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Mycobacterium tuberculosis and TLR2 Agonists Inhibit Induction of Type I IFN and Class I MHC Antigen Cross Processing by TLR9

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Abstract

Dendritic cells (DCs) cross process exogenous Ags and present them by class I MHC (MHC-I) molecules to $CD8^+$ T cells specific for Ags from viruses and bacteria such as *Mycobacterium tuberculosis*. Unmethylated CpG DNA signals through TLR9 to induce type I IFN (IFN-α/β), which enhances MHC-I Ag cross processing, but lipoproteins that signal through TLR2 do not induce IFN-α/β. In these studies we observed that *M. tuberculosis*, which expresses agonists of both TLR9 and TLR2, did not induce production of IFN-α/β or cross processing by murine DCs. Furthermore, *M. tuberculosis* and TLR2 agonists inhibited induction of IFN-α/β and DC cross processing by CpG DNA. Exogenous IFN-α/β effectively enhanced cross processing of *M. bovis* bacillus Calmette-Guérin expressing OVA, bypassing the inhibition of induction of endogenous IFN-α/β. In addition, inhibition of TLR9-induced cross processing of *M. bovis* bacillus Calmette-Guérin expressing OVA could be circumvented by pretreating cells with CpG DNA to induce IFN-α/β and MHC-I cross processing before inhibitory mycobacterial TLR2 agonists were present. Inhibition of the response to one TLR by another may affect the ultimate response to pathogens like *M. tuberculosis* that express agonists of multiple TLRs, including TLR2 and TLR9. This mechanism may contribute to immune evasion and explain why IFN-α/β provides little contribution to host immunity to *M. tuberculosis*. However, downregulation of certain TLR responses may benefit the host by preventing detrimental excessive inflammation that may occur in the presence of persistent infection.

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After aerosol infection with *Mycobacterium tuberculosis*, T cells play a critical role in limiting the growth of bacteria in host lungs (1,2). Although $CD4^+$ T cells are critical for host survival, $CD8^+$ T cells are also important for protection (3–9). Mice are more susceptible to mycobacterial infection if they lack the ability to produce a class I MHC (MHC-I)-restricted T cell response (1,6,10,11). Ag-specific $CD8^+$ T cells can be found in both mice (7,12,13) and humans (9,14) infected with *M. tuberculosis*, resulting in the eventual development of central memory T cells (15). Adoptive transfer of $CD8^+$ T cells improves host responses to *M. tuberculosis* infection (16).

Mycobacteria evade host immune responses in many ways, and one mechanism to counteract the protective effects of T cells is by inhibiting Ag processing and limiting T cell activation. *M. tuberculosis* inhibits the class II MHC Ag processing pathway and expression of related molecules, including class II MHC molecules themselves (17–23). Some data indicate that MHC-I cross processing, which enables the presentation of exogenous or vacuolar Ags by MHC-I molecules, is inhibited by *M. tuberculosis* (24), but relatively little information is available regarding the mechanisms by which *M. tuberculosis* may inhibit MHC-I Ag processing mechanisms.

Multiple molecules expressed by mycobacteria signal through innate immune receptors to induce a variety of cytokines, including type I IFN (IFN-α/β) (25), which promotes priming of $CD8^+$ T cell responses (26). Unmethylated mycobacterial DNA is immunostimulatory (27) due to recognition of unmethylated CpG motifs by TLR9; consequent TLR9 signaling induces IFN-α/β and other cytokines. *M. tuberculosis* also can induce IFN-α/β through intracellular innate immune receptors (28). TLR9-induced IFN-α/β enhances cross priming and phenotypic maturation of $CD8^+$ T cells in vivo (29,30). IFN-α/β induces cross processing in dendritic cells (DCs) (31,32) and activates cytolytic $CD8^+$ T cells (33). In contrast, mycobacterial lipoproteins are agonists of TLR2 (34–36), a receptor that does not generally induce IFN-α/β (37,38), although with certain cell types and ligands TLR2 may participate in induction of IFN-α/β (39). Mice deficient in both TLR2 and TLR9 have diminished survival relative to that of either single knockout when infected with *M. tuberculosis* (40), indicating that TLR2 and TLR9 play nonredundant roles in host protection.

IFN-α/β signaling may have both beneficial and detrimental effects for the host in mycobacterial infection. Although some studies have suggested that treatment with IFN-α/β may be clinically useful (41–43), others contend that it may be deleterious (44), and clinical utility is not established. Strains of *M. tuberculosis* that are associated with high IFN-α/β production are particularly virulent in mice (45). In murine aerosol infection with *M. bovis* bacillus Calmette-Guérin (BCG), IFN-α/β mediates partial innate immune control of early infection, although it does not have a major influence on later outcome of infection (46). The complexity of responses to IFN-α/β in *M. tuberculosis* infection may be due to different effects of IFN-α/β on different cells. IFN-α/β limits the ability of *M. tuberculosis*-infected monocytes to control bacterial growth (47), yet it may have beneficial effects in priming $CD8^+$ T cell responses to *M. tuberculosis*. Although IFN-α/β may have a mixed role during *M. tuberculosis* infection, it enhances the immune response of DCs in conjunction with BCG (48), and its importance in $CD8^+$ T cell priming may make it a crucial part of vaccine efforts (49).

M. tuberculosis has been reported to alter responsiveness of cells to IFN-α/β (50–52), but there is little known about whether *M. tuberculosis* alters production of IFN-α/β. Patients with tuberculosis have decreased levels of circulating plasmacytoid DCs (pDCs) and myeloid DCs (mDCs), and the DCs present have impaired IFN-α production ex vivo (53), but the mechanisms for this are unclear. The studies presented in this paper demonstrate that

M. tuberculosis inhibits induction of IFN-α/β in response to defined CpG oligodeoxynucleotide (ODN) TLR9 agonists or endogenous agonists expressed by *M. tuberculosis*. The ability of *M. tuberculosis* to inhibit IFN-α/β induction may alter host responses to *M. tuberculosis* infection, and these mechanisms may contribute to either immune evasion by the pathogen or host-beneficial control of immune responses.

Materials and Methods

Cells and media

Incubations were carried out at 37°C with 5% CO_2. Medium for DC growth was RPMI 1640 (Hyclone, Logan, UT) with L-glutamine and glucose, supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 1 mM sodium pyruvate, 10 mM HEPES buffer, and 1% penicillin/streptomycin (Hyclone). Medium for Ag processing assays was DMEM with L-glutamine and glucose (Hyclone), supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 1 mM sodium pyruvate, 10 mM HEPES buffer, and no antibiotics. Supplemented DMEM containing 1% penicillin/streptomycin was used for IL-2 bioassays with CTLL-2 cells. The CD8OVA 1.3 T hybridoma cell line, specific for SIINFEKL peptide ($OVA_{257–264}$):K^b, was used to detect MHC-I: peptide complexes in Ag processing assays.

Reagents and animals

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). $TLR9^{-/-}$, $TLR2^{-/-}$, and $MyD88^{-/-}$ mice (on C57BL/6 background) were provided by Shizuo Akira (Osaka, Japan). IFN-α/β$R^{-/-}$ mice (on 129/SvEv background) were from B&K Universal (Grimston, U.K.); 129/SvEv mice, congenic B6.SJL-*Ptprc*^{a}/BoyAiTac mice, and OT-I $RAG^{-/-}$ mice were from Taconic Farms (Germantown, NY). All of the mice were housed under specific pathogen-free conditions. Latex-OVA was made by conjugating OVA (Sigma-Aldrich, St. Louis MO) to 2-μm polystyrene beads (Polysciences, Warrington, PA) in 0.2 M sodium citrate buffer (pH 4.2; Fisher, Pittsburgh, PA). Endotoxin-free OVA was obtained from Hyglos (Regensburg, Germany). CpG-A ODN 2336 (5′-ggG GAC GAC GTC GTG ggg ggG-3′) and non–CpG-A ODN 2243 (5′-ggG GGA GCA TGC TGg ggg gG-3′) were from Coley Pharmaceutical Group (Wellesley, MA) for in vitro experiments or InvivoGen (San Diego, CA) for immunization experiments. Synthetic triacylated lipopeptide *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-[*R*]-cysteinyl-[*S*]-seryl-[*S*]-lysyl-[*S*]-lysyl-[*S*]-lysyl-[*S*]-lysine (Pam_3CSK_4) was from InvivoGen. Triacylated LpqH lipopeptide containing 15 aa of the N-terminal sequence of *M. tuberculosis* LpqH (19-kDa lipoprotein) was from EMC Microcollections (Tübingen, Germany). rIFN-α4 and rIFN-β were from PBL InterferonSource (Piscataway, NJ).

