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*J Immunol* 2011; 186:556-562; Prepublished online 22 November 2010;
doi: 10.4049/jimmunol.1001630
http://www.jimmunol.org/content/186/1/556

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TLR4 through IFN-β Promotes Low Molecular Mass Hyaluronan-Induced Neutrophil Apoptosis

Shaw-Wei Leu,*†‡ Liyun Shi,*†‡ Changqing Xu,* Yili Zhao,‡∥ Baoling Liu,‡∥ Yongqing Li,‡∥ Aviva Shiedlin,# Charlie Xiang,** Huaxiao Shen,‡∥∥ Deborah A. Quinn,*† Charles A. Hales,*† and Hang Zhao,*†‡

Intratracheal administration of low molecular mass (LMM) hyaluronan (200 kDa) results in greater neutrophil infiltration in the lungs of TLR4−/− mice compared with that in wild-type mice. In general, enhanced neutrophil infiltration in tissue is due to cell influx; however, neutrophil apoptosis also plays an important role. We have assessed the effects of TLR4 in the regulation of neutrophil apoptosis in response to administration of LMM hyaluronan. We found that apoptosis of inflammatory neutrophils is impaired in TLR4−/− mice, an effect that depends upon the IFN-β-mediated TRAIL/TRAILR system. IFN-β levels were decreased in LMM hyaluronan-treated TLR4-deficient neutrophils. The treatment of inflammatory neutrophils with IFN-β enhanced the levels of TRAIL and TRAILR 2. LMM hyaluronan-induced inflammatory neutrophil apoptosis was substantially prevented by anti-TRAIL neutralizing mAb. We conclude that decreased IFN-β levels decrease the activity of the TRAIL/TRAILR system in TLR4-deficient neutrophils, leading to impaired apoptosis of neutrophils and resulting in abnormal accumulation of neutrophils in the lungs of LMM hyaluronan-treated mice. Thus, TLR4 plays a novel homeostatic role in noninfected lung inflammation by accelerating the elimination of inflammatory neutrophils. The Journal of Immunology, 2011, 186: 556–562.

Neutrophils play a critical role in innate immunity and are rapidly recruited to sites of infection and injury; however, they use defense mechanisms that destroy exogenous substances that are potentially deleterious to tissues (1). Inflammatory neutrophils are cleared from inflamed sites by apoptosis, followed by macrophage phagocytosis; these actions promote resolution rather than persistence of tissue injury (2). Neutrophil apoptosis is widely accepted as a central mechanism in the resolution of lung inflammation, and attenuation of neutrophil apoptosis in the lung may contribute to severe and prolonged inflammatory responses, including acute respiratory distress syndrome (ARDS) and sepsis (3, 4).

Neutrophils are known to produce ligands of the TNF family, such as TNF-α, FasL, and TRAIL (5–7). In different situations, neutrophils are sensitive to these proapoptotic ligands (8). Endogenous TRAIL can be biologically effective not only as an integral membrane protein but also as a soluble cytokine that is released by neutrophils (6, 7). TRAIL/soluble TRAIL have been shown to eliminate senescent circulating neutrophils (9, 10) and to clear activated tissue neutrophils (6). TRAIL exerts its apoptotic activity by interacting with a complex system of two death receptors, TRAILR1 (death receptor 4; DR4) and TRAILR2 (death receptor 5). Data obtained by using blocking Abs to DR4 and DR5 indicated that DR5 rather than DR4 is a death-inducing TRAILR in neutrophils (9).

