American Journal of Pathology, Vol. 151, No. 2, August 1997

Intraepithelial Lymphocytes in Normal Human Intestine Do Not Express Proteins Associated with Cytolytic Function

Andreas Chott,*† Dirk Gerdes,* Amy Spooner,* Isabella Mosberger,† J. Alain Kummer,‡ Ellen C. Ebert,§ Richard S. Blumberg,‖ and Steven P. Balk*

From the Cancer Biology Program, Hematology-Oncology Division, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston Massachusetts; the Department of Clinical Pathology,† University of Vienna, Vienna, Austria; the Department of Pathology,‡ Free University Hospital, Amsterdam, The Netherlands; the Department of Medicine,§ Robert Wood Johnson Medical School, New Brunswick, New Jersey; and the Gastroenterology Division,‖ Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts*

Human small intestine contains a very large population of intraepithelial T lymphocytes (IELs) that are oligoclonal, appear functionally to be cytolytic T cells, and may contribute to the normal and pathological turnover of intestinal epithelial cells. This report addresses the cytolytic function of IELs in normal small intestine by examining their expression of molecules that carry out cell-mediated cytolysis. Immunohistochemical analyses of granzyme B, perforin, Fas ligand, and tumor necrosis factor-α demonstrated these proteins were not expressed by small intestinal IELs *in situ*. These proteins also were not expressed by colonic IELs or by lamina propria lymphocytes in the small or large intestine. Granzyme A, however, was expressed by a large fraction of IELs. In contrast to these *in situ* results, isolated and *in vitro* activated IELs were shown to express effector proteins consistent with cytolytic T cells, including granzyme B, Fas ligand, tumor necrosis factor-α, and interferon-γ. These results are most consistent with the vast majority of IELs in normal human small intestine being resting cytolytic T cells and suggest that these cells do not contribute to the apoptotic cell death of epithelial cells in normal intestine. *(Am J Pathol 1997, 151:435–442)*

The intestinal mucosa contains two populations of anatomically distinct lymphocytes, intraepithelial lymphocytes (IELs), and lamina propria lymphocytes (LPLs).[1–3] The human small intestine contains a very large number of IELs, estimated to be approximately 1 IEL for every 6 to 10 epithelial cells.[4] The majority (>90%) of these small intestinal IELs express CD8 and the αβ T cell antigen receptor (TCR).[2,5–7] $CD8^+$ αβ T cells are also the major population in human colon, but the number of IELs in the colon is much less than in the small intestine, and other T cell subsets are more represented.[8,9] Intestinal IELs express a number of markers indicative of previous or current activation, including CD45RO, CD69, and the $\alpha^E\beta_7$ integrin.[5,9,10] TCR analyses have further shown that the majority of human intestinal IELs are derived from the expansion of a small number of clones.[11–15] These observations indicate that IELs are responding to a small number of intestinal antigens, but the identity of these antigens and the effector functions mediated by these IELs in response to antigen remain to be determined.

Intestinal epithelial cells are noteworthy for their extremely high rate of turnover in normal intestine, undergoing apoptotic cell death several days after their generation.[16,17] This process is accelerated in a number of pathological conditions, some of which may involve T cell activation in the intestinal mucosa.[16,18–20] The mechanisms that regulate epithelial cell turnover in normal intestine are poorly understood. In particular, whether the very large number of IELs in small intestine participate in the regulation of normal epithelial cell turnover by apoptosis is unclear.

Functional studies have shown that IELs from human intestine proliferate poorly in response to TCR-mediated signals but do proliferate in response to stimulation by CD2 antibodies[21–23] or interleukin (IL)-7.[24] These observations suggest that the majority of intestinal IELs may be activated *in situ*. Several lines of evidence indicate that murine intestinal IELs are activated cytolytic T cells, including their large granular lymphocyte morphology and cytolytic activity in redirected lysis assays.[25–29] In contrast, most human intestinal IELs do not have large cytotoxic granules and appear to have only weak cytolytic activity when freshly isolated.[30,31] Intestinal IELs do ex-

Supported by National Institutes of Health grants AI33911 and DK44319 to S. P. Balk and R. S. Blumberg and DK42166 to E. C. Ebert. A. Chott was supported by Schrodinger Grant J935. D. Gerdes was supported by a research fellowship from the Crohns and Colitis Foundation of America.

Accepted for publication April 22, 1997.

Address reprint requests to Dr. Steven Balk, Hematology-Oncology Division, HIM Building Room 1047, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215.

press TIA-1, which may be associated with cytotoxic granules, but expression of this protein is not a marker of activated cytolytic T cells.[31] Moreover, recent data indicate that these cells *in situ* in normal intestine do not express granzyme B, a component of the cytotoxic granules that is clearly a marker of cytolytic activity.[32] Therefore, whether human intestinal IELs are active cytolytic T cells *in situ* in normal intestine remains to be determined.

Perforin and granzyme B are stored in T cell cytotoxic granules and are the primary proteins mediating cytolysis by cytolytic T cells.[33–36] Studies using perforin-deficient mice have uncovered a second cytolytic pathway mediated by Fas ligand expressed on activated T cells binding to Fas,[37–39] which is expressed constitutively on many epithelial cells, including those in human intestine.[40] In contrast to the perforin/granzyme B cytotoxic granule-based system, the Fas ligand system can be rapidly induced and results in apoptosis of target cells. Finally, tumor necrosis factor (TNF)-α expressed by activated T cells may also mediate apoptosis of target cells expressing TNF receptors.[36,41]

This report addresses the biological function of IELs in normal small intestine by examining their expression of molecules that carry out cell-mediated cytolysis. Isolated small intestinal IELs were found to express granzyme B, Fas ligand, and TNF-α. However, immunohistochemical studies demonstrated that none of these proteins, or perforin, were expressed by intestinal IELs *in situ*. These results indicate that the vast majority of intestinal IELs are not actively cytolytic in normal intestine and, therefore, are unlikely to participate in the normal apoptotic cell death of epithelial cells. They also suggest that the weak cytolytic activity of isolated human IELs may reflect *in vitro* activation.

