

# Monocyte and M1 Macrophage-induced Barrier Defect Contributes to Chronic Intestinal Inflammation in IBD

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**Background:** Macrophages are key players in inflammatory bowel diseases (IBD). This study aimed to determine site-specific effects of defined macrophage subtypes on the integrity of the intestinal epithelial barrier.

**Methods:** Macrophage subtypes in situ in intestinal specimens of patients with IBD were visualized by immunohistochemistry. In vitro polarization of human peripheral CD14<sup>+</sup> cells yielded M1 or M2 macrophages. The influence of primary monocytes or macrophage subtypes on epithelial barrier integrity was analyzed by transepithelial resistance measurements, Western blot analysis, confocal laser scanning microscopy, and cytometric bead array in a coculture model of primary human macrophages and layers of intestinal epithelial cell lines.

**Results:** The lamina propria of the inflamed intestine in patients with IBD, predominantly in Crohn's disease, is massively infiltrated by CD68<sup>+</sup> cells also positive for inducible nitric oxide synthase and tumor necrosis factor (TNF)  $\alpha$ . The presence of M1 macrophage shifted the balance in the local macrophage compartment towards a proinflammatory state. In the coculture model, monocytes and M1 macrophages reduced transepithelial resistance as a marker for epithelial barrier integrity. The mechanisms for paracellular leakage included intracellular relocalization of tight junction proteins like claudin-2 and epithelial cell apoptosis. Determined by specific cytokine blockade, M1 macrophages exerted their deleterious effect mainly through TNF- $\alpha$ , whereas monocyte-mediated damage was driven by the inflammasome effector cytokines, interleukin-1 $\beta$  and interleukin-18.

**Conclusions:** Lamina propria monocytes and M1 macrophages invading intestinal tissues directly contribute to disrupting the epithelial barrier through deregulation of tight junction proteins and induction of epithelial cell apoptosis, thus driving intestinal inflammation in IBD.

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**Key Words:** inflammatory bowel diseases, Crohn's disease, macrophage subtypes, epithelial barrier

Inflammatory bowel diseases (IBD) result from deregulated responses of the innate and the adaptive immune system. While the latter has been studied extensively in the past, the significance of the innate system returned into the spotlight when a decade ago the risk for developing Crohn's disease (CD) was correlated to distinct mutations within an intracellular pattern recognition receptor namely within the caspase recruitment domain-containing protein 15 (CARD15/NOD2).<sup>1</sup> Accordingly, clinical studies provide evidence that stimulation of cells of the innate immune system, e.g., by granulocyte-macrophage

colony-stimulating factor, decreases intestinal inflammation and thus disease severity in patients with CD.<sup>2,3</sup>

Tumor necrosis factor (TNF)  $\alpha$ , a monocyte-, macrophage- and T-cell-derived cytokine,<sup>4</sup> is a key mediator of inflammation in IBD.<sup>5</sup> It impairs epithelial barrier function by altering structure and function of the tight junctions.<sup>6</sup> Disruption of the epithelial barrier represents the initiating step of intestinal inflammation.<sup>7</sup> Besides in intact tight junctions, the integrity of the intestinal epithelial barrier depends on a well-controlled rate of epithelial cell apoptosis that is significantly increased in patients with IBD but effectively restored to normal in patients responding to anti-TNF therapy.<sup>8</sup> In vitro data using the macrophage-like THP-1 cell line suggest a direct macrophage-mediated effect on epithelial cell apoptosis and necrosis through TNF receptor I and the nuclear factor kinase  $\beta$ .<sup>9</sup>

A milestone in treating IBD has been the introduction of anti-TNF- $\alpha$  agents, like infliximab, in the 1990s.<sup>10</sup> Direct neutralization of soluble TNF- $\alpha$ , binding to membranous TNF- $\alpha$  and consecutive induction of apoptosis in various immune cells<sup>11</sup> as well as initiation of a specialized regulatory macrophage subset have been suggested as potential mechanisms of action for infliximab.<sup>12</sup> These macrophages, also referred to as "alternatively activated" or M2 macrophages expressing large amounts of interleukin (IL)-10, scavenger-, mannose- and galactose-type receptors, are involved in T-helper (Th) 2-cell activation and regulate extracellular matrix molecule synthesis, wound

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repair, and tumor progression.<sup>13</sup> In contrast, “classically activated” M1 macrophages produce proinflammatory cytokines such as TNF- $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6, and effector molecules like reactive oxygen species or nitric oxide. They regulate Th1-cell activation and mediate acute inflammation.<sup>13</sup> Mice with deficient polarization of macrophages towards the regulatory M2 phenotype display a higher susceptibility to dextran sulfate sodium–induced colitis.<sup>14</sup> Recruited to the tissue, factors from the local stroma shape now resident intestinal monocyte cells to a phenotype with high phagocytic and bactericidal activity but lacking proinflammatory cytokine production.<sup>15</sup> However, the activation pattern and the significance of polarized macrophage subtypes in IBD, their distinct effects on human intestinal epithelial cells, and the associated barrier function are only partially understood. Aim of this study was to define macrophage subtypes present within the lamina propria of patients with IBD and their potential specific contribution to the effects on intestinal epithelial cells and intestinal barrier function.

## MATERIALS AND METHODS

### Patient Samples and Cell Lines

Formalin-fixed and paraffin-embedded surgical specimens from patients with active ileocecal or colonic CD (n = 11; age 18–50 yr, median 35 yr) or treatment-refractory ulcerative colitis (n = 8; age 20–47 yr, median 32 yr) and from patients with noninflammatory dysplasia (n = 4; age 43–71 yr, median 65.5 yr), herein referred to as controls, were obtained from the archives of the Institute of Pathology, Charité–Universitätsmedizin Berlin. Heparinized whole-blood samples were obtained from healthy volunteers. The study was approved by the local ethical committee (Approval number: EA4/059/10). The following human intestinal epithelial cell lines were used: HT-29/B6, i.e., the B6 subclone from cell line HT-29, as characterized previously,<sup>16</sup> Caco-2 (ATCC HTB-37),<sup>17</sup> and T84 (ATCC CCL-248).<sup>18</sup> All cells were maintained in the culture medium consisting of RPMI 1640 with 10% fetal bovine serum (Linaris, Bettingen, Germany), 2 mM L-glutamine, 10 U/mL penicillin, and 10 mg/mL streptomycin (all from PAA Laboratories, Cölbe, Germany).

