Identification of the Mycobacterial Subcomponents Involved in the Release of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand from Human Neutrophils[∇]

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Intravesical administration of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) continues to be a successful immunotherapy for superficial bladder cancer. Recently, workers in our laboratory observed expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on neutrophils in voided urine following BCG therapy. Neutrophils released a soluble and functional form of TRAIL when they were stimulated in vitro with BCG, and the activity was localized predominantly to the cell wall fraction. In this study, we examined the ability of individual mycobacterial components to stimulate TRAIL release from neutrophils. Our results demonstrated that cell wall-derived lipoarabinomannan (LAM), mycolyl arabinogalactan-peptidoglycan complex, and a Triton X-114 (Tx114)-solubilized protein pool were effective agonists of TRAIL release from neutrophils. Mycobacterial DNA was also an agonist of TRAIL release from neutrophils. Furthermore, purified antigen 85 ABC complex and alpha-crystallin (HspX), two major cell wall antigens present in the Tx114 pool, induced TRAIL release from neutrophils. The Tx114 pool stimulated HEK-293 cells expressing either Toll-like receptor 2/1 (TLR2/1) or TLR2/6, but only HspX was able to stimulate TLR2/6-expressing cells. TLR4/MD2/CD14-expressing cells responded only to LAM. Collectively, these results suggested that TRAIL release from neutrophils was induced through the recognition of multiple mycobacterial components by TLR2 and TLR4.

Since it was first described in 1976, intravesical administration of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) has been the treatment of choice for superficial bladder cancer (30). However, the mechanism of BCG immunotherapy has not been clearly defined, which has limited rational improvement to this treatment strategy. Instillation of BCG results in an early influx of neutrophils into the bladder wall, followed by an influx of mononuclear cells consisting primarily of CD4⁺ cells. A proinflammatory, Th1 cytokine (interleukin-2 [IL-2], IL-12, gamma interferon [IFN- γ]) response predominates following BCG stimulation, and the generation of this Th1 response is often associated with a favorable outcome of treatment (5, 9, 21). BCG establishes a localized infection in the bladder by attachment to and internalization in urothelial cells (both malignant and normal) (3, 28). The urothelium releases IL-1, -6, and -8 and granulocyte-macrophage colony-stimulating factor in response to bacterial pathogens (20). IL-8 is chemotactic for neutrophils, and a high level of early IL-8 production is associated with better clinical responses to BCG (42). The net effect of chemokine signals is escalating recruitment of neutrophils and monocytic leukocytes into the bladder with each successive BCG instillation (33). Within 4 to 6 h after late-cycle clinical BCG instillation, it is common to find massive pyuria with more than 10⁷ white blood cells/ml of urine (M. O'Donnell, personal communication). More than 75% of

these cells are neutrophils; macrophages account for approximately 5 to 10% of the cells, and only 1 to 3% of the cells are T cells or NK cells (8).

Neutrophils are professional phagocytes that migrate to sites of inflammation and ingest microorganisms by phagocytosis (11, 17, 31). Neutrophils kill microorganisms by the combined activity of antimicrobial proteins and reactive oxygen species. Initiation of these effector functions is dependent upon recognition of conserved molecular patterns of microorganisms. The Toll-like receptor (TLR) family is an evolutionarily conserved group of proteins responsible for mediating innate immune reactions through recognition of pathogen-associated molecular patterns (1, 38). Ten TLRs have been identified in humans, and human neutrophils express mRNA for all the known human TLRs except TLR3 (19). The expression of multiple TLRs gives neutrophils the potential to recognize multiple pathogen-associated molecular patterns and respond to a broad range of microorganisms.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor superfamily of cytokines that can induce apoptosis in a variety of tumor cell types (45). We recently observed expression of TRAIL on neutrophils in voided urine following BCG therapy (27). In vitro studies demonstrated that BCG serves as a secretagogue for human neutrophils, causing them to release intracellular stores of soluble TRAIL that exhibits cytotoxic activity (25). Furthermore, the stimulatory activity of BCG was localized to the cell wall fraction. The mechanisms of BCG-induced TRAIL release by neutrophils have not been defined. Therefore, the goals of the present study were to identify the myco-

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bacterial components that stimulate the release of TRAIL from neutrophils and to determine the role of TLRs in this response.

