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Antigenic stimulation activates T cells and simultaneously destines them to die by Fas-mediated apoptosis. In this study, we demonstrated that various pathogen-associated molecular patterns up-regulate Fas expression in macrophages and sensitized them specifically to Fas ligand (FasL), but not to other apoptosis-inducing agents such as TNF-α, etoposide (VP-16), and staurosporine. Toll-like receptor, NF-κB, and p38 mitogen-activated protein kinase mediated these responses. LPS stimulation induced the expression of Fas, caspase 8, cellular FLIP Bh-I/A1, and Bcl-x, but not FasL, TNFR p55, Bak, Bax, and Bad at the transcriptional level. Thus, LPS selectively induced the expression of apoptotic molecules of the Fas death pathway (except for cellular FLIP) and antiapoptotic molecules of the mitochondrial death pathway. However, the kinetics of macrophage disappearance following Escherichia coli-induced peritonitis was similar between wild-type and Fas-deficient mice, suggesting that Fas is not essential for the turnover of activated macrophages in T cell-independent inflammation. In contrast, LPS-activated macrophages produced a large amount of IL-1β upon FasL stimulation. Thus, unlike the activation-induced cell death of T cells, the sensitization of macrophages to FasL by pathogen-associated molecular patterns seems to be a proinflammatory rather than an anti-inflammatory event. The Journal of Immunology, 2003, 171: 1868–1874.

Macrophages play a key role in innate immunity against a variety of pathogens. They produce diverse products, including enzymes, complement components, coagulation factors, and cytokines, and link innate immunity and acquired immunity by presenting Ags to T lymphocytes. Just like other immune effector cells, activated macrophages have to be removed by apoptosis when they have accomplished their task, and the mechanisms for their removal are not well understood. In contrast, macrophages often serve as habitats for intracellular pathogens such as Mycobacterium tuberculosis, and induction of apoptosis in infected macrophages by cell-mediated cytotoxicity is suggested to be an important mechanism for killing such pathogens (1).

It has been well demonstrated that Fas ligand (FasL) plays an essential role in the regulation of the apoptosis of T and B cells, and thereby in immunological self-tolerance and the homeostasis of lymphocyte number (2–5). More recently, roles of Fas and FasL in the innate immune system have been studied. We previously demonstrated that FasL simultaneously induces apoptosis and the release of mature IL-1β in inflammatory neutrophils (6). Similarly, macrophages die and produce cytokines upon FasL stimulation (7, 8). These activities of FasL can explain its proinflammatory function in vivo (9–11) and suggest that apoptosis of inflammatory cells is not necessarily an anti-inflammatory event. In addition, FasL has been shown to contribute to the killing of intracellular M. tuberculosis-infected macrophages (12).

The innate immune system distinguishes pathogens based on their molecular characteristics called pathogen-associated molecular patterns (PAMPs). The sensors for PAMPs consist of two groups of molecules, one with homology to the Drosophila Toll receptor (13) and the other with homology to the mammalian apoptosis regulator Apaf-1 and a class of plant disease-resistant genes (14). Previously, it was reported that LPS stimulation confers on human peripheral blood mononuclear cells resistance to spontaneous or Fas-mediated apoptosis (15, 16). Elsewhere, it was reported that LPS up-regulates Fas expression, but does not affect susceptibility to Fas-mediated apoptosis in murine peritoneal exudate macrophages (17). In contradiction to these reports, in this study we show that LPS and other PAMPs sensitize murine macrophages to FasL-induced apoptosis. We also investigated the molecular mechanisms and biological consequences of this phenomenon. The possible physiological and pathological significance of this finding are discussed.