### Immunohistochemistry

Immunohistochemistry was performed as described previously.<sup>19</sup> Briefly, after heat-induced epitope retrieval, sections (2  $\mu$ m) were blocked with Donkey serum (Dako, Hamburg, Germany) and incubated with rabbit-derived antibodies specific for CD68 (PG-M1; Dako), inducible nitric oxide synthase (iNOS; polyclonal; Abcam, Cambridge, United Kingdom), TNF- $\alpha$  (P-T2; Abcam), CD163 (10D6; Novocastra, Berlin, Germany), or stabilin-1 (polyclonal; Sigma-Aldrich, Deisenhofen, Germany). Biotinylated donkey anti-rabbit (Dianova, Hamburg, Germany) was visualized using the REAL Detection System with alkaline phosphatase/RED (Dako). Nuclei were counterstained with hematoxylin and images acquired by light microscopy (Carl Zeiss MicroImaging, Oberkochen, Germany). Positive cells were quantified and averaged from high power fields (0.237 mm<sup>2</sup>).

### Generation of Monocytes, M1 or M2 Macrophages

Monocytes were isolated and polarized into macrophages as described earlier.<sup>19</sup> In brief, CD14<sup>+</sup> cells were prepared from peripheral blood mononuclear cells by Ficoll density gradient centrifugation (GE Healthcare, Freiburg, Germany) and subsequent magnetic cell sorting using CD14 MACS MicroBeads (Miltenyi Biotech, Bergisch-Gladbach, Germany). Preparations contained >98% CD14<sup>+</sup> cells as determined by flow cytometry. Inducing polarization to M1 or M2 macrophages, cells were cultured in the presence of 50 U/mL human granulocyte-macrophage colony-stimulating factor or 50 U/mL human macrophage colony-stimulating factor (both from PeproTech, Rocky Hill, CT) for 6 days. Preserving monocytes, CD14<sup>+</sup> cells were cultured in medium without additional stimuli for 1 day. Resulting cell populations could be distinguished by appearance in phase contrast microscopy (see Fig. A, Supplemental Digital Content 1, <http://links.lww.com/IBD/A814>, which demonstrates morphology of monocytic cell populations). Surface marker expression as assessed by flow cytometry revealed high expression of HLA-DR on M1 macrophages, whereas a CD163<sup>high</sup>CD14<sup>high</sup> phenotype was characteristic for M2 macrophages (see Fig. B, Supplemental Digital Content 1, <http://links.lww.com/IBD/A814>, which demonstrates expression of surface markers of monocytic cell populations).

### Flow Cytometric Assessment of Surface Markers and Cytokines

Cells were washed twice with buffer consisting of phosphate-buffered saline (PAA Laboratories) with bovine serum albumin (5%). The following fluorochrome-coupled antibodies were applied: anti-CD14 (61D3), anti-CD163 (GHI/61), and anti-HLA-DR (LN3; all from eBioscience, San Diego, CA). Dead cells were excluded by propidium iodide staining. Samples were assessed by flow cytometry using a FACSCanto II device and the FACS Diva software (version 6; both from BD Biosciences, Heidelberg, Germany). Supernatants of the cultures were tested for TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and IL-10, using the Human Inflammatory Cytokine Bead Array (BD Biosciences). Quantification ranges were from 20 to 5000 pg/mL.

### Coculture of Monocytes/Macrophages with Epithelial Cells and Monitoring of Transepithelial Resistance

As previously described,<sup>17</sup> Caco-2, HT-29/B6, or T84 cells in standard culture medium were plated on Millicell PCF filters (0.4  $\mu$ m; Merck Millipore, Billerica, MA) at an average density of  $7 \times 10^5$  cells per square centimeter and grown as monolayers until confluence. On days 10 to 14 after plating for Caco-2 and T84 cells or on days 7 to 9 after plating for HT-29/B6 cells, filters were transferred to 12-well plates containing monocytes, M1 or M2 macrophages ( $3 \times 10^5$  cells per well). The upper compartment was filled with 500  $\mu$ L and the lower with 2 mL culture medium. If applicable, 100 ng/mL lipopolysaccharide (LPS) from *Escherichia coli* (Invivogen, Toulouse, France), 10  $\mu$ g/mL chimeric anti-TNF- $\alpha$  (infliximab; Remicade; MSD, Haar, Germany),

15  $\mu\text{g/mL}$  recombinant, nonglycosylated human IL1 receptor antagonist (Anakinra; Kineret; Swedish Orphan Biovitrum, Langen, Germany), or 1  $\mu\text{g/mL}$  monoclonal anti-human IL-18 (125-2H; MBL, Nagoya, Japan) were added to the basolateral chamber.

Transepithelial resistance of monolayers was assessed using 2 fixed pairs of electrodes (STX-2; World Precision Instruments, Sarasota, FL) connected with an impedance meter, whereas depth of immersion and position of the filters was standardized mechanically. Resistance values were corrected for the resistance of the empty filter and the bathing solution.<sup>6</sup> Transepithelial resistance was measured at various time points after initiating the coculture of the cells. For immunofluorescence studies, filters were fixed with 2% paraformaldehyde for 15 minutes at room temperature.

