MAJOR ARTICLE

Tetraacylated Lipopolysaccharide of *Yersinia pestis* Can Inhibit Multiple Toll-Like Receptor–Mediated Signaling Pathways in Human Dendritic Cells

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Background. *Yersinia pestis*, the causative agent of plague, showed a temperature-dependent change in lipid A composition, with a reduced degree of acylation when bacteria were grown at 37°C (tetraacylated) versus ambient temperature (hexaacylated).

Methods. Human monocytes and monocyte-derived dendritic cells (DCs) were exposed to *Y. pestis* grown at 26°C or 37°C, to their corresponding lipopolysaccharides (LPS-26°C or LPS-37°C), and to ligands of different Toll-like receptors (TLRs), such as LPS from *Escherichia coli* (TLR4), lipoprotein (TLR2), polyinosinic-polycytidylic acid (poly-IC) (TLR9), and their combinations. Production of cytokines was measured, along with expression of surface markers of DC maturation.

Results. *Y. pestis* grown at 37°C or LPS-37°C induced much lower production of cytokines (such as tumor necrosis factor α and interleukins 1β, 10, and 12) by DCs than did *Y. pestis* grown at 26°C or LPS-26°C. Expression of the surface markers HLA-DR, CD86, and CD40 by DCs was also reduced in response to treatment with LPS-37°C compared with LPS-26°C. Pretreatment of DCs with LPS-37°C inhibited subsequent stimulation with LPS-26°C, control LPS from *E. coli*, lipoprotein, or poly-IC.

Conclusions. LPS-37°C can inhibit stimulation of DCs not only via TLR4 signaling but also via TLR2 and TLR9.

Yersinia pestis, the agent of plague, alternates in nature between an ambient temperature within the flea arthropod vector and the mammalian host at 37°C. Plague bacilli can multiply in the midgut of the flea after the insect takes a blood meal from an infected animal and can be transmitted to a new host via the flea bites. Therefore, a temperature shift is one of the first environmental signals that *Y. pestis* encounters during the transmission process. In addition, colonization of the flea digestive tract by *Y. pestis* depends on the initial bacterial inoculum, and a high bacteremia level is a selective advantage for the transmission of the pathogen [1]. Interestingly, it was shown recently that lipid A of the lipopolysaccharide (LPS) molecule of *Y. pestis* differed in the degree of acylation, depending on the temperature at which *Y. pestis* was grown. The lipid A obtained from bacteria cultivated at ambient temperature was normally more acylated than that isolated from bacteria grown at 37°C. LPS from bacteria grown at 37°C (LPS-37°C) was shown to be a weaker stimulant of tumor necrosis factor α (TNF-α) in macrophages, and this difference in biological activity was more pronounced in human than in murine macrophages [2].

Because LPS is considered a major mediator of pathology, endotoxic shock, and death, a less toxic form of LPS might offer *Y. pestis* time to reach higher numbers, prolonging the survival of a bacteremic host. Thus, although the phenomenon of reduced acylation in LPS from *Y. pestis* at mammalian body temperature remains to be confirmed in vivo, the production of less acylated

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lipid A at 37°C can be beneficial for plague transmission. The increased acylation of lipid A has been shown to correlate with bacterial resistance to cationic antimicrobial peptides [3]. *Y. pestis* was significantly more resistant to these peptides when grown at ambient temperature [4–6] and less permeable to hydrophobic agents [7]. However, no direct correlation was found between the degree of LPS acylation and the survival of the plague microbe in the flea gut [8].

A temperature-dependent change in LPS composition of *Y. pestis* was first suspected because of an increase in the gel mobility of LPS-37°C on sodium dodecyl sulfate–polyacrylamide gel electrophoresis [9]. Currently, it is generally accepted that *Y. pestis* contains predominantly hexaacylated lipid A when grown at ambient temperature and predominantly tetraacylated lipid A when grown at 37°C [2, 4, 8, 10]. Comparison of the *Yersinia* lipid A structures did not reveal a significant difference in general architecture compared with enterobacteria [11]. Like LPS from many gram-negative microorganisms, the LPS from *Y. pestis* has properties typical of endotoxin [12–14], which include the ability to produce gelation of limulus lysate [15, 16] and mitogenic activity that could be inhibited by polymyxin B sulfate [15].

Immunization of mice with LPS isolated from *Y. pestis* grown either at 28°C or 37°C, which resulted in the development of LPS-specific antibody, failed to protect animals from subsequent challenge with *Y. pestis* [12, 16, 17]. LPS obtained from *Y. pestis* grown at ambient temperature acted similarly to *Escherichia coli* LPS with regard to inducing in vitro cytokine production, but LPS-37°C had significantly reduced potential for such induction [2, 4, 14, 16, 18]. The in vivo toxic activity levels for LPS from bacteria grown at different temperatures correlated with the findings relative to acylation; that is, LPS-37°C was less toxic [19, 20], although no direct connection was found between the lethal toxicity of the LPS in vivo and its ability to induce TNF-α in vitro [14].

