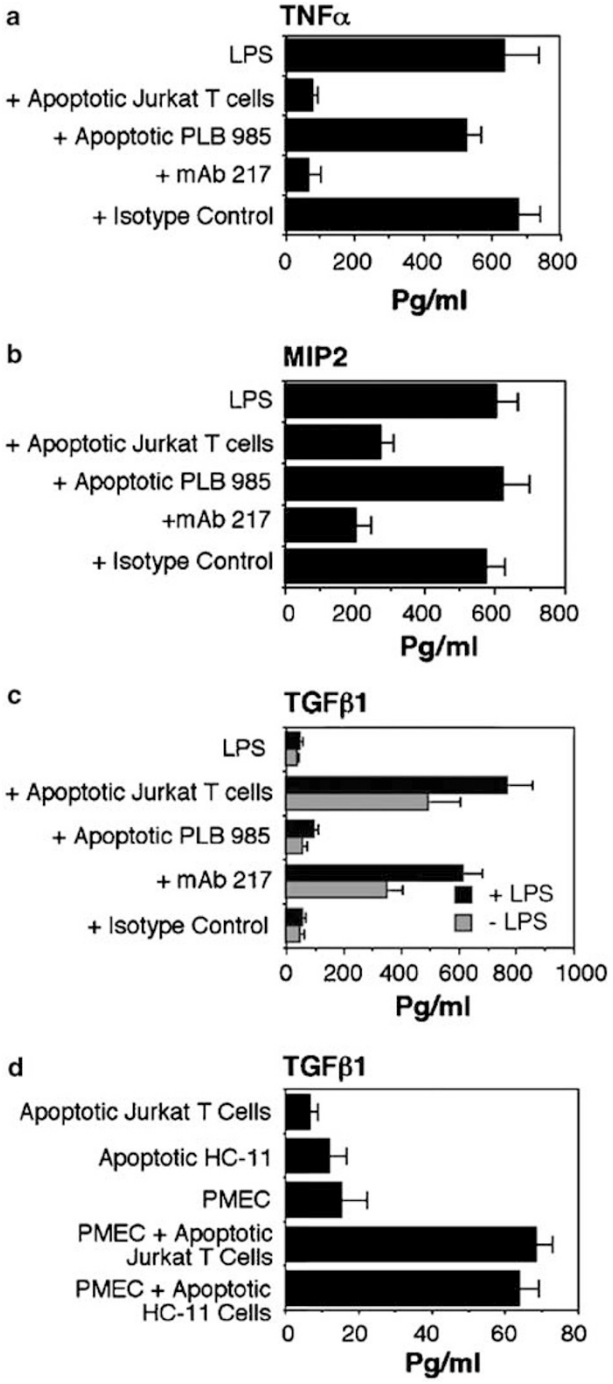

Figure 5 MEC release TGFβ and repress LPS-stimulated TNFα and MIP2 release following exposure to phosphatidylserine on apoptotic cells. (**a**) HC-11 cells were treated with LPS in the presence or absence of apoptotic Jurkat T cells (PtdSer positive by annexin V binding), apoptotic PLB 985 cells (PtdSer negative by annexin V), mAb 217, or its isotype control. After 24 h, the medium was collected and analyzed for TNFα by ELISA. TNFα was not measurable in the medium from HC-11 cells not stimulated by LPS (not shown). (**b**) HC-11 cells were treated as described for (**a**) and supernatants were evaluated for MIP2. MIP2 was not measurable in supernatants from HC-11 cells not stimulated by LPS (not shown). (**c**) HC-11 cells were treated as described for (**a**) and (**b**); supernatants were evaluated for TGFβ1 levels by ELISA. (**d**) Primary mammary epithelial cells (PMEC) were cultured as described in Materials and Methods. Apoptotic HC-11 cells and apoptotic Jurkat T cells were cultured alone or with the PMEC for 24 h, then supernatants collected and evaluated for the production of TGFβ by ELISA

clearance in the developing limb bud was delayed in the absence of macrophages, the limb developed normally. These observations suggest that while macrophage removal of apoptotic bodies may be more efficient, amateur phagocytes can and will engulf apoptotic cells when macrophages are absent. Macrophages appear to have many more receptors that they can dedicate for apoptotic cell engulfment when compared to amateur phagocytes, and these cells certainly have evolved to be highly efficient at phagocytosis of many particles. Yet, engulfment of apoptotic cells in animals such as the nematode worm *Caenorhabditis elegans* is efficient and is mediated by nonmacrophages, suggesting that removal of dying self is conserved throughout the animal kingdom. In fact, the mechanical aspects of apoptotic cell phagocytosis appear to be preserved, as mammalian homologues for many of the *C. elegans* engulfment genes have been identified and characterized.[50–56]