Cloning and expression of 6x His-tagged proteins

M. tuberculosis LprA was cloned previously (34). *M. tuberculosis* LprG was cloned as described (35) by amplification from *M. tuberculosis* H37Rv genomic DNA by PCR. The gene was ligated into the shuttle vector pVV16 (provided by J. Belisle, Colorado State University, Fort Collins, CO) behind the constitutively active heat shock protein 60 promoter and in-frame with a C-terminal 6x His tag. *M. smegmatis* strain MC2 1–2C (R. Wilkinson, Imperial College London, London, U.K.) was transformed and grown in Middlebrook 7H9 broth (Difco, Lawrence, KS), supplemented with 1% casamino acids (Fisher), 0.2% glycerol, 0.2% glucose (Fisher), 0.05% Tween 80, and 30 μg/ml kanamycin until bacteria were in late log-phase growth. Bacteria were isolated by centrifugation at 6000 × *g* for 20 min at 4°C.

Purification of 6x His-tagged proteins

LprA and LprG were expressed in *M. smegmatis* and purified as described (34). Cells were resuspended for 15 min at 37°C in lysis buffer (2.5 ml per liter of bacterial culture) consisting of 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole (pH 8), 2.5% protease inhibitor mixture (P8849; Sigma-Aldrich), 75 U/ml benzonase (70664-3; Novagen, Madison, WI), and 2.5 mg lysozyme (L-3790; Sigma-Aldrich), and bacteria were passed four times through a French press (2000 pounds per square inch). Insoluble material was removed by ultracentrifugation at 100,000 × *g* for 1 h at 4°C. Ni beads (1018244; Qiagen, Valencia, CA) were added to supernatants for 2–4 h at 4°C, then transferred to polypropylene columns, and washed three times with 25 volumes of wash buffer (50 mM NaH_2PO_4, 1 M NaCl, 20 mM imidazole, and 10% glycerol [pH 8]). Bound protein was dissociated with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 450 mM imidazole [pH 8]) and desalted into 20 mM Tris (pH 8) using PD-10 columns (17-085-01; GE Healthcare, Uppsala, Sweden). Proteins were purified further by anion-exchange chromatography using quaternary ammonium columns (17-5053-01; GE Healthcare), eluted by stepwise addition of 50, 150, 200, and 1000 mM NaCl, and concentrated using 10-kDa cutoff Centricon units (UFC801008; Millipore, Bedford, MA). Material eluted at 50–200 mM NaCl was used for all of the experiments. Protein purity was verified by SDS-PAGE with silver stain and anti-His_6 Western blot. Protein concentrations were determined by bicinchoninic acid protein assay (23225; Pierce, Rockford, IL).

DC culture

Bone marrow cells were isolated from mouse femurs and tibias, and RBCs were lysed with ACK lysis buffer (BioWhittaker, Walkersville, MD). Marrow cells were cultured in enriched RPMI 1640 medium containing 1 μg/ml Flt3 ligand-Ig fusion protein (Bioexpress, West Lebanon, NH) for 8–10 d in a six-well plate with 10 ml per well to produce a DC culture containing mDCs and pDCs. On days 3 and 6, half of the medium was removed and replaced with medium containing 2 μg/ml Flt3 ligand. On day 8, nonadherent cells were collected.

Bacteria

M. tuberculosis (strains H37Ra and H37Rv) and BCG were from the American Type Culture Collection (Manassas, VA). BCG-OVA, a strain of recombinant *M. bovis* BCG that expresses a C-terminal fragment of OVA (aa 230–359), was a kind gift from Dr. Subash Sad (University of Ottawa, Ottawa, Ontario, Canada) (54) and was kindly provided by Dr. Kevin Urdahl (University of Washington, Seattle, WA). *M. tuberculosis*, BCG, and BCG-OVA were grown in 7H9 medium (Difco), supplemented with 1% glycerol, 0.05% Tween 80 (Sigma-Aldrich), and 10% albumin/dextrose/catalase (BD Biosciences, Franklin Lakes, NJ) with 40 μg/ml kanamycin sulfate (Sigma-Aldrich) as selection for BCG-OVA. Bacteria were cultured with shaking at 37°C to mid-log phase (~1 wk) and harvested by centrifugation at 5000 × *g* for 30 min at 4°C. *M. tuberculosis* H37Ra and BCG were washed in RPMI 1640 medium, resuspended in RPMI 1640 medium supplemented with 10% FCS and 6% glycerol, and flash-frozen in a dry ice/ethanol bath. *M. tuberculosis* H37Rv was centrifuged at 2000 × *g* for 20 min, collected into 50-ml conical tubes (5 ml per tube) with 3-mm glass beads (Fisher), vortexed at high speed for 5 min to declump bacteria, centrifuged at 85 × *g* for 10 min, and frozen in 1-ml aliquots in bacterial medium. Prior to use in experiments, all of the bacteria were thawed at 37°C for 30–60 min. *M. tuberculosis* H37Ra and BCG-OVA were declumped with 10 passes through a 23-gauge needle and centrifuged at 200 × *g* for 2 min to pellet large clumps. *M. tuberculosis* H37Rv was declumped by adding 3-mm glass beads, vortexing for 5 min, and centrifuging for 5 min at 325 × *g* to remove large clumps. The resulting bacteria in suspension were used for experiments. Bacterial CFU values were quantified by performing serial dilutions of declumped bacteria in PBS supplemented with

0.05% Tween 80. Three spots of 10 μl per dilution were placed on 7H11 plates (7H11 agar [Difco], 5% glycerol, and 10% oleic acid, albumin, dextrose, catalase solution [BD Biosciences]) and allowed to grow at 37°C for quantification of colonies.

Ag presentation assay

DCs were placed into flat-bottom 96-well plates at 7.5×10^4–30×10^4 cells per well. Cells were incubated with Pam_3CSK_4, non–CpG-A ODN 2243, CpG-AODN 2336, or *M. tuberculosis* H37Ra for 16–18 h in medium without antibiotics, washed with fresh medium without antibiotics, and incubated with latex-OVA or BCG-OVA that was spun onto the plates by centrifugation at 900 × *g* for 10 min at 37°C. CD8OVA 1.3 T hybridoma cells (10^5 per well) were added and incubated for 24 h. Supernatants (100 μl) were harvested, frozen, thawed, and used in a CTLL-2 bioassay for IL-2. CTLL-2 cells (5×10^3 per well) were added and incubated for 24 h. Alamar blue (Invitrogen, Carlsbad, CA) was added (15 μl) for an additional 24 h. Alamar blue reduction was analyzed by reading the difference between OD at 570 nm and OD at 595 nm on a plate reader (model 680; Bio-Rad, Hercules, CA).

DC cytokine release

DCs (7.5×10^4–20×10^4 cells per well) were incubated in flat-bottom 96-well plates with Pam_3CSK_4, non–CpG-A ODN 2243, CpG-A ODN 2336, BCG, or *M. tuberculosis* H37Ra in medium without antibiotics for 24 h. Supernatants were frozen, thawed, and assessed by ELISA kits for IFN-α or IFN-β (PBL InterferonSource), IL-10 (BD Biosciences), IL-12, or TNF-α (R&D Systems, Minneapolis, MN).

