Macrophage Migration Inhibitory Factor Plays a Role in the Regulation of Microfold (M) Cell-Mediated Transport in the Gut

Angela L. Man, Federica Lodi, Eugenio Bertelli, Mari Regoli, Carmen Pin, Francis Mulholland, Abhay R. Satoskar, Michael J. Taussig and Claudio Nicoletti

*J Immunol* 2008; 181:5673-5680; doi: 10.4049/jimmunol.181.8.5673

http://www.jimmunol.org/content/181/8/5673

---

**References** This article cites 33 articles, 12 of which you can access for free at: http://www.jimmunol.org/content/181/8/5673.full#ref-list-1

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Macrophage Migration Inhibitory Factor Plays a Role in the Regulation of Microfold (M) Cell-Mediated Transport in the Gut

Angela L. Man,* Federica Lodi,† Eugenio Bertelli,‡ Mari Regoli,‡ Carmen Pin,* Francis Mulholland,* Abhay R. Satoskar,§ Michael J. Taussig,‡† and Claudio Nicoletti³§

It has been shown previously that certain bacteria rapidly (3 h) up-regulated in vivo microfold cell (M cell)-mediated transport of Ag across the follicle-associated epithelium of intestinal Peyer’s patch. Our aim was to determine whether soluble mediators secreted following host-bacteria interaction were involved in this event. A combination of proteomics and immunohistochemical analyses was used to identify molecules produced in the gut in response to bacterial challenge in vivo; their effects were then tested on human intestinal epithelial cells in vitro. Macrophage migration inhibitory factor (MIF) was the only cytokine produced rapidly after in vivo bacterial challenge by CD11c⁺ cells located beneath the M cell-rich area of the follicle-associated epithelium of the Peyer’s patch. Subsequently, in vitro experiments conducted using human Caco-2 cells showed that, within hours, MIF induced the appearance of cells that showed temperature-dependent transport of microparticles and M cell-specific bacterium Vibrio cholerae, and acquired biochemical features of M cells. Furthermore, using an established in vitro human M cell model, we showed that anti-MIF Ab blocked Raji B cell-mediated conversion of Caco-2 cells into Ag-sampling cells. Finally, we report that MIF⁻/⁻ mice, in contrast to wild-type mice, failed to show increased M cell-mediated transport following in vivo bacterial challenge. These data show that MIF plays a role in M cell-mediated transport, and cross-talk between bacteria, gut epithelium, and immune system is instrumental in regulating key functions of the gut, including M cell-mediated Ag sampling. The Journal of Immunology, 2008, 181: 5673–5680.

Passage of Ag across the epithelial barrier is the first step required to generate effective mucosal and systemic immune responses (1). The role of the highly specialized microfold cells (M cells)⁴ within the follicle-associated epithelium (FAE) in both GALT and nasal-associated lymphoid tissue in the transport of particulate Ags has been well documented (2). M cells are strategically located within the FAE and are capable of delivering antigenic material to the underlying lymphoid tissue, where the machinery is in place to start an immune response. Thus, the discovery of mechanisms underlying M cell formation and function could be beneficial in designing novel and more effective strategies for mucosal delivery of vaccines and therapeutics.

Previously, we reported that in vivo short-term challenge with a nonintestinal bacterium, Streptococcus pneumoniae R36a, up-regulated M cell-mediated transport of microparticles across the FAE of rabbit Peyer’s patch (PP) to the gut immune system and systemic circulation, and that this was due to an increase in the number of operational M cells within the FAE (3, 4). This experimental model has proved valuable to establish important features of the highly dynamic nature of the FAE of intestinal PPs. Indeed, although it did not distinguish whether the increase in number of functional M cells was due to rapid conversion of enterocytes into M cells upon interaction with lymphocytes and molecules of the immune system or rather to maturation of predetermined M cells already inhabiting the FAE (3, 5), it allowed us to demonstrate, for the first time, that the immunologically relevant Ag-sampling function of the FAE could be rapidly up-regulated in vivo. More recently, this model enabled others to conclude that the apparent increase in the numbers of operational M cells was due to maturation of predetermined M cells and not to a rapid conversion of enterocytes (6).

