ORIGINAL ARTICLE

Impaired migration of IgA-secreting cells to colon adenocarcinomas

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Abstract Local inflammation is a strong risk factor for the development of gastrointestinal adenocarcinomas. Mucosal regulatory T cells and IgA-secreting cells both contribute to reduce inflammatory responses, and their recruitment to tissues is dependent on local production of chemokines. More specifically, IgA-secreting cells are recruited to mucosal tissues by CCL28 signalling through CCR10. Here, we examined the recruitment of IgA-secreting plasma cells to tumor-associated mucosa in patients suffering from colon adenocarcinoma. Flow cytometric analyses of single cell suspensions from tumor-associated and unaffected colon mucosa showed a marked decrease in $CD19^+CD38^{high}IgA^+$ plasmablasts in the tumor-associated mucosa, while the total frequencies of B and T cells were similar. This finding was confirmed in ELISPOT assays, demonstrating a 64 % reduction in the frequencies of IgA-secreting cells among cells from the tumor-associated mucosa. The few IgA^+ plasmablasts present in the tumor did not express CCR10, and functional migration assays demonstrated that IgA-secreting cells from tumor-associated mucosa did not migrate in response to CCL28. Taken together, our results show an impaired migration of IgA-secreting cells to colon tumors, presumably caused by a decreased production of CCL28 in the tumor. The lack of local IgA antibodies may lead to impaired barrier function and increased bacterial colonization, driving further inflammatory responses and promoting tumor growth.

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Introduction

Colorectal cancer (CRC) is one of the most common tumor types worldwide. The tumor develops slowly over a long period of time, approximately 10–15 years, and risk factors include smoking, diet, and intestinal inflammation [1–3]. In particular, Crohn's disease and ulcerative colitis pose a strong risk for subsequent development of CRC. The gut microbiota is also different between CRC patients and healthy individuals, leading to the assumption that microbially induced inflammation may also promote tumor progression [4, 5]. In addition, the cyclooxygenase-2 inhibitor acetylsalicylic acid has a beneficial effect on tumor development and progression [6, 7]. Taken together, these observations lead to the conclusion that an inflammatory environment is beneficial for colorectal tumor initiation and progression.

IgA is produced in large amounts by plasma cells in the lamina propria of the large intestine [8]. The plasma cells are originally activated in mucosal inductive sites such as Peyer's patches and mesenteric lymph nodes and express the mucosal homing receptor integrin α4β7 upon egress to the peripheral circulation [9, 10]. Their migration to the intestinal lamina propria is initiated by binding to mucosal addressin cellular adhesion molecule-1 (MAdCAM-1), the endothelial counter receptor for α4β7 expressed on intestinal endothelial cells [11]], and recognition of the chemokine CCL28 (MEC). CCL28 is constitutively produced by mucosal epithelial cells, and signals via CCR10, which is expressed on $α4β7^+IgA^+$ plasma blasts generated in the gut [12–16]. CCR10 is also expressed on circulating T cells expressing the cutaneous lymphocyte antigen (CLA), but since these cells do not co-

express α4β7, they will not gain access to the intestinal lamina propria [17]. Polymeric IgA is secreted into the lamina propria and transported by endocytosis through the epithelial cell barrier by binding to the polymeric immunoglobulin receptor (pIgR) on the basolateral membrane of intestinal epithelial cells. When exposed on the apical membrane, enzymatic cleavage of pIgR leads to release into the lumen as secretory IgA (SIgA) [8]. SIgA acts to reinforce the gut barrier by neutralizing microbial binding to host cells and will thus preserve the intestinal tissue and prevent damage by immune overreaction to the commensal flora [8]. In the tissue, IgA will also compete with IgG and IgM to avoid activation of complement. In addition, binding of monomeric IgA to FcαR1 on monocytes and granulocytes will inhibit signalling in these cells, while antigen-IgA complexes will instead lead to their activation [18]. A recent study of immunized CRC patients actually showed that patients responding with higher IgA titers had a better survival, even though the relationship was only significant in univariate regression analyses [19]. Several in vitro studies also indicate that IgA can mediate tumor-specific cytotoxicity through NK cells or polymorphonuclear phagocytes [19–22].

