Infiltration by IL22-Producing T Cells Promotes Neutrophil Recruitment and Predicts Favorable Clinical Outcome in Human Colorectal Cancer

Nadia Tosti[1], Eleonora Cremonesi[2], Valeria Governa[2], Camilla Basso[3], Venkatesh Kancherla[1], Mairene Coto-Llerena[1], Francesca Amicarella[2], Benjamin Weixler[4], Silvio Däster[5], Giuseppe Sconocchia[6], Pietro Edoardo Majno[3], Dimitri Christoforidis[3], Luigi Tornillo[1], Luigi Terracciano[1], Charlotte K.Y. Ng[1,2], Salvatore Piscuoglio[1,7], Markus von Flüe[7,8], Giulio Spagnoli[2,6], Serenella Eppenberger-Castori[1], Giandomenica Iezzi[2,3], and Raoul Andre Droeser[8]

ABSTRACT

Immune cell infiltration in colorectal cancer effectively predicts clinical outcome. IL22, produced by immune cells, plays an important role in inflammatory bowel disease, but its relevance in colorectal cancer remains unclear. Here, we addressed the prognostic significance of IL22$^+$ cell infiltration in colorectal cancer and its effects on the composition of tumor microenvironment. Tissue microarrays (TMA) were stained with an IL22-specific mAb, and positive immune cells were counted by expert pathologists. Results were correlated with clinicopathologic data and overall survival (OS). Phenotypes of IL22-producing cells were assessed by flow cytometry on cell suspensions from digested specimens. Chemokine production was evaluated *in vitro* upon colorectal cancer cell exposure to IL22, and culture supernatants were used to assess neutrophil migration *in vitro*. Evaluation of a testing ($n = 425$) and a validation TMA ($n = 89$) revealed that high numbers of IL22 tumor-infiltrating immune cells were associated with improved OS in colorectal cancer. *Ex vivo* analysis indicated that IL22 was produced by CD4$^+$ and CD8$^+$ polyfunctional T cells, which also produced IL17 and IFNγ. Exposure of colorectal cancer cells to IL22 promoted the release of the neutrophil-recruiting chemokines CXCL1, CXCL2, and CXCL3 and enhanced neutrophil migration *in vitro*. Combined survival analysis revealed that the favorable prognostic significance of IL22 in colorectal cancer relied on the presence of neutrophils and was enhanced by T-cell infiltration. Altogether, colorectal cancer–infiltrating IL22-producing T cells promoted a favorable clinical outcome by recruiting beneficial neutrophils capable of enhancing T-cell responses.

Introduction

Colorectal cancer is the third most common cause of cancer-related death worldwide (1). Infiltration of immune cells into colorectal cancer tumors predicts clinical outcome more effectively than tumor–node–metastasis staging (2). Infiltration by cytotoxic T cells, Th type 1 cells, T regulatory cells, and neutrophils associates with favorable outcome in human colorectal cancer (3–6), whereas the role of IL17-producing T cells (Th17) is still debated (2, 7).

IL22 is a cytokine of the IL10 family produced by different cell types of the innate immune system, including group 3 innate lymphoid cells (ILC3), and of the adaptive immune system, including Th17, naïve CD4$^+$ T cells polarized upon exposure to TNFα and IL6, also known as Th22 cells (8), and CD8$^+$ T cells (9, 10). IL22 receptors, including IL22R αchain and IL10Rβ chain, are uniquely expressed by keratinocytes and a variety of epithelial cells, including intestinal cells (11). IL22 plays key roles in wound healing and tissue repair, and in the maintenance of the "barrier" functions of skin and of intestinal and bronchial epithelial layers (12–14). In these anatomic regions, IL22 synergizes with IL17 and TNFα to promote the expression of proteins involved in host defense (15–17) and innate immunity against bacterial infections. IL22 also induces epithelial cell proliferation and upregulation of genes encoding prosurvival molecules (18–21), and may protect the liver, intestine, and lungs from tissue destruction (19–24). Interestingly, IL22 also plays a role in the maintenance of host–microbiota symbiosis (25).

The fact that IL22 promotes inflammation and concomitantly prevents tissue destruction is intriguing. Notably, exogenous IL22 delivery is sufficient to promote inflammation in mice (26, 27). The functional relevance of IL22 depends on specific tissue microenvironment. For example, in the absence of IL17, IL22 promotes tissue repair in the lung (18). In the colon, IL22 protects against experimental chronic colitis and promotes intestinal wound healing following acute

[1]Institute of Pathology, University Hospital of Basel, University of Basel, Basel, Switzerland. [2]Department of Biomedicine, University of Basel, Basel, Switzerland. [3]Department of Surgery, Ente Ospedaliero Cantonale and Università Svizzera Italiana, Lugano, Switzerland. [4]Klinik für Allgemein-, Viszeral- und Gefäßchirurgie, Charité Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany. [5]Department of Colorectal Surgery, Royal Prince Alfred Hospital, Sydney, New South Wales, Australia. [6]Institute of Translational Pharmacology, National Research Council, Rome, Italy. [7]Department of Biomedicine, Visceral Surgery Research Laboratory, Clarunis, Basel, Switzerland. [8]University Center for Gastrointestinal and Liver Diseases, Clarunis, University of Basel, Basel, Switzerland.

Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

N. Tosti and E. Cremonesi, and G. Iezzi and R.A. Droeser contributed equally to this article.

Corresponding Authors: Raoul Andre Droeser, University Center for Gastrointestinal and Liver Diseases, Clarunis, 21 Spitalstrasse, Basel 4031, Switzerland. Phone: 41 61 777 7500; E-mail: raoulandre.droeser@clarunis.ch; and Giandomenica Iezzi, Department of Surgery, Ente Ospedaliero Cantonale and Faculty of Biomedicine, University of Southern Switzerland, c/o Institute of Research in Biomedicine, Via Murate 5, Bellinzona 6500, Switzerland. Phone: 41 91 821 00 62; E-mail: giandomenica.iezzi@eoc.ch

Cancer Immunol Res 2020;8:1452–62

doi: 10.1158/2326-6066.CIR-19-0934

intestinal injury (28). However, IL22 has also been suggested to play a role in the pathogenesis of a variety of human diseases, including psoriasis, arthritis, and inflammatory bowel disease (IBD; refs. 13, 29–31).

There is a paucity of data about the prognostic significance of IL22 in cancer (12, 32–34), in particular its role in human colorectal cancer remains unclear. In murine models, "uncontrolled" IL22 production promotes colorectal cancer development (33), possibly by direct effects on stem cells (35) or by enhancing cancer cell proliferation (36). Accordingly, in a murine model of colon cancer induced by administration of pathogenic *Helicobacter hepaticus* and carcinogenic azoxymethane in immunodeficient animals, IL22 produced by innate lymphoid cells mediates a protumorigenic effect via Stat3 activation in epithelial cells (37, 38). IL22 produced by human tumor-infiltrating lymphocytes also promotes colorectal cancer cell proliferation *in vivo* in a xenograft model (37). On the other hand, IL22 plays a key role in the control of genotoxic damage induced by carcinogens in colon epithelial stem cells, thereby limiting mutagenesis and cancer outgrowth (39).

