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Induction of Fas Ligand in Murine Bone Marrow NK Cells by Bacterial Polysaccharides

Öyvind Halaas, Randi Vik, and Terje Espevik

Bacterial polysaccharides have pleiotropic effects on the immune system, ranging from the beneficial alert of onset of an infection to the lethality of an endotoxic shock. Several organs and tissues are affected by LPS, especially the thymus, liver, and endothelium (1, 2). In sepsis or endotoxemia, a high degree of apoptosis is seen in thymocytes and liver cells, whereas less apoptosis occurs in spleen cells and bone marrow cells (BMC) (3–5). In vitro, LPS causes apoptosis in endothelial cells in synergy with heat shock inducers (6) but prevents neutrophils and monocytes/macrophages from undergoing apoptosis (7–9). The molecular mechanisms of LPS-induced apoptosis or prevention of apoptosis are not currently known.

The Fas (CD95, Apo-1) and Fas ligand (FasL, CD95L) apoptotic system is well established in regulation of immune cell development and selection as well as in immune responses. In hematopoietic cells, Fas is expressed on monocytes/macrophages (9–11), granulocytes (10, 11), T and B lymphocytes (12), NK cells (13), and hematopoietic progenitors (14–16). However, expression of Fas is not always sufficient to make cells susceptible to Fas-mediated apoptosis (10, 11, 17). The FasL effector molecule, originally thought to be expressed only in T cells, has now been shown to be widely expressed by hematopoietic cells. FasL is expressed on T cells (predominantly in Th0 and Th1 CD4+ helper cells and in CD8+ cytotoxic cells) (18), B cells (19), granulocytes (11), NK cells (13, 20), and macrophages (9). FasL is also abundantly expressed in nonhemopoietic immune-privileged tissues and in tissues with a high level of cell turnover (21). Northern blot studies have shown that only very low levels of FasL mRNA are constitutively expressed in murine bone marrow, and bone marrow-derived stromal cell lines are FasL negative (18). However, little is known about the regulation of FasL expression in the bone marrow or the role of Fas-FasL interactions in endotoxemia.

LPS is composed of a polysaccharide attached to a water-insoluble micelle-forming lipid moiety, while poly-β-(1–4)-d-mannurionate (mannuronan, poly-M) isolated from *Pseudomonas aeruginosa* is a well-defined polysaccharide with cytokine-inducing activity (22). Both LPS and mannuronan induce the production of TNF, IL-1, and IL-6 from human monocytes (22, 23), bind to membrane CD14 (24), and require either serum- or LPS-binding protein for TNF production at low concentrations (24, 25). While LPS can stimulate CD14-negative cells, mannuronan requires membrane CD14 for biologic activity (24). As opposed to LPS, mannuronan is not toxic in mice even at high concentrations (26). Mannuronan induces colony-stimulating activity from primary bone marrow stroma and is a synergistic factor in murine progenitor cell colony formation (27). Prophylactic administration of mannuronan rescues mice both from lethal irradiation (27) and lethal infection (Ref. 26 and T. Espevik, unpublished results) suggesting interesting therapeutic uses of this polymer.

In this report, we show that both LPS and a defined polymer such as mannuronan stimulate murine bone marrow NK cells to express FasL. Our results may contribute to the understanding of the molecular mechanisms of sepsis and LPS-associated graft-vs-host disease.

Materials and Methods

Medium and chemicals

Unless otherwise stated, medium refers to RPMI 1640 (Life Technologies, Paisley, Scotland) with 0.1 mg/ml l-glutamine and 20 μg/ml gentamicin
(Garamycin, Schering-Plough, Kenilworth, NJ). Complete medium contained 10% heat-inactivated (30 min, 56°C) FCS (Life Technologies).

Mannuronan was purified from *P. aeruginosa* as described elsewhere (25). LPS contamination was <10 ng/mg mannuronan, and no protein contamination could be detected in 1 mg mannuronan by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). LPS (*Escherichia coli* O26:B6) was purchased from Sigma (St. Louis, MO).

A fusion protein with murine Fas-linked to the constant and hinge region of human IgG1 (Fas-Fc) was purified from medium of Fas-Fc-secretting NIH-3T3 cells (kindly provided by Dr. P. Leder, Howard Hughes Medical Institute, Harvard Medical School, Boston, MA) on a protein A column as described elsewhere (17).