We recently demonstrated that the endothelium in colon tumors expresses less MAdCAM-1 than the surrounding unaffected mucosa, and a previous study has shown that CCL28 production is also reduced in colon tumors [23, 24]. These findings would indicate a lower migration of IgA-secreting cells to the tumor compared to surrounding tissues. However, there are studies showing a higher concentration of fecal IgA in CRC patients than controls, but also that the expression of pIgR and IgA is decreased at the tumor site [25–27]. Here, we hypothesized that the changes in MAdCAM-1 and CCL28 expression would lead to reduced plasma cell infiltration and IgA production in colon adenocarcinomas. To test this assumption, we used resection material from colon cancer patients to assess infiltration of IgA-secreting cells into colon tumors. Our results show substantially reduced frequencies of IgA-secreting cells in colon tumors compared to unaffected tissue, a virtual lack of CCR10 expression on IgA$^+$ plasmablasts in the tumor, and a severely impaired ability of tumor-derived IgA-secreting cells to migrate in response to CCL28.

Materials and methods

Patients

Eleven colon cancer patients (Table 1) undergoing colon tumor resection at Sahlgrenska University hospital were included in the study. The study was approved by the Regional Board of Ethics in Medical Research in west Sweden and was performed in accordance with the declaration of

Table 1 Characteristics of the colon cancer patients included in the study

	Females	Males
n	5	6
Age	45–78	68–84
Tumor location		
Ascending	2	3
Transverse	–	2
Descending	3	1
Differentiation grade		
High	–	–
Medium	5	5
Low	–	–
Mucinous	–	1
Tumor stage		
T1	–	–
T2	1	1
T3	4	3
T4	–	2
Lymph node spread	3	2
Distant metastases	–	1

Helsinki. All volunteers gave a written informed consent before participation. None of the patients had undergone radiotherapy or chemotherapy for at least 3 years prior to colectomy or suffered from autoimmune disease. During or immediately after colectomy, a strip of tumor tissue was collected together with unaffected mucosa, collected at least 5 cm away from the tumor. Information on tumor stage, differentiation grade, and metastases were retrieved from the routine pathology report.

Isolation of colon lymphocytes

Lamina propria lymphocytes (LPL) were isolated using collagenase/DNase enzymatic digestion after removal of epithelial cells, as previously described [28]. Briefly, the tissue samples were washed with phosphate buffered saline (PBS), and the muscle layers, fat, connective tissue, and blood vessels were carefully removed. The tissue was cut into 5 mm pieces and subjected to six rounds of EDTA/dithiothreitol treatment to remove epithelial cells and intraepithelial lymphocytes. The remaining tissue was digested with collagenase III (Sigma-Adrich Sweden AB, Stockholm, Sweden) for 2 h. The resulting single cell suspension was re-suspend in RPMI 1640 medium (GIBCO, Life technologies Europe BV, Stockholm, Sweden) containing 10 % fetal calf serum (FCS), 50 µg/ml of gentamicin, 50 U/ml of penicillin, and 50 µg/ml of streptomycin (GIBCO).

Chemotaxis assays

Analysis of migration toward CCL28 was performed in 24-well transwells with 5-μm polycarbonate membranes (Costar Corning Inc., Corning, NY, USA) as previously described [16], but after incubation overnight at 37 °C to allow for chemokine receptor re-expression; 5×10^5 mononuclear cells (MNC) were added to the upper chamber and allowed to migrate for 2.5 h at 37 °C into the lower chamber containing RPMI 1640 media with 0.5 % FCS supplemented with CCL28 (Peprotech EC Ltd, London, UK) or medium alone. In the initial experiments, 1–5 μg/ml of CCL28 was used [29], and it was found that 2.5 μg/ml induced optimal migration of colonic IgA-secreting cells. Treatment of MNC with CCL28 for 2.5 h at 37 °C did not influence the frequencies of IgA-secreting cells. Migrating cells were collected and the wells washed with ice cold PBS, the cells pooled and then analyzed in ELISPOT assays and by flow cytometry.

ELISPOT assay

Frequencies of IgA-secreting cells in untreated MNC suspensions, as well as in the cells migrating in response to CCL28, were determined in ELISPOT assays as previously described [30, 31]. Briefly, the total frequencies of IgA-secreting cells, irrespective of specificity, were determined in wells coated with goat antibodies to the Fab_2 fragment of human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Cells were incubated in the ELISPOT wells over night at 37 °C in a humidified atmosphere containing 5 % CO_2, and the assay was developed by the addition of horseradish peroxidase-conjugated goat antibodies to human IgA (Southern Biotech, Birmingham, AL, USA) for 3 h, followed by chromogen substrate. The number of spots, each representing the former position of an IgA-secreting cell, was determined under low magnification. The efficiency of migration was calculated by comparing the number of spots present in the input population with the number of spots present in the migrated population after subtraction of the number of cells migrating spontaneously to the medium control.