Materials and Methods

Reagents

Mouse soluble rFasL (previously termed WX1) was prepared, as described previously (18). The activity (units) of soluble FasL was determined by its cytotoxic activity against W4.5 cells overexpressing mouse Fas, as described previously (19). An anti-mouse Fas mAb (FLIM58) was established and purified, as described previously (20). LPS from Escherichia coli 026:B6, lipid A from E. coli F583 Rd mutant, and poly(I:C) were purchased from Sigma-Aldrich (St. Louis, MO). Phosphothioate-modified CpG (5′-tcctagctgctagct-3′) and GpC oligo DNA (5′-tcctagctgctagct-3′) (21) were purchased from Genset Oligos (La Jolla, CA). Peptidoglycan (Staphylococcus aureus) was purchased from Fluka (Buchs, Switzerland), Zymosan A (Saccharomyces cerevisiae) was purchased from MolecProbes (Eugene, OR). SN50 NF-κB inhibitor, SN50 M control substance, PD98059 extracellular signal-regulated kinase inhibitor, SB203580 p38 inhibitors, and SB202474 control substance were purchased from Calbiochem (La Jolla, CA).
Mice
Female C57BL/6, C57BL/6 jojo/Jr, C3H/HeN, and C3H/HeJ mice were purchased at 8 wk old from SLC (Shizuoka, Japan). The Kanazawa University Committee on Animal Welfare approved all animal protocols.

Cell lines
FBH, FFL, FDC2, and FFS were established and maintained, as described previously (19). RAW264.7 and J774A.1 mouse macrophage cell lines were cultured in DMEM supplemented with 10% FCS, 100 U/ml benzylenicilin potassium, and 100 μg/ml streptomycin sulfate (culture medium). NF-κB-luciferase reporter stable transfectants of RAW264.7 cells were generated by introducing pNF-κB-Luc (carrying a firefly luciferase cDNA driven by 5× NF-κB-binding sites; Stratagene, La Jolla, CA) together with pBLC-MyHC (carrying the myoglobin B resistance gene), by electroporation.

Thioglycollate-induced peritoneal macrophages
Thioglycollate-induced 4-day peritoneal exudate cells were prepared, as described previously (19). Peritoneal cells (2 × 10⁶) were then cultured in culture medium in a 96-well plate for 2 h, and nonadherent cells were then removed after gentle pipetting.

Assessment of FasL sensitivity
To assess the susceptibility to FasL-expressing cells, the effector cells were labeled with 100 μg/ml of FITC in PBS at 4°C for 10 min. FITC-labeled effector cells and unlabeled target cells (1 × 10⁶ cells each) were mixed in a round-bottom 96-well plate with 200 μl of culture medium, and cultured for 6 h. Cells were then stained with 10 μg/ml propidium iodide (PI) for 5 min on ice and analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA). The percentage of death of the target cells was calculated as the percentage of FITC- cells that were PI+. To assess the susceptibility to artificial soluble FasL (WXX1), the relative amount of viable cells (percentage of viable cells) was determined by a colorimetric assay using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) and 1-methoxy-5-methylphenol (for peritoneal macrophages), WST-1 and 1-methoxy-5-methylphenol (BD Biosciences) on ice. Cells were then analyzed using a FACSCalibur and the CellQuest software (BD Biosciences).

RNase protection assay
RNase protection assays were performed using a RiboQuant Kit with mouse apoptosis-related template sets mAPO-1 and mAPO-2 (BD PharMingen), according to the manufacturer’s protocol.

RT-PCR
For semiquantitative RT-PCR analyses, single-strand cDNA templates were prepared from 10 μg total RNA using oligo(dT)20 (Amersham, Piscataway, NJ) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Specific cDNAs were then amplified by PCR using the following primers and conditions: the long form of mouse cellular FLIP (c-FLIP), sense primer 5′-tgctgatctttgagg-3′, antisense primer 5′-gaagtgaagcactt-3′, annealing temperature (Ta) 55°C, 30 cycles; the short form of mouse c-FLIP, sense primer 5′-ggcctgtggtttt-3′, antisense primer 5′-tccttgagcttcact-3′, Ta 55°C, 30 cycles; mouse β-actin, sense primer 5′-tgcctgctactgcttg-3′, antisense primer 5′-ccgtgactcaaggaaag-3′, Ta 55°C, 15 cycles. The amounts of the template cDNA were adjusted so that a similar amount of a PCR fragment of β-actin was generated within the linear range of the PCR.

