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IL-10 Producing CD14^{low} Monocytes Inhibit Lymphocyte-Dependent Activation of Intestinal Epithelial Cells by Commensal Bacteria

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***Abstract*: Intestinal epithelial cell (IEC) activation by non-pathogenic, commensal bacteria was demonstrated to require the presence of immunocompetent cells. In this study, HT-29 and CaCO-2 transwell cultures, reconstituted with CD4$^+$ and CD8$^+$ T cells, CD19$^+$ B cells and CD14^{high} monocytes, were challenged with non-pathogenic Gram negative *Escherichia coli* and Gram positive lactobacilli. Cytokine expression was analysed by reverse transcription-polymerase chain reaction (RT-PCR) and enzyme linked immunosorbent assays (ELISA). Expression of tumour necrosis factor alpha (TNF-α) and interleukin (IL)-8 mRNA in *E. coli* or *L. sakei* challenged IEC was promoted by lymphocyte populations predominantly CD4$^+$ T cells, while monocytes failed to mediate an inflammatory cytokine response. The monocyte phenotype and function were further characterised by flow cytometry and mixed lymphocyte reaction (MLR). During the co-culture with IEC and bacterial stimulated IEC, CD14^{high} peripheral blood monocytes acquired a CD14^{low} CD16^{low} phenotype with reduced expression co-stimulatory (CD80, CD86, CD58) cell surface molecules. Immunosuppressive functions of IEC conditioned CD14^{low} monocytes were demonstrated by the predominant secretion of IL-10 and IL-1Ra and their reduced potential to trigger an allogeneic lymphocyte response. In conclusion, IEC contribute to the development of CD14^{low} CD16^{low} monocytes with immunosuppressive function and antagonised a lymphocyte-mediated activation of the intestinal epithelium in response to intestinal and food derived bacteria. These results strengthen the hypothesis that the gut epithelium constitutes an important functional element in the regulation of mucosal immune homeostasis towards commensal bacteria.**

***Key words*: Intestinal epithelial cells, CaCO-2 and HT-29 transwell co-cultures, TNF-α, IL-8, IL-10, CD14^{low} CD16^{low} monocytes, Lactobacilli, Commensal bacteria**

The indigenous microflora harbouring the human intestine is in close proximity to a large number of specialised, lymphoid and accessory cells present throughout the lamina propria, the follicles of the gut-associated-lymphoid tissue (GALT), and intraepithelial lymphocytes (27). The maintenance of local homeostasis in the intestinal mucosa to luminal bacterial antigens requires a fine tuning of immunological processes, particularly those involving T cell activation (31, 41). The lack of responsiveness or oral tolerance towards the autologous intestinal microflora is a physiological feature that, if impaired, may lead to intestinal inflammation (12, 13, 15, 18−20). It has been suggested that the hyporeactivity of lamina propria T cells to stimulation through the T cell receptor/CD3 complex could be one mechanism of the mucosal compartment securing immunoregulation at the antigen-specific level (43, 45, 58). This appears, at least in part, to be due to impaired signal transduction, probably mediated by soluble factors produced by intestinal epithelial cells (IEC) (11). Resident intestinal monocytes which are localised in the subepithelial region

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Abbreviations: ELISA, enzyme linked immunosorbent assays; IEC, intestinal epithelial cells; IL-1β, interleukin 1 beta; LPL, lamina propria lymphocytes; MLR, mixed lymphocyte reactions; PBL, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cells; RT-PCR, reverse transcription-polymerase chain reaction; TNF-α, tumour necrosis factor alpha.

also contribute to the suppression of lymphocyte activation suggesting that accessory cell function may be an additional target for the immunomodulatory effect of the mucosal environment (36, 44).

Intestinal epithelial cells which are the first cells in the frontline interacting with intestinal bacteria, are considered to participate in the initiation and regulation of mucosal immune response (5, 26, 34, 39). Experiments in germ-free animals have shown that lymphoid populations in the lamina propria are considerably reduced in the germ-free intestine, but assume its normal appearance of physiological inflammation after bacterial colonisation (4, 9, 28). We have recently shown that commensal non-pathogenic bacteria of different origin have the ability to elicit a characteristic cytokine response in leukocyte sensitised CaCO-2 cells *in vitro* and deliver a discriminative signal to underlying immunocompetent cells (25). We provided evidence that bi-directional cross talk between IEC and immunocompetent cells constitutes a crucial step in the response to non-pathogenic bacteria at the mucosal surface.

The aim of this study was to investigate the role of lymphoid and myeloid cell populations in the regulation of immune-mediated activation of IEC after the challenge with non-pathogenic food derived and intestinal bacteria. We provide evidence that IEC conditioned monocytes acquire a phenotype similar to lamina propria monocytes, which in turn, control inflammatory cytokine expression in IEC and modulate lymphocyte activation *in vitro*. The role of bacterial activated IEC in the development of immunosuppressive monocytes will be discussed in the context of pathological conditions, such as inflammatory bowel disease (IBD).

Materials and Methods

Bacteria and growth condition. The non-pathogenic *Escherichia coli* D2241 of human faecal origin was obtained from the Statens Serum Institute (International Escherichia and Klebsiella Center, Copenhagen, Denmark) and was grown in brain-heart-infusion broth (BHI) at 37 C. Intestinal lactobacilli, *Lactobacillus johnsonii* La1 (Strain collection, Nestlé Research Centre, Lausanne, Switzerland) and *Lactobacillus gasseri* (ATCC 33323) were grown in MRS broth (14) without acetate at 37 C. *Lactobacillus sakei* LTH 681 (Strain Collection, Hohenheim University; Institute of Food Technology, Stuttgart, Germany), isolated from fermented food, was grown in the same broth at 30 C. All bacteria were harvested by centrifugation (3,000$\times g$, 10 min) after 24 hr of cultivation at stationary growth phase. Bacteria were washed three times with phosphate buffered saline (PBS) (1×, pH 7.2, Gibco BRL) and subsequently diluted to obtain final cell densities of 1×10^6 and 1×10^7 colony forming units (cfu/ml) in RPMI 1640 medium (Gibco BRL).

Cell culture. Human intestinal epithelial cell (IEC) lines CaCO-2 and HT-29 (passage 40−60) were seeded (2×10^5 cells/well) on 10.5 mm cell culture inserts (0.4 μm nucleopore size; Becton Dickinson) and were placed in 12-well tissue culture plates (Nunc). CaCO-2 cells were cultured for 18−21 days at 37 C/10% CO_2 in DMEM (glutamine, high glucose; Amimed) supplemented with 20% decomplemented fetal calf serum (56 C, 30 min) (FCS, Amimed), 1% MEM non-essential amino acids (Gibco BRL) and 0.1% penicillin/streptomycin (10,000 IU/ml/10,000 UG/ml, Gibco BRL). HT-29 cells were cultured for 12−15 days at 37 C/5% CO_2 in DMEM (glutamine, high glucose, Amimed) and 0.1% penicillin/streptomycin (10,000 IU/ml/10,000 UG/ml, Gibco BRL). Transepithelial electrical resistance (TEER) (ohm/cm^2) was determined continuously in confluent CaCO-2 and HT-29 monolayers using a MultiCell-ERS electrode (voltmeter/ohmmeter).