The finding that certain bacteria can improve M cell transport across the intestinal barrier opened the way to identifying new means of achieving more effective mucosal delivery, e.g., of vaccines and therapeutics, and prompted us to investigate whether these events were mediated by soluble molecule(s) secreted either by the bacteria or by host cells. This question stemmed from previous in vivo and in vitro work, in which FAE epithelial cells showed morphological and functional features of M cells without being physically in contact with lymphocytes (4, 7, 8), suggesting...
that soluble mediator(s) might be involved in determining M cell phenotype. This issue was tackled by using a combination of proteomic analysis (two-dimensional (2D) gel electrophoresis and mass spectrometry), immunohistochemistry, and flow cytometry to identify molecules that were differentially expressed within intestinal luminal contents and tissues from rabbits challenged with bacteria, *S. pneumoniae* and *Escherichia coli*, that differed in their ability to regulate M cell transport (3, 4). We report in this study that in vivo challenge with *S. pneumoniae* induced a rapid increase of intraluminal levels of macrophage migration inhibitory factor (MIF) together with the appearance of MIF-producing CD11c+ cells located beneath the FAE of PPs and in close contact with the basolateral domain of epithelial cells of the FAE. We then assessed the effects of human rMIF on human intestinal epithelial Caco-2 cells, which have been extensively used to study M cell formation/ function in vitro (7, 8), a model that also offered the advantage of mimicking the precise spatial interaction between MIF and the basolateral domain of intestinal epithelial cells in vivo. We observed that MIF induced, within hours, the appearance of cells with biochemical and functional features of Ag-sampling M cells, and that similar effect of Raji B cells could be blocked by anti-MIF Ab. Furthermore, in vivo experiments with MIF-deficient mice showed that, in the latter, the rapid bacteria-mediated up-regulation of microparticle transport in the FAE of PPs was significantly reduced compared with wild-type mice.

**Materials and Methods**

**In vivo experimental procedures**

Bacterial challenge of rabbit intestinal PPs, using *S. pneumoniae* R36a or *E. coli* DH5α, as bacterial negative control (3, 4), or saline solution in intestinal isolated loops, was conducted, as described in detail elsewhere (3, 4, 6), to obtain a collection of luminal samples. These were used for the preparation of sterile, cell-free extracts. Luminal contents were centrifuged, filtered through 0.22-μm syringe filters, and stored at −80°C until used for proteomic analysis. MIF−/− mice were described, as previously developed (9), and backcrossed for >10 generations to a BALB/c genetic background; isolated intestinal loops were performed in MIF−/− and wild-type BALB/c mice, as described above. In each animal, pneumococci (Pn)- and *E. coli* DH5α-exposed PPs were excised, immersed in PBS/0.5 nM DTT for 10 min, and washed extensively to remove surface-bound microparticles. PPs were then cut into two pieces, one for immunohistochemistry and flow cytometry, and the other for proteomic analysis. MIF+/+/H9024 and wild-type mice were developed, as described previously (10), and backcrossed for more than 10 generations (11). Peptides generated from the tryptic digest were loaded onto a glass slide, stained with the specific primary and secondary Abs, and assayed by immunostaining with anti-zonula occludens (ZO)-1 mouse mAb (Caltag-Medsystem) and finally stained with FITC-labeled goat anti-mouse IgG Ab. Furthermore, in vivo experiments with MIF-deficient mice showed that, in the latter, the rapid bacteria-mediated up-regulation of microparticle transport in the FAE of PPs was significantly reduced compared with wild-type mice.

**Protemic analysis**

The 2D gel electrophoresis was carried, as described previously (11), by applying 25 μl of luminal extract samples to 375 μl of rehydration buffer before running on a 18-cm 3-10NL Immobiline DryStrip (Amersham Biosciences). After the second dimension, gels were stained with Sypro Ruby (12), according to manufacturer’s instructions (Bio-Rad), before imaging in a ProXpress Imager with Profinder software (PerkinElmer Life Sciences). The tiff images were analyzed using ProteinWeaver (Definiens) to assist in the manual identification of differentially expressed spots. Spots of interest were excised using a ProPick Excision robot (Genomic Solutions), and manual in-gel tryptic digestion was performed using modified porcine trypsin (Promega). Protein identification was conducted by de novo sequencing of the digest using a Q-ToF 2 Mass Spectrometer (Micromass) at the Joint Institute of Food Research—Norwich. Peptides generated from the tryptic digest were loaded onto a reverse-phase capillary column (75 μm internal diameter × 150 mm column, containing Symmetry C18 300 Å PEPmap packing; LC Packings) and eluted through a reverse-phase capillary column (75 μm internal diameter × 150 mm column, containing Symmetry C18 300 Å PEPmap packing; Waters) directly into the nano-electrospray ion source. Fragment ion spectrums were analyzed using the PepSeq de novo sequencing tool (Micromass). Sequence homology was determined by Protein-Protein BLAST searching against the National Center for Biotechnology Information nonredundant database.

