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Endogenous antigen presenting cell-derived IL-10 inhibits T lymphocyte responses to commensal enteric bacteria☆

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ABSTRACT

Interleukin-10 deficient (IL-10−/−) mice develop chronic T cell-mediated colitis when colonized with normal commensal bacteria, but germ-free (GF) IL-10−/− mice remain disease-free. Antigen presenting cells (APC) secrete regulatory cytokines that help determine T lymphocyte activation or tolerance. $CD4^+$ T cells from the mesenteric lymph nodes of inflamed IL-10−/− mice secrete more IFN-γ and IL-17 when cultured with cecal bacterial lysate-pulsed splenic APC from IL-10−/− mice than when cultured with normal control APC. GF IL-10−/− APC induce similar IFN-γ and IL-17 responses; therefore, the functional difference between normal and IL-10 deficient APC is inherent to the lack of IL-10 and not secondary to inflammation. Bacterial lysate-pulsed normal APC cultured with $CD4^+$ cells from colitic IL-10−/− mice or with exogenous IFN-γ secrete higher amounts of IL-10 compared to the same APC cultured with naïve T cells. APC enriched for $CD11c^+$ cells are potent activators of IFN-γ and IL-17 production by $CD4^+$ cells from IL-10−/− mice. These APC also produce IL-12/IL-23 p40 and IL-10. Recombinant IL-10 suppressed and anti-IL-10 receptor antibody increased IFN-γ, IL-17 and IL-12/IL-23 p40 production in bacterial lysate-pulsed APC and plus $CD4^+$ T cell co-cultures. Taken together, our results show that endogenous IL-10 produced by APC inhibits responses to commensal bacteria and influences the ability of APC to stimulate IFN-γ-producing effector lymphocytes, which reciprocally, induce IL-10 production by APC. Cytokines produced by APC are an important determinant of pathogenic versus protective mucosal immune responses to colonic bacterial stimulation.

1. Introduction

Inflammatory bowel diseases (IBD) are a constellation of disorders (Crohn's disease and ulcerative colitis) characterized by chronic, immune-mediated inflammation that affects the digestive tract. Although these are idiopathic conditions, dysregulated cell-mediated immune responses to nonpathogenic commensal enteric bacteria play a role in the pathogenesis of these disorders [1,2]. The mucosal immune system maintains a delicate balance; it must be able to defend against a pathogenic microbial threat yet remain unresponsive to resident bacterial and dietary antigens and adjuvants. Specifically in Crohn's disease, loss of tolerance to normal luminal bacteria is documented by increased antibody production and T lymphocyte responses to antigens of intestinal bacteria [3–5]. Patients with Crohn's disease exhibit high mucosal levels of certain cytokines including IL-23, IL-12, IL-18, IFN-γ, IL-17 and TNF [6–12] and respond to anti-TNF and anti-IL-12/IL-23 p40 monoclonal antibody treatment [13,14]. Further evidence of a role for bacteria in the pathogenesis is the attenuation of symptoms after antibiotic treatment [15,16].

Rodent models of intestinal inflammation, although not able to fully recapitulate human disease, provide important insights into the pathogenesis of IBD. First, chronic intestinal inflammation is T cell dependent; B cells are not required and in certain situations are protective [17–21]. The profile of cytokines in most models of chronic intestinal inflammation is Th1/Th17 [17,20,22,23]. IL-23, a heterodimeric cytokine composed of one unique component (p19) that associates with IL-12 p40, has recently emerged as a key inducer of IL-17-mediated inflammation [23]. However, recent work by Izcue et al. shows that IL-17 is not required for development of colitis in all model systems [24]. Second, in most, but not all models, germ-free (GF), sterile rodents do not develop chronic

Abbreviations: APC, antigen presenting cells; CBL, cecal bacterial lysate; CRF2, class II cytokine receptor family; DC, dendritic cells; GF, germ-free; IBD, inflammatory bowel diseases; IL-10−/−, IL-10 deficient; KLH, keyhole limpet hemocyanin; MACS, magnetic activated cell sorting; MLN, mesenteric lymph nodes.