### Immunostaining and Confocal Microscopy

Epithelial cell layers were stained using polyclonal rabbit anti-human zonula occludens (ZO) 1 and JAM-A (both from Life Technologies, Carlsbad, CA). Secondary anti-rabbit or anti-mouse immunoglobulin G labeled with AlexaFluor594 or AlexaFluor488 dyes (both from Life Technologies) were used. Nuclei were counterstained with 4',6-diamidino-2-phenylindole, and samples were analyzed by confocal laser scanning microscopy (Carl Zeiss Microimaging) as described previously.<sup>20</sup>

### Western Blotting

For Western blotting, the following primary antibodies of either rabbit or mouse origin were applied: claudin-1 and claudin-2 (Life Technologies), junctional adhesion molecule (JAM)-A (Merck Millipore), E-cadherin (BD Biosciences),  $\beta$ -actin (Sigma-Aldrich, St. Louis, MO), cleaved caspase-3 and caspase-8 (both from Cell Signaling Technology, Boston, MA). Secondary antibodies were peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (both from Life Technologies). Antibody binding was detected by chemiluminescence using the ECL system (GE Healthcare). Band densities from digitalized images were estimated using AIDA software (V.3.21; Raytest, Straubhardt, Germany) and are given as integral [linear arbitrary units] per area [in square millimeter].

### Statistics

Statistical significance was determined by Mann–Whitney U test using the GraphPad PRISM software (version 5.00 for Windows; GraphPad Software, San Diego, CA). Probability values  $P \leq 0.05$  were considered significant.

## RESULTS

### Monocyte/Macrophage Compartment Within the Lamina Propria of Patients with IBD Is Shifted to Proinflammatory Macrophage Subtypes

First, we asked for the spatial distribution of resident monocytes and macrophage subtypes in the intestine of controls and patients with IBD. CD68<sup>+</sup> monocytic cells were found within

the subepithelial layer of the lamina propria in all samples (Fig. 1A). However, in patients with CD or UC, the amount of CD68<sup>+</sup> cells was not only significantly higher compared with the controls but they were not restricted to the close proximity to the epithelium. Subsequent staining revealed the resident macrophages in normal colon tissue to be CD163<sup>+</sup> and stabilin-1<sup>+</sup>, suggesting a constant presence of the regulatory M2 subtype within the lamina propria of normal gut tissue (Fig. 1B). In both patients with CD and UC, M2 macrophages were increased in number. However, only in the inflamed gut, and predominantly in patients with CD, iNOS<sup>+</sup> ( $P < 0.001$ ) and TNF- $\alpha$ <sup>+</sup> ( $P < 0.001$ ) cells were massively accumulated in subepithelial areas (Fig. 1C). Thus, the balance of macrophage subpopulations was shifted towards a proinflammatory state, as indicated by a higher iNOS<sup>+</sup>/CD163<sup>+</sup> ratio in patients with CD ( $P < 0.001$ ) and UC ( $P < 0.01$ ) compared with normal intestinal tissues (Fig. 1D).

### Soluble Factors Released by Monocytes and Proinflammatory M1 Macrophages Mediate Disruption of Epithelial Integrity

To determine the effect of macrophage subtypes on the intestinal epithelial-barrier function, monocytes and in vitro generated M1 or M2 macrophages were cocultured with epithelial cell layers. In our coculture system, the intestinal epithelial cell lines were grown to confluence on a filter, whereas the monocytes or macrophages were placed at the bottom of the lower chamber, allowing communication only through soluble factors. Enumeration of living cells and analysis of surface marker expression after coculture revealed unaltered cell amounts (see Fig. A, Supplemental Digital Content 2, <http://links.lww.com/IBD/A815>) and polarization of the monocytic cell populations (see Fig. B, Supplemental Digital Content 2, <http://links.lww.com/IBD/A815>).

In the coculture model, the monocytotic cell populations differed regarding their capacity to decrease transepithelial resistance, the marker of epithelial integrity. Already after 24 hours, the distinct deleterious effect of monocytes and M1 macrophages on T84 cell layers in the presence of bacterial LPS was apparent (Fig. 2A). Maximum differences were found after 54 hours, the time point chosen for further experiments. Interestingly, LPS alone had no effect on transepithelial resistance and thus on functional epithelial cell integrity. Only M1 macrophages caused a profound decrease in transepithelial resistance in T84 and Caco-2 cell layers in the absence of LPS, compared with controls and monocytes or M2 macrophages (Fig. 2B). This effect was further aggravated in the presence of Toll-like receptor 4-mediated stimulation. The amplifying effect of LPS on the capacity to decrease transepithelial resistance was also seen to a lesser extent for monocytes and M2 macrophages.

In HT29/B6 cell layers, no effect of unstimulated monocytic cells on epithelial barrier function was seen. However, after LPS stimulation, monocytes featured the most pronounced capacity to decrease transepithelial resistance, followed by M1 macrophages (Fig. 2B).

