THE ROLE OF TGF-β RECEPTORS IN THE INDUCTION OF MUCOSAL TOLERANCE

by

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ABSTRACT

Oral tolerance, defined as the immunologic state of systemic and mucosal unresponsiveness to an antigen, is an essential function of the mucosal immune system. Although antigen presentation has been found to play an important role in oral tolerance, T cells are known to be the effector immune cells that perpetrate this state. Recent studies found that antigen-specific T regulatory (Treg) cells are important in the induction and maintenance of oral tolerance. The extrathymic development of these Tregs depends on the presence of TGF-β1 during the differentiation process. However, it has also been shown that oral tolerance could be induced in TGF-β1 knockout mice. Given this contradicting information, we sought to further the understanding of the role of TGF-β1 in high- and low-dose oral tolerance induction by focusing on the expression and function of TGFβRII, the signaling component of the oligomeric TGF-β receptor complex, on CD4⁺ T cells of the systemic (spleen) and relevant mucosal [Peyer’s patches (PPs), mesenteric lymph nodes (MLNs), and small intestinal lamina propria (iLP)] immune tissues. We hypothesized that TGFβRII on CD4⁺ T cells is required for both low- and high-dose oral tolerance induction and therefore focused on the role the expression of this receptor
by CD4$^+$ T cells plays at early time points after exposure to the tolerogenic dose(s) of antigen.

We found that the expression of TGFβRII by CD4$^+$ T cells is upregulated at the site of antigen uptake - the PPs - soon after exposure to the tolerizing dose of antigen. Further, we showed that these cells diminish in the PPs and increase in the MLNs and iLP soon after re-exposure to the antigen in the presence of mucosal adjuvant. Also, by employing the CD4 dominant negative TGFβRII transgenic mouse model in which TGFβRII signaling in CD4$^+$ T cells has been abrogated, we found that the proper function of TGFβRII is required for the induction of oral tolerance. Further analysis is necessary to tease out the exact function of these PP TGFβRII$^+$ CD4$^+$ T cells in the induction of oral tolerance.

Keywords: Mucosal immune system, oral tolerance, T cell, TGF-β, TGF-β receptor, T regulatory cell
TABLE OF CONTENTS

Page

ABSTRACT .......................................................................................................................... iii
DEDICATION ....................................................................................................................... v
ACKNOWLEDGMENTS ..................................................................................................... vi
LIST OF TABLES ........................................................................................................... viii
LIST OF FIGURES ........................................................................................................ ix
LIST OF ABBREVIATIONS ........................................................................................... xi
INTRODUCTION .............................................................................................................. 1

The Common Mucosal Immune system ................................................................. 1
Oral Tolerance ............................................................................................................... 2
Adaptive T Regulatory Cells in Oral Tolerance .................................................... 3
Role of TGF-β in Adaptive Treg Development and Oral Tolerance .......... 4

PRELIMINARY RESULTS ............................................................................................ 6

FUNCTIONAL TGF-β RECEPTOR EXPRESSION BY CD4⁺ T CELLS IN PEYER’S PATCHES IS ESSENTIAL FOR ORAL TOLERANCE INDUCTION .................................................. 24

SUPPLEMENTAL RESULTS ......................................................................................... 54

SUMMARY ..................................................................................................................... 61

LIST OF GENERAL REFERENCES .............................................................................. 64

APPENDIX: IACUC APPROVAL FORM ..................................................................... 68
DEDICATION

To my parents, Virginia and Larry, who have always
encouraged my curiosity about the natural world.
And, to my husband Robert without whose support, encouragement and love
I would never have made it to this point.
ACKNOWLEDGEMENTS

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ments he performed for me after the birth of my daughter that helped me to stay on track to graduate. I also wish to thank Dr. Shinichi “Jet” Sekine for assisting me with cell isolation and for teaching (and re-teaching) me FACS analysis.

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LIST OF TABLES

Table                                      Page

PRELIMINARY RESULTS

1  Increased IFN-γ and IL-4 production by mucosal T cells in CD4dnTGFβRII Mice.................................................................20
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>PRELIMINARY RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Increased total Ab levels in aging CD4dnTGFβRII mice ..................18</td>
</tr>
<tr>
<td>2</td>
<td>Increased total numbers of AFCs in aging CD4dnTGFβRII mice ..............19</td>
</tr>
<tr>
<td>3</td>
<td>Absence of IL-10 production by Th cells in iLP of aging CD4dnTGFβRII mice ...........................................................................21</td>
</tr>
<tr>
<td>4</td>
<td>OVA-Specific Ab responses in young adult CD4dnTGFβRII mice are similar to wild type mice..........................................................22</td>
</tr>
<tr>
<td>5</td>
<td>OVA-Specific Ab Responses in Young Adult CD4dnTGFβRII Mice................23</td>
</tr>
</tbody>
</table>

# FUNCTIONAL TGF-β RECEPTOR EXPRESSION BY CD4⁺ T CELLS IN PEYER’S PATCHES IS ESSENTIAL FOR ORAL TOLERANCE INDUCTION

<table>
<thead>
<tr>
<th>Figure</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Effect of large-dose OVA feeding on the occurrence of CD4⁺ TGFβRII⁺ T cells ..........................................................45</td>
</tr>
<tr>
<td>2</td>
<td>Effect of small-dose OVA feeding on the occurrence of CD4⁺ TGFβRII⁺ T cells ..........................................................47</td>
</tr>
<tr>
<td>3</td>
<td>Frequency of CD4⁺ TGFβRII⁺ T cells in tissues of tolerized C57BL/6 mice after single challenge with OVA plus CT..........................49</td>
</tr>
<tr>
<td>4</td>
<td>OVA-specific Ab responses in CD4dnTGFβRII mice after large-dose oral tolerance and oral challenge with OVA plus CT ..................50</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>OVA-specific Ab Forming Cells (AFCs) in CD4dnTGFβRII mice after large-dose oral tolerance and oral challenge with OVA plus CT ..................51</td>
</tr>
<tr>
<td>6</td>
<td>DTH responses in CD4dnTGFβRII mice after large-dose oral tolerance and oral challenge with OVA plus CT ..........................................................52</td>
</tr>
<tr>
<td>S1</td>
<td>Positive controls for FITC-hTGFβRII mAb .................................................................53</td>
</tr>
</tbody>
</table>

### SUPPLEMENTAL RESULTS

| 1      | CCR9 expression by CD4⁺TGFβRII⁺ T cells after a single oral challenge ..........59 |
| 2      | ααββ expression by CD4⁺TGFβRII⁺ T cells after a single oral challenge ..........60 |
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>AFC</td>
<td>antibody forming cell</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CMIS</td>
<td>Common Mucosal Immune System</td>
</tr>
<tr>
<td>CT</td>
<td>cholera toxin</td>
</tr>
<tr>
<td>DTH</td>
<td>delayed-type hypersensitivity</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>Foxp3</td>
<td>forkhead box P3</td>
</tr>
<tr>
<td>GALT</td>
<td>gut-associated lymphoreticular tissue</td>
</tr>
<tr>
<td>IC-FACS</td>
<td>intracellular FACS</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>i.g.</td>
<td>intragastric</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin-</td>
</tr>
<tr>
<td>iLP</td>
<td>small intestinal lamina propria</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------</td>
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<tr>
<td>LT</td>
<td>lymphotoxin</td>
</tr>
<tr>
<td>MLN</td>
<td>mesenteric lymph node</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer’s patch</td>
</tr>
<tr>
<td>S-IgA</td>
<td>secretory IgA</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>TGF-</td>
<td>transforming growth factor-</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory (cell)</td>
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<td>WT</td>
<td>wild-type</td>
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</tbody>
</table>
INTRODUCTION

The Common Mucosal Immune System

The surfaces of the gastrointestinal, respiratory, and urogenital tracts, as well as the conjunctiva of the eyes, are the sites of highest bacterial burden in the host. At these interfaces between the host and the outside environment lie the mucosae. Each individual tract is protected by mucous membranes and an underlying network of immune cells. These cells maintain a complex environment in which a swift response against pathogens can occur without causing injury to the host, a process called mucosal homeostasis. Through mucosal vaccine research it has been found that these mucosa-associated lymphoid tissues (MALT) are interconnected and have therefore been dubbed the Common Mucosal Immune System (CMIS) [1].

The mucosae are comprised of an epithelial barrier under which is a diffuse lymphoid tissue made up of antigen (Ag) presenting cells (APCs), antibody (Ab) secreting plasma cells, memory and effector T helper (Th) cells, and cytotoxic T cells. The main purpose of this tissue, termed lamina propria (LP), is to protect the host from pathogenic microorganisms that are inhaled or otherwise ingested by the host. Punctuating the lamina propria are lymphoreticular structures made up of APCs and naïve lymphocytes. Specialized epithelial cells overlying these structures, called M cells, allow for sampling of luminal Ags that are then processed and presented in the surrounding B and T cell zones. Depending on the microenvironment during Ag presentation these B and T cells then dif-
ferentiate into a myriad of Ag-specific effector cells and migrate out of the inductive sites, into the systemic circulation and then return to the mucosal effector sites of the lamina propria [2,3].

In addition to adaptive immune cells, the epithelium is also protected by innate factors, such as mucus and antimicrobial peptides, which are secreted into the luminal space. These innate factors serve to prevent harmful bacteria from coming into contact with the epithelial layer. One of the key components of the protective mucus is secretory (S)-IgA (reviewed in [4]). Polymeric IgA is produced by plasma cells in the lamina propria and is then transported via transcytosis across the epithelial layer and into the lumen where it is termed S-IgA [4,5]. S-IgA can be specific for a particular Ag but it can also act by nonspecifically binding to bacteria thus preventing them from invading the epithelium [4,5].

