Response of human intestinal lamina propria T lymphocytes to interleukin 12: additive effects of interleukin 15 and 7

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Abstract

Background/Aim—Interleukin (IL) 12 is involved in the mucosal response during intestinal inflammation but its role is not fully understood. The response of human lamina propria T lymphocytes (T-LPL) to IL-12 in terms of interferon γ (IFN-γ) release and proliferation was investigated, exploring whether IL-15 and IL-7 cooperate with IL-12. The role of accessory molecules (CD2 and CD28) was also investigated.

Methods—Unstimulated and phytohaemagglutinin preactivated T-LPL cultures were incubated with or without the initial addition of cytokines, anti-CD2 or anti-CD28 antibodies. IFN-γ mRNA was detected by reverse transcriptase polymerase chain reaction, and protein secretion was measured by enzyme linked immunosorbent assay (ELISA).

Results—IFN-γ mRNA was induced in T-LPLs by IL-12 and IL-15 but not IL-7, whereas IFN-γ was measured only in IL-12 stimulated T-LPL cultures. IL-12 induced IFN-γ release was not abrogated by neutralising anti-IL-2 antibody or by cyclosporin A. IL-12 synergised with either anti-CD2 or anti-CD28 antibodies in inducing IFN-γ synthesis. In preactivated T-LPLs, IL-7 enhanced IFN-γ release induced by both IL-12 and anti-CD2, whereas IL-15 potentiated only IL-12 induced IFN-γ synthesis. IL-12 did not induce proliferation of either unstimulated or preactivated T-LPLs and it did not enhance the CD2/CD28 stimulated T-LPL proliferative response. No transcript for IL-12 receptor β_1 subunit was detected in freshly isolated and activated T-LPLs whereas the β_2 subunit mRNA was consistently found in T-LPL samples.

Conclusions—IL-12 induces human T-LPLs to produce and release IFN-γ, and IL-15 and IL-7 cooperate with IL-12 in expanding the IFN-γ mucosal response.
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Keywords: interleukin 12; interleukin 7; interleukin 15; interferon γ; intestinal inflammation

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On activation with specific antigens, both CD4 and CD8 cells may differentiate into type 1 or type 2 subsets.[1][2] Interferon γ (IFN-γ) is the main product of Th$_1$ cells and it has undoubted physiological importance in regulating immune and inflammatory processes.[3] Evidence has been provided that interleukin 12 (IL-12), a heterodimeric cytokine produced by activated monocytes/macrophages, is prominently involved in Th$_1$ cell differentiation and IFN-γ synthesis.[4][5] Both resting and activated T cells are induced by IL-12 to release IFN-γ in vitro, and neutralising anti-IL-12 antibodies inhibit up to 80% of IL-12 induced IFN-γ production. IL-12 deficient mice have recently been generated and shown to be impaired in their ability to produce IFN-γ and mount a Th$_1$ immune response.[6] These observations indicate that IL-12 is the major physiological stimulus for IFN-γ production and release. An optimal response of IFN-γ-producing cells to IL-12 is, however, dependent in part on co-stimulatory signals.[5][7][8] IFN-γ released by T cells may in turn contribute to the regulation of IL-12 production by monocytes/macrophages, and suggestions have been made that the IL-12/IFN-γ loop plays a role in balancing the immune response during inflammation.[9]

Cell mediated immunity and T cell activation are important features of chronic intestinal inflammatory disorders such as Crohn's disease,[10–13] in which a Th$_1$ response and enhanced IFN-γ expression and release have been shown.[14–17] Bioactive IL-12 is expressed and released by lamina propria mononuclear cells (LPMCs) from patients with Crohn's disease, suggesting a pivotal role for this cytokine in this condition.[18] Furthermore, convincing experimental data have been provided that Th$_1$ mediated intestinal inflammation can efficiently be abrogated by blocking IL-12,[19] suggesting that, at the mucosal level, IL-12 is an essential requirement for a Th1 mediated inflammatory response.

It is therefore conceivable that human lamina propria T lymphocytes (T-LPLs) respond to IL-12. The physiological role of IL-12 in the intestinal mucosa compartment is, however, poorly understood. No direct evidence is available to show how in the human intestinal mucosa compartment IFN-γ expression and synthesis are induced and regulated by IL-12, and no data are available to indicate whether other cytokines cooperate with IL-12 in IFN-γ induction. The present study was therefore undertaken to investigate human T-LPL responsiveness to IL-12 in terms of IFN-γ production and proliferation and to relate it to the expression of IL-12 receptor β_1 and β_2 subunit (IL-12Rβ_1 and IL-12Rβ_2) mRNA.

IL-7 is locally released by epithelial cells in the human gut mucosa and exhibits different modulatory effects on mucosal T cells.[20] A syn-

ergistic effect of IL-7 and IL-12 on human T cell activation has been recently shown, and a modulatory effect of IL-7 on IFN-γ expression has been demonstrated using circulating T cells.[21 22] Like IL-7, the pleiotropic cytokine IL-15 is released by intestinal epithelial cells and may act as an intermediate molecule in determining a Th_1-like cytokine profile.[23 24] IL-7 and IL-15 share a receptor subunit (γ chain)[25] and it is conceivable that they may contribute to the regulation of intestinal Th_1 cell differentiation and function. The role of IL-15 and IL-7 in contributing to IFN-γ expression and release by human T-LPLs was therefore also examined.

We here report data from several experiments showing that: (*a*) IL-12 induces human T-LPL IFN-γ production; (*b*) IL-7 and IL-15 cooperate with IL-12 in expanding the IFN-γ response in T-LPLs, suggesting that at the mucosal level different mediators are involved in the expansion of the IL-12 driven Th_1 inflammatory response; (*c*) in human T-LPLs, $IL-12R\beta_2$ mRNA expression may not be sufficient to deliver all IL-12 mediated signals.