MATERIALS AND METHODS

Reagents. PAM3CSK4 and ultrapure lipopolysaccharide (LPS) from *Escherichia coli* (InvivoGen, San Diego, CA) were used in this study as agonists of TLR2 and TLR4, respectively. Agonists were resuspended in endotoxin-free water and diluted into tissue culture media at the time of the experiment.

***M. bovis* BCG and mycobacterial fractions.** MV261 BCG, a Pasteur strain, was obtained from Yi Luo (University of Iowa, Iowa City) and was used in all experiments. BCG was maintained at 37°C in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with 10% albumin dextrose concentrate (5% bovine serum albumin, 2% dextrose, 0.85% NaCl) and 0.05% Tween 80 (Sigma, St. Louis, MO). Mycobacterial fractions were obtained from the Mycobacteria Research Laboratories (Colorado State University, Ft. Collins) through a Tuberculosis Vaccine Testing and Research Materials contract. *M. bovis* and *Mycobacterium tuberculosis* are closely related organisms with >99.95% genomic identity, and no genes are unique to *M. bovis* (14). In addition, BCG (derived from *M. bovis*) has been used as a human vaccine for tuberculosis since the 1920s (2, 14), suggesting that many of the antigens recognized by immune cells are similar. Based on these similarities, the following fractions derived from *M. tuberculosis* were used in this study: irradiated *M. tuberculosis* whole cells (WC), *M. tuberculosis* cell walls (27,000 $\times$ -g fraction) (CW) isolated by centrifugation of whole-cell lysates, lipaarabinomannan (LAM) isolated by size exclusion chromatography, phosphatidylinositol mannosides (PIM) isolated by thin-layer chromatography, the mycolyl arabinogalactan-peptidoglycan complex (mAGP) isolated by sodium dodecyl sulfate extraction from the cell wall, a Triton X-114 (Tx114)-solubilized protein pool isolated from Tx114-solubilized cell wall fractions followed by acetone and phenol extractions, DNA isolated by organic solvent extraction of whole-cell lysates, native alpha-crystallin (HspX) isolated by size exclusion chromatography, and an antigen 85 complex isolated by high-performance liquid chromatography. The Tx114 fraction was dialyzed during isolation and did not contain detergent. All fractions were highly pure according to manufacturer quality controls (see <http://www.cvmbs.colostate.edu/microbiology/tb/sop.htm> for isolation and quality control protocols), and the lyophilized components were resuspended in dimethyl sulfoxide, phosphate-buffered saline, or endotoxin-free water according to product-specific recommendations of the manufacturer. *M. tuberculosis* mycolic acids (MA) and trehalose 6,6'-dimycolate (TDM) were purchased from Sigma (St. Louis, MO) and were resuspended in chloroform-methanol (9:1) according to the manufacturer's recommendations. Stock solutions were diluted in tissue culture media at the time of the experiment. All fractions were endotoxin free (<0.03 endotoxin unit/ml) after resuspension, as determined using the Pyrotel *Limulus* amoebocyte lysate assay (Associates of Cape Cod, Falmouth, MA).

Isolation of neutrophils. Heparinized blood was obtained from healthy human volunteers by using a protocol approved by the Institutional Review Board at The University of Iowa. Neutrophils were isolated by dextran sedimentation and Hypaque-Ficoll (Amersham) density gradient separation as previously described (6). Residual erythrocytes were removed by hypotonic lysis, and neutrophils were resuspended in RPMI supplemented with 10% fetal bovine serum, penicillin, streptomycin, 1 mM sodium pyruvate, 100 μ M nonessential amino acids, and 10 mM HEPES (complete RPMI). Isolation yielded $\geq 99\%$ neutrophils that were >95% viable as determined by trypan blue exclusion. The purity of neutrophils was determined on the basis of nuclear morphology using light microscopy and was confirmed by flow cytometry analysis of forward and side scatter and CD15 staining. In addition, the level of neutrophil viability was >95% at the time of stimulation, as determined by trypan blue exclusion.