Preparation of UV-killed E. coli
The E. coli K12-derived strain, DH5α, was propagated in L-broth and washed with PBS. The bacteria (2.5 × 10⁸ CFU of E. coli in 0.5 ml PBS) were spread in a 10-cm plastic dish without a lid and UV irradiated at a dose of 250 mJ/cm² using StrataLinker 2400 (Stratagene) equipped with five 15-W 254-nm UV light bulbs. The UV-killed E. coli was aliquotted and stored at −80°C until use.

Measurement of IL-1β
The concentration of IL-1β in the culture supernatant was measured using the OptEIA ELISA Kit (BD PharMingen), according to the manufacturer’s protocol.

Reproducibility of experiments
All experiments described in this work were repeated at least three times, and representative data are shown.

Results
LPS sensitizes macrophages to FasL-induced apoptosis
We investigated the effect of LPS stimulation on the susceptibility of the RAW264.7 macrophage cell line to the human FasL cDNA-transfected cell line FFL and the control cell line FBH (19) (Fig. 1, A and B). FFL cells killed ~30 and 95% of untreated and 4-h LPS-treated RAW cells, respectively. FBH cells did not induce significant cell death under either condition, considering that 10–15% of the RAW cells were always killed during their mechanical detachment from the culture well. FasL is synthesized as a membrane-bound protein, and released as a soluble protein by proteolytic cleavage (22, 23). Naturally processed mouse soluble FasL is essentially inactive in terms of cytotoxicity (18). However, human soluble FasL retains some cytotoxicity (22), although fewer cell types are sensitive to soluble FasL than to the membrane-bound form of FasL (24, 25). Therefore, we investigated the sensitivity of untreated and LPS-treated RAW cells to transfectants expressing uncleavable membrane-bound FasL (FDC2) or expressing soluble FasL of human origin (FFS). As shown in Fig. 1B, FDC2 cells, but not FFS cells, killed RAW cells, and LPS treatment enhanced the killing. These results indicate that LPS enhances the sensitivity of RAW cells to the membrane-bound form of FasL.

We then tested whether an artificial mouse soluble FasL that has a longer amino-terminal region than the natural soluble FasL could kill LPS-treated RAW cells, because we previously found that this form of FasL mimics the membrane-bound form rather than the soluble form of FasL (18, 24). As we expected, the purified artificial mouse soluble FasL protein killed LPS-treated, but not untreated RAW cells as efficiently as cells expressing membrane-bound FasL (Fig. 1C, upper). Thus, we used this FasL protein in the following experiments. LPS concentrations as low as 10 pg/ml exerted this effect on RAW cells (Fig. 1D). Similarly, LPS induced FasL susceptibility in another macrophage cell line, J774A.1 (Fig. 1C, lower).

To investigate whether more physiological macrophages are also sensitized to FasL by LPS treatment, we used thioglycollate-induced peritoneal macrophages. Although the peritoneal macrophages were resistant to FasL without LPS stimulation, they became sensitive to FasL after treatment with 100 ng/ml of LPS for 2 days (Fig. 1E). The range of FasL concentrations that induced apoptosis in LPS-activated macrophages was similar to the range that induces apoptosis in splenic T cells (18), suggesting that the observed FasL activity on LPS-activated macrophages is physiologically relevant.

It was previously reported that FasL induced not only apoptotic cell death, but also necrotic cell death in activated T cells (26). Therefore, we investigated whether the observed macrophage death was from apoptosis or necrosis. The presence of morphological features of apoptotic cells and of DNA degradation to nucleosomal units in FasL-treated RAW cells indicated that the form of cell death was apoptosis (Fig. 1, F and G).
LPS induces the expression of apoptotic molecules selectively for the Fas pathway

LPS sensitized RAW cells specifically to FasL, but the susceptibility of this cell line to TNF-α, etoposide, and staurosporine was not significantly altered by LPS pretreatment (Fig. 2). Therefore, we investigated the expression of Fas in macrophages. Fas expression on the macrophage surface was greatly up-regulated after LPS stimulation. Although it was reported previously that monocytes and macrophages express FasL under some circumstances (27, 28), we failed to detect FasL expression before or after the LPS stimulation (Fig. 3A). RNase protection analyses revealed that the Fas expression was up-regulated 6- to 30-fold at the transcriptional level (Fig. 3B, left, and data not shown). We did not detect FasL mRNA, irrespective of LPS treatment. We also found that LPS increased the expression of caspase 8 mRNA 2-fold.

