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# EGF module-containing mucin-like hormone receptor 2 and its role in human immune privilege

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**EGF MODULE-CONTAINING MUCIN-LIKE HORMONE RECEPTOR 2 AND ITS  
ROLE IN HUMAN IMMUNE PRIVILEGE**

by

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B.S., University of California – Santa Barbara, 2010

Submitted in partial fulfillment of the  
requirements for the degree of  
Master of Science

2014

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## **ACKNOWLEDGMENTS**

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**HELEN SONG**

ABSTRACT

**Purpose:** In the mouse, the macrophage adhesion G protein-coupled receptor (ad-GPCR) molecule, F4/80, is required for the development of regulatory T cells in two models of tolerance, the eye and gut. Since F4/80 is not expressed in humans, the purpose of this research is to determine the human analog of F4/80. F4/80 belongs to a novel family of Epidermal growth factor-seven transmembrane (EGF-TM7) molecules, which include the EGF module-containing mucin-like hormone receptor (EMR) molecules. In the human, EMR1 has sequential homology with F4/80 and EMR2 has shown immune suppressing function in tumor cells. Thus, we investigate the possible suppressor role of the EMR family in human ocular tolerance.

**Methods:** Human peripheral blood mononuclear cells (huPBMC) were treated with porcine TGF $\beta$ 2 and LPS or an antigenic stimulant for at least six hours to generate tolerogenic antigen presenting cells (APC). Cells were characterized by flow cytometric analysis for expression of CD14, CD40, PDL1, ILT3, and EMR2. Later, T regulatory cells were generated by incubating tolerogenic APCs with autologous huPBMC for five to seven days. Post culture, the T cells were stained and characterized for expression of CD4, CD25, and FoxP3.

**Results:** Post treatment of huPBMC with TGF $\beta$ 2 and antigen, the resulting tolerogenic APCs expressed PDL1, ILT3, and EMR2. CD40 remained unchanged and CD14 was constitutively expressed. Post five to seven day culture, tolerogenic APCs treated with TGF $\beta$ 2 increased the CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> lymphocyte populations.

**Conclusions:** The upregulation of EMR2 on human tolerogenic APCs suggests that EMR2 may have a role in inducing tolerance in humans. Much like its mouse counterpart, F4/80, EMR2 is an adhesion molecule that may facilitate the induction of naïve T lymphocytes to regulatory T lymphocytes. Once the F4/80 analog is established for humans, novel therapies may be developed to interfere or encourage signaling in the treatment of tumors or immune inflammatory diseases, respectively.

## TABLE OF CONTENTS

TITLE.....	i
COPYRIGHT PAGE.....	ii
READER APPROVAL PAGE.....	iii
ACKNOWLEDGMENTS .....	iv
ABSTRACT .....	v
TABLE OF CONTENTS .....	vii
LIST OF FIGURES .....	ix
LIST OF ABBREVIATIONS .....	x
INTRODUCTION .....	1
Anterior Chamber Associated Immune Deviation.....	3
EGF Module-Containing Mucin-Like Receptor 2 (EMR2).....	6
Specific Aims .....	10
METHODS .....	12
Collection of Human Blood Samples .....	12
Cell Isolation .....	12
Generation of Tolerogenic APC.....	13
Induction of T Regulatory Cells.....	14
Blocking EMR2 Ligand Binding .....	14



Humanized NOD SCID Mouse Model .....	15
Data Analyses.....	15
RESULTS .....	17
Surface Markers on Human Tolerogenic APC.....	17
Induced T Regulatory Cells .....	23
Blocking EMR2 Ligand Binding .....	30
In Vivo Model .....	34
DISCUSSION .....	36
Human Tolerogenic APC .....	36
Functions of Human Tolerogenic APCs.....	38
EMR2 and its Ligand, Chondroitin Sulfate Glycosaminoglycans (GAGs).....	39
REFERENCES .....	42
CURRICULUM VITAE.....	48

## LIST OF FIGURES

Figure	Title	Page
1	Synergistic processes of the ocular immune system	4
2	Molecular structures of three largest EGF-TM7 molecules	7
3	Phenotypic profile of tolerogenic APC.	17-18
4	Flow Cytometric APC Marker Profiles	20
5	Function of induced T regulatory cells	24-25
6	Flow cytometric dot plots of intracellular protein production	27
7	FoxP3 expression of T cells treated with soluble chondroitin sulfate	30
8	Flow cytometric EMR2 readout and dot plot post-soluble chondroitin sulfate treatment	31
9	DTH response in humanized SCID mice	34

## LIST OF ABBREVIATIONS

ad-GPCR.....	Adhesion G protein-coupled receptor
APC .....	Antigen presenting cell
APC-A.....	Allophycocyanin
CD.....	Cluster of Differentiation
DTH.....	Delayed type hypersensitivity
EGF-TM7.....	Epidermal growth factor-seven transmembrane
EMR.....	EGF module-containing mucin-like hormone receptor
FACS.....	Fluorescence-activated cell sorting
FBS.....	Fetal bovine serum
GAG.....	Glycosaminoglycan
GPCR.....	G protein-coupled receptor
huPBMC .....	Human peripheral blood mononuclear cell
IL.....	Interleukin
ILT3.....	Immunoglobulin-like transcript 3
IFN- $\gamma$ .....	Interferon gamma
ITS.....	Insulin/Transferrin/Selenium
LPS.....	Lipopolysaccharide
MHC.....	Major histocompatibility complex
NKT cells.....	Natural killer T cell
OVA.....	Chicken ovalbumin
PBMC.....	Peripheral blood mononuclear cell

PBS.....Phosphate buffered saline  
PD-L1.....Programmed cell death ligand 1  
PE-A.....Phycoerythrin  
siRNA.....Small interfering RNA  
TGF $\beta$  .....Transforming growth factor beta  
TT.....Tetanus toxoid

## INTRODUCTION

As the essential organ providing vision to living animals, eyes are critical to observing and analyzing the world around us. In the human, the eye is composed of three translucent layers: the fibrous externa, the inner nervous retina, and the middle vascular tunic. Within this middle layer lies the anterior chamber, filled with aqueous humor. The anterior chamber primarily regulates intraorbital pressure; however, the aqueous humor also contributes to the eye's unique ability to manipulate immunologic responses. Considering the immediate juxtaposition of the ocular surface to the outside world, the absence of recurrent immune reactions is paradoxical. Yet, the eye displays an important trait called ocular immune privilege, which allows it to coexist with the external environment without immune collateral damage (Abbas & Lichtman, n.d.). Many of the eye's cells are permanent resident cells that rarely undergo regeneration. Thus, inflammation and its products could be irreversibly damaging and counteractive for the survival of the eye (Stein-Streilein & Lucas, 2011).

The lack of well-defined lymphatic drainage from the eye and its blood-ocular barrier render it virtually isolated from the rest of the body in terms of immunologic surveillance. Immune privilege is very resilient and only transiently abrogated by infection, inflammation, or exogenous LPS. However, when the eye was subject to an injurious event, such as a retinal laser burn injury, immune privilege was lost and inflammation occurred (Lucas, Karamichos, Mathew,

Zieske, & Stein-Streilein, 2012). The autoantigens released have never been exposed to the body's T lymphocytes, thus they are viewed as foreign and subsequent immune responses can mount against the eye causing conditions like uveitis, sympathetic ophthalmia, and in some cases progress to blindness (Shao et al., 2007; Stein-Streilein & Lucas, 2011). On the contrary, introduction of a foreign antigen or alloantigen into the eye induces the ocular immune privilege response and an active suppression of inflammatory processes is observed (Forrester & Xu, 2012).

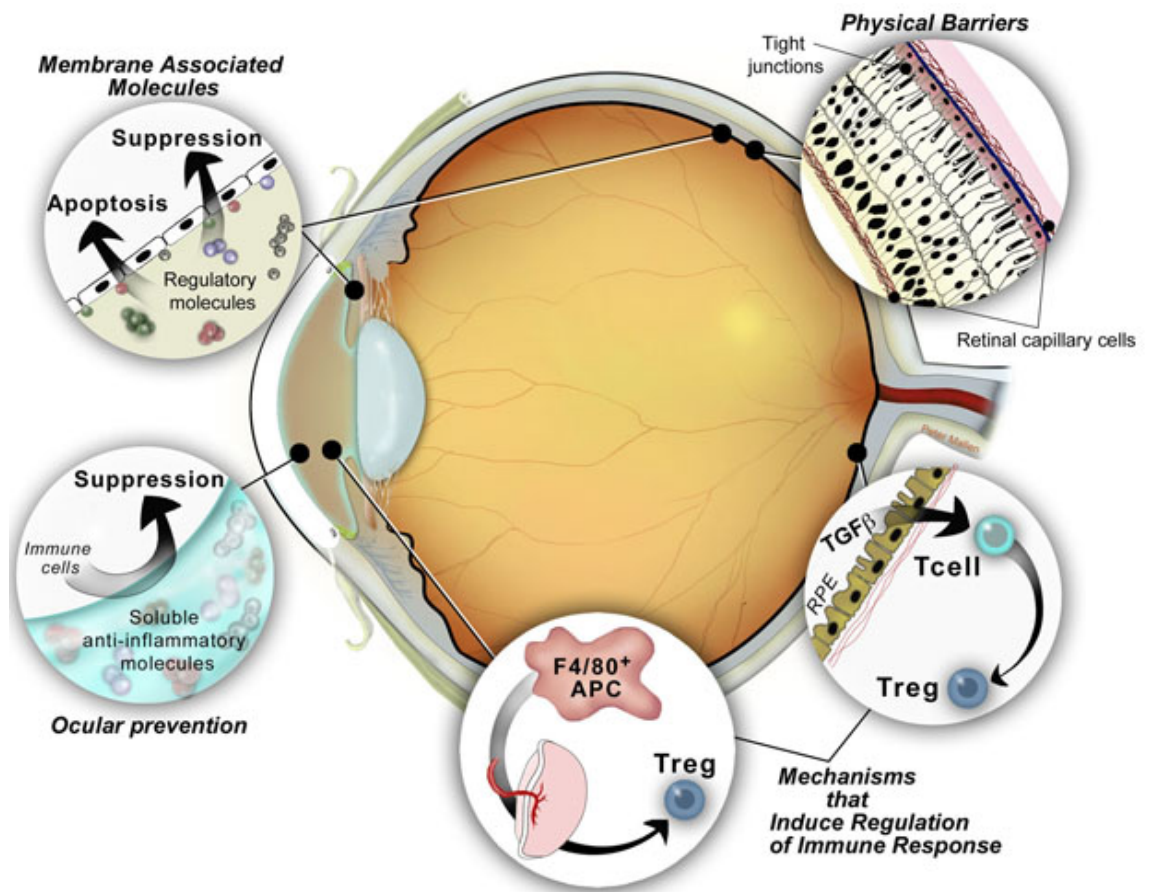
So how, then, does the eye evade excessive inflammation and display immune privilege? Immune privilege in the eye presents both conserved and unique mechanisms of tolerance. Soluble Fas ligand within the eye plays a role in limiting inflammatory activity by binding to Fas receptors on infiltrating immune cells and activating apoptosis (Gregory et al., 2011). Also, the blood-ocular barrier helps to physically reduce the number of incoming immunogenic cells. Inherently, the anterior chamber houses immunosuppressive factors that bathe ocular immune cells. These factors include alpha melanocyte stimulating hormone, neuropeptide Y, pigment epithelial growth factor, and many others that actively keep immune cells in check (Taylor, 2007). Of interest, transforming growth factor beta (TGF $\beta$ ), a cytokine known for promoting suppression of immune reactions, primes antigen presenting cells (APC) to change their function when come into contact with foreign antigen (D'Orazio & Niederkorn, 1998; Takeuchi et al., 2006).

