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Activated STAT4 and a Functional Role for IL-12 in Human Peyer's Patches[1]

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T cells in the Peyer's patches (PP) of the human ileum are exposed to a myriad of dietary and bacterial Ags from the gut lumen. Recall proliferative responses to common dietary Ags are readily demonstrable by PP T cells from healthy individuals, and the cytokine response is dominated by IFN-γ. Consistent with Th1 skewing, PP cells spontaneously secrete IL-12p70, and IL-12p40 protein can be visualized underneath the PP dome epithelium. In this study, we have analyzed IL-12 signaling in PP and investigated whether IL-12 plays a functional role. CD3$^+$ T lymphocytes isolated from PP and adjacent ileal mucosa spontaneously secrete IFN-γ with negligible IL-4 or IL-5. RNA transcripts for IL-12Rβ2, the signaling component of the IL-12R, are present in purified CD4$^+$ and CD8$^+$ T PP lymphocytes. Active STAT4, a transcription factor essential for IL-12-mediated Th1 differentiation, is readily detectable in biopsies from PP and ileal mucosa and STAT4-DNA binding activity is demonstrable by EMSA. Nuclear proteins from CD3$^+$ T PP lymphocytes contain STAT4 and T-bet, a transcription factor selectively expressed in Th1 cells. Stimulation of freshly isolated PP cells with staphylococcal enterotoxin B dramatically enhanced the production of IFN-γ, an effect which was largely inhibited by neutralizing anti-IL-12 Ab. These data show that IL-12 in human PP is likely to be responsible for the Th1-dominated cytokine response of the human mucosal immune system. *The Journal of Immunology,* **2003, 170: 300–307.**

Peyer's patches (PP),[3] the organized lymphoid tissues of the small intestine, are the sites where the immune system generates and regulates the immune response to foods, Ags of the normal flora, and pathogens (1, 2). As such, understanding the control of immune responses in these tissues is critical for our understanding of diseases such as food hypersensitivities (e.g., celiac disease), Crohn's disease, food allergy, and oral vaccines.

In recent years, animal studies have led to an enormous advance in our understanding of the mechanisms that regulate the mucosal immune response to gut lumenal Ags. Ingestion of soluble Ag leads to a state of specific systemic immunologic unresponsiveness (oral tolerance) (3–5) which is commonly ascribed to three nonmutually exclusive mechanisms, primarily determined by the dose of Ag. High doses of orally administered Ag facilitate anergy and/or deletion of reactive T cells in PP, while repeated low doses of Ag promote active suppression due to the induction of regulatory T cells which produce Th2-type cytokines such as IL-4 and TGF-β (6–9). After priming in PP, regulatory T cells migrate to the periphery where, upon Ag-specific reactivation, they downregulate potentially newly developing or established tissue-damaging inflammatory responses (10–12). Overall, many studies show that the T cell response in mouse PP is skewed toward the Th2 type (3–5, 13, 14), although recent studies have suggested that in certain situations, such as shortly after feeding or intra-PP IL-12 gene transfer, Th1 responses can be obtained (15–17).

Despite their obvious importance, there are only a few studies on immune responses in human PP. Freshly isolated mononuclear cells (MC) from human PP (PPMC) spontaneously secrete cytokines, with a marked Th1 dominance (18). Furthermore, in functional assays, we have shown that freshly isolated human PP T cells show in vitro proliferative responses to common dietary Ags such as β-lactoglobulin or casein of cow's milk, and again the response is dominated by IFN-γ and that IL-4, IL-5, IL-10, and TGF-β-producing cells are few. In seeking an explanation for this Th1 bias, we have shown that transcripts for IL-12p40 and p35 are readily detectable in human PP, that human PP cells spontaneously secrete IL-12p70, and that immunoreactive IL-12p40 can be localized to cells below the follicle-associated epithelium (19).

Therefore the aim of this study is to functionally link the presence of IL-12 in human PP with the intracellular signaling pathways associated with Th1 responses and to determine whether IL-12 plays a functional role in Th1 responses in human PP.

Materials and Methods

Patients and samples

The study received ethical approval from East London and City Health Authorities. Signed, informed consent was obtained from parents of all children studied and where possible the children themselves gave assent to the study. All PP biopsies were sampled from the terminal ileum as described previously (20). A maximum of two PP and two ileal biopsies were taken from each patient. Samples were obtained from 27 patients (14 male, 13 female; mean age 11.8 years, range 4.1–14.7 years). From each of these

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[3] Abbreviations used in this paper: PP, Peyer's patch; MC, mononuclear cell; IBD, inflammatory bowel disease; LP, lamina propria; PPL, PP lymphocyte; LPL, LP lymphocyte; SEB, staphylococcal enterotoxin B; SFC, spot-forming cell.

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patients, biopsy specimens were also collected from the terminal ileum adjacent to the PP. Twenty-three patients had no macroscopic and histologic abnormality. The indication for endoscopy was recurrent abdominal pain in 14 and rectal bleeding subsequently shown to be due to rectal polyps in 9 patients. All 23 patients were followed for a median time of 23 mo (range 12–35) after endoscopy and none have developed inflammatory bowel disease (IBD). Four of the 27 patients had IBD (one Crohn's colitis, two ulcerative colitis, and one indeterminate colitis). However, in all IBD cases, the ileum was macroscopically and histologically normal. After collection, PP and lamina propria (LP) biopsies were immediately analyzed by inverted microscopy. Follicles were easily identifiable as cream-colored solid structures (20), quite different from the highly vascular, translucent villi seen in the mucosal biopsies. Peripheral blood was also collected from 3 of 26 patients and 10 healthy volunteers. Because the cell or protein yields from the biopsies were limited, we were not able to measure every parameter in each patient. There was no selection for the analyses performed on any patient and the number of patient samples used in each analysis is stated in the figure legends or is clear from the figure itself.