Materials and methods

MUCOSAL SAMPLES

Mucosal samples were obtained from the macroscopically and microscopically unaffected areas of 12 surgical specimens of colon cancer. The samples were obtained from a group of four women and eight men (mean (SD) age 60 (6)). Adenocarcinoma was located in the left colon in all patients. Autologous peripheral blood mononuclear cells (PBMCs) were also obtained from seven patients. PBMCs from five healthy subjects were also available. The study was approved by the local department ethical committee.

LPMC AND PBMC ISOLATION AND PURIFICATION AND T CELL CULTURE

LPMCs were isolated by using the dithiothreitol-EDTA-collagenase sequence as previously described in detail.[18] Briefly, the dissected intestinal mucosa was freed of mucus and epithelial cells in sequential steps with dithiothreitol and EDTA and then digested with collagenase (all available from Sigma, St Louis, Missouri, USA). After collagenase digestion, the medium containing the mononuclear cells was collected and centrifuged at 400 *g* for 10 minutes. After two washes in calcium and magnesium free Hanks balanced salt solution (HBSS-CMF) (Sigma), the pellet was resuspended in a 40% Percoll solution (Pharmacia, Uppsala, Sweden). An isotonic Percoll solution, consisting of nine parts Percoll and one part 10 × HBSS-CMF (pH 7.4, 290 mOsm), was used to prepare dilutions with HBSS-CMF. In a glass tube, 2 ml each of 100, 60, 40, and 30% Percoll were layered. The tube was centrifuged at 400 *g* for 25 minutes, and LPMCs at the 60–40% Percoll layer interface were collected. The isolated cells were counted and checked for viability using 0.1% trypan blue (viability ranged from 86 to 94%).

PBMCs were isolated by density gradient centrifugation (Lymphoprep; Nycomed Pharma, Oslo, Norway) from 10 ml heparinised blood samples.

T lymphocytes were obtained after adherence of both LPMCs and PBMCs to plastic flasks (three hours, 37°C) by neuraminidase treated sheep red blood cell (SRBC) rosetting. The SRBCs were then lysed with 1 ml sterile distilled water. Both peripheral blood and lamina propria T cell preparations were consistently 92–95% CD3, with 3–4% CD56/CD16 and <4% contaminating monocytes and B cells. CD4 and CD8 T cell populations were purified by indirect panning. Briefly, goat anti-mouse immunoglobulin (Sigma) was diluted to 10 μg/ml in 0.05 M Tris/HCl, pH 9.5, and added to Petri dishes (overnight at 4°C). T cells (2 × 10^7) were resuspended in an appropriate amount of mouse anti-CD8 antibody (1:250 final dilution) (Sigma) and left for 30 minutes on ice, before incubation on the anti-mouse immunoglobulin-coated dish (60 minutes at 4°C). Non-adherent cells were collected by gently pipetting the fluid from the dish, and adherent cells were removed with a sterile scraper. The purity of the cell populations was consistently greater than 92% as indicated by FACS analysis.

After isolation, T cells were resuspended in RPMI 1640 supplemented with 10% fetal calf serum, 1% L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium) (all available from Sigma) at a concentration of 2 × 10^6 cells/ml, and cultured in 24-well culture plates with or without the initial addition of IL-12 (Sigma) and/or IL-7 and/or IL-15 (cytokine final concentration ranging from 10 to 10 000 pg/ml) and/or anti-CD28 antibody (clone CD28.2) (Sigma) (at a final concentration of 1 μg/ml) and/or the anti-CD2 antibody pair T11 $_{2,3}$ (kindly supplied by Dr Ellis Reinhertz, Dana-Farber Cancer Institute, Boston, Massachusetts, USA) (used at a dilution of 1:1000 each). All cultures were incubated in triplicate. Parallel experiments were also performed using T cells preactivated with phytohaemagglutinin (PHA) for five days. For this purpose, T cells (1 × 10^6 cells/ml) were resuspended in complete medium supplemented with 1 μg/ml PHA (Sigma) and cultured for five days. At the end of the culture period, cells were collected, washed three times with HBSS and checked for viability (viability ranged from 76 to 90%). PHA preactivated cells were then resuspended in complete medium at a concentration 2 × 10^6/ml and cultured in 24-well culture plates with and without the initial addition of the above stimuli. After 0, 12, 24, 48, and 72 hours, culture supernatants were collected and stored at −80°C until tested.

To examine whether T lymphocyte IFN-γ release induced by the above cytokines or anti-CD2/CD28 antibodies was IL-2 dependent, parallel PHA preactivated T cell cultures were incubated for 24 hours with or without the initial addition of a neutralising anti-IL-2 antibody (Sigma) (1:100 final dilution) or cyclosporin A (Sandoz-Wander Pharma SA, Berne, Switzerland) (0.1 μg/ml final concentration). To explore the role of counterbalancing

cytokines in IL-12 stimulated T-LPL IFN-γ release, in three separate experiments PHA preactivated T-LPLs were stimulated with 100 pg/ml IL-12 in the presence or absence of transforming growth factor β₁ (1 ng/ml final concentration) (Becton Dickinson Labware, Bedford, Massachusetts, USA) or IL-10 (1 ng/ml final concentration) (Sigma) for 24 hours.

Colonic biopsy specimens were also available for RNA analysis on freshly obtained whole tissue. Three biopsy samples were taken from the colon of three patients with irritable bowel syndrome undergoing colonoscopy for recurrent abdominal pain. The specimens were immediately placed in guanidinium thiocyanate buffer on ice, homogenised using a tissue homogeniser (Ystral GmbH, D-7801; PBI International, Dottingen, Germany) and used immediately for RNA extraction.