Hexaacylated LPS from *Y. pestis* had potent Toll-like receptor 4 (TLR4)–activating ability. In contrast, tetraacylated lipid A of *Y. pestis* had a significantly weaker capacity to activate cells via TLR4 signaling. Moreover, the stimulatory activity of LPS obtained from bacteria grown at 26°C (LPS-26°C) was inhibited by LPS-37°C [18]. These observations support the hypothesis that *Y. pestis* has a pathogenic mechanism that involves downregulation of the innate immune response during the early stages of infection [21]. In fact, *Y. pestis* that produced the hexaacylated form of lipid A via the constitutively expressed acyltransferase *lpxL* gene of *E. coli* exhibited reduced virulence in a murine model of bubonic plague [18].

In the present study, we compared *Y. pestis* grown at 26°C with that grown at 37°C and purified LPS-26°C with LPS-37°C for their ability to activate human monocytes and monocyte-derived dendritic cells (DCs). We showed that bacteria grown at 37°C, as well as LPS from these bacteria, are defective in activating monocytes and DCs. More importantly, we demonstrated for the first time that LPS-37°C can inhibit stimulation of DCs not only via TLR4 signaling by LPS-26°C but also via TLR2 and TLR9 stimulation by lipoprotein and polyinosinic-polycytidylic acid (poly-IC).

METHODS

Bacteria and reagents. Wild-type strain *Y. pestis* CO92 [22] was cultivated with aeration in a liquid heart infusion broth (HIB) at either 26°C or 37°C. Fresh HIB was inoculated with overnight cultures, and bacteria continued to grow at a corresponding temperature until they reached an optical density read at 600 nm (OD_{600}) of 1 ($\sim 5 \times 10^8$ colony-forming units/mL). At that time, bacteria were harvested, washed 3 times in cold phosphate-buffered saline (PBS), adjusted to an OD_{600} of 1, and used in monocyte or DC cultures at a differing multiplicity of infection (MOI). The actual number of colony-forming units added to the human cells was determined by plating.

LPS from *Y. pestis* KM 218, a plasmidless and pigmentation-negative derivative of live plague vaccine strain EV, was evaluated previously for chemical structure by mass spectrometry and endotoxic activity in mice [14, 19, 23]. Ultrapure LPS from *E. coli* O55:B5 and poly-IC were purchased from Sigma. Lipoprotein was purified from *Yersinia enterocolitica* as described elsewhere [24, 25].

DC and monocyte isolation. Ethylenediaminetetraacetic acid–treated blood from healthy human donors was handled under endotoxin-free conditions and diluted 1:1 with PBS, and peripheral blood mononuclear cells (PBMCs) were purified by centrifugation over a Ficoll–sodium diatrizoate solution (Ficoll-Paque; Pharmacia Fine Chemicals). Monocytes were purified from PBMCs by negative selection, using the magnetic column separation system from StemCell Technologies as described elsewhere [26]. Monocyte-derived DCs were generated from purified $CD14^+$ monocytes as described elsewhere [27]. Briefly, monocytes were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine, HEPES, sodium pyruvate, antibiotics (culture medium) plus granulocyte-macrophage colony-stimulating factor (100 ng/mL), and interleukin 4 (IL-4) (50 ng/mL). Monocytes were set up in 24-well tissue-culture plates at 1×10^6 cells/mL. Nonadherent immature DCs were obtained at day 7 of culture, and for experiments we used only homogeneous DC populations, characterized by high levels of CDla (>99% positive and completely negative for other cell phenotypes) and no CD83 expression. Viability was determined by trypan blue exclusion, and cells were used only when viability exceeded 95%.

DC and monocyte cultures. Human monocytes and monocyte-derived DCs were exposed to varying MOIs of *Y. pestis*

