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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 naso-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 C11C 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 developed, as described previously (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 PBS-treated loops were conducted. Following 3 h of bacterial challenge, fluorescent microparticles (0.5 mM Fluoresbrite YG Microspheres; Polysciences) were introduced into the loops for 30 min. Mice were sacrificed, and PPs were excised, immersed in PBS/0.5 nM 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 for 3 days (10). Finally, internalized microparticles were enumerated by cytometry (FC500; Beckman Coulter). Values were expressed as number of events/μl tissue. In additional mouse experiments, the luminal contents were collected at the end of bacterial challenge, centrifuged, and filtered sterile, and levels of MIF were determined by ELISA using a commercially available kit (USCN Life Sciences). All animal experiments were approved by the local ethics committee and conducted according to guidelines of the Animal Act 1986 (Scientific Procedures) of the United Kingdom.

**Proteomic 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), before running on 18-cm 3-10NL Immobiline DryStrip (Amersham Biosciences) for 2–3 min. The same treatment was conducted on Raji B cell suspensions for the cultures used for the Caco-2 cell experiments. Alternatively, Raji B cells were stained with mouse anti-human CD74, clone LN2 (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 –18 days, and SI activity was determined at various intervals in Caco-2 cells with cytokines was conducted only when TEER readings were stored throughout this period (Millicell-ERS; Millipore). Treatment of Caco-2 cells with cytokines was conducted only when TEER readings were 250 Ω·cm². MIF, IL-6, or IL-8 for control was added to the lower chamber and incubated at 37°C, 5% CO₂, 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, which after the Transwells were transferred to 4°C. Microparticle suspensions (0.5-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 nM 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 for 3 days (10). Finally, internalized microparticles were enumerated by cytometry (FC500; Beckman Coulter). Values were expressed as number of events/μl tissue. In additional mouse experiments, the luminal contents were collected at the end of bacterial challenge, centrifuged, and filtered sterile, and levels of MIF were determined by ELISA using a commercially available kit (USCN Life Sciences). All animal experiments were approved by the local ethics committee and conducted according to guidelines of the Animal Act 1986 (Scientific Procedures) of the United Kingdom.

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**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 primary anti-MIF Ab (Chemicon International) diluted 1/500 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. A solution of a CD11c−enriched cell population from rabbit PPs was conducted by magnetic beads coated 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 (Miltenyi Biotec) 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 for the cultures used for the Caco-2 cell experiments. Alternatively, Raji B cells were stained with mouse anti-human CD74, clone LN2 (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.
FIGURE 1. Overlays of 2D gels of intestinal luminal contents from rabbits challenged with PBS and \( S. pneumoniae \) (A) and \( E. coli \) and \( S. pneumoniae \) (B). Orange indicates proteins expressed in the luminal contents following challenge with \( S. pneumoniae \); blue indicates the proteins expressed following challenge with PBS (A) or \( E. coli \) (B), with common expression of protein spots in black. Differentially expressed spots were identified by mass spectrometry; MIF spot circled.

\[ \mu g/ml \text{ on day 1; 0.3 } \mu g/ml \text{ on day 2; 0.5 } \mu g/ml \text{ on day 3}. \] 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 nU/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 \( \mu g/ml \) 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:

\[ y(t) = \frac{y_{\text{max}}}{1 + e^{\alpha t}} + y_0 \]

where \( y = \text{ln}(F + 1) \), \( t = \text{time (minutes)} \), \( y_{\text{max}} \) 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/s/ml/g tissue) detected within PP challenged with Pn and PBS was estimated for each mouse. Both groups of measurements were compared by Student’s \( t \) test.