### ***Anterior Chamber Associated Immune Deviation***

The Streilein group published extensive results that show if a foreign antigen is placed into the anterior chamber of a mouse eye, systemic tolerance to that antigen occurs. To study this process, a model called anterior chamber associated immune deviation (ACAID) was developed. This model showed that antigen in the presence of anterior chamber soluble factors triggers ocular APC to induce the regulatory phenotype in peripheral T lymphocytes (Streilein, Masli, Takeuchi, & Kezuka, 2002). These T regulatory cells then actively suppress immune reactions against the presented antigen. Critical to this process, a molecule known as F4/80 is upregulated on mouse APCs in response to TGF $\beta$  and antigen treatment (Lin et al., 2005; Willbanks et al., 1997). It appears that once the antigen is placed into the eye, it is picked up by F4/80+ cells that then leave the eye and travel to the marginal zone of the spleen where they cluster and interact with resting T lymphocytes, marginal zone B cells, and NKT cells (Faunce, Sonoda, & Stein-Streilein, 2001; Sonoda et al., 2001; Streilein & Niederkorn, 2007). In the absence of the F4/80 adhesion molecule, peripheral tolerance is not induced via the eye, nor are T regulatory cells induced in vitro. This correlates with the above outlined ACAID model (Lin et al., 2005). Inducible, now regulatory, T cells begin to express FoxP3 and cluster of differentiation (CD)25 (T regulatory cell markers) in addition to constitutively expressing CD4 or CD8, T helper or Cytotoxic T markers respectively (Ji, Yin, & Yang, 2011). With

this phenotype, the inflammatory processes are downregulated in an antigen-specific fashion (Schmitt & Williams, 2013).

ACAID is a cooperation of the blood-ocular tight junction barrier, soluble factors of the anterior chamber, the tolerogenic F4/80<sup>+</sup> APC, the spleen, and peripheral induced T regulatory cells, outlined by Figure 1.



**Figure 1. Synergistic processes of the ocular immune system.** The ocular immune system includes multi-faceted defense mechanisms. Physical barriers,



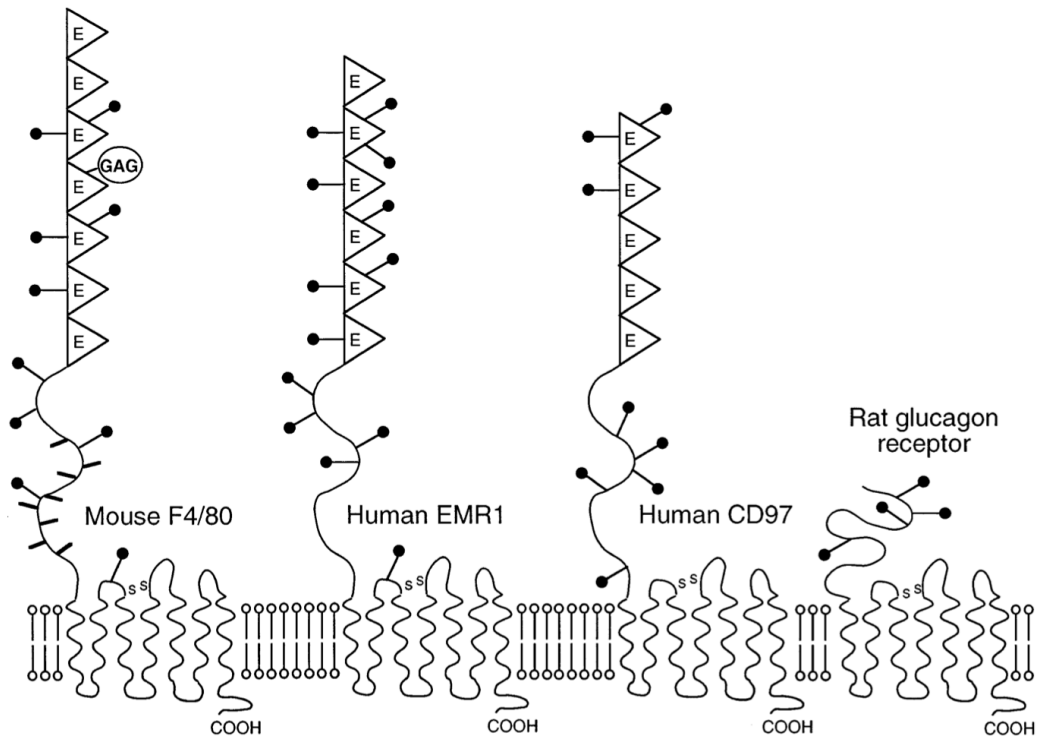
anti-inflammatory mediators, suppressive cell and humoral immunologic factors are among many contributors to ocular immune privilege (used with permission, Stein-Streilein, 2006).

As an experimental model outlined by Kaplan, JW Streilein, and Niederkorn (1977, 1978; 1983; 2007), ACAID is induced in mice by injecting a common antigen such as OVA (chicken ovalbumin) directly into the anterior chamber of the eye. Seven days post anterior chamber inoculation, the mouse was deliberately immunized with the same antigen and CFA. An additional seven days later, the mouse was challenged with antigen into the ear pinnae. The following day, the swelling of the ear was measured. This swelling response is known as a delayed type hypersensitivity response (DTH), a cell-mediated reaction in which CD4<sup>+</sup> T helper cells recognize major histocompatibility complex (MHC) class II - antigen complexes on APC. The CD4<sup>+</sup> cells proliferate, releasing interleukin (IL)-2 and interferon-gamma (IFN- $\gamma$ ) to potentiate inflammatory responses. In the mice with functional F4/80, the ear swelling is minimal since ACAID has been evoked and the delayed hypersensitivity response is controlled. However in the same experimental conditions without functional F4/80, ear swelling is significant as T regulatory cells were not induced by ACAID and immune responses prevail (Lin et al., 2005).

### ***EGF Module-Containing Mucin-Like Receptor 2 (EMR2)***

While extensive work on characterizing the ocular immune microenvironment has been done in the mouse, little progress has been made translating the molecular mechanisms to the human. Largely in part, F4/80 is a specific mouse macrophage marker not present in humans. Understanding the molecules that facilitate the induction of T regulatory cells is crucial in defining tolerance in the human.

F4/80 is an adhesion G protein-coupled receptor (ad-GPCR) belonging to a subfamily called epidermal growth factor-seven transmembrane receptors (EGF-TM7). This family also includes a molecule called EGF-like module containing mucin-like hormone receptor (EMR). There are four known types of EMR, molecules 1-4. EMR 1, 2, and 3, as well as another EGF-TM7 molecule, CD97, map to the same region of the human chromosome 19p13 (Lin, Stacey, Hamann, Gordon, & McKnight, 2000). F4/80 maps to a synonymous region in the mouse genome.



**Figure 2. Molecular structures of three largest EGF-TM7 molecules.** The basic skeleton of EGF-TM7 molecules is conserved throughout the subfamily. Triangles with “E” represent the EGF-like domains, darkened circles denote *N*-glycosylation sites, bars with no darkened circles represent known *O*-linked sugars. The absence of *O*-linked sugars on EMR1 and CD97 indicates these structures have not yet been studied. (Figure adapted from McKnight & Gordon, 1998)

As diagrammed by Figure 2, EMR1 is the structural homolog of F4/80 in the human (Hamann et al., 2007). With similar numbers of EGF-like domains, EMR, like F4/80, facilitates cell-cell adhesion. While the molecular structure has been determined, not much is known concerning the function of EMR1. While EMR1

may seem like the obvious F4/80 counterpart in the human, Hamann et al. (2007) have found EMR1 is a molecule specific for human eosinophils, co-expressed with CCR3 and immunoglobulin-like lectin Siglec-8. Thus, other EMR molecules were explored as a possibility of an F4/80 analog.

EMR3 is an EGF-TM7 molecule with only two EGF-like domains, as opposed to F4/80's seven domains and EMR1's six domains (Stacey, Lin, Hilyard, Gordon, & McKnight, 2001). While highly expressed on human myeloid cells, considerably neutrophils, monocytes, and macrophages, they only facilitate myeloid-myeloid adhesion interactions (Stacey et al., 2001). Important in other immune responses, EMR3 is unlikely to be a molecule required for the induction of ocular immune privilege due to its lack of myeloid-lymphoid binding ability.