Type I IFNs constitute a multimember cytokine family (IFN-α subtypes, β, ε, κ, ω, δ, and τ). IFN-β is represented by a single member, and although both the human and mouse genomes contain more than 20 different IFN-α genes, they share the same receptor, the IFN-α/β receptor (11). Type I IFNs are pleiotropic and have suppressive effects on immunity and inflammation. The suppressive effects of type I IFNs can predominate in certain pathological/physiological settings. For example, IFN-α not only enhances proapoptotic soluble TRAIL levels and mRNA expression in neutrophils in chronic myelogenous leukemia patients (12) and increases serum levels of anti-inflammatory factors, such as IL-1R antagonist (IL-1Ra), in patients with multiple sclerosis treated with weekly injections of IFN-β (13), but it also prevents the production of proinflammatory factors including IL-1β, IL-6, ICAM-1, and MMP-9 (14–16).

Previously, we reported that intratracheal administration of low molecular mass (LMM) hyaluronan (200 kDa) causes neutrophil infiltration into mouse lungs. A lack of TLR4 is associated with increased neutrophil counts in either bronchoalveolar lavage fluids (BALFs) or lung tissues (17). Enhanced pulmonary neutrophil counts can be attributed to increased neutrophil trafficking or decreased neutrophil apoptosis in the lungs (18–20). In the current study, we investigate the role of TLR4 and its downstream product, IFN-β, on regulating apoptosis of inflammatory neutrophils that have been activated via administration of LMM hyaluronan.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1001630
Materials and Methods

Mice and model description

C57BL/6J and Fas−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IFN-β−/− mice were provided by Dr. E.N. Fish (Toronto General Research Institute, Toronto, Ontario, Canada), and TLR4−/− mice were provided by Dr. S. Akira (Osaka University, Osaka, Japan). Routinely, mice 6–8 wk of age were used for experiments. All mice used in this report were housed in a pathogen-free rodent barrier facility. All animal experiments were approved in advance by the Massachusetts General Hospital Center for Comparative Medicine (Boston, MA). LMM hyaluronan (200 kDa, 65 mg/kg) was delivered directly to the trachea with a microsprayer (Penn-Century, Wyndmoor, PA).

BALF

The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge needle. BALF was obtained by instilling and collecting four 0.5-ml volumes of cold PBS through the incised trachea. A total of 1.8 ml lavage fluid was retrieved per mouse. Each BALF sample was centrifuged, and the supernatants were stored at −80°C until use. Total cell numbers in BALF were counted from each sample in a hemocytometer. BALF neutrophil counts were determined on cytospin preparations stained with a Diff-Quick staining kit (IMEB).

Generation of bone marrow chimeras

Recipient (C57BL/6J) or TLR4−/− mice were irradiated with 11 Gy. Next, 8 × 106 donor bone marrow cells were injected into the tail vein of recipient irradiated mice. The mice were kept in microisolator cages for 10 wk to allow for full hemopoietic reconstitution.

Preparation of neutrophils from mouse lung and cell culture

Mice were anesthetized, and the lungs were flushed in situ with 5 ml PBS via cannulation of the heart to remove the intravascular blood pool. Minced lung tissues were incubated at 37°C for 1 h on a rocker with 200 μg/ml collagenase D and 40 μg/ml DNase I (both from Roche) in 10 ml RPMI 1640 medium. Subsequently, the enzyme-digested lung tissues were passed through a stainless steel mesh. Apoptotic cells were removed using annexin V microbeads according to the manufacturer’s instructions (Miltenyi Biotech). Viable neutrophils were then prepared using the neutralization kit (Miltenyi Biotech) and resuspended in RPMI 1640 medium supplemented with 10% FBS and antibiotics. The cells were then stimulated for 20 h with 100 μg/ml LMM hyaluronan (200 kDa) or recombinant mouse IFN-β (100 U/ml; R&D Systems) in the presence or absence of anti-TLR4 neutralizing mAb (5 μg/ml; N2B2; eBioscience).

Myeloperoxidase assay

After BALFs were performed, whole lung homogenates were measured using a mouse myeloperoxidase (MPO) ELISA kit (Cell Sciences) according to the manufacturer’s protocol.

