Non-pathogenic bacteria elicit a differential cytokine response by intestinal epithelial cell/leucocyte co-cultures

D Haller, C Bode, W P Hammes, A M A Pfeifer, E J Schiffrin, S Blum

Abstract

***Background and aim*—Intestinal epithelial cells (IEC) are thought to participate in the mucosal defence against bacteria and in the regulation of mucosal tissue homeostasis. Reactivity of IEC to bacterial signals may depend on interactions with immunocompetent cells. To address the question of whether non-pathogenic bacteria modify the immune response of the intestinal epithelium, we co-cultivated enterocyte-like CaCO-2 cells with human blood leucocytes in separate compartments of transwell cultures.**

***Methods*—CaCO-2/PBMC co-cultures were stimulated with non-pathogenic bacteria and enteropathogenic *Escherichia coli*. Expression of tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-8, monocyte chemoattracting protein 1 (MCP-1), and IL-10 was studied by enzyme linked immunosorbent assays (cytokine secretion) and by semiquantitative reverse transcription-polymerase chain reaction.**

***Results*—Challenge of CaCO-2 cells with non-pathogenic *E coli* and *Lactobacillus sakei* induced expression of IL-8, MCP-1, IL-1β, and TNF-α mRNA in the presence of underlying leucocytes. Leucocyte sensitised CaCO-2 cells produced TNF-α and IL-1β whereas IL-10 was exclusively secreted by human peripheral blood mononuclear cells. CaCO-2 cells alone remained hyporesponsive to the bacterial challenge. *Lactobacillus johnsonii*, an intestinal isolate, showed reduced potential to induce proinflammatory cytokines but increased transforming growth factor beta mRNA in leucocyte sensitised CaCO-2 cells. TNF-α was identified as one of the early mediators involved in cellular cross talk. In the presence of leucocytes, discriminative activation of CaCO-2 cells was observed between enteropathogenic *E coli* and non-pathogenic bacteria.**

***Conclusion*—The differential recognition of non-pathogenic bacteria by CaCO-2 cells required the presence of underlying leucocytes. These results strengthen the hypothesis that bacterial signalling at the mucosal surface is dependent on a network of cellular interactions.**

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Keywords: CaCO-2 cells; leucocytes; enteropathogenic *E coli*; *Lactobacilli*; tumour necrosis factor; interleukin 1β; interleukin 10; chemokines

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Epithelial cells that line the intestinal tract are considered to participate in the initiation and regulation of the mucosal immune response to bacteria by interacting with immune cells of the gut associated lymphoid tissue, lamina propria lymphocytes (LPL), and intraepithelial lymphocytes (IEL).[1] It was shown that intestinal epithelial cells (IEC) may change phenotype as a consequence of stimulation by IEL derived soluble mediators such as interferon (IFN) γ.[2 3] This is in agreement with the concept that activated IEC express higher levels of HLA class II molecules,[4] classical class I, and non-classical HLA class Ib molecules, such as CD1d,[5 6] the adhesion molecule ICAM-1,[7] complement factors, and cytokine receptors.[8–11] Furthermore, human IEC lines secrete chemokines, interleukin (IL)-1, IL-6, IL-7, granulocyte macrophage-colony stimulating factor, tumour necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), and eicosanoids on stimulation with enteropathogenic *Escherichia coli*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Shigella flexneri*, and rotavirus but remain hyporesponsive to non-pathogenic commensal bacteria.[12–20] In the case of the colonised intestinal mucosa, however, protection against enteropathogens is mediated in part by the indigenous microflora.[21] The importance of microflora derived host protection is evident by the higher susceptibility of germ free animals to intestinal infections.[22 23] IEC may be implicated in the recognition of components of the intestinal microflora and the transduction of bacteria derived signals to resident mucosal immune cells.

To investigate the effect of non-pathogenic bacteria on modulation of the cytokine response by IEC in vitro, enterocyte-like CaCO-2 cells were co-cultured with human blood leucocytes in transwell cell cultures. In this study, we demonstrated that commensal non-pathogenic bacteria of different origins have the ability to induce a distinct cytokine/chemokine response in leucocyte sensitised

Abbreviations used in this paper: IEC, intestinal epithelial cells; PBMC, peripheral blood mononuclear cells; TNF-α, tumour necrosis factor alpha; IL-1β, interleukin 1 beta; IEL, intraepithelial lymphocytes; PBL, peripheral blood lymphocytes; LPL, lamina propria lymphocytes; RT-PCR, reverse transcription-polymerase chain reaction; TGF-β, transforming growth factor beta; PBS, phosphate buffered saline; TEER, transepithelial electrical resistance; LPS, lipopolysaccharide; bp, base pair; cfu, colony forming units; MCP-1, monocyte chemoattracting protein 1; mAb, monoclonal antibody; IBD, inflammatory bowel disease.

CaCO-2 cells and deliver a discriminative signal to underlying immunocompetent cells. We provide evidence that bidirectional cross talk between CaCO-2 cells and immunocompetent cells mediates recognition of non-pathogenic bacteria and is implicated in the maintenance of tissue homeostasis.

