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Differential expression and upregulation of interleukin-1α, interleukin-1β and interleukin-6 by freshly isolated human small intestinal epithelial cells

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***Background*: Small intestinal epithelial cells (SIEC) may contribute to local immune regulation.**
***Aim*: To examine production of interleukin (IL)-1α, IL-1β and IL-6 by freshly isolated human SIEC.**
***Methods*: IL-1α and IL-1β mRNA in epithelial layers (EL) prepared from small intestine and in intestinal epithelial cell (EC) lines were examined by reverse transcription-polymerase chain reaction. IL-1α, IL-1β and IL-6 protein expression by SIEC was examined by flow cytometry before and after activation with lipopolysaccharide and epithelial growth factor.**
***Results*: IL-1α and IL-1β mRNA was detected in EL and EC lines. Background expression of IL-1α and IL-1β protein by SIEC was observed, which did not increase even following activation. IL-6 protein was expressed by SIEC, in a proportion that increased in two out of three samples following activation.**
***Conclusions*: IL-6 expression and the presence of IL-1α and IL-1β mRNA suggest a role for SIEC in the regulation of local inflammation.**

Key words: Interleukin-1, Interleukin-6, Human, Small intestinal epithelial cells, Reverse transcriptase-polymerase chain reaction, Intra-cytoplasmic flow cytometric analysis, Lipopolysaccharide, Epithelial growth factor

Introduction

The small intestinal immune system is constantly exposed to harmless dietary antigens, ingested pathogens and a variety of commensal organisms, and must respond appropriately by activating cell-mediated or antibody responses or by inducing tolerance. The epithelial layer (EL), the first barrier encountered by antigens, is likely to play a role in determining whether and what types of immune responses are induced. A mechanism by which small intestinal epithelial cells (SIEC) could affect the function of the lymphocytes present in the intestinal EL and in the underlying lamina propria (LP) is through the production of cytokines. It has been shown that human colonic epithelial cell (EC) lines or freshly isolated colonic EC can produce the lymphocyte growth and activation factors IL-7 and IL-15,[1,2] multiple chemokines,[3–5] the pro-inflammatory cytokines tumour necrosis factor-α[6,7]and IL-18[8], and the regulatory cytokines transforming growth factor-β[9] and IL-10[10]. Due to a lesser availability of small intestinal tissue, less is known about SIEC. It is known that SIEC can produce IL-7,[1,11] transforming growth factor-β[12] and the lymphocyte-attracting chemokine thymus-expressed chemokine.[13]

IL-1 and IL-6 are pleiotropic cytokines, produced by many lymphoid and non-lymphoid cells, with functions both in general metabolic processes as well as in immune stimulation. Their levels are increased in a variety of diseases.[14–16] The two forms of IL-1, IL-1α and IL-1β, show 24% homology at the amino acid level, have similar three-dimensional structures, share biological properties and bind to the same receptors. They have direct stimulatory effects on lymphocytes and antigen-presenting cells (APC), as well as on the induction of acute phase proteins and the expression of chemokines.[14] IL-6 exerts multiple growth-inducing, growth-inhibitory and differentiation-inducing effects, depending on the nature of the target cells. IL-6 plays roles in T-cell and B-cell development, the induction of acute-phase protein production and synergises with IL-1 in inducing T-cell activation, proliferation and differentiation into effector cells.[14–16] More recently, IL-6 has also been described as a cytokine with anti-inflammatory effects.[17,18] IL-6 is involved in antibody production and isotype switching, and plays a critical role in the production

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and function of intestinal immunoglobulin (Ig)A.[19] Elevated levels of both IL-1β and IL-6 are found in inflammatory bowel disease,[20] and IL-6 has been shown to be required for the development of T helper $(Th)_1$-cell-mediated murine colitis and to be involved in the perpetuation of the inflammatory process during Crohn's disease.[21,22]

