T-cell Expression of IL10 Is Essential for Tumor Immune Surveillance in the Small Intestine

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Abstract

IL10 is attributed with immune-suppressive and anti-inflammatory properties, which could promote or suppress cancer in the gastrointestinal tract. Loss of IL10 exacerbates colonic inflammation, leading to colitis and cancer. Consistent with this, transfer of IL10-competent regulatory T cells (Treg) into mice with colitis or hereditary polyposis protects against disease, while IL10-deficient mice are predisposed to polyposis with increased colon polyp load. Little is known about the protective or pathogenic function of IL10 in cancers of the small intestine. We found CD4+ T cells and CD4+ Foxp3+ Tregs to be the major sources of IL10 in the small intestine and responsible for the increase in IL10 during polyposis in the APCfl/fl mouse model of hereditary polyposis. Targeted ablation of IL10 in T cells caused severe IL10 deficiency and delayed polyp growth. However, these polyps progressively lost cytotoxic activity and eventually progressed to cancer. Several observations suggested that the effect was due to the loss of IFNγ-dependent immune surveillance. IL10-incompetent CD4+ T cells failed to secrete IFNγ when stimulated with polyp antigens and were inefficient in T-helper-1 (TH1) commitment. By contrast, the TH17 commitment was unaffected. These findings were validated using mice whose T cells overexpress IL10. In these mice, we observed high intra-polyt cytotoxic activity and attenuation of polyposis. Thus, expression of IL10 by T cells is protective and required for immune surveillance in the small intestine.

Introduction

IL10 is a key immunosuppressive cytokine that is upregulated in cancer, and it has been attributed with a role in obstructing tumor lysis (1, 2) and tumor rejection (3, 4). However, reports also suggest a protective role for IL10 in cancer (5–7). The ambiguity suggests a complex role for IL10 that may need to be addressed in defined models of cancer, in a spatial and temporal manner. We have looked at the function of IL10 during the development of adenomatous polyps in the small intestine of APCfl/fl mice, a well-characterized mouse model of hereditary polyposis (8–11). Our findings support a protective role for IL10, specifically in preventing the adenoma to carcinoma transition.

The immune-suppressive functions of IL10 are associated with its effects on antigen-presenting cells as well as T cells. IL10 downregulates expression of costimulatory molecules by dendritic cells (DC) and induces T-cell anergy upon antigen encounter (1, 12). However, the inhibitory effects of IL10 are not always consistent; for example, IL10 hinders the proliferation of mouse CD4+ T cells but not necessarily their expression of cytokines, including IFNγ (13). IL10 has differential effects on CD8+ T cells, and unexpectedly it activates and expands tumor-resident T cells (14). The effects of IL10 on T cells may depend on their state of activation, which may explain both the enhancing and inhibitory effects observed after IL10 treatment in different in vivo experimental models (15). The timing of exposure to IL10 appears to be critical, inhibitory at antigen priming, and stimulatory at recall (16). Thus, transgenic expression of IL10 has disparate effects on T-cell response, inhibiting OVA peptide vaccination possibly at the T-cell priming level, while enhancing rejection of transplanted tumors in mice, potentially by influencing T-cell memory (17). Injection of soluble IL10 exacerbates graft-versus-host disease (18, 19). This observation is in line with more recent findings of critical needs for IL10 in cytotoxic T-cell differentiation (20) and IFNγ-dependent tumor lysis (14, 21, 22). The anti-inflammatory properties of IL10, particularly in the colon (23), suggest that the cytokine can suppress some immune responses while enhancing others. Effective immune intervention and therapy requires a better understanding of the functions of IL10 in specific tissue and disease settings (23). Here, we have examined the role of IL10 in small bowel cancer.

To assess the expression of IL10 in the small intestine during polyposis, we introduced into APCfl/fl mice (8) a sensitive reporter transgene that expresses Thy1.1 under the control of the
IL10 is needed for tumor immune surveillance.