*Oral Tolerance*

As mentioned above, a state of homeostasis is the ultimate goal of the CMIS. In humans, bacterial cells outnumber host cells by at least 10 fold. When food Ags, in the case of the gastrointestinal tract, are added into the equation, the enormity of the antigenic burden that the mucosal immune system must process can be truly appreciated. The task of finding and clearing pathogenic bacteria in this environment can be likened to finding the proverbial needle in a haystack. The CMIS must do so without reacting against food Ags or beneficial commensal bacteria, otherwise a state of chronic inflammation and tissue destruction would lead to severe diseases. For this reason, the default
immunologic state at the mucosal surfaces is one of unresponsiveness. In the gastrointestinal (GI) tract, this state of unresponsiveness is termed *oral tolerance*.

Oral tolerance has been studied extensively for decades. It has been primarily studied in the laboratory setting using rodents, but clinical studies have shown that the phenomenon exists in humans as well. In mice and other animals, many studies have found that prior feeding of an Ag can prevent reactivity against the Ag upon re-exposure, even in the presence of powerful adjuvants [6,7]. In human studies, it has been found that sensitivities to certain household allergens, such as dust mites or nickel [8,9], could be alleviated by feeding the susceptible individuals with doses of the offending allergens.

Unresponsiveness to an Ags is not the same as having no response. It was found that T cells isolated from orally tolerized mice were Ag-specific, but did not respond appropriately for a robust immune response to the Ag. One study showed that Th cells from tolerized mice could migrate after Ag re-exposure but did not provide sufficient cytokine production in order to elicit an Ab response [10]. Later, it was found that these T cells were activated by the Ag, but did not divide [11]. Further, in humans, T cells with food Ag specificity can be isolated from the Peyer’s patches (PPs) yet they are not pathogenic [12].

*Adaptive T Regulatory Cells in Oral Tolerance*

It is has been found that, in general, the dosage of the fed Ag affects how oral tolerance to the Ag is induced and maintained. The two primary methods for inducing oral tolerance in the laboratory setting are either to feed a large single dose of Ag, or to feed several small doses or continuous feeding of the Ag [13]. The persistent dogma sur-
rounding these two modes of oral tolerance induction is that a large single dose of protein induces clonal anergy and deletion of reactive T cells, while small doses induce active suppression of reactive T cells through cytokines produced by adaptive T regulatory cells (Tregs) [14]. However, new evidence shows that active suppression occurs during large-dose oral tolerance induction as well. Thus, ovalbumin (OVA)-specific Tregs could be cloned from PPs of mice fed a large dose of OVA [15]. Further, it was found that oral tolerance induced using OVA coupled to cholera toxin (CT) subunit B generated Tregs that induced deletion of OVA-specific T cells [16]. These studies indicate that there is interplay between the two modes of oral tolerance induction and that adaptive Treg are key players in both.

Role of TGF-β in Adaptive Treg Development and Oral Tolerance

Foxp3 (forkhead box P3) is a transcription factor that is expressed by natural and adaptive Treg subsets [17,18,19]. The induction of Foxp3 has been shown to rely on the presence of TGF-β1 during antigen presentation to CD4⁺ Foxp3⁻ T cells in vitro [20,21]. Further, it has been shown that TGF-β1 is necessary for the development of Foxp3⁺ Treg in vivo [21]. Since TGF-β1 is abundantly produced by cells in the GI tract [22], it is an ideal site for the development of Ag-specific adaptive Tregs which can in turn mediate oral tolerance. Accordingly, feeding OVA to mice has been shown to induce the development of OVA-specific Foxp3⁺ T cells in the PPs and mesenteric lymph nodes (MLNs) within 24 - 48 hours [23].

In addition to its roles in the development of Tregs, TGF-β1 is a potent suppressive cytokine that inhibits the differentiation of Th1 and Th2 cells by blocking the ex-
pression of their respective transcription factors, T-bet and GATA-3 [24]. TGF-β1 also plays a role in apoptosis [25] and therefore may be involved in the deletion of Ag-specific T cells after oral tolerance induction. However, it has been found that oral tolerance to proteins can be induced in TGF-β1 deficient mice [26]. Further analysis of the role of TGF-β1 in oral tolerance is necessary to clear up this contradiction.

TGF-β1 is recognized by a hetero-oligomeric receptor comprised of two type I and two type II receptors [27]. TGF-β receptor type II (TGFβRII) binds TGF-β1 and activates the type I TGF-β receptor through the kinase region of its cytoplasmic tail, initiating the TGF-β1 response [27]. Point mutations in the kinase domain of TGFβRII abrogate the TGF-β1 signal [28]. Further, deletion of the kinase domain of the receptor through the use of a dominant-negative form of TGFβRII has been used to study the effects of TGF-β1 signal abrogation in many cell types [29], including T cells [30]. In the latter, mice that express the dominant-negative TGFβRII protein on the surface of their T cells exhibit a phenotype very similar to that of TGF-β1 knockout (KO) mice in that they develop a lethal lymphoproliferative autoimmune syndrome [30,31]. However, because expression of the transgene is restricted to T cells, the development of this syndrome is delayed [30]. Therefore, these mice can be a useful tool for understanding the role that TGF-β1 signaling plays in T cells under physiologic conditions [32,33] and also experimental models of immunity and tolerance [34,35].
PRELIMINARY RESULTS
THE MUCOSAL PHENOTYPE AND IMMUNE RESPONSE OF THE CD4 DOMINANT NEGATIVE TGFβRII MOUSE

REBEKAH S. GILBERT

Unpublished Results
Background and Reasoning

Gorelik and Flavel designed and characterized the CD4dnTGFβRII transgenic mouse in 2000 [1]. These mice lack TGF-β signaling in T cells. Thus, they allow for analysis of the role of TGF-β1 in T cell immunology without the problems associated with TGF-β1 KO mice, which include developmental and severe immune problems [2]. The initial analyses of CD4dnTGFβRII mice were restricted to cytokine analysis and investigation of immune and non-immune organs for abnormal histology in young and aging transgenic mice and their nontransgenic littermates. In that study, it was found that CD4dnTGFβRII mice develop nonspecific activation of T cells as they age. T-helper (Th) cytokine production is dominated by IFN-γ, but there is also significant production of IL-4. Further, by 3 - 4 months of age, the animals develop a lymphoproliferative autoimmune disease marked by mononuclear cell infiltration into multiple organs and production of autoantibodies culminating in diarrhea and a wasting syndrome [1], similar to TGFβ1 KO mice.

We planned to utilize the CD4dnTGFβRII transgenic mouse in our investigation of the role of TGF-β1 and its receptors in oral tolerance induction. In order to do so, we first needed to extend Gorelik and Flavell’s observations to include the mucosal immune status of these mice, as this had not been specifically addressed in their study. Subsequently, it was necessary to determine whether CD4dnTGFβRII mice are capable of responding appropriately to standard oral challenge and oral tolerization protocols.

We initially determined the naïve mucosal phenotype of CD4dnTGFβRII mice at young (8 weeks old) and aging (6 months old) time points. Total immunoglobulin (Ig) levels and Ab-forming cells (AFCs) were determined using ELISA techniques. Further,
production of IFN-γ and IL-4 by T cells was analyzed using intracellular cytokine staining and flow cytometry (IC-FACS) analysis. CD4 IL-10 production in intestinal lamina propria (iLP) of 6 month-old mice was also determined using IC-FACS.

Next we subjected young CD4dnTGFβRII mice to a standard experimental protocol for inducing mucosal immunity. This protocol raises an Ag-specific mucosal and systemic immune response against OVA using three weekly intragastric gavages of OVA plus CT, a mucosal adjuvant. In C57BL/6 mice, the genetic background for CD4dnTGFβRII and therefore referred to as wild-type (WT), this protocol results in the production of OVA-specific IgM, IgG, and IgA in the plasma, as well as OVA-specific S-IgA Ab responses in the mucosal secretions (fecal extracts) [3]. The cytokine profile elicited is primarily Th2-type [4], however, cytokine analysis of immunized CD4dnTGFβRII mice was not performed as part of this preliminary study.

Finally, we performed a limited experiment to determine if experimental oral tolerance could be induced in CD4dnTGFβRII mice. This experiment was “limited” in that the number of animals used was small; their age was inappropriate for accurate interpretation of results; and lastly, because of time constraints, we did not use our standard oral challenge protocol (3 x OVA plus CT) but instead challenged subcutaneously with OVA in complete Freund’s adjuvant (CFA). Despite these shortfalls we felt that the results were promising, and warranted further examination under more ideal conditions. These subsequent results were submitted for publication as part of our main study.
Materials and Methods

Mice

Six- to 8-wk-old female C57BL/6 mice were purchased from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). Eight-wk to 6-month-old CD4dnTGF\(\beta\)RII mice or transgene negative littermates were obtained from an in-house colony maintained at the Immunobiology Vaccine Center, the University of Alabama at Birmingham (UAB). Upon arrival, mice were housed in microisolators, maintained in horizontal laminar flow cabinets, and provided sterile food and water as part of a specific-pathogen free facility at UAB. All of the mice used in these experiments were free of bacterial and viral pathogens. All of the mouse studies were performed in accordance with both NIH and UAB Institutional Animal Care and Use Committee (IACUC) guidelines.

Oral immunization procedure

Mice were immunized intragastrically (i.g.) three times at weekly intervals with 1 mg of OVA plus 15 \(\mu\)g of native cholera toxin (CT; List Biological Laboratories, Inc., Campbell, CA) as mucosal adjuvant in 0.25 ml sterile PBS (OVA plus CT).

