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Th1-Like Cytokine Induction by Heat-Killed Brucella abortus Is Dependent on Triggering of TLR9

Li-Yun Huang,* Ken J. Ishii,† Shizuo Akira,† Julio Aliberti,‡ and Basil Golding1*

In this report we provide evidence, for the first time, that bacterial DNA in the context of heat-killed Brucella abortus (HKBA) engages TLR9 in dendritic cells (DC), resulting in a Th1-like cytokine response. This is based on the findings that HKBA induction of IL-12p40 is: 1) abolished in DC from TLR9−/− mice; 2) blocked by suppressive oligodeoxynucleotides; 3) simulated by bacterial DNA derived from HKBA; and 4) abrogated by DNase or methylation of the DNA from HKBA. Furthermore, the effect of HKBA can be inhibited by chloroquine, indicating that endosomal acidification is required and supporting the notion that DNA from HKBA is interacting with TLR9 at the level of the endosome, as is the case with CpG oligodeoxynucleotides. In addition to DC, HKBA can elicit IL-12p40 secretion from macrophages, in which case the effect is wholly MyD88 dependent but only partially TLR9 dependent. This probably explains why HKBA effects in vivo are only partially reduced in TLR9−/− mice, but absent in MyD88−/− mice. Because of their intimate interactions with T cells, the DC response is most likely to be critical for linking innate and adaptive immune responses, whereas the macrophage reaction may play a role in enhancing NK cell and bystander immune responses. In addition to IL-12p40, HKBA induces other Th1-like cytokines, namely, IFN-α and IFN-γ, in a TLR9-dependent manner. These cytokines are important in protection against viruses and bacteria, and their induction enhances HKBA as a potential carrier for vaccines. The Journal of Immunology, 2005, 175: 3964–3970.
Materials and Methods
Mice and in vivo stimulation
Female C57BL/6 mice were obtained from The Jackson Laboratory. MyD88<sup>−/−</sup> and TLR9<sup>−/−</sup> were provided by Dr. S. Akira (Osaka University, Osaka, Japan) (31, 32). MyD88<sup>−/−</sup> and TLR9<sup>−/−</sup> were backcrossed six times to the C57BL/6 background and bred in a Center for Biologics Evaluation and Research (CBER) animal facility. Mice were used under protocols approved by the CBER Animal Care and Use Committee.

For in vivo stimulations, mice were injected i.p. with HKBA (10<sup>5</sup> organisms), CpG ODN (100 μg), or PBS. In some experiments, mice received suppressive or control ODN (150 μg) 5–10 min before HKBA injection. At the indicated time points after injection, sera were collected and stored at −20°C until assayed. In each in vivo experiment, at least three mice were used per stimulation and repeated at least twice. The SE bars represent variability between individual mice.

Reagents
HKBA 1119.3 was kindly provided by Dr. B. Martin at the U.S. Department of Agriculture (Ames, IA) and was washed extensively with PBS before use. Soluble Toxoplasma gondii tachyzoite Ag (STAg) was provided by Dr. A. Sher (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) and was prepared as previously described (33). LPS derived from Escherichia coli and chloroquine were obtained from Sigma-Aldrich.

Enzyme treatment of DNA
B. abortus DNA (DNA-BA) was extracted from B. abortus using the Qiagen genomic DNA protocol and reagents as previously described (34).

Methylation and DNase I treatment of DNA were performed as previously described (35, 36). In brief, DNA was treated with SssI CpG methylase (3 μl of DNA) in NE Buffer 2 supplement with S-adenosylmethionine (SAM; New England Biolabs) at 37°C. Methylation of DNA was confirmed by the loss of HpaII (New England Biolabs) cleavage susceptibility. Complete methylation was achieved after incubation and repeated replenishment of SAM and SssI methylase for 24 h. DNA was then extracted with phenol-chloroform-isomyl alcohol, precipitated with ethanol, and dissolved in PBS.

For DNase I treatment, DNA was treated with DNase I (6 μl of DNA) from Roche Applied Science at 37°C for 2–4 h. Before addition to spleen cells, genomic DNA was boiled for 5 min and quenched on ice for 5 min. Complete digestion of DNA by DNase I and methylation of DNA were confirmed by electrophoresis on 1% agarose gels.