Materials and Methods

Animals

B6 (C57BL/6J), CD4Cre, IL10+/−, and Rag1−/− mice were purchased from The Jackson Laboratory. We generated the APCΔ468 mice on a B6 background (26). IL10−/− mice were developed by Roers and colleagues (27). IL2pIL10 mice were developed by Hagenbaugh and colleagues (28). IL10Thy1.1 reporter mice were developed by Maynard and colleagues (25). IL10fl/fl mice were analyzed using ImageJ. Other anti-mouse antibodies used were Ki67 (SP6), Granzyme B, IgG, perforin (CB5.4), purchased from Abcam; Foxp3 (FJK-16s) was from eBioscience. Secondary antibodies used were AlexaFluor-488 and AlexaFluor-594 from Invitrogen. Apoptosis staining was performed using a TUNEL Apoptosis Detection Kit (Millipore). Light microscopy photographs were taken using the Leica DCC camera, and images were analyzed using the NIH software, ImageJ. Fluorescent microscopy images were taken using TissueGnostics microscope, and images were analyzed using ImageJ.

Gut mononuclear cell preparation

The small intestine was harvested, cleaned, and then minced. The minced tissue was then incubated in 25 mL complete RPMI-1640 with 10 units of collagenase type IV (Worthington Biochemical Corporation) for 25 minutes at 37°C with gentle agitation. Cells were enriched on a Percoll gradient (44%/67%), and gut mononuclear cells (MNC) at the interface were collected and washed prior to analysis.

Bone marrow reconstitution

Mice (4- to 6-week-old) were lethally irradiated (1000 rad; split dose). Lin-depleted bone marrow cells were injected into the retro-orbital (RO) sinus. Details are provided in Supplementary Materials.

Flow-cytometric staining and analysis

Cells in suspension were transferred to a 96-well round-bottom plate and stained with extracellular antibodies and Live/Dead Violet dye (Invitrogen) for 20 minutes. Cells were then washed and fixed with 1% PFA (10 minutes), permeabilized (0.3% saponin), and stained with intracellular antibodies (30 minutes). Additional details are provided in Supplementary Materials. Data were acquired on a FACSCanto II instrument and analyzed using FlowJo software (TreeStar).

Serum and tissue extracts

Mice were bled from the RO sinus. Blood was left for 15 to 30 minutes at room temperature to clot. Samples were centrifuged for 15 minutes, and supernatants were collected. Polyps were microdissected and separated from the surrounding healthy tissue. Tissue was minced and extruded in PBS on ice for 1 minute using a 16-gauge needle and syringe. Samples were centrifuged for 15 minutes, and the extracts were collected and filtered. Protein concentration was determined using the Bradford assay.

ELISA and multiplex ELISA

ELISAs for IL10 was performed according to the manufacturer’s instructions (eBioscience). Multiplex ELISA was performed according to the manufacturer’s instructions using a customized analyte detection set from Millipore. Data were acquired on a Luminex 100 instrument and analyzed with xPONENT software (Luminex Corporation).

IFNγ ELISPOT

Spleen-derived DCs were cultured for 1 week with X-Vivo medium containing mouse rec-GM-CSF (10 ng/mL; Millenium) at 37°C, 5% CO2. CD11c+ DCs (purified by Miltenyi) were pulsed with peptide extract (20 μg/100 μL) overnight before being cocultured with CS3+ MACS column–purified T cells for 40 hours. Anti–IFNγ-coated ELISPOT plates (Millipore) were analyzed using ImageJ. Other anti-mouse antibodies used were Ki67 (SP6), Granzyme B, IgG, perforin (CB5.4), purchased from Abcam; Foxp3 (FJK-16s) was from eBioscience. Secondary antibodies used were AlexaFluor-488 and AlexaFluor-594 from Invitrogen. Apoptosis staining was performed using a TUNEL Apoptosis Detection Kit (Millipore). Light microscopy photographs were taken using the Leica DCC camera, and images were analyzed using the NIH software, ImageJ. Fluorescent microscopy images were taken using TissueGnostics microscope, and images were analyzed using ImageJ.