Purification of leukocyte subpopulations from human peripheral blood mononuclear cells. Human peripheral blood mononuclear cells (PBMC) from healthy volunteers were purified from buffy coats (Blood Transfusion Center, Lausanne) using Ficoll-Hypaque 1077 (Pharmacia) gradient centrifugation (500$\times g$, 30 min). PBMC were incubated for 2 hr at 37 C and 5% CO_2 on 225-cm^2 tissue culture plates (Costar) to allow adherence. Nonadherent peripheral blood lymphocytes (PBL) were separated from adherent cells by aspiration. PBL were incubated with CD4, CD8, CD19 magnetic MicroBeads (Miltenyi Biotech) and separated by magnetic cell sorting (MACS) using positive selection technique. Monocytes were enriched from PBMC by depletion of T cells, B cells, NK cells, dendritic cells and basophils using indirect labelling with hapten-conjugated CD3, CD7, CD19, CD45RA, CD56, anti-IgE monoclonal antibodies (mAb) and MACS MicroBeads coupled to an antihapten monoclonal antibody (Monocyte Isolation Kit, Miltenyi Biotech). Finally, cells were diluted in RPMI 1640 containing 10% FCS (56 C, 30 min) and gentamicin (100 μg/ml, Gibco BRL) to a final density of 2×10^6 cells/ml. Where indicated lower cell numbers were used (5×10^5 to 1×10^6 cells/ml).

Flow cytometry. Cell staining was performed for 20 min at 4 C with saturating concentrations of the following mouse anti-human mAbs: FITC-conjugated anti CD4- (SK3) (IgG1, Becton Dickinson), CD8- (SK1) (IgG1, Becton Dickinson), CD19- (4G7) (IgG1, Becton Dickinson), CD58- (MY31) (IgG1, Becton Dickinson), CD33- (HIM3-4) (IgG1, Pharmingen), CD16- (3G8) (IgG1, Pharmingen), HLA-DR- (L243) (IgG2a, Becton

Dickinson), CD80- (L307.4) (IgG1, Pharmingen), CD86- (2331) (IgG1, Pharmingen) mAb; biotin-conjugated anti CD11b- (ICRF44) (IgG1, Pharmingen) mAb followed by Avidin-FITC (1:100, Pharmingen) and PE-conjugated anti-CD14 mAb (MøP9) (IgG2b, Becton Dickinson). Isotype controls: FITC-conjugated mouse IgM, IgG1, IgG2a mAb (G155-228, MOPC-21, G155-178, Pharmingen) and PE-conjugated mouse mAb (27-35, IgG2b, Pharmingen). The samples were analysed after double staining using a FACScan® (Becton Dickinson).

Cell viability. Cell viability was routinely tested by trypan exclusion. Discrimination between viable, apoptotic and necrotic cells was performed by flow cytometry using the Vybrant™ Apoptosis Assay Kit (Molecular Probes).

Intestinal epithelial cell/leukocyte co-cultures. Transwell cultures with confluent CaCO-2 or HT-29 monolayers were established as previously described (23). Freshly purified human PBMC, lymphocyte subpopulations or CD14^{high} monocytes were added to the basolateral compartment at cell densities of 2×10^6/ml. IEC/leukocyte co-cultures were stimulated with bacteria by adding 1×10^6 or 1×10^7 cfu/ml non-pathogenic *E. coli* or lactobacilli to the apical surface of IEC monolayers and incubated for 4, 16, 36 or 72 hr at 5% CO_2 and 37 C. To prevent bacterial growth Gentamicin (150 μg/ml) was added to the apical compartment of the cultures after 4 hr of incubation. Where indicated, neutralising anti-IL-10 mAb (10 μg/well, 500-M86, BioConcept) and recombinant human (rh) IL-10 (10 pg/well, R&D Systems) were added to the basolateral compartment of the co-cultures.

RNA extraction and amplification by RT-PCR. Total RNA from IEC was isolated using the acid guanidinium thiocyanate/phenol/chloroform method (Micro RNA Isolation Kit, Stratagene). Total RNA (0.5 μg), 1 mM of each dNTP, 2.5 U/ml MuLV reverse transcriptase (Perkin Elmer), and specific 3′ primers coding for the human cytokines TNF-α, IL-8 and β-actin were incubated at 42 C for 30 min. PCR amplification and subjection of PCR products to gel electrophoresis was performed as previously described (23).

ELISA. Quantification of IL-10, and IL-1Ra (R&D Systems) in the supernatants of IEC/leukocyte co-cultures was performed by ELISA.

Mixed lymphocyte reaction. Mixed lymphocyte reaction (MLR) was performed with freshly purified allogeneic monocyte depleted peripheral blood lymphocytes (PBL) and *in vitro* conditioned monocytes at varying concentrations (1×10^5, 5×10^4, 1×10^4, 5×10^3, 1×10^3 cells/well). Monocytes were harvested from transwell cultures after 3 days of culture on plastic (Mo 1), co-culture with HT-29 cells (Mo 2) and co-culture with *L. sakei* (10^7 cfu/ml) activated HT-29 cells (Mo 3). Finally, serial dilutions of the three differently matured monocytes from the same donor were incubated with a fixed number of allogeneic lymphocytes (2×10^5 cells/well). MLR cultures were maintained for 5 days at 5% CO_2 and 37 C. During the last 18 hr of cultures 1 μCi of [^{3}H]-thymidine (Amersham) was added to each well. Thereafter, cells were harvested on filter mats and [^{3}H]-thymidine incorporation was determined by liquid scintillation counting (TopCount, Packard).

Statistical analysis. Values are given as mean (SD) of triplicate measurements. All results were confirmed for at least three different donors in independent experiments. Significance was tested by Mann-Whitney U test (Statistica, Statsoft).

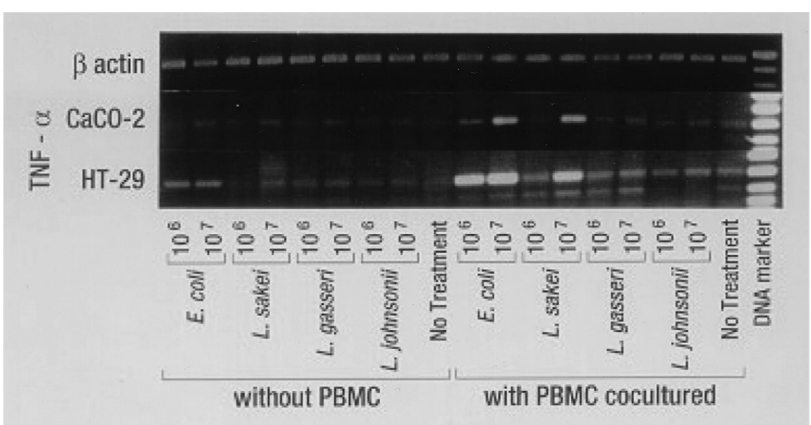

Fig. 1. Differential activation of CaCO-2 and HT-29 cells by non-pathogenic bacteria. Reverse transcription-polymerase chain reaction (RT-PCR) was used to determine tumour necrosis factor alpha (TNF-α) mRNA expression in CaCO-2 and HT-29 cells on stimulation of intestinal epithelial cells (IEC)/leukocyte co-cultures or IEC alone with non-pathogenic *E. coli*, *L. sakei*, *L. gasseri* and *L. johnsonii* (16 hr, 10^6 and 10^7 cfu/ml), respectively. Culture medium alone was used as a control. Results represent one of two independent experiments.

