Potential Role of *Mycoplasma hominis* in Interleukin (IL)–17–Producing CD4\(^{+}\) T-Cell Generation Via Induction of IL-23 Secretion by Human Dendritic Cells

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**Background.** *Mycoplasma hominis*, a human urogenital pathogen, is involved in genital and extragenital infections and arthritis, particularly in immunocompromised patients. The interleukin (IL) 23/T helper (Th) 17 axis is associated with inflammatory and autoimmune diseases. The aim of this study was to assess the IL-23 response to *M. hominis* in human dendritic cells (DCs) and the CD4\(^{+}\) T-cell differentiation in response to *M. hominis*–infected DCs.

**Methods.** Human monocyte–derived DCs were cultured with phosphate-buffered saline, lipopolysaccharide, or *M. hominis* PG21. Cocultures with heterologous T cells were performed. Extracts from *M. hominis* were separated and incubated with DCs. Isolates from different clinical syndromes were tested.

**Results.** *M. hominis* induced the maturation of human DCs with predominant IL-23 secretion in a Toll-like receptor 2–dependent manner. The in vitro immunomodulatory capacity of *M. hominis* was contained in a lipoprotein-enriched fraction from the mycoplasma. *M. hominis*–activated DCs induced IL-17–producing CD4\(^{+}\) T cells. Interestingly, clinical isolates differed in their ability to promote IL-23 secretion by DCs.

**Conclusions.** Taken together, our findings demonstrate a major role for the IL-23/Th17 axis in the defense against *M. hominis* and indicate a potential role for these bacteria in inflammatory and autoimmune diseases.
signal, DCs undergo a complex process of maturation into antigen-presenting cells. This happens while the DCs migrate from the periphery into the draining lymph node through the lymphatic system. Within the lymph nodes, through their ability to secrete different cytokines, DCs control T-cell priming [5]. Yet cytokines secreted to induce a specific immune response against an invading pathogen might interfere with DC homeostasis and induce an autoimmune response that can be responsible for tissue pathology. Indeed, clinical and epidemiologic studies have suggested a link between infectious agents and chronic inflammatory disorders, including autoimmune diseases [6].

Interleukin (IL) 12 and IL-23 are 2 heterodimeric cytokines composed of specific polypeptides, namely, p35 for IL-12 and p19 for IL-23, linked to a common p40 chain to form biologically active molecules. Studies using p19\(^{-/-}\) or p35\(^{-/-}\) mouse models of autoimmunity, tumors, and inflammatory bowel disease have identified IL-23, rather than IL-12, as the major factor responsible for lesions caused by chronic inflammation, partially acting through the induction of a CD4\(^{+}\) T lymphocyte subset producing IL-17 [7]. This T-cell subset has been recently named T-helper (Th) 17 and is identified based on the ability to produce IL-17, IL-21, and IL-22. These T cells have been shown to be major contributors to inflammatory and autoimmune diseases, including autoimmune diseases [6].

Table 1. Clinical Isolates of Mycoplasma hominis Used in This Study

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Year of isolation</th>
<th>Clinical syndrome</th>
<th>Specimen</th>
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<tbody>
<tr>
<td>1</td>
<td>1991</td>
<td>Psoriatic rheumatism</td>
<td>Synovial fluid</td>
</tr>
<tr>
<td>2</td>
<td>1991</td>
<td>Rheumatoid arthritis</td>
<td>Synovial fluid</td>
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<tr>
<td>3</td>
<td>1997</td>
<td>Arthritis in a patient with hypogammaglobulinemia</td>
<td>Synovial fluid</td>
</tr>
<tr>
<td>4</td>
<td>1999</td>
<td>Septic arthritis in an immunocompetent patient</td>
<td>Synovial fluid</td>
</tr>
<tr>
<td>5</td>
<td>2002</td>
<td>Osteoarthritis</td>
<td>Synovial fluid</td>
</tr>
<tr>
<td>6</td>
<td>2002</td>
<td>Arthritis in a patient with hypogammaglobulinemia</td>
<td>Synovial fluid</td>
</tr>
<tr>
<td>7</td>
<td>2005</td>
<td>Endometritis</td>
<td>Endometrial biopsy</td>
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carried out in sterile water. The lysate was centrifuged at 20,000 g for 2 hours at 4°C. The cytosolic fraction (supernatant) was cleared by centrifugation at 20,000 g for 1 hour at 4°C. The pellet corresponding to undisrupted cells and membrane vesicles was resuspended in PBS and submitted to ultrasonication 3 times on ice for 1 minute. Membranes were recovered by centrifugation (20,000 g for 1 hour) and washed 4 times in PBS. An aliquot of each fraction was analyzed by SDS-PAGE under reducing conditions. Proteins were concentrated by acetone precipitation and stored at −80°C.

