Published in final edited form as:

Microbes Infect. 2014 January ; 16(1): 67–72. doi:10.1016/j.micinf.2013.09.004.

FoxP3⁺ regulatory T cells are not important for rotavirus clearance or the early antibody response to rotavirus.

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Abstract

Regulatory T cells produce TGF- β that contributes to IgA induction by intestinal commensal bacteria but their importance in IgA responses to pathogens has not been determined. Immunity against the enteropathogen, rotavirus, is dependent on intestinal IgA, but whether FoxP3⁺ regulatory T cells contribute to this IgA is unknown. Infection with rotavirus increased the numbers of intestinal FoxP3⁺ regulatory T cells. Depletion of FoxP3⁺ regulatory T cells altered leukocyte activation but did not significantly alter rotavirus clearance or specific antibody levels. These data suggest FoxP3⁺ regulatory T cells are not critical for the early antibody response to rotavirus infection.

Keywords

rotavirus; regulatory T cells; IgA; FoxP3

1. Introduction

IgA is a key mediator of immunity within the gastrointestinal tract. Transforming growth factor β (TGF- β) is the major factor required for production of IgA. Multiple cell types in the gastrointestinal tract produce TGF- β , including macrophages, dendritic, T, epithelial, and stromal cells. However, the source of TGF- β required for IgA production in the intestine is unknown [1-4]. Regulatory T cells, which express the transcription factor FoxP3, are large producers of TGF- β and thus might play a role in production of IgA in the intestine [5,6].

The viral enteropathogen, rotavirus, is the leading cause of severe gastroenteritis in children worldwide resulting in approximately 400,000 deaths, particularly in low to moderate income countries [7]. It primarily infects the villus epithelium of the intestine and the induction of rotavirus-specific intestinal IgA coincides with viral clearance and correlates with protection [8]. Rotavirus pathogenesis and immunity in mice is similar to humans making the mouse model of rotavirus particularly suited to examine critical factors required for rotavirus immunity [8-10]. B cells are critical in rotavirus immunity as B cell knockout mice chronically shed rotavirus [11-13]. We recently showed that IgA is important for

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clearance and critical for protection against subsequent infections with rotavirus [10]. The mechanisms of rotavirus specific IgA production are unknown. We investigated whether Foxp3⁺ regulatory T cells are (1) induced in the intestinal immune tissue during rotavirus infection, and (2) essential to the induction of early rotavirus-specific IgA and rotavirus clearance.

2. Methods

2.1 Mice.

BALB/cJ and C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). FoxP3^{DTR} mice developed by Kim et al [14] were provided by Dr. Rongfu Wang (BCM Houston, TX) and bred in house. All experiments were performed in 4-6 week old female and male mice that were singly housed with nestlets in microisolator cages within a BSL2 facility. All animals were cared for under veterinary surveillance. All procedures were carried out within the provisions of the Guide for the Care and Use of Laboratory Animals, NIH Guide for Grants and Contracts and with approval from the Institutional Animal Care and Use Committee at Baylor College of Medicine.

2.2 Depletion of FoxP3 cells.

FoxP3 cells were depleted as described [14]. Briefly diphtheria toxin (50 µg/kg, Sigma-Aldrich, Milwaukee, Wisconsin) was injected intraperitoneally two consecutive days prior to rotavirus inoculation and then every other day for the duration of the experiment.

2.3 Viral infection, sample collection, and processing.

Murine rotavirus EC_{wt} was originally obtained from Harry Greenberg (Stanford University Medical School, Palo Alto, CA). At Baylor College of Medicine, intestinal lysates were prepared [15]. All mice were orally inoculated with equivalent amounts of virus, which was 10³ ID₅₀ for C57BL/6 or 10⁶ ID₅₀ for the BALB/c mice in 100 µl of PBS. Fecal pellets from individual mice were collected daily starting the day prior to inoculation (0 dpi) and continuing for the duration of the experiment. Fecal pellets were resuspended (10% weight/volume) and processed [15]. Terminal bleeds were performed at 11 dpi and serum was separated. All samples were stored at -20 °C.

