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NF- κ B hyper-activation in tumor tissues allows tumor-selective reprogramming of chemokine microenvironment to enhance the recruitment of cytolytic T effector cells

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Abstract

Tumor infiltration with effector CD8⁺ T cells (T_{eff}) predicts longer recurrence-free survival in many types of human cancer, illustrating the broad significance of T_{eff} for effective immunosurveillance. Colorectal tumors with reduced accumulation of T_{eff} express low levels of T_{eff}-attracting chemokines such as CXCL10/IP10 and CCL5/RANTES. In this study, we investigated the feasibility of enhancing tumor production of T_{eff}-attracting chemokines as a cancer therapeutic strategy, using a tissue explant culture system to analyze chemokine induction in intact tumor tissues. In different tumor explants, we observed highly heterogeneous responses to IFN γ or poly-I:C (a TLR3 ligand) when they were applied individually. In contrast, a combination of IFN γ and poly-I:C uniformly enhanced the production of CXCL10 and CCL5 in all tumor lesions. Moreover, these effects could be optimized by the further addition of COX inhibitors. Applying this triple combination also uniformly suppressed the production of CCL22/MDC, a chemokine associated with infiltration of T regulatory cells (T_{reg}). The T_{eff}-enhancing effects of this treatment occurred selectively in tumor tissues, as compared to tissues derived from tumor margins. These effects relied on the increased propensity of tumor-associated cells (mostly fibroblasts and infiltrating inflammatory cells) to hyper-activate NF- κ B and produce T_{eff}-attracting chemokines in response to treatment, resulting in an enhanced ability of the treated tumors to attract T_{eff} cells and reduced ability to attract T_{regs}. Together, our findings suggest the feasibility of exploiting NF- κ B hyper-activation in the tumor microenvironment to selectively enhance T_{eff} entry into colon tumors.

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Keywords

tumor microenvironment; immunomodulation; chemokines; effector T cells; regulatory T cells; colorectal cancer; indomethacin; celecoxib; PGE₂; COX2; interferons

Introduction

The ability of CD8⁺ T cells to infiltrate cancer lesions is essential for anti-tumor immunity, as evidenced by studies highlighting the prognostic value of effector T (T_{eff}) cells in multiple cancer types, including colorectal cancer (CRC) (1–4). In contrast, tumor infiltration with regulatory T cells (T_{regs}) predicts poor outcomes (5–8). Chemokines and their respective receptors are critical for T cell migration and homing (9–14). High levels of CCL5/RANTES (CCR5 ligand) and CXCL9/MIG and CXCL10/IP10 (ligands for CXCR3) in tumor tissues are associated with enhanced infiltration of CD8⁺ T cells in CRC (15), melanoma and gastric cancer (16, 17). In contrast to the benefits of intra-tumoral expression of CCL5 and CXCL9-11 (18), high levels of CCL22/MDC, the CCR4 ligand preferentially attracting T_{regs}, can be associated with reduced survival, as shown in ovarian cancer patients (19).

Several studies have indicated the propensity of colorectal tumors to over-express COX2 and its product PGE₂ (20, 21), the factor shown to promote the induction of CCL22 in dendritic cell (DC) cultures (22). Prompted by these reports, and by our observations of the reciprocal impact of IFN γ versus PGE₂ on the production of T_{eff}- and T_{reg}-attracting chemokines in isolated DCs (22), we tested the feasibility of using these factors to manipulate tumor microenvironment to enhance the production of T_{eff}-attracting chemokines in intact human tumor tissues. We used an *ex vivo* tumor/tissue explant culture system previously applied to study migration of DCs (23), to avoid spontaneous activation of the chemokine-producing cells in the process of tumor dissociation.

Guided by reports showing common hyper-activation of NF- κ B in cancer tissues (24–27), and the requirement for this factor in the induction of both T_{reg}- and T_{eff}-attracting classes of chemokines (28–30), we tested whether the selected PGE₂- and IFN γ -targeting strategies can be used to selectively enhance the production of T_{eff}-attracting chemokines in tumor tissues, rather than marginal tissues, in order to selectively direct T_{eff} cells to tumors.