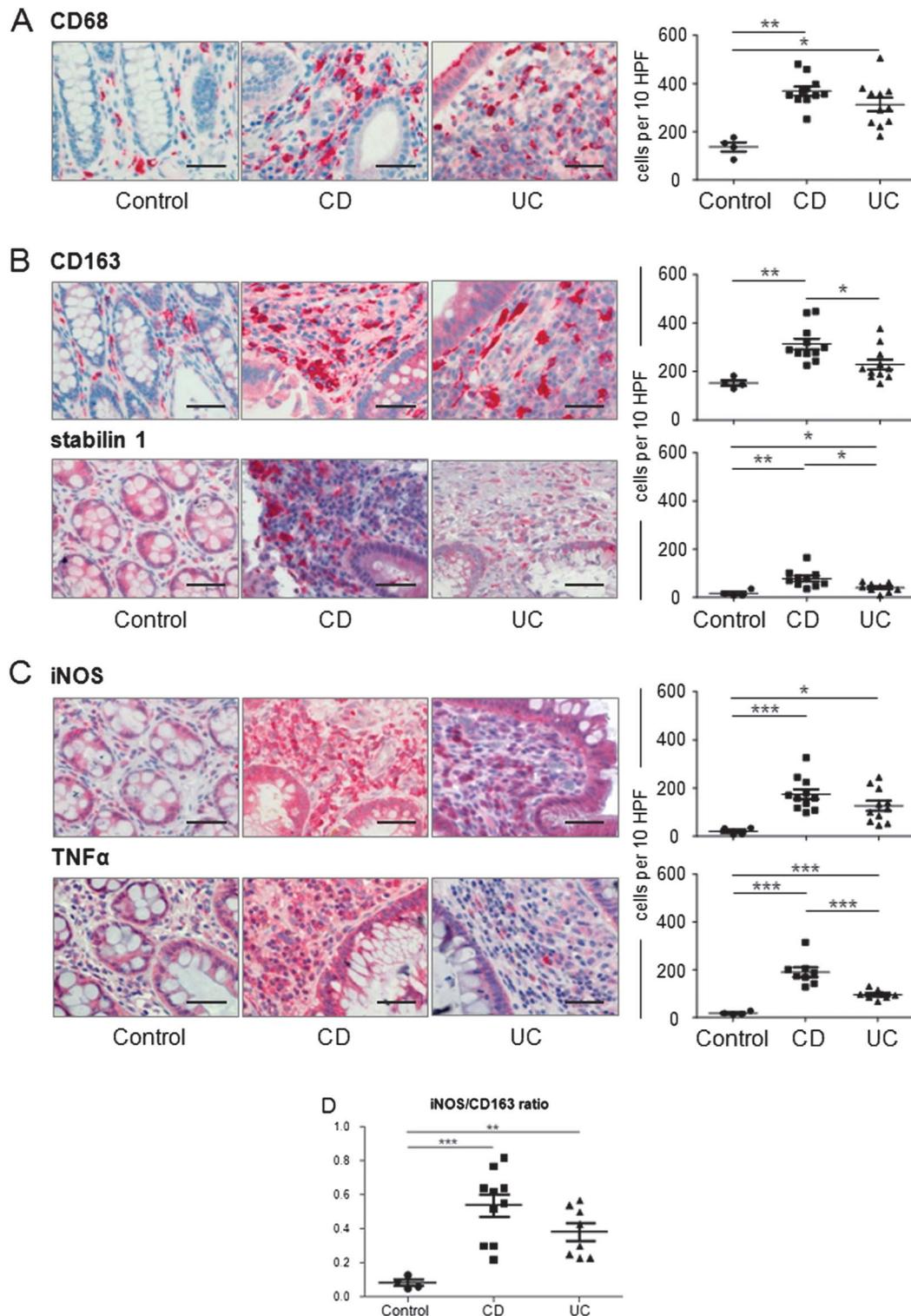


FIGURE 1. Monocytes and macrophage subpopulations in the LP of patients with IBD and normal intestinal tissue. Colon tissues samples from patients with CD, ulcerative colitis (UC), or normal tissue were stained by immunohistochemistry (A) for CD68 as a pan-macrophage marker, (B) the M2-macrophage markers CD163 and stabilin-1, and (C) for the M1 macrophage markers iNOS and TNF- $\alpha$ . Panels at the left hand side show representative images for immunohistochemical staining. Original magnification: 400-fold; bars represent 50  $\mu$ m. Positive cells appear in red. Panels at the right hand side summarize the respective positive cells counted in 10 high power fields. Median and range of n = 4 to 11. D, Ratio of iNOS<sup>+</sup> to CD163<sup>+</sup> cells as counted in 10 high power fields; \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

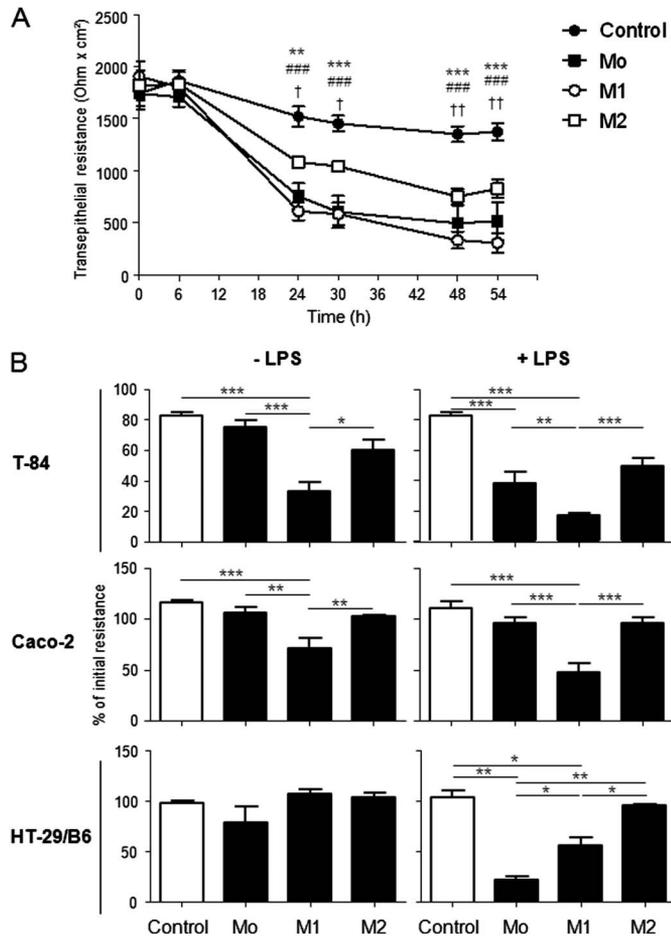


FIGURE 2. Effect of monocytes and macrophage subpopulations on the epithelial barrier. A, Monocytes (Mo), M1 or M2 macrophages were cocultured with the epithelial cell line T84 and LPS (100 ng/mL) was added to the lower chamber. Transepithelial resistance of the epithelial cell layer was measured over time after initiation of the coculture. Mean  $\pm$  SEM of  $n = 8$ .  $**P < 0.01$ ,  $***P < 0.001$  compared with cocultures with monocytes;  $###P < 0.001$  compared with cocultures with M1 macrophages;  $\dagger P < 0.05$ ,  $\dagger\dagger P < 0.01$ . B, Monocytes (Mo), M1 or M2 macrophages were cocultured with the intestinal epithelial cell lines T84, Caco-2, or HT-29/B6. If applicable, LPS (100 ng/mL) was added to the lower chamber. Transepithelial resistance of the epithelial cell layer was measured after 54 hours of coculture and is expressed as percent of initial resistance. Mean  $\pm$  SEM of  $n = 8$  to 22.  $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ .

### Monocyte-/Macrophage-mediated Disruption of the Epithelial Barrier Associates to Deregulated Tight Junction Proteins

To evaluate whether epithelial barrier function was disrupted through paracellular leakage, claudin-1, claudin-2, and JAM-A for tight junctions as well as E-cadherin for adhesion junctions were assessed from the epithelial cell layers of the coculture systems. Western blotting revealed significantly increased claudin-2 in epithelial cells exposed to M1 macrophages

or monocytes, compared with untreated controls or cells exposed to M2 macrophages (Fig. 3). Expression levels of claudin-1, JAM-A, and E-cadherin within the epithelial cells were not affected by monocyte-derived soluble factors. In untreated epithelial cell layers, integrity was defined by a homogeneously patterned tight junction mesh with ZO1 and JAM-A expression restricted to a small area along the lateral cell membrane (Fig. 4A). In epithelial layers incubated with monocytes or M1 macrophages, this structured architecture was severely disturbed with the junctional protein ZO1 being present in more basal parts of epithelial cells, compared with controls, as demonstrated in the confocal reconstructions along the x- and z-axes. This effect on ZO-1 relocalization was not as severe in epithelial cell layers incubated with M2 macrophages (Fig. 4B).

### Monocytic Cells Induce Apoptosis in Epithelial Cells

Next, we asked whether soluble factors secreted by monocytic cells cause epithelial cell apoptosis. M1 and M2 macrophages did not affect the viability of the cells within the epithelial cell layers as indicated by caspase-3 and caspase-8 levels that compared with the control of untreated epithelial cell layers. In contrast, upregulated caspase-3 was found in epithelial cells having been cocultured with monocytes (Fig. 5). Consistent significant increase of activated caspase-8 indicated that apoptosis was induced rather through the death receptor pathway than through mitochondrial regulation.

### Cytokine Blockade Partially Recovers Monocyte and M1 Macrophage-mediated Damage of Epithelial Cell Layer Integrity

The aggravating effect of LPS-stimulation of primary human monocytic cells on the extent of the damage within the epithelial cell layers suggested cytokine-dependent mechanisms. The monocytic cell populations differed regarding their cytokine profile (Fig. 6A and see Fig., Supplemental Digital Content 3, <http://links.lww.com/IBD/A816>). Most notably, high concentrations of TNF- $\alpha$  were found in the supernatants of M1 macrophages and, to a lesser extent, in monocytes. Only the latter expressed high amounts of the inflammasome-derived cytokine IL-1 $\beta$ . In contrast, the predominant cytokine for M2 macrophages was IL-10. In all conditions, cytokine expression was amplified in the presence of LPS.

Thus, candidate cytokines were TNF- $\alpha$  for the effect of monocytes and M1 macrophages, as well as IL-1 $\beta$  and IL-18 for that of monocytes. None of the blocking reagents directly affected the transepithelial resistance of the epithelial cell layer. Monocytes and M1 macrophages reduced this resistance by 50% to 80% and 80% in the presence of LPS, respectively. This was significantly recovered by inhibiting TNF- $\alpha$  using infliximab in the cultures with monocytes or M1 macrophages (Fig. 6B). Blocking the inflammasome-derived cytokines IL-1 $\beta$  and IL-18 with a recombinant IL-1 receptor antagonist or an IL-18-specific monoclonal antibody at least partially protected from the

