Macrophage Response to Apoptotic Cells Varies with the Apoptotic Trigger and Is Not Altered by a Deficiency in LRP Expression

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Abstract

Rapid engulfment of apoptotic cells in the absence of inflammation is required for maintenance of normal tissue homeostasis. The low-density lipoprotein receptor-related protein-1 (LRP/CD91) is a receptor mediating interactions between macrophages and apoptotic cells, but recent reports have challenged the requirement of this surface protein in this process. To explore the role of LRP in the recognition of apoptotic cells, target cells were generated with two distinct inducers of apoptotic cell death, etoposide and actinomycin-D. Jurkat T cells rendered apoptotic with etoposide exposed phosphatidylserine (PtdSer) and triggered engulfment by murine bone marrow-derived macrophages (BMDM), however they failed to suppress lipopolysaccharide-driven inflammatory cytokine secretion or, correspondingly, NF κ B-dependent or TNF α promoter-driven transcriptional activity in transfected RAW264.7 macrophages. In contrast, induction of apoptosis in either Jurkat cells or HeLa epithelial cells with actinomycin-D resulted in diminution of proinflammatory signaling from RAW264.7 cells and BMDM. Treatment of actinomycin-treated Jurkat cells with Q-VD-

OPh, an irreversible inhibitor of caspase activity, blocked apoptosis, as assessed by the inhibition of PtdSer exposure; however, the cells maintained anti-inflammatory activity. Anti-inflammatory signaling mediated by actinomycin-treated cells was not affected by a macrophage-specific deletion in LRP. Moreover, the presence of LRP on macrophages did not alter the efficiency of engulfment of apoptotic cells in vitro or in vivo. These data demonstrate that the method of induction of apoptosis of target cells influences subsequent macrophage responsiveness, and that LRP is not required for engulfment of apoptotic cells regardless of the method of induction.

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Introduction

Engulfment of apoptotic cells is a fundamental biological process required for embryonic development, resolution of immune response, and maintenance of normal tissue homeostasis. For example, approximately 10^{11} neutrophils are cleared from the circulation daily [1]. The prompt and efficient removal of apoptotic cells is thought to be important in preventing the loss of cellular integrity that accompanies cell death and the subsequent leakage of intracellular contents. The delayed clearance hy-

pothesis suggests that the release of intracellular contents results in presentation of autoantigens to the immune system and subsequent autoimmunity as seen, for example, in systemic lupus erythematosus [2]. This hypothesis has recently been challenged by Birge and Ucker [3], who suggest that instead of autoantigen presentation, appropriate recognition of apoptotic cells provides a mechanism for homeostatic regulation, and it is the absence of these recognition events that results in dysregulation of the immune response. These data are supported by the observation that apoptotic cells induce anti-inflammatory responses from engulfing cells: both professional and nonprofessional phagocytes [4–7].

Numerous receptor-ligand pairs have been implicated in the engulfment of apoptotic cells [reviewed in ref. 8]. It has been proposed that one of the key recognition elements is phosphatidylserine (PtdSer), a plasma membrane phospholipid normally confined to the inner leaflet of the plasma membrane but exposed on the cell surface when cells undergo apoptosis [9]. Three PtdSer receptors have recently been identified to mediate this recognition event: BAI1, TIM4 and stabilin-2 [10–12]. In addition, apoptotic cell recognition and clearance is facilitated by soluble bridging molecules that link apoptotic cells to phagocytes. For example, complement component 1 subcomponent C1q is a well-characterized bridging molecule for apoptotic cell removal [reviewed in ref. 13]. Calreticulin, or cC1qR, is a 60-kDa soluble endoplasmic reticulum lumen protein and is also reported to be found on the cell surface and to function as a C1q receptor [reviewed in ref. 13]. Calreticulin does not have a transmembrane domain and therefore is incapable of signaling for phagocytosis. Accordingly, calreticulin has been proposed to complex with the low-density lipoprotein receptor-related protein-1 (LRP/CD91), a large endocytic receptor, and the complex has been proposed to be required for ingestion of C1q-coated apoptotic cells [14, 15]. Genetic analyses in the nematode *Caenorhabditis elegans* have led to the identification of genes essential for engulfment of apoptotic cells, including CED-1 (a transmembrane protein required for recognition and clearance of apoptotic cells) and its intracellular binding partner CED-6/GULP [16]. It has been proposed that LRP is the mammalian homolog of CED-1, and the LRP cytoplasmic tail interacts with GULP [16] and facilitates phagocytosis [17]. Using mice deficient in the macrophage-specific expression of LRP, we recently confirmed that LRP facilitates ingestion of particles coated with known LRP ligands (receptor-associated protein and the thrombospondin heparin binding domain), however

LRP was not required for the C1q-dependent ingestion of apoptotic cells in the presence of human serum [18]. These data suggested that LRP is not a receptor for C1q-mediated engulfment or, alternatively, there may be a redundant receptor system.