ELISA

FasL, Fas, IL-1β, TNF-α, IL-6, MIP-2, keratinocyte cell-derived chemo- kine (KC), and IFN-β were determined using mouse ELISA assay kits (R&D Systems). Mouse TRAIL was measured by direct ELISA using an N2B2 Ab (eBioscience) in conjunction with a biotinylated Ab to mouse TRAIL (BAF112; R&D Systems).

Annexin V binding analysis and flow cytometry

The neutrophils used for FACS analysis were isolated from mouse lung cells by using the Anti-Ly-6G MicroBead Kit, two MS columns, and a MiniMACS Separator (Miltenyi Biotech). Neutrophil apoptosis was analyzed by a BD LSR II flow cytometer (BD Biosciences) using the annexin V–FITC and propidium iodide apoptosis detection kit (BD Biosciences) according to the manufacturer’s instructions. DR5 (PE) Ab was purchased from eBioscience. Data were analyzed by using FlowJo software (Tree Star).

Lung injury score

Severity of lung inflammation was semiquantitatively assessed according to methods previously described (21). Quantal assessment of injury was performed, in blinded fashion, by grading four histological findings: alveolar septae, alveolar hemorrhage, intra-alveolar fibrin, and intra-alveolar infiltrates. Lungs were fixed overnight in 10% buffered formalin. The fixed lung sections were dehydrated, cleared, and embedded in paraffin by conventional H&E staining. A minimum of five fields were examined for each section.

Caspase colorimetric assay

Caspase enzymatic activity was quantified in lysates prepared from neutrophils using caspase-3 and caspase-8 colorimetric assay kits (BioVision Research Products) according to the manufacturer's protocol. The chromophore p-nitroanilide light emission was quantified using a microtiter plate reader at a wavelength of 405 nm.

Western blot analysis and Abs

Neutrophil extracts were electrophoresed on 4––20% gradient SDS-PAGE gels (Invitrogen) and transferred to 0.22-μM nitrocellulose membranes. The blocked membranes were incubated with primary Ab in TBST containing 5% nonfat milk or BSA at 4°C overnight, followed by incubation with the appropriate secondary Abs (Cell Signaling Technology) according to the manufacturer’s instructions. Proteins were detected by ECL chemiluminescence (Pierce). Anti–β-actin was from Cell Signaling Technology, and Abs to TRAIL, DR5, and IFN-β were from Santa Cruz Bio-technology.

Quantitative real-time PCR

RNA was extracted from neutrophils using TRIZol Reagent (Invitrogen) following the manufacturer’s instruction. One microgram of total RNA was reverse-transcribed using SuperScript II RNase H− Reverse Transcriptase (Invitrogen). PCR was performed with the primers of Fas, 5′-ATGCAC- AACTTGGATGAA-3′; 5′-TTACGAGTCATCCTGTCTCC-3′; Fas ligand (RDP-58-025; R&D Systems); DR5, 5′-TGACGGGAAGAGGA- AACTGA-3′; 5′-GGCTTTGACATTGGATTGTA-3′; and TRAIL (RDP-63-025; R&D Systems).

Statistics

All data are expressed as mean ± SEM. Data for all experiments were analyzed by unpaired Student t test (2 groups) or ANOVA (>2 groups) using the Statview 6.0 software program (SAS Institute, Cary, NC). Comparisons between groups and tests of interactions were made assuming a two-factor analysis, with the interaction term testing each main effect and with the residual error testing the interaction. All comparisons were made using Fisher’s least significant difference procedure, so that multiple comparisons were made at the 0.05 level only if the overall F test from the ANOVA was significant at p < 0.05.