Quantitative real-time reverse transcription-PCR. Total RNA was isolated from 10^7 untreated neutrophils or 10^7 neutrophils stimulated with various concentrations of IFN- α (1 to 1,000 U/ml; PeproTech, Rocky Hill, NJ) along with TRIzol reagent (Life Technologies, Gaithersburg, MD). For the kinetic studies, 10^7 untreated neutrophils or 10^7 neutrophils were stimulated with IFN- α (100 U/ml) for 20 h. Total RNA (1 μ g) was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The real-time quantitative reverse transcription-PCR primer-probe sets for TRAIL mRNA (Hs00234356_m1) were purchased from PE Applied Biosystems (Foster City, CA). The rRNA probes were labeled with a second dye to analyze TRAIL and rRNA in the same reaction. Two hundred fifty nanograms of cDNA was used as a template for a TaqMan assay of TRAIL transcripts and an internal control of rRNA. The TaqMan PCR was carried out as described previously (43).

Flow cytometry. Neutrophils were stimulated with mycobacterial components, and surface CD62L expression was analyzed by flow cytometry. To stain cells, 100 μ l of cells was combined in a 96-well round-bottom plate with 5 μ l of DREG-56, fluorescein isothiocyanate (FITC)-conjugated immunoglobulin G1, anti-human CD62L (eBioscience, San Diego, CA), or immunoglobulin G1-FITC isotype controls (Caltag Laboratories, Inc., Burlingame, CA) and incubated at 4°C for 30 min. Following three washes with phosphate-buffered saline containing 2 mg/ml bovine serum albumin and 0.02% Na₂S₂O₃, cells were fixed with 2% paraformaldehyde and analyzed with a FACScan (Becton Dickinson, Franklin Lakes, NJ); more than 10^4 cells were analyzed for each sample.

IFN- α priming of neutrophils and stimulation with mycobacterial components. Neutrophils (2×10^6 to 3×10^6 cells/ml) were added to individual wells of a 24-well tissue culture plate, primed with 100 U/ml IFN- α for 6 h, and then stimulated with either live BCG (10 CFU/cell) or purified *M. tuberculosis* fractions at different concentrations for an additional 16 to 18 h (overnight). To ensure delivery of lipid fractions to neutrophils, polystyrene microspheres were coated with 50 μ g of LPS, MA, TDM, LAM, and PIM as described previously (23); uncoated beads were used as controls. Coated microspheres were added to neutrophils at a ratio of 10 beads/cell. Each suspension was centrifuged at $800 \times g$ for 5 min to induce contact and incubated overnight. Following stimulation, neutrophils were centrifuged, and the culture supernatants were analyzed for TRAIL using a sandwich enzyme-linked immunosorbent assay (ELISA) (Diacclone, Stamford, CT).