Cytokines used were recombinant human (rh)IL-6 (Sandoz, Basel, Switzerland), rhIFN-α (Glaxo, Geneva, Switzerland), murine rTNF (Genentech, South San Francisco, CA) and rhIL-2 (Cetus Oncology, Emeryville, CA).

**Cells and cell lines**

BMC flushed from the femora and tibia of 8- to 12-wk-old C57BL/6 mice (Bonnolt Gärö, Ry, Denmark) were used in all experiments except where indicated. C58/HenBom Fas/gld mice (gld) homozygous for nonfunctional mutated Fas (28) were purchased from Bonnolt Gärö for negative controls. L1210 (passage 1210-3) and Fas-transfected L1210-Fas cells expressing low and high levels of membrane Fas (29, 30), respectively, were generously provided by Dr. P. Golstein, INSERM, Marseille, France. d12S-P60C hybridoma (d12S) cells (provided by Dr. P. Golstein) stimulated with PMA (10 ng/ml) and ionomycin (500 ng/ml) (both from Sigma) were used as FasL-positive effector cells (29, 30). Jurkat T cells, which are sensitive to FasL, and the murine RAW macrophage cell line were purchased from American Type Culture Collection (Rockville, MD).

In some experiments, BMC were depleted of specific lineage marker cells by incubating 1 × 10⁶ BMC/ml with 10 µg/ml of rat IgG anti-mouse mAbs (CD2, CD4, CD8a, CD11b/Mac-1, CD45R/B220, Gr-1, or CD32/16; PharMingen, San Diego, CA) or mouse IgG2a anti-mouse NK1.1.1 (PharMingen) on ice for 30 min, washing three times in medium, and incubating with sheep anti-rat magnetic Dynabeads at bead-to-initial cell number ratios of 2:1. The yield of Lin⁻ cells was 91 to 98% of the BMC.

Bone marrow-derived macrophages were obtained by culturing nonadherent BMC in α-MEM (Life Technologies) supplemented with 0.3 µg/ml gentamicin, 100 µg/ml streptomycin (Life Technologies), 10% conditioned medium from L929 cells (source of CSF-1) (31), and 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT) for 1 wk at 37°C.

Primary stromal cell cultures were obtained by seeding 2 × 10⁶ BMC in 10 ml complete α-MEM with 20% horse serum (BioWhittaker, Walkersville, MD) and 100 µg/ml amphotericin B (Funakoshi, Bristol-Meyers Squibb, New York, NY; complete medium with amphotericin B), and 10 µg/ml hydrocortisone (Sigma) in 25-cm² flasks for 6 wk at 37°C with weekly semidetermination of nonadherent cells, then refeeding with 5 ml of complete medium with 20% horse serum and amphotericin B (without hydrocortisone).

**Cytotoxicity assay**

Chromium release cytotoxicity assays were performed essentially as described previously (17, 29). Briefly, 1 × 10⁶ 1210 or 1210-Fas cells/ml were labeled for 2 h with 18.5 MBq/ml (500 µCi/ml), and 6 × 10⁶ Jurkat cells/ml were labeled for 1.5 h with 22.2 MBq/ml (600 µCi/ml) with sodium ⁵¹Cr-chromate (sp. act., 18.5 GBq/mg (500 mCi/mg); DuPont NEN, DuPont, Boston, MA). The cells were washed three times in medium before 1 × 10⁷ target cells/well were added to effector cells with or without additions in a total volume of 200 µl in round-bottom 96-well plates. To ensure cell:cell contact, the plates were centrifuged at 200 × g for 2 min before the incubation started. Percentage of specific lysis was calculated from 100 × (experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm), where spontaneous cpm represents radioactivity released from target cells only, and maximum cpm represents radioactivity released from target cells lysed with 0.25% SDS. In some experiments, Fas-Fc fusion protein, anti-TNF mAb (TN3) 19.12, 50–100 µg/ml; CellTech, Slough, UK), anti-IFN-γ mAb (R4GA2, 50 µg/ml; PharMingen), anti-IL-6 mAb (20 µg/ml; Pharmingen), or anti-IFN-α mAb (20 µg/ml; HyCult Biotechnology, Uden, The Netherlands) were used for blocking of FasL, TNF, IFN-γ, IL-6, and IFN-α activities, respectively. Human IgG1 (Sigma) or rat IgG (Pierce, Rockford, IL) were used as controls. Non-specific binding of Fas-Fc to BMC was blocked with murine IgG (100 µg/ml; Caltag, So. San Francisco, CA). Control Abs alone or in combination with murine IgG did not affect specific lysis of L1210, L1210-Fas, or Jurkat cells. In some experiments, combinations between 1 × 10⁴ Jurkat target cells and 3 × 10⁵ BM effector cells was prevented by a 40-µm mesh Transwell (Life Technologies), and the viability of Jurkat cells was assessed by fluorescence microscopy after staining with acridin orange (yielding green fluorescence in viable cells) and ethidium bromide (red fluorescence in dead cells with damaged cell membranes). Jurkat cells cultured without effector cells showed >95% viability throughout these experiments.