**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 \( E. coli \) DH5\( \alpha \), 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), \( E. coli \) DH5\( \alpha \) 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 ( \mu g/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 ( \mu g/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 ± 90.0</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, $y_{\text{max}} - y_{\text{f}}$, 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 (C), and one untreated control (D); ●, 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 determined following challenge of Caco-2 cells with 0.3 μg/ml 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 μg/ml) was introduced into the upper chamber. In C, we show that addition of anti-MIF Ab to the culture (MIFAb) treated with 0.1 μg/ml MIF blocked particle transport. D shows that other proinflammatory cytokines IL-6 and IL-8 had no effect on M cell transport.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>μg/ml</th>
<th>No.</th>
<th>Mean</th>
<th>SD</th>
<th>p</th>
<th>Mean</th>
<th>SD</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>k (min⁻¹)</td>
<td>y_max - y₀</td>
<td></td>
<td>k (min⁻¹)</td>
<td>y_max - y₀</td>
<td></td>
</tr>
<tr>
<td>A: MIF</td>
<td>0.3</td>
<td>46</td>
<td>0.043</td>
<td>0.035</td>
<td>1.66</td>
<td>0.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>29</td>
<td>0.007</td>
<td>0.012</td>
<td>&lt;0.0001</td>
<td>0.52</td>
<td>0.48</td>
<td>&lt;0.0001</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</td>
<td>0.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>12</td>
<td>0.010</td>
<td>0.004</td>
<td>&lt;0.03</td>
<td>1.25</td>
<td>0.27</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>C: MIFAB</td>
<td></td>
<td>6</td>
<td>0.099</td>
<td>0.009</td>
<td>0.51</td>
<td>0.61</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</td>
<td>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</td>
<td>0.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>12</td>
<td>0.065</td>
<td>0.005</td>
<td>NS</td>
<td>0.51</td>
<td>0.41</td>
<td>NS</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</td>
<td>2.08</td>
<td></td>
<td></td>
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<tr>
<td>Con</td>
<td>11</td>
<td>0.043</td>
<td>0.026</td>
<td>&lt;0.01</td>
<td>0.02</td>
<td>0.99</td>
<td>&lt;0.004</td>
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</tbody>
</table>

Mean ± SD of the maximum relative rate of fluorescence increase (k) and the difference between the logarithms of the final and initial fluorescence intensities (y_max - y₀), determined following challenge of Caco-2 cells with 0.3 μg/ml (A) or 0.1 μg/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 μg/ml) was introduced into the upper chamber. In C, we show that addition of anti-MIF Ab to the culture (MIFAB) treated with 0.1 μg/ml MIF blocked particle transport. D shows that other proinflammatory cytokines IL-6 and IL-8 had no effect on M cell transport.

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. Fig. 4A shows microparticle transport in Caco-2 cells treated with 0.3 μg/ml MIF or left untreated as controls. Cells treated with MIF, at both 0.1 and 0.3 μg/ml (Table II, treatments A and B), showed significantly greater rates of increase in fluorescence (p = 0.02 and <0.0001 for 0.1 and 0.3 μg/ml, respectively) and greater differences between final and initial fluorescence values (p = 0.0132 and <0.0001 for 0.1 and 0.3 μg/ml, respectively) than their respective untreated controls, indicating that exposure to MIF induced Ag-sampling capability in Caco-2 cells. Smaller scale experiments showed that anti-MIF Ab blocked particle transport (Table II, treatment C). Importantly, other proinflammatory cytokines, such as IL-6 and IL-8, which also play a major role in the gut in response to bacteria, were used as controls, and these had no effect on M cell-mediated transport at similar concentrations (Table II, treatment D). To further confirm the involvement of active transcellular transport, experiments were also conducted whereby transport was monitored at 4°C and cells were grown on filters facing the lower chamber of the Transwells, as described (7) (Fig. 4B and Table II, treatment E). In the latter case, MIF was applied to the upper chamber, and transport of microparticles was monitored in the same compartment. Here too, cells treated with MIF showed significantly faster fluorescence uptake rates (p = 0.009) and greater differences between final and initial fluorescence values (p = 0.004). Fig. 4, C and D, shows examples of the rate of change of fluorescence and the difference between the final and initial fluorescence intensities 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.
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. The addition of anti-MIF Ab blocks Raji B cell-induced 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 the PPs challenged with Pn or PBS was determined in each mouse. Particle transport increased in wild-type following challenge with Pn, but there was no increase in MIF-deficient mice (Fig. 6). The natural log of the ratio was 1.33 ± 0.11 for wild-type and MIF−/− mice, respectively; the associated p value was 0.017. In addition, intraluminal levels of MIF were determined in mice treated with Pn or PBS. We observed that up-regulation of M cell transport was associated to a ~4-fold increase of intraluminal levels of MIF in mice challenged with S. pneumoniae. Levels of MIF ranged between 0.4 and 0.5 μg/ml in Pn-challenged mice compared with 0.1 μg/ml found in control groups.