Materials and Methods

Isolation of IELs and LPLs

Strips of grossly normal small intestine were obtained from surgical resections being performed for gastric bypass (jejunum) or pancreatic cancer. Colonic mucosa was obtained from normal margins in patients being resected for colon cancer. IELs and LPLs were isolated as described previously.[30,42] In brief, dissected mucosa was incubated for 20 minutes at 37°C in calcium- and magnesium-free Hanks' balanced salt solution containing 1 mmol/L dithiothreitol and 2% fetal calf serum (FCS). The IELs were then released by three 40-minute washes in Hanks' balanced salt solution containing 2% FCS and 1 mmol/L EDTA and pooled. The tissue was then incubated overnight at 37°C in 40 ml of RPMI-1640 containing 5.0 mg of collagenase D, 5.0 mg of trypsin inhibitor, and 0.01% deoxyribonuclease (Worthington Scientific, Malvern, PA) to release LPLs. Large fragments from both the IEL and LPL fractions were removed using a 100-μm mesh screen, and a 30%/44%/66% Percoll density gradient was used to separate lymphocytes from contaminating epithelial cells.

Peripheral blood mononuclear cells were obtained from normal donors by Ficoll-Hypaque centrifugation. Activated peripheral blood lymphocytes (PBLs) were generated by culturing freshly isolated peripheral blood mononuclear cells with phytohemagglutinin (PHA; 1 μg/ml) and IL-2 (10 U/ml) for 3 days in RPMI-1640 with 10% FCS. Purified small intestinal IELs and PHA-activated PBLs were formalin fixed and embedded in 0.5% agarose, and the agarose pellets were embedded in paraffin for immunohistochemical studies.

Tissue Samples

For routine microscopic examinations, formalin-fixed (7.5%, pH 7.4), paraffin-embedded tissues were stained with hematoxylin and eosin (H&E) and according to Giemsa. Samples from small bowel were taken from the distal resection margins of gastro-duodeno-pancreatectomies (Whipple procedures; n = 10) and from proximal margins of ileocolectomies for cecal adenoma or carcinoma (n = 5). Colonic samples were taken from distal margins of the ileocolectomies. Frozen sections were from small intestine (n = 5) or large intestine (n = 3). All samples studied contained microscopically normal mucosa without evidence of neoplasia or inflammation. In particular, the number of IELs in the small intestinal samples was in the normal range (9 to 39 IELs/100 enterocytes).[4]

Immunohistochemical Analysis

Immunostaining with the polyclonal anti-CD3 (Dako, Copenhagen, Denmark; 1:400), anti-Fas ligand (N-20, Santa Cruz Biotechnology, Santa Cruz, CA; 1:160), and TNF-α antibodies (Genzyme, Cambridge, MA; 1:200) and the monoclonal granzyme A and B antibodies (GrA-8 and GrB-4, respectively)[43] was done on the formalin-fixed, paraffin-embedded tissue. Staining with the monoclonal perforin antibody (T-Cell Diagnostics, Cambridge, MA; 1:100) was done on frozen sections after fixation in acetone for 10 minutes. Pretreatment for unmasking of antigens was done either by digestion with 0.05% protease (type XXIV, Sigma Chemical Co., St. Louis, MO) in Tris-buffered saline for 5 minutes at 37°C (for CD3 and TNF-α) or by microwaving in citrate buffer (10 mmol/L, pH 6.0) twice for 5 minutes each at 600 W (for Fas ligand, GrA-8, and GrB-4).

Endogenous peroxidase was blocked by incubation in 1% H_2O_2 in Tris-buffered saline or by incubation in a solution containing glucose (50 mg/ml) and glucose oxidase (Sigma). Application of the polyclonal antibodies was followed by incubation with biotinylated goat anti-rabbit Ig as a secondary antibody and then peroxidase-conjugated streptavidin (Super Sensitive HRP Label, Biogenex, San Ramon, CA). Staining was developed using 3-amino-9-ethylcarbazole as the chromogen (Sigma) in the presence of H_2O_2. The monoclonal antibody reactions used biotinylated horse anti-mouse IgG as the secondary antibody followed by Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA) and 3,3′-

diaminobenzidine as a chromogen (Fluka, Buchs, Switzerland) in the presence of H_2O_2. Nonspecific reactivity was assessed by omission of the primary antibodies.

For quantitation of IEL staining, serial sections were cut from each block. Sequential sections were then stained with anti-CD3 and GrB-4, GrA-8, Fas ligand, TNF-α, or perforin (the latter only on the frozen sections). The fraction of positive cells was then based upon the number of $CD3^+$ cells in the adjacent section.

Preparation of Anti-Fas Ligand Antiserum and Immunoprecipitation

Human Fas ligand cDNA was isolated by polymerase chain reaction (PCR) amplification based upon the published sequence.[44] A glutathione-S-transferase (GST)-Fas ligand fusion protein was made that contained the ectodomain of Fas ligand (codons 115 to 281). Antisera were generated in rabbits and tested for reactivity to the fusion protein by Western blotting. No reactivity was detected against TNF-α by enzyme-linked immunosorbent assay (ELISA).

For immunoprecipitations, freshly isolated cells (1×10^7)were surface labeled with sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin, Pierce Chemicals, Rockford, IL) using 0.5 mg/ml in 25 mmol/L HEPES, pH 8.0, 0.14 mol/L NaCl for 30 minutes at 4°C. After washing, cells were lysed in Tris-buffered saline containing 1% Triton X-100 and protease inhibitors. Lysates were then cleared by two to three incubations with nonimmune rabbit serum bound to protein A-Sepharose beads. They were then incubated with the anti-Fas ligand antiserum bound to protein A-Sepharose, eluted in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose, blotted with a Streptavidin-horseradish peroxidase conjugate and developed by ECL (Amersham, Arlington Heights, IL).