Histology and immunostaining

Cleaned intestines were fixed for 2 hours at room temperature with freshly prepared 2% paraformaldehyde (PFA). The fixed tissues were then washed for 5 minutes with PBS, and then incubated overnight at 4°C in 20% sucrose (in PBS). The next day, tissues were washed with PBS, then rolled in OCT, and frozen sections of 4 to 5 μm were prepared for analysis. Foxp3 was visualized using GFP reporter activity. IL10 was visualized using rat anti-mouse Thy1.1-biotin and streptavidin–Alexa Fluor 594. CD4 was visualized using mouse anti-mouse CD4 and anti-mouse-Cy5. Rabbit anti-mouse granzyme B or rabbit IgG control and rat anti-mouse perforin (Abcam) were incubated at 1:100 overnight at +4°C, and then visualized using secondary antibodies anti-rabbit Alexa Fluor-594 and anti-rat Alexa Fluor-488 (Invitrogen). Rabbit anti-mouse Foxp3 (Abcam) was incubated at 1:100 overnight at 4°C, then visualized using secondary anti-rabbit Alexa Fluor-594 (Invitrogen). Fluorescent microscopy images were taken using a TissueGnostics microscope, and images were analyzed using ImageJ. Other anti-mouse antibodies used were Ki67 (SP6), Granzyme B, IgG, perforin (CB5.4), purchased from Abcam; Foxp3 (FJK-16s) was from eBioscience. Secondary antibodies used were AlexaFluor-488 and AlexaFluor-594 from Invitrogen. Apoptosis staining was performed using a TUNEL Apoptosis Detection Kit (Millipore). Light microscopy photographs were taken using the Leica DCC camera, and images were analyzed using the NIH software, ImageJ. Fluorescent microscopy images were taken using TissueGnostics microscope, and images were analyzed using ImageJ.

Gene expression array

Microarray analyses were performed using CD4+ T cells isolated from the small intestine of healthy Foxp3GFP or Foxp3ΔFP APCΔ468 mice. Cells were subjected to negative selection (unprocessed T-cell isolation kit; Invitrogen), labeled for CD4, and double sorted on a Dako MoFlo cell sorter for expression of CD4 and exclusion of Foxp3 and dead cells (DAPI). Sorted populations (post-sort purity >98%) were used to prepare RNA for microarray analyses according to imrnugen.org. Array data were normalized for background. A GenePattern Expression Dataset file was generated from all array data. These were further compared for expression enrichment of a predetemined IL10 molecular pathway (Biocarta) using the Gene Set Enrichment program (Broad Institute). A heatmap of the IL10 pathway genes was generated using the ExpressCluster v1.3 module of the GenePattern package (Broad Institute).

Gut mucosa

Histology was performed as described (30). All animal work was approved and conducted according to the guidelines of the Animal Care and Use Committee (ACUC) of Northwestern University.

Gene promoters

We show that T cells are the major cellular source of IL10. By selectively ablating IL10 in T cells of bone marrow chimeric APCΔ468 mice (CD4ΔFPIL10fl/flAPCΔ468), we confirm that IL10 in the small intestine is almost entirely derived from T cells. By comparing the CD4ΔFPIL10fl/flAPCΔ468 mice with APCΔ468 mice that overexpress IL10 under the control of the IL2 promoter in their T cells (APCΔ468IL2pIL10), we show that expression of IL10 by T cells is indispensable for INFγ-dependent T-cell cytotoxicity and protective poly-specific immune surveillance. Our findings provide new support for the antitumor properties of IL10.
were visualized according to the manufacturer’s instructions (Mabtech). Data were acquired on a CTL-Immunospot instrument and analyzed using CTL ImmuNoSpot software (Cellular Technology Limited).

TH1 polarization

TH1 polarization was performed as described previously (31). Total cells were harvested from the MLN, and CD4+ responder cells were isolated by magnetic bead columns (Miltenyi). Spleens from syngeneic mice were harvested, red blood cells were lysed, and irradiated with 3,000 rad. Spleen cells and responder cells were cultured for 7 days with 5 ng/mL mouse rec-IL2 (eBioscience), 10 μg/mL monoclonal anti-IL4 (clone 11B11; BD Pharmingen), 10 ng/mL rec-IL12 (BD Pharmingen), 3 μg/mL anti-CD3 (eBioscience), and 3 μg/mL anti-CD28 (eBioscience).