Materials and Methods

Patients

72 colorectal patients were involved in the study. Tumors and marginal tissues were harvested during routine surgery. The patient profile is presented in Table 1. All patients signed a consent approved by the Institutional Review Board of the University of Pittsburgh for collection of tumor samples (UPCI 02-077).

Culture of macrophages, fibroblasts, HUVEC cells and colon cancer cell lines

For preparation of macrophages, monocytes were cultured in AIM-V with GM-CSF for 6 days. Fibroblasts (Cascade Biologicals, Portland, Oregon) and colon cancer cell lines CACO-2, HCT116, HT29, SW480 and SW620 (ATCC, Manassas, VA) were grown in IMDM+10% FBS, while HUVECs (AllCells, Emeryville, CA) were cultured in HUVEC complete media (Basal media supplemented with HUVEC stimulatory supplement; AllCells). All were washed, reseeded at 20,000 cells in 300 μ L in 96 well plates and treated with IFN γ , poly-I:C, and/or indomethacin as indicated for 48hrs, and supernatants were analyzed for chemokine production by ELISA.

Ex vivo cultures of tumor- and marginal tissue explants

Using a 4mm biopsy puncher, the cubes of tumor or marginal tissue were prepared and placed in antibiotic-containing IMDM/10% FBS (typically 3 cubes/well in 24 well plates) for 24–48 hours, as indicated. When indicated, the tissues were treated with 10,000 units of IFN γ , 20 μ g/mL of poly-I:C, 50 μ M of indomethacin or 10 μ M of celecoxib. Biopsies were harvested at 0 and 24hrs for mRNA analysis and confocal microscopy analysis. Culture supernatants were harvested at 24–48 hours, as indicated (all groups in a single type of experiment were harvested at the same time point) for ELISA and chemotaxis assays. The detailed work flow is depicted in Supplementary Fig. S1A. The system used, based on our previously-developed *ex vivo* whole tissue culture system (23), allowed us to avoid spontaneous induction of chemokine production by the process of tumor dissociation (Supplementary Fig S1B and data not shown)

Taqman analysis of mRNA expression in tumors and marginal tissues

4mm biopsies were placed in lysing Matrix E tubes (MP Biologicals, Solon, OH) containing RLT buffer (RNeasy kit, Qiagen, Valencia, CA), and agitated using a FPI20 homogenizer (MP Biologicals). Debris-free supernatant from the lysis matrix tubes were transferred into new tubes and the total RNA was extracted using the RNeasy kit. 1 μ g of RNA extracted by the above described method was used for cDNA synthesis, and 25–50 ng of subsequent cDNA was used to perform mRNA expression analysis by Taqman analysis on the Step One Plus system (Applied Biosystems). All the primers used for the analysis were standard, purchased from Applied Biosystems.

ELISA analysis of chemokines in tumor ex vivo culture supernatants

Culture supernatants from tumor *ex vivo* cultures were analyzed by ELISAs for the presence of chemokine proteins CCL5, CCL22 and CXCL10, using primary and secondary antibodies from Peprotech, Rocky Hill, NJ. Detection was done using Streptavidin-HRP conjugate and TMB substrate from Pierce Biotechnology Inc, Rockford, IL.

Isolation of tumor infiltrating CD8⁺ T cells

Tumor infiltrating lymphocytes were isolated as described by Dudley et al (31), with the following modifications: Tumor was cut into 4mm cubes using a biopsy punch, and each 4mm tumor piece cultured in 1mL of IMDM + 5% human AB serum with 1000U/mL IL-2 for 2 weeks. Medium was changed twice a week, until lymphocytes were extruding from tumor and formed proliferating clusters.

Chemotaxis

Chemotaxis assays were performed in 24 transwell plates with 5 μ m pore size polycarbonate filters (Corning Inc, Corning, NY). The lower chambers were filled with 600 μ L of tumor supernatants. As indicated, 2x10⁵ of either isolated tumor-infiltrating lymphocytes or DC1-activated CD8⁺ T_{eff} cells (32), in 200 μ L of IMDM 10% FCS, were added to the upper chambers and incubated for 3hrs at 37°C. Migrated cells were harvested from the lower chambers and stained for CD8. Cell counts were performed by a 60 second limited run on a BD Beckman Coulter XL cytometer. For analysis of T_{reg} cell migration, bulk CD4⁺ T cells were isolated by negative selection using EasySep CD4 enrichment kits (StemCell), and 1x10⁶ of the isolated cells in 200 μ L were allowed to migrate towards 600 μ L of tumor supernatants in the bottom chambers. The migrated cells in the bottom chambers were harvested and FOXP3/GITR frequencies were determined by Taqman analysis or flow cytometry.

