ROLE OF SPLENIC B CELLS AND GAMMA DELTA T CELLS IN THE INDUCTION OF PERIPHERAL TOLERANCE ELICITED THROUGH THE ANTERIOR CHAMBER OF THE EYE

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DEDICATION

First and foremost, I thank God who makes everything possible. I would like to thank my mentor, Dr. Jerry Niederkorn, for accepting me in his lab and for being a wonderful mentor and advisor on both scientific and personal levels throughout the time I spent in his lab. Dr. Niederkorn has always been an ideal person to work with. I would like to extend this appreciation to all members of the Niederkorn lab, both past and present. You have all been very helpful and nice people to work with. I would like to thank the members of my Graduate Committee for constant advice and support; Dr. Nancy Street, Dr. Nitin Karandikar, Dr. John Schatzle, and Dr. Clay Beauregard. Finally, I would like to thank my parents, Dr. Mohamed Seif Ashour and Dr. Fatma Elsakhawy and my siblings, Dr. Tarek Ashour and Dr. Rehab Ashour for their continuous moral support and encouragement. To everyone I mentioned above, I will never forget what y'all have done for me.

ROLE OF SPLENIC B CELLS AND GAMMA DELTA T CELLS IN THE INDUCTION OF PERIPHERAL TOLERANCE ELICITED THROUGH THE ANTERIOR CHAMBER OF THE EYE

by

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Anterior chamber-associated immune deviation (ACAID) is a form of peripheral tolerance that is induced by introducing antigens into the anterior chamber (AC) of the eye, and is maintained by antigen-specific regulatory T cells (Tregs). ACAID regulates harmful immune responses that can lead to irreparable injury to innocent bystander cells that are incapable of regeneration. ACAID is a product of complex cellular interactions that involve $F4/80^+$ ocular antigen presenting cells (APC), NK T cells, B cells, gamma delta ($\gamma\delta$) T cells, $CD4^+CD25^+$ Tregs, and $CD8^+$ Tregs. Antigens injected into the AC are processed by $F4/80^+$ antigen presenting cells (APC), which migrate to the thymus and spleen.

In the spleen, ocular APC induce the development of antigen-specific B cells that act as ancillary APC and are required for ACAID induction. Results show that ocular APC elicit the generation and expansion of antigen-specific splenic B cells that induce ACAID. However, direct cell contact between ocular APC and splenic B cells is not necessary for the induction of ACAID B cells. Peripheral tolerance produced by ACAID requires the participation of ACAID B cells, which induce the generation of both $CD4^+$ Tregs and $CD8^+$ Tregs. Using *in vitro* and *in vivo* models of ACAID, we demonstrate that ACAID B cells must express both MHC class I and class II molecules for the generation of Tregs. This suggests that ACAID requires antigen presenting B cells that simultaneously present antigens on both MHC class I and class II molecules. Gamma delta T cells are also crucial for the generation of ACAID, but their function in ACAID is largely unknown. Several hypotheses were proposed for determining the functions of γδ T cells in ACAID. The results indicate that γδ T cells neither suppress DTH directly nor do they act as antigen presenting cells. However, γδ T cells were shown to secrete IL-10, thus facilitating the generation of ACAID Tregs. In addition, the contribution of γδ T cells in ACAID generation could be replaced by adding exogenous rmIL-10 to ACAID spleen cell cultures lacking γδ T cells. Throughout this dissertation, we have shed light on the roles of B cells and γδ T cells in ACAID and have found that B cells need to express both MHC I and MHC II in order to induce the generation of Tregs and that γδ T cells need to secrete IL-10 for ACAID to be generated.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

1-Ashour H. M. and J. Y. Niederkorn. Peripheral Tolerance Via the Anterior Chamber of the Eye: Role of B Cells in MHC Class I and II Antigen Presentation. J Immunol 2006; 176 (10): 5950-5957

2-Ashour H. M. and J. Y. Niederkorn. γδ T Cells Promote Anterior Chamber-Associated Immune Deviation and Immune Privilege Through Their Production of IL-10. J Immunol 2006; 177 (12): 8331-8337

LIST OF FIGURES

LIST OF TABLES

LIST OF DEFINITIONS

α-MSH	Alpha-melanocyte stimulating hormone
AC	Anterior chamber
ACAID	Anterior chamber-associated immune deviation
APC	Antigen presenting cells
ARVO	Association for Research in Vision and Opthalmology
β2m	Beta-2-microglobulin
BCR	B cell receptor
bp	Base pair
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene-related peptide
cm	centimeter
CTL	Cytotoxic T lymphocyte
DTH	Delayed-type hypersensitivity
FACS	Fluorescein-activated cell sorting
FasL	Fas-ligand
FITC	Fluorescien isothiocyanate
HBSS	Hanks' balanced salt solution
HEL	Hen egg lysosyme
IC	Intracameral
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon

Ig	Immunoglobulin
IL	Interleukin
IP	Intraperitoneal
IV	Intravenous
KO	Knockout
LAT	Local Adoptive Transfer
LPS	Lipopolysaccharide
mg	Milligram
μg	Microgram
MHC	Major histocompatibility complex
MIF	Macrophage inhibitory factor
MIP-2	Macrophage inflammatory protein-2
ml	Milliliter
μl	Microliter
mm	Millimeter
ng	Nanogram
NH_4Cl	Ammonium chloride
NK	Natural Killer
OVA	Ovalbumin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEC	Peritoneal exudates cells

rm	Recombinant mouse
SC	Subcutaneous
TAP	Transporter in antigen processing
TCR	T cell receptor
Tg	Transgenic
TGF-β	Transforming growth factor-beta
Th	T helper
VIP	Vasoactive intestinal peptide
^{o}C	Degrees centigrade

CHAPTER ONE

Introduction

IMMUNE TOLERANCE VIA THE AC OF THE EYE

Immune privilege

The eye is an immune privileged site and thus, is protected from immune-mediated inflammation. Tissues in the eye normally have little or no regenerative properties, which makes the eye vulnerable to inflammatory immune responses, such as delayed-type hypersensitivity (DTH). DTH responses produce extensive injury to innocent bystander cells. However, antigens encountered in the eye elicit a form of peripheral tolerance that is maintained by antigen-specific regulatory T cells (Tregs) (1-3). This tolerance is associated with the anterior chamber (AC) of the eye and is thus called anterior chamber-associated immune deviation (ACAID) (4). ACAID is characterized by an antigen-specific downregulation of DTH responses which serves as a protective mechanism against immune mediated inflammatory damage. ACAID is the product of a complex series of cellular interactions that begin in the AC of the eye where antigen is captured and processed by resident $F4/80^{+}$ antigen presenting cells (APC), which are bathed in the aqueous humor cytokines and immunosuppressive factors causing these cells to express different cytokines and cell surface molecules (1, 2).

Cellular interactions in ACAID

Migration of $F4/80^+$ ocular APC to the spleen initiates complex cellular interactions that ultimately generate $CD4^+CD25^+$ Tregs and $CD8^+$ Tregs (5-11). These interactions involve $F4/80^+$ ocular antigen presenting cells (APC), B cells, γδ T cells, NK T cells, $CD4^+CD25^+$ Tregs, and $CD8^+$ Tregs (4). While $CD4^+CD25^+$ Tregs inhibit the induction or afferent component of the immune response, $CD8^+$ Tregs inhibit the expression of DTH by previously sensitized T cells (i.e., the efferent component of the immune response) (4, 12).

B cells

Splenic B cells are among the cells that are required for the generation of ACAID. Previous studies have shown that B cells capture and then process antigens released by $F4/80^+$ ocular APC after their migration into the spleen (13, 14). First, capturing the antigen occurs through the B cell receptor (BCR), then follows internalization and processing through acidified lysosomes (13, 14). This is followed by antigen presentation to T cells (14). The details of antigen presentation by B cells have yet to be fully discovered. In this study, we show that ACAID B cells present antigens on both MHC class I and II molecules during the establishment of peripheral tolerance. Results also show that the antigen-specific B cell population expands following ACAID induction and that this expansion is crucial for the development of ACAID.

Gamma delta T cells

Gamma delta T cells are crucial for the generation of ACAID and for corneal graft survival (13-15). However, the functions of γδ T cells in ACAID are not yet fully understood. To determine the function of γδ T cells in ACAID, several hypotheses were proposed and tested. The results indicate that γδ T cells do not cause direct suppression of DTH, nor do they act as tolerogenic antigen presenting cells. On the other hand, γδ T cells were shown to secrete IL-10 and facilitate the generation of ACAID Tregs. Moreover, the contribution of γδ T cells to ACAID generation could be replaced by adding exogenous rmIL-10 to ACAID spleen cell cultures lacking γδ T cells.

CHAPTER TWO

Review of the Literature

IMMUNE TOLERANCE VIA THE AC OF THE EYE

Anatomy of the eye

The thrust of this dissertation is on one important aspect of immune privilege of the eye, namely anterior chamber associated immune deviation (ACAID). This phenomenon is initiated in the anterior chamber (AC) which is part of the anterior segment of the eye. In addition to the AC, the anterior segment also includes the cornea, iris, lens, and ciliary body. The AC is anatomically located between the cornea and the iris as shown in **FIGURE 1**. The posterior segment of the eye is composed of the retina, the choroid, the vitreous, and the optic nerve (15).

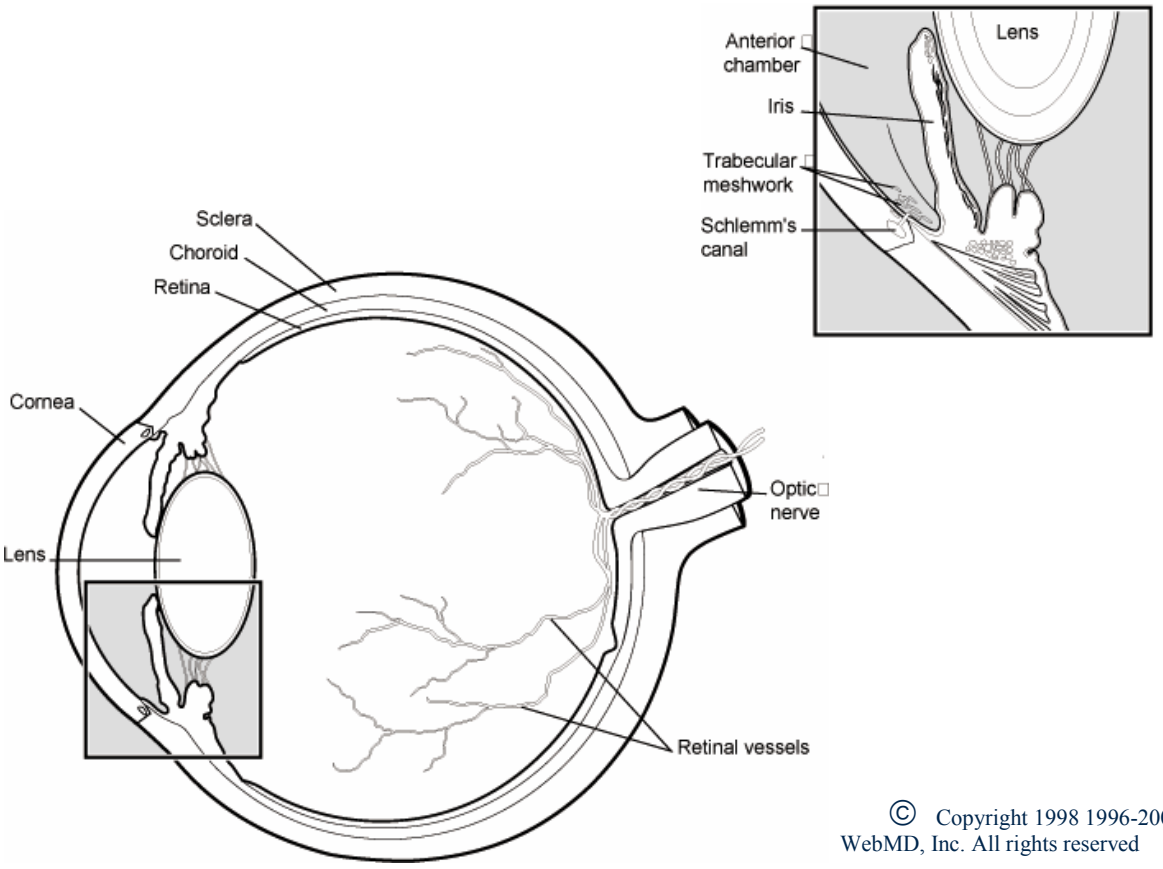


FIGURE 1. Anatomy of the eye

The AC of the eye allows light rays to pass through into the posterior segment of the eye. The cornea is the first cellular component of the AC that is exposed to the light rays. The cornea is composed of three layers of cells: the outermost corneal epithelium, which rests upon the stroma which is above the innermost layer, the endothelium. The endothelial cells are the corneal cells that are in contact with the aqueous humor in the AC. The epithelium and the stroma are capable of withstanding minor damage to the eye causing only minor edema. By contrast, the corneal endothelial cells are extremely sensitive to damage due to their inability to regenerate. This explains why in many cases, serious damage to the endothelial layer of the cornea results in blindness (16).

The fluid that fills the AC of the eye is called the aqueous humor. It is a clear fluid that is produced by the ciliary body and constantly bathes the corneal endothelium. In the human eye, the aqueous volume is approximately 250 µl and is turned over at a rate of 1.5 µl/minute. The aqueous humor leaves the AC and ultimately reaches the systemic circulation. This occurs through the trabecular meshwork that leads to the canal of Schlemm and finally enters the venous circulation. The implications of this will be discussed in more detail later.

The ciliary body, which is the source of the aqueous humor, extends into a structure called the iris. The iris divides the AC from the posterior segment of the eye and is juxtaposed to the anterior surface of the biconvex lens of the eye. The structures in the anterior segment of the eye serve to focus the light rays onto the retina and the posterior segment of the eye. These light signals are then processed in the retina and transmitted to the brain.

As explained before, preservation of an intact corneal endothelium is required for normal vision . This underlines the importance of the immune privilege in the eye. The following sections explain the term immune privilege, the factors that contribute to immune privilege, and how it is manifested by the phenomenon of ACAID.

Immune privilege of the eye

What is immune privilege?

Immune privilege is the condition in which the full array of immune processes is inhibited or excluded. Immune privileged sites include: testis, uterus, central nervous system (CNS), and eye (17). Immune responses directed toward an antigen can lead to inflammation. On one hand, inflammation can be beneficial, as it can lead to the destruction of a threatening pathogen. On the other hand, inflammation can injure normal bystander cells. If these bystander cells are incapable of regeneration, as is the case with the corneal endothelium, inflammation can be deleterious and cause permanent damage, and loss of function to an organ. Given that the corneal endothelium serves as an osmotic pump that dehydrates the cornea by removing fluid from the stroma, any imbalance in this process will cause edema that will culminate in corneal opacity. In this case, even simple inflammation that affects the corneal endothelium can pose a direct threat to vision. To guard against all these serious complications, the eye has developed immune privilege.

The eye is not the only organ that expresses immune privilege as a safeguard mechanism against undesirable immune responses. In fact, the brain, testes, and pregnant uterus also use this mechanism to protect their sensitive tissues and the allogeneic fetus from immune-mediated damage. It is important to mention that immune privilege does not universally silence all immune responses in an organ or a body site, but instead suppresses those immune responses that cause injury to 'innocent bystander' cells while preserving less harmful immune responses (4). Studying the eye as a model of immune privilege can give useful insights into other forms of immune privilege.

Initial observations of immune privilege were made in 1873 by van Dooremaal who noted prolonged survival of mouse skin grafts placed into the AC of dog eyes (18). Several decades passed until Greene confirmed the initial observations of van Dooremaal by showing that human tumors transplanted into the AC and brain of different animal species could still survive and grow progressively (19). Shortly afterwards, Medawar showed that the eye and the brain permitted prolonged skin graft survival compared to other graft beds. He coined the term “immune privilege” to describe this absence of immunological rejection. He explained this immune privilege by the lack of lymphatic drainage from the eye or the brain which leads to sequestration of the antigen from the immune system (20). This simple “immunological ignorance” explanation was later challenged by Streilein and colleagues who demonstrated that immune privilege is the product of multiple anatomical, physiological, and immunoregulatory processes (21) . They showed that alloantigenic cells placed into the AC were perceived by the immune system with its humoral and cellular components. Whereas

the humoral arm of the immune system responded by generating hemagglutinating antibodies against donor antigens, the cellular immune response was impaired. This was demonstrated by the delayed rejection of orthotopic donor-specific skin grafts in the AC-primed hosts (22, 23). These observations proved that immune privilege in the eye is not the result of immunological ignorance. Subsequent studies on CNS models of immune privilege also showed that antigens injected into the CNS gain access to peripheral lymph nodes and induce antibody production (24, 25).

Layers of immune privilege in the eye

The role of Fas ligand

Immune privilege in the eye proved to be a highly sophisticated mechanism that has many layers, each of which contributes to the overall process. One layer is manifested through Fas ligand (FasL; also called CD95L), which is a molecule that is constitutively expressed by many tissues in the eye (26). FasL can induce apoptosis of FAS^+ invading immune cells especially neutrophils and activated T cells, and thus protect the eye from immunological damage (27). This method is very effective in the elimination of lymphocytes in the ocular environment without causing much inflammation or tissue necrosis (28). Since the unusually favorable outcome of corneal transplantation is a consequence of mechanisms of immune privilege of the eye, the function of FasL on corneal allografts was tested (29). BALB/c mice received orthotopic corneal transplants from C57BL/6 (B6, $FasL^+$) and B6-gld ($FasL^-$) donor

mice (29). The rejection rate in the gld group was significantly higher than in the $FasL^+$ control group (29). This indicates that FasL is crucial for preventing immune rejection of corneal allografts (4, 29). Although the corneal endothelium is Fas^+, it can still escape apoptotic injury (30).

It is noteworthy that the ocular microenvironment influences the function of FasL. In immune-privileged sites, FasL behaves as an anti-inflammatory molecule. By contrast, in non-privileged sites FasL has proinflammatory effects. This was proven by Chen *et al*. who demonstrated that colon carcinoma cells transfected with wild type FasL were proinflammatory when injected into a non-privileged site, but the same tumor cells failed to induce inflammatory responses when injected into the AC of the eye (31). This may be due to the plethora of immunosuppressive factors present in immune-privileged sites, but not in non-privileged sites.

Expression of MHC molecules

The absence of MHC class I molecules on many ocular cells protects the eye from class I-restricted cytotoxic T lymphocyte (CTL)-mediated lysis in the event of a viral infection (21). This mechanism is particularly important with cells that have limited or no capacity of regeneration, such as the retina and corneal endothelial cells. Damage to the retina leads to retinal detachment and blindness. Since corneal endothelial cells function as osmotic pumps that are crucial for maintaining corneal deturgescence and thus, clarity, damage to these cells leads to impaired vision and, in severe cases, blindness (32). However, decreased classical

MHC class I expression can enable viruses to infect cells within the eye and escape immune elimination. In CNS, which is another immune privileged site, deficiency in classical MHC class I expression leads to virus establishment in the neurons and chronic infection (33).

In contrast to the decreased expression of classical MHC molecules, there is an upregulated expression of non-classical class Ib MHC, which contributes to the immune privilege of the eye by preventing NK cell-mediated destruction (34). Studies have shown expression of Qa-2 , a non-classical class I MHC molecule, on the corneal epithelium, endothelium, and ciliary body (34). This is important as it was shown that some MHC class Ib molecules send inhibitory signals to NK cells to protect class Ia negative cells (such as the corneal endothelial cells) from NK cell-mediated lysis (35). MHC class Ib molecules can also present antigens from invading intracellular bacteria or parasites to CTL, and thus, bring these invading organisms to the attention of the host immune system. Recognition by the immune CTL leads to their elimination, thus protecting the eye and host from their deleterious effects.

The role of TRAIL

TNF-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily that is expressed in the eye, and is important for inducing ACAID (36). TRAIL can induce apoptosis in a large number of tumors and inflammatory cells (4, 36). Studies by Lee *et al* revealed that TRAIL mRNA and protein are constitutively expressed on numerous ocular

structures, including the cornea and retina (36). Moreover, TRAIL expressed on ocular tissues was fully functional as determined by in vitro killing of TRAIL-sensitive tumor cell lines (36). These observations were supported by studies of Wang *et al* who demonstrated that $CD4^+$ T cells and corneal endothelial cells express TRAIL that induces apoptosis of syngeneic tumor cells that express DR5 receptor for TRAIL and thus, are susceptible to TRAIL-induced apoptosis (37). To confirm the role of TRAIL, they further showed that apoptosis induced by either $CD4^+$ T cells or corneal endothelial cells can be blocked with anti-TRAIL Ab (37). All of this strongly suggests a role for this molecule in ocular tumor surveillance and immune privilege of the eye.

