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Phagocytosis Triggers Macrophage Release of Fas Ligand and Induces Apoptosis of Bystander Leukocytes

Simon B. Brown1 and John Savill

Human monocyte/macrophages (Mφ) exposed to nonparticulate stimuli can express cell surface Fas ligand (FasL) and release active soluble FasL (sFasL). We now report that monocyte/Mφ-ingesting opsonized zymosan released sFasL and conditioned supernatants so that these triggered Fas-mediated apoptosis of “bystander” monocytes and FasL-negative neutrophils. Furthermore, identical results were seen with Mφ taking up apoptotic neutrophils, whereas medium conditioned by Mφ phagocytizing latex beads had no proapoptotic effects upon neutrophils despite the presence of sFasL. These data suggest the hitherto unrecognized existence of a feedback loop requiring soluble factors in addition to sFasL that may promote resolution of inflammation-phagocytic clearance of apoptotic cells leading to Fas-mediated killing of bystander leukocytes by phagocytizing macrophages.


Cells of the monocyte/macrophage (Mφ)2 lineage are multifunctional, orchestrating many aspects of inflammation and tissue repair. A key property of the monocyte/Mφ, first recognized over a century ago by Metchnikoff (1), is the capacity to phagocytize particulate debris, invading microorganisms and effete cells. Indeed, there are now persuasive data to show that a critical event in resolution of inflammation is nonphlogistic safe phagocytotic clearance of intact leukocytes undergoing deletion by apoptosis, also known as programmed cell death (2–11).

In addition to clearing away apoptotic cells, important new data (12, 13) suggest that monocyte/Mφ can trigger apoptosis in other cells by regulated surface expression of Fas ligand (FasL) and by release of soluble FasL (sFasL). Since neutrophils, eosinophils, lymphocytes, and monocytes themselves all express the Fas “death receptor”3 and may, in certain circumstances, be susceptible to apoptosis triggered by cross-linking of Fas (12, 14–18), it has been suggested that monocyte/Mφ expression of FasL may be an important factor in regulating leukocyte populations at inflamed sites (12, 13). However, to date there has been little study of stimuli that control FasL expression/release by monocyte/Mφ, although there are reports that monocyte/Mφ FasL is up-regulated by nonparticulate stimuli such as soluble immune complexes, superantigen, phytohemagglutinin, and Ab-mediated cross-linking of monocyte/Mφ CD4 (13, 19, 20). In this report, we have studied monocyte/Mφ expression of FasL after phagocytosis of opsonized zymosan or apoptotic neutrophils, asking whether these stimuli induced release of sFasL from phagocytizing monocyte/Mφ and triggered Fas-mediated apoptosis in “bystander” neutrophils and monocytes.

Materials and Methods

Materials

All chemicals were of analytical reagent (AR) grade and were purchased from Sigma (Poole, U.K.), including zymosan (catalogue No. Z4250), latex particles (catalogue No. LB-8; Lot 11H0622), calcium ionophore A23187 (catalogue No. C7522), and the phorbol ester TPA (catalogue No. P8139), unless stated otherwise. Percoll was obtained from Pharmacia Biotech (St Albans, U.K.); sodium citrate solution from Pharma Hamelio (Hanover, Germany); culture media (HBSS, RPMI-1640, Iscove’s modified Dulbecco’s medium), and supplements (penicillin, streptomycin, glucose, FCS) from Life Technologies (Paisley, U.K.); FITC-conjugated anti-CD16 (mAb 3G8) from Caltag Laboratories (Bradure Biologicals, Shepshed, U.K.); FITC-conjugated annexin-V from BioWhitaker U.K. (Wokingham, U.K.); anti-Fas mAbs CH-11 and ZB4 from TCS Biologicals (Botolph Clayton, U.K.); anti-FasL mAb (clone 33) from Transduction Laboratories (Affiniti Research Products, Manhead, U.K.); and anti-Fas pAb (Ab-1) from Calbiochem-Novabiochem (U.K./Nottingham, U.K.). Opsonized zymosan (OpsZ) was prepared by washing zymosan particles exhaustively with endotoxin-free HBSS (without calcium and magnesium) before and following a 30-min incubation at 5 mg/ml with human clotted sera pooled from 10 different donors.