Most of these studies have addressed direct effects of IL22 on tumor cells, whereas its potential ability to condition the colorectal cancer tumor microenvironment has not been explored in comparable detail (40). Prognostic significance of IL22 expression in human colorectal cancer, the overwhelming majority (>90%) of which is not associated to IBD or to clinically relevant chronic colitis, has not been investigated in large cohorts of patients. To fill this knowledge gap, in this study we used two tissue microarrays (TMA), collectively including >500 clinically annotated colorectal cancer specimens, to investigate the impact of IL22 on clinical outcome and its possible influence on tumor microenvironment composition.

Materials and Methods

TMA construction

The TMAs were constructed at the Pathology Biobank at the University Hospital of Basel (Basel, Switzerland). Unselected, nonconsecutive, formalin-fixed, paraffin-embedded primary colorectal cancer tissue blocks were used as donor blocks. Tissue cylinders with a diameter of 1 mm were punched from morphologically representative areas of each donor block and brought into one recipient paraffin block (30 × 25 mm), using the TMA GrandMaster (TMA-GM; 3D-Histech Ltd, Sysmex AG) technology. Each punch was derived from the center of the tumor in an area with no necrosis so that each TMA spot consisted of more than 50% tumor cells. Approval by the local ethics committee (Ethik Kommission beider Basel, EKBB) for the use of this clinically annotated TMA was obtained in advance, as stated in previous publications (6, 7, 41).

Clinicopathologic features

Clinicopathologic data for patients included in the TMAs were collected retrospectively in a nonstratified and nonmatched manner. The larger TMA set was a subset of a previously published TMA (42) including patients undergoing surgery from 1987 to 1996. The validation TMA was built with surgical specimens of patients who underwent surgery at the University Hospital of Basel (Basel, Switzerland) in the years from 2007 to 2012. Clinicopathologic characteristics are listed in Supplementary Tables S1 and S2. Overall survival (OS) was defined as primary endpoint. Available follow-up data for the testing and validation cohort had a mean event-free follow-up time of 115 and 36 months, respectively.

IHC

IHC IL22 staining was assessed on two TMAs consisting of 538 and 100 colorectal cancer specimens, respectively. After excluding samples for which the tissue punch was absent or had poor staining quality, 425 and 89 colorectal cancers samples, respectively, were available. Staining was performed using the BenchMark ULTRA IHC System (Ventana Medical Systems, Inc.), following the manufacturer's instructions, with an IL22-specific mAb (Creative Diagnostic #DCABH-2900, clone JNH9G22F3, dilution 1:100) and iVIEW-DAB as chromogen. Immunoreactivity was scored as number of tumor-infiltrating $IL22^+$ immune cells by experienced pathologists (L. Tornillo and L. Terracciano). IL17 and CD66b staining protocols have been reported in previous studies (6, 7).

Clinical specimen collection and processing

Freshly excised clinical specimens were collected from patients undergoing surgical treatment at University Hospital of Basel (Basel, Switzerland) and St. Claraspital (Basel, Switzerland). Informed written consent was obtained from all patients whose specimens were analyzed for this study.

Tumor or healthy tissue fragments were snap frozen for RNA extraction or enzymatically digested (2 mg/mL collagenase IV, Worthington Biochemical Corporation and 0.2 mg/mL DNAse I, Sigma-Aldrich, for 1 hour at 37°C) to obtain single-cell suspensions. In addition, peripheral blood mononuclear cells from healthy donors were isolated by density gradient centrifugation (Histopaque-1077, Sigma-Aldrich; 400 × *g* for 30 minutes at room temperature, without break). Use of human samples in this study was approved by local ethical authorities (Ethikkommission Nordwest und Zentralschweiz, EKNZ 2014–388).

The Cancer Genome Atlas analysis

Gene expression data [as fragments per kilobase of transcript per million mapped reads values (FPKM)] from 597 colorectal cancer samples and 51 normal colorectal mucosa specimens, were obtained from The Cancer Genome Atlas (TCGA) Genomics Data Commons harmonized data portal using *TCGA biolinks* R package (43). Clinical information regarding 597 colorectal cancers (see Supplementary Table S3) was retrieved from the Human Protein Atlas (44). After normalization, expression (FPKM values) of genes encoding IL22 and a panel of cytokines, chemokines, and immune markers was retrieved and correlations between immune markers and patients' OS were evaluated.

Flow cytometry and cell sorting

Cell suspensions obtained from colorectal cancer and tumor-free colonic mucosa were incubated with 50 ng/mL phorbol 12-myristate 13-acetate, 1 μg/mL ionomycin, and 5 μg/mL Brefeldin A (Sigma-Aldrich) for 5 hours. Cells were fixed with the Intracellular Fixation and Permeabilization Buffer Set (eBioscience), following the manufacturer's instructions, and surface stained with fluorochrome-conjugated antibodies specific for human CD3 (clone SK7), CD4 (clone SK3), CD8 (clone SK1), and CD56 (clone B159), all from BD Biosciences. Following permeabilization, intracellular staining with antibodies specific for human IFNγ (BD Bioscience, clone 25723.11), IL22 (R&D Systems, clone 142928), and IL17 (eBioscience, clone 64DEC17) was performed. Cells were analyzed by FACSCalibur or Fortessa Flow Cytometers (BD Biosciences). Primary colorectal cancer cells were sorted from tumor cell suspensions by magnetic microbeads conjugated to EpCAM-specific Antibodies (MACS MicroBeads, Miltenyi Biotec, catalog no. 130–061–101), following the manufacturer's

instructions. Cell purity was >97%, as evaluated by flow cytometry. Data were analyzed using FlowJo Software (Tree Star).

Real-time reverse transcription PCR assays

Total RNA was extracted from stored colorectal cancer tissues or sorted cell populations using NucleoSpin RNA Kit (Macherey-Nagel, catalog no. 740955.50), following the manufacturer's instructions, and quantified by Spectrophotometry (NanoDrop, Thermo Fisher Scientific). RNA was reverse transcribed using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen). Quantitative PCR was performed in the ABI prism 7700 sequence detection system, using SYBR Green (Roche) or TaqMan Universal Master Mix, No AmpErase UNG (Applied Biosystems), and commercially available primer sequences. All primers are listed in Supplementary Table S4. Each gene was assayed in duplicate wells, using 20 ng template each. Expression of individual genes was analyzed by using the $2^{-\Delta\Delta C_t}$ method (45), as relative to the expression of GAPDH housekeeping gene.

Cell lines

LS180, HT29, Colo205, HCT15, SW480, SW620, and DLD-1 human colorectal cancer cell lines were purchased from the European Collection of Cell Cultures (period 2013–2015), and immediately stored in liquid nitrogen. Cells used for individual experiments were thawed from original cryopreserved aliquots and maintained in culture, for a maximum of 10 passages, in RPMI1640 (Gibco) or, for HT29 in McCoy's 5A Medium (Sigma-Aldrich) or, for SW480 and SW620 in L15 Medium (Leibovitz, Sigma-Aldrich), supplemented with 10% FBS, GlutaMAX-I, and Kanamycin (Gibco). Absence of *Mycoplasma* contamination in cultured cells was verified by PCR testing prior to investigations.

