Human Small-Intestinal Epithelium Contains Functional Natural Killer Lymphocytes

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**Background & Aims:** CD3− non-T lymphocytes constitute the second most abundant lymphoid subset in the human small-bowel epithelium, and these CD3− intraepithelial lymphocytes are virtually absent in active celiac disease. Phenotypically, they resemble natural killer cells and have been termed natural killer–like intraepithelial lymphocytes. Because of the limited availability of appropriate human samples, functional studies have not yet been reported, and it is not yet clear whether these are true natural killer cells. **Methods:** We used magnetic bead–based purification and flow cytometry to study several aspects of normal human small-bowel natural killer–like intraepithelial lymphocytes: intracellular cytokine content (basally and after activation); ability to lyse natural killer–sensitive K562 target cells; and expression of perforins, Fas ligand, and other functional markers. **Results:** CD3− intraepithelial lymphocytes cultured in interleukin-2 showed a higher lymphokine-activated killer activity than CD3+ intraepithelial lymphocytes (48%–83% lysis exerted by CD3− intraepithelial lymphocytes at an effector–target cell ratio of 2:1 vs. 8%–18% by CD3+ intraepithelial lymphocytes). Perforin content correlated with this lytic potential (75% ± 4% in CD3− vs. 5% ± 4% in CD3+ intraepithelial lymphocytes). Both CD3− and CD3+ cells displayed a type I cytokine profile (interferon-γ > tumor necrosis factor-α > interleukin-2; undetectable interleukin-4 and interleukin-10). In addition to their activated phenotype, subsets of natural killer–like intraepithelial lymphocytes expressed CD8αα and intracellular CD3ε chain, showing the existence of heterogeneity within this cell lineage. **Conclusions:** This is the first demonstration of functional natural killer cells within the human gut epithelium. These cells might play an important role in innate mucosal immunity (host defense and tumor surveillance) and tolerance.

**Abbreviations used in this paper:** APC, allophycocyanin; CM, complete medium; D10, 3,3′-diododecylxocarbocyanine; FCM, flow cytometric analysis; FITC, fluorescein isothiocyanate; i-IL, intraepithelial interleukin; IFN, interferon; i-NK, intraepithelial intestinal NK cells; LAK, lymphokine-activated killer; mAb, monoclonal antibody; NK, natural killer; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; PMA, phorbol myristate acetate; PI, propidium iodide; TCR, T-cell receptor; TNF, tumor necrosis factor.

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IL-15 (lymphokine-activated killer [LAK] assays).\textsuperscript{19,20} IEL cytokine production has been also investigated, with a prominent type 1 profile (interferon [IFN]-γ and tumor necrosis factor [TNF]-α)\textsuperscript{14,21–23} predominant over nearly undetectable IL-4\textsuperscript{22} and IL-10 production.\textsuperscript{14} The evidence thus supports a potential role of IEL in mucosal surveillance against infections\textsuperscript{24,25} and tumors,\textsuperscript{17,26} as well as in epithelial growth\textsuperscript{27} and in immunomodulation and maintenance of oral tolerance.\textsuperscript{28,29} Such diverse functions are likely exerted by different IEL subsets. So far, there is no evidence of a direct implication of any IEL subset in mucosal enteropathy.

In this article, we discuss some aspects of phenotypic heterogeneity in the NK-like IEL subset and functionally characterize their cytotoxic potential and cytokine production. Our results are in agreement with the functional properties of similar NK IEL subsets that have been recently characterized in the mouse,\textsuperscript{30} chicken,\textsuperscript{31} and rat\textsuperscript{32} and show the existence of a functional NK IEL subset in human intestinal mucosa (i-NK).

**Materials and Methods**

**Mucosal Specimens**

Histologically normal biopsy samples (1–2 mm\textsuperscript{2}) were obtained from children and adults undergoing endoscopic examination for the investigation of small-bowel disease in which the final diagnoses excluded the involvement of the intestine. Duodenal specimens (4–6 cm\textsuperscript{2}) were obtained from healthy adult individuals undergoing Scopinaro operations for morbid obesity. Jejunal and proximal ileal mucosa were also obtained from the resected intestine of a child with microvillous inclusion disease who was undergoing intestinal transplantation. In total, samples from 47 individuals—20 children and 27 adults—were used. All the specimens were obtained after informed, written consent was given. This study was approved by the local Ethics Committee of Ramón y Cajal Hospital.