Mouse immunization

To obtain OT-I T cells, spleens were disrupted with the plunger of a 3-ml syringe, and splenocytes were passed over a 70-μm filter to produce a single-cell suspension. RBCs were lysed with ACK lysis buffer, and cells were washed twice with 10 ml PBS. Cells were labeled in 2 μM CFSE/DA (Invitrogen) in PBS at 2×10^6–5×10^6 cells per milliliter at room temperature for 15 min. Labeling was quenched in twice the labeling volume of ice-cold PBS supplemented with 10% FCS. Cells were washed twice with 10 ml ice-cold PBS and resuspended in ice-cold HBSS. A total of 1×10^6–5×10^6 labeled OT-I cells were injected i.v. retro-orbitally into congenic B6.SJL mice (200 μl per mouse). After 3 d, mice were injected bilaterally in the dorsum of the foot with 20 μg endotoxin-free OVA in PBS with or without CpG-A 2336 (25 μg), Pam_3CSK_4 (25 μg), or a combination of CpG-A 2336 (25 μg) and Pam_3CSK_4 (25 μg). After an additional 2 d, popliteal lymph nodes were harvested and up to 10^7 cells were washed in PBS with 0.1% BSA (Sigma-Aldrich) and incubated for 15 min on ice in Fc block (anti-CD16 and anti-CD32; BD Biosciences) at a dilution of 1:50 in 250 μl PBS with 0.1% BSA. An additional 250 μl allophycocyanin-conjugated anti-CD45.2 (BD Biosciences) was added at a final dilution of 1:100 for 30 min on ice. Cells were washed three times with PBS with 0.1% BSA, fixed in 400 μl PBS with 0.1% BSA and 1% paraformaldehyde (Polysciences), and analyzed on a BD LSR-II flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo, version 8.8.6 (Tree Star, Ashland, OR), and proliferation was determined using the FlowJo proliferation calculator.

Results

IFN-α/β–dependent MHC-I cross processing is induced by TLR9 agonists but not TLR2 agonists

M. tuberculosis expresses agonists of both TLR9 and TLR2, so we studied the effects of TLR2 and TLR9 stimulation on cross processing. For TLR9 stimulation, we used CpG-A ODN 2336, an unmethylated type A CpG ODN that is known to induce IFN-α/β (55). Non–

CpG-A ODN 2243 was used as a negative control. Consistent with prior data (32,55), CpG-A ODN 2336 enhanced MHC-I cross processing by DCs in a manner dependent on IFN-α/β signaling, and this effect was lost in DCs from mice lacking the IFN-α/βR (Fig. 1*A*). The latter observation also indicates that IFN-α/β regulation of the T hybridoma cells was not the cause of the effect. These data demonstrate that induction of cross processing and presentation by CpG ODN are dependent on sensitivity of DCs to CpG-induced IFN-α/β. In contrast, MHC-I cross processing was not induced by the synthetic lipopeptide TLR2 agonist Pam_3CSK_4 (Fig. 1*B*). Although *M. tuberculosis* expresses TLR9 agonist CpG DNA, it also failed to induce MHC-I cross processing (Fig. 1*B*).

We performed ELISAs to determine whether functional differences in Ag processing corresponded to differences in production of IFN-α/β by DCs. In early studies, results with IFN-α and IFN-β were similar (data not shown), and IFN-β was chosen as the primary target for analysis. CpG-A ODN 2336 induced production of high levels of IFN-β, whereas Pam_3CSK_4 did not (Fig. 2*A*). Mycobacteria (*M. tuberculosis* H37Ra or BCG) induced only low levels of IFN-β in wild-type DCs (Fig. 2*A*, 2*B*). *M. tuberculosis* TLR9 agonists were capable of inducing TLR9 signaling, because the IFN-β that mycobacteria induced was dependent on TLR9 (absent in $TLR9^{-/-}$ cells; Fig. 2*B*) and MyD88 (absent in $MyD88^{-/-}$ cells; data not shown). *M. tuberculosis*-induced IFN-β was increased notably in $TLR2^{-/-}$ DCs, suggesting that TLR2 signaling by *M. tuberculosis* suppresses induction of IFN-α/β by *M. tuberculosis* TLR9 agonists. Although *M. tuberculosis* induced less IFN-β than CpG-A ODN in wild-type DCs, the IFN-β levels induced in $TLR2^{-/-}$ cells (Fig. 2*B*) were similar to those induced by lower-efficacy CpG-B ODN and CpG-C ODN (data not shown). Both IL-12p40 (Fig. 2*C*) and TNF-α (data not shown) were highly induced by CpG, Pam_3CSK_4, and mycobacteria. Thus, mycobacteria induce TLR9 signaling, but simultaneous induction of TLR2 signaling interferes with TLR9 induction of IFN-α/β. Overall, agonists that enhanced cross processing also strongly induced production of IFN-β.

TLR2 agonists inhibit TLR9 induction of IFN-α/β and MHC-I cross processing

We considered the hypothesis that mycobacterial TLR2 agonists inhibit induction of IFN-α/β and IFN-α/β–dependent MHC-I cross processing by mycobacterial TLR9 agonists (Fig. 2*B*). Accordingly, we tested whether TLR2 signaling by Pam_3CSK_4 can inhibit induction of cross processing by CpG-A ODN. When added simultaneously with CpG-A ODN 2336, Pam_3CSK_4 inhibited induction of cross processing at concentrations as low as 0.1 nM and produced strong inhibition at concentrations of 10–1000 nM (Fig. 3*A*, 3*B*). Pam_3CSK_4 did not inhibit Ag processing induced by rIFN-α (Fig. 3*C*), suggesting that the inhibition was at the level of IFN-α/β production rather than signaling. TLR2 signaling not only failed to induce cross processing but actually inhibited induction of MHC-I cross processing by CpG-A ODN.

Because TLR2 inhibited induction of cross processing by TLR9 but not by rIFN-α, we considered the hypothesis that inhibition may be a result of decreased IFN-α/β production. To determine whether TLR2 ligands inhibit production of IFN-β, we coincubated Pam_3CSK_4 and CpG-A ODN 2336 with DCs. Pam_3CSK_4 (0.1–10 nM) inhibited induction of IFN-β by CpG-A ODN (Fig. 4*A*), with complete inhibition at a concentration of 10 nM, and similar inhibition was observed for induction of IFN-α (data not shown). Moreover, induction of IFN-β by CpG-A ODN also was inhibited by mycobacterial TLR2 agonists, including *M. tuberculosis* lipoprotein LprA (Fig. 4*B*), *M. tuberculosis* lipoprotein LprG (Fig. 4*C*), and a lipopeptide derived from *M. tuberculosis* lipoprotein LpqH (Fig. 4*C*). Pam_3CSK_4 and LprG were more potent inhibitors of IFN-β induction than LprA or the LpqH lipopeptide (Fig. 4*D*), consistent with their greater potency for induction of other TLR2-mediated responses (35) (M.G. Drage and C.V. Harding, unpublished observations). Thus,

TLR2 agonists, including those expressed by *M. tuberculosis*, prevent TLR9 enhancement of cross processing by inhibiting production of IFN-α/β.

Direct versus indirect inhibition of cross processing and IFN-α/β production

In humans, pDCs express TLR9 but not TLR2, whereas mDCs express TLR2 but not TLR9 (56), with some exceptions in the literature (57,58). Thus, if TLR2 were to inhibit TLR9 induction of IFN-α/β in human leukocytes, then the mechanism would be indirect, via cytokines secreted by TLR2-expressing cells. In contrast, murine mDCs express both TLR2 and TLR9 (59), allowing the possibility of inhibition through either direct intracellular mechanisms or indirect cytokine effects. To investigate the possibility of indirect mechanisms, we sought to recapitulate the human system by mixing $TLR2^{-/-}$ DCs with $TLR9^{-/-}$ DCs. Accordingly, $TLR9^{-/-}$ cells (which cannot respond to TLR9 agonists but can secrete cytokines in response to TLR2 agonists) were mixed with $TLR2^{-/-}$ cells (which can respond to TLR9 agonists but can only be inhibited by TLR2 agonists by indirect mechanisms involving factors produced by the $TLR9^{-/-}$ cells). Control studies showed that Pam_3CSK_4 inhibited CpG-induced cross processing in wild-type DCs alone (Fig. 5*A*) but not in $TLR2^{-/-}$ cells alone (Fig. 5*B*). $TLR9^{-/-}$ cells did not recognize CpG-A ODN and thus did not produce IFN-β (Fig. 5*C*). Pam_3CSK_4 inhibited CpG-induced cross processing in a culture of mixed $TLR2^{-/-}$ and $TLR9^{-/-}$ cells (Fig. 5*D*), although the degree of inhibition was much less in the mixed culture than that in the wild-type system, suggesting a partial effect. The partial inhibition of cross processing seen in the mixed culture suggests that the inhibition may occur in part through indirect means, such as cytokine production. The small magnitude of this effect suggests either that the indirect mechanism was not recapitulated with the mixed system or that the direct intracellular signaling mechanisms provide a major contribution.

