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Toll-Like Receptor 4-Mediated Innate IL-10 Activates Antigen-Specific Regulatory T Cells and Confers Resistance to *Bordetella pertussis* by Inhibiting Inflammatory Pathology[1]

Sarah C. Higgins, Ed C. Lavelle, Chantelle McCann, Brian Keogh, Edel McNeela, Patricia Byrne, Brian O'Gorman, Andrew Jarnicki, Peter McGuirk, and Kingston H. G. Mills[2]

Signaling through Toll-like receptors (TLR) activates dendritic cell (DC) maturation and IL-12 production, which directs the induction of Th1 cells. We found that the production of IL-10, in addition to inflammatory cytokines and chemokines, was significantly reduced in DCs from TLR4-defective C3H/HeJ mice in response to *Bordetella pertussis*. TLR4 was also required for *B. pertussis* LPS-induced maturation of DCs, but other *B. pertussis* components stimulated DC maturation independently of TLR4. The course of *B. pertussis* infection was more severe in C3H/HeJ than in C3H/HeN mice. Surprisingly, Ab- and Ag-specific IFN-γ responses were enhanced at the peak of infection, whereas Ag-specific IL-10-producing T cells were significantly reduced in C3H/HeJ mice. This was associated with enhanced inflammatory cytokine production, cellular infiltration, and severe pathological changes in the lungs of TLR4-defective mice. Our findings suggest that TLR-4 signaling activates innate IL-10 production in response to *B. pertussis*, which both directly, and by promoting the induction of IL-10-secreting type 1 regulatory T cells, may inhibit Th1 responses and limit inflammatory pathology in the lungs during infection with *B. pertussis*. ***The Journal of Immunology,* 2003, 171: 3119–3127.**

Pathogen recognition receptors, including the Toll-like receptors (TLRs),[3] allow the innate immune system to detect conserved patterns of molecules on pathogens and to respond in the first line of defense against infection by producing inflammatory cytokines (1). Furthermore, the innate immune response to pathogens can shape the adaptive immune response, through the stimulation of dendritic cells (DCs), which act as APCs, but also direct the differentiation of naive T cells. A range of pathogen-derived molecules has been identified as ligands for TLRs. TLR4 was first implicated in LPS recognition (2), although more recent studies have shown that structurally distinct LPS from certain bacteria can signal through TLR2 (3, 4). The role of TLR4 in LPS signaling was identified using LPS-hyporesponsive C3H/HeJ mice, which have a point mutation in the cytoplasmic region of TLR4 (2). Recognition of LPS is initialized by the cooperative interplay between the LPS-binding protein, CD14, and the TLR4-MD2 complex (5). Upon activation by LPS, TLR4 signals via a pathway involving the kinases of the IL-1R-associated kinase family, TNFR-associated factor-6 and NF-κB (6).

Binding of pathogens to TLRs on DCs results in their maturation, characterized by up-regulation of MHC class II, CD80, CD86, and CD40, but also activation of proinflammatory cytokine production, including TNF-α and IL-12. The mature DCs migrate from the tissue to the lymph nodes, where they present Ag to naive T cells. Evidence is emerging that DCs activated by distinct pathogen-derived molecules can selectively promote the induction of distinct T cell subtypes. Many pathogen molecules, including *Escherichia coli* LPS (2), CpG motifs in bacterial DNA (7), flagellin (8), and viral dsRNA (9) that bind TLRs and stimulate IL-12 production by innate cells, such as DCs, direct the induction of Th1 cells. Other pathogen-derived molecules, such as yeast hyphae (10), helminth components (11), cholera toxin (12), and *Porphyromonas gingivalis* LPS (3), can activate DCs that drive the differentiation of naive T cells to a Th2 phenotype. Finally, we have recently reported that filamentous hemagglutinin (FHA) from *Bordetella pertussis* can activate innate IL-10 production and stimulate DCs that selectively activate type 1 regulatory T (Tr1) cells (13, 14).

B. pertussis is a Gram-negative bacterium that causes whooping cough, a protracted respiratory disease in young children. Recovery from infection in both children and mice is associated with the development of *B. pertussis*-specific Th1 cells (15, 16). Adoptive transfer of Th1 cells from convalescent mice can confer protection (15), and IFN-γ receptor knockout mice develop lethal disseminating infection (17). Athymic or SCID mice fail to clear the infection, but the bacteria do not disseminate from the lung (15, 18), suggesting that innate responses may prevent bacterial dissemination before the development of adaptive immunity. However, induction of IgG and Th1 responses is suppressed during the acute stages of infection (19), and we have recently shown that Ag-specific Tr1 clones can be generated from the respiratory tract of *B. pertussis*-infected mice (14). The Tr1 cells were shown to suppress *B. pertussis*-specific Th1 responses in vitro and in vivo. We concluded that induction of Tr1 cells may represent an evasion strategy by the pathogen to subvert protective Th1 responses, but also speculated that they may have a role in limiting immunopathology in the lungs (14).

In the present study, we set out to examine the role of TLR4 in the pathogenesis of *B. pertussis* infection, specifically the role of

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[3] Abbreviations used in this paper: TLR, Toll-like receptor; BAL, bronchoalveolar lavage; DC, dendritic cell; FHA, filamentous hemagglutinin; iDC, immature dendritic cell; MIP, macrophage-inflammatory protein; PT, pertussis toxin; Tr, regulatory T.

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TLR4 in innate and consequently adaptive immunity to this respiratory pathogen. Our findings demonstrate that *B. pertussis* LPS stimulates DC IL-10 production, in addition to proinflammatory cytokine production and maturation, through TLR4. *B. pertussis* infection was more severe in TLR4-defective mice, despite an enhancement in Ab- and Ag-specific IFN-γ production. However, Ag-specific IL-10 production by T cells was significantly reduced and inflammatory pathology was enhanced in TLR4-defective mice, suggesting that TLR4-mediated IL-10 production promotes the generation of Tr1 cells and confers host resistance to the infection by limiting inflammation and collateral damage in the lungs.

Materials and Methods

Mice

Specific pathogen-free BALB/c, C3H/HeN, and C3H/HeJ mice were obtained from Harlan (Bicester, Oxon, U.K.). Mice were maintained according to the regulations and guidelines of the Irish Department of Health.

Cytokine/Chemokine secretion by DCs and macrophages

Bone marrow-derived immature DC (iDC) cells were generated by culturing bone marrow cells for 7 days in medium with 5–10% GM-CSF cell supernatant, as described (14). Bone marrow-derived DCs (10^6/ml) were cultured in 24-well tissue culture plates at 37°C for 24 h with live, heat-killed, or sonicated *B. pertussis* (10 bacteria to 1 DC or equivalent, unless otherwise stated), *B. pertussis* LPS (1 μg/ml). In certain experiments, anti-IL-10 (BD PharMingen, Oxford, U.K.) or anti-TLR4 (Alexis, Nottingham, U.K.) Abs (10 μg/ml) were added to the cultures. Supernatants were removed after 0.5–48 h for analysis of cytokine and chemokine concentrations. IL-12p70, IL-10, IL-6, TNF-α, macrophage-inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), and MIP-2 (CXCL1) concentrations were measured by immunoassay using pairs of Abs specific for mouse cytokines/chemokines (BD PharMingen or R&D Systems, Abingdon, U.K.), as described (20).