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.

Table III. Effects of anti-MIF Ab on particle transport by Caco-2 cells cocultured with Raji B cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>k (min⁻¹)</th>
<th>ymax − y0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raji</td>
<td>0.168(a)</td>
<td>4.24(a)</td>
</tr>
<tr>
<td>Raji + αMIF</td>
<td>0.061(b)</td>
<td>1.53(b)</td>
</tr>
<tr>
<td>None</td>
<td>0.003(b)</td>
<td>0.19(b)</td>
</tr>
<tr>
<td>Expt. B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raji</td>
<td>0.034(a)</td>
<td>1.934(a)</td>
</tr>
<tr>
<td>Raji + αMIF</td>
<td>0.010(b)</td>
<td>1.035(b)</td>
</tr>
<tr>
<td>None</td>
<td>0.003(b)</td>
<td>0.424(b)</td>
</tr>
</tbody>
</table>

* Mean ± SD of the maximum relative rate of fluorescence increase (k), and the difference between the logarithms of the final and initial fluorescence intensities (ymax − y0), of particles transported by Caco-2 cells cultured alone (None), or with Raji B cells either in the presence of 0.1 μg/ml (Expt. A) or 0.2 μg/ml (Expt. B) or absence (Raji) of anti-MIF Ab. Equal letters denote means that are not significantly different according to a Tukey test (99% confidence). Means indicated with “a” are significantly different from means indicated with “b” (No. = number of Transwells).

FIGURE 6. Reduced levels of bacteria-induced up-regulation of microparticle transport in MIF−/− mice. The natural logarithm (Ln) of the ratio (FEspec/FEPBS) between the fluorescence (microparticles/ml/mg tissue) 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.
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 fol-
licle, but contain very few lymphocytes (19). Finally, in vivo stud-
ies 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-regu-
lation of M cell transport suggested that any soluble factor in-
volved would probably be secreted as the result of rapid posttrans-
lation 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 chal-
lenge with bacteria (S. pneumoniae), which up-regulated the num-
ber 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 intesti-
nal mucosa, including Caco-2 cells (20). However, in contrast to
other cytokines that are secreted by epithelial cells in the basolat-
eral direction, production of MIF was characterized by a highly
polared secretion with nearly 80% released apically into the lu-
men and the remaining amount (~1 ng/ml) in the basolateral di-
rection (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 hu-
mans. Addition of MIF to the basolateral compartment of Caco-2
cell Transwell cultures induced the appearance of cells with func-
tional 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 intro-
duction 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 charac-
terized by the absence of high rate of transport.

Although the exact sequence of events remains to be fully de-
termined, 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 + den-
ritic cells within the lymphoid tissue of the PPs, inducing the pro-
duction of MIF by these cells (23). The latter event can be
triggered by Gram-positive bacteria-derived exotoxins that are po-
tent 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 trans-
port. 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 suggest-
ing 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-spe-
cific 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 termi-
nally differentiated enterocytes undergo a conversion into M cells
in vivo. In fact, it has been argued that the lack of terminal dif-
ferentiation 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 par-
ticipating in the genesis or de novo conversion 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 micro-
particles across the FAE following in vivo challenge with Pn com-
pared 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 pas-
sage across the intestinal barrier. This may also provide a tool to
improve delivery of vaccines and therapeutics to the mucosal im-
une system through increased M cell-mediated transport. Indeed,
although MIF long-term overexpression in the gut has been asso-
ciated 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 vac-
cines and therapeutics to the gut immune system.

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Disclosures
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