IL-6 is constitutively expressed by many APC and its production is upregulated by numerous signals including bacterial lipopolysaccharide (LPS). In contrast, IL-1 production by APC usually requires different activating factors, depending on the APC type.[23] As for human small intestinal EL, IL-1 has not been so far detected, and whether IL-6 can be produced by SIEC remains controversial. Conflicting reports have described the presence[24,25] or absence[26] of IL-6 production by human SIEC. In the present study, we have used polymerase chain reaction amplification of reverse-transcribed mRNA (RT-PCR) to ascertain whether IL-1α and IL-1β mRNA are produced by freshly isolated EL from the human small intestine, and have used flow cytometry to quantify the percentages of SIEC that express IL-1α, IL-1β and IL-6. We have also investigated whether the production of these cytokines by SIEC could be induced or upregulated by bacterial LPS (known to induce IL-6 expression on other EC[27] and IL-1 on rat intestinal EC[28]) and epithelial growth factor (EGF) (a trophic factor for duodenal EC[29]). Our results show that IL-6 is constitutively produced by freshly isolated SIEC and that it can be upregulated by LPS + EGF activation. IL-1α and IL-1β mRNA is present on the small intestinal EL but IL-1 protein was not expressed or was detected only in a very small percentage of SIEC, even following activation.

Materials and methods

Human tissue specimens

Duodenal biopsies (four or five biopsies per individual) were obtained from consenting patients (n = 6) undergoing oesophagogastro-duodenoscopy. Surgical specimens of uninvolved ileal tissue were obtained from patients (n = 4) undergoing resection for colonic cancer. Bone marrow (BM) from healthy donors (n = 3) was obtained from discarded BM bags immediately after BM transplant. Blood was obtained from healthy volunteers and from the patients from whom biopsies had been obtained. Ethical approval for this study was obtained from the Ethics Committee in St Vincent's University Hospital.

Cell lines and antibodies

Human colonic tumour EC lines Caco-2 and T84 were obtained from the American Type Culture Collection (Rockville, MD, USA). Anti-BerEP4 (Dako, Galstrup, Denmark) monoclonal antibody was used to identify EC. The anti-cytokeratin antibody CAM 5.2 (Becton-Dickinson, Oxford, UK) was used for intra-cytoplasmic EC staining. Mouse anti-human IL-1α and IL-1β were purchased from Immunotech (Marseille, France), and mouse IgG2a anti-IL-6 monoclonal antibody (clone 1927.311) was a gift of R&D Systems (Abingdon, UK). fluorescein isothiocyanate-conjugated or phyco-erythrin-conjugated rat anti-mouse IgG was used as the secondary antibody. Appropriate isotype-matched antibodies were used as negative controls.

Preparation of tissue samples

Small intestinal (ileal) surgical specimens were dissected to fragments smaller than 1 cm^2. EL cell suspensions from these surgical fragments (used for mRNA detection) or from duodenal biopsies (used for protein detection) were prepared by a method previously described.[30] This method has been shown not to disrupt the basement membrane separating the EL from the underlying LP. Briefly, samples were placed in ion-free Hanks' Balanced Salt Solution (Gibco-BRL, Paisley, UK) + 1 mM ethylenediamine tetraacetic acid + 1 mM dithiothreitol + 5% foetal calf serum, and were agitated at 37°C for 60–75 min, causing the release of single cells into the supernatant. The tissue remaining after this EL removal was considered LP. BM transplant bags were rinsed with Hanks' Balanced Salt Solution and mononuclear cells prepared by Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation. Peripheral blood mononuclear cells (PBMC) were isolated using Lymphoprep. Cell yield and viability was assessed using ethidium bromide/acridine orange.

IL-1α and IL-1β RT-PCR

Total RNA was extracted by the standard guanidinium isothiocyanate-phenol-chloroform method,[31] either from LP tissue or from pelleted EL and BM cells, and contaminating genomic DNA was removed by DNase. One microgram of RNA was reverse transcribed to give 20 μl of cDNA using standard procedures,[32] and the cDNA was purified by extraction with phenol/chloroform/isoamyl alcohol. PCR amplification was performed in 25 μl reaction mixtures. IL-1α-specific and IL-1β-specific primers were designed based on the cDNA published sequences.[33] The specific primers for IL-1α were 5′-TGGCCAAAGTTCCAGACATG-3′ and 5′-TTCCAGTATCTGAAAGTCAG-3′, and those for IL-1β were 5′-AGGAGAATGACCTGAGCACC-3′ and 5′-ATCATCTTTCAACACGCAGG-3′. PCR amplification of β-actin, to confirm the presence of cDNA, used the primers 5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′ and 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′ (Clontech, Palo Alto, CA, USA). All PCR reactions consisted of 94°C for 30 sec, 56°C for