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colitis and continuous exposure to luminal bacteria is necessary to maintain disease [25–27]. Furthermore, T cells from mice with chronic intestinal inflammation respond to components of commensal enteric bacteria [17,20,26,28–31]. Therefore, endogenous nonpathogenic colonic bacteria provide the antigenic and adjuvant stimulation necessary to maintain intestinal inflammation. This loss of tolerance in genetically susceptible hosts can be due either to an overly aggressive effector cell activity, to defective regulatory cell function, or to inadequate response to regulatory signals. Regulatory cells producing TGFβ and IL-10 mediate tolerance in normal hosts [32].

IL-10 deficient mice spontaneously develop progressive colitis in specific pathogen-free (SPF) conditions [33], while GF mice develop neither histologic evidence of disease nor immune activation [25]. GF IL-10 deficient mice develop progressively more aggressive colitis 1–4 weeks after colonization with SPF fecal bacteria; however wild type controls colonized with the same bacteria do not exhibit colitis or immune activation [25,34]. This clearly identifies the role of IL-10 in maintaining immunologic tolerance to the normal enteric microorganisms.

IL-10 is a key immunosuppressive cytokine that acts directly on antigen presenting cells (APC) to inhibit IL-12 secretion and down regulate the expression of MHC Class II and costimulatory molecules such as CD80 and CD86 [35]. This direct action on APC indirectly inhibits T cell activation. IL-10 is produced by multiple cell types, including T cells, dendritic cells (DC), macrophages, and B cells [35–37]. Regulatory T cells are a well-documented source of IL-10 and have been shown to prevent onset of colitis in several murine models. After transfer of $CD45RB^{high}$ cells, syngeneic *scid* recipients develop colitis. Cotransfer of $CD45RB^{lo}$ cells, however, prevents disease [38]. If the $CD45RB^{lo}$ population is derived from IL-10 deficient mice, this population cannot prevent development of colitis [39]. Furthermore, transfer of IL-10 secreting enteric bacterial-responsive regulatory T cell lines can prevent disease in the C3H/HeJBir cotransfer model [40]. However, IL-10 regulatory cell function has been described for other cell populations as well, including DC and B lymphocytes in models of pulmonary or intestinal inflammation [19,41,42].

Despite the proven importance of IL-10 as an immunosuppressive agent both *in vivo* and *in vitro*, the relative roles of T cell-derived and non-T cell-derived IL-10 remain unclear. The purpose of this investigation is to explore the ability of intrinsic IL-10 produced by APC versus $CD4^+$ T lymphocytes to regulate bacterial antigen-induced IFN-γ, IL-17, and IL-12/IL-23 p40 responses. We demonstrate that APC-derived IL-10 is an important regulator of T cell responses to physiologic colonic bacterial stimulation.

2. Materials and methods

2.1. Mice

IL-10 deficient (IL-10−/−) mice on a 129S6/SvEv background and normal and 129S6/SvEv (129 wild type) mice were maintained GF in Trexler isolators in the Gnotobiotic Core of the Center for Gastrointestinal Biology and Disease at the College of Veterinary Medicine, North Carolina State University, Raleigh, NC. Sterility was confirmed by aerobic and anaerobic fecal cultures every 2 weeks. A SPF colony of 129S6/SvEv mice was established from mice obtained from Taconic Laboratories, Germantown, NY and known to be free of *Helicobacter* species. GF → SPF mice used for the source of $CD4^+$ mesenteric lymph nodes (MLN) cells were transferred from GF isolators to the SPF facility at 8–14 weeks of age and euthanized 8 weeks after being colonized with the fecal contents from the SPF 129S6/SvEv mice described above. The North Carolina State University Institutional Animal Care and Use Committee (IACUC) approved all animal protocols.