It has been reported that the FasL susceptibility of T cells is inversely correlated with the expression level of c-FLIP (29, 30). c-FLIP is expressed in either a long form, which contains tandem death effector domains and a caspase-like domain, or a short form, which contains the death effector domains only. RT-PCR analyses indicated that macrophages constitutively express mRNA for both forms of c-FLIP, and that the mRNA for the short form was up-regulated after LPS stimulation (Fig. 3C).

The expression of mRNAs for the antiapoptotic members of the Bcl-2 family, Bfl-1 and Bcl-xL (but not Bcl-2), was enhanced after LPS stimulation, while mRNAs for the proapoptotic members, Bak, Bax, and Bad, were unaffected (Fig. 3B, right). These results indicate that LPS selectively induces the expression of apoptotic molecules that are used in the Fas-induced cell death pathway and antiapoptotic molecules that are involved in the mitochondrial cell death pathway.

(n = 3). F, RAW264.7 cells were precultured with or without 100 ng/ml LPS for 4 h, then cultured with or without 4000 U/ml of FasL for 6 h. Chromosomal DNA was then extracted and analyzed by 1% agarose gel electrophoresis. G, Photomicrographs of cells cultured as in F. Cells sequentially treated with LPS and FasL showed apoptotic membrane blebbing (arrowheads).
Toll-like receptor 4 (TLR4) mediates the LPS signal that sensitizes macrophages to FasL. C3H/HeJ mice are defective in the LPS response because of a point mutation in TLR4 (31, 32). However, involvement of some other molecules in the LPS responses has been suggested (14). Therefore, we stimulated wild-type (C3H/HeN) and TLR4-mutant (C3H/HeJ) macrophages with lipid A, the bioactive component of LPS. Lipid A stimulation induced Fas expression and FasL susceptibility in the wild-type, but not the TLR4 mutant macrophages (Fig. 4, A and B). These results indicate that TLR mediated the observed responses to LPS.

Various PAMPs induce Fas expression and FasL susceptibility in macrophages

We tested whether various PAMPs for distinct TLRs have similar activity. CpG DNA, which is an activator for TLR9 (33, 34), enhanced Fas expression and FasL susceptibility, but a control DNA (GpC) did not (Fig. 4, C left, and E). Peptidoglycan and zymosan, which activate TLR1/2 or 2/6 complex, and poly(I:C) dsRNA, which activates TLR3, did the same (Fig. 4, C right, D, and E).
NF-κB and p38 mitogen-activated protein (MAP) kinase mediate the LPS-induced Fas expression

NF-κB has been implicated in the induction of Fas expression by PMA plus ionomycin or TNF-α stimulation (35–37). TLRs transduce the PAMPs signal into NF-κB activation pathways (38). TLRs are also reported to signal through members of the MAP kinase family (39). Therefore, we investigated whether inhibitors for NF-κB and MAP kinase pathways interfere with the LPS-induced up-regulation of Fas expression. First, RAW cells transfected with NF-κB-luciferase reporter gene were pretreated with SN50, a peptide inhibitor of NF-κB nuclear translocation, or SN50M control peptide before LPS stimulation. As expected, SN50, but not SN50M, inhibited NF-κB activation by LPS stimulation, as revealed by luciferase activity (Fig. 5A). In parallel, SN50, but not SN50M, inhibited LPS-induced up-regulation of Fas expression. First, RAW cells transfected with NF-κB-luciferase reporter gene were pretreated with SN50, a peptide inhibitor of NF-κB nuclear translocation, or SN50M control peptide before LPS stimulation. As expected, SN50, but not SN50M, inhibited NF-κB activation by LPS stimulation, as revealed by luciferase activity (Fig. 5A). In parallel, SN50, but not SN50M, inhibited LPS-induced up-regulation of Fas expression. First, RAW cells transfected with NF-κB-luciferase reporter gene were pretreated with SN50, a peptide inhibitor of NF-κB nuclear translocation, or SN50M control peptide before LPS stimulation. As expected, SN50, but not SN50M, inhibited NF-κB activation by LPS stimulation, as revealed by luciferase activity (Fig. 5A). In parallel, SN50, but not SN50M, inhibited LPS-induced up-regulation of Fas expression.

