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Bystander-mediated stimulation of proteolipid protein-specific regulatory T (T_{reg}) cells confers protection against experimental autoimmune encephalomyelitis (EAE) via TGF-β

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ABSTRACT

To assess the potency of regulatory T (T_{reg}) cells induced against an irrelevant Ag, mice were orally vaccinated with *Salmonella* expressing *Escherichia coli* colonization factor antigen I fimbriae. Isolated $CD25^+$ and $CD25^-CD4^+$ T cells were adoptively transferred to naive mice, and T_{reg} cells effectively protected against experimental autoimmune encephalomyelitis (EAE), unlike T_{reg} cells from *Salmonella* vector-immunized mice. This protection was abrogated upon in vivo neutralization of TGF-β, resulting in elevated IL-17 and loss of IL-4 and IL-10 production. Thus, T_{reg} cells induced to irrelevant Ags offer a novel approach to treat autoimmune diseases independent of auto-Ag.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) serves as a rodent model for multiple sclerosis (MS) and shares some aspects of this human neurodegenerative disease and T cell dependency (Sospedra and Martin, 2005; Baxter, 2007). EAE can be actively induced upon immunization with encephalitogenic peptides (Sospedra and Martin, 2005; Baxter, 2007) or by passive transfer of myelin auto-Ag reactive T cells (Linington et al., 1993; Miller et al., 1995; Williams et al., 2011). EAE pathology is characterized by the massive CNS infiltration of myelin-reactive $CD4^+$ T cells (Olsson, 1995; Fife et al., 2000), along with other inflammatory cells, such as macrophages and neutrophils (Juedes et al., 2000; Kroenke et al., 2008; Li et al., 2011), causing CNS inflammation and variable paralysis.

The major CNS-invading $CD4^+$ T cells are T helper type 1 cells (Th1) and Th17 cells, which produce inflammatory cytokines IFN-γ and IL-17, respectively. Although IFN-γ can contribute to disease (Olsson et al., 1995; Karpus et al., 1998; Juedes et al., 2000), Th17 cells have been found to be the major encephalitogenic T cells (Hofstetter et al., 2005; Komiyama et al., 2006; Sutton et al., 2006), which can be induced by IL-23 (Cua et al., 2003). In fact, IL-23p19$^{-/-}$ mice have been found to be resistant to EAE (Cua et al., 2003; Langrish et al., 2005). IL-23 exerts its role in EAE through proliferation of Th17 cells (Aggarwal et al., 2003; Langrish et al., 2005), and adoptively transferred IL-23-driven Th17 cells have been found to be highly potent in promoting EAE (Langrish et al., 2005). Recent findings have shown that IL-23 also stimulates GM-CSF-producing autoreactive $CD4^+$ T cells, which in turn induce Th17 cells, causing an exacerbation of disease because of myeloid cell recruitment to the CNS (Codarri et al., 2011; El-Behi et al., 2011). Alternatively, Th17 cells can also be induced upon concomitant stimulation by TGF-β and IL-6 (Veldhoen et al., 2006; Kimura et al., 2007; Okamoto et al., 2010).

Stimulation of anti-inflammatory Th2 cells has been shown to confer protection against EAE. This Th2 cell-dependence is demonstrated by the production of the anti-inflammatory cytokines, IL-4 (Falcone et al., 1998; Inobe et al., 1998) and IL-13 (Offner et al., 2005; Ochoa-Repáraz et al., 2008). However, regulatory T cells are the predominant T cell subset responsible for protection via IL-10 (Faria et al., 2003; Stephens et al., 2009), TGF-β (Chen et al., 2003; Faria et al., 2003; Zheng et al., 2008), and now IL-35 (Collison et al., 2010; Kochetkova et al., 2010). These regulatory T cells can vary phenotypically (Stephens et al., 2009; Kochetkova et al., 2010; Sakaguchi et al., 2010; Campbell and Koch, 2011), but most often the inducible regulatory $CD4^+$ T cells express CD25 and FoxP3 (Stephens et al., 2009; Campbell and Koch, 2011) and are commonly referred to as T_{reg} cells. While T_{reg} cells are induced during EAE (McGeachy et al., 2005; Rynda-Apple et al., 2011), these often lack the ability to ameliorate disease. Thus, alternative means to induce T_{reg} cells are sought. Along these lines, Ag-specific T_{reg} cells are preferential, and

Abbreviations: CFA/I, colonization factor antigen I; EAE, experimental autoimmune encephalomyelitis; HNLNs, head and neck lymph nodes; MS, multiple sclerosis; PLP, proteolipid protein; T_{reg} cell, regulatory T cell.