Results

Apoptosis of TLR4-deficient neutrophils is impaired

Apoptosis, in response to LMM hyaluronan administration, was monitored in pulmonary neutrophils from TLR4−/− and wild-type (WT) mice 24 h after intratracheal administration of LMM hyaluronan. Whole lung neutrophils were stained with FITC-labeled annexin V and then analyzed by flow cytometry. The percentage of cells stained positive with annexin V was markedly decreased in TLR4-deficient neutrophils compared with that in WT neutrophils (Fig. 1A, 1B), indicating that TLR4 deficiency hampered neutrophil apoptosis. Next, we determined whether caspase activation is also decreased in TLR4-deficient neutrophils. The activities of caspase-3 and caspase-8 were markedly decreased in TLR4-deficient neutrophils compared with that in WT neutrophils (Fig. 1C, 1D), indicating that TLR4 deficiency attenuated neutrophil apoptosis via a caspase-dependent process.

TRAIL/DR5 pathway is involved in decreased TLR4-deficient neutrophil apoptosis

Caspase-8 activation is associated with death receptor apoptotic signaling and typical death receptor systems include TRAIL/DR5 and FasL/Fas systems. Here we describe that TLR4 deficiency impairs caspase-8 activity and, therefore, TRAIL/DR5 pathway.

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LMM hyaluronan-induced neutrophil apoptosis, Fas$^{-/-}$ and WT mice were intratracheally administered LMM hyaluronan. After 24 h, there was no significant difference in either BALF neutrophil counts or lung tissue MPO activity between these two groups (data not shown). Thus, LMM hyaluronan-induced neutrophil apoptosis does not require the FasL/Fas system.

Because TRAIL is expressed on a variety of tissues and cells (23), we addressed the relative importance of TLR4 on hematopoietic cells and/or parenchymal cells involved in TRAIL production in this murine model. We created mixed chimeras by performing bone marrow transplantation to lethally irradiated mice. Twenty-four hours after intratracheal administration of LMM hyaluronan, TRAIL levels were significantly reduced in WT and TLR4$^{-/-}$ recipient mice that were reconstituted with TLR4$^{-/-}$ bone marrow compared with those in WT and TLR4$^{-/-}$ recipient mice that were reconstituted with WT bone marrow (Fig. 2B). Thus, TRAIL production in the lung is dependent upon TLR4 signaling from cells of hematopoietic origin. We found that LMM hyaluronan only increased the number of neutrophils in BALFs (17). Taken together, our data indicate that neutrophils may be a predominant source for TRAIL production.

Next, we examined DR5 and TRAIL mRNA levels in lung neutrophils 24 h after intratracheal administration of LMM hyaluronan. We found that DR5 and TRAIL mRNA levels were significantly decreased in TLR4-deficient neutrophils compared with those of WT (Fig. 2C), indicating that TLR4 is required for transcription of these genes. Consistent with these observed differences in gene transcription, DR5 and TRAIL protein levels in TLR4$^{-/-}$ bone marrow compared with those in WT and TLR4$^{-/-}$ recipient mice that were reconstituted with WT bone marrow (Fig. 2D).

**FIGURE 1.** Apoptosis is decreased in TLR4-deficient neutrophils. Mice were intratracheally administered LMM hyaluronan (200 kDa, 65 mg/kg) and sacrificed after 24 h. Whole lung neutrophils were then prepared using the neutrophil isolation kit as described in Materials and Methods. A and B, The numbers of apoptotic neutrophils were evaluated by staining with annexin V. C and D, The activities of caspase-3 (C) and caspase-8 (D) in protein extracts of whole lung neutrophils were assayed using a caspase-8 and a caspase-3 colorimetric assay kit, respectively. Data represent mean ± SEM from three independent experiments.

