

CUTTING EDGE

Cutting Edge: In Vivo Induction of Integrated HIV-1 Expression by Mycobacteria Is Critically Dependent on Toll-Like Receptor 2¹

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Mycobacterial infection has been implicated as a possible factor in AIDS progression in populations where HIV-1 and Mycobacterium tuberculosis are coendemic. In support of this concept, we have previously shown that HIV-1-transgenic (Tg) mice infected with mycobacteria display enhanced viral gene and protein expression. In this study, we demonstrate that the induction of HIV-1 observed in this model is dependent on Toll-like receptor 2 (TLR2), a pattern recognition receptor known to be involved in mycobacteria-host interaction. Spleen cells from HIV-1-Tg mice deficient in TLR2 (Tg/TLR2^{-/-}) were found to be completely defective in p24 production induced in response to live M. tuberculosis or Mycobacterium avium as well as certain mycobacterial products. Importantly, following in vivo mycobacterial infection, Tg/TLR2^{-/-} mice failed to display the enhanced HIV-1 gag/env mRNA and p24 protein synthesis exhibited by wild-type Tg animals. Together, these results argue that TLR2 plays a crucial role in the activation of HIV-1 expression by mycobacterial coinfections. The Journal of Immunology, 2003, 171: 1123–1127.

Mycobacterium tuberculosis and HIV-1 are coendemic in many areas of the world and patients simultaneously infected with these two agents exhibit greater morbidity and mortality than individuals harboring either pathogen alone (1, 2). Although AIDS clearly leads to heightened susceptibility to mycobacterial infection, there is also evidence suggesting that *M. tuberculosis* and *Mycobacterium avium* may accelerate the progression of HIV disease through a process of immune activation of viral expression (1–4). In support of this hypothesis, live mycobacteria as well as mycobacterial products have been shown to stimulate HIV-1 gene expression or replication in human peripheral blood monocytes, lymphocytes, or cell lines in vitro (5–7). Nevertheless, it has been difficult to assess the relevance of, and to study the mech-

anism(s) of, such mycobacterial-induced immune activation in vivo (1, 2).

We have used a transgenic (Tg)³ mouse expressing complete DNA copies of the HIV-1 genome to investigate the influence of coinfecting pathogens on latent viral expression in vivo (8). Upon exposure to *Toxoplasma gondii* (8), *Plasmodium chabaudi* (9), or *M. avium* (10), these animals display enhanced expression of HIV-1 transcripts as well as p24 (a capsid protein encoded by the *gag* gene) and assemble small numbers of infectious viral particles detected by coculture with human T cell lines. In the case of *T. gondii* infection, such activation of HIV-1 gene expression was found to be markedly reduced in control Tg mice in which the NF- κ B region of the long-terminal repeat (LTR) (3) was inactivated by sequence substitution (8). Interestingly, in this transgenic mouse model, APC (macrophages, dendritic, and B cells) rather than T cells provide the major source of increased HIV-1 production. Indeed, the former cells show elevated p24 secretion when exposed to microbial products such as LPS or mycobacterial extracts (10, 11).

Recent evidence indicates that the innate and adaptive immune response to mycobacteria is highly dependent on signaling through Toll-like receptors (TLR) (12, 13). Two members of this conserved family, TLR2 and TLR4, have been shown to be involved in triggering proinflammatory cytokine production and NF- κ B activation in response to *M. tuberculosis* or subcellular products from a number of different mycobacterial species (14–18). In particular, TLR2 is required for the induction of cytokine responses to the mycobacterial cell wall components lipoarabinomannan (14), 19-kDa lipoprotein (19), and monosylated phosphatidylinositol (20). In addition, mice deficient in this receptor show impaired resistance to high doses of *M. tuberculosis* (17). These findings suggest that TLR2 may play an important role in the immune activation of HIV-1 by mycobacterial infection and, in particular, from non-T cell sources of the virus.