2.4 Fragment cultures.

Fragment cultures were performed as described [16]. Briefly, all intestinal Peyer's patches (PP) and mesenteric lymph nodes (MLN) were removed and washed in complete RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS), 200 mM L-glutamine, 10,000 U/ml penicillin and streptomycin, 50 mM 2-ME, and 5% NCTC-109 medium (BioWhittaker). Washed tissues were then incubated in 96-well plates containing 200 µl of GALT medium [Ex-Cell Hybri-Max Medium (SAFC Biosciences)] containing 10 mM HEPES, 10% FBS, 4 mM glutamine, 100 U/ml penicillin and streptomycin and amphotericin B (Gibco) at 37 °C with 95% humidity and 5% CO₂. Four days later, supernatants were removed and immediately tested for rotavirus specific antibody by ELISA.

2.5 ELISA to measure rotavirus antigen.

Rotavirus shedding in stool suspensions was measured by ELISA [15]. Briefly, 96-well polyvinylchloride plates (VWR, Batavia, IL) were coated with mouse monoclonal anti-VP6 antibody (6E7, rotavirus strain SA11) and incubated overnight at room temperature. Plates were blocked with 5% milk (Carnation from Nestle, Solon, OH) for 2 hours at 37 °C. Stool suspensions were pretreated with 0.05 M EDTA. Following one hour incubation, rotavirus

was detected by hyperimmune guinea pig anti-serum to rotavirus (strain ALA) followed by horseradish peroxidase (HRP)-conjugated goat anti-guinea pig IgG (Sigma-Aldrich Corp., St. Louis, MO) in buffer containing 2.5% FBS. Values with an optical density 0.1 were considered positive.

2.6 ELISA to measure anti-rotavirus antibody.

Total rotavirus-specific antibody (IgA, IgG, and IgM) or rotavirus-specific IgA was measured by ELISA [15]. Briefly, plates were coated with anti-SA11 hyperimmune guinea pig serum and incubated overnight at room temperature. Plates were blocked with 5% milk (Carnation from Nestle) for 2 hours at 37 °C and then incubated with SA11 infected MA104 cell lysate treated with 0.05 M EDTA. Samples were added and followed by incubation with HRP-conjugated goat anti-mouse IgA, IgG, and IgM or anti-mouse IgA (Kirkegaard and Perry) diluted in buffer containing 5% normal guinea pig serum (Rockland Immunochemicals Inc., Gilbertsville, PA). Samples with an optical density 0.1 were considered positive.

2.7 Flow cytometry to measure FoxP3⁺ cells and leukocyte activation.

All small intestinal PP and MLN were harvested, and single cell suspensions of each tissue were generated [16]. Two million cells were stained in 100 µl of complete RPMI. For leukocyte activation, cells were stained with PE-labeled CD69 (0.06 µg/10⁶ cells) and either APC-labeled CD4 (0.06 µg/10⁶ cells), CD19 (0.06 µg/10⁶ cells), or CD11c (0.06 µg/10⁶ cells), or Cy-Chrome-labeled CD8 (0.06 µg/10⁶ cells) (BD Biosciences, San Jose, CA) for 30 minutes in the dark. Cells were washed, fixed in 500 µl 4% paraformaldehyde, and stored at 4°C until analyzed. To detect the FoxP3⁺ cells the R&D Systems mouse regulatory T cell 3 color flow kit was used following manufacture's directions (FMC014, Minneapolis, MN). Samples were analyzed using a LSRII (BD Biosciences) flow cytometer and at least 10,000 events were collected.

