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Differential distribution of B7.1 (CD80) and B7.2 (CD86) costimulatory molecules on mucosal macrophage subsets in human inflammatory bowel disease (IBD)

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SUMMARY

The molecules B7.1 and B7.2 deliver costimulatory signals of critical importance to naive T cells, and may thus be involved in abrogation of oral tolerance in IBD. Functional disparity apparently exists among antigen-presenting cells *in vivo*. We wanted to examine if differential B7 expression occurs on mucosal macrophage subsets. Cryosections of bowel specimens from patients with IBD and normal controls were subjected to immunofluorescence and immunoperoxidase staining. In normal mucosa, selective subepithelial accumulation of $B7.2^{+}$ cells was found. In inflamed IBD mucosa, however, subsets appeared consisting of both $B7.2^{hi}$ and $B7.1^{hi}$ cells as well as $CD14^{hi}$ macrophages. Notably, outside lymphoid aggregates the prominent fraction of recently recruited $CD14^{hi}$ macrophages comprised most ($\approx 80\%$) of the $B7.1^{hi}$ cells, whereas most ($\approx 70\%$) $B7.2^{hi}$ cells were identified as resident mucosal macrophages ($CD14^{lo}$ or $CD14^{-}$). Differential expression of B7.1 and B7.2 on two functionally different subsets of intestinal macrophages implies separate immunoregulatory roles for the two molecules. This finding is in keeping with recent experimental data demonstrating that monocyte-derived cells are crucial for immune responses at mucosal surfaces. Preferential B7.1 up-regulation might be critical in breaking the immunological tolerance to luminal antigens in IBD, but it cannot be excluded that it is a secondary pathogenic event.

Keywords human intestinal mucosa macrophages costimulatory molecules ulcerative colitis Crohn's disease

INTRODUCTION

Effective activation of naive T cells requires engagement of the T cell receptor with MHC–peptide complex, in parallel with costimulation via accessory molecules that ligate the antigen-presenting cell (APC) to the T cell. Both signals need to be delivered by the same cell for optimal T cell responses to occur [1]. Recent work has demonstrated that CD28 is the primary costimulatory signal receptor on T cells, and B7.1 (CD80) and B7.2 (CD86) are its ligands on APC [2–4]. Blocking costimulatory interactions through CD28 inhibits T cell activation *in vitro* and may rather induce a state of unresponsiveness, termed T cell anergy [5]. Both murine and human $CD4^{+}$ T cell clones can be divided into two major subsets based on their cytokine profiles. In the mouse, Th1 cells mainly produce IL-2, interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α), whereas Th2 cells produce IL-4, IL-5, IL-6 and IL-13 as well as IL-10 [6]. In humans, polarization is best documented for IFN-γ (Th1) *versus* IL-4 and IL-5 (Th2). These two profiles favour cell-mediated and humoral immune responses, respectively [7,8].

The two $CD4^{+}$ T cell subsets are only extremes of an array of possible phenotypes occurring *in vivo*. Clones producing Th1 and Th2 cytokines in various combinations are often found, therefore designated Th0 cells [6]. In this respect, the functional necessity for the two CD28 counter-receptors is currently unknown, although several studies have suggested that B7.1 and B7.2 can differentially skew T cell responses in a Th1-like or Th2-like direction, respectively [9–12]. Thus, B7.2 has been found to be critical for the induction of Th2 and Th0 responses in naive T cells, but not for maintaining these responses. Conversely, B7.1 may provide a more neutral signal, although its later kinetics probably favours maintenance of Th1 responses that depend on continuing CD28 engagement [9,12,13]. Furthermore, a similar polarizing effect of the two CD28 ligands on the cytokine profiles of $CD8^{+}$ T cells has recently been reported [8,14].

The roles for B7.1 and B7.2 in differential T cell activation *in*

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vivo remain controversial [12], but the two molecules appear to exert quite different functions in disease processes [10,11,15–17]. Nevertheless, B7.1 and B7.2 may mediate similar signals under certain *in vitro* conditions [18,19], although the latter molecule shows only 26% amino acid identity with the former [12]. Such disparity between *in vivo* and certain *in vitro* findings could partly result from differential expression of B7.1 and B7.2 on separate APC subsets *in vivo* [12]. In this respect, a differential distribution of B7.1 and B7.2 on APC in human intestinal mucosa might be of critical importance in the modulation of local T cell responses and induction or break of oral tolerance to luminal antigens.

It has recently been suggested that IBD results from abrogation of mucosal tolerance to the indigenous microbial gut flora [20,21]. We therefore examined *in situ* whether the expression profiles of B7.1 and B7.2 on different putative APC subsets, particularly the newly recruited and the resident macrophages, are altered in IBD lesions compared with the situation in normal intestinal mucosa. Striking inflammation-associated cellular up-regulation was observed for both B7.1 and B7.2; notably, outside lymphoid aggregates and follicles, B7.1 was preferentially expressed on newly recruited macrophages. To our knowledge, this is the first direct demonstration of differential B7 expression on subsets of APC *in situ* associated with inflammatory diseases and preferential up-regulation of B7.1 *versus* B7.2 on macrophages newly recruited to the lesion.

MATERIALS AND METHODS

Tissue specimens

Tissue specimens were obtained from macroscopically inflamed mucosa of 10 patients with clinically and histologically well established ulcerative colitis (UC) and 23 patients with Crohn's disease (CD); the latter included ileal surgical specimens from 12 and colonic specimens from 11, all from different patients. In addition, we also obtained specimens from histologically uninflamed colonic mucosa from three of the CD patients and three of the UC patients. For comparison, four surgical and four biopsy specimens from histologically normal colonic mucosa, and two surgical and eight biopsy specimens from histologically normal jejunal mucosa, were selected as control material. Regrettably, no fresh material from non-IBD inflamed intestinal mucosa was available at our hospital.

The tissue specimens were immediately transported to the laboratory in ice-chilled isotonic saline and, after excising small tissue blocks from the surgical material, the samples were orientated on thin slices of carrot, embedded in ornitine carbamyl transferase tissue compound (OCT; Tissue Tek, Miles Labs, Elkhart, IN), and snap-frozen in liquid nitrogen. Cryosections were cut at 4 μm perpendicular to the mucosal surface and air-dried overnight before they were fixed in acetone (10 min, 20°C) and wrapped in aluminium foil and stored at –20°C until use.

The sex and age distributions of the patients were as follows: UC, four men and six women, median 40 years (range 32–46 years); colonic CD, four men and seven women, median 31 years (24–37 years); ileal CD, six men and six women, median 31 years (26–46 years); normal colon from four men and four women, median 55 years (range 25–66 years); and normal small intestine from three men and seven women, median 35 years (range 4–66 years).

Several IBD patients received immunosuppressive therapy: one with CD and one with UC were treated with cyclosporin A (CsA; 100 mg and 60 mg/day, respectively), one CD patient received azathioprin (100 mg/day), and seven CD patients and three UC patients received steroids (10–45 mg/day). The normal control specimens from colon were from two patients operated for obstipation, one for fistula to the intestine from a newly implanted graft on the abdominal aorta, and five patients subjected to endoscopy for abdominal symptoms but with normal endoscopic and histological findings. The normal control specimens from the small intestine were from three organ donors in whom the peripheral circulation was artificially maintained, and from seven patients investigated for different intestinal symptoms but with normal endoscopic and histological findings.