TLR cell lines. HEK-293 cells were maintained in Dulbecco modified Eagle medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 1 mM sodium pyruvate, 100 μ M nonessential amino acids, and 10 mM HEPES (complete DMEM) at 37°C with 5% CO₂. HEK-293 cells stably transfected with TLR2/6 were purchased from InvivoGen (San Diego, CA) and were maintained in complete DMEM with 10 μ g/ml blasticidin (InvivoGen, San Diego, CA). HEK-293 cells stably transfected with TLR2 and HEK-293 cells stably transfected with TLR4/MD-2/CD14 were gifts from Jerrold Weiss (University of Iowa, Iowa City) and were maintained in complete DMEM with 10 μ g/ml blasticidin and in complete DMEM with 10 μ g/ml blasticidin and 100 μ g/ml hygromycin B (Invitrogen, Carlsbad, CA), respectively. 293-TLR1/2 cells were generated by transient transfection with the Eugene 6 transfection reagent (Roche, Indianapolis, IN) using plasmid pDUO-hTLR1/TLR2 (InvivoGen, San Diego, CA), and successful expression was assessed by determining responsiveness to the TLR2 agonist PAM3CSK4 (parent HEK-293 cells are unresponsive to TLR agonists). For stimulation experiments, 1×10^5 cells were seeded into individual wells of a 24-well tissue culture plate and allowed to adhere overnight. Fresh medium (without selective antibiotics) was added, and the cells were stimulated with purified *M. tuberculosis* fractions or TLR agonists at the concentrations indicated below for 18 h. Culture supernatants were collected, and IL-8 was analyzed using the IL-8 DuoSet ELISA system from R&D Systems (Minneapolis, MN).

Statistical analysis. A statistical analysis was performed using SigmaStat v3.5 (Systat Software, San Jose, CA). The data were obtained in at least three individual experiments and are expressed below as means and standard errors of the means. Statistically significant differences ($P < 0.05$) were determined by a one-way analysis of variance, followed by a Dunnett's posttest with comparison to the untreated or control group.

RESULTS

IFN- α increased the amount of TRAIL released from neutrophils in response to BCG. Both IFN- α and IFN- γ are potent inducers of TRAIL expression on multiple immune cells, including neutrophils (10, 18, 24, 40), and work in our laboratory demonstrated that compared to unprimed neutrophils, IFN-primed neutrophils contained higher intracellular levels of TRAIL and were able to secrete significantly more TRAIL after stimulation with BCG (25). In our previous studies, neutrophils were primed with IFN- α (100 or 1,000 IU/ml) for 20 h. Because human neutrophils are short-lived cells, we determined the optimal dose of IFN- α and the incubation time necessary to stimulate transcription of the TRAIL (TNFSF10) gene in order to maximize cell viability. Neutrophils were incubated with different amounts of IFN- α (1 to 1,000 IU/ml) for 20 h, and TRAIL mRNA levels were determined by quantita-

FIG. 1. IFN- α increased the amount of TRAIL released from neutrophils in response to BCG. Neutrophils were primed for 20 h with different doses of IFN- α (A) or with 100 IU/ml IFN- α for different times up to 8 h (B), and TRAIL mRNA levels were determined by quantitative reverse transcription-PCR. Unprimed neutrophils (shaded bars) and IFN- α -primed neutrophils (100 IU/ml for 6 h) (solid bars) were challenged with BCG (ratio, 10:1) for 18 h, and IL-8 (C) and TRAIL (D) levels in culture supernatants were determined by ELISA. The bars indicate the means of at least three independent experiments with different donors, and the error bars indicate the standard errors of the means. Values that are statistically significant ($P < 0.05$) are indicated by asterisks.

tive reverse transcription-PCR (Fig. 1A). The level of TRAIL mRNA peaked with a dose of 100 IU/ml IFN- α (Fig. 1A). Next, neutrophils were stimulated with 100 IU/ml IFN- α for different periods of time, and the TRAIL mRNA levels steadily increased with increasing time (Fig. 1B). Finally, we determined the impact of IFN- α priming on both IL-8 and TRAIL secretion by neutrophils. Unprimed and primed neutrophils released similar amounts of IL-8 in response to BCG stimulation (Fig. 1C), but fourfold more TRAIL was released from IFN- α -primed neutrophils than from unprimed neutrophils (Fig. 1D), consistent with our previous findings (25). Based on these results, all subsequent experiments were performed with neutrophils primed with 100 IU/ml IFN- α for 6 h prior to stimulation with the mycobacterial fractions.