**Isolation and Purification of Intestinal Intraepithelial Lymphocyte Subsets**

**Mechanical dispersion of the mucosa.** Single cell suspensions were prepared from the epithelial layer of small-intestinal biopsy samples or fragmented surgical specimens (cut into fragments of approximately 1–5 mm\textsuperscript{3}) by using a previously described protocol\textsuperscript{13} with minor modifications. Briefly, IEL and epithelial cells were liberated from the mucosal specimens by incubation for 1 hour under intense stirring with 1 mmol/L dithiothreitol and 1 mmol/L EDTA in RPMI 1640 medium (Gibco BRL Life Technologies, Vienna, Austria) supplemented with 10% fetal calf serum and antibiotics (complete medium [CM]). The suspension of released cells was then washed and further processed according to their further use: (1) labeled with the appropriate conjugated monoclonal antibodies (mAb) for 3- or 4-color flow cytometric analysis (FCM), (2) kept in culture, or (3) subjected to further purification of IEL by Percoll density gradients.

The density of IELs per 100 epithelial cells in control biopsy samples was 9.7% ± 4.3% (mean ± SD). The proportion of NK-like IEL was 44% ± 16% in children and 30% ± 12% in adults (mean ± SD). We did not observe phenotypic or functional differences with respect to age. On average, the mechanical dispersion procedure yielded 50,000–150,000 IEL per biopsy. Cell clumps were disaggregated by passage through a 25-gauge needle.

**Cell cultures.** IEL were cultured in CM. When required, phorbol myristate acetate (PMA, 5 ng/mL; Sigma, St. Louis, MO) and calcium ionophore (ionomycin, 100 ng/mL; Sigma) or recombinant human IL-2 (R&D Systems, Minneapolis, MN) was added as indicated.

**Purification of intraepithelial lymphocyte subsets.** First, Percoll density gradients containing 20–40–55% Percoll solution (Amersham Pharmacia Biotec, Uppsala, Sweden) in CM were used to enrich IEL after mechanical disruption of the mucosa. The cell fraction between 40% and 55% Percoll was collected, and IEL were further purified by immunomagnetic methods. We took advantage of the high expression of phosphatidyl serine at the cell surface of enterocytes at this step of the purification protocol (>95% annexin V\textsuperscript{+} enterocytes with 9000 means fluorescence channel vs. <15% annexin V\textsuperscript{+} IEL with 200 means fluorescence channel) and used annexin V coated to magnetic microbeads (Dead Cell Removal kit; Miltenyi Biotec, Bergich Gladbach, Germany) to retain dead cells in MiniMACS magnetic columns (Miltenyi Biotec). Eluted IEL (>95% pure and viable) were stained with anti-CD3/fluorescein isothiocyanate and separated into the CD3\textsuperscript{+} and CD3\textsuperscript{−} fractions by using anti-fluorescein isothiocyanate–coated magnetic beads (Miltenyi Biotec). The resulting fractions were >90% pure after 2–3 rounds of separation. Limitations in the size of biopsy specimens and the low yields obtained with this lengthy approach did not allow us to obtain enough freshly isolated CD3\textsuperscript{−} cells to perform the cytotoxicity assays.