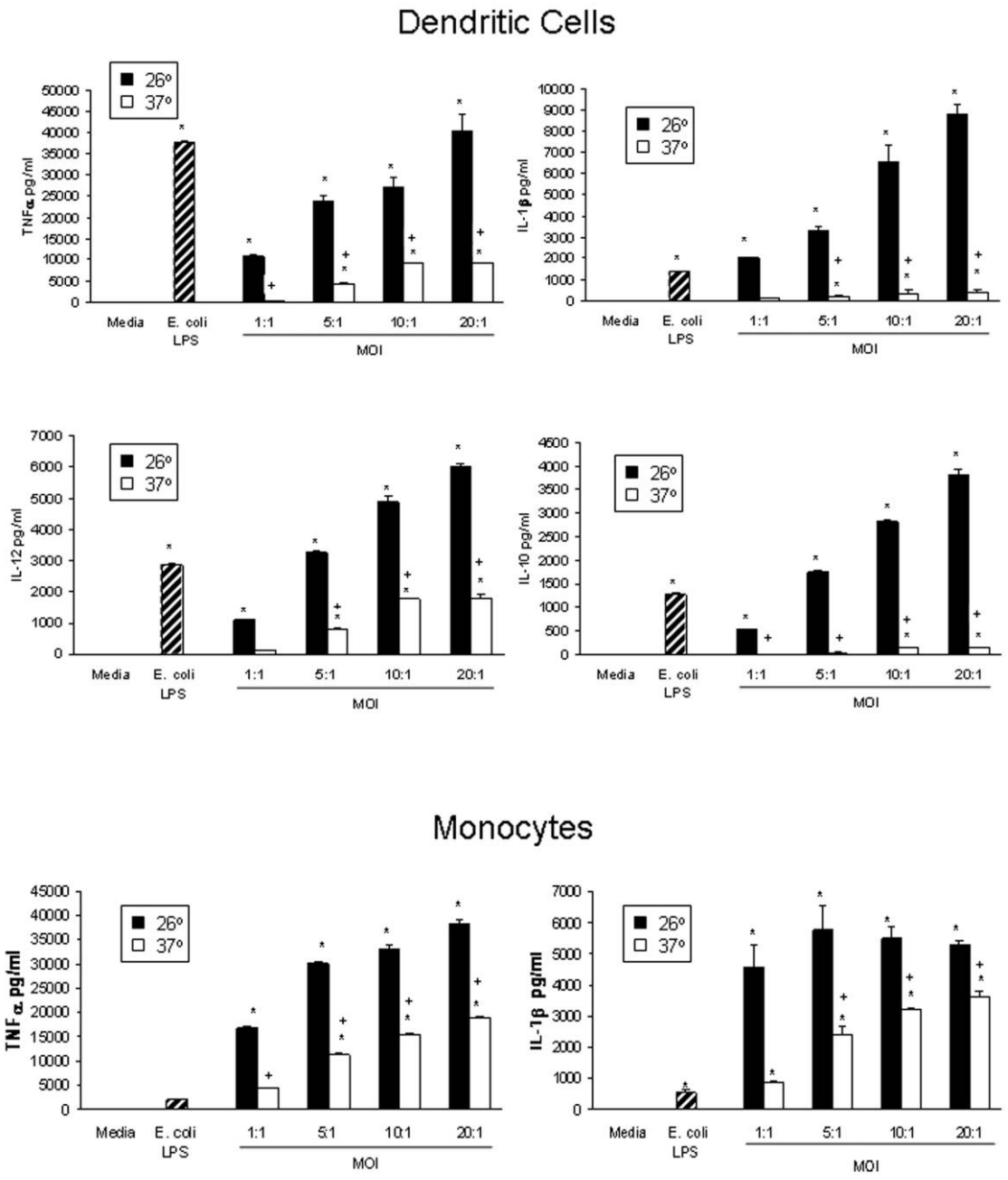

Figure 1. Secretion of tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 10 (IL-10), and interleukin 12 (IL-12) by human dendritic cells (*A*) and of TNF-α and IL-1β by human monocytes (*B*) 24 h after infection with *Yersinia pestis* cells grown at either 26°C or 37°C. In each case, a different multiplicity of infection (MOI) was used. Stimulation with 1 ng of lipopolysaccharide (LPS) from *Escherichia coli* was used as a positive control in each set of experiments. $^{*}P < .05$ for 26°C versus 37°C; $^{+}P < .05$ for experimental versus control or untreated.

CO92 grown at 26°C versus 37°C. For these experiments, monocytes or DCs were at a cell density of 1×10^5–5×10^5 cells/mL. Gentamicin (100 μg/mL) was added simultaneously with bacteria, and incubation was continued overnight at 37°C in 5% CO_2. At ~24 h, supernatants were collected, filter sterilized, and used for enzyme-linked immunosorbent assays (ELISAs).

LPS stimulation of cells. Human monocytes and monocyte-derived DCs were cultured in 96-well plates at a cell density of 1×10^6 cells/mL and exposed to one of the following: (1) medium alone, (2) LPS from *E. coli*, (3) LPS-26°C versus LPS-37°C, (4) poly-IC, or (5) lipoprotein. In some experiments, cells were exposed to different stimuli for 1 h and then exposed to varying stimuli for 24 h. Supernatants were collected at 24 h and assessed for the levels of different cytokines. DC viability was assessed by trypan blue staining before surface staining

