Activated Eosinophils Exert Antitumorigenic Activities in Colorectal Cancer

Hadar Reichman1, Michal Itan1, Perri Rozenberg1, Tal Yarmolovsky1, Eli Brazowski2, Chen Varol2, Nathan Gluck2, Shiran Shapira3, Nadir Arber3, Udi Qimron1, Danielle Karo-Atar3, James J. Lee4,5, and Ariel Munitz1

Abstract

Immunotherapies targeting T lymphocytes are revolutionizing cancer therapy but only benefit a subset of patients, especially in colorectal cancer. Thus, additional insight into the tumor microenvironment (TME) is required. Eosinophils are bone marrow–derived cells that have been largely studied in the context of allergic diseases and parasite infections. Although tumor-associated eosinophilia has been described in various solid tumors including colorectal cancer, knowledge is still missing regarding eosinophil activities and even the basic question of whether the TME promotes eosinophil recruitment without additional manipulation (e.g., immunotherapy) is unclear. Herein, we report that eosinophils are recruited into developing tumors during induction of inflammation-induced colorectal cancer and in mice with the ApcMin/+ genotype, which develop spontaneous intestinal adenomas. Using adoptive transfer and cytokine neutralization experiments, we demonstrate that the TME supported prolonged eosinophil survival independent of IL5, an eosinophil survival cytokine. Tumor-infiltrating eosinophils consisted of degranulating eosinophils and were essential for tumor rejection independently of CD8+ T cells. Transcriptome and proteomic analysis revealed an IFNγ-linked signature for intratumoral eosinophils that was different from that of macrophages. Our data establish antitumorigenic roles for eosinophils in colorectal cancer. These findings may facilitate the development of pharmacologic treatments that could unleash antitumor responses by eosinophils, especially in colorectal cancer patients displaying eosinophilia.

Introduction

The tumor microenvironment (TME) has been recognized as a critical factor in tumor biology (1). The importance of the immune system in the TME is nicely exemplified by the crucial functions that have been uncovered for cytotoxic T cells in tumor cell elimination (2), which have been utilized for therapy using immune-checkpoint blockade (3). However, the TME often promotes T-cell suppression, which complicates targeting of T cells, immune-checkpoint blockade (3). Nonetheless, most of the experimental data assessing eosinophil function in cancer have been gathered using tumor cell lines that secrete eosinophil-promoting cytokines (e.g., IL5 and CCL11; refs. 6, 7), genetically modified tumors that polarize a type 2 cytokine environment (8), responses to immunotherapy (e.g., IL4, GM-CSF, IL33, and TSLP) or in the absence of T regulatory cells (9–12). Thus, translation of such data into insights about human disease is difficult.

Here, we studied eosinophils in cancer with experimental models that fulfilled three conditions: (i) The models addressed tumor types with clinically reported data on increased tumor-infiltrating eosinophils; (ii) tumor development occurred in an anatomically relevant tissue for eosinophils; and (iii) tumor progression occurred gradually without exogenous tumor cell injections, a process that allowed eosinophils to adapt to their changing microenvironment. Using these criteria led us to investigate eosinophils in colorectal cancer. Several clinical studies reported increased tissue eosinophilia in colorectal cancer patients and that eosinophilia was associated with favorable prognosis (13–15). The largest homeostatic niche for eosinophils is the gastrointestinal (GI) tract; and eosinophil accumulation in the GI tract is a feature of numerous inflammatory GI diseases, in which eosinophils have key functions (16, 17). Finally, several chronic experimental models exist for colorectal cancer, which mimic the human disease, at least in part (18).

We show herein that the TME in human and experimental colorectal cancer is characterized by eosinophil recruitment, prolonged survival, and degranulation. Eosinophils possessed antitumorigenic activities that were independent of CD8+ T cells.
Unbiased RNA-seq and proteomics revealed that intratumoral eosinophils were characterized by an IFNγ signaling signature. Consistent with this signature and the in vivo role for eosinophils in colorectal cancer, activation of eosinophils with IFNγ in vitro potentiated eosinophil-mediated killing of colorectal cancer cells. Collectively, our data established antitumorigenic functions for eosinophils in colorectal cancer and describe the phenotypic landscape of intratumoral eosinophils. Thus, pharmacologic approaches that target eosinophils have the potential to unleash antitumor activities.

Materials and Methods

Human tissue arrays

Human tissue arrays as well as data on patients’ characteristics were obtained from US Biomax, Inc. The following arrays were used: CO601, CO602, CO703, CO702B, CO952, CO953, CO992A, TO54B, and TO55, and a total of 351 human samples were obtained from US Biomax, Inc.. The following arrays were Human tissue arrays.

Mice

Wild-type (WT) C57BL/6 mice were originally obtained from Harlan Laboratories and grown in-house. CD3-IL5 transgenic mice (NJ.1638, IL5Tg) were kindly provided by Dr. Jamie Lee (Mayo Clinic, Scottsdale, AZ). AdblGATA mice were kindly provided by Dr. August Avery (Cornell University, Ithaca, NY). Apc<Min>/−/AdblGATA mice were generated by mating male Apc<Min>/− mice with female AdblGATA. All experiments were reviewed and approved by the Animal Care Committee of Tel Aviv University (Number M-14-061, M-15-001) and were performed in accordance with its regulations and guidelines regarding the care and use of animals for experimental procedures. All of the experiments were conducted in the specific pathogen-free facilities of Tel Aviv University. In all experiments, age-, weight-, and sex-matched mice were used.