In the present study, we have used our HIV-1 Tg mouse model to assess the role of TLR2 in the induction of HIV-1 expression by mycobacteria in vitro as well as in vivo. We show

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³ Abbreviations used in this paper: Tg, transgenic; LTR, long-terminal repeat; TLR, Toll-like receptor; CFP, culture filtrate protein; PIM, phosphatidylinositol mannoside; MAG, soluble *M. avium* extract.

that the entire response to mycobacterial pathogens by cultured Tg cells is TLR2-dependent, and that live mycobacterial infection fails to induce HIV-1 transcription and p24 production in vivo in Tg mice deficient in TLR2. These observations support the concept that TLR2 signaling is a major driving force in the immune activation of HIV-1 by mycobacterial coinfections.

Materials and Methods

Experimental animals

The transgenic mouse line 166 which contains multiple copies of the complete proviral genome of HIV-1 strain NL4-3 was derived as previously described (8). TLR2^{-/-} mice (21) on a partially crossed 129/Ola × C57/BL6 background were kindly provided by Drs. D. T. Golenboch and S. Akira (Osaka University, Osaka, Japan). HIV-1 Tg mice were crossed with either TLR2-deficient or wild-type 129/Ola × C57/BL6 mice (The Jackson Laboratory, Bar Harbor, ME); these F₁ animals were then intercrossed to derive homozygous Tg/TLR2^{-/-} and Tg/TLR2^{+/+} mice identified by PCR of tail snips as previously described (21). The homozygous animals were then bred as lines in an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility. Mice of both sexes between 6- and 12-wk old were used in all experiments.

Pathogens and experimental infections

Stocks of *M. tuberculosis* strains H37Rv and H37Ra (10⁸ CFU/ml) and *M. avium* strain 2-151 SmT (1.2 × 10⁸ CFU/ml) were prepared in liquid broth and stored at -70°C. In some experiments, mycobacteria were killed by heating at 60°C for 30 min and the loss in viability confirmed by the failure to form colonies on agar. For in vivo infection, mice (4–6/group) were injected i.p. with 10⁷ CFU/animal of *M. avium* suspended in PBS. To assess bacterial load, spleens were harvested at 1-wk postinfection and single cell suspensions were diluted in buffered saline and cultured on agar plates as previously described (22). Control mice in the same experiments were infected with 30 cysts of the avirulent ME49 *T. gondii* strain by i.p. inoculation.

Induction of HIV-1 expression in vitro

Spleens from mice were disrupted through a nylon mesh (40 μm) to obtain single cell suspensions and depleted of RBC by osmotic lysis. The cells were then suspended in RPMI 1640 culture medium as previously described (22) and stimulated with live or heat-killed *M. tuberculosis*, *M. avium*, or soluble *M. avium* extract (MAG; Ref. 23). Additionally, cultures were stimulated with the following bacterial components: culture filtrate protein (CFP), phosphatidylinositol mannoside (PIM)_{1,2} from *M. tuberculosis* (both provided by the National Institute of Allergy and Infectious Diseases Tuberculosis Research Materials and Vaccine Testing Contract N01AI-75320 at Colorado State University, Fort Collins, CO), LPS (*Escherichia coli* 0117:B08; Sigma-Aldrich, St. Louis, MO), and a synthetic lipoprotein S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-Lys⁴-OH, trihydrochloride (Pam3Cys; EMC Microcollections, Tübingen, Germany). TNF-α and p24 were then assayed by ELISA in supernatants at 24 or 48 h, respectively, using commercial kits (TNF-α: R&D Systems, Minneapolis, MN; p24: Beckman Coulter, Miami, FL).

Measurement of HIV-1 gene expression by real-time RT-PCR

Total RNA was isolated from spleen as previously described (22). Real-time PCR was performed on an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix after reverse transcription of 1 μg of RNA. The relative amount of PCR product was determined by the comparative cycle threshold method as described by the manufacturer, in which each sample was normalized to hypoxanthine guanine phosphoribosyltransferase (*hprt*) and expressed as a fold increase vs untreated controls. The following primer pairs were used: for *hprt*, GTTGGTTACAG GCCAGACTTTGTTG (forward) and GAGGGTAGGCTGGCCTATAGv GCT (reverse); for *env*, GGGGACCAGGGAGAGCATT (forward) and TGGGTCCCCTCCTGAGGA (reverse); for *gag*, CCAGATGAGAGAACC AAGGG (forward) and TTGTGAAGCTTGCTCGGCTCT (reverse).