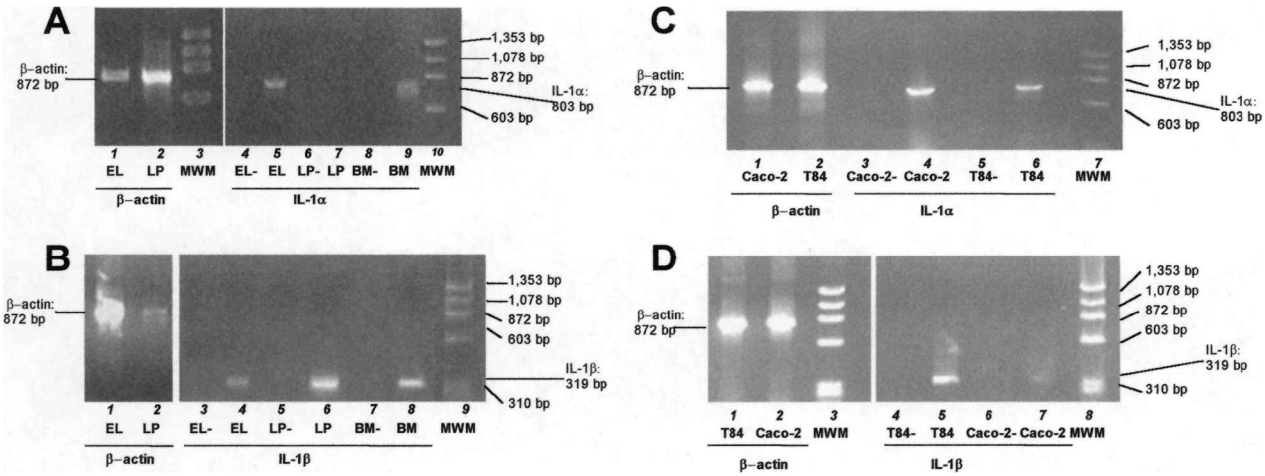

FIG. 1. Detection of (A, C) IL-1a mRNA and (B, D) IL-1b mRNA in (A, B) freshly isolated human small intestinal (ileal) specimens and (C, D) human colonic EC lines by RT-PCR. (A) Lanes 1 and 2, b-actin in the EL and LP from the same patient; lane 3, molecular weight marker (MWM); lane 4, negative control for lane 5 (no reverse transcriptase added to RNA during reverse transcription stage); lane 5, EL IL-1a; lane 6, negative control for lane 7; lane 7, lack of IL-1a in LP; lane 8, negative control for lane 9; lane 9, BM IL-1a; lane 10, MWM. (B) Lanes 1 and 2, b-actin in EL and LP from the same patient; lane 3, negative control for lane 4; lane 4, EL IL-1b; lane 5, negative control for lane 6; lane 6, IL-1b in LP; lane 7, negative control for lane 8; lane 8, BM IL-1b; lane 9, MWM. (C) Lanes 1 and 2, Caco-2 and T84 b-actin; lane 3, negative control for lane 4; lane 4, Caco-2 IL-1a; lane 5, negative control for lane 6; lane 6, T84 IL-1a; lane 7, MWM. (D) Lanes 1 and 2, T84 and Caco-2 b-actin; lane 3, MWM; lane 4, negative control for lane 5; lane 5, T84 IL-1b; lane 6, negative control for lane 7; lane 7, Caco-2 IL-1b mRNA; lane 8, MWM. Bp, base pairs.

40 sec and 72°C for 30 sec for 16 cycles, and a further 18 cycles consisting of 94°C for 30 sec, 54°C for 40 sec and 72°C for 40 sec, and were carried out in a Hybaid (Teddington, UK) thermocycler. In all cases where a PCR product was not detected, 2–4 μl of the reaction product were subjected to a second round of PCR amplification.