Colitis-associated cancer

Mice were injected intraperitoneally with 12.5 mg/kg azoxy-methane (AOM, Sigma). At 5 days after injection, mice were given 2.25% dextran sulfate sodium (DSS) in drinking water for 5 days, followed by 16 days of normal drinking water. DSS treatment was repeated for 2 additional cycles of 2.25% and 2% DSS, and mice were euthanized at 10 to 13 weeks after AOM injection.

Adoptive transfer experiments

Spleens were extracted from IL5<sup>−/−</sup> mice and crushed through a 70-μm strainer. Red blood cells were lysed, and the remaining white blood cells were subjected to lymphocyte depletion using Dynabeads conjugated to antibodies against Thy1.2 and B220 (Thermo Fisher Scientific). The purity of eosinophils was determined using flow cytometry and was consistently >95%. Thereafter, AdblGATA mice were injected intravenously with 50 x 10<sup>6</sup> eosinophils on day 4 of the DSS treatment or at the age of 6 weeks in the Apc<sup>Min>/−</sup> mouse model.

Orthotopic injections

Mice were anesthetized using Ketamine/Xylazine. Submucosal injections of MC38 colorectal cancer cells were accomplished using flexible stainless steel, 8 inch long, 30 gauge, 45 degree bevel hypodermic needles custom made according to our specification (Cadence, Inc.). The needle was inserted through Luer lock (Söllner, GmbH) screwed on the working channel of the endoscope to avoid air leakage. After the scope was inserted into the mouse colon and the colon inflated, the needle was brought through the working channel to the scope’s front. The colorectal cancer cell implantation procedure required two persons: one to navigate the colonoscopy while the other performed the injection. The injection consisted of a very gentle submucosal penetration with the open side of the bevel heading up at a flat angle. A volume of 50 μL colorectal cancer tumor cells in saline was then injected into the colonic submucosa. Mice were euthanized on day 21 and histologic specimens were prepared.

IL5 neutralization

Apc<sup>Min>/−</sup> mice were treated for a period of up to 5 months with anti-IL5 (TRFK5, 50 μg/mouse, twice a week) or isotype control antibodies. Thereafter, mice were euthanized, and eosinophil levels were determined in the peripheral blood and intestines.

Punch biopsies

Colons were flushed with phosphate-buffered saline and opened along a longitudinal axis; 3 mm<sup>2</sup> punch biopsies were incubated for 24 hours in RPMI supplemented with 10% fetal calf serum and antibiotics. Supernatants were collected and assessed for cytokine expression by ELISA (19).

Coculture experiments

Primary eosinophils were isolated from the peritoneal lavage of IL5<sup>−/−</sup> mice and enriched by lymphocyte depletion through use of either a MACS cell separation system with antibodies against CD90.2 and CD45R (Miltenyi Biotec) or Dynabeads conjugated to antibodies against Thy1.2 and B220 (Thermo Fisher Scientific). Eosinophils were then seeded together with CT26 or MC38 cells at 800,000 cells per well at varying ratios. In several experiments, eosinophils were cultured in the presence of colorectal cancer cell–conditioned media, which was obtained from the cells after they reached confluence. Eosinophils were cultured with tumor cell–conditioned media in the presence of anti-IL5 (clone TRFK5, 0.15 μg/ml) or isotype control.

Enzymatic digestion of gastrointestinal lamina propria cells

Colon tissue was excised and flushed with 1 mL of calcium- and magnesium-free HBSS (CMF-HBSS). The colon was dissected longitudinally and shaken (250 RPM) in 5 mL CMF-HBSS containing 5% FCS, 2 mmol/L EDTA, and 1 mmol/L DTT (Dithiotheritol) for 40 minutes at 37°C in order to remove epithelial cells and intraepithelial lymphocytes. Then, the colonic tissue was vortexed and strained through 70-μm gray mesh. The remaining tissue or isolated tumors were washed in PBS and then incubated and shaken (250 RPM) with complete PBS containing calcium and magnesium) supplemented with 5% FCS, 1 mg/mL collagenase A (Roche), and 0.1 mg/mL Dnase I (Sigma) for 40 minutes at 37°C. The cell suspension was filtered using gauze (70-μm mesh) and suspended in flow cytometry staining buffer (HBBS, 1% FCS).

Flow cytometry

Single-cell suspensions of mouse cells were stained using the following antibodies: anti-CD45-APC, anti-CD45-APC-eFluor780, anti-CD11b-PerCP-Cy5.5, anti-Gr1-PE, anti-CD8a-PE (obtained from eBioscience), anti-CD3e-PE-Cy7 (obtained from BioLegend), anti-CCR3-FTC (obtained from R&D Systems).
anti-Siglec-F-PE (BD Biosciences), and DAPI (Sigma). Eosinophils were identified as CD45+/CD11b+/Siglec-F+/Ly6c-/Ly6g+/MHC-II+ (colon and ileum); CD45+/Siglec-F+/CCR3+/CD8+/CD45+/Gr1+/CD11b+ cells, respectively.

IHC
Tissues were fixed, embedded, sectioned, and stained with anti-EPX or anti-major basic protein (MBP) (kindly provided by Dr. Jamie Lee, Mayo Clinic, Scottsdale, AZ), as described previously (19). For anti-CD31 and anti-ki67 staining, slides were trypsinized with 0.1% trypsin at 37°C for 5 minutes (BD Difco), incubated with 180 mL methyl alcohol and 3 mL of 30% hydrogen peroxide, blocked with 2% rabbit serum/PBS/Triton for 2 hours, and incubated overnight with rat anti-mouse CD31 (Blood and bone marrow). T cells and myeloid-derived suppressor cells were identified as CD45+/CD3+/CD8- and CD45+/Gr1+/CD11b+ cells, respectively.