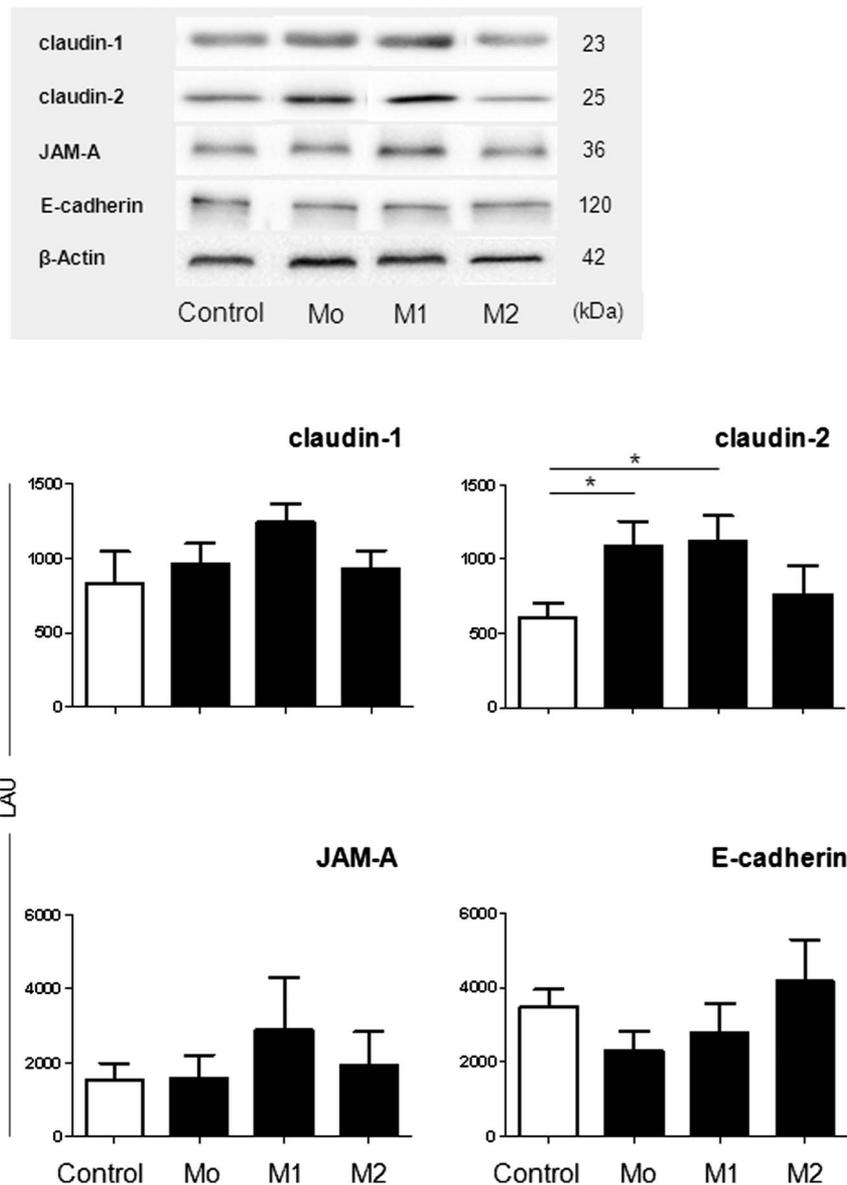


FIGURE 3. Effect of monocytic cells on tight junction proteins. After coculture with monocytes or macrophage subpopulations or without monocytic cells for control for 54 hours without LPS, claudin-1, claudin-2, JAM-A, and E-cadherin were detected in T84 cells by Western blot analysis.  $\beta$ -actin served as loading control. Upper panels show representative blots; lower panels show the corresponding densitometric analysis. Mean  $\pm$  SEM of  $n = 4$ ;  $**P < 0.01$ .

monocyte-mediated loss of epithelial resistance. Thus, human monocytes and M1 macrophages mediated their effect on the epithelial integrity mainly by these proinflammatory cytokines.

## DISCUSSION

Macrophages are constantly present within the mucosa of the normal and the inflamed intestinal tract of all mammals but the role of different macrophage subtypes in homeostasis or inflammation and especially their distinct effect on the barrier function of the intestinal epithelium is not completely understood yet.<sup>21</sup>

Our *in vitro* model to study effects of human primary monocytic cells on human intestinal epithelial cells excluded effects by other lamina propria cell populations. Also focusing on soluble factors derived from freshly isolated monocytes or macrophages polarized *in vitro* from peripheral CD14<sup>+</sup> cells, these cells were cocultured with confluent layers of intestinal epithelial cell lines on a transwell membrane. Monocytes and proinflammatory M1 macrophages are significantly augmented cell populations in the lamina propria of patients with IBD. Applying the coculture model, we here provide evidence that release of IL-1 $\beta$ , IL-18, and/or TNF- $\alpha$  by these cell types directly contributes to paracellular

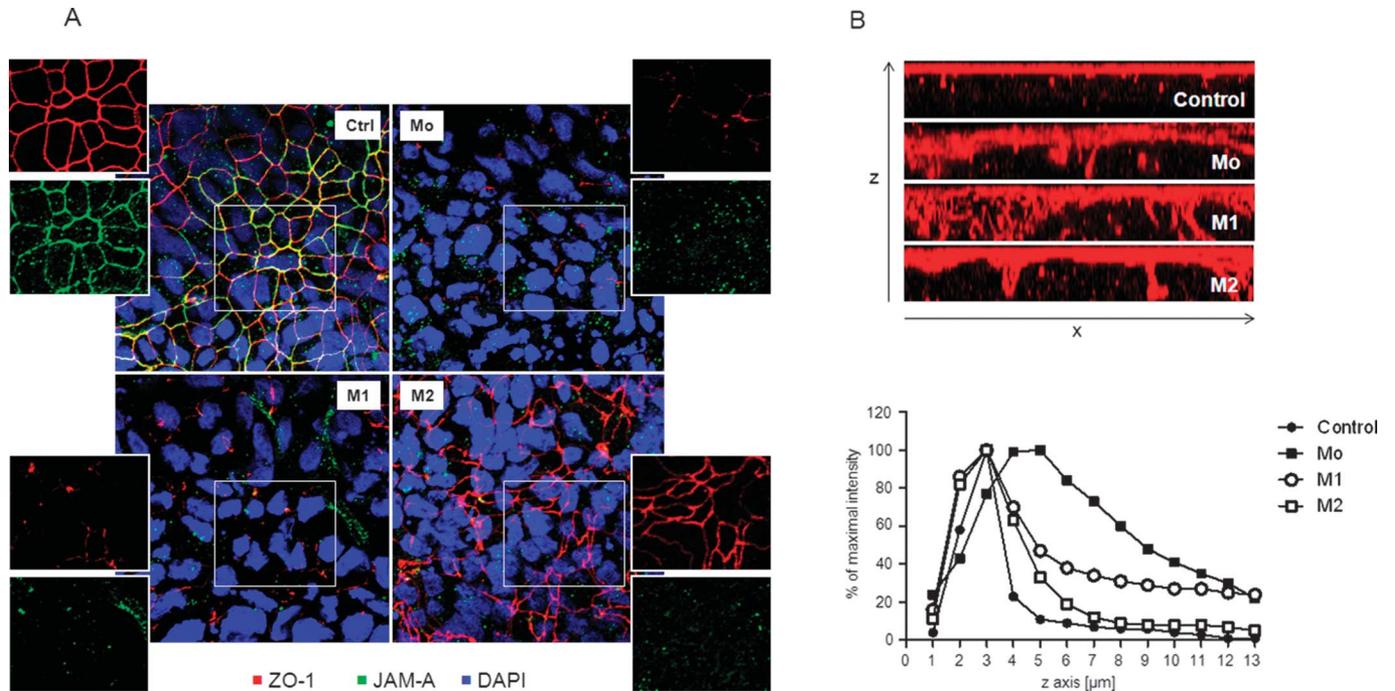


FIGURE 4. Effect of monocytic cells on the integrity of the epithelial layer. T84 cells cocultured with monocytes (Mo), M1 or M2 macrophages or without immune cells (control, ctrl) were immunostained for ZO-1 (red) and JAM-A (green). Nuclei were stained with DAPI (blue). A, Panels show collapsed z-stack projections (n = 4). In areas of interest, stainings are shown in single color mode. Bars represent 10 μm. B, Distribution of ZO-1 within the cell layer is shown in XZ sections of immunostained T84 cell layers. Upper panels show representative images. Lower panels show the corresponding quantification of ZO-1, expressed as percent of maximum intensity in thirteen horizontal sections of the epithelial cell layer (from apical to basal).

leakage within the epithelium and consecutively induces a breakdown of the epithelial barrier.

This study not only translates previous findings with the monocytic THP-1 cell line to primary human monocytic cells<sup>9</sup> but extends them to specifics of macrophages subtypes. More importantly, it underlines the role of monocytic cells in the development of IBD-associated barrier defects, which is well established to be caused by deregulation of tight junction proteins and induction of apoptosis.<sup>7,8,22</sup> Claudin-2, a pore-forming tetraspanning protein that integrates into the tight junction, thus allowing a cation flux through the intestinal barrier<sup>23</sup> was specifically upregulated in M1- and monocyte-exposed epithelial cell layers. It is known from morphological studies on various chronic intestinal inflammations that not only the total amount but also the subcellular localization of integral tight junction proteins determine mucosal barrier function.<sup>20,24</sup> In accordance, cellular architecture of epithelial cell layers that had been incubated with monocytes or M1 macrophages was destroyed and of the tight junction proteins claudin-2 and ZO-1 redistributed to the cytoplasm or basal parts of the cells. This phenomenon was not found for epithelia cocultured with the IL-10-producing M2 macrophages.

Soluble factors, like IL-1β, induce increased intestinal permeability through porous tight junctions.<sup>25</sup> The 2 inflammasome-derived cytokines IL-1β and IL-18 are increased in inflamed intestinal tissue.<sup>26,27</sup> Our findings suggest monocytes in close

proximity to the epithelium as an important source because only these nonpolarized cells constitutively express active caspase-1 to release these tightly controlled cytokines.<sup>28</sup> Resident macrophages are unresponsive to inflammation and as a part of the niche support colonic epithelial progenitors intermittently regenerating the epithelial cell layer.<sup>15,29</sup> Hence, our findings support the suggestion of a constant recruitment of peripheral monocytes and M1 macrophages to the lamina propria in CD. Rates of epithelial apoptosis were elevated in the presence of monocyte-derived soluble factors and were driven through the death receptor pathway, potentially through TNF-α and its interaction with the TNF-receptor I. This again translated findings with the nonpolarized THP-1 cell line<sup>9</sup> to cells isolated ex vivo. Polarized macrophages had no effect on apoptosis, illustrating that M1 macrophages mainly alter tight junctions. The monocyte-/M1 macrophage-mediated epithelial disruption was to some extent reversed by interfering with TNF-α through infliximab. Although infliximab facilitates the induction of regulatory CD206<sup>+</sup>/CD68<sup>+</sup> macrophages in vitro and in vivo,<sup>12,30</sup> in our hands, the polarized macrophage subsets showed stable surface marker expression. Despite the capacity of anti-TNF-α treatment to induce apoptosis in monocytes,<sup>31</sup> macrophages<sup>32</sup> and T cells,<sup>33</sup> we again did not find the number of viable macrophages changed. Infliximab alone exerted no effect on epithelial resistance, arguing against a significant direct effect on epithelial cells.

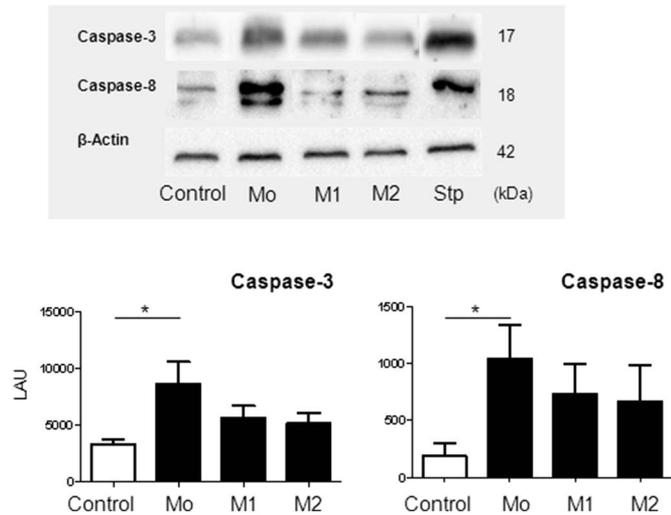


FIGURE 5. Induction of apoptosis in epithelial cells. After coculture with monocytes or macrophage subpopulation or without monocytic cells for control for 54 hours without LPS, cleaved caspase-3 and -8 were detected in T84 cells by Western blot analysis with  $\beta$ -actin as loading control. Staurosporine (Stp) served as positive control for apoptosis. Upper panels show representative blots; lower panels show the corresponding densitometric analysis. Mean  $\pm$  SEM of  $n = 4$ ;  $^{**}P < 0.01$ .