**FIGURE 2.** TRAIL and DR5 are decreased in TLR4$^{-/-}$ mouse lungs and TLR4-deficient neutrophils. A, Twenty-four hours after intratracheal administration of LMM hyaluronan (200 kDa, 65 mg/kg). TRAIL levels in lung homogenates were measured by ELISA. B, Ten weeks after reconstitution, LMM hyaluronan at 65 mg/kg was administered intratracheally into bone marrow chimeric mice. Mice were sacrificed 24 h after LMM hyaluronan treatment; TRAIL levels in lung homogenates were determined in various chimeric mice. C, mRNA was prepared from whole lung neutrophils 24 h after intratracheal administration of LMM hyaluronan (200 kDa, 65 mg/kg). DR5, TRAIL, Fas, and FasL mRNA levels were determined by quantitative PCR. Values in WT controls were set to 100%. Results are expressed as percentage of decrease of DR5, TRAIL, Fas, and FasL mRNA. D, Twenty-four hours after intratracheal administration of LMM hyaluronan (200 kDa, 65 mg/kg). DR5 and TRAIL levels in whole lung neutrophils were assessed by Western blot analysis. Data represent mean ± SEM from three independent experiments. LMM HA, LMM hyaluronan.
were also decreased in TLR4-deficient neutrophils compared with those of their corresponding WT controls (Fig. 2D). However, we were unable to detect any change in cell surface TRAIL between TLR4-deficient neutrophils and WT neutrophils 24 h after intratracheal administration of LMM hyaluronan (data not shown).

IFN-β−/− mice develop severe lung injury

Because type I IFNs are the downstream signaling pathway from TLR4 (24, 25), we investigated whether type I IFNs are involved in LMM hyaluronan-induced lung injury. Analysis of BALF neutrophil counts showed similar results between TLR4−/− and IFN-β−/− mice 24 h after intratracheal administration of LMM hyaluronan (data not shown).

FIGURE 3. Lungs from TLR4−/− and IFN-β−/− mice show similar patterns of inflammation 24 h after intratracheal administration of LMM hyaluronan (LMM HA, 200 kDa, 65 mg/kg). A, Neutrophil counts in BALF were performed. B, After neutrophil counts in BALF were performed, the MPO of lung tissue homogenates was measured. C, Top panels, Lung sections were stained with H&E (original magnification ×200). Bottom panels, Diff-Quick–stained cytospins of BALF neutrophils (original magnification ×400). The BALF dilution in TLR4−/− or IFN-β−/− mice plus LMM hyaluronan was 1/4. Representative data from multiple mice (n = 4 to 6 per group) are shown. D, Lung injury score. E, IL-1β, TNF-α, IL-6, MIP-2, and KC levels in BALF were measured by ELISA. F, IFN-β levels in two strains of TLR4 deficient/mutant mice (C57BL/6J, C3H/HeJ) and their respective controls (C57BL/6J, C3H/HeOuJ) were measured by ELISA. Data represent mean ± SEM from three independent experiments.

TLR4−/− mice, a total of 1 × 10⁸ U/mouse of IFN-β (R&D Systems) was injected s.c. 30 min before intratracheal administration of LMM hyaluronan (200 kDa). BALF neutrophils and MPO were then measured, as illustrated in Fig. 3A and 3B. TLR4−/− mice had a higher response to LMM hyaluronan compared with that of WT mice, and this increase was significantly reduced by pretreatment with IFN-β (Fig. 3A, 3B). The histological examination of the lungs from TLR4−/− and IFN-β−/− mice revealed obvious severe inflammatory infiltrates in the alveolar space within 24 h after intratracheal administration of LMM hyaluronan (Fig. 3C). Quantal scoring of the severity of histological lung injury showed that the lung injury score significantly increased in TLR4−/− and IFN-β−/− mice (Fig. 3D). LMM hyaluronan-induced lung injury in TLR4−/− and IFN-β−/− mice correlated with a marked increase in proinflammatory IL-1β.

