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TLR4 Signaling via MyD88 and TRIF Differentially Shape the CD4+ T Cell Response to *Porphyromonas gingivalis* Hemagglutinin B

Dalia E. Gaddis,* Suzanne M. Michalek,* and Jannet Katz†

Recombinant hemagglutinin B (rHagB), a virulence factor of the periodontal pathogen *Porphyromonas gingivalis*, has been shown to induce protective immunity against bacterial infection. Furthermore, we have demonstrated that rHagB is a TLR4 agonist for dendritic cells. However, it is not known how rHagB dendritic cell stimulation affects the activation and differentiation of T cells. Therefore, we undertook the present study to examine the role of TLR4 signaling in shaping the CD4+ T cell response following immunization of mice with rHagB. Immunization with this Ag resulted in the induction of specific CD4+ T cells and Ab responses. In TLR4−/− and MyD88−/− but not Toll/IL-1R domain-containing adapter inducing IFN-β-deficient (TRIF−/−) mice, there was an increase in the Th2 CD4+ T cell subset, a decrease in the Th1 subset, and higher serum IgG1/IgG2 levels of HagB-specific Abs compared with those in wild-type mice. These findings were accompanied by increased GATA-3 and Foxp3 expression and a decrease in IFN-γ and IL-17 production by memory CD4+ T cells. To our knowledge, these results demonstrate for the first time that TLR4 signaling, via the downstream MyD88 and TRIF molecules, exerts a differential regulation on the CD4+ T cell response to HagB Ag. The gained insight from the present work will aid in designing better therapeutic strategies against *P. gingivalis* infection.


*Porphyromonas gingivalis* is a Gram-negative, black-pigmented, anaerobic bacterium that is one of the main causative agents of adult chronic periodontitis and has been isolated from lesions of >80% of patients suffering from destructive periodontitis (1–4). This disease is characterized by the continuous inflammation of the gingival tissues, which ultimately results in alveolar bone resorption and loss of teeth. In addition to periodontal diseases, *P. gingivalis* has also been implicated in a variety of systemic disorders, including atherosclerosis and cardiovascular diseases (5–8). Because individuals may carry *P. gingivalis* without developing disease, it is thought that disease progression is due to an imbalance between the bacterial biofilm and the immune response (9). Thus, it is of great importance to understand the interactions that occur between *P. gingivalis*, its virulence factors, and the host’s immune defense system.

*P. gingivalis* has several virulence factors that allow the bacteria to escape the immune system, including fimbriae, cysteine proteases known as gingipains, LPS, and hemagglutinins (1, 10–15). Hemagglutinins are nonfimbrial adhesins that agglutinate erythrocytes and facilitate bacterial attachment to the host tissue (16). Several *P. gingivalis* hemagglutinins have been identified, including hemagglutinin A, B, C, and D (HagA, HagB, HagC, and HagD, respectively) (17–20). However, HagB has been the most studied and has been shown to activate mouse peritoneal macrophages and bone marrow-derived dendritic cells (DC) (21, 22). Immunization of mice with HagB resulted in the production of Abs that prevented the adherence of *P. gingivalis* to coronary endothelial cells and rendered protection against bone loss (23–29). Thus, HagB is considered to be an important Ag with therapeutic applications.

TLRs are pattern recognition receptors expressed on various immune cells and are important in immune recognition by their ability to distinguish conserved pathogen-associated molecular patterns (30–32). Several TLRs have been discovered and their ligands identified (30–32). For example, TLR4 recognizes LPS from enteric bacteria and a number of viral and bacterial proteins (22, 33–36). TLR2 heterodimerizes with TLR1 or TLR6 and recognizes triacylated or diacylated forms of lipoproteins, respectively (37). Two main signaling adaptor molecules, MyD88 and Toll/IL-1R domain-containing adapter inducing IFN-β (TRIF), are downstream of TLRs. All TLRs use MyD88 except TLR3, which signals through TRIF (38–41). TLR4 is the only TLR known to use MyD88 and TRIF molecules. Once a ligand interacts with its cognate TLR, a sequence of events follows including the recruitment of adaptor molecules, phosphorylation of signaling molecules, and activation and translocation of transcription factors, ultimately resulting in the expression of genes required for cell activation and immunity. This process is essential for DC to initiate the adaptive immune response by priming naive T cells, ultimately leading to the activation of B cells and Ab production (42–50).
CD4+ T cells play a central role in the adaptive immune response as they provide help for 1) Ab production by B cells (51), 2) CD8 T cell memory development (52), and 3) macrophage activation (53). Different subsets of CD4+ T cells have been described, including Th1, Th2, Th17, and regulatory T cells (Tregs), depending on the cytokines they produce and their effector functions (54). TLR signaling influences the CD4+ T cell and Ab response, since in the absence of TLR signaling, the adaptive immune response is either diminished or altered (43–50). Although evidence shows that TLR4 signaling results in a Th1 type response and TLR2 signaling leads to a Th2 or an anti-inflammatory T regulatory type 1 response (44, 55, 56), others have shown a dependency of Th2 response on TLR4 signaling (57–59), making the exact role of TLR4 signaling in CD4+ T cell subset development inconclusive.

We have previously shown that an endotoxin-free rHagB preparation stimulates DC in a TLR4-dependent manner and that MyD88 and TRIF are required for their optimal activation (22), suggesting that priming of a CD4+ T cell response may occur following immunization of mice with rHagB. Because the development of periodontitis is thought to result from an ineffective T cell response (9), and immunization with rHagB has shown promise as a successful vaccine, it is imperative to determine whether a T cell response is induced following rHagB immunization, as well as to understand the role played by TLR4 signaling in shaping this T cell response. In this study, we set out to investigate the type of T cell subsets induced following immunization, as well as to understand the role played by TLR4 signaling in mediating a CD4+ T cell response, and the mechanisms by which both MyD88 and TRIF participate in the immune response. The results from this study provide valuable information that will be beneficial in the development of effective therapeutics design to protect the host against P. gingivalis infection and the detrimental consequences in the periodontium and systemic disorders.