Mycobacterial fractions induce TRAIL release from IFN-primed neutrophils. CD62L is expressed on the surface of neutrophils and is released by endoproteolysis upon cellular activation (34, 41). Thus, we examined CD62L shedding in response to the mycobacterial components to determine the optimal amount of each fraction needed to stimulate neutrophils. CD62L shedding was observed with live BCG, irradiated *M. tuberculosis* whole cells, the cell wall fraction, the Tx114-soluble fraction, mAGP, and DNA (Fig. 2). No CD62L shedding was observed with neutrophils treated with PIM, LAM, mycolic acids, or TDM. The doses of TLR ligands and myco-

FIG. 2. Mycobacterial fractions stimulated CD62L shedding from neutrophils. Neutrophils were stimulated with the mycobacterial fractions at different concentrations and incubated for 4 h at 37°C. Cells were stained using FITC-conjugated anti-CD62L antibody or an isotype control and were analyzed by flow cytometry. Live BCG (BCG), WC, CW, the Tx114-soluble fraction (Tx114), mAGP, MA, TDM, LAM, and PIM were examined. The data are representative of three independent experiments with different donors.

bacterial components that resulted in CD62L shedding from neutrophils were used in subsequent experiments.

Next, we examined the amounts of TRAIL protein present in the culture supernatants of IFN-primed neutrophils stimulated with the mycobacterial components. Stimulation of neutrophils with the mycobacterial fractions demonstrated that

FIG. 3. Neutrophils released TRAIL in response to mycobacterial fractions. Neutrophils were primed with 100 U/ml IFN- α for 6 h and then stimulated with the mycobacterial fractions for 18 h at 37°C. TRAIL levels in culture supernatants were determined by ELISA (A). The doses of fractions were as follows: PAM3CSK4, 10 μ g/ml; LPS, 100 ng/ml; BCG, 10:1 ratio; WC, 100 μ g/ml; CW, 10 μ g/ml; PIM, 10 μ g/ml; Tx114-soluble fraction (Tx114), 10 μ g/ml; LAM, 10 μ g/ml; mAGP, 10 μ g/ml; MA, 10 μ g/ml; TDM, 10 μ g/ml; DNA, 10 μ g/ml; Ag85ABC, 10 μ g/ml; and HspX, 10 μ g/ml. (B) Polystyrene microspheres were coated with the mycobacterial cell wall lipids as described in Materials and Methods and were added to IFN- α -primed neutrophils at a 10:1 ratio. The levels of TRAIL in culture supernatants after 18 h were determined by ELISA. The bars indicate the means of at least five independent experiments with different donors, and the error bars indicate standard errors of the means. Values that are statistically significant ($P < 0.05$) are indicated by asterisks.

live BCG, irradiated *M. tuberculosis* whole cells, the cell wall fraction, the Tx114-soluble fraction, LAM, mAGP, and DNA all resulted in release of TRAIL (Fig. 3A). In contrast, MA, TDM, and PIM did not induce TRAIL release from neutrophils (Fig. 3A), even when they were coated on polystyrene microspheres to ensure delivery of the lipids to the cells (Fig. 3B). LPS- and LAM-coated microspheres stimulated TRAIL release from neutrophils more than uncoated controls stimu-

lated TRAIL release, suggesting that microspheres were effectively coated with each of the compounds.

Of all the mycobacterial fractions tested, the Tx114-soluble protein pool was the most potent stimulus of TRAIL release from neutrophils, suggesting that a protein(s) from the cell wall had high agonistic activity. The cell wall of *Mycobacterium* sp. contains an abundance of proteins and lipoproteins (36). The antigen 85 ABC (Ag85ABC) complex and alpha-crystallin (HspX) are two predominant cell wall antigens of both BCG and *M. tuberculosis* (7, 29, 44) and are present in the Tx114-soluble protein pool. Thus, we examined the ability of Ag85ABC and HspX to induce TRAIL release from IFN- α -primed neutrophils (Fig. 3A). Our findings demonstrated that both Ag85ABC and HspX were potent agonists of TRAIL release from neutrophils. In addition, TRAIL-inducing activity was eliminated when either Ag85ABC or HspX was digested with proteinase K (data not shown). These results demonstrate that alpha-crystallin and the antigen 85 complex are agonists of TRAIL release from neutrophils and suggest that other mycobacterial cell wall proteins have potential agonist activity.