***In situ* hybridization**

Tissue specimens were fixed in 4% para-formaldehyde, processed and pre-treated as described (33), except that tissues were sectioned on a cryostat at 5µm. Gene-specific riboprobes were synthesized by *in vitro* transcription using a Maxiscript SP6/T7 kit (Ambion) and unincorporated nucleotides were removed using RNA Mini Quick Spin Columns (Roche). *In situ* hybridization with ³⁵S-labeled riboprobes was performed as described (33, 34), with 0.1M dithiothreitol included in the hybridization mix. Hybridizations were performed at 50°C overnight. Tissue sections were coated with NTB emulsion (Kodak) and exposed at 10°C for 7–14d. Simultaneous *in situ* hybridization and immunohistochemistry were performed as described (33, 34), except that the dithiothreitol concentrations were 0.01M in the hybridization mix and 1mM in the washes. An antibody against HLA-DR (Dako) was used at a dilution of 1:25.

Confocal microscopy analysis of tumor and marginal tissues

4mm tumor punches, either untreated or treated, were embedded in OCT medium-containing cryomolds and immediately frozen in 2-methyl-butane. 6µm frozen sections of the tissues were made using the cryostat and layered on superfrost® plus slides (Thermo Scientific, Rockford, IL). The slides were incubated in 4% para-formaldehyde for 15 minutes, washed and blocked for 60 minutes at RT. The slides were then stained for 3hrs at RT with antibodies for P65 (ab16502) or for CD8 (ab4055), CXCL10 (ab8098) and CCL5 (ab10590; both AbcamCambridge, MA). The slides were washed 3 times with 1x PBS and incubated with secondary antibodies anti-rabbit (Alexa 647), anti-mouse (Alexa 488; both Cell Signal, Danvers, MA) and anti-goat (Alexa 488; Invitrogen, Carlsbad, CA) for 30 minutes at RT. The slides were washed 3 times with 1x PBS and once with high salt PBS. Cover slips were mounted on the sections using prolong gold anti-fade solution (Invitrogen). Confocal analyses of stained slides were performed using a LEICA TCS SL DMRE Microsystems. To quantify the numbers of cells showing nuclear NF- B translocation, images were taken of 10 different fields (63x magnification) of the tumor and marginal tissue sections (untreated or treated). To identify the cells showing P65 translocation and chemokine production in response to treatment, tumor tissues were stained with CD45 (H130, BioLegend, San Deigo, CA), CD326 (9C4, BioLegend), fibroblast marker (TE-7, EMD Millipore, Billerica, MA) and CCL5 (ab9679, Abcam) and CXCL10 (ab9807, Abcam), and cells were enumerated as described above.

Statistical analysis

Pearson rank correlations between the chemokine genes and T cell markers were calculated on logarithmically transformed data. In situations where significant between-batch variation was observed, the correlation was adjusted for the batch effect by performing a multivariate analysis of variance (MANOVA) of each pair of variables on batch, and deriving the correlation from the MANOVA residual matrix. Comparisons of continuous variables between groups were performed by two-tailed paired *t* tests. *P* < 0.05 was considered significant. Analyses were performed using SAS v9.2 (SAS Institute, Cary, NC) or GraphPad Prism 5 software.

Results

The expression of effector T cell (T_{eff})-recruiting chemokines in colorectal tumor samples correlates with effector CD8⁺ T cell markers

Using resected tumor material from 72 patients with advanced colorectal cancer (metastatic in 68 patients), we observed that local expression of two T_{eff} cell markers (CD8 and Granzyme B; GZMB) is strongly correlated with the expression of two T_{eff}-attracting

chemokines, CCL5 and CXCL10 (Fig. 1A). In contrast, the T_{reg} markers FOXP3 and GITR were correlated with CCL22 (Fig. 1B), a known T_{reg} attractant (19, 22). Additional correlations were observed between CXCL9 (alternative CXCR3 ligand) and T_{eff} markers and between CCL22 and the CCL22-inducing factor (22) COX2 (Supplementary Fig. S2A–B). Confocal microscopy analysis of the tumor sections revealed that all CXCL10-producing cells (Supplementary Fig. S2D; right panel) and a significant proportion of CCL5-producing cells (left panel) were CD8-negative, arguing against the possibility that the above correlations result from the production of these chemokines by CD8⁺ T cells themselves, instead suggesting their causative role in mediating CD8⁺ T cell infiltration.