Intra-cytoplasmic flow cytometry

The expression of IL-1a, IL-1b and IL-6 by freshly isolated EC was analysed by intra-cytoplasmic flow cytometric analysis. For IL-1a and IL-1b, duodenal samples from six patients were analysed. For IL-6, three of those patients were analysed. Peripheral blood monocytes from the same individuals were used as controls for cytokine expression. Monoclonal antibody staining for flow cytometric analysis was performed as previously described.[34] Briefly, cells were adjusted to 2×10^6 cells/ml, fixed for 15 min in 2% paraformaldehyde and split into two fractions: one for permeabilisation with 0.05% saponin, and the other as a control. Surface staining with BerEP4 antibody was performed to identify a region that would contain > 95% EC, to be subsequently analysed for cytokine expression. Data were acquired for flow cytometric analysis on a Becton-Dickinson FACScan. Anti-cytokeratin staining was used to test the efficiency of the permeabilisation and intra-cytoplasmic staining procedure. As positive controls for the detection of IL-1a, IL-1b and IL-6, a region was analysed that contained the monocyte population in the PBMC preparations obtained from the same patients.

LPS and EGF activation

EL single-cell suspensions from three of the samples were cultured at densities of 1×10^6 cells/ml in the presence of 10 ng/ml of LPS and 20 ng/ml of EGF (Sigma, Poole, UK), at 37°C in 5% CO_2. Cytokine analysis was performed at time zero (t_0) and 4 h after activation. Monocytes from PBMC activated with 10 ng/ml of LPS were used as the positive control for cytokine upregulation.

Statistical analysis

Percentages are expressed as the mean ± standard error of the mean.

Results

IL-1a and IL-1b mRNA expression by human SIEC

RT-PCR was performed to detect expression of mRNA specific for IL-1a and IL-1b. In all BM samples used as positive controls, DNA products of 803 and 319 base pair products were amplified when IL-1a-specific (Fig. 1A) or IL-1b-specific (Fig. 1B) primers were used, respectively. IL-1a mRNA was detected in EL from three uninvolved surgical specimens out of four samples examined. In two of these three surgical specimens, IL-1a mRNA was not detected in the LP fractions, indicating that positive results obtained for EL are not due to contamination from the LP. IL-1b mRNA was detected in all EL and LP samples from surgical specimens examined (Fig. 1B). The b-actin mRNA was readily detected in all samples.

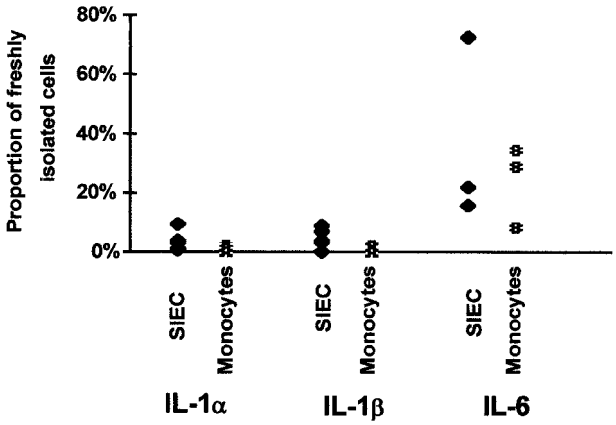

FIG. 2. Intracellular expression of IL-1a (n = 6), IL-1b (n = 6) and IL-6 (n = 3) by SIEC freshly isolated from human duodenal biopsies. Results are expressed as percentages of SIEC expressing each cytokine, as assessed by flow cytometry, and are compared with those obtained for monocytes from the same patients.

IL-1a and IL-1b mRNA expression by human intestinal EC lines

The human colonic EC lines Caco-2 and T84 expressed both IL-1a mRNA (Fig. 1C) and IL-1b mRNA (Fig. 1D), reinforcing the hypothesis that the detection of IL-1 mRNA in the freshly isolated small intestinal EL could be due to the EC and not to potential contaminating cells in the preparations.