Isolation and culture of PP and ileal LPMC

Biopsies were incubated for 30 min at 37°C in calcium- and magnesium-free HBSS containing 1 mmol/l EDTA (Sigma-Aldrich, Dorset, U.K.) to remove the epithelium. Following dissection with hypodermic needles, PPMC and LPMC suspensions were prepared by collagenase digestion as described (18). As previously (20), cell yields from PP biopsies were considerably higher (median 1.7×10^6; range $1.2–2.9 \times 10^6$ cells/biopsy) than from ileal biopsies (median 0.8×10^6; range $0.3–1.2 \times 10^6$ cells/biopsy), giving independent confirmation that PP biopsies contained lymphocyte-rich follicles. $CD3^+$ PP and LP lymphocytes (PPL and LPL) were isolated from each of all eight subjects and used for analysis of cytokine-secreting cells by ELISPOT. PPL and LPL T cells were purified by incubating PPMC and LPMC with immunomagnetic beads coated with monoclonal anti-CD3 Ab according to the instructions of the manufacturer (Dynal, Oslo, Norway). $CD4^+$ and $CD8^+$ PPL and LPL were also purified from three of eight subjects using anti-CD4 or anti-CD8 Abs (Sigma-Aldrich) and immunomagnetic beads. In addition, PBMC were isolated using Ficoll-Hypaque gradients and used to purify either $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes as indicated above. $\beta7^+$ and $CD45RA^+$ T lymphocytes were purified using immunomagnetic beads coated first with a monoclonal anti-CD3 Ab and then with monoclonal anti-$\beta7$ or CD45RA Ab (both from BD Biosciences, Oxford, U.K.).

PPMC (1×10^6 cells/ml) were resuspended in RPMI 1640 medium supplemented with a serum replacement reagent HL-1 (BioWhittaker, Wokingham, U.K.) and preincubated with or without a neutralizing rabbit anti-human IL-12 Ab (10 μg/ml final concentration; R&D Systems, Abingdon, U.K.) or a rabbit control IgG (10 μg/ml final concentration; Sigma-Aldrich) for 4 h, and then stimulated with 1 μg/ml staphylococcal enterotoxin B (SEB; Sigma-Aldrich) for a further 20 h, as it is well-known that both PP and LP cells respond to SEB stimulation with enhanced cytokine secretion (19, 21).

Enumeration of cytokine-secreting cells by ELISPOT

ELISPOT assays for IFN-γ-, IL-4-, and IL-5-secreting cells were performed as previously described (18) using both PPMC and $CD3^+$ purified PPL and LPL. Because the frequency of IFN-γ ELISPOTs was so high, 5,000 cells per well gave around 50 ELISPOTs per well which was high enough to identify individual spots but large enough for it to represent a reasonable sample of the cells. However for IL-4, IL-5, and IL-10 spot-forming cells (SFC), 50,000 cells were added per well because of the low frequency of SFC. The frequency of SFC was calculated per 10^5 cells.

Cytokine concentrations in culture supernatants

The cytokines IL-2, IL-4, IL-5, IL-10, and IFN-γ were measured in culture supernatants using the BD Biosciences cytometric bead array as per the manufacturer's instructions. Bead particles of six different fluorescent intensities with an emission wavelength of 650 nm (FL-3) were coupled to an Ab against one of the six cytokines (IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ). Each bead represents a discrete population, unique due to its FL-3 intensity. The Ab-bead complexes serve as a capture for a given cytokine in the panel, and can be detected simultaneously in the supernatant. The captured cytokines are detected via direct immunoassay using six different Abs coupled to PE which emits at 585 nm (FL-2). The standards, ranging from 0 to 10,000 pg/ml, are a mixture of all six cytokines, giving six standard curves.

For each cell culture supernatant and cytokine standard mixture, 50 μl were added to a mixture of 25 μl of capture Ab bead reagent and 25 μl of detector Ab-PE reagent. The mixture was subsequently incubated for 3 h at room temperature, and washed to remove unbound detector Ab-PE reagent before acquisition using flow cytometry.

Two-color flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences), and the data was acquired and analyzed using BD Biosciences cytometric bead array software. Forward vs side scatter gating was used to only include the 7.5-μm beads. Eighteen-hundred events were collected for each sample. Standard curves were plotted of cytokine standard concentration vs mean fluorescence intensity using a four-parameter logistic curve-fitting model. If a sample had a cytokine concentration below the detection limit for the assay, it was given a value of 0.

Protein extraction and Western blotting

Tissue samples were homogenized in lysis buffer containing 10 mmol/L HEPES (pH 6.8) (Life Technologies, Paisley, U.K.), 1 mmol/L EDTA, 60 mmol/L KCl, 0.2% Igepal CA-630, 1 mmol/L sodium fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mmol/L DTT, and 1 mmol/L PMSF (all from Sigma-Aldrich). After cell lysis, the sample was centrifuged at $4000 \times g$ for 40 min (4°C) and the supernatant was collected and stored at −70°C until assay. Approximately 500 μg of protein could be obtained from a PP or mucosal biopsy.

For the detection of IL-12p70, 120 μg of total proteins were separated on a 8% SDS-polyacrylamide gel under nonreducing conditions and were electrophoretically transferred onto a nitrocellulose membrane for 1 h at room temperature. A rabbit anti-human IL-12p70 Ab (2 μg/mL; R&D Systems) followed by a HRP-conjugated goat anti-rabbit IgG mAb (1/2500 dilution; DAKO, High Wycombe, U.K.) were used, and immunoreactivity was detected using a chemiluminescence kit (Amersham, Amersham, U.K.). To confirm the equal loading and transfer of proteins, membrane was stained with Ponceau S. For the detection of IFN-α and IL-18, 120 μg of total protein were separated on a 15% SDS-polyacrylamide gel under reducing conditions. In each experiment, recombinant human IFN-α (National Institute for Biological Standards and Control, Potters Bar, U.K.) and recombinant human IL-18 (R&D Systems) were used as positive controls. IFN-α was detected by using a rabbit anti-human IFN-α Ab (1/100 final dilution; Immunokontact, Frankfurt, Germany) followed by a HRP-conjugated goat anti-rabbit IgG Ab (1/2500 dilution, DAKO). After detection of IFN-α, blots were stripped by incubation for 30 min at 50°C in stripping medium (2% SDS, 0.05 M Tris, 0.1M 2-ME) and then incubated with an Ab against IL-18 (Santa Cruz Biotechnology, Santa Cruz, CA) as previously reported (21).