2.8 Statistical Analysis

Differences between FoxP3 depleted and non-depleted groups were calculated using the non-parametric, Mann Whitney U two tailed significance using SPSS statistical software version 17.0 (IBM, Armonk, NY). ANOVA followed by protected T test was used to examine differences between FoxP3 depleted and non-depleted and rotavirus infected and uninfected groups. A p value 0.05 was considered significant.

3. Results

3.1 Numbers of regulatory T cells increase upon rotavirus inoculation.

Percentages and numbers of regulatory T cells increased in the spleen and less frequently in the MLN of suckling mouse pups after infection with rotavirus [17] but whether this occurs in PP has not been examined. To determine whether FoxP3⁺ regulatory T cells are increased following rotavirus inoculation in adult mice concurrently with B cell activation [11,16-18], single cell suspensions of PP and MLN collected 2-4 days post inoculation (dpi) from CD-1 mice were analyzed by flow cytometry. Total numbers of FoxP3⁺CD4⁺ cells were significantly increased in the MLN 4 dpi and in the PP 2-4 dpi of rotavirus infected compared to control mice (Fig. 1). Therefore, rotavirus infection of adult mice increases the total number of FoxP3⁺ regulatory T cells in gut associated lymphoid tissue (GALT).

3.2 Depletion of regulatory T cells does not alter rotavirus clearance

To determine whether the presence of regulatory T cells impacted clearance of rotavirus, we used mice that express the diphtheria toxin receptor under the control of the FoxP3 promoter

[14], which allows specific depletion of FoxP3⁺ regulatory T cells by diphtheria toxin. After administration of diphtheria toxin, FoxP3⁺ CD4⁺ cells decreased 98% (2.55% to 0.05%) in the MLN and 90% (0.75% to 0.075%) in the PP. Following virus inoculation, rotavirus antigen was detected in the stool as early as 1 dpi and peaked at 5 dpi in both the regulatory T cell depleted and non-depleted mice (Fig. 2A). While none of the non-depleted mice were shedding detectable levels of rotavirus after 7 dpi, two of the FoxP3⁺ cell depleted mice shed rotavirus in their stool through 10 dpi. However, the mean days of virus shedding was not significantly different between the groups (6.8 vs. 7.9 days, respectively) (Fig. 2B). These findings indicate that regulatory T cells play little to no role in rotavirus clearance.

3.3 Depletion of regulatory T cells has no effect on early rotavirus-specific antibody titers

We next determined whether the depletion of FoxP3⁺ regulatory T cells during rotavirus infection altered early rotavirus-specific antibody levels. Rotavirus-specific antibody titers in stool and serum were similar ($p>0.5$) between depleted and non-depleted groups at 10 and 11 dpi, respectively (Fig. 2C and 2D). Assessment of IgA titers beyond 10-11 dpi was not feasible because mice depleted of FoxP3⁺ regulatory T cells developed severe autoimmunity [14] requiring humane euthanasia. To examine whether regulatory T cells affected local levels of IgA production in the intestinal immune tissues, MLN and PP fragment cultures were established at 4 dpi to allow comparison of antibody induction onset and titers prior to development of severe autoimmunity. Rotavirus specific antibody developed by 4 dpi and levels were similar ($p>0.5$) between depleted and non-depleted mice (Fig. 2E). Thus, the depletion of regulatory T cells during a primary rotavirus infection does not alter the induction of early rotavirus-specific intestinal antibody.