We also used a mixed cell culture to determine whether TLR9 induction of IFN-β is inhibited directly by intracellular TLR2 signaling or indirectly by TLR2-induced factors that produce intercellular signaling. Pam_3CSK_4 profoundly inhibited IFN-β induction by CpG-A ODN in wild-type cells (Fig. 6*A*). $TLR2^{-/-}$ cells were not inhibited by TLR2 agonists (Fig. 6*B*), whereas $TLR9^{-/-}$ cells did not respond to CpG-A ODN to produce IFN-β (Fig. 6*C*). Pam_3CSK_4 produced only slight inhibition of IFN-β in mixed $TLR2^{-/-}$ and $TLR9^{-/-}$ cells (Fig. 6*D*), suggesting that there is little contribution of indirect signaling mechanisms. $TLR2^{-/-}$ cells were more responsive to CpG-A ODN, producing higher levels of IFN-β (Fig. 6*B*) than wild-type cells (Fig. 6*A*). These results suggest that TLR2 inhibition of TLR9 induction of IFN-β occurs largely through direct intracellular signaling mechanisms.

M. tuberculosis inhibits TLR9 induction of IFN-α/β and MHC-I cross processing

Because *M. tuberculosis* expresses TLR2 agonists, we tested its effect on induction of MHC-I cross processing and IFN-α/β. In wild-type cells, CpG-A ODN induction of MHC-I cross processing was inhibited by *M. tuberculosis* H37Ra (multiplicity of infection [MOI] of 1; Fig. 7*A*). *M. tuberculosis* inhibition of cross processing was TLR2-dependent, because it was reversed in $TLR2^{-/-}$ cells (Fig. 7*B*), although the degree of TLR2 dependence was not complete in all of the experiments. The level of inhibition in wild-type cells depended on the dose of *M. tuberculosis* (MOI 0.01–1; Fig. 7*C*). Incubation of wild-type DCs with *M. tuberculosis* H37Ra (MOI 0.3–3) inhibited IFN-β production (Fig. 8*A*, 8*B*). *M. tuberculosis* H37Rv also inhibited IFN-β production in wild-type DCs (Fig. 8*C*), and this effect was reversed in $TLR2^{-/-}$ cells (Fig. 8*D*), confirming that this effect occurs with virulent *M. tuberculosis*. These results indicate that *M. tuberculosis* signaling through TLR2 inhibits induction of IFN-α/β, decreasing MHC-I cross processing, which may reduce priming of *M. tuberculosis*-specific $CD8^+$ T cells.

Exogenous IFN-α/β or prior induction of IFN-α/β can overcome M. tuberculosis inhibition and induce MHC-I cross processing of mycobacterial Ag

Because mycobacterial TLR2 agonists inhibit the ability of TLR9 agonists from *M. tuberculosis* to induce IFN-α/β, the potential for *M. tuberculosis*-induced TLR9 signaling to activate MHC-I cross processing of mycobacterial Ag may be diminished in the course of infection. We tested whether other sources of IFN-α/β may still be effective for induction of MHC-I cross processing by mycobacteria-infected cells. We assessed MHC-I cross processing of BCG-OVA for presentation to CD8OVA T hybridoma cells (Fig. 9); in this scenario, BCG-OVA is both providing the Ag for cross processing and potentially regulating induction of endogenous IFN-α/β that can promote cross processing activity. Incubation of DCs with BCG-OVA alone produced only a low level of cross presentation, but addition of exogenous IFN-α at the same time or prior to addition of mycobacteria resulted in strong cross processing (Fig. 9*A*). Similar results were achieved with exogenous IFN-β (data not shown). Although addition of mycobacteria at the same time as CpG-A ODN inhibited induction of MHC-I cross processing (Figs. 8, 9*B*), addition of CpG-A ODN prior to addition of mycobacteria enabled MHC-I cross processing of BCG-OVA, and longer preincubations with CpG-AODN resulted in more efficient cross processing (Fig. 9*B*). When incubated without any IFN-α/β stimulus other than that endogenously induced by BCG-OVA, we found that $TLR2^{-/-}$ DCs processed mycobacterial Ag more efficiently than wild-type or $TLR9^{-/-}$ DCs (Fig 9*C*). Although *M. tuberculosis* inhibits endogenous production of IFN-α/β that can induce MHC-I Ag cross processing, IFN-α/β from other sources or prior induction of endogenous IFN-α/β can overcome mycobacterial inhibition and induce MHC-I cross processing of Ag expressed in mycobacteria.

TLR2 agonists in an adjuvant model inhibit CpG induction of cross priming in vivo

We examined a potential role for interactions between TLR2 and TLR9 signaling in the regulation of vaccine responses in vivo. CFSE-labeled OT-I cells (which express CD45.2) were adoptively transferred (1×10^6–5×10^6 cells) into B6.SJL mice (which express CD45.1). The recipient animals then were immunized with OVAwith or without CpG-A 2336, Pam_3CSK_4, or a combination of CpG-A and Pam_3CSK_4. Although TLR2 agonists induced a low level of OT-I T cell proliferation, they effectively inhibited induction of higher levels of OT-I proliferation by TLR9 agonists (Fig. 10). These results indicate that TLR2 agonists may regulate responses to CpG ODN in vivo, particularly induction of $CD8^+$ T cell responses. Reduced $CD8^+$ T cell responses may result from TLR2 inhibition of TLR9-induced IFN-α/β–dependent MHC-I cross processing. Moreover, these results indicate that the inclusion of TLR2 agonists in vaccine adjuvants or the presence of TLR2 agonists in vaccine vectors (e.g., BCG) may have an important influence on the nature of vaccine-induced T cell immunity.

Discussion

Pathogens may express agonists of more than one TLR or innate immune receptor, and innate immune recognition by multiple receptors may produce a distinct response pattern. *M. tuberculosis* is a human pathogen that expresses agonists of both TLR9 (DNA present in the bacterial cytoplasm) and TLR2 (lipoproteins and glycolipids present in the cell wall, some of which are shed from the bacteria). Our studies demonstrate that TLR9 agonists induce IFN-α/β and MHC-I Ag cross processing, whereas TLR2 agonists and whole mycobacteria do not. Furthermore, TLR2 agonists inhibit TLR9 induction of both IFN-α/β and MHC-I cross processing. Infection of DCs with mycobacteria resulted in TLR2-dependent inhibition of IFN-α/β expression, resulting in decreased induction of IFN-α/β–dependent MHC-I cross processing. Thus, even though *M. tuberculosis* expresses TLR9 agonists, it may prevent TLR9 induction of IFN-α/β and MHC-I cross processing,

potentially reducing induction of CD8 T cell responses. Although the host may often benefit from integration of signaling by multiple TLRs to produce responses unique to the pattern of TLR stimulation, some patterns of stimulation may produce signaling outcomes that are detrimental to host responses to certain pathogens. Successful pathogens like *M. tuberculosis* may use such mechanisms to promote persistence in the host. However, the host may have evolved such mechanisms to provide feedback inhibition to prevent induction of excessive inflammation. In the absence of this feedback inhibition, inflammation may be increased in a way that is ineffective or even detrimental to the host in the presence of persistent infection (60).