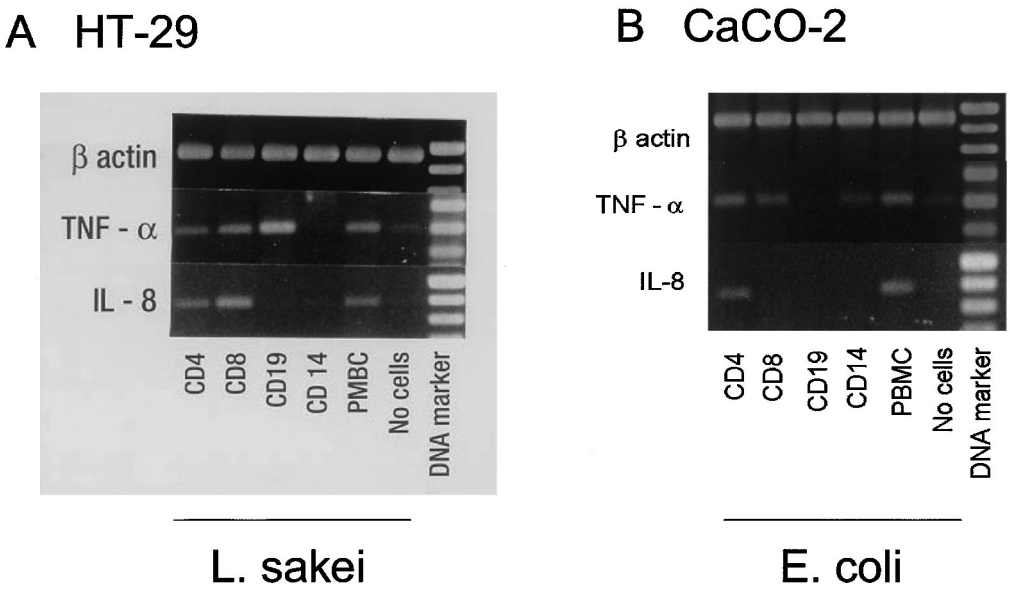

Fig. 2. Peripheral blood lymphocytes drive activation of HT-29 cells after bacterial stimulation. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to determine tumour necrosis factor alpha (TNF-α) and interleukin (IL)-8 mRNA expression in HT-29 and CaCO-2 cells after stimulation with *L. sakei* and *E. coli* (16 hr, 1×10^7 cfu/ml), respectively. (A) HT-29 and (B) CaCO-2 cells were reconstituted with $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells, $CD14^+$ monocytes and peripheral blood mononuclear cells (PBMC) (2×10^6 cells/ml). The absence of immunocompetent cells (no cells) was used as a control. Results represent one of two independent experiments.

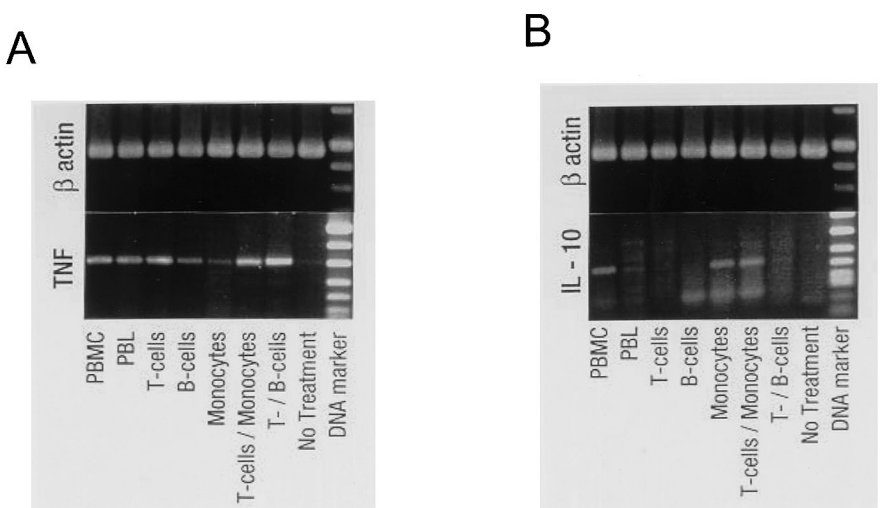

Fig. 3. Differential expression of TNF-α and IL-10 mRNA in leukocyte populations of CaCO-2 transwell cultures. CaCO-2 cell transwell cultures were apically stimulated with *E. coli* (16 hr, 1×10^7 cfu/ml). Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to determine tumour necrosis factor alpha (TNF-α) (A) and interleukin (IL)-10 (B) in underlying PBMC, PBL, T cells ($CD4^+$:$CD8^+$ ratio 1:1), B cells ($CD19^+$), monocytes ($CD14^+$), as well as combinations of T cells and monocytes ($CD4^+$:$CD8^+$:$CD14^+$ ratio 1:1:1) or T/B cells ($CD4^+$:$CD8^+$:$CD19^+$ ratio 1:1:1) (2×10^6 total cells/ml). The absence of immunocompetent cells (no cells) was used as a control. Results represent one of two independent experiments.

Results

Peripheral Blood Lymphocytes Drive Activation of IEC after Challenge with Non-Pathogenic Bacteria

Previous results (25) demonstrated that the stimulation of CaCO-2/PBMC co-cultures with *E. coli* and *L. sakei* induced TNF mRNA and IL-8 mRNA expression in IEC after 12 hr of incubation. The strong activation of IEC determined between 14–20 hr of incubation was completely down-regulated after 36 hr. In this study, the stimulation of CaCO-2 and HT-29 transwell cultures by non-pathogenic *E. coli*, *L. sakei*, *L. gasseri* and *L. johnsonii* for 16 hr resulted in a characteristic activation

pattern of IEC, when PBMC (2×10^6/well) were present in the basolateral compartment. As shown in Fig. 1, TNF-α mRNA expression in IEC was strongly induced by 10^7 cfu/ml Gram negative *E. coli* and Gram positive *L. sakei*, while the inflammatory cytokine response in HT-29 and CaCO-2 cells induced by Gram positive intestinal lactobacilli such as *L. johnsonii* or *L. gasseri* remained at low levels. *E. coli* induced low levels of TNF-α mRNA expression in HT-29 cells but not in CaCO-2 cells, even in the absence of immunocompetent cells. These results are in accordance with our recently published data (25).

To identify the leukocyte population involved in IEC activation, MACS purified CD4$^+$ and CD8$^+$ T cells, CD19$^+$ B cells or CD14^{high} monocytes (2×10^6/well) were used to reconstitute HT-29 and CaCO-2 transwell cultures. As shown in Fig. 2A, HT-29 stimulation with *L. sakei* (10^7 cfu/ml) for 16 hr resulted in the expression of TNF-α mRNA in IEC, when either total PBMC, CD4$^+$, CD8$^+$ T cells or CD19$^+$ B cells were present in the basolateral compartment. Induction of IL-8 mRNA was mediated by PBMC, CD4$^+$ and CD8$^+$ T cells, but not by CD19$^+$ B cells after bacterial stimulation. Notably, purified CD14^{high} monocytes failed to mediate activation of HT-29 cells after bacterial stimulation. Figure 2B shows *E. coli*-induced TNF-α mRNA expression in CaCO-2 cells in the presence of total PBMC, CD4$^+$ and CD8$^+$ T cells. Low TNF-α mRNA expression was induced by CD14^{high} monocytes, whereas CD19$^+$ B cells failed to mediate epithelial cell activation. Induction of IL-8 mRNA was only induced, when either total PBMC or CD4$^+$ T cells were present in CaCO-2 co-cultures. In summary, total PBMC and CD4$^+$ T cells consistently induced TNF-α and IL-8 mRNA expression in both IEC lines, whereas CD14^{low} monocytes and CD19$^+$ B cells revealed a low potential to trigger an inflammatory cytokine response in bacteria stimulated IEC. Unstimulated IEC/leukocyte co-cultures were not activated suggesting that alloantigens released by IEC are not responsible for the activation of IEC (data not shown).

Constitute an Immunoregulatory Subpopulation in Bacteria Challenged IEC/Leukocyte Co-Cultures

To further dissect the function of different leukocyte subpopulations in IEC co-cultures, we stimulated IEC with bacteria and investigated TNF-α and IL-10 mRNA expression in underlying immune cells. First, CaCO-2 cells were stimulated for 16 hr with *E. coli* (10^7 cfu/ml) and RT-PCR analysis of IEC co-cultured leukocyte populations was performed. Figure 3A shows low expression of TNF-α mRNA in monocytes, a slightly higher expression in B cells, and strong expression in total PBMC, PBL, T cells as well as T cell combinations with B cells and monocytes. On the other hand, RT-PCR analysis of the same samples showed IL-10 mRNA expression in PBMC, monocytes as well as a combination of T cells and monocytes (CD4:CD8:CD14 ratio 1:1:1), but not in T cells (CD4:CD8 ratio 1:1), B cells and the combination of T/B cells (CD4:CD8:CD19 ratio 1:1:1) (Fig. 3B).