Generation of DCs and M. hominis Stimulation

Human monocyte-derived DC generation was performed as described elsewhere, using magnetic CD14 microbeads (Miltenyi Biotec) [11]. The purity and viability of DC preparations were >95%. After 5 days of culture, monocyte-derived DCs were transferred into 96-well plates at 2 × 10⁵ cells per well along with different doses of M. hominis (from 10⁵ to 10⁸ CCU/mL, depending on the experiments) and incubated at 37°C for 24–48 hours. DCs were also cultured with 50 μL of PBS (negative control) or in the presence of LPS from *Escherichia coli* 026:B6 (Sigma, positive control) at a final concentration of 10 ng/mL. In some experiments, cells were exposed to the LPS inhibitor polymyxin B sulfate (Sigma) at 10 μg/mL or to human anti-Toll-like receptor (TLR) 2 blocking antibody (eBiosciences) for 1 hour at 37°C and then exposed to *M. hominis* for 24 hours. DCs were analyzed using a fluorescence-activated cell sorter (FACS) after 24 hours of incubation with *M. hominis*. Supernatants were harvested after 24 or 48 hours of coincubation and were stored at −80°C until analysis. They were assessed for cytokine content using Ready-Set-Go enzyme-linked immunosorbent assay (ELISA) kits for the detection of IL-12p70, IL-10, IL-23p19, and tumor necrosis factor (TNF) α (eBiosciences). Data were processed with the Biolise 2.0 software.

FACS Analysis of DC Cell Surface Molecules

For immunophenotyping, DCs were washed in PBS and incubated at 4°C with one of the following human monoclonal antibodies: anti–CD80–phycoerythrin (PE), anti–CD-86–PE, anti–HLA-DR–PE–cyanine (Cy) 5, anti–CD40–PE, anti–CD83–PE, or anti–CD1a–fluorescein isothiocyanate (FITC) (eBiosciences). The appropriate isotype controls were included. After washing, 10⁴ cells were analyzed on a FACSca-libur flow cytometer (Becton Dickinson). Data were processed with the CellQuest cytometry software (BD Biosciences). The expression of the cell surface molecules was either shown as 1-parameter histograms or evaluated using mean fluorescence intensity.

Mixed-Lymphocyte Reaction

DCs coincubated with PBS, LPS, or *M. hominis* were harvested after 24 hours of incubation, and free stimuli were removed by washing 2 times with RPMI1640. Mixed-lymphocyte reactions using viable, cryopreserved total allogenic T lymphocytes were conducted as described elsewhere [11].

Intracellular Flow Cytometry Detection of Cytokine Production by Allogenic CD4⁺ T Cells

CD4⁺ T cells were purified from the CD14-negative fraction using indirect magnetic cell labeling with an anti–CD3–FITC antibody and anti–FITC microbeads (Miltenyi Biotec). Monocyte-derived DCs coincubated with PBS, LPS, or *M. hominis* PG21 for 24 hours were used to stimulate allogenic T cells at a ratio of 1:1 for 5 days.