Histological grading of inflammation was performed on a 0–4 scale [22], as follows: 0, normal mucosa; 1, architectural distortion of crypts, mucosal atrophy, no active inflammation in the lamina propria; 2, mild active inflammation in the lamina propria, some crypt abscesses, mild goblet cell depletion, architectural changes; 3, moderate active inflammation in the lamina propria, crypt abscesses, more evident goblet cell depletion, superficial erosions, architectural changes; and 4, severe active inflammation in the lamina propria, significant epithelial destruction with ulceration. The distribution of the degree of inflammation in the mucosal specimens from the different IBD patients was as follows: UC, median 4 (3–4); colonic CD, median 3·3 (2·5–4); and ileal CD, median 2·5 (2·5–3·5).

Lymphoid aggregates or follicles were found in only three of the eight specimens from normal control colon and two of the six specimens of uninflamed IBD colon; no apparent germinal centres were present. Lymphoid aggregates were not found in the specimens from normal control small intestine, which were all from the jejunum. The inflamed IBD specimens contained a variable number of relatively large lymphoid aggregates (range 0–4); they were present in all of the 10 specimens from UC, in nine of the 11 colonic CD specimens, and in eight of the 12 ileal CD specimens. Overt germinal centres were histologically found in three of the inflamed UC specimens and in two of the inflamed colonic CD specimens, but follicular dendritic cells (FDC) were found in more of them (see Results). For brevity, lymphoid aggregates and lymphoid follicles, including their adjacent T cell zones, will be called lymphoid structures (Fig. 1).

Immunohistochemistry—methodological considerations

Characterization of APC subsets in lamina propria. The immunohistochemical staining protocols are described in the following sections. All antibody reagents and immunofluorescence fluorochrome conjugates are listen in Table 1, together with the specifications of applied working dilutions. To our knowledge, no generally accepted alternative to CD68 [23,24] as a pan-macrophage marker is available. In cryosections this marker is according to our experience only satisfactorily revealed by immunostaining when applying the MoAb KP1, which is of the murine IgG1 subclass. Adequate paired immunofluorescence staining with this MoAb was therefore precluded in combination with the two available MoAbs that performed satisfactorily for *in situ* demonstration of B7.1 and B7.2, both being of the IgG1 subclass as well. However, MoAb RFD1 (murine IgM), reported to identify a unique MHC class II antigen expressed preferentially by dendritic cells, has been shown to decorate most macrophages both in normal and IBD mucosa [25–27]. In accordance with this, we found by paired staining with MoAb RFD1 in combination with MoAb KP1 or the macrophage marker detected by MoAb RFD7 [25] that the vast

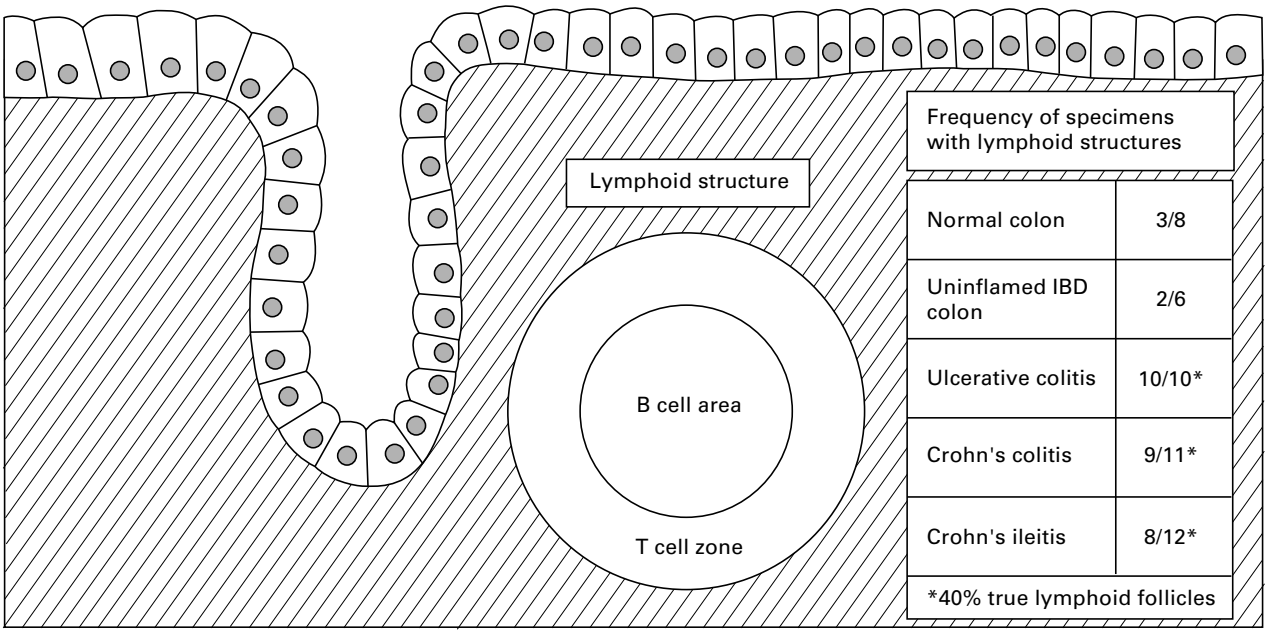

Fig. 1. Schematic depiction of the two tissue compartments of intestinal mucosa investigated: lymphoid structures (for further characterization, see text) and the remaining lamina propria (hatched area). The frequency of specimens with lymphoid structures is indicated in the panel.

majority (>80%) of RFD1$^+$ cells outside lymphoid structures co-expressed CD68 (Fig. 2a) and were also positive for RFD7 (Fig. 2b). Outside the lymphoid structures (Fig. 1), only scattered CD1c$^+$ dendritic cells [28] and CD20$^+$ B cells were present, therefore making their contribution to the total number of APC in the lamina propria negligible.

On the basis of these results, it seemed justified to apply RFD1 to identify mucosal macrophages in lamina propria by paired two-colour immunofluorescence staining for B7.1 or B7.2, allowing application of a MoAb with sufficient B7 isoform affinity irrespective of its IgG subclass; the use of subclass-specific secondary antibody conjugates (that generally provide reduced staining sensitivity) could thereby be avoided. In this manner a satisfactory immunofluorescence detection level was obtained for B7 isoform expression in paired staining with MoAb RFD1 by applying biotinylated goat anti-mouse IgG (affinity-purified to avoid cross-reaction against mouse IgM); the achieved detection level appeared to be between that of the highly sensitive immunoperoxidase (ABC, streptavidin–biotin complex; Dako, Glostrup, Denmark) method and subclass-specific paired immunofluorescence staining (see below).

Definition of the variable expression levels of B7.1, B7.2 and CD14. When immunofluorescence staining for B7.1, B7.2 and CD14 was performed with relevant IgG subclass-specific secondary reagents, the expression levels revealed were arbitrarily defined as high (B7.1^{hi}, B7.2^{hi} and CD14^{hi}); this seemed justified because the subclass-based staining approach, contrary to the ABC method, did not reveal any of these leucocyte markers in normal intestinal mucosa or in uninflamed specimens of IBD mucosa except for scattered CD14^{hi} cells and B7.2^{hi} cells in the latter. Therefore, the expression levels visualized solely by the highly sensitive ABC method were defined as low (B7.1^{lo}, B7.2^{lo} and CD14^{lo}).