Chemokine induction in colorectal cancer cell lines

Colorectal cancer cells from LS180 and HT29 established cell lines were plated in 24-well plates (Sigma-Aldrich, 3.5×10^5 cells/well in 0.5 mL) in culture media and then incubated with 10 and 100 ng/mL concentrations of IL22 (R&D Systems) at 37°C. After 4 hours, chemokine expression was assessed by quantitative PCR.

ELISA

Chemokine content in culture supernatants was assessed by ELISA using CXCL1 and CXCL3 DuoSet ELISA (R&D Systems, catalog nos. DY275 and DY801, respectively), following the manufacturer's instructions. Results were collected by Spectrophotometer (BioTek Instruments) using the SoftMax Pro 6 software.

Cell proliferation assay

Colorectal cancer cells from LS180 and HT29 established cell lines were plated in 24-well plates (Sigma-Aldrich, 10^5 cells/well in 0.5 mL) in RPMI1640 and McCoy's 5A Medium (Sigma-Aldrich), respectively, and then incubated with 10 or 100 ng/mL recombinant human IL22 (R&D Systems, catalog no. 782-IL/CF) for 4 days at 37°C. Cell proliferation was quantified by CyQUANT Cell Proliferation Assay (Thermo Fisher Scientific, catalog no. C7026), following the manufacturer's instructions.

Migration assay

$CD8^+$ T cells and neutrophils were sorted from peripheral blood of healthy donors by Magnetic Microbeads (MACS MicroBeads, Miltenyi Biotec, catalog no. 130-045-201, and EasySep Human Neutrophil Isolation Kit, Stemcell Technologies, catalog no. 17957, respectively), according to the manufacturer's instructions, to a purity of >98%, as confirmed by flow cytometry. Chemotaxis assays were performed using 96-well transwell plates with 5-μm pore size membranes (Corning Costar). Supernatants from LS180 or HT29 cells, left untreated or treated overnight with 10 or 100 ng/mL of IL22, were added to the bottom chambers (250 μL/well). In specific experiments, colorectal cancer cell line supernatants were depleted of CXCL1 and/or CXCL3, prior to use, by using specific capture antibodies (R&D Systems, clone 20326, catalog no. MAB275 and clone 49801, catalog no. MAB160, respectively). $CD8^+$ T cells and neutrophils (1.5×10^4 cells/well in 80 μL) were placed in the top chamber and allowed to migrate for 60 minutes at 37°C. The number of cells that migrated into the bottom chamber was quantified by flow cytometry. The extent of cell migration was expressed as a migration index, calculated as number of cells migrated toward supernatants/number of cells migrated toward control medium.

Statistical analysis

$IL22^+$ tumor-infiltrating immune cells were counted on each of the 425 and 89 colorectal cancer plus 16 nonmalignant cores, respectively. After having proven an association between the number of $IL22^+$ infiltrating cells and OS by univariate Cox regression, an optimal threshold was estimated by regression tree analysis (rpart Statistical Package Software R package, version 3.4.1, 2017-06-30). The obtained threshold was found to be almost equal to the median value. Subsequently, continuous values were dichotomized subdividing half of the collective as colorectal cancer with low or high IL22 immune infiltration. χ^2 or Fisher exact tests were used to determine the association between IL22 infiltration and clinicopathologic discrete features, as well as the Wilcoxon signed rank-sum test for comparison with continuous values. Survival curves were depicted according to the Kaplan–Meier method and compared with the log-rank test. Moreover, individual survival curves were compared one by one and the *P* values were adjusted according to the Benjamini–Hochberg method, which controls the FDR and the expected proportion of false discoveries among the rejected hypotheses (Survminer R package).

$IL17^+$ and $CD66b^+$ cells were evaluated as reported previously (6, 7). Upon staining with antibodies specific for IL17 (goat polyclonal anti-human IL17, R&D Systems) or CD66b (BioLegend, clone G10F5), numbers of positive cells per punch were scored by experienced pathologists. By regression tree analysis (rpart package), cut-off values for both markers, IL17 and CD66b, were set at 10 cells per punch. After dichotomization, Kaplan–Meier curves were plotted, and compared by log-rank test.

The assumption of proportional hazards was verified for all markers by analyzing the correlation of Schoenfeld residuals and the ranks of individual failure times. Any missing clinicopathologic information was assumed to be missing at random.

All *P* values were two-sided and considered significant at $P < 0.05$. Analyses were performed by using the Statistical Package Software R (version 3.4.1, 2017-06-30, http://www.r-project.org or higher) and GraphPad Prism 7 Software (GraphPad Software).

Results

High density of $IL22^+$ cells is associated with favorable prognosis in human colorectal cancer

IL22 protein expression was first evaluated by IHC on a testing TMA set including 425 primary colorectal cancer specimens (Supplementary Table S1). As expected, positive staining was clearly detected on infiltrating immune cells. However, a more diffuse staining was also