3.4 Leukocyte activation during immune responses to rotavirus is altered by loss of FoxP3 cells

Rotavirus infection induces activation of PP and MLN B cells and dendritic cells, but not CD4⁺ or CD8⁺ T cells, within 2-3 dpi [16,18]. To determine whether depletion of FoxP3⁺ regulatory T cells altered leukocyte activation following rotavirus exposure, the numbers of activated (CD69⁺) leukocytes were examined in PP and MLN of FoxP3⁺ regulatory T cell depleted and non-depleted mice at 2 dpi. Leukocyte activation within the PP and MLN was significantly increased in FoxP3⁺ depleted compared to non-depleted animals (Fig. 3). In the MLN, rotavirus infection significantly increased activation of CD8⁺ T and CD19⁺ cells only in FoxP3⁺ regulatory T cell depleted mice. In the PP, rotavirus infection significantly increased activation of CD19⁺ cells in both FoxP3⁺ regulatory T cell non-depleted and depleted mice and CD11c cells only in depleted mice and significantly decreased CD4⁺ T activation but only in the FoxP3⁺ non-depleted mice (Fig 3). These data indicate that depletion of FoxP3⁺ regulatory T cells globally enhances leukocyte activation and alters leukocyte activation after rotavirus infection.

4. Discussion

Regulatory T cells enhance production of both total and commensal bacteria specific IgA via TGF- β [5]. We examined whether FoxP3⁺ regulatory T cells contributed to the control of infection, induction of early rotavirus-specific antibody, and leukocyte activation by rotavirus in GALT. We found small but significant increases in the numbers of FoxP3⁺ regulatory T cells in response to infection and altered leukocyte activation in the GALT following rotavirus infection in mice depleted of regulatory T cells. However, depletion of regulatory T cells did not significantly alter intestinal or systemic antibody induction during infection or clearance of rotavirus. Therefore, FoxP3⁺ regulatory T cells are not critical in the induction phase of intestinal antibody responses to a viral enteropathogen that induces a profound mucosal IgA response.

A limitation of our study is that it was not feasible to examine the role of regulatory T cells on achievement of peak rotavirus specific antibody titers 3-4 weeks post infection because high levels of regulatory T cell depletion result in early mortality [14]. Use of lower diphtheria toxin doses would have enhanced survival time but at the expense of depletion levels [19,20] and depletion of CD25⁺ T cells, which depleted 60-70% FoxP3⁺ cells in the MLN, had no effect on rotavirus immunity [17]. Therefore, we chose a model in which we could both specifically deplete FoxP3⁺ regulatory T cells, which is a more specific marker than CD25 [17,21-23], and achieve a >90% depletion of these cells. We cannot exclude the possibility that depletion of FoxP3⁺ regulatory T cells affects peak antibody titers to rotavirus but depletion of these cells did not significantly impact early intestinal or serum antibody titers or rotavirus clearance.

We also examined whether depletion of FoxP3⁺ regulatory T cells altered leukocyte activation in GALT. As expected, the loss of regulatory T cells globally enhanced leukocyte activation levels [14] but had a more limited impact on rotavirus-induced leukocyte activation. Rotavirus infection of wild type mice activates both B and dendritic cells but not T cells in PP and MLN by 2-3 dpi with onset and peak activation in MLN lagging that in PP by one day [16,18]. In the current study, depletion of FoxP3⁺ regulatory T cells appeared to advance the onset of MLN B cell and PP dendritic cell activation. The significance of these differences is unclear as they were not reflected by differences in antibody titers. Rotavirus infection of wild type mice does not induce T cell activation in GALT [16,18]. Our data as well as that of Kim et al [17] suggest that FoxP3⁺ or CD25⁺ regulatory T cells may suppress rotavirus activation of MLN CD8 or CD4 and CD8 T cells, respectively. Yet, release of this suppression had no apparent phenotype in either depletion model in terms of antibody responses or viral clearance. We cannot exclude the possibilities that this lack of phenotype is due to global or rotavirus induction of (i) increased cytokine production by activated CD4⁺ T and dendritic cells that compensate for and maintain IgA production in the absence of the TGF- β produced by regulatory T cells, and/or (ii) enhanced activation of CD8⁺ T cells that maintains normal viral clearance. However, in FoxP3⁺ replete mice, the individual absence or depletion of T cell receptor, CD8⁺ T cells, B cells, or dendritic cells significantly impairs viral clearance or IgA [11,12,16,18]. Because depletion of either FoxP3⁺ (this study) or CD25⁺ [17] regulatory T cells had no significant effect on either viral clearance or IgA, it seems unlikely that regulatory T cells are as important in the immune response to rotavirus as these other cell types.