RNA AND cDNA PREPARATION
Total RNA was extracted from both unstimulated and stimulated T cells cultured for 0, 6, 12, and 24 hours. For RNA preparation, cells were lysed in 1 ml guanidinium thiocyanate buffer and subjected to phenol/chloroform extraction as described by Chomczynski and Sacchi.[26] The sample obtained was quantified by measuring absorbance at 260 nm. RNA integrity was assessed by electrophoresis on a 1.5% agarose gel. cDNA was synthesised from 0.5 μg total RNA using 0.2 U murine leukaemia virus reverse transcriptase (Promega, Madison, Wisconsin, USA), 2.5 μM random hexamers (Boehringer-Mannheim, Mannheim, Germany), 1mM dNTP (Boehringer-Mannheim), 2 U RNase inhibitor (Promega) in a total volume of 20 μl. The reaction was performed at 37°C for 60 minutes.

REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION (RT–PCR)
Before examining transcripts for IFN-γ, IL-12Rβ₁, and IL-12Rβ₂, sample cDNA content was normalised on the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal. For this purpose, 2 μl cDNA was incubated in a PCR mixture for 18, 21, 23, and 25 cycles with GAPDH specific primers. IFN-γ, IL-12Rβ₁, and IL-12Rβ₂ primers were assayed on all samples by incubating an equivalent amount of cDNA for 35 cycles. PCR was performed in a total volume of 50 μl in the presence of 1 U *Taq* DNA polymerase (Boehringer-Mannheim), 200 μmol dNTPs, and 25 pmol/l 5' and 3' primers. PCR was carried out in a Robocycler thermal cycler (Stratagene, La Jolla, California, USA) (denaturation one minute at 94°C; annealing for one minute at 55°C for both IFN-γ and IL-12Rβ₂, and for one minute at 59°C for IL-12Rβ₁; extension for one minute at 72°C).

PCR primers (Genosys, Cambridge, UK) were as follows:

IFN-γ: 5'-AATGCAGGTCATTCAGATG-3' and 3'-AACTGACTTGAATGTCCAA-5'; IL-12Rβ₁: 5'-CCGGCGTCCTAAAG-GAGTATG-3' and 3'-TGGAGACAGGTG-CAAGGCC-5'; IL-12Rβ₂: 5'-GGATGCTC-ATTGGCATTTAT-3' and 3'- TTGTCT-GCAAACTGGCCTG-5'; GAPDH: 5'-CACCATCTTCCAGGAGCGAG-3' and 3'-TCCGGGAAACTGTGGCGTGA-5'.

In order to exclude contamination of the samples with amplified genomic DNA, experiments were also performed using RNA as substrate for the PCR assay. A 10 μl sample of PCR product was combined with 1 μl loading buffer and electrophoresed on a 1.5% agarose gel (in Tris/EDTA buffer). A 123 bp ladder was used to assess sample size.

The specificity of the PCR products for both IL-12R subunits was validated by restriction enzyme analysis. For this purpose the amplified IL-12Rβ₁ PCR product was digested with *Hga*I (Amersham International, Slough, Bucks, UK) into two fragments of 303 and 254 bp. For the IL-12Rβ₂ PCR product restriction analysis, the enzyme *Bam*HI (Boehringer-Mannheim) was used. This enzyme cleaves the amplified PCR product into two fragments of 185 and 113 bp.

IFN-γ ASSAY
IFN-γ was measured in T-LPL and T-PBL culture supernatants using a sensitive enzyme linked immunosorbent assay (ELISA) (Amersham International). According to the manufacturer, the minimum detectable IFN-γ concentration is 10 pg/ml.

PROLIFERATION ASSAYS
For the proliferation assay, 1×10^5 cells were cultured in 0.2 ml complete medium with different stimuli in flat-bottomed 96-well microtitre plates for one, two, three, and four days. At 18 hours before termination of the culture, 1 μCi [³H]thymidine was added to each well; cells were then collected on a glass filter and radioactivity was measured. All cultures were incubated in triplicate.

STATISTICAL ANALYSIS
Student's *t* test was used for statistical analysis of the data.

Results

IFN-γ mRNA EXPRESSION BY T-LPLs AFTER EXPOSURE TO IL-12
IFN-γ mRNA was detected in four of 12 unfractionated LPMC samples. In all four samples, transcripts for both IL-12 subunits (p40 and p35) were found. Since IL-12/p40 is required to generate a functionally bioactive IL-12, T-LPLs used in subsequent experiments were purified from the eight LPMC samples not expressing IL-12/p40 mRNA. No transcript for IFN-γ was found in these T-LPL samples, but the cells were fully capable of expressing transcripts for IFN-γ in response to various stimuli—for example, PHA, anti-CD2/anti-CD28 antibodies. IFN-γ mRNA was detected in the IL-12 stimulated T-LPL samples. All IL-12 concentrations tested (from 10 to 10 000 pg/ml) efficiently induced transcripts for IFN-γ independently using CD4 or CD8 purified T-LPLs. IFN-γ mRNA accumulation was detectable as early as six hours after IL-12 stimulation, and it was not affected by the initial addition of 10 μg/ml cycloheximide
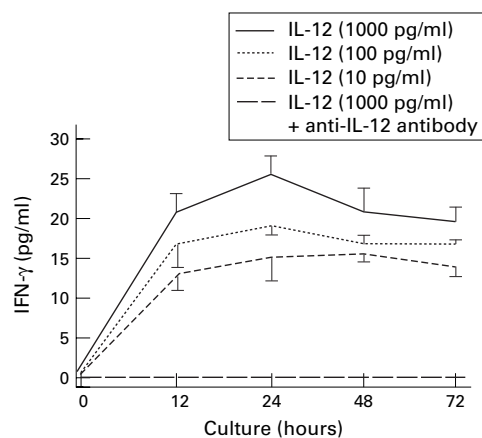
Figure 1 Interferon γ (IFN-γ) release in three day cultures of both interleukin (IL) 12- and IL-12 (1000 pg/ml final concentration) plus anti-IL-12 antibody-stimulated lamina propria T lymphocytes. Each point on the curve represents the mean of all experiments; vertical bars indicate 1 SD. At each time interval, IL-12 induced IFN-γ release in a dose dependent manner (p = 0.01).