Analysis of Cytokine Expression

RNA was extracted using RNAzol-B (TM Cinna Scientific, Friendswood, TX), and cDNA was synthesized using oligo-dT and MMTV reverse transcriptase (New England Biolabs, Beverly, MA). cDNAs were PCR amplified with Fas ligand primers (sense, 5′-GATGTTTCAGCTCTTCCACCTAC; antisense, 5′-CAACATTCTCGGTGCCTGTAAC) in exons 1 and 4, respectively. PCR amplification for expression of other mediators was done using primers for IL-1α, IL-2, IL-4, IL-5, IL-7, IL-10, IL-13, interferon (IFN)-γ, macrophage colony-stimulating factor (M-CSF), TNF-α, and granulocyte/macrophage (GM)-CSF from Clontech (Palo Alto, CA). Additional primers for keratinocyte growth factor (KGF) were synthesized (sense, 5′-CCTGCCAACTTTGCTCTACAGAT; antisense, 5′-CCTCCGTTGTGTGTCCATTTAGCT). For ELISAs, cells were cultured at 1×10^6/ml without further stimulation or with PHA (1 μg/ml), plate-bound anti-CD3ϵ (Immunotech, Westbrook, ME), or IL-2 (10 U/ml). TNF-α and IFN-γ were measured after 1 to 3 days by ELISA (Endogen, Cambridge, MA).

Results

Immunohistochemical Analysis of Granzyme A, Granzyme B, and Perforin Expression

Previous data have shown that isolated human small intestinal IELs have cytolytic activity.[30,31] IEL expression of granzyme B, a component of the T-cell granule-based cytolytic pathway, was assessed by immunohistochemistry to determine whether this pathway may contribute to cytolytic activity. Using purified small intestinal IELs, weak staining with the granzyme B monoclonal antibody was observed in approximately 15% of the cells. Figure 1A shows a field containing one of these weakly stained cells. In contrast, intense granzyme B staining was seen in a large fraction (approximately 30%) of PHA-activated PBLs (Figure 1B).

Although granzyme B was detected in isolated IELs, a recent report indicated that it was not expressed by IELs *in situ*.[32] Therefore, subsequent studies were carried out in tissue sections from normal intestine rather than purified cells. Sections were analyzed for granzyme B expression to determine whether the weak staining by a minority of isolated IELs could be detected *in situ*. A representative section of small intestinal mucosa, examined initially by immunohistochemistry with an anti-CD3 antiserum, is shown in Figure 1C. Specific staining of numerous T cells could be clearly visualized in the epithelium and lamina propria. In contrast, staining for granzyme B revealed only rare positive cells in the epithelium or lamina propria. A field containing a rare granzyme-B-positive cell in the lamina propria is shown in Figure 1D. Quantitative comparisons between $CD3^+$ *versus* granzyme-B-positive cells in normal small intestine from a series of 10 cases demonstrated that <2% of IELs expressed detectable levels of granzyme B. Granzyme-B-positive cells were similarly rare or absent from normal colon in a series of five cases.

Additional frozen sections from normal small intestine (five cases) were analyzed with an anti-perforin monoclonal antibody. Similar to the granzyme B results, only rare positive cells were seen. Figure 1E shows a field containing one of these positive cells in the lamina propria. Quantitatively, a comparison of CD3- *versus* perforin-positive cells indicated that, similar to granzyme B, <2% of T cells expressed perforin. Perforin-positive cells were also rare or absent in frozen sections from normal colon (three cases). The rare perforin-positive cells and granzyme-B-positive cells above had the morphology of lymphocytes, consistent with their being T cells or NK cells.

Interestingly, staining for granzyme A revealed that approximately 30% of IELs were positive. The proportion of positive cells and the staining intensity varied along the crypt to villous axis. The lower and intermediate villous regions contained approximately 10 to 15% weakly granzyme-A-positive IELs, whereas between 30 and 60% of