Leukocyte purification and isolation

Human neutrophils and monocytes were isolated from freshly drawn venous blood following centrifugation, dextran sedimentation, and discontinuous plasma-Percoll density gradient centrifugation, as previously described (3, 20, 21). Percoll density separation resulted in two distinct leukocyte layers, a neutrophil-enriched fraction (>98% purity), containing eosinophils as the only major contaminant, and a mixed monocyte/lymphocyte fraction. The neutrophil fraction was washed free of plasma and incubated at 37°C in Iscove’s modified Dulbecco’s medium containing antibiotics and supplemented with 10% autologous platelet-rich plasma-derived serum (PRPDS). In all experiments, monocytes were further purified by counterflow centrifugation (12–21; Beckman Instruments, Palo Alto, CA) to yield preparations greater than 95% purity (22). Monocyte-derived macrophages (Mφ) were obtained by the standard technique (3, 23) of culture of adherent monocytes in Iscove’s medium plus 10% PRPDS for 3 or 6 days.

Jurkat cell culture

The human T lymphoblastoid cell line Jurkat (kindly provided by Dr. C. Gregory, University of Birmingham) was maintained in RPMI 1640 supplemented with l-glutamine, 5 U/ml penicillin, 5 µg/ml streptomycin, and 10% FCS. Jurkat cells were free of mycoplasma contamination as assessed routinely by PCR. Expression of FasL and its secretion by Jurkat cells required stimulation with the calcium ionophore A23187 (2 µg/ml) and the phorbol ester TPA (10 ng/ml), with Jurkat cells typically treated for 4 h in serum-free media.
Assessment of apoptosis

In the present study, monocyte and neutrophil apoptosis was routinely quantified by flow cytometry using FITC-conjugated annexin V (24) and using a Becton Dickinson FACSscan (Oxford, UK), as previously described (25). We have extensively validated this technique against apoptosis as quantified by morphology on Giemsa-stained cytopsins (3) and by shedding of CD16 (26), and we agree with published data (24) that, in myeloid cells, these different approaches yield closely comparable results (25).

Phagocytic stimulation of monocytes and macrophages

Mo6 cultured in Costar plates (Wycombe, UK) or monocytes cultured in teflon-lined wells were coincubated with washed particles of OpsZ at a final concentration of 0.5 mg/ml for up to 7.5 h in Iscove’s medium in the absence of autologous serum. Typically 4 x 10^9 Mo6 or 2 x 10^6 monocytes in a final volume of 400 μl were used per equivalent experiment. Aliquots of conditioned media were removed at times indicated and clarified by sequential centrifugation at 300 x g for 5 min to remove intact cells and cell debris and at 1200 x g for 5 min to remove all traces of OpsZ before being used to resuspend freshly isolated but untreated peripheral blood monocytes or neutrophils. Monocytes were resuspended to a density of 2 x 10^9/ml in teflon wells and incubated at 37°C for up to 19 h in the absence of autologous serum. Neutrophils, on the other hand, were cultured at 5 x 10^6/ml in the presence of 10% autologous PRPDS to undergo constitutive apoptosis without significant necrosis (3). In addition to OpsZ, 6-day Mo6 were also incubated with latex beads at a final concentration of 0.5 mg/ml or with apoptotic neutrophils at 1 x 10^6/ml. When indicated, assays also contained the Fas-antagonist Ab ZB4 or an isotype-matched control negative Ab at an equivalent concentration.

FasL expression as determined by flow cytometry

Monocytes, maintained in the presence of 10% autologous PRPDS and cultured in teflon-lined “wells,” were assessed for FasL protein expression by indirect immunofluorescent labeling and flow cytometric analysis. Cells (1 x 10^6), resuspended in 100 μl of 10% newborn calf serum, were co-incubated with 100 ng of an anti-FasL mAb, clone 33 (or a control isotype-matched mAb), at 4°C for 60 min. Cells were washed and recovered by centrifugation at 300 x g for 5 min before resuspending in 10% newborn calf serum containing an FITC-conjugated F(ab’)2 fragment of a sheep anti-mouse IgG polyclonal Ab. Cells were then analyzed by a Becton Dickinson FACSscan flow cytometer for cell-associated fluorescence.