For intracellular cytokine staining, cells were incubated for 5 hours with phorbol myristate acetate (50 ng/mL; Sigma), ionomycin (500 ng/mL; Sigma), and brefeldin A (10 μg/mL; Sigma). Cells were stained for surface CD4 expression by incubation for 15 minutes at 4°C with an anti–CD4–PE–Cy5 antibody (eBioscience). Cells were fixed and permeabilized for 20 minutes at 4°C using the Cytofix/Cytoperm buffer set (Becton Dickinson) and were stained for 30 minutes at 4°C in permeabilization buffer with anti–interferon (IFN) γ–FITC and anti–IL-17A–PE antibodies (eBiosciences). The cells were analyzed using a Becton Dickinson FACSCANTO instrument. Data were analyzed using the FACSDIVA 6.1.3 software.

Taqman Real-Time Polymerase Chain Reaction for IL-23p19 and IL-12p35 RNA

Total RNA was isolated from 1 × 10⁵ to 10⁶ cells using a Total RNA Isolation Reagent kit (Ademtech). RNA was precipitated in isopropanol, resuspended in 30 μL of RNase-free water and quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). In summary, 1–10 pg of total RNA was reverse-transcribed to complementary DNA (cDNA) with the Verso cDNA Kit (Thermo Scientific ABgene), according to the manufacturer’s instructions. The cDNA was amplified and quantified using the Taqman real-time polymerase chain reaction assay described for human IL-12p35 and IL-23p19 by Lee et al [12]. The real-time polymerase chain reaction mixture consisted of 10 μL of 2× LightCycler 480 Probes Master (Roche Diagnostics), 0.6 μL of each primer (10 μmol/L), 0.8 μL of each of the 3 probes (5 μmol/L), and 3 μL of cDNA as template. We used a Roche LightCycler 480 thermocycler for 15 minutes at 95°C, followed by 45 cycles of 10 seconds at 95°C and 60 seconds at 60°C. Each sample was normalized to the human acidic ribosomal protein gene (hARP) by obtaining the difference in threshold cycle (Ct) number between hARP and the cytokines. Relative RNA (messenger RNA [mRNA]) expression levels were calculated using the formula 2−ΔΔCt, where the Ct of the target gene is normalized to the gene expression of the control condition.

Statistical Analyses

Data are expressed as means ± standard deviations, and the significance of differences between 2 series of results was
determined using Student unpaired $t$ test. Differences were considered significant at $P \leq .05$.

**RESULTS**

**Morphologic, Phenotypic, and Functional Maturation of Human DCs by *M. hominis* PG21**

To analyze the potential immunomodulatory activity of *M. hominis* toward human DCs, preparations of PBS, LPS, or *M. hominis* PG21 were added to 5 day-cultured monocyte-derived DCs. Different culture conditions were tested to find the optimal dose and incubation time. *M. hominis* was used live or heat-inactivated at several concentrations, 10^5–10^8 CCU/mL, and incubated with DCs for 24 or 48 hours. No significant difference was seen between live and heat-inactivated *M. hominis* or between different incubation times (data not shown). The conditions used in further experiments were live *M. hominis* cells at 10^8 CCU/mL for 24 hours. After incubation, DCs were collected and observed after centrifugation by light microscopy. Both LPS and *M. hominis* induced the development of widespread cytoplasmic projections called dendrites (Figure 1), reflecting a morphologic maturation. The surface expression of HLA-DR, CD80, CD83, CD86, and CD40 were analyzed by FACS (Figure 1B, left panel). Compared with PBS, *M. hominis* induced a significant up-regulation of CD86 ($P < .01$), CD83 ($P < .05$), CD40 ($P < .05$), and a trend toward an increase of HLA-DR and CD80 expression (Figure 1B, right panel). These results indicate that *M. hominis* induced phenotypic maturation of DCs. To further assess the effect of *M. hominis* on the function of monocyte-derived DCs, PBS-, LPS- or *M. hominis*-activated DCs were...
cocultured with allogenic total lymphocytes for 5 days at a 1:100 ratio. LPS and *M. hominis* activation of DCs markedly enhanced lymphocyte proliferation (*P* < .01) (Figure 1C).