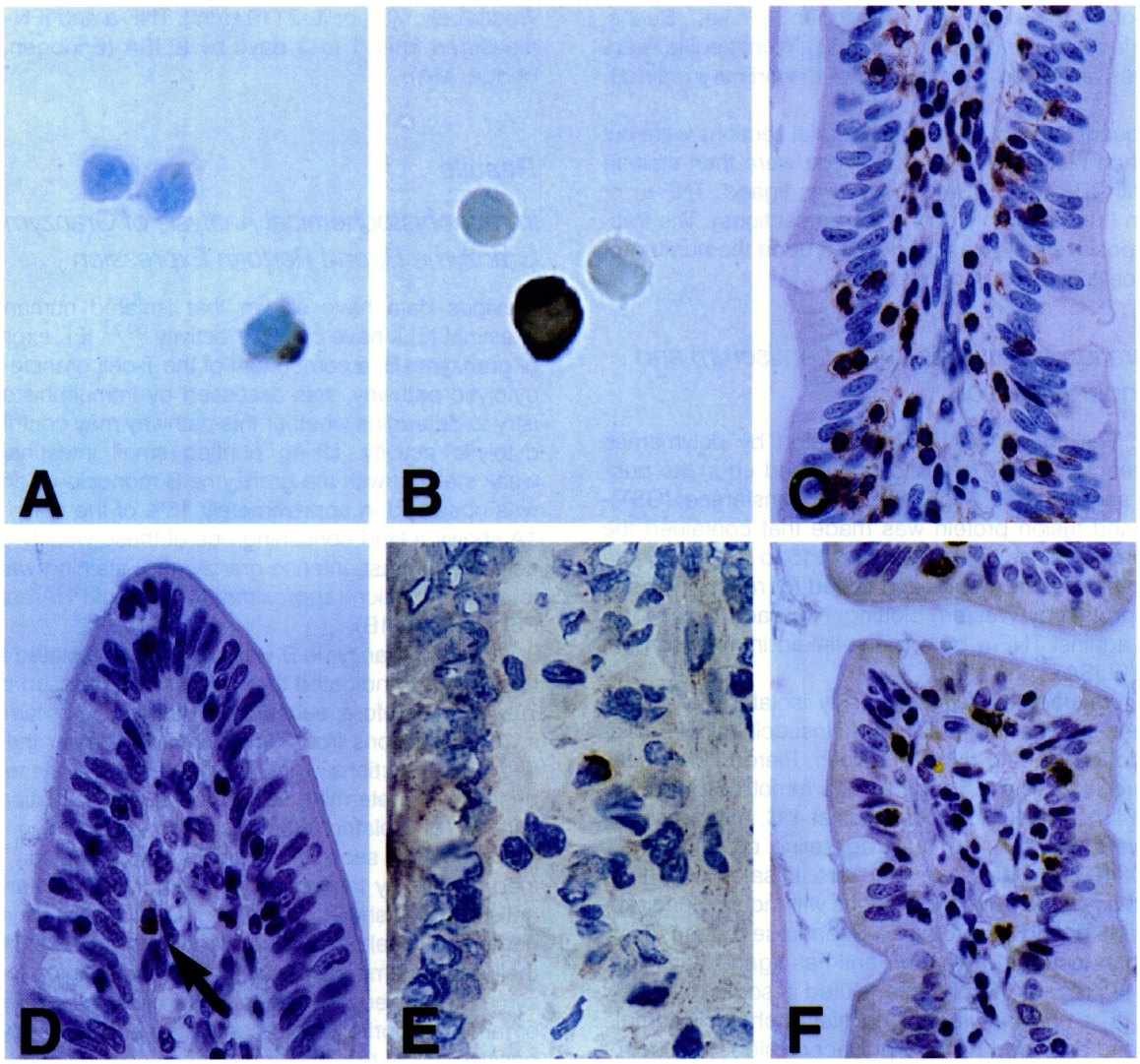

Figure 1. Immunohistochemical analyses of granzyme and perforin expression. Formalin-fixed, purified cells or tissue sections were stained with granzyme-B-, CD3-, perforin-, or granzyme-A-specific antibodies. **A:** Isolated small intestinal IEL preparation stained with granzyme B antibody showing a field with a weakly positive cell. **B:** PHA-activated PBLs stained with granzyme B antibody showing strong staining by a representative positive cell and negative cells in the same field as an internal control. **C:** Section of small intestine stained with CD3 antibody showing staining by numerous IELs at the basolateral surface of enterocytes. **D:** Section of small intestine stained with granzyme B antibody showing a rare positive LPL. **E:** Section of small intestine stained with perforin antibody showing a rare positive LPL. **F:** Section of small intestine stained with granzyme A antibody showing several positive IELs.

IELs in the upper regions close to the villous tips displayed moderate to strong immunoreactivity (Figure 1F). Moreover, the most pronounced granzyme A staining was found in upper villous regions of single circumscribed areas of 1 to 3 mm in diameter in four samples (two jejunal and two ileal samples). This localized increase of granzyme A expression was not accompanied by a dramatic increase in granzyme B reactivity, based upon analyses of serial sections. However, it should be noted that the rare granzyme-B- and perforin-positive IELs were most commonly found in the upper villous regions (not shown).

Fas Ligand Expression by IELs

A second pathway through which T cells may mediate cytotoxicity is by Fas ligand expression. Fas ligand message was detectable by PCR amplification in each of four small-intestinal IEL preparations (data not shown). Immunoprecipitations were carried out to determine whether Fas ligand protein was expressed by isolated IELs. IELs and LPLs isolated from normal small intestine were surface biotinylated and immunoprecipitated with nonimmune rabbit antiserum or a rabbit antiserum generated against a Fas ligand-GST fusion protein. A specific band of approximately 36 kd, consistent with the size of glycosylated Fas ligand, was observed in the lysates from the IELs and from activated PBLs but not from the LPLs (Figure 2). This result was consistent with the PCR analysis above and indicated that Fas ligand may be expressed at higher levels by isolated IELs *versus* LPLs.

Although Fas ligand was expressed by isolated small-intestinal IELs, this protein can be rapidly induced by T cell activation *in vitro*.[45] Therefore, immunohistochemistry was used to determine whether Fas ligand was expressed by small-intestinal IELs *in situ*. Figure 3A shows

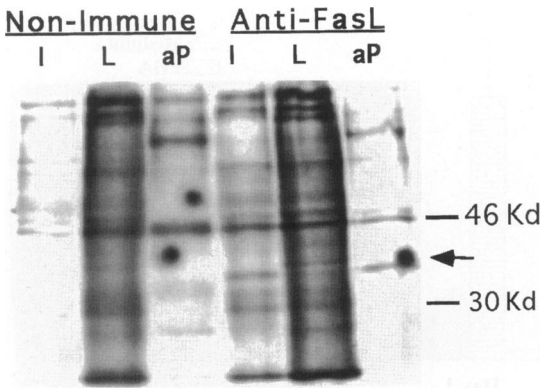

Figure 2. Immunoprecipitation of Fas ligand from freshly isolated small intestinal IELs. Isolated IELs (I) and LPLs (L) from the same sample of normal small intestine and PHA-activated PBLs (aP) were analyzed. Cells were surface biotinylated, lysed, and immunoprecipitated with nonimmune rabbit antiserum (Non-immune) or an anti-Fas ligand antiserum (Anti-FasL) made in rabbits against a Fas ligand-GST fusion protein. Proteins were visualized using horseradish-peroxidase-conjugated streptavidin and ECL.

the expression of Fas ligand by several mononuclear cells in the lamina propria, most of which morphologically appear to be macrophages or dendritic cells. However, in contrast to the results *in vitro*, these studies failed to detect Fas ligand expression by small intestinal or colonic IELs. Faint staining of some lymphoid cells in basally located lymphoid aggregates was seen, and low to moderate granular staining was seen in Paneth cells (not shown). Paneth cells were recently reported to express Fas ligand message based upon *in situ* hybridization.[46]

Expression of Other Effector Proteins by Isolated Intestinal IELs

The expression of additional effector molecules by intestinal IELs was examined. As an initial screen, intestinal IEL cDNA (four from normal small intestine and one from normal colon) were amplified for 40 cycles with primers specific for IL-1α, IL-2, IL-3, IL-4, IL-5, IL-7, IL-10, IL-13, TNF-α, IFN-γ, GM-CSF, M-CSF, and KGF. No transcripts encoding IL-3, IL-4, IL-5, IL-7, or IL-13 were detected in any of the five cDNA samples examined (data not shown). KGF was detected only in an IEL sample from normal colon and was presumably derived from TCR-$\gamma\delta$ cells, which are more abundant in the colon and have been shown to produce KGF.[47]

TNF-α and IFN-γ expression by isolated IELs and LPLs from normal small intestine was further assessed by ELISA. In the absence of added stimuli, TNF-α was produced at low levels. PHA stimulated the rapid production of TNF-α from IELs and substantially higher levels from LPLs (Figure 4, A and B). Anti-CD3 and IL-2 were significantly less effective than PHA at stimulating TNF-α production. IFN-γ production was also stimulated by PHA in both the IELs (Figure 4C) and LPLs (Figure 4D), with anti-CD3 stimulation again being less effective. In the absence of added stimuli, there was no detectable IFN-γ produced by the IELs, whereas low-level production by the LPLs was detectable.