Previous results showed that the Gram negative *E. coli* but not Gram positive *L. sakei* induced IL-10 expression in PBMC (24). To further elucidate whether monocytes exert immunoregulatory functions in IEC co-cultures, we measured the secretion of regulatory mediators such as IL-10 and IL-1 receptor antagonist (IL-1Ra) in the basolateral compartment of IEC/monocyte co-cultures after the stimulation with *L. sakei*. HT-29 cells were stimulated with *L. sakei* (10^7 cfu/ml) for 4, 16 and 36 hr.

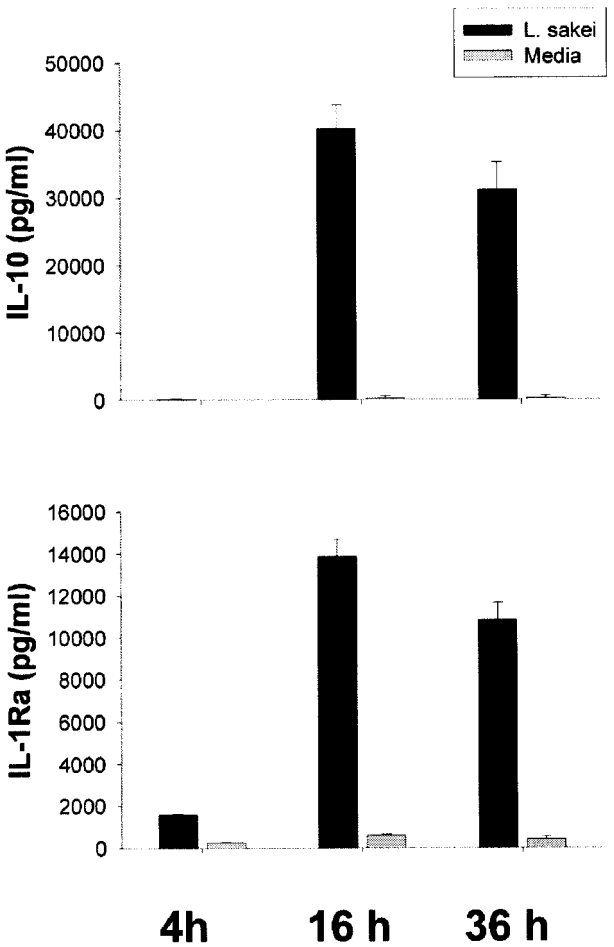

Fig. 4. IL-10 and IL-1Ra secretion in HT-29/monocyte co-cultures after stimulation with non-pathogenic bacteria. HT-29/monocyte co-cultures were stimulated with *L. sakei* (1×10^7 cfu/ml) (black bar) or culture medium (no treatment) (gray bar). Secretion of IL-10 and IL-1 receptor antagonist (Ra) was determined (pg/ml) in the basolateral compartment of transwell cultures after 4, 16 and 36 hr. Data are mean (SD) of triplicate values and represent one of three independent experiments.

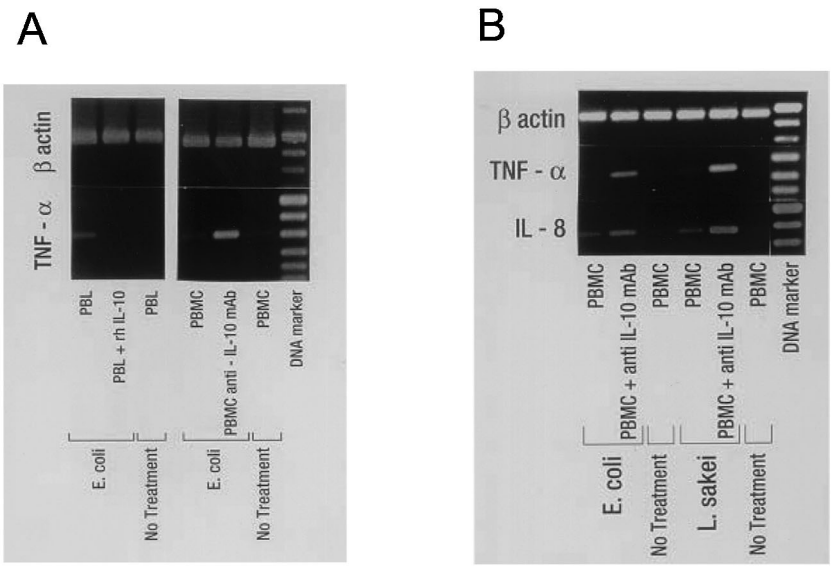

Fig. 5. Monocyte-derived IL-10 down-regulates bacterial induced activation of IEC. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to determine tumour necrosis factor alpha (TNF-α) and interleukin (IL)-8 mRNA expression in CaCO-2 and HT-29 cells after bacterial stimulation. (A) CaCO-2 cells were co-cultured with monocyte-depleted peripheral blood lymphocytes (PBL, left panel) and peripheral blood mononuclear cells (PBMC, right panel) and stimulated with *E. coli* (36 hr, 1×10^7 cfu/ml). (B) HT-29 cells were co-cultured with PBMC and stimulated with *L. sakei* (36 hr, 1×10^7 cfu/ml) or *E. coli* (36 hr, 1×10^7 cfu/ml). Where indicated (A) recombinant human (rh) IL-10 (10 pg/ml) or (B) neutralising anti-IL-10 monoclonal antibodies (mAb) (10 μg/ml) were added to the basolateral compartment. Controls were performed in the absence of anti-IL-10 mAb or rh IL-10. Isotype control antibodies did not change the results. Results represent one of three independent experiments.

As shown in Fig. 4, IL-1Ra secretion was already induced in HT-29/monocyte cultures after 4 hr of bacterial stimulation, however maximal secretion of IL-1Ra (13,850 pg/ml) and IL-10 (40,325 pg/ml) was detectable after 16 hr. The concentrations of IL-1Ra (10,825 pg/ml) and IL-10 (31,200 pg/ml) remained at relatively high levels after 36 hr incubation. These results demonstrate that bacterial activated CaCO-2 and HT-29 cells induce the expression of immunoregulatory mediators in co-cultured monocytes. *L. sakei* was used in this study to ensure that not only translocated bacterial products mediate IL-10 induction in IEC/monocyte co-cultures.

Monocyte-Derived IL-10 Down-Regulates Proinflammatory Cytokine Expression in Bacteria-Activated IEC after 36 hr Stimulation

We showed that monocytes have low potential to induce a bacteria-dependent activation of proinflammatory cytokine expression in IEC and at the same time, produce immunoregulatory cytokines such as IL-10. Our previous study showed that the expression TNF-α mRNA in *E. coli* stimulated CaCO-2 cells was first induced after 12−20 hr and down-regulated after 36 hr (25). To demonstrate that monocyte-derived IL-10 down-regulates a bacteria induced epithelial cell activation, we depleted PBMC from monocytes (peripheral blood lymphocytes, PBL) and stimulated CaCO-2/PBL co-cultures for 36 hr with *E. coli*. As shown in Fig. 5A (left panel), TNF-α mRNA expression in bacteria stimulated IEC was sustained in the absence of monocytes (CaCO-2/PBL cultures). The addition of rh IL-10 (10 pg/ml) to CaCO-2/PBL co-cultures inhibited the expression of TNF-α mRNA in CaCO-2 cells, demonstrating the pivotal role of IL-10 in the regulation of epithelial cell activation. In the same experiment (Fig. 5A, right panel), we demonstrated that the presence of neutralising anti-IL-10 mAbs (10 μg/ml) in *E. coli* stimulated CaCO-2/PBMC co-cultures sustained the expression of TNF-α mRNA at late time points (36 hr). Similarly, when neutralising anti-IL-10 mAbs (10 μg/ml) were added to the basolateral compartment of *L. sakei* or *E. coli* (10^7 cfu/ml) stimulated HT-29/PBMC co-cultures, TNF-α and IL-8 mRNA expression in IEC was sustained at 36 hr. These results may demonstrate that monocyte-derived IL-10 plays an important regulatory role in bacteria stimulated IEC/leukocyte cultures. Antibodies alone did not induce IEC activation. Specificity for the effects of anti-IL-10 mAb was controlled using Ig isotype control antibodies.