Flow cytometric analyses

To determine the cell composition of the MNC suspensions isolated from tumor and unaffected tissue, cells were stained with anti-CD19-FITC or anti-CD19-PE, CD14-PE, CD4-PerCP, CD8-APC, CD38-PerCP, CD69-FITC (all from BD Biosciences, San Jose, CA), IgA-PE (Miltenyi Biotec Norden AB, Lund, Sweden), and CCR10-APC (R&D systems Europe Ltd., Abingdon, UK) and

appropriate isotype controls. Plasmablasts were defined as large $CD14^-CD19^+CD38^{high}$ lymphocytes and resting B cells as small $CD19^+$ lymphocytes. Migrating cells collected from the lower chamber were stained with antibodies to CD19, CD38, CCR10, and CD69 or IgA. Flow cytometry was performed using a FACSCalibur and analyzed using Flowjo 7.5 software.

Statistical analyses

All analyzes were performed using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA). Two-tailed Wilcoxon signed rank test was used for statistical analyses of paired data. A p value of less than 0.05 was considered to be significant.

Results

Composition of cells in unaffected and tumor affected tissue

LPL were isolated from unaffected tissue and colon tumors by collagenase digestion, and the isolated cells were analyzed for the presence of $CD19^+$, $CD4^+$, and $CD8^+$ lymphocytes and $CD14^+$ monocytes/macrophages using flow cytometry (Fig. 1a). There were no major differences in the percentage of B and T cells or $CD14^+$ myeloid cells isolated from the unaffected and tumor tissues. The small lymphocyte population, containing mainly resting memory or naïve lymphocytes, had a high percentage of $CD4^+$ cells, while the large lymphocyte population had a high percentage of $CD19^+$ (Fig. 1b). However, the tumor tissue contained significantly fewer large $CD19^+$ lymphocytes compared to unaffected lamina propria ($p < 0.05$; Fig. 1c). The large $CD19^+$ lymphocytes were also $CD38^{high}$ and thus defined as plasmablasts. Of these large $CD38^+$ plasmablasts, 83 ± 7 % (mean ± SD) expressed IgA in the unaffected mucosa, whereas only 55 ± 22 % of plasmablasts in the tumor expressed IgA ($p < 0.05$).

The presence of IgA-secreting cells in unaffected and tumor affected tissues was further analyzed using ELISPOT analysis. Strikingly, the tumor tissue had significantly reduced frequencies of IgA-secreting cells compared to the unaffected tissue ($p < 0.01$; Fig. 1d, e). On average, the frequencies of IgA-secreting cells in the tumors were only 36 % of those found in the unaffected tissue. Taken together, these results demonstrate that there is a large reduction in IgA-secreting plasmablasts and plasma cells in colon tumors compared to the surrounding, unaffected mucosa.

