Diverse populations of T cells with NK cell receptors accumulate in the human intestine in health and in colorectal cancer

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T cells expressing NK cell receptors (NKR) display rapid MHC-unrestricted cytotoxicity and potent cytokine secretion and are thought to play roles in immunity against tumors. We have quantified and characterized NKR$^+$ T cells freshly isolated from epithelial and lamina propria layers of duodenum and colon from 16 individuals with no evidence of gastrointestinal disease and from tumor and uninvolved tissue from 19 patients with colorectal cancer. NKR$^+$ T cell subpopulations were differentially distributed in different intestinal compartments, and CD161$^+$ T cells accounted for over one half of T cells at all locations tested. Most intestinal CD161$^+$ T cells expressed $\alpha\beta$ TCR and either CD4 or CD8. Significant proportions expressed HLA-DR, CD69 and Fas ligand. Upon stimulation *in vitro,* CD161$^+$ T cells produced IFN-γ and TNF-α but not IL-4. NKT cells expressing the Vα24Vβ11 TCR, which recognizes CD1d, were virtually absent from the intestine, but colonic cells produced IFN-γ in response to the NKT cell agonist ligand α-galactosylceramide. NKR$^+$ T cells were not expanded in colonic tumors compared to adjacent uninvolved tissue. The predominance, heterogeneity and differential distribution of NKR$^+$ T cells at different intestinal locations suggests that they are central to intestinal immunity.

1 Introduction

A minor subset of both murine and human T cells expresses NK cell receptors (NKR) and displays functional characteristics of NK cells and classical T cells [1–4]. NKR$^+$ T cells become activated rapidly after stimulation, are capable of MHC-unrestricted cytotoxicity *in vitro* and can secrete large amounts of cytokines including IFN-γ, TNF-α, IL-2 and IL-4 [5–7]. They account for about 5% of peripheral blood lymphocytes but are enriched in liver and bone marrow, accounting for 35–50% of lymphocytes in these organs [8, 9]. NKR can act as inhibitory molecules on cloned T cells as shown by *in vitro* models in CD3-TCR mediated cytolysis [10, 11]. Recently, NKR$^+$ T cells that recoginize HLA-E molecultes in association with peptides derived from MHC class I molecules or viruses were described [12] Although the mechanisms that control the expression of NKR on T cells are not well understood, these receptors are believed to function as costimulatory molecules or to regulate effector T cell activity [13–16].

In mice, the majority of NKR$^+$ T cells are NKT cells. NKT cells express NK1.1 and an invariant TCR α-chain, Vα14Jα18 (formerly Vα14Jα281), which preferentially pairs with a limited number of β chains and recognizes glycolipid antigens presented by the MHC class I-like protein CD1d [17–19]. The natural ligand(s) for NKT cells is unknown, but they respond to α-galactosylceramide (α-GalCer), an extract of the marine sponge *Agelas mauritanius*, in a CD1d-restricted manner [20, 21]. NKT cells play key roles in immunity against tumors in some murine models. Injection of mice with either IL-12 or α-GalCer can result in tumor rejection by a mechanism that is dependent on IFN-γ production and/or anti-tumor cytotoxicity by NKT cells [22, 23]. Furthermore, mice deficient in NKT cells fail to mediate IL-12-induced rejection of tumors [24].

CD1d-restricted NKT cells expressing invariant Vα24Jα18 TCR α-chains (formerly Vα24JαQ) paired with Vβ11 have been identified in humans [25, 26], but they are found in much lower numbers in blood (~0.02% of lymphocytes), liver and bone marrow (<1%) compared to mice [9, 27]. However, human T cells expressing various NKR, including CD56, CD57, CD161, CD94 and killer Ig-like receptors, have been reported to share functional similarities with invariant NKT cells, such as rapid MHC-unrestricted cytotoxicity and potent cytokine secretion [3, 4, 7]. Human NKR^{+} T cells are also thought to play roles in anti-tumor immunity [5, 28, 29].

The human gastrointestinal tract contains several phenotypically and functionally distinct populations of T cells [30–33]. Many of these cells are thought to play roles in anti-tumor immunity. T cells isolated from the epithelial layer and the lamina propria (LP) of the human colon are cytotoxic and kill carcinoma cell lines *in vitro* [34, 35], and T cell infiltration into tumor tissue is associated with improved prognosis of colonic carcinoma [36, 37]. Recent studies have indicated that NKR^{+} T cells are also present in murine and human intestine [38–40], but to date no studies have addressed the potential roles of these cells in intestinal cancer.