Analysis of CG dinucleotide content in genomic DNA
B. abortus (9–941) was sequenced in its entirety and is available from GenBank. The genome is 3.3 Mb and composed of two circular chromosomes; Chr I (2.12 Mb) and Chr II (1.16 Mb) are AE017223 and AE017224, respectively (37). The frequency of CpG dinucleotide content in genomic DNA of B. abortus was determined by Gene Runner version 3.05 for Windows (free DNA program, www.genenumber.com).

ODNs
Phosphorothiate-modified ODNs were synthesized at the CBER core facility (Bethesda, MD). The following ODNs were used: immunostimulatory ODN 1555 (CpG) (GCTAGACGTAGCCT), suppressive ODN H514 (sODN) (CTCAAGCTTTGAGGG), and control ODN 1471 (cODN) (TCAAGCTTTGA) (38).

Cell isolation and in vitro stimulation
Splenic DC were purified as previously described (19, 39). Briefly, Spleens harvested from mice were digested with Liberase CI (Roche Applied Science). Low-density leukocytes were then obtained after centrifugation on a dense-BSA gradient. Enriched DC were further purified using anti-CD11c magnetic beads (Miltenyi Biotec). The cells purified from the column were routinely 70–85% DC.

For in vitro stimulation, low-density cells or enriched DC were cultured in RPMI medium (19) at a concentration of 1–5 × 10<sup>5</sup> cells per milliliter or splenocytes at a concentration of 10<sup>7</sup> cells per milliliter. These cultures were incubated in the presence or absence of different stimuli in RPMI 1641 supplemented with 10% heat-inactivated FBS, penicillin-streptomycin, HEPEs buffer, 2-ME, nonessential amino acids, pyruvate, and glutamine. In some experiments, cells were pretreated with chloroquine or sODN for 1–3 h before the addition of HKBA or CpG. After stimulation, culture supernatants were collected after overnight incubation and stored at −20°C until assayed.

Detection of cytokines
The cytokines IL-12p40 and IFN-γ was measured by using commercial ELISA kits from R&D Systems or Pierce Endogen, whereas IFN-ω was measured by using commercial ELISA kits from PBL Biomedical Laboratory. Values are expressed as the means with the SD for duplicate or triplicate samples.

Statistical analyses
Results were expressed as the mean ± SE (for in vivo experiments) or mean ± SD (for in vitro experiments). Data were analyzed by the unpaired t tests using GraphPad Prism version 3.00 for Windows (GraphPad). Differences were considered statistically significant at p < 0.05.

Results
Secretion of IL-12p40 after HKBA stimulation of murine splenic DC in vitro is MyD88 and TLR9 dependent
Previously, using WT and TLR-deficient mice, we showed that HKBA-induced IL-12p40 production in murine DC was MyD88 dependent but TLR2/4 independent. To determine whether TLR9 played a role, we added HKBA to DC from TLR9<sup>−/−</sup> mice and found that IL-12p40 was reduced to background levels, indicating that IL-12p40 induction by HKBA is TLR9 dependent (Fig. 1). In contrast, stimulation of IL-12p40 by STAg, which is CCR5 and MyD88 dependent was unaffected (40).

Because HKBA retains the bacterial DNA component, it was of interest to examine the role of this DNA in the induction of IL-12p40 by DC. DNA purified from HKBA and CpG ODN were able to stimulate IL-12p40 from DC from WT mice but the response was abrogated in TLR9<sup>−/−</sup> mice (Fig. 1).

Methylation and DNase I treatment abrogate the ability of DNA from HKBA to induce IL-12p40
Bacterial DNA, unlike eukaryotic DNA, contains a high frequency of unmethylated CpG motifs (41). Based on analysis of ~10% of the genome (330,000 bp), the frequency of CpG dinucleotide content in B. abortus was determined to be 0.0916 (see Materials and Methods). This is higher than the expected CpG dinucleotide frequency for bacterial DNA, ~1 in 16 bases (0.0625) (41, 42), and higher than that reported for E. coli as 0.0747 (43). Thus based on CpG content, DNA from HKBA should be immunostimulatory to an extent similar to E. coli.