Measurement of p24 levels in plasma and ex vivo culture supernatants

At 1 wk postinfection, mice were bled from the tail vein into EDTA-treated vacutainer tubes (BD Biosciences, Franklin Lakes, NJ). Plasma was separated and kept frozen at -40°C to assay for p24 antigenemia by ELISA as described above. To control for individual variations in basal p24 levels (range 200–300 pg/ml) between animals, antigenemia was calculated as the fold increase in p24 vs the preinfection level for each individual mouse. The means and SE were

then calculated on the pooled values from all animals assayed. In additional experiments, ex vivo production of p24 by freshly isolated spleens from infected and control mice was assessed by incubating single cell suspensions (5 × 10⁶/ml) for 48 h at 37°C in culture medium as previously described (22).

Statistical analysis

Nonparametric statistical analysis was determined by two-tailed, Mann-Whitney *t* test at 95% confidence intervals.

Results and Discussion

Transgenic spleen cells stimulated with *M. tuberculosis* or *M. avium* induce HIV-1 p24 expression in vitro

To assess the ability of live mycobacteria to stimulate HIV-1 expression in vitro, we exposed Tg spleen cells to different doses of a virulent (H37Rv) or avirulent (H37Ra) strain of *M. tuberculosis* or *M. avium* and measured p24 production in supernatants 48 h later. As shown in Fig. 1A, all three mycobacterial isolates stimulated comparable p24 levels. Heat killing of the mycobacteria did not diminish the levels of p24 induced in vitro (Fig. 1B), arguing that infection of host cells by live bacteria is not required for HIV-1 induction. Similar results to those shown in Fig. 1 were observed when highly enriched peritoneal macrophages were used instead of bulk spleen cells (data not shown).

TLR2 interaction is required for the induction of HIV-1 p24 by mycobacterial components as well as live bacteria in vitro

Because mycobacteria have previously been shown to synthesize molecules that can stimulate cellular function through TLR2 (13), we hypothesized that such TLR2 ligands present in the heat-killed mycobacteria were responsible for the induction of p24 observed. To test this concept, we generated HIV-1 Tg mice deficient in TLR2 by crossing HIV-1 Tg animals with

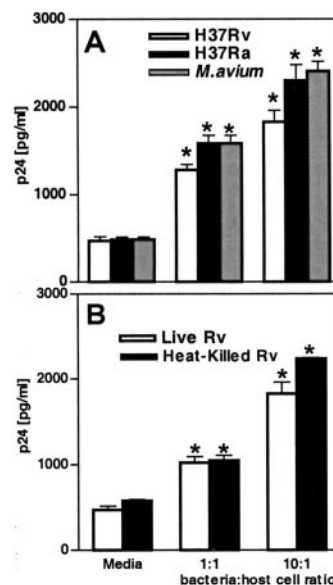


FIGURE 1. Mycobacteria induce HIV-1 expression in Tg spleen cells in vitro. *A*, HIV-1-Tg spleen cells were exposed to different bacterial doses of either the virulent H37Rv or avirulent H37Ra strains of *M. tuberculosis* or a virulent 2-151 SmT strain of *M. avium*. *B*, Similar cultures were exposed to live or heat-killed H37Rv mycobacteria. After 48 h, p24 was measured in supernatants by ELISA. The experiment shown is representative of three performed. *, Values significantly different ($p < 0.01$) from those obtained from unexposed control cells.

