Toll-Like Receptor 2-Dependent Inhibition of Macrophage Class II MHC Expression and Antigen Processing by 19-kDa Lipoprotein of Mycobacterium tuberculosis

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Toll-Like Receptor 2-Dependent Inhibition of Macrophage Class II MHC Expression and Antigen Processing by 19-kDa Lipoprotein of Mycobacterium tuberculosis


Mycobacterium tuberculosis (MTB) induces vigorous immune responses, yet persists inside macrophages, evading host immunity. MTB bacilli or lysate was found to inhibit macrophage expression of class II MHC (MHC-II) molecules and MHC-II Ag processing. This report characterizes and identifies a specific component of MTB that mediates these inhibitory effects. The inhibitor was extracted from MTB lysate with Triton X-114, isolated by gel electrophoresis, and identified with Abs to be MTB 19-kDa lipoprotein. Electroelution- or immunoaffinity-purified MTB 19-kDa lipoprotein inhibited MHC-II expression and processing of both soluble Ags and Ag 85B from intact MTB bacilli. Inhibition of MHC-II Ag processing by either MTB bacilli or purified MTB 19-kDa lipoprotein was dependent on Toll-like receptor (TLR) 2 and independent of TLR 4. Synthetic analogs of lipopeptides from Treponema pallidum also inhibited Ag processing. Despite the ability of MTB 19-kDa lipoprotein to activate microbial and innate immune functions early in infection, TLR 2-dependent inhibition of MHC-II expression and Ag processing by MTB 19-kDa lipoprotein during later phases of macrophage infection may prevent presentation of MTB Ags and decrease recognition by T cells. This mechanism may allow intracellular MTB to evade immune surveillance and maintain chronic infection. The Journal of Immunology, 2001, 167: 910–918.

Materials and Methods

Cells and media

Unless otherwise specified, all experiments were performed at 37°C in 5% CO2 atmosphere and standard medium composed of DMEM (BioWhittaker, Walkervillie, MD) supplemented with 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 50 μM 2-ME, 2 mM t-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, nonessential amino acids, and antibiotics. CBA/J (H-2k), C3H/HeJ (H-2k), C3H/HeOuJ (H-2k), and C57BL/6 (H-2b) female retired breeder mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions. H2-DM knockout mice were generously provided by L. Van Kaer (Vanderbilt University, Nashville, TN) and bred at Case Western Reserve University under specific pathogen-free conditions. TLR 2 knockout mice (15) were generously provided by O. Takeuchi and S. Akira (Osaka University, Osaka, Japan) and bred onto C57BL/6 background. Unless otherwise specified, macrophages were derived from bone marrow precursors that were harvested from femur marrow and differentiated in bacterial grade dishes for 7 days in standard medium supplemented with 10% L929 cell-conditioned medium. The resultant macrophages were used during the following week. Con A-elicited peritoneal macrophages were used for initial characterization of BB7 T hybridoma cells. Bovine ribonuclease A (RNase; Sigma, St. Louis, MO) or hen egg lysozyme (HEL;
Sigma) peptide-MHC-II complexes were detected with T hybridomas obtained from P. Allen and E. Unanue (Washington University, St. Louis, MO): TS12, specific for RNASae–26–I-Ak; A6, specific for HEL24–45–I-Ak; 3A1, specific for HEL149–158/I-Ak; DOB8 T hybridoma cells (17) were used to detect OVA233–243–I-Ak complexes. BB7, a T hybridoma cell line specific for Ag85B241–256/I-Ak complexes, was generated by fusion of BW5147 cells with lymph node cells from C57BL/6 mice immunized with MTB Ag 85B (18).

Abs and reagents
The World Health Organization mAb bank at Colorado State University (Fort Collins, CO) provided supernatants containing IgG anti-19-kDa lipoprotein mAbs. Abs IT-12 (19) and IT-19 (19, 20), and the IT-19 cell line (with permission of J. Ivanyi, King’s College, London, U.K.). B8-24-3 Abs were purified from culture supernatants by protein G affinity chromatography. DalCult, Bangkok, CA, was the source of a rabbit anti-M. bovis bacillus Calmette-Guérin (BCG) serum that recognizes ~100 BCG Ags, many of which are common to other mycobacteria. MTB 85B Ag was obtained through the Tuberculosis Research Materials and Vaccine Testing Contract at Colorado State University. LPS from Escherichia coli 055:B5 was obtained from Sigma. The synthetic Trepomemon pallidum lipoprotein 47L and its nonacetylated control peptide 47 were synthesized as described (21).