The role of Iris/Ciliary body

Iris/Ciliary body (I/CB) cells have been shown to significantly contribute to immune privilege of the eye. First, they can directly suppress both T cell proliferation and IFN-γ secretion through a contact-dependent mechanism (38). Moreover, Yoshida *et al* have shown that T cells exposed to cultured I/CB PE (pigment epithelial) cells were induced to secrete active and latent TGF-β, which led to the generation of Tregs that inhibited the differentiation and the effector function of Th1 cells. This suggests that I/CB contribute to immune privilege by the induction of TGF-β-secreting Tregs that inhibit both T cell proliferation and DTH (39). It was also suggested that the ability of I/CB tissues to produce immunosuppressive factors contributes to the success of orthotopic corneal transplants (40). The role of TGF-β in immune suppression will be discussed later in more detail.

Anatomical factors that contribute to ocular immune privilege

It is noteworthy that one of the early classical explanations for immune privilege in the eye was based on the erroneous assumption that there is an absolute lack of lymphatic drainage in the AC of the eye, which prevents ocular antigens from reaching organized regional lymphoid tissues. Although there is very little lymphatic drainage, there is some evidence that AC-injected antigens reach the submandibular lymph nodes in the mouse (41). Also, the work of Kaplan and Streilein argues against afferent blockade, since AC injection of allogeneic cells leads to production of alloantibodies and the generation of antigen-specific Tregs (22).

Factors in the aqueous humor that contribute to ocular immune privilege

In addition to the aforementioned layers of immune privilege of the eye, ocular immune privilege is also supported by anti-inflammatory and immunosuppressive molecules present in the aqueous humor that act on both the innate and adaptive immune responses (4). TGF-β2 was one of the first immunomodulatory molecules discovered in the aqueous humor and has multiple anti-inflammatory and immunosuppressive properties. TGF-β2 was shown to inhibit the production of proinflammatory cytokines, and also counteract the effects of inflammatory cytokines (42). Other studies have demonstrated that TGF-β2 can inhibit the cytolytic activity of CTL and NK cells (43). In addition, TGF-β2 is known to regulate many cellular processes such as cell proliferation, migration, morphogenesis, extracellular matrix production, and apoptosis (44). Therefore, the presence of TGF-β2 in the aqueous humor of

the eye significantly contributes to ocular immune privilege on several levels.

The aqueous humor contains numerous other factors that contribute to the immune privilege of the eye. One of these factors is the neuropeptide alpha-melanocyte stimulating hormone (α-MSH), which inhibits cell proliferation and the production of inflammatory cytokines such as IFN-γ (45). Somatostatin, another neuropeptide present in the aqueous humor, contributes to immunoregulation by stimulating the production of immunosuppressive cytokines such as α-MSH and causing the activation of Tregs (46, 47).

The aqueous humor also contains vasoactive intestinal peptide (VIP), which at physiologically relevant concentrations has immunosuppressive properties and contributes to ocular immune privilege (48). VIP is also abundant in the nerves of the iris and ciliary body (49). VIP suppresses IFN-γ production and partially suppresses antigen-stimulated T cell proliferation (50). This serves to dampen inflammatory responses, and favor an immunosuppressive microenvironment in the eye (48).

Calcitonin gene-related peptide (CGRP) is another neuropeptide that is found in the aqueous humor. CGRP can suppress both nitric oxide and peroxide generation by macrophages (51). It can also block the antigen presenting capacity of macrophages and dendritic cells (52, 53). Accordingly, it inhibits immunogenic inflammation and *in situ* DTH expression that can result from efficient antigen presentation (51, 54).

MIF is another soluble factor in the aqueous humor. MIF protects MHC-deficient

corneal endothelial cells from NK cell-mediated lysis (32). In normal situations, inhibitory KIR molecules bind target cell MHC class I molecules and prevent NK cell attack on normal cells (55). However, if MIF were not present, ocular cells would be subjected to lysis by NK cells. This is because of the low level of MHC expression on ocular cells renders these cells easily recognizable by NK cells as described in the "missing-self" hypothesis which suggests that NK cells kill targets such as tumor and virally infected cells by being activated when it encounters a cell that is missing self class I MHC receptors.

Finally, soluble FasL, which has both anti-inflammatory and immunosuppressive properties, is present in the aqueous humor (56, 57). The numerous factors that are present in the aqueous humor and that support immune privilege in the eye are summarized in TABLE 1. The complex nature of the aqueous humor suggests that numerous factors contribute to the immune privilege in the AC and this, in turn, supports the importance of controlling inflammation in this organ (58).

Factor	Effect on immune privilege
TGF-β_2	Inhibition of T cell proliferation Inhibition of DTH Inhibition of NK cells
VIP	Inhibition of T cell proliferation Inhibition of DTH
CGRP	Inhibition of DTH
α-MSH	Inhibition of DTH
MIF	Inhibition of NK cells
Somatostatin	Stimulation of the production of immunosuppressive cytokines such as α-MSH Activation of regulatory T cells
Soluble FasL	Inhibits neutrophil recruitment and activation
IDO	T cell apoptosis
Complement regulatory proteins	Inhibition of complement cascade

TABLE 1. Soluble factors in the aqueous humor that support ocular immune privilege

ANTERIOR CHAMBER-ASSOCIATED IMMUNE DEVIATION (ACAID)

ACAID is an important aspect of immune privilege that not only affects the local environment of the eye, but acts systemically. The term ACAID was initially coined by Streilein and Niederkorn to identify this unique form of peripheral tolerance that is induced via the AC of the eye (59). They showed that DBA/2 mastocytoma cells (P815) survive and grow in the AC of BALB/c mice, but are rejected if transplanted to other sites. When these mice were subsequently grafted with skin from the same DBA/2 donors, the hosts primed in the AC with P815 cells failed to reject the DBA/2 skin grafts. However, these mice were still capable of rejecting skin grafts from third party donors, suggesting that the immune suppression was antigen-specific. This antigen-specific delay in graft rejection was associated with a systemic down-regulation of DTH (59-61).

ACAID is induced when an antigen is injected into the AC of the eye, either by intracameral (IC) injection or by corneal transplantation (**FIGURE 1**). ACAID can be induced with viral (62), soluble (63), or histocompatibility antigens (64). It has been demonstrated with every soluble antigen tested so far. After IC injection of the antigen, an antigen-specific suppression of DTH ensues due to the generation of Tregs that are systemically distributed and can be found in peripheral lymph nodes and the spleen (65). ACAID also results in the generation of alloantibodies, and a significant delay in the rejection of orthotopic donor-specific skin allografts in hosts primed in the AC (22, 23). Thus, cell-mediated immune responses are impaired, while humoral immune responses are largely preserved. However, the humoral immune response involves a shift from

complement-fixing antibody isotypes (IgG2a) toward the synthesis of non-complement fixing antibodies (IgG1) (66, 67).

The systemic consequences of AC introduction of antigen suggests that there is a connection between the ocular environment and systemic circulation. It is believed that antigens escape from the AC by passing through the trabecular meshwork, then through the canal of Schlemm and episcleral blood vessels into the systemic blood vasculature of the host (17). Ocular APC migrate to the spleen and the thymus where they interact with cells in both organs. It was demonstrated that an intact functional spleen was required for the generation of ACAID (59). Similarly, removal of the thymus also prevents the induction of ACAID (68). It was recently reported that an intact sympathetic nervous system is also involved in the induction of ACAID (69). In this study, chemical sympathectomy inhibited the induction of ACAID (69). This may be due to the disruption of the dense sympathetic innervations that are present in the eye, thymus, or spleen (69). Thus, the induction of ACAID involves multiple cell populations in at least 4 organ systems.

Organs and cells involved in ACAID

The four main organs that are involved in ACAID are: 1) eye (70); 2) thymus (68); 3) spleen (59); and 4) sympathetic nervous system (69). The importance of the eye has been demonstrated by experiments in which the eye was removed within three days of IC injection of the antigen; that is, the enucleated mice did not develop ACAID (70-72). However, removal of the eye seven days after IC injection did not prevent the generation of ACAID

(70, 71). This demonstrated that an intact eye is needed only during the first three days after IC priming with antigen, but is dispensable thereafter. In the eye, the antigen is captured by ocular F4/80^{+}macrophages, which are bone marrow-derived cells present in the stroma of the I/CB tissues (73, 74). These ocular APC are subjected to a plethora of immunosuppressive factors in the intraocular environment. TGF-β2 is the most critical factor in the aqueous humor that affects the behavior of the F4/80^{+} ocular APC (macrophages) (75, 76). Treating normal APC with TGF-β2 at concentrations present in the aqueous humor (2 ng/ml) endows them with ACAID-inducing properties (77). As few as 20 of these ACAID-inducing APC are capable of inducing ACAID when adoptively transferred to naïve recipients (73). This suggests that there is significant amplification of the tolerizing response that is initiated by the ACAID-inducing APC (4) .

ACAID-inducing APC have reduced expression of CD40 as compared to regular (non-ACAID-inducing) APC (78). In addition, ACAID APC secrete lower amounts of the pro-inflammatory cytokine IL-12, but produce higher amounts of the immunosuppressive cytokine IL-10 than non-ACAID APC (78, 79). Production of IL-10 by ACAID-inducing APC is crucial for ACAID to be generated as APC from IL-10 KO mice cannot induce ACAID, suggesting a major role for IL-10 secretion by the ocular APC in the generation of ACAID. In addition, AC injection of OVA into IL-10 KO mice elicited normal DTH responses rather than ACAID (63).

Antigen-bearing ocular APC then migrate from the eye to the thymus and then to the spleen (68). Within 3 days of entering the thymus, ocular APC elicit the generation of CD4^{-}

$CD8^-$ NK T cells, which carry the immunoregulatory activity from the thymus to the spleen (6, 9). Once in the spleen, $CD4^-$ $CD8^-$ NK T cells contribute to the generation of splenic Tregs (80). The $CD4^-$ $CD8^-$ NK T cells are also capable of transferring ACAID to naïve recipients (9). Although it is not clear how the ocular APC interact with cells in the thymus to generate ACAID, it is certain that the thymus is necessary for ACAID, as thymectomy prevents the generation of ACAID (68).

Antigen-bearing ocular APC are also believed to migrate directly from the eye to the spleen (74, 81), where they interact, either directly or indirectly, with $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK T cells, and γδ T cells. These interactions lead to the production of antigen-specific Tregs that down-regulate DTH. (81). Collectively, these findings support the existence of a cameral-splenic axis that links the ocular environment with the splenic environment for the generation of ACAID Tregs (65).

Many studies on the induction of ACAID have focused on the spleen, as this is the organ where most cellular interactions take place during the induction of ACAID (59). It is also the organ where the Tregs are generated following IC injection of the antigen Moreover, Tregs isolated from the spleen will adoptively transfer ACAID to naïve recipients (12). ACAID Tregs are antigen-specific and inhibit the expression of DTH responses for the antigens that were used for the AC priming (12).

Regulation of DTH by ACAID Tregs

Within the AC, $F4/80^+$ macrophages are bathed in a variety of immunosuppressive and

anti-inflammatory cytokines, including TGF-β2, which influence their antigen presentation and tolerance-inducing behavior (74, 82). Antigen-bearing $F4/80^+$ ocular APC enter the venous circulation within 48 hr of introducing the antigen into the AC (9, 74). After capturing antigens in the AC, $F4/80^+$ ocular macrophages migrate from the eye into the thymus and spleen by way of the blood vascular route, as the eye has no patent lymphatic drainage channels (17, 83). These ACAID macrophages have been shown to possess the ability to secrete IL-10 (79). It is possible that this IL-10 production by ACAID APC influences the generation of Tregs in the spleen. These Tregs can act at two potential phases of DTH responses:

1-The **Afferent** or **Sensitization** Phase: This is the phase in which the antigen is processed by APC and presented to naïve T cells to prime antigen-specific T cells. Inhibition of the afferent arm of the immune response prevents the antigen from being perceived and processed by the immune system.

2- The **Efferent** or **Expression** Phase: This occurs upon a second exposure to the antigen and results in inflammation and antigen-specific DTH.

Induction of ACAID involves the participation and interaction of $CD4^+$ afferent Tregs and $CD8^+$ efferent Tregs (84). The $CD4^+$ afferent Tregs are believed to prevent perception of the antigen, and thus prevent the generation of DTH responsiveness (84-86). On the other hand, the $CD8^+$ efferent regulatory cells suppress the expression of an immune response by inhibiting DTH responsiveness in previously immunized hosts (84-86). There is some sort of cross-talk between these two cell populations, as it was shown that $CD4^+$ T cells are also

required for the generation of $CD8^+$ efferent Tregs (10). However, $CD8^+$ efferent Tregs do not influence the development of $CD4^+$ afferent Tregs (84-86).

$CD4^+$ Tregs

$CD4^+$ Tregs might inhibit the generation of T cell immunity by preventing the clonal activation, expansion, or proliferation of antigen-specific T cells. The $CD4^+$ afferent Tregs might accomplish this by inducing apoptosis of the antigen-specific T cells or by elaborating cytokines that prevent T cell proliferation. The latter hypothesis was examined by Skelsey *et al* who showed that IL-10, but not IL-4, secretion by ACAID $CD4^+$ Tregs was necessary and sufficient for the generation of $CD8^+$ Tregs. In addition, they demonstrated that direct cell contact between $CD4^+$ Tregs and $CD8^+$ Tregs was not required for ACAID (10). Earlier studies have demonstrated that during ACAID, $CD4^+$ T cells secrete low insignificant levels of IL-2 and IFN-γ, but high levels of IL-10 (87). Other studies have confirmed the finding that IL-10, and not IL-4, is crucial for the development of ACAID. It was also shown that expression of the CD25 molecule (the IL-2α receptor) on $CD4^+$ T cells was necessary for the generation of $CD8^+$ efferent Tregs (10, 88).

$CD8^+$ Tregs

As indicated before, $CD8^+$ efferent Tregs can inhibit the efferent phase of the immune response. Efferent Tregs can be generated *in vivo* by IC injection of antigen. Efferent Tregs can be also produced *in vitro* by incubating antigen-pulsed ACAID APC with naïve spleen cells for 5-7 days. This *in vitro* system mimics the spleen of an ACAID-induced

animal and has been previously characterized (13, 14, 89, 90). Both *in vivo* and *in vitro*-generated $CD8^+$ Tregs can be demonstrated in a local adoptive transfer (LAT) assay. In this assay, the ear pinna of a naïve mouse receives a mixture of putative ACAID regulatory cells and immune effector cells that can produce DTH lesions when co-injected with relevant antigen (91). The presence of $CD8^+$efferent regulatory cells is demonstrated by the inhibition of an ear swelling DTH response that is normally produced by immune effector cells that are co-injected with relevant antigen in the ear.

$CD8^+$ efferent ACAID Tregs generated *in vitro* secrete high levels of TGF-β (92). However, efferent ACAID Tregs treated with anti-TGFβ2 prior to injecting them in a LAT assay are still capable of inhibiting DTH, indicating that the suppressive function of $CD8^+$ efferent ACAID Tregs is not mediated through TGFβ2(93). Other studies have shown that $CD8^+$ efferent Tregs do not mediate suppression by inducing apoptosis of immune cells through a Fas-FasL interaction (94). Thus, the mechanisms used by efferent Tregs to inhibit the expression of DTH is still not known. Moreover, the precise cellular mechanisms that lead to the production of $CD8^+$ efferent Tregs are not fully understood.

As mentioned before, ACAID involves interactions of many different cell populations. To gain a better understanding of efferent suppression in ACAID, the roles of each of these cells and the cross-talk between each of these cell populations needs to be fully investigated. The next sections will discuss briefly what is known about different spleen cell populations that contribute to the generation of $CD8^+$ efferent Tregs in ACAID.

NK T cells

The initial observation of increased numbers of NK T cells, and not conventional NK cells, in spleens of ACAID mice have suggested the involvement of NK T cells in the generation of ACAID (6). The requirement of NK T cells for ACAID was demonstrated by the inhibition of ACAID generation in CD1 KO mice that lack NK T cells (95). Furthermore, reconstitution of these mice with NK T cells and $CD1^+$ APC led to the restoration of ACAID. Thus, CD1d-reactive NK T cells are needed for the induction of ACAID, but do not act directly as efferent regulatory cells (6, 96, 97).

ACAID-inducing $CD4^-$ $CD8^-$ $NK1.1^+$ cells are produced in the thymus before migrating to the marginal zone of the spleen (9, 97, 98). Recruitment of the NK T cells to the spleen requires the chemokine, macrophage inflammatory protein-2 , MIP-2 (97). NK T cell deficient mice cannot develop ACAID when reconstituted with NK T cells from IL-10 KO mice (96). This suggests that NK T cells must produce IL-10 in order to generate ACAID efferent Tregs in the spleen (96). Faunce *et al* examined frozen tissue sections from the spleens of mice at various days after AC-inoculation of OVA (97). Confocal microscopical examination revealed that after 5-7 days, NK T cells colocalized in the marginal zones with the $F4/80^+$ cells and conventional marginal zone T cells (97). In a subsequent study, Faunce *et al* demonstrated that CD1d stimulation of NK T cells induced them to produce RANTES, which is another chemokine that attracts additional $F4/80^+$ APC and $CD8^+$ T cells, resulting in the formation of discrete cell clusters in the marginal zone of the spleen (98). Expression of RANTES mRNA in NK T cells of AC-injected B6 mice

pretreated with anti-CD1d mAb was significantly less than their littermates that received control rat IgM pretreatment suggesting that CD1d-NK T cell interaction is needed for NK T cells to secrete RANTES during induction of peripheral tolerance(98). Treating mice with anti-RANTES mAb abolished the induction of Tregs in ACAID indicating that release of RANTES with the subsequent recruitment of $F4/80^+$ APCs and potential $CD8^+$ T cells is crucial for the differentiation of $CD8^+$ Tregs in ACAID (98).

B cells

B cells play a crucial role in other forms of antigen-specific suppression by acting as tolerogenic APC (13, 89, 90). This is consistent with their proposed contribution to the development of ACAID. Indeed, studies have shown that B cells are needed for the generation of ACAID, as animals treated with anti-IgM antibody (89) and B cell KO animals (90) cannot express ACAID (89, 90). Subsequent studies have shown that antigens introduced into the AC induce the formation of a B-cell population that can transfer ACAID to naive recipients, which suggests that ACAID B cells, like ACAID ocular APC, deliver an antigen-specific signal that elicits the development of Tregs (13).

Additional studies by Skelsey *et al* have shown that B cells act as ancillary APC in the generation of ACAID (14). They showed that B cells treated with chloroquine (to inhibit antigen processing and presentation) were unable to contribute to the generation of ACAID Tregs (14). This demonstrated that phagolysosomal acidification of internalized antigen is essential before TAP-independent presentation of antigen to Tregs can occur (14). These

results supported the hypothesis that B cells contribute to the generation of ACAID by internalizing and processing antigenic moieties that are released from ACAID-inducing ocular APC. Skelsey *et al* further showed that the B cell receptor (BCR) is necessary for the induction of ACAID, and that the BCR conveys antigen specificity for the generation of ACAID Tregs (14). According to this model, B cells capture antigen that is released from ocular APC, internalize the antigenic moieties through the BCR, and then presents the antigen to Tregs. This model is in line with previous studies that demonstrated that macrophages in other body sites, such as the lung and peritoneum, can process antigen and regurgitate peptide fragments that can be captured by a second population of APC, which can then present the peptides to T cells (99-101).

Gamma delta T cells

The vast majority of T cells express a T-cell receptor (TCR) composed of αβ heterodimers. However, there is a group of T cells that expresses a γδ heterodimer TCR (102). These cells have been called γδ T cells, and are typically $CD4^-/CD8^-$ (103). However, $CD8^+$ γδ T cells and $CD4^+$ γδ T cells have also been reported (104, 105). γδ T cells are involved in several forms of immune tolerance, such as oral tolerance (106), testicular tolerance (107), nasal tolerance (104, 108), and tumor-associated tolerance (109-111). γδ T cells also contribute to the immune privilege of allogeneic fetuses during pregnancy (112, 113). In addition, γδ T cells are crucial for the development of ACAID (114). ACAID cannot be generated in mice treated with anti-TCRδ chain antibody or in γδ KO mice (114). It has also been shown that γδ T cells are required for corneal allograft survival through their role in the corneal allograft's

induction of ACAID (114). However, the exact role of γδ T cells in the downregulation of Th1 immune responses such as DTH remains unclear.