SDS-PAGE and protein blotting

Twelve percent SDS slab gels were prepared and run according to the method of Laemmli (27), with the exception that gels were run with 1 mM thioglycollic acid added to the cathode buffer and with the anode buffer diluted twofold. Each sample was prepared on the basis of equal numbers of extracted cells rather than on protein content. A typical 100 x 100 x 1.5 mm gel was run at 25 mA for 2 h and transferred to polyvinylidene difluoride (PVDF) membranes at 2 mA/cm² for 3 h at 4°C according to the methodology of Towbin et al. (28). Membranes were then blocked with 5% milk powder (Marvel, 99% fat-free) in PBS containing 0.2% Tween 20 for 1 h before probing overnight with either an anti-FasL mAb (clone 33) or pAb (Ab-1), with all steps maintained at 4°C. The presence of Tween 20 in blots to maintain immunoblotting conditions at 4°C was essential in minimizing cross-reactivity of clone-33 with b-actin (our unpublished observations). Blots were then washed with ice cold PBS before probing with a peroxidase-conjugated secondary pAb of appropriate specificity and detection with 4-chloro-1-naphthol.

Results

Phagocytosis of opsonized zymosan stimulates monocyte/Mφ expression of surface FasL and release into supernatants

In keeping with earlier reports (16, 20), we were unable to detect surface expression of FasL by normal human peripheral blood monocytes prepared by elutriation of mixed mononuclear cells obtained from plasma-Percoll density gradient centrifugation of dextran-sedimented blood (Fig. 1A). Furthermore, we were also able to confirm (13) that freshly isolated monocytes did not release FasL into supernatants during 3-h culture, as assessed by immunoblotting (Fig. 1B). However, monocytes are believed to contain intracellular pools of FasL that can be rapidly mobilized to the cell surface by soluble stimuli, including immune complexes (13, 20).
controls for untreated monocyte or 3-day M
up to 19 h. Apoptosis was assessed by annexin-V labeling and confirmed
f
tants freshly obtained from monocyte/M
Preliminary experiments (data not shown) indicated that superna-
usceptible to apoptosis induced by Fas ligation (12, 14, 17, 28).
Freshly isolated monocytes cultured in the absence of serum are
might have similar effects. By 3 h, we observed increased surface
phagocytic removal of apoptotic cells by M
and incubate fresh peripheral blood monocytes at 2
tration of 1200 ng/ml. Clarified supernatants were then used to resuspend
inflammatory response that did not exhibit Fas/FasL fratricide.

We examined whether uptake of opsonized zymosan (OpsZ), a
well-established model of Ig/complement-mediated phagocytosis,
might have similar effects. By 3 h, we observed increased surface
expression of FasL by monocyte/Mφ and release of FasL into the
supernatant by monocytes or monocyte-derived Mφ taking up
OpsZ (Fig. 1, A and B).

Supernatants from monocyte/Mφ phagocytizing OpsZ promote
Fas-mediated apoptosis of monocytes
Freshly isolated monocytes cultured in the absence of serum are susceptible to apoptosis induced by Fas ligation (12, 14, 17, 28).

Preliminary experiments (data not shown) indicated that superna-
tants freshly obtained from monocyte/Mφ taking up OpsZ could
induce apoptosis in “target” monocyte populations; a supernatant
transfer approach was taken in preference to coculture to avoid underestimation of apoptosis in the target population because of
phagocytic removal of apoptotic cells by Mφ. Unfortunately, the
proapoptotic effects of media conditioned by monocyte/Mφ-
ingesting OpsZ were poorly preserved by freezing or storage at 4°C,
despite no significant changes in sFasL protein as assessed by
Western blot analysis (data not shown). Therefore, we designed an
experiment (Fig. 2A) in which two populations of monocytes (“do-
nor” and “target”) were prepared at the same time from any given
donor. As previously reported (29), monocytes in the target popu-
lation cultured in the absence of serum underwent apoptosis
(~30% at 19 h), and this could be partially inhibited by the func-
tion-blocking Fas mAb ZB4 (Fig. 2A, left bars), in keeping with
data implicating Fas/FasL-mediated fratricide in such cultures (12).
However, when supernatants conditioned by donor mono-
cytes ingesting OpsZ were transferred to target monocytes at the
end of the conditioning period (either 2.5 h or 7.5 h), increased
apoptosis in the target monocytes was observed when the experi-
ment was terminated at 19 h after cell isolation (Fig. 2A, center and
right bars). With increasing conditioning time the proapoptotic
effect of supernatants from monocytes ingesting OpsZ increased.
Nevertheless, stimulated apoptosis of target monocytes was par-
tially abrogated by Fas blockade with ZB4. Supernatants condi-
tioned by donor monocytes cultured in medium alone had no pro-
apoptotic effect (Fig. 2A, right bar). Essentially similar results
were obtained when 3-day monocyte-derived Mφ were employed as
the donor population (Fig. 2B), except that medium conditioned by Mφ cultured without OpsZ protected monocytes against con-
stitutive apoptosis (Fig. 2B, right bar).