**Flow cytometry**

All steps were done at 0 to 4°C. For detection of surface Fasl, cells were preincubated with murine IgG (100 µg/ml) in PBS + 0.1% BSA (Sigma) for 30 min to reduce nonspecific binding before 50 µg/ml mouse IgG2b anti-mouse Fas mAb Kay-10 (PharMingen) was added for 30 min. Bound FasL, Abs were detected with 20 µg/ml biotinylated anti-mouse IgG2b (PharMingen) and streptavidin-phycocerythrin (Becton Dickinson, Mountain View, CA). Cells incubated with mouse IgG and subjected to staining in the second and third step served as controls. The cells were analyzed on a FACScan cytometer (Becton Dickinson). Surface Fasl was also detected by staining with Fas-Fc (50 µg/ml) for 30 min using FITC-conjugated mouse anti-human IgG1 (20 µg/ml; Zymed Laboratories, San Francisco, CA) as a second step.

**Reverse transcriptase-PCR**

RNA was extracted from 10⁷ BMC with TRizol reagent (Life Technologies) as described by the manufacturer. cDNA was synthesized from 2 µg RNA using M-MLV reverse transcriptase (Life Technologies) as previously described (13). PCR was performed on a 1:2 dilution of the cDNA as described (13). Each PCR cycle consisted of a denaturation step at 94°C for 1 min, annealing for 2 min (at a primer-specific temperature), polymerization at 72°C for 1 min, and after completion of the cycles, one 7-min polymerization at 72°C. Primers were (5' to 3'): murine β-actin 3'-end, GAT CTT GAT CTT CAT GGT AGG; 5'-end, TTG TTA CCA ACT AGG ACC ACA TGG (annealing at 67°C, 20 cycles); murine Fasl 3'-end, CCA CCG CCA TCA CAA CC; 5'-end, GGG GCT GGT TGT TGC AA (annealing at 67°C, 28–30 cycles); murine Fas 3'-end, AAC TGC ACC CTG ACC ACA ACC; 5'-end, CAG CAC AGG AGC AGG TG (annealing at 69°C, 30 cycles); all primers were synthesized at Kebo Lab, Spånga, Sweden. PCR products were dissolved in a 1.8% (w/v) agarose gel, stained with ethidium bromide, and analyzed on a GelDoc 1000 with Molecular Analyst software (Bio-Rad Laboratories).

**Cytokine analysis**

TNF was determined by its cytotoxic effects on the fibrosarcoma cell line WEHI 164 clone 13 measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (32) as previously described (33) using murine rTNF as standard. IL-1 was determined using a two-step bioassay employing the murine thymocyte cell line EL-4 NOB-1, which responds to IL-1 with IL-2 production as previously described (34). rhIL-6 was included as standard. IL-2 from IL-1-stimulated NOB-1 cells or from LPS-stimulated BMC was determined in an MTT assay using the IL-2-dependent murine T cell line HT-2 as described (35). IL-6 was determined in an MT assay using the IL-6-dependent mouse hybridoma cell line B 13.29 clone 9 as described previously (36). rhIL-6 was included as a standard.