RT-PCR for IL-12Rβ2

IL-12Rβ2 transcripts were semiquantitatively assessed in $CD3^+$, $CD4^+$, and $CD8^+$ PPL and LPL by Southern blotting of RT-PCR products as previously reported (22).

Determination of STAT4, STAT6, and T-bet

Total proteins (500 μg/sample) were incubated with anti-STAT4 or STAT6 (Santa Cruz Biotechnology) at 4°C for 2 h. Immune complexes were collected by incubation with protein A/G agarose (Santa Cruz Biotechnology), washed three times with lysis buffer, and heated for 5 min in a boiling water bath in sample buffer for SDS/PAGE. Immunoprecipitates from extracts containing the same amount of protein were analyzed by Western blotting with anti-phosphotyrosine (p-Tyr) (Santa Cruz Biotechnology) and subsequent incubation with HRP-conjugated goat anti-mouse IgG Ab (Santa Cruz Biotechnology). Ab reaction was detected with a chemiluminescence detection kit (Amersham). After p-Tyr analysis, blots were stripped as indicated above and then incubated with Ab against STAT4 or STAT6 to ascertain equal loading of the lanes. To determine whether PP and ileal LP T lymphocytes contained active STAT4 and STAT6, cells were first lysed in buffer A containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.2 mM EGTA, and 0.5% Nonidet P40. After removing the cytosolic proteins, nuclear extracts were prepared by solubilizing the remaining nuclei in buffer C containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and 10% glycerol. Both buffers were supplemented with 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF (all reagents were from Sigma-Aldrich). Eight micrograms of nuclear protein per sample were separated on a 8% SDS/PAGE gel, and then incubated with rabbit anti-human STAT4 or rabbit anti-human STAT6 (both Abs were from Santa Cruz Biotechnology, 1/300 final dilution) followed by a HRP-conjugated goat anti-rabbit IgG mAb (1/2500 dilution; DAKO). As internal loading control, a goat anti-human histone-1 Ab (Santa Cruz Biotechnology 1/300 final dilution) followed by a HRP-conjugated rabbit anti-goat IgG Ab (1/2500 dilution; DAKO) was used.

T-bet was analyzed in six PPL, six LPL, three $\beta7^+$, and three CD45RA$^+$ PBL samples. Twelve micrograms of nuclear proteins were separated on a 8% SDS/PAGE gel, and then incubated with goat anti-human T-bet (1/500 final dilution; Santa Cruz Biotechnology) followed by rabbit anti-goat IgG mAb (1/2500 dilution; DAKO). Immunoreactivity was visualized using chemiluminescence (Amersham).

EMSA

EMSA was used to detect specific binding of activated STAT4 to DNA. Nuclear protein-DNA binding studies were carried out for 20 min at room temperature in a 20-μl reaction volume containing 10 mM Tris, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, 1 μg poly(dI-dC) (all the reagents were from Sigma-Aldrich), 50 fmol biotin-labeled probe containing a binding site for STAT4 (5′-GAGCCTGATTTCCCCGAAATGAT GAGCTAG-3′), and 5 μg nuclear proteins. The DNA probe was prepared by annealing the two consensus STAT4 oligonucleotides, which were labeled at the 3′ end with biotin using a commercially available kit (Pierce, Rockford, IL). The binding specificity was confirmed by incubating the nuclear protein samples with unlabeled-specific STAT4 oligonucleotide or an unrelated oligonucleotide spanning an AP-1 binding site (5′-CGCTT GATGACTCAGCCGGAA-3′) in 30-fold molar excess to compete binding. In an Ab blocking assay, a rabbit anti-human STAT4 or control rabbit IgG Ab (both used at a concentration of 2 μg) were incubated with the nuclear proteins for 15 min before adding the DNA probe. A 6% nondenaturing polyacrylamide gel was used for electrophoretic separation. After blotting to a membrane, labeled oligonucleotides were detected with a chemiluminescence EMSA kit (Pierce).

Statistical analysis

In each group, data points were first analyzed to determine whether they conformed to a sample from a normally distributed population. Comparison between all groups was made by two-tailed Mann Whitney U test, $p < 0.05$ being considered significant.

Results

Frequencies of IL-4-, IL-5-, and IFN-γ-secreting cells in human PPL and LPL

To confirm the dominance of Th1 cytokines in human PP, CD3$^+$ PPL, and LPL isolated from PP and ileal mucosal biopsies ($n = 8$) which were macroscopically and histologically normal were analyzed for IL-4-, IL-5-, and IFN-γ-secreting cells by ELISPOT. Large numbers of IFN-γ-secreting cells were detected in each of the eight patients, four to eight times greater than the number of IL-4- and IL-5-secreting cells (Fig. 1).

IL-12p70, a Th1-inducing cytokine, is produced in human PP

The in vivo differentiation of T cells along Th1 or Th2 pathways is dependent on the environment in which Th0 cells are exposed to the Ag (23). There is compelling evidence that in humans, IL-12 and IFN-α promote T cell differentiation along the Th1 pathway (24, 25). Proteins were extracted from PP and ileal mucosal biopsies with no macroscopic and histologic abnormalities and analyzed using Abs which specifically recognize the functional active IL-12 heterodimer or IFN-α, respectively. Immunoreactivity for IL-12p70 was seen in 11 of 13 PP and in 3 of 13 ileal LP samples (Fig. 2, *upper blot*). In contrast, IFN-α was not seen in any sample (Fig. 2, *middle blot*).