Collectively, these results demonstrated that the primary agonists of TRAIL release from neutrophils were a mycobacterial cell wall-derived protein(s), mAGP, LAM, and DNA, with the cell wall lipids making lesser contributions. Together, the combined recognition of all these structures may contribute to the potent stimulation of TRAIL release from neutrophils treated with whole mycobacteria.

Mycobacterial cell wall components that induce TRAIL release from neutrophils are agonists of TLR2 and TLR4. Previous work in our laboratory demonstrated that purified agonists of TLR2 and TLR4 stimulated TRAIL release from neutrophils (25). To identify the TLRs involved in the recognition of mycobacterial cell wall structures, we used HEK-293 cell lines expressing TLR2, TLR2/1, TLR2/6, or TLR4/MD-2/CD14. We limited our focus to TLR2- and TLR4-expressing cells, because PAM3CSK4 and LPS were the only purified TLR agonists to stimulate TRAIL release from neutrophils. In addition, based on the types of ligands recognized by TLR3, TLR5, TLR7, and TLR9, these TLRs are unlikely to be activated by the mycobacterial cell wall components. Cells were stimulated with either mycobacterial fractions or specific TLR agonists as controls, and IL-8 levels in culture supernatants were determined by ELISA (Fig. 4). Parental HEK-293 cells lacking TLRs failed to respond to any of the mycobacterial fractions or TLR agonists (data not shown). In contrast, HEK-293 cells expressing either TLR2 and TLR1 or TLR2 and TLR6 responded to PAM3CSK4, irradiated *M. tuberculosis* whole cells, cell walls, and the Tx114-soluble fraction (Fig. 4A and B). Similar to the Tx114-soluble fraction, HspX stimulated cells expressing TLR2/1 and TLR2/6. Surprisingly, Ag85ABC did not stimulate TLR2/1 or TLR2/6 cells. TLR4/MD-2/CD14-expressing cells responded to only LPS and LAM (Fig. 4C). These results suggested that the mycobacterial fractions stimulated cells by both TLR2 and TLR4 and that a cell wall protein(s) was the primary TLR2 agonist.

DISCUSSION

TRAIL is a potent inducer of apoptosis in tumor cells and exhibits little cytotoxicity against normal cells (45). Previous

FIG. 4. Mycobacterial fractions stimulated TLR2- and TLR4-expressing cell lines. HEK-293 cells expressing TLR2/1 (A), TLR2/6 (B), and TLR4/MD-2/CD14 (C) were stimulated with TLR agonists as controls or with mycobacterial fractions for 18 h. Culture supernatants were collected, and IL-8 was analyzed by ELISA. The doses of agonists and fractions were as follows: PAM3CSK4, 10 μ g/ml; LPS, 100 ng/ml; WC, 100 μ g/ml; CW, 10 μ g/ml; PIM, 10 μ g/ml; Tx114-soluble fraction (Tx114), 10 μ g/ml; LAM, 10 μ g/ml; mAGP, 10 μ g/ml; MA, 10 μ g/ml; TDM, 10 μ g/ml; DNA, 10 μ g/ml; Ag85ABC, 10 μ g/ml; and HspX, 10 μ g/ml. The bars indicate the means of at least three independent experiments, and the error bars indicate the standard errors of the means. Values that are statistically significant ($P < 0.05$) are indicated by asterisks.