TLR2 knockout mice. Consistent with their expected phenotype, these Tg/TLR2^{-/-}, in contrast to Tg/TLR2^{+/+} animals, failed to produce TNF- α when stimulated with a known non-mycobacterial TLR2 ligand (the synthetic lipoprotein Pam3Cys) while responding normally to the TLR4 ligand, LPS (Fig. 2B). Similarly, while both Pam3Cys and LPS induced p24 production from Tg/TLR2^{+/+} spleen cells in vitro, the response to Pam3Cys (but not LPS) was completely ablated in the Tg/TLR2^{-/-} cells arguing that TLR2 signaling is a potent stimulus for HIV-1 induction (Fig. 2A).

When tested in the same assay, the purified *M. tuberculosis* cell wall component PIM also stimulated a p24 response (Fig. 2A) which was TLR2-dependent, consistent with its previously described activity as a TLR2 ligand (20). Importantly, the p24 responses to unfractionated mycobacterial preparations such as CFP from *M. tuberculosis* and MAg were also found to be completely abolished in Tg/TLR2^{-/-} spleen cells, suggesting that HIV-1 induction by mycobacteria occurs predominantly through TLR2 interaction (Fig. 2A). Indeed, a similar total dependency on TLR2 was observed when the same analysis was repeated using live mycobacteria instead of bacterial components (Fig. 2C). Interestingly, while the p24 response to the live pathogen was inhibited, Tg/TLR2^{-/-} spleen cells still produced significant quantities of TNF- α (Fig. 2D) as well as a 3- to 6-fold elevation in TNF- α mRNA (data not shown). These data suggest the involvement of other TLR and/or the existence of alternative pathways for TNF- α induction by mycobacteria. In support of the latter possibility, a reduced but nevertheless significant TNF- α response was detected when splenocytes from MyD88^{-/-} mice were exposed to the same live bacterial stimulus (data not shown).

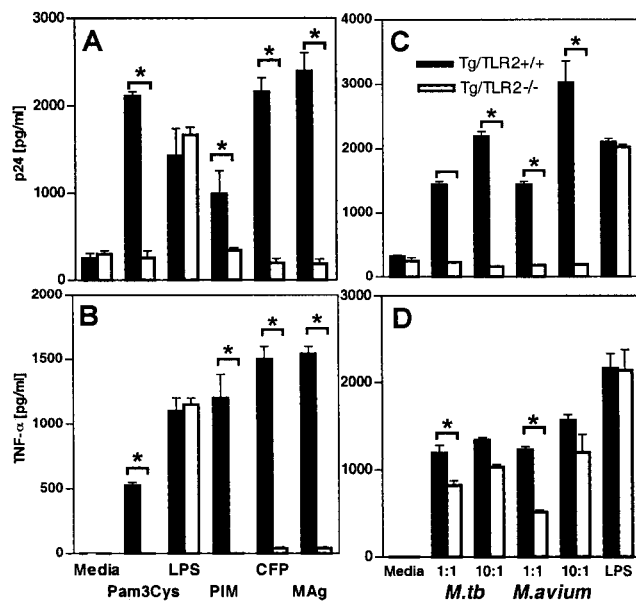


FIGURE 2. TLR2 is required for HIV-1 induction by mycobacterial components as well as live bacteria in vitro. *A* and *B*, Spleen cells from Tg/TLR2^{+/+} (■) or Tg/TLR2^{-/-} (□) mice were left untreated (media) or exposed to mycobacterial components (CFP, PIM, or MAg = 10 μ g/ml), or bacterial stimuli (Pam3Cys or LPS = 100 ng/ml). *C* and *D*, Similar cultures were exposed to different doses of live *M. tuberculosis* (H37Rv strain) or *M. avium*. p24 (*A* and *C*) and TNF- α levels (*B* and *D*) were measured in the supernatants at 48 and 24 h, respectively. The means \pm SE of measurements from quadruplicate wells are shown. The experiment shown was performed twice with similar results. *, Values significantly different ($p < 0.01$) between exposed and unexposed cultures.