Macrophage subtypes represent a spectrum of various activation phases of these cells. Especially, M2 macrophages comprise a heterogeneous group of subtypes.<sup>19</sup> Our in vitro and in situ findings suggest the presence of a rather proinflammatory IL-10<sup>high</sup> IL-12<sup>low</sup> M2b phenotype, providing an explanation for our finding that M2 macrophage in the presence of Toll-like receptor stimulation can affect epithelial integrity to a minor extent. Macrophage subtypes are believed to switch phenotypes dependent on the microenvironment.<sup>13,15,34</sup> We provide evidence that macrophage subtypes in the lamina propria are shifted towards the proinflammatory M1 subtype in patients with CD and less prominent also in patients with ulcerative colitis. Whether this is due to a primary failure in cellular differentiation programs of patients with IBD or due to the local milieu of cytokines and chemokines regulating cell differentiation remains incompletely understood at this point. Recently, we showed that regulatory M2 macrophages invading the fat tissue surrounding the inflamed part of the gut in patients with CD are highly influenced by the local cytokine and adipokine milieu.<sup>19</sup> Interestingly, the responses of Caco-2 and T84 cells to coculture with primary monocytic cells were similar but mucus producing HT-29/B6 cells only displayed a substantial loss of epithelial integrity in the presence of simultaneous Toll-like receptor 4 stimulation mimicking inflammatory conditions after the epithelial barrier is already disrupted.

Taken together, our coculture system with primary human monocytes or in vitro polarized macrophage subtypes and human epithelial cell lines reflecting important aspects of the in vivo situation of human intestinal inflammation allows for better comprehension of steps relating to epithelial integrity in

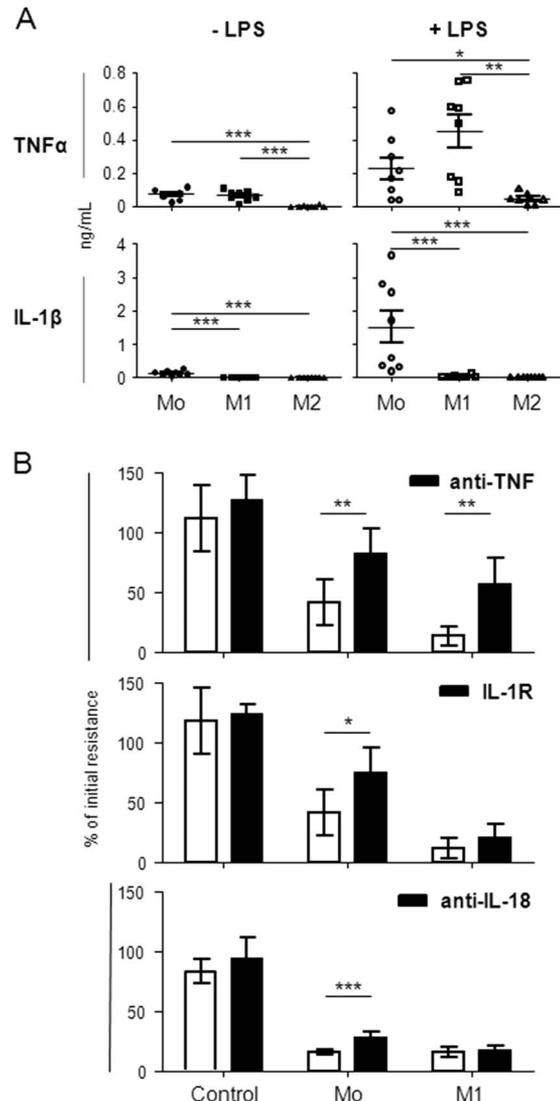


FIGURE 6. Effect of cytokine inhibition on monocyte/macrophage-mediated disruption of epithelial barrier. A, CD14<sup>+</sup> cells cultured for 1 day (Mo; monocytes) or polarized to M1 and M2 macrophages for 6 days were subsequently stimulated with LPS (100 ng/mL) for additional 24 hours. Cytokine production was determined in supernatants by cytometric bead array. Median and range of  $n = 8$  to 10;  $^{*}P < 0.05$ ;  $^{**}P < 0.01$ ;  $^{***}P < 0.001$ . B, Monocytes (Mo), M1 or M2 macrophages were cocultured with Caco-2 cells. LPS (100 ng/mL) and anti-TNF- $\alpha$  (10  $\mu$ g/mL), a soluble IL-1R (15  $\mu$ g/mL) or anti-IL-18 antibody (1  $\mu$ g/mL) were present in the lower chamber. Transepithelial resistance of the epithelial cell layer after 54 hours of coculture is expressed as percent of initial resistance. Mean  $\pm$  SEM of  $n = 5$  to 12;  $^{*}P < 0.05$ ;  $^{**}P < 0.01$ ;  $^{***}P < 0.001$ .

the pathogenesis of IBD. Earlier best described in mouse model systems,<sup>35,36</sup> our findings underline the crucial role of the IL-10 receptor/IL-10 signaling by macrophages in the lamina propria for human intestinal homeostasis through fine-tuning of epithelial barrier function and allow to place the contribution of monocytic cell types within the course of developing IBD. In acute inflammation, monocytes infiltrating the lamina propria

modify tight junctions and induce epithelial cell apoptosis through IL-1 $\beta$  and IL-18. In the second step, proinflammatory M1 macrophages recruited to the lamina propria further deregulate the tight junction integrity mainly by TNF- $\alpha$ . Eventually, intraluminal content entering the lamina propria through paracellular leakage attracts immune cell subsets that perpetuate chronic inflammation. Inspecting intestinal specimens of patients with chronic states of intestinal inflammation in IBD mostly reveals this diversity of immune cells within the lamina propria with a dominance of proinflammatory cells in active disease.

Better understanding of shifts within the macrophage compartment in terms of a milieu indicator will serve to explain varying efficacy of cytokine blockade used in IBD treatment and result in patient stratification according to histopathological findings: Patients with large amounts of unpolarized monocytic cells in the lamina propria might benefit from strategies blocking the inflammasome, while these agents lose effectiveness upon chronic inflammation with a shift to M1 macrophages, the situation most frequently encountered in the clinic and successfully treated by blocking TNF- $\alpha$ .

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