LETTER

# Characterisation of innate lymphoid cell subsets infiltrating colorectal carcinoma

We read with great interest the paper by de Vries *et al*[1] which provides an interesting and unbiased characterisation of the immune contexture of colorectal cancer (CRC), the third most common cancer worldwide.[2] By mass cytometric analysis together with single cell RNA sequencing, the authors identified several clusters of immune cells infiltrating CRC. They show that natural killer (NK) cells (identified as $CD127^{-}CD56^{+}CD45RO^{+}$) are the prevalent innate lymphoid cell (ILC) population. NK cells are cytotoxic cells and can be distinguished from the non-cytotoxic $CD127^{+}$ 'helper' ILC subsets (hILCs), which are specialised in the secretion of different sets of cytokines.[3] Because of the low numbers of $CD127^{+}$ hILCs, the authors did not characterise further hILC subsets. However, given their capacity to rapidly respond to environmental signals and pathogenic challenges, hILCs serve as important sentinels of mucosal tissue homeostasis.[4] Therefore, their possible role both in CRC pathogenesis and in antitumour response has yet to be determined. Data on the ILC subset composition in CRC may allow improvement in therapeutic strategies for the control of this tumour by either suppressing or harnessing ILC function.

In this context, we analysed ILC subsets in samples from 33 resected primary CRC (online supplementary table 1) in comparison with matched normal mucosa (from the same patient), sampled 10 cm distant from the tumour (see online supplementary data). Single cell suspensions were analysed by multiparametric flow cytometry (online supplementary figure 1). No difference was found in the number of leukocytes ($CD45^{+}$ cells) infiltrating the normal or tumour tissues (figure 1A). Among lineage (CD34, CD123, CD3, CD19, CD14) negative cells, we gated on $CD56^{+}CD127^{-}$ cells (NK cells), and on both $CD56^{+}$ and $CD56^{-}$ $CD127^{+}$ cells (hILCs). While NK cell numbers were similar in the normal and tumour tissues (figure 1B), a significant decrease in the overall number and frequency of hILCs among the total innate lymphocytes was detected (figure 1B–E). In agreement with the conventional classification of ILCs,[3] we further gated hILCs into $CD117^{-}CRTH2^{-}$ (ILC1s), $CD117^{bimodal}CRTH2^{+}$ (ILC2s) and $CD117^{+}CRTH2^{-}$ (ILC3s). As shown in figure 1D–F, the hILC subset composition was different between the normal and tumour samples. In the normal colon mucosa, ILC3s represented the most abundant subset, followed by ILC1s and ILC2s (the least represented subset among hILCs). In tumour samples, there was a marked reduction in total hILCs that was mainly reflecting a loss of interleukin (IL)-22-producing ILC3s (figure 1D–F). The ILC2 frequency was also low in tumour tissues, while the ILC1/ILC3 proportion was altered, reflecting both the decrease of ILC3s and the increase of ILC1s (figure 1D,E). Immunohistochemical analysis of epithelial crypts revealed higher numbers of infiltrating ILC3s ($RORγt^{+}CD3^{-}$) in the normal as compared with the tumour tissues