**Spontaneous intraepithelial lymphocyte release from mucosal specimens and their expansion with interleukin-2.** Only mucosal fragments with more than 30% CD3\textsuperscript{−}CD103\textsuperscript{−} IEL were cultured ex vivo without processing, and the cells that were detached after 24–48 hours of culture in CM were harvested and tested for CD103, CD3, and CD19 expression to assess their intraepithelial origin and the level of any eventual lamina propria or peripheral blood contamination. Only samples with less than 10% non-IEL contamination were further processed. An initial IEL phenotype after dithiothreitol/EDTA de-epithelialization in a parallel sample was performed as a reference of the mucosa in the study. IEL were cleared of cellular debris and expanded through successive passages in CM supplemented with 10 ng/mL recombinant human IL-2 (R&D Systems). Expanded CD3\textsuperscript{+} and CD3\textsuperscript{−} subsets were positively and negatively selected by immunomagnetic procedures by using goat anti-mouse immunoglob-
ulun (IgG-coated magnetic microspheres (Dynabead M-450; Dynal, Oslo, Norway) previously incubated with anti-CD3 mAb (Beckman Coulter, Brea, CA), by following the commercial instructions. This procedure of IEL expansion rendered low cell recoveries (<10%) of a high purity (>95%) on both selected subsets (as controlled by FCM analysis), but there were enough cells to perform cytotoxic assays with CD3+ and CD3− IEL. Peripheral blood NK cells were purified by immunomagnetic procedures from healthy donor blood: peripheral blood mononuclear cells were plated on plastic for 1.5 hours at 37°C, and the nonadherent cells were depleted of T and B cells by incubation with anti-CD3 and anti-CD19 antibodies (BD Pharmingen, San Diego, CA) followed by goat anti-mouse IgG-coated magnetic microspheres (Dynal), by following the manufacturers’ protocols. The purity of the NK cells was assessed as >95% by CD45 and CD56 staining, and the cells were used as a positive control for the lysis assays after stimulation with IL-2 (50 ng/mL) for 7 days.

**Flow Cytometric Analysis**

The expression of surface markers on IEL was analyzed by 3- or 4-color FCM by using a FACScan or a FACSCalibur (BD Biosciences, San Diego, CA) after staining with fluorochrome-conjugated mAbs (see below), and data were processed with Lysis II and Cell Quest software (BD Biosciences). Acquisition of multiparameter data was performed with an appropriate forward-scatter threshold to exclude erythroid cells and debris. IEL cells were selected from the entire population of epithelial cells after gating on the basis of their low 90° light scattering, and their intraepithelial localization was confirmed by the expression of CD103 and by the absence of CD19+ B cells in the suspension. Perforin intracellular staining was performed after cell fixation (2% paraformaldehyde in phosphate-buffered saline) and permeabilization (0.1% saponin; Sigma). The rest of the intracytoplasmic stainings (cytofixation) were performed with the commercial kit Cytofix/Cytoperm (BD Pharmingen). Fluorochrome-conjugated isotype control Igs were used for all analyses. The specificity of the anti-CD95L/FasL staining was investigated by blockade with recombinant human CD95L: 1 μg of recombinant human CD95L (rhFasL; Alexis, CA) per 1 μg of anti-CD95L.

**Monoclonal antibodies.** For FCM, fluorochrome- or biotin-conjugated mAbs directed against CD3ε (clone Leu4), CD3ε (UCHT1), CD8 (SK1), CD10 (H110a), CD11a (G-25.2), CD16 (NKP15), CD18 (L130), CD28 (L293), CD29 (MAR-4), CD31 (L133.1), CD44 (L178), CD45 (2D1), CD45 (H130), CD69 (L78), CD80 (L307.4), CD86 (IT2.2), CD95 (Dx2), CD103 (BerACT8), CD117 (104D2), CD152/CTLA-4 (BN13), TCR-αβ (WT31), TCR-γδ (11F2), IL-2 (M91-17H), IL-4 (MP4-25D2), IL-10 (JES5-19F1), IFN-γ (4S.B3), TNF-α (Mab11), perforins (8G9), and isotype IgG controls were obtained from BD Pharmingen; mAbs against CD4 (T4), CD8α (T8), CD8β (2ST8.5H), and CD56 (NKH1) were obtained from Beckman Coulter, mAbs against CD3 (OKT3) and CD54 (6.5B5) were obtained from DAKO Diagnostics (Glostrup, Denmark); and mAbs against CD95 ligand (NOK-1), CD120a (2H10), and CD120b (4D1B10) were obtained from CALTAG (Burlingame, CA). PE-conjugated streptavidin was obtained from BD Pharmingen.