Culture and biochemical fractionation of bacteria
MTB H37Ra (American Type Culture Collection) was grown to log phase in Middlebrook 7H9 medium (Difco, Detroit, MI) with albumin, dextrose, and catalase enrichments (DiReco), harvested, and frozen at −70°C as described (22). Bacterial titer was determined by counting CFU on 7H10 medium (Difco). Unless otherwise specified, MTB lysate refers to a MTB cytosol preparation. This preparation was obtained by resuspending MTB H37Ra in deionized water containing 7.5 mM EDTA, 0.75 g/ml leupeptin, 0.2 mM PMSF (Sigma), 0.7 μg/ml pepstatin A (Sigma), 10 μM DNase (Sigma), and 25 U/ml RNase A (Boehringer Mannheim, Indianapolis, IN), passing the suspension through a French press 2–3 times, centrifuging the suspension for 1–2 h at 100,000 × g, and harvesting the supernatant. Protein concentration was estimated by an assay (Bio-Rad, Richmond, CA) with BSA standards. For Triton X-114 (TX114) extraction (23), MTB lysate was incubated at 4°C for 1 h with ice-cold 2% TX114 (Sigma) in 50 mM sodium phosphate, pH 7.4 (total protein concentration, 0.5–1 mg/ml), warmed to 37°C for 15 min, and centrifuged at 37°C for 10–15 min at 2400 g to separate aqueous and TX114 (detergent) layers. The aqueous layer was reduced, cold 50 mM phosphate buffer was added to the detergent layer, and the tube was incubated on ice until the phases merged and centrifuged as described above. The TX114 layer was washed 3–5 times in this manner and then precipitated by overnight incubation at −20°C with 10 volumes cold acetone (Fisher Chemicals, Fair Lawn, NJ). The pellet was washed once with cold 80% acetone and then resuspended in PBS, DMSO (Sigma), or 6–8 M urea (Sigma) to produce a detergent-free “TX114 fraction” that could be added to cell cultures (with prior dialysis in some cases). For purification of lipoproteins by electrophoresis, the aceton precipitate was solubilized in reducing SDS-PAGE sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 700 mM 2-ME, 0.01 μg/ml bromophenol blue), boiled for 5 min, loaded onto a 12% SDS polyacrylamide preparative gel, and electrophoresed using a Bio-Rad model 491 Prep Cell. Fractions were collected every 8 min for 20 h in elution buffer (25 mM Tris, 192 mM glycine, pH 8.3, flow rate 0.1 ml/min), and the fraction containing the most bromophenol blue dye marker was designated fraction 0. For immunoaffinity purification of MTB 19-kDa lipoprotein, the TX114 phase from extraction of 1.5 mg MTB lysate was passed over either IT-19 (anti-19-kDa lipoprotein) or B8-24-3 (control Ab) linked to AminoLink gel matrix (Pierce, Rockford, IL). The columns were washed with 0.1% TX114 in PBS, and bound material was eluted with 0.1% TX114 in 0.1 M glycine, pH 2.5. Each eluted fraction was neutralized, and the TX114 phase was aceton precipitated and solubilized in water.

SDS-PAGE and Western blot analysis
Samples were loaded in SDS-PAGE sample buffer under reducing conditions, electrophoresed on 12% SDS polyacrylamide gels, and stained using the Bio-Rad Silver Stain Plus kit. For Western blotting, proteins were transferred onto 0.2 μm nitrocellulose membranes (Bio-Rad), incubated for 1 h in room temperature blocking buffer (5% Carnation nonfat dry milk (Nestle, Solon, OH) in PBS with 0.1% Tween 20), and probed with primary Ab in blocking buffer for 1 h at room temperature or overnight at 4°C. Blots were washed extensively, incubated with HRP-labeled secondary Ab (Amersham Pharmacia, Piscataway, NJ) for 1 h at room temperature, and developed with a Supersignal West Pico chemiluminescence kit (Pierce). Blots were stripped with Re-blot Western Blot Recycling kit (Chemicon, Temecula, CA) and reprobed.

