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

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Abbreviations used in this paper: α-GalCer, α-galactosylceramide; DN, double negative $CD4^-CD8^-$; E, epithelial; LP, lamina propria; NKR, NK cell receptor.

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Summary

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 characterised 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 αβ-TCRs 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 $V\alpha24V\beta11^+$ TCRs, that recognize CD1d, were virtually absent from the intestine, however, 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 murine and human T cells express NK cell receptors (NKRs) and display 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]. NKRs 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 which recognise HLA-E molecules in association with peptides derived from class I MHC molecules or viruses were described [12]. Although the mechanisms that control the expression of NKRs on T cells are not well understood, these receptors are believed to function as co-stimulatory 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 recognises 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 upon 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 $V\alpha24J\alpha18^+$ TCR α-chains (formerly VαJαQ) 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 NKRs, 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 characterised 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 Compartmentalisation 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 were found 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%) or the lamina propria (12.2%).

The high frequency of CD161$^+$ cells in both epithelial layer and LP of human colon and the co-localization of CD161 and CD3 was confirmed by immunohistochemistry and double immunofluorescence (Fig. 1 C and D).

2.2 Phenotypic characterisation 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. 2 A and 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 and 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 αβ TCRs while less than 3% expressed γδ TCRs (Fig. 2 A and B). CD56 was expressed by <20% of $CD161^+$ T cells at all intestinal locations tested (Fig 2 A and 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 compared with $CD161^-$ T cells (14.5% *vs* 1.8%; $P<0.05$; Fig. 2C).

2.3 $V\alpha24^+V\beta11^+$ 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 $V\alpha24J\alpha18^+$ NKT cells [25,26, 41,42]. We used flow cytometry to detect $V\alpha24J\alpha18^+$ NKT cells in human colon. The percentage of colonic $CD3^+$ T cells that express 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 $V\alpha24^+$ T cells did not co-express the Vβ11 chain. In the liver, up 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 $V\alpha24V\beta11^+$ TCRs, these invariant NKT cells are present in extremely low numbers in the human colon.

$V\alpha24V\beta11^+$ NKT cells specifically recognize α-GalCer presented by CD1d [41,42], which is expressed and is functionally active in the murine and human intestine [43-46]. We examined reactivity to α-GalCer in the colonic cell preparations by culturing $1x10^6$ cells with α-GalCer and analysing the culture supernatants for IFN-γ production. Levels of IFN-γ were significantly upregulated 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% *versus* 21.2%; $p<0.05$) and LP (69.7% *versus* 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 $CD161^+$ T cells and $CD161^-$ T cells that produced IL-4 upon PMA/I stimulation was <5% in all donors examined (Fig. 4B).

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

Immunofluorescence studies on paired tumor and uninvolved colon tissue sections from 6 patients with colonic carcinoma (Fig. 1D) revealed that T cell numbers were increased in the tumors in 4 patients and decreased in 2. Medians of 640 and 487 $CD3^+$ cells/mm^2 of tissue were counted in tumor and uninvolved tissue, respectively, from 5 patients (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 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 significantly differ in tumors compared with adjacent uninvolved mucosa (medians 187 vs 239 and 27 vs 68 cells per mm^2, respectively; Fig. 5B). The proportions and absolute numbers of $CD57^+$ T cells were also unchanged in colonic tumors (Fig. 5B).

$V\alpha24^+$ T cells were also compared in tumor tissue and adjacent mucosa in matched tissue specimens from 5 patients. The percentages of $V\alpha24^+$ 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 predominantly localise in peripheral tissues and functionally have the capacity to rapidly kill tumor cells and to 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 lamina propria layers. In confirmation of previous studies [38,39], we found that $CD161^+$ T cells are amongst 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 both the duodenum and colon. Compared to peripheral blood, $CD56^+$ T cell numbers are elevated in the LP of the duodenum and colon and the epithelial layer of the colon only. $CD57^+$ T cells were found at similar frequencies in blood and intestinal compartments.