IL-18 shares biological activities with IL-12 in driving the development of Th1 cells (26). In all PP and ileal LP samples, anti-IL-18 Ab detected a protein with a molecular size of 24 kDa (Fig. 2, *lower blot*). Because we used a polyclonal Ab reacting with an epitope corresponding to an amino acid sequence mapping at the C terminus of the human IL-18 precursor, the 24 kDa band represents the IL-18 propeptide precursor. In contrast, no band corresponding to the mature IL-18 was seen in tissue homogenates from all PP and LP samples (Fig. 2, *lower blot*). Gut epithelial cells express the 24 kDa form of IL-18 (27), so it is likely that these cells are the source of IL-18 seen in Western blots of both PP and ileal mucosa.

IL-12Rβ2 and STAT4, two essential components of IL-12-mediated Th1 differentiation, are expressed in human PP

The T cell response to IL-12 is dependent on the expression of the high affinity IL-12R composed of two subunits, IL-12Rβ1 and IL-12Rβ2 (28), with the IL-12Rβ2 chain being the critical signaling component (29–35). IL-12Rβ2 RNA was consistently detected in CD4$^+$ and CD8$^+$ PPL and LPL, but not PBL (Fig. 3).

The effect of IL-12 on Th1 cell differentiation depends specifically on rapid phosphorylation and activation of STAT4 (36, 37).

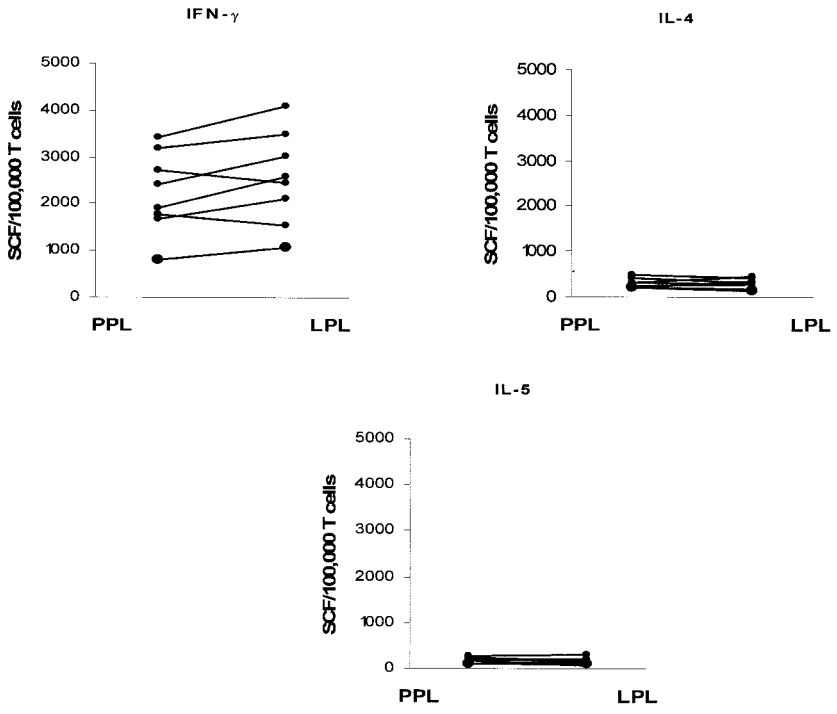

FIGURE 1. IFN-γ-, IL-4-, and IL-5-secreting cells in PP and ileal LP CD3$^+$ cells. ELISPOT assays for cytokines were performed using freshly purified CD3$^+$ PPL and LPL, and the frequency of SFC was calculated per 10^5 total viable cells. Median frequencies of IFN-γ-secreting cells were 2150 (range = 820–3420) and 2525 (range = 1060–4100)/10^5 T cells in PPL and LPL, respectively, and significantly higher than those of IL-4 (280 (range = 200–480, in PPL) and 305 (range = 140–460 in LPL)) and of IL-5 (172.5 (range = 98–270, in PPL) and 165 (range = 175–310 in LPL)) $p < 0.008$.

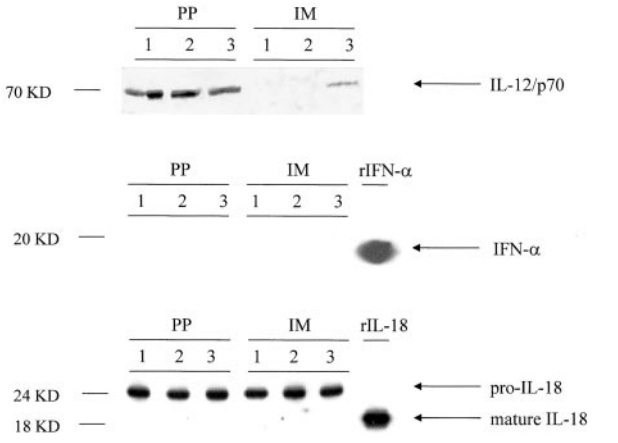

FIGURE 2. IL-12, a Th1-inducing cytokine, is detectable in human PP. *Upper blot*, Western blot analysis of IL-12 in freshly obtained PP and ileal mucosal (IM) samples. Total proteins (120 μg/sample) were separated on a 8% SDS-polyacrylamide gel under nonreducing conditions, and IL-12p70 was detected using a rabbit anti-human IL-12p70 Ab followed by a HRP-conjugated goat anti-rabbit IgG mAb. Anti-IL-12 detects a protein of ~70 kDa in three of three PP and one of three IM. One representative experiment of four independent experiments is shown, in which 13 PP and 13 IM samples were analyzed in total. *Middle blot*, Western blot analysis of IFN-α in freshly obtained PP and IM samples. Total proteins (120 μg/sample) were separated on a 15% SDS-polyacrylamide gel under reducing conditions. IFN-α was detected using a rabbit anti-human Ab followed by a HRP-conjugated goat anti-rabbit IgG Ab. No protein corresponding to the size of human recombinant IFN-α was detected in any sample. *Lower blot*, Western blot analysis of IL-18 in freshly obtained PP and IM samples. After IFN-γ detection, the membrane was stripped and subsequently incubated with a goat anti-human IL-18 Ab followed by a rabbit anti-goat Ab conjugated to HRP. Anti-IL-18 Ab detected a protein corresponding to the size of the human IL-18 precursor (pro-IL-18) in all PP and IM samples. In contrast, no protein corresponding to the size of human mature IL-18 was detected. Sizes of protein standards are given in kilodaltons. One representative experiment of four independent experiments is shown, in which 13 PP and 13 IMS were analyzed in total.