Materials and Methods

Mice

C57BL/6 wild-type (WT), TLR4−/−, MyD88−/−, and TRIF−/− mice were bred and maintained in an environmentally controlled, pathogen-free animal facility at the University of Alabama at Birmingham. The original TLR4−/− and MyD88−/− breeding pairs were obtained under a Materials Transfer Agreement from Dr. Shizuo Akira (Osaka University, Osaka, Japan). The original TRIF−/− breeding pairs were obtained from The Jackson Laboratory (Bar Harbor, ME). Female mice were 6–12 wk of age when used in the studies. All experiments were done according to the guidelines of the National Institutes of Health. Protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

Antibodies

Fluorescent-labeled Abs against CD4 (clone RM4-5), CD8α (clone 53-6.7), CD25 (clone PC61.5), CD44 (clone IM7), CD69 (clone H12F3), T-bet (clone eBio4B10), GATA-3 (clone TWAJ), retinoic acid-related orphan receptor-γt (RORγt) (clone AFKJS-9), Foxp3 (clone FJ5-165), CTLA-4 (clone 1D4) (UC10-4B9), and IFN-γ (clone XMG1.2) were all purchased from eBioscience (San Diego, CA). Mouse anti-STAT5 (pY694) was purchased from BD Biosciences (San Jose, CA).

rHagB purification and immunization

rHagB was purified and tested for endotoxin contamination as previously described (22). WT, TLR4−/−, MyD88−/−, or TRIF−/− mice were immunized with rHagB (25 μg/mouse) and the GFI-101 (GPI) adjuvant (a semisynthetic saponin analog derived from hydrolyzed Quillaja saponins) (60–62) (100 μg/mouse; Galenica Pharmaceuticals, Birmingham, AL) in PBS by the s.c. route at day 0. A booster dose of rHagB (25 μg/mouse) alone was administered s.c. on day 14. For controls, WT, TLR4−/−, MyD88−/− or TRIF−/− mice received only GPI at day 0 and PBS at day 14. All mice were bled and sacrificed on day 21 or days 42 or 44 following initial immunization. Spleens and axillary and brachial lymph nodes were harvested for analysis. Blood samples were collected from the retro-orbital plexus using heparinized capillary tubes. The serum was obtained after centrifugation at 15,000 rpm and stored at −20°C until further analyzed.

Cell purification and stimulation

Spleenocytes and lymph node cell suspensions from rHagB immunized or control mice were erythrocyte-depleted and used to purify total CD4+ T cells using a MACS CD4+ T cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. For the preparation of feeder cells, spleenocytes and lymph node cell suspensions from naïve WT, TLR4−/−, MyD88−/−, or TRIF−/− mice were depleted of CD4+ T cells using MACS CD4 beads (Miltenyi Biotec) according to the manufacturer’s instructions and irradiated with 3000 rad. Equal numbers of CD4+ T cells and irradiated feeders were cultured in 96-well plates in RPMI 1640 media supplemented with 10% heat-inactivated FCS, penicillin (50 U/ml), streptomycin (50 mg/ml), l-glutamine (2 mM), and 2-ME (50 mM) at 37°C in a humidified 7.5% CO2 incubator. Cultures were stimulated with increasing concentrations of rHagB (0.01, 0.1, 1, or 10 μg/ml; see Results) or were left unstimulated as negative controls. Unless otherwise indicated, cells were stimulated for 36 h for assessing the expression levels of activation markers or transcription factors and for 5 d for assessing proliferation or cytokine production.

Cell surface marker expression analysis

To determine the expression of surface markers on CD4+ or CD8+ T cells ex vivo, splenocytes and lymph node cell suspensions were stained with CD4, CD8, CD44, or CD152 (CTLA-4) Abs in PBS buffer supplemented with 2% BSA and 0.1% sodium azide (FACS buffer) for 30 min on ice. Cells were washed and fixed with PBS containing 2% paraformaldehyde. Samples were acquired using a FACS Calibur (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

Intracellular cytokine analysis

CD4+ T cells from immunized or control mice were purified and stimulated for 36 h as described above. GolgiPlug (brefeldin A; BD Biosciences) was added to cells at 1 μl/ml cell culture for an additional 10 h stimulation. Cells were harvested and then stained with CD4 Abs for 30 min on ice. Cells were washed, fixed, and permeabilized using IC Fixation Buffer (eBioscience) for 20 min, washed, and then stained with IFN-γ Abs in permeabilization buffer (eBioscience) for 30 min. Cells were finally washed and fixed in paraformaldehyde and samples were analyzed by flow cytometry as described above.

Transcription factor and activation marker expression analysis

To assess expression of transcription factors and activation markers, purified CD4+ T cells from immunized or control mice were stimulated and harvested as described above and stained with CD4 and CD25 or CD69 Abs for 30 min on ice. Cells were washed, fixed, and permeabilized using Foxp3 staining buffer (eBioscience) for 20 min, washed, and then stained with IFN-γ Abs in permeabilization buffer (eBioscience) for 30 min. Cells were finally washed and fixed in paraformaldehyde and samples were analyzed by flow cytometry as described above.

CFSE labeling and proliferation assay

CD4+ T cells were purified from immunized or control mice as described above. Cells were labeled with 5 μM CFSE (Invitrogen, Carlsbad, CA) in PBS at 25°C for 5 min. CFSE labeling was quenched with the addition of FCS to a final concentration of 30% (v/v). Labeled cells were washed, centrifuged, and cultured with irradiated feeders of the appropriate genotype and stimulated with rHagB or left unstimulated as previously described. Cultures were harvested 5 d later and stained with CD4 Abs in FACS buffer for 30 min. Samples were analyzed by flow cytometry.

Cytokine ELISA

Culture supernatants were harvested 2 d following detection of IL-2 or 5 d following stimulation and were assessed for the levels of IFN-γ and IL-2 (eBioscience) and IL-4, IL-5, IL-10, and IL-17 (R&D Systems, Minneapolis, MN) by ELISA, according to the manufacturers’ instructions.

Ab ELISA

Serum samples were assessed for Ab activity against rHagB by ELISA. Briefly, 96-well flat-bottom MaxiSorp microtiter plates (Nunc International, Roskilde, Denmark) were coated with rHagB (2 μg/ml) or with optimal concentrations of unlabeled goat anti-mouse total IgG (IgG1, IgG2a, IgG2b, IgG3), and incubated overnight at 4°C. Plates were blocked with 1% BSA in PBS-T for 1 h. Standards and samples were diluted in 1% BSA-PBS-T and added to the plates in triplicate. After 1 h incubation at room temperature, plates were washed and incubated with Anti-IgG HRP conjugate for 1 h. Plates were washed and developed with 3,3′,5,5′-tetramethylbenzidine (Sigma, St. Louis, MO) and 2 M H2SO4. The absorbance was read at 450 nm. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA).