IEC and Bacterial Stimulated IEC Modulate Cell Surface Antigen Expression of Monocytes

To investigate the influence of IEC on the phenotype of co-cultured monocytes, the expression cell surface

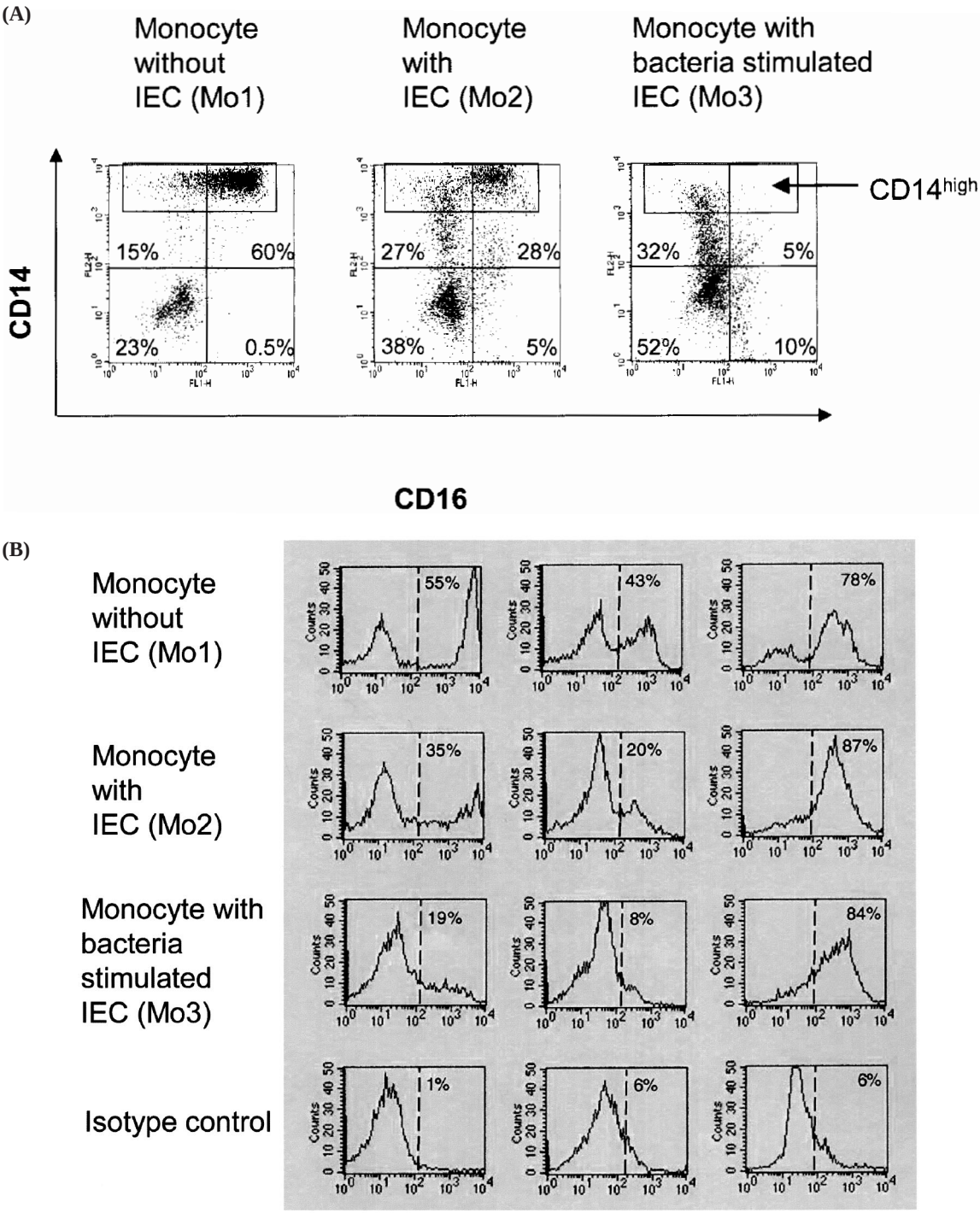

Fig. 6. Intestinal epithelial cells induce phenotype changes in peripheral blood monocytes. Peripheral blood CD14^{high} monocytes were co-cultured with HT-29 cells for 36 hr (A) and 72 hr (B) in the presence of culture medium (Mo 2) or *L. sakei* (1×10^7 cfu/ml) (Mo 3). Control monocytes were cultured in 12-well plates in the absence of HT-29 cells (Mo 1). FACS analysis was used to determine cell surface expression of CD14, CD16 and CD11b (% positive cells). Isotype controls (IgG2b, IgG1, IgG2a) were performed. Results represent one of three independent experiments.

markers on monocytes were analysed after their culture in the absence of IEC (Mo 1), their culture in the presence of IEC (Mo 2), and their culture in the presence of bacterial stimulated IEC (Mo 3). Figure 6A shows the change of CD14^{high} monocytes to a CD14^{low} CD16^{low} phenotype in IEC co-cultures at 36 hr. In the absence of IEC (Mo 1) the predominant monocyte phenotype was CD14^{high} CD16^{high}, whereas in the presence of HT-29 cells (Mo 2) the CD14/CD16 double positive population was markedly reduced (60% to 28%). This phenotype

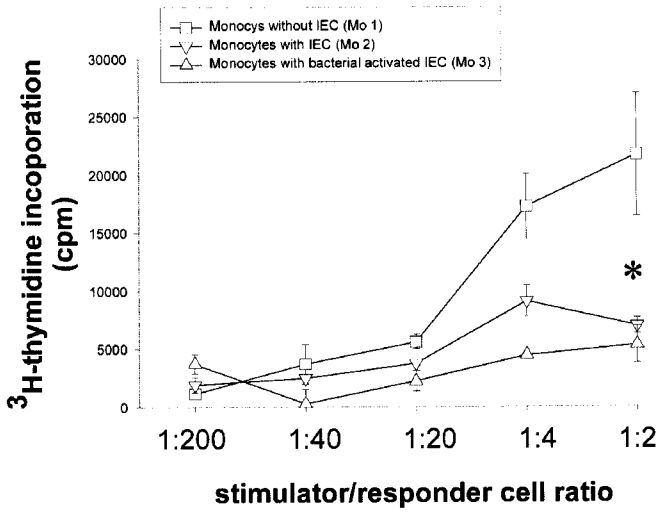

Fig. 7. Capability of monocytes to stimulate allogeneic lymphocyte response. Peripheral blood CD14 positive monocytes were co-cultured with HT-29 cells for 3 days in the presence of culture medium (triangle down) or *L. sakei* (1×10^7 cfu/ml) (triangle up). Control monocytes were cultured in 12-well plates in the absence of HT-29 cells (square). Thereafter, mixed lymphocyte reaction (MLR) was performed with allogeneic peripheral blood lymphocytes (PBL) (2×10^5 cells/well) and monocytes at varying concentrations (1×10^5, 5×10^4, 1×10^4, 5×10^3, 1×10^3 cells/well). MLR was maintained for 5 days and [^{3}H]-thymidine incorporation was determined (cpm). Data are mean (SD) of triplicate values and represent one of three independent experiments.

changes were even stronger pronounced when HT-29 cells were stimulated with *L. sakei* (60% to 5%) (Mo 3). Although the distinct $CD14^{high}$ monocyte population changed to a more $CD14^{low}$ phenotype, the total percentage of CD14 positive cells remained at relatively high numbers (55% for Mo 2 and 37% for Mo 3).