Ag processing and presentation assays
Macrophages were removed from bacteriologic grade dishes with trypsin-verseine (BioWhittaker), plated in 96-well flat-bottom plates (BD Biosciences, Franklin Lakes, NJ) at 5 × 10^4 macrophages/well, and incubated with 2 ng/ml IFN-γ (Genzyme, Cambridge, MA) for 20–24 h. Cells were incubated for an additional 22–26 h (or in some cases 48 h) with 2 ng/ml rIFN-γ with or without bacteriologic preparations, washed, incubated with RNASae, HEL, or HEL 1–3–V fixed in 1% paraformaldehyde, washed extensively, and incubated with T hybridoma antibody (0.05% well for 20–24 h). In assays using MTB bacilli as Ag, macrophages were infected in medium without antibiotics for 2 h as previously described (6), washed, fixed, and processed as described for soluble Ag. To monitor infection, parallel cultures of macrophages in Lab-Tek chamber slide systems (Nalge Nunc International, Naperville, IL) were infected, fixed with 100% methanol, and stained for acid-fast bacilli by the cold Kinyoun method (24); percentage of infection was determined from the mean of three individual slides, counting 100 cells/slide. Supernatants from the T hybridoma assay were assessed for IL-2 using a CTL-2 cell bioassay with a colorimetric determination (25, 26) using Alamar Blue (Alamar Biosciences, Sacramento, CA) and a Bio-Rad 550 microplate reader (Bio-Rad). Unless otherwise noted, all data points represent the mean response from triplicate wells ± SD (when error bars are not visible they are smaller than the symbol width).

Detection of I-Ak and I-Aβ by flow cytometry
Macrophages were plated at 2 × 10^6 cells/100-mm petri dish, cultured for 24 h with IFN-γ (R&D Systems, Minneapolis, MN), and then cultured for 24 or 48 h with IFN-γ with or without MTB 19-kDa lipoprotein. Macrophages were removed with trypsin plus 0.02% EDTA (Life Technologies, Grand Island, NY) and placed in V-bottom 96-well plates at 2 × 10^5/well. Cells were incubated with 10% normal mouse serum (Sigma) and 1% PBS (HyClone Laboratories) in PBS and then stained with biotinylated 10.3.6-2 anti-I-Ak, biotinylated Y3P anti-I-Ab, or biotinylated IgG2a isotype control Ab (BD PharmMingen, San Diego, CA) at 5 μg/ml. Cells were washed, incubated with streptavidin-cychrome (1:100; PharMingen), and resuspended in 1% paraformaldehyde. Analysis was performed with a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA).

Results
Ag processing inhibitory activity partitions into the detergent phase upon TX114 extraction
To investigate inhibition of MHC-II Ag processing by MTB, murine macrophages were infected with MTB H37Ra for 2 h, washed, cultured (generally for 22–26 h), pulsed with soluble Ag, and fixed with paraformaldehyde to prevent further processing (6). Presentation of peptide-MHC-II complexes was determined with T hybridoma cells. Infection of macrophages for 12 h or more inhibited subsequent processing of model Ags, including RNASae (Fig. 1A) and HEL (data not shown). Inhibition of Ag processing was observed after prolonged (>12 h) MTB infection of macrophages (detected first after 12 h of infection and maximal after 20–24 h of infection). Inhibition did not result from loss of cell viability, decreased Ag endocytosis, or decreased Ag degradation (6). Similar inhibition was produced by exposure of macrophages to MTB H37Ra lysate (soluble fraction of MTB homogenate) (6) for 24 h (Fig. 1A), indicating that the inhibitor was a constitutive component of MTB. Inhibitory activity was found in different fractions of MTB bacilli, including preparations of MTB cytosol, cell wall, cell membranes, and culture filtrate (data not shown). Lysates from virulent MTB H37Rv, avirulent MTB H37Ra, and M. avium had similar inhibitory activity (data not shown and Ref. 6).

To purify the inhibitor, MTB H37Ra lysate was extracted with TX114 (23) (Fig. 1B). The sample was incubated at 37°C, above the cloud point of TX114, to separate aqueous and TX114 (detergent) phases. The TX114 phase was precipitated with acetone and solubilized to produce a detergent-free TX114 fraction. Both the
aqueous and TX114 fractions then were added to macrophages to determine their ability to inhibit the processing of RNase to RNAS25,56-I-A\(^2\) complexes (using a colorimetric bioassay for IL-2 production). Means of triplicate wells are shown with SD values.

B. Diagram of fractions from TX114 extraction of MTB lystate. C. IFN-\(\gamma\)-stimulated CBA/J macrophages were incubated for 24 h in 0.1 ml control medium (none) or medium containing 0.3 \(\mu\)g MTB lystate (MTB lysi), a proportion of aqueous fraction (Aq fx), or TX114 fraction (TX114 fx) derived from 3 \(\mu\)g MTB lystate or an equivalent volume of TX114-extracted lysis buffer (TX114 ctrl). The TX114 fraction acetonate precipitate was resuspended in 7 M urea and dialyzed against PBS before use in cell cultures. Ag processing was assessed as in A. D. Western analysis of MTB cytosol (2 \(\mu\)g protein), aqueous fraction (6 \(\mu\)g protein), and TX114 fraction (1 \(\mu\)g protein) probed with polyclonal anti-BCG rabbit serum (Fig. 1D). Whole MTB lystate and aqueous fraction contained numerous bands, whereas the TX114 fraction contained only 8–10 dominant bands, at least two of which were enriched in this fraction relative to MTB lystate. We conclude that a small number of species, including an MTB-derived inhibitor of Ag processing, were enriched in the TX114 fraction.