**M. hominis** PG21 Increases DC Inflammatory Cytokine Production Including Predominant IL-23 Secretion in a TLR2-Dependent Manner

Because DCs are known to play key roles in the integration of environmental signals and polarization of the adaptive immune response, the production of several cytokines (TNF-α, IL-10, IL-12, and IL-23) by *M. hominis*-activated DCs was tested. No bioactive cytokines were detectable in the cell culture supernatants of PBS-stimulated DCs. *M. hominis* PG21 consistently induced TNF-α, IL-10, and IL-23. There was significantly higher production of IL-23 by *M. hominis*-treated DCs as compared with LPS-treated DCs (Figure 2A) (*P* < .05). Interestingly, in the presence of *M. hominis* PG21, very low IL-12 production by DCs was detected, whereas LPS induced the secretion of both IL-23 and IL-12 (Figure 2A) (*P* < .01). These results were further confirmed by the detection of IL-23 mRNA, but not IL-12 mRNA, in DCs incubated with *M. hominis* (Figure 2B). A previous study showed that *M. hominis* induced TNF-α production by human monocytes in a TLR2- but not TLR4-dependent manner [13]. Therefore, we wanted to determine whether TLR2 was involved in IL-23 production by human DCs activated by *M. hominis*. As shown in Figure 2C, blocking anti-TLR2 antibodies inhibited *M. hominis*-induced IL-23 production.

**In Vitro Immunomodulatory Capacity of M. hominis** Residing in Membrane-Associated Proteins

Different *M. hominis* protein extracts were assayed for their ability to induce cytokine production by human DCs. The protein fractions that were tested corresponded to the membrane-enriched and cytosolic fractions isolated by differential centrifugation and to the detergent-soluble and aqueous phases separated by TX-114 phase partitioning. The complementarity of the electrophoresis profiles obtained from the TX-114-soluble and aqueous phases was examined (Figure 3A). Similar to the whole cells of *M. hominis*, the detergent-soluble and membrane-enriched phases induced a significant increase in production of TNF-α, IL-10, and IL-23 by human DCs. In contrast, the cytosolic and TX-114 aqueous phases only weakly modulated the cytokine profiles (Figure 3B). Hence, *M. hominis* membrane–associated amphiphilic proteins were mainly responsible for the induction of cytokine production by DCs. To exclude potential LPS contamination, the effects of prior treatment with polymyxin B, an inhibitor of LPS-mediated stimulation, on the ability of the different extracts to activate DCs were assessed. The activity of membrane and detergent-enriched fractions was not reduced by polymyxin B, whereas it dramatically inhibited LPS activity by about 95% (Figure 3C) (*P* < .01).

**Induction of IL-17–Producing CD4+ T Cells by M. hominis** PG21–Activated DCs

Considering the prominent secretion of IL-23 by *M. hominis* PG21–stimulated DCs, we next examined whether these DCs were able to induce IL-17–secreting cells. This ability was assessed by measuring intracellular IFN-γ and IL-17 production by gated CD4+ T cells among CD3+ T cells cultured along with *M. hominis* PG21–stimulated DCs. Figure 4A shows a representative result obtained from 1 experiment of 7. In comparison with PBS- or LPS-treated DCs, DCs activated by *M. hominis* induced a significantly higher percentage of IL-17–positive CD4+ T cells (Figure 4B) (*P* < .05).