To establish the role of methylation in the ability of Brucella DNA to stimulate IL-12p40, the DNA from HKBA was methylated. Complete methylation was confirmed by resistance to HpaII.
treatment, and complete DNase I digestion of DNA were shown in Fig. 2A. Methylated or DNase I-treated DNA from HKBA were no longer able to induce IL-12p40 from mouse splenocytes (Fig. 2B). These results confirm that the stimulatory ability of DNA from HKBA resides in unmethylated CpG motifs contained in the DNA.

sODN inhibit HKBA induction of IL-12p40 secretion
sODN have been shown to block the signaling cascade initiated by CpG DNA (38, 44–48). There are two classes of sODNs, those that suppress broadly (45, 48) and other ODNs that are selective for interaction of CpG with TLR9 (38, 44, 47). To determine whether HKBA mediated IL-12p40 secretion is a consequence of the interaction between bacterial DNA and TLR9, H154 sODN that is selective for CpG suppression was used. It inhibited induction of IL-12p40 secretion by HKBA in a manner similar to that seen with CpG ODN. These data support the notion that DNA from HKBA is indeed responsible for the secretion of IL-12p40, probably via engagement of TLR9 (Fig. 3).

Recognition of HKBA in DC by TLR9 requires endosomal acidification
HKBA exists as particles of various sizes. To access TLR9, which is situated intracellularly and translocates from the endoplasmic reticulum to endosomes after activation (49, 50). HKBA would have to be phagocytosed and phagolysosomes would need to fuse with endosomes. To examine whether HKBA stimulation of IL-12p40 required endosomal acidification, chloroquine was added and shown to inhibit CpG and HKBA-induced IL-12p40 in a dose-dependent manner in WT mice (Fig. 4). This suggests that HKBA and CpG have similar requirements for stimulation of TLR9 and IL-12p40 secretion and localize to endosomes where they engage TLR9.

HKBA stimulation of IL-12p40 in vivo in mice is MyD88 dependent but only partially TLR9 dependent
As shown previously, IL-12p40 secretion after HKBA stimulation in vivo is MyD88 dependent, but TLR2/4 independent. When injected in vivo, HKBA induced detectable serum IL-12p40 in WT mice, but not in mice with defective MyD88 expression, as was seen in vitro (Fig. 5). However, when TLR9−/− mice were used, IL-12p40 levels were only partially reduced throughout the time points examined, unlike the complete TLR9 dependency observed in vitro with DC (Fig. 1). In contrast, IL-12p40 secretion induced by CpG was totally abolished in TLR9−/− mice (Fig. 5).

To further characterize the in vivo response, WT mice were injected with HKBA in the presence and absence of sODNs. Unlike what was observed in vitro, sODNs only partially inhibited HKBA-induced IL-12p40 in WT mice, but totally inhibited CpG-induced IL-12p40. The finding that sODN had no effect on IL-12p40 secretion after HKBA stimulation in vivo in TLR9−/− mice was expected and attests to the specificity of H154 sODN to inhibit CpG interaction with TLR9 but not other pathways for IL-12p40 induction. Nonstimulatory ODNs were used as a control (Fig. 6). As an additional control, LPS E. coli stimulation of IL-12p40 induction in vivo was shown to be unaffected by sODN (data not shown).

These findings suggest that HKBA-induced IL-12p40 production in vivo is likely to be dependent on at least two distinct TLR pathways, only one of which is TLR9 dependent.

HKBA stimulation of macrophages, but not B cells, in vitro elicits IL-12p40 secretion, which is MyD88 dependent, but only partially TLR9 dependent
The discrepancy between DC and in vivo responses to HKBA could be due to the fact that, in addition to DC, other cell types,
e.g., macrophages and B cells, that express TLR9, could respond to HKBA in vivo in a TLR9-independent manner. Stimulation of peritoneal macrophages from WT mice resulted in IL-12p40 production. This effect was abolished in MyD88−/− mice but only partially decreased in TLR9−/− mice (Fig. 7). This indicates that macrophages, unlike DC, are only partially dependent on TLR9 for induction of IL-12p40 after stimulation with HKBA. In contrast, induction of IL-12p40 by LPS E. coli, which acts via TLR4, was not reduced in TLR9−/− mice. Previously we showed that induction of IL-12 p40 by HKBA in macrophages is TLR2/4-independent (Ref. 19 and our unpublished data). These observations may provide an explanation for the difference between stimulation by HKBA of DC in vitro (TLR9 dependent) and mice in vivo (partially TLR9 dependent).