ACAID VERSUS IV-INDUCED TOLERANCE

Intravenous (IV) injection of an antigen before immunization is a potent way to induce immune regulation at the T-cell level. Because there are no patent major lymph vessels that drain the AC, the bulk of the antigen introduced into the AC must leave via the blood vasculature. Thus, it was thought by some that ACAID was merely another form of IV-induced tolerance (115, 116). Although both ACAID and IV-induced tolerance elicit systemic suppression of DTH, these two forms of tolerance are different. For instance, B cells were shown to play a critical role in the induction of ACAID, but their involvement in IV-induced tolerance is still controversial (89, 90). Furthermore, efferent Tregs, blood borne-APC, NK T cells, IL-10, and β_2-microglobulin are all unique to ACAID, whereas IL-4 is unique to IV immune deviation (54). The requirement of CD1d-NK T cell interaction, and the need for cluster formation between NK T cells, $F4/80^+$ APC, and $CD8^+$ T cells in the marginal zone of the spleen are all characteristics of ACAID, but not of IV-induced tolerance (97, 98). TABLE 2 summarizes the major differences between ACAID and IV-induced tolerance.

Requirement	ACAID	IV-Induced Tolerance
IL-4	No	Yes
IL-10	Yes	No
Blood-borne APC	Yes	No
NK T cells	Yes	No
Efferent regulatory cells	Yes	No
β2 microglobulin	Yes	No

TABLE 2. Differences between ACAID and IV-induced tolerance

SIGNIFICANCE OF ACAID

ACAID has many clinical implications, as it protects the eye from immune-mediated damage and contributes to corneal allograft survival (117). Injection of donor cells into the AC before corneal transplantation enhances corneal allograft survival (117, 118). On the other hand, preventing the induction of ACAID by splenectomy results in increased corneal allograft rejection (117, 118). Loss of immune privilege in the eye might cause idiopathic uveitis, and sympathetic ophthalmia although this has not been formally proven (4). Ocular infections do not induce ACAID and instead elicit DTH responses that might produce blindness due to immune-mediated injury to innocent bystander tissues in the cornea (119). Keratitis caused by *Herpes simplex* virus infection of the cornea and trachoma caused by *Chlamydia trachomatis* infection of the conjunctiva are examples of DTH-mediated diseases of the eye. In both cases, strong DTH responses to microbial antigens lead to rampant ocular inflammation that culminates in blindness (119, 120).

CURRENT INVESTIGATIONS

The work described in this dissertation focuses on two spleen cell populations that are crucial for the generation of efferent Tregs in ACAID, namely B cells and γδ T cells. The purpose was to determine how each of these cell populations contributed to the generation of Tregs in an ACAID model. In order to determine the roles of B cells and γδ T in inducing T cell tolerance, some of the events occurring upstream or downstream of these cells were

examined. Unraveling cellular and molecular splenic interactions and cross-talk between B cells and γδ T cells in relation to other cells involved in ACAID will permit us to better dissect the mechanism of induction of ACAID. This will also facilitate studying the induction of T cell tolerance in other models that may share the same fundamental mechanisms with ACAID.

Since the numbers of splenic B cells with a BCR specific for any given antigen are too few to be able to participate efficiently in the induction of ACAID, we examined the expansion of B cells following the initiation of ACAID with an AC injection of antigen. The question of whether expansion of an antigen-specific B cell population was required for the generation of ACAID was also considered. In order to better analyze the participation of different cellular components involved in ACAID, splenic cellular interactions upstream and downstream of B cells were studied. Upstream of B cells, we looked at the importance of cell-cell contact between ocular APC and B cells in the generation of Tregs. We believed that this experimental approach would shed some light on whether soluble factors are the main inducers of the tolerogenic signal originating from the $F4/80^+$ APC to the B cells. Downstream of B cells, we examined the antigen presentation functions of B cells during the induction of ACAID. Puzzled by the observations that both $CD4^+$ afferent regulatory cells and $CD8^+$ efferent Tregs can work together to mediate antigen specific generation of T regulatory cells, we proposed a model of antigen presentation of B cells in ACAID. The central hypothesis, in this line of investigations, is that B cells use both MHC class I and MHC class II molecules to present antigens to $CD4^+$ T cells (in the context of MHC class II)

and to $CD8^+$ T cells (in the context of MHC class I). A second hypothesis examined the contribution of γδ T cells in the generation of efferent ACAID Tregs. Several hypotheses were proposed for the functions of γδ T cells in ACAID. The hypothesis in which γδ T cells are the end stage Tregs that inhibit the expression of DTH was examined. We also tested if γδ T cells act as APC in the initiation of ACAID. A final hypothesis proposed that γδ T cells acted as cytokine producers. We tested whether γδ T cells were a crucial source of IL-10, IL-4, or IFN-γ that was necessary for ACAID to be produced.

CHAPTER THREE

Materials and Methods

Animals

C57BL/6 (H-2^b) mice; C57BL/6 B-cell receptor transgenic (BCR-Tg) mice (H-2^b), C57BL/6-Tg (IghelMD4) 4Ccg/J; B6.129P2-*β2m*^{tm1Unc}/J (β2 microglobulin knockout, KO, or class I-deficient) mice; B6.129S2-*Igh-6*^{tm1Cgn}/J B cell-deficient (B-cell KO) mice; δ-chain knockout mice (TCR δ KO) (C57BL/6J-*Tcrd*^{tm1Mom}), IL-4 knockout mice (B6.129P2-*Il4*^{tm1Cgn}/J**),** IFN-γ knockout mice (B6-*IFNγ*^{tm1Ts}/J**)**, and IL-10 knockout mice (B6.129P2-*IL-10*^{tm1Cgn}/J) were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129-H2-*Ab1*^{tm1Gru} N12 (MHC class II deficient) mice were purchased from Taconic (Hudson, NY). All animals were housed and cared for in accordance with the guidelines of the University Committee for the Humane Care of Laboratory Animals, the NIH Guidelines on Laboratory Animal Welfare, and the "Association for Research in Vision and Ophthalmology" Statement for the Use of Animals in Ophthalmic and Vision Research.

Screening BCR-Tg mice

BCR-Tg mice (C57BL/6-Tg (IghelMD4)4Ccg/J) are hemizygotic for the hen egg lysozyme (HEL) transgene expressed on B cell receptors (121). More than 90% of the splenic B cells display the proper allotype, and about 60% to 90% can bind HEL (94, 121). PCR was used to screen DNA isolated from tails of their offspring to detect the expression of the BCR transgene according to The Jackson Laboratories protocol. The PCR products were visualized on a 1.5% agarose gel containing ethidium bromide. A 430 bp band indicated a BCR-Transgene–positive animal.

Antibodies

Anti-gamma delta (GL3) Ab was produced from hybridoma cells and then purified by protein A columns. The hybridoma was graciously provided by Dr. Leo Lefrancois (University of Connecticut, Farmington, CT). This Ab blocks the δ chain of the TCR and thus inhibits the function of γδ T cells (122). Animals were treated IP with 500 µg GL3 Ab on days –3, +4, and +11. This *in vivo* depletion protocol was slightly modified from the one used by Skelsey *et al* (114). In other experiments, γδ T cells were depleted *in vitro* using purified anti-mouse γδ TCR (UC7-13D5) (BD Biosciences, San Jose, CA) together with complement (Cedarlane Laboratories, Ontario, Canada). $CD8^+$ T cells were depleted *in vitro* using purified rat anti-mouse CD8a (Ly-2) (BD Biosciences) together with complement. Two antibody isotype controls were used: Hamster IgG3 kappa for the UC-7, and rat IgG2a

kappa for the Ly-2 (BD Biosciences). Phycoerythrin-conjugated anti-mouse γδ T-cell receptor (GL3) ab (BD Biosciences) was used to specifically label γδ T cells from the B cell-depleted spleen cell population of normal C57BL/6 mice, and in the various KO mice (IL-10 KO mice, IL-4 KO mice, and IFN-γ KO mice) prior to sorting of the γδ T-cell population at the flow cytometric core facility of U.T Southwestern Medical Center.

Subcutaneous immunization

Mice were immunized by subcutaneous (SC) injection of 250 μg of ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS). OVA was emulsified 1:1 (v/v) in complete Freund's adjuvant (CFA; Sigma). Each animal received a total volume of 200 μl.

Anterior Chamber (AC) injection

Mice were anaesthetized with 0.133 mg/Kg ketamine hydrochloride (Fort Dodge Laboratories, Fort Dodge, IA) and 0.006 mg/Kg xylazine (Bayer Corporation, Shawnee Mission, KS) given IP. A glass micropipette was fitted into a sterile infant feeding tube (no. 5 French, Professional Medical Products Inc., Greenwood, SC) and mounted onto a 0.1 ml Hamilton syringe (Hamilton Co. Inc., Whittier, CA). The Hamilton automatic dispensing apparatus was then used to inject 100 μg (in 5 μl) of either OVA or HEL into the AC as previously described (14).

Delayed-type hypersensitivity (DTH) assay

An ear swelling assay was used to measure DTH to either OVA or HEL as previously described (10). Results are expressed as: specific ear swelling = (24 hr measurement – 0 hr measurement) for experimental ear – (24 hr measurement – 0 hr measurement) for negative control ear.

Isolation of peritoneal exudate cells (PEC)

Naïve C57BL/6 mice were injected IP with 2 ml of aged 3% thioglycolate (Sigma) that had been stored at room temperature for 1-4 months to increase the yield of peritoneal macrophages (123). Mice were sacrificed 3 to 5 days later. The peritoneal cavities of these mice were washed twice with HBSS in order to isolate peritoneal exudate cells. The isolated cells were plated on Primaria Petri dishes (Becton Dickinson Labware, Franklin Lakes, NJ). The cells were cultured for 1.5 hours at 37^{o}C and 5% CO_2. Cold HBSS was used to gently wash away the non-adherent cells, and a rubber cell scraper was used to remove the adherent macrophages. Cells are then suspended in RPMI + 2-ME and ready for use.

Generation of ACAID-inducing APC

ACAID APC were generated *in vitro* using a previously described protocol that has been used extensively for analyzing Tregs in ACAID (3, 5, 6, 96, 124-126). Macrophages were collected from C57BL/6 mice (as described above) and cultured overnight (2 x 10^6 cells/ml) in complete RPMI medium supplemented with 10 mg/ml OVA and 2 ng/ml human TGF-β2 (R&D Systems, Minneapolis, MN), which is the isoform that is exclusively produced within the eye. These macrophages act as APC and induce peripheral tolerance that is identical to ACAID (5, 8, 14).

Generation of ACAID-inducing B cells

An *in vitro* culture system was used to generate B cells that are capable of inducing Tregs that express the same phenotype as those induced by AC injection of the antigen (8, 9, 124). OVA-pulsed ACAID APC generated *in vitro* (as described above) were washed three times in HBSS, then co-cultured for 48 hr with B cells isolated from the spleens of normal C57BL/6 mice using CD45R (B220) microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany). B220 antigen is expressed on all mouse B cells. The idea of MACS bead separation relies on the fact that anti-B220 antibody-labeled magnetic beads can positively select B cells from among a mixture of other cells. After 15 min. of incubation at 4^{o}C, the mixed cell suspension (treated with the beads) is then passed through the MACS column where the magnetically labeled beads are retained on the MACS Column in the magnetic

field of a MACS separator, while the unlabelled non-B cells pass through the column. The column can then be released from the magnetic field and the positively selected B cells can be eluted with a degassed bead buffer (PBS is supplemented with 0.5% bovine serum albumin and 2 mM EDTA then the buffer is degassed by applying vacuum). The purity of B cell separation by microbeads was checked by FACS analysis and was shown to be >95%. Cells were collected and plated on Primaria (Becton Dickinson) Petri dishes and incubated at 37^{o}C in 5% CO_2 for 1.5 hr. The non-adherent B cell population was collected and treated with anti-F4/80^{+} antibody (Accurate Chem., Westbury, NY) + complement (Cedarlane Laboratories, Ontario, Canada) to kill any residual macrophages. These ACAID-inducing B cells were then adoptively transferred (By harvesting the cells followed by IV injection) into either normal C57BL/6 mice or γδ T cell KO mice (4 x 10^6 B cells/mouse). In some experiments, B cells were treated with 50 μg/ml mitomycin-C (Sigma) or γ-irradiation (2400 rad) prior to incubation with OVA-pulsed ACAID APC. Mitomycin-C and γ-irradiation were used to inhibit proliferation of B cells. In order to titrate the minimum number of ACAID B cells required for ACAID generation, experiments were performed so that different numbers (2.5 x 10^5, 5 x 10^5, 10^6, 2 x 10^6, 4 x 10^6) of ACAID B cells were injected into normal C57BL/6 mice. The B cells were collected and then adoptively transferred into normal C57BL/6 mice as described above. The viability of the B cells was always determined by trypan blue exclusion immediately prior to the adoptive transfer and was always > 95%.

Tritiated thymidine incorporation assay

A tritiated thymidine incorporation assay was used to quantify B cell proliferation. B cells ($3x10^5$) were added to a 96-well plate and incubated with either ACAID APC ($2.5x10^4$; described above), or Non ACAID APC ($2.5x10^4$; APC incubated with OVA, without adding TGF-β2) in a 100-150 μl total volume for two, three , or five days. As a positive control for proliferation, 25 μg/ml lipopolysaccharide, from *Escherichia coli* serotype 055:B5 (Sigma), was added to the B cells. B cells alone were used as a negative control. Tritiated thymidine (0.5 microcuries; ICN Biomedicals, Inc; Irvine, California) was added 12 hours before the end of the incubation period. At the end of the incubation period, the non-adherent B cells were collected and radioactivity was counted in a beta-counter.

Flow cytometric analysis

BCR-Tg mice were injected in the AC with either OVA (Sigma), HEL (Sigma), or PBS. Seven days later, spleen cells were harvested and B cells purified using CD45R (B220) microbeads (Miltenyi Biotec). B cells were then stained with HEL-Biotin (Abcam, Cambridge, MA) followed by streptavidin FITC (BD Biosciences). A single-color flow cytometry was performed to assess proportions of BCR specific for HEL antigen. To analyze the data, CellQuest Pro software was used on a FACSCalibur instrument (BD Biosciences, San Jose, CA).

***In vitro* ACAID model of T regulatory (Treg) cell generation**

We used an *in vitro* spleen cell culture system that generates Tregs that express the same properties and surface markers as Tregs produced by AC injection (5, 8, 10, 13, 14, 90). These *in vitro*-generated Tregs are antigen-specific $CD8^+$ T cells that can directly inhibit DTH (14). The use of an *in vitro* model for generation of Tregs in ACAID has proven very useful in permitting us to dissect cellular interactions of individual cell populations. ACAID APC (5×10^6) were added to a large Petri dish (Falcon 3003; BD Biosciences) containing 5×10^7 spleen cells harvested from either normal C57BL/6 mice or γδ T cell KO mice. In some experiments, cell populations were cultured in transwell chambers separated by a semipermeable membrane (Costar, Corning, NY; pore size 0.4 μm). Spleen cell cultures were incubated for 5 to 7 days at 37°C before being tested for the presence of Tregs. Viability of the *in vitro*-generated Tregs was always >95% as assessed by trypan blue exclusion. Trypan blue stains dead cells only. Thus, cells that will not get the blue stain are viable cells.

Reconstitution of the *in vitro* ACAID culture

In some experiments, ACAID APC were co-cultured with B cell-depleted spleen cell suspension that were reconstituted with B cells from C57BL/6 mice, MHC class II deficient mice, or β2 microglobulin KO mice. To ensure depletion of APC from spleen cells, non-adherent cells were further treated with anti-$F4/80^+$ antibody (Accurate) + complement

(Cedarlane laboratories). In other groups, 10 ng/ml of recombinant mouse IL-10 (R&D Systems) was added to the culture media.

In other experiments, two spleen equivalents of γδ T cells (sorted from spleen cells of C57BL/6 mice, MHC I KO mice, or MHC II KO mice) were used to reconstitute spleen cell cultures from γδ T cell KO mice. In our hands, the yield of γδ T cells from two spleens ranged from 5 x 10^5 to 1 x 10^6 γδ T cells. One group of the reconstituting γδ T cells was treated with chloroquine (80 μM/ 2x10^5 cells) (Sigma-Aldrich), checked for viability by trypan blue exclusion, and then added to reconstitute the *in vitro* spleen cell cultures. Chloroquine was used as it inhibits endosomal and lysosomal acidification required for efficient MHC II presentation. In other groups, 10 ng/ml of either recombinant mouse IL-10 (R&D Systems) or recombinant mouse IL-4 (R&D Systems) was added to the culture medium.

Local adoptive transfer (LAT) Assay

This assay was used to test for Tregs in ACAID (13, 14). Tregs can be generated using the *in vitro* ACAID model or by AC injection of the antigen. To perform the assay, immune spleen cells were harvested 14 days after SC immunization of naïve C57BL/6 mice with 250 μg of OVA in PBS emulsified in CFA. Putative Tregs were co-injected (1x10^6 cells in 10 μl PBS) with immune spleen cells (1x10^6 cells in 10 μl PBS) collected from SC immunized donors, and 10 mg/ml of OVA or HEL into the left ear pinna of a naïve mouse. The right ear

pinna was injected with either 10mg/ml of OVA or HEL without cells as a negative control. Ear swelling was measured 24-48 hr later with a Mitutoyo engineer's micrometer and compared to the measurement of the ears of naïve C57BL/6 mice immediately prior to challenge. Results were calculated as: specific ear swelling = (24 hr measurement – 0 hr measurement) for experimental ear – (24 hr measurement – 0 hr measurement) for negative control ear. Results are expressed as ear swelling x 10^{-4} inches. The presence of Tregs was demonstrated by the suppression of the ear swelling responses mediated by immune spleen cells.

Statistics

All experimental and control groups contained 5 animals per group. Statistical significance of DTH was determined using the Student's t-test. P values < 0.05 were considered to be statistically significant.

CHAPTER FOUR

Results

B CELLS AND GAMMA DELTA T CELLS IN ACAID

A TYPICAL ACAID EXPERIMENT

ACAID can be induced by AC injection of the antigen or via corneal transplantation. In a typical ACAID experiment, OVA antigen was introduced into the AC of normal C67BL/6 mice on day 0. Seven days later, the mice received SC immunization of OVA+ CFA. On day 14, mice received intradermal injections of OVA in the ear pinnae. On day 15, ear swelling was measured. Whereas a positive ear swelling indicates a positive DTH response, no ear swelling indicates a suppressed DTH response. The down-regulation of DTH response in ACAID is antigen-specific. Positive controls in a typical ACAID experiment consist of mice receiving only SC immunization with OVA+CFA followed by intradermal injection of the antigen into the ear pinna one week later. This leads to ear swelling indicative of a positive DTH response. Negative controls in a typical ACAID experiment are mice that receive antigen in the ear pinna without any previous exposure to the antigen. Five mice were typically used per group. Results of such an experiment are shown in **FIGURE 2.**

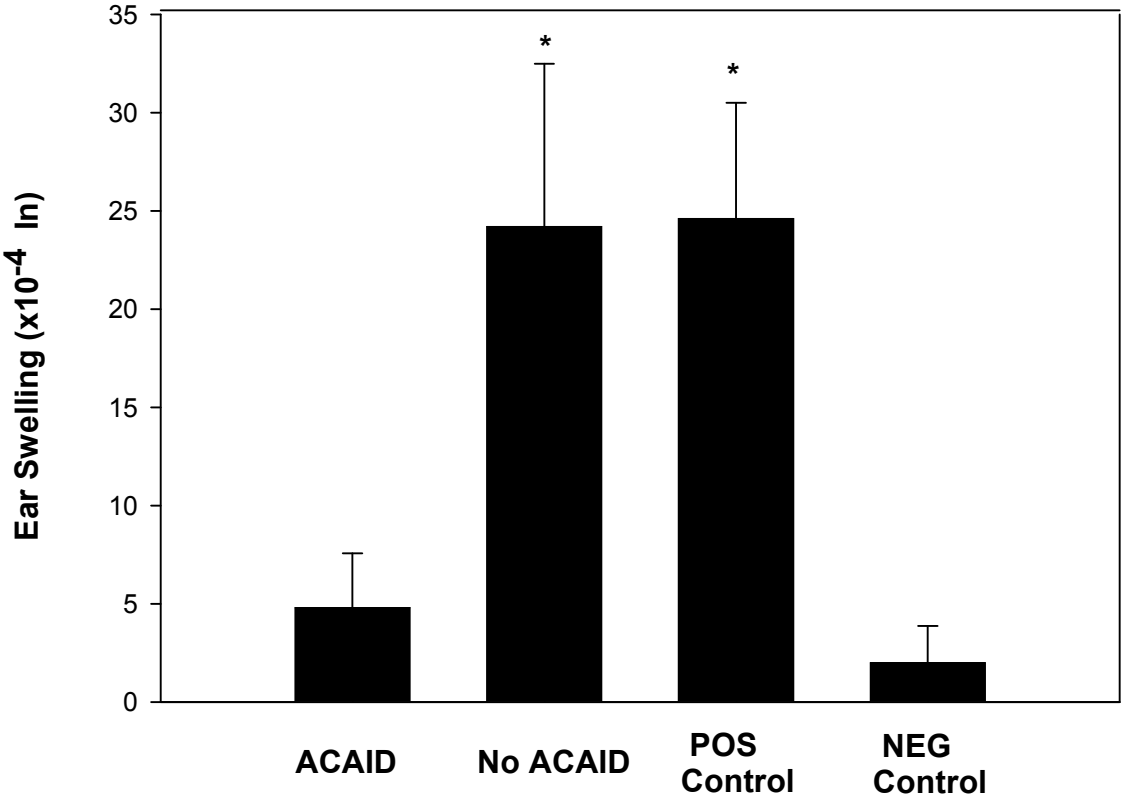


FIGURE 2. Typical ACAID result

ACAID was generated by injecting soluble antigen, OVA into the AC of the eyes of C57BL/6 mice. AC injected mice were immunized SC on day 7. On day 14, antigen was injected intradermally into the ear pinnae of mice to test for ear swelling response. Contralateral ears received PBS only. Positive control mice were SC immunized on day 7, and tested for DTH with antigen on day 14. Negative control mice received antigen on day 14 only. Ear swelling was measured 24 hours after intradermal injection into the ear pinnae. (P value <0.01)

CELLULAR INTERACTIONS UPSTREAM OF B CELLS IN THE INDUCTION OF ACAID

Previous work has suggested that during the induction of ACAID, ocular APC capture and process antigen, but do not directly present the antigen to T cells. Instead, ocular APC release antigenic peptides to splenic B cells, which in turn present the antigen to T cells (13, 14). Taking this into consideration, experiments were performed to determine if cell contact between ocular APC and splenic B cells was needed for the generation of ACAID Tregs. In the past, we generated ACAID Tregs *in vitro* by culturing naive spleen cells with OVA-pulsed ACAID APC. However, in these experiments, a transwell culture system was used in which ACAID APC and $F4/80^+$ APC-depleted spleen cells were separated by a semi-permeable membrane to prevent direct contact between the two cell populations, yet permit soluble factors to pass through the membrane. After 5-7 days in culture, a LAT assay was performed on the spleen cells to detect ACAID Tregs. The LAT assay is based on the principle that OVA-specific $CD8^+$ ACAID Tregs, if generated, will directly suppress the expression of DTH responses produced by OVA-immunized T cells that normally induce an ear swelling response when mixed with OVA and injected into the ears of naïve mice. The results of a typical LAT assay showed that ACAID Tregs were generated whether direct contact between ACAID APC and spleen cells was inhibited or allowed **(FIGURE 3)**. This demonstrated that direct contact between ocular APC and spleen cells is not needed for ACAID induction. Importantly, culturing spleen cells in the presence of TGF-β alone did not lead to the development of ACAID Tregs. This demonstrated that the generation of Tregs

required ACAID APC and was not simply an effect of the immunosuppressive factor TGF-β that was added to the cell cultures.