Oral tolerance induction and systemic challenge

To establish oral tolerance to a high dose of oral protein, mice were fed 50 mg of OVA (Sigma-Aldrich) i.g. dissolved in 0.25 ml of PBS; control mice received PBS only. One week later, mice were then challenged subcutaneously with OVA in CFA (DIFCO).
Sample collection and cell isolation

Plasma and fecal samples were collected one week after the final challenge with OVA plus CT, or two weeks after subcutaneous injection with OVA plus CFA. Fecal extracts were isolated from homogenates of feces in sterile PBS (1 g/ml). Mononuclear cells were isolated from spleen and MLNs by a mechanical dissociation method using gentle teasing through stainless steel screens as described previously [5,6]. PPs were carefully excised from the small intestinal wall and dissociated using the neutral protease enzyme collagenase type IV (0.5 mg/ml; Sigma-Aldrich) in RPMI 1640 (Cellgro Mediatech, Washington, DC) containing 2 % fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, Georgia) to obtain single-cell preparations [6,7]. Mononuclear cells in the iLP were isolated after removal of PP and intraepithelial lymphocytes from the small intestine using a combination of enzymatic dissociation and discontinuous Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden). Mononuclear cells in the interface between the 40 % and 75 % layers were removed, washed and resuspended in complete medium (RPMI 1640, Cellgro Mediatech, Washington, DC) containing 10 % FCS, 1 % L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, and 40 µg/ml gentamicin.

Isotype-specific Ab assays

Plasma and fecal extracts of naïve mice were determined using isotype-specific ELISA. Briefly, microtiter plates (BD Falcon) were coated with 200 µg of unlabeled-anti-Ig(H+L) (Southern Biotech). After blocking with 1 % bovine serum albumin (BSA, Sigma-Aldrich) in PBS, serial dilutions of plasma or fecal extracts, as well as an Ig isotype standard (100 ng/ml) for IgG, IgM or IgA (Southern Biotech), were applied to the
plates and incubated overnight at 4° C. Plates were then washed and incubated with dilutions of horseradish peroxidase (HRP)-labeled secondary antibodies against IgG, IgM and IgA (Southern Biotech). Color-change reaction was developed using ABTS buffer [0.5M phosphate-citric acid, 0.6 mg/ml ABTS powder (Calbiochem), 3mM 30 % H$_2$O$_2$ (Sigma-Aldrich)]. Development was halted when the titer for the highest concentration of standard reached an optical density at 415 nm (OD$_{415}$) of ~ 2.0 units above background control values. DeltaSoft3 software for Macintosh was used to create standard curves and interpolate the concentrations of IgG, IgM and IgA in the samples.

Mononuclear cells obtained from mucosal and systemic lymphoid tissues were subjected to isotype-specific ELISPOT assay to detect numbers of total AFCs per 10$^6$ lymphoid cells. MultiScreen™ HTS plates (Millipore) were coated with 200 µg of unlabeled-anti-Ig(H+L) (Southern Biotech). After blocking with 1 % BSA in PBS, serial dilutions of mononuclear cells in complete medium were incubated at 37° C for 4 hours. Plates were then washed and incubated with secondary HRP-Abs as described above. Spots were developed using AEC buffer [0.05M Acetate (Sigma-Aldrich), 0.3M N,N-dimethylformamide (Sigma-Aldrich), 0.278 mg/ml AEC powder (Pierce), 4mM 30% H$_2$O$_2$ (Sigma-Aldrich)] for 45 minutes. Spots were counted using ImmunoSpot ELISPOT reader.

*OVA-specific Ab assays*

OVA-specific Ab levels in plasma and fecal extracts (FE) of immunized mice were determined by ELISA. Microtiter plates (BD Falcon) were coated with 100 µg of OVA. After blocking with 1 % BSA-PBS, serial dilutions of plasma or fecal extracts were applied to the plates and incubated overnight at 4° C. Plates were then washed and
incubated with dilutions of HRP-labeled secondary detection antibodies against IgG, IgM or IgA (Southern Biotech). Color-change reaction was developed using ABTS buffer for 15 minutes. Endpoint titers were expressed as the last dilution yielding an optical density at 415 nm (OD$_{415}$) of > 0.1 units above background control values.

Mononuclear cells obtained from mucosal and systemic lymphoid tissues were subjected to an ELISPOT assay to detect numbers of OVA-specific AFCs per 10$^6$ total lymphoid cells. MultiScreen™ HTS plates (Millipore) were coated with OVA (1 mg/ml in PBS) before being blocked with 1 % BSA-PBS. Procedure then followed as described above for isotype-specific ELISpot.

OVA-specific CD4$^+$ T cell proliferation assay

CD4$^+$ T cells from spleens were purified by use of an automated magnetic-activated cell sorter system (Miltenyi Biotec, Auburn, CA), as described previously [8]. The purified CD4$^+$ T-cell fraction (>97% pure, >99% viable) was then suspended in complete RPMI 1640 (4 x 10$^6$ cells/mL) and cultured with or without 1 mg/ml of OVA in the presence of T-cell–depleted, irradiated (3000 rad) splenic APCs taken from nonimmunized mice for 5 days. To assess OVA-specific T-cell proliferative responses, an aliquot of 0.5 μCi of tritiated [$^3$H]-TdR (Amersham Biosciences, Arlington Heights, IL) was added during the final 18 hours of incubation, and the amount of [$^3$H]-TdR incorporation was determined by scintillation counting.

Flow cytometric analysis

Mononuclear cells from mucosal and systemic tissues were isolated and incubated with 50 ng/ml phorbol myristate acetate (PMA) plus 1 μg/ml ionomycin for 4 hours at
37° C. The cells were then washed and preincubated with purified CD16/32 mAb (Fc Block, 2 µg/ml; BD Pharmingen, San Diego, CA). Samples were then stained with a combination of FITC-labeled anti-CD3α, allophycocyanin (APC)-conjugated anti-CD4, and PE-tagged anti-IL-4, -IL-10, or -IFN-γ (BD Pharmingen).

Statistical Analysis

The results are expressed as the mean ± SEM. Non-parametric data were analyzed using an unpaired Mann-Whitney U test using InStat software for Macintosh by GraphPad. p values < 0.05 were considered significant, and < 0.001 were considered highly significant.

Results and Discussion

Aging CD4dnTGFβRII mice have increased total Ab production

Eight-week- and 6-month-old CD4dnTGFβRII and age-matched WT mice were analyzed for the production of total IgG, IgM and IgA in plasma, and S-IgA Abs in FE. Eight-week-old mice from both groups showed similar levels of Abs in plasma and FE. By contrast, by 6 months of age, CD4dnTGFβRII mice displayed a significant increase in IgG and IgA in plasma and S-IgA Ab titers in FE (Fig. 1). These findings were further confirmed at the cellular level. Thus, 6-month-old CD4dnTGFβRII mice had significantly higher numbers of AFCs than their 8-wk-old counterparts (Fig. 2). Interestingly, total numbers of IgM-producing cells in spleens of aging CD4dnTGFβRII were increased; yet concentrations of IgM in plasma were not significantly different than those of control mice. These data indicate that aging CD4dnTGFβRII mice have a dysregulated Ab response in both the systemic and mucosal compartments. This dysregulation may
have negative effects on the outcomes of mucosal immunization and tolerization studies. To this end, aging mice are not ideal for use in our studies.

*Aging CD4dnTGFβRII mice have an altered T cell cytokine profile*

We next determined IFN-γ production by 12-wk- and 6-mo-old CD4dnTGFβRII mice and 6-mo-old WT mice. Aging WT mice showed a low frequency of IFN-γ+ T cells in spleen, MLNs and PPs. In stark contrast to this finding, aging CD4dnTGFβRII mice displayed a dramatic increase in IFN-γ+ T cells; with nearly one quarter of all PP T cells, ~50% of T cells in MLNs and iLP, and almost 90% of splenic T cells scoring as positive for IFN-γ (Table 1). Six-month-old CD4dnTGFβRII mice also showed an increase in the frequency of IL-4+ T cells in all tissues, when compared to age-matched WT mice (Table 1). Interestingly, IL-10 production by CD4+ T cells was largely absent in the iLP of aging CD4dnTGFβRII mice (Fig. 3). These data provide further evidence of the disruption of the mucosal and systemic immune homeostasis in aging CD4dnTGFβRII mice. In order to reduce the interference of this dysregulation, these mice must be immunized at as young an age as possible. Based on this cytokine data and combined with the total-Ig data, we estimated that 4 - 5 weeks-of-age is the most ideal age for experimental purposes.

*Young adult CD4dnTGFβRII mice respond normally to oral immunization with OVA plus cholera toxin*

After determining that young adult (8-wk-old) CD4dnTGFβRII mice have naïve mucosal and systemic immune phenotypes similar to WT mice, we next examined how these transgenic animals respond to a standard oral immunization protocol comprised of three weekly gavages of 1 mg of OVA plus 15 µg of CT (OVA plus CT). It is of utmost
importance that the mice respond appropriately to OVA plus CT since this is the method by which we test the experimental induction of mucosal and systemic tolerance. One week after the final immunization with OVA plus CT, OVA-specific Ab titers in plasma and FE were determined and AFCs from spleen and PPs were enumerated using ELISA and ELISPOT, respectively. OVA-specific IgM, IgG and IgA Ab titers in plasma from CD4dnTGFβRII mice were slightly elevated but not significantly different from WT controls (Fig. 4). Similarly, OVA-specific S-IgA Ab titers in FE were not different from WT (Fig. 4). Examination of OVA-specific AFCs confirmed these data at the cellular level (data not shown). These data indicate that the ability to mount Ag-specific systemic and mucosal immune responses to OVA using CT as adjuvant is intact in young adult CD4dnTGFβRII mice. Therefore, these mice can be used in future oral tolerance experiments.

