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Short Communication

Interleukin-10 prevents epithelial cell apoptosis by regulating IFNγ and TNFα expression in rhesus macaque colon explants

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ABSTRACT

Interleukin-10 (IL-10) is an important immunomodulatory cytokine that plays an obligate role in regulating inflammatory responses. Here we demonstrated the role of IL-10 in regulating crypts length and breadth as well as maintaining the survival of epithelial cells using rhesus colon explant cultures. Anti-IL-10 antibody treatment of colon explant cultures induced increased production of inflammatory cytokines/molecules like IFNγ, TNFα, CD107a and perforin as well as increased epithelial cell apoptosis compared to media controls tested. Our results suggest that IL-10 plays a crucial role in maintaining mucosal homeostasis by regulating mucosal IFNγ and TNFα cytokine production.

1. Introduction

Intestinal epithelial cells (ECs) play an important role in the immune system, both as a barrier and as a first-line pathogen recognition system [1]. Increased permeability due to compromised barrier function could be relevant to mucosal transmission of HIV and generalized HIV-induced immune-cell activation and disease progression. We recently have shown increased intestinal EC apoptosis in both acute and chronically SIV-infected rhesus macaques (RMs) [2]. Interleukin-10 (IL-10) was first described as an inhibitory factor for the production of Th1 cytokines [3]. IL-10 signaling is mediated by the interaction of IL-10 and IL-10 receptor (IL-10R consists of IL-10R1, IL-10R2), resulting in tyrosine phosphorylation of JAK1 and Tyk2 that finally activates latent transcription factors like stat1, stat3 or stat5, which are essential for regulating IL-10 anti-inflammatory activities [4]. *In vivo* and *in vitro* studies with recombinant IL-10 (rIL-10) protein and neutralizing IL-10 monoclonal antibodies (MAbs) have shown pleiotropic activity of IL-10 on T-cells, natural killer cells, B-cells, activated macrophages/monocytes, mast cells, dendritic cells, and keratinocytes [4,5]. IL-10 also has inhibitory functions on several costimulatory molecules and cytokine synthesis, nitric oxide production, and MHC classes I and II expression [6]. Studies with IL-10 deficient mice have shown that resident enteric bacteria are necessary for the development of spontaneous colitis and activation of immune system [7]. Similarly, studies with Th1-mediated colitis in SCID mice have provided evidence that IL-10 plays an essential role in the function of regulatory T-cells that control intestinal inflammatory responses [8]. A recent report also has shown increased IFNγ gene expression along with increased EC apoptosis in anti-IL-10 antibody treated colon explant cultures collected from patients with colon carcinoma [9]. Several studies demonstrate that IL-10 associated immune defects contribute to intestinal inflammation in inflammatory bowel disease including Crohn's disease and Ulcerative Colitis where inflammatory T-cell responses were detected against harmless bacterial antigens [10,11]. Despite all these studies, the detailed role of IL-10 in regulating intestinal homeostasis of normal healthy RMs is poorly documented, where the RM model is well recognized for understanding HIV/SIV pathogenesis, drug development and vaccine design.

Abbreviations: AC-3, active caspase-3; IL-10, interleukin-10; HIV, human immunodeficiency virus; Th1, T-helper 1; JAK1, janus kinase 1; Tyk2, tyrosine kinase 2; SCID, severe combined immunodeficiency; EC, epithelial cells; RM, rhesus macaque; MAb, monoclonal antibody; rIL-10, recombinant IL-10; SIV, simian immunodeficiency virus.

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In this study, we have examined the role of IL-10 in regulating intestinal ECs survivability by colon explant cultures using either anti-IL-10 MAbs or rIL-10 proteins. We have quantified mucosal cytokine(s) and degranulation molecule producing cells and correlated with the total apoptotic ECs. We present evidence that mucosal IL-10 plays an important role in maintaining intestinal mucosal integrity by regulating the expression of IFNγ and TNFα cytokines in intestinal lamina propria (LP).

2. Materials and methods

2.1. Animals and ethical statement

Eight healthy, uninfected, normal male and female Indian RMs (*Macaca mulatta*) between 4.6–7.5 years of age and negative for HIV-2, SIV, type D retrovirus and STLV-1 infection were used for this study. Animals were housed at the Tulane National Primate Research Center (TNPRC) and under the full care of TNPRC veterinarians in accordance with the standards incorporated in the Guide to the Care and Use of Laboratory Animals. All animal procedures were performed only with sedated animals with the approval of the Tulane Institutional Animal Care and Use Committee. Colon specimens were collected during the time of their necropsy.