LRP has also been proposed to function directly as a receptor for apoptotic cells by engaging calreticulin on the apoptotic cell surface in the absence of C1q [19]. Our previous studies focused on the contribution of LRP to C1q-mediated activation of phagocytosis but did not thoroughly investigate the C1q-independent engulfment of apoptotic cells. Since LRP is widely recognized as a macrophage receptor critical for the engulfment of apoptotic cells, we sought to extend these initial studies to assess the contribution of LRP to the C1q-independent engulfment of apoptotic cells.

While intense effort has been directed towards identifying the receptors and ligands required for engulfment of apoptotic cells, relatively little is known about the recognition components required for the alteration in cytokine production following exposure to apoptotic cells. PtdSer has been demonstrated to be required for some anti-inflammatory properties of apoptotic cells, such as the production of anti-inflammatory TGF β [5]; however, PtdSer-independent inhibition of proinflammatory signaling by apoptotic cells has also been reported [7]. Our previous studies using LRP-deficient macrophages utilized Jurkat cells treated with etoposide as apoptotic targets [18] following established protocols [20, 21]. Apoptotic Jurkat cells were readily engulfed by macrophages, however we did not explore the modulation of inflammation in response to the targets. Here we use two distinct inducers of apoptotic cell death, etoposide and actinomycin-D, to demonstrate that the trigger leading to apoptotic cell death influences the proinflammatory response from the macrophage. Etoposide, a topoisomerase II inhibitor that blocks DNA repair, failed to generate an apoptotic cell capable of modulating macrophage cytokine synthesis. Actinomycin-D treatment, leading to the inhibition of transcription, led to generation of apoptotic target cells that both triggered macrophage engulfment and were anti-inflammatory, however the anti-inflammatory activity was independent of caspase activation and PtdSer exposure. LRP-deficient macrophages (Mac-LRP $^{-/-}$) engulfed actinomycin-D- and etoposide-treated Jurkat cells at similar levels as wild-type (WT) macrophages both in vivo and in vitro, and actinomycin-D-treated Jurkat cells inhibited lipopolysaccharide (LPS)-induced TNF α production in the presence and absence of LRP. These data provide a system to investigate

macrophage responsiveness to apoptotic cells, and highlight the importance of the method of induction of apoptosis on macrophage responsiveness. Using this system, we demonstrate that LRP is dispensable for engulfment of apoptotic cells in vitro and in vivo.

Materials and Methods

Reagents and Antibodies

All reagents were purchased from Fisher (Pittsburgh, Pa., USA) unless otherwise indicated. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, trypsin-EDTA, and carboxyfluorescein succinimidyl ester (CFSE) were purchased from Gibco/Molecular Probes/Invitrogen (Carlsbad, Calif., USA). CFSE was reconstituted to 5 mM in DMSO and stored at -20°C . Etoposide, actinomycin-D and 2-nitrophenyl β -D-galactopyranoside were obtained from Sigma (St. Louis, Mo., USA). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, Utah, USA) and heat inactivated for 30 min at 56°C . Annexin V-FITC apoptosis detection kit was obtained from BioVision (Mountain View, Calif., USA). Rat anti-mouse CD11b-phycoerythrin (PE) and Ig2b-PE antibodies were purchased from Beckman (Fullerton, Calif., USA). LPS (*Escherichia coli* 0111:B4) was purchased from List Biological Laboratories (Campbell, Calif., USA). Q-VD-OPh, a general caspase inhibitor, was purchased from R&D Systems (Minneapolis, Minn., USA).

Mice

Mice deficient in macrophage LRP were generated using loxP/Cre-mediated recombination as described [18]. LRP expression on MacLRP $^{-/-}$ macrophages was reported previously (2.6–20.4% of WT levels [18]); a representative flow-cytometric plot is provided online (supplementary figure 1; www.karger.com/doi/10.1159/000295790). All studies were approved by the Institutional Animal Care and Use Committees of the University of Notre Dame and the Indiana University School of Medicine.

Cell Culture and Death Induction

RAW264.7 murine macrophages stably expressing TNF α or NF κ B luciferase reporter gene were a kind gift from Dr. Jeff Schoenly (University of Notre Dame) and have been described previously [22]. Cells were cultured at 37°C in a humidified, 5% (v/v) CO_2 atmosphere in DMEM supplemented with FBS (10% v/v) and 400 $\mu\text{g}/\text{ml}$ G418 sulfate. Mouse bone marrow-derived macrophages (BMDM) were isolated as described previously [23]. Briefly, bone marrow from femurs and tibia were collected and, following removal of adherent cells, cultured in BMDM media [DMEM with 10% FBS, 100 units/ml penicillin G, 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate (Pen/Strep) and 10 mM HEPES, pH 7.4, supplemented with 15% L929 conditioned media] at 5% CO_2 for 7 days prior to use. The Jurkat human acute T-leukemia cell clone A3 was obtained from ATCC (Manassas, Va., USA) and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, Pen/Strep and 10 mM HEPES (pH 7.4) at 5% CO_2 . HeLa human cervical carcinoma cells were grown in DMEM supplemented with 10% FBS, Pen/Strep and 10 mM HEPES (pH 7.4) at 5% CO_2 .