Immunofluorescence
Tissues were placed in OCT (Tissue-TEK) and snap-frozen over dry ice. Tissue sections were cut, air dried, fixed, and blocked. Thereafter, tissues were stained with anti-MBP (kindly provided by Dr. Jamie Lee, Mayo Clinic, Scottsdale, AZ) and anti-cleaved caspase-3 (Cell Signaling) followed by the following secondary antibodies: donkey anti-rat AlexaFluor 488 (1:300, Jackson ImmunoResearch) and goat anti-rabbit AlexaFluor 546 (1:500, Life Technologies). Slides were stained with DAPI (Sigma) and mounted using gel-mount (Sigma). Images were captured using an LSM 800 confocal microscope (Zeiss).

RNA-seq
RNA was extracted using TRIzol (Invitrogen) according to the manufacturer’s instructions. Samples were prepared with CEL-seq and sequenced using Illumina HiSeq 2500. Sample preparation, sequencing, quality control, and differential expression analyses were conducted by the "Technion Genome Center," Life Science and Engineering Interdisciplinary Research Center, Technion, Haifa, Israel.

Proteomics
To identify differentially expressed proteins and phospho-proteins, eosinophils and macrophages were subjected to protein isolation using scioExtract Pro (Sciomics) using adapted protocols. All samples to be analyzed were normalized to have equal protein yields according to the respective cell counts. Individual eosinophil cell sorting batches were pooled to yield at least 229,000 to 235,000 cells in total. The individual samples were extracted in a cascade fashion, meaning that the first samples were treated with scioExtract and the whole solution was carried to the next sample tube. Three individual samples were pooled to yield a sample for analysis. For all other samples, individual samples were extracted according to standard Sciomics protocols with scioExtract Pro. The complete resulting protein fraction was labeled with scioDye 1 (Sciomics) and purified with low cell count additives (Sciomics) during the purification step. All samples were incubated for three hours on scioDiscover arrays containing 1,130 antibodies against 900 proteins (Sciomics) according to standard protocols for microarray analysis for protein expression and phosphorylation analysis. Subsequently, slides were washed and dried. Slides were scanned using a Powerscanner (Tecan) to obtain fluorescence values. The resulting raw data were analyzed using the linear models for microarray data (LIMMA) package of R-Bioconductor for differential protein expression including normalization (Cyclic Lowess) and P value as well as log2fc calculation. The false discovery rate was controlled according to Benjamini and Hochberg (20).

Proliferation assays
Cell proliferation was assessed using the Click-iT EdU kit (Invitrogen) according to the manufacturer’s instructions.

Statistical analysis
Data were analyzed by analysis of variance followed by Tukey post hoc test or Student t test using GraphPad Prism 5. Survival curves were analyzed using the Gehan–Breslow–Wilcoxon test and the log-rank (Mantel–Cox) test. Data are presented as mean ± SEM, and values of P < 0.05 were considered statistically significant; P values for differential expression of RNA-seq data were corrected for multiple testing according to the Benjamini–Hochberg procedure (20).

Results
Accumulation of eosinophils in human colorectal cancer inversely correlates with tumor stage
To define the role of eosinophils in colorectal cancer, we first analyzed intratumoral eosinophils using tissue arrays containing biopsies from 275 patients and healthy individuals (see the patient characteristics in Supplementary Table S1). Tissues were stained with anti-eosinophil peroxidase (EPX), a commonly used method determine human eosinophil numbers and degranulation in situ (21). Stained biopsies were assessed using computerized analyses and divided into four groups based on the numbers of intratumoral eosinophils: <10 eosinophils/mm²; 10–40 eosinophils/mm²; 40–100 eosinophils/mm²; and >100 eosinophils/mm² (Fig. 1A). Segregation of the biopsies according to the tumor stage revealed an inverse correlation between tumor stage and intratumoral eosinophils (Fig. 1B), which was not due to fluctuations in eosinophil numbers in the adjacent healthy tissue (Fig. 1C). Most specimens, independent of tumor grade, contained degranulated eosinophils as observed by extracellular deposition of eosinophil granule content (Fig. 1D, blue arrows) as well as intact cells (Fig. 1D, black arrows).

Next, we asked whether eosinophils were also present in draining lymph node metastases. To this end, eosinophils in biopsies from 91 patients who displayed lymph node metastasis were assessed (Fig. 1E). Prominent eosinophilia (i.e., more than 10 eosinophils/mm²) was observed in ~27% of the biopsies (Fig. 1F).

Eosinophils are recruited and activated in experimental colorectal cancer
To delineate the roles of eosinophils in colorectal cancer, we investigated whether eosinophils accumulate in the GI tract...
During experimental colorectal cancer, using two models that represent distinct etiologies: chronic inflammation (i.e., colitis-associated cancer; CAC) and genetic predisposition (Apcmin/þ mice, resembling familial adenomatous polyposis patients—who do not always have an inflammatory phenotype; ref. 22).

Anti-eosinophil MBP stain revealed a gradual elevation in colonic eosinophils following induction of CAC (Fig. 2A). Early stages of this model (weeks 3 and 7) showed elevated eosinophilia, and the colon was highly infiltrated with eosinophils by week 11. Intratumoral eosinophils were readily detected (Fig. 2B) and extracellular MBP, a marker of eosinophil degranulation, was observed in the colonic lamina propria (Fig. 2C).