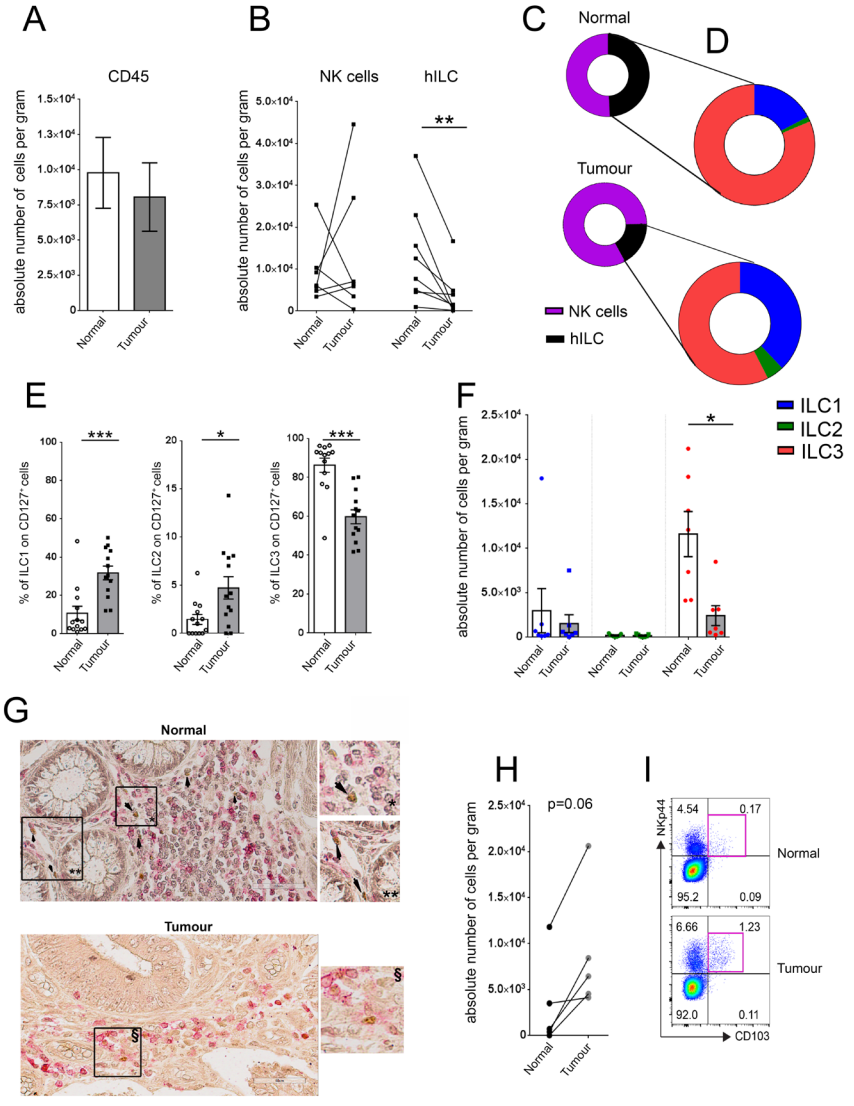


**Figure 1** hILCs infiltrate CRC. After Percoll, single cell suspensions of normal and tumour colon biopsies from patients with CRC were stained and analysed by flow cytometry. (A, B) Absolute number of $CD45^{+}$ cells (n=10), NK cells and hILC ($CD127^{+}$) (n=8) per gram. (C, D) Proportion of NK cells (purple) and hILC (black) among live $CD45^{+}$ cells and proportion of ILC1 (blue), ILC2 (green) and ILC3 (red) among hILCs (n=13). (E) Frequency of ILC1, ILC2 and ILC3 among live/$CD45^{+}$/$lin^{-}$ (CD3, CD19, CD14, CD34, CD123) $CD127^{+}$ cells (n=13). (F) Absolute number of ILC1, ILC2 and ILC3 (n=7) per gram. (G) Immunohistochemical analysis of normal and tumorous specimens from a patient with CRC using RORγt (brown) and CD3 (red). The arrows indicate $RORγt^{+}CD3^{-}$ ILC3. Scale bars: 50 mm. (H) Absolute number of ieILC1 per gram (n=5) and (I) representative dot plots showing ieILC1s gated as live/$CD45^{+}$/$lin^{-}$ (CD3, CD19, CD14) $NKp44^{+}CD103^{+}$ cells. CRC, colorectal cancer; hILC, helper ILC; ieILC1, intraepithelial ILC1; ILC, innate lymphoid cell; NK, natural killer; RORγt, RAR-related orphan receptor gamma. * p<0.05, ** p<0.01, *** p<0.001.

(figure 1G). ILC1s are characterised by the secretion of the proinflammatory cytokines interferon (IFN)-γ and tumour necrosis factor-α,[5] while ILC3s are thought to play a protective role against cancer as suggested by different studies.[6 7] Thus, their sharp reduction, together with the increase of ILC1s (which may favour chronic intestinal inflammation), could play a role in malignant transformation/tumour progression. Notably, the altered ILC1/ILC3 balance may be, at least in part, dependent on the plasticity of ILC3s driven by the CRC microenvironment in which cytokines such as IL-1β, IL-15 and IL-12 may convert ILC3s into IFN-γ-producing ILC1-like cells.[5 8] In addition, another subset of IFN-γ-producing ILC1 ($NKp44^+CD103^+CD127^-$), called intraepithelial ILC1 and previously identified in intestinal lamina propria,[9] was also enriched in the tumour as compared with the normal tissues (figure 1H,I).