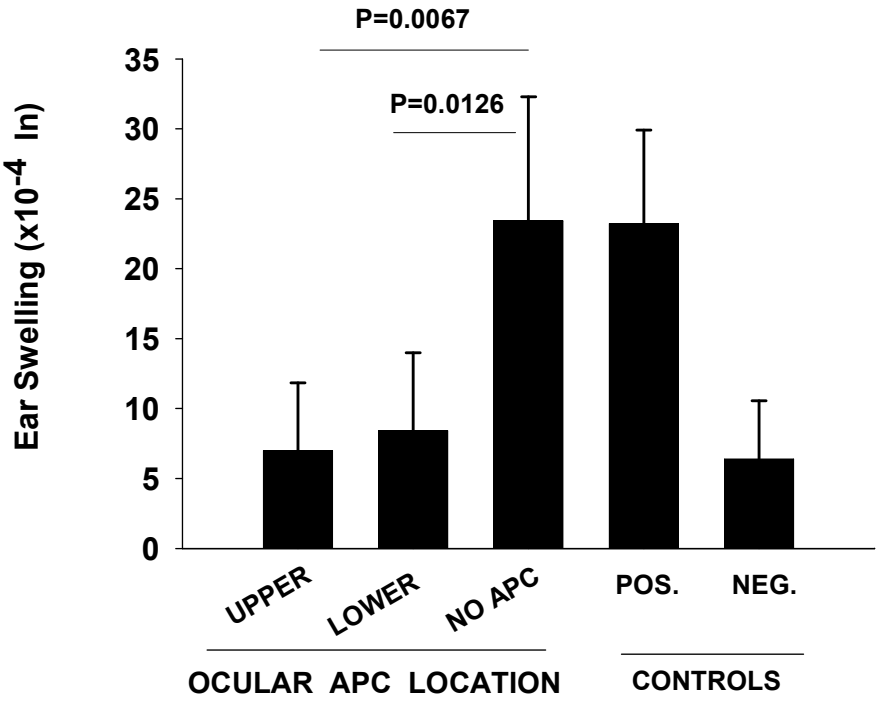


FIGURE 3. Direct contact between ocular APC and spleen cells is not needed for ACAID induction

OVA-pulsed ACAID APC were cultured either in the upper chamber or the lower chamber of transwell culture plates. Spleen cell suspensions depleted of $F4/80^+$ cells were placed in the lower chamber of transwell culture plates. After 5-7 days, the spleen cell suspensions were tested in a LAT assay for their capacity to suppress DTH responses to OVA. *$P<0.01$.

To confirm that splenic B cells, rather than splenic APC, were responsible for the induction of ACAID, additional transwell culture experiments were performed to test the hypothesis that cell-cell contact between ocular APC and splenic B cells is not a requirement for the induction of ACAID. In these transwell experiments, ACAID APC were pulsed with either OVA or hen egg lysozyme (HEL) and placed in the upper chambers of transwell culture plates. B cell suspensions were placed into the lower chambers. Two days later, B cells were adoptively transferred to naïve C57BL/6 mice. Seven day later, the B cell recipients were immunized SC with OVA emulsified in CFA. Seven days after the SC immunization, ear swelling responses to the inducing OVA antigen and an irrelevant HEL antigen were assessed. The results demonstrate that B cells did not have to be in direct contact with the ocular APC in order for them to induce ACAID when adoptively transferred to naïve hosts (**FIGURE 4**). In addition, B cells co-cultured with ACAID APC pulsed with an irrelevant antigen (HEL) or untreated ACAID APC did not induce suppression of OVA-specific DTH response. Moreover, APC not treated with TGF-β (regular APC), but pulsed with OVA did not elicit the generation of ACAID-inducing B cells. In conclusion, ocular APC deliver an antigen-specific signal to B cells that renders them tolerogenic and capable of inducing antigen-specific tolerance in third-party hosts. Moreover, the transmission of the tolerogenic signal of APC does not require direct cell to cell contact between the ACAID APC and the B cell.

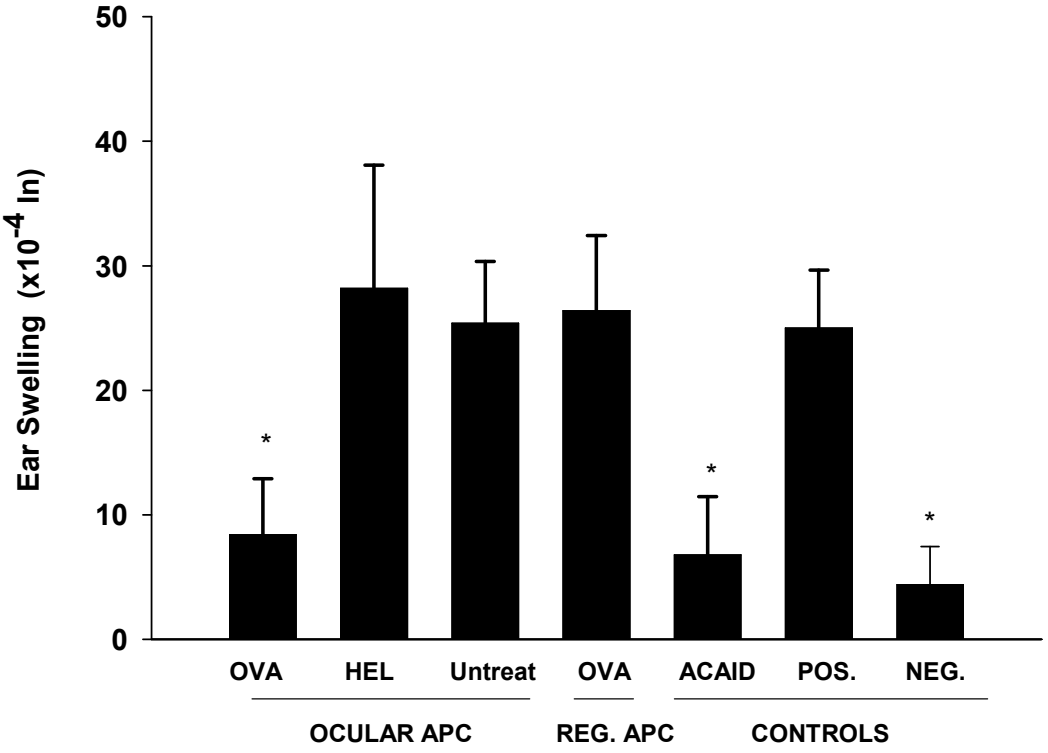


FIGURE 4. Direct contact between ocular APC and B cells is not needed for ACAID induction

ACAID APC or non-ACAID APC were placed in the upper chamber of transwell culture plates and B cell suspensions were placed in the lower chambers. ACAID APC were pulsed with OVA, HEL, or no antigen. Non-ACAID regular (Reg.) APC were pulsed with OVA. ACAID-inducing B cells (ACAID control) were generated by co-culturing OVA-pulsed ACAID APC with B cells. Two days later, B cells from the bottom chambers and the ACAID control B cells were injected i.v. into panels of naïve C57BL/6 mice. Seven days later, all mice were immunized SC with OVA + CFA. DTH responses to OVA were assessed in a conventional ear-swelling assay seven days after the SC immunization with OVA + CFA. Positive controls consisted of C57BL/6 mice immunized SC with OVA + CFA. Naïve C57BL/6 mice served as negative controls. *$P<0.01$.

To further confirm that OVA was processed by ocular APC and released to B cells for additional processing and presentation to T cells, the following experiment was performed. OVA-pulsed ACAID APC and ACAID APC not pulsed with OVA were thoroughly washed in HBSS and incubated with splenic B cells for 48 hr. As a control, other splenic B cell suspensions were co-cultured with free OVA (in the absence of ACAID APC) for 48 hr. Both populations of B cells were washed thoroughly with HBSS and injected IV into naïve C57BL/6 mice. After 7 days, the mice were immunized SC with OVA emulsified in CFA. Seven days later, a conventional ear swelling assay was used to measure DTH responses to OVA. Results shown in **FIGURE 5** demonstrate that B cells incubated with ACAID APC that had been pulsed with OVA induced the generation of Tregs in naïve mice, as shown by the suppressed DTH responses to OVA. On the other hand, B cells incubated with OVA in the absence of ACAID APC did not induce suppression of DTH responses when adoptively transferred to naïve recipient mice. Similarly, ACAID APC not pulsed with OVA did not induce the generation of ACAID-inducing B cells. Thus, processing of OVA by ocular APC is necessary for the induction of tolerogenic B cells.

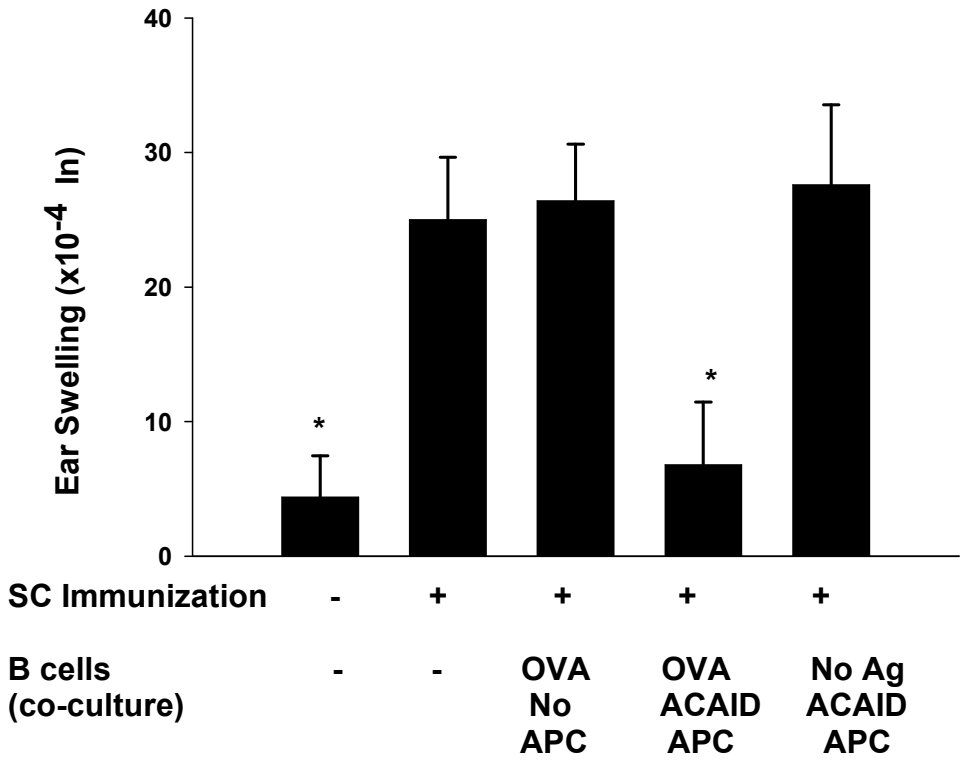


FIGURE 5. Ocular APC process OVA and render B cells tolerogenic

ACAID APC were pulsed either in the absence ("No Ag Ocular APC") or presence of OVA ("OVA Ocular APC") for 24-48 hr in the presence of TGF-β, washed thoroughly, and co-cultured for 48 hr with B cells. Other B cell suspensions were cultured with OVA alone for 48 hr ("OVA No APC"). B cell suspensions were treated with anti-F4/80 antibody plus complement and injected IV into normal C57BL/6 mice that were immunized SC with OVA + CFA 7 days later. DTH responses to OVA were assessed 7 days after SC immunization using an ear swelling assay. *P<0.01.

Clonal expansion of ACAID B cells

Titration of the Minimal Number of B Cells Required for ACAID Generation

Wilbanks *et al* has shown that as few as 20 of the blood-borne F4/80^{+} ocular APC will induce ACAID when adoptively transferred into naive recipient mice (73). This suggests that very few ocular APC can significantly amplify the tolerizing response. Since the splenic B cell population also has an antigen presentation function, in the induction of ACAID, it was important to determine the minimum number of ACAID B cells that are required for the generation of ACAID. ACAID B cells were generated *in vitro* by incubating B cells with ACAID-inducing APC for 48 hr. This was followed by the adoptive transfer of varying numbers of ACAID B cells into groups of normal C57BL/6 mice. One week later, recipient mice were injected SC with OVA emulsified in CFA. On day 14, mice were intradermally challenged with OVA and an ear swelling assay was performed on day 15. Results in **FIGURE 6** show that $\geq 1 \times 10^{6}$ ACAID B cells were needed for the induction of ACAID. Thus, the number of B cells needed to transfer ACAID was, in fact, about 5 logs more than the number of ocular APC required to transfer ACAID. This suggests that an expansion of splenic B cells is needed for the induction of tolerance via the AC of the eye. This may be due to the fact that only the select few of B cells that express the relevant BCR can process antigen and induce ACAID.

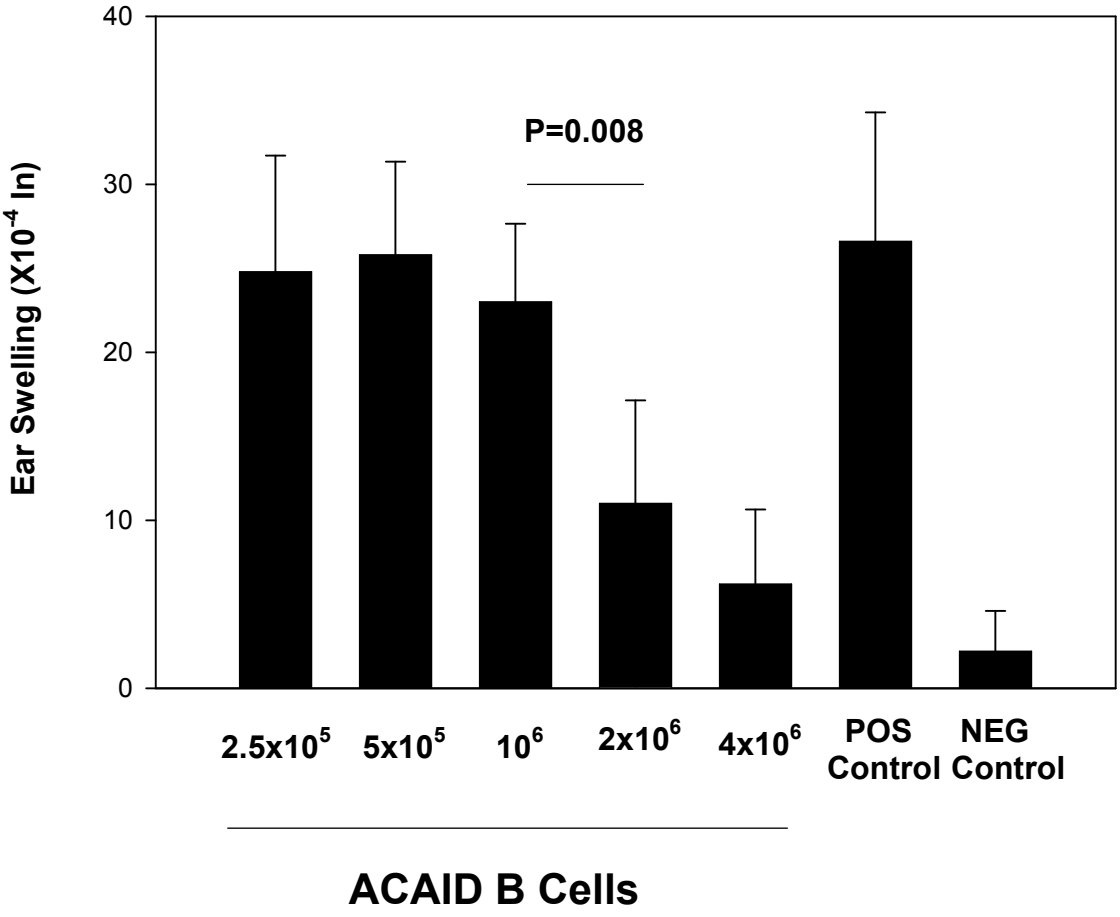


FIGURE 6. Number of adoptively transferred B cells required for ACAID generation

ACAID B cells were generated *in vitro*. About 2.5 x 10^5, 5 x 10^5, 10^6, 2 x 10^6, and 4 x 10^6 ACAID B cells were adoptively transferred into different groups of normal C57BL/6 mice. On day 7, recipient mice were injected with OVA emulsified in CFA. On day 14, mice were intradermally challenged with OVA . On day 15, an ear swelling DTH assay was performed.

Clonal Expansion is Required for the Generation of Tolerogenic B Cells

Previous studies have demonstrated that the induction of ACAID requires that the host's B cells express the BCR that recognizes the antigen that is introduced into the AC. That is, mice bearing the BCR transgene for HEL will develop ACAID if HEL is injected into the AC, but will not develop ACAID if other antigens are used (14). Considering the small number of B cells expressing the BCR for OVA, we tested the hypothesis that ACAID APC stimulate the expansion of B cells. Splenic B cells were co-cultured with either OVA-pulsed ACAID APC or OVA-pulsed non-ACAID APC for 2,3, or 5 days, and B cell proliferation was assessed by the uptake of ^{3}H-thymidine. The results revealed that OVA-pulsed ACAID APC stimulated a spike in B cell proliferation within 48 hr. The proliferation rate was significantly higher than that with non-ACAID APC after 2 days. However, there was no significant difference between the two groups after 3 days. Moreover, after 5 days B cells incubated with non-ACAID APC proliferated at a higher rate than B cells incubated with ACAID APC (**FIGURE 7**).