FIGURE 4. IFN-β deficiency is associated with decreased TRAIL/DR5 expression and neutrophil apoptosis 24 h after intratracheal administration of LMM hyaluronan (200 kDa, 65 mg/kg). A, TRAIL levels in lung homogenates were measured by ELISA. B, DR5 and TRAIL levels in whole lung neutrophils were assessed by Western blot analysis. C, The percentage of annexin V-positive (apoptotic) whole lung neutrophils was analyzed by flow cytometry. D, The activity of caspase-3 in protein extracts of whole lung neutrophils was assayed. Data represent mean ± SEM from three independent experiments. LMM HA, LMM hyaluronan.
TNF-α, IL-6, MIP-2, and KC (Fig. 3E). Importantly, we found a lack of TLR4-impaired IFN-β production in the lungs 24 h after intratracheal administration of LMM hyaluronan (Fig. 3F). To determine whether the observed impairment in IFN-β production in TLR4−/− mice is strain dependent, additional studies were performed using TLR4−/− mutant (C3H/HeJ) mice and their control (C3H/HeOuJ). We found that IFN-β levels in BALF did not differ between the two strains of TLR4-deficient/mutant mice (Fig. 3F), indicating that mouse strain does not influence the observed phenotypes.

Next, we investigated whether TRAIL and DR5 are involved in IFN-β–mediated neutrophil apoptosis. TRAIL levels in the lung homogenates of IFN-β−/− mice were significantly decreased compared with those of WT mice 24 h after intratracheal administration of LMM hyaluronan (Fig. 4A). DR5 and TRAIL protein levels were also decreased in IFN-β–deficient neutrophils compared with those of their corresponding WT controls (Fig. 4B). The percentages of cells stained positive with annexin V and caspase-3 activity were both markedly decreased in IFN-β–deficient neutrophils compared with those of WT neutrophils (Fig. 4C, 4D).

IFN-β upregulates TRAIL/DR5 in neutrophils

Given the finding that IFN-β levels were decreased in TLR4-deficient neutrophils compared with those of WT neutrophils 24 h after intratracheal administration of LMM hyaluronan (200 kDa, 65 mg/kg) (Fig. 5A), we investigated whether exogenous IFN-β could induce DR5 and TRAIL in neutrophils isolated from LMM hyaluronan-stimulated mouse lungs. After 20 h of incubation with IFN-β, DR5 protein levels in TLR4-deficient neutrophils were upregulated, as shown by Western blot analysis (Fig. 5B). Exogenous IFN-β also induced a significant increase in TRAIL protein levels in both TLR4-deficient neutrophils and in cell culture supernatant (Fig. 5C, 5D). However, LMM hyaluronan did not increase TRAIL and DR5 levels in TLR4-deficient neutrophils (Fig. 5C, 5E) or in TLR4-deficient neutrophil culture supernatant (Fig. 5D). We found that TRAIL induction was defective in IFN-β–deficient neutrophils in response to LMM hyaluronan (Fig. 5C). This defect was due to a loss of IFN-β, as we observed that IFN-β was able to rescue TRAIL levels in IFN-β–deficient neutrophils (Fig. 5C).

**FIGURE 5.** LMM hyaluronan-mediated IFN-β up regulates TRAIL and DR5 levels in neutrophils. A, IFN-β levels in whole lung neutrophils were assessed by Western blot analysis 24 h after intratracheal administration of LMM hyaluronan (200 kDa, 65 mg/kg). B–E, Whole lung neutrophils were isolated from mice 24 h after intratracheal administration of LMM hyaluronan (200 kDa, 65 mg/kg) as described in Materials and Methods, and neutrophils (2 × 10⁶/ml) were cultured with recombinant mouse IFN-β (100 U/ml) or LMM hyaluronan (100 μg/ml). After 20 h of culture, DR5 levels in neutrophils treated with IFN-β (100 U/ml) were assessed by Western blot (B). TRAIL levels in neutrophil cell lysate (C) and culture supernatant (D) were measured by ELISA. DR5 levels in neutrophils treated with LMM hyaluronan (100 μg/ml) were assessed by Western blot (E). Representative data of three independent experiments with similar results are shown. *p < 0.05. LMM HA, LMM hyaluronan.