Our results indicate that almost all intestinal $CD161^+$ T cells express αβ-TCRs. 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 expressing the double negative $CD4^-CD8^-$ phenotype commonly associated with NKT cells [17-19] were rarely found in all intestinal compartments tested. Colonic $CD161^-$ and $CD161^+$ T cells showed similar frequencies of expression of the T cell activation markers CD25, CD69 and HLA-DR, but CD178 or Fas ligand, was found to be expressed by higher frequencies of $CD161^+$ than $CD161^-$ T cells in the colon. Colonic $CD161^+$ like $CD161^-$ T cells are 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 invariant $V\alpha24V\beta11^+$ TCR chains, that are 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 $V\alpha14J\alpha18^+$ TCRs 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 IELs has also recently been 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 $V\alpha24J\alpha18^+$ 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 recognise 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 $V\alpha24V\beta11^+$ NKT cells [41,42], which would argue against the notion of non-invariant hepatic NKT cells recognising 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 antitumor immune responses, being capable of potent antitumor 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 were significantly reduced in tumors. While, their overall numbers were also found to be reduced in tumors of 4 of 5 patients, this reduction was not significant. We also found that $CD161^+$ T cells more frequently expressed Fas ligand and produced more TNF-α than $CD161^-$ T cells, suggesting that they are specialised for anti-tumor cytotoxicity. Our observations that NKR^+ T cells are not expanded in tumours are not consistent

with a role for these cells in tumor immunity, however, 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. Previously, apoptosis of tumor infiltrating lymphocytes was 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 compared with 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. These cells frequently express Fas ligand and produce IFN-γ and TNF-α in response to stimulation. Very small numbers of these cells express invariant $V\alpha24V\beta11^+$ TCRs but reactivity against α-GalCer is detectable amongst 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 lamina propria were prepared from colonic and duodenal biopsy specimens as previously described [51,52]. Briefly, biopsy samples were rotated for 1 hr at 37^{o}C in calcium- and magnesium-free Hanks Balanced Salt Solution (Gibco-BRL, Paisley, UK) supplemented with 5% FCS, 1 mM DTT and 1 mM EDTA. This results in the removal of the epithelial layer leaving the lamina propria 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 of supplemented RPMI-1640 medium containing 130 U/ml collagenase (Type 1A, Sigma-Aldrich, Ireland) and rotated for 3 hr 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 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.

Peripheral blood mononuclear cells were prepared by standard density gradient centrifugation (Lymphoprep, Nycomed Pharma, Oslo, Norway).

4.3 Antibodies and flow cytometry

Fluorochrome labelled monoclonal antibodies (mAbs) specific for human CD3, CD4, CD8, CD56, CD57, CD161, αβTCR, γδ TCR, HLA-DR, CD25, Fas ligand, IFN-γ, TNF-α, IL-4 and isotype matched controls were obtained from BD Pharmingen (Oxford, UK). Monoclonal anti-Vα24 and anti-Vβ11 antibodies were obtained from Coulter Immunotech (Marseilles, France). Single cell suspensions (1×10^5) derived from intestinal tissue and peripheral blood were stained using specific mAbs as previously described [8, 51, 52] and analysed by flow cytometry using a FACScan and CellQuest lysis 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 hours at 37ºC 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 permeabilised before staining with mAb specific for the cytokines and analysing by flow cytometry, as described previously [9].

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 x 5 mins at 750W in buffer containing 0.1M sodium citrate and 0.1M citric acid, pH 6), rinsed in PBS and then incubated with monoclonal anti-CD161 (5 μg/ml) (Serotec, Oxford, UK), or with isotype-matched control antibodies. Staining was revealed with a Vectstain *Elite* kit (Vector laboratories, Peterborough, UK) 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 5μm, were labelled with monoclonal 50 μg/ml anti-CD161, 50 μg/ml polyclonal anti-CD3 (Dako, Ely, UK) or with 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

Reactivity to 10^6 intestinal cells were cultured in 24-well tissue culture plates in the presence of 100 ng α-GalCer (Kirin Pharmaceutical Research Laboratory, Gunma, Japan) [13-15], PHA, or vehicle as control. After 48 hours incubation, culture supernatants were collected and IFN-γ and production were assayed by ELISA according to the manufacturer's instructions (R&D Systems, Oxon, UK).

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 analyse results. *P* values of less than 0.05 were considered significant.

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Legends to figures

Figure 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 expression by gated lymphocytes from a non-diseased colon specimen. The numbers in the upper right hand 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 lamina propria (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**, Localisation of $CD161^+$ cells by immunohistochemical staining in normal colonic mucosa. $CD161^+$ cells are indicated by brown staining (x200 magnification). **D**, Immunofluorescence staining for $CD3^+CD161^+$ T cells in normal colon. Positive staining is indicated by the greenish-yellow staining (x400 magnification).

Figure 2. Phenotypic characterisation of $CD161^+$ T cells freshly isolated from human intestine. **A and B**, Percentages of $CD3^+CD161^+$ lymphocytes that express CD8, CD4, double negative $CD8^-CD4^-$ (DN), αβTCR, γδTCR and CD56 in the epithelial and lamina propria (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.