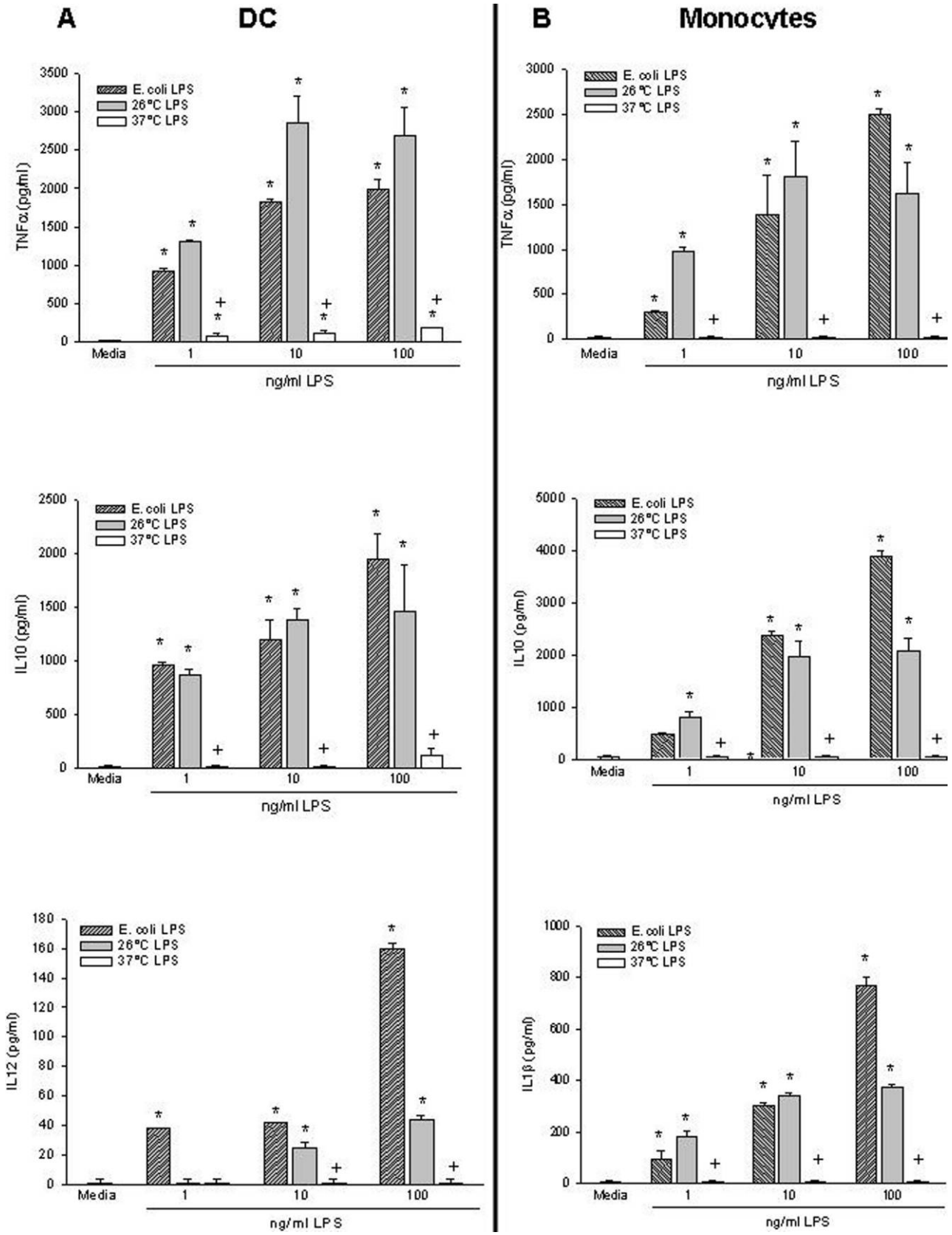

Figure 2. Stimulation of human dendritic cells (DCs) (*A*) and monocytes (*B*) by different doses of lipopolysaccharide (LPS) from *Escherichia coli* or from *Yersinia pestis* cultivated at 26°C or 37°C. Level of cytokines were measured in the supernatants of cell culture medium 24 h after stimulation. IL, interleukin; TNF, tumor necrosis factor. $^{*}P < .05$ for 26°C versus 37°C; $^{+}P < .05$ for experimental versus control or untreated.