LMM hyaluronan-mediated TRAIL induces neutrophil apoptosis in vitro

WT and TLR4-deficient neutrophils treated with IFN-β were significantly more apoptotic than cells treated with medium alone at 20 h postadministration (Fig. 6A). The acceleration of neutrophil apoptosis when stimulated with IFN-β was accompanied by increased expression of DR5 (Fig. 5B). The elevated expression of DR5 might enhance the sensitivity of neutrophils to IFN-β–mediated TRAIL–induced apoptosis in vitro. However, LMM hyaluronan only increased the apoptosis and DR5 levels of WT neutrophils and not TLR4-deficient neutrophils (Figs. 6A and 5E).

To demonstrate that LMM hyaluronan-treated neutrophil-generated endogenous TRAIL is able to induce neutrophil apoptosis, we added anti-TRAIL neutralizing mAb into cell cultures and evaluated the neutrophil apoptosis. The addition of anti-TRAIL

**FIGURE 6.** Effects of anti-TRAIL Ab on neutrophil apoptosis in vitro. Whole lung neutrophils were isolated from mice 24 h after intratracheal administration of LMM hyaluronan (200 kDa, 65 mg/kg). A. The isolated neutrophils (2 × 10⁶/ml) were incubated in complete medium or with recombinant mouse IFN-β (100 U/ml) or LMM hyaluronan (200 kDa, 100 μg/ml) for 20 h. The percentage of annexin V-positive neutrophils was analyzed by flow cytometry. B, The isolated neutrophils (2 × 10⁶/ml) were incubated in complete medium or with LMM hyaluronan (100 μg/ml) in the presence of 5 μg/ml normal mouse IgG or 2 or 5 μg/ml anti-TRAIL neutralizing mAb (N2B2) for 20 h. The percentage of annexin V-positive neutrophils was analyzed by flow cytometry. Representative data of three independent experiments with similar results are shown. *p < 0.05. LMM HA, LMM hyaluronan.
neutrophils in the lung to initiate inflammatory responses via the IL-1β–MyD88 dependent pathway (17). In contrast, LMM hyaluronan also induces production of the anti-inflammatory/ proapoptotic mediator IFN-β via the TLR4–MyD88 independent pathway. Thus, IL-1β induced by LMM hyaluronan within the lung triggers a proinflammatory response. However, this innate response must be dampened within the microenvironment of the lung to limit excessive lung inflammation as its dysregulation can result in lung injury. We have found that LMM hyaluronan-induced IFN-β is one of these inhibiting components. IFN-β is able to induce neutrophils to release TRAIL, and expression of DR5 in neutrophils is increased in the presence IFN-β. The elevated expression of DR5 might enhance the sensitivity of neutrophils to TRAIL-induced apoptosis. Meanwhile, IFN-β plays an immunosuppressive role through the induction of the anti-inflammatory mediator, IL-1Ra (data not shown). IL-1Ra acts as a negative regulator of the IL-1β signal (17). Thus, IFN-β also inhibits neutrophil trafficking into the lung.

Although IFN-β is effective at delaying spontaneous apoptosis in human peripheral blood neutrophils (26), the importance of IFN-β in apoptosis of inflammatory neutrophils is not fully understood. We found that TLR4-mediated IFN-β upregulates neutrophil apoptosis in our setting. The discrepancy between our description of the responsiveness of neutrophils to IFN-β and that of Wang et al. (26) may be due to differences in the location where the neutrophils were studied. When neutrophils migrate from circulating blood into various tissues, some remarkable phenotypical changes take place. For example, the life span of inflammatory lung neutrophils is increased compared with that of peripheral blood neutrophils (27). These opposite effects of IFN-β on neutrophil apoptosis are important for various neutrophil functions. The ability of IFN-β to prolong the survival of peripheral blood neutrophils allows the neutrophils to more efficiently phagocytose inflammatory stimuli. Once their functions are complete, the number of activated neutrophils needs to be tightly controlled to avoid excessive inflammation and tissue damage. Thus, it is sensible that IFN-β would induce neutrophil apoptosis in a later stage of inflammation.