**Cytotoxicity assessment.** The lysis of the NK-sensitive K562 cell line exerted by IEL subsets was measured by using a previously described protocol14 with minor modifications. Briefly, purified subpopulations of IEL, either incubated with recombinant human IL-2 (10 ng/mL) for 12 hours if freshly isolated from biopsy samples or directly added to the assay if obtained from in vitro expanded cultures, were mixed at the indicated effector–target ratios, centrifuged at low speed (800 rpm) to favor cell contact, and incubated for 4 hours. After washing, cells were stained with propidium iodide (PI; Molecular Probes, Eugene, OR). K562 target cells, a human erythroleukemic cell line (American Type Culture Collection, Rockville, MD), were cultured to log phase growth. For assays performed with freshly isolated IEL (which showed more debris than cultured IEL), K562 cells were previously labeled with the lipophilic membrane dye 3,3′-dihexadecyloxacarbocyanine (DiO; Molecular Probes) at a concentration of 30 μmol/L for the 18 hours before the assay. DiO emits green fluorescence and allows for the identification of K562 in the fluorometric biparametric analysis of DiO/PI staining. The mortality index was calculated as the percentage of compromised K562 PI+ in the assay.

**Intracellular cytokine detection.** This analysis was performed on IEL stimulated (or not) with PMA (5 ng/mL; Sigma) and ionomycin (100 ng/mL; Sigma) for 12 hours in CM in the presence of monensin (GolgiStop; BD Pharmingen). After stimulation, cells were washed and labeled with conjugated mAb (PerCP– and APC–) against surface antigens. They were then fixed and permeabilized (Cytosfix/Cytoperm) for 20 minutes, washed with WashPerm (BD), and labeled with cytokine-specific FITC- and PE-conjugated mAbs and isotype-matched Ig controls. IEL were gated by their side scatter and CD45+ profiles.

**Results**

**Phenotypic Heterogeneity Within the Natural Killer–Like Intraepithelial Lymphocytes (CD3−CD7+ Intraepithelial Lymphocytes)**

**Signs of cellular immaturity.** The characteristics of the NK-like IEL8 strongly suggested that they were indeed NK cells, but we sought to investigate the potential coexistence of CD7+CD3− NK/T precursor cells among them. We first analyzed the intracellular expression of the CD3ε chain by FCM and found it in 10%–25% of the NK-like IEL (n = 10), which are by definition negative for surface membrane staining of CD3 (Figure 1B). None of the characteristic NK markers—CD2, CD16 and CD56, or CD8α—showed a selective distribution between the intracellular CD3ε+ or intra-
cellular CD3ε− fractions (Figure 1C–F). The immature-cell markers CD1a, CD117, and CD10 were expressed in CD3+ IEL at very low proportions (5% ± 4% for CD1a, 2% ± 2% for CD117/c-kit, and 3% ± 3% for CD10). Terminal deoxynucleotidyl transferase and CD34 were negative. No immature-cell markers were found in the CD3− subset (Figure 1G–J).

**Extrathymic maturation markers.** The expression of the CD8αα homodimer is attributed to an extrathympic ontogeny of IEL. Only a minor fraction of NK-like IEL expressed CD8 (10% ± 6%), but these were predominantly CD8αα (83% ± 5% of the CD8+ NK-like IEL were CD8αα; Figure 1K and L). By contrast, the CD8αα homodimer represented less than half of the CD8+ TcR-γδ+ IEL (45% ± 6%) and a minor percentage of the CD8+ TcR-αβ+ IEL (15% ± 7%; Table 1).

**Activation antigens and accessory molecules.** In agreement with previous reports, we noticed a major expression of CD69 on CD3+ IEL, most of which were

<table>
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<tr>
<th>Variable</th>
<th>TcR-αβ+ IEL</th>
<th>NK-like IEL</th>
<th>TcR-γδ+ IEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8αa</td>
<td>73 ± 20</td>
<td>10 ± 6</td>
<td>48 ± 5</td>
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<tr>
<td>CD8ααb</td>
<td>15 ± 7</td>
<td>83 ± 5</td>
<td>45 ± 6</td>
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<tr>
<td>CD8αβb</td>
<td>85 ± 7</td>
<td>17 ± 5</td>
<td>55 ± 6</td>
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*Percentage of CD8α cells for each of the 3 major IEL subsets.