Little is known about human EMR4. There has been work detailing two alternatively spliced human EMR4 transcripts, which are strikingly similar to the mouse in regards to the intron positions (Caminschi et al., 2006). Although the genetic sequences of EMR4 contain highly conserved regions similar to other EGF-TM7 family members, Caminschi et al. propose that expression of the TM7 domain proteins is likely lost. While genetic analysis has been done, translation of EMR4 into a stable protein has not yet been detected ("EMR4P egf-like module containing, mucin-like, hormone receptor-like 4 pseudogene [Homo sapiens (human)] - Gene - NCBI," n.d.). Thus, the function of EMR4 is still unknown.

Lastly, EMR2 is the final EMR molecule of the EGF-TM7 subfamily. More extensively studied, it has been shown that EMR2 and its family member CD97 are highly conserved in terms of their molecular structure (Lin et al., 2000; McKnight & Gordon, 1998). CD97, found constitutively on most immune cells of hematopoietic origin, is upregulated following immunogenic activation of lymphocytes (Abbott et al., 2007). Lin et al. (Lin et al., 2000) have highlighted the identical gene organization of EMR2 and CD97 to conclude that the two genes likely resulted from duplication. However, the two proteins do not share major cellular binding ligands. While CD97 is well known to interact with CD55 to regulate the complement cascade, EMR2 has a binding affinity to CD55 many-fold lower than CD97 and thus postulated to have a different function and signaling pathway from CD97 (Lin et al., 2000). More recently, EMR2 has been found to be expressed in human breast tumor cells (Davies et al., 2011). The prevalence of EMR2 in the survival of neoplastic epithelial cells indicates a role for evading or down-regulating immunogenic responses. With these factors combined and a known ligand to block EMR2 signaling (Stacey et al., 2003), EMR2 was chosen as the molecular target for establishing an F4/80 analog in the human.

Due to its highly conserved nature and purported immunosuppressive properties, we hypothesized that EMR2 functions as the human analog of F4/80. This suggests that the molecule will be upregulated on tolerogenic APCs and be critical in the induction of T regulatory cells.

### **Specific Aims**

In this study, we hypothesize that EMR2 is the F4/80 analog in the human. We suggest that this molecule serves the same function as F4/80 in facilitating the development of CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> T regulatory cells and that its expression is critical for the function of the tolerogenic APCs. After the induction of these lymphocytes into T regulatory cells, they actively suppress immunologic responses to the specific antigen presented by the APC.

We plan to test this hypothesis by the following aims:

- 1) Compare and contrast the surface molecules on APC exposed to antigen with or without TGF $\beta$  to characterize the phenotype of APCs from the human after *in vitro* treatment.
- 2) Test the ability of the human tolerogenic APC to induce T regulatory cells when co-cultured with naïve lymphocytes using the expression of immunosuppressive markers and functions to define the T regulatory cells.
- 3) Determine the role of EMR2 in the development of T regulatory cells by blocking EMR2 signaling prior to analysis of FoxP3 expression from T regulatory cells

We expect these experiments to show:

- 1) EMR2 is upregulated in human APC post treatment with aqueous humor type immunosuppressive factors, TGF $\beta$ .

- 2) An increase of CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> lymphocyte populations post-treatment with tolerogenic APCs.
- 3) Blocking EMR2 with a soluble ligand specific to EMR will prevent the induction of CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> T regulatory cells.

By identifying a major molecular mechanism in ocular immune privilege, novel therapies may be developed to induce or abrogate immune privilege not only in the eye but also in the periphery to treat inflammatory or neoplastic diseases, respectively.

## METHODS

### ***Collection of Human Blood Samples***

Blood samples were purchased from the Massachusetts General Hospital Blood Transfusion Service donor pool. Human donor protocols were considered exempt and were in compliance as defined by Massachusetts Eye and Ear Infirmary Institutional Review Board procedures and in approval with Partners Subcommittee on Human Studies.

### ***Cell Isolation***

Peripheral blood mononuclear cells (PBMC) were collected from anti-coagulated Leukopacs and washed twice with RPMI-1640 (Lonza, Basel, Switzerland), supplemented with 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA), 0.1 mM non-essential amino acids (Invitrogen, Carlsbad, CA), 100 U/mL Penicillin/Streptomycin (Invitrogen, Carlsbad, CA), and 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA). Monocytes were enriched by depletion of non-monocyte cells with Negative Monocyte Isolation Kit II (Miltenyi Biotec, Auburn, CA). Monocyte purity (~90-95%) was assessed by fluorescence-activated cell sorting (FACS) and analyzed for the constitutive CD14 monocyte surface marker (BD Pharmingen, San Diego, CA).



### ***Generation of Tolerogenic APC***

Isolated cells were plated on sterile petri dishes (BD Falcon, Franklin Lakes, NJ) in serum-free culture medium, RPMI-1640 supplemented with Glutamax (Invitrogen, Carlsbad, CA), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/mL Penicillin/Streptomycin, 10 mM HEPES (Invitrogen, Carlsbad, CA), Insulin/Transferrin/Selenium (ITS) (Invitrogen, Carlsbad, CA), and 0.1% BSA (Sigma-Aldrich, St. Louis, MO). Cells were cultured with or without 1 ng/mL porcine TGF $\beta$ 2 (R&D Systems, Minneapolis, MN) for at least six hours. Cells were then pulsed with or without tetanus toxoid (TT) antigen (List Biological Laboratories, Campbell, CA) in serum-free media and incubated overnight in 37°C, 5% CO<sub>2</sub>.

APC were analyzed by flow cytometry to confirm the expression of myeloid and tolerogenic markers. CD14 (constitutive on monocyte-derived cells), Immunoglobulin-like transcript 3 (ILT3, cell surface immunoglobulin that co-ligates to APC stimulatory receptors to downregulate activation signals (Cella et al., 1997)), Programmed cell death 1 ligand 1 (PD-L1, well-known immunosuppressive ligand), and EMR2 were measured. Monoclonal antibodies to ILT3 and PD-L1 were purchased from eBioscience (San Diego, CA) and the monoclonal antibody to EMR2 was purchased from R&D Systems (Minneapolis, MN).

### ***Induction of T Regulatory Cells***

Tolerogenic APC were washed twice with serum-free media to remove all of the exogenously added TGF $\beta$ . Cells were then cultured with an autologous T cell sample, obtained by Human T cell Enrichment Columns (R&D Systems, Minneapolis, MN). Antigen is then reintroduced to the cell co-culture prior to incubation at 37°C, 5% CO<sub>2</sub> for five days. Following the incubation period, the cells were detached from the dishes by incubating with cold 2mM EDTA solution in phosphate buffered saline (PBS) for 10-15 minutes. Co-culture cells were collected and immunostained for CD4 (T helper cell marker), CD25, and FoxP3 (in conjunction, regulatory T cell markers). In parallel, cells were tested for functionality by measuring intracellular production of IFN- $\gamma$  (pro-inflammatory cytokine) and IL-10 (anti-inflammatory cytokine). All antibody markers and intracellular staining kit were purchased from eBioscience.

### ***Blocking EMR2 Ligand Binding***

Monocytes were cultured as outlined above. Soluble chondroitin sulfate from shark cartilage (Sigma-Aldrich, St. Louis, MO) was used to bind to EMR and block signaling at a concentration of 1.5 mg/mL. CD25 and FoxP3 expression as well as intracellular cytokine functionality was tested.

### ***Humanized NOD SCID Mouse Model***

Fresh human PBMC were collected and were injected into the peritoneal cavity 12-15 week old NOD. Cg-Prkdcscid Il2rgtm1Wjl/SzJ female mice (Jackson Laboratory). This humanized the SCID mice. The following day, mice were anesthetized with 120 mg/kg of Ketamine and 20 mg/kg of Xylazine. Equal volumes of TT at 0.5 ug/mL and Complete Freund's Adjuvant (Sigma-Aldrich, St. Louis, MO) were mixed into an emulsion and 100 ul was injected subcutaneously on the nape of the mouse neck using a 19G needle. Seven days later, mice were anesthetized with 120 mg/kg of Ketamine and 20 mg/kg of Xylazine. The thickness of the mice ears was measured using an engineer's micrometer (Mitutoyo). Then a volume of 10 ul consisting of either  $5 \times 10^5$  fresh autologous PBMCs with 1 ug/mL TT or  $2.5 \times 10^5$  fresh autologous PBMCs with  $2.5 \times 10^5$  T regulatory cells with 1 ug/mL TT was injected intradermally into the ear pinnae. 24 Hours later, mice were anesthetized with 120 mg/kg of Ketamine and 20 mg/kg of Xylazine. The thickness of the same ear was measured by an engineer's micrometer and compared to original thickness.

### ***Data Analyses***

Flow cytometry data was collected on a BD LSR II via BD FACSDiva software. Flow cytometry data was analyzed using FlowJo software (Tree Star, Ashland, OR). All data were compiled and statistically analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA). For statistical analyses, \* $p \leq 0.05$ , \*\* $p$

$\leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$  Differences between two groups were considered significant at  $p \leq 0.05$ .