Immunoperoxidase staining

All incubation steps described below were followed by gentle washing of the tissue sections for 2–3 min in PBS pH 7·6, if not otherwise stated. The primary antibodies (Table 1) were applied on cryosections for 1 h at room temperature, followed by a brief rinse in PBS and fixation in paraformaldehyde (1%) lysine-periodate fixative (PLP 1%, 10 min, 4°C) before application of biotinylated horse anti-mouse IgG for 1·5 h. The tissue sections were then incubated with 0·6% H_2O_2 in methanol for 5 min to block endogenous peroxidase activity and then with the ABC (Dako) solution for 30 min. This last incubation was followed by a rinse in Tris-buffered (0·01 M, pH 7·6) isotonic saline for 5 min before addition of a solution of 0·05% (w/v) diaminobenzidine and 0·015% H_2O_2 in Tris-buffered saline as chromogen substrate for 10 min. Counterstaining was performed with haematoxylin for 1 min.

It was decided to use the immunoenzyme method only for single staining, because co-expression of two markers at the same location (e.g. on the cell surface membrane) cannot be reliably visualized by this approach; critical detection of variable double expression therefore has to rely on paired immunofluorescence staining [29].

Paired immunofluorescence staining

Cryosections were treated as described above, and primary as well as secondary antibody reagents are listed in Table 1 together with the respective working dilutions. The sections were incubated with various pairs of primary antibodies (different isotypes) for 1 h at room temperature, followed by the appropriate pair of secondary reagents for 1 h. For staining of cells with the phenotype CD14^{hi} B7.1^{hi}, CD14^{hi} B7.2^{hi}, CD20 B7.1^{hi} or CD20 B7.2^{hi} (see above), both B7.1^{hi} and B7.2^{hi} cells (detected by MoAbs of the IgG1 subclass) were visualized by additional incubation for 30 min with Cy3-conjugated (red signal) goat anti-mouse IgG1, whereas CD20 or CD14^{hi} cells were visualized by biotinylated subclass-specific secondary antibody followed by FITC-labelled (green signal) streptavidin—the latter being co-incubated with the Cy3-conjugated anti-mouse IgG1. The green signal for CD14 was further amplified by a

Table 1. Characteristics of immunoreagents used in the study

Clone	Specificity	Immunoglobulin class/ subclass	Origin of antibody	Conjugate/ labelling	Final dilution	Producer/ source
Primary antibodies						
RM052	CD14	IgG2a	Mouse	None	3 μg/ml	Biosys, Compiegne, France
KP1	CD68	IgG1	Mouse	None	7 μg/ml	Dako, Glostrup, Denmark
RFD1	''Dendritic cells''	IgM	Mouse	None	Supernatant, 1:250 (~4 μg/ml)	Serotec, Oxford, UK
RFD7	''Tissue macrophages''	IgG1	Mouse	None	Supernatant, 1:250 (~4 μg/ml)	Serotec
L26	B cells (CD20)	IgG2a	Mouse	None	2 μg/ml	Dako
M241	CD1c	IgG1	Mouse	None	Ascitic fluid 1:1000	Courtesy Dr R.S. Blumberg, Brigham and Women's Hospital, Boston, MA
L307.4	CD80	IgG1	Mouse	None	10 μg/ml	Becton Dickinson, San Jose, CA
Fun-1	CD86	IgG1	Mouse	None	5 μg/ml	Pharmingen, San Diego, CA
Ki-M4	Follicular dendritic cells	IgG	Mouse	None	5 μg/ml	BMA Biomedicals AG, Augst, Switzerland
55/8	Irrelevant (control)	IgM	Mouse	None	*	Courtesy Dr R. Burns, Scottish Agricultural Sci. Agency, Edinburgh, UK
60/3.4	Irrelevant (control)	IgG1	Mouse	None	*	Courtesy Dr R. Burns
42/2	Irrelevant (control)	IgG2a	Mouse	None	*	Courtesy Dr R. Burns
Secondary antibodies, tertiary antibodies and fluorochrome conjugates						
	Mouse IgG2a		Goat	Biotinylated	2·5 μg/ml	Southern Biotechnology Assoc. Inc., Birmingham, AL
	Mouse IgG1		Goat	Biotinylated	2·5 μg/ml	Southern Biotechnology
	Mouse IgG		Horse	Biotinylated	20 μg/ml	Vector Labs, Burlingame CA
	Mouse IgG1		Goat	Cy3	2·5 μg/ml	Southern Biotechnology
	Biotin			Cy3-conjugated streptavidin	0·5 μg/ml	Jackson ImmunoResearch Lab., Inc. West Grove, PA
	Mouse IgG		Rabbit	Biotinylated	6 μg/ml	Jackson
	Streptavidin		Goat	Biotinylated	2·5 μg/ml	Vector
	Streptavidin		Goat	FITC	5 μg/ml	Vector
	Mouse IgM		Rabbit	FITC	40 μg/ml	Zymed Labs, Inc. San Francisco, CA
	Biotin			FITC-conjugated streptavidin	20 μg/ml	Boehringer Mannheim, Mannheim, Germany

* Comparable to that of the relevant antibody.

subsequent incubation for 30 min with FITC-conjugated goat anti-streptavidin.

For simultaneous staining with MoAb RFD1 together with MoAb against CD68, CD20, CD1c, RFD7, B7.1 or B7.2 the secondary incubations included biotinylated rabbit anti-mouse IgG (affinity-purified against mouse IgM) for 1 h, followed by 30 min with FITC-conjugated rabbit anti-mouse IgM together with Cy3-conjugated streptavidin for 30 min. For amplification of the B7.1 and B7.2 signals, two additional 30-min incubations were included, consisting of biotinylated goat anti-streptavidin and then Cy3-conjugated streptavidin.

Negative control stainings were obtained for all markers by applying isotype- and concentration-matched primary murine MoAbs of irrelevant specificity (Table 1).

Microscopy and histomorphometry

Counting of cells stained by the immunoperoxidase method was performed by ordinary light microscopy. At least 3 mm mucosal length at full thickness of the lamina propria were examined in each section at ×400 magnification. The numbers of B7.1^{+} and B7.2^{+} cells within lymphoid structures (Fig. 1) of IBD specimens were recorded on a semiquantitative scale from 1 to 4, defining 1 as scattered few positive cells, 2 as > 20–40 positive cells, 3 as > 40–100 positive cells, and 4 as > 100 positive cells. The staining results for the two markers in the same lymphoid structure were compared in adjacent serial sections mounted on the same glass slide.

Sections subjected to paired immunofluorescence staining were examined as earlier described [30] with a Leitz DMRDXE microscope camera system (Leitz, Wetzlar, Germany) equipped with a Ploem-type vertical illuminator and DIC optics. When examining areas of lamina propria outside lymphoid structures, at least four high-power fields (×400 magnification) were included per section. The fraction of B7.1 or B7.2 cells co-expressing CD14^{hi} or the RFD1 antigen in these areas was determined only in sections where > 100 B7.1^{hi} or B7.2^{hi} cells were found.