Insufficient proteins could be isolated from PP T cells for immunoprecipitation, so instead we analyzed proteins isolated from whole biopsies. As shown in Fig. 4*A*, active (phosphorylated) STAT4 was seen in all the PP and ileal LP biopsies, but not in freshly isolated human PBMC. In contrast, active STAT6, a transcription factor induced by IL-4 and associated with the development of Th2 cells (38), was weakly expressed in only two of eight PP and three of eight ileal LP biopsies (Fig. 4*B*). We next determined whether nuclear extracts from $CD3^+$ PPL and LPL contained STAT4 and STAT6 because after phosphorylation, STAT4

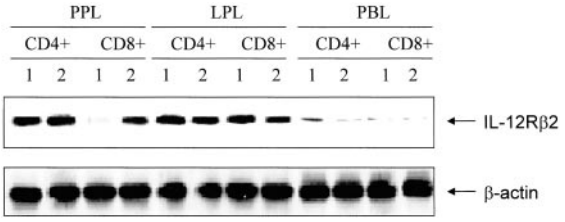

FIGURE 3. RNA transcripts for IL-12Rβ2 and β-actin in PP, ileal LP and peripheral blood (PB) $CD4^+$ and $CD8^+$ lymphocytes. cDNA (1.5 and 2 μl for β-actin and IL-12Rβ2, respectively) was incubated with specific primers for β-actin and IL-12Rβ2 for 22 and 28 cycles, respectively, and the RT-PCR products were analyzed by Southern blotting. One of two representative experiments is shown, in which PPL, LPL, and PBL purified from three patients were analyzed.

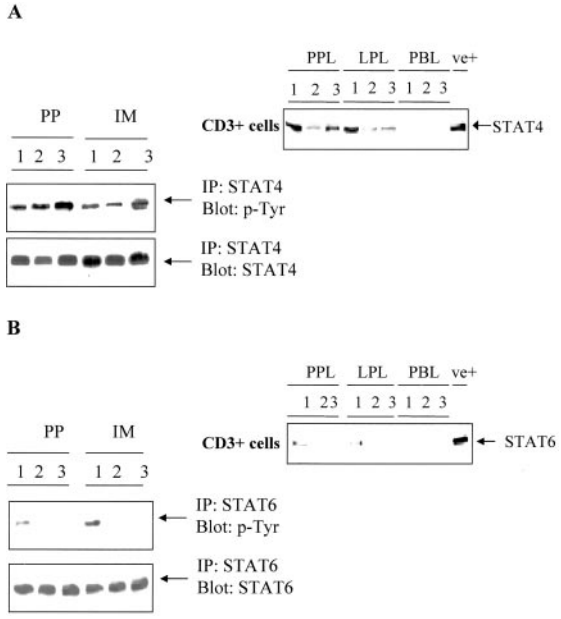

FIGURE 4. Representative expression of both active (phosphorylated) and inactive STAT4 (*A*) and STAT6 (*B*) in samples from three PP biopsies and three ileal mucosa (IM) biopsies. Total proteins were immunoprecipitated (IP) with STAT4 or STAT6 Ab, run on 8% SDS-PAGE under reducing conditions, and immunoblotted with antiphosphotyrosine Ab (p-Tyr). After detection of p-Tyr-STAT4 and STAT6 proteins, the membrane was stripped and reblotted with another STAT4 or STAT6 Ab to ascertain equivalent loading of the lanes. Phosphorylation of STAT4 is seen in all PP and IM samples, whereas active (phosphorylated) STAT6 is weakly seen in only one of three PP and one of three IM samples. The example is representative of three experiments in which eight PP and eight IM samples were analyzed in total. *Right insets*, STAT4 (*A*) and STAT6 (*B*) in nuclear proteins extracted from three PP, three ileal LP, and three peripheral blood (PB) $CD3^+$ lymphocyte samples. The example is representative of two experiments in which six PPL and six LPL and three PBL samples were analyzed. Nuclear proteins extracted from PBMC pretreated with anti-CD3 (5% final dilution) for 3 days and then stimulated with IL-12 (5 ng/ml) or IL-4 (10 ng/ml) for 30 min were used as positive controls (ve+).

migrates into the nucleus where it binds to specific DNA sequences (36). Consistent with the data on phosphorylated STAT4 in immunoprecipitates, all $CD3^+$ PPL and LPL nuclear samples (six of six) contained readily detectable STAT4 (Fig. 4*A*, *inset*), whereas STAT6 was barely detectable (Fig. 4*B*, *inset*). Furthermore, EMSA analysis demonstrated specific STAT4 binding to target DNA in both PPL and LPL (Fig. 5).

T-bet, a master switch factor for the Th1 differentiation, is expressed in human PP

Recent studies have led to the identification of a new transcription factor, termed T-bet, which is sufficient in initiating Th1 differentiation and repressing Th2 cell responses (39, 40). Therefore, we analyzed whether PPL and LPL also express T-bet. As shown in Fig. 6, all the PPL and LPL samples were positive. Furthermore, T-bet was detected in $\beta7^+$, but not $CD45RA^+$, T PBL (Fig. 6, *inset*). The anti-β7 Ab we used recognizes both $\alpha4\beta7$ and $\alpha_E\beta7$. However, since very few blood T cells express $\alpha_E\beta7$, $\beta7$ staining is a reliable indicator of $\alpha4\beta7$ expression.