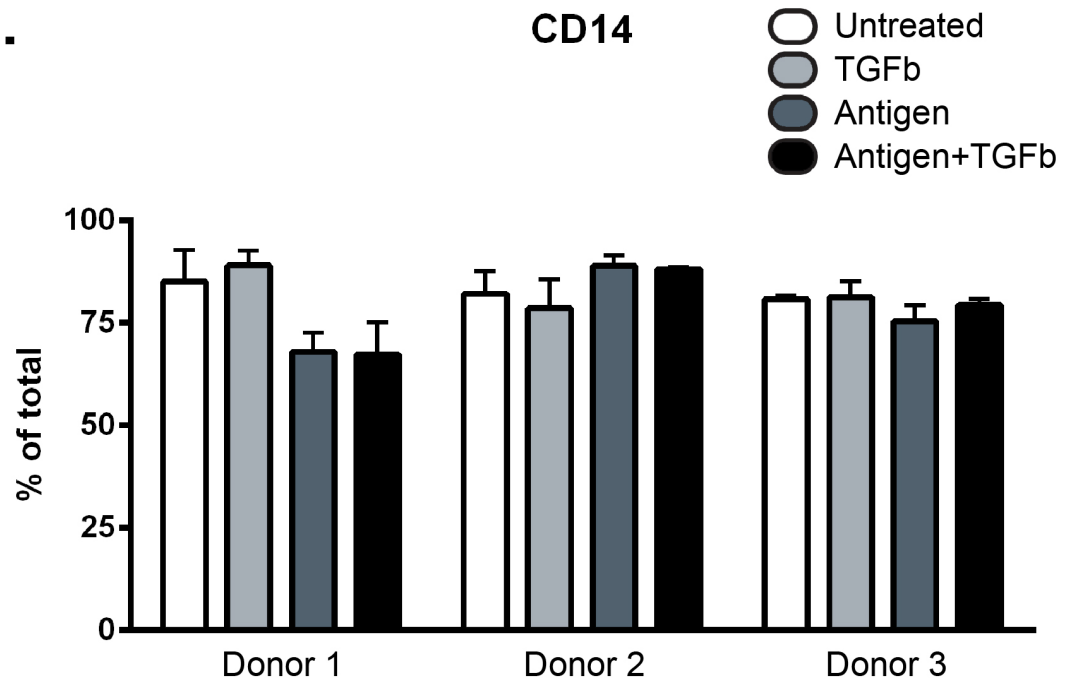
## RESULTS

### ***Surface Markers on Human Tolerogenic APC***

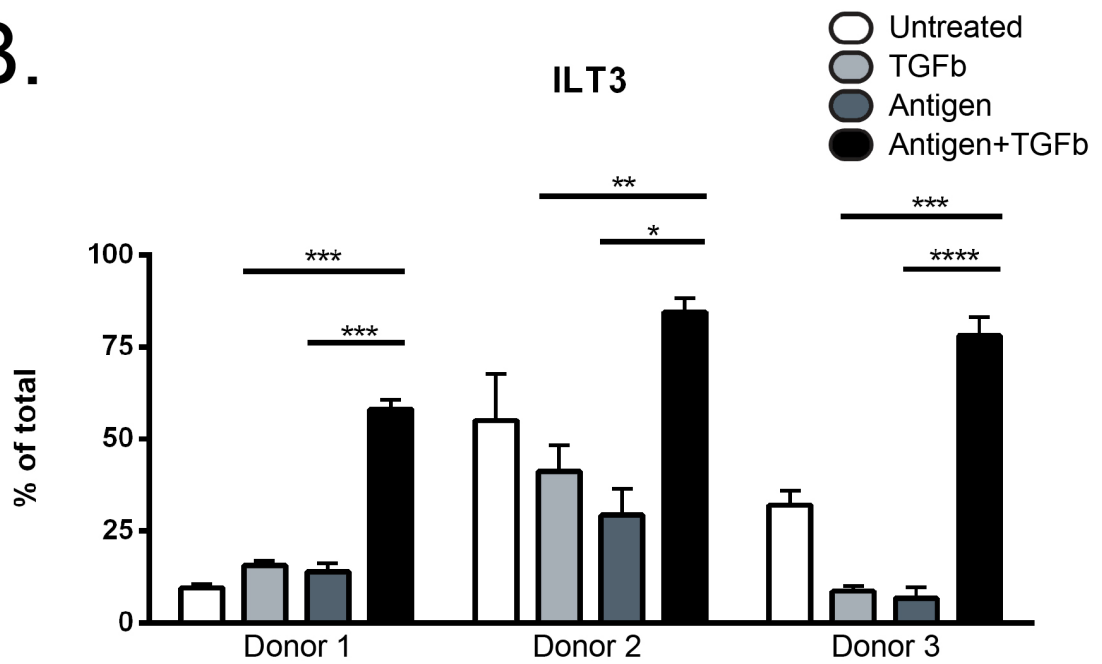
When treated with aqueous humor factors, namely TGF $\beta$ 2, mouse F4/80 macrophages acquire the ability to induce T regulatory cells. When treated with TGF $\beta$ 2, human APCs upregulate inhibitory co-receptors as opposed to classical co-stimulatory molecules, such as CD40 (data not shown). Here, we compared and contrasted the surface markers expressed on APCs post-exposure to TGF $\beta$ 2 and antigen. CD14 remained constitutively expressed on all cell treatment groups (Figure 3A). PD-L1, an inhibitory co-receptor, and ILT3, a novel immunosuppressive molecule, were significantly ( $p \leq 0.05$ ) upregulated in the presence of both TGF $\beta$ 2 and antigen when compared to antigen or TGF $\beta$ 2 alone (Figure 3D, 3B). The potential F4/80 analog, EMR2, was also upregulated (Figure 3C). Significance was assessed by one-way ANOVA for each cell surface marker. A difference between two groups was considered significant if  $p \leq 0.05$ .

Flow cytometric data showed differences in surface marker expression in comparison to unstained control (Figure 4).

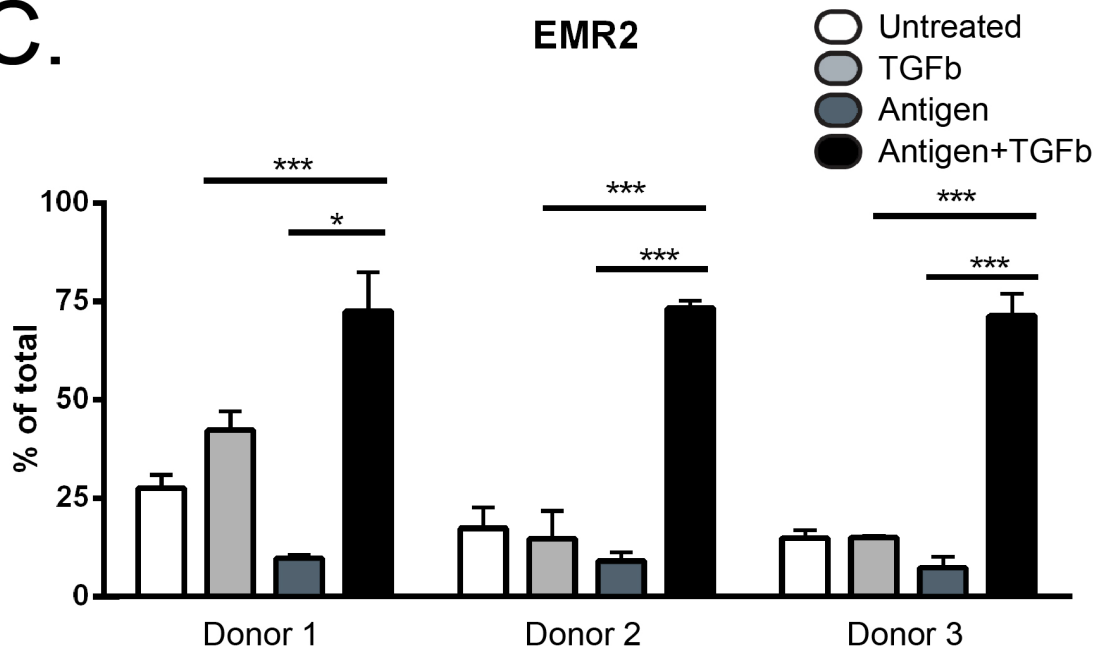
A.



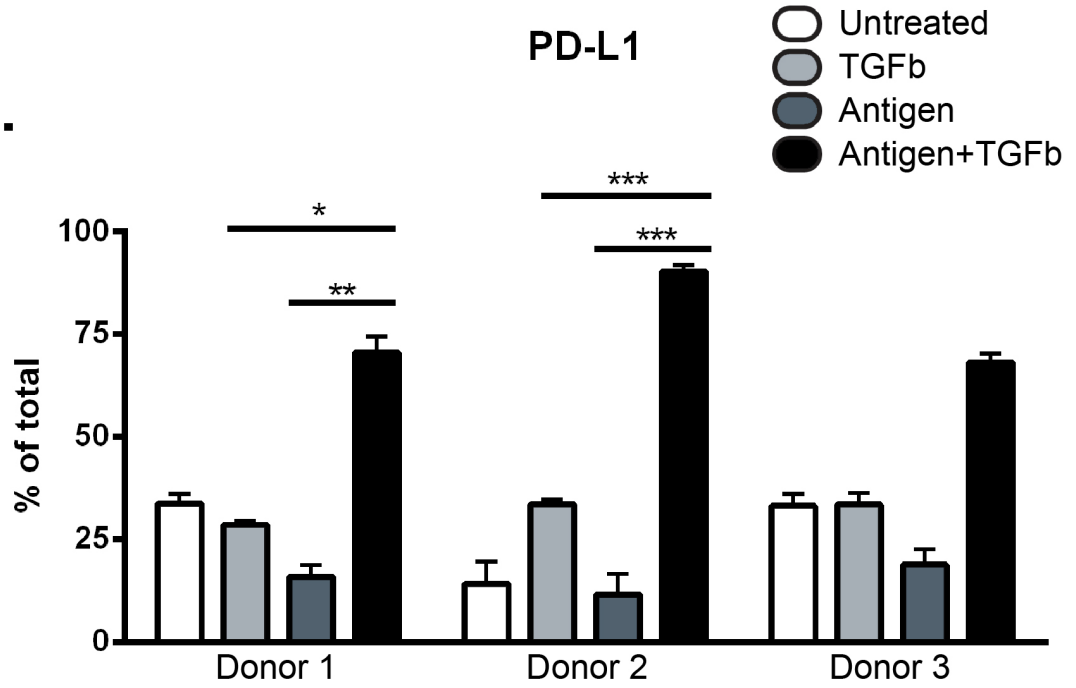
B.