Material and methods

BACTERIA AND CULTURE CONDITIONS

Enteropathogenic *Escherichia coli* (EPEC) E2348/69, EPEC E2348/69 *eaeA* deletion mutant CVD206 (kindly provided by Professor Dr J Hacker, Institute of Molecular Biology of Infections, University Würzburg, Germany), and the non-pathogenic *E coli* LTH 634 (Strain Collection, Institute of Food Technology, Hohenheim University, Germany) which lacks virulence factors commonly distributed in the species *E coli*,[24] were grown in brain-heart infusion broth at 37°C. *Lactobacillus johnsonii* La 1 (Strain Collection, Nestlé Research Centre, Lausanne, Switzerland), a human intestinal isolate, was grown in MRS broth[25] without acetate at 37°C. *Lactobacillus sakei* LTH 681 isolated from fermented food was grown in the same broth at 30°C. All bacteria were harvested by centrifugation (3000 *g*, 15 minutes) after 24 hours of cultivation at the stationary growth phase. For some experiments bacteria were heat killed (95°C, 30 minutes). Bacteria were washed three times with phosphate buffered saline (PBS) (1×, pH 7.2, Gibco BRL, Basel, Switzerland) 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 enterocyte-like CaCO-2 cells (passage 60-65) were seeded at a density of 2.5×10^5 cells/ml on 25 mm cell culture inserts (0.4 μm nucleopore size; Becton Dickinson, Basel, Switzerland). The inserts were placed into six well tissue culture plates (Nunc) and cultured 18–22 days at 37°C/10% CO_2 in DMEM (glutamine, high glucose; Amimed, Allschwil, Switzerland) supplemented with 20% decomplemented fetal calf serum (56°C, 30 minutes; Amimed), 1% MEM non-essential amino acids (Gibco BRL), 10 μg/ml gentamycin (Gibco BRL), and 0.1% penicillin/streptomycin (10 000 IU/ml/10 000 UG/ml; Gibco BRL). Cell culture medium was changed every second day until the cells were fully differentiated (day 21). Transepithelial electrical resistance (TEER) was determined continuously in confluent CaCO-2 monolayers using a MultiCell-ERS electrode (voltmeter/ohmmeter).

ISOLATION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

Human peripheral blood mononuclear cells (PBMC) from healthy volunteers were purified from buffy coats (Blood Transfusion Centre, Lausanne, Switzerland) using Ficoll-Hypaque (1077, Pharmacia, Dübendorf, Switzerland) gradient centrifugation (500 *g*, 30 minutes). PBMC were taken from the interface and washed five times with RPMI 1640 and finally diluted (2×10^6 cells/ml) in RPMI 1640 containing 20% decomplemented human AB serum (56°C, 30 minutes; Sigma, St Louis, Missouri, USA) and gentamycin (150 μg/ml), henceforth referred to as culture medium. Gentamycin free culture medium was used where indicated.

CaCO-2/LEUCOCYTE CO-CULTURE MODEL

Tissue culture inserts covered with CaCO-2 cell monolayers were washed twice with prewarmed RPMI 1640 medium and transferred to six well tissue culture plates. Culture medium (2 ml) was added to the apical and basolateral compartments of the transwell cell culture system. In CaCO-2/leucocyte co-cultures, 2 ml of freshly purified PBMC (2×10^6 cells/ml) were added to the basolateral part of the culture plates.

BACTERIAL STIMULATION OF CaCO-2/LEUCOCYTE CO-CULTURES

The apical surface of CaCO-2 monolayers was challenged by addition of 1×10^6 or 1×10^7 cfu/ml of non-pathogenic *E coli*, *L johnsonii*, *L sakei*, or lipopolysaccharide (LPS) (1 μg/ml, Serotype O111:B4, Sigma), respectively. Gentamycin was added apically (150 μg/ml) to the cell culture where indicated. In accordance with Canil and colleagues,[26] infection of CaCO-2 cells with EPEC strains E2348/69 and CVD206 was performed for four hours in the absence of gentamycin. Thereafter, gentamycin was added (150 μg/ml) to prevent (i) bacterial growth and (ii) secretion of bacterial metabolites. Cytokine expression in CaCO-2 cells was controlled by stimulation with IL-1β (10 ng/ml, Sigma). Culture medium (no treatment) was used as a negative control. After stimulation for 6–36 hours (37°C, 10% CO_2), the CaCO-2 cells were collected, washed once with cold PBS (1×, pH 7.2), and lysed in denaturation solution for RNA extraction (Micro RNA Isolation Kit, Stratagene). Cellular lysates were stored at −20°C. Cell culture supernatants were collected separately from the apical and basolateral compartments and frozen at −20°C until further analysis. The immune response of CaCO-2 cells and underlying PBMC was monitored by analysis of cytokine gene transcription (TNF-α, IL-8, monocyte chemoattracting protein 1 (MCP-1), TGF-β, IL-12, IFN-γ, IL-4, IL-10) using a reverse transcription-polymerase chain reaction (RT-PCR) technique and determination of cytokine secretion in cell culture supernatants using an ELISA technique.

To investigate the role of TNF-α and IL-1β in the cellular cross talk of CaCO-2/PBMC co-cultures, challenging experiments were performed in the presence of neutralising anti-TNF-α (10 μg/ml, IKD9, ImmunoKontakt, Frankfurt, Germany) or anti-IL-1β (500-M01B, BioConcept, Allschwil, Switzerland) monoclonal antibodies (mAbs) in the basolateral compartment. The experiments included isotype control antibodies. Thereafter, CaCO-2 cells were analysed for TNF-α and IL-8 mRNA expression using RT-PCR.

To control the potential migration of immunostimulatory bacterial products into the basolateral compartment, different concentrations of LPS (0, 0.1, 1, 100 μg/ml) were added to the cell culture inserts in the absence and presence of CaCO-2 cells. Measuring TEER (Ω cm^2) monitored the intact barrier function of CaCO-2 cell monolayers.