Statistical analysis

Box and whisker histograms depict median flanked by the upper and lower 25% quartiles with whiskers showing maximum and minimum data points. Statistical analyses were performed with the use of the Prism 4 software.

Histograms depict mean ± SEM

Statistical significance was determined using a two-tailed paired t test (*, P < 0.01; **, P < 0.001; ***, P < 0.0001; ns, not statistically significant).

Results

Conventional CD4+ T cells and Tregs are the major source of IL10 in the small intestine

IL10 was elevated during polyposis, reaching levels that were 10- to 20-fold higher in polyps and in healthy intestine tissue of APCΔM68 mice than in age-matched wild-type (WT) B6 mice (Fig. 1A). The cellular source of IL10 was visualized using a sensitive IL10 mice reporter (25). To distinguish conventional T cells from Tregs, we crossed Foxp3-GFP reporter mice (32) with IL10Thy1.1 mice, and then generated bone marrow chimeric APCΔM68 mice that expressed Thy1.1 stably under the control of the IL10 promoter. Lethally irradiated Thy1.2 B6 and Thy1.2 APCΔM68 mice were reconstituted with bone marrow from IL10Thy1.1 Foxp3GFP dual-reporter mice. The efficiency of bone marrow chimerism was 76% or better (Supplementary Fig. S1). Multicolor immunofluorescence staining of jelly-roll preparations of the small intestine allowed us to visualize CD4+ Foxp3+ Tregs and CD4+ Foxp3+ conventional T cells (Supplementary Fig. S2A). Expression of IL10 was visualized by staining for Thy1.1 (Supplementary Fig. S2B and S2C). No staining for Thy1.1 was detected in the intestines of conventional T cells (Supplementary Fig. S2A). Isotype control antibodies were used to validate these findings (Supplementary Fig. S3). Strikingly, conventional CD4+ T cells and Tregs accounted for nearly all IL10-expressing cells infiltrating the small intestines of healthy (B6) as well as APCΔM68 mice (margin and polyp; Fig. 1B). In 4-month-old poly-p-riden APCΔM68 mice, the frequency of IL10-expressing cells was higher in the margin than that inside the polyps (Fig. 1C). This was largely accounted for by the accumulation of conventional CD4+ T cells in the polyp margin (Fig. 1D) and the increase in their IL10-competent fraction (inside the polyps, 22%; in the margin, 27%) relative to that in the healthy B6 intestine (16%; Fig. 1D). By contrast, Tregs preferentially accumulated inside the polyps and produced less IL10 (inside the polyps, 30%; in the polyp margins, 44%) than in the healthy B6 intestine (58%; Fig. 1D). We analyzed gene expression downstream of the IL10 receptor (IL10R) were mainly in conventional T cells and Tregs responding to their own IL10 in an autocrine fashion during polyposis.

T-cell ablation of IL10 delays polyposis but facilitates cancer progression

To determine the contribution of T cells to the content and function of IL10, we specifically ablated IL10 in the T cells of...
CD4\textsuperscript{+}IL10\textsuperscript{-/-}APC\textsuperscript{fl/fl} mice. In parallel, for comparison we generated IL10\textsuperscript{-/-}APC\textsuperscript{fl/fl} mice that were completely deficient for IL10. Ablation in T cells reduced IL10 concentration in the intestine to nearly undetectable levels (Fig. 1A). Together, the reporter and ablation experiments demonstrated that IL10 content of the small intestine was almost entirely derived from the T-cell compartment.