Live cells refer to target cells that were not treated with an apoptosis-inducing agent; however, some dead cells will exist in these samples due to repeated washing and handling of the cells (fig. 1). Apoptosis was induced by treatment of HeLa and Jurkat cells with 2 $\mu\text{g}/\text{ml}$ actinomycin-D for 12–18 h, or by treatment of Jurkat cells with 40 μM etoposide for 12 h. Apoptosis was inhibited with concurrent treatment with 10 μM Q-VD-OPh. Necrotic cell death was induced by incubation at 55°C for 20 min (until trypan blue uptake indicated compromise of membrane integrity).

Induction of apoptosis and necrosis was routinely confirmed using the Apoptosis Detection Kit (BioVision, Mountain View, Calif., USA) according to the manufacturer's protocol and analyzed by flow cytometry using a Beckman FC500. The kit utilizes annexin V to stain PtdSer and propidium iodide (PI) as a vital dye.

Luciferase Assays

RAW264.7 murine macrophages stably expressing an NF κ B or a TNF α promoter luciferase reporter gene were harvested and plated in 12-well plates at 2×10^5 cells/well in serum-free media (HL-1 + Pen/Strep + 1% L-glutamine) for 2 h. Apoptotic, necrotic and viable Jurkat or HeLa cells were washed three times with HBSS++ and resuspended in serum-free media. Macrophages were incubated with or without indicated target cells (at different target cell/macrophage ratios) and/or LPS (100 or 1,000 ng/ml; both doses provided robust macrophage proinflammatory responses). Supernatants were collected for TNF α measurement and monolayers were washed with PBS. Cells were lysed with reporter lysis buffer (Promega, Madison, Wisc., USA), and luciferase activities from cells in each well were measured using the luciferase assay system (Promega) in a GloMax 20/20 luminometer. Results are presented as luciferase activities in LPS-treated macrophages relative to the non-treated control population and are the mean $\pm$ SD of triplicate determinations.

Quantification of Cytokine Release

Cytokine production was assessed following incubation of macrophages with target HeLa or Jurkat cells in the presence or absence of LPS. Culture supernatants were withdrawn from wells after 4 h of incubation and assessed quantitatively for secreted TNF α . Cytokines were assayed by the mouse TNF α ELISA Ready-Set-Go! kit (eBioscience, San Diego, Calif., USA) according to manufacturer's protocol; 100 μl of diluted samples and standards were added to wells in duplicate.

Phagocytosis Assay

In vitro phagocytosis assays were performed essentially as described previously [18]. Jurkat T cells and human epithelial HeLa cells were labeled with 5 μM CFSE for 30 min at 37°C , washed twice with complete media and resuspended at $5 \times 10^5/\text{ml}$. Target cells were then induced to undergo apoptotic or necrotic cell death, or left untreated. Apoptotic, necrotic and live cells were washed three times with HBSS++ and resuspended in phagocytosis buffer (DMEM supplemented with 5 mM MgCl_2 and Pen/Strep). Murine BMDM were harvested with PBS 10 mM EDTA, washed three times with HBSS++ and resuspended in phagocytosis buffer. Macrophages (2.5×10^5) were incubated with 7.5×10^5 apoptotic, necrotic or viable Jurkat or HeLa cell targets for 15–60 min at 37°C in 4-well Lab Tek chamber slides (Nunc, Rochester, N.Y., USA). Non-adherent cells were removed by washing with PBS and adherent cells were harvested by trypsinization. Cells were washed twice

Fig. 1. Apoptotic cells are preferentially engulfed compared to live cells. Jurkat or HeLa cells were left untreated (live) or treated with 40 μ M etoposide for 12 h (a), or 2 μ g/ml actinomycin-D for 12–18 h (b, c, respectively). Cells were stained with annexin V and PI to detect PtdSer exposure and viability, respectively (column one and two). CFSE-labeled live or apoptotic cells (Jurkat-E, Jurkat-

ActD and HeLa-ActD) were cocultured with adherent BMDM for 15 and 30 min at 37°C. Macrophages were stained with anti-CD11b-PE and analyzed by flow cytometry. Percent phagocytosis was calculated by dividing CFSE, CD11b double-positive cells by the total number of CD11b-positive cells and multiplying by 100 (last column). Shown is a representative experiment ($n \geq 3$).

in FACS buffer (HBSS++, 0.2% BSA and 0.2% sodium azide) at 4°C and stained with anti-CD11b-PE. Cells were then washed twice in FACS buffer at 4°C and immediately analyzed by flow cytometry. Cells that were double positive for CFSE/CD11b represented phagocytes that had engulfed targets. Percent phagocytosis was calculated by dividing CFSE/CD11b-positive cells by the total number of CD11b-positive cells and multiplying by 100.