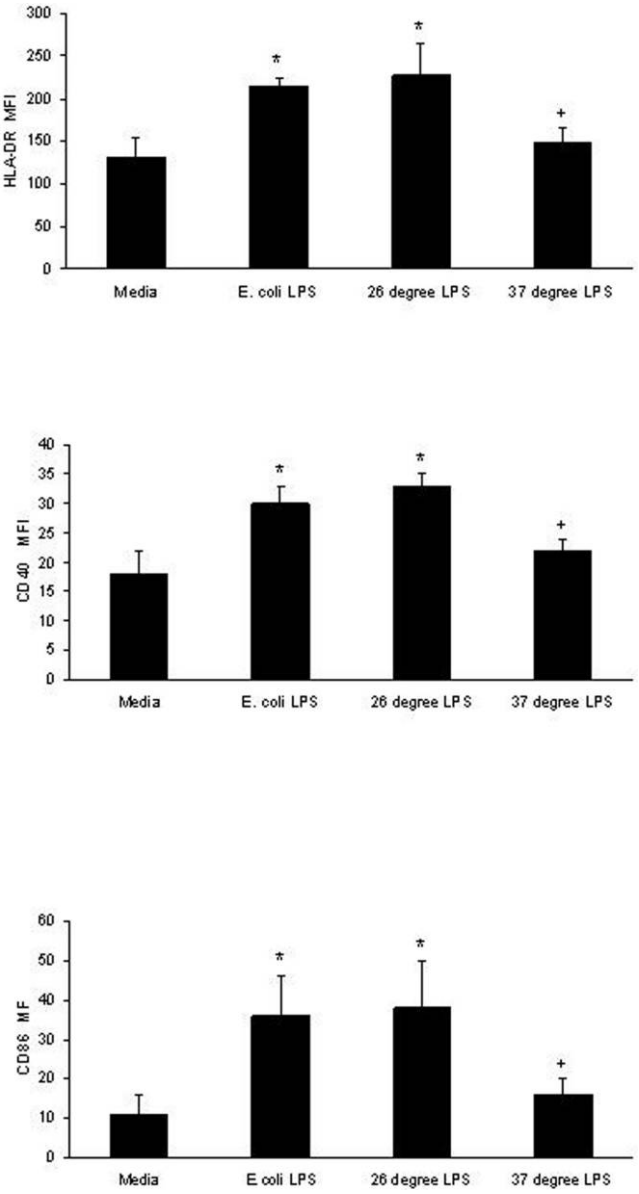

Figure 3. Induction of the expression of extracellular markers CD40, CD86, and HLA-DR in human dendritic cells with 1 ng of lipopolysaccharide (LPS) from *Escherichia coli* or from *Yersinia pestis* cultivated at 26°C or 37°C, determined by flow cytometry. Results of representative experiments are shown. MFI, median fluorescence intensity. $^{*}P < .05$ for 26°C versus 37°C; $^{+}P < .05$ for experimental versus control or untreated.

and supernatant collection and was always found to be identical to that in medium control cultures.

Flow cytometry. DCs were assessed for enhanced expression of surface HLA-DR, CD40, and CD86 by standard 1-color flow cytometry with fluorescein isothiocyanate–conjugated monoclonal antibody (BD Biosciences and Caltag). Briefly, aliquots of DCs (2×10^5–5×10^5) were incubated with the indicated antibodies or an isotype control antibody for 30 min at 4°C. After incubation, the cells were washed 3 times with PBS containing 2% FBS and then fixed with paraformaldehyde (1% in PBS). Fixed samples were then analyzed using a FACScan flow cytometer (BD Biosciences).

Cytokine measurements. Cytokine levels present in monocyte or DC culture supernatants were determined using commercially purchased ELISAs. The cytokines measured were as follows: interleukin 10 (IL-10) (50–10,000 pg; BD Biosciences), interleukin 12p70 (IL-12p70) (50–20,000 pg; BD Biosciences), TNF-α (50–10,000 pg; BD Biosciences), and interleukin 1β (IL-1β) (5–500 pg; eBiosciences). The standard errors of the mean and the statistical significance of differences between treatments or conditions were determined by analysis of variance, followed by the Tukey-Kramer comparison of means.

RESULTS

Decreased cytokine production by human monocytes and monocyte-derived DCs exposed to* Y. pestis *grown at 37°C. Human DCs were exposed to varying numbers of *Y. pestis* that had been grown at either 26°C or 37°C. As controls, cells were stimulated with commercial LPS from *E. coli* (positive) or left in medium only (negative). Levels of TNF-α, IL-1β, IL-10, and IL-12 in culture supernatants were then determined 24 h after exposure. High levels of all 4 cytokines were observed in cultures where cells were exposed to *Y. pestis* grown at 26°C (Figure 1*A*). A prominent dose-dependent effect of several-fold magnitude was seen when the MOI was increased from 1 to 20. In contrast, very low levels of all cytokines were present in cultures that had been exposed to *Y. pestis* grown at 37°C. In fact, barely detectable levels of either IL-1β or IL-10 were present even for the highest MOI of 20. *Y. pestis* grown at 37°C did induce significant levels of TNF-α and IL-12, but these levels were notably lower than those induced by *Y. pestis* grown at 26°C. These findings were not unique to DCs; similar results were found when using monocytes. Monocytes exposed to *Y. pestis* grown at 37°C produced significantly less TNF-α and IL-1β than did monocytes exposed to *Y. pestis* grown at 26°C (Figure 1*B*). Thus, *Y. pestis* grown at 37°C was significantly less efficient at inducing cytokine production in both DCs and monocytes than *Y. pestis* grown at 26°C.

***Induction of cytokine production in monocytes and DCs by hexa- and tetraacylated LPS from* Y. pestis.** Because LPS-37°C of *Y. pestis* has been shown to be less stimulatory than LPS-26°C for this pathogen [2, 4, 14, 16, 18], we next compared these types of LPS for their ability to activate monocytes versus DCs for cytokine production (TNF-α, IL-1β, IL-10, and IL-12). Results from a representative experiment are presented in Figure 2. LPS-26°C, which contained a hexaacylated lipid A, induced high levels of cytokine production in both DCs and monocytes. These were comparable to the cytokine levels induced by commercial LPS from *E. coli*. Both of these LPS molecules induced, with similar potency, a strong dose-depen-