Ab ELISA

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IgG_{1}, or IgG_{3} (SouthernBiotech, Birmingham, AL) in borate buffer saline (100 mM NaCl, 50 mM boric acid, 1.2 mM Na_{2}B_{4}O_{7} [pH 8.2]) overnight at 4°C. Nonspecific binding sites were blocked with 1% BSA in borate buffer saline for 3 h at room temperature. Serum samples from immunized mice were diluted at 1:40,000 (IgG_{1}), 1:20,000 (IgG_{3}), 1:4,000 (IgG_{2a}, IgG_{2b}), and 1:1,000 (IgG_{3}) in 1% BSA in borate buffer saline. Diluted samples were added in duplicate wells and further subjected to six 2-fold serial dilutions. To generate standard curves, 2-fold serial dilutions of a mouse Ig reference serum (ICN Biomedicals, Costa Mesa, CA) or IgG_{3} reference serum (BedOx Laboratories, Montgomery, TX) were added to two rows of wells in each plate that were coated with the appropriate unlabeled anti-mouse IgG subclass reagent. Samples and standards were incubated overnight at 4°C. Plates were then washed and incubated with HRP-conjugated goat anti-mouse IgG_{2a}, IgG_{2b}, IgG_{3}, or IgG_{1} Abs (SouthernBiotech) for 3 h at room temperature. Plates were then washed and incubated with o-phenylenediamine substrate with hydrogen peroxide. Color development was stopped with 2N H_{2}SO_{4} and plates were read at 490 nm. The concentration of the Abs in all serum samples was calculated by the interpolation on the standard curves generated using the mouse reference serum, using the SoftMax Pro software v5.0.1 (Molecular Devices, Menlo Park, CA).

Statistical analysis
The data from two groups were subjected to a two-tailed unpaired Student t test, whereas data from three groups or more were subjected to an unpaired ANOVA, followed by post hoc analysis with the Tukey–Kramer multiple comparison test. All data were analyzed using the GraphPad InStat version 3.0a (GraphPad Software, San Diego, CA). Differences between groups were considered significant at the level of p < 0.05.

Results
Immunization with HagB and GPI-0100 results in a HagB-specific CD4+ T cell response and Ab production
CD4+ T cells play an important role in the immune response during F. tularensis infection. Because HagB induces DC activation, this suggested that it might also prime CD4+ T cells. To determine whether immunization with HagB induces a CD4+ T cell response, WT mice were immunized with rHagB and GPI-0100 adjuvant as described (see Materials and Methods). GPI-0100 acts as an adjuvant by increasing the Ag uptake of APCs and decreasing the inhibitory signal delivered to the T cells through CTLA-4 (60). Twenty-one days following immunization, we observed a significant increase in the percentages and numbers of CD4+ T cells expressing high levels of CD44 ex vivo in the immunized mice compared with controls (Fig. 1A). This increase was detected on CD4+ T cells and not CD8+ T cells. Additionally, there was a dose-dependent production of IFN-γ, IL-17, IL-4, IL-5, and IL-10 by CD4+ T cells isolated from immunized mice (Fig. 1B), indicating the induction of a mixed CD4+ T cell subset response. As expected, no cytokine response was detected in the culture supernatants of CD4+ T cells isolated from control mice or from CD8+ T cells isolated from immunized mice (Fig. 1B and data not shown), suggesting that the cytokine response produced by CD4+ T cells isolated from immunized mice is mediated by effector or memory CD4+ T cells and that CD8+ T cells do not participate in the response to rHagB. This CD4+ T cell response was specific to rHagB, as stimulation of CD4+ T cells purified from immunized mice with an irrelevant protein, DnaK, a heat shock protein from Francisella tularensis (34), did not induce cytokine production (Supplemental Fig. 1A). Furthermore, the response required Ag presentation, since blocking of MHC class II cytokine production (Supplemental Fig. 1B). HagB was not a superantigen, as stimulation of naive CD4+ T cells with rHagB did not show spontaneous massive activation and proliferation as seen with staphylococcal enterotoxin B superantigen (data not shown). In vitro stimulation and in vivo immunization experiments with rHagB did not show a great expansion of particular Vβ TCR CD4+ T cell clones as seen with staphylococcal enterotoxin B (data not shown). Addi-
tionally, upon stimulation of rHagB-primed CD4+ T cells with live *P. gingivalis*, a significant increase in IFN-γ, IL-17, and IL-10 cytokines was detected in culture supernatants from purified CD4+ T cells from immunized mice compared with those from control mice (Supplemental Fig. 1C), suggesting that rHagB-primed CD4+ T cells respond to *P. gingivalis* upon stimulation.

Upon examination of serum Ab levels, most of the rHagB-specific Abs were of the IgG1 and IgG2c subclasses, with lesser amounts of IgG2b and IgG3 (Fig. 1C). Trivial amounts of serum IgA and IgE were detected (data not shown). IgG Ab activity persisted for up to 100 d following immunization (data not shown). Taken together, these results demonstrate that immunization of WT mice with rHagB and GPI-0100 induces a CD4+ T cell response.
(days 21 and 42) rHagB-specific Ab levels in WT and TLR4−/− mice (data not shown and Fig. 2C), a significant decrease in IgG2b and IgG2c was detected in TLR4−/− mice compared with WT mice (Fig. 2C). This decrease in IgG2 resulted in an increase in the relative ratio of rHagB-specific IgG1/IgG2 in TLR4−/− mice over time following immunization (Fig. 2D), confirming our previous results that the absence of TLR4 signaling in rHagB immunized mice results in a shift to Th2 over Th1 response.

MyD88 and TRIF play differential roles in the induction of CD4+ T cell responses following rHagB immunization

Because signaling through TLR4 utilizes both MyD88 and TRIF, we next investigated the role of these molecules in inducing the CD4+ T cell response following rHagB immunization. CD4+ T cells from MyD88−/− and TRIFLps2, similar to WT and TLR4−/−, upregulated CD44 following HagB immunization (data not shown). CD4+ T cells isolated from rHagB immunized MyD88−/− mice showed a severe reduction in their capacity to produce IFN-γ, no capacity to produce IL-17, and an increased capacity to produce IL-4 and IL-5 at both 21 and 44 d following immunization compared with CD4+ T cells isolated from WT mice (Fig. 3A). IL-10 production in MyD88−/− CD4+ T cells was higher than in WT at day 21, but it was significantly lower at 42 d following immunization (Fig. 3A). Meanwhile, CD4+ T cells isolated from TRIFLps2 immunized mice showed a decrease in IFN-γ and IL-17, an increase in IL-5, and no difference in IL-4 production at day 21 following immunization when compared with CD4+ T cells isolated from WT immunized mice (Fig. 3A). However, this decrease in IFN-γ and IL-17 production was reversed over time, that is, 44 d following immunization (Fig. 3A). The difference in the capacity of CD4+ T cells to produce IFN-γ was further confirmed by intracellular cytokine staining (Fig. 3B, Supplemental Fig. 2). These differences in the ability of MyD88−/− and TRIFLps2 CD4+ T cells to produce cytokines suggest that MyD88 regulates the inflammatory side of the CD4+ T cell response, whereas TRIF is responsible for counterbalancing this effect by decreasing IFN-γ and IL-17 while upregulating IL-4 production. By examining the serum Ab levels, there was a significant decrease in IgG1, IgG2b, and IgG2c levels in MyD88−/− mice compared with WT mice at day 21 (data not shown and Fig. 3C). Although this was accompanied by a decrease in the levels of IgG1, the IgG1/IgG2 ratio was still higher in MyD88−/− mice compared with WT or TRIFLps2 mice.