The phenotypic analysis of CD8⁺ tumor-infiltrating lymphocytes (TIL) obtained from colon cancer patients (see M&M) revealed that the majority of CD8⁺ TILs are CCR5⁺ CXCR3⁺ (Supplementary Fig. S2E), and Granzyme B⁺ (Supplementary Fig. S2F), which further indicates that the intra-tumoral expression of the CCR5- and CXCR3-ligands was responsible for recruiting the effector T cells into the tumor.

Combination of IFN α , indomethacin and poly-I:C selectively enhances the production of T_{eff}-recruiting chemokines in tumor tissues and suppresses T_{reg}-recruiting chemokines

In order to test the possibility of correcting the chemokine environment in the tumors with low ratios of T_{eff}-to T_{reg}-attracting chemokines, we tested in pilot studies the feasibility of modulating their production using different combinations of IFN γ , indomethacin (COX1/2 inhibitor) and poly-I:C in individual populations of tumor-relevant cells, such as colon cancer cells, macrophages, fibroblasts and HUVECs. We observed strong synergy between IFN γ and poly-I:C in the induction of CCL5 and CXCL10, and a strong suppressive effect of IFN γ on the production of CCL22 in macrophages and fibroblasts (Supplementary Fig. S3A–B). These desirable effects were further potentiated in the presence of indomethacin (Supplementary Fig. S3B). In contrast, none of the long-term-cultured colon cancer cell lines tested (CACO-2, HCT116, HT29, SW480 and SW620) or HUVECs produced any detectable CCL5, CXCL10 or CCL22 (data not shown).

In order to test the feasibility of using these factors to manipulate the complex microenvironment of whole tumor tissues, involving all the above cell types and their interactions, we used an *ex vivo* tumor/tissue explant culture system previously developed to study migration of DCs (23). This system allowed us to avoid nonspecific activation of the chemokine-producing cells during tumor dissociation (see Supplementary Figure 1B and data not shown).

As shown in Figure 2, different tumor tissues treated with IFN γ or poly-I:C alone showed variable chemokine expression, falling into three different patterns: minimal induction of CCL5 and CXCL10; minimal induction of CCL5 but significant induction of CXCL10; or significant induction of both CCL5 and CXCL10 (Fig. 2A). This heterogeneity was observed between tumors from different patients, and even between different lesions within a single patient (Fig. 2A and Supplementary Fig. S3C). However, combining IFN γ and poly-I:C resulted in uniformly high expression of both CCL5 and CXCL10 in all tumors tested (Fig. 2A and Supplementary Fig. S3C).

Additional exposure to indomethacin (which blocks COX1 and COX2) further enhanced the production of CCL5 and CXCL10 induced by the combination IFN γ and poly-I:C and reduced CCL22 in whole tumor tissues (Fig. 2B), with similar results obtained using a selective COX2 blocker, celecoxib (Supplementary Fig. S3D).

Based on these data, we selected the triple combination of IFN γ , poly-I:C and indomethacin as the preferred treatment for all subsequent experiments. This combination consistently

enhanced CXCL10 and CCL5 production and suppressed the production of CCL22 in all tumor samples, as shown by individual chemokine gene expression at the single cell level using in-situ hybridization (ISH; Fig. 3A) and at the level of chemokine secretion, using ELISA (Fig. 3B). Similar observations were also made in case of CXCL9 (data not shown).

The dual staining for HLA-DR (immunohistochemistry) and chemokine mRNA (ISH) demonstrated that CCL22 was expressed predominantly by HLA-DR⁺ APCs, while CXCL10 and CCL5 were expressed by both HLA-DR⁺ and HLA-DR⁻ cells (Supplementary Fig. S4), indicating the contribution of multiple tumor-associated cell types to the production of T_{eff}-recruiting chemokines within the tumor microenvironment.