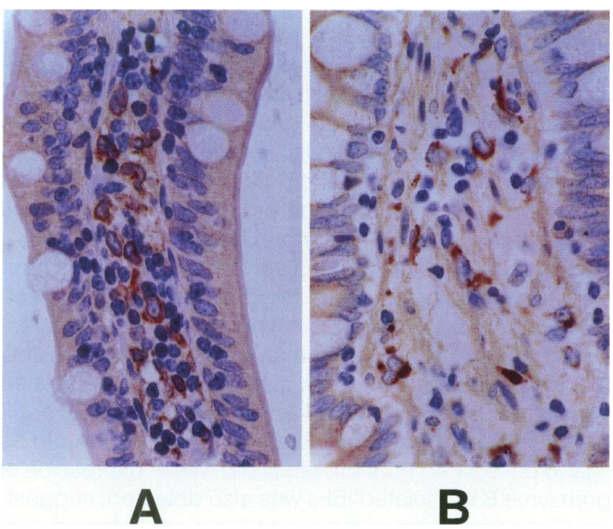

Figure 3. Immunohistochemical analyses of Fas ligand and TNF-α expression. Sections of normal small intestine were stained with Fas ligand (**A**) or TNF-α (**B**) antibodies. In **A**, Fas-ligand-positive cells are seen in the lamina propria, which morphologically represents macrophages and potentially dendritic cells. No IEL or LPL staining is detected. In **B**, several TNF-α-positive mononuclear cells are seen in the lamina propria, but no IEL staining is detected.

TNF-α Expression by Small Intestinal IELs in Situ

TNF-α is the final cytolytic pathway mediator, in addition to perforin/granzyme B and Fas ligand, through which T cells are known to mediate cytolysis of specific target cells.[36,41] The studies above demonstrated that TNF-α was expressed at low levels by isolated intestinal IELs and that production of TNF-α by these cells could be rapidly induced with activation. To determine whether TNF-α was expressed by intestinal IELs *in situ*, possibly at high levels by a subset of activated IELs, a series of immunohistochemical analyses were carried out on normal intestine.

In three samples (one from jejunum and two from ileum), strong staining for TNF-α was found in mast cells, macrophages, and fibroblasts (Figure 3B), and moderate to light staining was observed in granulocytes and Paneth cells. Interestingly, germinal centers of Peyer's patches showed a TNF-α-positive network that appeared to represent follicular dendritic cells processes (not shown). In the remaining 12 samples (7 from normal small intestine and 5 from normal colon), only mast cells showed consistent strong TNF-α expression whereas the other cell types mentioned above showed weak or no staining. No immunoreactivity was seen in IELs or other lymphoid cells in any of the specimens examined. These results indicated that TNF-α, similar to the other known mediators of cytolysis examined, was not expressed by IELs *in situ* in normal intestine.

Discussion

The human small-intestinal epithelium contains a very large population of $CD8^+$ TCR-$\alpha\beta$ IELs that express sev-

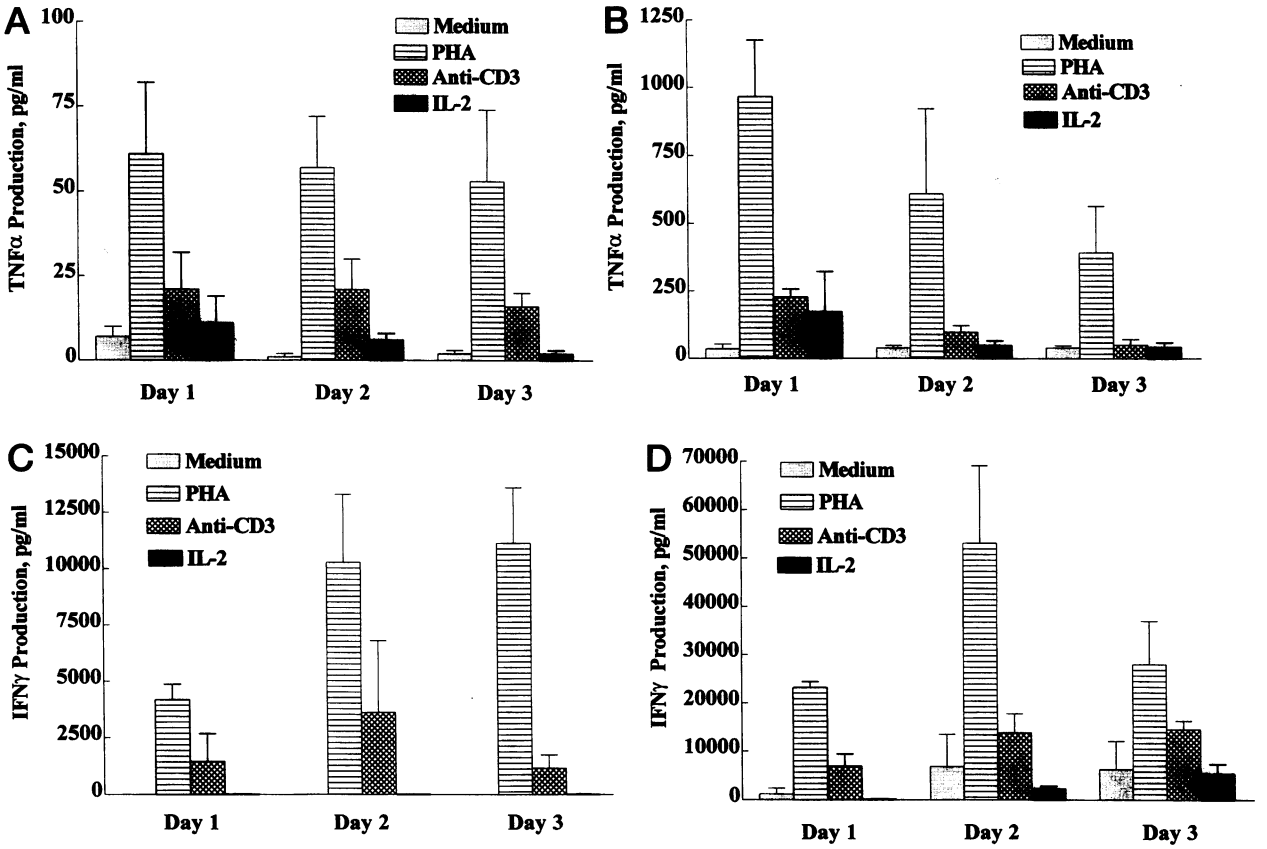

Figure 4. Expression of TNF-α and IFN-γ protein by ELISA. Freshly isolated IELs (A and C) and LPLs (B and D) from normal jejunum were cultured without stimulation or with PHA (1 μg/ml), plate-bound anti-CD3ε, or IL-2 (10 U/ml). TNF-α (A and B) or IFN-γ (C and D) were then measured after 1 to 3 days by ELISA.

