NF-κB Hyperactivation in Tumor Tissues Allows Tumor-Selective Reprogramming of the Chemokine Microenvironment to Enhance the Recruitment of Cytolytic T Effector Cells

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Abstract

Tumor infiltration by effector CD8+ T cells (Teff) predicts longer recurrence-free survival in many types of human cancer, indicating the broad significance of Teff for effective immunosurveillance. Colorectal tumors with reduced accumulation of Teff express low levels of Teff-attracting chemokines such as CXCL10/IP10 and CCL5/RANTES. In this study, we investigated the feasibility of enhancing tumor production of Teff-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 IFNα or poly-I:C (a TLR3 ligand) when they were applied individually. In contrast, a combination of IFNα 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 (Treg). The Teff-enhancing effects of this treatment occurred selectively in tumor tissues, as compared with tissues derived from tumor margins. These effects relied on the increased propensity of tumor-associated cells (mostly fibroblasts and infiltrating inflammatory cells) to hyperactivate NF-κB and produce Teff-attracting chemokines in response to treatment, resulting in an enhanced ability of the treated tumors to attract Teff cells and reduced ability to attract Treg cells. Together, our findings suggest the feasibility of exploiting NF-κB hyperactivation in the tumor microenvironment to selectively enhance Teff entry into colon tumors. Cancer Res; 72(15): 3735–43. ©2012 AACR.

Introduction

The ability of CD8+ T cells to infiltrate cancer lesions is essential for antitumor immunity, as evidenced by studies highlighting the prognostic value of effector T (Teff) cells in multiple cancer types, including colorectal cancer (CRC; refs. 1–4). In contrast, tumor infiltration with regulatory T cells (Treg) 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 intratumoral expression of CCL5 and CXCL9-11 (18), high levels of CCL22/MDC, the CCR4 ligand preferentially attracting Treg cells, can be associated with reduced survival, as shown in patients with ovarian cancer (19).

Several studies have indicated the propensity of colorectal tumors to overexpress COX2 and its product prostaglandin E2 (PGE2; refs. 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 IFNα versus PGE2 on the production of Teff- and Treg-attracting chemokines in isolated DCs (22), we tested the feasibility of using these factors to manipulate tumor microenvironment to enhance the production of Teff-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 hyperactivation of NF-κB in cancer tissues (24–27), and the requirement for this factor in the induction of both Treg- and Teff-attracting classes of chemokines (28–30), we tested whether the selected PGE2- and IFNα-targeting strategies can be used to selectively...
enhance the production of T_{eff}-attracting chemokines in tumor tissues, rather than marginal tissues, to selectively direct T_{eff} cells to tumors.

Materials and Methods

Patients

Seventy-two patients with CRC 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 (Pittsburgh, PA) 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 granulocyte macrophage colony-stimulating factor for 6 days. Fibroblasts (Cascade Biologicals) and colon cancer cell lines CACO-2, HCT116, HT29, SW480, and SW620 (American Type Cell Culture) were grown in Iscove’s Modified Dulbecco’s Medium (IMDM) with 10% FBS, whereas human umbilical vein endothelial cells (HUVEC; AllCells) were cultured in HUVEC complete media (Basal media supplemented with HUVEC stimulatory supplement; AllCells). All cell lines were washed, reseeded at 20,000 cells in 300 μL in 96-well plates, and treated with IFNγ, poly-I:C, and/or indomethacin as indicated for 48 hours, and supernatants were analyzed for chemokine production by ELISA.

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<th>Table 1. Demographic profile and clinical status of the 72 patients with CRC involved in the study</th>
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Ex vivo cultures of tumor and marginal tissue explants

Using a 4-mm biopsy puncher, the cubes of tumor or marginal tissue were prepared and placed in antibiotic-containing IMDM with 10% FBS (typically 3 cubes/well in 24-well plates) for 24 to 48 hours, as indicated. When indicated, the tissues were treated with 10,000 U of IFNγ, 20 μg/mL of poly-I:C, 50 μmol/L of indomethacin, or 10 μmol/L of celecoxib. Biopsies were harvested at 0 and 24 hours for mRNA analysis and confocal microscopy analysis. Culture supernatants were harvested at 24 to 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 workflow is depicted in Supplementary Fig. S1A. The system used, on the basis of 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

Four-millimeter biopsies were placed in Lysing Matrix E Tubes (MP Biologicals) containing RLT buffer (RNeasy Kit; Qiagen), and agitated using a FP120 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. One microgram of RNA extracted by the earlier described method was used for cDNA synthesis, and 25 to 50 ng of subsequent cDNA was used to conduct mRNA expression analysis by TaqMan analysis on the StepOnePlus 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 ELISA for the presence of chemokine proteins CCL5, CCL22, and CXCL10, using primary and secondary antibodies from PeproTech. Detection was done using Streptavidin-horseradish peroxidase conjugate and 3,3',5,5'-tetramethylbenzidine substrate from Pierce Biotechnology, Inc.