We have phenotypically and functionally characterized NKR^{+} T cells in the epithelial and LP layers of non-diseased human duodenum and colon and evaluated changes in their numbers and phenotypes in patients with colorectal cancer. Our results indicate that distinct subsets of NKR^{+} T cells, but not invariant NKT cells, accumulate at different intestinal locations. Many of these cells express Fas ligand and markers of activation and secrete inflammatory cytokines upon stimulation *in vitro*. Colonic CD56^{+}, CD57^{+} and CD161^{+} T cells were not expanded in tumor tissue compared with adjacent uninvolved tissue.

2 Results

2.1 Compartmentalization of NKR^{+} T cells in human intestine

Flow cytometric analysis of single-cell suspensions prepared from human colon and duodenum revealed differential distribution of NKR[+] T cell populations in the epithelium and LP (Fig. 1A). Approximately one half of all T cells in both the epithelial (median 44.6%) and LP (58.4%) layers of the colon expressed CD161 (Fig. 1B). CD56 was expressed by smaller proportions of colonic T cells, being present on a median of 35.1% of epithelial layer T cells and on significantly lower numbers (22.4%; $p<0.05$) of LP T cells. CD57 was expressed by similar proportions of colonic epithelial layer (8.2%) and LP (9.8%) T cells as in peripheral blood (9.6%) (Fig. 1B).

When T cell subpopulations in duodenal tissue samples were examined, CD161 was also found to be expressed by the majority of epithelial layer (58.5%) and LP (57.5%) T cells (Fig. 1B). Smaller proportions of T cells expressed CD56, with significantly higher levels found in the LP (19.6%) compared with the epithelium (10.9%; $p<0.05$). T cells expressing CD57 were less frequently detected in both the epithelium (4.3%) and the LP (12.2%). The high frequency of CD161^{+} cells in both the epithelial layer and LP of human colon and the co-localization of CD161 and CD3 were confirmed by immunohistochemistry and double immunofluorescence (Fig. 1C, D).

2.2 Phenotypic characterization of intestinal CD161^{+} T cells

In both human colon and duodenum, CD8 was expressed by the majority of epithelial layer CD161^{+} T cells (89.5% in colon and 59.9% in duodenum; Fig. 2A, B), while smaller frequencies expressed CD4. In contrast, CD4 was expressed more frequently by CD161^{+} T cells in the LP (58.5% in colon and 81.2% in duodenum; Fig. 2A, B). Less than 5% of epithelial layer and LP CD161^{+} T cells in both colon and duodenum expressed neither CD4 nor CD8, a double-negative phenotype commonly associated with NKT cells [17]. Analysis of TCR phenotypes indicated that almost all CD161^{+} T cells in both duodenal and colonic epithelium and LP layers express $\alpha\beta$ TCR, while less than 3% expressed $\gamma\delta$ TCR (Fig. 2A, B). CD56 was expressed by <20% of CD161^{+} T cells at all intestinal locations tested (Fig. 2A, B).

The majority of total colonic CD161^{+} T cells expressed the early activation marker CD69 (median 78.2%), and up to 38.2% (median 18.4%) expressed the late activation marker HLA-DR (Fig. 2C). CD25 was expressed by a smaller proportion of colonic CD161^{+} T cells (median 7.2%). Significant populations of intestinal CD161^{−} T cells also displayed activated phenotypes (Fig. 2C). Expression of Fas ligand (CD95L) was significantly more frequent in CD161^{+} T cells than in CD161^{−} T cells (14.5% *vs.* 1.8%; $p<0.05$; Fig. 2C).