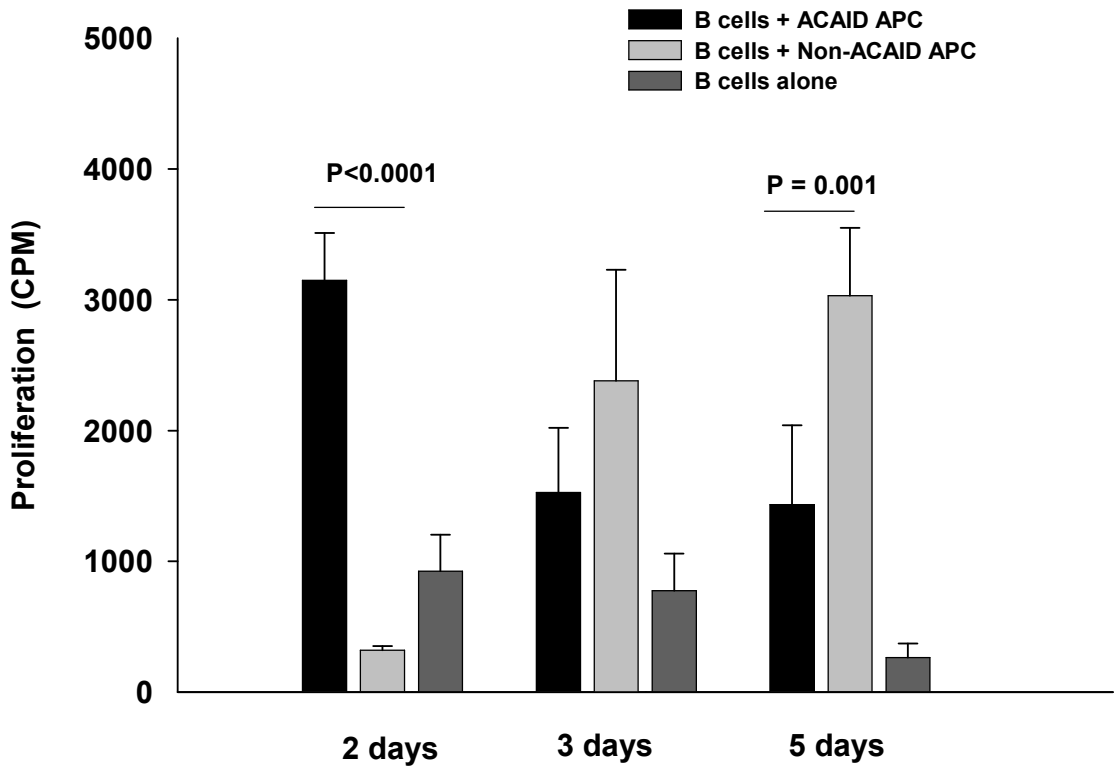


FIGURE 7. ACAID APC stimulate B cell proliferation *in vitro*

Splenic B cells ($3x10^5$) were co-cultured with OVA-pulsed ACAID APC ($2.5x10^4$) or OVA-pulsed Non- ACAID APC ($2.5x10^4$) in a 96-well plate. After two, three , or five days. Tritiated thymidine (0.5 microcuries) was added 12 hours before the end of the incubation period. At the end of the incubation period, the non-adherent B cells were collected and radioactivity was counted in a beta-counter.

Expansion of Antigen-Specific BCR on B Cells Following AC Injection of Antigen

The requirement of the BCR for B cells to participate in the induction of ACAID was demonstrated by using anti-Ig antibody that prevented the generation of ACAID Tregs *in vitro* (14). Considering the small number of B cells expressing BCR specific for the AC-injected antigen, we hypothesized that AC priming with an antigen stimulates the expansion of antigen-specific splenic B cells. To test this hypothesis, HEL, OVA, or PBS was injected into the AC of HEL BCR transgenic mice. Seven days later, splenic B cell suspensions from each group of mice were stained with HEL-Biotin followed by streptavidin FITC and then examined by flow cytometry for the relative number of BCR on B cells that bound HEL. The results indicate that there was a dramatic increase in the number of B cells that bound HEL in the HEL BCR mice that had been injected with HEL (**FIGURE 8**). However, AC injection of an irrelevant antigen such as OVA or AC injection of PBS (to control against trauma to the eye) did not result in an increase in the number of HEL-binding B cells. This result fits well with results from Skelsey *et al* who demonstrated that B cells expressing HEL-specific BCR do not induce ACAID when used in spleen cell cultures with OVA-pulsed ACAID APC (14).

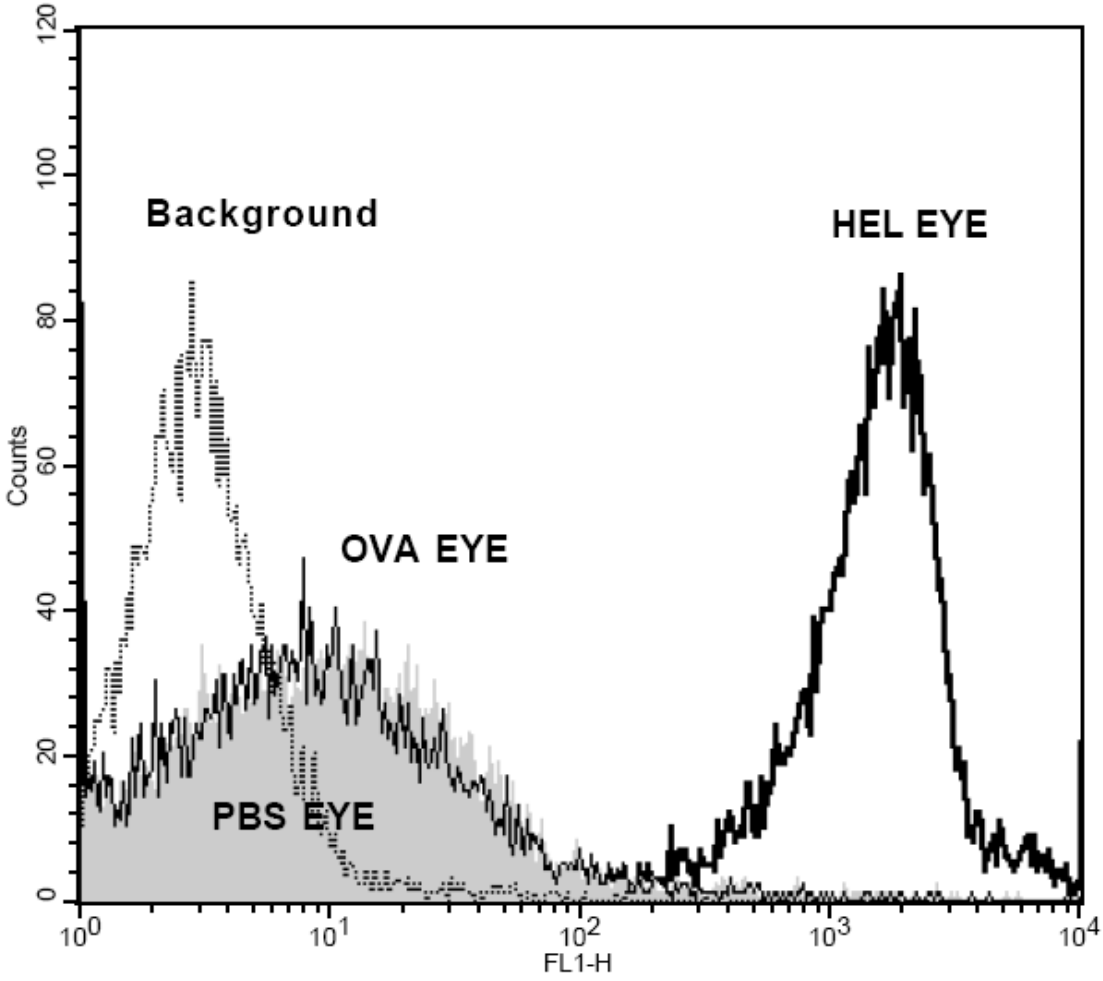


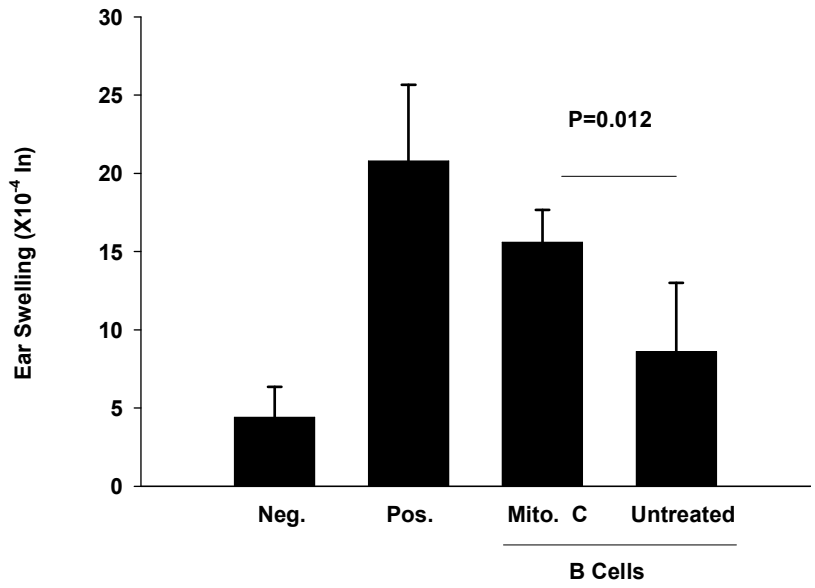
FIGURE 8. Expansion of HEL-binding B cells in mice primed in the AC with HEL

HEL BCR transgenic mice were injected in the AC with OVA, PBS, or HEL. Seven days later, splenic B cells were isolated, incubated with biotinylated HEL and the number of HEL-binding B cells was determined by flow cytometry using FITC-labeled avidin.

Inhibition of B cell proliferation prevents the induction of ACAID

ACAID-inducing B cells can be generated *in vitro* by co-culturing OVA-pulsed ACAID APC with B cells for two days. These ACAID B cells can induce the generation of $CD4^+$ and $CD8^+$ Tregs when adoptively transferred to naïve recipients (14). The *in vitro* and *in vivo* expansion of B cell populations in response to ACAID APC and AC injection of antigen respectively, suggested that B cell proliferation was intimately associated with the induction of ACAID. To test this, ACAID B cell suspensions were either untreated or treated with either mitomycin-C or γ-irradiation prior to adoptively transferring them to naïve mice. Mice were immunized SC with OVA + CFA 7 days after the B cell adoptive transfer and DTH was measured 7 days later. Inhibition of B cell proliferation by treatment with either mitomycin-C or γ-irradiation prevented the induction of ACAID by B cells (**FIGURE 9**). By contrast, untreated ACAID B cells were able to induce ACAID.

A.


Ear Swelling (X10-4 In)
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25
20
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0
P=0.012
Neg.
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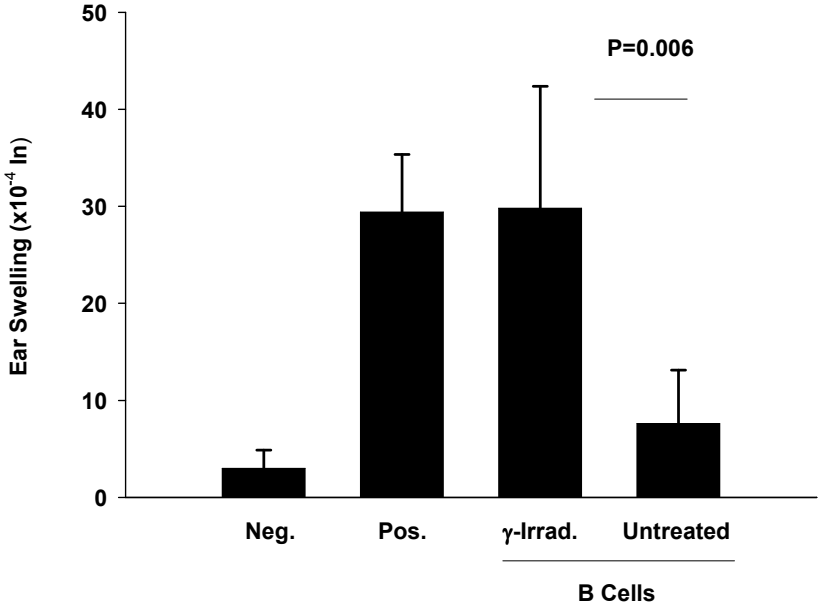
B.


Ear Swelling (x10-4 In)
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40
30
20
10
0
P=0.006
Neg.
Pos.
γ-Irrad.
Untreated
B Cells

FIGURE 9. Inhibition of B cell proliferation prevents the induction of ACAID.

OVA-pulsed ACAID APC were co-cultured for 5 days with untreated B cells or B cells treated with: **A**) mitomycin-c or **B**) γ-irradiation. B cell suspensions were treated with anti-F4/80 antibody plus complement to remove residual macrophages and were injected i.v. into normal C57BL/6 mice that were immunized SC with OVA + CFA on day 7. DTH responses to OVA were measured 7 days later.

ROLE OF MHC EXPRESSION ON B CELLS IN THE INDUCTION OF ACAID

Role of MHC Class II-Bearing B Cells in the Induction of ACAID

ACAID is a form of peripheral tolerance that requires the participation of two independent populations of Tregs; one is $CD4^+$ and promotes the development of the second population, which is $CD8^+$ and suppresses the expression of DTH response. When adoptively transferred to naïve mouse recipients, ACAID B cells are capable of inducing both Treg populations and thus the inhibition of DTH (13, 14). The time-honored principle that antigen is presented to $CD4^+$ T cells via MHC class II and to $CD8^+$ T cells via MHC class I coupled with our knowledge that B cells are needed for the generation of both populations of Tregs (14), led us to test the hypothesis that ACAID B cells present antigens on both MHC class I and II molecules during the establishment of ACAID.

Adoptive Transfer with putative ACAID B Cells from MHC II KO Donors Does Not Induce ACAID

In order to determine if ACAID B cells presented antigen via MHC class II for the generation of MHC class II-restricted $CD4^+$ Tregs, we tested the hypothesis that B cells lacking MHC class II determinants would not be capable of presenting antigen to $CD4^+$ Tregs and as a result, ACAID would not be induced. ACAID B cells were generated *in vitro* as described earlier. However, B cells were obtained from either normal mice or MHC class II

KO mice. After *in vitro* culture with OVA-pulsed ACAID APC, the putative ACAID B cells were injected IV into normal mice. After 7 days, mice were immunized with OVA emulsified in CFA. Seven days later, DTH to OVA was measured. As expected, adoptive transfer of normal B cells co-cultured with OVA-pulsed ACAID APC induced suppression of DTH responses to OVA. By contrast, B cells from MHC class II KO mice that were co-cultured with OVA-pulsed ACAID APC were unable to induce the generation of ACAID Tregs that suppressed DTH responses when adoptively transferred to normal mice **(FIGURE 10**).

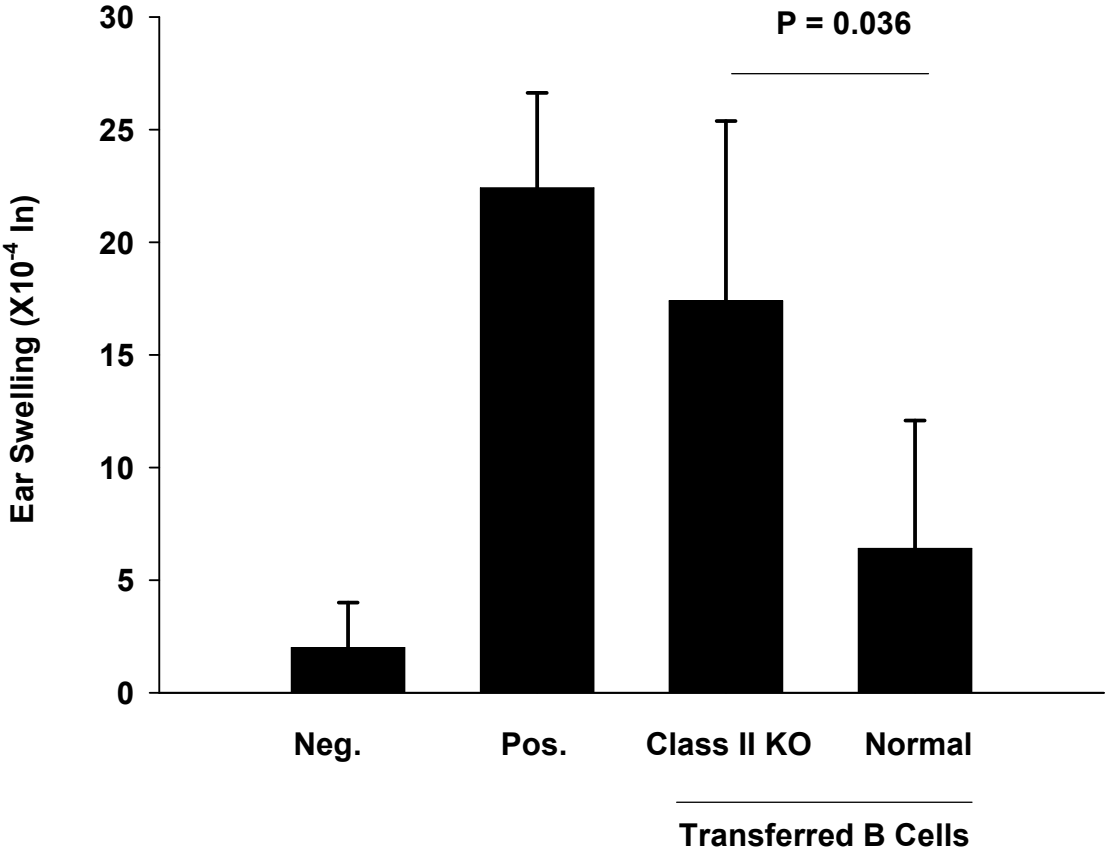


FIGURE 10. B cells must express MHC class II molecules in order to induce ACAID

B cells from either normal or MHC class II KO mice were co-cultured for 48 hr with OVA pulsed ACAID APC prepared from normal mice. B cell suspensions were treated with anti-F4/80 antibody plus complement and injected IV into normal C57BL/6 mice that were immunized SC with OVA + CFA 7 days later. DTH responses to OVA were assessed 7 days after SC immunization using an ear swelling assay.

Reconstitution of B Cell KO Mice with B Cells from MHC Class II KO Donors Does Not Restore the Capacity to Induce ACAID

To further support this requirement for MHC Class II expression on B cells in the induction of ACAID, B cell KO mice were reconstituted with B cells from either normal mice or MHC class II KO mice to test the hypothesis that ACAID cannot be generated in B cell KO mice reconstituted with B cells from MHC II KO mice due to the lack of an efficient MHC II antigen presentation pathway. Thus, B cell-reconstituted mice were primed in the AC with OVA. Seven days later, the mice were immunized SC with OVA emulsified in CFA. Seven days after the SC immunization, assessment of DTH responses to OVA revealed that, as expected, reconstitution of B cell KO mice with B cells from normal donors restored the generation of ACAID. On the other hand, B cells from MHC class II KO mice failed to restore the generation of ACAID in the B cell-deficient hosts (**FIGURE 11**).

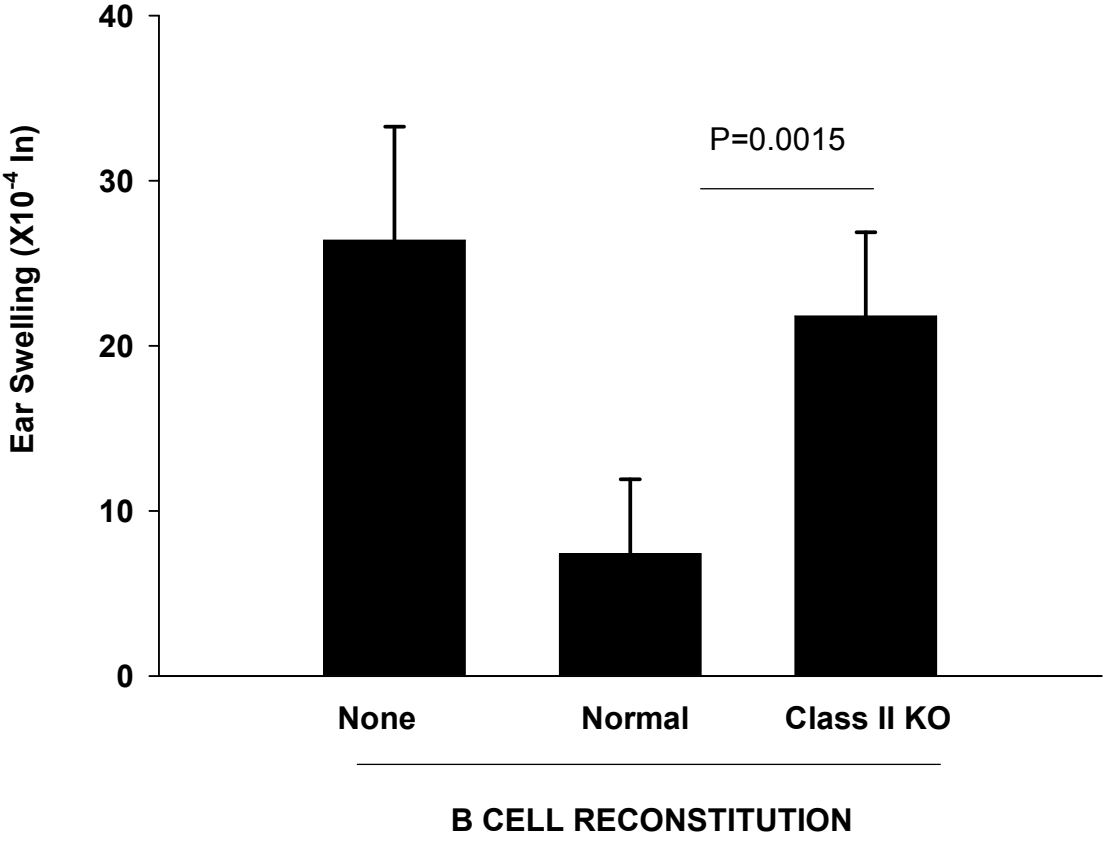


FIGURE 11. B cells used for reconstitution of B cell KO mice must express MHC class II molecules in order to induce ACAID

B cell KO mice were reconstituted with a splenic equivalent of B cells from either normal mice or MHC class II KO mice. B cell-reconstituted mice were injected in the AC with OVA 7 days prior to being immunized SC with OVA emulsified in CFA. DTH responses to OVA were assessed 7 days after the SC immunization using an ear swelling assay.