_Systemic challenge shows that CD4dnTGFβRII mice appear to be incapable of oral tolerance induction_

Our final preliminary experiment was to simply try to induce oral tolerance in a small number of CD4dnTGFβRII mice as a proof-of-concept. If oral tolerance were to be induced, there would be no need for further experimentation using these mice. Unfortunately, at this point in the pre-dissertation period, conditions were not ideal for this experiment. The transgenic mice on-hand were older than the 4 - 5 weeks of age that we had determined would be best for experimental purposes, and the time frame was not sufficient for using a mucosal challenge protocol. Thus, the CD4dnTGFβRII mice used in this preliminary oral tolerance experiment were nearly 4 months old at the time of large-dose (50 mg) OVA feeding. One week later, a subcutaneous challenge of OVA in CFA,
rather than oral OVA plus CT, was used to determine the state of systemic unresponsiveness to OVA. Two weeks following this subcutaneous challenge, OVA-specific IgG titers in plasma were examined and a CD4\(^+\) T cell proliferation assay was performed. OVA- or PBS-fed WT mice were used as controls. PBS-fed CD4dnTGFβRII controls for the proliferation assay were not determined. As expected, OVA-fed WT mice showed a significant decrease in OVA-specific IgG Ab titers in plasma when compared with PBS-fed WT mice. In contrast to this, CD4dnTGFβRII mice fed a large dose of OVA did not have decreased OVA-specific plasma IgG Ab responses when compared with PBS-fed controls (Fig. 5A). Further, CD4\(^+\) T cells taken from spleens of OVA-fed CD4dnTGFβRII mice proliferated similarly to PBS-fed WT mice when re-exposed to OVA in vitro (Fig. 5B). Although these results were limited, they indicated that further examination of oral tolerance induction in CD4dnTGFβRII mice was warranted.

References


Figure 1. Increased total Ab levels in aging CD4dnTGFβRII mice. Plasma and fecal extracts of 8-wk- and 6-mo-old naïve CD4dnTGFβRII or C57BL/6 (WT) mice were subjected to isotype-specific ELISA to determine IgM, IgG, IgA (plasma) or S-IgA (fecal extract) Ab responses. Concentrations were interpolated from standard curves generated from dilutions of internal standards of each isotype. Values are expressed as the mean ± SEM. * p < 0.05.
Figure 2. Increased total numbers of AFCs in aging CD4dnTGFβRII mice. Mononuclear cells were isolated from spleen and PPs of 8-wk- and 6-mo-old naïve CD4dnTGFβRII mice. Cells were then subjected to an ELISPOT assay to detect numbers of isotype-specific AFCs / 10^6 cells. * p < 0.05.
Table 1. Increased IFN-γ and IL-4 production by mucosal T cells in CD4dnTGFβRII mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>The Frequency of IFN-γ⁺ T cells § (%)</th>
<th>The Frequency of IL-4⁺ T cells (%)</th>
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<tbody>
<tr>
<td></td>
<td>12 wk TβRII</td>
<td>6 mo TβRII</td>
</tr>
<tr>
<td>SP</td>
<td>44 ± 3.0</td>
<td>86 ± 2.3</td>
</tr>
<tr>
<td>MLN</td>
<td>24 ± 2.1</td>
<td>78 ± 8.5</td>
</tr>
<tr>
<td>PP</td>
<td>17 ± 0.5</td>
<td>26 ± 2.9</td>
</tr>
<tr>
<td>iLP</td>
<td>37 ± 2.2</td>
<td>57 ± 2.0</td>
</tr>
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§ CD3⁺ T cells from each mouse group were analyzed by IC-FACS analysis
ND, not determined
*CD4dnTGFβRII
Figure 3. Absence of IL-10 production by Th cells in iLP of aging CD4dnTGFβRII mice. Mononuclear cells were isolated from iLP of naïve 6-mo-old CD4dnTGFβRII or C57BL/6 (WT) mice. Cells were then stained with FITC-conjugated anti-CD3, APC-labeled anti-CD4, and PE-tagged anti-IL-10. Samples were subjected to flow cytometry analysis by FACSCalibur®. Values given are averages of three mice, representative FACS plots are shown.
Figure 4. OVA-specific Ab responses in young adult CD4dnTGFβRII mice. One week after the last challenge, plasma and fecal extracts from CD4dnTGFβRII or C57BL/6 (WT) mice were subjected to OVA-specific ELISA to determine IgM, IgG, IgA (plasma) or S-IgA (fecal extract) Ab responses. Endpoint titers are expressed as the last dilution yielding OD415 of > 0.1 units above background control values. Values are expressed as the mean ± SEM.
Figure 5. Oral tolerance cannot be induced in CD4dnTGFβRII mice. A. Two weeks after subcutaneous challenge, OVA-specific IgG titers in plasma of each mouse group were determined. Values represent the mean of 5 mice ± SEM. * p < 0.05. B. Further, splenic CD4⁺ T cells from each group were purified using an AutoMACS system, resuspended in complete medium and incubated with or without 1 mg/ml OVA for 5 days. Eighteen hours prior to culture harvesting, 0.5 µCi of tritiated thymidine was added to each well. Stimulation index was calculated by dividing cpm of OVA+ wells by cpm of OVA- wells. Values represent average of two mice ± SEM. WT, wild-type; ND, not determined.
FUNCTIONAL TGF-β RECEPTOR EXPRESSION BY CD4+ T CELLS IN PEYER’S PATCHES IS ESSENTIAL FOR ORAL TOLERANCE INDUCTION

by

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Abstract

Our previous studies have shown that PPs play a key role in the induction of oral tolerance. Therefore, we hypothesized that PPs are an important site for TGF-β signaling and sought to prove that this tissue is of importance in oral tolerance induction. We found that expression of TGFβRII by CD4+ T cells increases and persists in the PPs of normal C57BL/6 mice after either high- or low-dose feeding of OVA when compared to MLNs and spleen. Approximately, one-third of these TGFβRII+ CD4+ T cells bear the Foxp3 gene. Interestingly, the number of TGFβRII+ CD4+ T cells in PPs decreased when OVA-fed mice were orally challenged with OVA plus native CT. In contrast, numbers of TGFβRII+ CD4+ T cells were increased in the iLP of these challenged mice. Further, these PP CD4+ TGFβRII+ T cells up-regulated Foxp3 within 2 hours after OVA plus CT challenge. Mice fed PBS and challenged with OVA plus CT did not reveal any changes in TGFβRII expression by CD4+ T cells. In order to test the functional property of TGFβRII in the induction of oral tolerance, CD4dnTGFβRII transgenic mice, in which TGFβRII signaling is abrogated from all CD4+ T cells, were employed. Of importance, these mice could not develop oral tolerance to OVA. Our studies show a critical, dose-independent, role for TGFβRII expression and function by CD4+ T cells in the gut-associated lymphoid tissues; further underlining the vital role of PPs in oral tolerance.

Introduction

Oral tolerance is a function of the mucosal immune system by which the host is protected from deleterious immune responses to innocuous gut Ags [1,2]. Large doses of Ag or prolonged exposure to small doses of Ag induce a state of mucosal and systemic
unresponsiveness that is characterized by reduced Ag-specific IgG and helper T cell responses in the presence of protective S-IgA Ab production [1,2,3]. When oral tolerance is disrupted, allergy and inflammatory bowel diseases can occur. Conversely, it has been proposed that harnessing oral tolerance can be an effective means of treating various diseases, from allergy to autoimmunity [1,4,5,6,7,8,9]

Although dendritic cells (DCs) have been shown to be involved both directly and indirectly in the induction of oral tolerance [10,11,12,13,14,15,16], it is primarily agreed that oral tolerance is established and maintained at the T cell level [5,17,18]. The magnitude of the dose of Ag determines how the tolerance is mediated. Large doses of Ag are understood to induce anergy - a failure to respond to the Ag - and/or deletion of Ag-specific T cells, while small recurrent doses of Ag lead to the development of Ag-specific Tregs which in turn suppress surrounding T cells by the production of inhibitory cytokines, such as TGF-β and IL-10 [1,2,19,20,21,22]. In addition to these mechanisms, recent studies have suggested that anergy is also important in small-dose oral tolerance [13] and vice versa, that active suppression can play a role in large-dose oral tolerance [13,23].

TGF-β1 plays important roles in the induction and maintenance of tolerance. In the absence of IL-6, TGF-β1 induces the expression of Foxp3 in naïve CD4+ cells in vitro [19,24,25,26] and in vivo [25,27]. Further, TGF-β1 has been shown to be necessary for the maintenance of Foxp3 expression in adaptive CD4+ CD25+ Tregs [28]. In addition to its roles in adaptive Treg differentiation and function, TGF-β1 suppresses Ag-specific effector T cells in vitro and in vivo [19,25,29].

TGF-β1 is recognized by a type I-type II hetero-oligomeric receptor [30]. TGF-β receptor type II (TGFβRII) binds TGF-β1 and activates the type I TGF-β receptor
through the kinase region of its cytoplasmic tail, initiating the TGF-β1 response [30]. Point mutations in the kinase domain of TGFβRII abrogate the TGF-β1 signal [31]. Further, deletion of the kinase domain of the receptor through the use of a dominant-negative form of TGFβRII has been used to study the effects of TGF-β1 signal abrogation in many cell types, including mammary cells [32], osteoblasts [33], skin cells [34], and T cells [35]. In the latter, mice that express the dominant-negative TGFβRII protein on the surface of their T cells exhibit a phenotype very similar to that of TGF-β1 knockout mice in that they develop a lethal lymphoproliferative autoimmune syndrome [35]. Although these mice have thymus-derived natural Tregs, their effector T cells ultimately escape suppression [36].