*Percentage of CD8αα+ and CD8αβ+ cells within the CD8+ fraction of each major IEL subset.
CD25⁻ (not shown), and this marker was also present in NK-like IEL (97% ± 2% were CD69⁺CD25⁻). Only 2% ± 2% of the CD3⁻ IEL expressed major histocompatibility complex II, whereas 39% ± 25% of the CD3⁺ IEL did, as reported by others. The transferrin receptor, CD71, was negative on all IEL (not shown).

IEL are more completely activated by ligation of their co-receptors than by stimulation of the T-cell receptor. CD28 and CTLA-4 were undetectable on NK-like IEL, whereas these co-stimulatory molecules could be readily detected on CD3⁺ IEL (25% ± 19% CD28⁺, n = 20; 5% ± 4% CTLA-4⁺, n = 10; Figure 2).

The pairs B7/CD80 and B7.2/CD86 are ligands for CD28 and CTLA-4. CD80 was not detected on CD3⁺ (as reported previously) or on NK-like IEL (Figure 2D), and CD86 was scarcely expressed on both CD3⁺ (4% ± 4%) and NK-like IEL (10% ± 9%; n = 10; Figure 2C).

CD44 and lymphocyte function–associated antigen-1, two adhesion molecules involved in co-stimulation, were mainly expressed in the CD3⁺ IEL subset (95% ± 4% and 45% ± 10%, respectively, while undetected on the NK-like IEL), whereas ICAM-1 was predominantly localized in the CD3⁻ IEL fraction (86% ± 6% vs. 34% ± 16% in the CD3⁺ IEL; not shown). These differences tended to disappear when IEL were cultured with IL-2: CD44 and lymphocyte function–associated antigen-1 were induced up to 80% in the CD3⁻ fraction, whereas intercellular adhesion molecule 1 was found all through the IEL compartment (not shown). CD31/PECAM-1, a molecule that has been implicated in NK cell adhesion to endothelium, was present in a small fraction of CD3⁺ IEL (57% ± 9%; n = 4), but not in NK-like IEL (not shown).

In summary, we found differences in the expression of markers associated with ontogeny, homing, and activation requirements among IEL subsets, suggesting distinct functional roles for T and NK-like IEL.

Lytic Potential of the Natural Killer–Like Intraepithelial Lymphocytes

Natural killer cytotoxic assays (K562 as target): cytolitic capacity of freshly isolated CD3⁺ intraepithelial lymphocytes. We performed 3 independent NK lytic assays with freshly isolated IEL (from duodenal biopsies) after 12 hours of preincubation with IL-2. We achieved high IEL purity (>(90%) but low yields (<10%), and we could not obtain enough freshly isolated CD3⁻ IEL. The lytic capacity of CD3⁺ IEL was modest, in the range of 3% to 8% (percentage of PI⁺ K562) at an effector–target ratio of 1:1 and 4%⁻10% at a ratio of 5:1 (Figure 3).

Cytolytic capacity of CD3⁺ and CD3⁻ intraepithelial lymphocytes cultured with interleukin-2. We performed 4 independent NK lytic assays with IEL harvested from cultured mucosal specimens and expanded in culture for 2–3 weeks with IL-2 (as described in Materials and Methods). After fractionation into CD3⁺ and CD3⁻ subsets, the purity of the fractions was >93%.

Figure 4 shows a representative 2-parameter cytogram analysis of the cell populations involved in an NK assay, performed with the CD3⁻ (Figure 4F and G) and CD3⁺ (Figure 4H and I) IEL fractions expanded from ileal mucosa. The percentage of compromised PI⁺ K562 was superior when CD3⁻ (NK-like) IEL and purified IL-2–cultured peripheral blood NK cells were used as effectors. Increasing the effector–target ratio up to 50 CD3⁺ IEL per target cell implied a dose–response cytotoxicity that reached a plateau in the range of 10%–25% IP⁺ K562 (n = 2; not shown). Table 2 shows the range of the lytic capacity displayed by each IEL fraction and com-
pared with that of peripheral blood NK cells. As can be seen, NK-like IEL possess a cytotoxic LAK activity that is clearly superior to that of CD3+ IEL.