In conclusion, our data complement the study by de Vries *et al*,[1] providing information on the hILC populations present in CRC tissues. Our data may offer a clue for therapeutic strategies, for example, harnessing ILC3s and/or targeting ILC1s. It will also be important to define whether altered ILC1/ILC3 proportions in CRC samples may have a prognostic value.

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**Contributors** PC designed the research, performed the experiments and revised the manuscript. PO performed the immunohistochemical experiments. LQ and NT analysed the data and wrote the manuscript. RV, RB and AP selected and processed the samples and revised the manuscript. SS provided samples from the patients. MCM critically revised the manuscript. LM wrote the manuscript. PV conceived and supervised the research and wrote the paper.

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## REFERENCES

1 de Vries NL, van Unen V, Ijsselsteijn ME, *et al*. High-Dimensional cytometric analysis of colorectal cancer reveals novel mediators of antitumour immunity. *Gut* 2019. doi:10.1136/gutjnl-2019-318672. [Epub ahead of print: 03 Jul 2019].
2 Brenner H, Chen C. The colorectal cancer epidemic: challenges and opportunities for primary, secondary and tertiary prevention. *Br J Cancer* 2018;119:785–92.
3 Vivier E, Artis D, Colonna M, *et al*. Innate lymphoid cells: 10 years on. *Cell* 2018;174:1054–66.
4 Atreya I, Kindermann M, Wirtz S. Innate lymphoid cells in intestinal cancer development. *Semin Immunol* 2019;41:101267.
5 Bernink JH, Krabbendam L, Germar K, *et al*. Interleukin-12 and -23 Control Plasticity of CD127(+) Group 1 and Group 3 Innate Lymphoid Cells in the Intestinal Lamina Propria. *Immunity* 2015;43:146–60.
6 Carrega P, Loiacono F, Di Carlo E, *et al*. NCR(+)ILC3 concentrate in human lung cancer and associate with intratumoral lymphoid structures. *Nat Commun* 2015;6:8280.
7 Tumino N, Martini S, Munari E, *et al*. Presence of innate lymphoid cells in pleural effusions of primary and metastatic tumors: functional analysis and expression of PD-1 receptor. *Int J Cancer* 2019;145:1660–8.
8 Cella M, Gamini R, Sécca C, *et al*. Subsets of ILC3-ILC1-like cells generate a diversity spectrum of innate lymphoid cells in human mucosal tissues. *Nat Immunol* 2019;20:980–91.
9 Fuchs A, Vermi W, Lee JS, *et al*. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-γ-producing cells. *Immunity* 2013;38:769–81.

| Code | TNM stage | Dukes stage | Grade | MSI | Localization | Sex |
|---|---|---|---|---|---|---|
| #1 | IIIc | C2 | 3 | | RT | F |
| #2 | IIa | B2 | 4 | yes | TR | M |
| #3 | IIIb | C2 | 2 | | RT | F |
| #4 | IIa | B2 | 2 | | DX | F |
| #5 | IIa | B2 | 2 | yes | DX | M |
| #6 | IIIb | C2 | 2 | | RT | M |
| #7 | IIa | B2 | 2 | | SG | M |
| #8 | IIb | B2 | 2 | | SG | M |
| #9 | IIa | B2 | 2 | yes | DX | F |
| #10 | IIa | B2 | 2 | | DX | M |
| #11 | IIa | B2 | 2 | | SG | M |
| #12 | IIa | B2 | 2 | | RT | M |
| #13 | IIb | B2 | 2 | | TR | M |
| #14 | IIb | B2 | 2 | | DX | M |
| #15 | IIa | B2 | 3 | | DX | M |
| #16 | IIa | B2 | 2 | | SG | M |
| #17 | IIIb | C2 | 2 | | SG | M |
| #18 | IIa | B2 | 2 | yes | DX | F |
| #19 | IIa | B2 | 1 | | SG | M |
| #20 | IVb | D | 3 | yes | DX | M |
| #21 | IIa | B2 | 2 | | SG | M |
| #22 | IIIc | C2 | 3 | | RT | M |
| #23 | IIIb | C2 | 2 | | RT | M |
| #24 | IIa | B2 | 2 | | SG | F |
| #25 | IIa | B2 | 1 | | RT | F |
| #26 | IIIb | C2 | 2 | | SG | M |
| #27 | IIIb | C2 | 2 | | RT | M |
| #28 | IIa | B2 | 2 | | TR | M |
| #29 | IIIc | C2 | 3 | | DX | F |
| #30 | IIa | B2 | 3 | yes | DX | F |
| #31 | IIa | B2 | 2 | | RT | M |
| #32 | IIa | B2 | 2 | | RT | M |
| #33 | IIIb | C2 | 3 | yes | DX | F |