**FIGURE 5.** An NF-κB inhibitor interferes with up-regulation of Fas expression and sensitization to FasL in response to LPS treatment. A, RAW264.7 cells stably transfected with an NF-κB-luciferase reporter gene were precultured in the presence or absence of 36 μM NF-κB inhibitor peptide SN50 or control peptide SN50M for 90 min, then cultured with or without 1 ng/ml of LPS for 4 h. Luciferase activity in the cells was measured and normalized to the protein concentration. B, RAW264.7 cells were cultured, as described in A, then stained with PE-conjugated anti-Fas (thin lines, without LPS; bold lines, with LPS), or an isotype-matched control Ab (dotted line). LPS stimulation did not affect the staining profile of the control Ab. C, RAW264.7 cells were cultured, as described in A, and then cultured with or without 4000 U/ml FasL for 12 h. The percentage of viable cells in the culture without FasL was set as 100% for each culture condition, and the values in the culture with FasL are shown. D, RAW264.7 cells were precultured in the presence (shaded profiles) or absence (open profiles) of indicated inhibitors (5 μM) for 60 min, then cultured with or without 1 ng/ml of LPS for 4 h. Cells were then stained with PE-conjugated anti-Fas (thin lines, without LPS; bold lines and shaded profiles, with LPS), or an isotype-matched control Ab (dotted line). E, RAW264.7 cells were cultured, as described in D, and then cultured with or without 4000 U/ml FasL for 12 h. The percentage of viable cells after the FasL treatment is shown, as described in C, C and E, Vertical lines indicate SD (n = 3).

Turnover of activated macrophages in lpr mice is normal

Fas and FasL play an essential role in the peripheral clonal deletion of activated T cells. To investigate whether Fas-mediated apoptosis is involved in the deletion of activated macrophages in vivo, we compared the time course of disappearance of activated peritoneal macrophages in wild-type and lpr mice after inoculation with UV- killed *E. coli*. There was no significant difference between the wild-type and lpr mice in the rate with which the number of peritoneal macrophages decreased (Fig. 6).

**FIGURE 6.** Turnover of activated macrophages in lpr mice is normal. Wild-type and lpr/lpr C57BL/6 mice were injected with 5 × 10⁷ UV-killed *E. coli* i.p. on day 0. At the indicated time points, peritoneal exudate cells were recovered by peritoneal lavage. The average numbers of macrophages (Mac-1-high Gr-1-dull cells) of five mice each were plotted. Each point represents average of five mice. Vertical lines indicate SD (n = 5).
effect of LPS pretreatment on the FasL-induced IL-1β production in macrophages. Without LPS pretreatment, FasL stimulation alone was not sufficient to induce IL-1β production in thioglycollate-induced peritoneal macrophages. However, IL-1β secretion from LPS-treated macrophages was greatly up-regulated by FasL in a dose-dependent manner (Fig. 7A). Similarly, a large amount of IL-1β was detected in the culture supernatant of LPS-treated RAW cells, but only when they were subsequently cultured with transfectants expressing the membrane-bound forms of FasL, FFL, and FDC2 (Fig. 7B).