Constitutive IL-6 protein expression by human SIEC but lack of IL-1a or IL-1b protein

Freshly isolated EC from six patients were examined for IL-1a and IL-1b, and three of these were also analysed for IL-6 protein expression by intracytoplasmic flow cytometry. The yield of viable SIEC, as assessed by ethidium bromide/acridine orange nuclear staining, ranged from 0.4×10^6 to 3.2×10^6. The efficiency of the permeabilisation process was validated by staining SIEC with anticytokeratin antibody (results not shown). Only BerEP4-positive cells stained positive for cytokeratin following permeabilisation (results not shown), indicating that the intracellular staining was specific. IL-1a and IL-1b were found to be expressed by only very small percentages of resting SIEC (mean ± SE of six samples, 3.3 ± 1.4% for IL-1a and 3.8 ± 1.4% for IL-1b) and monocytes (0.9 ± 0.4% for IL-1a and 0.8 ± 0.3% for IL-1b; n = 6) (Fig. 2). In contrast, IL-6 was detected in a considerably higher proportion of permeabilised SIEC from all three patients examined, with 15.8, 72.5 and 22.0% SIEC expressing IL-6 in the different EC samples (Fig. 2). Resting monocytes from the same individuals (n = 3) also expressed intra-cytoplasmic IL-6 (7.9, 34.3 and 29.1%).

Effect of LPS and EGF activation on IL-1 and IL-6 protein expression

The effect of LPS (10 ng/ml) and EGF (20 ng/ml) on cytokine production by SIEC was investigated (n = 3). Fig. 3A-I show flow cytometric staining for these cytokines in one patient, for freshly isolated SIEC, for SIEC after 4 h activation with LPS + EGF, and for monocytes from the same individuals after stimulation for 4 h with 10 ng/ml of LPS. Fig. 3J-O show a summary of results from all three patients. IL-1a and IL-1b expression by SIEC was not induced by activation with LPS and EGF (Fig. 3D,E,J,K). Only a small proportion of SIEC appeared to express IL-1a (6.2, 0.4 and 6.7%) (Fig. 3J) and IL-1b (3.3, 2.5 and 8.6%) (Fig. 3K) after 4 h of activation. This was in contrast with the induction of these cytokines in a significant proportion of monocytes from the same individuals following a 4-h incubation (44.5, 55.0 and 55.1% for IL-1a and 39.3, 64.7 and 17.5% for IL-1b, respectively) (Fig. 3G,H,M,N). This finding appears to indicate that IL-1 is not expressed by SIEC at any significant level, and that this expression is not inducible by LPS, unlike in the case of monocytes. IL-6 expression by EC was modulated by activation with LPS and EGF (Fig. 3F,L). In two of three patients, the percentage of SIEC expressing intracellular IL-6 was upregulated (from 15.8 to 84.8% in the first patient, and from 22 to 32.1% in the second patient) following a 4-h incubation. In the third patient, 72.5% of EC were already expressing IL-6 at t_0, and this percentage fell to 53.6% after 4 h activation (Fig. 3L). The percentage of monocytes expressing IL-6 was consistently increased after a 4-h activation (Fig. 3I,O) (from 7.9 to 73.6%, from 34.3 to 77.0%, and from 29.1 to 74.1%). Thus, upregulation of IL-6 by SIEC appeared to follow a similar pattern to that of monocytes following LPS activation, except in the case when a high percentage of resting EC were already expressing the protein.

Discussion

Immune responses in the intestinal mucosa are partly controlled by cytokines released in response to environmental stimuli. The present study shows that significant proportions of freshly isolated human SIEC constitutively express IL-6 and, while mRNA for IL-1a and IL-1b was detectable in normal small intestinal EL samples, only a small proportion of SIEC, considered background levels, appeared to express IL-1 protein. IL-6 production has been shown by other groups in histological sections of small intestinal epithelium using immunohistochemistry and immunofluorescence.[24,25] In freshly isolated EC from normal human colon, IL-6 mRNA and protein have previously been detected.[7,35] However, these findings are in contrast to those of other groups, who detected IL-6 protein[36] or mRNA[37] only in colonic EC from patients with