PPs play key roles in oral tolerance. Our previous studies showed that the presence of PPs was required for oral tolerance to proteins to occur [37]. Thus, PP-null mice fed a large dose of OVA and subsequently challenged systemically developed OVA-specific Abs and helper T cell responses [37]. In addition to this, targeted delivery of Ag to PPs using M-cell targeting protein σ1 fused with OVA greatly reduces the amount of Ag needed to induce tolerance by up to 1,000 fold [38]. Others have shown that Ag-specific CD4+ CD25+ T cells with suppressive functions can be cloned from PPs of orally tolerized mice [23]. Despite these findings, the topic of a PP requirement in oral tolerance induction remains controversial. Subsequent studies of oral tolerance induction in PP-null mice showed that oral tolerance could be achieved [39], and that only the removal of the MLNs prior to tolerization could prevent oral tolerance induction [40]. Early studies in germ-free mice linked the impairment of oral tolerance induction in these animals to the reduced number T cells in their PPs [41]. Yet, more recent studies found that adoptively
transferred Ag-specific T cells isolated from high- and low-dose Ag-fed germ-free recipients proliferated the same as those from specific pathogen-free animals [42], suggesting again that PPs play a secondary role to MLNs.

The present study aims to provide further evidence for the vital role of PPs in early events in the induction of oral tolerance. We provide evidence that PPs are uniquely populated with T helper cells that are equipped to respond to TGF-β1 and may therefore contribute to the suppressive environment necessary for tolerance initiation. This study is the first to examine the expression of TGF-β1 receptors in mucosal and systemic inductive tissues in the early time points during the induction of oral tolerance.

Materials And Methods

Mice

Six- to 8-wk-old female C57BL/6 mice were purchased from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). Five-wk-old CD4dnTGFβRII mice or WT littermates were obtained from an in-house colony maintained at the Immunobiology Vaccine Center, the UAB. Upon arrival, mice were housed in microisolators, maintained in horizontal laminar flow cabinets, and provided sterile food and water as part of a specific-pathogen free facility at UAB. All of the mice used in these experiments were free of bacterial and viral pathogens. All of the mouse studies were performed in accordance with both NIH and UAB Institutional Animal Care and Use Committee (IACUC) guidelines; Animal protocol number 100908212.
**Induction of oral tolerance and oral challenge procedure**

To establish oral tolerance to a high dose of oral protein, mice were gastrically intubated with 30 mg of OVA (Sigma-Aldrich) dissolved in 0.25 ml of PBS; control mice received PBS only. To establish low-dose oral tolerance, mice were continuously fed 1 mg / ml of OVA *ad libitum* in drinking water for 7 days; control groups received water only. To determine TGFβRII expression after oral challenge, mice were challenged intragastrically (i.g.) 7 days after tolerization with 1 mg of OVA plus 10 µg of CT (List Biological Laboratories, Inc., Campbell, CA) as mucosal adjuvant in 0.25 ml sterile PBS (OVA plus CT). To examine the induction of oral tolerance to OVA, transgenic and WT mice were challenged i.g. with OVA plus CT on days 7, 14 and 21 after an initial 30 mg dose of OVA or PBS as control [43].

**Sample collection and cell isolation**

Plasma and fecal samples were collected one week after the final challenge with OVA plus CT. Fecal extracts were isolated from homogenates of feces in sterile PBS (1 g / ml). Mononuclear cells were isolated from spleen and mesenteric lymph nodes (MLNs) by a mechanical dissociation method using gentle teasing through stainless steel screens as described previously [38,43]. PPs were carefully excised from the small intestinal wall and dissociated using the neutral protease enzyme collagenase type IV (0.5 mg/ml; Sigma-Aldrich) in RPMI 1640 (Cellgro Mediatech, Washington, DC) containing 2 % fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, Georgia) to obtain single-cell preparations [38,44]. Mononuclear cells in the iLP were isolated after removal of PP and intraepithelial lymphocytes from the small intestine using a combination of enzymatic dissociation and discontinuous Percoll gradients (Pharmacia Fine Chemicals, Uppsala,
Sweden). Mononuclear cells in the interface between the 40 % and 75 % layers were removed, washed and resuspended in complete medium (RPMI 1640, Cellgro Mediatech, Washington, DC) containing 10% FBS, 1 % L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, and 40 µg/ml gentamicin. [38,44].

**OVA-specific Ab assays**

OVA-specific Ab levels in plasma and fecal extracts were determined by ELISA as previously described [38,43,44,45]. Endpoint titers were expressed as the last dilution yielding an optical density at 415 nm (OD_{415}) of > 0.1 units above background control values. Mononuclear cells obtained from mucosal and systemic lymphoid tissues were subjected to an ELISPOT assay to detect numbers of OVA-specific AFCs per 10⁶ total lymphoid cells [38,43,45].

**DTH responses**

Ag-specific DTH responses were measured 7 days after the last oral challenge with OVA plus CT as described above. Briefly, PBS (20 µl) containing 10 µg OVA was injected into the left ear pinna of the mice, whereas the right ear pinna received PBS as control. Ear swelling was measured 24 h later with a dial-thickness gauge (Ozaki Manufaturing, Tokyo, Japan). The DTH response was expressed as the increase of ear swelling after OVA injection minus the swelling in the control site [37].

**Flow cytometric analysis**

Mononuclear cells from mucosal and systemic tissues were isolated at various time points and preincubated with purified CD16/32 mAb (Fc Block, 2 µg / ml; BD PharMingen, San Diego, CA). Samples were then stained with a combination of FITC-
labeled anti-human TGFβRII mAb (clone # 25508; R&D Systems, Minneapolis, MN), APC-conjugated anti-CCR9 or -CD4, PE-tagged anti-CD8α, -CD4, or -LPAM, and biotin-conjugated anti-CD3 mAbs (BD Pharmingen, San Diego, CA) followed by PerCP-Cy™5.5-conjugated streptavidin (BD Pharmingen, San Diego, CA). CD4dnTGFβRII mice over-express a non-functional TGF-βRII molecule on all CD4⁺ T cells; therefore CD4⁺ T cells were isolated from MLN and PP of CD4dnTGFβRII mice and stained as described above for use as positive controls for the staining of FITC-hTGFβRII mAb (Fig. S1). In some experiments, intracellular Foxp3 expression was detected using a Foxp3 staining buffer kit and PE-conjugated anti-hFoxp3 (eBioscience, San Diego, CA).

Statistical Analysis

The results are expressed as the mean ± one SEM. Non-parametric data were analyzed using an unpaired Mann-Whitney U test using InStat software for Macintosh by GraphPad. Parametric data were analyzed using two-way ANOVA using Prism software for Windows by GraphPad. p values < 0.05 were considered significant, and < 0.001 were considered highly significant.

Results

TGFβRII expression is induced and persistent in the Peyer's patches after OVA feeding

We initially determined the expression of the TGFβRII on CD4⁺ T cells in the PPs, MLNs and spleen of C57BL/6 mice using flow cytometry. We found that TGFβRII is not expressed on CD4⁺ T cells of naïve mice (data not shown). We next assessed the kinetics of this expression after feeding large or small doses of oral OVA or PBS as a negative control. Interestingly, TGFβRII expression by CD4⁺ T cells increases within 24
h after feeding a 30 mg dose of OVA when compared to those in tissues from PBS-fed mice. PPs of mice fed 30 mg OVA had significantly higher frequencies of CD4$^+$ TGFβRII$^+$ T cells than mice fed PBS alone, 4% versus 0.6%, respectively. These frequencies were maintained for seven days after Ag feeding, the point at which an oral challenge protocol would begin (Fig. 1A and 1B). In contrast, the frequencies of CD4$^+$ TGFβRII$^+$ T cells in MLNs and spleen fluctuated and returned to basal levels over this time course, and did not exceed 2% of the CD4$^+$ T cell population.

When fed a low dose of OVA (1mg/ ml in drinking water for 7 days), a similar pattern of TGFβRII expression was observed; however, it was not as pronounced as with 30 mg of OVA feeding, with PP CD4$^+$ TGFβRII$^+$ T cell frequency reaching 3.3% of all CD4$^+$ T cells (Fig. 2A and 2B). Although the magnitude of CD4$^+$ TGFβRII$^+$ T cell increase in PP of small-dose fed mice was not as great as in high-dose recipients, it was significantly higher than in PP of mice that drank only water. Further, the frequencies of CD4$^+$ TGFβRII$^+$ T cells in MLNs and spleens of small-dose recipients were not significantly different from those of water-fed control mice. These data indicate that TGF-β1 signaling is occurring and is sustained to a higher degree in PPs for initiating the potential development of Treg cells when compared to systemic lymphoid tissues during large- and small-dose oral tolerance induction.