(Sigma). No transcript for IFN-γ was detectable in unstimulated T-PBLs, while IFN-γ mRNA was induced by exposure to IL-12. Again, transcripts for IFN-γ were detectable as early as six hours after the beginning of stimulation with IL-12 (concentrations ranging from 10 to 1000 pg/ml).

T-LPL SECRETION OF IFN-γ AFTER EXPOSURE TO IL-12

No IFN-γ was measured in the culture supernatants of unstimulated T-LPLs. In contrast, IFN-γ was consistently detected in the culture supernatants of IL-12 stimulated T-LPLs containing IFN-γ transcripts. As shown in fig 1, IL-12 induced T-LPL IFN-γ production in a dose-dependent manner. The amount of IFN-γ released by T-LPLs after IL-12 exposure was low but comparable with that measured by others in IL-12 stimulated PBMC cultures.[5] T-LPL IFN-γ secretion was detectable as early as 12 hours after the start of IL-12 stimulation and reached a plateau at 24 hours, with no further increase over the culture period (fig 1). The initial addition of a neutralising anti-IL-12 antibody (Sigma) (1:100 final dilution) to the IL-12 stimulated T-LPL cultures completely inhibited the IFN-γ release (fig 1). No inhibition was found with a non-relevant control antibody (anti-IL-4 antibody) (R&D Systems, Minneapolis, Minnesota, USA) (1:100 final dilution). To test

whether the IL-12 induced IFN-γ production was dependent on endogenous IL-2, T-LPL cultures were incubated for 24 hours with IL-12 (100 pg/ml) in the presence or absence of a neutralising anti-IL-2 antibody (1:100 final dilution). In these cultures, the anti-IL-2 antibody did not abrogate the IL-12 induced IFN-γ release (18 (1.2) vs 19 (0.55) pg/ml; mean (SD)). In addition, the IL-12 stimulated IFN-γ production was not inhibited by 0.1 μg/ml cyclosporin A (18 (1.2) vs 18.25 (0.8) pg/ml after 24 hours).

When PHA preactivated T-LPLs were used, the amount of IFN-γ measured in the 24 hour supernatants of cells exposed to IL-12 was significantly higher than that released by freshly isolated IL-12 stimulated T cells (p<0.001). This was evident for each IL-12 concentration tested (table 1). The initial addition of transforming growth factor β_1 to the PHA preactivated T-LPL cultures significantly decreased the IFN-γ release induced by IL-12 (65 (6.0) vs 20 (4.0) pg/ml; p<0.001). Similarly, the IL-12 stimulated T-LPL IFN-γ release was affected by IL-10 (65 (6.0) vs 32 (5.5) pg/ml; p<0.01).

No IFN-γ was measured in the culture supernatants of unstimulated T-PBLs. Similarly, no measurable IFN-γ was found in T-PBL cultures stimulated with 10 or 100 pg/ml IL-12. In contrast, IFN-γ was consistently detectable in the culture supernatants of T-PBLs after exposure to 1000 pg/ml IL-12 (table 1). As with T-LPLs, the IL-12 induced IFN-γ release was evident after 12 hours of T-PBL stimulation (16 (2.0) pg/ml) and reached a maximal level at 24 hours (18 ± 2.8 pg/ml), with no further increase over the culture period (16 (1.0) pg/ml at 48 hours, 15 (0.89) pg/ml at 72 hours).

EFFECT OF CD2 AND CD28 ON THE RESPONSIVENESS OF INTESTINAL T-LPLs TO IL-12

Both anti-CD2 and anti-CD28 antibodies were capable of inducing IFN-γ secretion (table 2). The amount of IFN-γ released by T-LPLs after exposure to anti-CD2 was 15 times that released after exposure to anti-CD28 (table 2). To examine the role of CD2 and CD28 in T-LPL responsiveness to IL-12, unstimulated and PHA preactivated T-LPLs were incubated with graded concentrations of IL-12 in the presence or absence of anti-CD2 or anti-CD28 antibodies. A synergistic IFN-γ inducing effect was observed when T-LPLs were co-stimulated with graded doses of IL-12

Table 1 Interferon γ (IFN-γ) release in culture supernatants of both unstimulated and phytohaemagglutinin (PHA) preactivated lamina propria T lymphocytes (T-LPLs) and autologous peripheral blood T lymphocytes (T-PBLs) after 24 hours of exposure to graded doses of interleukin 12 (IL-12)

| | IFN-γ secretion (pg/ml) | | | |
| | Unstimulated lymphocytes | | PHA activated lymphocytes | |
	T-LPLs	T-PBLs	T-LPLs	T-PBLs
Medium alone	0	0	16 (4.1)	23 (3.0)
IL-12 (pg/ml)				
10	14 (2.9)	0	46 (2.5)	48 (5.0)
100	18 (1.2)	0	65 (6.0)	74 (6.0)
1000	24 (2.0)	18 (2.8)	190 (10)	260 (10)

Results are expressed as mean (SD) from six representative experiments.