a

b

Small lymphocytes

Large lymphocytes
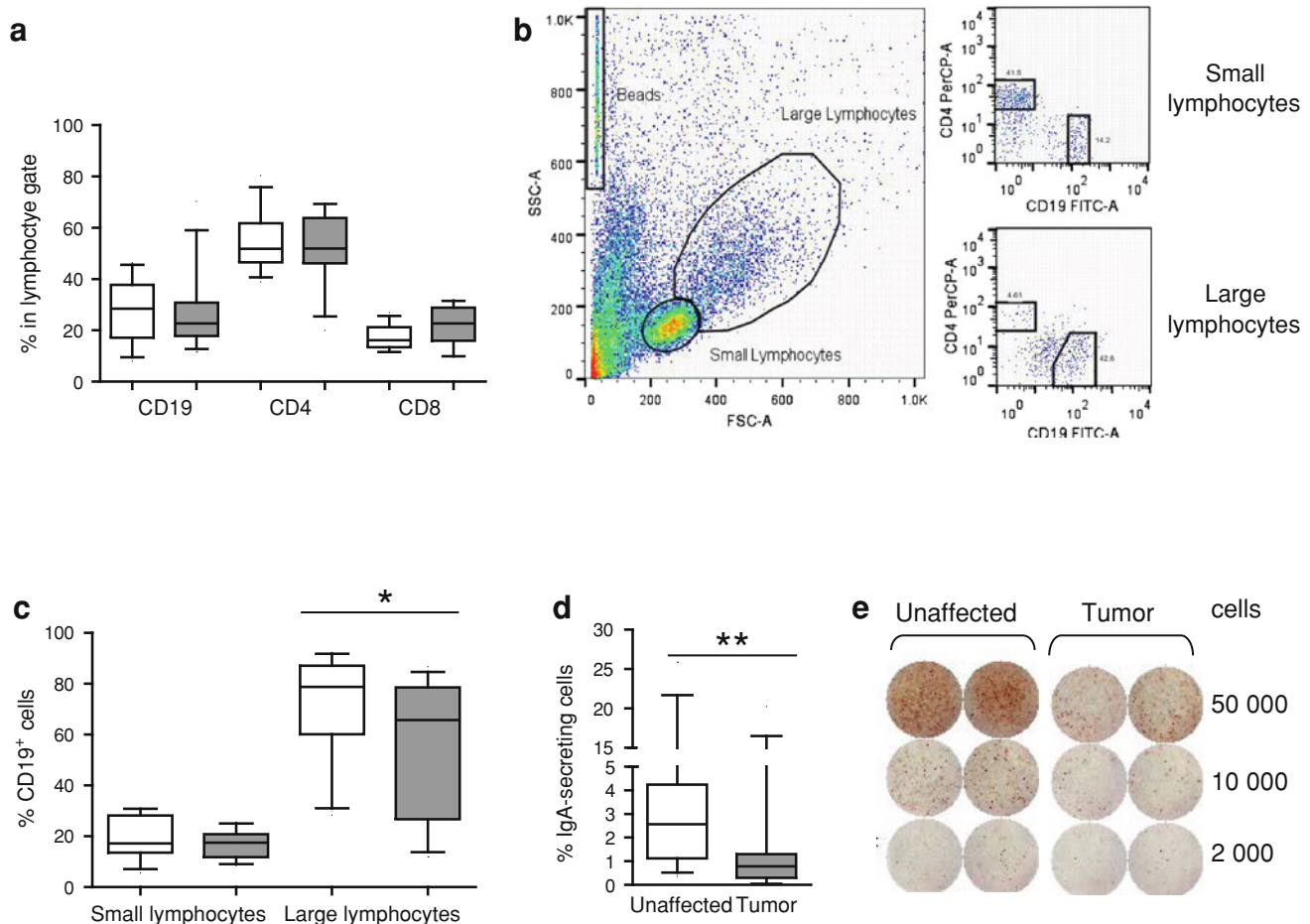
c

d

e

Fig. 1 Lymphocyte subsets in colon tissue. Colon lamina propria cells were isolated from colon cancer patients and analyzed by flow cytometry and ELISPOT assays. **a** Frequencies of CD19^{+}, CD4^{+}, and CD8^{+} lymphocytes in lamina propria cell suspensions isolated from unaffected tissue (*white bars*) and tumors (*gray bars*) determined by flow cytometry. **b** Illustration of the gating strategy used to divide small and large colon lamina propria lymphocytes, and the expression of CD19 and CD4 in the two populations. **c** Frequencies of CD19^{+} cells in the small and large lymphocyte populations from unaffected tissue (*white bars*) and tumors (*gray bars*) determined by flow cytometry. **d** Frequencies of IgA-secreting cells in colon lamina propria cell suspensions determined by ELISPOT. **e** Illustration of ELISPOT results from one individual. *Horizontal lines* show median values, *boxes* show the 25–75 ‰, and whiskers the 10–90 ‰. ($n = 11$). *$p < 0.05$, **$p < 0.01$

Decreased migration of large CD19^{+} lymphocytes from tumors to CCL28

Next, we wanted to determine if the reduced frequencies of IgA-secreting cells were due to reduced migration. Thus, isolated LPL were allowed to migrate toward CCL28 in a transwell system. The migrating cells were collected and analyzed by flow cytometry, and the fold increase in the migration of cells toward CCL28 compared to medium alone was calculated. CD4^{+} and CD8^{+} cells from both unaffected and tumor affected tissue migrated very poorly in response to CCL28, and there was no difference between the unaffected and tumor affected tissue (data not shown). Similarly, no difference in migration was observed in the case of small CD19^{+} lymphocytes (Fig. 2a). In contrast, the large CD19^{+}