**Potential mechanisms of intraepithelial lymphocyte–mediated cytotoxicity: perforin content.** Freshly isolated NK-like IEL showed a greater content of intracellular perforins (75% ± 4%; n = 7) than their CD3+ counterparts (5% ± 4%, similar to what has been recently reported16; Figure 5A). When IEL were expanded in culture with IL-2, the perforin content remained high in the CD3+ subset, particularly in the CD56+CD3+ IEL (Figure 5C), and increased up to 25% in the CD3+ IEL subset (Figure 5D).

**CD95L (FasL) expression.** CD95L expression on freshly isolated IEL was low (6% ± 3% in CD3+ and 5% ± 3% in NK-like IEL; n = 10; Figure 5E) but specific, as suggested by its blockade with recombinant human CD95L protein (Figure 5, histograms). This contrasts with the widespread expression on IEL of its receptor, CD95 (Fas)8,46 (Figure 5F).

The expression of other members of the TNF receptor superfamily implicated in apoptosis and cell death was also evaluated. CD120a (TNF receptor 1)18 was hardly detected in the IEL compartment (Figure 5G), whereas CD120b (TNF receptor 2)18 was highly expressed in CD3+ (61% ± 2%) and more variably in NK-like IEL (31% ± 27%; Figure 5H). Other members of the family, such as the activation antigens CD30 and CD70, were negative.

In conclusion, CD3+ IEL were capable of strong lytic activity after in vitro expansion with IL-2, comparable to IL-2–stimulated peripheral blood NK cells. In contrast, CD3+ IEL were weaker effectors against K562.

**Table 2.** NK Lytic Activity of IL-2–Expanded IEL Subsets From Human Small Intestine Versus IL-2–Cultured Peripheral Blood NK Cells

<table>
<thead>
<tr>
<th>Effector—target cell ratio</th>
<th>Peripheral blood NK (n = 2)</th>
<th>NK-like (CD3+) IEL subset (n = 4)</th>
<th>CD3+ IEL subset (n = 4)</th>
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</thead>
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<tr>
<td>1:1</td>
<td>16, 49</td>
<td>19, 27, 44, 78</td>
<td>3, 5, 13, 17</td>
</tr>
<tr>
<td>2:1</td>
<td>29, 60</td>
<td>48, 50, 58, 83</td>
<td>8, 10, 19, 19</td>
</tr>
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NOTE. Data represent the proportion of PI+ K562 cells in each sample.
Interferon-γ and Tumor Necrosis Factor-α Are the Main Cytokines Produced by Natural Killer–Like Intraepithelial Lymphocytes

The intracellular content of type I (IFN-γ, TNF-α, and IL-2) and type II (IL-10, IL-4, and IL-5) cytokines was determined by FCM on freshly isolated IEL. Basal cytokine content in IEL, without in vitro stimulation, was difficult to evaluate because of the low frequencies of cytokine-producing cells, which were in the limit of the sensitivity threshold of the FCM method of analysis. In general, IEL had a negligible spontaneous production of IFN-γ and TNF-α (fewer than 2% cytokine-producing cells), whereas IL-2, IL-4, IL-10, and IL-5 were undetectable (Figure 6).

The production of cytokines by CD3 TcR-αβ+ and -γδ+ and NK-like IEL subsets was then investigated after activation with a phorbol ester (PMA) and ionomycin. Type I cytokines were readily detectable in all IEL subsets, but not type II ones (Figure 6). The frequency of IFN-γ–, TNF-α–, and IL-2–producing cells was higher in the CD3 TcR-αβ+ IEL subset than in the TcR-γδ+ or NK-like IEL. The results are summarized in Table 3 and indicate that (1) IFN-γ was mainly synthesized by

Figure 5. Flow cytometric analysis of some potential cytolytic mechanisms of IEL. (A) Representative analysis of intracellular perforin content of freshly isolated IEL from a duodenal biopsy, with the corresponding isotype-matched IgG-PE control (B). Intracellular perforin content on CD3– (C) and CD3+ (D) IEL subsets after in vitro IL-2 expansion for 2–3 weeks. Representative dot plots of CD95L (FasL) (E), CD95 (Fas) (F), CD120a (G), and CD120b (H) expression on CD3+ and CD3– IEL subsets. The histograms on the right suggest the specificity of the fluorometric CD95L-PE signal by its partial blockage with recombinant human CD95L (rh-CD95L) protein. PE, phycoerythrin.