ADHERENCE ASSAY

To investigate the potential of non-pathogenic bacteria and EPEC strains to attach to CaCO-2 cells, adherence assays were performed as described previously by Bernet and colleagues.[27] Briefly, bacteria were metabolically labelled for 24 hours by addition of [^{14}C] acetic acid (94 mCi/mmol, 100 μCi/10 ml tube; Amersham) for non-pathogenic *E coli* and [^{3}H] adenine (24 Ci/mmol, 100 mCi/10 ml tube; Amersham) for *L johnsonii* and *L sakei*. CaCO-2 cells were incubated for one hour with bacteria (1×10^8), washed three times with PBS and dissolved in 0.2 N NaOH. The rate of bacterial adhesion to CaCO-2 cells in the absence and presence of leucocytes after one hour of incubation was evaluated by liquid scintillation counting or plating on agar. Invasion of CaCO-2 cells was controlled according to the method described by Canil and colleagues.[26] Briefly, CaCO-2 cell monolayers were challenged for four hours with live bacteria in the absence of antibiotics. The CaCO-2 cell monolayer was washed twice with PBS (1×) and culture medium containing gentamycin (150 μg/ml) was added. After one hour of incubation the CaCO-2 cell monolayer was washed three times with PBS and lysed. Bacterial numbers were determined by plating on agar.

CYTOKINE SECRETION BY CONDITIONED CaCO-2 CELLS

To determine to what extent CaCO-2 cells participate in the secretory cytokine response of the co-culture system, transfer experiments were performed. CaCO-2 cells were incubated with bacteria in CaCO-2 monocultures and CaCO-2/leucocyte co-cultures for 12 hours. Thereafter the CaCO-2 cell monolayers on the cell culture inserts were washed twice basolaterally and transferred to fresh culture medium. Cell culture supernatants of CaCO-2 cells and leucocyte sensitised CaCO-2 cells were collected after another 24 hours and analysed for secretion of cytokines.

RNA EXTRACTION AND AMPLIFICATION BY REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA from CaCO-2 cells and PBMC was isolated using the acid guanidinium thiocyanate/phenol/chloroform method according to the supplier's recommendation (Micro RNA Isolation Kit, Stratagene). Total RNA (0.5 μg), 1 mM of each dNTP, 2.5 U/μl MuLV reverse transcriptase (Perkin Elmer, New Jersey, USA), and specific 3' primers coding for the human cytokines TNF-α, IL-1β, IL-8, MCP-1, IL-12, IFN-γ, IL-4, IL-10, TGF-β, and β-actin were incubated at 42°C for 30 minutes. PCR amplification was performed in a total volume of 50 μl using Taq polymerase (Perkin Elmer) and specific cytokine primers. After initial denaturation at 94°C for one minute, 30 cycles of denaturation (94°C, one minute), primer annealing (60°C, one minute), and extension (72°C, one minute) were performed (PTC-100 Thermocycler, MJ Research Inc, Naltham, USA). PCR products were subjected to gel electrophoresis on 2% agarose gels containing 1×Tris borate EDTA and visualised by staining with ethidium bromide. The oligonucleotide primers used were as follows: TNF-α (5') 5'-CAGAGGGAAGAGTTCCCCAG-3' (3') 5'-CCTTGGTCTGGTAGGAG-ACG-3' (product length 324 base pairs (bp)); IL-1β (5') 5'-GATCATCTGTCTCTGAATCA-3' (3') 5'-TCCAGATTATGTAATGCAGC-3' (387 bp); IL-8 (5') 5'-CTGGCCGTGGCTCTC TTGGCAG-CCTTCCTG-3' (3') 5'-GGCAA CCCTACAACAGACCCACACAATA-CA-3' (395 bp); MCP-1 (5') 5'-TCTGTGCCTG CTGCTCATAGC-3' (3') 5'-GGGT-AGAAC TGTGGTTCAAGAGG-3' (510 bp); IL-12 p40 (5') 5'-CGTAGAATTGGA-TTGGTAT CCGG-3' (3') 5'-GCTCTTGC-CCTGGAC CTGAACGC-3' (702 bp); IFN-γ (5') 5'-ATATCTTGGCTTTTCAGCTC-3' (3') 5'-CTCCTTTTTCGCTTCCCTGT-3' (489 bp); IL-4 (5') 5'-GTTCTTCCTGCTAGC ATGTG-3' (3') 5'-ATTTCTCTCTCATGA TCGTC-3' (411 bp); IL-10 (5') 5'-TGATGTCTGGGTC-TTGGTTC-3' (3') 5'-GCCTAACATGCT-TCGAGATC-3' (204 bp); TGF-β (5') 5'-CTC-CGAGAAGCGG TACCTGAAC-3' (3') 5'-CACTTGCAGTG TGTTATCCCT-3' (288 bp); and β-actin (5') 5'-GGCGACGAGGCCCAGGAGCAAGAG AGGCATC-3' (3') 5'-CGATTTCCC-GCTC GGCCGTGGTGGTGAAGC-3' (460 bp).

ELISA

Quantification of TNF-α, IL-1β, IL-10, IL-4, IFN-γ, and IL-12 (ImmunoKontact) was performed by enzyme linked immunosorbent assay (ELISA) according to the manufacturer's protocols.

STATISTICAL ANALYSIS

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

Results

MONOLAYER INTEGRITY AND BACTERIAL ADHERENCE

The integrity of the CaCO-2 cell monolayers was a prerequisite for studying the recognition of non-pathogenic bacteria by IEC/leucocyte co-cultures. As shown in table 1, TEER (Ω cm^2) was kept constant after bacterial challenge of CaCO-2 cells in the absence and presence of underlying PBMC, thus confirming the intact barrier function of IEC during the course of the experiments. Although TEER decreased slightly for *L johnsonii*, this was not significant ($p>0.05$). Cell viability in CaCO-2 cell monolayers was >95% on bacterial challenge (24

Table 1 Transepithelial electrical resistance of CaCO-2 cell monolayers

	Mean of TEER (Ω cm²)[a] (% of no treatment)[b]		
	E coli	*L johnsonii*	*L sakei*
CaCO-2			
0 hours	620 (111)	591 (104)	616 (110)
24 hours	583 (114)	425 (83)	561 (110)
CaCO-2/PBMC			
0 hours	632 (110)	598 (104)	691 (121)
24 hours	549 (108)	478 (94)	613 (121)

[a]Values for transepithelial electrical resistance (TEER, Ω cm²) are given as the mean of four independent experiments. [b]Changes in TEER values on bacterial stimulation of CaCO-2 cells are given relative to control values (% of no treatment).
Time (0, 24 hours) is after bacterial stimulation of CaCO-2 cells.
PBMC, peripheral blood mononuclear cells.