The ability of MEC to engulf apoptotic bodies and their ability to secrete anti-inflammatory mediators in response suggest an important role for amateur phagocytes in the gland. During mammary involution, significant cell death occurs prior to dissolution of the basement membrane supporting the alveolar epithelial cells. During the first 4 days of murine involution, we have found that the sole phagocyte is the epithelial cell (Monks J *et al*., submitted for publication). After that time, macrophages have migrated into the tissue and are believed to participate both in the removal of residual apoptotic bodies and remodeling of the tissue.[14] Thus, it is entirely possible that mediators secreted by MEC following exposure to apoptotic cells helps to call in macrophages, facilitate remodeling, and inhibits the migration or activation of inflammatory neutrophils into the tissue. Activation of a full-blown immune response, as seen in dairy cows with mastitis, leads to tissue damage, the inability of the gland to fully regenerate, and decreased milk production. Thus, the response of the epithelium to apoptosis of its neighbors is crucial to both the immune barrier and the subsequent lactational function of the gland. In a broader sense, these data support the hypothesis that during normal cell turnover in epithelial tissues, epithelial phagocytes are major contributors to dead cell removal, and that they therefore contribute significantly to tissue homeostasis.

Materials and Methods

Cell culture

HC-11 cells were cultured according to published protocols,[57] in RPMI 1640 medium with 10% fetal calf serum, penicillin, streptomycin supplemented with 5 μg/ml insulin and 10 ng/ml epidermal growth factor, and passed twice per week, before confluency. EpH4 cells were grown in Dulbecco's modified Eagle's medium (high glucose) with 5% fetal calf serum, penicillin, and streptomycin. PMEC were isolated from day 15 pregnant CD1 mice as described previously,[58] thawed and cultured on rat tail collagen for 5–7 days prior to use in experiments.

Induction of apoptosis

HC-11 cells were induced to undergo apoptosis by culturing them in the absence of growth factors (in RPMI containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine + 2% BSA as protein source) in Teflon plates to prevent adherence. This was previously shown to induce apoptosis in this cell line.[59] Apoptosis was assessed by morphology using light microscopy and by ability to bind to FITC-conjugated annexin V (Pharmingen). In all, 70 ± 18% cells were apoptotic by morphological assessment, with 77 ± 20% annexin positive, at 4 h; 8% were trypan blue positive; therefore, these were used for subsequent phagocytosis and cytokine assays. Apoptosis was induced in Jurkat T cells (a human T lymphocyte cell line) and PLB 985 cells (a human monomyelocytic cell line) using UV irradiation as described previously.[48] Apoptosis was assessed by morphology using light microscopy; loss of phospholipid asymmetry and exposure of phosphatidylserine on the outer leaflet of the plasma membrane was assessed by the ability to bind Alexa-488-conjugated annexin V (Molecular Probes). As we have shown previously, Jurkat T cells express phosphatidylserine externally following induction of apoptosis, and PLB 985 cells do not.[48]

Histochemistry

Visualization of uptake: HC-11 cells were induced to undergo apoptosis as described above. After 10 h, the nonadherent cells were labeled with Texas red-X-SE (Molecular Probes, Eugene, OR, USA) and fed to Cell-Tracker green (Molecular Probes, Eugene, OR, USA) labeled HC-11 cells on glass coverslips. After a 4-hour incubation, unbound cells were washed away and the coverslips were moved into ice-cold fixative: 3% paraformaldehyde, 3% sucrose in Dulbecco's PBS ($+Ca^{2+}/Mg^{2+}$). The cells were counterstained with Hoechst 33452 at 0.5 μg/ml for 30 min, rinsed with PBS and mounted onto slides with a glycerol-based mounting medium with *o*-phenylenediamine (opda) antifade.

Immunohistochemistry for expression of PtdSerR

Staining for surface receptors was performed without permeabilization of the cells. Briefly, HC-11 or EpH4 cells were rinsed with HBSS and incubated with mAB 217G8E9 or mouse IgM isotype control in HBSS for 30 min at 4°C. Cells were rinsed with ice-cold PBS and fixed for 20 min on ice. The cells were permeabilized with TX100 as above and blocked with 1% ovalbumin in PBS. Fluorescent secondary antibody was added with DAPI or Hoechst 33258 to visualize nuclei. The cells were incubated with the fluors for 45 min, soaked in PBS overnight and mounted with opda/glycerol mounting medium.

Staining of mammary tissue

Mice were anaesthetized with Nembutal (sodium pentobarbital at 0.1 mg/g body weight) and intracardially perfused with ice-cold Dulbecco's PBS, followed by 2%, then 4% formaldehyde solution in PBS. Tissues were dissected, further immersion fixed for 4 h and frozen in methylbutane cooled with liquid nitrogen. Frozen sections, 8-μm thick, were collected onto Cell-Tak-coated coverslips and staining proceeded as described for the cultured cells above. Cytodeath, an antibody to caspase-cleaved keratin-18, was used according to the manufacturer's instructions (Roche Molecular Biochemicals).

Imaging: Imaging was carried out on an epifluorescent, multifluor imaging, deconvolution and analysis system with SlideBook software (Intelligent Imaging Innovations, Inc., Denver, CO, USA).