Subsequently, we aimed to determine whether eosinophils accumulate in the ileum of Apcmin/þ mice, which spontaneously develop intestinal polyps. Our analyses revealed that eosinophils infiltrate the lamina propria (Fig. 2D) and the tumor (Fig. 2E). Flow-cytometric analysis of excised adenomas revealed that intratumoral eosinophils (defined as CD45+/CD11b+/MHC-II+/Siglec-F+/Ly6g+/Ly6c-/SSC−) constituted up to 13% of all intratumoral leukocytes (Fig. 2F) and ~30% of all intratumoral myeloid cells (i.e., CD11b+ cells). Degranulating eosinophils and extracellular MBP were observed in 5-month-old Apcmin/þ mice, which display multiple adenomas at that time point (Fig. 2G, blue arrows).

Figure 1.
Eosinophils accumulate in human colorectal cancer and are inversely correlated with the tumor stage. A, Human tissue arrays from patients with colorectal cancer (n = 275) were stained with anti-eosinophil EPX. B, Stained tissues were divided according to the presence of eosinophils per mm², ranging from <10 to >100 eosinophils/mm² and assessed for their tumor stage (I–IV). C, The number of eosinophils in nontumor, healthy areas was determined and assessed for tumor grade. D, A representative anti-EPX stained slide demonstrating degranulating and intact eosinophils (blue and black arrows, respectively) is shown. Eosinophil numbers were determined in colorectal cancer lymph node metastasis using anti-EPX staining (n = 91, E) and divided according to the presence of eosinophils per mm², ranging from <10 to >100 eosinophils/mm² (F).
The accumulation of eosinophils in the GI tract of mice undergoing CAC and in 5-month-old Apcmin/+ mice was associated with fewer eosinophils in the peripheral blood (Fig. 2H and I) and, to lesser extent, in the bone marrow (Supplementary Fig. S1). Finally, syngeneic intracolon injection of MC38 colorectal cancer cells resulted in the peritumoral accumulation of eosinophils (Supplementary Fig. S2).

Increased expression of CCL11/eotaxin-1 in colorectal cancer
Assessment of the eosinophil-specific chemokines CCL11/eotaxin-1 and CCL24/eotaxin-2 in GI punch biopsies revealed a specific increase in CCL11, but not CCL24 (Supplementary Fig. S3). Thus, eosinophil homing signals are present in the TME, and accumulation of eosinophils in colorectal cancer is independent of mode of disease induction.

The TME in colorectal cancer promotes eosinophil recruitment
To demonstrate that eosinophils were actively and specifically recruited into the TME, purified splenic eosinophils from Il5Tg mice were adaptively transferred into ΔdblGATA mice undergoing CAC (Fig. 2I, top) or into Apcmin/+ mice that lack eosinophils (Apcmin/+ΔdblGATA; Fig. 2I, bottom). The colons of ΔdblGATA mice, which received no eosinophils (No Eos), or naive ΔdblGATA mice that were injected once with eosinophils (+ Eos), displayed few eosinophils (defined as CD45+/CD11b+/Siglec-F+/Ly6c-/Ly6G+/MHC-II+/SSChi cells; Fig. 2K). In contrast, a single injection of eosinophils into ΔdblGATA mice undergoing CAC resulted in preferential and rapid homing of eosinophils into the colon (Fig. 2K and I). Similarly, a single injection of eosinophils into Apcmin/+ΔdblGATA mice resulted in significantly more eosinophils in comparison with ΔdblGATA mice (Fig. 2M and N).

Prolonged eosinophil survival in the TME is independent of IL5
The observation that eosinophils were still present in the colon of ΔdblGATA mice undergoing colitis-associated cancer and the ileum of Apcmin/+ΔdblGATA mice, respectively, for up to three months (Fig. 2K–N), following a single injection (Fig. 2I), suggests that as well as recruiting eosinophils, the TME supports prolonged eosinophil survival. Indeed, 14 days following adaptively transferring eosinophils, we could not detect peripheral blood eosinophils nor observe any eosinophil proliferation in situ (Supplementary Fig. S4).

IL5 is an eosinophil survival factor that regulates eosinophil differentiation, maturation, and survival (23). To determine whether eosinophil survival in colorectal cancer is regulated by IL5, Apcmin/+ mice were treated with anti-IL5 neutralizing antibodies for 5 months, at which point peripheral blood and colonic eosinophils were assessed. Neutralization of IL5 in Apcmin/+ mice significantly decreased peripheral blood eosinophils in comparison with isotype control–treated mice (Supplementary Fig. S5). Nonetheless, eosinophils were detected in the colons of anti–IL5-treated Apcmin/+ mice, and no difference was observed between the anti–IL5-treated and isotype control–treated mice (Supplementary Fig. S5). Consistently, conditioned media of MC38 colorectal cancer cells increased eosinophil survival in vitro (Fig. 2O). Neutralization of IL5 had no effect on eosinophil survival in response to MC38–conditioned media (Fig. 2O).

Eosinophils prevent the development of colorectal cancer
To address the in vivo function of eosinophils in colorectal cancer, CAC was induced in WT and ΔdblGATA mice. The frequency of colitis-associated cancer mortality in WT mice was 13.3% (n = 30 mice). In contrast, in the absence of eosinophils, the mortality rose by 4.2-fold, and 56.2% of the mice died (n = 32 mice, P < 0.05; Fig. 3A). Eosinophil-deficient mice undergoing CAC had an increased tumor load (Fig. 3B), as determined by the increased total tumor counts and size (Fig. 3C and D). Despite our efforts to assess whether eosinophil reconstitution will decrease tumor load, and similar to previously published data (24), maximum eosinophil reconstitution reached only 50% in comparison with the levels of eosinophils in WT mice undergoing CAC. Thus, as an alternative approach, we examined tumor load in hypereosinophilic Il5Tg mice. Il5Tg mice undergoing CAC displayed decreased tumor burden following the induction of CAC (Supplementary Fig. S6).