In vivo phagocytosis assays were performed essentially as described previously [24]. WT and MacLRP^{-/-} mice were injected with 1 ml of sterile 4% Brewers thioglycollate, and 96 h after thioglycollate injection, 1×10^7 CFSE actinomycin-D-treated or untreated cells were injected into the peritoneal cavity in a volume of 200 μ l. After 20 min, peritoneal cells were collected by lavage with 5 ml of HBSS++ 5 mM EDTA. Cells were washed as above

and counted, and 1×10^6 cells were stained with anti-CD11b-PE for analysis by flow cytometry.

Transfection

Murine RAW264.7 macrophages stably expressing an NF κ B luciferase reporter gene were transiently transfected with a reporter plasmid expressing β -galactosidase driven by the CMV promoter (kindly provided by Dr. Tracy Vargo-Gogola, Indiana University School of Medicine – South Bend) using Lipofectamine (Invitrogen) according to the manufacturer's protocol. Briefly, macrophages were plated at 1×10^6 cells per well into 6-well dishes. The following day, mixtures of 1 μ g DNA and 6 μ l Lipofectamine were complexed for 35 min in serum-free DMEM, and complexes were incubated with cells for 5 h. Complexes were replaced with complete media and cells were used the following morning.

β -Galactosidase Assay

RAW264.7 macrophages transfected with pNF κ B luciferase reporter gene and CMV β -galactosidase plasmid were lysed with reporter lysis buffer (Promega). β -Galactosidase activity was measured using *o*-nitrophenyl β -D-galactopyranoside as a substrate. In a 96-well plate, 50 μ l of assay buffer (200 mM sodium phosphate buffer, pH 7.2, 2 mM MgCl₂, 1.33 mg/ml ONPG and 100 mM β -mercaptoethanol) were added to 50 μ l of cell lysate and incubated at room temperature for 45–60 min or until a faint yellow color developed. The reaction was stopped by adding 1 M sodium carbonate. The assay was quantified by spectrophotometry at 420 nm with correction at 550 nm.

Results

Apoptotic Cells Are Preferentially Engulfed Compared to Live Cells

The ability of macrophages to ingest apoptotic Jurkat cells was confirmed using a flow cytometry-based phagocytosis assay as described previously [18]. Apoptosis was induced in Jurkat cells by treatment with 40 μ M etoposide for 12 h (Jurkat-E) or induced in Jurkat cells and HeLa cells with 2 μ g/ml of actinomycin-D for 12–18 h (Jurkat-ActD and HeLa-ActD, respectively). Extensive cell death was confirmed by annexin V and PI staining. Using etoposide, 79% of the Jurkat cell population were annexin V positive (average of $59.6 \pm 10.5\%$, $n = 9$) with the majority of the apoptotic cells excluding PI (fig. 1a and data not shown). Fewer Jurkat-ActD exposed PtdSer; 31% of the cells were annexin V positive, with an average of $30.4 \pm 7.0\%$ (fig. 1b and data not shown, $n = 13$). Similar to Jurkat-E, 67% (average of $64.2 \pm 13.3\%$, $n = 5$) of HeLa cells treated with actinomycin-D were apoptotic (annexin V positive) and the majority excluded PI (fig. 1c and data not shown). Apoptosis following etoposide and actinomycin-D treatment was also confirmed by caspase 3-de-

Fig. 2. Jurkat cells rendered apoptotic with etoposide do not alter macrophage proinflammatory cytokine production. RAW264.7 cells transfected with a TNF α luciferase reporter plasmid were treated with 100 ng/ml LPS (**b**) or transfected with an NF κ B reporter plasmid and treated with 1,000 ng/ml LPS (**c**) for 4 h in the presence or absence of increasing numbers of live, Jurkat-E or necrotic Jurkat cells. **a** Cell supernatants in **b** were analyzed by ELISA for TNF α production. Shown is the average from 4 wells in a representative experiment $\pm$ SD ($n = 2$). **b**, **c** Cells were lysed and reporter plasmid activity was measured (**b**: results from a single trial; **c**: 1 representative experiment of 4). Results are presented as luciferase activities in LPS-treated macrophages relative to the nontreated control population (means $\pm$ SD of triplicate determinations).

Fig. 3. Jurkat cells rendered apoptotic with actinomycin down-regulate LPS-stimulated macrophage activation. **a** RAW264.7 cells transfected with an NFκB reporter plasmid were treated with 100 ng/ml LPS for 4 h at 37°C in the presence or absence of increasing numbers of Jurkat cells rendered apoptotic with actinomycin-D. **a** Supernatants were analyzed by ELISA for TNFα production. Each point represents the average from 4 wells $\pm$ SD ($n = 1$). **b** Cells were lysed and reporter plasmid activity measured as described (1 representative experiment of 2). Results are presented as luciferase activities in LPS-treated macrophages relative to the nontreated control population (means $\pm$ SD of triplicate determinations).