Purification of the inhibitory activity and identification of MTB 19-kDa lipoprotein as an inhibitor of MHC-II expression and Ag processing

For further purification, the TX114 fraction was eluted electrophoretically from a preparative 12% SDS-polyacrylamide gel, and fractions were tested for inhibition of Ag processing (Fig. 2A). At 1/2000 dilution, peak inhibitory activity was found in fractions 10–18 (Fig. 2B). No inhibition was produced by elution buffer (1/20 dilution) or fractions eluted before the dye front (fraction –15) or after fraction 110. Analytical SDS-PAGE and silver staining showed that peak inhibitory activity correlated with a prominent band of ~20 kDa (Fig. 2C, peak level in fractions 12 and 16) that reacted with anti-BCG polyclonal serum (Fig. 2D, peak level in fractions 12 and 16).

The discovery of a 20-kDa, TX114-extractable inhibitor suggested MTB 19-kDa lipoprotein as a candidate for this molecule, because mycobacterial lipoproteins partition into TX114 (27). Western analysis with two mAbs specific for MTB 19-kDa lipoprotein (19) showed that fractions 6–20 reacted intensely with both IT-12 (Fig. 2E) and IT-19 (data not shown). Much lower levels of MTB 19-kDa lipoprotein were detected in some other fractions, consistent with the distribution of the 20-kDa band seen in silver-stained gel (Fig. 2C) and Western blot with anti-BCG polyclonal serum (Fig. 2D). Peak levels of both MTB 19-kDa lipoprotein and inhibitory activity coincided in fractions 10–18. Based on total protein determination, MTB 19-kDa lipoprotein from fraction 14 produced inhibition at a final concentration of 2.4 nM (Fig. 2B).

Earlier studies indicated that exposure to MTB or MTB lystate for 24–48 h inhibited expression of MHC-II molecules by macrophages (6). To determine the effect of purified MTB 19-kDa lipoprotein on MHC-II expression, IFN-\(\gamma\)-stimulated macrophages were treated with or without 7.5 nM electroeluted MTB 19-kDa lipoprotein for 24–48 h, and I-A\(^b\) expression was assessed by flow cytometry (Fig. 3A). After 24 h, the mean fluorescence value (MFV) of I-A\(^b\) staining was 747 without MTB 19-kDa lipoprotein vs 408 with MTB 19-kDa lipoprotein. After 48 h, MFV was 835 without MTB 19-kDa lipoprotein vs 154 with MTB 19-kDa lipoprotein. Thus, MTB 19-kDa lipoprotein produced substantial inhibition of MHC-II expression by macrophages.

To confirm its role as an inhibitor of Ag processing, MTB 19-kDa lipoprotein was purified from the TX114 fraction by immunoaffinity chromatography with IT-19 (anti-19-kDa lipoprotein). Material eluted from the IT-19-conjugated column inhibited Ag processing by macrophages, whereas material eluted from a column conjugated with an isotype-matched control mAb (B8-24-3) had no detectable inhibitory activity (Fig. 3B). Therefore, both electroelution and immunoaffinity purification identified the MTB 19-kDa lipoprotein as an inhibitor of MHC-II expression and Ag processing by macrophages.

MTB 19-kDa lipoprotein is a potent inhibitor of processing of multiple antigenic epitopes

The ability of MTB 19-kDa lipoprotein to produce generalized inhibition of processing of multiple antigenic epitopes was determined by incubating macrophages with or without 19-kDa lipoprotein for 24 h before the addition of soluble Ags. MTB 19-kDa lipoprotein (19) showed that fractions 6–20 reacted intensely with both IT-12 (Fig. 2E) and IT-19 (data not shown). Much lower levels of MTB 19-kDa lipoprotein were detected in some other fractions, consistent with the distribution of the 20-kDa band seen in silver-stained gel (Fig. 2C) and Western blot with anti-BCG polyclonal serum (Fig. 2D). Peak levels of both MTB 19-kDa lipoprotein and inhibitory activity coincided in fractions 10–18. Based on total protein determination, MTB 19-kDa lipoprotein from fraction 14 produced inhibition at a final concentration of 2.4 nM (Fig. 2B).