Table 2 Adhesion of non-pathogenic bacteria to CaCO-2 cell monolayers

	Mean of adhesion (log cfu)[a] (% of total)[b]		
	E coli	*L johnsonii*	*L sakei*
CaCO-2	6.5[a] (3.9)	6.6 (3.8)	6.2 (1.6)
CaCO-2/PBMC	6.6 (4.2)	6.6 (3.8)	6.3 (2.3)

[a]Values for bacterial adhesion to CaCO-2 cells are given as number of attached bacteria per CaCO-2 monolayer (log cfu/log 5.6 CaCO-2 cells) and percentage (%) of total (10^8 cfu/ml) after one hour of incubation. Results are given as the mean of three independent experiments.
PBMC, peripheral blood mononuclear cells.

hours), as determined by trypan blue exclusion. Adhesion assays performed in the presence and absence of underlying PBMC revealed a similar ability of non-pathogenic *E coli*, *L johnsonii*, and *L. sakei* to attach to CaCO-2 cells (table 2).

BACTERIA MEDIATED CYTOKINE mRNA EXPRESSION IN LEUCOCYTE SENSITISED Caco-2 CELLS

Differentiated CaCO-2 monolayers were challenged for 16 hours with non-pathogenic *E coli*, *L johnsonii*, and *L sakei*. In the absence of PBMC, non-pathogenic bacteria did not induce mRNA expression of the proinflammatory chemokines IL-8 or MCP-1. The soluble gram negative cell wall component LPS failed to induce chemokine mRNA expression. In contrast, IL-1β (10 ng/ml) significantly induced IL-8 and MCP-1 mRNA expression, demonstrating the general responsiveness of CaCO-2 cells to soluble mediators (fig 1). Remarkably, when CaCO-2 cells were co-cultured in the presence of PBMC, a differential response to non-pathogenic bacteria was observed. Challenge of leucocyte sensitised CaCO-2 cells using 10^6 or 10^7 cfu/ml *E coli* or *L sakei*, respectively, elicited IL-8 and MCP-1 mRNA expression. The inductive effect of *L johnsonii* was lower compared with the other non-pathogenic bacteria. IL-12, IL-10, IL-4, or IFN-γ mRNA was not induced by non-pathogenic bacteria or IL-1β (data not shown).

BACTERIA INDUCED TNF-α AND IL-1β RESPONSE IN Caco-2/PBMC CO-CULTURES

TNF-α and IL-1β are primary mediators of immune stimulation and inflammatory responses which have been implicated in both beneficial and injurious conditions. As shown in fig 2, TNF-α and IL-1β mRNA expression was strongly increased in leucocyte sensitised CaCO-2 cells only after challenge with non-pathogenic *E coli* and *L sakei* (1×10^7 cfu/ml). In contrast, *L johnsonii* induced neither TNF-α nor IL-1β mRNA expression above control levels. Notably, CaCO-2 cells alone remained unresponsive to bacterial challenge (data not shown).

Secretion of TNF-α and IL-1β (fig 2, bar charts) followed the same induction pattern as shown at the level of mRNA expression. TNF-α and IL-1β were exclusively detected in the basolateral compartment and significantly increased on challenge of co-cultures with non-pathogenic *E coli* and *L sakei*. *L johnsonii* did not induce TNF-α or IL-1β secretion above control levels. The variation in cytokine

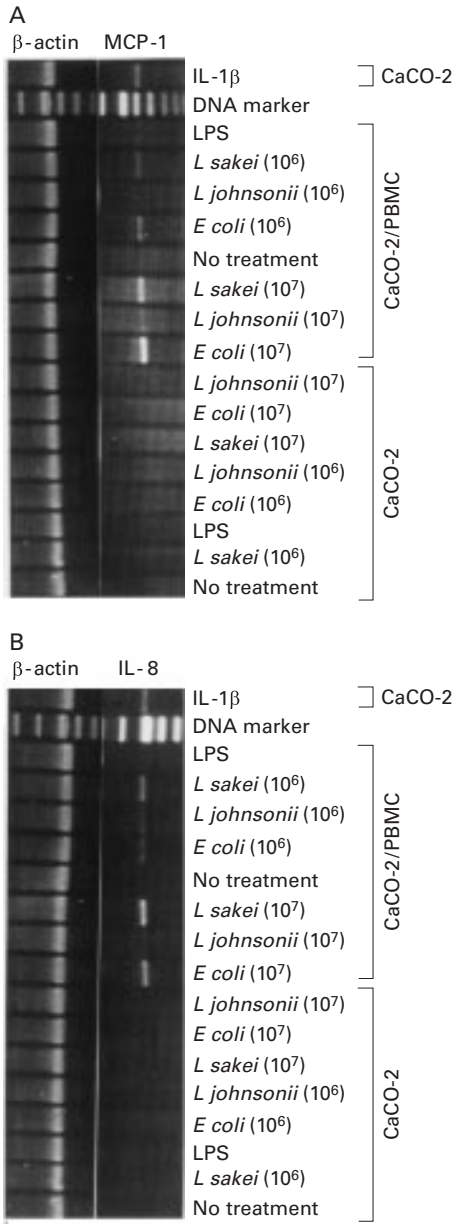

Figure 1 Differential chemokine expression in CaCO-2 cells. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to determine monocyte chemoattracting protein 1 (MCP-1) (A) and interleukin (IL)-8 (B) mRNA expression in CaCO-2 cells on stimulation of CaCO-2/leucocyte co-cultures or CaCO-2 cells alone with non-pathogenic E coli, L johnsonii, and L sakei (16 hours, 10^6 and 10^7 cfu/ml), respectively. Lipopolysaccharide (LPS, 1 μg/ml), IL-1β (10 ng/ml) and culture medium (no treatment) were used as controls. Results represent one of three independent experiments.