2.3 Vα24^{+}Vβ11^{+} NKT cells in human colon

It has previously been shown that, in most tissues, the co-expression of the Vα24 and Vβ11 TCR chains defines invariant CD1d-restricted Vα24Jα18^{+} NKT cells [25, 26, 41, 42]. We used flow cytometry to detect Vα24Jα18^{+} NKT cells in human colon. The percentage of colonic CD3^{+} T cells that expressed Vα24 ranged from 0.3% to 3.3% (median 1.0% in epithelium and 1.6% in LP; Fig. 3A). However, these colonic epithelial and LP Vα24^{+} T cells did not co-express the Vβ11 chain. In the liver, up
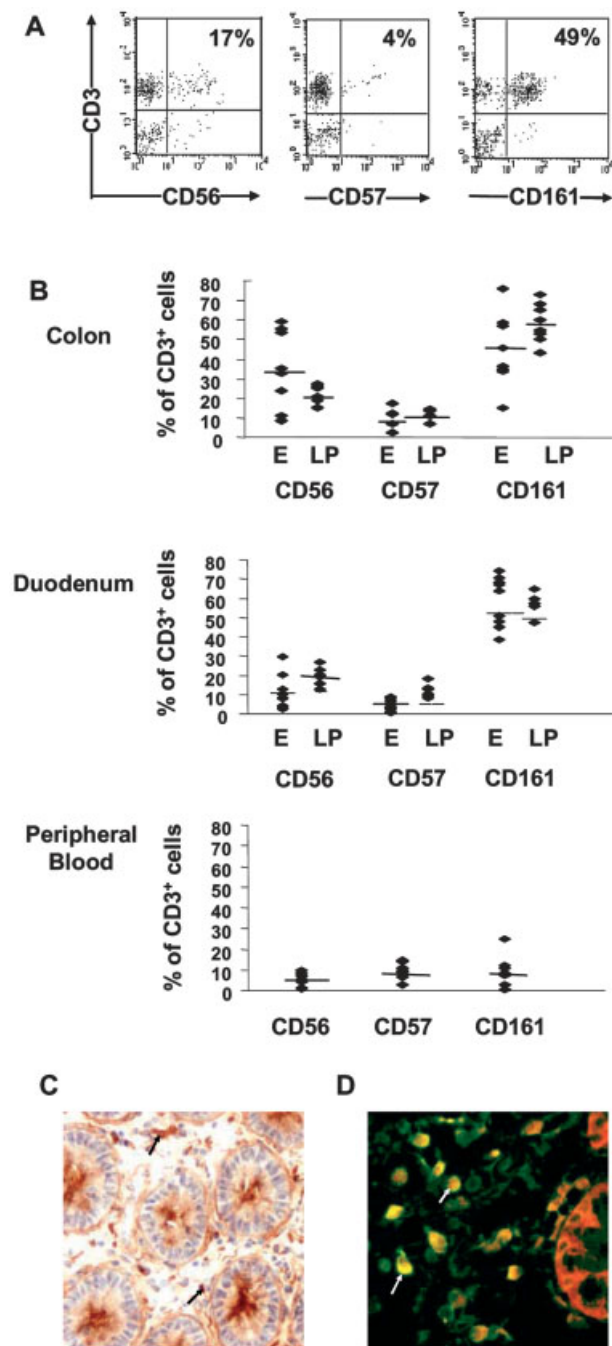
◄ *Fig. 1.* NK receptor-positive T cells accumulate in the human intestine. (A) Flow cytometric analysis of the co-expression of CD3 with CD56, CD57 or CD161 on gated lymphocytes from a non-diseased colon specimen. The numbers in the upper right quadrants show the percentages of $CD3^+$ cells that express CD56, CD57 or CD161. (B) Percentages of $CD3^+$ cells that express CD56, CD57 or CD161 in the epithelial (E) and LP layers of human colon (top, *n*=8) and duodenum (middle, *n*=10) and in peripheral blood (bottom, *n*=7). Horizontal bars indicate the median values. (C) Localization of $CD161^+$ cells by immunohistochemical staining in normal colonic mucosa. $CD161^+$ cells are indicated by brown staining (×200). (D) Immunofluorescence staining for $CD3^+CD161^+$ T cells in normal colon. Positive staining is indicated by the greenish-yellow staining (×400).

[43–46]. We examined reactivity to α-GalCer in the colonic cell preparations by culturing 1×10^6 cells with α-GalCer and analyzing the culture supernatants for IFN-γ production. Levels of IFN-γ were significantly up-regulated by α-GalCer beyond levels detected in unstimulated cultures (Fig. 3C).