Isolation of tumor-infiltrating CD8+ T cells

Tumor-infiltrating lymphocytes were isolated as described by Dudley and colleagues (31), with the following modifications: Tumor was cut into 4-mm cubes using a biopsy punch, and each 4-mm tumor piece cultured in 1 mL of IMDM with 5% human AB serum with 1,000 U/mL interleukin (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 conducted in 24 Transwell plates with 5-μm pore size polycarbonate filters (Corning Inc.). The lower chambers were filled with 600 μL of tumor supernatants. As indicated, 2 × 10⁵ of either isolated tumor-infiltrating lymphocytes or αDC1-activated CD8+ T_{eff} cells (32), in 200 μL
μL of IMDM with 10% fetal calf serum, were added to the upper chambers and incubated for 3 hours at 37°C. Migrated cells were harvested from the lower chambers and stained for CD8. Cell counts were carried out by a limited 60-second run on a BD Beckman Coulter XL Cytometer. For analysis of Treg cell migration, bulk CD4+ T cells were isolated by negative selection using EasySep CD4 Enrichment Kits (StemCell), and 1 × 10^6 of the isolated cells in 200 μL were allowed to migrate towards 600 μL 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% paraformaldehyde, processed, and pretreated as described (33), except that tissues were sectioned on a cryostat at 5 μm. 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 conducted as described (33, 34), with 0.1 mol/L dithiothreitol included in the hybridization mix. Hybridizations were conducted at 50°C overnight. Tissue sections were coated with NTB emulsion (Kodak) and exposed at 10°C for 7 to 14 days. Simultaneous in situ hybridization and immunohistochemistry were conducted as described (33, 34), except that the dithiothreitol concentrations were 0.01 mol/L in the hybridization mix and 1 mmol/L 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

Four-millimeter tumor punches, either untreated or treated, were embedded in optimum cutting temperature (OCT) medium containing cryomolds and immediately frozen in 2-methyl-butane. Six-micrometer frozen sections of the tissues were made using the cryostat and layered on SuperFrost Plus Slides (Thermo Scientific). The slides were incubated in 4% paraformaldehyde for 15 minutes, washed, and blocked for 60 minutes at room temperature. The slides were then stained for 3 hours at room temperature with antibodies for P65 (ab16502) or for CD8 (ab4055), CXCL10 (ab9807, Abcam), and CCL5 (ab10590; both Abcam). The slides were washed 3 times with 1 × PBS and incubated with secondary antibodies anti-rabbit (Alexa 647), anti-mouse (Alexa 488; both Cell Signal Technology), and anti-goat (Alexa 488; Invitrogen) for 30 minutes at room temperature. The slides were washed 3 times with 1 × PBS and once with high-salt PBS. Cover slips were mounted on the sections using ProLong Gold antifade solution (Invitrogen). Confocal analyses of stained slides were conducted using a LEICA TCS SL DMRE Microsystems. To quantify the numbers of cells showing nuclear NF-κB translocation, images of 10 different fields (×63 magnification) of the tumor and marginal tissue sections (untreated or treated) were taken. To identify the cells showing P65 translocation and chemokine production in response to treatment, tumor tissues were stained with CD45 (H130, BioLegend), CD326 (9C4, BioLegend), fibroblast marker (TE-7, EMD Millipore), 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 conducting a multivariate ANOVA (MANOVA) of each pair of variables on batch and deriving the correlation from the MANOVA residual matrix. Comparisons of continuous variables between groups were conducted by 2-tailed paired t tests. P < 0.05 was considered significant. Analyses were conducted using SAS v9.2 (SAS Institute) or GraphPad Prism 5 software.

Results

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

Using resected tumor material from 72 patients with advanced CRC (metastatic in 68 patients), we observed that local expression of 2 T_{eff} cell markers (CD8 and Granzyme B; GZMB) is strongly correlated with the expression of 2 T_{eff}-attracting chemokines, CCL5 and CXCL10 (Fig. 1A). In contrast, the Treg 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 CCL2 and the CCL22-inducing factor (22) COX2 (Supplementary Fig. S2A and S2B). Confocal microscopy analysis of the tumor sections revealed that all CXCL10-producing cells (Supplementary Fig. S2D, right) and a significant proportion of CCL5-producing cells (left) were CD8+ , 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 patients with colon cancer (see Materials and Methods) revealed that the majority of CD8+ TILs are CCR5+ , CXCR3+ (Supplementary Fig. S2E), and GZMB+ (Supplementary Fig. S2F), which further indicates that the intratumoral expression of the CCR5 and CXCR3 ligands was responsible for recruiting the T_{eff} into the tumor.

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

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 IFNα, 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 IFNα and poly-I:C in the induction of CCL5 and CCL10, and a strong suppressive effect of IFNα on the production of CCL22 in macrophages and fibroblasts (Supplementary Fig. S3A and S3B). 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, CCL10, or CCL22 (data not shown).