Photographs (single and double exposures) were taken on

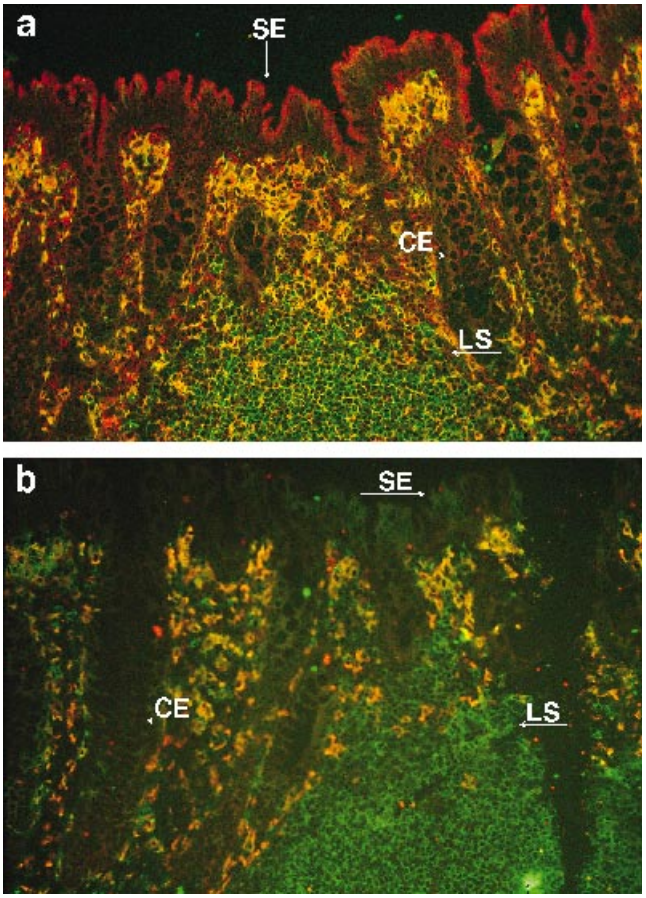

Fig. 2. Paired immunofluorescence staining of two acetone-fixed cryosections from the same inflamed ulcerative colitis specimen. Dense subepithelial accumulations of macrophages that appear yellow because they co-express the RFD1 antigen (green) and (a) CD68 (red) or (b) RFD7 antigen (red). Interdigitating dendritic cells or B cells (green) in the periphery of a large lymphoid structure are identified by RFD1 and are not decorated for CD68 and RFD7 antigen. Note that CD68 detected by KP1 is also typically expressed apically by the epithelium. CE, Crypt epithelium; SE, surface epithelium; LS, lymphoid structure. Original mag. $\times 250$.

Kodak Elite II 400 ISO daylight film pushed to 800 ISO for fluorescence.

Statistical analysis

The data were considered to be non-parametric, and comparisons between groups were therefore performed by the Mann–Whitney *U*-test for unpaired samples (two-tailed). Results are presented as median and 95% confidence interval if not otherwise stated. When the sample size is $< n = 8$, the dispersion is reported as the observed range.

RESULTS

Lymphoid structures

Characterization. Lymphoid structures were frequently seen in inflamed IBD specimens, but were scant in normal control mucosa (Fig. 1). Such structures often contained a peripheral dense population of $CD1c^+$ dendritic cells intermingled with T cells, mimicking parafollicular zones adjacent to true lymphoid follicles. By immunostaining with anti-CD14 [32] and Ki-M4 [33], the latter

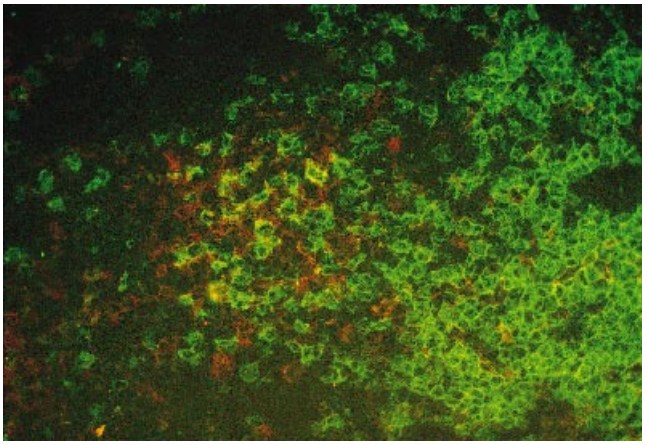

Fig. 3. Paired immunofluorescence staining of acetone-fixed cryosection of Crohn's colitis specimen showing the periphery of a lymphoid structure. The central area is packed with $CD20^+$ B cells (green), whereas the border zone to the left contains cells that appear yellow either because they co-express B7.1 (red) and CD20, or because $B7.1^+$ putative interdigitating dendritic cells (red) are in intimate contact with B cells. Original mag. $\times 400$.

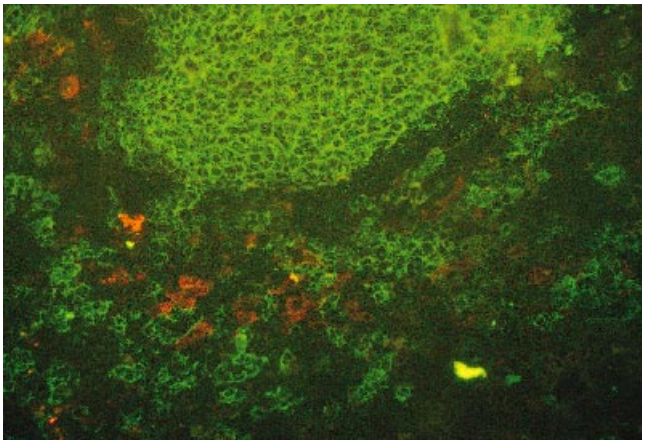

Fig. 4. Paired immunofluorescence staining of acetone-fixed cryosection of Crohn's colitis specimen showing the periphery of a lymphoid structure. In the B cell area (top), numerous packed cells appear variably yellow because they co-express CD20 (green) and B7.2 (red), or because B cells have intimate contact with dendritic cells that express B7.2. In the adjacent T cell zone (bottom), only $CD20^-$ cells express B7.2, probably representing interdigitating dendritic cells. Original mag. $\times 400$.

selectively detecting FDC, it was substantiated that median 40% (range 0–70%) of the lymphoid structures in IBD were true follicles, with no apparent difference between UC and CD.

Only a few scattered $CD1c^+$ cells were seen in each section outside the lymphoid structures in IBD and normal mucosa. The vast majority ($> 90\%$) of the $CD1c^+$ cells were co-stained with RFD1, as were the various B cell subsets in overtly organized lymphoid follicles. This result was confirmed by co-staining for the specific B cell marker CD20. Lymphoid structures generally contained only a few scattered $CD68^+$ cells (tingible body macrophages) and showed little or no co-expression ($< 1\%$) when RFD1 was combined with CD68 or RFD7 (Fig. 2a,b).