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lipoprotein inhibited processing of all Ag epitopes studied, including an epitope from RNase (Fig. 4A), two different epitopes from HEL (Fig. 4B), and an epitope from OVA. The potency of the inhibitor was assessed using different dilutions of MTB 19-kDa lipoprotein. Processing was inhibited at a 1/2000 dilution of a fraction containing 90 μg/ml protein, indicating that 2.4 nM MTB 19-kDa lipoprotein was sufficient to inhibit Ag processing.

MTB 19-kDa lipoprotein inhibits the processing of MTB Ag 85B by MTB-infected macrophages

To test the impact of MTB 19-kDa lipoprotein on processing of an Ag expressed by intact MTB bacilli, we generated MTB-specific murine T hybridomas after immunization of C57BL/6 mice with MTB Ag 85B (18, 28). Of the resulting Ag 85B-reactive hybridomas, BB7 was characterized and showed specific, dose-dependent secretion of IL-2 in response to Ag 85B presented by macrophages (Fig. 5A), responding to as little as 10 ng/ml of purified Ag. To map the epitope recognized by BB7, we used overlapping 16-mer synthetic peptides that spanned ~60% of the MTB Ag 85B sequence and included previously reported T cell epitopes in M. bovis BCG (29). BB7 responded to two overlapping peptides, Ag 85B236–251 and Ag 85B241–256 (Fig. 5A, inset), suggesting that the core epitope contained residues 241–251 of MTB Ag 85B. Because C57BL/6 mice do not express I-E, I-A^b was the only potential MHC-II restriction element, and BB7 recognition of Ag 85B241–256 was blocked by two anti-I-A^b Abs, Y-3P and AF6-120.1.2, confirming that this peptide was presented by I-A^b (data not shown). BB7 also responded to macrophages that were infected with viable MTB bacilli for 1–2 h (Fig. 5B), indicating that the BB7 epitope was processed and presented in the course of infection with MTB. Processing of this epitope from MTB bacilli was inhibited by chloroquine, ammonium chloride, or the use of macrophages from H2-DM knockout mice (data not shown).
studies established that BB7 recognizes an MTB Ag 85B epitope processed from both soluble Ag and intact bacilli.

To determine whether MTB 19-kDa lipoprotein inhibits processing of Ags expressed by intact MTB bacilli, macrophages were incubated with TX114 fraction (data not shown) or MTB 19-kDa lipoprotein (Fig. 5B) for 24 h. Macrophages were then infected with MTB for 2 h, fixed, and assessed for presentation of MTB Ag 85B to BB7 T hybridoma cells. TX114 fraction and purified 19-kDa lipoprotein both inhibited processing of this epitope from intact bacilli. The inhibitor did not alter the number of MTB bacilli internalized per macrophage (data not shown). TX114 fraction also inhibited processing of MTB bacilli by a human macrophage cell line (THP-1) for presentation of Ag 85B to HLA-DR1-restricted T cells, confirming this effect for human macrophages (data not shown).

Thus, 19-kDa lipoprotein inhibits processing of Ag expressed by intact MTB bacilli in infected cells and may inhibit processing of MTB Ags during chronic infection.

MTB 19-kDa lipoprotein and other microbial lipopeptides share a common ability to inhibit Ag processing via a TLR 4-independent mechanism

Because MTB 19-kDa lipoprotein and other bacterial lipoproteins may represent a pathogen-associated molecular pattern (PAMP) (30) that is associated with many bacterial species, we investigated the ability of synthetic analogs of lipopeptides from a different bacterium, T. pallidum, to inhibit Ag processing. Synthetic lipopeptide Tp47L, a hexapeptide derived from T. pallidum 47-kDa lipoprotein, inhibited Ag processing (Fig. 6, A and B) at concentrations similar to those that induce other responses (e.g., NF-κB-dependent gene transcription and cytokine secretion) (21). Non-acylated control peptide Tp47 had no inhibitory activity (Fig. 6, A and B), demonstrating that acylation is required for this activity. Tp17L, a similar T. pallidum lipopeptide with the hexapeptide sequence CGSSHH, gave similar results but was less potent than Tp47L (data not shown).

Immunomodulatory activities of many pathogen-associated molecular patterns (PAMPs) are mediated by binding to TLRs. LPS acts primarily through TLR 4 (15, 31), whereas Gram-positive organisms and lipoproteins act primarily through TLR 2 (15, 32–39). Tp47L, MTB 19-kDa lipoprotein, MTB lysate, and LPS (16) all inhibited Ag processing by macrophages from C3H/HeOuJ mice, and all except LPS inhibited Ag processing by C3H/HeJ macrophages, which lack functional TLR 4 (Fig. 6). Thus, inhibition of Ag processing by MTB 19-kDa lipoprotein, MTB lysate, and Tp47L was not due to LPS contamination and was independent of TLR 4.