M. tuberculosis inhibits Ag processing through many mechanisms, and TLR2 may be one of many pathways exploited by the bacteria to inhibit MHC-I cross processing and presentation to $CD8^+$ T cells. *M. tuberculosis* and purified TLR2 agonists inhibit both cross processing and IFN-α/β production, but inhibition by *M. tuberculosis* is only partially reversed in $TLR2^{-/-}$ cells, suggesting that *M. tuberculosis* inhibits IFN-α/β through other pathways in addition to TLR2. Mycobacteria express a variety of molecules that drive host responses through different innate immune receptors, some of which may regulate induction of IFN-α/β in a manner similar to TLR2.

Murine mDCs express both TLR2 and TLR9, allowing for the possibility that inhibition may occur directly through intracellular signaling by TLR2 and TLR9 in the same cell or indirectly (e.g., through cytokines induced by TLR2 in one cell that act upon another cell). In contrast, human mDCs express TLR2 but not TLR9 (56), with some exceptions in the literature (57,58), and human pDCs express TLR9 but not TLR2, suggesting that TLR2 inhibition of TLR9-induced responses may occur through indirect mechanisms in humans. For example, in human PBMCs hepatitis C virus core protein inhibits TLR9 induction of IFN-α/β through TLR2 signaling that produces inhibitory cytokines (61). We investigated indirect mechanisms in the murine system by mixing $TLR2^{-/-}$ DCs with $TLR9^{-/-}$ DCs. Any inhibition observed in this system must be derived indirectly by one set of cells responding to TLR2 agonists and inhibiting other cells that respond to TLR9 agonists. We found that inhibition occurs in this system, but it is less efficient than that in wild-type murine DCs. This suggests that a secreted factor may be responsible for at least part of the inhibition by TLR2 in a manner similar to that of human PBMCs. The small magnitude of inhibition in this system, however, suggests that inhibition may occur partly or largely through intracellular signaling in wild-type cells. Alternatively, the mechanism in wild-type cells may involve cytokines in a manner that is incompletely recapitulated by mixing $TLR2^{-/-}$ and $TLR9^{-/-}$ cells. This may be due to highly efficient autocrine signaling in the wild-type cells that is replaced by less efficient paracrine signaling in the mixed cell system. Because we add TLR2 and TLR9 agonists simultaneously, there may be insufficient time for production of cytokines to produce a strong indirect inhibitory effect, and the magnitude of inhibition in wild-type cells despite the simultaneous addition of both signals suggests that direct signaling may be an important factor.

During mycobacterial infection in vivo, IFN-α/β may produce a balance of beneficial effects (e.g., by enhancing priming of $CD8^+$ T cells) and harmful effects [e.g., increasing bacterial proliferation in infected cells (47) and organism burden in vivo (45) or decreasing induction of Th1 immunity (45)]. In *M. tuberculosis* infection, it is unclear whether inhibition of the IFN-α/β pathway is of greater benefit to the pathogen (as a means of immune evasion) or the host (as a means to limit pathogen-beneficial effects or host-detrimental excessive inflammation). Some virulent *M. tuberculosis* strains have been reported to induce higher levels of IFN-α/β than avirulent mycobacterial strains (45), suggesting that IFN-α/β may promote progression of infection with these strains. This could result from greater expression of IFN-α/β-inducing molecules by these virulent *M. tuberculosis* strains.

Alternatively, our results suggest that these strains may express lower levels of TLR2 agonist activity, resulting in reduced inhibition of IFN-α/β induction and greater levels of IFN-α/β expression.

In the setting of vaccination, however, the strategic balance of IFN-α/β effects may be different, because innate immune effects of IFN-α/β that increase organism burden during *M. tuberculosis* infection may not be of significance during vaccination, and the dominant mechanisms may be those that affect T cell responses to vaccination. We observed that prior induction of IFN-α/β signaling allows DCs to effectively cross process and present Ags expressed by mycobacteria in vitro, and this mechanism could enhance T cell responses to mycobacterial vaccine strains. Our vaccination studies further substantiate that TLR2 responses can inhibit TLR9 induction of $CD8^+$ T cell responses in vivo, a mechanism of potential significance for responses to bacterial infection and bacterial vaccine strains. The BCG vaccine uses an organism that activates an immune response but inhibits production of IFN-α/β; strategies that enhance IFN-α/β effects might enhance induction of T cell immunity by BCG. Another possibility is vaccination with a TLR9 agonist and *M. tuberculosis* Ags to prime strong $CD4^+$ and $CD8^+$ responses that could be further enhanced by subsequent BCG vaccination. Our studies indicate the importance of considering outcomes that are determined by combinations of signals from different receptors that differentially regulate immune responses (e.g., production of IFN-α/β). Furthermore, the complexity of such mechanisms may influence responses to infection and vaccination in different ways.

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Abbreviations used in this paper

BCG	*Mycobacterium bovis* bacillus Calmette-Guérin
BCG-OVA	a strain of recombinant *M. bovis* bacillus Calmette-Guérin that expresses a C-terminal fragment of ovalbumin (aa 230–359)
DC	dendritic cell
IFN-α/β	type I IFN
mDC	myeloid dendritic cell
MHC-I	class I MHC
MOI	multiplicity of infection
Mtb	*M. tuberculosis*
ODN	oligodeoxynucleotide
Pam_3CSK_4	synthetic triacylated lipopeptide *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-[*R*]-cysteinyl-[*S*]-seryl-[*S*]-lysyl-[*S*]-lysyl-[*S*]-lysyl-[*S*]-lysine
pDC	plasmacytoid dendritic cell

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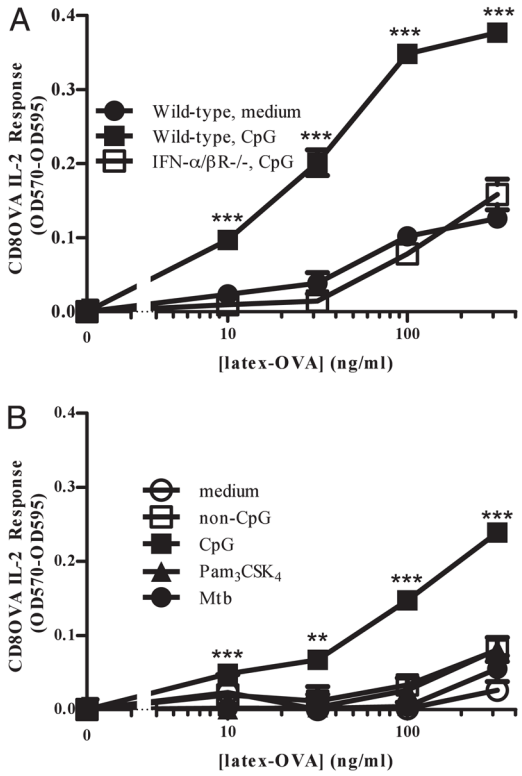

FIGURE 1.

TLR9 signaling induces MHC-I cross processing through IFN-α/β, but TLR2 signaling does not. DCs were incubated with or without agonists for 16–18 h, washed, and incubated for 24 h with latex-OVA and CD8OVA 1.3 T hybridoma cells. *A*, Induction of cross processing by CpG-A ODN 2336 (300 nM) in wild-type versus IFN-α/β$R^{-/-}$ DCs. *B*, DC cross processing of latex-OVA after incubation with non–CpG-A ODN 2243 (300 nM), CpG-A ODN 2336 (300 nM), Pam_3CSK_4 (10 nM), or *M. tuberculosis* (MOI 1). Statistical significance was determined for comparison of responses with CpG-A ODN relative to responses by wild-type cells without CpG-A ODN or with non–CpG-A ODN at the same Ag concentration. $^{**}p < 0.01$; $^{***}p < 0.001$. Data points represent the means of triplicate samples ± SD. Results are representative of three or more independent experiments.