Lymphoid structures in IBD. In the inflamed IBD specimens, the frequency of lymphoid structures was significantly increased

Table 2. Expression of B7.1 and B7.2 in lymphoid structures

Nos of specimens evaluated*	Specimen category	Expression score†	
		B7.1	B7.2
$n=3$	Normal colon	0	1 (1–2)‡
$n=10$	Ulcerative colitis	2 (1–3)	3 (1–4)
$n=9$	Crohn's colitis	2 (0–3)	3 (1–4)
$n=8$	Crohn's ileitis	2 (1–4)	3·5 (1–4)

* See Fig. 1.

† Semiquantitative comparison of the number of cells positive for B7.1 and B7.2 was performed after parallel staining of adjacent sections for the two markers (see Materials and Methods).

‡ Median and observed range.

(Fig. 1); and because such structures were generally larger and exhibited a density of B7.2$^+$ and B7.1$^+$ cells at least matching that of comparable structures in normal control mucosa, the number of aggregated cells expressing these costimulatory molecules in IBD was evidently increased. Because these structures generally contained only a few scattered CD68$^+$ cells, macrophages did not represent any significant contribution to their B7.1 or B7.2 expression. The small number of lymphoid structures in normal control mucosa precluded comparison concerning possibly altered expression levels of B7.1 relative to B7.2.

Reliable determination of the identity of B7$^+$ cells in B cell areas of lymphoid structures by paired immunofluorescence staining was generally prohibited by the fact that FDC and B cells were in intimate contact with each other (Fig. 3). However, in the adjacent T cell zones the vast majority (>90%) of both B7.1^{hi} and B7.2^{hi} cells co-expressed the RFD1 antigen, which was also co-expressed by most (>90%) CD1c$^+$ cells found in this location (Fig. 1). Furthermore, because most of them were CD20$^-$, they were deemed to be interdigitating dendritic cells (Fig. 4).

The semiquantitative scoring of the number of cells stained in the lymphoid structures by the immunoperoxidase method demonstrated that the relative expression levels of the two CD28 ligands in these compartments did not appear to differ significantly between UC and CD (Table 2). Too few (a total of three) such structures were present in the normal colonic mucosal specimens for statistical comparison, but the B7 markers appeared to be up-regulated in the inflamed IBD specimens (Table 2).

Lamina propria outside lymphoid structures

Normal control mucosa. A subepithelial accumulation of B7.2^{lo} large cells, often showing blunt projections, was detected by immunoperoxidase staining in most (five of eight) of the colonic specimens, and often (eight of 10) also in the villi of the small intestine (Fig. 5). Because of their extremely low B7 expression, they could not be clearly and consistently revealed by any of the paired immunofluorescence staining protocols described above. The same was true for a population of cells with faint CD14 expression, most numerous in the colon (Fig. 5). However, immunoperoxidase staining for CD68 (macrophages), CD20 (B cells) and CD1c (dendritic cells) in adjacent serial sections suggested that virtually all of these B7.2^{lo} and CD14^{lo} cells were CD68$^+$ macrophages. No B7.1$^+$ cells were found in any of the normal control specimens.

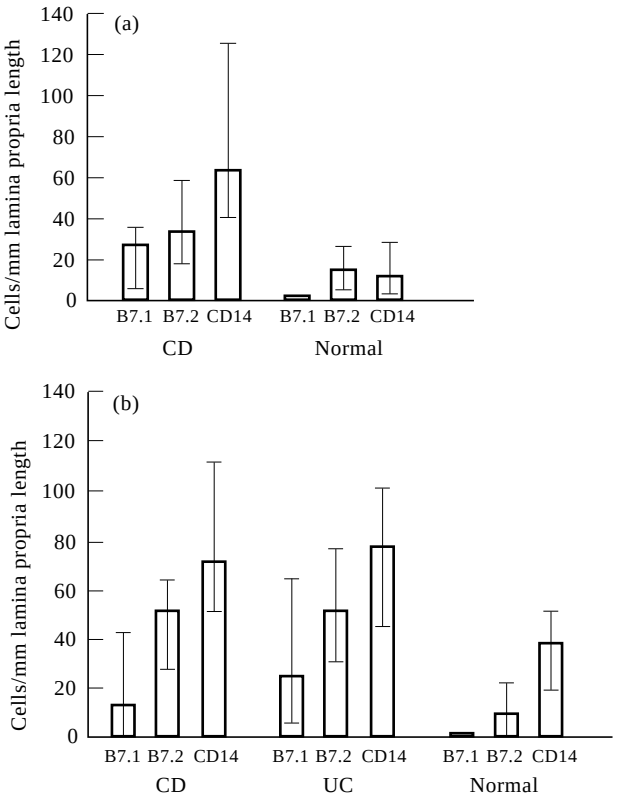

Fig. 5. Cellular expression of B7.1, B7.2 and CD14 in mucosal lamina propria outside lymphoid structures (that includes the adjacent T cell zones) as detected by immunoperoxidase staining of cryosections from (a) inflamed ileal Crohn's disease (CD) specimens ($n=12$) and normal jejunum ($n=10$), as well as (b) inflamed colonic CD specimens ($n=11$), ulcerative colitis (UC) specimens ($n=10$), and normal colon ($n=8$). The number of positive cells per mm length unit of lamina propria sectioned perpendicular to the mucosal surface was counted. Median and 95% confidence intervals are indicated.

Uninflamed IBD mucosa. There was no apparent increase in the expression of B7.1, B7.2 or CD14 compared with normal controls, as judged by immunoperoxidase staining ($n=6$, data not shown). As in normal control mucosa, faintly CD14$^+$ and B7.2$^+$ cells were relatively common, but B7.1$^+$ cells were rare. By the less sensitive immunofluorescence staining protocol (see above), only a few scattered B7.2^{hi} and CD14^{hi} cells were detected, while B7.1^{hi} cells were absent.

Inflamed IBD mucosa. Cells deemed to show B7.2^{hi} expression occurred in all CD specimens from the small intestine and colon as well as in all UC specimens. Most of these cells were located in the upper parts of the lamina propria, mainly densely packed below the surface epithelium. Parallel to the up-regulated expression of this marker on individual cells, the number of positive cells detected by immunoperoxidase staining per mm length unit of lamina propria at full mucosal height was significantly increased ($P<0{\cdot}001$) compared with normal (Fig. 5).

By paired immunofluorescence staining, it was shown that most (range 60–95%) of the B7.2^{hi} cells in the IBD specimens co-expressed the RFD1 antigen (Fig. 6), which identified the vast majority of macrophages outside lymphoid structures (see above)—that is, median 80%, 65% and 90% for CD specimens

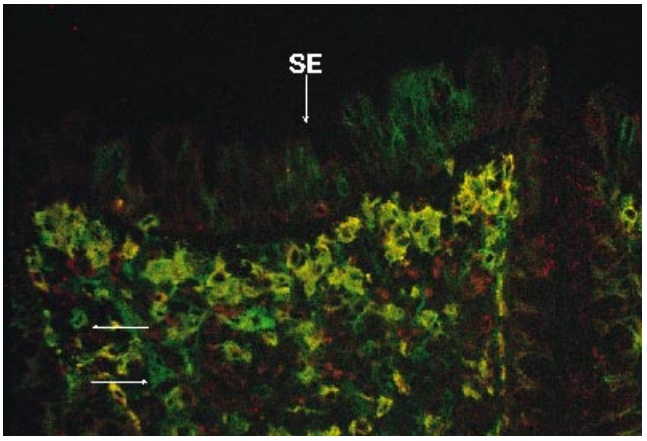

Fig. 6. Paired immunofluorescence staining of acetone-fixed cryosection of ulcerative colitis specimen shows subepithelial accumulation of macrophages that appear yellow because they co-express RFD1 antigen (green) and B7.2 (red). Only a few purely RFD1$^+$ cells are seen (arrows). RFD1 is known to decorate the vast majority of the prominent subepithelial resident macrophage population. Faint orange-red cells in the background represent non-specific fluorescence of eosinophils. SE, Surface epithelium. Original mag. ×400.