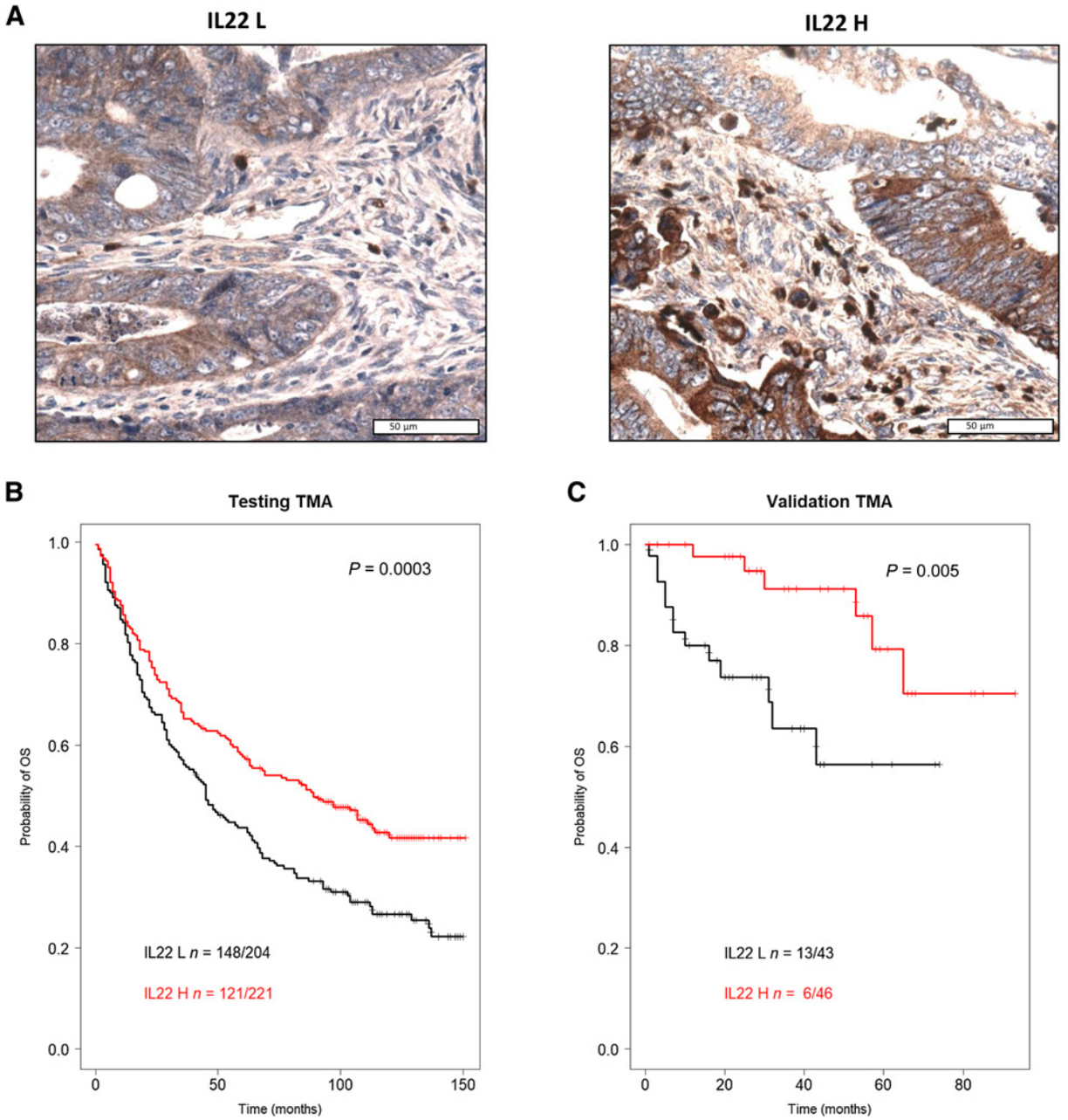

Figure 1.

High density of IL22$^+$ cells is associated with favorable prognosis in human colorectal cancer. **A,** Representative pictures of colorectal cancers with low (left) or high (right) infiltration by IL22$^+$ immune cells (scale bar, 50 µm). **B** and **C,** Kaplan–Meier curves depicting the probability of OS of patients dichotomized according to low or high number of IL22$^+$ colorectal cancer–infiltrating immune cells in the test TMA ($n = 425$) and in the validation TMA ($n = 89$). Statistical significance was assessed by log-rank test. H, high; L, low.

detectable on tumor cells (**Fig. 1A**). To clarify this issue, IL22 gene expression was investigated in colorectal cancer cells isolated from established cell lines with consistently negative results (Supplementary Fig. S1), suggesting that colorectal cancer cell positivity might be due to the IL22 fraction bound to its specific receptor on epithelial cells.

Therefore, we focused our analysis on tumor-infiltrating immune cells. IL22$^+$ cells were counted in each TMA core. IL22$^+$ cells were detectable within both normal colonic tissues and colorectal cancer, although in the latter case at a significantly higher density ($P = 0.039$). Observed continuous values ranged between 0 and 300 cells per core with median and mean values of 20 and 39 cells per core, respectively. Median value was used to dichotomize tumor specimens into IL22-low and -high groups. Representative examples of colorectal cancer displaying low and high infiltration are displayed in **Fig. 1A**. As

Table 1. Correlation of IL22$^+$ cell density with clinicopathologic features in $n = 425$ colorectal cancer specimens.

Characteristics	IL22 low		IL22 high		P^a
	$N = 221$	(100%)	$N = 204$	(100%)	
Age					
Years (median, mean)	71, 69.5	(40–90)	71, 70.5	(40–96)	$P = 0.318$
Sex					
Female	117	(52.9)	104	(51.0)	$P = 0.687$
Male	104	(47.1)	100	(49.0)	
Diameter					
mm (median, mean)	50, 52.4	(10–170)	50, 51.0	(7–160)	$P = 0.527$
Tumor location					
Left sided	136	(61.5)	138	(67.6)	$P = 0.167$
Right sided	85	(38.5)	65	(31.9)	
Histologic subtype					
Mucinous	9	(4.1)	19	(9.3)	$P = 0.494$
Nonmucinous	212	(95.9)	185	(90.7)	
pT stage					
pT1	7	(3.2)	8	(3.9)	$P = 0.043^b$
pT2	26	(11.8)	32	(15.7)	
pT3	140	(63.3)	138	(67.6)	
pT4	39	(17.6)	21	(10.3)	
pN stage					
pN0	99	(44.8)	112	(54.9)	$P = 0.059^c$
pN1	64	(29.0)	51	(25.0)	
pN2	48	(21.7)	35	(17.2)	
Tumor grade					
G1	7	(3.2)	2	(1.0)	$P = 0.255$
G2	193	(87.3)	185	(90.7)	
G3	11	(5.0)	12	(5.9)	
Vascular invasion					
Absent	150	(67.9)	155	(76.0)	$P = 0.099$
Present	62	(28.1)	44	(21.6)	
Tumor border					
Pushing	54	(24.4)	64	(31.4)	$P = 0.151$
Infiltrating	156	(70.6)	135	(66.2)	
PTL inflammation					
Absent	167	(75.6)	145	(71.1)	$P = 0.162$
Present	45	(20.4)	54	(26.5)	
Microsatellite stability					
Deficient	30	(13.6)	33	(16.2)	$P = 0.452$
Proficient	191	(86.4)	171	(83.8)	
5-year survival rate					
(95% CI)	0.43	0.36–0.50	0.58	0.51–0.65	$P = 0.004$

Abbreviations: CI, confidence interval; PTL, peritumoral lymphocytic.
[a]Correlation between the IL22-low and IL22-high subgroup. Percentage may not add to 100% due to missing values of some variables.
[b]Overall difference in subgroup analysis due to T4.
[c]Overall difference in subgroup analysis due to N0.

summarized in **Table 1**, the presence of high numbers of IL22$^+$ cells was slightly associated with lower T stage ($P = 0.043$). Instead, no significant association was found with histologic subtype ($P = 0.494$), lymph node metastases ($P = 0.059$), tumor grade ($P = 0.255$), vascular invasion ($P = 0.099$), tumor border configuration ($P = 0.151$), and microsatellite instability ($P = 0.452$).

Survival analysis at 5 years showed that patients with tumors characterized by high numbers of IL22$^+$ infiltrating cells had significantly higher survival probability than those with tumors displaying low IL22$^+$ cell numbers [OS, 58%; confidence interval (CI), 51%–65.0% vs. 43%; CI, 36%–50%; $P = 0.004$; **Table 1**].

Kaplan–Meier survival curve analysis revealed that the favorable prognostic effect of IL22$^+$ cell infiltration remained constant over time ($P = 0.0003$; **Fig. 1B**). The positive prognostic impact of high IL22$^+$ cell infiltration was confirmed upon staining of a TMA with an independent validation cohort (Supplementary Table S2) of 89 patients with colorectal cancer ($P = 0.005$; **Fig. 1C**). In this second cohort, for which data regarding adjuvant treatment were also available, multivariate analysis showed that IL22 retained an impact on OS irrespective of adjuvant chemotherapy ($P = 0.02$; Supplementary Table S5).