Mycobacterial-induced HIV-1 activation in vivo is totally dependent on TLR2

We have previously shown that infection of Tg mice with *M. avium* results in up-regulated expression of HIV-1 mRNA and p24 protein (10). To determine whether TLR2 in addition to its role in vitro is critical for the induction of HIV-1 by mycobacteria in vivo, we infected both Tg/TLR^{+/+} and Tg/TLR2^{-/-} mice i.p. with *M. avium* and compared virus expression 1 wk later. At this time point, the TLR2-deficient animals displayed a small, but significant, increase in splenic bacterial CFU vs the TLR2-sufficient mice (Fig. 3A). As a control for the experiment, Tg/TLR^{+/+} and Tg/TLR2^{-/-} animals were infected with *T. gondii*, a pathogen that also induces HIV-1 transgene expression but which appears to trigger APC function by a TLR2-independent mechanism (24). As shown in Fig. 3B, *M. avium* infection induced a >2.0-fold elevation in plasma p24 levels in Tg/TLR2^{+/+} animals but no detectable increase in the Tg/TLR2^{-/-} mice. Similarly, *M. avium* infection triggered a significant 3.0-fold augmentation in ex vivo p24 production by spleen cells from TLR2-sufficient mice, but again no apparent increase in the TLR2-deficient animals (Fig. 3C). Finally, whereas spleens from *M. avium*-infected Tg/TLR2^{+/+} showed significant elevations in both *env* and *gag* mRNA expression, no gene induction was evident in the same tissue from TLR2-deficient animals despite the increased in vivo bacterial load (Fig. 3D). Importantly, *T. gondii* also triggered increases in p24 production and viral mRNA levels in the TLR2-sufficient animals,

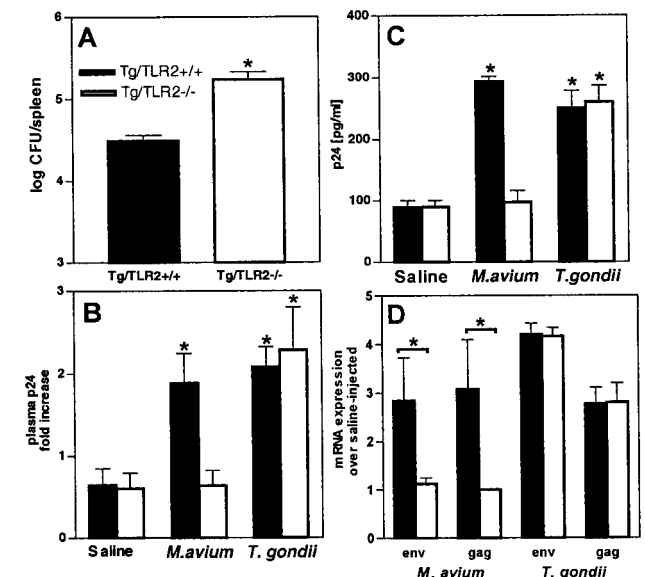


FIGURE 3. In vivo mycobacterial-induced HIV-1 expression is critically dependent on TLR2. *A*, Bacterial loads in spleens of Tg/TLR2^{+/+} and Tg/TLR2^{-/-} mice at 1 wk postinfection with *M. avium*. *B*, Plasma p24 levels in Tg/TLR2^{+/+} and Tg/TLR2^{-/-} mice 1 wk following inoculation with saline, *M. avium*, or *T. gondii*. The data shown are the ratios between the prebleed and postinfection values as described in *Materials and Methods*. *C*, Ex vivo p24 production by splenocytes from the same groups of mice shown in *B* measured at 48 h of culture. *D*, *env* and *gag* mRNA levels in spleens of *M. avium*- and *T. gondii*-infected Tg/TLR2^{+/+} and Tg/TLR2^{-/-} mice as a ratio with saline-injected control mice as determined by real-time RT-PCR (see *Materials and Methods*). Bars in each panel are means \pm SE of measurements on four individual mice. The experiments shown were performed twice with similar results. *, Values significantly different ($p < 0.05$) from uninfected controls values (*B* and *C*) or between measurements on Tg/TLR2^{+/+} vs Tg/TLR2^{-/-} animals (*A* and *D*).

while no difference in HIV-1 expression was observed in the Tg/TLR2^{-/-} mice (Fig. 3, B–D). The latter finding confirms that the Tg/TLR2^{-/-} animals do not have a generalized defect in their HIV-1 response to microbial challenge.