Table 2   Interferon γ (IFN-γ) release in both unstimulated and phytohaemagglutinin (PHA) preactivated lamina propria T lymphocytes (LPLs) and autologous peripheral blood lymphocytes (PBLs) after 24 hours of culture

	IFN-γ secretion (pg/ml)			
Stimulus	Unstimulated LPLs	PHA stimulated LPLs	Unstimulated PBLs	PHA stimulated PBLs
Medium alone	0	16 (4.1)	0	23 (3.0)
Anti-CD2 antibody	430 (40)	500 (40)	320 (10)	2500 (84)
Anti-CD28 antibody	28 (3.5)	30 (5)	20 (7.0)	34 (2.2)

Results are expressed as mean (SD) from six representative experiments.
Cells were cultured with or without the initial addition of anti-CD2 (1:1000 final dilution) or anti-CD28 antibody (at a final concentration 1 µg/ml).
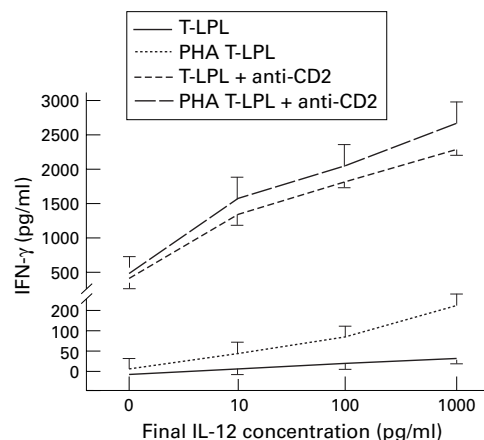

Figure 2   Interferon γ (IFN-γ) release in both lamina propria T lymphocytes (T-LPLs) and phytohaemagglutinin (PHA) preactivated T-LPLs after 24 hours of culture. Both T-LPLs and PHA preactivated T-LPLs were incubated with graded doses of interleukin (IL) 12 in the presence or absence of anti-CD2 antibody. Each point on the curve represents the mean of six representative experiments; vertical bars indicate 1 SD. Values of IFN-γ measured after co-stimulation with IL-12 and anti-CD2 antibody were significantly higher than observed after stimulation with IL-12 (p<0.0001) or anti-CD2 antibody alone (p<0.0001).

and anti-CD2 antibody. The amount of IFN-γ released after co-stimulation with IL-12 and anti-CD2 antibody was 100 times that observed after stimulation with IL-12 and three times that observed after stimualtion with anti-CD2 antibody alone (p<0.0001) (fig 2). The co-stimulatory effect of anti-CD28 antibody with IL-12 was much less pronounced (40 (6.0) pg/ml IFN-γ after 10 pg/ml IL-12), although a significant increase was observed in T-LPLs with 100 and 1000 pg/ml IL-12 in comparison with anti-CD28 antibody alone (350 (42) and 400 (10) respectively vs 28 (3.5) pg/ml) (p<0.0001).

To investigate the role of endogenous IL-2 in the synergistic effect of IL-12 and CD2/CD28 molecules, PHA preactivated cells were cultured in the presence of either IL-12 (at a final concentration of 100 pg/ml) or anti-CD2 or anti-CD28 antibodies with or without the initial addition of a neutralising anti-IL-2 antibody (1:100 final dilution) or cyclosporin A. As shown in table 3, the IL-12 induced IFN-γ release in T-LPLs and T-PBLs after 24 hours culture was not affected by anti-IL-2 antibody or cyclosporin A. In contrast, the addition of anti-IL-2 antibody to CD2 or CD28 stimulated T-LPL or T-PBL cultures resulted in a significant reduction in IFN-γ release similar to (but less pronounced than) that observed after exposure to cyclosporin A (table 3).

ROLE OF IL-7 AND IL-15 IN T-LPL IFN-γ PRODUCTION

No transcript for IFN-γ was detected in either T-LPL or T-PBL samples stimulated with IL-7. Each IL-7 concentration used (ranging from 10 to 10 000 pg/ml) failed to induce T lymphocyte IFN-γ mRNA accumulation. IFN-γ mRNA was induced in T-LPLs and T-PBLs by IL-15, but only when used at a final concentration higher than 50 pg/ml. IFN-γ mRNA accumulation was detectable six hours after IL-15 stimulation and it was not abrogated by the addition of a neutralising anti-IL-2 antibody or cyclosporin A.

No IFN-γ was measurable in the culture supernatants of either unsimulated T-LPLs or T-PBLs exposed to either IL-7 or IL-15. To investigate whether IL-7 and IL-15 are able to influence mitogen stimulated IFN-γ production, PHA preactivated cells were cultured for 24 hours in the presence or absence of either IL-7 (10 ng/ml) or IL-15 (10 ng/ml) with the addition of one of the following: IL-12 (100
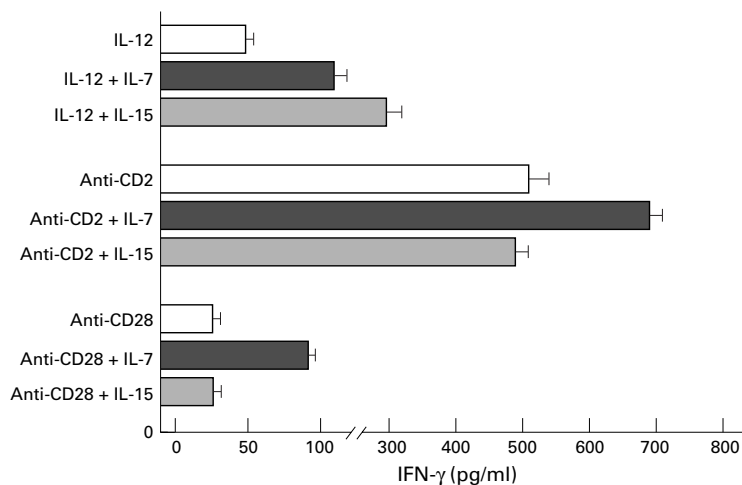
Table 3   Phytohaemagglutinin (PHA) stimulated interferon γ (IFN-γ) release in lamina propria T lymphocytes (LPLs) and peripheral blood lymphocytes (PBLs) after 24 hours of culture

	IFN-γ secretion (pg/ml)					
	Medium alone		Medium + anti-IL-2		Medium + CsA	
Stimulus	T-LPLs	T-PBLs	T-LPLs	T-PBLs	T-LPLs	T-PBLs
Medium + anti-CD2	500 (40)	2500 (84)	120 (40)	1600 (65.5)	50 (10)	42 (10)
Medium + anti-CD28	30 (5.5)	34 (2.2)	ND	25 (2.8)	ND	ND
IL-12 (100 pg/ml)	65 (6.0)	74 (6.0)	68.5 (3.8)	79 (3.5)	64.6 (4.2)	72 (6.6)

Results are expressed as mean (SD) from five representative experiments.
PHA stimulated lymphocytes were cultured with or without the initial addition of interleukin (IL)-12 (100 pg/ml) or anti-CD2 (1:1000 final dilution) or anti-CD28 (at a final concentration 1µg/ml) antibody, in the presence or absence of cyclosporin A (CsA) (0.1 µg/ml final concentration) or anti-IL-2 antibody (1:100 final dilution).
ND, not detectable.