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tolerization mechanisms surely enhance their development (Rynda et al., 2008; Rynda et al., 2010; Getts et al., 2011; Rynda-Apple et al., 2011). Alternatively, we (Jun et al., 2005; Ochoa-Repáraz et al., 2007; Kochetkova et al., 2008; Ochoa-Repáraz et al., 2008; Kochetkova et al., 2010) and others (Falcone and Bloom, 1997; Fiori et al., 1997) have shown that stimulation of bystander immunity can be effective in treating autoimmunity. Specifically, the expression of enterotoxigenic *Escherichia coli* (ETEC) colonization factor antigen 1 (CFA/I) by an attenuated *Salmonella* Typhimurium vaccine vector (*Salmonella*-CFA/I) alters *Salmonella*'s properties. When macrophages are infected by the *Salmonella*-CFA/I, the stimulation of inflammatory cytokines is suppressed (Pascual et al., 2002). Upon oral immunization, the observed anti-inflammatory properties of *Salmonella*-CFA/I could, via bystander immunity, prevent (Jun et al., 2005; Ochoa-Repáraz et al., 2007) or treat EAE (Ochoa-Repáraz et al., 2008) or collagen-induced arthritis (Kochetkova et al., 2008). Subsequently, it has been learned that *Salmonella*-CFA/I could stimulate diverse subsets of regulatory T cells, depending on the disease model: T_{reg} cells for EAE (Ochoa-Repáraz et al., 2007) and $CD39^+CD4^+$ T cells for arthritis (Kochetkova et al., 2008). An advantage of this immunization approach is that it eliminates the need to develop Ag-specific tolerogens for each disease and instead can utilize this nonspecific approach to render Ag-specific T_{reg} cells, as well as Th2 cells during EAE challenge (Jun et al., 2005; Ochoa-Repáraz et al., 2008) without loss of anti-*Salmonella* protection (Walters et al., 2005). Both the Th2 and T_{reg} cells, when adoptively transferred into EAE-challenged mice, could confer protection, but the level of protection is significantly greater using the vaccine-induced T_{reg} cells. Since production of TGF-β is similar for both $CD4^+$ T cell subsets (Ochoa-Repáraz et al., 2008), we inquired into the significance of this finding. From the evidence presented here, clearly the production of TGF-β by the T_{reg} cells has a greater impact than Th2 cells. Adoptive transfer of vaccine-induced T_{reg} cells and subsequent TGF-β neutralization rendered the complete loss of protection. Similar adoptive transfer of Th2 cells only conferred partial protection against EAE and also showed loss of efficacy as a consequence of TGF-β neutralization. Nonetheless, these results demonstrate that the production of TGF-β is essential for protection mediated by *Salmonella*-CFA/I-induced T_{reg} and Th2 cells.

2. Materials and methods

2.1. Mice and oral immunizations

Female SJL mice (6 week-old) were obtained from The Jackson Laboratory (Bar Harbor, MA) or Frederick Cancer Research Facility, National Cancer Institute, (Frederick, MD). All mice were maintained at the Montana State University Animal Resource Center in individual ventilated cages under HEPA-filtered barrier conditions and fed sterile food and water ad libitum. All procedures were compliant with institutional policies for animal health and well-being.

Mice were pretreated with a 50% saturated sodium bicarbonate solution by intragastric gavage and then orally immunized with 5×10^9 CFU *Salmonella* Typhimurium-CFA/I (strain H696) or its isogenic *Salmonella* vector (H647; lacks the *cfa*/I operon), as previously described (Pascual et al., 1999; Jun et al., 2005).

2.2. Adoptive transfer, EAE challenge, and TGF-β neutralization

To isolate T_{reg} cells, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were obtained from spleens and head and neck lymph nodes (HNLNs) from H696- or H647-vaccinated mice. On day 14 post-immunization, $CD4^+$ T cell subsets were isolated by sequential application of lymphocytes to magnetic beads using a Dynal® Mouse CD4 Negative Isolation Kit (Invitrogen-Life Technologies, Carlsbad, CA), and to isolate $CD25^+$ T cells, CELLection™ Biotin Binder Kit (Invitrogen-Life Technologies); >98% and 96% cell purity were obtained, respectively.