Neutralization of IL-12 inhibits SEB-stimulated IFN-γ production by PP cells

A specific neutralizing Ab to IL-12p70 was used to determine whether IL-12 in human PP plays an important functional role in

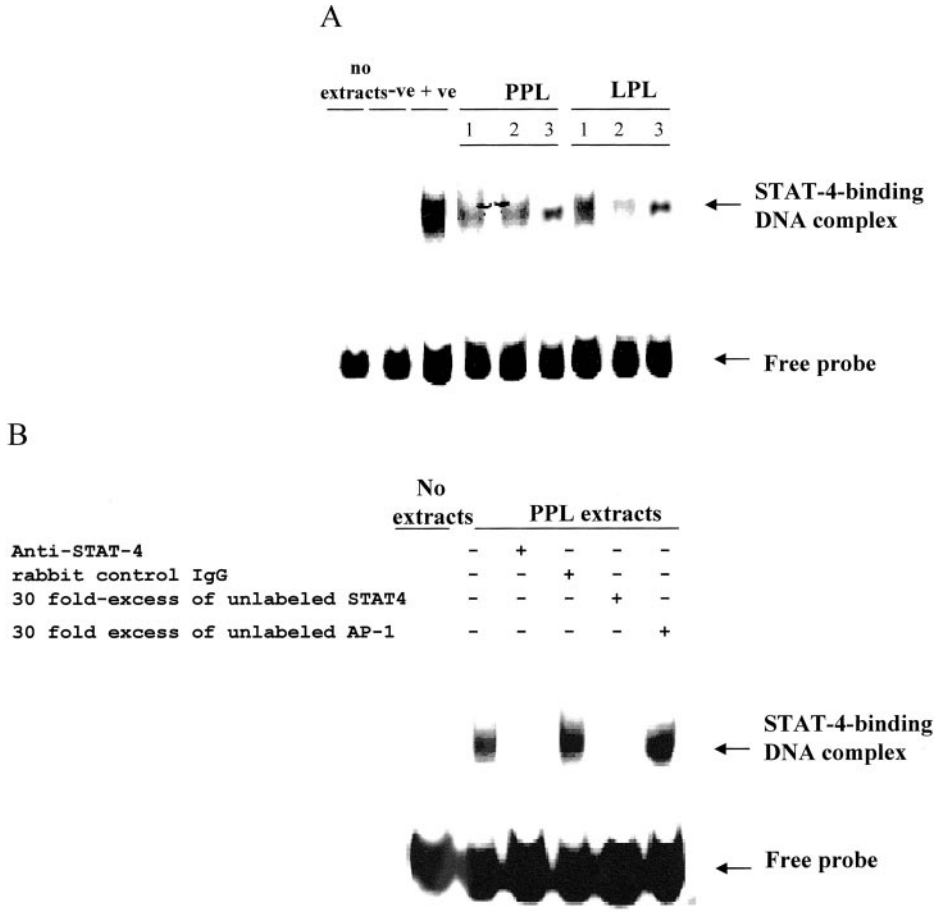

FIGURE 5. *A*, Representative EMSA blot showing STAT4-binding DNA complex in PPL and LPL. $CD3^+$ T PPL and LPL were isolated from three patients and used for extracting nuclear proteins as indicated in Materials and Methods. Protein extracted from PBMC pretreated with anti-CD3 for 3 days and then stimulated with IL-12 (5 ng/ml) for 30 min was used as a positive control (+ve). Unstimulated PBMC were used as negative control (−ve). In the first lane, no nuclear extract was loaded. STAT4-binding DNA complex is retarded, whereas unbound (free) DNA probe migrates to the bottom of the electropherogram. One of two separate experiments analyzing PPL and LPL from five patients is shown. *B*, Representative EMSA blot showing the specificity of the STAT4-binding DNA complex. Incubation of nuclear proteins, extracted from PPL, with a rabbit STAT4 Ab or excess of specific STAT4 probe, but not with a nonrelevant control Ab or excess of nonspecific competitor (AP-1), leads to inhibition of binding.

the dominant IFN-γ response. As shown in Fig. 7, IFN-γ ELISPOTs were detected in PPMC cultured overnight in medium alone (median number of ELISPOTs per 10^5 cells = 880; range = 700–1220). Stimulation of PPMC with SEB significantly increased the number of IFN-γ-secreting cells (median = 2016; range = 1560–3500, $p = 0.03$) (Fig. 7). The addition of a neutralizing IL-12 Ab to the SEB-stimulated PPMC cultures resulted in a significant inhibition in the number of IFN-γ-ELISPOTs (median = 1200; range = 720–1700, $p = 0.03$) (Fig. 7) to a value which was not different from the unstimulated cells. In contrast, no inhibition in SEB-stimulated IFN-γ induction was seen when a control rabbit IgG was used (median of IFN-γ-ELISPOTs = 1900; range = 1620–3460, not shown on the figure). Stimulation of PPMC with SEB did not result in any increase in the number of IL-4-secreting cells (mean = 220, range = 110–320 in unstimulated PPMC vs 265, range = 110–312 in SEB-stimulated PPMC) and IL-5-secreting cells (mean = 126, range = 70–195 in unstimulated PPMC vs 160, range = 50–220 in SEB-stimulated PPMC) (Fig. 7).