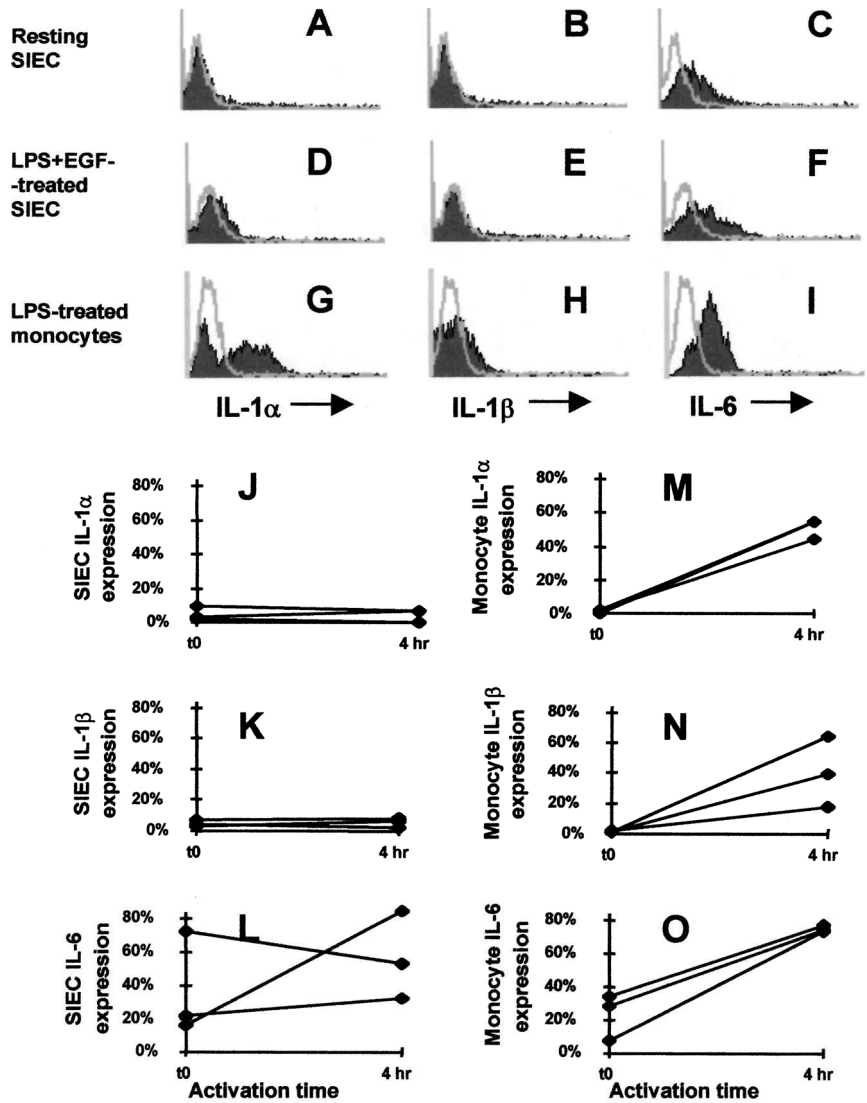

FIG. 3. IL-1_a, IL-1_b and IL-6 expression at time zero before activation (t_0) and 4 h following activation. (A-I) Flow cytometric analysis histograms showing intracellular IL-1_a, IL-1_b and IL-6 expression (A-C) by resting SIEC, (D-F) by SIEC after 4 h activation, and (G-I) by activated monocytes from the same patient. Open histograms represent control background fluorescence, filled histograms show intra-cytoplasmic cytokine expression. (J-O) Summary of percentages of freshly-isolated SIEC expressing (J) IL-1_a, (K) IL-1_b and (L) IL-6 at t_0 and at 4 h ($n = 3$). Results are compared with those obtained for monocytes from the same patients for (M) IL-1_a, (N) IL-1_b and (O) IL-6.