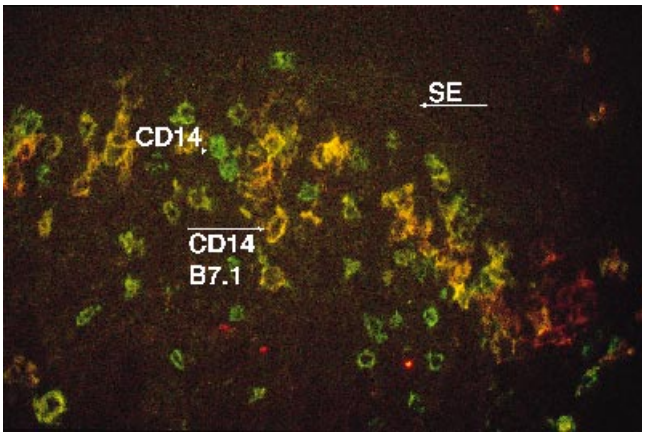

Fig. 7. Paired immunofluorescence staining of acetone-fixed cryosection of inflamed colonic Crohn's disease specimen showing that most cells positive for B7.1 (red) co-express CD14^{hi} (green) and therefore appear yellow. SE, Surface epithelium. Original mag. ×400.

from the ileum and colon and from UC specimens, respectively. About one-third of the B7.2^{hi} cells also co-expressed CD14 in both UC and CD. Such co-expression apparently paralleled the relative size of the CD14^{hi} subset as a fraction of the total macrophage population in IBD mucosa [30,31]. Less than 2% of the B7.2^{hi} cells co-expressed CD20 in these areas. Furthermore, because dendritic cells identified by CD1c were found only as scattered cells by immunoperoxidase staining outside the lymphoid structures, such cells could not contribute significantly to the B7-expressing population in the lamina propria.

In addition to the strikingly increased population of B7.2^{hi} cells, immunoperoxidase staining revealed the appearance of a prominent fraction of B7.1$^+$ cells, together with a significantly ($P < 0{\cdot}02$) increased population of CD14$^+$ cells in nearly all IBD specimens (Fig. 5). Paired immunofluorescence staining demonstrated the emergence of a population of B7.1^{hi} cells in both diseases, most of these also being CD14^{hi} (median 74%; colonic

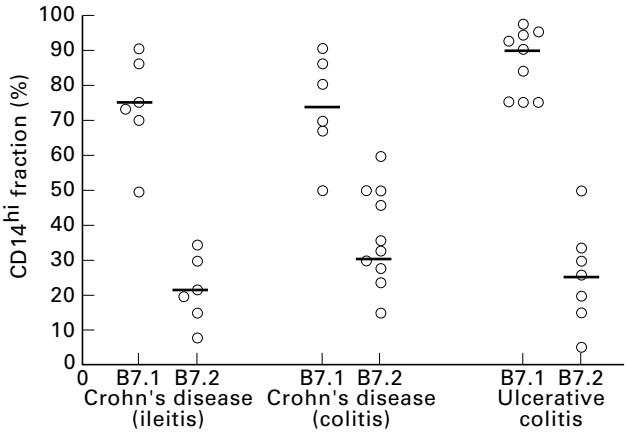

Fig. 8. Scatter diagram depicting fraction (%) of B7.1^{hi} or B7.2^{hi} cells co-expressing CD14^{hi} in mucosal lamina propria outside lymphoid structures (that includes the adjacent T cell zones) in inflamed IBD specimens.

CD 75%; and UC 90%), in contrast to the relatively moderate representation of this phenotype among B7.2^{hi} cells (Figs 7 and 8). In CD, similar phenotypic changes also extended into the deeper parts of the submucosa.

The relation between B7.1 and B7.2 expression by lamina propria macrophages did not seem to differ significantly between UC and CD colitis (Fig. 5b).

DISCUSSION

This study examined, apparently for the first time, the detailed cellular distribution of the CD28 ligands B7.1 and B7.2 in human intestinal mucosa. The up-regulated expression of these molecules on mucosal macrophages in inflamed IBD lesions suggested a significant change in their costimulatory potential via CD28. This pathway is believed to play a critical role in immune responses, both with regard to cytokine production and avoidance of anergy in naive T cells [2,5,8,13], which are known to extravasate increasingly in IBD lesions [34].

It was of special interest that B7.1 remained undetectable in normal mucosa even with the highly sensitive immunoperoxidase method applied in this study. The appearance of B7.1^{hi} on CD14^{hi} cells in active IBD lesions might at least partly be a response to invading Gram-negative bacteria [35,36], triggered through the lipopolysaccharide (LPS) receptor CD14 [37–39], on such recently recruited macrophages [31]. The inverse relation between the expression of B7.1 and B7.2 found on these CD14^{hi} macrophages, compared with the remaining macrophage population, could in fact reflect the function of CD14 as a dominant trigger of B7.1 expression [37]. This result agreed with the reported dominant role of up-regulated B7.1 for the APC and IFN-γ-inducing functions of monocytes [40]. Furthermore, CD14^{hi} macrophages have recently been shown to possess a prominent potential for IL-10 production [38]; this cytokine is reported to selectively down-regulate B7.1 on APC [41]. Therefore, an autoregulatory effect of IL-10 secreted by recently recruited mucosal macrophages might contribute to down-regulation of B7.1 (and thus a preferential B7.2 profile) as they gradually differentiate when approaching the surface epithelium in the normal situation, and to a lesser extent also in active IBD.

Considerable controversy exists in the literature concerning

possible differences between UC and CD lesions with regard to local production of immunoregulatory cytokines [42,43], including IL-2 and IFN-γ [44–49]. However, similar and rather modest production of IL-4 in CD, UC and normal controls has been reported in two recent studies [49,50]. To our knowledge, only two studies have presented combined data for both Th1 and Th2 cytokines, allowing evaluation of the balance between Th1 and Th2 clones operating in the actual lesions; both reports document a predominant IFN-γ response and a minimal or decreased IL-4 response in UC as well as in CD [48,49]. A selectively increased IL-5 production was shown for CD4$^+$ T cells from UC, but its level was extremely small compared with that of IFN-γ [49]. Interestingly, we did not find any apparent disparity between the two types of lesion in terms of the balance between B7.1 and B7.2 expression on lamina propria macrophage subsets or on the prominent populations of APC in lymphoid structures. Thus, our results might well be in keeping with a possible role of the two CD28 ligands in determining the balance between Th1 and Th2 cytokine responses *in vivo* [8–12].