IL22 was expressed by polyfunctional T cells predictive of prolonged survival

We next investigated phenotypes and functions of colorectal cancer–infiltrating IL22$^+$ cells. Flow cytometric analysis of single-cell suspensions obtained from colorectal cancer clinical specimens revealed that IL22$^+$ cells were mostly found within conventional CD3$^+$

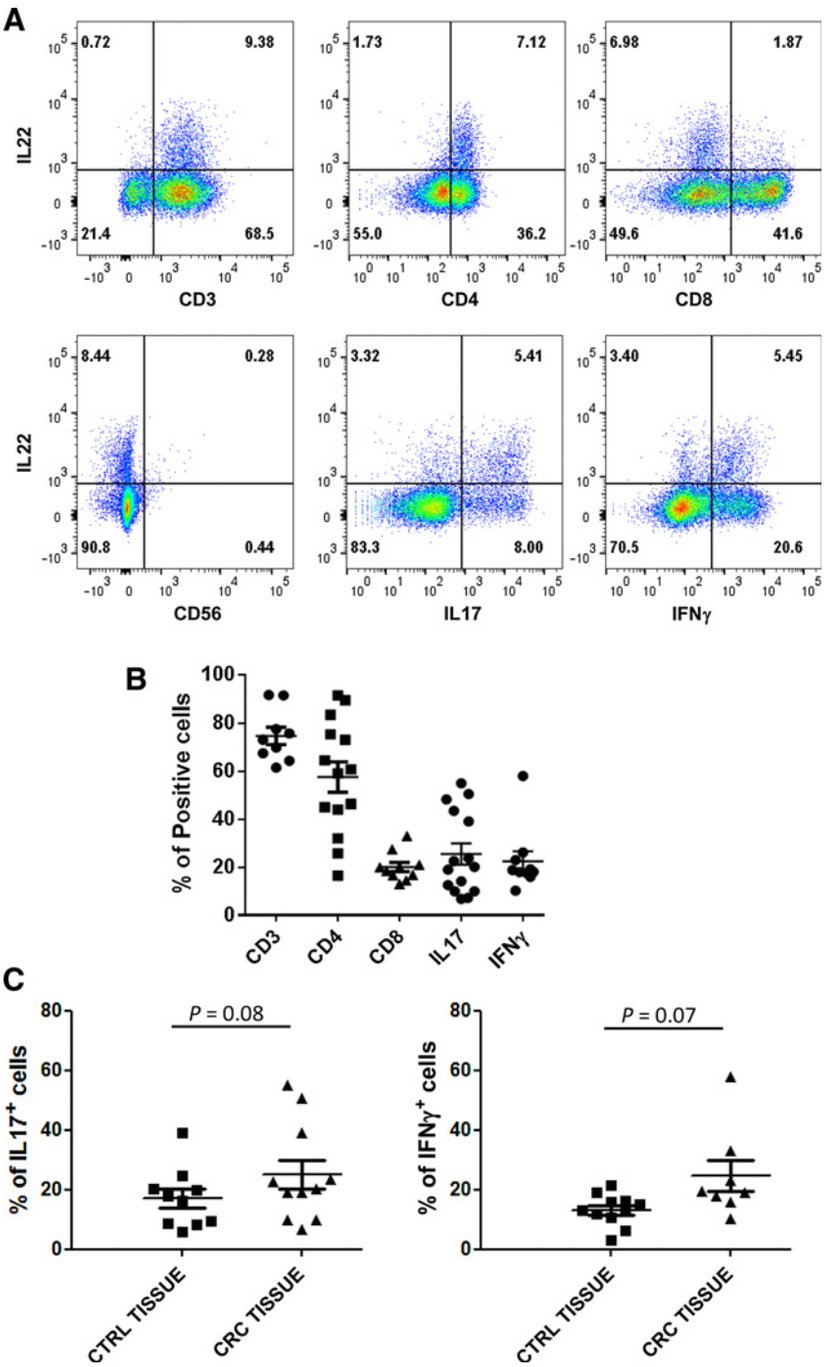

Figure 2.

IL22 is expressed by conventional polyfunctional T cells. Single-cell suspensions obtained from freshly excised clinical specimens of colorectal cancer and tumor-free colonic tissues were surface stained with antibodies specific for CD3, CD4, CD8, and CD56 and intracellularly stained for IL17 and IFNγ, and analyzed by flow cytometry. **A,** Representative phenotypic analysis. **B,** Percentages of cells positive for the indicated marker or cytokine within the IL22$^+$ gate ($n \leq 15$). **C,** Percentages of IL17$^+$ or IFNγ$^+$ cells within the IL22$^+$ cell gate in colorectal cancer (CRC TISSUE) and corresponding tumor-free colonic tissues (CTRL TISSUE). Means and SDs are indicated by bars. Statistical significance was assessed by the Wilcoxon signed rank test.

T cells and expressed CD4 and, to lower percentages, CD8 molecules (**Fig. 2A** and **B**). Expression of IL22 at the single-cell level, based on intracellular cytokine staining, was not significantly increased in cells infiltrating tumors as compared with those present in normal tissues (median fluorescence intensity within gated CD3$^+$ IL22$^+$ cells in tumors vs. nontumor tissues: 54 ± 32 vs. 53 ± 47; $P = 0.88$ and mean fluorescence intensity within CD3$^+$ IL22$^+$ cells in tumors vs. nontumor tissues: 125 ± 97 vs. 85 ± 63; $P = 0.15$). However, a large fraction of IL22$^+$ cells also showed production of additional cytokines, such as IL17 and IFNγ, thus indicating that they were polyfunctional T cells (**Fig. 2C**), as reported previously (7, 46). Notably, this subset of IL22-producing cells showed a trend toward an increase in colorectal cancer as compared with control tissues (**Fig. 2C**).

Consistent with their "Th17-like" phenotype, numbers of infiltrating IL22$^+$ cells were significantly higher within colorectal cancer characterized by high infiltration by IL17$^+$ cells (Supplementary Fig. S2A). In TCGA cohort, *IL22* gene expression significantly correlated with that of *IL17* gene ($r = 0.578$; $P < 0.0001$, see **Table 2**). In the TMA cohort, tumors displaying high densities of both IL22$^+$ and

Table 2. Correlations between expression of *IL22* gene and selected cytokine/chemokine genes in TCGA cohort ($n = 597$).

	Spearman correlation coefficient	P
IL17A	0.578	<0.00001
CXCL2	0.447	<0.00001
CXCL1	0.401	<0.00001
CXCL3	0.393	<0.00001
CXCL8	0.152	0.0002

IL17$^+$ cells were characterized by enhanced survival probability (Supplementary Fig. S2B).

IL22 stimulated colorectal cancer cells to release neutrophil-recruiting chemokines

TMA staining data indicated that colorectal cancer infiltration by IL22-producing T cells was associated with improved survival. This was an unexpected finding, because in murine colorectal cancer models, IL22 has mostly been shown to play a direct protumorigenic role, by enhancing tumor cell proliferation (35).