To further establish the antitumorigenic activity of eosinophils in colorectal cancer, we aimed to determine their role in Apcmin/+ mice. Apcmin/+ΔdblGATA mice displayed approximately a 3-fold increase in cancer-associated mortality (Fig. 3E). Apcmin/+ mice displayed a mortality frequency of 13.8% (n = 41 mice), whereas Apcmin/+ΔdblGATA mice displayed 40.9% (n = 44 mice; Fig. 3E, P < 0.01). Increased mortality in Apcmin/+ΔdblGATA mice was accompanied by an increase in tumor load (Fig. 3F and G).

Antitumorigenic activities of eosinophils in colorectal cancer are independent of CD8+ T cells
Eosinophils may orchestrate tumor rejection in part by enhancing the infiltration of CD8+ T cells (11). However, immune phenotyping of the cellular infiltrate that was present in the colonic tissue in ΔdblGATA mice undergoing CAC and in Apcmin/+ΔdblGATA mice revealed that increased tumor load in the absence of eosinophils was not associated with any alterations in CD8+ T cells or myeloid-derived suppressor cells (Supplementary Fig. S7). We assessed whether the antitumorigenic activities of eosinophils in colorectal cancer were dependent on CD8+ T cells.
Therefore, \( Apc^{min/+} \) and \( Apc^{min/+}/\Delta dblGATA \) mice were treated with antibodies to deplete CD8\(^{+}\) T cells (25). Depletion of CD8\(^{+}\) T cells in \( Apc^{min/+} \) mice (Supplementary Fig. S8) resulted in 16\% tumor-associated death in comparison with isotype control-treated mice, who showed no mortality (Fig. 3H). Isotype control–treated \( Apc^{min/+}/\Delta dblGATA \) mice displayed 50\% tumor-associated death, which was further increased to 66\% upon depletion of CD8\(^{+}\) T cells (Fig. 3H). Tumor burden was increased in anti–CD8-treated \( Apc^{min/+}/\Delta dblGATA \) mice in comparison with anti–CD8-treated \( Apc^{min/+} \) mice (Fig. 3I). Collectively, these data indicate that eosinophils display antitumorigenic activities in colorectal cancer independent of CD8\(^{+}\) T cells.

Transcriptome signatures of intratumoral eosinophils and macrophages

To identify potential mechanisms that mediate the antitumorigenic activities of eosinophils in colorectal cancer, we defined their transcriptional signature. We compared the transcriptional signatures of intratumoral eosinophils with those of macrophages, which have been better characterized. To this end, naïve colonic eosinophils and macrophages as well as intratumoral eosinophils and macrophages were sorted (purity of both cell types was ≥95\%) and subjected to RNA-seq following induction of CAC. Principal component analysis (PCA) revealed four distinct cellular populations: naïve colonic eosinophils, naïve colonic macrophages, intratumoral eosinophils, and intratumoral macrophages (Fig. 4A). In comparison with naïve eosinophils, intratumoral eosinophils displayed multiple differentially expressed transcripts. Of these, 587 transcripts were upregulated and 438 downregulated [fold change >2; \( \text{P}\) value adjusted for false discovery rate (FDR) <0.05; Fig. 4B; Supplementary Tables S2 and S3]. Unbiased STRING analysis, which identifies known and predicted protein interactions (26), revealed that the transcriptome signature of intratumoral eosinophils was associated with a proinflammatory phenotype and was divided into three clusters (Fig. 4C; Supplementary Tables S4–S6). Cluster 1 included enrichment of transcripts that represent a response to interferons and regulation of TLR signaling (e.g., \( Irf1, Irf7, Irf9, Irf14, Iftm1, Nox2 \), and \( Stat1 \); FDR = \( 2.37 \times 10^{-13} \)). Cluster 2 was enriched with transcripts associated with chemokines and cell migration. Cluster 3 comprised transcripts associated with inflammatory and innate immune responses (e.g., \( Cd2, Cd14, Il12b, Thr2, Il17a \), and \( Traf1 \)). Consistent with our STRING analysis, visualization and integrated discovery (DAVID) annotation analysis of
gene ontology (GO) pathways revealed enrichment of inflammatory pathways in intratumoral eosinophils including defense and innate immune responses to various stimuli involving bacteria, stress, lipids, and external stimuli such as cytokines, specifically IFNγ (Fig. 4D). Intratumoral eosinophils also displayed increased mRNA expression of the antiapoptotic molecule Bcl2l1 and decreased mRNA expression of the proapoptotic molecule Casp3 (Supplementary Tables S2 and S3).

A similar analysis of the macrophage transcriptome revealed that in comparison with naïve macrophages, intratumoral macrophages displayed 1,392 transcripts that were differentially expressed. Of these, 654 were upregulated and 738 were downregulated (fold change > 2; P value adjusted for FDR < 0.05; Supplementary Tables S7 and S8). Intratumoral eosinophils and macrophages displayed distinct phenotypes, because only 270 (26% of the eosinophils and 19% of the macrophage transcript signatures, respectively) were shared between eosinophils and macrophages (Fig. 4E; Tables S7–S9). Analysis of these 270 shared transcripts in intratumoral eosinophils and macrophages revealed discrete expression patterns between the two cells (Fig. 4F and G). For example, expression of eosinophil-associated ribonuclease 2 (Ear2) was increased in eosinophils and decreased in macrophages, whereas vascular endothelial growth factor (Vegf) decreased in eosinophils but increased in macrophages (Fig. 4G).