eral markers indicative of activation. A substantial fraction of IELs in normal murine intestine are activated cytolytic T cells,[25–29] and weak spontaneous cytolytic activity by freshly isolated human small-intestinal IELs has been demonstrated.[30,31] These observations suggest that IELs in normal human small intestine may also be activated cytolytic T cells, possibly contributing to the normal rapid turnover of the epithelium. However, this report demonstrates that the vast majority of IELs in normal human small intestine and colon do not express any of the proteins known to mediate cytolysis by T cells.

Consistent with a previous report, *in situ* immunohistochemistry showed that cells expressing granzyme B were rare in the epithelium and lamina propria of normal small intestine and colon.[32] Absence of the granule-mediated cytolytic system was confirmed by an analysis of perforin expression. Similar to granzyme B, perforin-expressing cells were rare in the epithelium and lamina propria of the small and large intestine. These results demonstrate that, in contrast to murine intestine, the vast majority of IELs are not activated cytolytic T cells in normal human intestine.

T cells may also mediate cytolysis through Fas ligand or TNF-α pathways. Fas ligand message expression was reported previously in the rat small intestine by Northern blot analysis[48] and recently in human Paneth cells by *in situ* hybridization.[46] A recent study using purified intestinal cells suggested that Fas ligand protein may be expressed by human LPLs.[49] In the current study, Fas ligand message and protein were detected in isolated small-intestinal IELs. However, immunohistochemical analyses of Fas ligand in normal intestine demonstrated protein in macrophages, dendritic cells, and Paneth cells but not in IELs or LPLs. This result suggests that the Fas ligand expression observed with freshly isolated small-intestinal IELs may reflect *in vitro* activation. TNF-α expression by intestinal IELs and LPLs *in situ* was also undetectable. Consistent with previous reports, TNF-α was detected *in situ* in Paneth cells and in a number of other cells types in the lamina propria.[50–52] These *in situ* analyses of granzyme B, perforin, Fas ligand, and TNF-α demonstrate that the vast majority of intestinal IELs (and LPLs) do not express any of the proteins known to mediate cytolysis by T cells, indicating that IELs are not actively cytolytic in normal human intestine. It is possible that previous reports of weak cytolytic activity by isolated intestinal IELs[30,31] reflected *in vitro* activation of Fas ligand expression, as suggested by the detection of Fas ligand on isolated IELs but not *in situ*. Weak expression of granzyme B by isolated IELs was also detected, suggesting that early *in vitro* activation of the granule-based system may contribute to cytolysis.

Although the immunohistochemical analyses *in situ* were negative for granzyme B and perforin, significant staining for granzyme A was detected. The biological function of granzyme A is not clear. The roles of perforin

and granzyme B in cytolysis have been shown directly using mice with targeted deletions in these genes,[33–35] whereas lack of granzyme A does not affect the cytolytic potential of T cells.[53] A recent report suggests the interesting possibility that granzyme A may play a role in clearing infections with viruses that can inactivate granzyme B.[54] Whether granzyme A expression by intestinal IELs *in situ* reflects this or another effector function remains to be determined.

Although the vast majority of IELs do not appear to be actively cytolytic in normal intestine, several lines of evidence indicate that they do function as cytolytic T cells when activated. In this report, the spectrum of transcripts detected by PCR amplification in isolated IELs was indicative of cytolytic T cells, as was the production of TNF-α and IFN-γ after PHA activation *in vitro*. These data are consistent with observations made in celiac disease, where a marked increase of activated small-intestinal IELs expressing granzyme B was demonstrated.[32] A recent report analyzing freshly isolated and *in vitro* activated intestinal IELs also found a cytokine profile consistent with cytolytic T cells.[55] Interestingly, this latter report examined IFN-γ expression by immunohistochemistry and found approximately 8% of jejunal IELs to be positive *in situ*.[55] In contrast, in the current study, IFN-γ protein expression by freshly isolated jejunal IELs was undetectable, although IFN-γ message could be detected by PCR.

The majority of small-intestinal IELs are $CD8^+$, TCR-$\alpha\beta^+$ T cells derived from the expansion of a relatively small number of T cell clones,[11–15] but the target antigens recognized by these cells are presently unknown. This limited TCR repertoire and resting cytolytic T cell phenotype are most consistent with one of two functions for the majority of human small-intestinal IELs, although additional novel functions are certainly possible. First, they may be conventional cytolytic T cells specific for one or a very small number of related and very prevalent intracellular pathogens. Alternatively, they may be specific for cellular antigens the expression of which on intestinal epithelial cells is induced by certain types of cellular damage. In either case, given the normal short life span of intestinal epithelial cells, it seems most likely that IELs would target abnormalities that prolong the life span of the epithelial cells. Finally, the very low frequency of IELs expressing cytolytic effector proteins *in situ* in normal intestine suggests that the clonal expansion of these cells may occur through activation in response to multiple acute insults to the intestinal epithelium over time rather than more chronic stimuli.