**Variable Ability of M. hominis** Clinical Isolates to Induce IL-23 Secretion by DCs

Seven strains isolated from different clinical syndromes were studied (Table 1). Six isolates were obtained from synovial fluids of patients suffering from inflammatory or noninflammatory rheumatologic diseases (isolates 1–6), and the last isolate was obtained from a patient with endometritis (isolate 7). IL-23, IL-10, IL-12, and TNF-α production were measured in the supernatants of DCs cultured with each of the 7 isolates by ELISA after 24 hours of incubation. Secretion of TNF-α and IL-10 was equivalent in all isolates (data not shown), and the IL-12 secretion was even lower than that induced by PG21 for all isolates except for the osteoarthritis isolate (isolate 5). Interestingly, compared with PG21, the IL-23 secretion induced by the clinical isolates was very variable (Figure 5A) ranging from 172 ± 59 pg/mL for isolate 5 to 1946 ± 300 pg/mL for isolate 7. When the IL-23/IL-12 ratio was considered, differences among these isolates were strongly reinforced. As seen in Figure 5B, this ratio was significantly lower for the osteoarthritis isolate (isolate 5) and higher for the endometritis isolate (isolate 7) than for *M. hominis* PG21. Moreover, it was significantly higher for the hypogammaglobulinemia arthritis isolate (isolate 6) than for the osteoarthritis isolate (isolate 5) (*P* ≤ .05). No significant difference was shown between *M. hominis* PG21 and the 5 other clinical isolates. Taken as a whole, this set of data suggests that different *M. hominis* strains have different abilities to activate the innate immune system.

**DISCUSSION**

We studied the effects of *M. hominis* PG21 on the maturation and activation of human monocyte-derived DCs. We observed that the production of IL-23 by DCs was enhanced by *M. hominis* PG21 in a TLR2-dependent manner, thereby promoting the skewing of T cells toward IL-17–producing cells. We also showed that this biologic effect was mainly contained in the membrane lipoproteins of *M. hominis*. Unexpectedly, when testing the IL-23/IL-12 production by several clinical
isolates, the results differed according to the isolate origin, suggesting differential ability to induce IL-17–producing cells.

Here, we not only confirmed that *M. hominis* promoted secretion of IL-10 and low IL-12 by human monocytes [13] and DCs [14] but extend the observation with the strong ability of *M. hominis* to induce IL-23 secretion in a TLR2-dependent fashion. This is in accordance with the description by Peltier et al [13] of a TLR2-dependent secretion of TNF-α by *M. hominis*–stimulated human monocytes. TLR2 ligands, including Pam2C, are known to inhibit IL-12 and increase IL-23 production by monocyte-derived DCs. Accordingly, TLR2 can be viewed as a key pattern recognition receptor that differentially regulates the expression of these 2 cytokines [15]. Interestingly, other mycoplasma species have been described to activate either DCs
or monocytes through TLR2. As an example, *M. arthritidis*, an arthritis inducer in rodents, also presents antigenic lipoproteins, including *M. arthritidis* mitogen (MAM), that induce TNF-α secretion by murine DCs through TLR2 [16]. It should be noted that no homolog of the MAM gene has been identified in the complete genome sequence of *M. hominis* [17].

*Mycoplasma pneumoniae*, a human respiratory pathogenic mycoplasma, harbors lipid-associated membrane proteins on its surface that drive murine monocytes to secrete IL-23 via a heterodimer formed by TLR2 and TLR1 or TLR6 [18, 19]. The macrophage-activating lipopeptide-2 (MALP-2) of *Mycoplasma fermentans*, a human urogenital mycoplasma, induces proinflammatory cytokine secretion by human monocytes and IL-10 secretion by DCs via TLR2 and TLR6 [20, 21].

Phase partitioning of *M. hominis* components with TX-114 indicated that the activity of *M. hominis* could be mainly attributed to the fraction containing amphiphilic, membrane-associated proteins. In mycoplasmas, TX-114-soluble fractions are enriched in lipoproteins [10], and the active fraction for stimulating DCs may correspond to one or several lipoproteins. Indeed, the immunomodulatory activity of lipoproteins has already been observed for *M. hominis* [13] and other *Mycoplasma* species [16, 20]. Additional experiments will be required to identify the human DC-activating components of *M. hominis*.

Unexpectedly, we observed that *M. hominis* from different clinical isolates displayed different potentials to promote the secretion of IL-12 and IL-23 by DCs. This difference in virulence has already been observed in other *Mycoplasma* strains.