To investigate the role of B cells in the partial in vivo IL-12p40 response to HKBA, mouse B cells were purified from splenocytes by using CD19 MACS beads (Miltenyi Biotec). WT and TLR9−/− mouse B cells were stimulated with HKBA and CpG, and low levels of IL-12p40 protein were induced in WT cells, but not in TLR9−/− cells. However, the HKBA and CpG induced high levels of IL-6 in WT B cells confirming that the B cells were functional (data not shown). These results indicate that B cells contribute minimally to the partial IL-12p40 response of TLR9−/− mice to HKBA in vivo.

**HKBA induction of IFN-γ and IFN-α in vivo is TLR9 dependent**

WT and TLR9−/− mice were inoculated i.p. with HKBA, and IFN-γ and IFN-α levels were measured in serum samples at various time points (Fig. 8A). In WT mice, maximal IFN-γ responses were observed at 6–8 h but these were markedly reduced in TLR9−/− mice (Fig. 8A) and were absent in MyD88−/− mice (data not shown). These results indicate that the IL-12p40 detected in vivo at 2–8 h (Fig. 5) represents functional IL-12 that precedes and likely induces the subsequent IFN-γ secretion. Previously, we have shown that HKBA stimulates DC to migrate to T cell areas and secrete IL-12p40. Increases in IL-12 and IFN-γ provide an environment that would favor Th1-like responses.

Type 1 IFNs have been associated with viral infections, but IFN-α can also be induced by certain bacteria (29) and by D-type ODNs, but not K-type ODNs (24, 51, 52). In this report, we show that HKBA can also induce IFN-α as measured in the serum (Fig. 8B). Serum IFN-α was detected within 3 h after HKBA injection, and the level was absent from MyD88−/− mice (data not shown) and markedly reduced in the serum from TLR9−/− mice (Fig. 8B). To our knowledge, this is the first demonstration that a bacterium induces IFN-α by a TLR9-dependent mechanism.

**Discussion**

Previously we showed that *B. abortus* activated cells of the innate immune system and more specifically via TLR triggering in DC and macrophages. In vitro and in vivo data from MyD88−/− mice indicate that HKBA induction of both TNF and IL-12p40 secretion require an intact MyD88 pathway. MyD88 is an adaptor molecule that participates in all known TLR pathways (except TLR3) and also functions in IL-1 and IL-18 receptor signaling (20, 31). Interestingly, based on experiments with targeted deletion of TLR2 or mutation of TLR4, induction of TNF and IL-12p40 involve different TLRs, such that TLR2 is required for TNF, but not IL-12p40 production. TLR4 is not required for either TNF or IL-12p40 induction by HKBA (19).

In the present study, we show that HKBA interacts with TLR9 to induce IL-12p40 secretion in murine DC. This effect is likely to be mediated by bacterial DNA from HKBA, because it was also seen with DNA extracted from HKBA and was inhibited by sODN, selective for CpG suppression. Scanning both chromosomes of the *Brucella* genus revealed that this species contains higher levels of CpG dinucleotide (0.0916) than average bacteria (0.0625) and even higher levels than *E. coli* (0.0747) (41–43). Moreover, when DNA from HKBA was treated with DNase I or methylation, it lost its ability to stimulate IL-12p40.

TLR9 was recently shown to translocate from the endoplasmic reticulum to the endosome after cell activation (53, 54). Chloroquine has been shown to interfere with endosomal acidification which is required for CpG-mediated signaling of TLR9 (52, 55, 56). Thus, the fact that chloroquine blocked the effect of HKBA on IL-12p40 secretion by DC, supports the notion that DNA derived from HKBA and TLR9 probably interacts in endosomes. Wortmannin, another inhibitor of CpG uptake into TLR9-containing endosomes (57), was also effective in blocking HKBA from eliciting IL-12p40 secretion (data not shown). Even though triggering of TLR9 by synthetic unmethylated CpG-bearing ODNs is well known, this is the first demonstration that a bacterium can enter a cell and stimulate TLR9 via its DNA.