These data strongly suggested that FasL and/or related mole-
cules released from monocyte/Mφ phagocytizing OpsZ accounted
for the proapoptotic effect of conditioned medium. However, the
possibility remained that some other factor might have up-regu-
lated Fas/FasL-mediated fratricide in the target monocyte popula-
tion. Therefore, we went on to seek target cells of relevance to the
inflammatory response that did not exhibit Fas/FasL fratricide.

Supernatants from monocyte/Mφ-ingesting OpsZ also induced
Fas-mediated death in FasL-negative neutrophils
Neutrophils are widely recognized to express Fas and to be moder-
ately sensitive to apoptosis induced by Fas ligation (14). We
confirmed that relatively high concentrations of the agonistic Fas
mAb CH-11 were able to accelerate constitutive apoptosis in highly purified neutrophils prepared from normal human blood by
plasma-Percoll density gradient centrifugation (Fig. 3A). However,
by contrast with monocytes prepared by the same techniques (Fig. 2), there was no evidence of Fas/FasL-mediated fratricide, in that
Fas blockade with mAb ZB4 had no effect on constitutive apopto-
sis of neutrophils. This was despite the ability of ZB4 being able
to block CH-11-induced apoptosis (Fig. 3A). Furthermore, ZB4 did
not inhibit constitutive apoptosis in neutrophils cultured in the
absence of serum (data not shown), indicating that serum was not
masking Fas/FasL-mediated fratricide.

In addition, we were unable to detect FasL protein by neutro-
phils from 16 different healthy donors using techniques simulta-
neously proven to reveal FasL expression by activated Jurkat T
cells or stimulated/matured monocytes (Fig. 3B). Furthermore, we
were unable to detect sFasL in the supernatants of highly purified
neutrophil preparations (data not shown), important evidence
against the possibility that failure to detect FasL might reflect
cleavage from the cell surface. Previous reports that have used
different granulocyte isolation protocols (16, 20) suggest that
neutrophils may express FasL, in a manner that may be dependent
upon techniques of preparation (20). However, no FasL expression
could be detected by immunoblotting of highly purified neutro-
phils subjected to deliberate activation by adherence to plastic,
exposure to various concentrations of FMLP and LPS, or phago-
cytosis of OpsZ (Fig. 3B), data independently confirmed by flow
cytometry and where the presence or absence of serum (human or
bovine) had no effect (data not shown). Furthermore, medium con-
ditioned by cultured neutrophils that possessed no sFasL by West-
ern blot analysis had no proapoptotic effect when used to resus-
pend neutrophils, monocytes, or Jurkat T cells (data not shown).
Nevertheless, it was possible to detect FasL in neutrophil lysates/
culture supernatants when these were deliberately contaminated by
as few as 1% monocytes (not shown), evidence against the possi-
ability that degradation of FasL by neutrophil proteases might mask
FasL expression.

When highly purified, FasL-negative neutrophils were em-
ployed as a “target” population, essentially similar results to those
observed with monocyte targets were obtained: medium condi-
tioned by “donor” monocyte/Mφ taking up OpsZ accelerated con-
stitutive apoptosis in target neutrophils in a manner that was par-
ially inhabitable with Fas-blocking ZB4 (Fig. 3, C and D; compare
with Fig. 2). Again, supernatants conditioned by donor Mφ not
The in vivo relevance of these data needs very cautious interpretation, but the findings suggest a hitherto unrecognized role for monocyte/Mϕ-derived factors in regulating apoptosis in neutrophils that have also been summoned to inflammatory sites. Since there is now compelling evidence that granulocytes undergoing apoptosis at inflamed sites are phagocytosed by monocyte/Mϕs (3–5, 7, 8), we went on to examine whether Mϕ ingestion of apoptotic neutrophils might also promote release of FasL.