Indeed, we found that human colorectal cancer cells do express IL22 receptors, that is, IL22 Rα and IL10Rβ chains (Supplementary Fig. S3A and S3B), thus potentially responding to direct IL22-mediated effects. However, when we evaluated the direct effects of IL22 on LS180 and HT29 colorectal cancer cell lines, we did not observe any significant impact of IL22 on colorectal cancer cell proliferation *in vitro* (Supplementary Fig. S3C and S3D).

In contrast, IL22 treatment consistently increased gene expression of CXCL1, CXCL2, CXCL3 neutrophil-recruiting chemokines in two different colorectal cancer cell lines (**Fig. 3A**; Supplementary Fig. S4A). CXCL1 and CXCL3 protein release was also detectable in culture supernatants (**Fig. 3B**; Supplementary Fig. S4B), and enhanced neutrophil migration *in vitro* was accordingly observed (**Fig. 3C**).

Increased expression of genes encoding T-cell–recruiting chemokines, such as CCL22, CXCL9, and CXCL11, was also boosted by IL22 in LS180, but not in HT29 cells (**Fig. 3A**; Supplementary Fig. S4A).

IL22$^+$ T cells displayed cross-talk with neutrophils in colorectal cancer samples

On the basis of these findings, we hypothesized that a cross-talk between tumor and beneficial immune cell populations could underlie the favorable prognostic significance of IL22$^+$ cell infiltration in colorectal cancer.

Consistent with our *in vitro* data, we found a significant positive correlation between expression of *IL22* gene and that of *CXCL1* ($r = 0.401$; $P < 0.00001$), *CXCL2* ($r = 0.447$; $P < 0.00001$), and *CXCL3* ($r = 0.393$ and $P < 0.00001$) genes in colorectal cancer specimens from TCGA database (**Table 2**). Upon *ex vivo* analysis on primary colorectal cancer cells sorted from clinical specimens, we found that expression of genes encoding neutrophil-recruiting chemokines was significantly enhanced in tumor cells derived from samples displaying high IL22 expression as compared with those purified from tumors displaying no or low IL22 expression (**Fig. 3D**). Accordingly, increased CD66b expression, consistent with higher neutrophil densities, was detected in IL22-high versus IL22-low tumors (**Fig. 3E**).

In our TMA cohort, we observed that high neutrophil densities, as defined by CD66b marker–specific staining, were significantly ($P < 0.00001$) associated with higher IL22$^+$ colorectal cancer–infiltrating cell numbers (**Fig. 4A**) and high *IL22* gene expression (Supplementary Fig. S5A).

The positive prognostic significance of high IL22$^+$ cell infiltration in colorectal cancer was lost in the absence of CD66b$^+$ cells, thus indicating that the beneficial effect of IL22-producing cells required neutrophil recruitment (**Fig. 4B**). A similar trend ($P = 0.09$) was also detectable in TCGA data (Supplementary Fig. S5B).

Neutrophils costimulate antigen-driven activation of colorectal cancer–infiltrating CD8$^+$ T cells (6). Remarkably, the presence of high CD8$^+$ or CD3$^+$ cell infiltration, in addition to IL22$^+$ and CD66b$^+$ cells, further enhanced their prognostic significance (**Fig. 4C**; Supplementary Fig. S6).

Discussion

Tumor infiltration by immune cell populations characterized by different cytokine production profiles heavily impacts clinical outcome of human colorectal cancer (3, 4, 47, 48); however, the role of immune cells producing IL22, a cytokine involved in tissue repair processes at mucosal surfaces, has remained elusive.

In experimental murine models, direct effects of IL22 on epithelial stem cells and tumor cells associate with tumor progression. IL22 also plays a role in IBD pathogenesis, potentially resulting in colorectal cancer outgrowth (37, 38). However, only ≤2% of sporadic human colorectal cancers are associated with clinically relevant IBD (49, 50). The prognostic significance of tumor infiltration by IL22$^+$ cells in the majority of colorectal cancer cases, diagnosed in the absence of a clinically significant chronic colitis/inflammation, has not been thoroughly investigated. High expression of DOTL1, an IL22-induced methyltransferase, associates with poor patient survival (35). However, no data regarding the prognostic impact of *IL22* gene or protein expression were provided.

This is the first study evaluating the impact of colorectal cancer–infiltrating IL 22$^+$ cells on patient survival. Upon analysis of two independent cohorts including 425 and 89 primary colorectal cancer cases, respectively, we unexpectedly observed that high infiltration by IL22$^+$ cells was associated with favorable prognosis. Evaluation at transcriptional level in TCGA cohort also provided data in-line with our findings of protein expression from TMA. Thus, these independent analyses concur in indicating IL22 expression as favorable predictive factor in human colorectal cancer.

Phenotypic analysis revealed that IL22-producing cells mostly comprised of polyfunctional Th17 and CD8$^+$ T cells, producing IL17 and IFNγ, in addition to IL22. These findings are in-line with previous studies reporting IL22 production by Th17 cells in human sporadic colorectal cancers (35, 46). The role of IL17 and Th17 cells in colorectal cancer is still debated. IL17 is suggested to be protumorigenic in mice and negatively influences colorectal cancer prognosis in humans (51). However, colorectal cancer–infiltrating Th17 cells might play a dual role depending on their tissue localization (7). Here, we showed that IL17 was positively associated with favorable prognosis only in the presence of high density of IL22$^+$ tumor-infiltrating cells, thus indicating a distinct role of polyfunctional Th17 cells as compared with cells producing IL17 only. On the other hand, in accordance with previous studies (35, 40), CD3$^-$ IL22$^-$ cells, possibly including ILCs, appeared to represent a minor fraction of human colorectal cancer–infiltrating IL22-producing cells. In a previously reported IBD-associated colorectal cancer mouse model (35), IL22-producing ILCs sustained tumor progression in immunodeficient animals. It is conceivable that in colorectal cancer subtypes driven by distinct pathogenesis, IL22 is produced by different cell populations, possibly endowed with distinct functional roles and prognostic significance.

The observed association between infiltration by IL22$^+$ cells and improved prognosis contradicts previous studies showing a