**FIGURE 3.** MyD88 and TRIF differentially regulate the CD4+ T cell response toward rHagB following immunization. A, Cytokine production by splenic and lymph node-purified CD4+ T cells from WT, MyD88−/−, or TRIFLps2 immunized or control mice 21 or 44 d following immunization. Cells were stimulated as previously described (see Materials and Methods) and culture supernatants were harvested 5 d later and assessed for the levels of IFN-γ, IL-17, IL-4, IL-5, and IL-10 by ELISA. Results are expressed as the mean ± SE of triplicate cultures from one of three independent experiments. B, IFN-γ production by intracellular cytokine staining. CD4+ T cells from WT, MyD88−/−, or TRIFLps2 were stimulated with 10 μg/ml rHagB or left unstimulated as controls for 36 h and cytokine secretion was blocked for an additional 10 h. IFN-γ production was detected by intracellular cytokine staining. Numbers represent percentages of CD4+ T cells that produce IFN-γ.

C, Serum levels of rHagB-specific IgG1, IgG2b, and IgG2c from WT, MyD88−/−, and TRIFLps2 mice. Each symbol represents an individual mouse and the horizontal lines represent the mean of the response. D, Ratio of rHagB-specific IgG1/IgG2b+2c in serum of WT, MyD88−/−, and TRIFLps2 mice. Ratios of IgG1 to IgG2b+2c were calculated for each individual mouse sample in C prior to the calculation of the mean and SE. ***p < 0.001, **p < 0.01, *p < 0.05 compared with control groups.
mice (Fig. 3D). This indicated that similar to TLR4−/− mice, the absence of MyD88 resulted in a higher rHagB Th2 response. No difference in IgG1 levels between WT and MyD88−/− mice was detected (data not shown). Additionally, there was no difference in HagB-specific Ab levels between TRIF−/− and WT mice, suggesting that TRIF plays no role in Ab subclass isotype switching following rHagB immunization.

**TLR4 signaling regulates the expression of transcription factors T-bet and GATA-3**

Depending on the major cytokines produced, effector CD4+ T cells have been divided into Th1, Th2, and Th17 cells, producing mainly IFN-γ, IL-4/IL-5, and IL-17, respectively. Each of these cytokines is regulated by a master transcription factor that plays an essential role in the production of the cytokine in question, with T-bet for IFN-γ, GATA-3 for IL-4/IL-5, and RORγt for IL-17 (54, 63). Because we observed differences in the cytokine production pattern following stimulation of rHagB-primed CD4+ T cells isolated from WT, TLR4−/−, MyD88−/−, and TRIF−/− mice, we next examined the expression levels of the above transcription factors in rHagB-stimulated CD4+ T cells. Our results showed a significant decrease in T-bet expression, a slight increase in GATA-3 expression, and no difference in RORγt expression in rHagB-stimulated CD4+ T cells isolated from WT, TLR4−/−, MyD88−/−, and TRIF−/− mice, (data not shown and Supplemental Fig. 3A). Similar results were detected in CD69+CD4+ T cells and in CD4+ T cells isolated 42 d following immunization (data not shown and Supplemental Fig. 3A).

In contrast, whereas no difference was seen in the percentages of activated CD25+CD4+ T cells that express T-bet in MyD88−/− CD4+ T cells, there was a significant increase in GATA-3 expression of pSTAT5 in stimulated CD4+ T cells from both WT and TRIF−/−CD4+ T cells 44 d following immunization (Supplemental Fig. 3B). These results suggest that the increase in Th2 phenotype in both TLR4−/− and MyD88−/− mice could be attributed to a differential regulation in the transcription factors T-bet and GATA-3.

**IL-2 signaling is more pronounced in rHagB-primed TLR4−/− CD4+ T cells**

IL-2 plays a critical role in the generation of Th2 T cell subsets (64–67), especially in the absence of TLR signaling (68). Because both TLR4−/− and MyD88−/− immunized mice showed an increase in Th2 subset development, we examined the role played by IL-2 signaling in this response. Our results showed a significant increase in IL-2 production by TLR4−/− and MyD88−/− CD4+ T cells isolated from immunized mice compared with those from WT mice at day 21 (Fig. 5A, 5B) and at day 42 (Supplemental Fig. 4A), whereas TRIF−/− CD4+ T cells showed an increase in IL-2 production at day 44 only (Supplemental Fig. 4A). Upon examining the upregulation of IL-2Rα-chain (CD25) following rHagB stimulation, a significant increase in CD25 was seen in TLR4−/− CD4+ T cells compared with WT cells (Fig. 5C, Supplemental Fig. 4B). However, this was not evident in MyD88−/− or TRIF−/− CD4+ T cells at days 21 and 44 following immunization (Fig. 5D, Supplemental Fig. 4C). Examination of the levels of phosphorylated STAT5, a major signal transduction molecule in the IL-2 signaling pathway, revealed higher levels of pSTAT5 in activated CD69+CD4+ T cells from TLR4−/− immunized mice compared with WT cells (Fig. 5E, Supplemental Fig. 4D), whereas expression of pSTAT5 in activated CD4+ T cells from both MyD88−/− and TRIF−/− mice was decreased at day 21 (Fig. 5F). However, at day 42, there was an increase in the expression of pSTAT5 in activated CD69+CD4+ T cells from MyD88−/− and TRIF−/− compared with WT mice (Supplemental Fig. 4E). These results suggest that the sustained increase in IL-2 signaling may contribute to the enhanced Th2 phenotype observed in TLR4−/− CD4+ T cells, but less in MyD88−/−/CD4+ T cells, and that perhaps the increased GATA-3 expression is the major factor contributing to the enhanced Th2 response in MyD88−/− CD4+ T cells.