Enhanced activation of tumor-associated NF- κ B by the chemokine-modulatory regimen results in preferential induction of CXCL10 in tumors, rather than marginal healthy tissues

Using matched tissue samples from 10 patients with metastatic colon cancer, we compared the responsiveness to the chemokine-modulating regimen between liver-metastatic tumor tissues and marginal tissues. As shown in Fig. 4A (and Supplementary Fig. S5), while the baseline differences in chemokine production between the untreated liver-metastatic tumors and marginal liver tissues did not reach significance ($P=0.12$), tumor treatment with the combination of IFN γ , poly-I:C and indomethacin induced much more pronounced secretion of CXCL10 by tumor tissues compared to the marginal tissues ($P<0.01$). Similar observations at the protein and chemokine gene expression level were made in the case of CCL5 (Supplementary Fig. S5A&B). This increased responsiveness of tumors compared to marginal tissues was not due to decreased survival of the marginal tissues, as determined by undisturbed expression levels of glycogen phosphorylase (Supplementary Fig. S5C). Similarly, the differences in the responsiveness to the chemokine-modulatory regimen between tumors and marginal tissues could not be explained by potential differences between their expression of the IFN γ receptor, TLR3, IRF1, IRF3, or the differential infiltration with APCs or NK cells, which were all similar between tumors and marginal tissues (data not shown).

Driven by the previously reported key role of NF- κ B in the induction of CXCL10 and other chemokines (28–30), and the ubiquitous enhancement of the NF- κ B signaling in cancer lesions critically needed for tumor survival and growth (24–27), we tested whether potential differences in NF- κ B activation could be responsible for the differential ability of the tumors versus marginal tissues to respond to the chemokine modulatory regimen.

In accordance with this possibility, we observed that the colorectal cancer tissues showed not only elevated baseline levels of NF- κ B activation (measured by the rate of its nuclear translocation; see Fig. 4B, left), but an even more pronounced ability to further activate NF- κ B after the IFN γ /poly-I:C/indomethacin treatment (Fig. 4B, right). The key role of NF- κ B in CXCL10 production by tumor tissues was validated by using an NF- κ B inhibitor, CAY10470, which completely abrogated CXCL10 induction (Fig. 4C).

CCL5 regulation showed a similar pattern (treatment-induced up-regulation in tumors, rather than in marginal tissues) and was also blocked by CAY10470 (Supplementary Fig. S5B), showing the general role of the tumor-associated NF- κ B deregulation in the selective induction of T_{eff}-attracting chemokines by the chemokine-modulating regimen. CAY10470, used in these experiments (at 20 μ M), was non-toxic, as shown by similar expression of glycogen phosphorylase mRNA in untreated and treated tissues (Supplementary Fig. S5C).

Interestingly, our confocal microscopy analysis revealed that most of the cells that showed nuclear translocation of NF- κ B and produced CCL5 and CXCL10 represented CD45⁺ infiltrating inflammatory cells and (TE-7-binding) tumor-associated fibroblasts, with only

some of the CD326/EpCAM⁺ cancer cells producing CCL5 (Fig. 4D; *also see Supplementary Fig. S5D for example of single-color analyses*).

IFN α /poly-I:C/indomethacin-treated colorectal tumors preferentially attract effector CD8⁺ T cells

In order to demonstrate that the modulation of chemokine achieved by the combination of IFN α , poly-I:C and indomethacin is indeed sufficient to affect the ability of tumors to attract different subsets of T cells, we used an *ex-vivo* chemotaxis assay involving the supernatants from differentially-treated tumors and either expanded tumor-infiltrating CD8⁺ T cells (TILs; see Supplementary Fig. S2F) or polyclonal *ex-vivo*-induced effector CD8⁺ T cells induced by superantigen-loaded DC1s (32). As shown in Fig. 5A, each type of effector CD8⁺ T cell showed strongly enhanced migratory responsiveness uniformly to all the IFN α / poly-I:C/indomethacin-treated tumors. In contrast, CD4⁺FOXP3⁺ T cells preferentially migrated to untreated tumors, as determined by Taqman analysis of the migrated blood-isolated CD4⁺ T cells (Fig. 5B), or flow cytometry (Supplementary Fig. S6A). As expected, Taqman analysis of another T_{reg} marker, GITR, yielded similar results (Supplementary Fig. S6B).