In Vitro Reconstitution with B Cells from MHC II KO Donors Does Not Restore the Capacity to Induce ACAID

In vitro spleen cell cultures were used to confirm that expression of MHC class II is required for the induction of ACAID Tregs. OVA-pulsed ACAID APC prepared from normal donors were co-cultured with B cells from either normal or MHC class II KO mice and with spleen cells depleted of B cells and $F4/80^+$ APC. Seven days later, the spleen cells were tested in a LAT assay for the presence of Tregs that suppressed OVA DTH responses. As in previous studies, spleen cells from normal mice co-cultured with OVA-pulsed ACAID APC in the presence of B cells from normal mice inhibited the expression of DTH by spleen cells collected from normal mice that were immunized with OVA emulsified in CFA. By contrast, B cells from MHC class II KO mice did not promote the generation of ACAID Tregs (**FIGURE 12**).

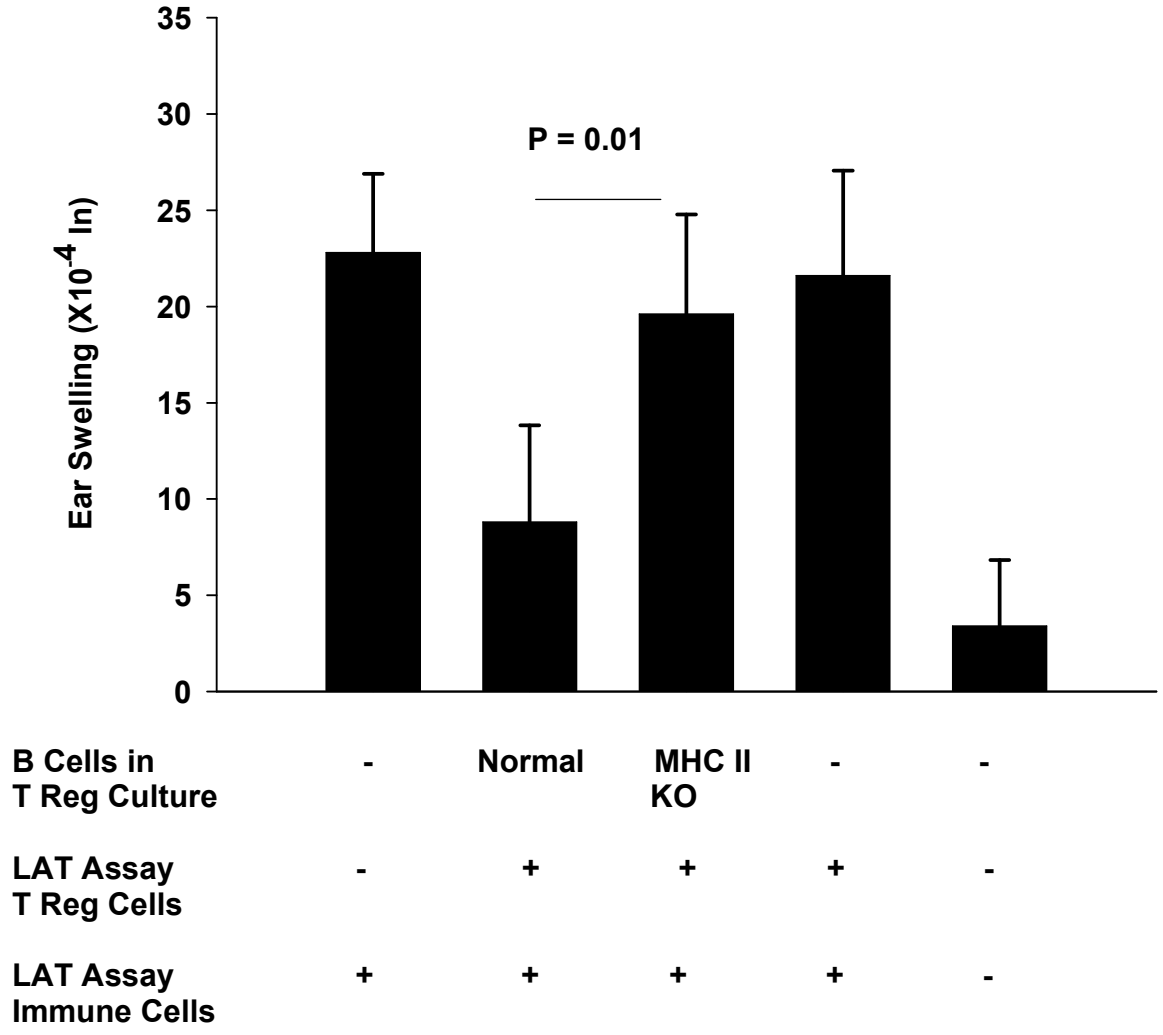


FIGURE 12. ***In vitro*-cultured B cells must express MHC class II molecules in order to induce ACAID**

ACAID Tregs were generated using *in vitro* spleen cell cultures. The three spleen cell culture groups (Bars # 2,3&4) contained OVA-pulsed ACAID APC and spleen cell suspensions that were depleted of B cells and $F4/80^{+}$ APC. Spleen cell cultures were supplemented with B cells from normal mice or MHC Class II-deficient mice. As a control, one spleen cell culture was not supplemented with B cells. All three spleen cell cultures were incubated for 5-7 days. Each spleen cell suspension was tested in a LAT assay for Tregs that suppressed OVA-specific DTH.

IL-10 can substitute for MHC class II-bearing B cells in the induction of ACAID

It has been previously reported that $CD4^+$ Tregs are required for the development of $CD8^+$ end-stage efferent ACAID Tregs (11). Using the *in vitro* spleen cell culture model, it was shown that the $CD4^+$ Tregs in ACAID must produce IL-10 in order to promote the development of $CD8^+$ Tregs (10). In addition, $CD4^+$ T cells from IL-10 KO mice are unable to support the generation of ACAID, either *in vitro* or *in vivo* (10). However, exogenous IL-10 is able to replace $CD4^+$ T cells, but not $CD8^+$ T cells, for the *in vitro* induction of ACAID (10). Therefore, it was highly probable that MHC class II expression on ACAID B cells was required for the generation of $CD4^+$ Tregs, whose primary function was to produce IL-10. To examine this hypothesis, B cell-depleted, $F4/80^+$ APC-depleted spleen cell suspensions from normal mice were reconstituted with B cells from MHC class II KO donors and were co-cultured with OVA-pulsed ACAID APC from normal mice in the presence or absence of exogenous IL-10. Seven days later, the spleen cell cultures were tested in a LAT assay for the presence of ACAID Tregs. As before, spleen cell cultures containing B cells from MHC class II KO mice failed to promote the development of ACAID Tregs that suppressed OVA DTH responses. However, addition of exogenous IL-10 to MHC class II KO B cell cultures restored the generation of ACAID Tregs that suppressed OVA DTH responses. Importantly, IL-10 alone did not induce the development of Tregs **(FIGURE 13).**

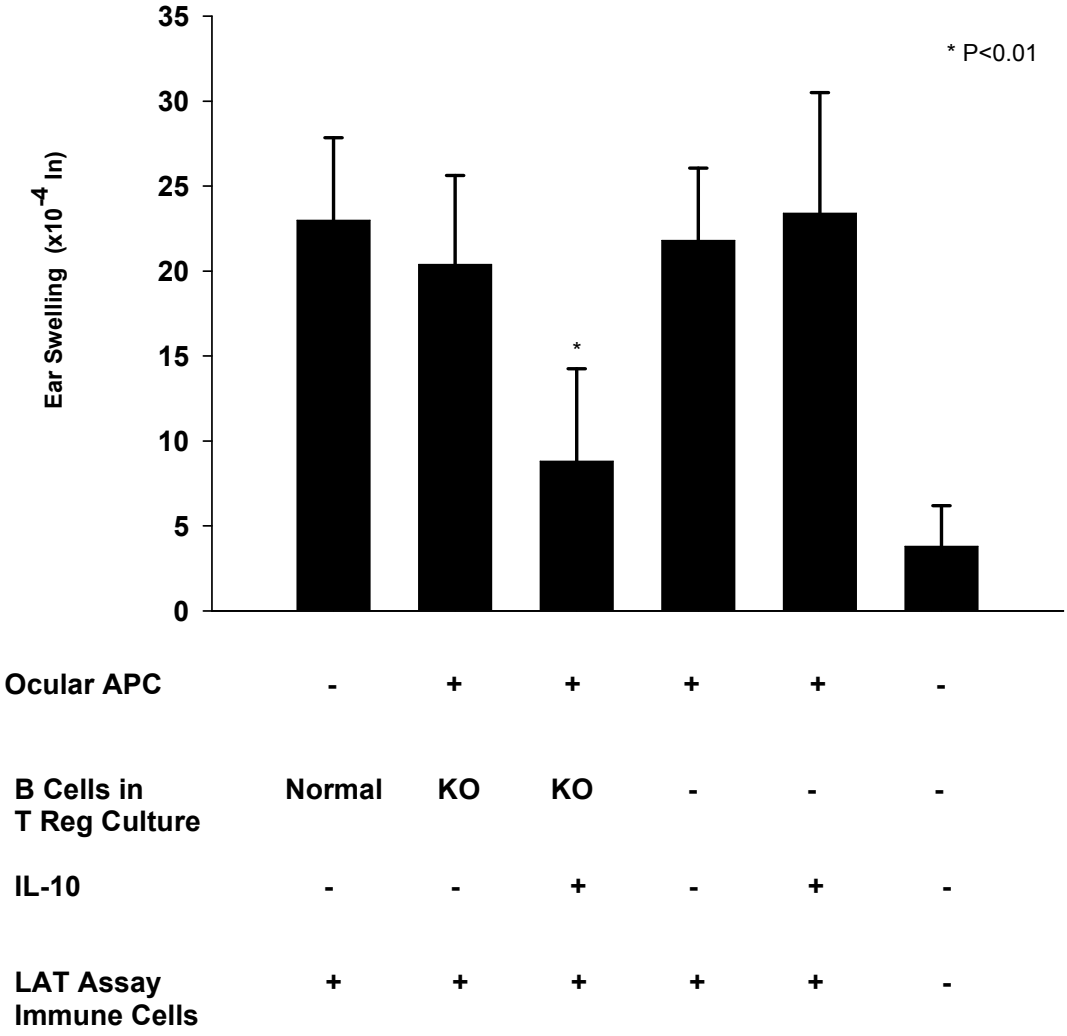


FIGURE 13. IL-10 can substitute for MHC class II-bearing B cells in the induction of ACAID

B cell-depleted spleen cell suspensions that were treated with anti-F4/80 antibody plus complement, were reconstituted with B cells from MHC class II KO mice or B cells from normal mice and co-cultured for 5 days with OVA-pulsed ACAID APC in the presence or absence of exogenous IL-10 (10 ng/ml). Spleen cells were then tested for their capacity to suppress OVA-specific DTH in a LAT assay.

Role of MHC Class I-Bearing B Cells in the Induction of ACAID

Adoptive Transfer with putative ACAID B Cells from β2M KO Donors Does Not Induce ACAID

In order to determine if ACAID B cells presented antigen via MHC class I for the generation of MHC class I-restricted $CD8^+$ Tregs, we tested the hypothesis that B cells lacking MHC class I determinants would not be capable of presenting antigen to $CD8^+$ Tregs and as a result, ACAID would not be induced. To examine this hypothesis, Tregs were generated *in vitro* by co-culturing OVA-pulsed ACAID APC with B cells from either normal mice or β_2 microglobulin (β2M) KO mice that have very low MHC class I expression due to the deficiency in the expression of the β2M chain which is part of the heterodimer for the MHC class I (127). Two days later, B cells were isolated from the cultures and adoptively transferred to naïve normal recipients. Seven days later, the recipient mice were SC immunized with OVA emulsified in CFA. Seven days after the SC immunization, DTH responses to OVA were assessed. As expected, B cells from normal mice were able to induce ACAID. By contrast, B cells from β2M KO mice were unable to induce ACAID Tregs (**FIGURE 14**).

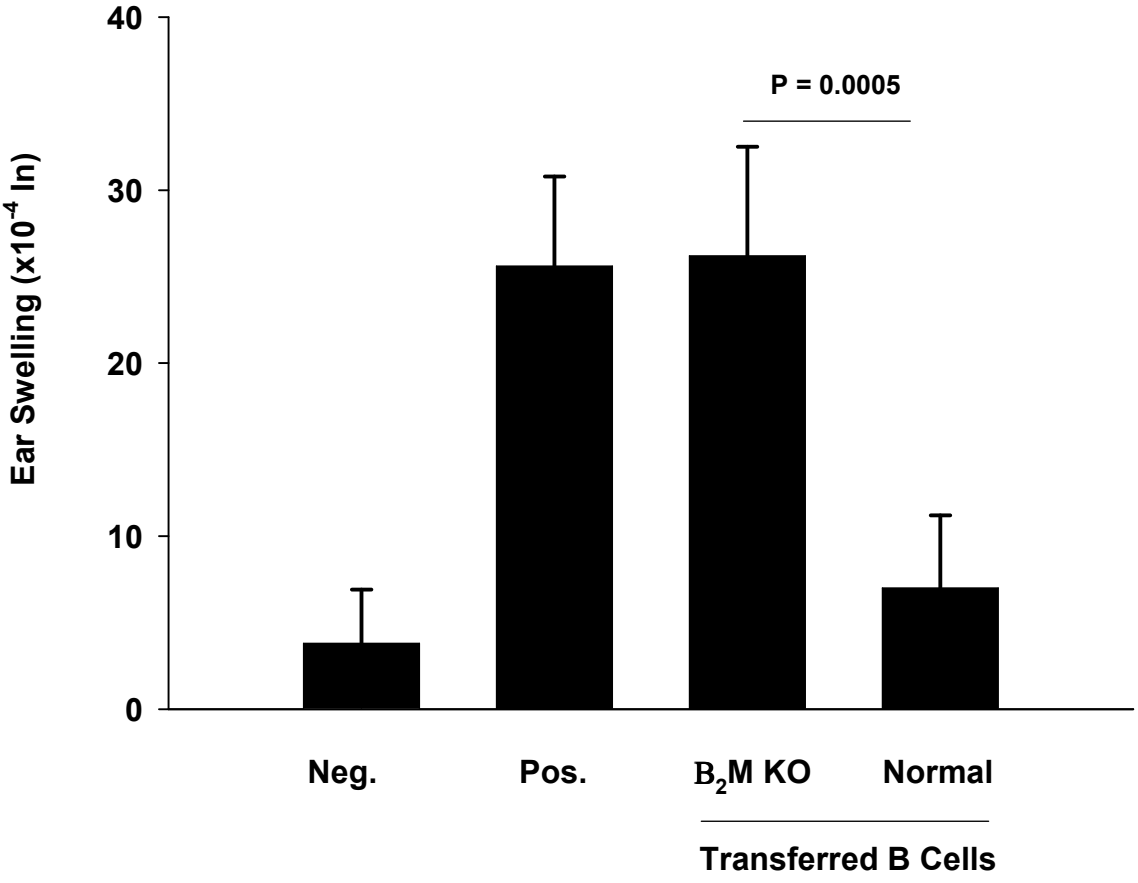


FIGURE 14. B cells must express MHC class I molecules in order to induce ACAID

B cells from either normal or MHC class I-deficient β2M KO mice were co-cultured for 48 hr with OVA-pulsed ACAID APC prepared from normal mice. B cell suspensions were treated with anti-F4/80 antibody plus complement to remove residual macrophages and were injected IV into normal C57BL/6 mice that were immunized SC with OVA emulsified in CFA 7 days later. DTH responses to OVA were assessed 7 days after the SC immunization using an ear swelling assay.

Reconstitution of B Cell KO Mice with B Cells from β2M KO Donors Does Not Restore the Capacity to Induce ACAID

To further test the requirement for MHC Class I expression on B cells in the induction of ACAID, B cell KO mice were reconstituted with B cells from either normal or β2M KO mice. The reconstituted mice were primed in the AC with OVA. Seven days later, mice were immunized SC with OVA emulsified in CFA. Seven days later, DTH responses to OVA were assessed. Results demonstrated that B cells from normal mice were able to reconstitute B cell KO mice and restore the development of ACAID in these hosts. On the other hand, B cell KO mice reconstituted with B cells from β2M KO mice failed to develop suppression and instead demonstrated positive DTH responses to OVA confirming that B cells must express MHC class I determinants for the induction of ACAID. These results are consistent with the hypothesis that B cells act as ancillary APC in the induction of ACAID by presenting antigens on both MHC class I and MHC class II molecules (**FIGURE 15**).

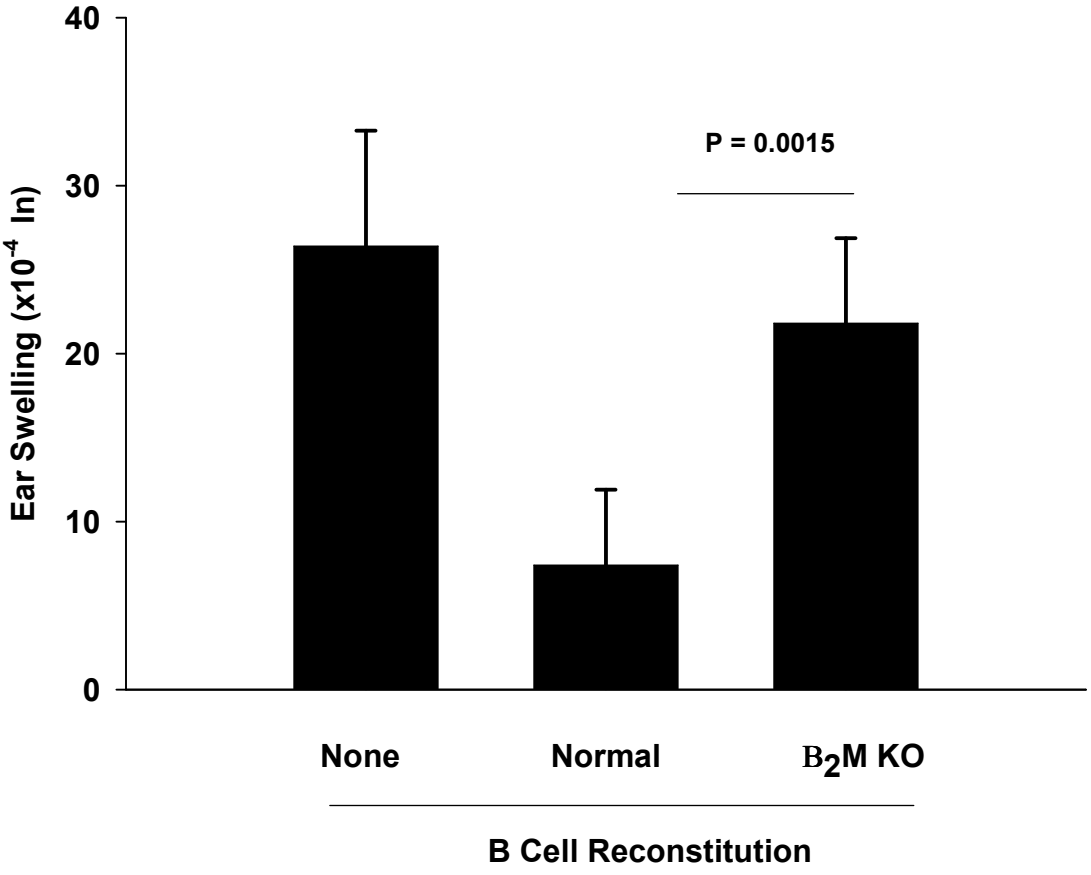


FIGURE 15. B cells used for reconstitution of B cell KO mice must express MHC class I molecules in order to induce ACAID

B cell KO mice were reconstituted with a splenic equivalent of B cells from either normal mice or β2M KO mice. B cell-reconstituted mice were injected in the AC with OVA 7 days prior to being immunized SC with OVA emulsified in CFA. DTH responses to OVA were assessed 7 days after the SC immunization using an ear swelling assay.