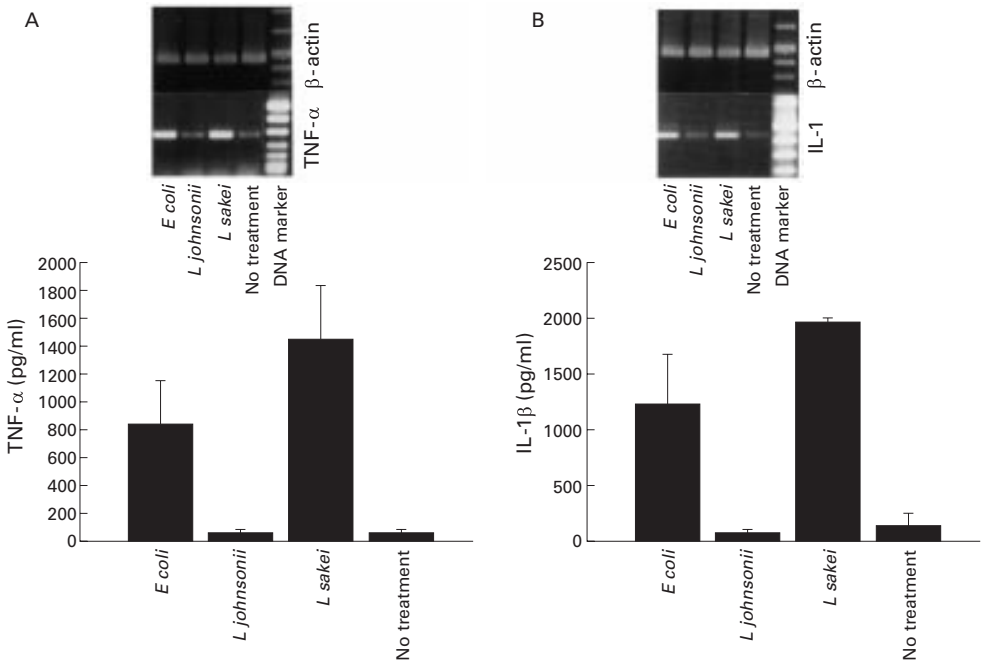

Figure 2 Bacteria induced tumour necrosis factor alpha (TNF-α) and interleukin (IL)-1β response by CaCO-2/leucocyte co-cultures. Stimulation of CaCO-2/leucocyte co-cultures with non-pathogenic E coli, L johnsonii, and L sakei (16 hours, 10^7 cfu/ml). Secretion of TNF-α (A) and IL-1β (B) into the basolateral compartment was determined (bar charts). Culture medium (no treatment) was used as a control. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to determine expression of TNF-α (A) and IL-1β (B) mRNA in leucocyte sensitised CaCO-2 cells. Data are mean (SD) of triplicate values and represent one of three independent experiments.

response obtained with different blood donors (n=3) ranged between 33% and 128% depending on the bacteria used for stimulation.

By challenging the CaCO-2/PBMC co-cultures with non-pathogenic *E coli* (1×10^7 cfu/ml) for 12 hours and thereafter separating the two cell compartments for another 24 hours (transfer experiment), secretion of TNF-α and IL-1β by conditioned CaCO-2 cells and PBMC was studied. As shown in fig 3, TNF-α was produced in significant levels by PBMC sensitised CaCO-2 cells (708 pg/ml) whereas only low levels of IL-1β were detected (55 pg/ml). Increased production of TNF-α (2492 pg/ml) and IL-1β (859 pg/ml) was detected in conditioned PBMC (data not shown). Thus CaCO-2 cells substantially contributed (1:4) to the total amount of TNF-α. Culture medium alone did not induce cytokine secretion. No cytokine secretion occurred after bacterial stimulation of CaCO-2 cells alone, suggesting that cytokine expression by CaCO-2 cells in response to certain non-pathogenic bacteria may be induced by PBMC derived soluble mediators, as cell to cell interactions between IEC and leucocytes did not occur. No TNF-α or IL-1β was detectable in the apical cell culture supernatants, demonstrating polarised secretion by CaCO-2 cells. Moreover, activation of CaCO-2 cells by non-pathogenic bacteria did not induce secretion of IFN-γ, IL-4, or IL-12 by underlying PBMC (data not shown).