**Immunohistochemistry and flow cytometry**

Frozen sections (7–8 μm) were obtained using a cryostat (Cryocut E; Reichert-Jung), and immunohistochemistry was conducted by incubation with biotin anti-MIF Ab. Anti-MIF Ab (M-CSF Biotechnology) diluted 1/500 v/v in PBS/1% BSA for 2 h at room temperature, followed by incubation with FITC-labeled mouse anti-goat Ig as secondary Ab for an additional 2 h. Immunohistochemistry was conducted by incubation with mouse anti-rabbit CD11c Ab (Chemicon International) following preparation of cell suspension. Briefly, PPs were treated with serum-free medium containing DTT, HEPEs, and 5 mM EDTA in HBSS for 90 min at room temperature to remove epithelial cells. Cells were then isolated using MACS separation column (Milteny Biotech) subsequently treated with Fix & Perm solution (Caltag-Medsystem) and finally stained with FITC-labeled N-20 anti-MIF Ab for flow cytometry analysis. Expression of CD74 on Caco-2 cells was also analyzed. Caco-2 cells were harvested with 0.25% trypsin and 1 mM EDTA for 2–3 min. The same treatment was conducted on Raji B cell suspensions of cultured cells for 15.0°C or Raji B cells were stained with mouse anti-human CD74, clone L2 (Biolegend), or mouse anti-human CD3, as negative control, for 1 h at room temperature. The cells were washed and incubated with FITC-conjugated goat anti-mouse IgG Ab for 1 h at room temperature and then analyzed by flow cytometry.

**In vitro model**

Caco-2 cells were obtained from the European Collection of Cell Cultures and used between passages 39 and 49. The cells were seeded onto the upper face of Transwell inserts (6.5 mm diameter, 3.0 μm pore size; Corning Glass, Costar) and grown on the filters for 14 days at 37°C, 5% CO2, until fully differentiated. Transepithelial electric resistance (TEER) was monitored throughout this period (Millicell-ERS; Millipore). Treatment of Caco-2 cells with cytokines was conducted only when TEER readings were above 300 Ω·cm2. MIF, IL-6, or IL-8 for control was added to the lower chamber and incubated at 37°C, 5% CO2, for up to 5 h at 0.1–0.3 μg/ml. In smaller scale experiment, Transwells were treated with a solution containing 0.1 μg/ml MIF previously saturated with 0.1 mg/ml human anti-MIF Ab. Fresh medium was added to the cultures, after which the Transwells were transferred to 4°C. Microparticle suspensions (0.5–5 μm Fluoresbrite YG Microspheres; Polysciences) were introduced into the loops for 30 min. Mice were sacrificed, and PPs were excised, immersed in PBS/0.5 mM DTT for 10 min, and washed extensively to remove surface-bound microparticles. PPs were weighed and individually solubilized in 0.5 ml of 15% potassium hydroxide (10) for 3 days (10). Finally, internalized microparticles were enumerated by CytoSoft (FCS500; Coulter Electronics) and flow cytometry (Coulter EPICS Altra; Coulter Beckman). Parallel experiments were conducted by seeding Caco-2 cells onto the lower face of the filters following an established procedure (7), culturing overnight, and then returning into the Transwell device, where they grew facing the lower chamber. In such cases, MIF was introduced into the upper compartment of the Transwell to interact with the basolateral domain of Caco-2 cells (24 mm diameter, 3.0 μm pore size). Microparticles was monitored in the same compartment. In some experiments, *Vibrio cholerae* N16961, resuspended in DMEM medium supplemented with 1% FCS and 1% nonessential amino acids, was added to the apical chamber of the Transwell (1.0 × 105 CFU/well) following challenge with MIF (3–5 h), and incubated for 1 h at 4°C, followed by 1 h at 37°C. At the end of reincubation periods, microparticle dimensions of the basolateral medium were isolated out onto Luria-Bertani agar plates and incubated at 37°C overnight. MIF-treated and control filters were washed and processed for transmission electron microscopy (TEM).

Polarization of the monolayer following challenge with MIF was assessed by immunostaining with anti-α-zonula occludens (ZO)–1 mouse mAb T8-754 (13), followed by incubation with anti-mouse IgG-FITC-labeled Ab. In the latter cases, filters were excised from the Transwell insert, placed onto a glass slide, stained with the specific primary and secondary Abs, and examined under a fluorescence microscope (JVC, Olympus BX60). Scurase isomaltase (SI) activity was determined at various intervals in Caco-2 cells either untreated, treated with MIF, or cocultured in the presence of M cell-inducing Raji B cells for 4–6 days, as described (8), as positive control. In this case, Caco-2 cells were grown on Transwell inserts (24 mm diameter, 3.0 μm pore size) for 14–18 days, and SI activity was determined after challenge with MIF (0.1 or 0.3 μg/ml) for 5–24 h, or alternatively exposed to increasing doses of MIF for 4 days (0.1 μg/ml on day 0; 0.2
µg/ml on day 1; 0.3 µg/ml on day 2; 0.5 µg/ml on day 3). The filters were excised from the plastic support, and SI activity was measured on cell homogenates using a glucose assay kit (Sigma-Aldrich). Specific enzyme activity was expressed as µM/mg of protein.

Anti-MIF Ab-blocking experiments

The above-described Caco-2/Raji B cell coculture model (8) was also used to determine the ability of anti-MIF Ab to block Raji B cell-mediated induction of M cells in vitro. To this end, anti-MIF polyclonal Ab (R&D Systems) was added to the coculture system at 0.1 or 0.2 µg/ml for the duration of the coculture period (4 days), and microparticle transport was monitored, as described above. Constitutive production of MIF by Raji B cells was determined in culture supernatant by Western blot using standard protocols.