Expression of TLR 2 is required for inhibition of MHC-II expression and Ag processing by MTB 19-kDa lipoprotein

Because lipoproteins require TLR 2 to induce NF-κB-dependent gene transcription and cytokine secretion (36, 37), we investigated...
whether lipoprotein-induced inhibition of MHC-II expression and Ag processing also depends on signaling via TLR 2. Macrophages were prepared from TLR 2 knockout (TLR 2−/−) mice (on C57BL/6 background), their heterozygous (TLR 2+/−) littermates, and C57BL/6 mice. Macrophages were incubated for 48 h with or without electrophoresis-purified MTB 19-kDa lipoprotein, and I-Aβ expression was evaluated by flow cytometry. MTB 19-kDa lipoprotein decreased expression of MHC-II by C57BL/6 and TLR 2+/− mice but not TLR 2−/− mice (Fig. 7A). We also assessed the role of TLR 2 in the effects of lipoprotein on Ag processing with a T cell readout (in a H-2b system with DOBW T hybridoma cells due to the background of the TLR 2 knockout mice). C57BL/6, TLR 2+/−, and TLR 2−/− macrophages were incubated for 24 h with or without MTB 19-kDa lipoprotein, exposed to OVA for 1 h, and fixed. Presentation of OVA was assessed with DOBW T hybridoma cells. MTB 19-kDa lipoprotein inhibited processing and presentation of OVA by C57BL/6 and TLR 2+/− macrophages but not TLR 2−/− macrophages (Fig. 7B). MTB 19-kDa lipoprotein also failed to inhibit processing of MTB bacilli by TLR 2−/− macrophages for presentation to BB7 T hybridoma cells (processing tested as in Fig. 5B, data not shown). Thus, TLR 2 was required for MTB 19-kDa lipoprotein to inhibit expression of MHC-II molecules and their use in the processing of Ag for presentation to T cells.

Inhibition of macrophage MHC-II Ag processing by infection with MTB is primarily mediated through TLR 2

MTB has been shown to contain agonists for both TLR 2 and TLR 4 (40, 41). We also observed that a TLR 4 agonist, e.g., LPS, can inhibit MHC-II Ag processing (Fig. 6 and Ref. 16) similar to a TLR 2 agonist, e.g., MTB 19-kDa lipoprotein. Thus, it was important to determine the relative importance of TLR 4 and TLR 2 in MTB-mediated inhibition of MHC-II Ag processing. TLR 4-defective C3H/HeJ and wild-type C3H/HeOuJ macrophages were infected with MTB (multiplicity of infection (MOI) of 10:1) for 24 h and then tested for the ability to process exogenous model Ag (as in Fig. 1A). Infection with MTB produced similar inhibition of MHC-II Ag processing by macrophages from both mouse strains (Fig. 8A), indicating that TLR 4 is not essential for inhibition of MHC-II Ag processing by MTB. In contrast, macrophages from TLR 2−/− mice were substantially more resistant to MTB inhibition of MHC-II Ag processing than macrophages from C57BL/6 mice (Fig. 8B), although a slight TLR 2-independent decrease in MHC-II Ag processing was observed with TLR 2−/− macrophages (mediated through unknown receptors potentially including other TLRs or non-TLRs). In addition, MHC-II Ag processing by TLR 2−/− macrophages was not inhibited by a lysate of MTB that inhibited MHC-II Ag processing by C57BL/6 macrophages (Fig. 8C). This observation confirms the dominant effect of TLR 2 in the inhibition and indicates that TLR 4 does not contribute substantially to this effect (TLR 4 agonists may still be present in MTB and the lysate preparation without impacting on these mechanisms). Macrophages from TLR 2−/− and C57BL/6 macrophages responded similarly to LPS (Fig. 8D), indicating that TLR 2−/− macrophages are capable of responding to a TLR 4 agonist. In summary, the experiments described in this section compare the effects of purified MTB 19-kDa lipoprotein to the effects of infection with viable MTB bacilli, confirming that the effects and TLR signaling mechanisms of the two agents are similar. Taken together, these data also establish that MTB inhibits MHC-II Ag processing by TLR 2-dependent signaling that does not require TLR 4, although these observations do not exclude roles for TLR 4 or other TLRs in different responses to MTB.