Electron microscopy

Apoptotic HC-11 cells were incubated with HC-11 cell phagocytes for 4 h at 37°C. Unbound cells were washed away with ice-cold HBSS and cell

dissociation solution, consisting of HBSS ($-Ca^{2+}/Mg^{2+}$) + 10 mM EDTA, was added. After 15 min, on ice, the cells were lifted with a cell lifter and tritrated to further dissociate. The cells were then pelleted from the dissociation solution and resuspended in 1.5% glutaraldehyde in cacodylate buffer. The cells were fixed overnight at 4°C and then processed for electron microscopy as previously described.[60]

Similarly, formaldehyde-perfused tissue was further fixed, overnight in 1% glutaraldehyde, 0.5% acrolein in cacodylate buffer and processed for electron microscopy.

Flow cytometry

HC-11, EpH-4 cells, and PMEC were plated in 100 mm dishes and cultured in growth medium. Cells were harvested when the confluency reached 40–50% by incubating in Hank's BSS ($-Ca^{2+}/Mg^{2+}$) containing 5 mM EDTA and gentle scraping. Following washing in HBSS, 0.5 million cells per condition were resuspended in 100 μl HBSS containing 2% FCS, plus primary antibodies. Those used included mAb 217G8E9 or its isotype control (mouse IgM, 200 μg/ml),[38] anti-mouse CD 51 (anti-α_v, hamster IgG, Pharmingen, 20 μg/ml), anti-mouse CD61 (β_3, hamster IgG Pharmingen, 20 μg/ml) anti-calreticulin N-terminal (ABR, chicken polyclonal, 1 : 10), anti-CD91α (mouse IgG1, American Diagnostica, 10 μg/ml), anti-mouse CD36 (mouse IgA, Cascade Biosciences, 10 μg/ml). All isotype controls were used at same concentration as the specific primary antibodies. Secondary antibodies were Cy3-conjugated F(ab)'$_2$ antibodies from Jackson ImmunoResearch or whole immunoglobulin from Pharmingen (FITC-conjugated anti-mouse IgA).

Phagocytosis assays

Apoptosis was induced in HC-11 cells as described above. Then, 3–5 million cells from the apoptotic population were added in a total volume of 1 ml of mammary epithelial cell growth medium (see above, contains 10% FCS) to 24-well plates containing viable HC-11 cells, viable EpH4 cells, or PMEC, which had been preincubated for 30 min with anti-PtdSerR (mAb 217) or its isotype control (mouse IgM) at 200 μg/ml, anti-CD91α (American Diagnostica) or its isotype control (mouse IgG1, Pharmingen) at 50 μg/ml, anti-mouse CD36 (Cascade Biosciences) or its isotype control (mouse IgA, Pharmingen) at 100 μg/ml, anti-α_v (anti-mouse CD51, Pharmingen, at 100 μg/ml, C1q at 25 μg/ml, mannose-binding protein (MBP) at 25 μg/ml. Viable and apoptotic cells were incubated for 3 h. The cells were then washed three to four times with ice-cold PBS, fixed with 1% bufferend normal formalin and stained with Diff-Quik (Baxter Healthcare Corp.). Phagocytosis was scored by light microscopy. A minimum of 200 cells was counted in each duplicate well. The number of epithelial cells containing phagocytosed material was counted, and the percentage of positive cells was determined. Results from duplicate samples were averaged to generate the mean for each condition.

Cytokine assays

HC-11 cells were plated at 0.25 million cells per well in 24-well plates in growth medium (RPMI + 10% FCS + EGF (10 ng/ml) + insulin (5 μg/ml) + glutamine (2 mM) + penicillin (100 μg/ml) + streptomycin (100 μg/ml)). After overnight incubation, the medium was replaced with serum-free medium (*X-Vivo*, Biowhittaker) containing EGF (10 ng/ml) and insulin (5 μg/ml). Apoptotic Jurkat T cells (3 million per well), apoptotic PLB 985 cells (3 million per well), mAb 217G8E9 (200 μg/ml), or its isotype control, mouse IgM (200 μg/ml) were added in the presence or absence of LPS (*E.coli* O11 : B4, 1 μg/ml). The cells were then cultured for an additional 24 h. Supernatants were collected, centrifuged to remove particulate matter, and frozen at −70°C until analyzed by ELISA for TNFα, MIP2, IL-10 (R & D Systems), and TGFβ1.[61] PMEC were obtained from day 15 pregnant CD1 outbred mice as described above. For these experiments, the normal growth medium was removed and replaced with *X-vivo* containing EGF and insulin (same concentrations used for HC-11 as described above). Then, either apoptotic Jurkat T cells or apoptotic HC-11 cells were added (5 million of each per well) and the PMEC were cultured for an additional 24 h. Supernatants were collected, centrifuged to remove particulate debris, and frozen at −70°C until analyzed for TGFβ1 by ELISA.[61]

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