In Vitro Reconstitution with B Cells from β2M KO Donors Does Not Restore the Capacity to Induce ACAID

In vitro spleen cell cultures were used to confirm that expression of MHC class I was required for the induction of ACAID Tregs. OVA-pulsed ACAID APC prepared from normal donors were co-cultured with spleen cell suspensions depleted of B cells (by negative selection), and of $F4/80^+$ APC (by treating with anti $F4/80^+$ APC + complement). The spleen cell cultures were reconstituted with B cells prepared from either normal or β2M KO mice. Seven days later, the spleen cells were tested for their capacity to suppress OVA DTH responses in a LAT assay. As in previous studies, spleen cells from normal mice co-cultured with OVA-pulsed ACAID APC in the presence of B cells from normal mice developed Tregs that were able to inhibit the expression of DTH by spleen cells collected from normal mice that had been immunized with OVA emulsified in CFA. As in previous experiments, B cells from β2M KO mice did not promote the generation of ACAID Tregs (**FIGURE 16**).

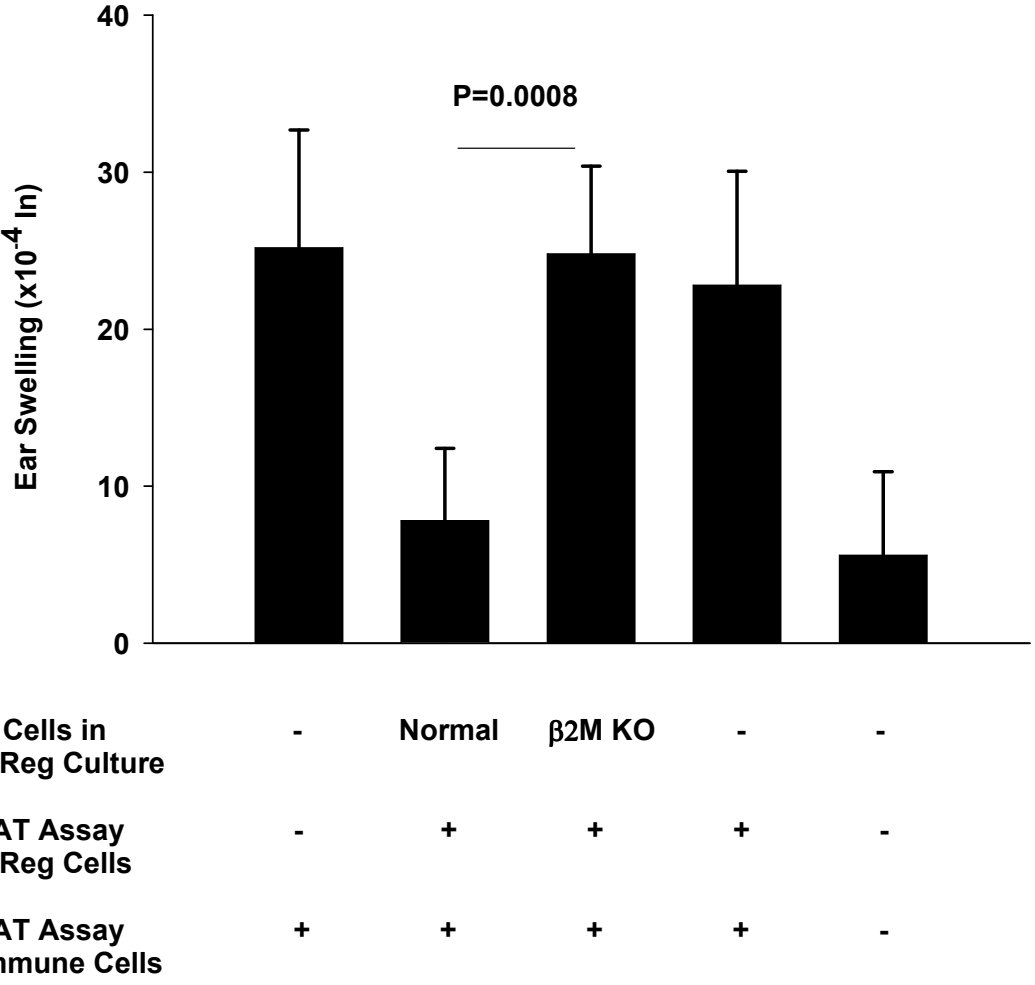


FIGURE 16. ***In vitro*-cultured B cells must express MHC class I molecules in order to induce ACAID**

ACAID Tregs were generated using *in vitro* spleen cell cultures. The spleen cell culture groups (Bars # 2,3&4) contained OVA-pulsed ACAID APC and spleen cell suspensions that were depleted of B cells and $F4/80^+$ APC. Spleen cell cultures were either not reconstituted with B cells or were supplemented with B cells from either normal mice or β2 microglobulin KO mice. All spleen cell cultures were incubated for 5-7 days. Each spleen cell suspension was tested in a LAT assay for its capacity to suppress OVA-specific DTH.

Reconstitution of B Cell KO Mice with B Cells from MHC Class II KO Donors and β2M KO donors Restores the Capacity to Induce ACAID

If MHC class I-expressing B cells and MHC class II-expressing B cells are both needed for the induction of ACAID, it should be possible to restore peripheral tolerance by reconstituting B cell KO mice with B cells from MHC class II KO donors, which express normal MHC class I, and B cells from β2M KO donors, which express normal MHC class II. This hypothesis was examined by reconstituting B cell KO mice with equal numbers of B cells from MHC class II KO donors and β2M KO donors. Reconstituted B cell KO mice were primed in the AC with OVA. Seven days later, mice were SC immunized with OVA emulsified in CFA. Seven days after the SC immunization with OVA, DTH responses to OVA were assessed. The results demonstrate that reconstitution with a mixture of B cells from MHC class II KO donors and β2M KO donors restored ACAID in B cell KO mice (**FIGURE 17**).

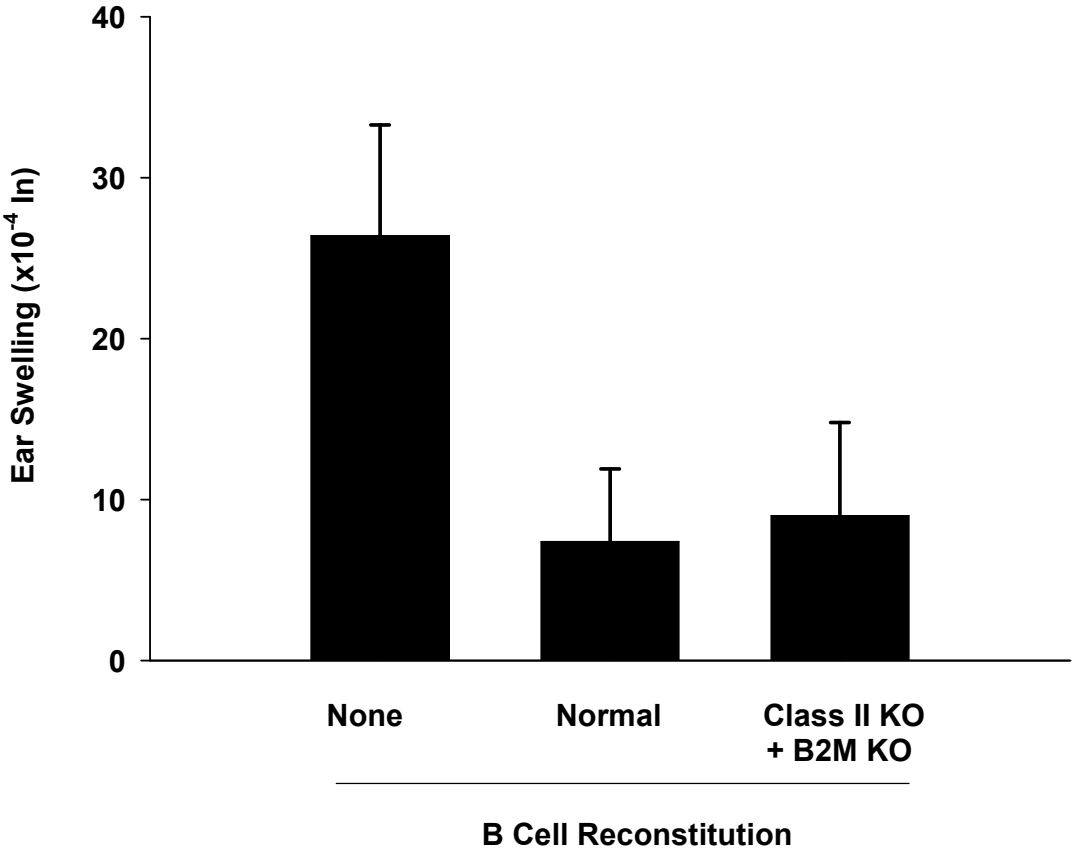


FIGURE 17. B cells from MHC class II KO mice and β2M KO mice reconstitute ACAID in B cell KO mice

B cell KO mice were reconstituted with a splenic equivalent of a B cell suspension containing equal numbers of B cells from MHC class II KO mice and β2M KO mice. OVA was injected into the AC 7 days after B cell reconstitution. Mice were immunized SC with OVA emulsified in CFA 7 days after the AC injections with OVA. DTH responses to OVA were assessed 7 days after the SC immunization using an ear swelling assay.

Role of Gamma delta T cells in ACAID

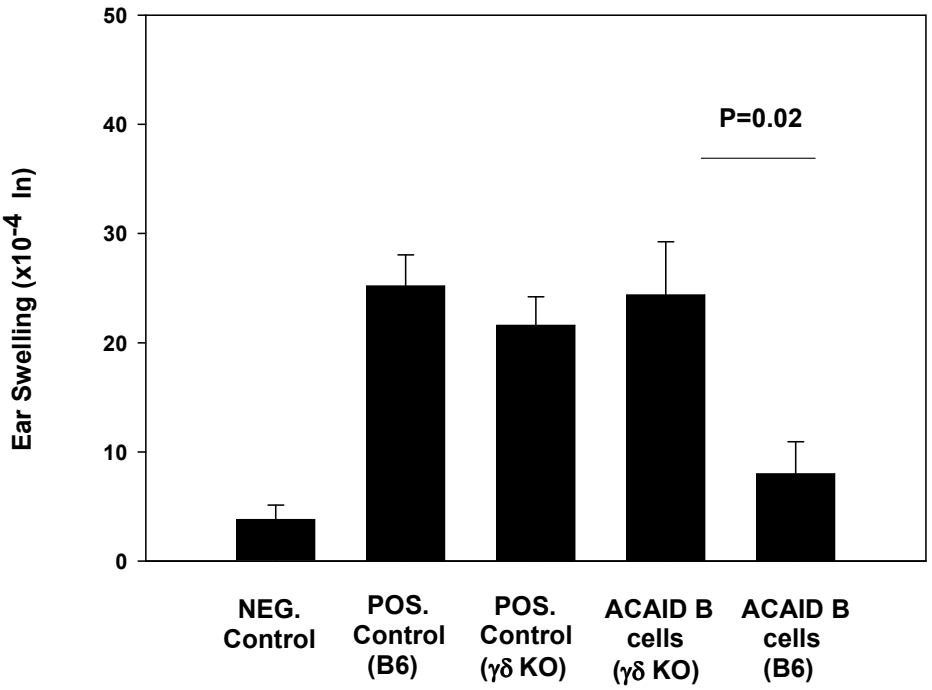
As mentioned before, ACAID is a sequential process that requires the presence of multiple functional cell populations in the spleen (4, 119). The antigen presenting B cell population and γδ T cell population are two of these cell populations that are crucial for the development of ACAID (14, 114). The requirement of γδ T cells in the induction of ACAID and the development of $CD8^+$ Tregs was previously established using both γδ KO mice and mice treated with anti-TCRδ chain antibody (114). Moreover, γδ T cells are required for corneal allograft survival, which is intimately linked to ACAID (114).

ACAID B cells cannot induce ACAID in γδ KO mice or γδ T cell-depleted mice

In order to determine the role of γδ T cells in the induction of ACAID, it was important to determine if they acted upstream or downstream of B cells following AC injection of the antigen. The initial steps in the induction of ACAID can be recapitulated *in vitro* by co-culturing antigen-pulsed, ACAID APC with spleen cells from normal mice. After 5-7 days of *in vitro* culture, $CD8^+$ Tregs are generated that have the same properties of Tregs induced by AC injection of the antigen. To determine if γδ T cells acted downstream from B cells in the induction of ACAID, we tested the capacity of ACAID-inducing B cells to generate ACAID in mice deficient in γδ T cells. ACAID-inducing B cells were generated *in vitro* by co-culturing OVA-pulsed, ACAID APC with purified B cell suspensions for 2 days. B cells generated in such cultures will induce ACAID when adoptively transferred to naïve mice (14, 90, 126). Accordingly, ACAID B cells were

adoptively transferred into either γδ T cell KO mice or wild-type C57BL/6 mice depleted of γδ T cells using anti-γδ T cell antibody. The recipients of adoptively transferred B cells were immunized SC with OVA emulsified in CFA and subsequently tested for OVA-specific DTH. The results indicated that, as expected, recipients of ACAID-inducing B cells developed ACAID as demonstrated by suppressed DTH responses to OVA (even though these mice had been immunized SC with OVA+CFA). By contrast, both categories of γδ T cell-deficient mice did not develop ACAID and as a result, mounted DTH responses comparable to the positive control mice. These results suggest that γδ T cells act downstream from B cells during the induction of ACAID (**FIGURE 18**)

A.


50
40
30
20
10
0
Ear Swelling (x10-4 In)
P=0.02
NEG. Control
POS. Control (B6)
POS. Control (γδ KO)
ACAID B cells (γδ KO)
ACAID B cells (B6)

B.

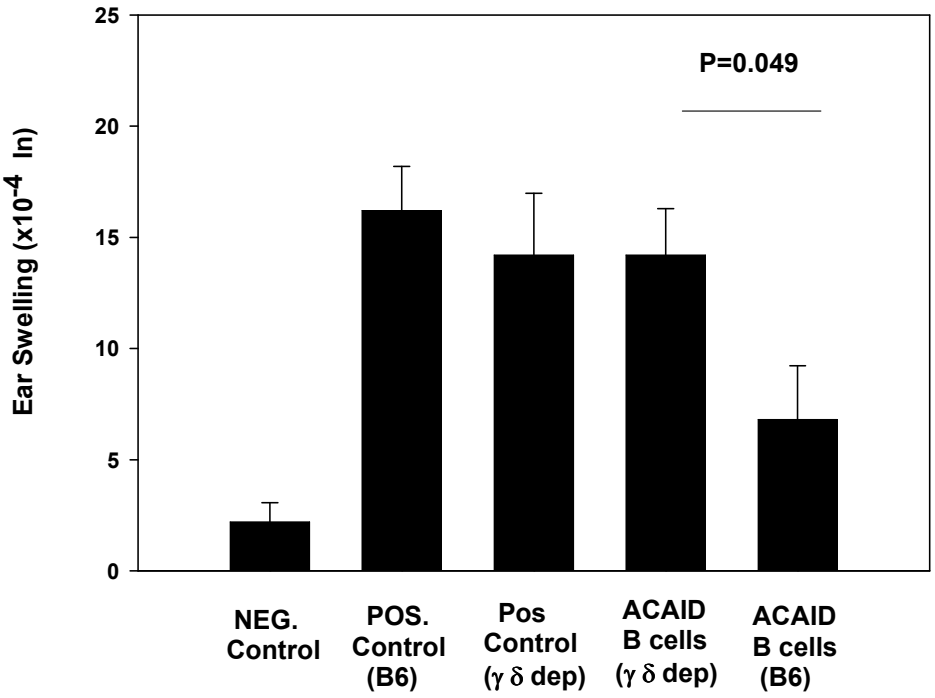

25
20
15
10
5
0
Ear Swelling (x10-4 In)
P=0.049
NEG. Control
POS. Control (B6)
Pos Control (γ δ dep)
ACAID B cells (γ δ dep)
ACAID B cells (B6)

FIGURE 18. ACAID B cells cannot induce ACAID in γδ KO mice or γδ T cell-depleted mice

A) ACAID B cells were generated *in vitro*, injected IV ($4x10^6$ cells/mouse) into γδ KO mice or wild-type C57BL/6 (B6) mice. Mice were then immunized SC with OVA + CFA 7 days later. DTH responses to OVA were assessed 7 days after SC immunization using an ear swelling assay. B) ACAID B cells were generated *in vitro*, injected IV ($4x10^6$ cells/mouse) into either γδ T cell-depleted B6 mice or normal B6 mice. Mice were then immunized SC with OVA + CFA 7 days later. DTH responses to OVA were assessed 7 days after SC immunization using an ear swelling assay.

FUNCTION OF GAMMA DELTA T CELLS IN ACAID

Do Gamma delta T cells act as ACAID Tregs?

γδ T cells do not mediate efferent suppression of DTH

The splenic phase of ACAID involves the interactions between ocular APC, B cells, NK T cells, $CD4^+$ T cells, $CD8^+$ T cells, and γδ T cells. Since γδ T cells act downstream from ACAID B cells (as shown above) , it is possible that γδ T cells are, in fact, the end stage Tregs that suppress the expression of DTH. To examine this hypothesis , ACAID Tregs were generated *in vitro* and spleen cell cultures were depleted of γδ T cells immediately prior to testing for Treg cell activity in a LAT assay. Previous studies have demonstrated that ACAID Tregs are $CD8^+$ (84). Therefore, as a control, the *in vitro* generated Tregs were treated with anti-CD8 antibody together with complement to remove Treg activity. The results indicated that, as expected, removal of $CD8^+$ T cells abolished Treg activity and allowed full expression of OVA-specific DTH responses. On the other hand, depletion of γδ T cells did not remove Treg activity and indicated that γδ T cells do not act as the end stage Tregs in ACAID. The use of isotype controls for each of the depleting antibodies did not interfere with the generation of Tregs as expected (**FIGURE 19)**.

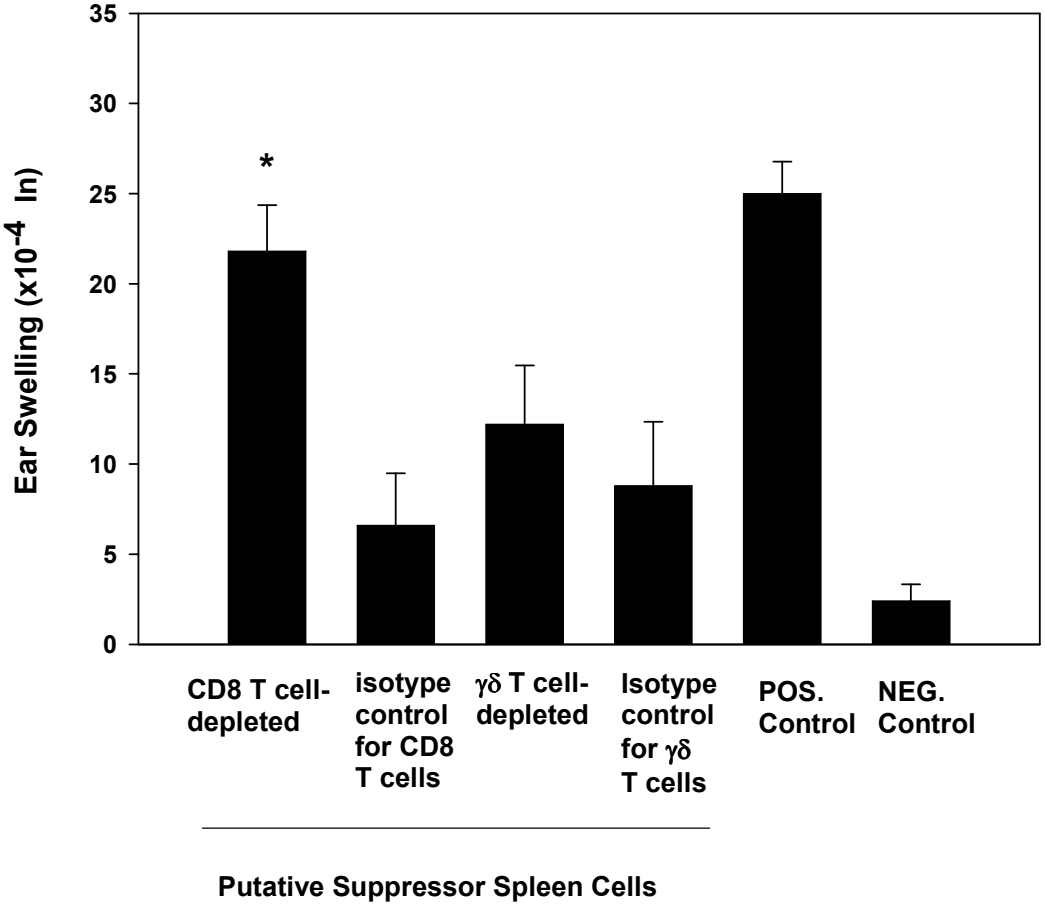


FIGURE 19. γδ T cells do not mediate efferent suppression of DTH

ACAID APC were generated *in vitro*, incubated with spleen cells for 5-7 days to generate ACAID Tregs. Spleen cell suspensions were treated with purified anti-mouse γδ TCR (or isotype control antibody) + complement immediately prior to use in a LAT assay. Other spleen cell suspensions were treated with purified rat anti-mouse CD8a (or isotype control antibody) + complement immediately prior to use in a LAT assay. A LAT assay was performed using OVA + OVA-immune spleen cells.
* $P<0.01$ (compared to all other groups except positive control)

Do Gamma delta T cells function as APC in the induction of ACAID?