![FIGURE 4](http://www.jimmunol.org/)  

**FIGURE 4.** TLR4 signaling regulates the expression of T-bet and GATA-3. Expression of T-bet, GATA-3, and RORγt by rHagB-stimulated purified CD4+ T cells from spleens and lymph nodes of WT and TLR4−/− (A) or WT, MyD88−/−, and TRIF−/− (B) immunized or control mice 21 d following immunization. Cells were stimulated with rHagB (10 μg/ml) or left unstimulated as controls. Cells were harvested at 36 h poststimulation and stained with CD4, CD25, T-bet, GATA-3, or RORγt Abs. Numbers represent the percentage of activated CD25+CD4+ T cells that express a particular transcription factor. Results are expressed as the mean ± SE of two to four independent experiments. ***p < 0.001, **p < 0.01, *p < 0.05 compared with unstimulated cultures from rHagB immunized mice; /p < 0.05 compared with stimulated cultures from rHagB immunized mice.
FIGURE 5. Lack of TLR4 results in an increase in IL-2 signaling following rHagB stimulation. A and B, IL-2 production by rHagB-stimulated purified CD4+ T cells from spleens and lymph nodes of WT and TLR4−/− (A, C, E) or WT, MyD88−/−, and TRIF−/− mice (B, D, F) immunized or control mice 21 d following immunization. Cells were stimulated with rHagB (10 μg/ml) or left unstimulated as controls. Culture supernatants were harvested 36 h poststimulation and were assessed for the levels of IL-2 by ELISA. Results are expressed as the mean ± SE of triplicate cultures from one of two independent experiments. C and D, Expression of CD25 (IL-2Rα) by rHagB-stimulated CD4+ T cells. Cells were stimulated with rHagB (10 μg/ml) or left unstimulated as controls, harvested at 36 h following stimulation, and stained with CD4 and CD25 Abs. Numbers represent the percentages of CD25+CD4+ T cells. Results are expressed as the mean ± SE of at least triplicate cultures from two to four independent experiments. (E, F) Expression of pSTAT5 by rHagB-stimulated purified splenic and lymph node CD4+ T cells. Cells were stimulated as above, harvested at 36 h poststimulation and stained with CD4, CD69, and pSTAT5 Abs. Numbers represent percentages of activated CD69+CD4+ T cells that express pSTAT5. Results represent one of two independent experiments. ***p < 0.001, compared with unstimulated cultures from HagB immunized mice; ###p < 0.001, **p < 0.01 compared with HagB-stimulated cultures from HagB immunized mice.

TLR4−/− and MyD88−/− CD4+ T cells from immunized mice express high levels of Foxp3

In addition to promoting Th2 differentiation, IL-2/IL-2R signaling plays an important role in the maintenance of regulatory T cells and the expression of transcription factor Foxp3 (69, 70). Foxp3 has been associated with the development of both natural and inducible Tregs and is essential for their inhibitory functions (71, 72). Therefore, we next compared the levels of Foxp3 expression in WT, TLR4−/−, MyD88−/−, or TRIF−/− CD4+ T cells. Upon rHagB stimulation, there was an increase in the percentages of CD25+ Foxp3+ TLR4−/− CD4+ T cells (~2-fold) at 21 and 42 d following immunization compared with WT cells (Fig. 6A). Because TLR4−/− CD4+ T cells had higher levels of CD25+ than did WT cells (Fig. 5C), we normalized the CD25+CD4+ T cell population to 100% and calculated the proportion of CD4+ T cells expressing Foxp3 as a percentage of the total CD25+CD4+ T cells. Whereas rHagB-stimulated CD4+ T cells from WT immunized mice showed ~40% Foxp3+, stimulated TLR4−/− CD4+ T cells showed a significant increase of Foxp3 (~55%) (Fig. 6B), suggesting a more regulatory phenotype of the TLR4−/− CD4+ T cells.

Although there was no difference in the percentages of CD25+ Foxp3+ WT and MyD88−/− CD4+ T cells (Fig. 6C), there was an increase in the proportion of CD25+Foxp3+CD4+ T cells out of the total CD25+CD4+ T cell population in MyD88−/− cells compared with WT CD4+ T cells, following rHagB stimulation (65% versus 43.5%, respectively) (Fig. 6C, 6D). These results suggest that the increase in Foxp3+CD4+ T cells following rHagB stimulation can be the factor leading to the decrease in IFN-γ production in the absence of TLR4 signaling, which is regulated mainly through MyD88 and not TRIF.

No difference in Foxp3 expression was detected in CD4+ T cells isolated from TRIF−/−/− mice when compared with WT cells at day 21 (data not shown); however, the proportion of CD25+Foxp3+CD4+ T cells was lower in TRIF−/−/− CD4+ T cells than in WT cells (Fig. 6D). This resulted in an increase in the relative ratio of T-bet/Foxp3 and RORγt/Foxp3 on day 44, but not on day 21, in TRIF−/−/− compared with WT CD4+ T cells (Fig. 6E, 6F). The change in the relative ratio of T-bet and RORγt to Foxp3 in TRIF−/−/− CD4+ T cells compared with WT may provide an explanation for the increased IFN-γ and IL-17 response detected at day 44 compared with day 21 following immunization (Fig. 3A).

TLR4 signaling differentially regulates CD4+ T cell activation and proliferation

Several factors control the activation and the proliferation of CD4+ T cells, including the expression of costimulatory molecules, innate cytokine production, and IL-2 levels. Because our results showed an increase in IL-2 production and Foxp3 expression in CD4+ T cells from TLR4- and MyD88-deficient mice compared with WT cells, we next examined the role TLR4 signaling plays on the activation and the proliferation capacity of CD4+ T cells. By evaluating CD69 expression, a significantly lower upregulation of CD69 was seen on TLR4−/− CD4+ T cells compared with WT cells at both 21 and 42 d following immunization (Fig. 7A). Similarly, there was less upregulation of CD69 expression on MyD88−/− CD4+ T cells compared with WT and TRIF−/−/− rHagB-stimulated cells, which was more significant at 44 d following immunization (Fig. 7B). These results further confirmed that TLR4 and MyD88 are required for the optimal activation of CD4+ T cells.

Upon assessing the proliferation potential of CD4+ T cells, our results showed a decrease in the proliferation capacity of rHagB-stimulated TLR4−/− CD4+ T cells compared with WT cells at 21 d following immunization (Fig. 8A). Unexpectedly, rHagB-stimulated MyD88−/− CD4+ T cells showed a similar proliferation capacity to WT CD4+ T cells 21 d following immunization (Fig. 8B). TRIF-deficient CD4+ T cells showed a slight increase in the proliferative activity than did CD4+ T cells isolated from WT immunized mice. These results suggest that while blocking TLR4 signaling results in a general decrease in the ability of the CD4+ T cells to proliferate, MyD88 and TRIF molecules independently play a minimal role in regulating the proliferation of CD4+ T cells.

CTLA-4 is upregulated in TLR4−/− but not MyD88−/− or TRIF−/−/− CD4+ T cells

CTLA-4 is a CD28 homolog that competes against CD28 for CD80/CD86 costimulatory molecules, thus preventing activation...
signals being delivered by APCs to T cells (73). CTLA-4 signaling can also inhibit proliferation of T cells. Because of the differences in the proliferating ability of TLR4−/−, MyD88−/−, and TRIF−/− mice, we next determined whether there is a differential regulation in the expression of CTLA-4 on CD4+ T cells following immunization. No significant difference in the percentages of ex vivo CD4+ T cells showing the percentages of cells expressing CD25 and Foxp3. Results represent one of two or three independent experiments. E and F, Purified CD4+ T cells from immunized or control WT, MyD88−/−, and TRIF−/− mice compared with those from WT mice is due to a differential regulation of CTLA-4 expression.