Figure 3 Interferon γ (IFN-γ) release by phytohaemagglutinin preactivated lamina propria T lymphocytes (T-LPLs) after 24 hours of culture. Cells were cultured with interleukin (IL) 12 (100 pg/ml final concentration) or anti-CD28 (1 μg/ml final concentration) or anti-CD2 (1:1000 final dilution) antibody in the presence or absence of IL-7 (10 ng/ml final concentration) or IL-15 (10 ng/ml final concentration). Data are the mean of six representative experiments; horizontal bars indicate 1 SD. The co-stimulation with IL-7 significantly enhanced T-LPL IFN-γ release induced by IL-12 (p<0.0001) or anti-CD28 (p<0.0001) or CD2 (p<0.0001) antibody. IL-15 potentiated IL-12 stimulated T-LPL IFN-γ release (p<0.0001) but not that induced by anti-CD28 or CD2 antibody.

pg/ml), anti-CD2 (1:1000 final dilution), or anti-CD28 antibody (1μg/ml). As shown in fig 3, IL-7 enhanced IFN-γ production by T-LPLs induced by both IL-12 and anti-CD2 or anti-CD28 antibody. In contrast, IL-15 was capable of potentiating only the IL-12 induced IFN-γ release, with a fourfold increase over the values of cultures exposed to IL-12 only (fig 3).

In T-PBL cultures, IFN-γ release induced by IL-12 or anti-CD2 or CD28 antibody was significantly increased by IL-7 stimulation (p<0.0001) (table 4). Again, IL-15 enhanced T-PBL IFN-γ release induced by IL-12 (p<0.0001) but not that stimulated via the CD2 or CD28 signals (table 4).

PROLIFERATION RESPONSE OF T-LPLs TO IL-12
No significant change over baseline in[^{3}H]thymidine uptake (7.5 (1.0) × 10^{3} cpm) was seen in T-LPL cultures stimulated with increasing concentrations of IL-12 (5.5 (1.0) × 10^{3} , 7.5 (1.8) × 10^{3}, and 5 (0.9) × 10^{3} cpm in cultures provided with 10, 100, and 1000 pg/ml IL-12 respectively). T-LPLs did, however, show a proliferative response to anti-CD2/CD28 antibody (22 (3.0) × 10^{3} cpm) as well as to IL-7 (17 (2.5) × 10^{3} cpm). No proliferative effect could be attributed to IL-12 when PHA stimulated T-LPLs were used as cell samples (7.4 (0.2) × 10^{3} cpm in unstimulated cells vs

8.0 (0.16) × 10^{3}, 6.8 (0.26) × 10^{3}, and 8.8 (1.1) × 10^{3} cpm in cell cultures provided with 10, 100, and 1000 pg/ml IL-12). In addition, no IL-12 mediated proliferation was observed in PHA preactivated unfractionated LPMC cultures. PHA preactivated T-LPLs did proliferate in response to either anti-CD2 (30 (2) × 10^{3} cpm) or anti-CD28 (13 (0.45) × 10^{3} cpm) antibody with no further additive effect.

IL-12 exhibited no proliferative effect on freshly isolated T-PBLs (8.2 (1.4) × 10^{3} cpm in unstimulated cells vs 6.0 (0.8) × 10^{3}, 7.0 (2.4) × 10^{3}, and 5.6 (1.0) × 10^{3} cpm in cell cultures provided with 10, 100, and 1000 pg/ml IL-12), but it induced a dose-dependent proliferation of PHA preactivated cells (6.2 (1.2) × 10^{3} cpm in unstimulated cells vs 12.5 (1.0) × 10^{3}, 14.0 (1.1) × 10^{3}, and 18 (0.8) × 10^{3} cpm in cell cultures provided with 10, 100, and 1000 pg/ml IL-12). The proliferation induced by 1000 pg/ml IL-12 on activated T-PBLs was lower than that induced by anti-CD2 antibody (30 (1.9) × 10^{3} cpm) but higher than that by anti-CD28 (12 (0.45) × 10^{3}) (p<0.0001). In these cell cultures, 1000 pg/ml IL-12 and anti-CD2 exert an additive effect in promoting T-PBL proliferation (49 (1.65) × 10^{3} cpm), whereas IL-12 and anti-CD28 appeared to synergise (44 (1.2) × 10^{3} cpm).

IL-12R SUBUNIT mRNA EXPRESSION
To correlate the IL-12 biological effects with IL-12R subunits, the expression of both IL-12R β$_1$ and β$_2$ mRNA was assayed. No IL-12Rβ$_1$ mRNA was found in the homogenised colonic tissue samples or in four of four freshly isolated T-LPL samples (fig 4). In addition, no transcript for IL-12Rβ$_1$ was detected in T-LPLs stimulated by either PHA or anti-CD2/CD28 antibody (fig 4). Finally, no IL-12Rβ$_1$ mRNA was evident in T-LPL samples stimulated for 24 hours with IL-7 or IL-15. In contrast, IL-12Rβ$_2$ mRNA was consistently detected in the homogenised tissue samples and in T-LPL samples (fig 4).

No IL-12R subunit was expressed in freshly obtained T-PBLs (fig 4). However, these cells were fully capable of expressing transcripts for both IL-12R subunits after both PHA and anti-CD2/antiCD28 antibody stimulation (fig 4).