Data analysis

In vitro transport of fluorescent microparticles in MIF- and Raji B cell-treated and control Caco-2 cells was monitored by measuring fluorescence intensity, i.e., fluorescence events per second, F, in each Transwell at several sampling times. A logistic function (14) was used to model the dependence of F on time, t, for each Transwell, as follows: \( y(t) = y_{\text{max}} - \ln(1 + e^{-(y_{\text{max}} - y_0)}t + \ln(F + 1), t = \text{time (minutes)}, y_{\text{max}} \) and \( y_0 \) are the maximum and minimum value of \( y \), respectively, and \( k \) is the maximum relative fluorescence increase rate, i.e., \( k = \max(\Delta F/\Delta t) \). The model was fitted with the observed data by nonlinear regression using the least squares method. Two main parameters were recorded for each Transwell, as follows: the fluorescence rate, \( k \), and the difference between the final and initial fluorescence intensities, \( y_{\text{max}} \) and \( y_0 \). Statistical analysis was conducted with the logarithm of the rates. Student’s \( t \) test was applied to compare these parameters between MIF-treated Transwells and untreated controls. For the in vitro Ab-blocking experiments in the Caco-2/Raji B cell model, a Tukey test for multiple samples comparison was applied.

For in vivo data from MIF- and wild-type mice, the natural logarithm of the ratio between the fluorescence (events/ml/g tissue) detected within the gut lumen and within PP was determined. We and others have previously shown that \( S. \) pneumoniae R36a, in contrast to \( E. \) coli DH5α, rapidly increased M cell-mediated transport in vivo (3, 4, 6). This prompted us to compare the luminal contents of \( S. \) pneumoniae- and \( E. \) coli-challenged gut to identify soluble cytokine(s) underlying this event. Proteomic analysis by 2D gel electrophoresis and mass spectrometry of luminal contents following short-term challenge with \( S. \) pneumoniae R36a (Pn), \( E. \) coli DH5α as negative bacterial control (3, 4), or saline solution was conducted. Overlay of gels from \( E. \) coli- and \( S. \) pneumoniae-challenged samples revealed a variety of differentially expressed proteins 3 h after the application of the bacterial stimulus (Fig. 1A, overlay Pn vs PBS; Fig. 1B, Pn vs \( E. \) coli), and MIF was the only cytokine identified. Detectable levels of MIF were secreted into the lumen upon challenge with R36a, but not with \( E. \) coli or PBS.

Results

In vivo bacterial challenge induced secretion of MIF into the gut lumen and within PP

We and others have previously shown that \( S. \) pneumoniae R36a, in contrast to \( E. \) coli DH5α, rapidly increased M cell-mediated transport in vivo (3, 4, 6). This prompted us to compare the luminal contents of \( S. \) pneumoniae- and \( E. \) coli-challenged gut to identify soluble cytokine(s) underlying this event. Proteomic analysis by 2D gel electrophoresis and mass spectrometry of luminal contents following short-term challenge with \( S. \) pneumoniae R36a (Pn), \( E. \) coli DH5α as negative bacterial control (3, 4), or saline solution was conducted. Overlay of gels from \( E. \) coli- and \( S. \) pneumoniae-challenged samples revealed a variety of differentially expressed proteins 3 h after the application of the bacterial stimulus (Fig. 1A, overlay Pn vs PBS; Fig. 1B, Pn vs \( E. \) coli), and MIF was the only cytokine identified. Detectable levels of MIF were secreted into the lumen upon challenge with R36a, but not with \( E. \) coli or PBS.

### Table I. TEER of MIF-treated Caco-2 cell monolayers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. Transwell</th>
<th>t = 0</th>
<th>t = 120</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: MIF (0.3 µg/ml)</td>
<td>46</td>
<td>429.9 ± 33.5</td>
<td>490.6 ± 22.9</td>
</tr>
<tr>
<td>Control</td>
<td>29</td>
<td>424.7 ± 27.9</td>
<td>494.6 ± 35.4</td>
</tr>
<tr>
<td>B: MIF (0.3 µg/ml)</td>
<td>23</td>
<td>577.8 ± 83.9</td>
<td>510.2 ± 79.0</td>
</tr>
<tr>
<td>Control</td>
<td>11</td>
<td>528.5 ± 99.0</td>
<td>465.5 ± 99.6</td>
</tr>
<tr>
<td>C: Raji + αMIFAb</td>
<td>12</td>
<td>394.8 ± 26.5</td>
<td>340.1 ± 23.2</td>
</tr>
<tr>
<td>Raji - αMIFAb</td>
<td>12</td>
<td>408.3 ± 27.0</td>
<td>322.5 ± 85.2</td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>400.3 ± 37.0</td>
<td>382.5 ± 90</td>
</tr>
</tbody>
</table>