Addition of LPS (0, 0.1, 1, 10 μg/ml) to the cell culture inserts in the absence of CaCO-2 cells induced secretion of TNF-α by PBMC (17, 2793, 2175, 1717 pg/ml, respectively). In contrast, LPS (0.1, 1, 10 μg/ml) did not induce secretion of TNF-α by PBMC in the presence

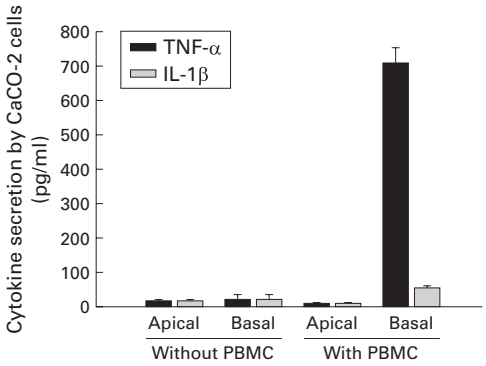

Figure 3 Polarised secretion of tumour necrosis factor alpha (TNF-α) and interleukin (IL)-1β by leucocyte sensitised CaCO-2 cells. Stimulation of CaCO-2/leucocyte co-cultures or CaCO-2 cells alone with non-pathogenic E coli (10^7 cfu/ml). After 12 hours of incubation, CaCO-2 cells were transferred to fresh culture medium. After an additional 24 hours of incubation, secretion of TNF-α and IL-1β by leucocyte sensitised CaCO-2 cells was determined in the apical and basolateral compartments (transfer experiment). Controls (no treatment) did not induce cytokine secretion. Data are mean (SD) of triplicate values and represent one of three independent experiments.

of CaCO-2 cell monolayers suggesting that migration of bacterial products across the epithelial barrier did not occur.

INDUCTION OF REGULATORY CYTOKINES BY CaCO-2/LEUCOCYTE CO-CULTURES

The balance between inflammatory and regulatory cytokines is of crucial importance in the control of normal homeostasis and gut immunity. TGF-β and IL-10 are important inhibitory cytokines, shown to downregulate the inflammatory cytokine response. In contrast with IL-10, low levels of mRNA coding for the regulatory cytokine TGF-β were induced in

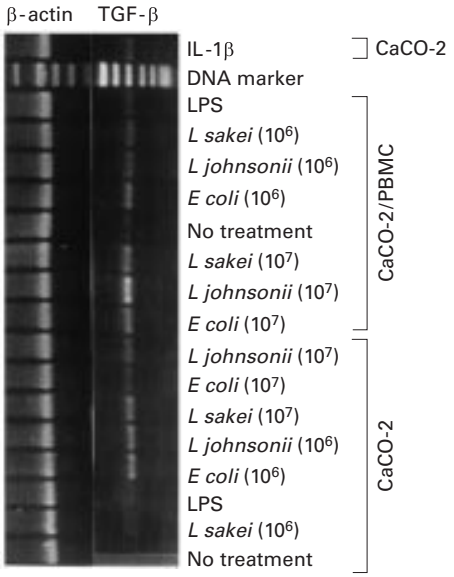

Figure 4 Differential expression of transforming growth factor beta (TGF-β) mRNA in CaCO-2 cells. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to determine TGF-β mRNA expression in CaCO-2 cells on stimulation of CaCO-2/leucocyte co-cultures or CaCO-2 cells alone with non-pathogenic E coli, L johnsonii, and L sakei (16 hours, 10^6 and 10^7 cfu/ml), respectively. Lipopolysaccharide (LPS, 1 μg/ml), interleukin (IL)-1β (10 ng/ml), and culture medium (no treatment) were used as controls. Results represent one of three independent experiments.

CaCO-2 cells, regardless of the bacteria used. In leucocyte sensitised CaCO-2 cells, however, discernible enhanced TGF-β mRNA expression was detected exclusively with *L johnsonii* (10^7 cfu/ml). Culture medium and LPS did not induce TGF-β mRNA expression above control levels (fig 4). Moreover, the presence of underlying leucocytes did not induce expression of IL-10 mRNA in activated CaCO-2 cells (data not shown). Thus IL-10 was secreted exclusively by PBMC after challenge of CaCO-2 cells with non-pathogenic *E coli* (511 pg/ml) and *L sakei* (1221 pg/ml). Although *L johnsonii* upregulated IL-10 at the level of mRNA expression, protein secretion was not observed (fig 5A).

THE ROLE OF TNF-α AND IL-1β IN THE ACTIVATION OF CaCO-2 CELLS

As TNF-α and IL-1β were secreted from conditioned CaCO-2 cells and PBMC after challenge with non-pathogenic *E coli* and *L sakei*, the role of these cytokines in cellular cross talk was investigated. CaCO-2/PBMC co-cultures were challenged with the non-pathogenic *E coli* (1×10^7 cfu/ml) in the presence and absence of neutralising anti-TNF-α or anti-IL-1β mAb (10 μg/ml). Remarkably, the presence of anti-TNF-α mAb completely abolished *E coli* mediated expression of TNF-α and IL-8 mRNA in CaCO-2 cells (fig 6A, B). The presence of anti-IL-1β mAb weakly reduced TNF-α and IL-8 gene transcripts, demonstrating that TNF-α was the predominant cytokine implicated in the cellular cross talk. Isotype control antibodies did not affect cytokine mRNA expression in CaCO-2 cells (data not shown).

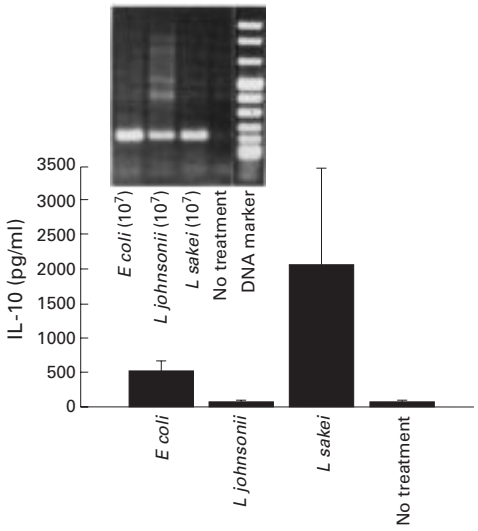

Figure 5 Induction of interleukin (IL)-10 response by CaCO-2/leucocyte co-cultures. Secretion of IL-10 was determined in the basolateral compartment on stimulation of CaCO-2/leucocyte co-cultures with non-pathogenic E coli, L johnsonii, and L sakei (16 hours, 10^7 cfu/ml), respectively (bar charts). Culture medium (no treatment) was used as a control. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to determine IL-10 mRNA expression in conditioned PBMC. Data are mean (SD) of triplicate values and represent one of three independent experiments.