Previous studies have established the ability of TLR ligands such as LPS and CpG-DNA to activate HIV expression in virus-infected, transfected, or transgenic cells (11, 25). In the present report, we have used a transgenic mouse model to investigate the role of TLR signaling in the in vivo induction of proviral gene expression by live mycobacterial pathogens frequently associated with HIV-1 infection in humans. Although both TLR2 and TLR4 ligands were capable of triggering an HIV-1 response in transgenic cells in vitro, the absence of TLR2 was sufficient to completely ablate HIV-1 expression induced by live *M. tuberculosis* and *M. avium* in vitro and by *M. avium* infection in vivo. This profound effect of TLR2 deficiency is unlikely to be the result of a loss in TNF- α production, because Tg/TLR2^{-/-} cells showed only a partial impairment in the secretion of that cytokine. Moreover, the addition of neutralizing anti-TNF- α Abs failed to inhibit mycobacterial-induced p24 production in vitro and no stimulation of p24 synthesis was observed when exogenous TNF- α at concentrations as high as 10 ng/ml was added to Tg spleen cell cultures (data not shown).

Recent studies have shown that mycobacteria contain ligands capable of triggering several TLR in addition to TLR2 (reviewed in Ref. 13). For example, the mycobacterial glycolipids PIM₄₋₆ have been demonstrated to be TLR4 agonists (18) while mycobacterial DNA in common with other bacterial DNA should contain CpG oligonucleotide motifs able to stimulate TLR9 (reviewed in Ref. 26). Moreover, we and our colleagues (11) have recently demonstrated additive effects of TLR2, TLR4, and TLR9 agonists on HIV-1 expression in transgenic spleen cells in vitro. The finding reported in this study that the induction of HIV-1 by live mycobacteria both in vitro and in vivo is totally dependent on TLR2 suggests that mycobacterial TLR2 ligands such as PIM_{1,2}, lipoarabinomannan, and the 19-kDa lipoprotein are the dominant TLR agonists presented to APC by live bacteria. However, because Tg/TLR2^{-/-} spleen cells stimulated with mycobacteria generated a significant TNF- α response while totally defective in p24 production, it is also possible that on a quantitative basis TLR2 ligands may preferentially trigger the pathway(s) leading to HIV-1 LTR activation as opposed to proinflammatory cytokine production. Several examples of such associations between specific TLR triggering and differential effector responses have now been documented (20, 27). Because TNF- α production is retained in TLR2-deficient mice, it is possible that TLR2, in addition to triggering NF- κ B, stimulates an NF- κ B-independent pathway essential for the induction of the Tg LTR. Indeed, a number of transcription factors distinct from NF- κ B have previously been shown to participate in HIV LTR induction in infected human cells (reviewed in Ref. 28).

Although studies in TLR2^{-/-} mice have established a role for this receptor in host resistance to both *M. tuberculosis* and *M. avium* (Ref. 17 and C. Feng, C. Scanga, C. Collazo-Custodio, A. Cheever, S. Hieny, P. Caspar, and A. Sher, manuscript in preparation) in each case, the effect of TLR2 deficiency is partial either manifesting itself only at high bacterial inocula (17) or resulting in delayed mortality relative to MyD88-defi-

cient animals (C. Feng, C. Scanga, C. Collazo-Custodio, A. Cheever, S. Hieny, P. Caspar, and A. Sher, manuscript in preparation). On the basis of this evidence, one can speculate that it may be possible to design interventions targeting TLR2 that interfere with *M. tuberculosis*-induced HIV-1 expression without jeopardizing host control of bacterial infection. Further studies using aerogenic infection of Tg/TLR2^{-/-} mice with *M. tuberculosis* are planned to systematically investigate the differential effects of TLR2 deficiency on host resistance and HIV-1 induction with the goal of exploring this strategy.

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