*Expression of TGFβRII on CD4$^+$ T cells decreases after oral challenge with OVA plus CT*

Following a typical oral challenge protocol of three weekly feedings of 1 mg of OVA plus 10 μg of CT (OVA plus CT) starting one week after large-dose tolerance induction, we found that there was no significant difference in expression of TGFβRII on
CD4+ T cells between mucosal and systemic lymphoid tissues. The frequency of CD4+ TGFβRII+ T cells in the PPs, MLNs and spleen had returned to basal levels by one week after the final challenge (data not shown). Upon further analysis, this drop in expression was observed by 24 h after a single oral challenge with OVA plus CT. Therefore, we focused on early time points after the first oral challenge in order to examine the kinetics of this observed decrease. Within 30 min of OVA plus CT administration, numbers of CD4+ TGFβRII+ T cells decreased in the PPs (Fig. 3A). Mice fed PBS showed essentially no change in CD4+ TGFβRII+ T cells after oral challenge. When TGFβRII expression in the iLP was also examined in mice fed OVA, a significantly increased number of CD4+ TGFβRII+ T cells were seen 4 h following oral challenge. Further, MLNs and spleen showed increased numbers of CD4+ TGFβRII+ T cells. These data suggest that the CD4+ TGFβRII+ T cells may be dispatching from the PPs to effector tissues via the draining lymph nodes shortly after challenge. Importantly, by two hours after oral challenge with OVA plus CT, CD4+ TGFβRII+ T cells from the PPs of mice fed a large dose of OVA have significantly increased intracellular Foxp3 (Fig. 3B). In contrast, CD4+ TGFβRII+ Foxp3+ T cells decreased in iLP, MLNs and spleen during this time. Further, Foxp3 expression in CD4+ TGFβRII+ T cells decreased in all tissues over this period of time (data not shown). It is interesting to note that these results also indicate a direct affect of OVA plus CT challenge on CD4+ T cells, since this time frame would preclude Ag processing and presentation by APCs.
Abrogation of TGFβRII signaling in CD4\(^+\) T cells fails to elicit mucosal unresponsiveness

Our findings thus far indicate that TGFβRII expression by CD4\(^+\) T cells is influenced by large- and small-dose Ag feeding. However, it is important to determine whether the function of this receptor is required for oral tolerance induction. To this end, we next examined the role of functional TGFβRII signaling in the induction of oral tolerance in a mouse model. For these experiments, we utilized CD4dnTGFβRII mice in which TGF-β1 signaling is abrogated in all CD4\(^+\) T cells. WT littermates served as controls. CD4dnTGFβRII mice fed 30 mg of OVA prior to oral challenge show no significant decrease in OVA-specific plasma IgG and IgA Ab responses when compared with those of mice fed PBS (Fig. 4A, B). Thus, the levels of anti-OVA IgG and IgA Abs in plasma were essentially the same as those Ab responses seen in WT mice fed PBS (Fig. 4A and 4B). In contrast, plasma of WT mice fed OVA contained significantly reduced levels of OVA-specific IgG and IgA Ab responses. These results were further confirmed at the cellular level. Thus, numbers of anti-OVA IgG and IgA AFCs in spleen of CD4dnTGFβRII mice were not significantly different from PBS-fed CD4dnTβRII (Fig. 5A and 5B).

Since this oral OVA plus CT challenge system is designed for examining unresponsiveness in mucosal effector tissues, we next determined OVA-specific S-IgA Ab responses in the GI tract of mice fed OVA. CD4dnTGFβRII mice fed 30 mg of OVA prior to oral challenge with OVA plus CT showed essentially no reduction in OVA-specific S-IgA Ab responses in fecal extracts (Fig. 4C). Further, numbers of anti-OVA IgA AFCs in MLNs of CD4dnTGFβRII mice fed OVA were comparable to those of PBS-fed CD4dnTGFβRII mice (Fig. 5C). On the other hand, WT mice fed OVA showed
significant reductions in OVA-specific S-IgA Ab responses in fecal extracts (Fig. 4C) and MLNs when compared with those of PBS-fed WT mice (Fig. 5C). These data clearly indicate that no down-regulation of OVA-specific mucosal and systemic Ab responses were induced in mice without functional TGFβRII expression by CD4⁺ T cells.

*DTH responses are not reduced in OVA-fed CD4dnTGFβRII mice*

In order to further confirm lack of oral tolerance induction in CD4dnTGFβRII mice at the T cell level, we assessed OVA-specific DTH responses in PBS- and OVA-fed CD4dnTGFβRII and WT mice. CD4dnTβRII mice fed 30 mg OVA showed robust DTH responses 24 h after the challenge. This response was similar to DTH responses seen in both PBS-fed CD4dnTGFβRII and WT mice and was significantly greater than the response of 30 mg OVA-fed C57BL/6 mice (Fig. 6). Combined with our Ag-specific Ab data, these results further underline the importance of intact TGF-β1 signaling in CD4⁺ T cells for mucosal and systemic unresponsiveness.

**Discussion**

In this study we showed that within 24 h of inducing oral tolerance, TGFβRII expression by CD4⁺ T cells in the PPs is significantly up-regulated. This up-regulation was independent of fed Ag dosage. This receptor is part of the oligomeric receptor complex that recognizes TGF-β1 and it has been shown that this molecule is required for TGF-β1 signaling [30]. Many studies have shown that TGF-β1 plays a central role in the development of acquired-type Ag-specific Tregs, through the induction and maintenance of Foxp3 expression in CD25⁻ and CD25⁺ T cells, respectively [25,26,27,46]. Therefore, the up-regulation of TGFβRII by CD4⁺ T cells indicates an increased ability to respond to
TGF-β1 and could therefore predisposition these cells to differentiate into Ag-specific Tregs [24].

After administering a high-dose oral tolerance induction protocol (i.e. 30 mg OVA), we found that CD4⁺ T cells in the PPs maintained a high frequency of TGFβRII expression. CD4⁺ T cells from other lymphoid tissues either did not increase TGFβRII expression, or did not maintain an elevated expression of the receptor—as in the spleen or MLNs, respectively. TGFβRII analysis during a low-dose oral tolerance induction protocol (ad lib 1 mg/ml OVA in water) confirmed that TGFβRII is up-regulated by PP CD4⁺ T cells regardless of the magnitude of the dose of OVA. Further, MLN and splenic CD4⁺ T cells failed to up-regulate TGFβRII expression during the low-dose protocol. These novel findings indicate that PPs are a uniquely populated with CD4⁺ T cells that are primed to respond to TGF-β1 after a tolerogenic event.

When tolerized mice were orally challenged after seven days with OVA plus CT, expression of TGFβRII on CD4⁺ T cells rapidly decreased in the PPs and increased in the MLNs and iLP. These data indicate that after challenge, this cell population may mobilize and migrate into other mucosal inductive and effector sites. Interestingly, by 2 h after a single OVA plus CT challenge, Foxp3 expression in PP CD4⁺ TGFβRII⁺ T cells nearly doubles, but decreases in the other tissues by up to 50%. In contrast, CD4⁺ TGFβRII⁻ cells, Foxp3 decreases over this period of time (data not shown). This finding shows that the increased expression of TGFβRII leads to preferential up-regulation of Foxp3 in this cell population. Further, it indicates that the PPs are playing an important, non-redundant role in oral tolerance induction by being sites for TGF-β1-dependent, Ag-specific Treg differentiation.
We next examined the role of this receptor in oral tolerance by using a large dose of OVA in CD4dnTGFβRII mice. These mice express a signaling deficient TGFβRII molecule on all T cells and therefore these cells fail to respond to TGF-β1 [35,36]. The importance of TGF-β1 in the peripheral differentiation of naïve T cells into Treg cells has been described (34-36). Indeed, a recent study found that reduced expression of TGFβRII was correlated with impaired differentiation of peripheral (Ag-specific) Tregs (37). When compared to WT mice, CD4dnTβRII mice have an increased frequency of CD4⁺ TGFβRII⁺ T cells. This increase is due to the loss of function of the receptor. However, despite this increased expression, when young adult CD4dnTGFβRII mice were fed a 30 mg dose of OVA prior to oral challenge with OVA plus CT, oral tolerance could not be induced. Thus, these mice revealed high levels of Ag-specific IgG and IgA Ab responses in plasma and fecal extract which are comparable to PBS fed CD4dnTGFβRII mice challenged with OVA plus CT. Further, the OVA-specific DTH response was not reduced in CD4dnTGFβRII mice fed OVA. These novel findings indicate that both expression and proper function of TGFβRII are critical to oral tolerance induction. The lack of oral tolerance induction in CD4dnTGFβRII mice is most likely not due to the loss of Treg cell development but not the absence of TGF-β1 signaling in effector CD4⁺ T cells. To support this view, it has been shown that TGF-β1 producing cells were not increased in mice orally tolerized with OVA [44,47,48]. In order to further confirm this point, we are currently testing whether adoptive transfer of OVA-induced Treg cells induce systemic unresponsiveness in CD4dnTGFβRII mice.

The role of PPs in the induction of oral tolerance has proved to be a controversial subject. Our previous studies showed that PPs are indeed required for the development
oral tolerance to protein Ags. Thus, mice treated *in utero* with lymphotoxin (LT)βR-Ig, and were therefore PP-null, could not develop oral tolerance to OVA [37]. In response to this study, another group reported that PP-null mice could be induced to oral tolerance and, further, that MLNs were instead the key organ for oral tolerance induction [39,40]. Although the MLNs are the immunological portal for nearly all gut-derived Ags [1], it is more likely that the PPs and MLNs work together during a tolerance event, since recent evidences for the importance of PPs in oral tolerance have come to light. Thus, it was shown that Ag-specific Tregs can be cloned from the PPs of orally-tolerized mice [23], while others found that PPs are required for oral tolerance to experimental autoimmune encephalitis in mice by way of myelin basic protein feeding [49]. Further, our recent work indicates that by targeting the M-cells of the follicle-associated epithelium of PPs using Ag fused with the σ1 protein of reovirus, the dosage of Ag needed to induce oral tolerance is reduced by 1000 fold [38]. The present study further underlines the importance of PPs in the very early time points of oral tolerance induction by showing that these tissues are populated with T helper cells that are primed to respond to TGF-β1 and that these cells mobilize shortly after re-exposure to the tolerizing Ag.