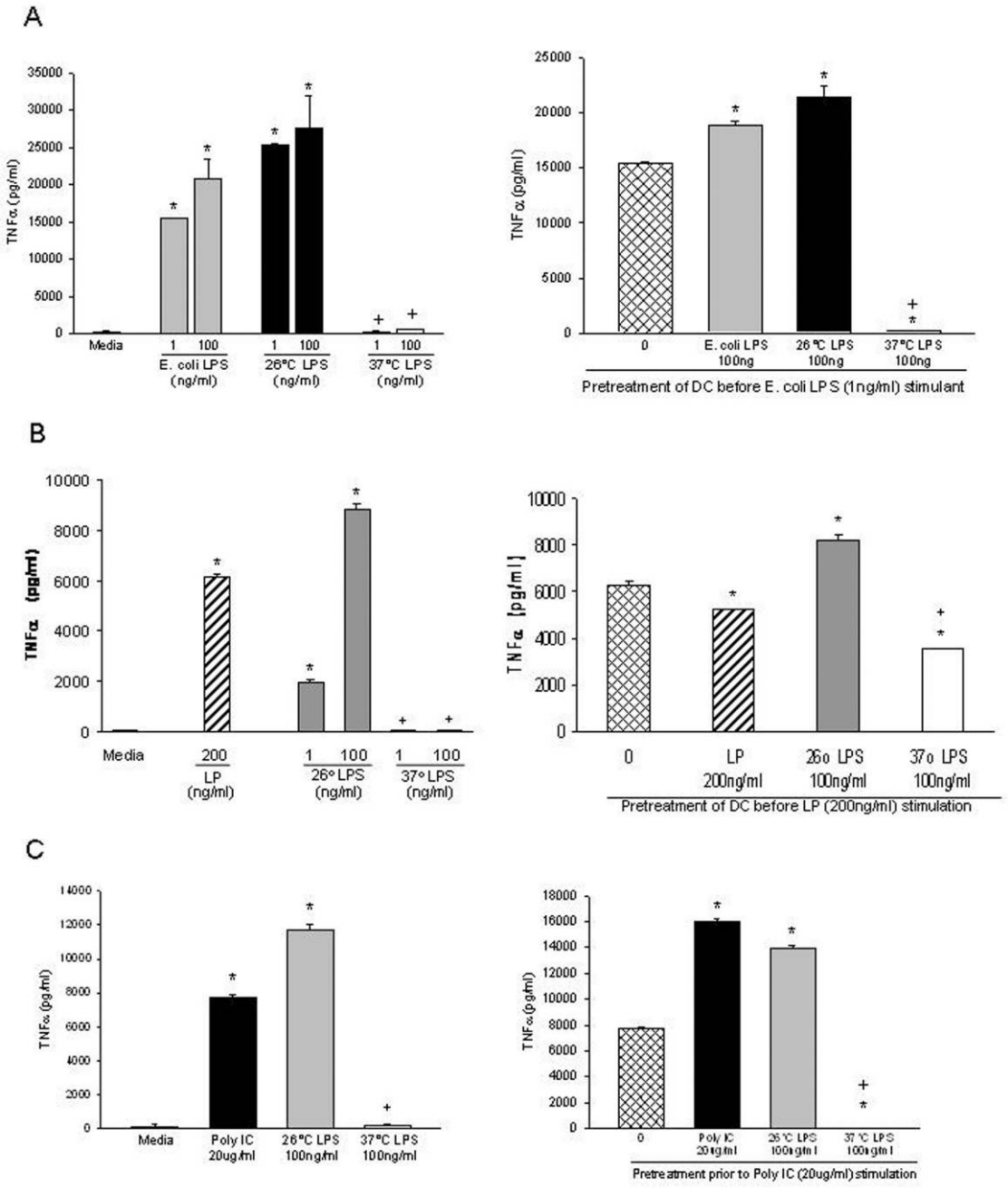

Figure 4. Production of tumor necrosis factor α (TNF-α) in human dendritic cells (DCs) after treatment with lipopolysaccharide (LPS) from *Escherichia coli* (*A*), lipoprotein (LP) (*B*), or polyinosinic-polycytidylic acid (poly-IC) (*C*). The left and right parts of each panel show results of stimulation without or with pretreatment of DCs using different stimuli. 26°C LPS and 37°C LPS, LPS from *Yersinia pestis* grown at 26°C or 37°C, respectively. $^{*}P < .05$ for 26°C versus 37°C; $^{+}P < .05$ for experimental versus control or untreated.

dent secretion of cytokines 24 h after stimulation. In contrast, when a tetraacylated LPS, isolated from *Y. pestis* grown at 37°C, was used for stimulation, little to no cytokine production was detected. Barely measurable levels of TNF-α and IL-10 were detected in DCs (Figure 2*A*), and monocytes failed to respond to this type of LPS (Figure 2*B*). Thus, the effects of LPS-37°C correlated with the diminished ability of *Y. pestis* grown at 37°C to induce cytokine production in both DCs and monocytes.