Figure 3. TX-114 extraction of *Mycoplasma hominis* PG21 indicates the presence of the dendritic cell (DC)–activating compounds in the detergent phase (Tx) containing the membrane lipoproteins and is not due to lipopolysaccharide (LPS) contamination. A, Proteins from *M. hominis* PG21 whole cells (Mh), cytosolic fraction (Cyt), membranes (Mb), aqueous phase isolated after TX-114 partitioning (Aq), and TX-114-enriched fraction (Tx) were separated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Molecular masses are indicated on the left. B, Cytosol and membranes (upper panel) or Tx and Aq phases (lower panel) were assayed for the induction of tumor necrosis factor (TNF) α, interleukin (IL) 10, and IL-23 by human DCs after 24 hours. Results are expressed as a percentage of the secretion obtained after incubation with the whole *M. hominis* PG21 and are representative of 3 experiments. C, Effect of polymyxin B on *M. hominis* extracts. Polymyxin B (10 μg/mL) was incubated with DCs for 1 hour at 37°C before adding reagents. Means ± SD of IL-23 production are from 3 experiments; **P < .01.
Cole et al [16] have used *M. arthritidis* strains exhibiting different degrees of virulence to show that the virulent strain of *M. arthritidis* was more potent in activating murine DCs than an avirulent strain. Interestingly, the highest DC-induced IL-23/IL-12 ratio was found with isolate 7 obtained from an inflammatory syndrome, an endometritis, and was significantly higher than that obtained with *M. hominis* PG21. In contrast, the lowest IL-23/IL-12 ratio, significantly lower than that of PG21, was obtained from a noninflammatory noninfectious clinical disease (isolate 5, osteoarthritis). It should be noted that all the other tested isolates induced a DC-secreted IL-23/IL-12 ratio not significantly different from that of *M. hominis* PG21. A mixture of virulent and less virulent strains cannot be eliminated, and the different isolates should be cloned to confirm our findings. Moreover, these observations question the consequences of the different IL-23/IL-12 ratios with regard to the ability of these isolates to induce IL-17 production by T lymphocytes. We are currently investigating these particular issues.

IL-23, in combination with other cytokines such as IL-6 and transforming growth factor (TGF) β, is involved in Th17 differentiation that is essential for the defense against fungi, extracellular bacteria, and *Mycobacterium tuberculosis* [22–24]. Recent findings in mice showed that Th17 cells could be generated directly by IL-23 without TGF-β [25]. IL-23 might also

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**Figure 4.** Human dendritic cells (DCs) primed by *Mycoplasma hominis* PG21 (Mh) induce interleukin (IL) 17–producing cells. A, Frequency of IL-17– and interferon (IFN) γ–positive cells among CD4+/T cells cultured for 5 days with phosphate-buffered saline–, lipopolysaccharide–, or *M. hominis*–activated DCs. Cytokine content was measured by intracellular staining after 5 hours of phorbol myristate acetate/ionomycin activation. One representative of 7 experiments is shown. Numbers in quadrants are percentages of positive cells. B, Cumulative data from 7 independent experiments. Boxes include median and 25th and the 75th percentiles; bars outside boxes represent 10th and 90th percentiles. Data are from 7 independent experiments; *P < .05, **P < .01.
to contribute to the defense against *M. pneumoniae* [18] and *M. arthritidis* [26] in mice, but little is known about the role of this cytokine in the defense against mycoplasmas in humans. Thus, our results confirm the ability of *M. hominis*–induced IL-23 production to promote the up-regulation of IL-17 in humans.

There is compelling evidence for the involvement of Th17 cells in mouse and human autoimmune diseases. Mycoplasmas have been suspected to be involved in inflammatory chronic autoimmune diseases, including RA, through their antigenic plasticity and/or through molecular mimicry [27]. The fact that mycoplasmas can be found in synovial specimens from patients with RA and the ability of mycoplasmas to induce IL-17A production necessitates to consider again mycoplasmas in the pathogenic landscape of inflammatory disorders [28]. Collectively, our findings delineate a major role for the IL-23/Th17 axis in the defense against *M. hominis* and point to the potential implication of some isolates of *M. hominis* in the occurrence of flares in IL-17–mediated chronic disorders.

**Notes**

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