Figure 4.
Intratumoral eosinophils and macrophages display distinct transcriptome signatures. Naïve colonic and intratumoral eosinophils and macrophages were sorted from mice undergoing colitis-associated cancer and subjected to RNA-seq (n = 2 for naïve groups and 6 for intratumoral groups). PCA of the different experimental groups is shown (A). The transcriptome signature of naïve (N1–2) colonic eosinophils and intratumoral (C1–6) eosinophils is shown (B; fold change > 2, P value adjusted for FDR < 0.05). Significantly upregulated transcripts were subjected to STRING analysis (C). Furthermore, the transcriptome signature of intratumoral eosinophils was subjected to bioinformatics analysis using the database for annotation, visualization, and integrated discovery (DAVID) and annotation of gene ontology (GO) pathways (D). The transcriptomic signature of intratumoral eosinophils was compared with that of macrophages using a Venn diagram (E) and heat plot analyses (F, G). A comparison of the expression of selected transcripts from intratumoral eosinophils and macrophages is shown. The transcriptome signature of intratumoral macrophages was subjected to bioinformatics analysis using the DAVID and the annotation of GO pathways (H).
increased to a greater extent in eosinophils, whereas transcripts that were associated with tissue repair, such as matrix metalloproteinase 7 (Mmp7) and arginase 2 (Arg2), increased to a greater extent in macrophages. The resistin-like molecule a (Retnla), a hallmark of alternatively activated macrophages, which resemble tumor-associated macrophages (27), was decreased in eosinophils and macrophages, although it was downregulated to a greater extent in macrophages (Fig. 4H; a complete transcript list of genes that are differentially regulated in macrophages and eosinophils can be found in Supplementary Table S8). Bioinformatics analysis, using the DAVID annotation of GO pathways, revealed enrichment of pathways that are associated with developmental and tissue repair processes in intra-tumoral macrophages (Fig. 4H).

IFNγ as a key activator of eosinophils in colorectal cancer

Next, we characterized the proteomic profile of intra-tumoral eosinophils following CAC by means of antibody array (28). PCA analysis and subsequent hierarchical clustering segregated the samples according to cell type and treatment (Fig. 5A). Hierarchical clustering revealed that all four cell groups segregated distinctly (Fig. 5A, bottom tree cluster). However, naïve colonic eosinophils were distinctly separated from naïve macrophages as well as intra-tumoral macrophages and eosinophils (Fig. 5A, bottom tree cluster). Intra-tumoral and naïve eosinophils differentially expressed 155 proteins. Of these proteins, 49 were abundant in naïve eosinophils and 106 were abundant in intra-tumoral eosinophils (Fig. 5B and see list in Supplementary Table S10). STRING analysis of the differentially regulated proteins in intra-tumoral eosinophils revealed that they were grouped into three unique clusters (Fig. 5C). Cluster 1 comprised proteins associated with cell survival (e.g., BAX, BCL2, caspase-3); cluster 2 included the enrichment of various cytokines and cell-surface receptors (e.g., IL4, IFNγ, IL12b, CD44, and CD79); cluster 3 comprised growth factors and enzymes that can regulate the integrity of the extracellular matrix (e.g., MMP7, TIMP-1, and FGF7). Comparison of the proteomic signature, which was retrieved for eosinophils, revealed that only 40 of 155 proteins (25% of the eosinophil proteomic signature) were shared between intra-tumoral macrophages and eosinophils (Fig. 5D; Supplementary Tables S1–S13). Although these 40 proteins were the majority of the proteins detected in macrophages, further analysis revealed that the expression pattern (i.e., increased vs. decreased expression) of 90% of them (36 proteins) was higher in intra-tumoral eosinophils in comparison with macrophages (Fig. 5E). Consistent with our transcriptome data, unbiased bioinformatics analysis, with DAVID annotation of GO pathways, revealed that the pathway most enriched in intra-tumoral eosinophils was associated with IFNγ signaling (Fig. 5F).
IFNγ potentiates eosinophil-mediated colorectal cancer cell killing

The increased abundance of IFNγ-regulated pathways in intra-tumoral eosinophils suggested a role for IFNγ in their antitumorigenic activities. Coculture of eosinophils with MC38 or CT26 cells resulted in increased colorectal cancer cell death without eosinophil activation (Fig. 6A). Nonetheless, IFNγ enhanced the ability of eosinophils to kill colorectal cancer cells in vitro (Fig. 6B). Increased cytotoxic activities of eosinophils in response to IFNγ were not...
due to increased eosinophil death in vitro, because nonstimulated and stimulated eosinophils displayed ~12% cell death. Increased cytotoxicity of eosinophils toward colorectal cancer cells was specifically enhanced by IFNγ because stimulation of eosinophils with additional stimuli such as E. coli, poly IC, TNFα, and f-met-leu-phe (FMLP) did not increase eosinophil cytotoxicity (Fig. 6C). Eosinophil-mediated cytotoxicity is likely not generalized to all tumor cells, because eosinophils did not induce cytotoxicity in vitro toward PyMT breast cancer cells (Fig. 6D).