Figure 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 lamina propria (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). *$P<0.05$

Figure 4. Intracellular cytokine production by $CD161^+$ and $CD161^-$ T cells from normal colonic epithelium (E) and lamina propria (LP). **A**, Representative flow cytometry dot plot showing IFN-γ, TNF-α and IL-4 expression by colonic $CD161^+$ T cells stimulated for 4 hours 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 lamina propria layers of 9 normal colonic specimens that express IFN-γ, TNF-α and IL-4 upon stimulation. *P<0.05.

Figure 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 specimens 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.

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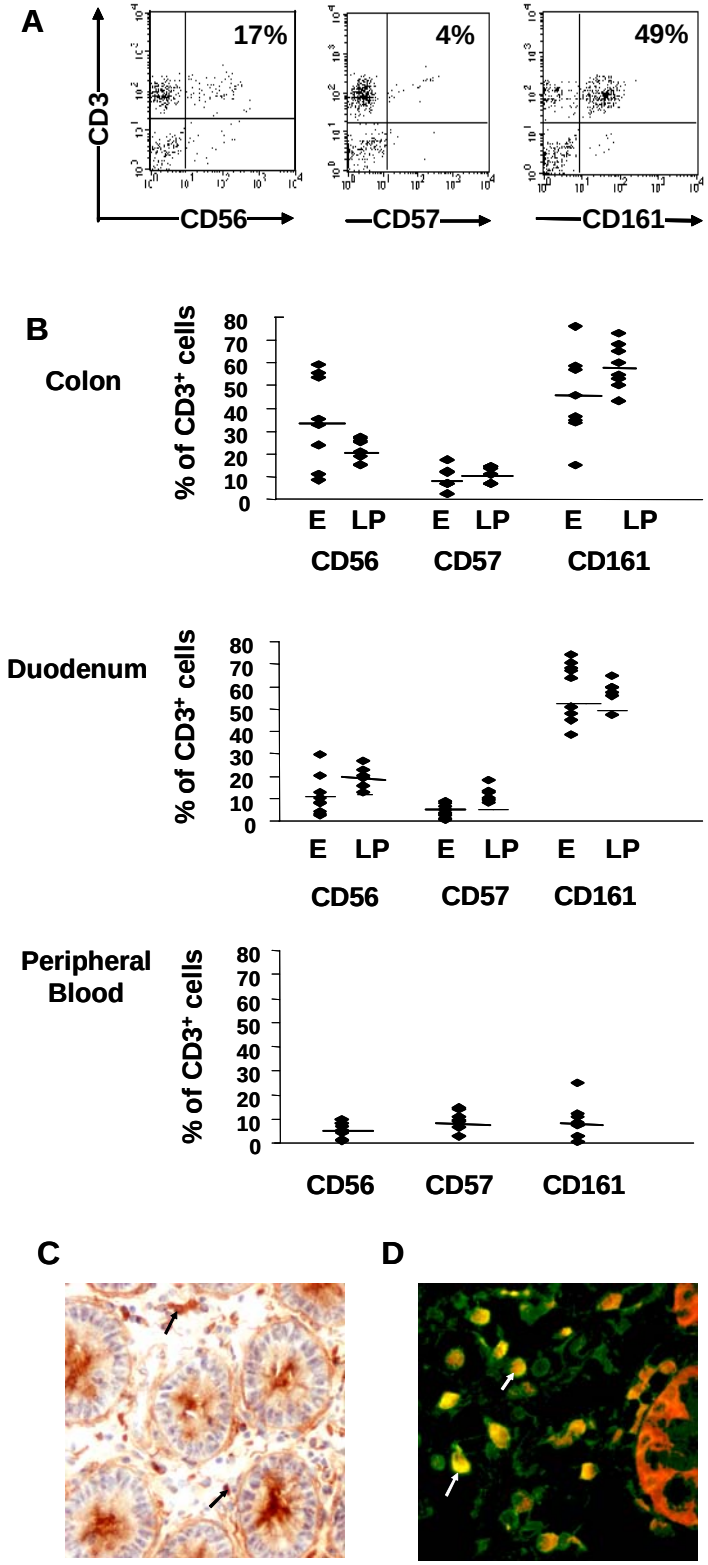
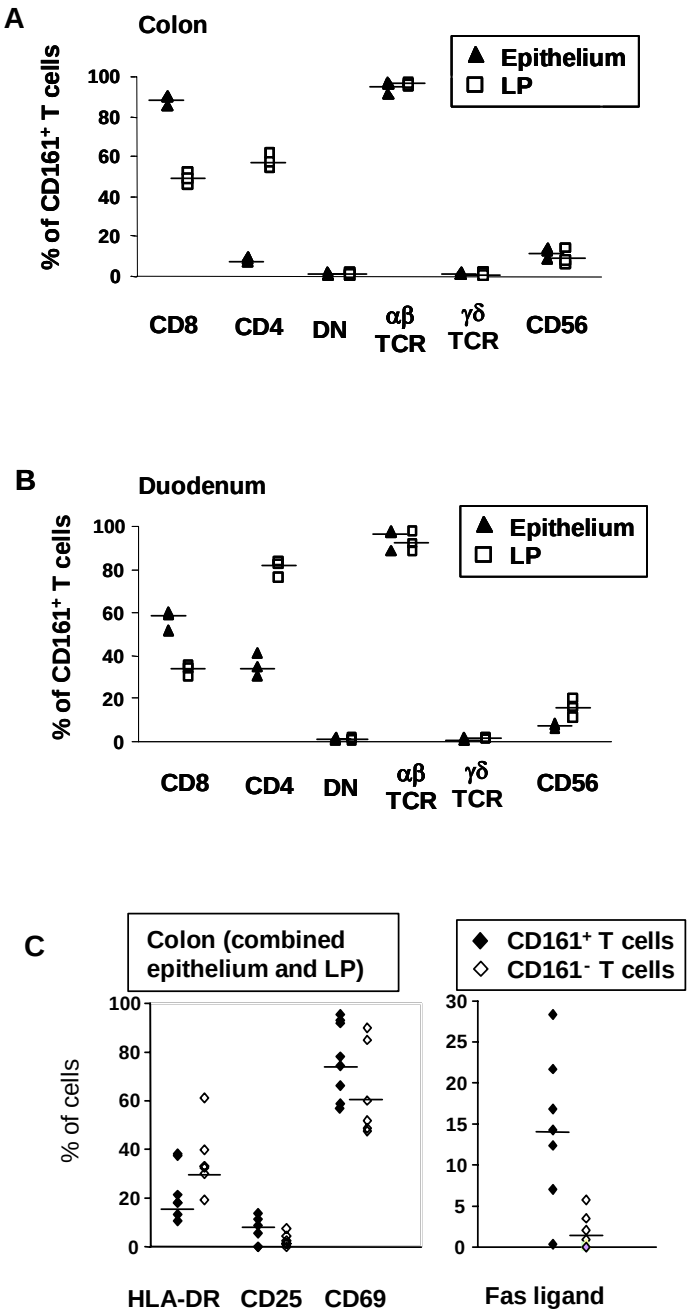
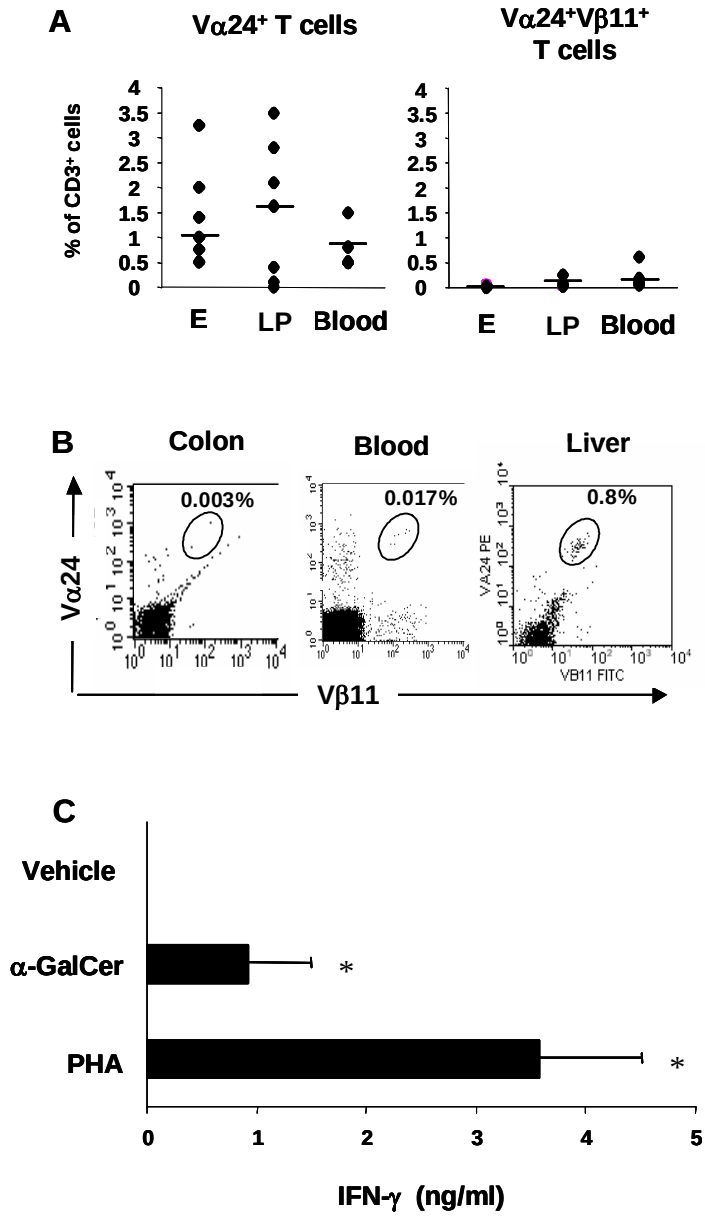
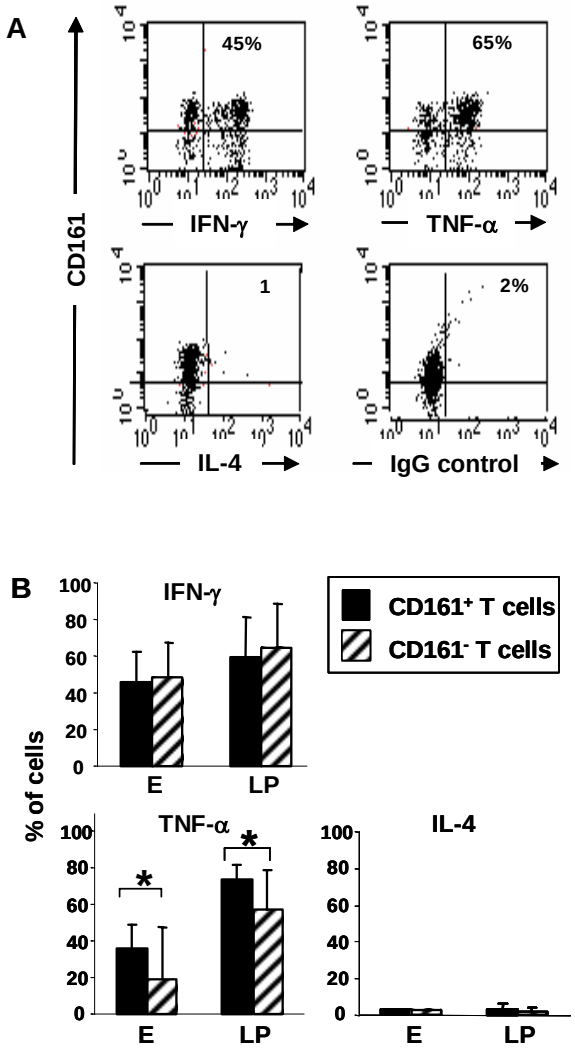
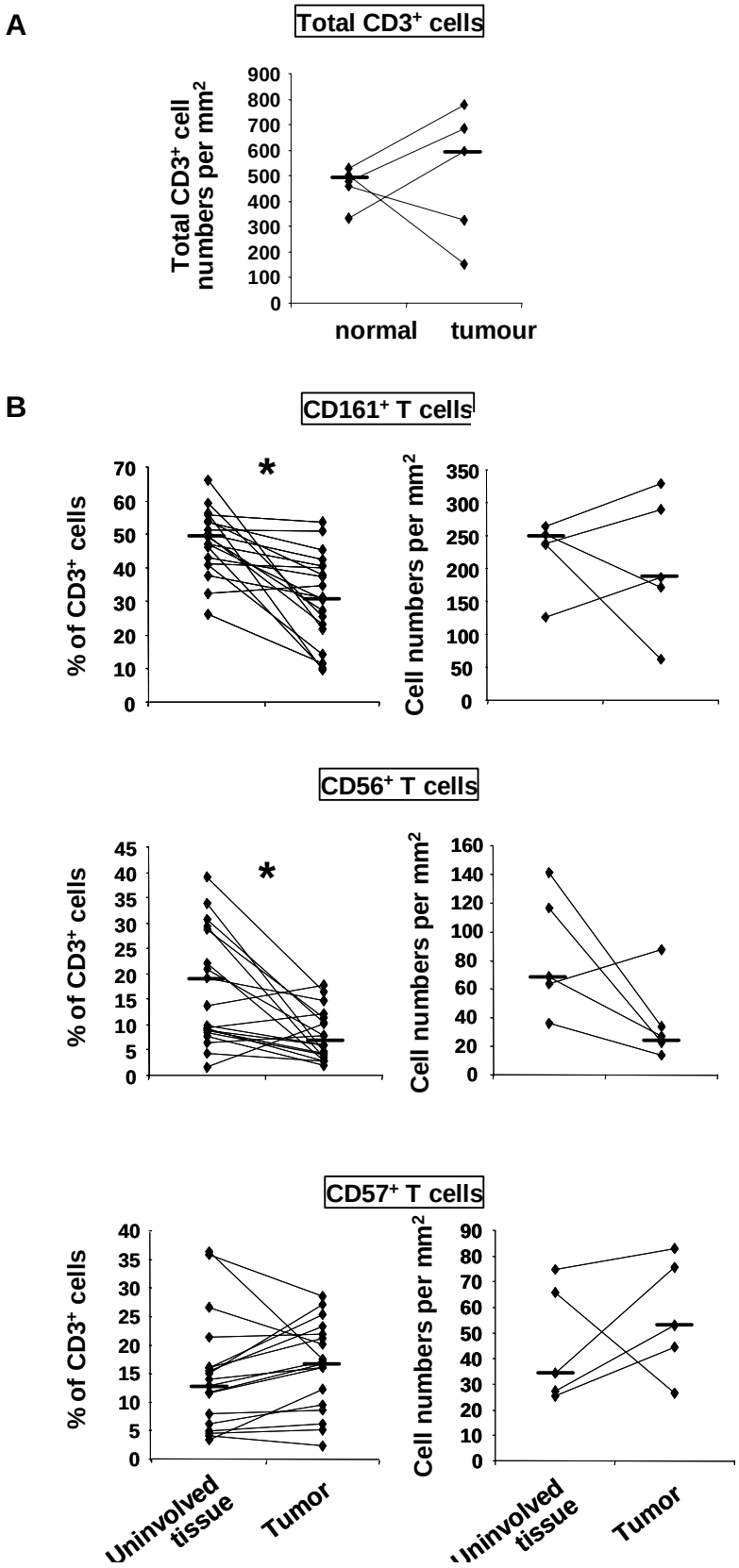
Figure 1