* TEER of the Caco-2 cell cultures was monitored at the beginning and end of each experiment. Cells were seeded onto the upper (A) or lower (B) face of Transwell inserts until fully differentiated, and then challenged with MIF or left untreated (control). In additional experiments (C), Caco-2 cells were cocultured with Raji B cells in the presence or absence of anti-MIF Ab (αMIFAb). Values represent mean ± SD.
Subsequent immunohistochemistry confirmed that challenge with S. pneumoniae rapidly (3 h) induced production of MIF in the PPs (Fig. 2). Cells stained with anti-MIF Ab were observed within lymphoid follicles of PPs challenged with S. pneumoniae (Fig. 2A), but not in E. coli-treated tissues (Fig. 2B). Flow cytometry analysis showed that ~20% of CD11c+ cells isolated from PPs expressed MIF after challenge with Pn (Fig. 2D), and that this was not detected in control (E. coli) PPs (Fig. 2C). The distribution of MIF-producing cells is noteworthy, being located in the peripheral area of the FAE, which is rich in M cells (4); in contrast, no MIF-positive cells were observed in the dome area, which is M cell deficient. These data taken together pointed to MIF as a potential candidate molecule with the ability to affect M cell-mediated transport.

Human rMIF converts human Caco-2 cells into microparticle- and bacteria-sampling cells

The human intestinal Caco-2 cell line has been used extensively as an experimental model to study lymphocyte-mediated in vitro differentiation of M cells in various systems (7, 8, 15). Unchanged values of TEER (Table I) of the Caco2 cell culture (ranging between 300 and 450 Ω·cm²) and detailed analysis of anti-ZO-1 (16) expression (Fig. 3) demonstrated both the integrity and normal polarization of the monolayer following challenge with MIF.

FIGURE 3. Detailed immunohistochemistry analysis with anti-ZO-1 mAb was used to assess polarization of the Caco-2 cell monolayer following basal challenge with human rMIF. Correct expression of ZO-1 and unchanged TEER (Table I) were maintained in Caco-2 cell monolayer during exposure to different doses of MIF.

FIGURE 4. MIF-induced particle transport in Caco-2 cells. Maximum relative fluorescence-increase rate, k, plotted against the difference between the logarithms of the final and initial fluorescence intensities, \( y_{\text{max}} - y_{\text{in}} \), determined following 3 h of treatment with MIF (○) or control (●) of Caco-2 cells grown on the upper side of Transwell filters. A, Shows microparticle transport in Caco-2 treated with 0.3 μg/ml MIF. B, Caco-2 cells were grown with the apical domain facing the lower chamber of the Transwell culture system and treated with MIF (0.3 μg/ml). For summary of the data, see Table I. C and D show examples of the fluorescence intensities measured from two individual Transwells: one treated with MIF (○), and one untreated control (●); ○, indicate the fluorescence measurements in time, and the lines the fitted logistic model. Temperature-dependent transport of microparticles by Caco-2 cell following basolateral challenge with MIF for 5 h (E). Transport was absent at 4°C, but started rapidly when cultures were placed at 37°C. Values represent means of fluorescent events/s ± SD of seven selected Transwells as determined by flow cytometry. Both F and inset show TEM of intracellular V. cholerae (within box). Caco-2 cells were treated with MIF for 5 h, and then V. cholerae were added to the apical compartment of the Transwell for 60 min. Arrowheads indicate profound alteration of the brush border, typical of M cells.
Transport of fluorescent microparticles through the intestinal epithelial cell monolayer was analyzed by describing the fluorescence intensity detected in the basolateral compartment as a function of time in each Transwell. From the model, two parameters were derived, as follows: 1) the rate of change of fluorescence; 2) the difference between the final and initial fluorescence intensities \((\gamma_{\text{fin}} - \gamma_0)\), determined following challenge of Caco-2 cells with 0.3 \(\mu\text{g/ml}\) (Table II, treatment A and B) or 0.1 \(\mu\text{g/ml}\) (B) MIF or left untreated (Con). Treatments A and B were carried out with cells seeded on the upper surface of the Transwell insert, and transport was monitored in the lower chamber. In E, cells were seeded on the lower surface of the Transwell insert, facing the lower chamber, and transport was monitored in the upper chamber; MIF (0.3 \(\mu\text{g/ml}\)) was introduced into the upper chamber. In C, we show that addition of anti-MIF Ab to the culture (MIF\(\text{Ab}^{B}\)) treated with 0.1 \(\mu\text{g/ml}\) MIF blocked particle transport. D shows that other proinflammatory cytokines IL-6 and IL-8 had no effect on M cell transport (No. = number of Transwells).