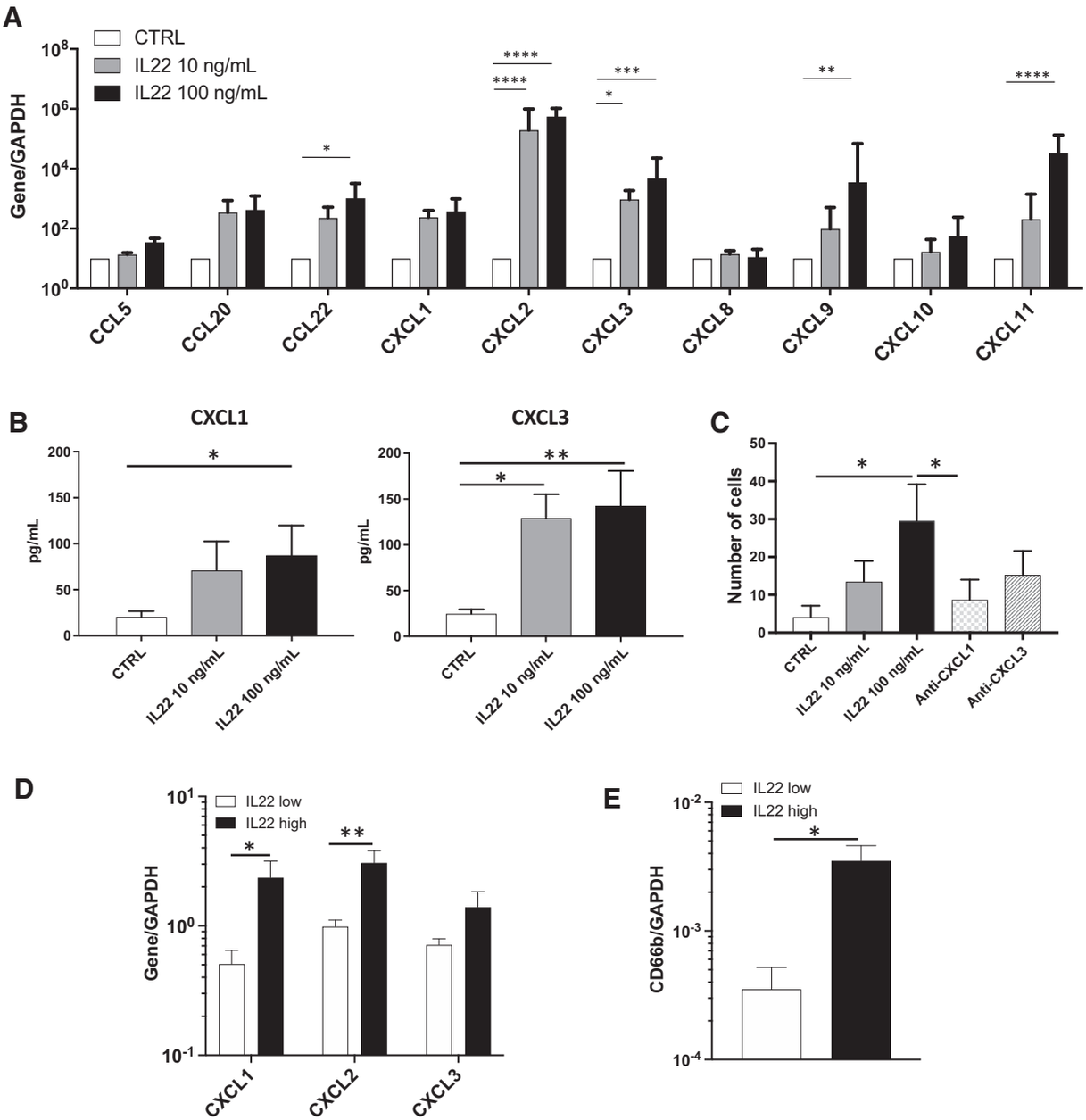

Figure 3.

IL22 enhances the release of neutrophil-recruiting chemokines by colorectal cancer cells. Colorectal cancer cells from the LS180 cell line were treated with IL22 (10 or 100 ng/mL), as indicated. **A,** After 4 hours, expression levels of genes encoding the indicated chemokines were analyzed by qRT-PCR, using *GAPDH* as reference gene. Means and SD from five independent experiments are shown. Statistical significance was assessed by two-way ANOVA test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). **B,** After an overnight culture, CXCL1 and CXCL3 chemokine contents in culture supernatants were measured by ELISA. Means and SD from three independent experiments are shown. Statistical significance was assessed by Mann–Whitney test (*, $P < 0.05$; **, $P < 0.01$). **C,** Migration of neutrophils, isolated from healthy donors, toward culture supernatants from LS180 cells, untreated (CTRL) or exposed to the indicated doses of IL22 for an overnight period, was assessed in the presence or absence of CXCL3- and CXCL1-blocking reagents. Means and SD from three independent experiments are shown. Statistical significance was assessed by one-way ANOVA test (*, $P < 0.05$). **D,** Expression of CXCL1, CXCL2, and CXCL3 genes was evaluated in primary colorectal cancer cells sorted, based on EpCAM expression, from cell suspensions obtained upon enzymatic digestion of fresh colorectal samples. IL22 gene expression was assessed in corresponding whole colorectal cancer tissues, and, using the median of detected values as cutoff, tumors were classified as IL22 high or IL22 low. Expression of the indicated chemokines genes in colorectal cancer cells from IL22-high versus IL22-low tumors is depicted. **E,** Expression of the neutrophil marker CD66b in whole colorectal cancer tissues of IL22-high versus IL22-low tumors. All indicated *P* values were assessed by Mann–Whitney test (*, $P < 0.05$; **, $P < 0.01$).