**Results**

**Time- and dose-dependent induction of BMC-mediated cytotoxicity by LPS and mannuronan**

We analyzed the induction of cytotoxicity against the human T cell line Jurkat by murine BMC stimulated with LPS and mannuronan. Addition of LPS (100 ng/ml) or mannuronan (100 µg/ml) to cultures of BMC and ³¹Cr-labeled Jurkat cells at an E:T ratio 100:1 resulted in a time-dependent increase in the lysis of Jurkat cells reaching 50 and 60%, respectively, at 24 h. Unstimulated BMC did not induce lysis of Jurkat cells. LPS or mannuronan did not affect the spontaneous lysis of Jurkat cells, showing that BMC were required for lysis of Jurkat cells. Both LPS and mannuronan contamination could be detected in 1 mg mannuronan by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). LPS (*Escherichia coli* O26:B6) was purchased from Sigma (St. Louis, MO).
L1210-Fas cells compared with Jurkat cells, but both time and dose dependency for LPS and mannuronan were similar. The specific lysis of L1210-Fas cells by the FasL<sup>+</sup> d12S hybridoma at an E:T ratio 3:1 was 80%, while specific lysis of L1210 cells was 20%, suggesting that L1210 cells expressed low levels of Fas. Also, flow cytometric analysis showed a low level of expression of Fas by L1210 cells. Expression of Fas by L1210 cells has also been shown by others (30, 37). Hence, the presence of Fas on the L1210 parent cell line may account for the susceptibility to lysis by BMC stimulated with LPS or mannuronan (Fig. 1).

C3H/HeN gld/gld mice have a point mutation in the FASL gene resulting in a defective FasL protein that is unable to bind to and signal through Fas (28). BMC from C3H/HeN gld/gld mice were not able to lyse L1210, L1210-Fas, or Jurkat cells in the presence of LPS (Fig. 2), confirming that FasL is the major cytotoxic molecule in LPS-stimulated BMC.

In addition, BMC-mediated cytotoxicity was inhibited by a Fas-Fc fusion protein. The inhibition of cytolyis of L1210-Fas cells with Fas-Fc (100 mg/ml) was 64 and 47% for mannuronan and 57 and 61% for LPS at E:T ratios of 50:1 and 100:1, respectively (Fig. 3). Also, lysis of Jurkat cells by LPS-stimulated BMC was inhibited 40% by Fas-Fc (60 mg/ml) at an E:T ratio of 100:1 (not shown). The blocking anti-FasL mAb Kay-10 (20 mg/ml) also inhibited the specific lysis of L1210-Fas cells 50% when LPS-stimulated BMC at an E:T ratio of 50:1 were used as effector cells (not shown). Fas-Fc (10 mg/ml and 50 mg/ml) completely inhibited the cytolytic activity of FasL<sup>+</sup> d12S cells against L1210-Fas in short term <sup>51</sup>Cr release assays (4 h), but only by 55 to 60% in long term assays (24 h).

These experiments provide evidence that BMC stimulated with LPS or mannuronan express FasL, which is a major cytotoxic effector molecule against L1210-Fas and Jurkat cells.

Induction of surface FasL and FasL mRNA in murine BMC by LPS

The expression of FasL was further analyzed by flow cytometry and RT-PCR. Adding LPS to unfractionated BMC for 24 h resulted in a marked increase in the expression of surface FasL (Fig. 4). The histogram shown in Figure 4B suggests that FasL is expressed mainly by a subpopulation of LPS-stimulated BMC. Only low levels of surface FasL were detected on freshly isolated BMC (data not shown). Similar results were obtained when Fas-Fc was used to detect surface FasL.

RT-PCR showed that the FASL gene was constitutively transcribed in BMC (Fig. 5). FasL mRNA levels increased in BMC stimulated with LPS for 6 and 12 h, but after 24 h with LPS, the FasL mRNA level was reduced to that of freshly isolated BMC. BMC incubated in medium alone retained the FasL mRNA level until a drop after 24 h of culture (Fig. 5). Fas, however, was not regulated at the transcriptional level (data not shown). The data show that both transcription of the FASL gene and induction of surface FasL occur in LPS-stimulated BMC, which is in agreement with the bioassays.

Involvement of bone marrow NK cells in LPS-stimulated BMC-mediated cytotoxicity

We next characterized the cell type responsible for BMC-mediated cytotoxicity. Macrophages respond readily to LPS, but neither macrophages derived from bone marrow nor murine RAW macrophages had any cytotoxic activity against L1210-Fas or Jurkat cells in the presence of LPS (data not shown). We performed immunomagnetic depletion of lineage specific cells from fresh BMC before initiating the cytotoxicity assays. All of the cytotoxic activity resided in the CD2 population (Fig. 6A). In the mouse, CD2

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FIGURE 1. Involvement of FasL in LPS- or mannuronan-induced BMC cytotoxicity. The specific lysis of L1210 cells (low Fas expression; upper panel) or L1210-Fas cells (high Fas expression; lower panel) by BMC after 22 h of coculture in the absence (ctrl) or presence of LPS (100 ng/ml) or mannuronan (50 mg/ml) at different E:T ratios. The results are mean ± SEM of 2 to 5 independent experiments in triplicates. *p < 0.05, **p < 0.005, and ***p < 0.001 by paired Student’s t test.