inflammatory bowel disease but not from normal colons.[26,36,37] Using flow cytometry on freshly isolated human duodenal epithelial layers, we have confirmed previous reports[24,25] that IL-6 is constitutively produced by SIEC, and shown for the first time that the proportions of SIEC that express IL-6 are comparable with those of monocytes taken from blood of the same individuals. Our results also show that, when only a small proportion of freshly isolated SIEC express IL-6, it is possible to induce the '*in vitro*' expression of IL-6 in other SIEC by treatment for 4 h with EGF + LPS. The discrepancies found in the different groups studying IL-6 production by intestinal EC may relate to differences in the sensitivities of the techniques used. Alternatively, the discrepancies may arise as a result of differences in the tissues studied: duodenal samples were used in the present study for protein detection, while other groups used ileal and colonic samples, suggesting possible differences in IL-6 production by EC from different regions of the intestine. The duodenum has both digestive and absorptive functions, while the ileum, which forms the distal portion of the small intestine, and the colon are predominantly absorptive. Immunological specialisation of different regions of the intestine has recently been demonstrated by Kunkel *et al.*[13] This group found that the lympho-attractant chemokine thymus-expressed chemokine is expressed by small intestinal but not large intestinal EC, suggesting that EC of the duodenum, ileum and colon may also differ in the production of other cytokines. In support of this notion, Shirota *et al.*[24] reported that IL-6 staining

was more abundant in the small intestinal than the colonic EC. Future comparisons of cytokine production by duodenal and ileal EC may point to important differences in the roles of these regions of the small intestine in immune activation and oral tolerance.

The present study is consistent with a previous report that IL-1 is not produced by SIEC,[26] even after activation by a combination of EGF and LPS. In contrast, LPS-stimulated monocytes showed a significant upregulation of IL-1α and IL-1β expression, thus confirming the reliability of our flow cytometric technique for IL-1 detection. Moreover, EGF + LPS upregulated IL-6 expression by the SIEC, indicating that the activation conditions were efficient for SIEC. However, specific stimulatory conditions, different to those used for IL-6, might be needed to selectively induce the expression of IL-1 by SIEC. In contrast with the protein results, mRNA for IL-1α and IL-1β was detectable in ileal EL samples, indicating the potential of SIEC to produce IL-1 protein. The different results obtained for IL-1α for the EL and LP from the same individual indicates that the IL-1 mRNA detection in the EL is not due to contamination from LP cells. The detection of IL-1α and IL-1β mRNA in colonic EC lines (used here as a model for intestinal EC) further supports the notion that the IL-1 mRNA detected in the small intestinal EL would be produced by the EC themselves. The search for IL-1 in human colonic EC by other groups has yielded before controversial results. Jarry *et al.*[38] have reported IL-1α and IL-1β secretion by freshly isolated human colonic EC. However, other studies have failed to detect IL-1 mRNA or protein in human colonic EC,[7,26,35,37] even after bacterial invasion[7] or LPS, interferon-γ, tumour necrosis factor-α or IL-1β activation.[35]

The present findings that human SIEC can produce IL-6, and potentially IL-1, strongly suggest a role for SIEC in the activation or regulation of immune responses in the small intestine. The small intestine needs to maintain both immunity against ingested pathogens and tolerance to dietary antigens and commensal bacteria. IL-6 can polarise the differentiation of naïve $CD4^+$ T cells into Th_2 cells[17,39] and is essential in the development of IgA, a first line of defence against many pathogens[19] at mucosal surfaces. IL-6 production by SIEC points to an essential role by these cells in the maintenance of a Th_2, antibody-mediated environment in the small intestine. The upregulation of IL-6 production by bacterial LPS in two out of three cases favours the notion that SIEC may also have a role in antibacterial immunity. IL-6 has been implicated in Th_1 responses and in inflammatory process in human Crohn's disease and murine models of colitis,[21,22] suggesting that IL-6 may potentiate the inflammatory role elicited by other cytokines such as IL-1. The presence of IL-1α and IL-1β mRNA in SIEC makes these cells potential inducers of inflammatory responses. Our failure to detect significant expression of IL-1 protein by SIEC suggests that these cells have distinct functions to other cells involved in immune activation such as monocytes. Alternatively, SIEC may produce IL-1 but require different stimulatory signals than other cells. Future, more extensive, analyses of SIEC activation may confirm whether these cells can produce IL-1. Given that the main function of the small intestine is digestion and absorption of nutrients, the environment is predisposed to dampen an inflammatory response. We suggest that, under certain conditions *in vivo*, such as disease or infection, SIEC may be induced to produce IL-1 protein and be key cells in the initiation of inflammatory immune responses against harmful antigens in the small intestine.

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