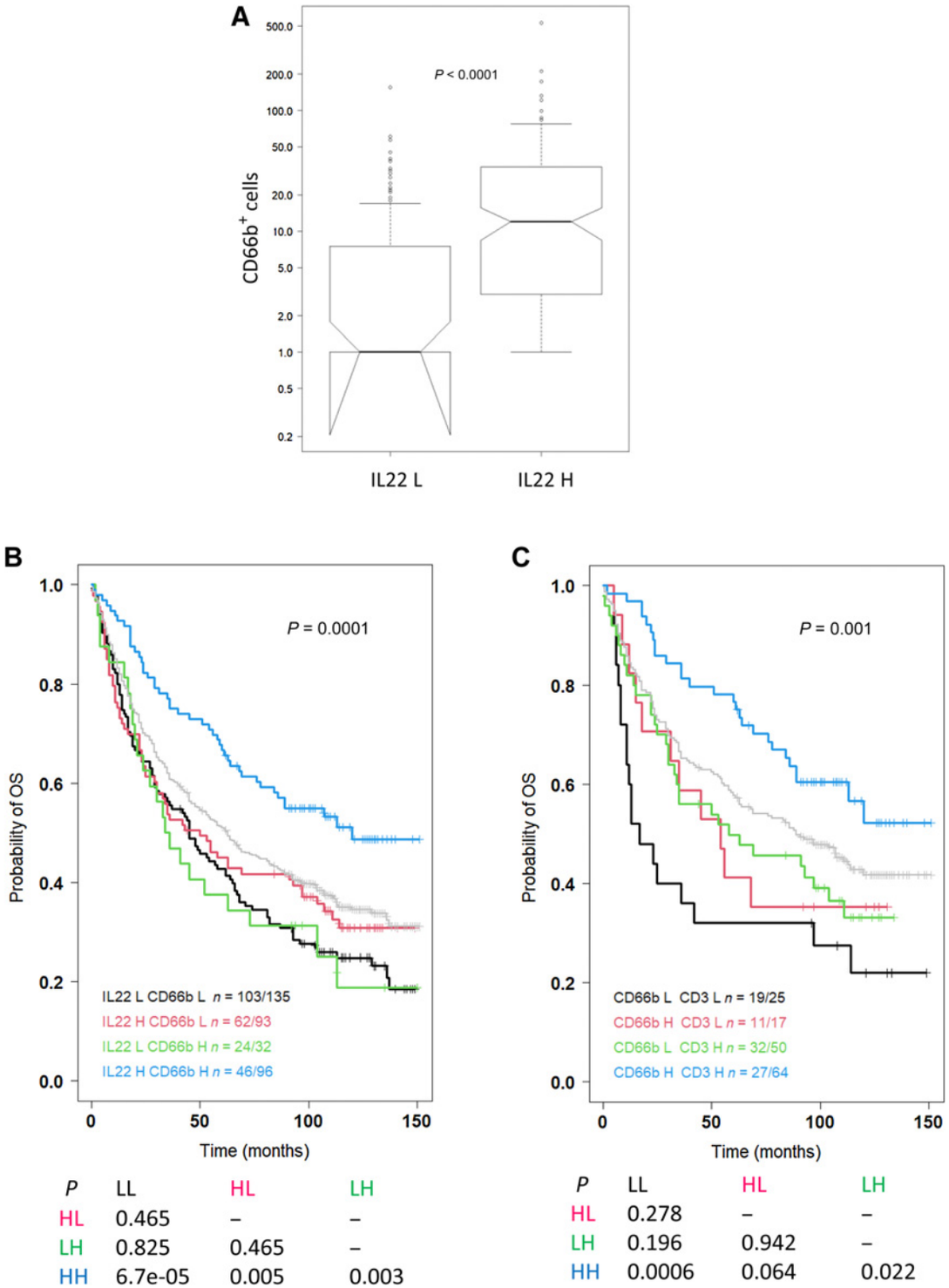

Figure 4.

The positive prognostic effect of IL22$^+$ cells depends on tumor-infiltrating neutrophils and on their interplay with T cells. **A,** Distribution of CD66b$^+$ cells according to the low or high IL22$^+$ colorectal cancer–infiltrating cells. **B,** Kaplan–Meier curves depicting the probability of OS in the entire collective (gray line) and in patients stratified according to combinations of IL22$^+$ and CD66b$^+$ cell density in the overall testing TMA ($n = 356$ instead of 425 because of CD66b missing values). **C,** Kaplan–Meier curves depicting the probability of OS in patients with tumors characterized by IL22$^+$-high density in the entire collective (gray line) and stratified according to combinations of CD66b$^+$ and CD3$^+$ cell infiltration ($n = 156$ instead of 221 because of missing CD66$^+$ and/or CD3$^+$ values). H, high; L, low.

protumorigenic direct effect of IL22 on tumor cells (52, 53). Although we did not observe any significant effect when we evaluated the impact of IL22 on colorectal cancer cell proliferation *in vitro* according to published protocols (52, 53), it is possible that IL22 plays a dual role during different stages of the disease. While IL22 might contribute to tumor development during early phases of oncogenesis, in clinically detectable tumors with an established microenvironment, it might help to recruit beneficial immune cell populations.

Here, exposure of colorectal cancer cells to IL22 resulted in increased secretion of neutrophil-recruiting chemokines, including CXCL1, CXCL2, and CXCL3, and enhanced neutrophil migration *in vitro*. Consistently, in human colorectal cancer samples, expression of IL22 positively correlated with expression of neutrophil-recruiting chemokines in whole-tumor tissues and in purified tumor cells. This was associated with a higher expression of the CD66b neutrophil marker. The positive prognostic effect of $IL22^+$ cells was dependent on the presence of neutrophils.

Neutrophils are key players in the immune response against infectious challenges. However, their role in tumor immunobiology has long been neglected, possibly due to important differences in the granulocyte compartment between humans and experimental mice (54). A number of studies indicate that neutrophils may exert antitumor effects, potentially of high clinical relevance (55). In previous works, we and others (6, 55–57) showed that neutrophil infiltration associates with favorable prognosis in colorectal cancer. In addition, colorectal cancer–associated neutrophils costimulate antigen-triggered tumor-infiltrating T cells (6). Our data further underline the critical prognostic relevance of neutrophil infiltration in colorectal cancer microenvironment and unravel a previously unsuspected ability of IL22 to shape its composition. By stimulating production of neutrophil-recruiting chemokines in tumor cells, IL22-producing T cells might favor colorectal cancer infiltration by neutrophils, ultimately enhancing T-cell activation and expansion, and favoring a more positive clinical outcome.

In this scenario, the nature of stimuli favoring differentiation and recruitment of IL22-producing T cells and their antigenic specificities remains to be clarified. Also, it must be noted that the magnitude of IL22-induced effects ultimately depends on responsiveness of cells from individual tumors. Indeed, expression and functionality of IL22 receptors on tumor cells or their chemokine production capacity are determined by their genetic and epigenetic make-up (3, 47). This likely accounts for the variability of IL22-mediated effect across individual cases and the possible lack of neutrophilic infiltration in spite of high IL22 expression.

In conclusion, upon analysis of a large cohort of colorectal cancer cases, we found an unexpected positive prognostic effect of tumor-infiltrating IL22-producing T cells and showed the capacity of IL22 to recruit beneficial neutrophils into the tumor microenvironment by triggering the production of specific chemokines by colorectal cancer cells. This knowledge may be exploited for more precise tumor prognostication and may suggest the development of innovative treatments aimed at enhancing colorectal cancer infiltration by beneficial immune cell populations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

N. Tosti: Resources, data curation. **E. Cremonesi:** Resources, data curation. **V. Governa:** Data curation. **C. Basso:** Data curation, formal analysis, data acquisition for the revision. **V. Kancherla:** Formal analysis. **M. Coto-Llerena:** Data curation, formal analysis. **F. Amicarella:** Supervision, writing–review and editing. **B. Weixler:** Writing–review and editing. **S. Däster:** Writing–review and editing. **G. Sconocchia:** Funding acquisition, writing–review and editing. **P.E. Majno:** Writing–review and editing. **D. Christoforidis:** Writing–review and editing. **L. Tornillo:** Supervision, validation, writing–review and editing. **L. Terracciano:** Supervision, writing–review and editing. **C.K.Y. Ng:** Formal analysis, methodology, writing–review and editing. **S. Piscuoglio:** Formal analysis, supervision, validation, writing–review and editing. **M. von Flüe:** Writing–review and editing. **G. Spagnoli:** Conceptualization, supervision, writing–review and editing. **S. Eppenberger-Castori:** Data curation, formal analysis, writing–review and editing. **G. Iezzi:** Conceptualization, supervision, funding acquisition, methodology, writing–original draft. **R.A. Droeser:** Conceptualization, supervision, funding acquisition, methodology, writing–original draft.

Acknowledgments

The authors are grateful to Prof. Dr. Fabio Grassi (Institute of Research in Biomedicine, Bellinzona, Switzerland) and to Prof. Dr. Andreas Diefenbach (Charité - Universitätsmedizin Berlin, Germany) for critical comments and discussion. This study was supported by the Swiss Cancer League (KFS-3528-08-2014), the Werner and Hedy Berger-Janser – Foundation for cancer research, the "Stiftung zur Krebsbekämpfung," and by the Swiss National Science Foundation (grant no. 310030_185234; to G. Iezzi). G. Sconocchia was supported by the Italian Association for Cancer Research grant N IG17120.

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Received November 23, 2019; revised June 19, 2020; accepted August 12, 2020; published first August 24, 2020.

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