2.4 Cytokine production by $CD161^+$ T cells

The ability of freshly isolated colonic epithelial and LP layer $CD161^-$ and $CD161^+$ T cells to produce cytokines upon stimulation *in vitro* was assessed by flow cytometric analysis of permeabilized cells. Significant proportions of $CD161^+$ T cells produced IFN-γ and TNF-α, but not IL-4, upon activation with PMA and ionomycin (Fig. 4A). TNF-α was produced by significantly higher frequencies of $CD161^+$ T cells compared with $CD161^-$ T cells from colonic epithelium (39.8% *vs.* 21.2%; $p<0.05$) and LP (69.7% *vs.* 57.1%; $p<0.05$). In contrast, the frequencies of colonic epithelial and LP $CD161^+$ and $CD161^-$ T cells that produced IFN-γ in response to stimulation were similar. The proportions of both $CD161^+$ and $CD161^-$ T cells that produced IL-4 upon PMA/ionomycin stimulation was <5% in all donors examined (Fig. 4B).

2.5 NKR^+ T cells are not expanded in colonic tumor tissue

Immunofluorescence studies on paired tumor and uninvolved colon tissue sections from six patients with colonic carcinoma (Fig. 1D) revealed that T cell numbers were increased in the tumors in four patients and decreased in two. The median numbers of $CD3^+$ cells/ mm^2 in tumor and uninvolved tissue from five patients were 640 and 487, respectively (*p* not significant; Fig. 5A). Flow cytometric analysis of these T cells revealed that NKR^+ T cells were not expanded in tumors. The proportions of T cells that expressed CD161 and

to 90% (median 64.2%) of $V\alpha24^+$ T cells expressed Vβ11 (Fig. 3B) [27]. These data indicate that, while up to 1.6% of hepatic T cells express Vα24Vβ11 TCR, these invariant NKT cells are present in extremely low numbers in the human colon.

Vα24Vβ11$^+$ NKT cells specifically recognize α-GalCer presented by CD1d [41, 42] which is expressed and is functionally active in the murine and human intestine
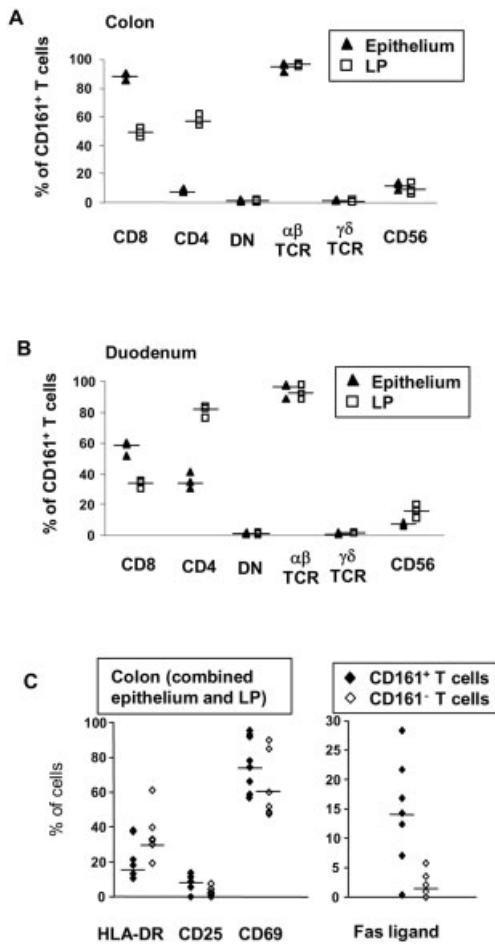
Fig. 2. Phenotypic characterization of CD161⁺ T cells freshly isolated from human intestine. (A, B) Percentages of CD3⁺CD161⁺ lymphocytes that express CD8, CD4, double-negative CD8⁻CD4⁻ (DN), αβ TCR, γδ TCR and CD56 in the epithelial and LP layers of human colon (A, *n*=7) and duodenum (B, *n*=5). (C) Percentages of total CD3⁺CD161⁺ and CD3⁺CD161⁻ lymphocytes from normal colon (*n*=7) expressing HLA-DR, CD25, CD69 and Fas ligand. Horizontal bars indicate median values.

Fig. 3. Invariant NKT cells do not accumulate in the human colon. (A) Percentages of CD3⁺ cells that express Vα24 (left) and Vα24Vβ11 (right) TCR chains in epithelial (E) and LP layers of human colon (*n*=7) and blood (*n*=4). Horizontal bars indicate median values. (B) Representative flow cytometry dot plots showing Vα24 and Vβ11 TCR chain expression by gated CD3⁺ cells freshly isolated from normal colon, blood and liver. Numbers show percentages of CD3⁺ cells that express the Vα24Vβ11 TCR. (C) Mean IFN-γ levels released by unstimulated, α-GalCer-stimulated and PHA-stimulated total colonic cells (*n*=5). **p*<0.05.