### Table II. Microparticle transport in MIF-treated Caco-2 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(\mu\text{g/ml})</th>
<th>No.</th>
<th>Mean SD</th>
<th>(k) (min(^{-1}))</th>
<th>(\gamma_{\text{fin}} - \gamma_0)</th>
<th>Mean SD</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: MIF</td>
<td>0.3</td>
<td>46</td>
<td>0.043</td>
<td>0.035</td>
<td>1.66 ± 0.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>0</td>
<td>29</td>
<td>0.007</td>
<td>0.012 &lt; 0.0001</td>
<td>0.52 ± 0.48 &lt; 0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B: MIF</td>
<td>0.1</td>
<td>23</td>
<td>0.040</td>
<td>0.042</td>
<td>1.92 ± 0.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>0</td>
<td>12</td>
<td>0.010</td>
<td>0.004 &lt; 0.03</td>
<td>1.25 ± 0.27 &lt; 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C: MIF(\text{Ab})</td>
<td>0.1</td>
<td>6</td>
<td>0.009</td>
<td>0.009</td>
<td>0.51 ± 0.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>0</td>
<td>6</td>
<td>0.031</td>
<td>0.021 NS</td>
<td>1.36 ± 0.57 NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D: IL-6</td>
<td>0.1</td>
<td>12</td>
<td>0.002</td>
<td>0.004</td>
<td>0.25 ± 0.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>0.1</td>
<td>12</td>
<td>0.006</td>
<td>0.009</td>
<td>0.54 ± 0.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>0</td>
<td>12</td>
<td>0.005</td>
<td>0.005 NS</td>
<td>0.51 ± 0.41 NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E: MIF</td>
<td>0.3</td>
<td>23</td>
<td>0.830</td>
<td>0.049</td>
<td>4.66 ± 2.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>0</td>
<td>11</td>
<td>0.043</td>
<td>0.026 &lt; 0.01</td>
<td>0.02 ± 0.99 &lt; 0.004</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(\gamma_0\): initial fluorescent intensity in two individual Transwells, one treated with MIF (C) and one left untreated (D). Also, MIF-induced transport of microparticles was temperature dependent, as shown by the absence of transport when filters were incubated at 4°C (Fig. 4E). Indeed, Caco-2 monolayers exposed to MIF acquired transcytotic activity only when transferred to 37°C, with a rapid accumulation of microparticles into the basolateral compartment after 30 min. This result, along with the lack of alteration of both TEER and cell polarization, demonstrated that MIF induced active transcellular transport. Finally, evidence of transcellular transport was provided by TEM analysis of Caco-2 cells treated with MIF and subsequently challenged with \(V.\) cholerae (Fig. 4F), an organism known to be specifically transported by M cells in vivo (17) and in vitro (7). Following treatment with MIF, the number of \(V.\) cholerae organisms translocated increased nearly 50-fold.

**FIGURE 5.** Western blot (A) showing spontaneous secretion of MIF in Raji B cell culture supernatant (lane 1, Raji supernatant; lane 2, culture medium; lane 3, human rMIF). Levels of SI did not decrease in Caco-2 cells exposed to MIF (0.3 \(\mu\text{g/ml}\)) for either 5 or 24 h (B, MIF, 5 and 24 h, respectively). In contrast, levels of SI decreased after 3 days (MIF3d) in the presence of increasing concentrations of MIF (0.1 \(\mu\text{g/ml}\) on day 0; 0.2 \(\mu\text{g/ml}\) on day 1; 0.3 \(\mu\text{g/ml}\) on day 2; 0.5 \(\mu\text{g/ml}\) on day 3) in a manner similar to that observed in a Caco-2/Raji B cell coculture M cell model (raji). The addition of anti-MIF (0.1 \(\mu\text{g/ml}\), C, or 0.2 \(\mu\text{g/ml}\), D) Ab to the in vitro Caco-2/Raji cocultures significantly reduced microparticle transport in this coculture model. Maximum relative fluorescence-increasing/decreasing rate, \(k\), vs the difference between the logarithm of the final and initial fluorescence intensity, \(\gamma_{\text{fin}} - \gamma_0\). C and D, Show particle transport by Caco-2 cells in coculture with Raji B cells (C) or in coculture with Raji B cells and anti-MIF Ab (D) and the respective untreated controls (○). For summary of the data, see Table II.
from 7 ± 0.61 × 10^3 in controls (n = 5) to 3.45 ± 1.3 × 10^5 in MIF-treated cultures (n = 5), whereas no translocation was observed at 4°C. Again, in this case, no significant alteration of TEER was observed following challenge with MIF or the subsequent incubation with *V. cholerae*.

**Anti-MIF Ab blocks Raji B cell-induced conversion of Caco-2 cells into Ag-sampling cells**

Coculture with Raji B cells induced the conversion of Caco-2 cells into functional M cells even when these two cell types were not in physical contact (8). We found that Raji cells constitutively produced MIF in culture (Fig. 5A), which prompted us to test the hypothesis that MIF was involved in Raji-mediated conversion of Caco-2 cells. The addition of anti-MIF at 0.1 or 0.2 µg/ml Ab (Fig. 5, C and D, respectively; summary in Table III) for the duration of the coculture significantly suppressed transport of microparticles. A Tukey test for multiple samples comparison showed that particle transport, measured as fluorescence rates and the differences between final and initial fluorescence values, did not differ between Caco-2 cells cultured alone or cocultured with Raji B cells in the presence of anti-MIF Ab (99% confidence). In contrast, the same parameters were significantly higher (p < 0.01) in Caco-2/Raji cocultures in the absence of anti-MIF Ab.