FIGURE 6. MTB 19-kDa lipoprotein and other microbial lipopeptides inhibit Ag processing independent of TLR 4. IFN-γ-stimulated bone marrow macrophages from C3H/HeOuJ (A, C, and E) or C3H/HeJ (B, D, and F) mice were incubated for 24 h with medium alone (none) or medium with 3.5 μM synthetic T. pallidum lipopeptide 47L (Tp47L), 3.5 μM nonacylated peptide 47 (Tp47), 5 μg/ml MTB lysate (MTB lys), MTB 19-kDa lipoprotein fraction 12 (see Fig. 2) at 10 nM, or 10 ng/ml LPS. RNase was added, and Ag processing was assessed as in Fig. 1. Means of triplicate wells are shown with SD values.
LIPOPROTEIN INHIBITS MHC CLASS II AG PROCESSING

Discussion

We previously reported that a constitutive component of MTB inhibits the ability of macrophages to form new peptide-MHC-II complexes by a mechanism that includes decreased synthesis of MHC-II molecules (6). In this study, we have specifically identified MTB 19-kDa lipoprotein as an inhibitor of MHC-II Ag processing. MTB 19-kDa lipoprotein belongs to a widely distributed family of bacterial lipoproteins characterized by a distinctive N-terminal lipo-amino acid, N-acyl-S-diacylglyceryl cysteine. Lipoproteins and their corresponding lipopeptides have immunomodulatory activities (e.g., induction of cytokine secretion (21, 42), NO production (37, 43), apoptosis (36), and dendritic cell maturation (44)). We now demonstrate that MTB 19-kDa lipoprotein inhibits MHC-II expression and Ag processing, consistent with an earlier study that suggested modulation of MHC-II expression by a different synthetic lipopeptide (42). Exposure of macrophages to either purified MTB 19-kDa lipoprotein or synthetic T. pallidum lipopeptide Tp47L decreased formation of new peptide-MHC-II complexes. Studies with the T. pallidum lipopeptide demonstrated that this inhibitory activity, like other immunomodulatory activities of lipoproteins, required N-terminal acylation (Fig. 6).

One interesting question is whether MTB 19-kDa lipoprotein, shown here to inhibit MHC-II expression and MHC-II Ag processing, has more general inhibitory effects on Ag processing and presentation, e.g., by MHC-I molecules. Other studies from our laboratory (N. S. Potter, R. K. Pai, W. H. Boom, and C. V. Harding, manuscript in preparation) show that the inhibitory effect of MTB 19-kDa lipoprotein is specific to MHC-II. MTB 19-kDa lipoprotein did not inhibit MHC-I expression (by flow cytometry) or function in alternate MHC-I Ag processing of exogenous particulate Ags, e.g., E. coli HB101.Crl-OVA (expressing a fusion protein containing OVA sequence and the SIINFEKL K\(^{\alpha}\) epitope) for presentation to T cells. Because alternate MHC-I Ag processing in this system (45, 46) depends on MHC-I molecules, phagocytosis, phagolysosomal function, and lysosomal proteases, this finding implies that MTB 19-kDa lipoprotein does not generally inhibit Ag uptake, proteolytic processing, or the expression of MHC-I molecules. In addition, we have previously demonstrated that Ag uptake and catabolism are not affected by MTB (6). These observations suggest that the inhibitory effects of MTB and 19-kDa lipoprotein are largely attributable to decreased MHC-II expression.