Although the functions of B7.1 and B7.2 *in vivo* remain controversial [12], and a dominating role of CD28-mediated signals in Th subset differentiation has been questioned [51], the strikingly preferential expression of B7.1 on newly recruited CD14^{hi} *versus* CD14^{lo} or CD14$^-$ resident macrophages in IBD is strongly suggestive of different roles played by the two CD28 ligands, as also reflected by recent *in vitro* studies of monocytes [40]. Thus, the appearance of the CD14^{hi} B7.1^{hi} monocyte-like subset in IBD lesions may represent a major change favouring a significant shift away from a normal anergic mucosal state towards enhanced Th1-type local immune activation [52,53], including a break of tolerance against the indigenous bacterial flora [20,54]. CD14^{hi} macrophages, in addition, possess an increased potential for release of other tissue-aggressive factors in the IBD lesion, such as the proinflammatory cytokines IL-1 [55] and TNF-α [38], as well as reactive oxygen metabolites [31]. IL-1 and TNF-α, moreover, have been reported to synergize with IL-12 in inducing a Th1-like response [54].

In the normal state, as well as in IBD, CD14$^+$ peripheral blood monocytes recruited to the intestinal mucosa are supposedly a major source of potent APC, because recent reports suggest that monocytes can differentiate into classical dendritic cells under certain *in vitro* conditions [56,57]. In the human intestinal mucosa such cells probably share functional properties with dendritic cells that are reported to traffic and populate mucosal surfaces in the rat [58–60]. Near the epithelial surfaces of the intestine, monocyte-derived CD14$^+$ cells probably differentiate to APC, with suppressive effects on untoward T cell activation induced by luminal antigens [61–64]. However, the potential importance of mucosal macrophage subsets in the modulation of tolerance to the luminal flora needs to be investigated further by relevant functional studies, because information to this end appears not to exist. Although an excessive accumulation of CD14$^+$ B7.1$^+$ macrophages in IBD might represent a secondary event following epithelial damage and microbial invasion, it could nevertheless be important for the chronicity and aggravation of both UC and CD [42].

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REFERENCES

1 Janeway CJ, Bottomly K. Signals and signs for lymphocyte responses. Cell 1994; **76**:275–85.
2 Allison JP. CD28–B7 interactions in T-cell activation. Curr Opin Immunol 1994; **6**:414–9.
3 Robey E, Allison JP. T-cell activation: integration of signals from the antigen receptor and costimulatory molecules. Immunol Today 1995; **16**:306–10.
4 Linsley PS, Ledbetter JA. The role of the CD28 receptor during T cell responses to antigen. Annu Rev Immunol 1993; **11**:191–212.
5 Lenschow DJ, Bluestone JA. T cell co-stimulation and *in vivo* tolerance. Curr Opin Immunol 1993; **5**:747–52.
6 Kelso A. Th1 and Th2 subsets: paradigms lost? Immunol Today 1995; **16**:374–9.
7 Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989; **7**:145–73.
8 Mosmann R, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 1996; **17**:138–46.
9 Freeman GJ, Boussiotis VA, Anumanthan A *et al*. B7-1 and B7-2 do not deliver identical costimulatory signals, since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4. Immunity 1995; **2**:523–32.
10 Lenschow DJ, Ho SC, Sattar H *et al*. Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse. J Exp Med 1995; **181**:1145–55.
11 Kuchroo VK, Das MP, Brown JA *et al*. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. Cell 1995; **80**:707–18.
12 Thompson CB. Distinct roles for the costimulatory ligands B7-1 and B7-2 in T helper cell differentiation? Cell 1995; **81**:979–82.
13 Webb LM, Feldmann M. Critical role of CD28/B7 costimulation in the development of human Th2 cytokine-producing cells. Blood 1995; **86**:3479–86.
14 Sad S, Marcotte R, Mosmann TR. Cytokine-induced differentiation of precursor mouse CD8$^+$ T cells into cytotoxic CD8$^+$ T cells secreting Th1 or Th2 cytokines. Immunity 1995; **2**:271–9.
15 Liblau RS, Singer SM, McDevitt HO. Th1 and Th2 CD4$^+$ T cells in the pathogenesis of organ-specific autoimmune diseases. Immunol Today 1995; **16**:34–38.
16 Matulonis U, Dosiou C, Freeman G *et al*. B7-1 is superior to B7-2 costimulation in the induction and maintenance of T cell-mediated antileukemia immunity. Further evidence that B7-1 and B7-2 are functionally distinct. J Immunol 1996; **156**:1126–31.
17 Windhagen A, Newcombe J, Dangond F *et al*. Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. J Exp Med 1995; **182**:1985–96.
18 Lanier LL, O'Fallon S, Somoza C *et al*. CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. J Immunol 1995; **154**:97–105.
19 Levine BL, Ueda Y, Craighead N, Huang ML, June CH. CD28 ligands CD80 (B7-1) and CD86 (B7-2) induce long-term autocrine growth of CD4$^+$ T cells and induce similar patterns of cytokine secretion *in vitro*. Int Immunol 1995; **7**:891–904.
20 Duchmann R, Kaiser I, Hermann E *et al*. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin Exp Immunol 1995; **102**:448–55.
21 Macpherson A, Khoo U, Forgacs I, Philpott-Howard JIB. Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria. Gut 1996; **38**:365–75.
22 O'Morain CA, Abelow AC, Chervu LR, Fleischner GM, Das KM. Chromium 51-ethylenediaminetetraacetate test: a useful test in the