Failure of LPS-37°C to up-regulate costimulatory molecules in DCs. Monocyte-derived DCs express low levels of major histocompatibility complex (MHC) class II molecules and the costimulatory molecules CD40 and CD86. After exposure to bacteria, LPS, or inflammatory mediators, DCs undergo a maturation process characterized by cytokine production and increased expression of MHC and costimulatory molecules. To explore further the defects associated with the tetraacylated

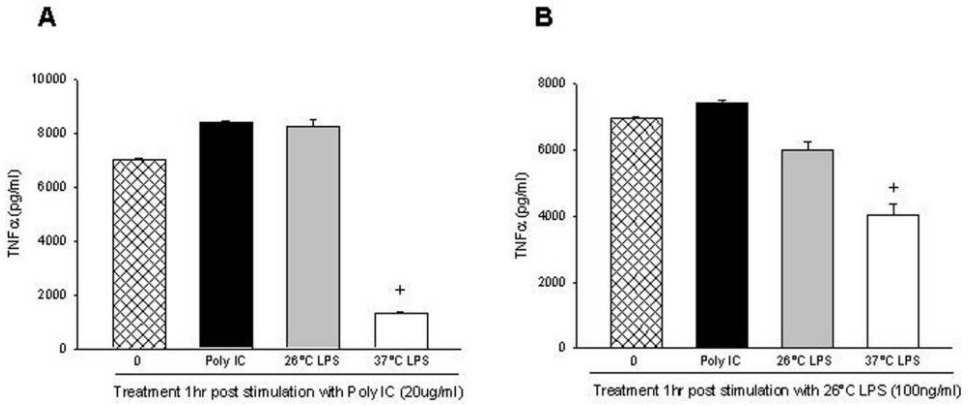

Figure 5. Production of tumor necrosis factor α (TNF-α) in human dendritic cells (DCs) after pretreatment with different types of lipopolysaccharide (LPS) (100 ng/mL) or polyinosinic-polycytidylic acid (poly-IC) (20 ng/mL) followed by restimulation with 20 ng/mL poly-IC (*A*) or 100 ng/mL LPS from *Yersinia pestis* grown at 26°C (*B*). $^{+}P < .05$ for experimental versus control (untreated).

LPS-37°C, we assessed DCs for increased expression of HLA-DR, CD40, and CD86 after 24 h of exposure to LPS-26°C versus LPS-37°C. Data from a representative experiment are presented in Figure 3. All 3 surface proteins were up-regulated after exposure to the LPS from *E. coli* or hexaacylated LPS-26°C. In contrast, there was little to no up-regulation of these surface proteins of DCs after exposure to tetraacylated LPS-37°C.

Effect of pretreatment with tetraacylated LPS-37°C on LPS, lipoprotein, or poly-IC stimulation of DCs. The weak ability to induce cytokine production in DCs or monocytes by tetraacylated LPS-37°C could result from either the low stimulatory potential of this type of LPS or a specific inhibition of TLR-mediated signaling. Therefore, we designed experiments in which we pretreated human DCs for 1 h with different stimuli, followed by restimulation with homologous or heterologous molecules affecting different TLR-mediated signaling pathways. TNF-α production was then assessed 24 h after secondary stimulation. Results from a representative experiment are shown in Figure 4. As expected, DCs exposed to LPS from *E. coli* or LPS-26°C produced high levels of TNF-α. In contrast, DCs exposed to tetraacylated LPS-37°C failed to produce significant levels of TNF-α. Moreover, DCs pretreated with 100 ng/mL LPS-37°C were shown to be significantly suppressed in their ability to respond to either LPS obtained from *E. coli* or LPS-26°C.

One possible explanation for this suppressed response is simple competition for TLR4, which could result from pretreating DCs with LPS-37°C. To investigate this possibility, we pretreated DCs with LPS-37°C versus LPS-26°C and then exposed these cells to poly-IC (TLR9 ligand) or lipoprotein (TLR2 ligand) and assessed TNF-α production at 24 h. As seen in Figure 4*B* and 4*C*, pretreatment of DCs with LPS-37°C also significantly inhibited DC activation via completely different TLR receptor ligands, poly-IC and lipoprotein. To investigate this suppressive effect further, we initiated experiments to test whether LPS-37°C could alter cytokine production by DCs if added 1 h after activation by LPS-26°C or by poly-IC, a TLR9 ligand. Results from these experiments are presented in Figure 5. LPS-37°C also significantly inhibited cytokine production in LPS- or poly-IC–activated DCs, even when added 1 h after exposure to these activators. These results suggest that tetraacylated LPS-37°C can mediate a generalized suppression of TLR signaling and that this effect is neither unique to LPS and TLR4 signaling nor easily explained by simple competition for TLR receptors.