## SUPPLEMENTAL INFORMATION

### Materials and Methods

### Patients and cells

Tissue samples were obtained from 33 CRC patients undergoing therapeutic intervention at the Unit of Oncological Surgery, IRCCS-AOU San Martino-IST, Genoa, provided informed consent (the study was approved by the institutional and regional ethical committee, PR163REG2014). All patients gave consent in according to the Declaration of Helsinki. Samples were used for preparation of cell suspensions and immunohistochemistry. Cells suspensions were obtained as previously described [1]. Lymphocytes were obtained by density Percoll (Sigma-Aldrich) gradient centrifugation.

### Flow cytometry analyses and monoclonal antibodies

Cells were stained with the following mAbs: CD294 (CRTH2), CD117, CD123 and purchased from Miltenyi; CD19 and CD45 purchased from BD, CD94-FITC; CD127-BrilliantViolet421; NKp44, CD117, CD103 and CD127 purchased from BioLegend: CD56, CD34, CD14, CD19, CD3 and NKp44 purchased from IL-Beckman Coulter; and Live/dead fixable aqua dead purchased from Invitrogen/Molecular Probes. All samples were analyzed on Gallios (Beckman Coulter). Data analysis was done using FlowJo software (TreeStar Inc.) and Cytexpert (Beckman Coulter).

### Immunohistochemistry

Paraffin-embedded samples from 20 CRC patients were analyzed. Immunohistochemistry (IHC) was performed on 4-µm-thin sections, deparaffinized in xylene, and treated with Peroxo-Block (Thermo Fisher Scientific) to quench endogenous peroxidase. An anti CD3 (SP7) rabbit polyclonal antibody (Thermoscientific) and anti RORγt mAb (Millipore) were used as primary antibodies on a Leica Bond RX immunostainer and detected with ChromoPlex 1 Dual Detection for BOND (Leica). Images of slides were acquired using Aperio AT2 scan (Leica).

### Statistical analyses

The data obtained in multiple experiments are reported as absolute number or percentages ± SEM (standard error of the mean). Statistical analyses were performed with GraphPad Prism 7 software

(La Jolla, CA). Wilcoxon paired non-parametric test was used. We considered significant p-values of less than 0.05 (*), less than 0.01 (**), less than 0.001 (***) or less than 0,0001 (****). Where not indicated, data were not statistically significant.