Similar to our findings with mouse eosinophils, human peripheral blood eosinophils cocultured with SW480 colorectal cancer cells induced cell death, an effect potentiated by activation of eosinophils with IFNγ (Fig. 6E).

**Antitumorogenic activities of eosinophils are associated with cytotoxicity**

Dual immunofluorescence staining of MBP and cleaved caspase-3 in WT mice undergoing CAC demonstrated intratumoral eosinophils in the vicinity of apoptotic (i.e., active caspase-3+) tumor cells (Fig. 6F and G). The majority of eosinophils (>99%) were negative for active caspase-3 staining, confirming our observation regarding prolonged eosinophil survival in the TME (Fig. 2).

Moreover, tissues from WT and ΔdblGATA mice undergoing CAC as well as tissues from Apcmin+/− and ΔdblGATA/Apcmin+/− mice were obtained and stained with anticleaved caspase-3. The number of cleaved caspase-3+ cells was decreased in the absence of eosinophils both in colitis-associated cancer and in Apcmin+/− mice (Fig. 6H–I). No differences, however, were observed in the numbers of Ki67+ epithelial cells (Supplementary Fig. S9) and/or CD31+ blood vessels (Supplementary Fig. S9).

**Discussion**

Our perceptions regarding the roles of eosinophils in health and disease have changed because functions for these cells have been identified in settings that are beyond classic type-2 immunity (29, 30). In this study, we dissected the roles of eosinophils in colorectal cancer. We demonstrate that elevated eosinophila in human colorectal cancer was associated with an improved disease stage, suggesting beneficial roles for eosinophils in colorectal cancer. Experimentally, eosinophils were recruited to the TME, which supported prolonged eosinophil survival and CD8+ T-cell–independent antitumorogenic activities. Transcriptome and proteomic analysis of intratumoral eosinophils revealed an activated eosinophil phenotype, which was associated with IFNγ signaling. These data provide insight into the transduction mechanisms unleashing antitumor activities from eosinophils and identify these cells as targets for future immunotherapy especially in colorectal cancer.

Our analyses revealed that tumor eosinophilia in colorectal cancer was inversely correlated with tumor grade, in line with studies that have associated tumor eosinophilia with improved overall and/or colorectal cancer–specific survival (13, 14, 31–34). Furthermore, we demonstrated that 27% of colorectal cancer patients with lymph node metastasis displayed lymph node eosinophilia. Although previous reports in colorectal cancer did not directly assess eosinophils in metastatic sites such as the lymph node, eosinophila in the primary site was inversely correlated with local recurrence and distant metastases, and tumors with more tumor eosinophilia displayed less metastasis (35). Future studies will be required to assess the role of eosinophils in lymph nodes and distant metastatic sites during tumor progression in colorectal cancer.

We established that eosinophils display prolonged survival in the TME independently of IL5 and confirmed this with adoptive transfer experiments and the sparse number of active caspase-3+ eosinophils in the GI tract. Consistent with previous reports in murine models of allergic GI inflammation and IBD (36–38), the eosinophil-specific chemokine CCL11/eotaxin-1 (but not CCL24/eotaxin-2) was increased during the progression of colorectal cancer. Increased CCL11/eotaxin-1 was associated with accumulation of eosinophils in human colorectal cancer (39), although CCL24/eotaxin-2 may also be involved (40). The finding that the TME supports eosinophil accumulation and survival suggests that eosinophil degranulation in vivo and eosinophil-mediated tumor elimination is not necessarily a consequence of eosinophil cell death.

Eosinophils can eliminate tumor cells by direct and indirect mechanisms (41). Under appropriate settings, eosinophils are highly suited for eliminating tumors (42–46). It is unclear, however, what signaling mechanisms induce eosinophils to display antitumorogenic functions. Our unbiased empirical approach, subjecting isolated primary intratumoral eosinophils to RNA-seq and proteomics, revealed that tumor-infiltrating eosinophils in colorectal cancer displayed an IFNγ–associated signature with multiple innate immune–signaling components, such as pattern recognition receptors and IFNγ-dependent genes (e.g., Stat1, Ifi202b, Fpr1, Fpr2, Rtp4, Nos2, S100a9, Ifi1b12, Ifi201m, and Ifi34). Unbiased proteomic and subsequent bioinformatics analysis substantiated the IFNγ–associated signature by revealing that the top-enriched pathway (with a P value of 7.83×10−14) was IFNγ-dependent signaling. A reported microarray analysis of eosinophils differentiated ex vivo with recombinant IL18, which induces IFNγ production, suggests a distinct gene-expression signature characteristic of "inflammatory eosinophils" (47). These inflammatory eosinophils display increased expression of several transcripts that we observed in intratumoral eosinophils, including Cd274, Saa3, Ly6a, Ifi3, Oasl2, Spp1, and Rtp4. Moreover, Il18bp, a negative regulator of IL18, which is induced by IFNγ (48), is also increased in intratumoral eosinophils. The IFNγ signature, which we identified by means of RNA-seq and proteomics, was functionally validated by activation of mouse and human eosinophils with IFNγ, and consequently, the cytotoxicity toward colorectal cancer cells increased. The finding that IFNγ–activated eosinophils displayed tumoricidal activities suggests that, in colorectal cancer, eosinophils display an activated phenotype that resembles that of classically activated macrophages (also termed M1 cells), which also have the ability to kill tumor cells (49). Indeed, IFNγ–activated eosinophils are capable of releasing reactive oxygen species, mitochondrial DNA, and nitric oxide, which are capable of killing tumor cells (17, 50). Comparing the transcriptome and proteome signature of tumor-infiltrating eosinophils to that of macrophages revealed that although these two cell types were exposed to similar stimuli in the TME, their responses differ. Eosinophils were polarized into an inflammatory state, with increased proinflammatory cytokines, chemokines, and signaling pathways. In contrast, macrophages displayed a phenotype that was associated with tissue repair and organ development, with increased expression of growth factors and matrix metalloproteinases. Future research is needed to determine whether, while retaining their
antitumorigenic activities, eosinophil-derived IL4/IL13 contributes to the suppressive function of macrophages in the TME.