lymphocytes from the tumor displayed a significantly lower migration ($p < 0.01$) toward CCL28 compared to cells from the unaffected colon (Fig. 2b). To further define the differences between migrating cells from tumors and unaffected colon, the migrating CD19^{+} B cells were analyzed for their expression of IgA. These analyses show that small IgA^{+} B cells migrate to CCL28 to a certain extent, but that there is no difference between cells derived from tumor or unaffected mucosa (Fig. 2c). In contrast, IgA^{+} cells in the large, plasmablast-containing gate migrate efficiently toward CCL28 when isolated from unaffected tissue, while the migration of tumor-derived large IgA^{+} cells was much lower ($p < 0.05$; Fig. 2d). Taken together, these results show that while colon T cells migrate poorly toward CCL28, B cells, and in particular large IgA^{+} B cells from unaffected colon tissue,
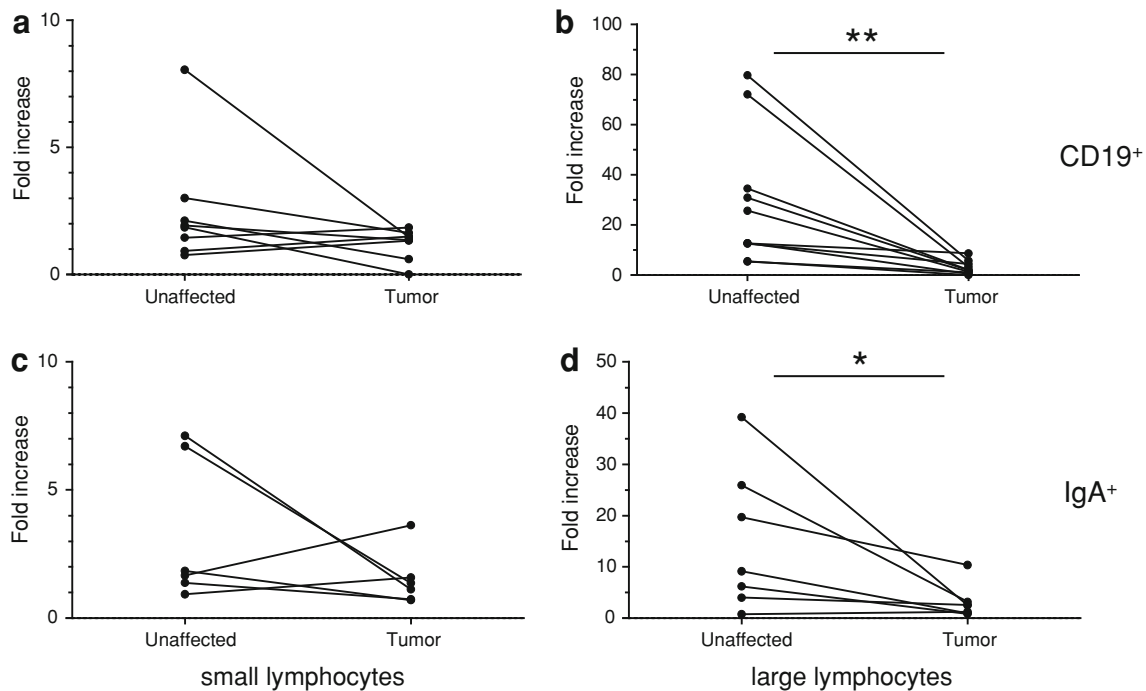
Fig. 2 Migration of B cells from colon tissue to CCL28. Cells from unaffected tissue and tumors were allowed to migrate toward CCL28 and the migrating cells analyzed by flow cytometry. Graphs show the fold increase in migration toward CCL28 compared to control wells with medium alone in small and large CD19$^+$ B cells (**a–b**) and IgA$^+$ B cells (**c–d**). *Symbols* represent individual values and *lines* connect unaffected tissue and tumor from one individual. ($n = 6$–10). *$p < 0.05$, **$p < 0.01$

migrate efficiently toward CCL28. This ability, however, is missing in the large IgA$^+$ B cells residing in the tumor.

IgA-secreting cells from tumors migrate poorly to CCL28

The above results show that large CD19$^+$IgA$^+$ colonic B cells from unaffected tissues migrated well in response to CCL28, as would be predicted from the literature [12, 13]. Since these cells probably comprise the IgA-secreting population, we wanted to further investigate whether these migrating cells actually secrete IgA. Thus, migrating cells were transferred to ELISPOT plates to identify IgA-secreting cells. The specific migration of IgA-secreting cells from the unaffected colon was small, but still significantly higher in response to CCL28 than to the medium ($p < 0.01$; Fig. 3a). On the other hand, IgA-secreting cells from tumor affected tissue did not migrate significantly compared to the spontaneous migration in assays with medium alone (Fig. 3b). When we compared the fold increase in migration toward CCL28 compared to medium alone, there was always a higher migration of IgA-secreting cells from unaffected colon compared to tumors ($p < 0.01$; Fig. 3c). The migrating cells were also analyzed for their expression of the activation marker CD69. However, the migration of cells from both the unaffected colon and the tumor tissue did not depend on their activation state

(Data not shown). These data confirm the lack of CCL28-dependent migration among IgA-producing plasmablasts and plasma cells located in colon tumors.