Indeed, our study further adds to the growing evidence that the two traditional modes of oral tolerance are linked. Early studies on the mechanisms of oral tolerance have led to the development of a dogma that how oral tolerance is induced, mediated and maintained is determined by the size and frequency of Ag dose [1,2,3,19]. It is commonly held that large doses of protein Ag cause anergy and/or deletion of Ag-specific T cells, while small frequent doses or continual exposure to Ag induce the differentiation of regulatory T cells that mediate suppression by the Ag-specific production of suppressive
cytokines [19]. However, more recent studies have found that these are not mutually exclusive occurrences; that Ag-specific Tregs can be found after feeding large doses of Ag [13,23] and that anergy may also play a role in oral tolerance to small doses of Ag [13]. The increase in TGFβRII expression on CD4+ T cells in PPs could indicate that these cells are primed to differentiate into Treg cells [24,50]. Finally, our results may indicate an increase in the necessity for CD4+ T cells to respond to TGF-β1 after large doses of Ag, indicating a link between active suppression and this form of oral tolerance.

In conclusion, our current study shows that significant and persistent increases in TGFβRII expression on CD4+ T cells occur in the PPs immediately after tolerizing events, independent of Ag dose. When challenged these PP CD4+ TGFβRII+ cells up-regulate Foxp3 expression. Expression of the TGFβRII alone is insufficient, as is indicated by the lack of oral tolerance induction in mice lacking functional TGFβRII on CD4+ T cells even in the presence of increased expression of the receptor. These increases may indicate that the PPs are important sources of tolerogenic Tregs that begin differentiating in early time points after Ag feeding. Further study is needed to determine if these cells are indeed Tregs or perhaps precursors of Tregs, and to examine the mechanism of their development and function after challenge with Ag and mucosal adjuvant. To this end, experiments to determine the function of these PP-derived CD4+ TGFβRII+ T cells are currently being undertaken in our laboratory.
Acknowledgements

The authors wish to thank Dr. Jerry R. McGhee for his constructive comments and discussion. The authors also wish to thank Dr. Keiko Fujihashi and Dr. Yoshiko Fukuyama for their assistance with tissue and cell preparation.

References


**Figure 1. Effect of large-dose OVA feeding on the occurrence of CD4^+ TGFβRII^+ T cells.** Mononuclear cells were isolated from PPs, MLNs, and spleens of naïve C57BL/6 mice (0) and at 24 h time points after 30 mg of OVA feeding. Cells were then stained with FITC-conjugated anti-hTGFβRII, APC-labeled anti-CD4, and biotin-tagged anti-CD3 mAbs followed by PerCP-Cy^5.5-conjugated streptavidin. Samples were subjected to flow cytometry analysis by FACSCalibur®. A. Plots are representative of naïve tissues and tissues analyzed 7 days after OVA feeding. B. Time course of CD4^+ TGFβRII^+ T cells in PPs, MLNs, and spleen 1, 2, 3, 5 and 7 days after 30 mg of OVA feeding, n = 20 mice per time point, *p < 0.05 or **p < 0.001 when compared to sham-tolerized control group.
A

Naive

High Dose OVA + 7 Days

Peyer’s Patches

Mesenteric Lymph Nodes

Spleen

CD4

TGFβRII

B

% CD4+TGFβRII+ T Cells

Time (days)

- PP
- MLN
- SP

* **
Figure 2. Effect of small-dose OVA feeding on the occurrence of CD4⁺ TGFβRII⁺ T cells. Mononuclear cells were isolated from PPs, MLNs, and spleen of naïve C57BL/6 mice (0) and at time points during ad libitum OVA feeding (1 mg / ml). Cells were then stained with FITC-conjugated anti-hTGFβRII, APC-labeled anti-CD4, and biotin-tagged anti-CD3 mAbs followed by PerCP-Cy™5.5-conjugated streptavidin. Samples were subjected to flow cytometry analysis by FACSCalibur®. A. Plots are representative of naïve tissues and tissues analyzed 7 days after beginning OVA feeding. B. Time course of CD4⁺ TGFβRII⁺ T cells frequency in PPs, MLNs, and spleen 3, 5 and 7 days after 30 mg of OVA feeding, n = 10 mice per time point, **p < 0.01 when compared to sham-tolerized control group.
A

Naive

Low Dose OVA
+ 7 Days

Peyer’s Patches

Mesenteric Lymph Nodes

Spleen

B

% CD4+ TGFβRII+ T Cells

Time (days)

PP

MLN

SP

**
Figure 3. Frequency of CD4$^+$ TGFβRII$^+$ T cells in tissues of tolerized C57BL/6 mice after single challenge with OVA plus CT. A. Mononuclear cells were isolated from PP, MLNs, iLP and spleen of C57BL/6 mice 7 days after 30 mg of OVA feeding (0) and at 0.5, 2 and 4 h after single challenge with 1 mg of OVA plus 10 µg of CT (OVA plus CT). Cells were then stained with FITC-conjugated anti-hTGFβRII, APC-labeled anti-CD4, and biotin-tagged anti-CD3 mAbs followed by PerCP-Cy5.5-conjugated streptavidin. B. Cells were isolated as described above and stained with FITC-conjugated anti-hTGFβRII, APC-labeled anti-CD4, PE-labeled anti-hFoxp3, and biotin-tagged anti-CD3 mAbs followed by PerCP-Cy5.5-conjugated streptavidin. Samples were subjected to flow cytometry analysis by FACSCalibur$^\text{®}$. n = 5 mice per time point, *p < 0.05 compared to sham-tolerized and OVA plus CT challenged control.
Figure 4. OVA-specific Ab responses in CD4dnTGFβRII mice after large-dose oral tolerance and oral challenge with OVA plus CT. A-C. One week after the last challenge, plasma and fecal extracts were subjected to OVA-specific ELISA to determine IgG, IgA (plasma; A, B) or S-IgA (fecal extract; C). Endpoint titers are expressed as the last dilution yielding OD415 of > 0.1 units above background control values. For CD4dnTGFβRII experiments: n = 8 OVA-fed, n = 5 PBS controls. For WT experiments: n = 19 OVA-fed, n = 8 PBS controls. **p < 0.001.
Figure 5. OVA-specific Ab Forming Cells (AFCs) in CD4dnTGFβRII mice after large-dose oral tolerance and oral challenge with OVA plus CT. A-C. Mononuclear cells were isolated from MLNs and spleen one week after the final challenge. Cells were then subjected to an ELISPOT assay to detect numbers of OVA-specific AFCs. For CD4dnTGFβRII experiments: n = 8 OVA-fed, n = 5 PBS controls. For WT experiments: n = 19 OVA-fed, n = 8 PBS controls. **p < 0.001.
Figure 6. DTH responses in CD4dnTGFβRII mice after large-dose oral tolerance and oral challenge with OVA plus CT. Six days after final immunization, the ear pinnae of mice were injected with 20 µg OVA or PBS. Ear swelling was measured 24 h later. DTH response is expressed the difference in thickness between left and right ears. For CD4dnTGFβRII experiments: n = 6 OVA-fed, n = 6 PBS controls. For WT experiments: n = 19 OVA-fed, n = 4 PBS controls. **p < 0.001.
Supplemental Information

**Figure S1. Positive controls for FITC-hTGFβRII mAb.** Mononuclear cells were isolated from MLN and PP of naive CD4dnTGFβRII mice. Cells were then stained with FITC-conjugated anti-hTGFβRII, APC-labeled anti-CD4, and biotin-tagged anti-CD3 mAbs followed by PerCP-Cy5.5-conjugated streptavidin. Analysis is gated on CD4+ T cells. Representative FACS plots are shown.
SUPPLEMENTAL RESULTS
THE EXPRESSION OF MIGRATORY MARKERS BY CD4⁺TGFBRII⁺ T CELLS
AFTER A SINGLE ORAL CHALLENGE

REBEKAH S. GILBERT

Unpublished Results
**Background**

Our previous findings showed that CD4\(^+\) TGF\(\beta\)RII\(^+\) T cells in PP of tolerized mice decreased after a single oral challenge with OVA plus CT. At the same time, this cell population increased in the MLN and iLP (See “Functional TGF-\(\beta\) Receptor Expression by CD4\(^+\) T Cells in Peyer’s Patches is Essential for Oral Tolerance Induction”). We hypothesized that this cell population may migrate after it is exposed to the tolerizing antigen in the presence of adjuvant.

To investigate whether migration of CD4\(^+\) TGF\(\beta\)RII\(^+\) cells is occurring, we examined changes in the expression of the gut imprinting markers CCR9 and \(\alpha_4\beta_7\) by this population through FACS analysis. CCR9 is the receptor for CCL25, which is a chemokine expressed in the small intestine and thymus. CCR9 is expressed on various lymphoid cells including thymic T cells, intraepithelial lymphocytes and IgA-secreting plasma cells. CCR9 is also highly expressed by T cells that are in the process of homing to the iLP [1]. The integrin \(\alpha_4\beta_7\) is often co-expressed with CCR9 by this latter population of cells.

**Materials and Methods**

**Mice**

Six- to 8-wk-old female C57BL/6 mice were purchased from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). Upon arrival, mice were housed in micro isolators, maintained in horizontal laminar flow cabinets, and provided sterile food and water as part of a specific-pathogen free facility at the UAB. All of the mice used in these experiments were free of bacterial and viral pathogens. All of the
animal studies were performed in accordance with both NIH and UAB institutional
guidelines.

*Induction of oral tolerance and oral challenge procedure*

To establish oral tolerance, mice were i.g. with 30 mg of OVA (Sigma-Aldrich)
dissolved in 0.25 ml of PBS. Seven days after tolerization mice were challenged i.g. with
1 mg of OVA plus 10 µg of native CT (List Biological Laboratories, Inc.) as mucosal ad-
juvant in 0.25 ml sterile PBS (OVA + CT). Control mice received PBS only.