Discussion

In the present study, we provide evidence that immunization with rHagB induces a specific CD4+ T cell and Ab response. HagB-primed CD4+ T cells respond to P. gingivalis stimulation by the production of cytokines, mainly IFN-γ. The pronounced immunogenic effect can be attributed to the dual ability of rHagB to act as a TLR4 agonist and an immunogen rather than a superantigen. Several studies have shown that the association of a TLR agonist with an immunogen is more effective than one alone for inducing a specific immune response.
The lack of TLR4 results in a decrease in the proliferation capacity of CD4^+ T cells, CFSE-labeled CD4^+ T cells from WT and TLR4^−/− (A) or WT, MyD88^−/−, and TRIF^−/− (B) immunized or control mice 21 d following immunization. Cells were stimulated with rHagB (10 μg/ml) or left unstimulated as controls, harvested 5 d poststimulation, and stained with CD4 and CD69 Abs. Histogram plots gated on CD4^+ T cells show the percentages of CD69^+CD4^+ cells. Results are expressed as the mean ± SE of at least triplicate cultures from two or three independent experiments.

Ab induction, and the levels of transcription factors. Although TLR4 signaling was not required for the initiation and maintenance of the response, it was important for the regulation of CD4^+ T cell subsets, the ability of CD4^+ T cells to proliferate, and the array of Ab subtypes induced. Several studies have shown that the lack of TLR4 signaling leads to a prominent Th2 response (44, 77), or a decrease in the Th1 type response (76, 78), perhaps by decreased IL-12 production required for Th1 priming. However, this explanation fails to account for the results obtained in asthma models or helminthes infections, where the deficiency of TLR4 results in decreased Th2 responses (57–59). This difference could be attributed to 1) the type of Ag used in the studies, 2) the type of T cell response induced by a particular Ag, or 3) the quantity of the Ag available in the system. A study by Eisenbarth et al. (79) showed that limiting the amount of LPS used as an adjuvant during OVA sensitization resulted in a Th2-sensitized allergic airway inflammation, whereas the use of high quantities of LPS resulted in a Th1 response. Another study by Sun et al. (68, 80) showed that limiting the concentration of TLR2/4/9/3 agonist resulted in an increase in the Th2 response, whereas increasing the concentration blocked the Th2 response and augmented a Th1 response. These findings are in agreement with our results showing that lower concentrations of rHagB induced lower levels of IFN-γ by TLR4^−/− and MyD88^−/− CD4^+ T cells compared with higher doses. Additionally, although we could only detect a decrease in IgG2a anti-HagB Ab activity in TLR4^−/− mice when they were immunized with rHagB alone (data not shown), indicating that the decrease in the Th1 response in the absence of GPI-0100 was more pronounced. Although the use of GPI-0100 as an adjuvant is advantageous, as it increases the efficiency of rHagB immunization to prime CD4^+ T cells (~30- and 100-fold increase in cytokine and Ab response, respectively) (data not shown) and relies on a non-TLR signaling mechanism to exert its adjuvant effect (60), GPI-0100 may have increased the availability of rHagB as an Ag, thus augmenting the response detected even in the absence of TLR4 signaling. A study by Hou et al. (81) showed that the use of a cationic lipid DOTAP as an adjuvant with CpG stimulation resulted in an increased response of MyD88^−/− cells. Although such response was partially dependent on TLR signaling, it was not, however, completely di-

FIGURE 7. TLR4 signaling differentially regulates CD69 expression. Expression of CD69 by rHagB-stimulated purified splenic and lymph node CD4^+ T cells from WT and TLR4^−/− (A) or WT, MyD88^−/−, and TRIF^−/− (B) immunized or control mice 21, 42, or 44 d following immunization. Cells were stimulated with rHagB (10 μg/ml) or left unstimulated as controls, harvested at 36 h poststimulation, and stained with CD4 and CD69 Abs. Numbers represent the percentages of CD69^+CD4^+ cells. Results are expressed as the mean ± SE of at least triplicate cultures from two or three individual experiments. ***p < 0.001 compared with unstimulated cultures from rHagB immunized mice; *p < 0.05 compared with rHagB-stimulated cultures from rHagB immunized mice.

FIGURE 8. The lack of TLR4 results in a decrease in the proliferation capacity of CD4^+ T cells, CFSE-labeled CD4^+ T cells from WT and TLR4^−/− (A) or WT, MyD88^−/−, and TRIF^−/− (B) immunized or control mice 21 d following immunization. Cells were stimulated with rHagB (10 μg/ml) or left unstimulated as controls, harvested 5 d poststimulation, and stained with CD4 Abs. Histogram plots gated on CD4^+ T cells show the percentages of cells that diluted CFSE. Results are expressed as the mean ± SE of at least triplicate cultures from two or three individual experiments.

FIGURE 9. TLR4 differentially regulates CTLA-4 expression on CD4^+ T cells. Expression of CTLA-4 on CD4^+ T cells ex vivo from WT and TLR4^−/− (A) or WT, MyD88^−/−, and TRIF^−/− (B) immunized or control mice 21 d following immunization. Numbers represent the percentages of CD4^+ T cells expressing CTLA-4. Results are expressed as the mean ± SE of one or two of three individual experiments. *p < 0.05 compared with control groups; #p < 0.05 compared with WT rHagB immunized mice.
minimized in the absence of MyD88 (81), further supporting the notion that Ag availability plays an important role in influencing the role TLR signaling plays in CD4^+ T cell responses.

Our results highlight that CD4^+ T cell responses induced in the absence of TLR4 or MyD88 exhibit quantitative and qualitative differences. First, the loss in IFN-γ was more prominent in MyD88^−/− than in TLR4^−/− cells, which could be attributed to the involvement of the MyD88 molecule in IL-1β and IL-18 receptor signaling (82). Because IL-18 augments IFN-γ production (83), the lack of MyD88 may result in a failure of IL-18 signaling, leading to a further decrease in IFN-γ, although further evidence is required to prove this hypothesis. Second, the increase in IL-4 production by memory MyD88^−/− CD4^+ T cells over TLR4^−/− cells could be attributed to the significant increase in GATA-3 expression in MyD88^−/− CD4^+ T cells in comparison with TLR4^−/− cells. Because rHagB stimulation upregulates CD86 expression on MyD88^−/− DC, but not TLR4^−/− DC (22), and the upregulation of CD86 has been shown to play an important role in Th2-mediated responses (84–86), this could have augmented the Th2 response in the MyD88^−/− immunized mice over the TLR4^−/− mice.