**SI is reduced in MIF-treated Caco-2 cells**

Reduction of SI is used to demonstrate conversion of Caco-2 cells into Ag-sampling cells (7, 15). We analyzed the levels of SI activity in Caco-2 cell monolayers, either treated with MIF, left untreated, or cocultured in the presence of Raji B cells. Although no significant changes were observed after 5 to 24 h of exposure to MIF (0.3 µg/ml), Caco-2 cells cultured for 3 days in the presence of increasing concentrations of MIF (0.1 µg/ml on day 0; 0.2 µg/ml on day 1; 0.3 µg/ml on day 2; 0.5 µg/ml on day 3) showed a decline of SI activity similar to that observed following coculture with Raji B cells (Fig. 5B). These data suggested that MIF induced the rapid appearance of M cell-specific functions, such as transcytosis of particles and bacteria, which preceded the acquisition of specific biochemical features. No change in villin distribution (18) was observed in MIF-treated cells (data not shown), suggesting that the alteration of actin-based cytoskeleton, which is not related to M cell transport (7), is likely to be the result of mechanical interaction with lymphocytes.

**Bacteria-mediated up-regulation of particle transport in the FAE is absent in MIF−/− mice**

Finally, the role of MIF in the events underlying bacteria-mediated up-regulation of particle transport in the gut was assessed in MIF−/− mice. The ratio between the number of particles detected by flow cytometry within PP challenged with *S. pneumoniae* R36a or PBS was estimated for each MIF−/− (n = 6) or wild-type (WT) (n = 12) mouse. The ratio for MIF−/− was −0.11 ± 1.10, and the wild-type was 1.32 ± 1.44 (linear scale 0.89 and 3.66, respectively). Both groups of measurements were compared by Student’s t test; the associated p value was 0.0173.

**Discussion**

In this study, we report in vivo and in vitro experiments demonstrating a role for the cytokine MIF in the regulation of M cell-mediated transport in the intestine. It is accepted that M cells of the FAE of PP play a pivotal role in mucosal immune surveillance by continuously transporting material from the lumen to the underlying lymphoid tissue. The finding that certain bacteria can rapidly up-regulate M cell-mediated transport in vivo (3, 4, 6) led us to investigate whether these events were mediated by soluble molecules released following host-bacteria interaction. Data from several groups have suggested previously that molecule(s) produced by the immune system may play a role in the induction of M cell phenotype. Indeed, conversion of Caco-2 cells into M cells by coculture with lymphocytes was not limited to cells obviously in contact with B cells (7), and later, the concept of a soluble mediator was further highlighted by the observation that Raji B cells converted Caco-2 cells when the two cell types were cocultured.
but were not physically in contact (8). Furthermore, the dome-associated crypts possess a specialized epithelium on the dome side, in which the development of M cells and the suppression of the generation of other cell types (e.g., goblet cells) take place. These regions are in contact with the lymphoid tissue of the follicle, but contain very few lymphocytes (19). Finally, in vivo studies showed cells with M cell phenotype, which were not in contact with lymphocytes (4). Thus, the sum of in vitro and in vivo studies suggested the presence of soluble factor(s) that plays a critical role in lymphoepithelial cross-talk in the gut. However, the nature and origin of such molecule(s) remained to be determined.

The short time required for in vivo bacteria-mediated up-regulation of M cell transport suggested that any soluble factor involved would probably be secreted as the result of rapid posttranslational events, which would not be apparent from analysis of mRNA expression. In this study, proteomic analysis of the luminal contents and immunohistochemistry revealed that short-term challenge with bacteria (S. pneumoniae), which up-regulated the number of functioning M cells, induced the production of MIF. CD11c+ cells producing MIF were located within the lymphoid tissue of PP in close proximity to the basal domain of epithelial cells in an area rich in M cells (4) and, as such, strategically placed to impact on their function. Physiological production of low levels of MIF in the gut has been observed in healthy gastric and intestinal mucosa, including Caco-2 cells (20). However, in contrast to other cytokines that are secreted by epithelial cells in the basolateral direction, production of MIF was characterized by a highly polarized secretion with nearly 80% released apically into the lumen and the remaining amount (~1 ng/ml) in the basolateral direction (20). This, together with our immunohistochemistry data, suggested that the spatial interaction between MIF and the basal side of the FAE might be critical for the MIF-epithelial cell interaction.