Lipoproteins belong to a larger group of microbial molecules categorized as PAMPs that contain molecular motifs characteristic of microbes and absent from mammalian hosts (30). Recognition of PAMPs, such as LPS and lipoproteins, can initiate or enhance microbicidal mechanisms (including production of toxic intermediates such as NO), cytokine secretion, and other functions that enhance both innate and acquired immunity. PAMPs trigger these responses by binding to pattern recognition receptors, e.g., TLR 2 and TLR 4 (30, 47). Inhibition of macrophage MHC-II expression and Ag processing appears to be another consequence of the recognition of multiple diverse PAMPs, because such inhibition is produced by LPS and bacterial CpG DNA as well as lipoproteins (16). Like other immunomodulatory activities of LPS, the inhibition of MHC-II expression and Ag processing by LPS is abrogated in TLR 4 mutant macrophages (Fig. 6F). However, the lipoprotein-induced inhibition of Ag processing is independent of TLR 4 (Fig. 6, B and D). Lipoproteins signal via TLR 2 to induce NF-\(\kappa\)B-dependent gene transcription and cytokine secretion (36, 37). Our studies with macrophages from TLR 2 knockout mice show that lipoprotein-induced inhibition of MHC-II Ag processing also involves signaling via TLR 2. In addition, macrophages that lack TLR 2 were largely resistant to inhibition of MHC-II Ag processing following infection with MTB bacilli (Fig. 8). These studies indicate that MTB-mediated inhibition of MHC-II Ag processing is dependent on TLR 2 and does not require TLR 4. Because inhibitory activity was largely limited to fractions containing MTB 19-kDa lipoprotein (data not shown and Figs. 1C and 2), we conclude that MTB-mediated inhibition of MHC-II Ag processing was mediated primarily by MTB 19-kDa lipoprotein signaling through...
FIGURE 8. MTB-mediated inhibition of Ag processing is dependent on TLR 2. A, IFN-γ-stimulated macrophages were prepared from C3H/HeOuJ and C3H/HeJ mice, incubated for 2 h with or without MTB (MOI = 10:1), washed, incubated for 22 h, exposed to RNase for 1 h, and fixed with paraformaldehyde. RNase 42–56 :I-Ak complexes were detected with TS12 hybridoma cells. B, IFN-γ-stimulated macrophages were prepared from C57BL/6 and TLR 2−/− mice, incubated for 2 h with or without MTB (MOI 10:1), washed, incubated for 22 h, exposed to OVA for 1 h, and fixed. OVA 223–239 :I-Ak complexes were detected with DOBW T hybridoma cells. C, C57BL/6 and TLR 2−/− macrophages were prepared as above, incubated for 24 h with 30 μg/ml MTB lysate (MTB lysis), exposed to OVA for 1 h, and fixed for the DOBW assay. D, C57BL/6 and TLR 2−/− macrophages were prepared as above, incubated for 24 h with 10 ng/ml LPS or 30 nM MTB 19-kDa, exposed to OVA for 1 h, and fixed for the DOBW assay.

TLR 2 (although other PAMPs and receptors may be involved in different responses to MTB).

Although inhibition of Ag processing after chronic microbial stimulation may benefit the host under some conditions, MTB may exploit this mechanism to evade T cell effector responses and maintain chronic infection inside host cells. MTB peptide-MHC-II complexes are presented by macrophages shortly after infection, leading to CD4+ T cell responses and control of infection. However, small numbers of bacilli survive and persist inside macrophages. Inhibition of MHC-II Ag processing by MTB 19-kDa lipoprotein may allow MTB residing in chronically infected macrophages to avoid detection by effector CD4+ T cells, promoting persistence of MTB infection. Recent evidence suggests that TLR 2 can sample material present in phagosomal compartments (35, 48). Thus, phagosomal MTB 19-kDa lipoprotein may chronically activate TLR 2 in cells that are infected with MTB, leading to decreased MHC-II Ag processing. Inhibition of MHC-II Ag processing by 19-kDa lipoprotein may also explain why vaccination with recombinant M. vaccae or M. smegmatis expressing 19-kDa lipoprotein resulted in less protection against MTB than vaccination with nonrecombinant strains of these bacteria (49, 50). We speculate that the efficacy of live mycobacterial vaccines, e.g., BCG or attenuated MTB, may be improved by the development of vaccine vectors that lack molecules that inhibit Ag processing.

Inhibition of macrophage MHC-II Ag processing by PAMPs, including LPS (16), bacterial CpG DNA (16), and lipopolysaccharides (Figs. 3–6), seems discordant with the ability of PAMPs to enhance microbicidal functions and stimulate innate and acquired immunity, but the delayed kinetics of inhibition suggest a resolution to this paradox. Upon immediate exposure of macrophages to microbes, microbial Ags will be processed before inhibition. Subsequent inhibition of Ag processing may be accompanied by stabilization of existing peptide-MHC-II complexes, enhancing the half-life of a cohort of peptide-MHC-II complexes containing microbial peptides. This would freeze the selection of Ags presented and prolong the presentation of a frame of microbial Ags, a model we term the “freeze-frame hypothesis.” The ability of PAMPs to induce dendritic cell maturation (51–53) is consistent with this hypothesis. In addition, inhibition of Ag processing may limit unnecessary enhancement of Ag presentation beyond the initial period of microbial exposure, contributing to down-regulation of immune responses when infection has been controlled. Thus, our results are consistent with a model wherein PAMPs, e.g., MTB 19-kDa lipoprotein, initially activate microbicidal mechanisms and innate immunity (also impacting on specific immunity) but later cause inhibition of certain immune processes, e.g., MHC-II Ag processing. In the context of tuberculosis, we propose that inhibition of MHC-II Ag processing by MTB 19-kDa lipoprotein decreases presentation of MTB Ag to CD4 T cells, allowing intracellular bacilli to evade immune surveillance and promoting chronic infection.

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