Reduced expression of CCR10 on IgA$^+$ cells from colon tumors

The recruitment of IgA-secreting cells to mucosal tissues is partly dependent on the expression of CCR10 [12, 13]. Hence, we examined if the decreased migration of cells from the tumor affected tissue was related to the expression of CCR10. First, cells from unaffected colon tissue and colon tumors were analyzed for their expression of CCR10. The CD38$^+$IgA$^+$ plasmablasts from unaffected tissue had a much higher expression of CCR10 compared to the corresponding cells isolated from tumors in all patients ($p < 0.05$; Fig. 4a, b). Next, the migrating cells from unaffected colon and tumors were examined for CCR10 expression. There was a clear accumulation of CCR10$^+$ cells among the cells from the unaffected tissue that had migrated toward CCL28, as 77 ± 10 % of the migrating cells expressed CCR10 (Fig. 4c). Among the few cells from the tumor cell suspension that did migrate to CCL28, only few expressed CCR10 even in the migrating subpopulation (Fig. 4c). Taken together, these data suggest that the reduced production of CCL28 in colon tumors results in an inability to recruit CCR10$^+$ plasmablasts, leading to the observed deficiency in IgA-secreting cells.
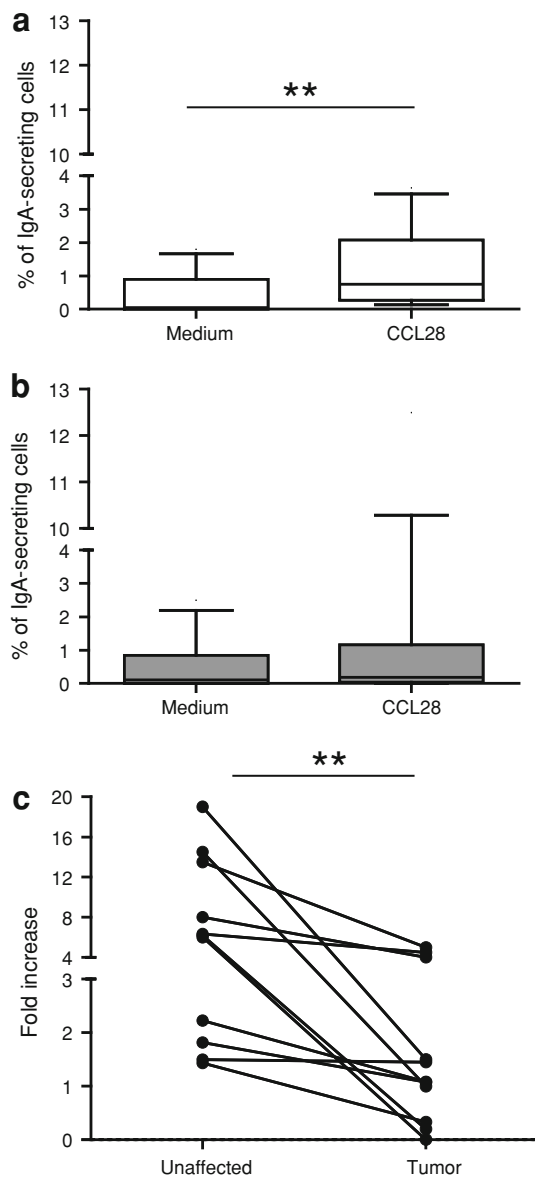
Fig. 3 Migration of IgA-secreting cells from colon tissue to CCL28. Cells from unaffected tissue (**a**) and tumors (**b**) were allowed to migrate toward CCL28, and the migrating cells were analyzed by ELISPOT to detect the frequencies of IgA-secreting cells. The percentage of IgA-secreting cells was calculated based on the frequencies of IgA-secreting cells in the original cell suspension. **c** Fold increase in migration toward CCL28 compared to control wells with medium alone of cells from unaffected and tumor tissue. *Horizontal lines* show median values, boxes show the 25–75 ‰, and whiskers the 10–90 ‰. *Symbols* represent individual values and *lines* connect unaffected tissue and tumor from one individual. ($n = 11$). $*p < 0.05$, $**p < 0.01$

Discussion

In this study, we show a marked reduction in the frequencies of IgA-secreting cells in human colon adenocarcinomas compared to the surrounding, unaffected tissue.