Next, we determined the outcomes of IL10 deficiency in the small intestine. APC\textsuperscript{fl/fl} mice and CD4\textsuperscript{Cre}IL10\textsuperscript{-/-}APC\textsuperscript{fl/fl} mice at 2 months of age had comparable numbers of aberrant crypts and microscopic polyps, detected using a dissection microscope (Fig. 3A and B). By 4 months of age, APC\textsuperscript{fl/fl} mice developed extensive visible polyposis in the small intestine, but IL10\textsuperscript{-/-}APC\textsuperscript{fl/fl} and CD4\textsuperscript{Cre}IL10\textsuperscript{-/-}APC\textsuperscript{fl/fl} mice had very few, if any, visible polyposis (Fig. 3A). However, by 7 months of age, CD4\textsuperscript{Cre}IL10\textsuperscript{-/-}APC\textsuperscript{fl/fl} and IL10\textsuperscript{-/-}APC\textsuperscript{fl/fl} mice became cachexic. The intestines contained large and invasive lesions (Fig. 3B and C). Thus, the inability of T cells to produce IL10 changed both the kinetics and nature of polyposis in the small intestine, favoring cancer progression as the mice aged.

Loss of intra-polyp cytotoxicity in mice with IL10-ablated T cells

APC\textsuperscript{fl/fl} mice exhibit ongoing intra-polyp cytotoxic activity and polyp-specific TH1 responses (33). To better understand the impact of ablation of IL10 in T cells on anti-polyp immunity, we measured intra-polyp cytotoxic activity by staining histologic sections for granzyme B and perforin (34, 35). By 2 months of age, the aberrant-crypts and micro-adenomas of CD4\textsuperscript{Cre}IL10\textsuperscript{-/-}APC\textsuperscript{fl/fl} mice had nearly twice as many granzyme B\textsuperscript{+} and perforin\textsuperscript{+} infiltrating cells as in the lesions of APC\textsuperscript{fl/fl} mice (Fig 4A and B; Supplementary Fig. S3), but the cytotoxic activity declined sharply by 4 months and was completely extinguished by 7 months of age. By comparison, intra-polyp cytotoxicity increased with age in the IL10-competent APC\textsuperscript{fl/fl} mice (Fig. 4A and B; Supplementary Fig. S3). There were no significant differences or change in cytotoxic activities within the healthy margin tissues of the IL10-competent versus IL10-defective mice (Fig. 4A and B). Furthermore, at all ages examined, CD8 T cells were preferentially excluded from the polyps of CD4\textsuperscript{Cre}IL10\textsuperscript{-/-}APC\textsuperscript{fl/fl} mice and were not cytotoxic (Fig. 4C). We detected consistent drops in polyp-associated apoptotic events as CD4\textsuperscript{Cre}IL10\textsuperscript{-/-}APC\textsuperscript{fl/fl} mice aged (Fig. 5A and B). Thus, we conclude that IL10 is essential for anti-polyp cytotoxic activity and tumor lysis, but cannot be sure that the actual effectors are CD8\textsuperscript{+} T cells.

Mice with IL10-ablated T cells have systemic TH1 defects

The above findings resonate with earlier reports that IL10 is required for antitumor IFN-dependent cytotoxic response (21, 22). The gradual loss of cytotoxicity implied defects in T-cell help. Therefore, we used ELISPOT assays to compare polyp-specific TH1 responses in 4-month-old APC\textsuperscript{fl/fl} mice with responses in age-matched CD4\textsuperscript{Cre}IL10\textsuperscript{-/-}APC\textsuperscript{fl/fl} mice. T cells from the spleens were cocultured with DCs that had been
pulsed with polyp extracts, and IFNγ expression by the T cells was measured. Strong IFNγ responses were detected in T cells isolated from APCfl/fl mice, but significantly less (P < 0.001, Student t test) response was detected in T cells isolated from APCfl/− mice (Fig. 6A). To determine if this was a T-cell inherent defect, we tested TH1 polarization of IL10-proficient and IL10-deficient T cells. CD4 T cells isolated from CD4CreIL10fl mice demonstrated poor TH1 commitment compared with T cells from healthy B6 mice (Fig. 6B and C). These findings are consistent with an earlier report that expression of IL10 by T cells is required for TH1 commitment (22). We tested the ability of CD4 T cells to commit to the TH17 lineage by stimulating them with anti-CD3 in vivo as described previously (36). These cells readily committed to the TH17 lineage (data not shown), indicating that the drop in TH17 cytokines in 4-month-old mice may have been more likely a secondary result of attenuation of polyposis at this age.
IL10 Is Needed for Tumor Immune Surveillance