work in our laboratory revealed higher levels of TRAIL in the urine of patients that responded well to BCG therapy than in the urine of poor responders, suggesting that TRAIL plays an important role in the antitumor outcome of BCG immunotherapy (27). Despite the worldwide acceptance of BCG for the treatment of patients with bladder cancer, only limited information about the immunological basis of this therapy is available. BCG immunotherapy could be viewed as a method of inducing repeated mycobacterial infections in the bladder and the immunological response to infection secondarily includes a tumoricidal component. The bladder is not normally infiltrated with large numbers of immune cells. However, after repeated instillations of BCG, there is an early accumulation of neutrophils, followed by infiltration of macrophages and lymphocytes into the bladder (4, 8, 21). Whereas the role of T cells and NK cells in BCG immunotherapy is well accepted, there is limited information regarding the role of neutrophils in this response. However, recent evidence suggests that neutrophils have an essential role in the success of BCG therapy in mice (37). Recent work in our laboratory revealed that neutrophils were a major source of soluble TRAIL that was cytotoxic against tumor cells and present in the urine of bladder cancer patients undergoing BCG therapy (25, 27). Both live and heat-killed BCG, as well as the mycobacterial cell wall fraction, elicited TRAIL release from neutrophils stimulated in vitro (25). These findings point to the role of neutrophils in the accumulation of active TRAIL in the urine after BCG therapy and suggest that individual cell wall components of mycobacteria can potentially stimulate TRAIL release from neutrophils.

The antitumor activity is unique to BCG, and it is unclear what molecular cues of BCG distinguish it from other microorganisms capable of establishing bladder infections. In this study, we wanted to further characterize the ability of mycobacterial cell wall components to stimulate the release of TRAIL from neutrophils. *M. bovis* and *M. tuberculosis* are closely related organisms with >99.95% genomic identity, and no genes are unique to *M. bovis* (14). In addition, BCG (derived from *M. bovis*) has been used as a human vaccine for tuberculosis since the 1920s (2, 14), suggesting that many of the antigens recognized by immune cells are similar. Based on these similarities, we obtained subcellular fractions and cell wall components derived from *M. tuberculosis* from the Mycobacteria Research Laboratories at Colorado State University that were readily available for research use. In support of this, we found that both live BCG and irradiated *M. tuberculosis* whole cells stimulated neutrophils to release equivalent amounts of TRAIL, suggesting that the same stimulatory antigens were present in both organisms. Furthermore, our results demonstrated that the cell wall fraction, the Tx114-soluble protein pool, purified LAM, and mAGP were agonists of TRAIL secretion from neutrophils. In contrast, the mycobacterial cell wall lipids (MA, TDM, and PIM) did not stimulate TRAIL release from neutrophils, even when they were coated on polystyrene microspheres. Studies have demonstrated that these mycobacterial cell wall lipids are adjuvants of immune cells (22, 26, 32). In addition, PIM stimulates cells through a TLR2-dependent mechanism (15, 22). Comparisons of our experimental methods and the methods used the previous studies revealed no striking differences in the handling of PIM and delivery of this component to the cells. However, the

major difference in our study is that we specifically examined the ability to induce the release of TRAIL from human neutrophils. Our results do not rule out the possibility that mycobacterial cell wall lipids play a role in TRAIL release, but they suggest that the major factors involved in TRAIL release from neutrophils are the cell wall proteins, mAGP, and LAM. It is possible that the cell wall lipids may have an indirect role in the amount of TRAIL released by neutrophils through cell recruitment of immune cells and/or stimulation of cytokines, such as IFN, which augments TRAIL secretion from neutrophils.

The Tx114-soluble protein pool had the highest TRAIL-inducing activity of all the fractions tested. Ag85ABC and alpha-crystallin are cell wall antigens of both BCG and *M. tuberculosis* (7, 13, 22, 29, 44), and Ag85ABC comprises up to 15% of the total protein found in *M. bovis* and *M. tuberculosis*. Our findings indicated that both Ag85ABC and HspX stimulated TRAIL release from neutrophils and may be major factors contributing to the potent activity of the Tx114-soluble fraction. However, there are many proteins in the cell wall of *Mycobacterium* sp., some of whose immunostimulatory activity has been characterized (39), so an important focus of future studies will be to determine if another mycobacterial cell wall protein(s) can stimulate TRAIL release from neutrophils.