Our results indicate that the shift from a Th1 to Th2 response in the TLR4^−/− and MyD88^−/− CD4^+ T cells correlated with a decrease in T-bet expression and an increase in GATA-3, respectively. Although other studies have examined this correlation, it is still uncertain whether this correlation exists in all models studied. De Luca et al. (87) showed that CD4^+ T cells from MyD88^−/− mice infected with Candida albicans produced less IFN-γ and expressed less T-bet than did CD4^+ T cells from WT mice. This was accompanied by an increase in the production of IL-10, the expression of Foxp3, and Treg activity in the MyD88^−/− mice. Furthermore, Rivera et al. (78) showed that in MyD88^−/− mice infected with Aspergillus fumigatus, IFN-γ production was reduced without an increase in the Th2 response. However, CD4^+ T cells from MyD88^−/− mice showed similar expression levels of T-bet as did WT mice. The results from these studies and ours support a correlation between the shift from Th1 to Th2 response and the differential change in T-bet, GATA-3, or Foxp3 expression. However, in cases where there is no alteration in the expression of transcription factors, the decrease in Th1 response is not accompanied by an increase in a Th2 or Treg response. Whether these differences are driven by a specific Ag or occur regardless of the type of Ag still needs to be determined in future studies.

Less is known about the role of TRIF in the CD4^+ T cell response elicited to TLR ligands. Our findings suggest that TRIF counter-regulates MyD88 in its capacity to produce IFN-γ and IL-17, since the lack of TRIF molecules in memory CD4^+ T cells resulted in a higher level of inflammatory cytokines (IFN-γ and IL-17) when compared with WT or MyD88^−/− cells. However, our results are in conflict with two studies showing that TRIF-deficient mice had a decrease in both IFN-γ and IL-17 CD4^+ T cell responses following C. albicans infection (87) and staphylococcal enterotoxin A and LPS immunization (88). This discrepancy could be attributed to the earlier time points (2 wk and 21 d) following infection or immunization assessed in the reported studies. This is supported by our own findings showing decreased levels of IFN-γ and IL-17 cytokines in TRIF^−/− mice compared with those in WT mice at day 21. However, as demonstrated in our studies, the cytokine levels increased over time (day 44), probably due to an increase in the relative ratio of T-bet and RORγt to Foxp3, suggesting that TRIF plays an anti-inflammatory role in CD4^+ T cell memory development. Guo et al. (89) showed that TRIF^−/− mice developed more experimental autoimmune encephalitis with an increase in IL-17 and IFN-γ. Additionally, monophosphoryl lipid A, a less toxic derivative of LPS that preferentially utilizes TRIF signaling, results in substantially less inflammation and sepsis induction in mice than that seen with LPS (90).

Our results showed that P. gingivalis HagB mediates a Th17 response in addition to the outer membrane protein from P. gingivalis reported by Oda et al. (91). Although the role of TLR4 signaling has been studied in Th1 and Th2 differentiation (44, 57, 77), little is known about how it regulates Th17 responses. In this study, we show that the IL-17 response to rHagB is completely dependent on MyD88. This was not due to an intrinsic defect in the MyD88^−/− CD4^+ T cells since they are able to produce IL-17 under anti-CD3 stimulation with IL-6 and TGF-β in vitro (data not shown), but rather an Ag-dependent defect. Although the lack of recruitment of IL-1R–associated kinase, a downstream molecule of the MyD88 signaling pathway affecting Th17 development and RORγt expression (92), may explain the inability of MyD88^−/− to produce IL-17, the expression of RORγt by HagB-primed CD4^+ T cells suggests that there is another inhibiting factor contributing to the lack of IL-17 production by these cells. This could be due to 1) the increased expression of Foxp3 by MyD88^−/− CD4^+ T cells, which can bind to RORγt or Runx1 and prevents the differentiation of Th17 (93, 94), or 2) an increase in the expression of the transcriptional repressor growth factor independent 1, expressed in Th2 cells through the IL-4/STAT6 signaling pathway (95) and is able to interact with RORγt, preventing it from binding to the IL-17 promoter (96). Further evidence, however, is needed to rule out which mechanism is playing a role in this process.

In this study, we further showed that TLR4 signaling affects IL-2/STAT5 signaling following rHagB immunization. Several studies have shown that in addition to GATA-3 expression, Th2 differentiation is dependent on IL-2/STAT5 signaling (64–67). Although our results confirmed the observations made by other investigators (64–67), they also suggest that IL-2 plays a more prominent role in TLR4^−/− rather than MyD88^−/− CD4^+ T cells, since CD4^+ T cells from MyD88^−/− mice did not upregulate CD25 expression (days 21 and 44) or phosphorylate STAT5 (day 21) to the same levels as did TLR4^−/− CD4^+ T cells. This differential increase in IL-2/STAT5 signaling in TLR4^−/− CD4^+ T cells could be due to the availability of less rHagB Ag to the TLR4^−/− CD4^+ T cells than to their MyD88 counterparts, since in MyD88^−/− DC, rHagB can use the TLR4-TRIF signaling pathway to present Ag to T cells. Recently, it has been shown that TRIF is involved in MHC class II upregulation (97), thus allowing for Ag presentation in the case of MyD88^−/− DC, but not TLR4^−/− DC. This is further supported by an investigation showing that stimulation of CD4^+ T cells with limited concentration of TLR agonists results in an increase in IL-4 and IL-2 responses, both of which were abolished upon increasing the concentration of the agonist (68). Whether the increase in IL-2 signaling is an alternative pathway to overcome the decrease in the activation and proliferation exhibited by the CD4^+ T cells still remains to be determined. However, our results support this idea, since TLR4^−/− CD4^+ T cells were more susceptible to a decrease in activation when IL-2 signaling was blocked than WT CD4^+ T cells (data not shown), confirming that in the absence of TLR4, CD4^+ T cells are more dependent or sensitive to IL-2 signaling.
Additionally, to our knowledge, we have shown for the first time that TLR4 signaling cross-talks with the IL-2 signaling pathway, as well as influences the induction of inducible Tregs during rHagB immunization. Finally, these results have expanded our knowledge on the mechanisms involved in the immune response to the HagB Ag, which will provide us with valuable information that will affect our strategies in designing effective means to combat periodontal diseases and systemic disorders affected by *P. gingivalis* infection.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**

73. Foxp3 + T regulatory cell-mediated suppression.
Gaddis et al., Supplementary Figure 1
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Gaddis et al., Supplementary Figure 2
A

Percentage of activated CD4⁺ T cells expressing transcription factor (%)

Unstim.     rHagB  

T-bet: **  **  ***  
GATA-3:  
RORγt:  

WT GPI  
WT GPI+rHagB  
TLR4⁻/⁻ GPI  
TLR4⁻/⁻ GPI+rHagB  

B

Percentage of activated CD4⁺ T cells expressing transcription factor (%)