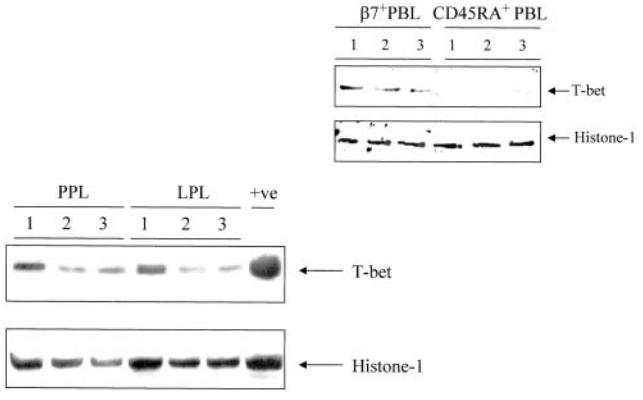

FIGURE 6. Representative expression of T-bet (*A*) and histone-1 (*B*) in nuclear proteins extracted from three PP and three ileal LP $CD3^+$ T lymphocyte samples. The example is representative of two experiments in which six PPL and six LPL purified from six patients were analyzed. An intestinal mucosal sample taken from a patient with Crohn's disease was used as positive control (+ve). The *inset* also shows a representative Western blot of T-bet (*upper panel*) and histone-1 (*lower panel*) in nuclear proteins extracted from three $\beta7^+$ PBL and three $CD45RA^+$ PBL samples, the former being used as a population of cells derived from PP homing to the mucosa and the latter as a negative control.

Because ELISPOTs do not measure total cytokine synthesis, we also used measured cytokine concentrations in the supernatants of the same cultures (Table 1). This also gave us the opportunity to measure other cytokines such as IL-2 and IL-10 which we did not study by ELISPOT. Without stimulation, PPMC released large amounts of IFN-γ (162–453 pg/ml) which increased ~10-fold (1150–8082 pg/ml) after activation with SEB. In contrast, spontaneous secretion of IL-4, IL-5, IL-10, and IL-2 were all below 5 pg/ml. IL-4, IL-5, and IL-10 secretion did not increase after SEB activation, but IL-2 increased 20-fold. Addition of anti-IL-12, but not a control IgG, dramatically inhibited SEB-induced IFN-γ production but had no effect on SEB-induced IL-2 production in the same cultures.

Discussion

We confirm that the profile of cytokines in the human intestinal mucosa is polarized toward the Th1 type. This finding is associated with expression of the functional IL-12p70 heterodimer in PP but

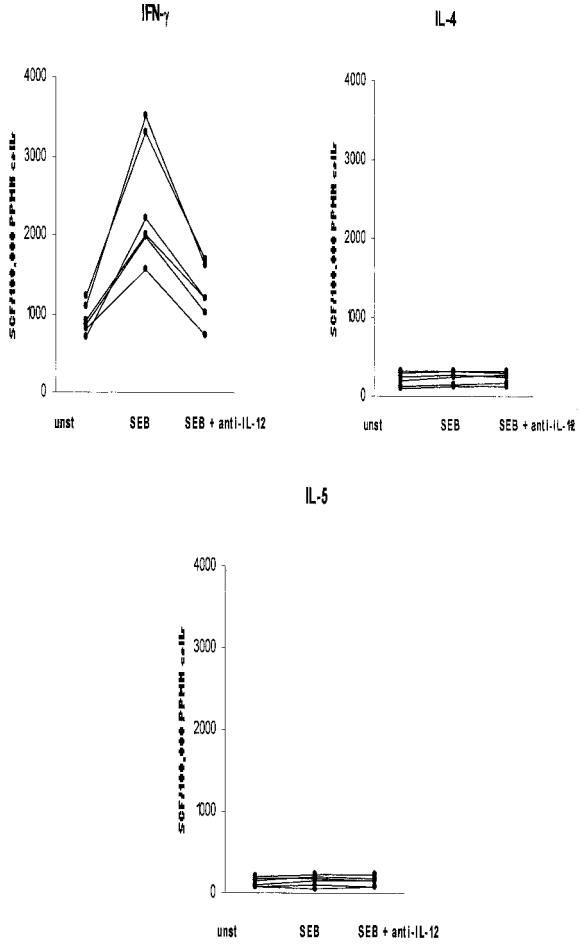

FIGURE 7. The number of IFN-γ-secreting PPMC induced by SEB is decreased by a neutralizing IL-12 Ab PPMC were preincubated in the presence or absence of a neutralizing IL-12 Ab (10 μg/ml) for 4 h, and then stimulated with 1 μg/ml SEB for 20 h. ELISPOT assays for cytokines were then performed, and the frequency of SFC was calculated per 10^5 total viable cells. Following SEB stimulation, a greater number of IFN-γ-secreting PPMC (median number of SFC per 10^5 MC = 2016; range = 1560–3500) was observed compared with the matched unstimulated PPMC (median = 880; range = 700–1220, $p = 0.03$). The addition of a neutralizing IL-12 Ab significantly reduced the number of IFN-γ-secreting cells induced by SEB (median = 1200; range = 720–1700, $p = 0.03$). In contrast, the number of both IL-4 (median number of SFC per 10^5 MC = 220; range = 110–320) and IL-5 (median number of SFC per 10^5 MC = 126; range = 70–195) was affected neither by SEB stimulation (median number of SFC per 10^5 MC = 265 and 160; range = 110–312 and 50–220 for IL-4 and IL-5, respectively), nor by incubation with the anti-IL-12 Ab (median number of SFC per 10^5 MC = 252 and 154; range = 120–312 and 70–215 for IL-4 and IL-5, respectively).

not in LP. Neither IFN-α nor active IL-18, another two cytokines known to be involved in promoting Th1 cell differentiation (25, 26), were detected in PP and LP. IL-12 is produced by APCs mostly in response to bacteria or bacterial products (24). It is likely that the abundant IL-12 in ileal PP is due to the resident bacterial flora in the ileum being transported across the M cells into the dome area and inducing IL-12 production by dendritic cells and macrophages.

IL-12 signals follow interaction of the p70 heterodimer with a specific receptor composed by two chains, IL-12Rβ1 and IL-12Rβ2 (28). The responsiveness to IL-12 of Th1 cells and the lack of responsiveness of Th2 cells correlates with the differential expression of the IL-12R chains (29–31). Th1 cells express both IL-12Rβ1 and, at high levels, the IL-12Rβ2 subunit, whereas Th2 cells express only the IL-12Rβ1 chain (30, 34, 35). Consistent with this, IL-12Rβ2 was seen in CD4 and CD8 PPL.