*Flow Cytometric Analysis*

Mononuclear cells from mucosal and systemic tissues were isolated at various
time points and preincubated with purified CD16/32 mAb (Fc Block, 2 µg / ml; BD
Pharmingen). Samples were then stained with a combination of FITC-labeled anti-human
TGFβRII mAb (R&D Systems), APC-conjugated anti-CCR9 or -CD4, PE-tagged anti-
CD4, or –LPAM-1 (α4β7), and biotin-conjugated anti-CD3 mAbs (BD Pharmingen) fol-
lowed by PerCP-Cy™5.5-conjugated streptavidin (BD Pharmingen).

*Statistical Analysis*

The results are expressed as the mean ± SEM. Non-parametric data were analyzed
using an unpaired Mann-Whitney *U* test using InStat software for Macintosh by Graph-
Pad. Parametric data were analyzed using two-way ANOVA using Prism software for
Windows by GraphPad. *p* values < 0.05 were considered significant, and < 0.001 were
considered highly significant.
Results

Changes in CCR9 expression by CD4+ TGFβRII+ T cells after single challenge

One week after feeding 30 mg of OVA, C57BL/6 mice were challenged with OVA plus CT. Control groups of mice were similarly tolerized, but challenged with PBS only. Expression of CCR9 and α4β7 by CD4+ TGFβRII+ T cells in spleen, MLNs, PPs and iLP was determined immediately (T0), 2 h and 8 h after challenge. After 2 h, there was a slight increase in CCR9 expression by CD4+ TGFβRII+ T cells in the spleen, PPs, and MLNs, but this change did not vary significantly from the PBS-challenged controls. CCR9 expression by CD4+ TGFβRII+ T cells in the iLP did not change in the first two hours (Fig. 1). However, at 8 hours after challenge with OVA plus CT, a highly significant decrease in CD4+ TGFβRII+ CCR9+ T cells in iLP was observed (Fig. 1). The frequency of CD4+ TGFβRII+ CCR9+ T cells in the spleen and MLNs continued to increase between 2 and 8 hours after challenge, but still did not reach significance.

Changes in α4β7 expression by CD4+ TGFβRII+ T cells after single challenge

The expression of α4β7 by CD4+ TGFβRII+ T cells in spleen, MLN, PP and iLP of tolerized mice given a single oral challenge was also determined at T0, 2 h and 8 h after challenge. A slight increase in the expression of this migratory marker was observed in the MLNs and iLP 2 and 8 hours after challenge, but this change was not significant (Fig. 2). There was no change in the frequency of CD4+ TGFβRII+ α4β7+ T cells in the spleen or PPs over the time course.
Discussion

Our previous findings indicated the possibility that CD4$^+$ TGFβRII$^+$ T cells may migrate from the PPs into the MLN and iLP after the tolerized animal was challenged with the tolerizing antigen plus mucosal adjuvant (i.e. OVA plus CT). If this were to be true, it could be hypothesized that these cells perhaps had a regulatory phenotype. Indeed one of our secondary hypotheses for this dissertation was that antigen priming creates a Treg “precursor” and thus subsequent challenge with the antigen initiates characteristic Treg functions by these cells (e.g. regulatory cytokine production). Indeed, one of our promising findings was that a large proportion of PP CD4$^+$ TGFβRII$^+$ T cells from tolerized mice up-regulated Foxp3 after a single oral challenge with OVA plus CT, indicating that this could be a possibility. However, these supplementary experiments show that CD4$^+$ TGFβRII$^+$ T cells in PPs are not up-regulating gut-homing markers. In fact, there was no significant change in the expression of these markers by PP CD4$^+$ TGFβRII$^+$ T cells. Due to this negative result, we must perform further experiments aimed at determining the function of these CD4$^+$ TGFβRII$^+$ T cells.

Reference

Figure 1. CCR9 expression by CD4\(^+\) TGF\(\beta\)RII\(^+\) T cells after a single oral challenge. Mononuclear cells were isolated from PPs, MLNs, iLP and spleen of C57BL/6 mice 7 days after 30 mg of OVA feeding (0) and at 2 and 8 h after single challenge with 1 mg of OVA plus 10 \(\mu\)g of CT (OVA plus CT). Cells were then stained with FITC-conjugated anti-hTGF\(\beta\)RII, PE-labeled anti-CD4, APC-labeled anti-CCR9, and biotin-tagged anti-CD3 mAbs followed by PerCP-Cy\(\text{TM}5.5\)-conjugated streptavidin. Values are expressed as the mean ± SEM of 12 mice. * \(p < 0.01\).
Figure 2. $\alpha_4\beta_7$ expression by CD4$^+$ TGF$\beta$RII$^+$ T cells after a single oral challenge. Mononuclear cells were isolated from PPs, MLNs, iLP and spleen of C57BL/6 mice 7 days after 30 mg of OVA feeding (0) and at 2 and 8 h after single challenge with 1 mg of OVA plus 10 $\mu$g of CT (OVA plus CT). Cells were then stained with FITC-conjugated anti-hTGF$\beta$RII, PE-labeled anti-LPAM-1 ($\alpha_4\beta_7$), APC-labeled anti-CD4, and biotin-tagged anti-CD3 mAbs followed by PerCP-Cy$^{TM}$5.5-conjugated streptavidin. Values are expressed as the mean ± SEM of 12 mice.
SUMMARY

In this dissertation research, three major findings were achieved: 1) CD4dnTGFβRII mice were found to be acceptable tools for use in the examination of TGF-β receptor function in oral tolerance induction, 2) the expression and function of TGFβRII by CD4+ T cells was found to be necessary for oral tolerance induction, and 3) Peyer’s patches were further shown to be essential players in the induction of oral tolerance via the presence of increased numbers of TGFβRII+ Th cells. Further, the migratory properties of CD4+ TGFβRII+ cells were examined, but the analysis revealed that further studies are necessary to fully understand the specific function of these cells in oral tolerance.

Analysis of the naïve mucosal phenotype of young adult and aging CD4dnTGFβRII mice revealed that, as they age, mucosal homeostasis breaks down. Evidence of this breakdown can be seen in the overproduction of effector cytokines by T cells and Abs by B cells in the PPs, MLN and iLP. However, young adult CD4dnTGFβRII mice displayed a relatively normal naïve mucosal phenotype, when compared to WT mice. Further, these young adult mice also responded normally to an oral immunization regime employing CT and designed to induce a robust immune response to OVA. Thus, the OVA-specific Ab responses in plasma and FE of young adult CD4dnTGFβRII mice were not significantly different from WT mice. Interestingly, a proof-of-concept experiment gave the intriguing result that a large oral dose of OVA was
not sufficient to induce tolerance to that antigen. With these results in hand, it was possible to utilize these CD4dnTGFβRII mice with confidence.

The main focus of this dissertation was in determining the roles TGF-β receptors play in oral tolerance induction. Through the analysis of TGFβRII at time points throughout oral tolerance induction (within one week of large dose feeding or of beginning a continuous feeding regimen) a pattern emerged in which TGFβRII expression by CD4+ T cells in the PPs significantly increased. This increase in CD4+ TGFβRII+ T cell frequency persisted until animals were challenged mucosally with OVA plus CT; after which, it decreased in PPs and increased in iLP and MLN before returning to basal levels in all tissues. Further, when young CD4dnTGFβRII mice were fed a large dose of OVA and were subsequently challenged with oral OVA plus CT, it was found that their OVA-specific immune response was intact, indicating a lack of oral tolerance induction. This study showed that both expression and function of TGFβRII are needed for the induction of oral tolerance. Further, PPs play a key role in oral tolerance induction by being the site of highest frequency of TGFβRII+ CD4+ T cells.

The reduction in CD4+ TGFβRII+ T cell frequency in PPs of tolerized mice after oral challenge with OVA plus CT suggested that this cell population might migrate after re-exposure to its cognate Ag. This could indicate that these cells are leaving the inductive tissue of the PPs in order to perform functions relative to oral tolerance, such as suppression. To explore this possibility, the expression of gut-homing- and Treg-associated molecules (α4β7/CCR9 and Foxp3, respectively) by these cells was examined. There was no significant change in the expression of α4β7 and CCR9 by PPs CD4+ TGFβRII+ T
cells. However, these cells did show an increase in Foxp3, indicating that these cells could have a regulatory function.

Further experiments are necessary to more fully understand what role these CD4\(^+\) TGFβRII\(^+\) T cells are playing in the induction and maintenance of oral tolerance. These experiments should include \textit{in vitro} analysis of the regulatory potential of CD4\(^+\) TGFβRII\(^+\) T cell through analysis of cytokine production and Ag-specific T cell suppression assays. Also, the ability of CD4\(^+\) TGFβRII\(^+\) T cells to transfer tolerance from orally-tolerized WT mice to CD4dnTGFβRII mice should be determined. Success of these studies would show that CD4\(^+\) TGFβRII\(^+\) T cells have a regulatory function and would further define their role in oral tolerance.
GENERAL LIST OF REFERENCES


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APPENDIX

IACUC APPROVAL FORM
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

Notice of Approval for Protocol Modification

DATE: November 3, 2010

TO: Kohtaro Fujihashi, D.D.S., Ph.D.
BBRB-761 2170
FAX: 975-4431

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Adenovirus-FLT3 Ligand Induces Nalt DCs for Salivary Gland S-IgA Ab Responses
Sponsor: NIH
Animal Project Number: 100908212

On November 3, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the modification as described. Additional Personnel to protocol: Daisuke Tokuhara
The sponsor for this project may require notification of modification(s) approved by the IACUC but not included in the original grant proposal/experimental plan; please inform the sponsor if necessary. The following species and numbers of animals reflect this modification.

<table>
<thead>
<tr>
<th>Species</th>
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Animal use is scheduled for review one year from September 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files.

Refer to Animal Protocol Number (APN) 100908212 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

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