pendent poly(ADP-ribose)polymerase cleavage (data not shown). As demonstrated previously [18], Jurkat-E were preferentially engulfed compared to live cells at both 15 and 30 min (fig. 1a). On average, macrophages ingested 3.3- and 3.9-fold more Jurkat-E than live Jurkat cells at 15 and 30 min, respectively ($n = 4$). We next examined the ability of macrophages to ingest Jurkat-ActD cells. Jurkat-ActD cells were preferentially ingested over live Jurkat cells after 15 and 30 min of incubation (fig. 1b). Macrophages ingested 3.0- and 1.9-fold more Jurkat-ActD cells

than live cells at 15 and 30 min, respectively ($n = 3$). To determine if preferential engulfment of apoptotic cells was cell type specific, HeLa cells were treated with actinomycin-D. HeLa-ActD cells were preferentially engulfed compared to live HeLa cells. On average, macrophages ingested 1.4-fold more HeLa-ActD cells than live cells at both 15 and 30 min ($n = 3$).

Jurkat-E Cells Do Not Block LPS-Dependent NFκB Activation

Our previous studies demonstrated that LRP was not required for the C1q-mediated enhancement of phagocytosis of apoptotic cells in the presence of complete serum, and these studies utilized Jurkat-E as apoptotic targets. Based on these findings, we hypothesized that Jurkat-E may lack critical ligands required for the LRP-dependent recognition of apoptotic cells. Cvetanovic and Ucker [6] and Cvetanovic et al. [7] demonstrated that apoptotic cells block the inflammatory response to a variety of stimuli including Toll-like receptor (TLR) ligands, and that inhibition of proinflammatory cytokine and chemokine production occurred at the level of gene transcription. More specifically, NFκB activation in response to TLR signaling (and other proinflammatory signaling pathways) was inhibited in the presence of apoptotic cells. To determine if Jurkat-E cells elicited anti-inflammatory responses, we tested their ability to modulate LPS (a TLR-4 ligand)-induced signaling from macrophages. The LPS responses from stable RAW264.7 macrophage transfectants harboring luciferase constructs dependent on a minimal NFκB promoter or the TNF-α promoter were measured following incubation with apoptotic, necrotic or viable Jurkat cells at different target/responder ratios. Surprisingly, TNF-α protein production from macrophages was not inhibited following interaction with apoptotic, necrotic or live Jurkat cells (fig. 2a). In accordance with this observation, neither NFκB nor TNFα reporter gene activity was altered by Jurkat-E following stimulation with LPS. Necrotic and live Jurkat cells also did not inhibit proinflammatory activity (fig. 2b, c), and an increase in proinflammatory response in the presence of live cells (fig. 2a, b) was not consistently observed.

Actinomycin-D-Treated Cells Alter Proinflammatory Activity in Macrophages

Numerous studies indicate that apoptotic cells modulate inflammatory signaling [reviewed in ref. 3] and are preferentially engulfed compared to live cells (fig. 1). In order to determine if the death-inducing stimulus influenced the modulation of proinflammatory response

Fig. 4. HeLa cells rendered apoptotic with actinomycin-D down-regulate LPS-stimulated macrophage activation. RAW264.7 cells transfected with an NFκB reporter plasmid were treated with 100 ng/ml LPS for 4 h at 37°C in the presence or absence of increasing numbers of HeLa-ActD cells. **a** Supernatants were analyzed by ELISA for TNFα production (1 representative experiment of 2, average $\pm$ SD from 4 wells). **b** Cells were lysed and reporter plasmid activity measured. Shown is a representative experiment ($n = 4$). Results are presented as luciferase activities in LPS-treated macrophages relative to the nontreated control population (means $\pm$ SD of triplicate determinations). **c** LPS-stimulated transfected RAW264.7 cells were treated with 100 ng/ml LPS in the presence of apoptotic, necrotic or live HeLa cells at a macrophage:target ratio of 1:5 (processed as described in **b**; average $\pm$ SD of 3 independent experiments; $n = 3$ for apoptotic and necrotic and $n = 2$ for live cells, * $p = 0.0001$, ANOVA).

from macrophages or if the difference was due to cell type, apoptotic cell targets, Jurkat-ActD and HeLa-ActD were assessed for their ability to dampen proinflammatory signaling. Unlike Jurkat-E, inhibition of proinflammatory macrophage responses by Jurkat-ActD was evident at the level of cytokine secretion. Jurkat-ActD inhibited the secretion of TNFα from macrophages; there was a 38% reduction in TNFα secretion from RAW264.7 cells when cocultured with Jurkat-ActD (macrophage/apoptotic cell ratio: 1:5; fig. 3a). Incubation with necrotic and live cells did not change the level of TNFα secretion from macrophages (fig. 3a). As expected, Jurkat-ActD inhibited LPS-dependent NFκB activation in a dose-dependent manner. Macrophage NFκB-dependent luciferase activity was reduced by 35.1% following coculture with Jurkat-ActD (macrophage/apoptotic cell ratio: 1:5; fig. 3b). Similar to Jurkat-ActD, HeLa-ActD were preferentially engulfed compared to live cells (fig. 1c), and inhibited proinflammatory signaling fig. 4).