Based on these results, we performed a more detailed analysis of the monocyte phenotype after 3 days of culture. As shown in Fig. 6B (Mo 2), co-cultivation of $CD14^{high}$ monocytes with HT-29 cells resulted in the down-regulation of CD14 (55% to 35% positive cells) and CD16 (43% to 20%), while CD11b remained at high levels (78% to 87%). The expression of co-stimulatory molecules such as CD80 (63% to 51%), CD86 (66% to 51%) and CD58 (49% to 31%) were also reduced (data not shown). The stimulation of HT-29 cells with *L. sakei* (Fig. 6A, Mo 3) resulted in a further down-regulation of CD14 (35% to 19%) and CD16 (20% to 8%), whereas the expression of CD11b (87% to 84%) and co-stimulatory molecules remained unchanged (CD80: 51% to 48%; CD86: 51% to 50%; CD58: 31% to 30%) (data not shown). IEC and bacterial stimulated IEC did neither influence the expression of CD33 (lineage marker) nor HLA-DR (data not shown). Thus, IEC co-cultured $CD14^{high}$ peripheral blood monocytes acquired a $CD14^{low}$ $CD16^{low}$ $CD11b^{high}$ $CD33^{high}$ $HLA\text{-}DR^{high}$ $CD80^{low}$ $CD86^{low}$ $CD58^{low}$. Cell viability after 3 days was $>95\%$. The number of apoptotic or necrotic cells did not exceed 5% (data not shown).

Decreased Capacity of IEC Co-Cultured Monocytes to Stimulate Allogeneic Lymphocyte Responses

One important function of antigen presenting cells (APC) is the stimulation of lymphocytes. Mixed-lymphocyte reactions (MLR) can be used to assess to potential of APC to stimulate proliferation of allogeneic lymphocytes (38). When the different *in vitro* matured monocytes (Mo 1−3) were investigated for their capacity to stimulate proliferation of alloreactive human PBL, only the plastic adherent monocytes (Mo 1) were able to stimulate significant proliferation (stimulator/responder cell ratio 1:4, 1:2). In contrast, those monocytes, matured in the presence of HT-29 or *L. sakei* activated HT-29 cells, could not trigger a substantial lymphocyte response (Fig. 7). Similar results were obtained when purified $CD4^+$ T cells were used as responder cells (data not shown).

Discussion

Intestinal monocytes, which constitute 10−20% of mononuclear cells in the lamina propria, differ markedly in phenotype and function from peripheral blood monocytes (16, 40). The classical monocyte-specific surface antigens CD14 (LPS co-receptor), CD11b (complement receptor 3, CR3) and CD16 (FcγIII receptor) are expressed at low levels in the normal intestinal mucosa. This is thought as one mechanism to prevent activation induced by trace amounts of translocated bacterial products (33, 46). Under pathological conditions, such as

active inflammatory bowel diseases (IBD), an increased fraction of monocytes with a monocyte-like phenotype appears in the lamina propria (1, 22, 23, 48, 49). Rugtveit et al. (50) showed that these CD14^{high} monocytes in IBD mucosa represent a newly recruited subset of intestinal monocytes extravasated from the peripheral blood, harbouring an increased potential for the production of proinflammatory cytokines compared to the resident CD14^{low} population, and therefore, may be involved the development of IBD (8).

In this *in vitro* study, we demonstrated that under the influence of intestinal epithelial cells (IEC), peripheral blood CD14^{high} monocytes changed to a CD14^{low} phenotype with immunoregulatory functions. A recent study from Spöttl et al. (56) showed for the first time that IEC cultured in multicellular spheroids induce the differentiation of CD14^{high} peripheral blood monocytes in intestine-like macrophage phenotype. In addition, we showed that the development of a CD14^{low} monocyte phenotype is further pronounced in the presence of bacteria activated IEC, suggesting that non-pathogenic bacteria/epithelial cell cross talk may help to maintain local immune homeostasis. The absence of increased numbers of apoptotic or necrotic cells during co-culture indicated that the loss of CD14^{high} expressing cells may be a consequence of monocyte differentiation and/or receptor shedding rather than cell death (3). This was supported by the observation that the panmyolilic differentiation marker CD33, reliable for the identification of intestinal tissue monocytes (46), and CD11b remained high. The development of CD14^{high} CD16^{high} tissue monocytes, observed under certain inflammatory conditions (6, 7, 17, 21, 30) was significantly suppressed under the influence of IEC and bacteria stimulated IEC, even though the kinetic in the development of a CD14^{low} and CD16^{low} phenotype differed for the two cell surface markers. Although soluble bacterial products such as lipopolysaccharide (LPS), peptidoglycan-polysaccharide (PG-PS), or formylated chemotactic oligopeptides (fMLP) (10, 55, 57), which have been shown to initiate inflammatory processes, may translocate to some extent through the epithelial cell monolayer, the stimulation of IEC with non-pathogenic *E. coli* or *L. sakei* facilitated the development of CD14^{low} CD16^{low} monocytes. The role of pattern recognition receptors such TLR4 and TLR2 in the initiation of non-pathogenic bacteria-induced IEC activation is under current investigation.

Lymphoid populations preferentially CD4^{+} T cells were shown to drive the activation of IEC by intestinal *E. coli* or food derived *L. sakei*, through a yet undefined mechanism, while monocytes were identified as a immunoregulatory cell population, secreting high amounts of IL-1Ra and IL-10. A previous study showed that *L. sakei* induced the secretion of IL-12 and IFN-γ but not IL-10 in PBMC cultures (24), demonstrating the capability of IEC to modulate immune responses. It was remarkable, that at later time points monocyte-derived IL-10 antagonised the lymphocyte-dependent activation of IEC with respect to TNF-α, and to a lesser extent IL-8 gene expression. The anti-inflammatory cytokine IL-10 has been reported to play an essential role in the control of normal gut homeostasis (2, 51). In active inflammatory bowel disease, associated with increased proinflammatory cytokines (42, 54), IL-10 secretion has been shown to down-regulate proinflammatory cytokine response in IBD derived lamina propria mononuclear cells and peripheral blood monocytes (52). In our model system, the IL-10 response was exclusively detected in monocytes and therefore might have inhibited TNF-α expression by an autocrine/paracrine, negative feed back regulation (35). Kucharzik et al. (29) showed that CaCO-2 cells prime lamina propria mononuclear cells to induce IL-10 secretion. The authors proposed that monocytes may be selectively targeted by epithelial cell derived mediators such as TGF-β1 or prostaglandin-E2 (PGE), resulting in the secretion of IL-10.