DIFFERENTIAL ACTIVATION OF CaCO-2 CELLS BY ENTEROPATHOGENS AND NON-PATHOGENIC BACTERIA: COMPARISON BETWEEN LIVE AND KILLED MICROORGANISMS

Enteropathogenic *E coli* were shown to be the major cause of infant diarrhoea.[28] Infection of IEC lines by enteropathogenic *E coli* induced

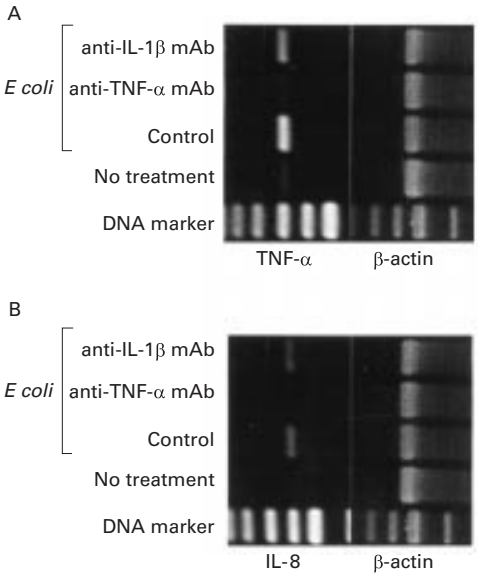

Figure 6 Neutralisation of tumour necrosis factor alpha (TNF-α) and interleukin (IL)-1β in CaCO-2/leucocyte co-cultures. CaCO-2/ leucocyte co-cultures were stimulated with non-pathogenic E coli (16 hours, 10^7 cfu/ml) in the presence of neutralising anti-TNF-α monoclonal antibody (mAb) or IL-1β mAb (10 μg/ml). A positive control was performed in the absence of neutralising antibodies. Challenge of CaCO-2/leucocyte co-cultures with culture medium alone was used as a negative control. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to determine TNF-α (A) and IL-8 (B) mRNA expression in CaCO-2 cells. Results represent one of three independent experiments.

expression of cytokines and chemokines in the model epithelium. To compare differences in bacterial induced activation of IEC, CaCO-2/leucocyte co-cultures were challenged with live/heat killed enteropathogenic *E coli* and commensal bacteria (1×10^7 cfu/ml). The initial interaction (four hours) between bacteria and CaCO-2 cells was allowed to take place in the absence of gentamycin. TNF-α mRNA expression was monitored in CaCO-2 cells after 6, 16, and 36 hours of incubation. Invasion of EPEC wild-type strain E23458/69 in CaCO-2 cells ranged between 10^4 and 10^5 cfu/ml/5×10^5 CaCO-2 cells. For the EPEC *eaeA* deletion mutant CVD206, which lacks the outer membrane protein intimin, its ability to invade CaCO-2 cells was significantly reduced (3 log). Non-pathogenic *E coli* and lactobacilli did not penetrate CaCO-2 cells.

Infection of CaCO-2/leucocyte co-cultures with both EPEC strains induced expression of TNF-α mRNA in CaCO-2 cells at the three times (6, 16, and 36 hours). In contrast, non-pathogenic bacteria (*E coli, L johnsonii, L sakei*) exclusively induced TNF-α mRNA expression after 16 hours of incubation. The cytokine pattern induced by live non-pathogenic bacteria was similar to that observed with gentamycin killed bacteria, although slightly increased TNF-α mRNA expression was observed for *L johnsonii*. Remarkably, heat killed bacteria did not induce cytokine mRNA expression (fig 7). Infection of CaCO-2 monocultures with EPEC strain E2348/69 and CVD206 induced TNF-α mRNA expression in IEC. No cytokine response was observed with non-pathogenic bacteria (data not shown).

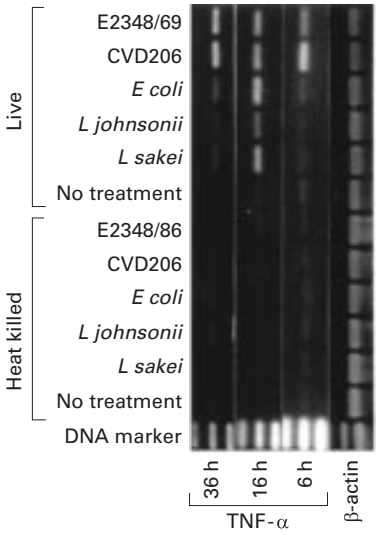

Figure 7 Differential induction of tumour necrosis factor alpha (TNF-α) mRNA by leucocyte sensitised CaCO-2 cells stimulated with enteropathogenic E coli and non-pathogenic bacteria. Stimulation of CaCO-2/leucocyte co-cultures with live or heat killed (95°C/30 minutes) EPEC strain E2348/69 and its eaeA deletion mutant CVD206, and non-pathogenic E coli, L johnsonii, and L sakei (10^7 cfu/ml) for four hours in the absence of gentamycin (150 μg/ml). Culture medium (no treatment) was used as a control. Expression of TNF-α mRNA in CaCO-2 cells was determined after 6, 16, and 36 hours of incubation by reverse transcription-polymerase chain reaction (RT-PCR) analysis. Results represent one of three independent experiments.