Discussion

Ashany et al. (17) previously reported that Th1 cell lines induce apoptosis in peritoneal exudate macrophages in a Fas-dependent manner. Consistent with their reports, we found that transfectants expressing the membrane-bound form of FasL (but not those expressing soluble FasL only) killed macrophages, albeit to a relatively small extent. However, in the same report, they concluded that LPS stimulation enhanced Fas expression, but not the susceptibility of macrophages to Fas-mediated apoptosis, which is in striking contrast to our observations. The contradictory results may be because they used an anti-Fas mAb, but not FasL-expressing cell lines, to investigate the effect of LPS, because anti-FasL mAb is a poor agonist compared with FasL under some circumstances (18). In contrast, our results described in this study clearly demonstrated that LPS sensitizes macrophages to FasL in a more physiological setting.

It has been shown that various PAMPs induce activation of NF-κB and p38 MAP kinase pathways through TLRs. In this study, we found that both of these signaling pathways are involved in the up-regulation of Fas expression. IL-1 and TNF-α also activate these signaling pathways. Consistent with a previous report (17), TNF-α enhanced Fas expression in macrophages (data not shown). However, IL-1β did not do so (data not shown). Therefore, activation of NF-κB and p38 may not be sufficient for Fas expression, although it is possible that macrophages are simply non- or low-responsive to IL-1.

In parallel with FasL sensitivity, LPS stimulation increased Fas and caspase 8 expression at the transcriptional level. Unexpectedly, it also up-regulated the expression of c-FLIP, which is an intracellular inhibitor of death receptor-mediated apoptosis (40–42). It has been reported that the FasL susceptibility of T cells is inversely correlated with the expression level of c-FLIP (29, 30). Recently, Lens et al. (43) established transgenic mice that over-express c-FLIP in T cells, and found that the T cells of these mice became resistant to soluble rFasL. However, the transgenic mice showed no lymphoproliferative disease and no abnormality in the activation-induced cell death of T cells. A possible explanation for these apparently controversial results is that c-FLIP can fully protect T cells from the soluble form of FasL, but not from its membrane-bound form. Furthermore, stimulation of B cells by CD40 ligand was shown to up-regulate Fas expression and FasL sensitivity (44), and it was recently reported that c-FLIP expression is also up-regulated by CD40 ligand stimulation (45). Therefore, c-FLIP expression alone seems not to be sufficient to confer resistance to the membrane-bound form of FasL, at least in B lymphocytes and macrophages.

In this study, we found that Fas was not essential for the clearance of activated macrophages at the end of E. coli-induced peritonitis. Instead, FasL dramatically augmented IL-1β production from LPS-activated macrophages. It was previously reported that FasL induces IL-18 production in macrophages isolated from Propionibacterium acnes-primed, but not untreated mice (7). Based on our results shown in this work, it is likely that P. acnes activated macrophages through its PAMPs in this model. Therefore, the up-regulation of Fas on macrophages by PAMPs stimulation seems to be an event that leads to enhancement of inflammation, rather than termination of inflammation. We previously demonstrated that FasL induces not only apoptosis, but also IL-1β production in inflammatory neutrophils (6). Similarly, it was reported that FasL induces the maturation of dendritic cells and their production of IL-1β (46). However, dendritic cells differ strikingly from macrophages and neutrophils in that they are resistant to FasL-induced apoptosis. In any case, FasL seems to play important roles in the activation of the innate immune system that are unlike its regulatory roles in the acquired immune system. This idea is consistent with the fact that FasL induces inflammation in vivo (9–11).

We previously demonstrated that Fas-deficient mice are resistant to LPS-induced lethality after P. acnes treatment (47). It has been reported that IL-1 plays an important role in this disease model (48), and that osteopetrotic (op/op) mice that have a defective macrophage/osteoclast lineage are resistant (49). Taken together, it is likely that FasL is involved in the pathogenesis of this disease model by inducing the production of IL-1 and possibly other inflammatory factors from LPS-activated macrophages.

Finally, recent reports have demonstrated that Fas- or FasL-deficient (lpr or gld) mice are highly susceptible to Leishmania major infection (50, 51). It was also reported that intracellular Mycobacterium tuberculosis is killed by FasL-induced apoptosis of infected macrophages (12). Therefore, PAMPs-induced sensitization of macrophages to FasL is likely to be a protective mechanism against intracellular pathogens.

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References

PAMPs SENSITIZE MACROPHAGES TO Fas LIGAND