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 a

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 Fig. S1B and data not shown).

As shown in Fig. 2, different tumor tissues treated with IFNα or poly-I:C alone showed variable chemokine expression, falling into 3 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 IFNα 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 IFNα 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).

On the basis of these data, we selected the triple combination of IFNα, 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) showed that CCL22 was expressed predominantly by HLA-DR⁺ antigen-presenting cell (APC), whereas 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 Tɛ-recruiting chemokines within the tumor microenvironment.

Enhanced activation of tumor-associated NF-κB 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 IFNα, poly-I:C, and indomethacin induced much more pronounced secretion of CXCL10 by tumor tissues compared with 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 and S5B). This increased responsiveness of tumors compared with marginal tissues was not due to...
NF-xB Imbalance Allows Tumor-Selective Chemokine Modulation

Figure 2. Heterogenous response pattern of different tumor tissues to individual chemokine modulators and their uniform response to the combination of IFNα, poly-I:C (pI:C), and indomethacin. A, fresh tumor samples from 11 patients with metastatic CRC were untreated or treated with IFNα 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 IFNα/poly-I:C, with or without indomethacin (indo).

NF-xB Imbalance Allows Tumor-Selective Chemokine Modulation

NF-xB imbalance influences the differential ability of tumors versus marginal tissues to respond to the chemokine-modulatory regimen.

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

CCL5 regulation showed a similar pattern (treatment-induced upregulation 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-xB deregulation in the selective induction of T_{off}.
-attracting chemokines by the chemokine-modulating regimen, CAY10470, used in these experiments (at 20 μmol/L), was nontoxic, 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-κB 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).

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

To show that the modulation of chemokine achieved by the combination of IFNα, 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 αDC1s (32). As shown in Fig. 5A, each type of effector CD8+ T cell showed strongly enhanced migratory responsiveness uniformly to all the IFNα/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 Treg marker, GITR, yielded similar results (Supplementary Fig. S6B).

Discussion

Our data show 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 Treg 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 IFNα, poly-I:C, and COX 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 Treg-attracting chemokine.

The IFNα/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...
NF-κB Imbalance Allows Tumor-Selective Chemokine Modulation

Figure 4. NF-κB–dependent selective enhancement of CXCL10 production in tumor tissues following exposure to the combination of IFNα, poly-I:C, and indomethacin. A, ELISA for CXCL10 expression in matched normal liver and liver metastatic tissues (Liver mets) from 10 different patients either untreated (left) or treated (right). 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). Representative images of each condition are shown on the left. C, ELISA analysis of CXCL10 production by the matched normal liver and liver metastatic CRC tissues, either untreated or treated (IFNα, poly–I:C, and indomethacin), in the absence or presence of 20 μmol/L CAY10470 (CAY; NF-κB inhibitor). D, CRC tissues from 3 patients with CRC were treated with indomethacin + IFNα + 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 3 experiments are shown. Left, representative sections of the activated tumor tissue. Right, numbers per vision field of the individual cells types showing p65 translocation and chemokine production. Data from 10 vision fields are expressed as average ± SEM.

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 αDC1 vaccine-induced CTLs). The known role of CXCR3 and CCR5 in the attraction of T11 cells and natural killer 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 hyperactivate NF-κB, but also respond to treatment with further enhanced levels of NF-κB activation. Because NF-κB 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-κB–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 IFNα (type 1 interferon), poly–I:C (TLR3 ligand), and either indomethacin (COX1 and COX2 inhibitor) or a selective COX2 inhibitor, celecoxib. While our data show that interferons and prostanooids...
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Figure 5. IFNκ, poly-I:C, and indomethacin-treated tumors show enhanced ability to attract T\textsubscript{reg}, but strongly reduced ability to attract T\textsubscript{eff} cells. A, ex vivo-generated T\textsubscript{reg} (left) or isolated CD8\textsuperscript{+} TILs (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\textsuperscript{+} T cells were allowed to migrate toward the treated or untreated tumor supernatants. Migrating cells were lysed and analyzed for FOXP3 expression by TaqMan. UD, undetectable.

Disclosure of Potential Conflicts of Interest

The methods of tumor-selective regulation of chemokine environment are the subject to a pending patent application by the University of Pittsburgh. None of the authors receives any form of remuneration related to these findings. P. Kalinski has ownership interest (including patents) in inventorship of a pending patent application relevant to the manuscript. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: R. Muthuswamy, P. Kalinski
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The combination of IFNκ, poly-I:C, and COX inhibition will be evaluated in clinical trials in patients with metastatic CRC, as a standalone treatment or in combination with αDC1 vaccines (32, 40), to enhance the numbers of circulating effector-type tumor-reactive CD8\textsuperscript{+} 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\textsubscript{eff} markers in patients with metastatic CRC also translate into differences in clinical course of the disease and patient survival, as predicted by studies in primary colon cancer (1–4).

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