**Supplementary Table 1. Clinical and pathological data of CRC patients**

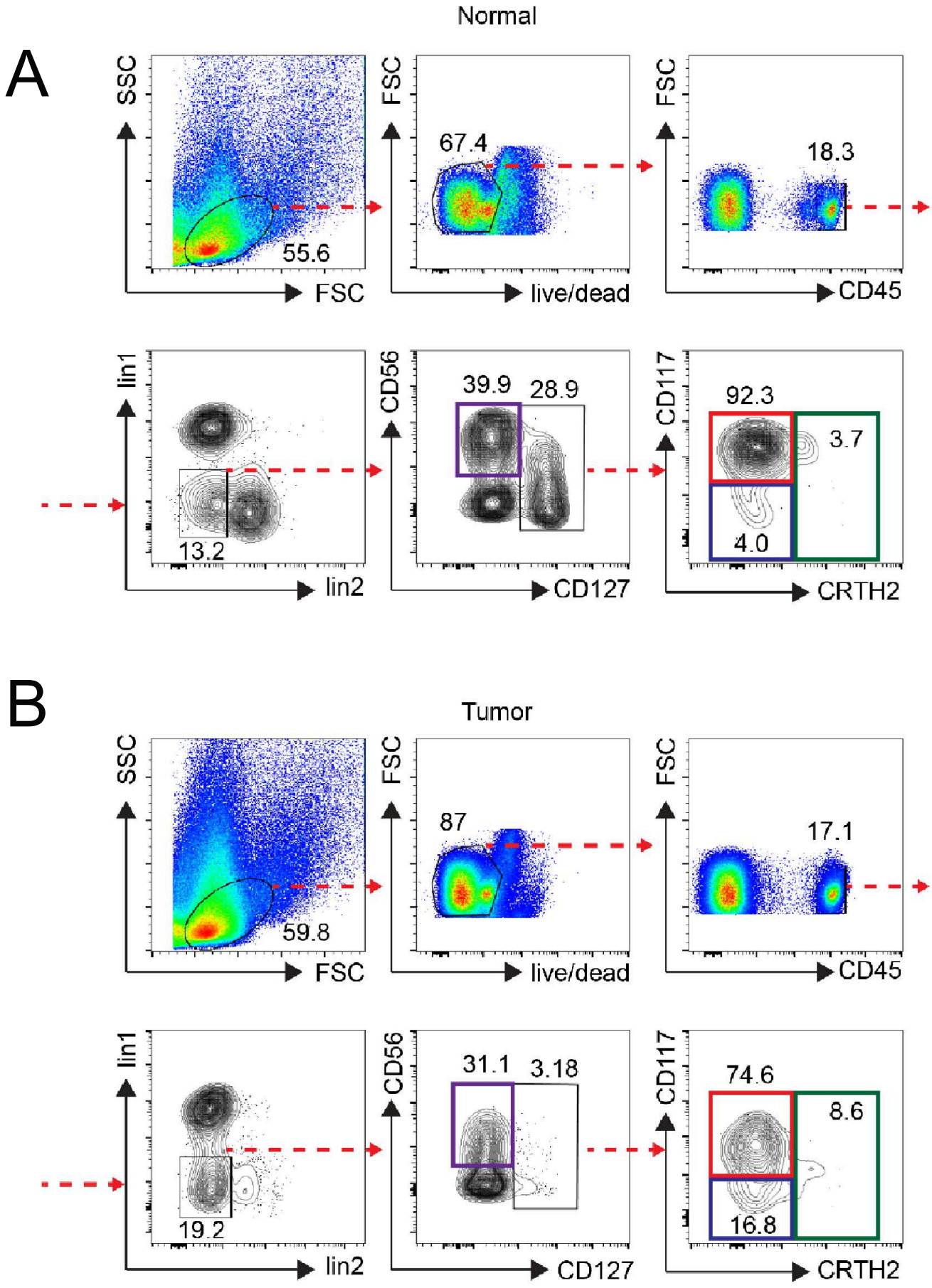
Clinical information about CRC cohort and surgical sampling. TNM and Dukes staging, tumor grade, microsatellite instability (MSI), anatomical localization of the tumor (DX: right or ascending; TR: transverse; SG: sigmoid; RT: rectum) and sex of patients are shown.

**Supplementary Figure 1**

Gating strategy to identify different ILC subsets in normal (A) and tumor (B) biopsies. NK cells are in purple, ILC1 in blue, ILC2 in green and ILC3 in red. Lin1 includes CD3 and CD14. Lin2 includes CD34, CD123 and CD19. Numbers indicate the frequency of different subsets in parent population.

**Supplemental Reference**

1 Costa D, Vene R, Benelli R, Romairone E, Scabini S, Catellani S*, et al.* Targeting the Epidermal Growth Factor Receptor Can Counteract the Inhibition of Natural Killer Cell Function Exerted by Colorectal Tumor-Associated Fibroblasts. Frontiers in immunology 2018;**9**:1150.


A
Normal
SSC
FSC
55.6
FSC
67.4
live/dead
FSC
18.3
CD45
lin1
13.2
lin2
CD56
39.9
28.9
CD127
CD117
92.3
3.7
4.0
CRTH2
B
Tumor
SSC
FSC
59.8
FSC
87
live/dead
FSC
17.1
CD45
lin1
19.2
lin2
CD56
31.1
3.18
CD127
CD117
74.6
8.6
16.8
CRTH2