To induce EAE, SJL females were given s.c. 200 μg of the encephalitogenic PLP peptide ($PLP_{139–151}$; HSLGKWLGHPDKF, HPLC-purified to >90%; Biosynthesis, Inc., Lewisville, TX) in 200 μl of complete Freund's adjuvant. On days 0 and 2, mice received i.p. 200 ng of *Bordetella pertussis* toxin (List Biological Laboratories; Campbell, CA), as previously described (Jun et al., 2005; Ochoa-Repáraz et al., 2007; Ochoa-Repáraz et al., 2008).

To determine the mechanism of protection induced by H696 vaccine, naïve SJL mice were given $CD25^-CD4^+$ or $CD25^+CD4^+$ T cells (5×10^5/mouse) derived from H696- or H647-immunized mice via tail vein injection (day −1). EAE was induced 1 day after adoptive transfer (day 0). To neutralize TGF-β in vivo, mice were given i.p. anti-TGF-β mAb (0.5 mg/dose) purified from hybridoma cell line (clone 1D11.16.8; American Type Culture Collection, Manassas, VA) on days −1 and 4 of EAE induction; for isotype control Ab, mouse IgG1 (MOPC-21 myeloma; 0.5 mg/dose; Abcam, Cambridge, MA) was given. Mice were monitored and scored daily for disease progression (Jun et al., 2005): 0, normal; 1, a limp tail; 2, hind limb weakness; 3, hind limb paresis; 4, quadriplegia; 5, death.

2.3. Cytokine ELISA

To assess the types of protective cytokines induced, spleens and HNLNs were harvested at the peak of the disease, and total mononuclear cells (5×10^6 cells/ml) were cultured without or with 30 μg/ml of $PLP_{139–151}$ for 3 days in a complete medium consisting of RPMI 1640 medium with the supplements (Invitrogen-Life Technologies): 1 mM sodium pyruvate, 1 mM nonessential amino acids, penicillin/streptomycin (10 U/ml), and 10% FBS (Atlanta Biologicals, Atlanta, GA). IL-4, IL-10, IL-13, IL-17, IFN-γ, and TGF-β were measured from culture supernatants, as previously described (Ochoa-Repáraz et al., 2007). Briefly, wells were coated with mouse cytokine-specific mAbs: IFN-γ (10 μg/ml; BD Pharmingen, San Diego, CA), IL-17 (2 μg/ml; BD Pharmingen), IL-4 (2 μg/ml; BD Pharmingen), IL-13 (4 μg/ml; R&D Systems, Minneapolis, MN), TGF-β (10 μg/ml; R&D Systems), and IL-10 (2 μg/ml; BD Pharmingen). Following blocking with PBS plus 1% BSA for 2 h at 37 °C, supernatants were incubated overnight at 4 °C. For detection, biotinylated cytokine-specific mAbs were added to wells for 1.5 h at 37 °C: IFN-γ (0.5 μg/ml; BD Pharmingen), IL-17 (0.5 μg/ml; BD Pharmingen), IL-4 (0.2 μg/ml; BD Pharmingen), IL-13 (0.2 μg/ml; R&D Systems), TGF-β (0.5 μg/ml; R&D Systems), and IL-10 (0.3 μg/ml; BD Pharmingen). Following washing, 1:1000 HRP-goat anti-biotin Ab (Vector Laboratories, Burlingame, CA) was added to each well for 1 h at room temperature, and developed with ABTS peroxidase substrate (Moss Inc., Pasadena, CA).

2.4. Flow cytometry analysis

Immunofluorescent staining for cell surface expression of CD4 and CD25 and intracellular Foxp3 was achieved using fluorochrome-labeled mAbs: CD4-FITC (BD Pharmingen), CD25-APC, and Foxp3-PE (eBioscience, San Diego, CA). For intracellular Foxp3 staining, cells were stained for cell surface Ags and then fixed with 2% paraformaldehyde, permeabilized with 0.2% saponin, and stained with anti-FoxP3-PE, similar to that previously described (Kochetkova et al., 2011). Cells were acquired using a LSR-II flow cytometer (Becton Dickinson, San Jose, CA) and analyzed with Flow Jo software (Tree Star Inc. Ashland, OR).

2.5. Statistical analysis

The Student t test was used to evaluate differences between variations in cytokine levels and flow cytometry data. The ANOVA followed by a posthoc Tukey test was applied to show differences in