Neutrophils express most of the TLRs described so far (TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR10), giving them the ability to respond to a variety of conserved molecular patterns (19). Previous findings demonstrated that BCG induced the transcription and secretion of IL-8 in neutrophils by signaling through TLR2 and TLR4 (16). Another study found that both TLR4^{-/-} and wild-type mice were able to successfully eliminate BCG after intraperitoneal challenge, but TLR2^{-/-} mice had 10-fold-higher bacterial loads in the lungs after similar challenges (22). These results demonstrate the importance of both TLR2 and TLR4 in the immune response to BCG. Previous work in our laboratory revealed that the TLR agonists PAM3CSK4 and LPS induce TRAIL secretion from neutrophils, suggesting that TLR activation has a role in TRAIL release.

Based on the correlation between TRAIL expression and TLRs, we wanted to determine if the TRAIL released from neutrophils by the mycobacterial fractions was the result of TLR activation. Initially, we attempted to block TRAIL release using two different neutralizing antibodies to TLR2 and TLR4, but we were unsuccessful. Neutrophils express many TLRs and other pattern recognition receptors, so it is possible that activation is the result of recognition by multiple receptors. In addition, neutrophils upregulate TLR expression after activation, which could result in the presence of new "unblocked" TLRs on the surface (19). Therefore, TLR-expressing HEK-293 cells were used to identify the TLRs on neutrophils that may be involved in the recognition of mycobacterial cell wall structures and subsequent TRAIL release. Our results showed that the various mycobacterial cell wall components were recognized by HEK-293 cells expressing either TLR2 or TLR4. The antigen 85 complex only weakly stimulated TLR2-expressing cells despite exhibiting potent activity in neutrophils, whereas HspX strongly activated TLR2/6 cells. Since Ag85ABC is a complex of three proteins, it may be recognized by multiple cellular receptors and/or coreceptors. Neutrophils express multiple pattern recognition receptors, whereas the

293-TLR cells are restricted to individual TLR receptor complexes and may be unable to recognize more complex structures. We limited our focus to cells expressing TLR2 and TLR4, because we observed TRAIL release only from neutrophils stimulated with agonists of TLR2 and TLR4 (25). Although mycobacterial DNA stimulated TRAIL release from neutrophils, TLR9 is involved only in the recognition of CpG-containing DNA motifs and is unlikely to be activated by mycobacterial cell wall structures. In support of this, preliminary experiments demonstrated that the individual mycobacterial cell wall components did not stimulate TLR5- or TLR9-expressing cells (data not shown). However, recognition of mycobacterial DNA by TLR9 after phagocytosis may contribute to enhanced TRAIL release by neutrophils. Surprisingly, mAGP failed to stimulate any of the TLR-expressing cells, despite being a potent agonist for neutrophils. The mAGP complex may not interact with TLRs and may be recognized by another cellular receptor, such as the NOD proteins that are involved in the recognition of bacterial peptidoglycans (12, 35). Collectively, our results suggest that cell wall components of mycobacteria are recognized by TLR2 and TLR4 and that these interactions contribute to the release of TRAIL from neutrophils.

Identification of antigens with TRAIL-inducing activity has important applications in the development of potential future vaccines for bladder cancer. BCG therapy is widely successful, but there are many associated risks and side effects, including cystitis and severe infections that occur in approximately 5% of patients (2). Although killed BCG is an effective agonist of TRAIL in vitro, only live BCG is effective in the treatment of bladder cancer (2), possibly due to the inability of killed BCG to establish a local infection. Developing new BCG-based therapies may be challenging, but our results identified several potential targets for enhancing the current BCG therapy regimen. In addition, our findings provide further insight into the mechanism of BCG-induced TRAIL release from neutrophils and further support the hypothesis that neutrophils have an important role in favorable responses to BCG therapy for bladder cancer.

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