Discussion

We provide here evidence that the IFN-γ gene is activated in normal human T-LPLs after exposure to recombinant human IL-12. Transcripts for IFN-γ were detected in all IL-12

Table 4 Phytohaemagglutinin (PHA) preactivated interferon γ (IFN-γ) release in peripheral blood T lymphocytes (T-PBLs) after 24 hours of culture

	IFN-γ secretion (pg/ml)		
Stimulus	*Medium*	*IL-7 (10 ng/ml)*	*IL-15 (10 ng/ml)*
Medium	23 (3.0)	28 (4.8)	29 (6.5)
Anti-CD2	2500 (84)	2900 (40)	2380 (80)
Anti-CD28	34 (2.2)	105 (12.0)	46 (5.6)
IL-12 (100 pg/ml)	74 (6.0)	700 (90)	900 (25)

Results are expressed as mean (SD) from five representative experiments.
PHA preactivated T-PBLs were cultured with or without the initial addition of IL-12 (100 pg/ml) or anti-CD2 (1:1000 final dilution) or anti-CD28 (at a final concentration 1μg/ml) antibody in the presence or absence of IL-7 (10 ng/ml final concentration) or IL-15 (10 ng/ml final concentration).
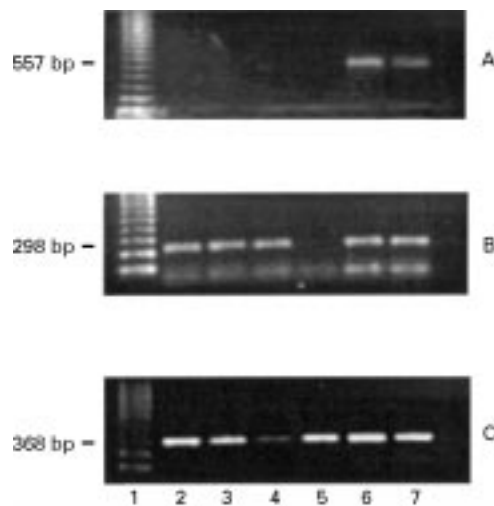
Figure 4   Agarose gel stained with ethidium bromide showing RT-PCR products for IL-12Rβ₁ (557 bp; A) (after 35 cycles), IL-12Rβ₂ (298 bp; B) (after 35 cycles), and glyceraldehyde-3-phosphate dehydrogenase (368 bp; C) (after 22 cycles) in lamina propria T lymphocytes (T-LPLs) and autologous peripheral blood T lymphocytes (T-PBLs) cultured with or without stimulation. Lane 1, 123 bp ladder; lane 2, unstimulated T-LPLs; lane 3, PHA (1μg/ml) stimulated T-LPLs; lane 4, anti-CD2/anti-CD28 antibody stimulated T-LPLs; lane 5, unstimulated T-PBLs; lane 6, PHA (1μg/ml) stimulated T-PBLs; lane 7, anti-CD2/anti-CD28 antibody stimulated T-PBLs.

stimulated T-LPL samples. IFN-γ mRNA expression was induced in both CD4 and CD8 T-LPLs by exposure to minute amounts—for example, a final concentration of 10 pg/ml—of IL-12, confirming previous data.[5] The IL-12 stimulated IFN-γ mRNA accumulation was not affected by the initial addition of cyclohex-imide to T-LPL cultures, suggesting that IL-12 induced IFN-γ gene activation is mediated by pre-existing proteins rather than being depend-ent on new protein synthesis.

IFN-γ was detected in the culture superna-tants of all T-LPL samples expressing mRNA, indicating that the effects of IL-12 on IFN-γ synthesis are mediated at both the transcrip-tional and post-transcriptional levels. T-LPL IFN-γ release was induced by IL-12 in a dose-dependent manner and was virtually abrogated by a neutralising anti-IL-12 antibody but not a non-relevant control antibody. The data sug-gest therefore that IL-12 is an important requirement for IFN-γ release in T-LPL culture supernatants. Through T-LPL IFN-γ synthesis, IL-12 may diminish IL-4 production,[27] thus polarising the response toward a Th₁ state.[28][29] When T-LPLs were pre-activated with PHA, a higher amount of IFN-γ was released in response to IL-12 than in unstimulated T-LPLs, possibly reflecting more rapid IFN-γ mRNA accumulation.[5]

An optimal T cell response to IL-12 seems to require the presence of accessory cell derived co-stimulatory molecules.[5] The present results confirm and expand on previous data showing a striking synergistic effect of IL-12 and anti-CD2 or anti-CD28 antibody in promoting IFN-γ production.[7][8] In both unstimulated and PHA preactivated T-LPL cultures, the amount of IFN-γ released after co-stimulation with IL-12 and anti-CD2 antibody was significantly

higher than that observed after stimulation with IL-12 or via CD2 alone. Similarly IL-12 synergised with anti-CD28 antibody in induc-ing T-LPL IFN-γ release. IFN-γ synthesis trig-gered by anti-CD2 or CD28 antibody was affected by the initial addition of cyclosporin A or anti-IL-2 antibody to either T-LPL or T-PBL cultures, whereas the IL-12 stimulated IFN-γ production was independent of endog-enous IL-2, supporting the existence of differ-ent intracellular biochemical signalling path-ways.