The decreased IgA production in tumors is probably caused by a reduced production of the mucosal chemokine CCL28 in the tumor tissue, as previously shown [24], which in turn was reflected by a virtual lack of CCR10 expression on tumor-associated IgA$^+$ plasmablasts and an inability to migrate in response to CCL28.

The healthy colon mucosa contains large numbers of IgA-secreting plasma cells, while IgG- and IgM-secreting cells are less numerous [32]. Furthermore, the frequencies of IgA$^+$ cells appear to be very similar in colon and rectal mucosa [32]. The frequencies of IgA-secreting cells that we find in the unaffected colon mucosa of cancer patients are also similar to what has previously been reported in rectal mucosa of healthy volunteers using the same ELISPOT technique [33]. Thus, it appears that the colon cancer patients do not have generally altered levels of intestinal IgA-secreting cells, but that there is a selective decrease at the tumor site. We have previously reported a similar finding in *Helicobacter pylori*-infected gastric cancer patients, which have a substantially decreased production of IgA not only in the tumor but also in the surrounding unaffected stomach mucosa when comparing to *H. pylori*-infected individuals with uncomplicated gastritis [34]. While our results show that both major types of gastrointestinal tumors are associated with decreased levels of IgA secretion, they cannot explain the general decrease in gastric IgA seen in gastric cancer patients.

Recent studies have used stool samples to correlate IgA secretion with colon tumor stage and could show that patients with advanced disease (Duke's stage D) had lower fecal IgA than patients with less advanced tumors [27]. However, in these studies, it was not possible to determine the exact distribution of IgA-secreting cells between tumors and unaffected mucosa. Earlier studies have shown lower IgA levels and a reduced density of IgA-containing cells within colon tumors compared to unaffected mucosa [25, 35, 36]. We now confirm and extend these observations by also determining the phenotype and migration capacity of tumor-associated IgA-producing cells, as well as providing an explanation to their low frequencies in the tumor. We thus found a striking reduction in the ability of IgA$^+$ plasmablasts from tumors to migrate in response to CCL28, as determined by both flow cytometry and ELISPOT techniques. Our finding that tumor-associated IgA$^+$ cells have a low expression of CCR10 compared to cells from unaffected colon mucosa probably explains their inability to migrate. It is also worth noting that low CCR10 expression and inability to migrate toward CCL28 was a general finding in all patients examined, even though the patients included in the study varied in their clinical presentation, age, and tumor stage. The few IgA-secreting cells present in the tumors may instead have been recruited by CCL20–CCR6 interactions, as CCR6 is expressed on

Fig. 4 Expression of CCR10 by IgA$^+$CD38$^+$ cells from colon tissues. Cells from unaffected and tumor tissue were allowed to migrate toward CCL28 and analyzed by flow cytometry before and after migration. **a** Expression of CCR10 on IgA$^+$CD38$^+$ freshly isolated lymphocytes from unaffected tissue and tumors. **b** Representative histograms of CCR10 staining (solid lines) compared to isotype staining (dotted lines) in cells from unaffected tissue (top panel) and tumor (lower panel) in the same individual. **c** Expression of CCR10 on the migrating subset of IgA$^+$CD38$^+$ lymphocytes from unaffected tissue and tumors. Symbols represent individual values and lines connect unaffected tissue and tumor from one individual. ($n = 6$). *$p < 0.05$
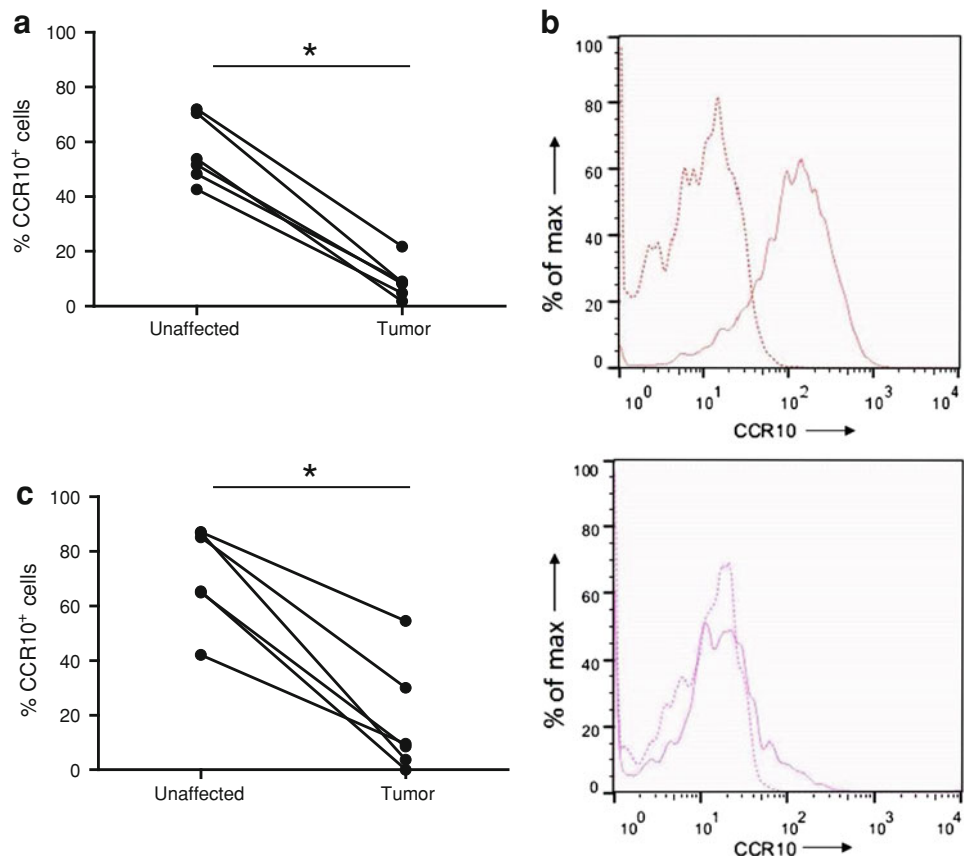
IgA$^+$ B cells and CCL20 produced in colorectal adenocarcinomas [37, 38].