CD56 were significantly reduced in tumors compared with adjacent uninvolved tissue (medians 31.9% *vs.* 49.7% for CD161⁺ T cells and 8.6% *vs.* 19.9% for CD56⁺ T cells; *p*<0.05; Fig. 5B). However, the absolute numbers of CD161⁺ and CD56⁺ T cells did not differ significantly (medians 187 *vs.* 239 and 27 *vs.* 68 cells/mm² in tumors and adjacent uninvolved mucosa, respectively; Fig. 5B). The proportions and absolute numbers of CD57⁺ T cells were also unchanged in colonic tumors (Fig. 5B).

Vα24⁺ T cells were also compared in tumor tissue and adjacent mucosa in matched tissue specimens from five patients. The percentages of Vα24⁺ T cells in normal
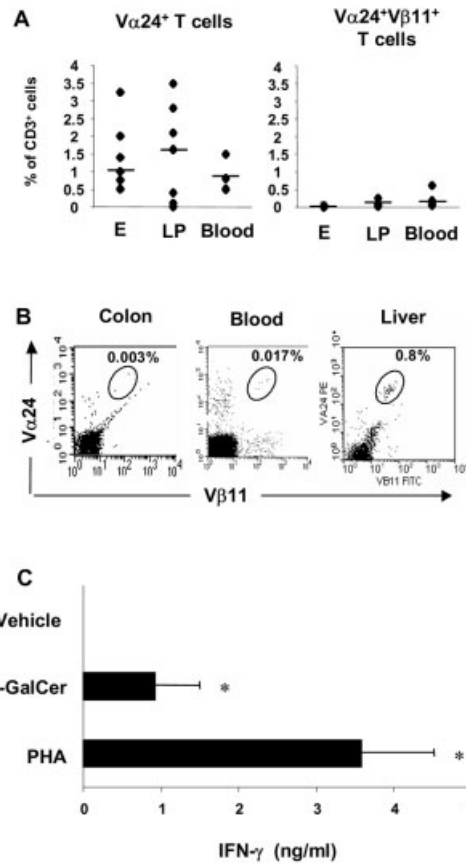
mucosa were low (0–2%), and the levels remained virtually undetectable in tumor tissues (data not shown).

3 Discussion

NKR⁺ T cells are predominantly localized in peripheral tissues and have the capacity to rapidly kill tumor cells and produce cytokines without the need for prior priming with antigen [1–9]. We report here that the healthy adult intestine is a site of accumulation of several distinct NKR⁺ T cell populations that are differentially distributed in the duodenum and colon and in the epithelial and LP layers. In confirmation of previous studies [38, 39], we
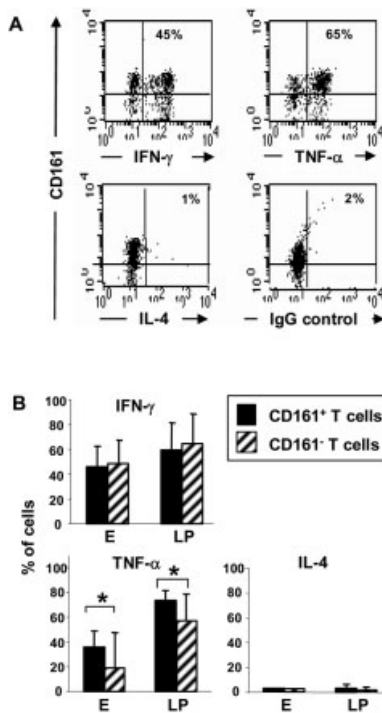
Fig. 4. Intracellular cytokine production by CD161^{+} and CD161^{−} T cells from normal colonic epithelium (E) and LP. (A) Representative flow cytometry dot plots showing IFN-γ, TNF-α and IL-4 expression by colonic CD161^{+} T cells stimulated for 4 h with PMA and ionomycin. The percentages of CD161^{+} cells that express the cytokines are indicated in the upper right quadrants. (B) Median percentages of CD161^{+} and CD161^{−} cells from epithelial and LP layers of 9 normal colonic specimens that express IFN-γ, TNF-α and IL-4 upon stimulation (*$p < 0.05$).

found that CD161^{+} T cells are among the most abundant NKR^{+} T cell population in the intestine. These cells were found to account for approximately one half of all T cells in both the epithelial and LP layers of the duodenum and colon. Compared to peripheral blood, CD56^{+} T cell numbers were elevated in the LP of the duodenum and colon and the epithelial layer of the colon. CD57^{+} T cells were found at similar frequencies in blood and the intestinal compartments.