Discussion

Prominent cellular sources of IL10 in mice are thought to be neutrophils (37) and macrophages (38, 39), as well as the intraepithelial lymphocytes of the small intestine and lamina-propria lymphocytes of the colon (40), which include T-repressive (Tr1) cells (41, 42), TH1 cells (43), and Tregs (25). Using a sensitive IL10 reporter transgene, we found that conventional T cells and Tregs are by far the major cellular sources of IL10 in the intestines of WT B6 mice as well as APC<sup>468</sup> mice. Ablation of IL10 in T cells reduced the levels of IL10, making the mice practically IL10 deficient.

Surprisingly, and in contrast with the colon where T-cell IL10 deficiency exacerbates polyposis (11), ablating IL10 expression in T cells initially attenuated polyposis in the small intestine. Because IL10 is anti-inflammatory, and polyposis growth in both the small and large intestines is inflammation driven (9), this observation can be interpreted as a demonstration of the difference in the nature of inflammation in the two sites. The difference could be in dependence on microbes. Polyposis is inhibited in the colon of germfree mice or mice treated with antibiotics (ref. 44; and unpublished observations), but not in the small intestine (45). The reduced rate in polyposis growth was a short-term benefit, and mice went on to develop large invasive tumors later. Tumor progression correlated with defects in T-cell function and intratumor cytotoxic activity. IL10-ablated T cells were less efficient in committing to the TH1 lineage than WT T cells, as assessed by in vitro lineage commitment. The mice had poor systemic TH1 responses to polyps, and intra-polytumor cytotoxic activity was extinguished with time. TH1 cells are

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The in vivo effects of upregulation or exogenous administration of cytokines are not always predictable from the outcome of deficiency of the same cytokine. For example, IL23 blockade or deletion reduces tumor incidence, yet exogenous administration of IL23 also causes tumor regression (52, 53). Using IL10-defective or IL10-overexpressing T cells, we demonstrated consistency in the antitumor effects of IL10. The indispensable role of IL10 in cancer immune surveillance is also highlighted by the pathology of patients who are born with IL10 deficiency. A third of the patients with IL10R deficiency have defective CD8 T-cell tumor immune surveillance and develop B-cell lymphomas in the first decade of their life (54). Together, these findings demonstrate that IL10 is not only required for tumor-specific immune surveillance but that its overexpression or targeted delivery may be therapeutic in the cancer setting.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: K.L. Dennis, H. Cheroutre, N.K. Egilmez, K. Khazaie Development of methodology: K.L. Dennis, C.T. Weaver, A. Roers, K. Khazaie Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.L. Dennis, A. Saadalla, N.R. Blatner, S. Wang, H. Cheroutre, N.K. Egilmez, K. Khazaie Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.L. Dennis, A. Saadalla, N.R. Blatner, S. Wang, H. Cheroutre, N.K. Egilmez, K. Khazaie Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.L. Dennis, S. Wang, H. Cheroutre, N.K. Egilmez Writing, review, and/or revision of the manuscript: K.L. Dennis, N.R. Blatner, S. Wang, H. Cheroutre, N.K. Egilmez

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Acknowledgments

The authors thank Dr. Steven Rosen for being a continuous source of inspiration and encouragement. Dr. Yang-Xin Fu and Dr. Bana Jabri are gratefully acknowledged for providing valuable advice and material. They thank Dr. Alan Pemberton from the University of Edinburgh for the generous donation of the anti-mouse mMCP6. The authors also thank Nicoleta Carapanceanu for excellent technical support.

Grant Support

K. Khazaie is the recipient of NIH grants 1R01CA160436-01 and 1R01AI108682-01. F. Gounari is supported by 1R01AI108682-01. N.K. Egilmez is supported by A1092133.

Received September 14, 2014; revised February 28, 2015; accepted March 23, 2015; published OnlineFirst April 8, 2015.

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