Our data indicate that the antitumorigenic activities of eosinophils in colorectal cancer are independent of CD8\(^{+}\) T cells and are associated with tumor cell death. In our models, the antitumorigenic function of eosinophils was more potent than that of CD8\(^{+}\) T cells. This conclusion is important because clinical data show that current T-cell–based immunotherapies have limited success for most colorectal cancer patients (42). The finding that the antitumorigenic activities of eosinophils are independent of CD8\(^{+}\) T cells is in contrast to a report showing that eosinophils coordinate antitumor immunity via recruitment of CD8\(^{+}\) T cells (11). In that system, eosinophils recruit CD8\(^{+}\) T cells and render macrophages antitumorigenic via secretion of CXCL9 and promotion of an environment characterized by elevated IFN\(\gamma\) and TNF\(\alpha\) (11). We also identified increased expression of IFN\(\gamma\), Cxcl9, and additional IFN\(\gamma\)-associated genes in intratumoral eosinophils. In addition, experimental models of GI infection also show increased activity of eosinophils in response to IFN\(\gamma\). Whereas in infectious disease settings, IFN\(\gamma\)-dependent expression of PDL-1 on eosinophils restricted Th1-induced immune responses (43), a collective view of these data highlight IFN\(\gamma\) as a key activator of eosinophils, especially in the GI tract.

The association between the antitumorigenic activities of eosinophils in response to IFN\(\gamma\) is consistent with previous reports regarding the role of IFN\(\gamma\) in colorectal cancer. Given the longevity of experimental models for colorectal cancer (3–5 months) and the accumulation of multiple cells that may differentially express IFN\(\gamma\), future experiments are required to identify the cellular source of the IFN\(\gamma\) and the \(\gamma \delta\) role of IFN\(\gamma\) signaling in the antitumorigenic activities of eosinophils. Nonetheless, the finding that eosinophils can mediate antitumor activities independent of CD8\(^{+}\) T cells may have therapeutic implications for combinatorial therapies targeting these cells.

One of the conclusions that we can draw from our studies is that the role of eosinophils in the TME is largely tissue- and context-dependent. Although this concept is accepted for other immune cells such as neutrophils and macrophages (44, 45), to date, eosinophils have been examined from a dichotomic point of view, leading to the overall notion that the roles of eosinophils in cancer are controversial (41, 46). On the basis of our data, we suggest an alternative explanation, whereby the differential roles of tumor immunology involving eosinophils (i.e., pro- vs. antitumorigenic activities) are dependent upon the TME. Under settings in which eosinophils are exposed to innate immune stimuli and in the presence of IFN\(\gamma\), they will be polarized to display antitumorigenic activities. In contrast, settings lacking IFN\(\gamma\) or innate immune activation may polarize eosinophils to produce tumor-promoting factors.

In summary, we provided evidence that, in colorectal cancer, eosinophils have antitumorigenic activity in vivo and their functions can be distinguished from cytotoxic T cells and intratumoral macrophages. These data enhance our understanding of the molecular pathways regulating tumor eosinophilia. Our findings have implications for cancer therapy, in particular for patients with colorectal cancer.

Disclosure of Potential Conflicts of Interest
A. Munitz is a consultant/advisory board member for GSK and Augmanity Nano Ltd. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: H. Reichman, M. I. Itan, N. Arber, A. Munitz, J. J. Lee
Development of methodology: H. Reichman, M. I. Itan, E. Brazowski, A. Munitz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Brazowski, C. Varol, N. Gluck, N. Arber
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Reichman, M. I. Itan, E. Brazowski, C. Varol, A. Munitz
Writing, review, and/or revision of the manuscript: H. Reichman, C. Varol, N. Arber, U. Qimron, D. Karo-Atar, J. J. Lee, A. Munitz
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Rozenberg, T. Yarmolovski, S. Shapira
Study supervision: A. Munitz

Acknowledgments
A. Munitz is supported by the US-Israel Bi-national Science Foundation (grant nos. 2009222 and 2011244), the Israel Science Foundation (grant no. 886/15), a project grant from the Israel Cancer Research Foundation, the Israel Cancer Association (grant no. 20150002), the Ministry of Health (grant no. 3-10117), and the Boaz and Varda Dotan Center Grant for Hemato-oncology Research. H. Reichman was funded in part by the Constantiner Institute for Molecular Genetics and performed this work in partial fulfillment of the requirements for a PhD degree at the Sackler Faculty of Medicine, Tel Aviv University, Israel. The authors wish to thank Professor Marc Rothenberg for critically reviewing this manuscript.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 23, 2018; revised October 14, 2018; accepted December 17, 2018; published first January 21, 2019.

References


Activated Eosinophils Exert Antitumorigenic Activities in Colorectal Cancer

Hadar Reichman, Michal Itan, Perri Rozenberg, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-18-0494

Cited articles
This article cites 50 articles, 12 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/7/3/388.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerimmunolres.aacrjournals.org/content/7/3/388.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.