Discussion

Our data demonstrate the feasibility of tumor-selective modulation of the chemokine environment, using clinically applicable combinations of pharmacologic and biologic factors to correct the balance between tumor-infiltrating T_{eff}⁺ and T_{reg}⁺ cells, the types of immune cells known to differentially affect the clinical course of cancer (1–8). Importantly for the clinical application of this strategy, we observed that while the responses of the individual tumor lesions (even in the same patient) to the individual chemokine-modulators were highly variable (consistent with the limited clinical effectiveness of such factors applied individually), the combination of IFN α , poly-I:C and cyclooxygenase inhibitors allowed for highly consistent and selective enhancement of T_{eff}⁺-attracting chemokines (CCL5 and CXCL9-10) within tumor lesions tested, with the concomitant uniform suppression of local CCL22, the T_{reg}⁺-attracting chemokine.

The IFN α /poly-I:C/indomethacin-induced production of T_{eff}⁺-attracting chemokines was highly tumor-selective, suggesting that even systemic administration of these chemokine-modulating factors can preferentially direct effector cells to tumors. While the attraction of different subsets of T cells to different tumor types is known to be regulated by a complex network of additional chemokines not included in our current analysis (35, 36) and can be subject to regulation at the level of chemokine receptor expression, for example by CCR5 polymorphism (37), our current functional data (Fig 5) indicate that the proposed regimen can uniformly promote the influx of effector CD8⁺ T cells (both spontaneously-arising TILs and DC1 vaccine-induced CTLs). The known role of CXCR3 and CCR5 in the attraction of Th1 cells and NK cells (12, 13, 38, 39) suggests that the proposed regimen may also be able to promote the entry of these additional types of desirable cells into tumors.

We observed that the tumor-selectivity of the proposed regimen depends on the propensity of tumor-associated fibroblasts and infiltrating inflammatory cells (with lesser involvement of tumor cells themselves) to not only spontaneously hyper-activate NF- κ B, but also respond to treatment with further-enhanced levels of NF- κ B activation. Since NF- κ B activation, critically involved in tumor survival and growth, represents an intrinsic feature of many tumor types (24–27), the current data suggest that the currently-described NF- κ B-targeting modulation of the tumor microenvironment may be applicable to multiple types of cancer. The currently-developed chemokine-modulating regimen consists of the combination of IFN α (type 1 interferon), poly-I:C (TLR3 ligand), and either indomethacin

(COX1 and COX2 inhibitor) or a selective COX2 inhibitor, celecoxib. While our data demonstrate that interferons and prostanoids differentially regulate the NF- κ B-driven production of T_{eff}, and T_{reg}-attracting chemokines, the specific mechanisms and the molecular level of interplay between these factors remain subjects of our current research. Our analyses performed so far did not reveal any differences between the expression of the IFN receptor, TLR3, IRF1, or IRF3 between tumors and marginal tissues (data not shown), but our current work focuses on the differential regulation of each of the pathways (poly-I:C, IFN and PGE₂ responsiveness) in whole tumor tissues and different types of tumor-associated cells. Similarly, we are also evaluating the mechanisms underlying the increased sensitivity of tumor-related cells to activate NF- κ B and the relative heterogeneity of different tumors with regard to the requirement for poly-I:C activation, which may help us to identify new strategies of chemokine regulation and of targeting NF- κ B in tumor therapy.

The combination of IFN, poly-I:C and COX inhibition will be evaluated in clinical trials in patients with metastatic colorectal cancer, as a standalone treatment or in combination with DC1 vaccines (32, 40), to enhance the numbers of circulating effector-type tumor reactive CD8⁺ T cells that respond to CCR5 and CXCR3 ligands (32) and enter tumor tissues. Our follow-up analyses will also allow us to determine whether the observed differences in the expression of chemokines and T_{eff} markers in patients with metastatic colorectal cancer also translate into differences in clinical course of the disease and patient survival, as predicted by studies in primary colon cancer (1–4).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Presence of T_{eff} and T_{reg} markers in tumors correlates with intra-tumoral expression of, respectively, T_{eff} or T_{reg} -attracting chemokines

Tumor biopsies from colon cancer patients were lysed, RNA extracted and Taqman analysis of various markers was performed. (A) Correlation between T_{eff} markers (CD8 and Granzyme B; GZMB) and T_{eff} -attracting chemokines (CCL5 and CXCL10) in tumor lesions. (B) Correlation between T_{reg} markers (FOXP3 and GITR) and the chemokine CCL22 in tumor lesions.