IL-12 binding is rapidly followed by tyrosine phosphorylation of STAT4. Active (phosphorylated) STAT4 was constitutively seen in PP biopsies. CD3 PPL also exhibited nuclear accumulation of STAT4 and STAT4 DNA binding activity was detected by EMSAS, all consistent with the notion that STAT4 is driving the PP Th1 response (41, 42). PPL also express T-bet, a recently described transcription factor present in Th1 but not Th2 cells (39). Although a recent study has shown that T-bet is capable of inducing IFN-γ through an IL-12/STAT4-independent mechanism (43), T-bet becomes optimally induced in response to IL-12-mediated STAT4 activation (44), thus establishing an autocrine positive feedback that drives polarization of the cytokine repertoire along the Th1 subtype. As expected, STAT6, a transcription factor typically expressed by Th2 cells (38), was barely detectable in PP. Despite this, we were able to detect GATA-3 in some PP samples (not shown), a factor which could trigger STAT6-independent Th2 cell differentiation (40). However, we feel that GATA-3 signaling is not a dominant pathway in human PP because production of IL-4 by PPMC was minimal.

Active STAT4 was also seen in all ileal mucosal samples, and nuclear protein preparations from $CD3^+$ LPL contained STAT4 and STAT4-DNA binding activity. High frequencies of IFN-γ-secreting cells were seen in the $CD3^+$ LPL samples, consistent with a large body of literature showing that T cells from the intestinal LP produce greater amounts of IFN-γ than other cytokines (45–48). However, these data are paradoxical because IL-12 was only rarely detected in LP biopsies. These results raise the question of what induces activation of STAT4 in LPL. It is unlikely to be IFN-α because we have previously shown that it is undetectable in the small bowel (49). LPL are derived from the PP where they are subjected to IL-12 stimulation but it is unlikely that active STAT4 will be maintained in T cells after they leave the PP and migrate to the LP, a process which takes several days. Further studies are

Table I. *Cytokine concentration (pg/ml)*[a]

	IFN-γ	IL-2	IL-4	IL-5	IL-10
CON	275 ± 76	1.4 ± 1.3	1.0 ± 0.3	0.8 ± 0.2	2.0 ± 1.2
SEB	4113 ± 1223	33.3 ± 9.1	1.8 ± 0.6	0.8 ± 0.4	3.7 ± 1.7
SEB+ anti-IL-12	885 ± 277	34.3 ± 10.7	2.4 ± 0.8	1.5 ± 0.4	3.9 ± 1.9
SEB+ control IgG	2380 ± 401	26.5 ± 14.0	0.9 ± 0.4	1.3 ± 0.6	3.0 ± 2.8

[a] Analysis of cytokines in the culture supernatants confirms that anti-IL-12 specifically inhibits SEB-induced IFN-γ production. Following activation with SEB, there was marked increase in IFN-γ ($p < 0.03$ compared to unstimulated cultures), which was inhibited by anti-IL-12 (885 pg/ml $p < 0.05$ compared to SEB-stimulated cells, and which was not different from control cultures). IL-4, IL-5, and IL-10 production was not elicited by SEB. IL-2 production was enhanced by SEB ($p < 0.005$ compared to control cultures), but this was not inhibited by anti-IL-12. Results are the average of data from six patients and are shown as mean ± 1 SE. The lower limit of sensitivity of the IL-4, IL-5, and IL-10 cytokine assays was 1 pg/ml. The mean values shown as <1 are because in a group of six samples, some were zero.

needed to determine whether LPL can continue to express active STAT4 when cultured on their own in vitro, which will establish whether STAT4 is being activated in LPL by exogenous factors or is indeed a remnant of IL-12 stimulation in PP.

As expected from the role of IL-12 in the differentiation of Th1 cells and induction of IFN-γ, the addition of a neutralizing IL-12 Ab to the PPMC cultures resulted in a significant decrease in the number of SEB-stimulated IFN-γ-secreting cells. The mechanism by which anti-IL-12 mediated this effect was not investigated in the present study. However, it is possible that the neutralization of IL-12 inhibits IFN-γ gene activation and/or enhances Th1 cell apoptosis (24, 50). Another possibility is that the anti-IL-12 Ab negatively regulates the expression of T-bet. Finally, it is also conceivable that in human PP, as reported in rodents, there is a direct and inverse relationship between the IL-12-dependent and TGF-β1-mediated response. In particular, anti-IL-12 treatment may associate with augmented TGF-β1 production which eventually leads to the suppression of IFN-γ-secreting cell development (15–17, 51). Studies aimed at investigating these mechanisms are now in progress.

The observation that the human PP response to lumenal Ags develops along the Th1 pathway could have important implications. The first is that human PP may be a site for induction of potentially harmful Th1 cells. Indeed, these cells, originating into the PP, traffic to and then populate the gut LP. However, these cells clearly do not normally cause disease perhaps because of their fate in the LP. First, the amount of costimulatory signals delivered at the time Ag re-exposure strongly influences the fate of Th1 cells retained in the gut. Ag-specific memory Th1 cells, arriving from the PP, would be capable of recognizing low amounts of intestinal Ags presented on LP APC but would be triggered only when high expression of costimulatory molecules were present (52). Secondly, in the LP, the PP-primed Th1 cells undergo apoptosis, as a consequence of their expression of Fas and low levels of the Bcl2 (53, 54). Finally, locally induced suppressive molecules (i.e. TGF-β, IL-10, PGE_2) counterregulate the inflammatory effects of Th1 cells (52, 55). However, if the PP Th1 cells are initially activated by peptides from pathogens, after migrating within the gut LP, they can drive a local protective cell-mediated immune response if peptides from the pathogen are presented again to the T cells in the LP (56).

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