Prompted a dose-dependent increase in BMC-mediated cytotoxicity, which reached a plateau level for lysis of Jurkat cells at 100 ng/ml LPS and 50 mg/ml mannuronan.

When cell:cell contact between BMC and Jurkat cells was prevented by a 0.4-μm mesh Transwell physical barrier, Jurkat cells were not killed after 48 h in coculture with BMC in the presence of LPS (100 mg/ml). Hence, LPS-induced BMC lysis of Jurkat cells is cell mediated and requires physical contact between the effector and target cells.

Involvement of FasL in LPS and mannuronan induced BMC cytotoxicity

We hypothesized that FasL was responsible for the cytotoxic activity, since Jurkat cells are sensitive to FasL but not to TNF (data not shown). The involvement of FasL was analyzed by employing Fas-transfected L1210-Fas cells and Fas<sup>−/−</sup> L1210 parent cells as target cells in <sup>51</sup>Cr release assays (29, 30). L1210-Fas cells were more susceptible than L1210 cells to lysis by stimulated BMC, suggesting that the main cytotoxic effector molecule is FasL (Fig. 1). LPS or mannuronan did not affect the spontaneous lysis of L1210 or L1210-Fas cells. Specific lysis was somewhat lower for

Involvement of bone marrow NK cells in LPS-stimulated BMC-mediated cytotoxicity

We next characterized the cell type responsible for BMC-mediated cytotoxicity. Macrophages respond readily to LPS, but neither macrophages derived from bone marrow nor murine RAW macrophages had any cytotoxic activity against L1210-Fas or Jurkat cells in the presence of LPS (data not shown). We performed immunomagnetic depletion of lineage specific cells from fresh BMC before initiating the cytotoxicity assays. All of the cytotoxic activity resided in the CD2 population (Fig. 6A). In the mouse, CD2

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is expressed on T, B, and NK cells, but since depletion of CD4+ or CD8a+ T cells did not affect the specific lysis and depletion of CD45R/B220+ B cells only modestly decreased the lysis of L1210-Fas cells, it seemed likely that NK cells were responsible for the BMC-mediated cytotoxicity. The NK1.1 Ag is considered a specific NK cell marker, and BMC depleted of cells expressing NK1.1 did not mediate any cytotoxicity in the presence of LPS even at high E:T ratios (Fig. 6A and B). CD32/16 (FcγRII/III) is expressed in high amounts on NK cells, and BMC depleted of cells expressing CD32/16 also displayed low levels of cytotoxicity (Fig. 6A). Depletion of cells expressing Gr-1 (primarily granulocytes) or CD11b/Mac-1 (primarily myeloid cells) resulted in a partial decrease in LPS-stimulated BMC-mediated cytotoxicity (Fig. 6A), suggesting that myeloid cells are involved in LPS-induced FasL-mediated cytotoxicity. Lin- cells, composed of stromal cells and

**FIGURE 2.** LPS-stimulated BMC from FasL-defective C3H/HeN gld/gld mice do not mediate cytotoxicity. The specific lysis of L1210 (left), L1210-Fas (middle), and Jurkat cells (right) (all 10^4 cells/well) by BMC from normal C57BL or FasL-defective C3H/HeN gld/gld mice after 22 h of coculture in the presence of LPS (100 ng/ml) at different E:T ratios. The results are the mean ± SEM of two to four independent experiments in triplicates. *p < 0.05, **p < 0.005, and ***p < 0.001 by Student’s t test.

**FIGURE 3.** Inhibition of BMC-mediated cytotoxicity by a Fas-Fc fusion protein. The specific lysis of L1210-Fas cells cocultured with BMC for 22 h with LPS (100 ng/ml) or mannuronan (50 μg/ml) in the presence or absence of Fas-Fc fusion protein (100 μg/ml). Cells were preincubated with murine IgG (100 μg/ml) to reduce unspecific binding. Addition of isotype-matched human IgG1 control did not affect specific lysis (–Fas-Fc in figure). Results are mean ± SD of triplicate determinations from one representative experiment repeated at least twice. *p < 0.05, **p < 0.01, ***p < 0.001 by paired Student’s t test.