Figure 6. Cytokine production by freshly isolated IEL. IEL isolated from duodenal biopsies were cultured for 12 hours with or without PMA and ionomycin in the presence of monensin, surface-labeled for different antigens (CD3 and CD45), and fixed and permeabilized before intracellular cytokine staining. Representative dot plots are shown.
CD3+ IEL T cells (45% ± 16% of TcR-αβ+ and 17% ± 3% of TcR-γδ+), but also by NK-like cells (11% ± 8%); (2) CD3+ IEL synthesized TNF-α (27% ± 13% of TcR-αβ+ and 4% ± 1% of TcR-γδ+), as did 11% ± 7% of the NK-like cells; (3) IL-2 was produced at lower percentages by both CD3+ (6% ± 2%) and NK-like IEL (4% ± 6%), whereas IL-4 and IL-10 were almost undetectable (less than 2%) in all subsets. Dual cytokine cell content for IFN-γ and TNF-α was also evaluated by 4-color FCM and was observed in approximately 30% of the positive cells (Figure 6, lower right dot plot).

Cytokine synthesis was also evaluated in IEL cultured with IL-2. IEL from 1 jejunal specimen and 1 ileal mucosal specimen, expanded in vitro with IL-2 (10 ng/mL) for 2–3 weeks, showed almost undetectable cytokine production without mitogenic stimuli (Figure 7A and C). After a 4–6-hour stimulation period with PMA/ ionomycin, the cytokine pattern in CD3+ IEL was similar to that observed in freshly isolated ones, but with higher frequencies of secreting cells (51%–80% produced IFN-γ, and 30%–70% produced IL-2; Figure 7B and D). Expanded NK-like cells produced IFN-γ (range, 10%–38%) and less IL-2 (<5%; Figure 7B and D). IL-4 and IL-10 were almost undetectable in both IL-2 cultured samples.

In conclusion, a significant fraction of the NK-like IEL displays a proinflammatory cytokine profile, which is compatible with a true NK nature.

**Discussion**

Since the initial observation made by Spencer et al.9 in 1989 of the presence of CD3+ CD7+ IEL cells in the intraepithelial compartment of human small intestine, few studies have referred to the existence of these cells,2,10 with a complete lack of information about their functional relevance. In previous works, we found that CD3+ CD7+ cells represented a numerically relevant fraction of small-intestinal IEL,11 characterized their phenotype as NK-like,7,8 and pointed out that these cells were drastically diminished in active celiac disease.9,11 We now extend these observations by showing that at least a subset of the NK-like IEL seem to constitute the intestinal NK cell lineage that has been recently described in other species,30–32 on the basis of their phenotype, their ability to lyse NK-specific targets, and their ability to secrete proinflammatory cytokines.