The modulation of lamina propria lymphocyte activation by local monocytes was reported to be partially based on their low expression of the co-stimulatory molecules CD80 (B7.1), CD86 (B7.2) and CD58 for T cell accessory receptors CD28/CTLA-4 and CD2 (44). The appearance of CD14^{high} CD80^{high} monocytes in IBD lesions was reported to change the normal anergic mucosal state towards an enhanced Th1-type of immune activation (47, 53), leading to a loss of tolerance against the indigenous bacterial flora. We demonstrated that under the influence of IEC, the percentage expression of co-stimulatory molecules, were down regulated, while expression of MHC class II molecules was conserved. In fact, IEC conditioned monocytes had reduced potential to trigger an allogeneic lymphocyte response. CD14^{high} peripheral blood monocytes, which are recruited to the intestinal mucosa, undergo differentiation near the epithelial surfaces. Thus, the influence of the intestinal epithelium on this particular monocyte population might be of critical importance in the modulation of the local T cell responses and induction of oral tolerance to luminal antigens (37). *In vitro*, this regulation is bidirectional, since the co-cultured PBMC inhibited bacterial induced TNF-α and IL-8 mRNA expression, a process mediated in part by monocyte-derived IL-10. Notably, commensal bacteria further promoted the induction of CD14^{low} CD16^{low} immunoregulatory monocytes by IEC, suggesting that bacterial cross talk at the mucosal surface could be beneficial for the host (32).

References

1) Allison, M.C., and Poulter, L.W. 1991. Changes in phenotypically distinct mucosal macrophage populations may be a prerequisite for the development of inflammatory bowel disease. Clin. Exp. Immunol. **85**: 504–509.
2) Autschbach, F., Braunstein, J., Helmke, B., Zuna, I., Schurmann, G., Niemir, Z.I., Wallich, R., Otto, H.F., and Meuer, S.C. 1998. *In situ* expression of interleukin-10 in noninflamed human gut and in inflammatory bowel disease. Am. J. Pathol. **153**: 121–130.
3) Bazil, V., and Strominger, J.L. 1991. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. J. Immunol. **147**: 1567–1574.
4) Berg, R.D., and Savage, D.C. 1972. Immunological responses and microorganisms indigenous to the gastrointestinal tract. Am. J. Clin. Nutr. **25**: 1364–1371.
5) Blumberg, R.S., Terhorst, C., Bleicher, P., McDermott, F.V., Allan, C.H., Landau, S.B., Trier, J.S., and Balk, S.P. 1991. Expression of a nonpolymorphic MHC class I-like molecule, CD1D, by human intestinal epithelial cells. J. Immunol. **147**: 2518–2524.
6) Blumenstein, M., Boekstegers, P., Fraunberger, P., Andreesen, R., Ziegler-Heitbrock, H.W., and Fingerle-Rowson, G. 1997. Cytokine production precedes the expansion of $CD14^+$ $CD16^+$ monocytes in human sepsis: a case report of a patient with self-induced septicemia. Shock **8**: 73–75.
7) Brauner, A., Lu, Y., Hallden, G., Hylander, B., and Lundahl, J. 1998. Difference in the blood monocyte phenotype between uremic patients and healthy controls: its relation to monocyte differentiation into macrophages in the peritoneal cavity. Inflammation **22**: 55–66.
8) Casini-Raggi, V., Kam, L., Chong, Y.J., Fiocchi, C., Pizarro, T.T., and Cominelli, F. 1995. Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. A novel mechanism of chronic intestinal inflammation. J. Immunol. **154**: 2434–2440.
9) Cebra, J.J. 1999. Influences of microbiota on intestinal immune system development. Am. J. Clin. Nutr. **69**: 1046S–1051S.
10) Chester, J.F., Ross, J.S., Matt, R.A., and Weitzmann, S.A. 1985. Acute colitis produced by chemotactic peptides in rats and mice. Am. J. Pathol. **121**: 284–290.
11) Christ, A.D., Colgan, S.P., Balk, S.P., and Blumberg, R.S. 1997. Human intestinal epithelial cell lines produce factor(s) that inhibit CD3-mediated T-lymphocyte proliferation. Immunol. Lett. **58**: 159–165.
12) Cong, Y., Brandwein, S.L., McCabe, R.P., Lazenby, A., Birkenmeier, E.H., Sundberg, J.P., and Elson, C.O. 1998. $CD4^+$ T cells reactive to enteric bacterial antigens in spontaneously colitic C3H/HeJBir mice: increased T helper cell type 1 response and ability to transfer disease. J. Exp. Med. **187**: 855–864.
13) Darfeuille-Michaud, A., Neut, C., Barnich, N., Lederman, E., Di Martino, P., Desreumaux, P., Gambiez, L., Joly, B., Cortot, A., and Colombel, J.F. 1998. Presence of adherent *Escherichia coli* strains in ileal mucosa of patients with Crohn's disease. Gastroenterology **115**: 1405–1413.
14) De Man, J.C., Rogosa, M., and Sharpe, M.E. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. **23**: 130–135.
15) D'Haens, G.R., Geboes, K., Peeters, M., Baert, F., Penninckx, F., and Rutgeerts, P. 1998. Early lesions of recurrent Crohn's disease caused by infusion of intestinal contents in excluded ileum. Gastroenterology **114**: 262–267.
16) Donnellan, W.L. 1965. The structure of the colonic mucosa. The epithelium and subepithelial reticulohistiocytic complex. Gastroenterology **49**: 496–514.
17) Draude, G., von Hundelshausen, P., Frankenberger, M., Ziegler-Heitbrock, H.W., and Weber, C. 1999. Distinct scavenger receptor expression and function in the human CD14(+)/CD16(+) monocyte subset. Am. J. Physiol. **276**: H1144–H1149.
18) Duchmann, R., Kaiser, I., Hermann, E., Mayet, W., Ewe, K., and Meyer, Z.B.K. 1995. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin. Exp. Immunol. **102**: 448–455.
19) Duchmann, R., May, E., Heike, M., Knolle, P., Neurath, M., and Meyer, Z.B.K. 1999. T cell specificity and cross reactivity towards enterobacteria, bacteroides, bifidobacterium, and antigens from resident intestinal flora in humans. Gut **44**: 812–818.
20) Duchmann, R., Schmitt, E., Knolle, P., Meyer, Z.B.K., and Neurath, M. 1996. Tolerance towards resident intestinal flora in mice is abrogated in experimental colitis and restored by treatment with interleukin-10 or antibodies to interleukin-12. Eur. J. Immunol. **26**: 934–938.
21) Fingerle, G., Pforte, A., Passlick, B., Blumenstein, M., Strobel, M., and Ziegler-Heitbrock, H.W. 1993. The novel subset of $CD14^+/CD16^+$ blood monocytes is expanded in sepsis patients. Blood **82**: 3170–3176.
22) Grimm, M.C., Pavli, P., Van de Pol, E., and Doe, W.F. 1995. Evidence for a $CD14^+$ population of monocytes in inflammatory bowel disease mucosa—implications for pathogenesis. Clin. Exp. Immunol. **100**: 291–297.
23) Grimm, M.C., Pullman, W.E., Bennett, G.M., Sullivan, P.J., Pavli, P., and Doe, W.F. 1995. Direct evidence of monocyte recruitment to inflammatory bowel disease mucosa. J. Gastroenterol. Hepatol. **10**: 387–395.
24) Haller, D., Blum, S., Bode, C., Hammes, W.P., and Schiffrin, E.J. 2000. Activation of human peripheral blood mononuclear cells by nonpathogenic bacteria *in vitro*: evidence for NK cells as primary targets. Infect. Immun. **68**: 752–759.
25) Haller, D., Bode, C., Hammes, W.P., Pfeifer, A.M., Schiffrin, E.J., and Blum, S. 2000. Non-pathogenic bacteria elicit a differential cytokine response by intestinal epithelial cell/leucocyte co-cultures. Gut **47**: 79–87.
26) Jung, H.C., Eckmann, L., Yang, S.K., Panja, A., Fierer, J., Morzycka-Wroblewska, E., and Kagnoff, M.F. 1995. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. J. Clin. Invest. **95**: 55–65.
27) Kagnoff, M., and Eckmann, L. 1997. Epithelial cells as sensors for microbial infection. J. Clin. Invest. **100**: 6–10.
28) Kenworthy, R. 1971. Observations on the reaction of the intestinal mucosa to bacterial challenge. J. Clin. Pathol. **24**: 138–142.
29) Kucharzik, T., Lugering, N., Winde, G., Domschke, W., and Stoll, R. 1997. Colon carcinoma cell lines stimulate