C.

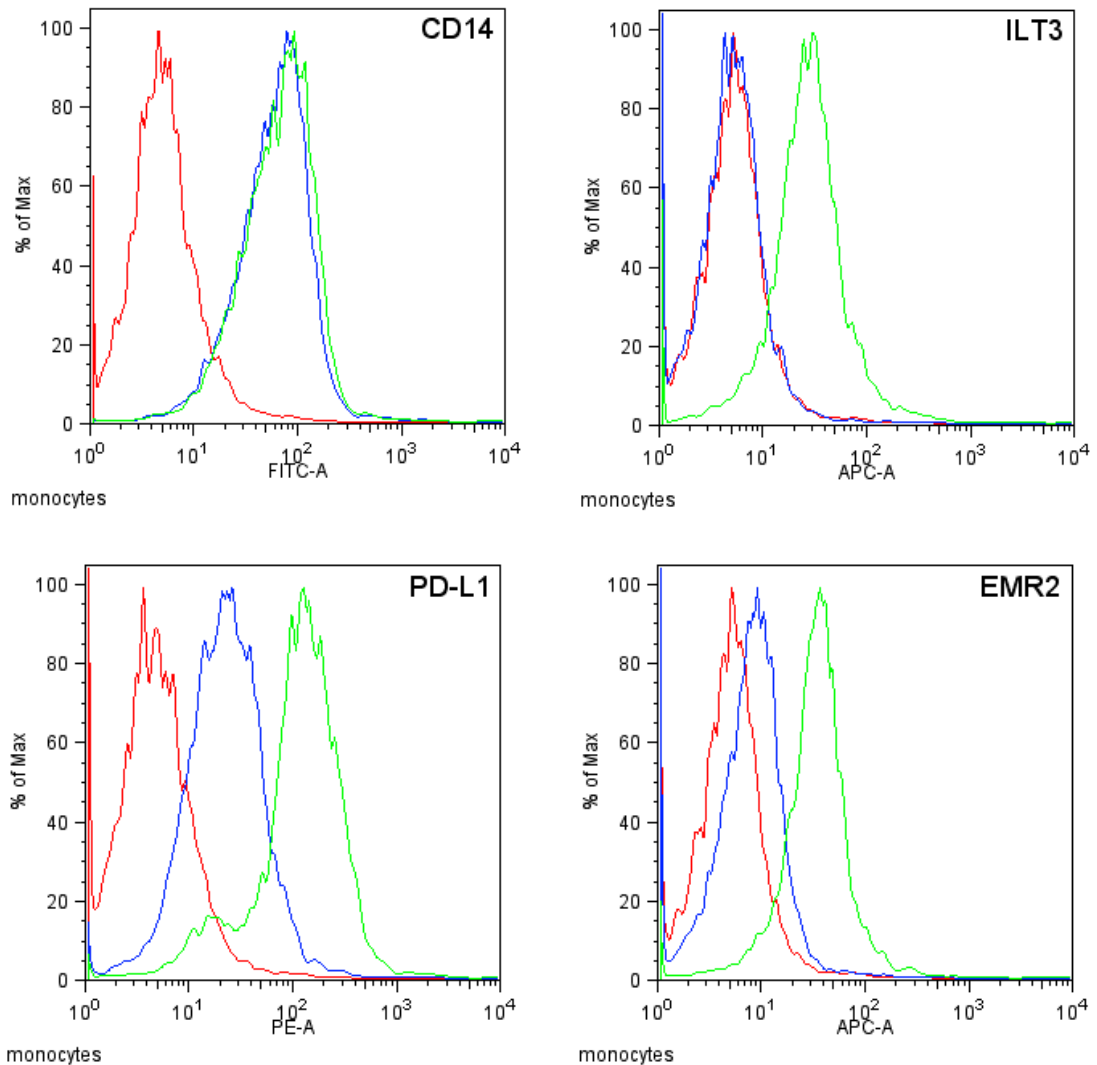


D.



**Figure 3. Phenotypic profile of tolerogenic APC.** Each bar shows the mean  $\pm$  SEM of three repeats. Y-axis represents percent of cells positive for each marker given the treatment condition.





**Figure 4. Flow Cytometric APC Marker Profiles.** Representative graphs for the four markers stained on the human tolerogenic APCs. Red corresponds to the unstained control; blue, cells treated with antigen alone; green, cells treated with antigen and TGF $\beta$ 2. TGF $\beta$ 2 alone showed similar fluorescence intensities as antigen alone (data not shown). ILT3, PD-L1, EMR2 were analyzed on a gated CD14 population to ensure majority monocytic cells for analysis. Y-axis

depicts percentage of cells out of total monocyte population. X-axis shows the fluorescence intensity of surface marker staining to the unstained control.

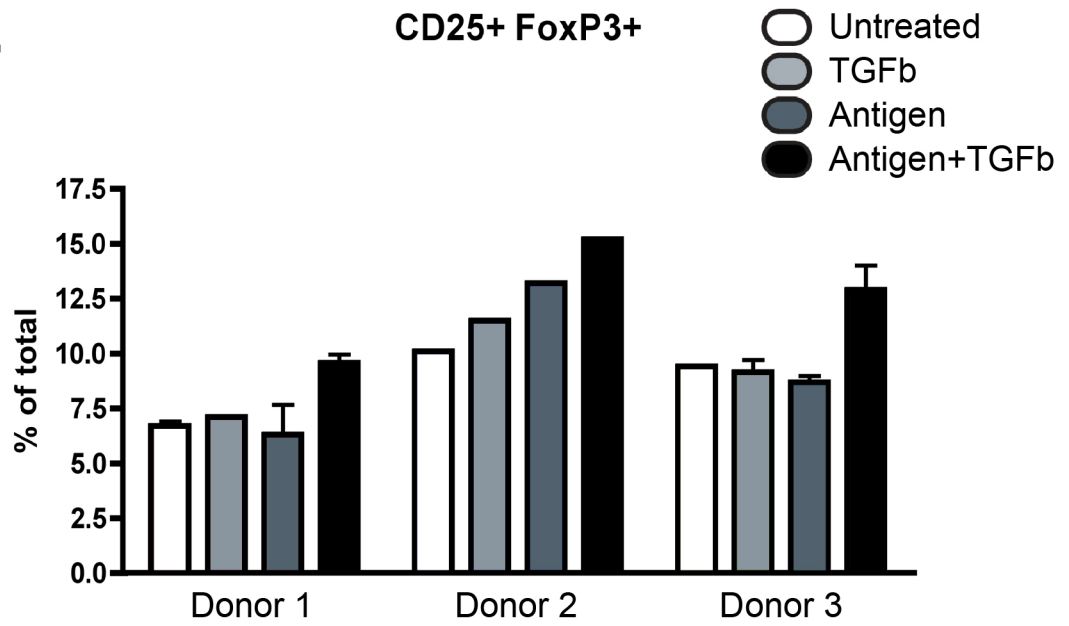
### ***Induced T Regulatory Cells***

Post antigen inoculation into the anterior chamber of the mouse, F4/80<sup>+</sup> APCs travel from the eye to the marginal zone of the spleen to facilitate cell aggregations. The interactions of cells within the aggregates induce T regulatory cells. To recreate the tolerance inducing environment for human cells, we essentially created a “spleen-in-a-dish” in which tolerogenic APCs were co-cultured with whole peripheral lymphocytes from the same blood sample. These lymphocytes were collected on a human T cell column in which unwanted cells attach to immunoglobulin or anti-immunoglobulin coated glass beads within the column. The resulting elution contained an enriched CD3<sup>+</sup> (a universal T cell marker) population. In the co-cultures, tolerogenic APC induced a population of CD25<sup>+</sup> FoxP3<sup>+</sup> lymphocytes. These non-adherent cells were collected and confirmed by surface staining the CD4 marker and analyzed for CD25 and FoxP3 (Figure 5). T regulatory cells also increased production of the anti-inflammatory cytokine, IL-10 and decreased production of the pro-inflammatory cytokine, IFN- $\gamma$ , also shown by figure 4.

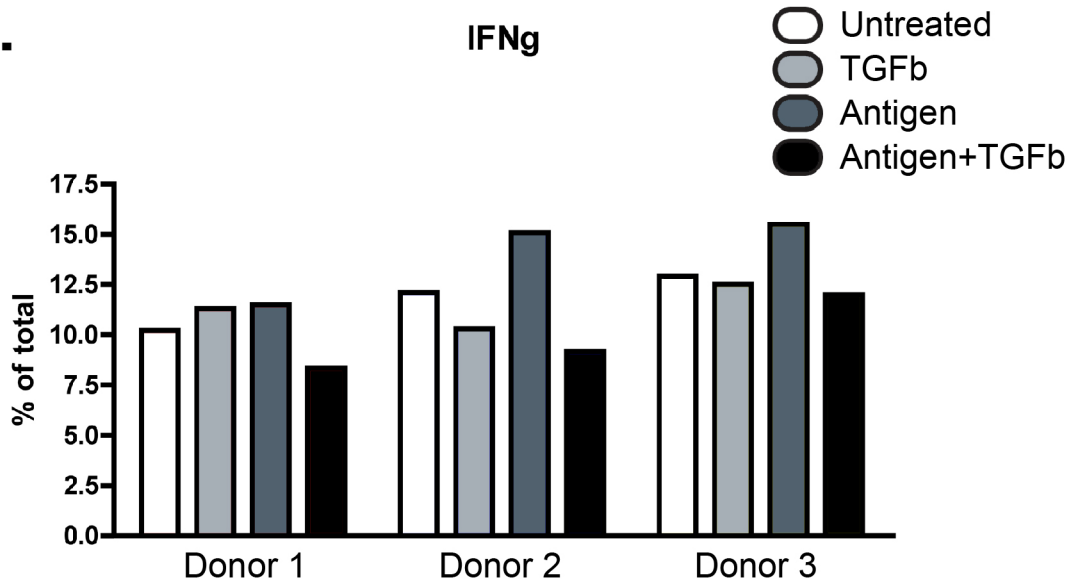
Flow cytometric analysis showed an increase of cells expressing both CD25 and FoxP3 from the cultures that contained the tolerogenic APC (Figure 6A). In the presence of TGF $\beta$  and antigen, the number of cells producing intracellular IL-10 increased and the number of cells producing intracellular IFN- $\gamma$  decreased (Figure 6B, 6C).