Figure 2. Heterogenous response pattern of different tumor tissues to individual chemokine modulators and their uniform response to the combination of IFN, poly-I:C and indomethacin
 (A) Fresh tumor samples from 11 patients with metastatic colorectal cancer were untreated or treated with IFN and poly-I:C either individually or in combination for 48 hours. The release of CCL5 and CXCL10 into culture media was analyzed by ELISA. Numbers indicate the prevalence of tumors with each chemokine pattern (respective patterns A, B or C). (B) ELISA analysis of CCL5, CCL22 and CXCL10 in tumors untreated or treated with IFN + pI:C, with or without indomethacin. *denotes $P < 0.05$ (the presence or absence of indomethacin).

Figure 3. Combination of IFN , poly-I:C and indomethacin, consistently up regulates T_{eff}-attracting chemokines and suppresses T_{reg}-attracting chemokines in tumor tissues
 (A) *In-situ* hybridization for respective chemokine mRNA (black grains) in tumor biopsies which were either left untreated or treated with the combination of indomethacin, IFN and poly-I:C (IAP). (B) ELISA analysis of the chemokine contents in the supernatants of 48 hour-cultured tumor tissues (untreated or treated) from 10 different patients.

Figure 4. NF-κB-dependent selective enhancement of CXCL10 production in tumor tissues following exposure to the combination of IFN, poly-I:C and indomethacin

(A) ELISA for CXCL10 expression in matched normal liver and liver metastatic tissues from 10 different patients either untreated (left panel) or treated (right panel). (B) Average number of cells counted per field (confocal microscopy; in a total of 10 fields) showing nuclear translocation of NF-κB in normal liver or liver metastatic tissues either untreated or treated (right panel). Representative images of each condition are shown in the left panel. (C) ELISA analysis of CXCL10 production by the matched normal liver and liver-metastatic colorectal cancer tissues, either untreated or treated (IFN, poly-I:C and indomethacin), in the absence or presence of 20μM CAY10470 (NF-κB inhibitor). (D) Colorectal cancer tissues from three colorectal cancer patients were treated with indomethacin + IFN + Poly-I:C for 30 minutes (p65 translocation) and 24 hours (chemokine production) and analyzed by confocal microscopy for the translocation of p65 and production of CCL5 and CXCL10 by infiltrating inflammatory cells (CD45⁺), tumor-associated fibroblasts (TE-7-binding cells) and cancer cells (CD326). Representative data from one of three experiments. *Left panel*: representative sections of the activated tumor tissue. *Right panel*: Numbers per vision field of the individual cells types showing p65 translocation and chemokine production. Data from 10 vision fields is expressed as average $\pm$ SEM.

Figure 5. IFN γ , poly-I:C and indomethacin-treated tumors show enhanced ability to attract T_{eff}, but strongly-reduced ability to attract T_{regs}

(A) *Ex vivo* generated T_{eff} (left) or isolated CD8⁺ tumor-infiltrating lymphocytes (right) (see Materials and Methods) were allowed to migrate towards supernatants from either untreated or treated tumors from 3 different patients in transwell chemotaxis assays. (B) Negatively-isolated total CD4⁺ T cells were allowed to migrate towards the treated- or untreated tumor supernatants. Migrating cells were lysed and analyzed for FOXP3 expression by Taqman. U.D.: undetectable.

Table 1

Demographic profile and clinical status of the 72 colorectal cancer patients involved in the study.

N=72		
Sex	Number	%
Male	38	52.78
Female	34	47.22
Median age at surgery	58.5	
Stage	Number	%
I	4	5.56
IIA	2	2.78
IIB	4	5.56
IIIA	1	1.39
IIIB	16	22.22
IIIC	6	8.33
IV	37	51.39
Metastasis sites	Number	%
Liver	30	41.67
Ovary	6	8.33
Peritoneal Carcinomatosis	14	19.44
Primary	4	5.56
Rectal recurrence	4	5.56
Other	14	19.44