**FIGURE 4.** Expression of surface FasL on BMC. Representative histograms of FasL expression in BMC cultured for 24 h without (A) or with LPS (100 ng/ml) (B). The cells were stained for FasL using mouse IgG2b anti-mouse FasL mAb Kay-10, biotinylated anti-mouse IgG2b, and streptavidin-phycocerythrin. Cells were preincubated with mouse IgG to reduce nonspecific binding. Cells incubated with mouse IgG and stained with the second and third step served as control.
hemopoietic progenitors, did not mediate cytotoxicity (Fig. 6A).

Six-week-old primary stromal culture cells stimulated with LPS or mannuronan also did not mediate cytotoxicity against L1210-Fas cells, although FasL transcripts were found by PCR (data not shown). These data demonstrate that NK cells are the major FasL effector cells in murine BMC-stimulated with LPS.

**IL-1, IL-2, TNF, IL-6, IFN-α, and IFN-γ are not involved in LPS-induced cytotoxicity**

Immunomagnetically separated NK cells responded poorly to LPS in induction of cytotoxicity compared with unseparated BMC, and myeloid cells did not seem to participate in the cytotoxic activity. It is possible, therefore, that LPS indirectly induces NK cell FasL expression through production of endogenous cytokines. LPS induced production of bioactive TNF, IL-1, and IL-6 from BMC. However, rTNF (10 ng/ml), IL-6 (10–100 ng/ml), or IL-1 (10 ng/ml) individually or combined did not significantly modulate BMC cytotoxicity alone or in combination with LPS. Moreover, neutralizing mAbs to TNF (50 ng/ml) or IL-6 (10 ng/ml) did not decrease LPS-induced BMC cytotoxicity. Furthermore, neutralizing mAbs to IFN-γ (50 μg/ml) or IFN-α (20 μg/ml) did not inhibit LPS-induced BMC cytotoxicity. IL-2, but not IL-7 and IL-12, has been shown to induce FasL expression in human NK cells (13). In agreement with this, IL-2 (at concentrations >4 U/ml) alone or in combination with LPS increased murine BMC-mediated Fas-based cytotoxicity (data not shown). The effect of IL-2 and LPS was additive or less than additive. However, the IL-2 level in BMC cultures (at 5 × 10^9 BMC/ml, corresponding to an E:T ratio of 100:1) stimulated with LPS was below the detection level of the assay (<1 U/ml). An IL-2 concentration of 20 to 100 U/ml was required to obtain a BMC-mediated lysis comparable to 100 ng/ml LPS. Hence, LPS-induced FasL expression in murine BMC is not likely to be mediated by the NK cell-activating cytokine IL-2 nor by the LPS-induced inflammatory cytokines IL-1, IL-6, TNF, IFN-α, or IFN-γ.

**Discussion**

In this report, we have shown that bacterial polysaccharides induce expression of FasL in murine bone marrow NK cells. BMC stimulated with LPS or mannuronan expressed a dose- and time-dependent cytotoxicity. The cytotoxicity was due to increased FasL expression, since 1) Fas-transfected L1210-Fas cells were more susceptible to lysis by LPS- or mannuronan-stimulated BMC than Fas<sup>low</sup>-expressing L1210 parent cells, 2) LPS-stimulated BMC from FasL-defective gld/gld mice were not cytolytic against L1210-Fas or Jurkat cells, and 3) LPS- or mannuronan-induced BMC cytotoxicity was inhibited by a Fas-Fc fusion protein. In addition, we demonstrated that FasL was up-regulated by LPS at the mRNA and surface protein level.