Since our organ of interest is the eye, and the eye is protected by a blood-ocular barrier, we have used serum-free culture medium in all of our experiments. Also, serum contains known inhibitors of TGF $\beta$ . Previously tested by the Stein-Streilein group, serum interferes with the signaling of many immunosuppressive factors in the aqueous humor. Thus, each experiment was done without serum. However, once the tolerogenic APCs are generated, the induction of T regulatory cells can occur in complete medium because signals that lead to T regulatory generation involve cell-cell contact and are not affected by the presence of serum. Thus, some experiments were conducted in complete medium for the latter portion of the APC-lymphocyte co-culture.

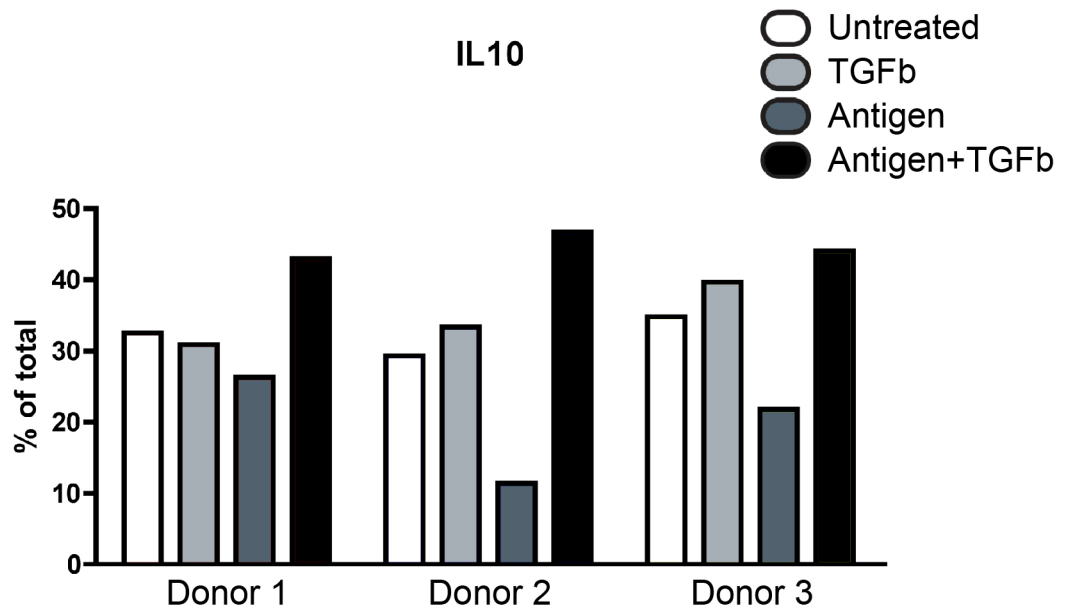
A.



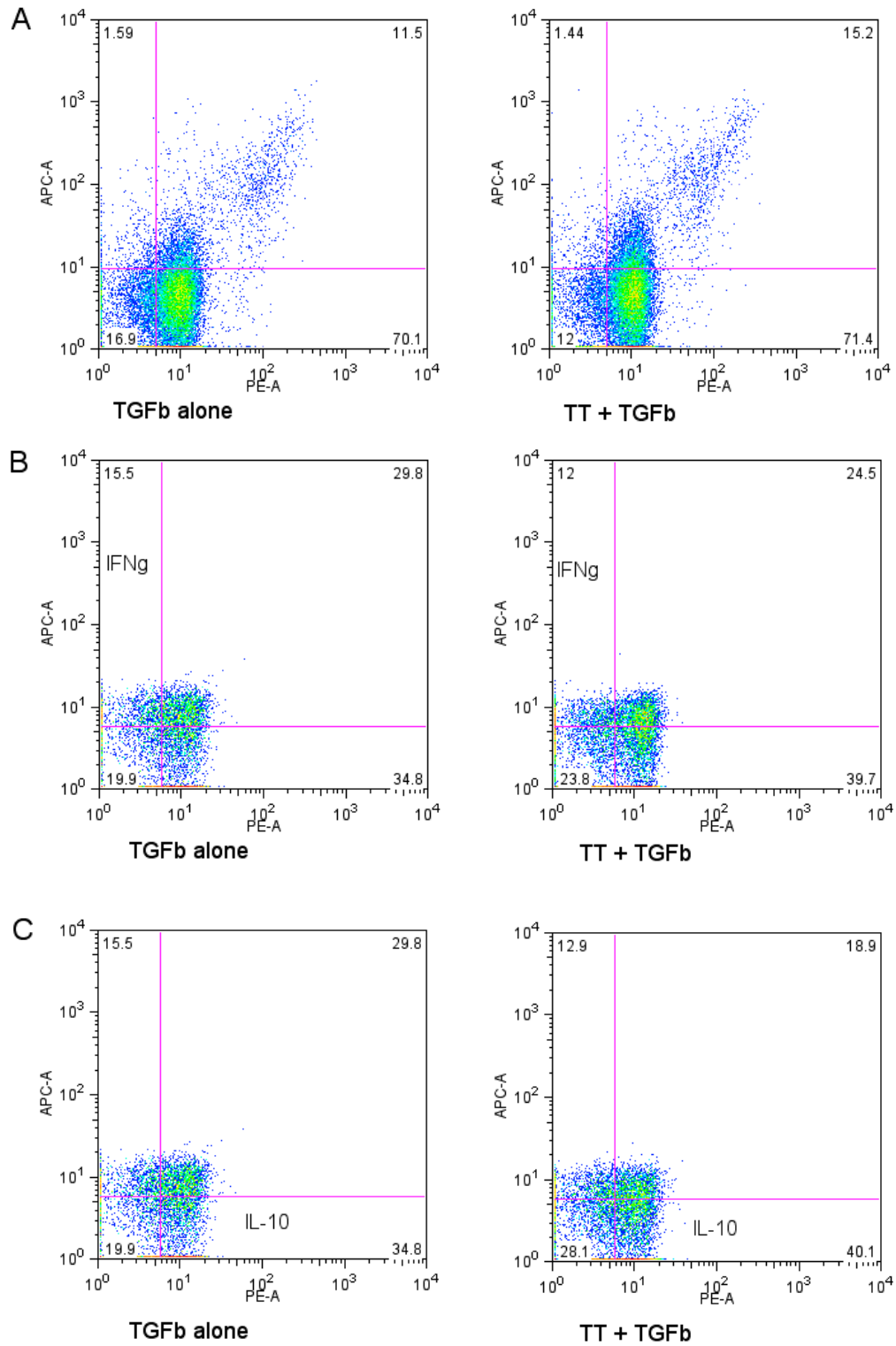
**B.**



**C.**



**Figure 5 Function of induced T regulatory cells.** Intracellular staining for FoxP3, IFN- $\gamma$ , and IL-10 of CD4<sup>+</sup> gated lymphocytes post co-culture with tolerogenic APC. (A) CD4<sup>+</sup> lymphocytes stained for surface CD25 and intracellular FoxP3. Each donor was repeated in duplicate. Y-axis represents percent total of cells positive for both CD25 and FoxP3 with regards to each treatment condition. (B, C) CD4<sup>+</sup> CD25<sup>+</sup> lymphocytes stained for intracellular IFN- $\gamma$  or IL-10. Y-axis represents percent total of cells positive for IFN- $\gamma$  or IL-10.