Our results indicate that almost all intestinal CD161^{+} T cells express $\alpha\beta$ TCR. They can express CD4 or CD8, but the majority in the epithelial layers of both the duodenum and colon are CD8^{+}, while CD4^{+} cells predominate among CD161^{+} T cells in the LP. CD161^{+} T cells with the double-negative (CD4^{−}CD8^{−}) phenotype commonly associated with NKT cells [17–19] were rarely found in the intestinal compartments tested. Colonic CD161^{−} and CD161^{+} T cells showed similar frequencies of expression of the T cell activation markers CD25, CD69 and HLA-
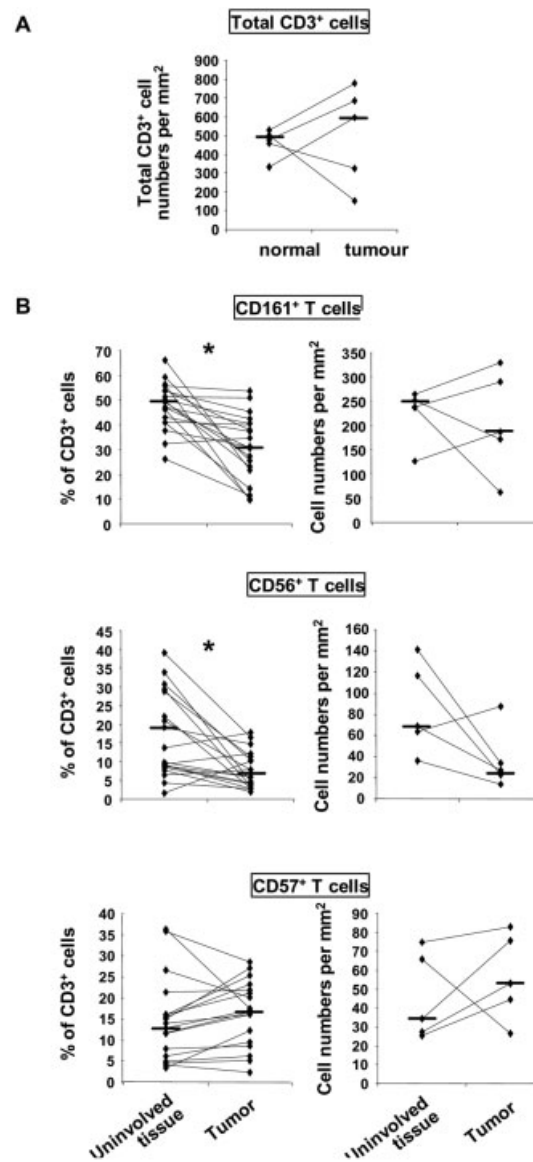

Fig. 5. Colonic NKR^{+} T cells are not expanded in colonic cancer. (A) Quantification of CD3^{+} cells per mm[2] of paired colonic carcinoma and adjacent uninvolved colonic mucosa tissue sections in 5 patients using immunohistochemistry. (B) Percentages (left) and absolute numbers (right) of CD3^{+} cells that express CD161, CD56 or CD57 in paired speimens of tumor-involved tissue and adjacent uninvolved mucosa. Horizontal bars indicate the median values (*n*=19 for percentages, *n*=5 for absolute numbers; *$p < 0.05$).

DR, but Fas ligand (CD178) was found to be expressed by higher frequencies of CD161^{+} than CD161^{−} T cells in the colon. Colonic CD161^{+} and CD161^{−} T cells were capable of rapid secretion of IFN-γ and TNF-α, but little IL-4, upon stimulation *ex vivo* with PMA and ionomycin. The percentage of CD161^{+} T cells capable of producing TNF-α was markedly higher when compared with

CD161^{-} T cells. These findings indicate that the majority of CD161^{+} T cells in the intestine produce cytokines of the Th1/Tc1 profile, consistent with a cytotoxic phenotype.