2.2. Colon explant experiments

Colon specimens approximately 8 cm in length were collected in ice-cold HBSS and were processed as described previously [12]. In brief, tissues were rinsed immediately and cut into approximately 2–4 mm^2 fragments and placed in ice-cold RPMI-1640. Finally explants were cultured for 6 h in 2 ml RPMI-1640 containing BSA (0.01%), fungizone (1%), HEPES (25 mM), and antibiotics (200 μg/ml streptomycin and 200 U/ml penicillin) in the presence of 5% CO_2 at 37 °C. Mucosal explants were treated with either anti-IL-10 MAbs (5 μg/ml, BioLegend), rIL-10 protein (50 ng/ml, BioLegend) or isotype control (5 μg/ml, BioLegend). Explants without any treatment or isotype controls were treated as media-only (negative) controls. Initially we cultured colon explant tissues collected from sacrificed normal Indian RMs for 0 h, 6 h, 12 h, and 24 h without any antibody/protein treatment and there were no significant changes in cell death or morphology between 6 h cultures compared to 0 h cultures. However, increased death and changes in morphology were evident in colon explants kept beyond 6 h (data not shown). Brefeldin A (Sigma) was added 1 h after treatment for *in situ* detection of cytokines and degranulation molecules. After incubation, explant cultures were either cryopreserved in OCT or embedded in paraffin after proper fixation as previously described [2]. Tissue sections of 5 μm thick were processed from paraffin blocks and stained with Hematoxylin and Eosin (H&E).

2.3. Immunofluorescence and immunoperoxidase staining

Tissue sections were processed for immunofluorescent staining with one or a combination of primary antibodies (Supplementary Table 1) as described earlier [2]. In brief, tissue sections were stained sequentially for 2–3 colors by incubating first with the primary antibody for 1 h, washed and stained further with Alexa Flour 488-conjugated secondary antibodies (1:1000 dilution, Invitrogen) for 30 min. Similarly, the slides were further stained with another primary antibody followed by Alexa Fluor 568-conjugated secondary antibodies (1:1000 dilution, Invitrogen). Nuclear staining was performed with anti-nuclear ToPro-3 antibodies (1 μM, Invitrogen). Stained tissue sections were mounted using Prolong® Gold antifade medium (Invitrogen) and scanned for imaging using a TCS SP2 confocal laser scanning microscope (Leica, Germany) equipped with three lasers. Negative control slides were incorporated in each experiment either by omitting the primary antibody or using isotype IgG1 and IgG (H + L) controls [2] (Supplementary Fig. 1). ImageJ (version 1.46, NIH, USA) and Adobe Photoshop CS5 Extended (USA) were used to assign colors to the channels collected. For quantification of intestinal apoptotic ECs, a minimum of 10 fields were imaged using Nuance FX multispectral imaging system at 500–720 nm spectral range and assigned color using Nuance Version 2.10 software (CRi, USA). Active caspase-3+ (AC3+, marker for apoptotic cells) enterocytes were expressed in percentages of the total enterocytes (ToPro-3+Cytokeratin+).

An average of five fields (400X magnification) were manually counted in each stained mucosal explant tissue for quantifying cytokines and degranulation molecules. The sites for all immunohistochemistry evaluations were selected randomly from each tissue and counted by two different individuals to avoid bias.

2.4. Morphometric analysis

Paraffin embedded colon explant tissues were used for morphometric analysis. Slides were stained for H&E and measured for crypt length and breadth using Image-Pro Plus, v4.5 software as outlined previously [13].

2.5. Statistics

Graphical presentation and statistical analysis of the data were performed using GraphPad Prism (Version 5.0f, GraphPad software, CA). Results between experimental groups were compared using nonparametric Kruskal–Wallis test. Dunn's multiple comparison test was used for post hoc analysis. The correlation between the frequency of cytokine expressing cells and percentages of apoptotic enterocytes from all treatments was calculated using nonparametric Spearman's rank correlation. Differences were considered statistically significant when the P value was <0.05.

3. Results and discussion

3.1. Intramucosal IL-10 blocking induces crypt morphological changes and apoptosis in colon explants

The lamina propria was mildly expanded with moderately increased lymphocytes and plasma cells in the anti-IL-10 MAb treated explants compared to controls (Fig. 1A). Neither isotype MAb or rIL-10 protein treated colonic explants showed any pathological changes. However, numbers of cells in apoptosis were increased both in LP and crypts, characterized by pyknotic nuclei and eosinophilic cytoplasm. Many goblet cells in crypts had moderate to severe cytoplasmic vacuolar degeneration. We also observed a significant dilatation of crypt breadth in anti-IL-10 MAbs treated explants ($P < 0.0001$) but no changes in crypt length ($P = 0.253$) compared to media controls (Fig. 1B) in three independent experiments. There were no morphological changes in explants treated with rIL-10 protein. These *in vitro* studies demonstrated that endogenous IL-10 in mucosal tissues is important in maintaining normal epithelial morphology.

3.2. Anti-IL-10 antibody treatment leads to increased expression of proinflammatory, Th1 cytokines and degranulation molecules in lamina propria lymphocytes and increased apoptosis of enterocytes

IL-10 is an important immunoregulatory cytokine that plays a key role in the control of inflammation [6,14]. Earlier studies in mice have shown that anti-mouse IL-10 MAbs neutralized IL-10