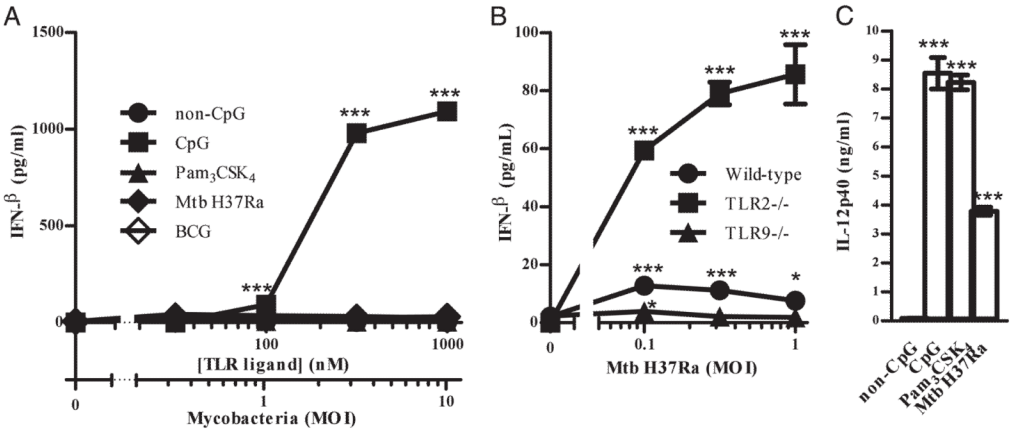

FIGURE 2.

Induction of IFN-α/β is through TLR9 and not TLR2. DCs were incubated for 24 h with ODN, Pam_3CSK_4, or mycobacteria, and supernatants were assessed for IFN-β by ELISA. *A*, Purified TLR agonists were added at various molar concentrations, or mycobacteria were added at various MOIs. *B*, DCs from wild-type, $TLR2^{-/-}$, or $TLR9^{-/-}$ mice were incubated for 24 h with Mtb. *C*, DCs were incubated for 24 h with TLR agonists (300 nM) or mycobacteria (MOI 3), and supernatants were assessed for IL-12 by ELISA. Statistical significance for *A* was determined for comparison of responses with CpG-A ODN relative to responses with non–CpG-A ODN at the same concentration. ***$p < 0.001$. Statistical significance for *B* and *C* was determined for comparison of responses with mycobacteria or agonists to responses with medium alone. *$p < 0.05$; ***$p < 0.001$. Data points represent the means of triplicate samples ± SD. Results are representative of three independent experiments. Mtb, *M. tuberculosis*.

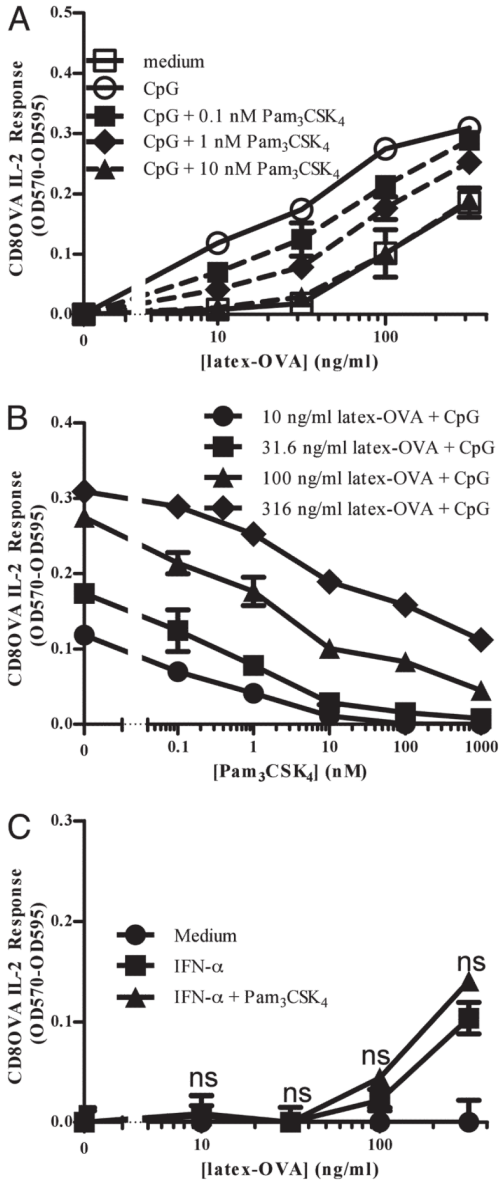

FIGURE 3.

TLR2 agonists inhibit TLR9 induction of cross processing. DCs were treated as in Fig. 1 with CpG-AODN 2336 (300 nM) or rIFN-α4 (1 ng/ml; 137.5 U/ml) and various doses of Pam_3CSK_4. *A*, Induction of cross processing after incubation with CpG-A ODN 2336 (300 nM) or inhibition with Pam_3CSK_4. T cell response is shown as a function of Ag dose with different lines for each Pam_3CSK_4 condition. Medium and CpG are represented with solid lines, and conditions with Pam_3CSK_4 are represented with dotted lines. *B*, T cell response is shown as a function of Pam_3CSK_4 concentration with different lines for each concentration of latex-OVA (drawn from the same data set as *A*). *C*, Induction of cross processing after incubation with IFN-α4 (1 ng/ml) and Pam_3CSK_4 (10 nM). Data points represent the means of triplicate samples ± SD. The addition of Pam_3CSK_4 produced a significant inhibition of MHC-I cross processing relative to CpG-A ODN alone. $p < 0.0001$ for all of the values with 100 nM Pam_3CSK_4; $p < 0.001$ for all of the values with 10 nM Pam_3CSK_4; $p < 0.005$ for all of the values with 1 nM Pam_3CSK_4; $p < 0.05$ for all of the values with 0.1 nM Pam_3CSK_4,

except at 316 ng/ml latex-OVA. Comparison of responses between cells treated with IFN-α4 in the presence or absence of $Pam3CSK_4$ resulted in a difference that was not significant. Data points represent the means of triplicate samples ± SD. Results are representative of three independent experiments.

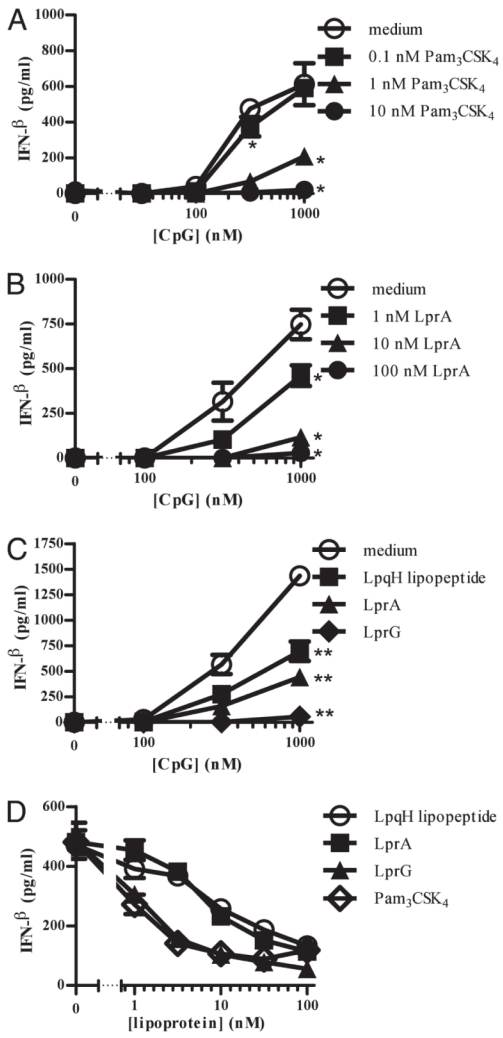

FIGURE 4.

TLR2 agonists inhibit TLR9 induction of IFN-α/β. DCs were treated as in Fig. 2. *A*, Various doses of CpG-AODN 2336 or Pam_3CSK_4 were added to DCs. *B*, Various doses of CpG-A ODN 2336 or LprA were added to DCs. *C*, Various doses of CpG-A ODN were combined with medium, LpqH lipopeptide, LprA, or LprG (TLR2 agonists all at concentrations of 10 nM). *D*, Various doses of Pam_3CSK_4, LpqH lipopeptide, LprA, or LprG were combined with 1 μM CpG-A ODN. Data points represent the means of triplicate samples ± SD. For *A*–*C*, the addition of lipoproteins produced a significant inhibition of IFN-β relative to that of CpG-A ODN alone. *$p < 0.05$ for all of the values; **$p < 0.01$ for all of the values; *$p < 0.05$ for 0.1 nM Pam_3CSK_4 at 100 and 316 nM CpG only. For *D*, the addition of lipoproteins produced a significant inhibition of IFN-β relative to that of CpG-A ODN alone (0 nM lipoprotein). $p < 0.01$ for LprA for 3.16–100 nM; $p < 0.01$ for Pam_3CSK_4, LpqH lipopeptide, and LprG at all of the values. Results are representative of four or more independent experiments for *A* and two or more independent experiments for each lipoprotein for *B*–*D*.

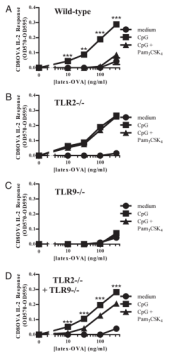

FIGURE 5.

TLR2 inhibition of TLR9-induced cross processing is partially indirect. DCs were treated as in Fig. 1 with CpG-A ODN 2336 (300 nM) and Pam_3CSK_4 (10 nM). *A*, Wild-type DCs (10^5 per well). *B*, $TLR2^{-/-}$ DCs (10^5 per well). *C*, $TLR9^{-/-}$ DCs (10^5 per well). *D*, $TLR2^{-/-}$ DCs and $TLR9^{-/-}$ DCs (5×10^4 of each per well). Statistical significance was determined for comparison of responses between cells treated with CpG with or without Pam_3CSK_4. $^{**}p < 0.01$; $^{***}p < 0.001$. Data points represent the means of triplicate samples ± SD. Results are representative of three independent experiments.

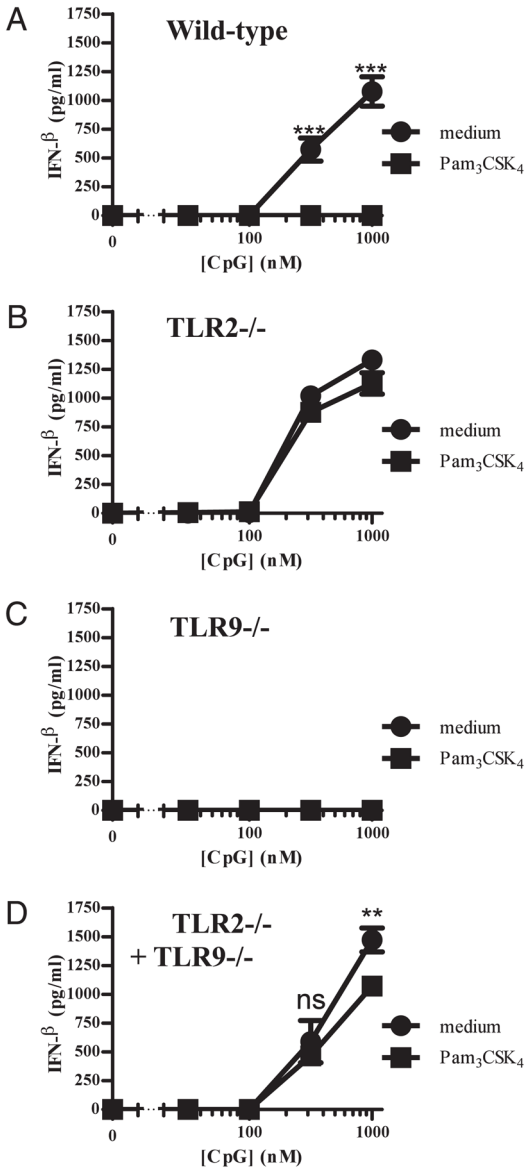

FIGURE 6.

TLR2 inhibition of TLR9-induced IFN-α/β production is partially indirect. DCs were treated as in Fig. 2 with various doses of CpG-A ODN 2336 and Pam_3CSK_4 (10 nM). *A*, Wild-type DCs (10^5 per well). *B*, $TLR2^{-/-}$ DCs (10^5 per well). *C*, $TLR9^{-/-}$ DCs (10^5 per well). *D*, $TLR2^{-/-}$ DCs and $TLR9^{-/-}$ DCs (10^5 of each per well). Statistical significance was determined for comparison of responses between cells treated with CpG-A ODN with or without Pam_3CSK_4. $^{**}p < 0.01$; $^{***}p < 0.001$. Data points represent the means of triplicate samples ± SD. Results are representative of three independent experiments.

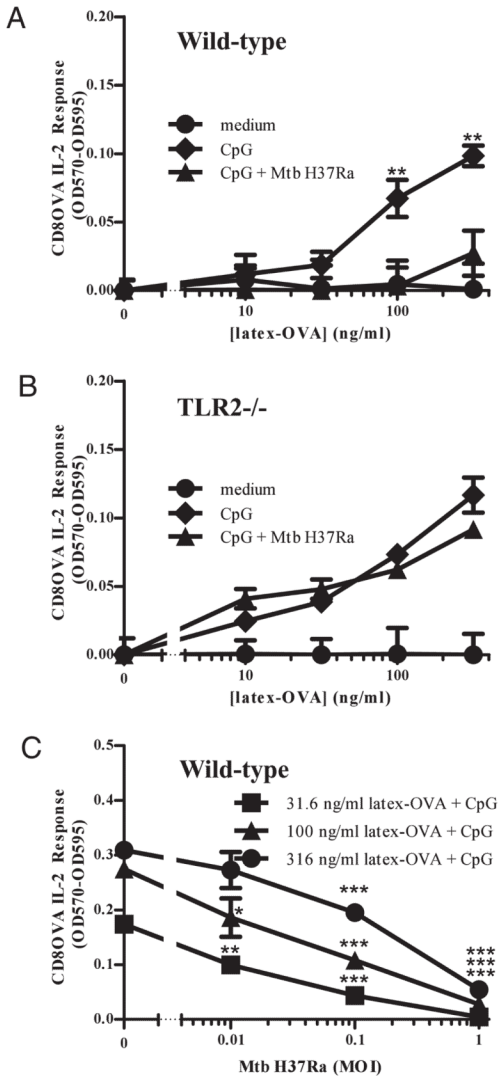

FIGURE 7.

M. tuberculosis inhibits TLR9 induction of cross processing. DCs were treated as in Fig. 1 with CpG-A ODN 2336 (300 nM) and various doses of Mtb H37Ra. *A*, Induction of cross processing in wild-type DCs by CpG-A ODN 2336 and inhibition by Mtb (MOI 1). *B*, Induction of cross processing in TLR2$^{-/-}$ DCs by CpG-A ODN 2336 and lack of inhibition by Mtb (MOI 1). *C*, Induction of cross processing by CpG-A ODN 2336 in wild-type DCs and inhibition by various doses of *M. tuberculosis*. For *A* and *B*, statistical significance was determined for comparison of CpG-treated cells with or without *M. tuberculosis* at the same Ag concentration. $^{**}p < 0.01$. For *C*, statistical significance was determined for comparison of cells treated with Mtb H37Ra at various MOIs to cells without Mtb H37Ra (MOI 0). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Data points represent the means of triplicate samples ± SD. Results are representative of three independent experiments. Mtb, *M. tuberculosis.*

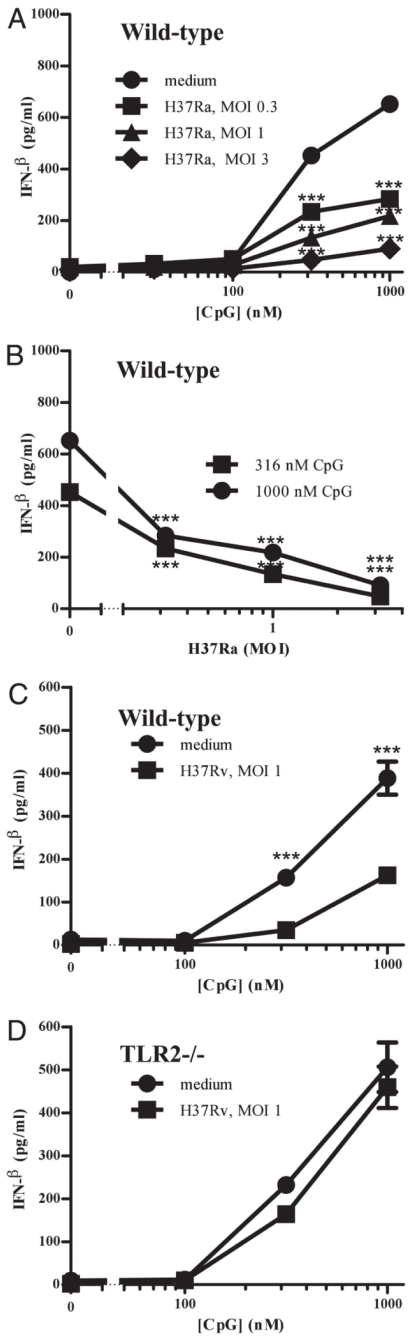

FIGURE 8.

M. tuberculosis inhibits TLR9 induction of IFN-α/β. DCs were incubated with CpG ODN and *M. tuberculosis* as in Fig. 2. *A* and *B*, Induction of IFN-β by CpG-A ODN 2336 is inhibited by *M. tuberculosis* H37Ra. *C*, Induction of IFN-β by CpG-A ODN 2336 is inhibited by *M. tuberculosis* H37Rv (MOI 1). *D*, *M. tuberculosis* H37Rv inhibition of IFN-β is dependent on TLR2. $TLR2^{-/-}$ DCs were incubated with CpG-A ODN 2336 with or without *M. tuberculosis* H37Rv (MOI 1). Statistical significance was determined for comparison of cells treated with *M. tuberculosis* H37Ra or H37Rv at various MOI to cells without *M. tuberculosis* at the same dose of CpG-A ODN. ***$p < 0.001$. Data points

represent the means of triplicate samples ± SD. Results are representative of three or more independent experiments for *A* and *B* and two independent experiments for *C* and *D*.

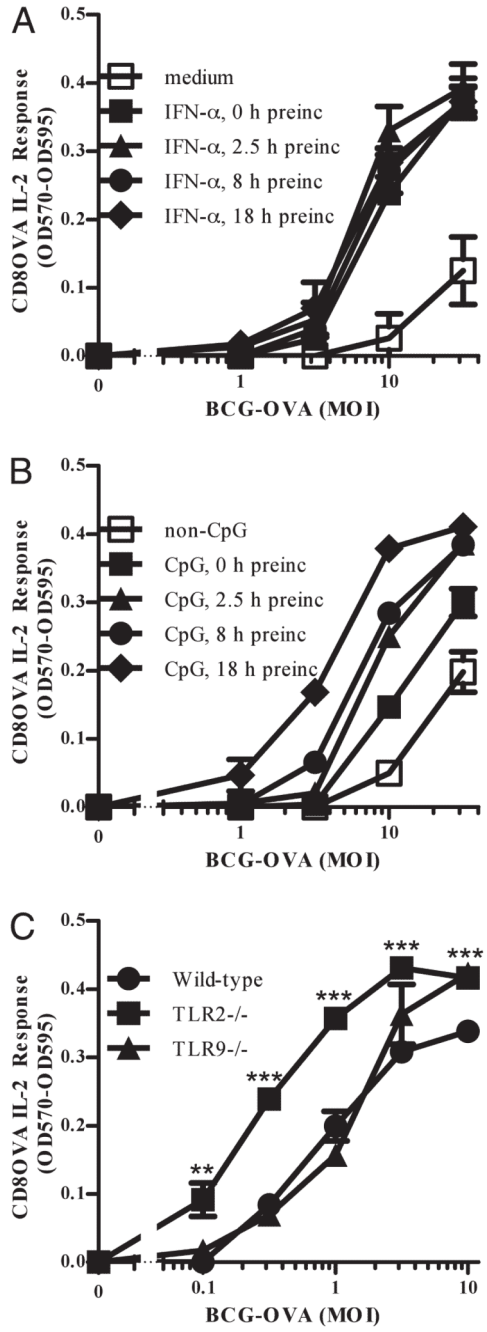

FIGURE 9.

Exogenous IFN-α/β or prior induction of IFN-α/β can overcome *M. tuberculosis* inhibition and induce MHC-I cross processing of mycobacterial Ag. DCs were incubated with medium, rIFN-α4 (1 ng/ml, 137.5 U/ml), rIFN-β (1 ng/ml; 28.75 U/ml), or CpG-A ODN 2336 (300 nM) for various periods of preincubation. BCG-OVA and CD8OVA 1.3 T hybridoma cells were added for 24 h (in continued presence of IFN or ODN at half of the prior concentration). *A*, Induction of cross processing of mycobacterial Ag by IFN-α4. At MOIs of 10 and 31.6, $p < 0.005$ for all of the points with IFN-α4 relative to medium. *B*, Induction of cross processing of mycobacterial Ag by preincubation with CpG-A ODN 2336. For comparison with the non-CpG condition, $p < 0.01$ for MOIs 10 and 31.6 at 0 h

CpG ODN preincubation; $p < 0.005$ for MOI 3.16 and $p < 0.0005$ at 2.5 h; $p < 0.0005$ for MOIs 3.16, 10, and 31.6 at 8 h; $p < 0.05$ for MOI 1 at 18 h). *C*, Induction of cross processing in 3×10^5 wild-type, TLR2$^{-/-}$, or TLR9$^{-/-}$ DCs that were incubated 24 h in V-bottom 96-well plates with BCG-OVA (MOI 0.1–10) and CD8OVA 1.3 T hybridoma cells. For *C*, statistical significance was determined for comparison of induction of cross processing in wild-type and TLR2$^{-/-}$ cells. $^{**}p < 0.01$; $^{***}p < 0.001$. Data points represent the means of triplicate samples ± SD. Results are representative of at least three independent experiments.

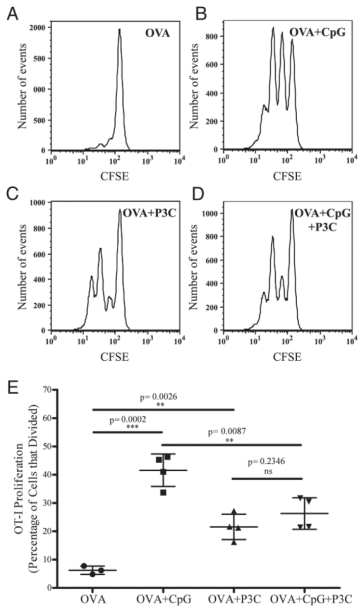

FIGURE 10.

TLR2 agonist inhibits TLR9-induced priming of $CD8^+$ T cells invivo. *A–D*, CFSE-labeled OT-I splenocytes (expressing CD45.2) were adoptively transferred into a congenic B6.SJL mouse (expressing CD45.1). After 72 h, mice were immunized in the dorsum of the foot with OVA (20 μg) with or without CpG-A 2336 (25 μg), Pam_3CSK_4 (25 μg), or a combination of CpG-A 2336 (25 μg) and Pam_3CSK_4 (25 μg). Two days after immunization, popliteal lymph nodes were collected and analyzed separately for each mouse. Cells were stained with allophycocyanin-conjugated anti-CD45.2, and gating for OT-I cells was based on CD45.2 expression. Proliferation was assessed by CFSE dye dilution. *E*, Percentage of cells that divided was determined by FlowJo software. Student *t* test was performed to compare T cell responses between groups of mice as indicated. In addition, analysis of combined data from three independent experiments revealed a significant enhancement of responses of mice immunized with OVA and CpG ($n = 12$) relative to that of mice immunized with OVA alone ($n = 11$, $p < 0.0001$), and responses of mice immunized with OVA, CpG, and Pam_3CSK_4 ($n = 12$) were significantly lower than those of mice immunized with OVA and CpG ($p = 0.0116$).