TNF-α/TNFαRs, FasL/Fas, and TRAIL/TRAILRIs are the best characterized death receptors expressed on neutrophils (22). We studied whether TNF-α/TNFαRs or FasL/Fas are also involved in LMM hyaluronan-induced pulmonary neutrophil apoptosis. In agreement with a previous report that neutrophils from Fas-deficient mice have been shown to undergo constitutively apoptotic apoptosis at the same rate as that of WT neutrophils (28), our results indicate that TLR4-mediated promotion of neutrophil apoptosis is independent of the FasL/Fas system. TNF-α has long been known to have a biphasic effect upon neutrophil apoptosis in vitro, with early induction followed by later (12 h onward in vitro) inhibition of apoptosis (29). Furthermore, the effects of TNF-α on neutrophil apoptosis were dose dependent: at low doses (100–1000 pg/ml) survival increased, but at higher doses (10,000 pg/ml) this effect was lost (30). The observed levels of TNF-α in TLR4−/− mouse BALF are ~210 pg/ml (17) 24 h after LMM hyaluronan treatment, which is in the range of TNF-α concentrations (100–1000 pg/ml) shown to prevent neutrophil apoptosis, indicating that TNF-α in TLR4−/− mice may play an anti-apoptotic effect in our setting.

TRAIL can selectively induce apoptosis of transformed and tumor cells but not of normal cells (31). Our data indicate that neutrophils in the lungs are TRAIL-sensitive under inflammatory conditions. Our finding is consistent with previous work that demonstrated the ability of TRAIL to induce neutrophil apoptosis, including aged neutrophils and activated tissue neutrophils (9, 10). Conversely, normal peripheral blood neutrophils are sensitive to FasL (32), and FasL only induces chemotaxis but not apoptosis in aged neutrophils (33, 34). Fresh peripheral blood neutrophils are sensitive to TNF-α (29), which paradoxically inhibits apoptosis of neutrophils cultured for >12 h in vitro (29). Taken together, these findings indicate that death receptors have different effects on neutrophil apoptosis, depending on the location and/or the time when neutrophils were studied.

In this study, the function of TLR4-mediated IFN-β is to balance inflammatory responses through upregulation of proapoptotic/anti-inflammatory molecules, such as TRAIL/DR5. TRAIL/TRD5 induces apoptosis of inflammatory neutrophils; TLR4 also downregulates proinflammatory cytokines (17), eventually leading to reduced inflammation. Consequently, the deficiency in TLR4-mediated IFN-β production or signaling pathways may result in generation of an inflammatory milieu that promotes excessive tissue inflammation. Thus, it is possible that some lung injury patients may not have the proper type of IFN-β induction or signaling during LMM hyaluronan-induced inflammation. Acute lung injury and ARDS affect ~200,000 patients per year in the United States with recent mortality rates being >40% (35). The BALF of ARDS patients contains a high percentage of LMM hyaluronans, whereas only high molecular mass hyaluronan is found in BALF of non-ARDS patients and normal controls (36, 37). LMM hyaluronan induces a dramatic infiltration of neutrophils into the interstitial and intra-alveolar compartment in mouse lung (17). Animal and clinical studies have indicated a pivotal role of neutrophils in the pathogenesis of acute lung injury and ARDS (38, 39). The current investigation suggests that treatment with the proapoptotic/anti-inflammatory mediators IFN-β or TRAIL may afford significant protection against LMM hyaluronan-induced lung injury.

Acknowledgments
We thank E.N. Fish, Division of Cell and Molecular Biology, Toronto General Research Institute (Toronto, Ontario, Canada), for IFN-β−/− mice and S. Akira, Department of Host Defense, Osaka University (Osaka, Japan), for TLR4−/− mice.

Disclosures
The authors have no financial conflicts of interest.

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