2.2. Cecal bacterial lysate

Cecal bacterial lysate (CBL) was prepared directly from the cecal contents of 129 wild type SPF mice according to the protocol of Cong [28]. Briefly, the cecum was isolated, placed in 1 ml of sterile RPMI, and vortexed thoroughly. After removal of the cecal tissue and the addition of 0.25 ml of MD solution (0.1 mg/ml DNase I, 0.02 mg/ml $MgCl_2$), this mixture was disrupted by 0.1 mm glass beads in a Minibead beater (Biospec Products, Bartlesville, OK) for 3 min. After centrifugation, the supernatant was filter-sterilized (0.45 μM filter) and the protein concentration was measured using a standard assay (Biorad Laboratories, Hercules, CA). Cecal bacterial lysate was either used immediately after isolation or was aliquoted and frozen at −80 °C.

2.3. Antigen presenting cell preparation

APC were prepared as previously described [26]. Briefly, spleens were isolated from 129 wild type or IL-10−/− mice. T cells were depleted by rabbit complement-mediated lysis using anti-Thy1.2 monoclonal antibody. The resulting population contained less than 6% $CD4^+$ and 1% $CD8^+$ cells. In select experiments, $B220^+$ and $CD11c^+$ cells were enriched by magnetic activated cell sorting (MACS). Briefly, T cell depleted splenocytes were incubated with magnetic beads coupled to antibodies and then passed through the magnetic column (Miltenyi, Auburn, CA). $B220^+$ cells were negatively selected using anti-CD11c and anti-CD11b magnetically labeled antibodies and passed through an LD column. $CD11c^+$ cells were enriched by the following two methods: (1) positive selection using anti-CD11c magnetically labeled antibodies and passed through an LS column. These cells were pulsed overnight with an unrelated antigen, keyhole limpet hemocyanin (KLH: Pierce, Rockford, IL), cecal bacterial lysate at 50 μg/ml, or cultured without antigen in complete medium (RPMI 1640 plus 5% heat inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mecaptoethanol, and 50 μg/ml gentamicin). (2) Cell sorting of spleen cells after incubation with FITC-labeled anti-mouse CD11c (BD Biosciences, San Diego, CA) using a DakoCytomation MoFlo High-Speed Cell Sorter (DakoCytomation, Fort Collins, CO). The 129 wild type and IL-10−/− MoFlo sorted $CD11c^+$ cells were 92.4% and 94.8% $CD11c^+$, respectively. Due to the low number of $CD11c^+$ APC obtained after MoFlo sorting, the cells were not pulsed overnight. Instead, 1×10^4 sorted cells were added directly to 96 well plates for co-culture as described in the following section.

2.4. $CD4^+$ T cell isolation and stimulation

$CD4^+$ cells were isolated and stimulated as previously described [26]. GF IL-10−/− mice were moved to SPF housing conditions and colonized with commensal microorganisms from 129 wild type mice that were free of *Helicobacter* species. After 8–14 weeks, the mesenteric lymph nodes were isolated and single cell suspensions were prepared. The $CD4^+$ T cells were separated by negative selection using MACS as follows: MLN cells were incubated with magnetic beads coupled to anti-CD8 and anti-B220 antibodies (Miltenyi), and passed through and LD column allowing $CD4^+$ cells to be enriched. The enriched cell population was greater than 95% $CD4^+$. APC were collected after overnight pulsing and washed twice to remove soluble antigens and other bacterial products. APC were cultured at 3×10^5 cells per well with $CD4^+$ cells at 2×10^5 cells per well in flat bottom 96 well plates (Costar 3595). The T cell number was selected based on our previously published results showing maximal responses using 2×10^5 $CD4^+$ cells per well in 200 μl [43]. In preliminary experiments for the current studies, in which we titrated APC numbers, we obtained higher amounts of IFN-γ and IL-17 produced by $CD4^+$ T cells co-cultured with 3×10^5