References

1. Selby WS, Janossy G, Jewell DP: Immunohistological characterisation of intraepithelial lymphocytes of the human gastrointestinal tract. Gut 1981, 22:169–176
2. Cerf-Bensussan N, Schneeberger EE, Bhan AK: Immunohistologic and immunoelectron microscopic characterization of the mucosal lymphocytes of human small intestine by the use of monoclonal antibodies. J Immunol 1983, 130:2615–2622
3. Ernst PB, Befus AD, Bienenstock J: Leukocytes in the intestinal epithelium: an unusual immunological compartment. Immunol Today 1985, 6:50–55
4. Ferguson A, Murray D: Quantitation of intraepithelial lymphocytes in human jejunum. Gut 1971, 12:988–994
5. Brandtzaeg P, Bosnes V, Halstensen TS, Scott H, Sollid LM, Valnes KN: T lymphocytes in human gut epithelium preferentially express the α/β antigen receptor and are often CD45/UCHL1-positive. Scand J Immunol 1989, 30:123–128
6. Trejdosiewicz LK, Smart CJ, Oakes DJ, Howdle PD, Malizia G, Campana D, Boylston AW: Expression of T-cell receptors TcR1 (γ/δ) and TcR2 (α/β) in the human intestinal mucosa. Immunology 1989, 68: 7–12
7. Jarry A, Cerf-Bensussan N, Brousse N, Selz F, Guy-Grand D: Subsets of $CD3^+$ (T cell receptor α/β or γ/δ) and $CD3^-$ lymphocytes isolated from normal human gut epithelium display phenotypical features different from their counterparts in peripheral blood. Eur J Immunol 1990, 20:1097–2103
8. Deusch K, Luling F, Reich K, Classen M, Wagner H, Pfeffer K: A major fraction of human intraepithelial lymphocytes simultaneously expresses the γ/δ T cell receptor, the CD8 accessory molecule and preferentially uses the V delta 1 gene segment. Eur J Immunol 1991, 21:1053–1059
9. Lundqvist C, Baranov V, Hammarstrom S, Athlin L, Hammarstrom ML: Intra-epithelial lymphocytes: evidence for regional specialization and extrathymic T cell maturation in the human gut epithelium. Int Immunol 1995, 7:1473–1487
10. Cerf-Bensussan N, Jarry A, Brousse N, Lisowska-Grospierre B, Guy-Grand D, Griscelli C: A monoclonal antibody (HML-1) defining a novel membrane molecule present on human intestinal lymphocytes. Eur J Immunol 1987, 17:1279–1285
11. Balk SP, Ebert EC, Blumenthal RL, McDermott FV, Wucherpfennig KW, Landau SB, Blumberg RS: Oligoclonal expansion and CD1 recognition by human intestinal intraepithelial lymphocytes. Science 1991, 253:1411–1415
12. Van Kerckhove C, Russell GJ, Deusch K, Reich K, Bhan AK, DerSimonian H, Brenner MB: Oligoclonality of human intestinal intraepithelial T cells. J Exp Med 1992, 175:57–63
13. Blumberg RS, Yockey CE, Gross GG, Ebert EC, Balk SP: Human intestinal intraepithelial lymphocytes are derived from a limited number of T cell clones that utilize multiple V β T cell receptor genes. J Immunol 1993, 150:5144–5153
14. Gross GG, Schwartz VL, Stevens C, Ebert EC, Blumberg RS, Balk SP: Distribution of dominant T cell receptor β chains in human intestinal mucosa. J Exp Med 1994, 180:1337–1344
15. Chowers Y, Holtmeier W, Harwood J, Morzycka-Wroblewska E, Kagnoff MF: The V delta 1 T cell receptor repertoire in human small intestine and colon. J Exp Med 1994, 180:183–190
16. Strater J, Koretz K, Gunthert AR, Moller P: In situ detection of enterocytic apoptosis in normal colonic mucosa and in familial adenomatous polyposis. Gut 1995, 37:819–825
17. Shibahara T, Sato N, Waguri S, Iwanaga T, Nakahara A, Fukutomi H, Uchiyama Y: The fate of effete epithelial cells at the villous tips of the human small intestine. Arch Histol Cytol 1995, 58:205–219
18. Bombi JA, Nadal A, Carreras E, Ramirez J, Munoz J, Rozman C, Cardesa A: Assessment of histopathologic changes in the colonic biopsy in acute graft-versus-host disease. Am J Clin Pathol 1995, 103:690–695
19. Kotler DP, Weaver SC, Terzakis JA: Ultrastructural features of epithelial cell degeneration in rectal crypts of patients with AIDS. Am J Surg Pathol 1986, 10:531–538
20. Lee FD: Importance of apoptosis in the histopathology of drug related lesions in the large intestine. J Clin Pathol 1993, 46:118–122
21. Ebert EC, Roberts AI, Brolin RE, Raska K: Examination of the low proliferative capacity of human jejunal intraepithelial lymphocytes. Clin Exp Immunol 1986, 65:148–157
22. Ebert EC: Proliferative responses of human intraepithelial lymphocytes to various T-cell stimuli. Gastroenterology 1989, 97:1372–1381
23. Pirzer UC, Schurmann G, Post S, Betzler M, Meuer SC: Differential responsiveness to CD3-Ti vs. CD2-dependent activation of human intestinal T lymphocytes. Eur J Immunol 1990, 20:2339–2342
24. Watanabe M, Ueno Y, Yajima T, Iwao Y, Tsuchiya M, Ishikawa H, Aiso S, Hibi T, Ishii H: Interleukin 7 is produced by human intestinal epithelial cells and regulates the proliferation of intestinal mucosal lymphocytes. J Clin Invest 1995, 95:2945–2953