LPS-induced BMC cytotoxicity was mediated mainly by NK cells, since all cytotoxic activity was removed by depleting BMC of NK1.1<sup>+</sup>, CD2<sup>+</sup>, or CD32/16<sup>+</sup> cells. Although other cell types present in the bone marrow also express FasL, their contribution seems to be of minor importance. CD4<sup>+</sup> and CD8<sup>+</sup> T cells, in which FasL was first found (18, 29), did not participate in cytolsis, either because they were not activated or because of their low frequency of occurrence in the bone marrow. Hahne et al. (19)
have shown that purified splenic B cells express FasL after stimulation with 50 μg/ml LPS for 3 to 4 days. We observed that BMC stimulated with 100 ng/ml LPS for less than 24 h mediated high cytotoxic activity. Furthermore, depletion of cells with the pan-B marker B220/CD45R from the BMC resulted in only a small and statistically insignificant reduction in the cytolytic activity. Both granulocytes (11) and monocytes/macrophages (9) express FasL, but depletion of Gr-1+ or CD11b+ cells only partially decreased the cytolytic activity. These data suggest that B cells, granulocytes, and macrophages contribute to a minor extent to the FasL activity, but that NK cells are the major cytotoxic cells in LPS-stimulated BMC. Both murine and human NK cells have been shown to express FasL (13, 20), and NK cell cytotoxicity is increased after stimulation with LPS (38). However, the contribution of FasL to LPS-stimulated NK cell cytotoxicity has not previously been demonstrated.

The mechanism by which LPS and mannuronic induce FasL on NK cells in the bone marrow is not known. CD14 is a receptor for both LPS and mannuronan (24). Soluble CD14 can substitute for membrane CD14 in LPS-induced effects on cells lacking membrane CD14; however, mannuronic does not activate cells through a soluble CD14 pathway (24). Thus, the biologic similarities between LPS and mannuronan are restricted to cells expressing membrane CD14. Since NK cells do not express CD14 (39) but are activated by mannuronan, CD14+ BMC may participate in the LPS/mannuronan response by producing cytokines that increase FasL expression in NK cells. As shown in Figure 6a, depletion of myeloid CD11b/Mac-1+ and Gr-1+ cells resulted in only a partial and statistically insignificant decrease in cytotoxic activity. Since other cells such as fibroblasts and endothelial cells also are able to produce inflammatory cytokines upon LPS stimulation (40), depletion of one cell type can be compensated for by the others. This may explain the low level of decrease in cytotoxic activity of BMC depleted of myeloid cells.

LPS and mannuronan induced IL-1, IL-6, and TNF production in murine BMC, but none of these cytokines affected Fas-mediated cytotoxicity individually or in combination. Furthermore, neutralizing mAbs to IL-6, TNF, IFN-α, and IFN-γ did not modulate LPS-induced cytotoxic activity. It is unlikely, therefore, that these inflammatory cytokines are responsible for the up-regulation of FasL. Although IL-2 alone increased FasL-based cytotoxicity in murine NK cells, LPS-induced cytotoxicity probably does not involve IL-2 for three reasons. First, <1 U/ml of IL-2 was detected in LPS-stimulated BMC cultures, while >4 U/ml of IL-2 was required for a detectable increase in cytotoxicity. Second, the effect of LPS and IL-2 was additive or less than additive even at low doses of IL-2, showing that substantial amounts of IL-2 are required for induction of cytotoxicity also in combination with LPS. Finally, the IL-2 dose equivalent for a 30% specific lysis of L1210-Fas cells (obtained with 100 ng/ml LPS) was 2 to 100 U/ml, while <1 U/ml was detected in LPS-stimulated BMC cultures. Whether other NK-activating cytokines, such as IL-15 (41) or IL-18 (42), participate in LPS-induced expression of FasL has yet to be determined.

The functional consequence of FasL expression on NK cells in the bone marrow may be increased apoptosis of Fas-expressing cells. While lymphoid cells are susceptible to apoptosis in response to LPS or infection, Fas-expressing myeloid cells appear to be rescued by LPS (3, 7–9, 43). Human bone marrow hematopoietic progenitor cells are sensitive to Fas-mediated apoptosis (14, 15), but whether progenitor and stem cells in the marrow are affected by LPS-induced FasL expression has yet to be studied. Importantly, ligation of Fas does not necessarily lead to apoptosis, since both intracellular proteins (44, 45) and cytokines such as granulocyte-CSF, granulocyte/macrophage-CSF, TNF, and IFN-γ (9, 11) can inhibit Fas-based apoptosis in various cell types. Further studies need to be done to clarify the physiologic significance of LPS-induced expression of FasL in the bone marrow.

The data shown in this report establish for the first time a link between LPS, NK cells, and FasL in the bone marrow. These are central mediators in the pathogenesis of acute graft-vs-host disease (43, 46, 47) and sepsis (1–4). Hence, our results may contribute to a better understanding of the molecular mechanisms at play in these disorders.

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