DISCUSSION

It is a well-established phenomenon that *Y. pestis* can modulate globally the expression of different virulence factors in response to changes in temperature mimicking a transition from the flea arthropod vector to the mammalian host [28, 29]. For example, lipid A is predominantly tetraacylated when the bacteria grow at 37°C and is hexaacylated when they grow at ambient temperature [2, 4, 8, 10, 23]. This decrease in acylation of LPS at the temperature of warm-blooded animals results in a more permeable outer membrane of the microorganism, which probably facilitates the exchange of hydrophobic nutrients and metabolites within host tissues [30]. However, temperature is not the only parameter that influences lipid A acylation in *Yersinia* species. In pathogenic but not environmental biotypes of *Y. enterocolitica*, the acylation was dramatically decreased when bacteria were grown at 37°C in acidic or calcium ion–restricted media [30]. More importantly, modulation of lipid A acylation is one of the factors that contributes to the endotoxic properties of this molecule [31, 32]. In general, the reduced endotoxicity obstructs detection of the pathogen by the host innate immune system, which works in concert with other *Yersinia* functions (such as the type 3 secretion system) to down-regulate innate immune responses during the early stages of infection [21].

We compared the ability of *Y. pestis* grown at either 26°C or 37°C and the corresponding purified LPS-26°C or LPS-37°C to induce cytokine production in human monocytes and monocyte-derived DCs. We found that hexaacylated LPS-26°C provided a dose-dependent induction of TNF-α, IL-1β, IL-10, and IL-12 comparable with that obtained using control hexaacylated LPS from *E. coli*. In contrast, the production of these cytokines by DCs was barely detectable or very low after exposure to tetraacylated LPS-37°C. Our results are in good agreement with previously published observations regarding the induction of proinflammatory cytokines by human and murine cells in response to LPS from *Y. pestis*. The LPS from *Y. pestis* grown at 28°C was shown to induce production of TNF-α and IL-6 by the murine macrophage-like cell line J774 [16]. LPS from *Y. pestis* grown at 27°C was also shown to be a better inducer of TNF-α than LPS-37°C in human (U937) and murine (RAW 264.7) macrophage cell lines [2], and LPS from *Y. pestis* grown at 21°C, but not at 37°C, stimulated human peripheral blood monocytes to secrete TNF-α in a dose-dependent fashion [4]. Moreover, high TNF-α–inducing activity of LPS from *Y. pestis* grown at 25°C for J774.A1 cells correlated with increased lipid A acylation in LPS samples prepared from a number of *Y. pestis* strains of different epidemic potential [14]. Finally, human and nonhuman primate PBMCs responded strongly to LPS-26°C, but not to LPS-37°C, in their production of TNF-α and IL-8. A similar result was also obtained for murine peritoneal macrophages, but murine cells appeared to respond to LPS-37°C compared with primate cells [18]. The effect of reduced stimulation of human DCs with *Y. pestis* LPS-37°C has not been reported previously.

Transition of DCs from the immature state to the mature state is induced by many factors, including LPS. This activation results in the production of cytokines and up-regulation of MHC-II and costimulatory molecules [33, 34]. We investigated whether these activation parameters were also reduced in DCs after exposure to LPS-37°C versus LPS-26°C. LPS-37°C failed to up-regulate HLA-DR, CD86, and CD40. In contrast, these surface proteins were significantly up-regulated by exposure to LPS-26°C.

The innate immune system recognizes pathogen-associated molecular patterns, such as LPS, unmethylated DNA, flagellin, lipoteichoic acids, peptidoglycan, lipoproteins, or double-stranded RNA by specific receptors, including the TLRs [35]. TLRs play an essential role in the control of the adaptive immune response via the critical linking between innate and adaptive immunity [36]. Stimulation of TLR4 by a variety of ligands, including LPS, results in an effective proinflammatory antibacterial response that helps fight infection. However, overstimulation or uncontrolled stimulation of TLR can be harmful to the host [37]. It was shown recently that LPS-26°C of *Y. pestis* is a potent stimulator of TLR4, whereas LPS-37°C is a weak stimulator of human PBMCs [18]. Moreover, LPS-37°C was shown to inhibit stimulation of PBMCs by LPS-26°C [18]. We now report that tetraacylated LPS-37°C fails to stimulate human monocytes or human DCs effectively and that this LPS can inhibit not only TLR4 signaling via LPS-26°C but also TLR2 and TLR9 signaling activated by other ligands. The inability to fully activate DCs and to inhibit DC maturation that might be achieved via *Y. pestis* factors other than LPS could have profound ramifications not only for the innate immune response but also for the development of an optimal adaptive immune response. The exact mechanism responsible for coordinated *Y. pestis* LPS inhibition of DC activation via multiple TLRs (TLR2, TLR4, and TLR9) is unclear but under investigation. We believe that the unique properties of LPS associated with *Y. pestis* growth at 37°C are important mechanisms for suppressing the innate and adaptive immune responses to human infection.

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Erratum

In the 1 December 2009 issue of the *Journal*, the article by Telepnev et al (Telepnev MV, Klimpel GR, Haithcoat J, Knirel YA, Anisimov AP, Motin VL. Tetraacylated lipopolysaccharide of *Yersinia pestis* can inhibit multiple Toll-like receptor–mediated signaling pathways in human dendritic cells. J Infect Dis 2009; 200:1694–1702) mistakenly referred to TRL9 throughout the text; all instances of "TRL9" should be "TRL3." The authors regret this error.

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