Regulatory T cells are critical for intestinal homeostasis, a source of intestinal TGF- β , and important for induction of total levels of IgA and IgA specific to commensal bacteria [5,24,25]. While it is known that IgA is critical in immunity against rotavirus, its mechanism of induction is unknown. We found that FoxP3⁺ regulatory T cells are not critical for early IgA induction or clearance of rotavirus. Whether this is a universal theme for the role of FoxP3⁺ regulatory T cells in immune responses to intestinal pathogens requires further investigation.

Acknowledgments

This work was supported in part by Public Health Service Grants NIH AI24998 (S.E.B., A.D.M., M.E.C), NIH AI07471 (A.D.M.), Merit review grant from the Office of Research and Development, Medical Research Service, Department of Veterans Affairs (M.E.C), and by the Cytometry and Cell Sorting Core at Baylor College of Medicine with funding from the NIH (AI036211, CA125123, and RR024574). Technical assistance was provided by Samuel Hardeman and Lara Berghammer.

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Figure 1. Total number of FoxP3⁺ regulatory T cells increase upon inoculation with rotavirus BALB/c mice were orally inoculated with gut homogenates containing 10⁶ ID₅₀ of EC_{wt} rotavirus (black bars) or control PBS gut homogenate (white bars). MLN (A) and PP (B) were collected from each mouse (n=3) 2-4 days post inoculation. Single cell suspensions of the tissues were analyzed for the presence of FoxP3⁺CD4⁺ regulatory T cells by flow cytometry. *, p=0.05 by Mann Whitney U two tailed significance compared to PBS inoculated mice at the same time point. Data representative of 4 repeat experiments.

Figure 2. FoxP3⁺ regulatory T cells are not required for rotavirus clearance or rotavirus specific antibody production

FoxP3⁺ regulatory T cells were depleted from FoxP3^{DTR} mice by intraperitoneal injection of diphtheria toxin (50 μ g/kg of body weight). Both depleted (black bar or dashed line) and non-depleted (white bar or solid line) mice were orally inoculated with gut homogenate containing 10³ ID₅₀ of EC_{wt} rotavirus. (A) Fecal pellets were collected every day and analyzed for the presence of rotavirus by ELISA. (B) Mean days of shedding were also calculated. (C) Rotavirus specific IgA was measured in stool samples 10 dpi using ELISA. (D) Rotavirus specific antibody was measured in serum samples collected 11 dpi. (E) MLN and PP were collected 4 days post inoculation and then cultured in vitro for 4 days. Supernatants were collected and analyzed for rotavirus specific antibody using ELISA.

Figure 3. Leukocyte activation is altered after depletion of FoxP3⁺ regulatory T cells
 FoxP3⁺ regulatory T cells were depleted from FoxP3^{DTR} mice by IP injection of diphtheria toxin (50μg/kg of body weight). Both depleted and non-depleted mice were orally inoculated with PBS gut homogenate or gut homogenate containing 10³ ID₅₀ of EC_{wt} rotavirus. MLN (A) and PP (B) were collected from each mouse (n=5) 2 days post inoculation. Single cell suspensions of the tissues were analyzed for the percentage of activated leukocytes by flow cytometry. #, p<0.05 by ANOVA followed by protected t test compared FoxP3⁺RV⁻ to FoxP3⁺RV⁺ at the same time point. *, p<0.05 by ANOVA followed by protected t test compared FoxP3⁻RV⁻ to FoxP3⁻RV⁺ at the same time point. All of the FoxP3⁻ samples were significantly different from the FoxP3⁺ samples at the same time points except for the PP CD11c samples and PP CD19 FoxP3⁺RV⁺ vs. FoxP3⁻RV⁻.