The dose of HKBA that elicited optimal IL-12p40 secretion in DC was 106 organisms. This dose contains ~500 ng/ml DNA, far less than the amount of purified DNA required, i.e., 25 μg/ml to elicit IL-12p40 secretion (34). This indicates that the DNA packaged in HKBA is more potent than purified DNA, possibly because the DNA from HKBA is released within the cells and is less vulnerable to degradation during processing. Alternatively, HKBA
provides other signals that enhance stimulation via TLR9. Previously we showed that, although DNA purified from HKBA lost activity after digestion with DNase, DNase or proteinase K digestions did not alter HKBA ability to induce cytokines in vitro (34), suggesting that the DNA in HKBA is protected from enzyme degradation. Taken together, these results support the hypothesis stated by Takeda and Akira (20) that bacteria are engulfed by DC and, after processing in phagosomes/lysosomes or endosomes/lysosomes, CpG DNA is exposed and interacts with TLR9.

FIGURE 7. In vivo induction of IL-12p40 by HKBA, unlike CpG ODN, is only partially reduced by sODN. HKBA (10⁶ organisms) or CpG ODN (100 μg of CpG1555) was injected into WT mice in the presence or absence of sODN or ODN. HKBA was also injected into WT and TLR9−/− mice in the presence or absence of sODN (150 μg of ODN H154) or cODN (150 μg of ODN 1471). Sera were harvested 3 h later and assayed for IL-12p40 production by ELISA. The mean values and SEs are shown. #, Values of p < 0.05. The data shown are representative of two similar experiments.

In vivo HKBA elicited secretion of IL-12p40 in a MyD88-dependent manner. However, unlike DC in vitro, this effect was only partially reduced in TLR9−/− mice. This discrepancy could be due to secretion of IL-12p40 in vivo by other cells, e.g., macrophages or B cells that can use other TLR pathways to secrete IL-12p40. B cells made little IL-12p40 in response to HKBA in vitro, and hence probably do not contribute to the partial IL-12p40 response observed in TLR9−/− mice in vivo. However, when macrophages were studied in vitro, IL-12p40 secretion after activation by HKBA was found to be MyD88 dependent but only partially dependent on TLR9. These findings suggest that other TLRs are involved in triggering macrophages to secrete IL-12p40 after HKBA stimulation, although we have previously excluded TLR2/4 (19).

The role of systemically increased IL-12 during an infection may be important in activation of NK cells and in bystander cell effects. However, it is likely that the local release of IL-12 by DC in T cell areas is critical for specific T cell and adaptive responses to the infectious agent. The IL-12 induced was probably functionally effective because IFN-γ was also observed in the serum of WT mice injected with HKBA. As expected, the IFN-γ induction was MyD88 and TLR9 dependent.

Because TLR9 ligation can result in IFN-α induction as a result of engagement by viral DNA or D-type CpG ODNs (24, 26, 58–60), it was of interest to determine the effect of HKBA on IFN-α secretion. IFN-α was observed within 3 h in the serum of WT, but not MyD88−/− or TLR9−/− mice injected with HKBA. To our knowledge, this is the first report indicating that HKBA induces IFN-α in a TLR9-dependent manner. This effect enhances the potential of HKBA as a Th1-like vaccine delivery system for viruses and bacteria.

The picture emerging from this and our previous studies is that HKBA acts on the immune system by stimulating DC via TLR2 to secrete TNF and undergo maturation followed by migration to T cell areas. As suggested by us (19) and confirmed by others, the TLR2 ligand on HKBA is lipoprotein (61). DC in T cell areas are stimulated by DNA from HKBA via TLR9 to secrete IL-12p40, which in turn activates nearby T cells to differentiate as Th1 cells and secrete IFN-γ. By chemically linking, or by expressing peptides or proteins, using recombinant technology, to HKBA, they can be internalized by DC and presented to appropriate T cells, so that desired specific Th1 cell responses can be achieved (9, 62).

Interestingly, stimulation of TLR2 has been shown by others to promote a Th2-like response (63, 64), whereas activation of TLR9 favors a Th1-like outcome. Because HKBA stimulates both TLR2 and TLR9, a mixed Th1/Th2 response may be expected. In fact, as we have shown previously, a Th1-like response predominates (62). This situation is probably operative with other microorganisms in that they may stimulate two or more TLRs and the outcome may be Th1- or Th2-like depending on the predominant pathway and inherent host differences (65).

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Disclosures

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