The vast majority of CD3- IEL share a common phenotype,7,8 but the heterogeneity we report now suggests that precursor cells7,3,48,50 may be present within the CD7+ CD3- IEL subset. We have observed intracellular expression of the CD3ε chain (found in NK and T progenitor cells51) in 10%–25% of the NK-like IEL, but these cells did not differ from the rest in terms of other surface markers. These intracellular CD3ε IEL may constitute the benign counterparts of the aberrant IEL found in intestinal T-cell lymphoma (enteropathy associated or not), which are usually surface-membrane CD3+ intracellular CD3ε+/CD2+/CD56+, and in refractory sprue.52,53 CD3ε has also been detected in fetal hepatic
NK cells and uterine NK cells, but not in peripheral blood NK cells unless induced by activation. Therefore, the presence of intracellular CD3ε could alternatively reflect an ontogenic or functional relationship among intestinal, uterine, and fetal hepatic NK cells. As a third possibility, it could be a sign of cellular hyperactivation. A second aspect of the heterogeneity of NK-like IEL is their partial expression of CD8α, a proposed marker of extrathymic intestinal ontogeny, as has been shown for CD8αα IEL in mice. We did not observe any significant expression of immature lymphoid cell markers (CD34, Tdt, or c-kit) on NK-like IEL, but most are CD16low−, which resembles uterine NK cells and a minor subset of peripheral blood NK that have been considered NK precursors. In addition, functionally immature human NK cells also lack CD16, reinforcing the hypothesis of an in situ pathway of NK differentiation in the intestinal epithelium. A final phenotypic aspect to note is that the unique pattern of adhesion molecules the NK-like IEL display is essentially lost after in vitro culture, indicating that it might respond to functional requirements that influence their migratory capacity, function, or both.66 Regarding the functionality of NK-like IEL, cytokine production by normal human IEL has infrequently been reported, and most studies have focused on CD3+ IEL, which show a predominant type I cytokine profile. In our study, the frequency of IEL that spontaneously produced cytokines was near the sensitivity threshold of FCM but also showed a proinflammatory T-helper type 1 bias, similar in magnitude to that in previous reports. Mitogenic stimulation of freshly isolated IEL clearly showed a type I cytokine pattern on both CD3+ and NK-like IEL subsets, with the former as the main source of IFN-γ and TNF-α. We did not find IL-4 or IL-10 production by normal IEL, even after mitogenic stimulation. To our knowledge, this is the first time that cytokine production by human intestinal intraepithelial NK-like cells has been reported. When stimulated with PMA and ionomycin, a significant fraction of NK-like cells produced IFN-γ (11% ± 8%), TNF-α (11% ± 7%), and some IL-2 (4% ± 6%), a type I pattern that was maintained after in vitro culture. We did not observe type II cytokine production by human NK-like IEL, unlike the reported IL-4 secretion detected by ELISPot (enzyme-linked immunospot assay) in rat intestinal NK IEL, although the latter cells show noticeable differences compared with human NK-like IEL (they are CD25+). Our data suggest an innate cytolytic effector function rather than an immunoregulatory or tolerogenic one for NK-like IEL, although we cannot exclude it.

Regarding the cytotoxic potential of IEL, most previous reports have focused on CD3+ IEL in redirected lysis assays or after cytokine stimulation. The limitations in the availability of healthy human small-bowel specimens to purify CD3− IEL prompted us to expand these cells with low concentrations of IL-2 to obtain enough to assess their function in vitro. By doing so, we observed that NK-like IEL lyse K562 cells far more efficiently than CD3+ IEL. This LAK activity parallels the intense cytotoxicity against K562 exerted by CD3−CD56−CD16− large granular lymphocytes obtained from unfractionated duodenal mucosa, as well as by uterine large granular lymphocytes and by circulating CD56+CD16− peripheral blood NK. Neither of these NK subsets displayed a significant baseline killing without IL-2 stimulation. Because K562 does not express Fas and because NK-like IEL possessed a higher perforin content, it seems plausible these molecules may have been responsible for the lysis, as has been shown in murine IEL. However, the role of other lytic co-stimulators, such as NKG2D or CD94, cannot be disregarded. Although our data do not directly support a regulatory or tolerogenic role of this NK-like subset in mucosal immune responses, this possibility is attractive because of their absence in celiac disease, a situation of gluten intolerance and oral tolerance, and to determine the reason for their absence in celiac disease.

In conclusion, this is the first functional description of human intestinal intraepithelial NK cells. These NK cells produce type I cytokines, mainly IFN-γ and TNF-α, and have a cytolytic LAK activity against NK targets. Further work is required to investigate the role of these cells in intestinal immune responses, possibly as innate lytic effectors and/or as regulators of homeostatic inflammation and oral tolerance, and to determine the reason for their absence in celiac disease.

References
5. Guy-Grand D, Rocha B, Mintz P, Malassis-Seris M, Selz F, Malis-


16. Melgar S, Bas A, Hammarström S, Hammarström ML. Human small intestinal mucosa harbors a small population of cytolytically active CD8+ \( \alpha\beta \) T lymphocytes. Immunology 2002;106:476–485.


37. Sillett HK, Southgate J, Howdle PD, Tredosievicz LK. Expression


73. Ferry BL, Starkey PM, Sargent JL, Watt GMO, Jackson M, Redman CWG. Cell populations in the human early pregnancy decidua: natural killer cell activity and response to interleukin-2 of CD56+ positive large granular lymphocytes. Immunology 1990;70:446–452.


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