In the present study, we show that while the human intestine contains large numbers of NKR^{+} T cells, very low proportions of these express the invariant Vα24Vβ11 TCR chain associated with CD1d restriction. Similar findings have been reported for murine NKR^{+} T cells defined by the co-expression of CD3 and NK1.1, which are rare in the small intestine but accumulate in the colon [40]. In contrast to murine liver, intestinal NKR^{+} T cells do not express invariant Vα14Jα18 TCR or require CD1d for their development [40]. However, CD1d is constitutively expressed by murine and human intestinal epithelial cells [43–46], suggesting that other non-invariant NKT cells may be present in the intestine. In support of this, we found that colonic mononuclear cells released significant amounts of IFN-γ after stimulation with α-GalCer *in vitro*. α-GalCer reactivity by human intraepithelial lymphocytes (IEL) has also been recently demonstrated by Van de Wal and co-workers [43], who showed that ligand recognition is CD1d-dependent. The α-GalCer-reactive T cells in human colon could either be invariant Vα24Jα18^{+} NKT cells that are present in very low numbers but can rapidly activate other cells, such as NK cells and conventional T cells, to produce IFN-γ. Alternatively, other non-invariant NKT cells that recognize α-GalCer presented by CD1d may be present in the colon and may be the primary source of IFN-γ. Non-invariant CD1d-restricted NKT cells that express CD56 and/or CD161 have been detected in human bone marrow [9] and in hepatitis C virus-infected liver [47]. However, recent studies using CD1d-α-GalCer tetramers have provided evidence that α-GalCer reactivity is restricted to Vα24Vβ11^{+} NKT cells [41, 42], which would argue against the notion of non-invariant hepatic NKT cells recognizing α-GalCer. NKT cells reactive with CD1 isotypes that are not found in mice, namely CD1a, CD1b and CD1c [18], may also reside within the CD56^{+}/CD161^{+} T cell compartment of the intestine.

CD56^{+} and CD161^{+} T cells can participate in anti-tumor immune responses, being capable of potent anti-tumor cytolytic activity and the production of large amounts of inflammatory cytokines *in vitro* [5–7]. Flow cytometric analysis of lymphocytes isolated from tumors and adjacent histologically normal colonic tissue from 19 patients showed that the proportions of T cells that express CD56 or CD161 are significantly reduced in tumors. However, while overall numbers were also reduced in tumors from 4 out of 5 patients, this reduction was not significant. We also found that CD161^{+} T cells more frequently express Fas ligand and produce more TNF-α than CD161^{-} T

cells, suggesting that they are specialized for anti-tumor cytotoxicity. Our observations that NKR^{+} T cells are not expanded in tumors are not consistent with a role for these cells in tumor immunity, but the reduction in the proportions of NKR^{+} T cells could result in insufficient activation of NK or cytolytic T cells required for efficient clearing of tumor cells [48]. Alternatively, it could be the result of activation-induced cell death of tumor-infiltrating lymphocytes; apoptosis of tumor-infiltrating lymphocytes was previously found to correlate with lymph node metastases and poor prognosis in primary colorectal cancer [49]. Changes in human NKR[+] T cell numbers, phenotypes and functions have also been described in other malignancies [27–29, 50], although their importance is less clear than in studies in mice [22–24].

In conclusion, this study has shown that the human intestine is a site where CD161^{+} T cells preferentially accumulate, suggesting that these cells are important in local immunity including resistance to tumor cell invasion. CD161^{+} T cells frequently express Fas ligand and produce IFN-γ and TNF-α in response to stimulation. Very small numbers of these cells express the invariant Vα24Vβ11 TCR, but reactivity against α-GalCer is detectable among colonic cells, suggesting the presence of other CD1d-restricted NKT cells.

4 Materials and methods

4.1 Subjects

Sixteen patients (9 females and 7 males; mean age 45 years; range 24–69 years) who were being investigated for upper gastrointestinal symptoms were studied. Small intestinal disease was excluded by endoscopy and routine histology. Nineteen patients (11 females and 8 males; mean age 69 years; range 56–84 years) with colorectal cancer were studied. All endoscopic samples were obtained with informed consent, and the study was approved by the Research and Ethics committee of St. Vincent's University Hospital, Dublin.