assessment of inflammatory bowel disease. J Lab Clin Med 1986; **108**:430–5.
23 Holness CL, Simmons DL. Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. Blood 1993; **81**:1607–13.
24 Pulford KA, Rigney EM, Micklem KJ *et al*. KP1: a new monoclonal antibody that detects a monocyte/macrophage associated antigen in routinely processed tissue sections. J Clin Pathol 1989; **42**:414–21.
25 Poulter LW, Campbell DA, Munro C, Janossy G. Discrimination of human macrophages and dendritic cells by means of monoclonal antibodies. Scand J Immunol 1986; **24**:351–7.
26 Allison MC, Cornwall S, Poulter LW, Dhillon AP, Pounder RE. Macrophage heterogeneity in normal colonic mucosa and in inflammatory bowel disease. Gut 1988; **29**:1531–8.
27 Selby WS, Poulter LW, Hobbs S, Jewell DP, Janossy G. Heterogeneity of HLA-DR-positive histiocytes in human intestinal lamina propria: a combined histochemical and immunohistological analysis. J Clin Pathol 1983; **36**:379–84.
28 Melian A, Beckman EM, Porcelli SA, Brenner MB. Antigen presentation by CD1 and MHC-encoded class I-like molecules. Curr Opin Immunol 1996; **8**:82–88.
29 Brandtzaeg P, Halstensen TS, Huitfeldt H, Valnes K. Immunofluorescence and immunoenzyme histochemistry. In: Johnstone AP, Turner MW, eds. Immunochemistry 2—a practical approach. Oxford: Oxford University Press, 1997:71–130.
30 Rugtveit J, Brandtzaeg P, Halstensen TS, Fausa O, Scott H. Increased macrophage subset in inflammatory bowel disease: apparent recruitment from peripheral blood monocytes. Gut 1994; **35**:669–74.
31 Rugtveit J, Haraldsen G, Hogasen AK, Bakka A, Brandtzaeg P, Scott H. Respiratory burst of intestinal macrophages in inflammatory bowel disease is mainly caused by $CD14^+L1^+$ monocyte derived cells. Gut 1995; **37**:367–73.
32 Schriever F, Freedman AS, Freeman G *et al*. Isolated human follicular dendritic cells display a unique antigenic phenotype. J Exp Med 1989; **169**:2043–58.
33 Parwaresch MR, Radzun HJ, Hansmann ML, Peters KP. Monoclonal antibody Ki-M4 specifically recognizes human dendritic reticulum cells (follicular dendritic cells) and their possible precursor in blood. Blood 1983; **62**:585–90.
34 Burgio VL, Fais S, Boirivant M, Perrone A, Pallone F. Peripheral monocyte and naive T-cell recruitment and activation in Crohn's disease. Gastroenterology 1995; **109**:1029–38.
35 Liu Y, van Kruiningen HJ, West AB, Cartun RW, Cortot A, Colombel JF. Immunocytochemical evidence of Listeria, *Escherichia coli*, and Streptococcus antigens in Crohn's disease. Gastroenterology 1995; **108**:1396–404.
36 Ohkusa T, Okayasu I, Tokoi S, Ozaki Y. Bacterial invasion into the colonic mucosa in ulcerative colitis. J Gastroenterol Hepatol 1993; **8**:116–8.
37 Schmittel A, Scheibenbogen C, Keilholz U. Lipopolysaccharide effectively up-regulates B7-1 (CD80) expression and costimulatory function of human monocytes. Scand J Immunol 1995; **42**:701–4.
38 Rugtveit J, Nilsen EM, Bakka A, Carlsen H, Brandtzaeg P, Scott H. Cytokine profiles differ in newly recruited and resident subsets of mucosal macrophages from inflammatory bowel disease. Gastroenterology 1997; **112**:1493–505.
39 Kielian TL, Blecha F. CD14 and other recognition molecules for lipopolysaccharide: a review. Immunopharmacology 1995; **29**:187–205.
40 Fleischer J, Soeth E, Reiling N, Grage-Griebenow E, Flad H-D, Ernst M. Differential expression and function of CD80 (B7-1) and CD86 (B7-2), on human peripheral blood monocytes. Immunology 1996; **89**:592–8.
41 Buelens C, Willems F, Pierard G, Delvaux A, Velu T, Goldman M. IL-10 inhibits the primary allogeneic T cell response to human peripheral blood dendritic cells. Adv Exp Med Biol 1995; **378**:363–5.
42 Brandtzaeg P, Haraldsen G, Rugtveit J. Immunopathology of human inflammatory bowel disease. Springer Semin Immunopathol 1997; **18**:555–89.
43 Sartor RB. Cytokines in intestinal inflammation: pathophysiological and clinical considerations. Gastroenterology 1994; **106**:533–9.
44 MacDonald TT, Hutchings P, Choy MY, Murch S, Cooke A. Tumour necrosis factor-alpha and interferon-gamma production measured at the single cell level in normal and inflamed human intestine. Clin Exp Immunol 1990; **81**:301–5.
45 Kusugami K, Matsuura T, West GA, Youngman KR, Rachmilewitz D, Fiocchi C. Loss of interleukin-2-producing intestinal $CD4^+$ T cells in inflammatory bowel disease. Gastroenterology 1991; **101**: 1594–605.
46 Brynskov J, Tvede N, Andersen CB, Vilien M. Increased concentrations of interleukin 1β, interleukin-2, and soluble interleukin-2 receptors in endoscopical mucosal biopsy specimens with active inflammatory bowel disease. Gut 1992; **33**:55–58.
47 Breese EJ, Braegger CP, Corrigan CJ, Walker SJ, MacDonald TT. Interleukin-2- and interferon-γ-secreting T cells in normal and diseased human intestinal mucosa. Immunology 1993; **78**:127–31.
48 Niessner M, Volk BA. Altered Th1/Th2 cytokine profiles in the intestinal mucosa of patients with inflammatory bowel disease as assessed by quantitative reversed transcribed polymerase chain reaction (RT-PCR). Clin Exp Immunol 1995; **101**:428–35.
49 Fuss IJ, Neurath M, Borivant M *et al*. Disparate $CD4^+$ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-γ, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. J Immunol 1996; **157**:1261–70.
50 Karttunnen R, Breese EJ, Walker SJ, MacDonald TT. Decreased mucosal interleukin-4 (IL-4) production in gut inflammation. J Clin Pathol 1994; **47**:1015–8.
51 Brown DR, Green JM, Moskowitz NH, Davis M, Thompson CB, Reiner SL. Limited role of CD28-mediated signals in T helper subset differentiation. J Exp Med 1996; **184**:803–10.
52 Schreiber S, MacDermott RP, Raedler A, Pinnau R, Bertovich MJ, Nash GS. Increased activation of isolated intestinal lamina propria mononuclear cells in inflammatory bowel disease. Gastroenterology 1991; **101**:1020–30.
53 Pirzer U, Schonhaar A, Fleischer B *et al*. Reactivity of infiltrating T lymphocytes with microbial antigens in Crohn's disease. Lancet 1991; **338**:1238–9.
54 Trinchieri G, Scott P. The role of interleukin-12 in the immune response, disease and therapy. Immunol Today 1994; **15**:460–3.
55 Casini RV, Kam L, Chong YJ, Fiocchi C, Pizarro TT, Cominelli F. Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. A novel mechanism of chronic intestinal inflammation. J Immunol 1995; **154**:2434–40.
56 Zhou L, Tedder T. $CD14^+$ blood monocytes can differentiate into functionally mature $CD83^+$ dendritic cells. Proc Natl Acad Sci USA 1996; **93**:2588–92.
57 Kiertscher S, Roth M. Human $CD14^+$ leucocytes acquire the phenotype and function of antigen-presenting dendritic cells when cultured in GM-CSF and IL-4. J Leukoc Biol 1996; **59**:208–18.
58 Maric I, Holt PG, Perdue MH, Bienenstock J. Class II MHC antigen (Ia)-bearing dendritic cells in the epithelium of the rat intestine. J Immunol 1996; **156**:1408–14.
59 McWilliam AS, Nelson D, Thomas JA, Holt PG. Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces. J Exp Med 1994; **179**:1331–6.
60 Holt PG, Haining S, Nelson DJ, Sedgwick JD. Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. J Immunol 1994; **153**:256–61.
61 Upham JW, Strickland DH, Bilyk N, Robinson BW, Holt PG. Alveolar macrophages from humans and rodents selectively inhibit T-cell proliferation but permit T-cell activation and cytokine secretion. Immunology 1995; **84**:142–7.
62 Chelen CJ, Fang Y, Freeman GJ *et al*. Human alveolar macrophages present antigen ineffectively due to defective expression of B7 costimulatory cell surface molecules. J Clin Invest 1995; **95**:1415–21.

63 Holt PG, Oliver J, Bilyk N *et al*. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells *in vivo* by resident alveolar macrophages. J Exp Med 1993; **177**:397–407.

64 Qiao L, Braunstein J, Golling M, Autschbach F, Muller P, Meuer S. Differential regulation of human T cell responsiveness by mucosal *versus* blood monocytes. Eur J Immunol 1996; **26**:922–7.