Monocyte/Mϕ ingestion of apoptotic neutrophils also stimulates FasL release and Fas-mediated apoptosis of FasL-negative neutrophils

“Donor” monocyte-derived Mϕ were cocultured with various particulate stimuli for 4 h, the medium was harvested and clarified, and then it was incubated with freshly isolated neutrophils for 15 h, after which time apoptosis in the “target” neutrophils was assayed (Fig. 4). Mϕ taking up apoptotic neutrophils not only released FasL into the supernatant but also yielded conditioned medium that accelerated neutrophil apoptosis to a degree almost completely inhibitable by Fas-blocking ZB4 (Fig. 4 (inset), lane iii), uptake of latex beads did cause FasL release (Fig. 4 (inset), lane iii), indicating that additional Mϕ-derived factors are required to contribute to the Fas-mediated proapoptotic effects of Mϕ-conditioned medium.

**Discussion**

The experiments described above add importantly to previous work implicating Fas and FasL in myeloid leukocyte homeostasis (12–14, 16, 17, 30). Using a supernatant transfer approach, the data demonstrate that monocyte/Mϕ- ingesting opsonized zymosan, a model of phagocytosis of Ab and complement-coated particles, releases FasL and triggers Fas-mediated apoptosis in “target” neutrophils. Furthermore, identical results were seen with Mϕ taking up apoptotic neutrophils, whereas medium conditioned by Mϕ- ingesting latex beads had no proapoptotic effect.

The in vivo relevance of these data needs very cautious interpretation, but the findings suggest a hitherto unrecognized role for phagocytizing macrophages in directing neutrophil (and monocyte) elimination from inflamed sites. It is particularly intriguing
saying that soluble FasL cleared from cell surfaces may not, in certain circumstances, promote apoptosis in “target” cells expressing Fas (35). Clearly, future studies will need to clarify the mechanisms by which monocytes/Mφ release FasL and related pro-apoptotic factors.

The current data clearly indicate that highly purified neutrophils did not express FasL at levels sufficient to be detected by immunofluorescence and immunoblotting or to induce Fas/FasL-mediated fratricide in culture. Preliminary RT-PCR experiments, employing appropriate positive controls, confirm lack of FasL mRNA expression by neutrophils (F. Salway and S. Brown, unpublished data). Different experimental conditions, as suggested by the studies of Mincheff et al. (20), could explain the apparent discrepancy between the current data and those of Liles et al. (16), particularly since that group’s own work demonstrated that, under some conditions, freshly isolated monocytes did not express FasL (16) whereas, under others, they did (12, 13). Furthermore, a recent report (36) suggests that the polyclonal Abs used in these later studies (12, 13) may be inappropriate for probing cell surface FasL expression by flow cytometric analysis (36). As for Western blot analysis, it may be pertinent that, in our experiments, neutrophil populations contaminated with as little as 1% mononuclear cells appeared to express FasL, a finding that also indicates that we were unlikely to be missing a possible intracellular pool of FasL in neutrophils (20). We conclude from the available data that, while FasL is not expressed at detectable levels by neutrophils under the conditions employed in this study, this does not discount the possibility of FasL expression under other conditions. However, debate over whether neutrophils are truly able to indulge in Fas/FasL-mediated fratricide may be somewhat academic, in that acutely inflamed tissues are rapidly infiltrated by Fas-bearing neutrophils and by monocytes, which are generally agreed to be capable of FasL expression.

Finally, the data also demonstrate that cultured unstimulated Mφ secrete factors that suppress apoptosis in both monocytes and neutrophils, consistent with previous data indicating that cytokines known to be elaborated by Mφ, such as granulocyte-macrophage (GM)-CSF, can inhibit apoptosis in both cell types (29, 37). These findings serve only to emphasize the potentially central importance of the macrophage in regulating elimination from inflamed sites of other leukocytes by apoptosis.

To conclude, our studies demonstrate that monocyte/Mφ uptake of OpSZ or apoptotic neutrophils release FasL and promote Fas-mediated apoptosis of monocytes and neutrophils. This may represent a hitherto unrecognized negative feedback loop serving to promote resolution of inflammation by accelerating deletion of leukocytes by apoptosis.

References