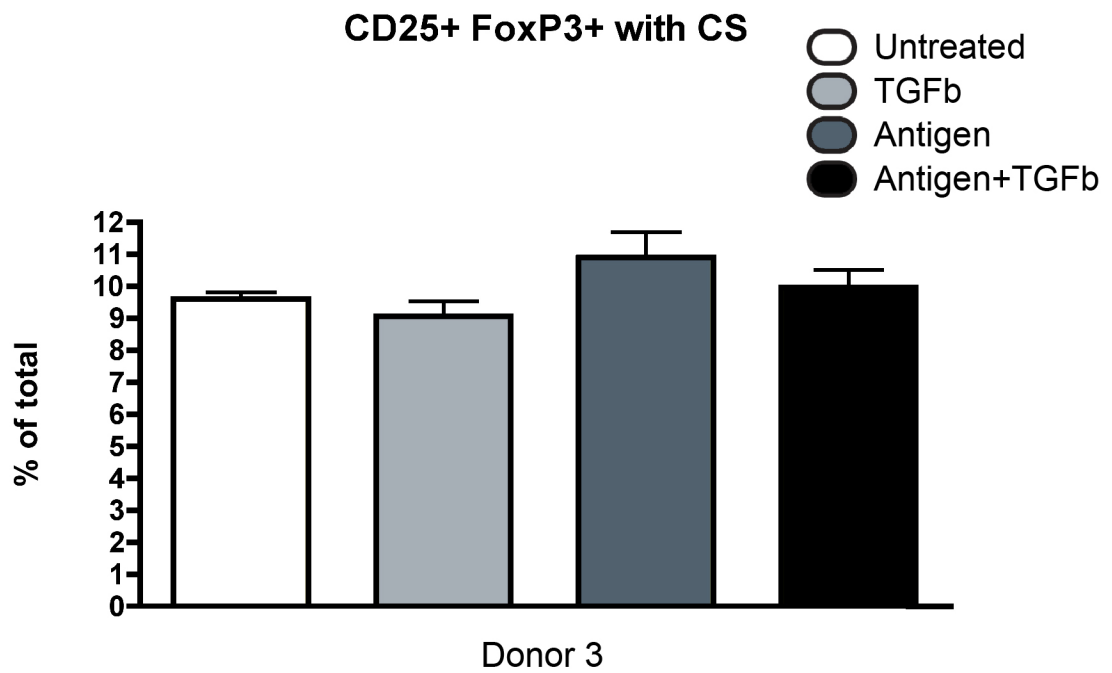
**Figure 6. Flow cytometric dot plots of intracellular protein production.** All quadrant gates were created on unstained untreated single color control populations. Quadrant II, as referenced in the text, is the upper right area of each graph which reflects populations that immunostained positive for both allophycocyanin and phycoerythrin fluorochromes. (A) Cells shown are CD4<sup>+</sup> lymphocytes, with TGFβ, and with or without antigen. Y-axis measures the CD25 marker by allophycocyanin (APC-A) fluorochrome, X-axis measures the intracellular FoxP3 marker by phycoerythrin (PE-A) fluorochrome. Both axes measure fluorescence intensity. Quadrant II (upper right corner) of each graph represents the CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> population. (B) Cells shown are CD4<sup>+</sup> CD25<sup>+</sup> lymphocytes, with TGFβ, and with or without antigen. Y-axis measures intracellular IFN-γ cytokine by allophycocyanin fluorochrome, X-axis measures intracellular IL-10 cytokine by phycoerythrin fluorochrome. Both axes measure fluorescence intensity. Panel B is a representative plot of decreased IFN- γ production in the presence of antigen. (C) Cells shown are CD4<sup>+</sup> CD25<sup>+</sup> lymphocytes, with TGFβ, and with or without antigen. Y-axis measures intracellular IFN-γ cytokine by allophycocyanin fluorochrome, X-axis measures intracellular IL-10 cytokine by phycoerythrin fluorochrome. Both axes measure fluorescence intensity. Panel C is a representative plot of increased IL-10 production in the presence of antigen. Control samples for untreated and antigen alone conditions not shown.

### ***Blocking EMR2 Ligand Binding***

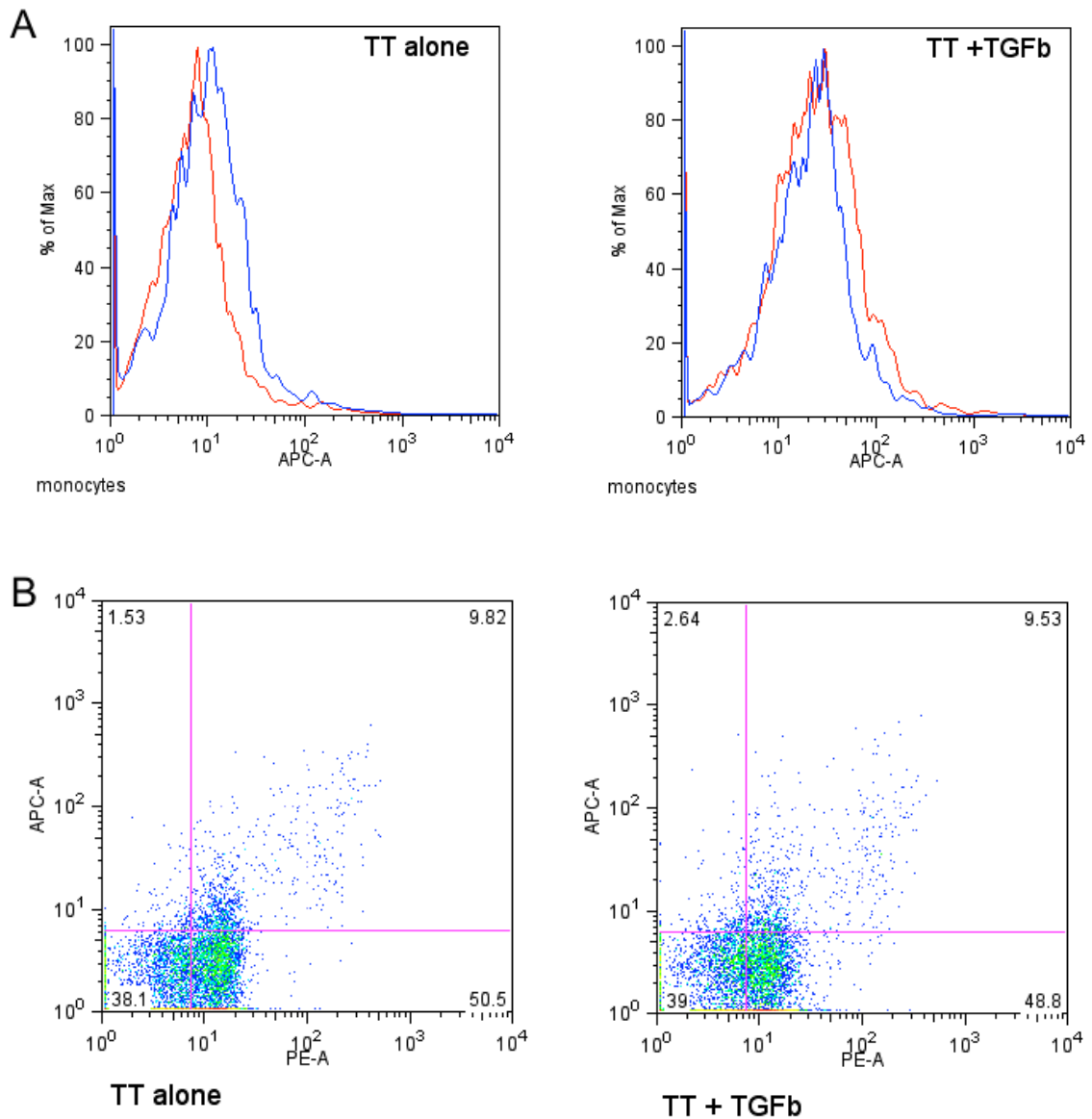
It has been found that the common ligand for EMR2 is chondroitin sulfate glycosaminoglycans (GAG) (Stacey et al., 2003). To mediate cell interactions, EMR2 binds to chondroitin sulfate GAGs on the opposing cell. To block EMR2 signaling and the induction of T regulatory cells, we added soluble chondroitin sulfate to the cell culture. We hypothesized saturating the culture with soluble chondroitin sulfate will competitively bind up available EMR2 molecules and subsequently block signaling from the APC to the lymphocytes.

We treated APC with soluble chondroitin sulfate (1.5 mg/mL) in the same conditions listed above. By using a dose curve, we found 1.5 mg/mL was the ideal concentration for saturating EMR2 receptors. Post co-culture with lymphocytes, we repeated surface and intracellular staining of lymphocytes to check for differences in FoxP3<sup>+</sup> cells (Figure 7).

Figure 8 shows flow cytometry readouts of the effect of using soluble chondroitin sulfate in the APC culture. In both figure 7 and figure 8, we see no increase in CD25<sup>+</sup> FoxP3<sup>+</sup> cell populations when treated with antigen and TGFβ.



**Figure 7. FoxP3 expression of T cells treated with soluble chondroitin sulfate.** Intracellular staining of FoxP3, surface staining of CD25 on CD4<sup>+</sup> gated lymphocytes post co-culture with tolerogenic APC. Y-axis represents percent of total lymphocytes positive for FoxP3 and CD25. Donor was repeated in duplicate.



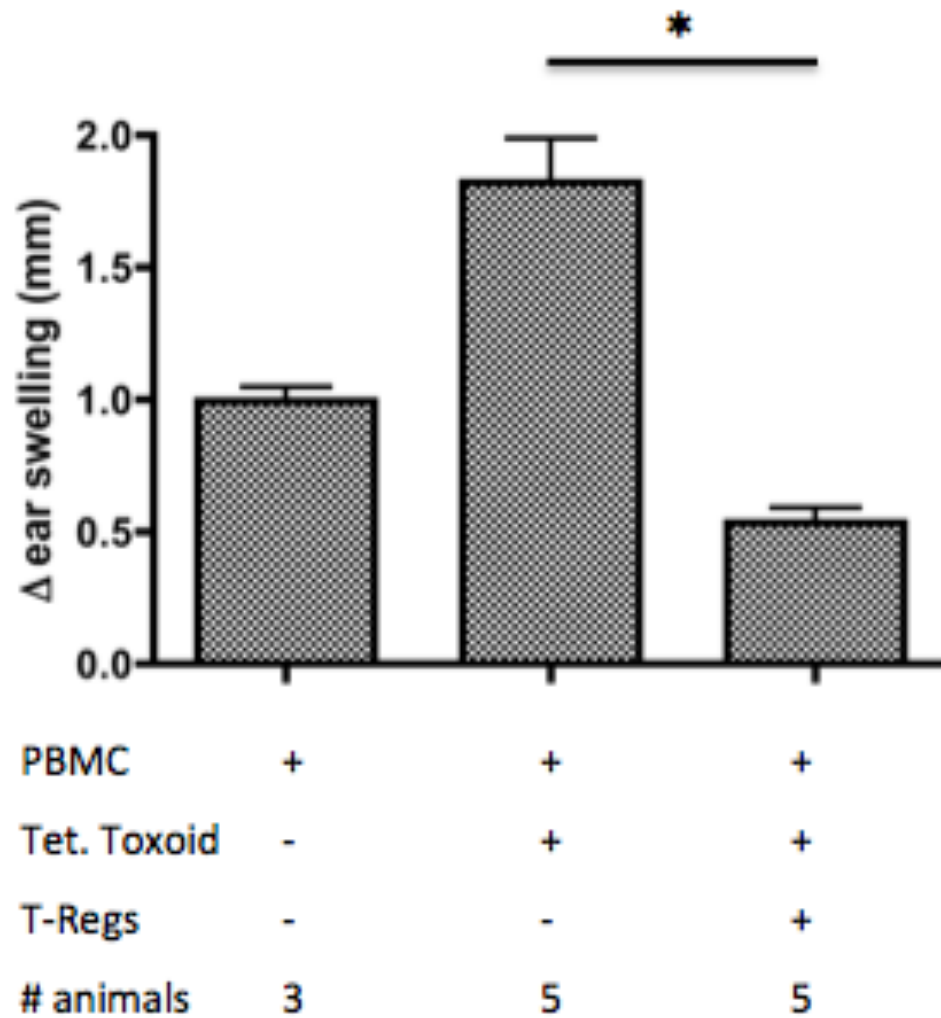
**Figure 8. Flow cytometric EMR2 readout and dot plot post-soluble chondroitin sulfate treatment.** Quadrant II, as referenced in the text, is the upper right area that reflects populations that immunostained positive of both APC-A and PE-A. (A) APC were cultured with antigen and with or without TGFβ. Both conditions received 1.5 mg/mL of soluble chondroitin sulfate from shark