Analysis of DC maturation by flow cytometry

DCs were washed, resuspended in PBS, and transferred to FACS tubes (Falcon). Nonspecific binding was prevented by incubating the cells with 2% normal mouse serum (Biological Labs, Ballina, Ireland) for 30 min on ice. Expression of surface markers was assessed using biotinylated anti-CD11c (clone HL3) with streptavidin PerCP, PE-conjugated anti-CD80 (16-10A1), FITC-conjugated anti-CD86 (clone GL1), and FITC-conjugated anti-CD40 (clone 3/23). Cells incubated with an isotype-matched directly conjugated Ab with irrelevant specificity acted as a control. All Abs were purchased from BD PharMingen. After incubation for 30 min at 4°C, cells were washed, and immunofluorescence analysis was performed using a FACScan (BD Biosciences, San Jose, CA) and analyzed using CellQuest software. Twenty thousand cells were analyzed per sample.

B. pertussis *respiratory challenge*

Respiratory infection of mice was performed by aerosol challenge, as previously described (15). The course of *B. pertussis* infection was followed by performing CFU counts on lungs from groups of four mice at intervals after challenge. The lungs were aseptically removed and homogenized in 1 ml of sterile physiological saline with 1% casein on ice. Undiluted and serially diluted homogenate (100 μl) from individual lungs was spotted in triplicate onto Bordet-Gengou agar plates, and the number of CFU was estimated after 4 days of incubation at 37°C. The limit of detection was ~0.6 $\log_{10}$ CFU per lung.

Generation of T cell lines and clones

$CD4^+$ T cell lines were generated from the spleens of mice 2 wk after aerosol challenge with *B. pertussis*, as described (14, 20). Briefly, unseparated spleen cells (2×10^6/ml) were cultured with FHA (5 μg/ml), and IL-2 (5 IU/ml) was added 5 days later. After a further 7 days, surviving T cells were cultured at 1×10^5/ml with Ag (FHA; 1 μg/ml) and APC (irradiated spleen cells; 2×10^6/ml); IL-2 was added after 5 days, and the stimulate-rest cycle continued every 10–14 days. Cells were tested for Ag specificity and cytokine production at the end of the rest stage, and were cloned by limiting dilution after two rounds of Ag stimulation.

T cell cytokine production

T cell lines or clones (1×10^5/ml) and APC (irradiated spleen cells 2×10^6/ml) or ex vivo spleen cells (2×10^6/ml) were cultured with medium only or *B. pertussis* Ag (1×10^5 killed bacteria/ml), FHA (1–5 μg/ml), or pertussis toxin (PT; 1–5 μg/ml). Cells were stimulated with anti-CD3 (2 μg/ml) and PMA (25 ng/ml) or medium only as positive and negative control stimuli, respectively. Spleen cells were also cultured with Ag in the presence or absence of anti-IL-10 Ab (10 μg/ml). Supernatants were removed from triplicate cultures after 72 h, and IL-4, IL-5, IL-10, and IFN-γ concentrations were determined by immunoassay, as described (20).

Ab response

Serum Ab responses to *B. pertussis* were quantified by ELISA using *B. pertussis* sonicate (5.0 μg/ml) to coat the plates. Bound Abs were detected using biotin-conjugated anti-mouse IgG1, IgG2a, or IgG2b Abs (BD PharMingen) and peroxidase-conjugated streptavidin. Ab levels are expressed as the mean endpoint titer ($\pm$SD), determined by extrapolation of the linear part of the titration curve to 2 SD above the background value obtained with nonimmune mouse serum.

Histopathology

Groups of four C3H/HeN and C3H/HeJ mice were sacrificed at 7, 14, and 21 days after *B. pertussis* infection. Mice were first anesthetized with halothane, and after initial perfusion with PBS, were perfused via the left ventricle with 10% formol saline for 5 min. The lungs were removed, and following overnight fixation in 10% formol saline, dehydrated, and embedded in paraffin wax. Sections (4 μM) were cut and routinely stained with H&E.

Detection of inflammatory response in the lungs

Bronchoalveolar lavage (BAL) fluids were obtained by injection and aspiration of 0.5 ml vol (total 4–5 ml) of warm RPMI 1640 medium via cannulation of the trachea (20). Cells from the lavage fluids were recovered by centrifugation at $300 \times g$ for 5 min, and resuspended in RPMI 1640 medium with 8% FCS. The cell composition in the lungs during the course of infection was followed by performing a total leukocyte count and microscopic examination of Diff-Quik (Thermo Shandon, Runcorn, Cheshire, U.K.)-stained cytospin preparations of BAL cells. Cytokine concentrations were determined in homogenized whole lungs. Lungs were homogenized in 1 ml of 1% casein solution, and frozen at −80°C for cytokine analysis. The concentrations of IL-1β, TNF-α, IFN-γ, IL-12p70, IL-10, IL-6, MIP-1α, and MIP-2 were determined by specific immunoassays (20). Cytokine concentrations in lung homogenates are expressed as pg or ng per lung.

Statistical analyses

One-way ANOVA was used to test for statistical significance of differences between more than two experimental groups. The Student's *t* test was used for analysis when two groups were compared. Statistical significance was recorded at $p < 0.05$.

Results

B. pertussis *stimulates proinflammatory cytokine and chemokine production from DCs, but also induces IL-10*

Bone marrow-derived DC from BALB/c mice were stimulated with a range of concentrations of killed *B. pertussis*, *B. pertussis* sonicated extract or *B. pertussis* LPS (1.0 μg/ml), and cytokine and chemokine production was measured 18 h later. *B. pertussis* and *B. pertussis* LPS activated DCs to secrete high levels of the proinflammatory cytokines, TNF-α (Fig. 1*A*), IL-12p40, IL-12p70, IL-6, and IL-1β (data not shown); inflammatory chemokines, MIP-1α (Fig. 1*A*), MIP-1β, and MIP-2 (data not shown); and the anti-inflammatory cytokine, IL-10 (Fig. 1*A*). Stimulation of DCs with as few as 1 bacterium to 10 DCs stimulated TNF-α production. Low concentrations of sonic extract from *B. pertussis* (which had not been heat inactivated) were more potent than the equivalent dose of heat-inactivated whole *B. pertussis*. At concentrations equivalent to 100-1000 bacteria to 1 DC, the sonic extract induced lower concentrations of cytokines than the heat-killed whole bacteria, probably due to the release of inhibitory heat-sensitive bacterial molecules. However, the doses of bacteria used did not affect DC viability.

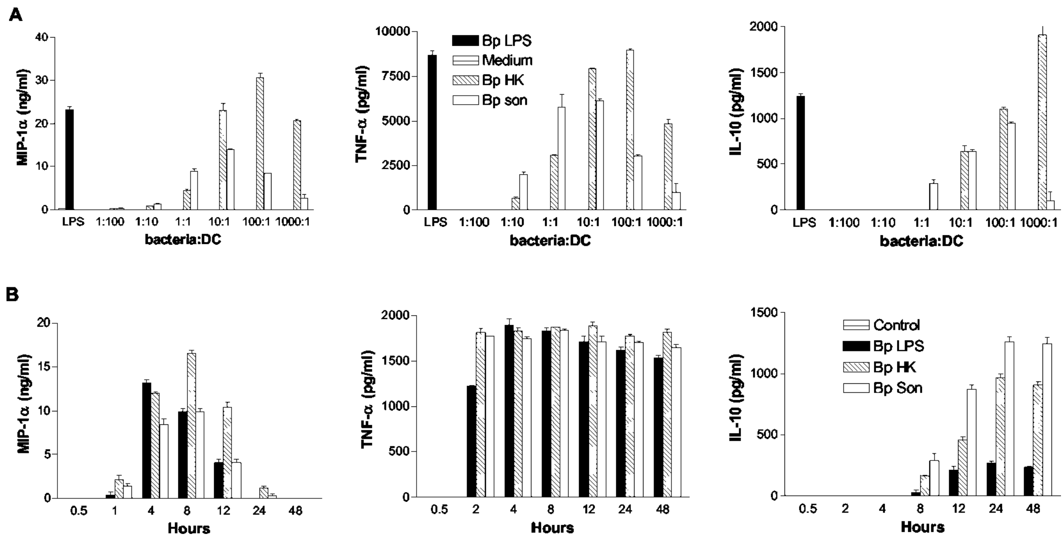

FIGURE 1. *B. pertussis* stimulates proinflammatory cytokines and chemokines and IL-10 production by DC. *A*, Bone marrow-derived DCs from BALB/c mice were stimulated with *B. pertussis* LPS (1 μg/ml), *B. pertussis* sonicate (Bp Son), or heat-killed *B. pertussis* (Bp HK) with ratios equivalent to 1 bacterium to 100 DCs to 1000 bacteria to 1 DC. Cytokine and chemokine concentrations were evaluated by immunoassay 18 h later. *B*, DCs were stimulated with *B. pertussis* LPS (1 μg/ml), *B. pertussis* sonicate (Bp Son), and heat-killed *B. pertussis* (Bp HK) at 1 DC to 10 bacteria or equivalent, and cytokine and chemokine concentrations were evaluated by immunoassay 0.5–48 h later.

To investigate the kinetics of cytokine production in response to *B. pertussis*, DCs were stimulated with *B. pertussis* sonicate and heat-killed *B. pertussis* at a ratio of 10 bacteria to 1 DC (or equivalent) or LPS (1.0 μg/ml), and supernatants were removed at various time points (0.5–48 h). TNF-α production occurred very rapidly; peak concentrations of TNF-α were detected at 2 h (Fig. 1*B*). IL-12 p70 (data not shown) and MIP-1α (Fig. 1*B*) were first detected at 2 h, but did not peak until 4–8 h after stimulation. In contrast, IL-10 induction was much slower, and was only detectable at 8 h with a peak at 24 h (Fig. 1*B*).

B. pertussis *induces DC pro- and anti-inflammatory cytokine and chemokine production by signaling through TLR4*

Cytokine and chemokine production was assessed 18 h after stimulation of DCs from C3H/HeN and C3H/HeJ mice with heat-killed *B. pertussis*, *B. pertussis* sonic extract, and live *B. pertussis* (10 bacteria to 1 DC or equivalent). The dose of bacteria used was established from dose range experiments with DC from C3H/HeN mice (data not shown) and was very similar to those observed for MIP-1α, TNF-α, and IL-10 for DC from BALB/c mice (Fig. 1). In addition, IL-12p40, IL-12p70, and IL-6 secretion for DC from C3H/HeN mice was optimal at 10 bacteria to 1 DC (data not shown). Comparable with DCs from BALB/c mice, DCs from C3H/HeN mice produced TNF-α, IL-12p70, IL-6, MIP-1α, MIP-2, and IL-10 in response to live, heat-killed, or sonicated *B. pertussis* (Fig. 2*A*). IL-12p70, MIP-1α (Fig. 2*A*), MIP-1β, and MCP-1 (data not shown) production was almost completely abrogated in the TLR4-defective C3H/HeJ DCs stimulated with *B. pertussis* ($p < 0.001$). Similarly, the production of IL-10 was significantly ($p < 0.001$) impaired in C3H/HeJ DCs stimulated with live or killed *B. pertussis* or *B. pertussis* sonicate. The TLR4 defect in C3H/HeJ mice did not have as significant an effect on TNF-α, IL-6, or MIP-2 production by DCs in response to *B. pertussis* (Fig. 2*A*), suggesting that components of the bacteria that do not signal through TLR-4 may also promote production of these cytokines. Similar concentrations of MIP-2 were produced by DCs from C3H/HeN and C3H/HeJ mice following stimulation with live *B. pertussis*. However, MIP-2 production in response to heat-killed *B. pertussis* was significantly lower in C3H/HeJ DCs compared with C3H/HeN DCs. To confirm that the reduction in cytokine production by DC from C3H/HeJ compared with C3H/HeN mice resulted from the TLR4 defect, rather than other genetic differences, we examined cytokine production by C3H/HeN in the presence of anti-TLR4 Ab. Addition of anti-TLR4 to *B. pertussis*-stimulated DC from C3H/HeN DC significantly reduced production of the two cytokines examined (TNF-α, 3.34 ± .04 to 0.77 ± .03 ng/ml, and IL-12p40, 11.12 ± 0.2 to 6.2 ± 0.3 ng/ml). We also assessed the influence of IL-10 on production of proinflammatory cytokine production by DC from C3H/HeN mice in response to *B. pertussis*, and found that addition of anti-IL-10 Ab to the cultures significantly augmented TNF-α (2.02 ± 0.07 to 4.07 ± 0.45 ng/ml) and IL-12p40 (38.0 ± 6.4 to 103.0 ± 22.4 ng/ml) production.

Because LPS from many, but not all, bacteria has been shown to signal through TLR4 (4–6), we examined the role of LPS in the inflammatory cytokine and chemokine responses to *B. pertussis*. The production of all proinflammatory cytokines and chemokines examined was significantly ($p < 0.01$ to $p < 0.001$) lower in the TLR4-defective compared with normal DCs in response to *B. pertussis* LPS (1 μg/ml). Furthermore, IL-10 production was almost completely abrogated in DCs (Fig. 2*B*) and macrophages (data not shown) from the C3H/HeJ mice.

B. pertussis *LPS induces maturation of DCs through TLR4 signaling, but* B. pertussis *bacteria can also induce maturation of DCs independently of TLR4*

Because signaling through TLRs also mediates the signaling events that lead to maturation of iDCs as well as induction of cytokine production, we examined the capacity of *B. pertussis* to induce maturation of murine DCs. Bone marrow-derived iDCs were cultured for 24 h with *B. pertussis* and then analyzed for surface expression of costimulatory molecules by immunofluorescence analysis with Abs specific for CD80, CD86, and CD40. Stimulation with live *B. pertussis* (Fig. 3) greatly enhanced CD80 and CD40 expression and moderately enhanced CD86 expression on DCs from C3H/HeN and C3H/HeJ mice (Fig. 3). Sonicated and heat-killed *B. pertussis* also promoted maturation of the DC in C3H/HeN and C3H/HeJ mice (data not shown). In contrast, *B. pertussis* LPS failed to up-regulate costimulatory molecule expression

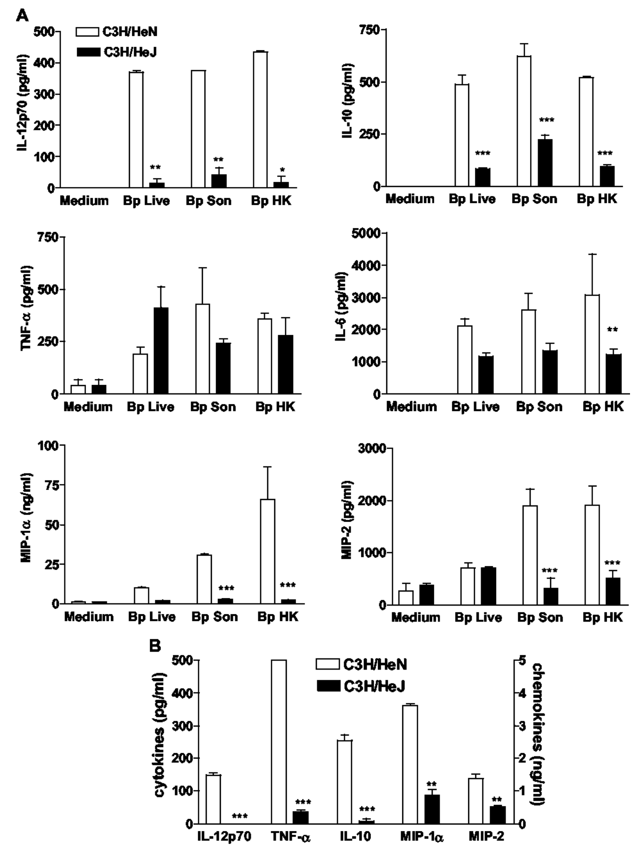

FIGURE 2. Impaired *B. pertussis*-induced cytokine and chemokine production by DC from TLR4-defective mice. Bone marrow-derived DCs from C3H/HeN mice and the TLR4-defective C3H/HeJ mice were stimulated with (*A*) live *B. pertussis* (Bp Live), *B. pertussis* sonicate (Bp Son), and heat-killed *B. pertussis* (Bp HK) at 10 bacteria to 1 DC (or equivalent) or with (*B*; below) 1 μg/ml *B. pertussis* LPS. Cytokine and chemokine concentrations were evaluated by immunoassay 18 h later. Values represent means ± SD of triplicate assays. $*$, $p < 0.05$; $**$, $p < 0.01$; $***$, $p < 0.001$ vs C3H/HeN. Data are representative of four similar experiments.

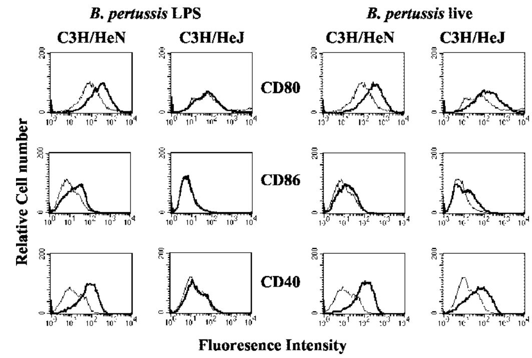

FIGURE 3. *B. pertussis* LPS induces maturation of DCs through TLR4, but the whole bacteria induce DC maturation independently of TLR4. Bone marrow-derived DCs from C3H/HeN and TLR4-defective C3H/HeJ mice were stimulated with *B. pertussis* LPS (1.0 μg/ml), live *B. pertussis* (10 bacteria:1 DC), or medium only. After 24 h, cells were washed, and immunofluorescence analysis was performed with Abs specific for CD80, CD86, or CD40 (bold line), or with isotype-matched control Abs (thin line). Results are representative of four experiments.

on DCs from TLR4-defective C3H/HeJ mice, but did activate maturation of iDCs from C3H/HeN mice; CD80, CD86, and CD40 were all up-regulated on iDCs from the C3H/HeN mice stimulated with LPS (Fig. 3). The findings demonstrate that *B. pertussis* LPS matures DCs by mediating signals through TLR4, but that other components of the bacterium also mature DCs independently of TLR4.

Protracted infection in TLR4-defective strain following B. pertussis *respiratory challenge*

Clearance of primary infection with *B. pertussis* appears to be mediated by Th1 cells, but there is also evidence of a role for innate immunity early in the infection (17, 18). In this study, we examined the role of TLR4 in protective immunity to *B. pertussis* in vivo. C3H/HeN and C3H/HeJ mice were challenged by exposure to an aerosol of *B. pertussis*, and the course of infection was monitored by counting bacteria in the lungs of mice at intervals after challenge. After an initial rise in bacterial counts, the C3H/HeN mice began to clear the bacteria from their lungs 7 days after challenge (Fig. 4). In contrast, the number of bacteria in the lungs of the TLR4-defective mice continued to increase until day 14; the CFU counts on days 14 and 21 were significantly higher ($p < 0.001$) than in the C3H/HeN strain (Fig. 4). The more severe course of infection in C3H/HeJ mice suggests that TLR4 plays a role in protective immunity to *B. pertussis*.

Differential inflammatory cytokine responses in lungs of B. pertussis-*infected C3H/HeN and C3H/HeJ mice*

Having demonstrated that cytokine production by innate cells is significantly compromised in TLR4-defective mice, we examined inflammatory cytokine and chemokine production in the lungs of C3H/HeJ and C3H/HeN mice following challenge with *B. pertussis*. *B. pertussis* infection of C3H/HeN mice was associated with rapid, but transient induction of proinflammatory cytokines and chemokines in the lungs (Fig. 5). In contrast, this early proinflammatory response was not detected in the C3H/HeJ strain; significantly higher concentrations of TNF-α, MIP-2 ($p < 0.001$), IL-1β, and MIP-1α ($p < 0.05$) were detected in the infected lungs of C3H/HeN compared with C3H/HeJ mice 3 h postchallenge. Transient induction of IL-12p70 was detected at day 7 in C3H/HeN mice, and levels were significantly ($p < 0.001$) higher than in the C3H/HeJ mice. This suggested that the early innate response to *B. pertussis* was compromised in the TLR4-defective mice. However, proinflammatory cytokines were significantly elevated much later (14 days postchallenge) in the TLR4-defective strain; IL-1β, IL-6, IL-12, and MIP-1α concentrations were significantly higher in the lungs of C3H/HeJ compared with C3H/HeN mice 14 days after *B. pertussis* challenge. IFN-γ concentrations also increased in the lungs after infection, and the levels at day 7 were significantly greater in the C3H/HeN mice. Overall, the concentrations of IFN-γ in the lungs were low in both strains; this is consistent with previous studies of suppressed IFN-γ production locally, but not systemically, during *B. pertussis* infection (19). We found high endogenous IL-10 production in the lungs of C3H/HeN and C3H/HeJ mice, and this increased after infection (Fig. 5). The concentrations at day 7 were higher, although not significantly, in C3H/HeN than in the C3H/HeJ mice. However, the concentrations declined to very low levels later in infection (Fig. 5) and returned to preinfection levels after bacterial clearance (data not shown).

Enhanced inflammatory infiltrate and exacerbated pathology in the lungs of TLR4-defective mice infected with B. pertussis

We examined the hypothesis that the increased bacterial load in TLR-4-defective mice may have resulted from enhanced local pathology as a result of defective innate, and as a consequence adaptive, IL-10 production. Lungs and BAL samples from *B. pertussis*-infected C3H/HeN and C3H/HeJ mice were examined for histopathological changes and inflammatory infiltration. A transient

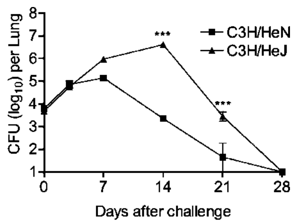

FIGURE 4. Increased bacterial burden in the lungs of TLR4-defective mice following *B. pertussis* respiratory challenge. C3H/HeN and C3H/HeJ mice were aerosol challenged with *B. pertussis*, and groups of four mice were sacrificed 3 h, or 3, 7, 14, or 21 days later. Lungs were removed, and the number of viable *B. pertussis* was determined by performing CFU counts on lung homogenates. ***, $p < 0.001$ vs C3H/HeN. Results are representative of two experiments.

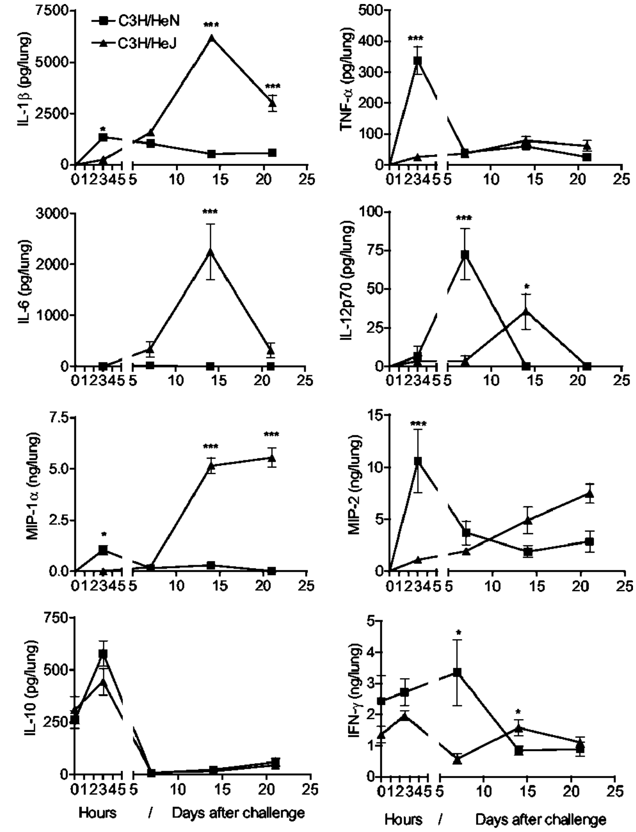

FIGURE 5. Differential inflammatory cytokine and chemokine responses in the lungs of C3H/HeN and C3H/HeJ mice infected with *B. pertussis*. Cytokine and chemokine analysis was performed on lung homogenates from C3H/HeN and C3H/HeJ mice 3 h, or 7, 14, or 21 days after aerosol challenge with *B. pertussis*. Results are mean (±SD) for four mice per group at each time point. *, $p < 0.05$; ***, $p < 0.001$ vs C3H/HeN. The results shown are representative of two experiments.

infiltrate of neutrophils was detected in BAL fluid from C3H/HeN mice soon after infection with *B. pertussis* (Fig. 6). However, this early cellular infiltrate was not observed in lungs of C3H/HeJ mice. In contrast, significantly higher numbers of neutrophils and lymphocytes were detected in the BAL fluid of C3H/HeJ mice 14 and/or 21 days after *B. pertussis* challenge (Fig. 6). Macrophages were the dominant cell type in the naive lung, and these levels increased immediately after challenge, especially in the C3H/HeJ mice.

On histopathological examination, the lungs of C3H/HeN mice appeared normal apart from mild lymphoid peribroncheolar cuffing 14 days postinfection (Fig. 7). In contrast, the lungs of the TLR4-defective strain exhibited exacerbated damage, with severe peribronchoalveolar cuffing and hyperplasia. A dense peribronchoalveolar leukocytic infiltrate of neutrophils and mononuclear cells was observed in the C3H/HeJ mice 14 days after challenge (Fig. 7). The intra-alveolar fibrin deposits observed are also indicative of severe damage in the lungs of these mice. This severe bronchopneumonia was still evident in the TLR4-defective strain 21 days postchallenge.

Reduced Ag-specific IL-10 and enhanced IFN-γ production in B. pertussis*-infected C3H/HeJ mice*

We examined the development of adaptive immune responses in TLR-defective mice and found that *B. pertussis*-specific IL-10 production by spleen cells was significantly lower ($p < 0.05$ to $p < 0.001$) in C3H/HeJ compared with C3H/HeN mice at 14–28 days post-*B. pertussis* challenge (Fig. 8*A*). In contrast, *B. pertussis*-specific IFN-γ was significantly higher in spleen cells from the C3H/HeJ strain compared with the C3H/HeN strain 14 days after challenge. However, similar concentrations of IFN-γ were observed in both strains 21–28 days postchallenge. *B. pertussis*-specific IL-4 could not be detected in spleen cells from C3H/HeJ or C3H/HeN mice in *B. pertussis*-infected mice at any time point examined (data not shown). Because the killed bacteria may have influenced the in vitro responses observed, we also examined the responses to purified *B. pertussis* Ags, FHA and PT. Ag-specific IL-10 production was higher, especially in response to PT, in spleen cells from C3H/HeN mice, whereas IFN-γ production was significantly stronger, especially in response to FHA, in spleen cells from C3H/HeJ mice (Fig. 8*B*). We also examined the influence of IL-10 on IFN-γ production by spleen cells from *B. pertussis*-infected C3H/HeN mice. Consistent with the experiments described in Fig. 8, *A* and *B*, Ag-specific IFN-γ was significantly lower in C3H/HeN than in C3H/HeJ mice 14 days after *B. pertussis* infection (Fig. 8*C*). However, addition of anti-IL-10 significantly augmented IFN-γ production by spleen cells from C3H/HeN mice in response to FHA or killed *B. pertussis*, suggesting that the defect in IFN-γ production by spleen cells from these mice was in part associated with IL-10 production.

To confirm that the cytokines detected were secreted by T cells, we established CD4$^+$ T cell lines from C3H/HeN and C3H/HeJ mice 14 and 21 days after challenge with *B. pertussis*. The T cell lines established 14 days after infection of C3H/HeN mice had profiles characteristic either of Tr1 cells (high IL-10, intermediate IL-5, low IFN-γ, and no IL-4) or Th1 cells (IFN-γ and no IL-4, IL-5, or IL-10), whereas all T cell lines from C3H/HeJ mice secreted only IFN-γ, characteristic of Th1 cells (Fig. 9*A* and data not shown). A similar pattern was observed with the T cell lines established 21 days after infection, except that IL-10 was detected at low levels in two of the four T cell lines from the C3H/HeJ mice (Fig. 9*B*). A number of these T cell lines were cloned; we successfully established four T cell clones from one of the T cell lines derived from a C3H/HeN mouse 14 days after *B. pertussis* infection. Each of these T cell clones secreted IL-10 and IL-5, but not IFN-γ or IL-4, a cytokine profile characteristic of Tr1 cells (Fig. 9*C*). We also quantified the number of IL-10-secreting CD4$^+$ T cells in Ag-restimulated spleen cells using intracellular staining and demonstrated that the frequency of IL-10-secreting Ag-specific CD4$^+$ T cells was considerably lower in *B. pertussis*-infected C3H/HeJ compared with C3H/HeN mice (1 vs 35%). These findings suggest that IL-10-secreting Tr cells are induced during acute infection of normal mice with *B. pertussis*, but that this response is impaired in TLR4-defective mice.

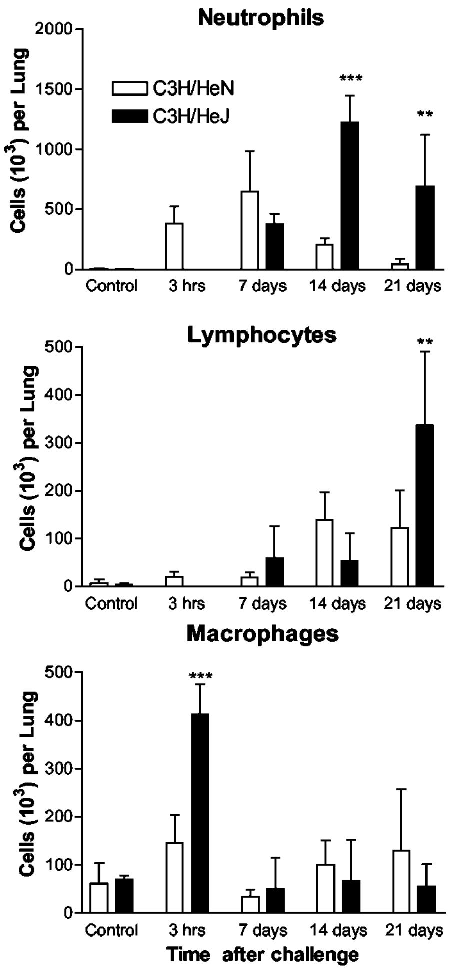

FIGURE 6. Enhanced cellular infiltrate in the lungs of TLR4-defective mice following *B. pertussis* respiratory challenge. BAL fluid was recovered from mice 3 h, or 7, 14, or 21 days after *B. pertussis* challenge, and assessed for cellular composition by microscopic examination of Diff-Quik-stained cytospins. Results are means (±SD) for four mice per group at each time point. **, $p < 0.01$; ***, $p < 0.001$ vs C3H/HeN. Results are representative of two experiments.

Enhanced Ab responses in C3H/HeJ mice infected with B. pertussis

We analyzed *B. pertussis*-specific Abs in the sera of *B. pertussis*-infected C3H/HeN and C3H/HeJ mice. *B. pertussis*-specific IgG2a was detected 14 days after challenge and reached a peak at day 21 in C3H/HeN mice (Fig. 10). IgG1 Abs were only detectable from day 21, and the titers were up to 10-fold lower than IgG2a. Ab responses were not compromised in TLR4-defective mice; higher titers of *B. pertussis*-specific IgG1, IgG2a, and IgG2b were detected in the C3H/HeJ strain when compared with the C3H/HeN strain (Fig. 10).

Discussion

The significant finding of this study is that signaling through TLR4 in response to *B. pertussis* activates IL-10 production from DCs and macrophages, which promotes IL-10-producing T cells and controls inflammatory pathology during acute infection of the lungs. Although IL-12 and other inflammatory cytokines were also induced by *B. pertussis* via TLR4, induction of Abs and Th1 responses were not compromised in TLR4-defective mice, suggesting that IL-10 had a dominant effect in vivo during acute infection.

Defective innate immune responses to bacterial molecules, including LPS, and increased sensitivity to infection have been reported in mice defective in TLRs (21–23). TLR4 plays a crucial role in activation of DCs and macrophages by LPS from *E. coli* and *Salmonella*. However, recent studies indicate that structurally distinct LPS from *Leptospira interrogans* (4) and *P. gingivalis* (3) use TLR2 for innate cell activation. In this study, we report that LPS from *B. pertussis* stimulates proinflammatory cytokine and chemokine production from DCs and macrophages and maturation of DCs through TLR4. Although TLR4 appears to be critical for *B. pertussis* LPS signaling in innate cells, our data suggest that other components of the bacteria stimulated DC maturation independently of TLR4. This finding is consistent with a recent report demonstrating that TLR4 is not required for full maturation of DCs by *Salmonella typhimurium* (24).

The defective innate response to *B. pertussis* in cells from TLR4-defective mice in vitro was reflected in an impaired inflammatory cytokine response in the lungs of C3H/HeJ mice soon after *B. pertussis* infection. The transient peak of IL-1β, TNF-α, MIP-1α, and MIP-2 observed in C3H/HeN mice within 3 h of *B. pertussis* challenge was not detected in the C3H/HeJ mice, demonstrating that the initial inflammatory response is much reduced in these mice. These inflammatory mediators play important roles in leukocyte recruitment in the protective cellular response to bacterial infections (25–27). *B. pertussis* can be taken up by macrophages and neutrophils, and it has been firmly established that Th1 cells can mediate protective cellular immunity to *B. pertussis* (15, 16). Rapid infiltration of neutrophils into the lungs was demonstrated following *B. pertussis* challenge of naive BALB/c (27) and C3H/HeN mice (present study), but this early cellular influx was not observed in TLR4-defective mice. Surprisingly, we observed a greater initial recruitment of macrophages in the C3H/HeJ mice, despite the lower levels of inflammatory chemokines in these mice early after challenge. It is possible that a mediator of macrophage recruitment is not affected by the TLR4 defect.

Consistent with the defective innate inflammatory cytokine and chemokine production soon after infection, we observed an increased bacterial burden in the TLR4-defective mice. Delayed clearance of respiratory infections such as *Haemophilus influenzae*, *Mycobacterium tuberculosis*, and respiratory syncytial virus has also been demonstrated in the C3H/HeJ mice (21–23), and in the case of the bacterial infections, this was attributed to defective innate immunity, in particular inflammatory cytokine production and neutrophil or macrophage recruitment (22, 23). However, the enhanced *B. pertussis* infection in the TLR4-defective mice was not associated with reduced inflammatory cell infiltrate into the lungs or a defect in the adaptive immune response. On the contrary, cellular inflammation and IL-1β, IL-6, IFN-γ, and MIP-1α production in the lungs were enhanced in the later part of the infection and were associated with a higher bacterial load in the TLR4-defective mice. In addition, serum Abs and *B. pertussis*-specific IFN-γ production by spleen cells were enhanced in the C3H/HeJ mice at the peak of infection. This may reflect a higher bacterial burden, but this is unlikely as IFN-γ and Ab responses are normally suppressed at the acute stage of *B. pertussis* infection (15, 19). Alternatively, the enhanced adaptive immune responses in C3H/HeJ mice may reflect the relative lack of Tr cells. Addition of anti-IL-10 in vitro enhanced IFN-γ production by spleen cells from *B. pertussis*-infected C3H/HeN mice. Furthermore, Ag-specific IFN-γ production was significantly higher and lung infection more severe in IL-10-defective (IL-$10^{-/-}$) mice (unpublished observations), providing support for our conclusion that exacerbated infection was related to the reduced innate and Ag-specific IL-10 production and as a consequence enhanced Th1 and inflammatory

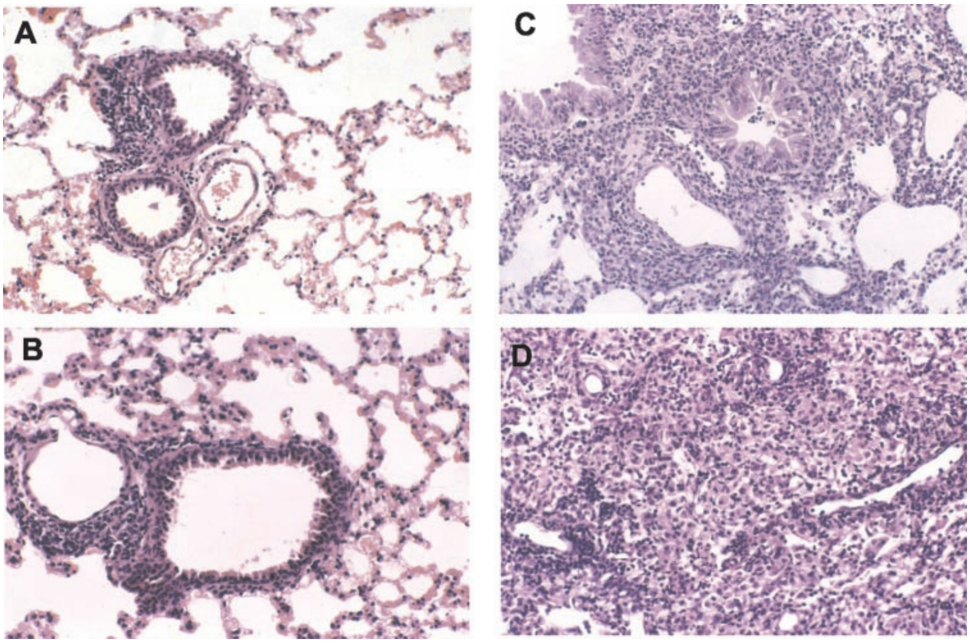

FIGURE 7. Enhanced pathological changes in the lungs of TLR4-defective mice infected with *B. pertussis*. *A*, C3H/HeN mouse, 14 days post-*B. pertussis* infection. Small numbers of lymphocytes in peribronchiolar and perivascular spaces. Alveoli appear normal. *B*, C3H/HeN mouse, 21 days postinfection. Mild peribronchiolar and perivascular lymphocte accumulations. *C*, C3H/HeJ mouse, 14 days postinfection. Perivascular neutrophil accumulations and hyperplasia of bronchiolar epithelium. Alveolar walls are thickened, and neutrophils and macrophages are present in alveolar spaces. Alveoli appear normal. *D*, C3H/HeJ mouse, 21 days postinfection. Intra-alveolar macrophages and neutrophils, and thickening of alveolar walls. Mild lymphocyte accumulations in perivascular areas (H&E, original magnification ×200).

responses. Although we did not detect major differences in IFN-γ or IL-10 in lung tissue, we did find significantly higher IL-12p70, IL-1β, IL-6, and MIP-1α concentrations in the lungs of C3H/HeJ compared with C3H/HeN mice 14 days after challenge. These cytokines and chemokines play a major role in inflammatory responses in the tissues and are enhanced by Th1 cell-derived IFN-γ or inhibited by IL-10. However, T cell cytokine production is more readily detected ex vivo in peripheral lymphoid organs rather than in the lungs (19).

Pathological examination of the lungs revealed a dense peribronchiolar infiltrate of neutrophils and lymphocytes in C3H/HeJ mice 14 days after challenge with *B. pertussis*. The intra-alveolar fibrin deposits observed are also indicative of severe damage in the lungs, and this severe bronchopneumonia was still evident in the TLR4-defective strain 21 days postinfection. In contrast, the C3H/HeN mice only showed mild lymphoid peribronchiolar cuffing on day 14 of infection. The exacerbated inflammation and lung pathology were associated with decreased Ag-specific IL-10 and normal or increased IFN-γ in the TLR4-defective mice. Although a recent report has suggested that TLR4 is required for optimal development of Th2 responses in an airway inflammation model (28), much of the reports to date have suggested that binding of pathogen-derived molecules to TLRs, including *E. coli* LPS binding to TLR4, enhances IL-12 production, leading to the selective activation of Th1 cells (1, 7–9). Consistent with these reports, we found that *B. pertussis* bacteria or LPS-stimulated IL-12 production was reduced in TLR4-defective mice. However, we have also demonstrated TLR4-mediated IL-10 production by both macrophages and DCs in response to *B. pertussis* LPS. LPS from *P. gingivalis*, which signals through TLR2, has been shown to enhance induction of Th2 cells, although IL-12, but not IL-4 or IL-10, production was stimulated in splenic DCs (3).

It has recently been reported that the low calcium response V Ag virulence factor from *Yersinia* can trigger IL-10 secretion from macrophages through CD14/TLR2 (29). TLR2-deficient mice were less susceptible to oral infection with *Yersinia enterocolitica*, suggesting that the bacteria exploit host innate PRRs to evade protective immune responses. It has been suggested that TLR ligands may induce delayed production of IL-10 by macrophages as a negative feedback to control inflammatory responses (30). Our study is in agreement with this suggestion; *B. pertussis* induced TNF-α and IL-12 secretion by DCs within 2–4 h of stimulation, whereas IL-10 was detectable after 8 h and peaked at 24 h. However, our data also suggest that reduced IL-10 production as result of defective TLR4 signaling has a more profound effect on the inflammatory response at the peak of infection and the bacterial burden than the reduction in TLR4-mediated IL-12. The course of infection and adaptive immune responses are similar in IL-12-defective and wild-type mice (unpublished observations). Studies with anti-IL-10 Abs suggested that IL-12 production by innate cells in vitro is tightly regulated by IL-10 (13).

We had previously demonstrated that FHA from *B. pertussis* stimulates IL-10 production by DCs and macrophages and promotes the development of Tr1 cells (13, 14). We also found that FHA enhances LPS-activated IL-10 from macrophages and DCs (unpublished observations). Thus, we propose that in normal C3H/HeN or BALB/c mice, LPS signaling through TLR4, and in cooperation with FHA, drives the production of IL-10, which transiently inhibits IL-12 and Th1 responses and promotes the development of Tr1 cells. We observed high levels of *B. pertussis*-specific IL-10 and modest levels of IFN-γ, but undetectable IL-4 in C3H/HeN mice 14–21 days after challenge with *B. pertussis*. In contrast, Ag-specific IL-10 was significantly lower and IFN-γ higher in TLR4-defective mice at the peak of infection. The cytokine profile of Ag-specific T cell lines confirmed the presence of both Th1- and Tr1-type cells in the *B. pertussis*-infected C3H/HeN mice, but almost exclusively Th1 type in the C3H/HeJ mice. We have previously reported that Ag-specific Tr1 cells can be isolated

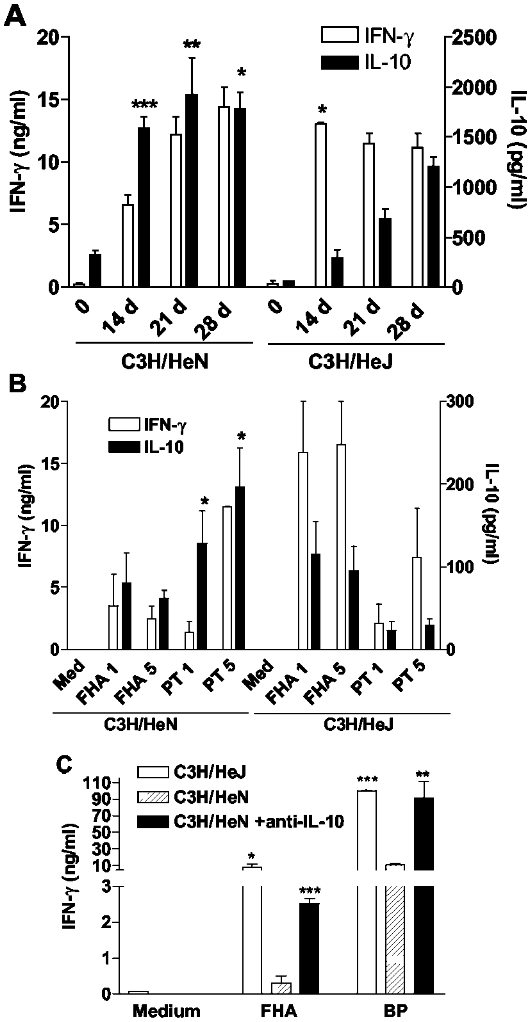

FIGURE 8. Reduced Ag-specific IL-10-producing T cells in TLR4-defective mice infected with *B. pertussis*. *A*, Spleen cells prepared from *B. pertussis*-infected C3H/HeN and C3H/HeJ mice 0–28 days postchallenge with *B. pertussis* were stimulated with Ag (1×10^5 killed *B. pertussis*/ml). Supernatants were removed after 3 days and tested for IFN-γ and IL-10 by immunoassay. Responses of cells to medium only were negative and are not shown. *B*, Spleen cells from C3H/HeN and C3H/HeJ mice 14 days after challenge with *B. pertussis* were stimulated with FHA or PT (1 or 5 μg/ml) or medium only; supernatants were removed after 3 days and tested for IFN-γ and IL-10. *C*, Spleen cells from C3H/HeN and C3H/HeJ mice 14 days after challenge with *B. pertussis* were stimulated with FHA (1 μg/ml) or killed *B. pertussis* (BP; 1×10^5 killed *B. pertussis*/ml) or medium only. Spleen cells from C3H/HeN mice were also cultured with Ags in the presence of anti-IL-10 (10 μg/ml). Supernatants were removed after 3 days and tested for IFN-γ. Results are mean (±SD) values for four mice per group and are representative of three experiments. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.001$ C3H/HeN vs C3H/HeJ or C3H/HeN + anti-IL-10.

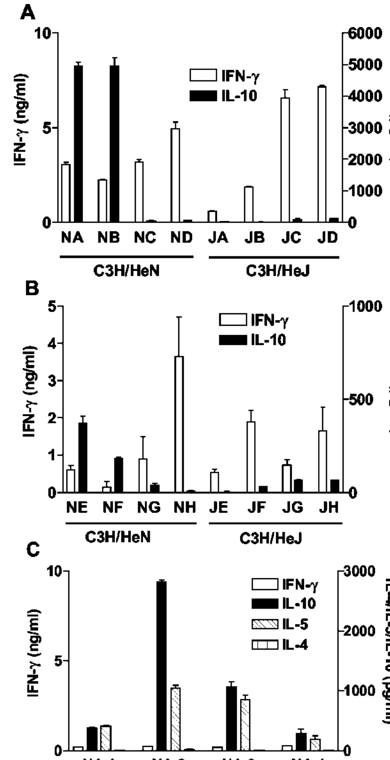

FIGURE 9. $CD4^+$ Tr1 cell lines and clones established from C3H/HeN mice. FHA-specific $CD4^+$ T cell lines were established from spleens of individual C3H/HeN and C3H/HeJ mice 14 (*A*) or 21 (*B*) days after challenge with *B. pertussis*. *C*, T cell clones were established from T cell line NA generated from a C3H/HeN mouse 14 days after *B. pertussis* challenge. T cell lines or clones were stimulated with APC + Ag and IFN-γ, IL-4, IL-5, and IL-10 tested in supernatants 3 days later. Results are mean values for triplicate cultures.

from BALB/c mice during acute infection with *B. pertussis*, and that these cells can suppress Th1 responses (14). Furthermore, we have generated Ag-specific Tr1 cell lines and clones from mice immunized with Ag in the presence of an adjuvant that enhances LPS-driven IL-10 production.[4] The Tr1-like cell lines had a cytokine profile similar to those generated from C3H/HeN mice in the present study, and were also found to suppress IFN-γ production by Th1 cells. Our findings suggest that innate IL-10 promotes the activation of IL-10-secreting Tr1 cells during acute infection of normal, but not TLR4-defective mice, and that these cells function to inhibit Th1 responses and limit immunopathology. This is consistent with recent reports demonstrating that $CD4^+$ $CD25^+$ Tr cells may also regulate pathogen-induced inflammation. $CD4^+$ $CD25^+$ T cells from *Helicobacter hepaticus*-infected mice block colitis where disease is induced by the transfer of *H. hepaticus*-specific T cells from IL-$10^{-/-}$ mice into $RAG^{-/-}$ mice (31). The suppression was mediated by IL-10 and was attributed to *H. hepaticus*-induced Tr cells. Furthermore, adoptive transfer of $CD4^+CD25^+$ T cells delays bacterial clearance in *Pneumocystis carinii*-infected mice, but also prevents development of lethal pneumonia by $CD4^+CD25^-$ T cells (32).

Previous studies have indicated that the induction of IL-10 by certain pathogens may represent an evasion strategy to inhibit protective Th1 responses. IL-$10^{-/-}$ mice are more resistant than wild-type mice to *Yersinia* (33) and *Mycobacterium bovis* BCG (34) infection, through enhanced TNF-α or cell-mediated immunity. However, it has also been demonstrated that the severity of diseases and inflammation, but not the bacterial burden, is exacerbated in IL-10-defective (IL-$10^{-/-}$) mice infected with *Listeria monocytogenes*, *E. coli*, or *H. hepaticus* (35–37). We observed increased bacterial load in *B. pertussis*-infected IL-$10^{-/-}$ mice, despite the enhanced IgG2a and Th1 responses at the peak of infection (unpublished observations). Our findings reveal that IL-10 is produced by cells of the innate immune system through TLR4, and as a consequence Tr cells are activated and this may serve as

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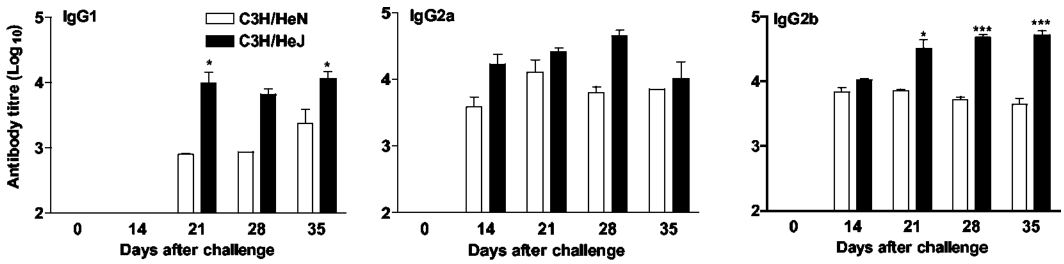

FIGURE 10. Enhanced Ab responses in TLR4-defective mice infected with *B. pertussis*. Ab titers in the sera of *B. pertussis*-infected C3H/HeN and C3H/HeJ strains were analyzed by ELISA 0–35 days after challenge. Results are the mean ± SD of subclass Ab titers for four mice per group, tested individually in triplicate. *, $p < 0.05$; ***, $p < 0.001$ vs C3H/HeN. Results are representative of two experiments.

a protective strategy adopted by the host to limit collateral damage mediated by pathogen-stimulated inflammatory responses.

Acknowledgments

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