A
Colon
% of CD161+ T cells
Epithelium
LP
100
80
60
40
20
0
CD8
CD4
DN
αβ TCR
γδ TCR
CD56
B
Duodenum
% of CD161+ T cells
Epithelium
LP
CD8
CD4
DN
αβ TCR
γδ TCR
CD56
C
Colon (combined epithelium and LP)
% of cells
HLA-DR
CD25
CD69
CD161+ T cells
CD161- T cells
30
25
20
15
10
5
0
Fas ligand


A
Vα24+ T cells
Vα24+Vβ11+ T cells
% of CD3+ cells
4
3.5
3
2.5
2
1.5
1
0.5
0
E
LP
Blood
B
Colon
Blood
Liver
0.003%
0.017%
0.8%
Vα24
Vβ11
VA24 PE
VB11 FITC
C
Vehicle
α-GalCer
PHA
*
*
0
1
2
3
4
5
IFN-γ (ng/ml)


A
CD161
45%
IFN-γ
65%
TNF-α
1
IL-4
2%
IgG control
B
% of cells
IFN-γ
CD161+ T cells
CD161- T cells
E
LP
TNF-α
IL-4
*
*


A
Total CD3+ cells
Total CD3+ cell numbers per mm2
900
800
700
600
500
400
300
200
100
0
normal
tumour
B
CD161+ T cells
% of CD3+ cells
70
60
50
40
30
20
10
0
*
Cell numbers per mm2
350
300
250
200
150
100
50
0
CD56+ T cells
% of CD3+ cells
45
40
35
30
25
20
15
10
5
0
*
Cell numbers per mm2
160
140
120
100
80
60
40
20
0
CD57+ T cells
% of CD3+ cells
40
35
30
25
20
15
10
5
0
Cell numbers per mm2
90
80
70
60
50
40
30
20
10
0
Uninvolved tissue
Tumor
Uninvolved tissue
Tumor