cartilage. Red corresponds to the unstained control, blue corresponds to the APC experimental conditions. Y-axis represents percentage of cells out of total monocyte population, X-axis represents fluorescence intensity of EMR2 staining compared to the unstained control. (B) Dot plot of TT alone vs TT and TGF $\beta$  with both conditions receiving 1.5 mg/mL of soluble chondroitin sulfate. Cells gated are CD4<sup>+</sup> lymphocytes; Y-axis measures CD25 by allophycocyanin fluorochrome and X-axis measures FoxP3 by phycoerythrin fluorochrome. Quadrant gates were created on unstained untreated single color controls. Control samples for untreated and TGF $\beta$  alone are not shown.

### ***In Vivo Model***

Once T regulatory cells were induced by our generated tolerogenic APCs, we began using NOD SCID mice to implement an *in vivo* model. SCID mice are unable to undergo somatic recombination of their T cell receptor genes, thus they are unable to form unique genetic sequences on their T cell receptors that recognize a bounty of antigenic proteins. As a result, these mice lack functional and mature T and B cells. In these mice, we applied our experimental conditions by adding fresh human PBMC into the animal system. We immunized the animal against the antigen of interest. Seven days later, the animal was challenged with the same antigen with or without autologous T regulatory cells generated *in vitro*. The following day, ear-swelling representative of the DTH response, was measured. With human cells, we were able to induce ACAID in an immunocompromised mouse with tetanus toxoid.

Compared to the control condition in which no T regulatory cells and no antigen were added, a large swelling response with the addition of TT antigen was observed. When TT antigen in the presence of autologous T regulatory cells was added to the animal, a significant decrease of swelling was observed. Thus, the *in vitro* generated T regulatory cells from EMR2<sup>+</sup> APCs were able to suppress DTH inflammation in the mouse ear.



**Figure 9. DTH response in humanized SCID mice.** Each bar is mean  $\pm$  SEM of 3-5 animals per treatment condition. Y-axis shows the change in ear swelling in millimeters. X-axis shows experimental condition of mice in each group.

## DISCUSSION

Immune privilege is a trait unique to certain parts of the body that are critical to life. In other areas exposed to the external environment or foreign antigens of the external environment, tolerance is necessary in order to keep excessive inflammation in check. The more we understand how the body regulates immune responses and induces tolerance, the possibility of manipulating characteristics of innate and adaptive immunity for therapeutic purposes becomes more plausible.

### ***Human Tolerogenic APC***

We determined that like F4/80 in the mouse, EMR2 is upregulated in human tolerogenic APCs post-treatment with aqueous humor-type immunosuppressive factors, namely TGF $\beta$ . Different from tolerogenic APCs, professional APCs present antigen and initiate T cell activation and proliferation. These two cell types differ phenotypically most notably in their co-stimulatory machinery. Professional APCs consist of dendritic cells, macrophages, and some B-cell types. Of the many types of dendritic cells, those myeloid cells that participate in T cell activation express CD11c and CD1a on their surface, which are classic dendritic cell markers. The professional macrophages express Mac-3 and CD11b. Both cell types constitutively display CD14, which indicate that they are of monocytic origin as well as the obligatory MHC class II and CD40 for co-



stimulation of the T cell. In the delayed type hypersensitivity response, both will secrete IL-12.

In immune privilege, tolerogenic APCs are of interest. Upon interaction with T cells, these APCs will induce the regulatory T cell phenotype and no T cell activation and expansion will occur. On the tolerogenic APCs, similar surface markers are present such as CD40 and MHC class II. However, these APCs express inhibitory molecules that compete with co-stimulatory molecules to prevent T cell activation. These inhibitory molecules include PD-L1 and ILT3, which downregulate or counter activating signals. In the presence of upregulated inhibitory molecules, EMR2 is also upregulated. Thus, EMR2 may be a marker for human tolerogenic APCs. While we did not explicitly test for other classic macrophage or dendritic cell markers listed above, these cells were constitutively CD14 positive and differentiated into tolerogenic APCs by showing upregulated inhibitory molecules that would be expected on suppressive monocytic cells interacting with T cells.

Our finding that EMR2 is upregulated on these tolerogenic APCs is novel. As an adhesion G-protein coupled receptor, EMR2 is a member of the EGF-TM7 subfamily and found on cells of myeloid origin. F4/80, also a member of the EGF-TM7 subfamily, is upregulated on mouse macrophages in the same experimental conditions with TGF $\beta$  and antigen. As our proposed F4/80 analog, the upregulation of EMR2 suggests that it functions in a synonymous manner to F4/80. Both are adhesion molecules capable of binding to glycosaminoglycans

and we postulate that EMR2 has a significant role in cell-cell interactions.

Through adhesion or signaling, EMR2 on the tolerogenic APCs may facilitate cell clustering with other T cells, B cells, and NKT cells to induce a T regulatory cell population, much like the role of F4/80.

### ***Functions of Human Tolerogenic APCs***

Second, we showed that a population of CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> lymphocytes increased post-culture with tolerogenic APC. The increase of this lymphocyte population in the mouse requires the presence of F4/80 on tolerogenic macrophages. When resting lymphocytes come into contact with F4/80 macrophages, the macrophages induce a peripheral lymphocyte population into the T regulatory phenotype, CD25<sup>+</sup> FoxP3<sup>+</sup>. Much like the mouse, human tolerogenic APCs with upregulated EMR2 induced a T regulatory cell population that were able to suppress inflammation due to a specific antigen *in vivo*.

The significance of these inducible T regulatory cells is that they perpetuate tolerance to an antigen of interest in the periphery. There are two types of T regulatory cells, natural and induced. While natural T regulatory cells develop naturally within the thymus through T cell receptor affinity selection, inducible ones do not and are subject to a multitude of conditions outside the thymus. Development of extrathymic inducible T regulatory cells can occur within instances of inflammation in which effector T cells as well as regulatory T cells

are developed simultaneously in a primary immune response. However, in conditions sans inflammation, inducible T regulatory cells can develop by antigen presentation from tolerogenic APCs. It is this latter condition in which our APCs induced other autologous peripheral lymphocytes into a population of CD25<sup>+</sup> FoxP3<sup>+</sup> cells. Thus, by priming our tolerogenic APCs with an antigen of interest, we induced tolerance in peripheral lymphocytes to become inducible T regulatory cells. In a living organism, the significance of an increased peripheral inducible T regulatory cell population indicates a systemic tolerance to that antigen from aqueous humor-derived tolerogenic APCs. This corroborates to the previously established mouse model and is the first step in translating tolerance and the mechanisms of immune privilege from the mouse to the human.

***EMR2 and its Ligand, Chondroitin Sulfate Glycosaminoglycans (GAGs)***

Lastly, we determined that blockade of EMR2 with a soluble ligand specific to EMR prevents the induction of CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> T regulatory cells. When F4/80 is blocked on mouse macrophages, they fail to produce T regulatory cells. When we blocked EMR2 ligand binding on human tolerogenic APCs, they similarly failed to produce T regulatory cells. From this, EMR2 is required for human tolerogenic APCs to induce a CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> T regulatory cell population. Without EMR2, peripheral tolerance cannot be induced to the antigen of interest because these T regulatory cells are not there to suppress inflammatory activity against that antigen. Thus the adhesion or signaling

between EMR2 from the APC and its glycosaminoglycan ligand on peripheral lymphocytes is necessary in order to produce T regulatory cells that uphold tolerance throughout the body.

This novel discovery opens up the possibility of manipulating mechanisms of immune privilege for future therapies. It is possible that EMR2 is a marker for tolerogenic APCs in the human. As a suppressor molecule, it is likely to be found in areas of the body displaying immune privilege. From a pathological standpoint, these are likely to be areas with neoplastic growth. As our immune system recognizes tumor cells and abnormal growths of tissue, the presence of molecules like EMR2 help the tumors survive by participating in tolerance induction and evading immune surveillance. Blocking EMR2 and its subsequent signaling could potentially assist self immune defenses against the tumor.

In contrast, hyperinflammation is a hallmark of many different diseases. As inflammatory products are damaging to cells and tissues within the area, immune regulation may need to be invoked to control excessive immune reactions. Augmenting EMR2 signaling through TGF $\beta$  may be protective because it will reduce inflammation and control damage to the surrounding tissues.

Through next-generation solutions like gene therapy, we may be able to manipulate the EMR2 gene construct and subsequent expression in an area of interest in order to induce or target immune privilege. While this may be far in the future, it has been shown extensively by the Streilein and Stein-Streilein groups that *in vitro* generated tolerogenic APCs are able to retain their functionality when

reintroduced into a living organism. Generating tolerogenic cells *in vitro* and re-educating immune cells to become regulatory before re-injecting them into the area of interest may be a near future method to target localized inflammation. TGF $\beta$  treatment into a local tissue may also have the same effect. Thus knowing that tolerogenic APCs express EMR2 may be a basis for future therapies regarding immune and inflammation-related diseases.

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## CURRICULUM VITAE