With this in mind, we used human intestinal Caco-2 cells as an established experimental model (7, 8) to test the role of MIF on M cell-mediated transport. The human in vitro system offered the possibility not only of reproducing faithfully the spatial interaction between MIF and the basolateral domain of the epithelial cells, but also the generation of data relevant to potential application in humans. Addition of MIF to the basolateral compartment of Caco-2 cell Transwell cultures induced the appearance of cells with functional and biochemical features of M cells, including temperature-dependent transcytosis of latex microparticles and M cell-specific bacteria (V. cholerae) and a reduction of SI. In contrast, the introduction of MIF into the apical compartment had no effect (our unpublished observation). In our experiments, transepithelial transport of microparticles by Caco-2 cells following challenge with MIF has been analyzed to evaluate both the transport rate and total amount of particles. In particular, the transport rate has an important biological significance in the context of M cell transport. In fact, a rapid transport (high rate) is a typical feature of M cell transport (21). This mode of transport is faithfully mimicked in Caco-2 cells exposed to MIF (see example in Fig. 4C), but not in control cultures (see example in Fig. 4D), the latter being characterized by the absence of high rate of transport.

Although the exact sequence of events remains to be fully determined, the combination of in vivo and in vitro data makes it plausible that after the rapid internalization and transport by FAE-associated M cells (22), S. pneumoniae interact with CD11c+ dendritic cells within the lymphoid tissue of the PPs, inducing the production of MIF by these cells (23). The latter event can be triggered by Gram-positive bacteria-derived exotoxins that are potent inducers of MIF secretion (24). Secretion of MIF is followed by its interaction with the basolateral domain of M or pre-M cells of the FAE, leading to the up-regulation of M cell-mediated transport. It has been reported that CD74 binds MIF on the surface of cells and initiates MIF signal transduction (25). We failed to detect CD74 expression on Caco-2 cells (data not shown), thus suggesting that this pathway is not involved in MIF-mediated regulation of M cell transport. However, CD74 is likely to be only one of the potential receptors for MIF; indeed, not all cells (i.e., neutrophils) targeted by MIF express surface CD74 (26). Also, it has to be stressed that a mechanism that bypasses the need for a MIF-specific receptor has been described (27).

The suggestion that MIF mediates the conversion of Caco-2 cells into M cell-like cells does not necessarily mean that terminally differentiated enterocytes undergo a conversion into M cells in vivo. In fact, it has been argued that the lack of terminal differentiation of Caco-2 closely mimics that of crypt stem cells more than that of fully mature and differentiated enterocytes (28). The notion that MIF is more likely to impact on the function of the M cells (or pre-M cells) already inhabiting the FAE, rather than participating in the genesis of de novo formation of enterocytes into M cells, comes from our observation on MIF−/− mice. These mice appeared to have physiological numbers of M cells, but they failed to show any rapid increase of transepithelial transport of microparticles across the FAE following in vivo challenge with Pn compared with wild-type mice. Also, the mechanisms underlying these events are likely to differ from those related to other examples of bacteria (Salmonella)-induced rapid de novo formation of M cells previously reported (29). Indeed, in vivo challenge with the Gram-negative Salmonella failed to induce production of MIF (A. Man, and C. Nicoletti, unpublished observation), suggesting that multiple signals and biochemical pathways are involved in the control of M cell formation/function. Interestingly, a close association between inflamed ileal mucosa and increased number of M cells has been described in humans (30); thus, it is possible in such a case that the inflammation-associated increment of M cell number might be linked to a local production of MIF. These findings have a direct bearing on several aspects of the biology of the intestinal immune system and its interaction with bacteria. They demonstrate that certain bacteria up-regulate M cell-mediated transport in the gut by inducing MIF production, and, in so doing, facilitate bacterial passage across the intestinal barrier. This may also provide a tool to improve delivery of vaccines and therapeutics to the mucosal immune system through increased M cell-mediated transport. Indeed, although MIF long-term overexpression in the gut has been associated with certain pathologies, such as sporadic human adenomas (31) and colitis (23), low levels of MIF are constitutively produced in healthy intestinal mucosa (20), suggesting that MIF plays a role in maintaining intestinal homeostasis. Furthermore, the finding that MIF showed a beneficial proapoptotic activity in colon cancer therapy (32) suggests that MIF could be safely delivered to the gut. Thus, it would be tempting to suggest that up-regulating M cell-mediated transport in the PP by targeted delivery of MIF via M cell-specific engineered MIF-producing bacterial vectors (33) or MIF-loaded biodegradable microparticles (34) may represent a novel and effective strategy to improve mucosal delivery of vaccines and therapeutics to the gut immune system.

Acknowledgments
We thank I. T. Johnson for his comments and continuous support throughout this project, and S. Strobob for helpful discussion. We are also grateful to M. Furuse for the anti-ZO-1 Ab, T. Hirst for the V. cholerae strain, E. Morlon for technical help, and P. Pople for his help in computer work.

Disclosures
The authors have no financial conflict of interest.
References