25. Lefrancois L, Goodman T: In vivo modulation of cytolytic activity and Thy-1 expression in TCR-$\gamma\delta^+$ intraepithelial lymphocytes. Science 1989, 243:1716–1718
26. Guy-Grand D, Malassis-Seris M, Briottet C, Vassalli P: Cytotoxic differentiation of mouse gut thymodependent and independent intraepithelial T lymphocytes is induced locally: correlation between functional assays, presence of perforin and granzyme transcripts, and cytoplasmic granules. J Exp Med 1991, 173:1549–1552
27. Gramzinski RA, Adams E, Gross JA, Goodman TG, Allison JP, Lefrancois L: T cell receptor-triggered activation of intraepithelial lymphocytes *in vitro*. Int Immunol 1993, 5:145–53
28. Viney JL, Kilshaw PJ, MacDonald TT: Cytotoxic α/β, and γ/δ^+ T cells in murine intestinal epithelium. Eur J Immunol 1990, 20:1623–1626
29. Sydora BC, Mixter PF, Holcombe HR, Eghtesady P, Williams K, Amaral MC, Nel A, Kronenberg M: Intestinal intraepithelial lymphocytes are activated and cytolytic but do not proliferate as well as other T cells in response to mitogenic signals. J Immunol 1993, 150:2179–2191
30. Taunk J, Roberts AI, Ebert EC: Spontaneous cytotoxicity of human intraepithelial lymphocytes against epithelial cell tumors. Gastroenterology 1992, 102:69–75
31. Russell GJ, Nagler-Anderson C, Anderson P, Bhan AK: Cytotoxic potential of intraepithelial lymphocytes (IELs): presence of TIA-1, the cytolytic granule-associated protein, in human IELs in normal and diseased intestine. Am J Pathol 1993, 143:350–354
32. Oberhuber G, Vogelsang H, Stolte M, Muthenthaler S, Kummer AJ, Radaszkiewicz T: Evidence that intestinal intraepithelial lymphocytes are activated cytotoxic T cells in celiac disease but not in giardiasis. Am J Pathol 1996, 148:1351–1357
33. Kagi D, Ledermann B, Burki K, Seiler P, Odermatt B, Olsen KJ, Podack ER, Zinkernagel RM, Hengartner H: Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature 1994, 369:31–37
34. Heusel JW, Wesselschmidt RL, Shresta S, Russell JH, Ley TJ: Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell 1994, 76:977–987
35. Walsh CM, Matloubian M, Liu CC, Ueda R, Kurahara CG, Christensen JL, Huang MT, Young JD, Ahmed R, Clark WR: Immune function in mice lacking the perforin gene. Proc Natl Acad Sci USA 1994, 91: 10854–10858
36. Kagi D, Ledermann B, Burki K, Zinkernagel RM, Hengartner H: Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis *in vivo*. Annu Rev Immunol 1996, 14:207–232
37. Kojima H, Shinohara N, Hanaoka S, Someya-Shirota Y, Takagaki Y, Ohno H, Saito T, Katayama T, Yagita H, Okumura K: Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes. Immunity 1994, 1:357–364
38. Lowin B, Hahne M, Mattmann C, Tschopp J: Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. Nature 1994, 370:650–652
39. Kagi D, Vignaux F, Ledermann B, Burki K, Depraetere V, Nagata S, Hengartner H, Golstein P: Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. Science 1994, 265:528–530
40. Leithauser F, Dhein J, Mechtersheimer G, Koretz K, Bruderlein S, Henne C, Schmidt A, Debatin KM, Krammer PH, Moller P: Constitutive and induced expression of APO-1, a new member of the nerve growth factor/tumor necrosis factor receptor superfamily, in normal and neoplastic cells. Lab Invest 1993, 69:415–429
41. Ratner A, Clark WR: Role of TNF-α in $CD8^+$ cytotoxic T lymphocyte-mediated lysis. J Immunol 1993, 150:4303–4314
42. Chott A, Probert CS, Gross GG, Blumberg RS, Balk SP: A common TCR β-chain expressed by $CD8^+$ intestinal mucosa T cells in ulcerative colitis. J Immunol 1996, 156:3024–3035
43. Kummer JA, Kamp AM, van Katwijk M, Brakenhoff JP, Radosevic K, van Leeuwen AM, Borst J, Verweij CL, Hack CE: Production and characterization of monoclonal antibodies raised against recombinant human granzymes A and B and showing cross reactions with the natural proteins. J Immunol Methods 1993, 163:77–83
44. Takahashi T, Tanaka M, Inazawa J, Abe T, Suda T, Nagata S: Human Fas ligand: gene structure, chromosomal location and species specificity. Int Immunol 1994, 6:1567–1574
45. Alderson MR, Tough TW, Davis-Smith T, Braddy S, Falk B, Schooley KA, Goodwin RG, Smith CA, Ramsdell F, Lynch DH: Fas ligand mediates activation-induced cell death in human T lymphocytes. J Exp Med 1995, 181:71–77
46. Moller P, Walczak H, Reidl S, Strater J, Krammer PH: Paneth cells express high levels of CD95 ligand transcripts: a unique property among gastrointestinal epithelia. Am J Pathol 1996, 149:9–13
47. Boismenu R, Havran WL: Modulation of epithelial cell growth by intraepithelial $\gamma\delta$ T cells. Science 1994, 266:1253–1255
48. Suda T, Takahashi T, Golstein P, Nagata S: Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 1993, 75:1169–1178
49. De Maria R, Boirivant M, Cifone MG, Roncaioli P, Hahne M, Tschopp J, Pallone F, Santoni A, Testi R: Functional expression of Fas and Fas ligand on human gut lamina propria T lymphocytes: a potential role for the acidic sphingomyelinase pathway in normal immunoregulation. J Clin Invest 1996, 97:316–322
50. Tan X, Hsueh W, Gonzalez-Crussi F: Cellular localization of tumor necrosis factor (TNF)-α transcripts in normal bowel and in necrotizing enterocolitis: TNF gene expression by Paneth cells, intestinal eosinophils, and macrophages. Am J Pathol 1993, 142:1858–1865
51. Murch SH, Braegger CP, Walker-Smith JA, MacDonald TT: Location of tumour necrosis factor α by immunohistochemistry in chronic inflammatory bowel disease. Gut 1993, 34:1705–1709
52. Beil WJ, Weller PF, Peppercorn MA, Galli SJ, Dvorak AM: Ultrastructural immunogold localization of subcellular sites of TNF-α in colonic Crohn's disease. J Leukocyte Biol 1995, 58:284–298
53. Ebnet K, Hausmann M, Lehmann-Grube F, Mullbacher A, Kopf M, Lamers M, Simon MM: Granzyme A-deficient mice retain potent cell-mediated cytotoxicity. EMBO J 1995, 14:4230–4239
54. Mullbacher A, Ebnet K, Blanden RV, Hla RT, Stehle T, Museteanu C, Simon MM: Granzyme A is critical for recovery of mice from infection with the natural cytopathic viral pathogen, ectromelia. Proc Natl Acad Sci USA 1996, 93:5783–5787
55. Lundqvist C, Melgar S, Yeung MM, Hammarstrom S, Hammarstrom M-L: Intraepithelial lymphocytes in human gut have lytic potential and a cytokine profile that suggest T helper 1 and cytotoxic functions. J Immunol 1996, 157:1926–1934