CCL28 signalling through CCR10 together with α4β7–MAdCAM-1 interactions is a pivotal part of plasmablast migration to intestinal sites, both in steady state and during vaccine-induced immune responses [9, 12, 13, 16]. Thus, the combination of reduced local MAdCAM-1 and CCL28 expression at the tumor site [23, 24], rather than a general down-regulation of CCR10 on plasmablasts, probably explains the observed lack of IgA-producing cells in tumors. CCL28 is constitutively produced by epithelial cells and also by colonic adenocarcinomas [14, 15, 24], but the production can be further increased by inflammatory mediators, hypoxia, or bacterial infection [29, 39–41]. No signal that actively down-regulate CCL28 production has yet been identified, and it is not clear at this stage if a local inhibitory factor in the tumor acts on the transformed epithelial cells to modulate CCL28 secretion, or if the transformation process as such leads to decreased CCL28 production. Overexpression of CCL28 has actually been reported in ovarian adenocarcinomas due to hypoxia [41], while pleomorphic adenomas of the salivary glands and mammary tumors usually expressed less CCL28 than the unaffected adjacent tissue [42, 43]. Thus, loss of CCL28 expression is not a general feature of all epithelium-derived tumors.

The lower IgA secretion that we demonstrate at the tumor site would lead to a reduced local barrier function and a higher risk of bacterial penetration into the tissues. CCL28 also has intrinsic antimicrobial activity [44], and a reduced CCL28 level in colon cancer tissue could thus contribute to bacterial persistence in colon tumors. Presence of bacteria would lead to inflammatory responses, including activation of local IL-17-secreting Th cells, as has been demonstrated in human colorectal tumors [45, 46]. Furthermore, in the APC$^{Min/+}$ murine model of intestinal adenocarcinoma, IL-17 was pivotal in driving inflammation-related tumor progression [47]. In most solid tumors, there is a negative correlation between accumulation of regulatory T cells (Treg) and patient outcome [48]. Intriguingly, in colon cancer patients, there is actually a positive correlation between high Treg numbers in the tumor and a good prognosis for the patient [49, 50]. The local Treg response in colon tumors may down-regulate bacterially induced inflammation and thus reduce inflammation-driven tumor progression [48]. Taken together, these findings indicate that Treg and IgA may act together to reduce bacteria-induced inflammation at the tumor site, and that a lack of IgA locally at the tumor site may lead to increase local inflammation and in this way promote tumor progression.

To conclude, we have found an impaired migration of IgA-secreting cells to colon tumors, presumably caused by a decreased production of CCL28 in the tumor. The lack of local IgA antibodies may lead to impaired barrier function and increased bacterial colonization, driving further inflammatory responses and promoting tumor growth.

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