monocytes and lamina propria mononuclear cells to produce IL-10. Clin. Exp. Immunol. **110**: 296–302.
30) Locher, C., Vanham, G., Kestens, L., Kruger, M., Ceuppens, J.L., Vingerhoets, J., and Gigase, P. 1994. Expression patterns of Fc gamma receptors, HLA-DR and selected adhesion molecules on monocytes from normal and HIV-infected individuals. Clin. Exp. Immunol. **98**: 115–122.
31) MacDonald, T.T. 1999. Effector and regulatory lymphoid cells and cytokines in mucosal sites. Curr. Top. Microbiol. Immunol. **236**: 113–135.
32) Macpherson, A.J., Gatto, D., Sainsbury, E., Harriman, G.R., Hengartner, H., and Zinkernagel, R.M. 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science **288**: 2222–2226.
33) Malizia, G., Calabrese, A., Cottone, M., Raimondo, M., Trejdosiewicz, L.K., Smart, C.J., Oliva, L., and Pagliaro, L. 1991. Expression of leukocyte adhesion molecules by mucosal mononuclear phagocytes in inflammatory bowel disease. Gastroenterology **100**: 150–159.
34) McCormick, B.A., Colgan, S.P., Delp-Archer, C., Miller, S.I., and Madara, J.L. 1993. *Salmonella typhimurium* attachment to human intestinal epithelial monolayers: transcellular signalling to subepithelial neutrophils. J. Cell Biol. **123**: 895–907.
35) Moore, K.W., O'Garra, A., de Waal, M., Vieira, P., and Mosmann, T.R. 1993. Interleukin-10. Annu. Rev. Immunol. **11**: 165–190.
36) Nathens, A.B., Rotstein, O.D., Dackiw, A.P., and Marshall, J.C. 1995. Intestinal epithelial cells down-regulate macrophage tumor necrosis factor-alpha secretion: a mechanism for immune homeostasis in the gut-associated lymphoid tissue. Surgery **118**: 343–350.
37) Newberry, R.D., Stenson, W.F., and Lorenz, R.G. 1999. Cyclooxygenase-2-dependent arachidonic acid metabolites are essential modulators of the intestinal immune response to dietary antigen. Nature **5**: 900–906.
38) Oehler, J.R., and Herberman, R.B. 1977. Interaction of T cells with allogeneic macrophages in mixed leukocyte cultures in rats. J. Immunol. **119**: 1785–1789.
39) Panja, A., Goldberg, S., Eckmann, L., Krishen, P., and Mayer, L. 1998. The regulation and functional consequence of proinflammatory cytokine binding on human intestinal epithelial cells. J. Immunol. **161**: 3675–3684.
40) Pavli, P., Maxwell, L., Van de Pol, E., and Doe, F. 1996. Distribution of human colonic dendritic cells and macrophages. Clin. Exp. Immunol. **104**: 124–132.
41) Phalipon, A., and Sansonetti, P.J. 1999. Microbial-host interactions at mucosal sites. Host response to pathogenic bacteria at mucosal sites. Curr. Top. Microbiol. Immunol. **236**: 163–189.
42) Pullman, W.E., Elsbury, S., Kobayashi, M., Hapel, A.J., and Doe, W.F. 1992. Enhanced mucosal cytokine production in inflammatory bowel disease. Gastroenterology **102**: 529–537.
43) Qiao, L., Schurmann, G., Autschbach, F., Wallich, R., and Meuer, S.C. 1993. Human intestinal mucosa alters T-cell reactivities. Gastroenterology **105**: 814–819.
44) Qiao, L., Braunstein, J., Golling, M., Schurmann, G., Autschbach, F., Moller, P., and Meuer, S. 1996. Differential regulation of human T cell responsiveness by mucosal versus blood monocytes. Eur. J. Immunol. **26**: 922–927.
45) Qiao, L., Schurmann, G., Betzler, M., and Meuer, S.C. 1991. Activation and signaling status of human lamina propria T lymphocytes. Gastroenterology **101**: 1529–1536.
46) Rogler, G., Hausmann, M., Vogl, D., Aschenbrenner, E., Andus, T., Falk, W., Andreesen, R., Scholmerich, J., and Gross, V. 1998. Isolation and phenotypic characterization of colonic macrophages. Clin. Exp. Immunol. **112**: 205–215.
47) Rugtveit, J., Bakka, A., and Brandtzaeg, P. 1997. Differential distribution of B7.1 (CD80) and B7.2 (CD86) costimulatory molecules on mucosal macrophage subsets in human inflammatory bowel disease (IBD). Clin. Exp. Immunol. **110**: 104–113.
48) Rugtveit, J., Brandtzaeg, P., Halstensen, T.S., Fausa, O., and Scott, H. 1994. Increased macrophage subset in inflammatory bowel disease: apparent recruitment from peripheral blood monocytes. Gut **35**: 669–674.
49) Rugtveit, J., Haraldsen, G., Hogasen, A.K., Bakka, A., Brandtzaeg, P., and Scott, H. 1995. Respiratory burst of intestinal macrophages in inflammatory bowel disease is mainly caused by $CD14^+L1^+$ monocyte derived cells. Gut **37**: 367–373.
50) Rugtveit, J., Nilsen, E.M., Bakka, A., Carlsen, H., Brandtzaeg, P., and Scott, H. 1997. Cytokine profiles differ in newly recruited and resident subsets of mucosal macrophages from inflammatory bowel disease. Gastroenterology **112**: 1493–1505.
51) Sartor, R.B. 1997. Pathogenesis and immune mechanisms of chronic inflammatory bowel disease. Am. J. Gastroenterol. **92**: 5S-11S.
52) Schreiber, S., Heinig, T., Thiele, H.G., and Raedler, A. 1995. Immunoregulatory role of interleukin-10 in patients with inflammatory bowel disease. Gastroenterology **108**: 1434–1444.
53) Schreiber, S., MacDermott, R.P., Raedler, A., Pinnau, R., Bertovich, M.J., and Nash, G.S. 1991. Increased activation of isolated intestinal lamina propria mononuclear cells in inflammatory bowel disease. Gastroenterology **101**: 1020–1030.
54) Schreiber, S., Raedler, A., Conn, A.R., Rombeau, J.L., and MacDermott, R.P. 1992. Increased *in vitro* release of soluble interleukin-2 receptor by colonic lamina propria mononuclear cells in inflammatory bowel disease. Gut **33**: 236–241.
55) Schwab, J.H. 1993. Phlogistic properties of peptidoglycan-polysaccharide polymers from cell walls of pathogenic and normal-flora bacteria which colonize humans. Infect. Immun. **61**: 4535–4539.
56) Spöttl, T., Hausmann, M., Kreutz, M., Peuker, A., Vogl, D., Schölmerich, J., Falk, W., Andreesen, R., Andus, T., Herfarth, H., and Rogler, G. 2001. Monocyte differentiation in intestine-like macrophage phenotype induced by epithelial cells. J. Leukoc. Biol. **70**: 241–251.
57) Sundberg, J.P., Elson, C.O., Bedigian, H., and Birkenmeier, E.H. 1994. Spontaneous, heritable colitis in a new substrain of C3H/HeJ mice. Gastroenterology **107**: 1726–1735.
58) Targan, S.R., Deem, R.L., Liu, M., Wang, S., and Nel, A. 1995. Definition of a lamina propria T cell responsive state. Enhanced cytokine responsiveness of T cells stimulated through the CD2 pathway. J. Immunol. **154**: 664–675.