Discussion

Changes in intestinal epithelial cells (IEC), lamina propria lymphocytes (LPL), and intraepithelial lymphocytes (IEL) have been described in response to modifications of the intestinal microflora.[29–31] This may occur in the newborn intestine when sterile mucosal sites become exposed to mainly non-pathogenic commensal bacteria. However, this also supports the hypothesis that the enteric microflora maintains a chronic and immunologically balanced intestinal inflammatory response.[32 33] The mechanisms of gut homeostasis are unknown but failure can lead to chronic inflammatory damage of the intestinal epithelium as seen in inflammatory bowel disease (IBD).[34–36] IEC are activated in response to proinflammatory mediators and enteric pathogens, and are therefore thought to play an important role in early activation of the mucosal immune system after intestinal infection.[12–20 37–39]

To address the question of whether non-pathogenic bacteria modify the immune homeostasis of the mucosa, a CaCO-2/leucocyte co-culture system was established. This is the first report demonstrating that non-pathogenic bacteria elicit a differential cytokine response by human IEC in the presence of human blood leucocytes. We demonstrated that leucocyte sensitised CaCO-2 cells in vitro can distinguish between signals delivered by different non-pathogenic bacteria and in turn can transduce a discriminative signal to underlying PBMC. The Gram positive food fermenting *L sakei* exerted a similar proinflammatory activation pattern as the Gram negative non-pathogenic *E coli* with respect to chemokine (IL-8 and MCP-1) and cytokine expression (TNF-α and IL-1β). It was recently shown that enteropathogenic *E coli* express proteins such as EspA, EspB, and EspC that induce signal transduction and cytokine secretion in CaCO-2 cells.[18] The non-pathogenic *E coli* used in our study lacks these and other virulence factors commonly distributed among the species *Escherichia coli*,[24] and therefore did not induce a cytokine response in CaCO-2 cells alone, confirming previous observations.[7 14 19] In the co-cultures, differences in the regulation of inflammatory immune response by IEC were observed between enteropathogenic *E coli* and non-pathogenic bacteria, suggesting that IEC transduced signals discriminated between enteropathogens and commensal bacteria. Recently, *Lactobacillus* species have been shown to prevent colitis in IL-10 gene deficient mice.[40] The authors showed that IL-10 gene deficient mice have decreased levels of colonic *Lactobacillus* species in the neonatal period. Normalising levels of colonic lactobacilli were associated with attenuated development of colitis. In vitro, *L johnsonii*, an intestinal isolate, had a low potential to induce a proinflammatory response but rather favoured induction of TGF-β expression in CaCO-2 cells. TGF-β is a key factor implicated in the regulation of intestinal barrier function[41 42] and is thought to mediate tolerance to the indigenous microflora via bystander

suppression.[43–45] It is known that there is tolerance towards the autologous microflora but its breakdown has been observed in IBD.[32]

The present study strengthens the hypothesis that bacterial signalling at the mucosal surface requires a network of cellular interactions. According to these results, sensitisation of CaCO-2 cells by neighbouring immunocompetent cells constitutes a crucial step for recognition of non-pathogenic bacteria as CaCO-2 cells alone remained hyporesponsive to non-pathogenic bacterial stimuli. CaCO-2 cells and human blood leucocytes were not co-cultured by direct cell to cell contact, therefore excluding interaction of surface molecules such as recognition of non-classical restriction elements.[46] Consequently, the sensitising effect on IEC function was mediated by a soluble factor. TNF-α and IL-1β are proinflammatory mediators which have been shown to induce chemokine/cytokine responses in human IEC. Here, non-pathogenic bacteria induced polarised secretion of TNF-α (and to a lesser extent IL-1β) by sensitised CaCO-2 cells to the subepithelial compartment. Thus both cytokines are reasonable candidates for cellular cross talk. Remarkably, when TNF-α was neutralised by anti-TNF-α mAb, expression of TNF-α and IL-8 in CaCO-2 cells was abolished. Neutralisation of IL-1β was less effective. Thus TNF-α was identified as one of the early mediators involved in the initiation of the immune response by IEC to non-pathogenic bacteria. Although TNF-α is a primary mediator of the immune response,[47] uncontrolled synthesis has been implicated in tissue damage,[48 49] induction of apoptosis in enterocytes,[50] and many human intestinal pathologies.[51] Hence TNF-α synthesis is tightly regulated by inhibitory cytokines such as IL-10. Notably, those bacteria (*E coli*, *L sakei*) which induced biosynthesis of TNF-α and IL-1β in CaCO-2/leucocyte co-cultures were also strong stimulators of IL-10 secretion from underlying PBMC, indicating that a negative feedback loop is activated. IL-10 is of crucial importance in the control of gut homeostasis and immunity.[35 36 52 53] Recently, Kucharzik *et al* reported that regulatory cytokines such as IL-10 downregulate chemokine responses in activated IEC.[54] Dysregulated secretion of IL-10 is thought to play a role in the pathogenesis of IBD.[55]

In conclusion, we have demonstrated a pivotal role for immune cells in the sensitisation of human differentiated CaCO-2 cells in recognising signals originating from non-pathogenic bacteria. Moreover, it was shown that IEC delivered a discriminative signal to neighbouring immune cells by secretion of soluble messengers. Although PBMC do not exactly represent intestinal mucosal immune cells, such as LPL or intestinal IEL,[56 57] lamina propria T cells have a similar CD4:CD8 ratio to that found in the periphery[58] and are predominantly αβTCR$^+$ T cells. Thus PBMC may be partially representative of LPL and have previously been used to study the effect of immunocompetent cells on the physiology of human IEC lines.[59] Hence the IEC/leucocyte co-culture model could be an additional[60] tool to characterise the role of the intestinal epithelium in response to components of the intestinal microflora.

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Non-pathogenic bacteria elicit a differential cytokine response by intestinal epithelial cell/leucocyte co-cultures

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Notes