To examine whether other molecules pro-duced by accessory cells are involved in the modulation of T-LPL IFN-γ production, T cell cultures stimulated with IL-12 or anti-CD2 or anti-CD28 antibodies were incubated in the presence or absence of IL-7 or IL-15. These two cytokines are produced by human intesti-nal epithelial cells and are involved in the modulation of intestinal mucosal immunity.[20][24] IL-15 seems to play a role in local defence mechanisms by contributing to T cell recruit-ment and activation at the sites of chronic inflammation.[30][31] IL-15 preferentially facili-tates the recruitment of CD45RO T cells[30] and this may be relevant to the immune regulation in the human intestinal mucosa where the vast majority of T cells bear the CD45RO molecule.[32]

IFN-γ mRNA was induced by IL-15 in both T-LPL and T-PBL samples. This effect was, however, detectable only when appropriate concentrations of IL-15—that is, >50 pg/ml—were used and was not followed by protein release. The addition of IL-15 to IL-12 stimu-lated cell cultures significantly enhanced IFN-γ release. Taken together, these observations seem to suggest that IL-12 is the pivotal cytokine in the T-LPL IFN-γ response, but cooperation with IL-15 is important for obtaining optimal production. Since IL-12 and IL-15 are produced by activated macrophages in response to bacteria or bacterial products, both cytokines may contribute to the early activation of T cells during the innate immune response. Interestingly, IFN-γ was shown to enhance both IL-12 and IL-15 production, thus facilitating an immunostimulatory loop able to perpetuate chronic inflammation within the intestinal tissue.[9][24] IL-15 mediates its activity through a heterotrimeric receptor con-sisting of a unique IL-15 receptor α chain in combination with the β and γ chains of IL-2R.[25] We here report no synergistic effect of IL-15 and CD2- or CD28-generated signals in inducing T-LPL and T-PBL IFN-γ production, whereas IL-2 was shown to be involved in such a synergistic effect.[7] A plausible explanation for this finding is that T-LPL cultures provided with anti-CD2/CD28 antibody contain amounts of IL-2 sufficient to completely satu-rate the β and γ receptor subunits.[17][33] The demonstration that IL-2 and IL-15 may be mutually redundant in potentiating cell functions[34] would support this hypothesis.

No IFN-γ mRNA and secretion were detected in IL-7 stimulated T-LPLs or T-PBLs, confirming the previous finding of undetect-able IFN-γ levels in human PBL cultures pro-

vided with IL-7.[21][35] IL-7 was, however, able to enhance IL-12 induced T-LPL IFN-γ production. The results of this study are in agreement therefore with recent observations showing an interaction between IL-7 and IL-12 in promoting T-PBL activation.[21] In T-LPLs, the mechanisms underlying the cooperation between IL-7 and IL-12 are not, however, related to the ability of IL-7 to upregulate the IL-12Rβ1 subunit.[21] In addition, IL-7 was capable of potentiating T-LPL IFN-γ production stimulated by either anti-CD2 or anti-CD28 antibody.

IL-7 and IL-15 belong to a group of cytokines that share a receptor subunit (γ chain). γ chain activation seems to be sufficient to bypass the anergy and prevent the apoptosis of T cells.[36] The ability of these two cytokines to potentiate IL-12 driven IFN-γ production may thus result in promotion and maintenance of Th_1 cell clone development in human intestine. This is also supported by the recent demonstration that IL-7 transgenic mice that develop chronic colitis show enhanced IFN-γ production in the colonic mucosa.[37]

No proliferation was observed in freshly isolated T-LPL cultures after exposure to graded doses of IL-12, in agreement with previous reports.[38] T-LPLs were, however, fully capable of proliferating when provided with anti-CD2/CD28 antibodies or IL-7, suggesting that the T-LPL unresponsiveness is restricted to IL-12. IL-12 seems therefore to be an effective stimulus for IFN-γ production but not for proliferation of normal T-LPLs. This is supported by the observation that, in murine systems, anergic T cells are induced to release IFN-γ but do not proliferate in response to IL-12.[39] Although Th_1 clones may exhibit IL-2 dependent proliferation in response to IL-12,[40] it is unlikely that defective T-LPL IL-2 production is the key factor in determining the unresponsiveness to IL-12, because no IL-12 mediated proliferative effect was documented in T-LPL cultures normally releasing IL-2—for example, T-LPLs stimulated by PHA and anti-CD2/CD28 antibody.[17] The demonstration that IL-12 did not exert any proliferative effect on LPMC samples suggests that the contact between T and antigen presenting cells is not sufficient to restore T-LPL IL-12 responsiveness. As with T-LPLs, IL-12 was not by itself significantly mitogenic for resting T-PBLs over the range of concentrations tested. T-PBLs exhibited, however, dose-dependent IL-12 mediated proliferation after activation. This finding has been associated with the upregulation of IL-12 receptor expression on activated cells.[5][41][42] In agreement with these observations, we were able to show in activated but not in resting T-PBLs transcripts for both IL-12R $β_1$ and $β_2$ subunits, the presence of which is required to obtain maximal T cell activation in response to IL-12.[42][43] In contrast, freshly isolated T-LPLs showed constitutive expression of IL-12Rβ₂ mRNA but not IL-12Rβ₁ mRNA. In addition, no IL-12Rβ₁ mRNA was detected in T-LPLs stimulated by either PHA or anti-CD2/CD28 antibody. In our study, IL-12Rβ₂ was only studied at the mRNA level.

It is, however, conceivable that T-LPLs also express IL-12Rβ₂ protein because these cells released IFN-γ after exposure to IL-12. Taken together, the data seem therefore to suggest that, at least in human intestine, the expression of β₂ subunit alone may not be sufficient to deliver IL-12 induced proliferative signals but it may mediate IL-12 stimulated IFN-γ release. Although both IL-12R subunits are required to generate a functional high affinity IL-12R, there is evidence that each subunit may encode both ligand binding and signal transducing functions. While this manuscript was in preparation, it was reported that IL-12Rβ₁ is required to mediate IL-12 responsiveness in murine systems.[44] In contrast, in other systems, IL-12Rβ₂ alone was shown to be capable of delivering IL-12 mediated T cell activation signals.[42][43] These observations are also supported by the demonstration that differential interactions take place between the cytoplasmic regions of the two IL-12R subunits and JAK2/Tyk2.[45]

In conclusion, the results of this study indicate that IL-12 facilitates T-LPL IFN-γ production and that other molecules are required in the expansion of the IL-12 driven Th_1 response in the human intestinal mucosa.

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