4.2 Preparation of intestinal cells

Single-cell suspensions of epithelial layer and LP were prepared from colonic and duodenal biopsy specimens as previously described [51, 52]. Briefly, biopsy samples were rotated for 1 h at 37°C in calcium- and magnesium-free Hanks Balanced Salt Solution (Gibco-BRL, Paisley, GB) supplemented with 5% FCS, 1 mM DTT and 1 mM EDTA. This results in removal of the epithelial layer, leaving the LP intact and attached to the basement membrane. The resulting single-cell suspension was washed in RPMI 1640 medium

supplemented with 10% FCS and antibiotics, and viable cells were enumerated by ethidium bromide and acridine orange staining. To obtain LP cells, the remaining mucosal tissue was placed in 5 ml supplemented RPMI 1640 medium containing 130 U/ml collagenase (Type 1A, Sigma-Aldrich, Ireland) and rotated for 3 h at 37°C. The resulting single-cell suspension was washed in RPMI 1640 medium, and viability counts were performed as above.

Surgically-resected intestinal tissue, which included uninvolved colonic mucosa as well as tumor tissue, was prepared similarly except that the tissue specimens were first cut into fine pieces using a sterile scalpel and then treated with 200 U/ml collagenase. The samples were subsequently filtered through a nylon mesh and washed in RPMI medium containing 10% FCS. Cell number and viability were determined as above. PBMC were prepared by standard density gradient centrifugation (Lymphoprep, Nycomed Pharma, Oslo, Norway).

4.3 Antibodies and flow cytometry

Fluorochrome-labeled monoclonal antibodies (mAb) specific for human CD3, CD4, CD8, CD56, CD57, CD161, $\alpha\beta$ TCR, $\gamma\delta$ TCR, HLA-DR, CD25, Fas ligand, IFN-γ, TNF-α, IL-4 and isotype matched controls were obtained from BD PharMingen (Oxford, GB). Anti-Vα24 and anti-Vβ11 mAb were obtained from Coulter Immunotech (Marseilles, France). Single-cell suspensions (1×10^5) derived from intestinal tissue and peripheral blood were stained using specific mAb as previously described [8, 51, 52] and analyzed by flow cytometry using a FACScan and CellQuest software (Becton Dickinson).

For measurement of intracellular cytokines (IFN-γ, TNF-α, IL-4), freshly isolated intestinal cell suspensions were stimulated with 10 ng/ml PMA and 1 μg/ml ionomycin (Sigma-Aldrich) for 4 h at 37°C in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich). Cells were stained for surface expression of CD161 and CD3 and subsequently fixed and permeabilized before staining with mAb specific for the cytokines and analysis by flow cytometry, as described previously [7].

4.4 Immunohistochemistry and immunofluorescence

For immunohistochemical staining, resected colonic tissue specimens were fixed in formalin and embedded in paraffin. Sections were subsequently dewaxed, microwave-heated (3–5 min at 750 W in buffer containing 0.1 M sodium citrate and 0.1 M citric acid, pH 6), rinsed in PBS and then incubated with monoclonal anti-CD161 (5 μg/ml) (Serotec, Oxford, GB) or isotype-matched control antibodies. Staining was revealed with a Vectstain *Elite* kit (Vector laboratories, Peterborough, GB) and indirect immunoperoxidase staining.

For immunofluorescence studies, biopsy specimens were mounted in cryopreservative embedding (OCT, Tissue Tec, Finetec Europe, The Netherlands) and snap-frozen in liquid nitrogen. Serial sections, cut at 5 μm, were labeled with 50 μg/ml monoclonal anti-CD161, 50 μg/ml polyclonal anti-CD3 (Dako, Ely, GB) or isotype-matched control antibodies. Secondary antibodies consisted of Cy3-labelled goat anti-biotin (Sigma-Aldrich) for CD161 and anti-rabbit FITC (Sigma-Aldrich) for CD3. Fluorescent microscopy was performed on a Nikon TE300 microscope.

4.5 Cytokine response to α-GalCer

Intestinal cells (1×10^6) were cultured in 24-well tissue culture plates in the presence of 100 ng α-GalCer (Kirin Pharmaceutical Research Laboratory, Gunma, Japan) [20–22], PHA or vehicle as a control. After 48 h incubation, culture supernatants were collected, and IFN-γ production was assayed by ELISA according to the manufacturer's instructions (R&D Systems, Oxon, GB).

4.6 Statistical analyses

Flow cytometric results were expressed as the median values and range. The Mann-Whitney U test for non-parametric data was used to analyze results. *p* values of less than 0.05 were considered significant.

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