Unstim.     rHagB  

T-bet:  **  **  ***  
GATA-3:  
RORγt:  

WT GPI  
WT GPI+rHagB  
MyD88⁻/⁻ GPI  
MyD88⁺/⁺ GPI+rHagB  
TRIF¹⁺/+ GPI  
TRIF¹⁻/⁻ GPI+rHagB  

Gaddis et al., Supplementary Figure 3
Gaddis et al., Supplementary Figure 4
Figure Legends for Supplemental material:

Sup. Fig. 1  Cytokine production by primed CD4+ T cells from rHagB immunized mice is specific to rHagB and requires antigen presentation. Purified CD4+ T cells from splenocytes and lymph node cells from immunized (closed bars (A) or squares (B)) or control (opened bars (A) or triangles (B)) mice were cultured with equal numbers of irradiated feeder cells and were stimulated with 10 μg/ml of rHagB, DnaK or left unstimulated as controls (A) or stimulated with 0.01, 0.1, 1 and 10 μg/ml of rHagB in presence of 10 μg/ml of anti-MHCII I-A/I-E (open symbols) or isotype control (closed symbols) (B). Culture supernatants were harvested 5 days later and assessed for the levels of IFN-γ, IL-4, IL-5 and IL-10 (A) or IFN-γ, IL-17, IL-4, IL-5 and IL-10 (B) by ELISA. (C) CD4+ T cells from rHagB immunized mice respond to *P. gingivalis* stimulation. Purified CD4+ T cells isolated from immunized and control mice, 21 days following immunization, were stimulated with 0.5 and 5 MOI of *P. gingivalis* 33277, or left unstimulated as controls. Culture supernatants were harvested and assessed for cytokine levels. Results are expressed as the mean ± SE of triplicate cultures from one of two independent experiments. ***, **, * Significant differences at $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively, compared to control groups.

Sup. Fig. 2  Lack of MyD88 signaling inhibits IFN-γ production by CD4+ T cells, while TRIF deficiency augments IFN-γ production. CD4+ T cells purified from immunized WT, MyD88−/− or TRIFLp−/− mice 42 days following immunization were stimulated with 10 μg/ml of rHagB or left unstimulated as controls for 36 h and cytokine secretion was blocked for an additional 10 h. IFN-γ production was detected by intracellular cytokine staining. Numbers represent percentages of CD4+ T cells that produce IFN-γ.
Sup. Fig. 3  TLR4 signaling regulates the expression of T-bet and GATA-3. Expression of T-bet, GATA-3 and RORγt by rHagB stimulated purified CD4+ T cells from spleens and lymph nodes of WT and TLR4−/− (A) or WT, MyD88−/− and TRIFLps2 (B) immunized or control mice, 42 or 44 days following immunization, respectively. Cells were stimulated with rHagB (10 μg/ml) or left unstimulated as controls. Cells were harvested at 36 h post-stimulation and stained with CD4, CD25, T-bet, GATA-3 or RORγt antibodies. Numbers represent the percentages of activated CD25+CD4+ T cells that express a particular transcription factor. Results are expressed as the mean ± SE of two to four independent experiments. ***, **, * Significant differences at \( P < 0.001, P < 0.01 \) and \( P < 0.05 \), respectively, compared to unstimulated cultures from rHagB immunized mice.

Sup. Fig. 4 Lack of TLR4 signaling results in an increase in IL-2 signaling following rHagB stimulation. (A) IL-2 production by rHagB stimulated purified CD4+ T cells from spleens and lymph nodes of WT and TLR4−/− (A, B and D) or WT, MyD88−/− and TRIFLps2 (A, C and E) immunized or control mice, 42 or 44 days following immunization. Cells were stimulated with rHagB (10 μg/ml) or left unstimulated as controls. Culture supernatants were harvested 36 h post-stimulation and were assessed for the levels of IL-2 by ELISA. Results are expressed as the mean ± SE of at least triplicate cultures from one of two independent experiments. (B, C) Expression of CD25 (IL-2Rα) by rHagB stimulated CD4+ T cells. Cells were stimulated with rHagB (10 μg/ml) or left unstimulated as controls, harvested at 36 h following stimulation, and stained with CD4 and CD25 antibodies. Numbers represent the percentages of CD25+CD4+ T cells. Results are expressed as the mean ± SE of at least triplicate cultures from two to four in-
dependent experiments. (D, E) Expression of pSTAT5 by rHagB stimulated purified splenic and lymph node CD4⁺ T cells. Cells were stimulated as above, harvested at 36 h post-stimulation and stained with CD4, CD69 and pSTAT5 antibodies. Numbers represent percentages of activated CD69⁺CD4⁺ T cells that express pSTAT5. Results represent one of two independent experiments. ***, **, Significant differences at $P < 0.001$ and $P < 0.01$, respectively, compared to unstimulated cultures from rHagB immunized mice. ###, ##, # Significant differences at $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively, compared to HagB stimulated cultures from HagB immunized mice.
SUPPLEMENTAL MATERIAL

Reagents and antibodies:
DnaK was purified and tested for endotoxin contamination in our laboratory, as previously described (1). For MHCII blocking, functional purified antibodies against MHCII I-A/I-E (clone M5/114.15.2) and matching isotype were purchased from eBioscience (San Diego, CA).

Porphyromonas gingivalis:
P. gingivalis ATCC 33277 was prepared as previously described with some modifications (2-5). Briefly, the bacteria were cultured and maintained on enriched Trypticase soy plates consisting of Trypticase soy agar supplemented with 1% yeast extract, 5% defibrinated sheep blood, hemin (5 mg/l), and menadione (1 mg/l) at 37°C in an anaerobic atmosphere of 10% H₂, 5% CO₂, and 85% N₂. For in vitro studies, the bacteria were harvested from the culture plate and resuspended in PBS. The estimated numbers of bacteria in the suspension were determined by reading the optical density at 580 nm and extrapolating from a standard curve.

T cell purification and stimulation:
To determine the specificity of the CD4⁺ T cell cytokine response, cultures were stimulated with rHagB, DnaK (10 µg/ml) or left unstimulated. To block antigen presentation, anti-MHCII I-A/I-E or isotype control antibody (10 µg/ml) were added together with the different concentration of rHagB and culture supernatants were harvested 5 days following stimulation and assessed for cytokines by ELISA. To test the capacity of rHagB primed CD4⁺ T cells to respond to P. gingivalis, CD4⁺ T cells and feeders were cultured as described above, without any antibiotics. Cells were stimulated with freshly prepared bacteria at a multiplicity of infection (MOI) of 0.5 and 5.
Penicillin and streptomycin were added to the cultures 4 h following stimulation.

SUPPLEMENTAL REFERENCES:


