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Human Monocytes Infected with *Yersinia pestis* Express Cell Surface TLR9 and Differentiate into Dendritic Cells

Kamal U. Saikh, Teri L. Kissner, Afroz Sultana, Gordon Ruthel, and Robert G. Ulrich

TLR9 recognizes DNA sequences containing hypomethylated CpG motifs and is a component of the innate immune system highly conserved during eukaryotic evolution. Previous reports suggested that the expression of TLR9 is restricted to plasmacytoid dendritic cells and B lymphocytes. Our results indicate that low levels of TLR9 are present on the cell surface of freshly isolated human monocytes, and expression is greatly increased by infection with *Yersinia pestis*. Enhanced cell surface TLR9 coincided with elevated levels of cytoplasmic TLR9 and recruitment of MyD88. Infected monocytes differentiated into mature dendritic cells, expressed IFN-α, and stimulated proliferative and cytotoxic T cell responses specific to *Y. pestis*. Furthermore, uninfected B cells and monocytes both increased cell surface TLR9, CD86, and HLA-DR in response to treatment with CpG-containing oligonucleotides, whereas cell surface TLR9 was down-modulated on infected dendritic cells by the addition of agonist oligonucleotide. Our results suggest that increased expression of TLR9 on the surface of infected cells may serve as a role as an activation signal to other cells of the immune system. *The Journal of Immunology*, 2004, 173: 7426–7434.
are important scavengers and hosts for intracellular infection with *Y. pestis*, they are also an abundant source of precursors for macrophages and DCs. Our results indicate that human monocytes express TLR9 and respond to infection by elevating cytoplasmic and cell surface levels of this innate immune receptor. In addition, cell surface TLR9 expression may function as a proinflammatory activation marker during the transition from monocyte to DCs.

**Materials and Methods**

**Reagents**

Pooled human AB sera were obtained from Pel-Freez Biologicals (Brown Deer, WI). Mouse anti-human CD14, CD19, and CD3 mAbs conjugated with magnetic beads were purchased from Miltenyi Biotec (Auburn, CA). The FITC-conjugated mAbs Leu M3 (anti-CD14), Leu HLA-DR (anti-DR), anti-human CD19, anti-CCR7, and Ig isotype control Abs were purchased from BD Biosciences (San Jose, CA). Goat anti-mouse IgG, FITC labeled, was purchased from Zymed Laboratories (San Francisco, CA). PE-conjugated anti-CD83 and FITC-conjugated anti-CD86 were purchased from BD Pharmingen (San Diego, CA). Primary mAbs for TLR9, TLR2, and TLR4 were obtained from Active Motif (Carlsbad, CA) or from eBiosciences (San Diego, CA). Anti-MyD88 Ab was obtained from NeoMarkers (Fremont, CA). IL-15 was purchased from PeproTech (Rocky Hill, NJ). Ficoll-Hypaque was purchased from Pharmacia (Uppsala, Sweden). CpG-NT 10103 is a previously reported (22) type B CpG oligonucleotide specifically optimized for vaccine applications and kindly provided by C. Roy (U.S. Army Medical Research Institute of Infectious Diseases). Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Burlingame, CA). Monoclonal (YPF19) mouse anti-F1 Ag *Y. pestis* was obtained from Research Diagnostics (Flanders, NJ).

**Cell isolation and culture**

PBMC were obtained from consenting healthy donors. Blood components for in vitro research use were collected from healthy, consented volunteers in accordance with the guidelines of an Institutional Review Board-approved research donor protocol. Mononuclear cells were isolated by standard density gradient centrifugation with Ficoll-Hypaque (Pharmacia). Mononuclear cells were harvested from the interface, washed, and suspended in RPMI 1640 medium. Monocytes (CD14⁺) and B cells (CD19⁺) were isolated, as previously described (23). Briefly, unseparated mononuclear cells were suspended (10⁷ cells/80 µl) in cold PBS supplemented with 2 mM EDTA and 0.5% BSA (fraction V; Sigma-Aldrich, St. Louis, MO). Paramagnetic beads
coated with anti-CD3 or anti-CD19 mAbs (Miltenyi Biotec) were mixed with the mononuclear cells (10^7 cells/20 µl). The Ab-labeled cells were incubated for 15 min (4°C), washed, and passed through a type LS or MS iron-fiber column placed within a strong magnetic field (Miltenyi Biotec). CD19^+ B cells bound to the column were eluted with buffer. Monocytes were isolated from cells that were not bound to the anti-CD3 column, using anti-CD14 mAb, followed by separation in a magnetic column (as above). CD14^+ monocytes were incubated 48 h with 100 ng/ml rIL-15 (Pepro-Tech) to prepare DCs, as previously described (23). Cell purities were >98% for isolated mononuclear cell subsets used in all reported experiments. Although representative results are presented, all experiments were performed at least three times.

**Bacterial strain and growth conditions**

The attenuated *Y. pestis* strain CO92, Pgm^-, Pla^+, was previously described (24). Bacteria were grown from a single colony in heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with 0.2% xylose and 2.5 mM CaCl2. The cultures were grown for 12 h at 37°C, and bacteria were pelleted by centrifugation and washed with RPMI 1640 medium before use in experiments.

**Infection of monocytes, intracellular staining, and confocal microscopy**

Freshly isolated CD14^+ monocytes were allowed to adhere to sterile glass coverslips in a 6-well cell culture plate (Corning Glass, Corning, NY) and were then incubated (1 h, 37°C) with Y. pestis suspension in RPMI 1640 medium. Cells were gently washed with PBS (pH 7.4, 37°C) to remove nonadherent cells and bacteria, and were fixed with 1% paraformaldehyde (Tousimis Research, Rockville, MD), 0.1% glutaraldehyde (Sigma-Aldrich) in PBS (5 min, 20°C). The fixed cells were washed with PBS containing 0.5% BSA and incubated (30 min, 20°C) in a permeabilization solution (BD Biosciences). Cells were then washed and incubated (15 min, 20°C) with PBS containing 5% BSA to block nonspecific Ab binding. Cells were incubated with labeled primary anti-F1 Ab for 1 h, washed (PBS, 0.5% BSA), followed by FITC-conjugated goat anti-mouse secondary Ab, and counterstained with DAPI to detect bacteria within the monocytes. Cells were mounted by inverting the coverslips on a slide, and labeled cells were imaged using a Bio-Rad (Hemel Hempstead, U.K.) Radiance 2000 MP laser scanning confocal system connected to a Nikon (Melville, NY) TE 300 inverted microscope.

**FIGURE 3.** Proinflammatory cytokine production by *Y. pestis*-infected monocytes. Monocytes (CD14^+) were infected with *Y. pestis* (50 bacteria/cell) for 1 h (37°C), washed, and cultured (24 h in RPMI 1640, 5% human AB serum). Cells were harvested, and culture supernatants were tested for cytokines. Results shown in the figure were from one representative experiment of three, using separate donors.

**FIGURE 4.** Monocytes and DCs infected with *Y. pestis* activate Ag-specific T cells. Monocytes (CD14^+) or mature DCs (HLA-A2) were infected with *Y. pestis*. Gentamicin (10 µg/ml) was added to cultures after 2 h of infection. Monocytes or DCs were cultured for 26 h and washed extensively to remove extracellular bacteria before adding autologous T cells to all cultures. Primary T cell cultures were then collected after 6 days of incubation. Proliferation to purified recombinant *Yersinia* F1 Ag presented by fresh autologous DCs was measured by measuring ATP remaining in metabolically active cells after CTL killing, followed by subtraction of experimental from maximum lysis of target cells. The data represent stimulation index or percent cytotoxicity ± SE of means of triplicate wells. Statistical analyses of proliferation of T cells (cpm) without or with F1 Ag (1, 10, and 100 µg/ml), the two-tailed p value from Student’s t test equals 0.0103, 0.0001, and 0.08, respectively. Statistical analyses of percent of cytotoxicity, i.e., cytolysis of target cells pulsed without or with F1 Ag at target:effector 1:1, 1:5, and 1:10 were all statistically significant, the two-tailed p value from Student’s t test <0.05. Results presented in the figure were representative of three experiments and three separate donors.
FIGURE 5. Increased expression of TLR9 and MyD88 in human monocytes infected with *Y. pestis*. A, Differential cell surface expression of CD14 and TLRs on infected or control monocytes treated with CpG NT. Monocytes (CD14^-/H11001^/) were infected with *Y. pestis* (50 bacteria/cell) for 1 h at 37°C, washed, and cultured for 48 h in RPMI 1640 containing 5% human AB serum. The monocytes were harvested and labeled with PE-conjugated anti-CD14, or alternatively, cells were labeled with anti-TLR2, anti-TLR4, or anti-TLR9 mAbs, followed by secondary labeling with an FITC-conjugated goat anti-mouse Ab, and cell surface expression was measured by flow cytometry. The thin line represents histogram for isotype-matched control Ab. The data shown are from a representative experiment (total of four experiments with different donors). B, Intracellular expression of TLR9 and MyD88 within infected monocytes. Monocytes (CD14^-/H11001^/) were infected with *Y. pestis* (10 bacteria per cell) for 1 h, noninternalized bacteria were removed by washing, and infected cells were cultured for 24 h. Cells were fixed, permeabilized, and labeled with primary anti-TLR9 Ab and primary anti-MyD88 Ab, followed by appropriate FITC- or PE-conjugated Ab. Cells were covered with mounting fluid containing DAPI for DNA staining, and examined using confocal microscopy. Bar = 10 μM. The data shown are from a representative experiment (total of three experiments, separate donors).
The capsular F1 protein of *Y. pestis* is a major protective Ag, and immunity to F1 Ag confers protection from plague infection by either the s.c. or respiratory route of exposure (26). We cocultured *Y. pestis*-infected monocytes or DCs with autologous T cells to evaluate *Yersinia*-specific T cell responses. T cells primed in vitro were isolated and tested with F1 Ag in proliferation and cytotoxicity assays. Our results showed that *Y. pestis*-infected DCs induced F1-specific T cell proliferation (Fig. 4). In addition to Th responses, monocytes and DCs infected with *Y. pestis* stimulated F1 Ag-specific CTL responses (Fig. 4). These results

**Cytokine analysis**

Cytokines in culture supernatants were measured using BD Cytometric Bead Array kit (BD Pharmingen) using capture beads coated with Abs specific for cytokines and flow cytometry analysis, according to the manufacturer’s method. Briefly, the cytokine standards were reconstituted in the assay dilution buffer for 15 min, and then were diluted by serial dilution from 5 to 5000 pg/ml. The cytokine capture beads were mixed together (10 µl/test) and then transferred, 50 µl of mixed beads to each assay tube. The PE detection reagent was added next to each assay tube (50 µl/test). The standard dilutions and test samples were added to the appropriate sample tubes (50 µl/test). The capture beads, PE-conjugated detection Abs, which form a sandwich complex, and recombinant standards or test samples were incubated together for 3 h at room temperature and protected from the light. After the 3-h incubation, the samples were washed with 1 ml of wash buffer and centrifuged for 10 min at ~200 × g. The supernatant was decanted, and 300 µl of wash buffer was added to each assay tube for analysis. After sample data acquisition by the BD FACsCalibur flow cytometer, the sample results were plotted in graphical and tabular format by the BD Cytometric Bead Array analysis software.

**Results**

**CD14+ monocytes differentiate into DCs in response to *Y. pestis* infection**

Resting monocytes were selected for our study from peripheral blood of volunteers, based on expression of the LPS-binding receptor CD14. Fresh cultures of *Y. pestis*, grown at 37°C under optimal conditions for production of bacterial capsules and most virulence factors, were washed in cell culture medium and mixed with monocytes. Bacteria labeled with an Ab reacting with the capsular protein F1 were clearly visible within the cytoplasm of infected monocytes by 1 h of culture (Fig. 1), and we observed a consistently high level of monocytes infected with bacteria (>87% average). The infected monocytes remained viable after 48 h of infection, and acquired a DC-like morphology, exhibited a loss of CD14, and increased cell surface expression of HLA-DR, CD86, and the DC maturation markers CD83 and CCR7 (Figs. 2A and 5A). These phenotypic changes indicated that infection resulted in differentiation of the monocytes into DCs. The *Y. pestis*-infected monocytes also expressed IFN-α (Fig. 2B). Significant levels of TNF-α, IL-1β, and IL-6 were produced by monocytes infected for 24 h (Fig. 3), suggesting a possible contribution of proinflammatory cytokines to maturation of the infected monocytes to DCs.

**T cell response to protective Ags from *Y. pestis* presented by infected monocytes and DCs**

The capsular F1 protein of *Y. pestis* is a major protective Ag, and immunity to F1 Ag confers protection from plague infection by either the s.c. or respiratory route of exposure (26). We cocultured *Y. pestis*-infected monocytes or DCs with autologous T cells to evaluate *Yersinia*-specific T cell responses. T cells primed in vitro were isolated and tested with F1 Ag in proliferation and cytotoxicity assays. Our results showed that *Y. pestis*-infected DCs induced F1-specific T cell proliferation (Fig. 4). In addition to Th responses, monocytes and DCs infected with *Y. pestis* stimulated F1 Ag-specific CTL responses (Fig. 4). These results

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**FIGURE 6.** Similar regulation of cell surface molecules on human monocytes and B cells responding to TLR9 agonist. A. Monocytes, CD14 and TLR9- B cells, CD19 and TLR9. Live cells were incubated with fluorochrome-conjugated Abs, fixed, and then analyzed by flow cytometry. B. Activation of B cells by TLR9 agonists. Cell surface expression of critical markers on B cells (CD19) or monocytes (CD14) treated with CpG NT. Cell surface receptors were labeled with FITC-conjugated mAbs specific to each Ag. Thick lines represent cell surface expression of Ags, and thin lines represent histogram for isotype-matched controls. Results are from a representative experiment (total of three experiments).
indicated that DCs derived from infected monocytes were functionally equivalent to DCs infected after maturation from cultured monocytes.

**Increased cell surface expression of TLR9 on monocytes infected with Y. pestis**

Infection of monocytes with *Y. pestis* resulted in reduced cell surface CD14, whereas levels of CD14 on cells treated only with CpG NT remained similar to untreated control cultures (Fig. 5A). Because molecular signatures of pathogens are recognized by TLRs, which in turn activate DCs, we next examined changes in expression of TLR9 on monocytes from multiple donors. Primary monocytes were infected for 48 h with *Y. pestis* and examined by flow cytometry. Infection with *Y. pestis* increased cell surface TLR9, whereas no change was observed for expression of TLR2 or TLR4 compared with levels in untreated cells (Fig. 5A). Similarly, treatment with CpG NT, containing motifs optimal for human biological activity, increased cell surface expression of TLR9. Adding CpG NT agonists of TLR9 to infected monocytes decreased cell surface TLR9 (Fig. 5A). A significantly increased colocalization of MyD88 with TLR9 was observed in the cytoplasm of cells harboring intracellular bacteria (Fig. 5B), whereas most uninfected monocytes retained a low, but detectable, level of TLR9 with little colocalization of MyD88. We also noted that levels of intracellular MyD88 were quite high compared with the noninfected controls (Fig. 5B), suggesting that neoexpression of the adaptor protein may be essential for the TLR9-mediated response to infection. These results indicated that TLR9 signaling was activated by infection and coincided with DC differentiation.

**Freshly isolated CD14+ monocytes express TLR9**

Because our results showed a significant increase in cell surface expression of TLR9 with *Y. pestis* infection, this prompted us to examine expression on freshly isolated human blood cells. By flow cytometry, cell surface TLR9 was observed on 5–7% of the CD14+ monocytes and B cells (Fig. 6A), varying from donor to donor (n = 16). Similar results were obtained with two independent mAbs against human TLR9 (data not shown). These results confirmed that freshly isolated monocytes are poised to respond to TLR9 ligands.

**B cells and monocytes exhibit similar regulation of TLR9 surface expression**

As expression of TLR9 is a prerequisite for CpG NT responsiveness in B cells, we next examined TLR9 in primary cultures of B (CD19+) lymphocytes, depleted of monocytes. A low level of TLR9 expression was observed by flow cytometry (Fig. 6), which appeared to be greater than that displayed by monocytes (Fig. 5A). We observed a dramatic increase in CD86 and HLA-DR, and a more modest increase in CD83 expression, in B cells responding to TLR9 agonist treatment (Fig. 6). Like monocytes, the change in surface markers in response to CpG NT was dose dependent (data not shown). Also similar to the results obtained with monocytes (Fig. 3), B cells treated with CpG NT (specific for human receptor) exhibited a significant increase in cell surface TLR9 (Fig. 6B). Because the CpG NT-induced increase in CD86 expression by B cells was so dramatic, we re-examined expression of this costimulatory molecule in monocytes (Fig. 6B). An increase in CD86 was observed in CD14+ cells treated with CpG NT, although the level was somewhat less than that obtained with B cells. These data also indicate that increased TLR9 cell surface expression was not dependent on phagocytosis. Collectively, our results suggest that agonist-induced changes in expression of costimulatory molecules and TLR9 are similar in monocytes and B lymphocytes.

**Discussion**

A thorough understanding of the initial interactions between host and pathogen is essential for devising methods of controlling the high mortality associated with *Y. pestis* infections. Our results demonstrate that human monocytes infected with *Y. pestis* differentiate into DCs, suggesting that adaptive immunity may still function during the intracellular stage of plague. Primary helper and CTLs were activated by monocytes matured to DCs by infection and by myeloid-derived DCs that were directly infected with *Y. pestis*. The capsular F1 protein, which is a major protective Ag and vaccine component (27), was recognized by lymphocytes responding to infected monocytes. Activation of cells expressing TLR9 by hypomethylated CpG NT motifs present in bacterial DNA stimulates a rapid inflammatory response (28). Our results also indicate that *Y. pestis* infection activates the TLR9 signal transduction pathway, resulting in a significant increase in expression of TLR9, both intracellularly and at the cell surface. Expression of intracellular MyD88 was also activated by infection, suggesting that increased amounts of this adaptor may be necessary to accommodate the elevated levels of TLR9 resulting from the host immune response to infection. Phagocytic internalization of bacteria is also strongly promoted by TLR9 (29). In addition, we conclude that both monocytes and myeloid DCs express functional TLR9, a property previously thought to be restricted to B cells and plasmacytoid DCs.

The recruitment of MyD88 to endosomes containing TLR9 confirmed that these receptors of innate immunity were functional in *Y. pestis*-infected monocytes. All TLR9-mediated responses are dependent on MyD88 (30), and colocalization of TLR9, MyD88, and CpG NT in late endosomes is necessary for signaling (17, 28). The colocalization of agonist, adaptor, and receptor was reported to be inhibited by wortmannin, suggesting a critical involvement of PI3K (31). Agonists of TLR9 bind to the plasma membrane (32), while other reports suggest that CpG NT-mediated signaling occurs in acidified endocytic vesicles (33, 34). The precise cellular mechanism delivering TLR9 to the cell surface is presently not known. Furthermore, it is possible that TLR9 turnover from cytoplasm to the cell surface is not critical to function and that signal transduction only occurs in endosomes. Macrophage phagosomes are formed by direct association and fusion of endoplasmic reticulum to the plasma membrane during early phagocytosis (35). Both endoplasmic reticulum and endosomes contribute elements to the phagosome membrane (35), bringing proteins from these intracellular compartments to the cell surface during phagocytosis. Therefore, it is possible that phagocytosis may cause TLR9 to cycle between endosomal and plasma membranes. However, phagocytosis of inert beads by monocytes did not result in activation of TLR9 expression or turnover, and a previous study of 293 cells transfected with a hemagglutinin-tagged TLR9 demonstrated receptor in the plasma membrane of these nonphagocytic cells (28). Taken together, these observations suggest a potential role for TLR9 in innate immune recognition of both intracellular and extracellular PAMPs, perhaps serving an additional regulatory function in vivo. Interestingly, TLR2 can bind Ags at the cell surface (36) and thus contribute to CD4+ T cell recognition of cognate Ag (36). In a similar manner, the high expression level of TLR9 on infected monocytes compared with the reduced basal level found on resting
monocytes may facilitate processing of stimulatory PAMPs or other Ags and serve as a signal of infection to other cells of the immune system.

Although the host activation signals produced during bacterial infection are likely to be complex, previous studies have demonstrated an involvement of TLRs in differentiation of DCs (37–39). In addition, the recent observation that CpG NT serve as maturation signals for activated myeloid DCs (40) supports our conclusion that functional TLR9 is also expressed by nonplasmacytoid human DCs. Agonists of TLR9 clearly function as differentiation signals for B cells, which also express this receptor on the cell surface. The rapid increase in expression of TLR9 and TLR10 by naive B cells triggered by cross-linking of cell surface Ig allows them to proliferate in response to CpG NT (41). In contrast, memory B cells express high levels of most TLRs, proliferate directly in response to CpG NT, and differentiate into Ig-secreting cells. Proliferation or secretion of IgG, IgM, IL-10, and IL-6 by CpG NT-treated B cells required costimulation by CD40L (42). Our results and previous reports (41) demonstrated increased cell surface expression of costimulatory molecules and HLA-DR on B cells activated with CpG NT. However, despite the many similarities between B cells and monocytes regarding TLR9 signaling, cell activation by CpG NT alone was not sufficient to drive differentiation of monocytes to DCs.

Many obligate and facultative intracellular bacteria are potent inducers of DC maturation in vitro (43). For example, maturation was reported to be induced by in vitro infection of human myeloid DCs with the intracellular organism Listeria monocytogenes (44). DC maturation during infection may be mediated by proinflammatory cytokines, for example TNF-α, for example TNF-α, which also express this receptor on the cell surface. The rapid increase in expression of TLR9 and TLR10 by naïve B cells triggered by cross-linking of cell surface Ig allows them to proliferate in response to CpG NT (41). In contrast, memory B cells express high levels of most TLRs, proliferate directly in response to CpG NT, and differentiate into Ig-secreting cells. Proliferation or secretion of IgG, IgM, IL-10, and IL-6 by CpG NT-treated B cells required costimulation by CD40L (42). Our results and previous reports (41) demonstrated increased cell surface expression of costimulatory molecules and HLA-DR on B cells activated with CpG NT. In a manner similar to our results with monocytes, cell surface TLR9 expression was also increased on B cells by treatment with CpG NT. However, despite the many similarities between B cells and monocytes regarding TLR9 signaling, cell activation by CpG NT alone was not sufficient to drive differentiation of monocytes to DCs.

Acknowledgment

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