Immunosuppression by Actinomycin-D-Treated Targets Is Independent of Caspase Activation

Since actinomycin-D inhibits transcription, we sought to determine if toxicity from actinomycin-D-treated target cells resulted in a global inhibition of transcription. Both Jurkat and HeLa cell lines treated with actinomycin-D were incubated with LPS-stimulated RAW264.7 cells stably expressing an NFκB reporter and transiently transfected with a β-galactosidase reporter plasmid. Jurkat and HeLa cells reduced NFκB levels to comparable degrees indicating that the immunosuppressive activity of actinomycin-D-treated cells was independent of the cell type. Compared to macrophages cultured with LPS in the absence of targets, there was a 63% reduction in NFκB activity following incubation with Jurkat-ActD and a 75% reduction in NFκB activity following incubation with HeLa-ActD (fig. 5a). As expected, Jurkat-E failed to inhibit LPS-dependent NFκB activity. β-Galactosidase activity was not altered in RAW264.7 cells following coculture with actinomycin- or etoposide-treated targets, suggesting that there was not a global inhibition of gene transcription or an alteration in macrophage viability that would be associated with a toxic effect from the targets (fig. 5b).

To further investigate the dependence of apoptotic cell death on anti-inflammatory signaling in this model, the contribution of caspase activation on the immunosuppressive effects of actinomycin-D-treated cells was assessed. Jurkat cells were treated with the global caspase inhibitor, Q-VD-OPh. Q-VD-OPh blocks the activation of caspases 1, 3, 8-10 and 12, and other hallmarks of apop-

Fig. 5. Actinomycin-D-treated cells do not alter transcription of β -galactosidase. RAW264.7 cells stably transfected with an NF κ B luciferase reporter plasmid were transiently transfected with a β -galactosidase expression plasmid (light gray column) or left untransfected (dark gray column) and treated with 100 ng/ml LPS for 4 h in the presence or absence of target cells (1:5). NF κ B luciferase (a) or β -galactosidase (b) were measured in cell lysates. Bars represent averages ($\pm$ SD) of duplicate plates from 1 representative experiment of 2.

toxicity, such as DNA laddering, exposure to PtdSer and cleavage of poly(ADP-ribose)polymerase [25]. Treatment with Q-VD-OPh inhibited exposure of PtdSer on Jurkat-ActD and Jurkat-E by an average of 83 and 79.9%, respectively (fig. 6a). Jurkat-ActD inhibited NF κ B activation by $55.8 \pm 5.8\%$. Similarly, Q-VD-OPh-Jurkat-ActD cells reduced NF κ B activation by $43.7 \pm 18.5\%$ (fig. 6b). Etoposide- and etoposide-Q-VD-OPh-treated cells both failed to inhibit NF κ B activation confirming Q-VD-OPh itself did not affect macrophage activation (data not shown). Importantly, these data demonstrate that apoptosis is not required for the anti-inflammatory signaling induced by actinomycin-D-treated target cells in this model.

Fig. 6. Immunosuppressive effect of Jurkat-ActD is independent of caspase activation. **a** Jurkat T cells were treated with 2 μ g/ml of actinomycin-D or 40 μ M etoposide in the presence or absence of 10 mM Q-VD-OPh for 12 h. After treatment, cells were stained with annexin-V FITC to quantify PtdSer exposure. Bars represent the average number of annexin V-positive cells $\pm$ SD ($n = 3$). * $p \leq 0.01$, unpaired Student's t test. RAW264.7 cells were cocultured with Jurkat cells in the presence of 100 ng/ml LPS (1 macrophage:5 Jurkat cells) for 4 h and luciferase activity was measured (average relative luciferase units of 3 independent experiments $\pm$ SD; **b**). LPS-treated macrophages were compared to LPS-treated macrophages in the presence of target cells $\pm$ Q-VD-OPh using an unpaired Student's t test, * $p \leq 0.02$. There was no significant difference between responses following treatment with Jurkat-ActD and Jurkat-ActD + Q-VD-OPh.

LRP-Deficient Macrophages Engulf Opsonized and Nonopsonized Apoptotic Cells at Levels Comparable to WT Macrophages

Since actinomycin-D and etoposide both induced apoptosis but elicited different macrophage responses, both dependent and independent of apoptosis, these different apoptotic target populations were utilized to assess the contribution of LRP to engulfment of apoptotic cells.

Fig. 7. LRP is not required for macrophage-mediated ingestion of apoptotic Jurkat cells. **a** Live, Jurkat-E and Jurkat-ActD in the presence or absence of serum were cocultured with WT (black column) and LRP-deficient BMDM (light gray column) at a 3:1 ratio. WT or MacLRP^{-/-} BMDM were incubated with live or apoptotic (Jurkat-E or Jurkat-ActD) CFSE-labeled Jurkat T cells for 30 min at 37°C in serum-free conditions or in the presence of 10% serum. Percent phagocytosis was calculated as described in figure 1. Each bar represents the average percent phagocytosis from three independent experiments $\pm$ SD ($n = 3$, $p = 0.03$). **b** Jurkat-ActD were cocultured with WT and MacLRP^{-/-} BMDM for 60 min at 37°C (see **a**) and percent phagocytosis was compared to ingestion of live Jurkat T cells and is indicated as fold change ($n = 3$). **c** CFSE-labeled Jurkat-ActD cells were injected into WT and MacLRP^{-/-} mice 96 h after induction of sterile peritonitis. Mice were euthanized after 20 min, and peritoneal cells were collected and stained with CD11b-PE. For each mouse, percent phagocytosis was calculated by dividing CFSE/CD11b double-positive cells by the total number of CD11b-positive cells and multiplying by 100. The average percent phagocytosis was calculated for each genotype, and points represent individual mice from 3 experiments $\pm$ SD.

WT and LRP-deficient macrophages (MacLRP^{-/-}) were cocultured with nonopsonized and serum-opsonized Jurkat-E or Jurkat-ActD, and phagocytosis was assessed. As expected, Jurkat-E exposed more PtdSer on the outer leaflet of the plasma membrane than Jurkat-ActD as indicated by annexin V staining (online suppl. fig. 2), and apoptotic cells were preferentially engulfed compared to live cells regardless of the method of induction of apoptosis (fig. 7a,

b). However, fewer Jurkat-ActD were ingested compared to Jurkat-E; following serum opsonization, 23 and 31% fewer Jurkat-ActD were ingested by WT and MacLRP^{-/-} macrophages, respectively. The diminished uptake of Jurkat-ActD correlated with a decrease in annexin V-positive and annexin V-positive/PI-positive cells. Interestingly, there was no significant difference in ingestion between WT and MacLRP^{-/-} macrophages under any condition

Fig. 8. LRP is not required for the Jurkat-ActD-induced inhibition of LPS-stimulated TNF α production from BMDM. BMDM from WT or macrophage LRP-deficient mice were cocultured with increasing numbers of apoptotic HeLa cells in the presence or absence of 100 ng/ml LPS. Data represent average and standard deviations of 4 wells from 1 representative experiment of 2. All points were made relative to the LPS-treated BMDM cultured in the absence of apoptotic cells.

tested (fig. 7a, b). While there was a trend to diminished uptake from MacLRP^{-/-} following engulfment of serum-opsonized Jurkat-ActD at both 30 and 60 min, the difference was not significant ($p > 0.1$). Serum contains C1q and other complement proteins, as well as other bridging molecules that influence engulfment of apoptotic cells. To further investigate the trend observed following engulfment of serum-opsonized Jurkat-ActD and approach more physiological conditions, we tested the requirement for LRP on ingestion of apoptotic cells in vivo using a previously established murine peritonitis model [24, 26]. Following induction of sterile peritonitis with thioglycollate, CFSE-labeled Jurkat-ActD were injected into the peritoneal cavity and after 20 min, peritoneal cavities were flushed and peritoneal macrophages analyzed by flow cytometry. Similar to the in vitro system, there was no difference in ingestion between WT mice and MacLRP^{-/-} mice (fig. 7c). These data demonstrate that macrophage LRP is not required to mediate engulfment of apoptotic cells in vivo. Moreover, incubation with HeLa-ActD led to a decrease in LPS-stimulated TNF α production from WT and MacLRP^{-/-} macrophages to similar degrees. With a 1:1 macrophage/HeLa-ActD ratio, there was a 71.5% reduction in TNF α production from WT macrophages and

an 83.7% reduction from MacLRP^{-/-} macrophages (fig. 8). Similarly, with a 1:5 macrophage/HeLa-ActD ratio, there was a 93% reduction in TNF α production from WT macrophages and a 94% reduction from MacLRP^{-/-} macrophages compared to LPS-stimulated macrophages without addition of target cells.

Discussion

This study demonstrates that the method of induction of apoptosis influences the subsequent macrophage cytokine response. Actinomycin-D-treated Jurkat or HeLa cells inhibited LPS-dependent proinflammatory signaling, but etoposide-treated Jurkat cells did not. Both are common methods of induction of apoptosis. Importantly, actinomycin-D-treated cells inhibited proinflammatory signaling independently of apoptotic cell death (Ptd-Ser exposure and caspase activation) in this model system. Mouse macrophages, both bone marrow-derived and RAW264.7 cells, failed to respond to Jurkat-E cells with an inhibition of proinflammatory signaling. These data suggest that anti-inflammatory signaling, well known to be associated with apoptotic cells, is cell type specific, reliant both on the phagocyte and the apoptotic target. However, BMDM readily engulfed apoptotic targets independent of target cell type or the method of induction of apoptosis, suggesting that this macrophage response is more highly conserved. We utilized the different apoptotic targets with macrophages deficient in LRP, a phagocytic receptor widely accepted as important for mediating engulfment of apoptotic cells, to demonstrate that this receptor is not required for engulfment of apoptotic cells in vitro or in vivo, nor is it required for the diminution of proinflammatory signaling by Jurkat-ActD cells. Combined, these data provide a system for further dissecting the molecular pathways required for engulfment of, and responsiveness to, apoptotic cells, and demonstrate that LRP is dispensable in this process.

LRP is suggested to be the mammalian homologue of CED-1, a transmembrane receptor in *C. elegans* required for engulfment of apoptotic cells. This hypothesis based on studies demonstrating that LRP was required for the C1q- and mannose-binding lectin-mediated enhancement of phagocytosis of apoptotic cells [27], and that the cytoplasmic tail of LRP interacted with GULP/CED-6, a CED-1 binding adaptor protein required for CED-1-mediated engulfment of apoptotic cells [16]. Numerous additional studies have further implicated LRP as the CED-1 homologue, and as such it has gained wide recognition

as a receptor for calreticulin, expressed either as a coreceptor for defense collagens that bridge apoptotic cells to phagocytes [14] or alternatively as a receptor for calreticulin that is tethered to the membrane of apoptotic cells [19]. These studies were largely performed using blocking reagents such as anti-LRP antibodies or the blocking ligand receptor-associated protein to interfere with interactions between LRP and its ligand. Recently, we utilized LRP-deficient macrophages generated using a tissue-specific loxP/Cre recombination to demonstrate that LRP was not required for the C1q-dependent enhancement of phagocytosis of antibody- or complement-opsonized particles, or the C1q-dependent engulfment of apoptotic cells in the presence of complete serum [18]. Since these results did not support the previous reports on the requirement for LRP in defense collagen mediated phagocytosis, we speculated that differences in the generation of the apoptotic targets may influence the behavior of the phagocyte and the requirement for specific receptor-ligand pairs.

There are numerous well-described methods for the induction of apoptosis including etoposide and actinomycin-D treatment, as used in this report. Serum starvation, UV irradiation and crosslinking FasL are additional commonly accepted methods for apoptosis induction. The significance of the method of apoptosis induction on subsequent phagocyte activity (engulfment or modulation of cytokine synthesis) has not been extensively investigated. We verified that Jurkat-E cells exposed PtdSer and stimulated engulfment of apoptotic cells, but these cells failed to inhibit proinflammatory signaling (fig. 1, 2). In contrast, induction of apoptosis with actinomycin-D treatment resulted in the generation of PtdSer-exposing targets that triggered macrophage engulfment and also downregulated proinflammatory signaling. The prophagocytic and anti-inflammatory properties of the apoptotic target were independent of the cell type as both Jurkat cells and HeLa cells stimulated macrophage responses. These data indicate that the method of induction of apoptosis has important consequences on phagocyte activity. While PtdSer exposure, thought to be critical for both engulfment and modulation of proinflammatory signaling, was observed for apoptotic cells generated with etoposide or actinomycin-D, Jurkat-E cells failed to inhibit proinflammatory signaling indicating that PtdSer exposure alone is not sufficient to regulate the macrophage response to apoptotic cells. This observation supports previous studies by Cvetanovic et al. [7] who demonstrated that apoptotic PLB-985 cells that fail to expose PtdSer still inhibit proinflammatory signaling. In addition, our study

suggests that the chemical trigger used to induce apoptosis may deliver an immunosuppressive activity independent of caspase activation. Treatment with Q-VD-OPh inhibited PtdSer exposure on Jurkat-ActD cells, and the cells retained their immunosuppressive activity. Steady-state levels of β -galactosidase were not altered in cells cocultured with actinomycin-D-treated target cells suggesting that the targets were not directly toxic (fig. 5b). However, future efforts should be directed at investigating the specificity of the immunosuppressive activity of target cells. Furthermore, the presence of late apoptotic or necrotic cells in apoptotic cell preparations (fig. 1) may influence the macrophage response.

The molecular characterization of apoptotic cells is required to harness the anti-inflammatory and prophagocytic potential of apoptotic cells for therapeutic benefit. These studies help to delineate the specific molecules involved in the regulation of macrophage activation by apoptotic cells, and highlight the contribution of the method of induction of apoptosis to macrophage responsiveness. Further characterization of the similarities and differences between Jurkat-E and Jurkat-ActD should provide insight into those molecules required for engulfment and regulation of inflammation. Jurkat-ActD expressed lower levels of PtdSer than Jurkat-E and were engulfed at lower levels from BMDM, and engulfment was not affected by a deletion of LRP (fig. 6a). Moreover, LRP-deficient macrophages ingested similar levels of apoptotic cells both in vitro and in vivo, indicating that the LRP pathway is not required for efficient ingestion of apoptotic cells. Future studies assessing the production of anti-inflammatory cytokines such as TGF- β and IL-10 from LRP-sufficient and -deficient macrophages in response to Jurkat-E and Jurkat-ActD, as well as the assessment of macrophage responsiveness to other apoptotic targets, will further these investigations. Ultimately, identification of the molecular requirements for apoptotic cell-induced phagocytosis and cytokine regulation should aid in the development of therapeutics to regulate inflammation.

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