Reconstitution with MHC-deficient γδ T cells restores ACAID in vivo

It has recently been demonstrated that γδ T cells can act as professional APC (128, 129). ACAID culminates in the generation of MHC class II-restricted $CD4^+$ Tregs and MHC class I-restricted $CD8^+$ Tregs and thus, requires APC that present antigen on MHC class I and MHC class II molecules (126). If γδ T cells act as APC for the induction of ACAID, then reconstituting γδ T cell KO mice with γδ T cells from either MHC class I-deficient or MHC class II-deficient donors should not restore ACAID in γδ T cell KO recipients. This hypothesis was tested by reconstituting γδ T cell KO mice with $5x10^5$ γδ T cells from wild-type C57BL/6 mice, MHC class I-deficient mice (β2M KO mice), or MHC class II-deficient mice. One week after reconstitution, OVA was injected into the AC of reconstituted γδ T cell KO mice and control mice. Seven days later, mice were immunized SC with OVA emulsified in CFA. OVA-specific DTH was assessed 7 days after the SC immunization. As anticipated, non-reconstituted γδ T cell KO mice failed to develop ACAID. However, reconstitution with γδ T cells restored the capacity of γδ T cell KO mice to develop ACAID. Importantly, reconstitution with γδ T cells from either MHC class I-deficient donors or MHC class II-deficient donors successfully restored ACAID, indicating that γδ T cells did not act as APC for the induction of ACAID (**FIGURE 20**).

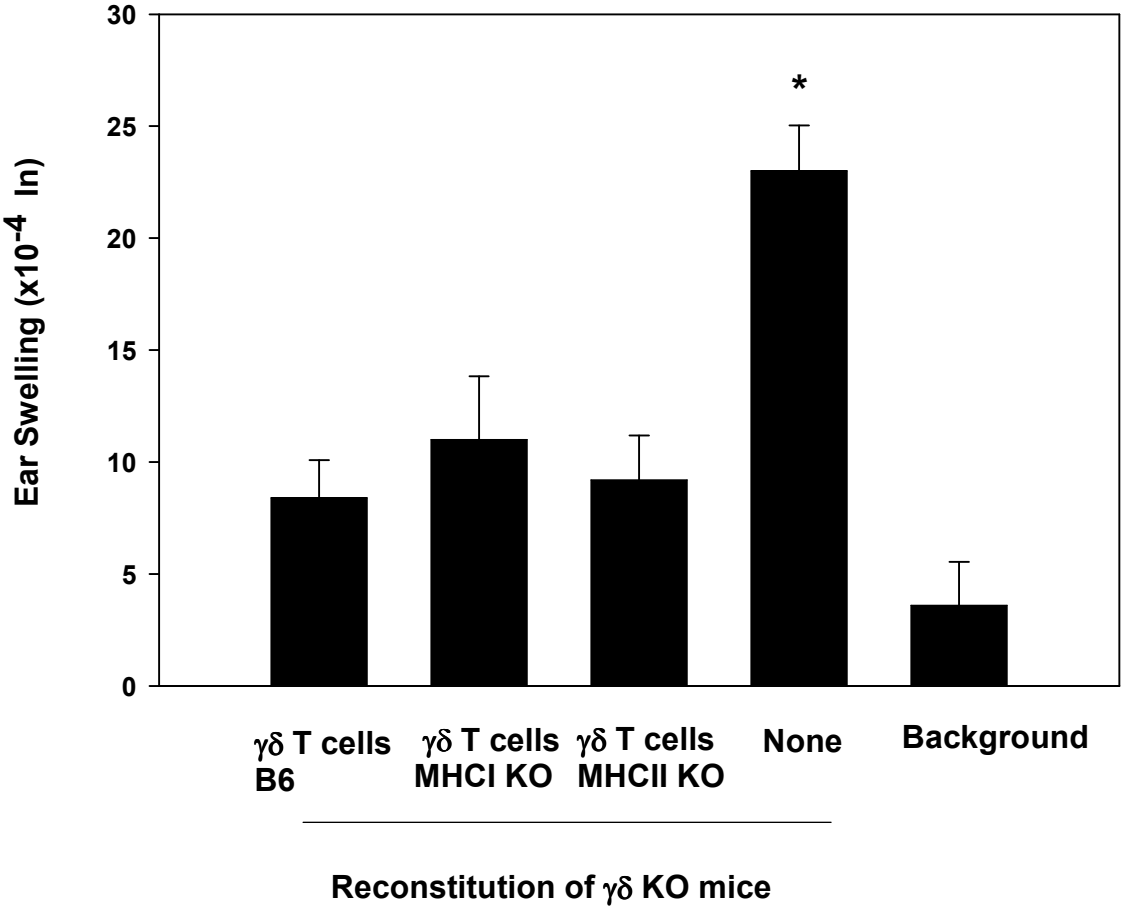


FIGURE 20. Reconstitution of γδ T cell KO mice with MHC-deficient γδ T cells restores ACAID *in vivo*

γδ T cell KO mice were reconstituted with $5x10^5$ γδ T cells from MHC I KO mice, MHC II KO mice or wild-type mice. OVA was injected into the AC 7 days after γδ T cell reconstitution. Mice were immunized SC with OVA + CFA 7 days after the AC injections with OVA. DTH responses to OVA were assessed 7 days after the SC immunization using an ear swelling assay. * $P<0.01$ (compared to each of the 3 groups of γδ T cell reconstituted mice)

The presence of γδ T cells is crucial for ACAID generation in vitro

In order to test for the requirement of the expression of MHC I and MHC II molecules on γδ T cell *in vitro*, it was necessary to first examine the effect of absence of γδ T cells on generation of regulatory cells *in vitro*. The hypothesis was that absence of γδ T cells will inhibit the generation of ACAID Tregs *in vitro*. To examine this hypothesis, ACAID-inducing APC from C57BL/6 mice were generated *in vitro*. ACAID APC were then incubated with spleen cells from either C57BL/6 mice or γδ T cell KO mice. After 5-7 days of incubation , the spleen cell suspensions were tested in a LAT assay for their capacity to suppress DTH responses to OVA. Results confirmed that γδ T cells were needed for the generation of ACAID Tregs *in vitro* (**FIGURE 21**).

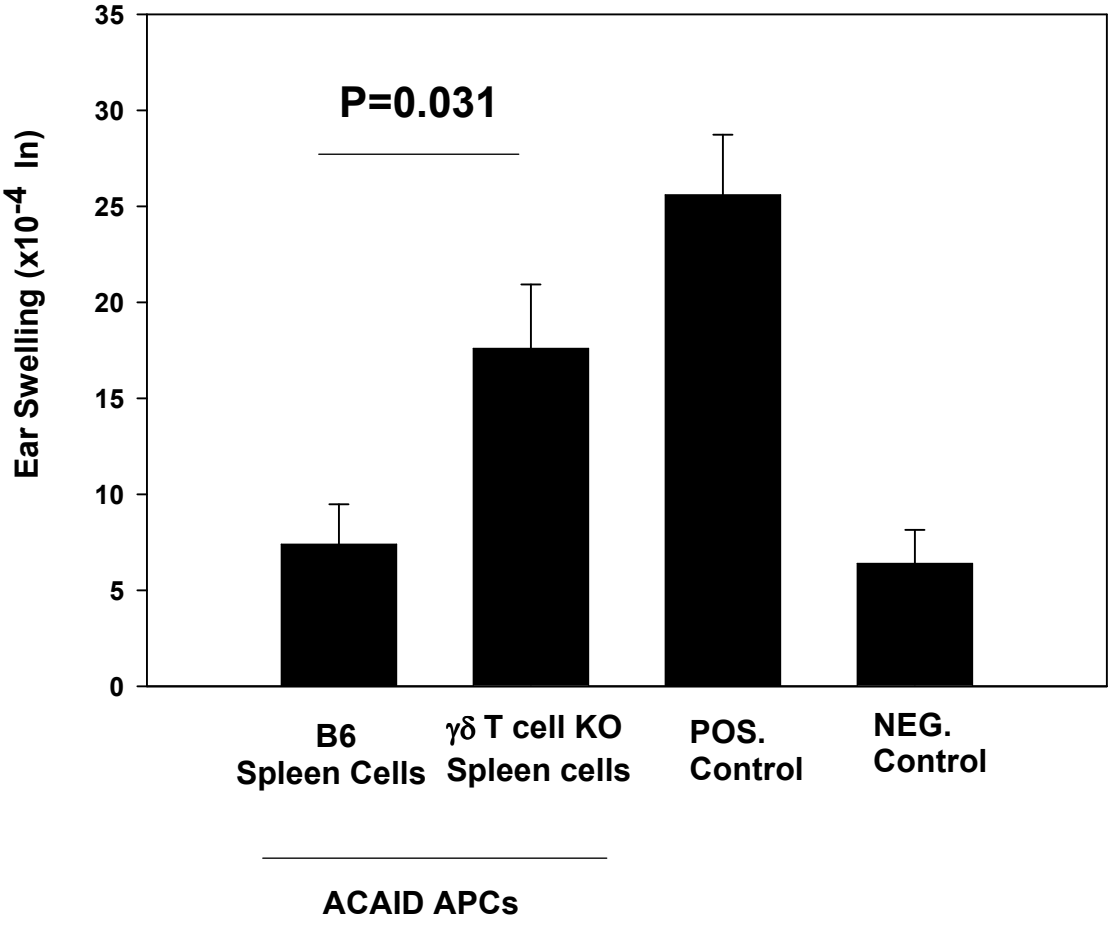


FIGURE 21. Absence of γδ T cells precludes the *in vitro* generation of ACAID Tregs

ACAID APC from B6 mice were generated *in vitro*, incubated ($5x10^6$) with spleen cells ($5x10^7$) from either B6 mice or γδ T cell KO mice. After 5-7 days, the spleen cell suspensions were tested in a LAT assay for their capacity to suppress DTH responses to OVA.

Reconstitution with MHC-deficient γδ T cells restores ACAID in vitro

As shown above, the absence of γδ T cells inhibits the *in vitro* generation of ACAID Tregs. Two approaches were used to explore the possibility that γδ T cells acted as APC for the *in vitro* generation of ACAID. The first approach was to examine the requirement for MHC I or MHC II expression on γδ T cells. If γδ T cells act as APC for the induction of ACAID, then reconstituting *in vitro* ACAID spleen cell cultures that lack γδ T cells with γδ T cells from either MHC class I-deficient or MHC class II-deficient donors should not restore ACAID.

The second approach was to inhibit the MHC Class II pathway for processing and presentation of exogenous antigens. Processing of exogenous antigens requires acidification of the lysosomes. Chloroquine prevents the acidification of lysosomes, thereby blocking the action of acid proteases necessary for antigen processing (130). Chloroquine treatment has also been shown to inhibit the antigen presenting function of γδ T cells in other systems (128), and was used to confirm that γδ T cells did not act as APC in the induction of ACAID in our system. If γδ T cells require phagolysosomal acidification, treatment with chloroquine should inhibit the γδ T cells' ability to induce ACAID. Accordingly, γδ T cells were isolated from wild-type C57BL/6 mice and were either untreated or treated with chloroquine (80 μM/ $2x10^5$ cells) before being used to reconstitute spleen cell cultures prepared from γδ T cell KO mice. Gamma delta T cells from either MHC I KO or MHC II KO mice were used to reconstitute similar spleen cell cultures. Spleen cell cultures were used to generate ACAID Tregs as before. Following 5 days in culture, Treg activity was examined in the previously

described LAT assay. The results indicated that neither chloroquine treatment nor deficiencies in MHC class I or II expression prevented γδ T cells from restoring ACAID in the spleen cell cultures prepared from γδ T cell KO mice (**FIGURE 22**). These results provided further evidence that γδ T cells did not function as APC in the induction of ACAID.

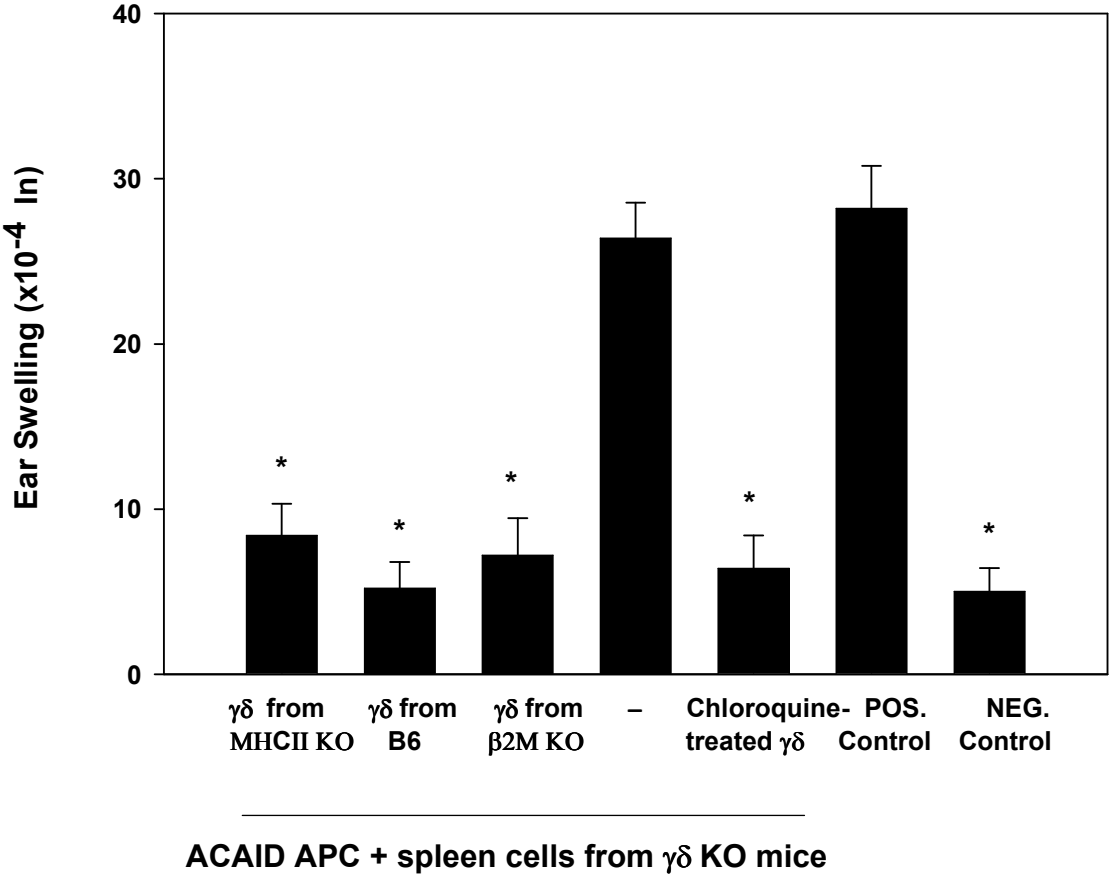


FIGURE 22. Reconstitution with MHC-deficient γδ T cells restores the generation of ACAID Tregs *in vitro*

ACAID-inducing APC were generated *in vitro*, incubated ($5x10^6$) with spleen cells ($5x10^7$) from γδ T cell KO mice without or with γδ T cells from B6 mice, MHC II KO mice, or MHC I KO mice. Another spleen cell culture from γδ T cell KO mice was incubated with ACAID-inducing APC and chloroquine-treated γδ T cells from C57BL/6 mice. After 5-7 days, the spleen cell suspensions were tested in a LAT assay for their capacity to suppress DTH responses to OVA. * $P<0.01$ (compared to positive control)

Do Gamma delta T cells need to secrete IL-10, IL-4 or IFN-γ in ACAID?

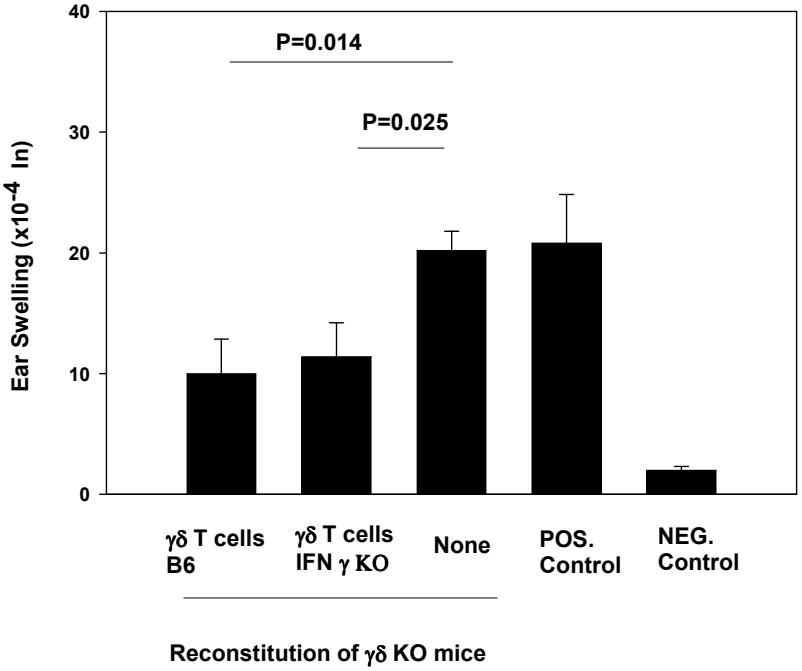
Gamma delta T cells must produce IL-10 to induce ACAID

Gamma delta T cells can produce a variety of cytokines including the immunosuppressive cytokine IL-10, the Th2 cytokine IL-4, and the Th1 cytokine IFN-γ (131-135). Moreover, reports have suggested that the immunosuppressive function of γδ T cells is mediated mainly by cytokines (133, 136). Since ACAID is a Th2-like phenomenon that inhibits Th1 immune responses, we hypothesized that γδ T cells need to secrete Th2 cytokines (such as IL-10 and IL-4) for the generation of efferent Tregs and down-regulation of DTH. Although IFN-γ is a signature cytokine for Th1 cells, it is also necessary for the generation of Tregs in some models and is known to mitigate some Th1-immune-mediated diseases (137). Therefore, the possibility that the production of IL-4, IL-10, or IFN-γ by γδ T cells was involved in the generation of ACAID was explored.

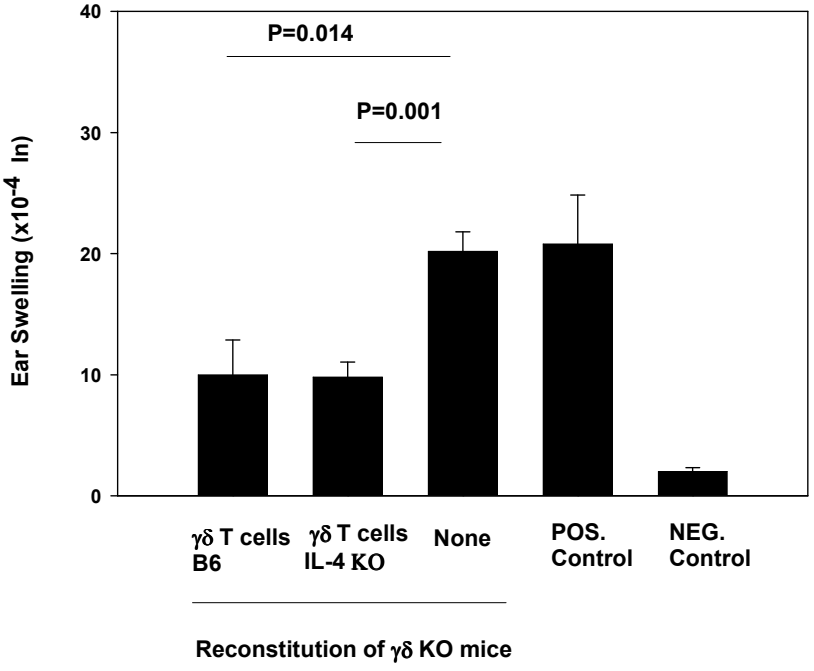
Gamma delta T cell KO mice were reconstituted with $5x10^5$ γδ T cells from IFN-γ KO mice, IL-4 KO mice, or IL-10 KO mice. One week after reconstitution, OVA was injected into the AC of reconstituted γδ T cell KO mice as well as the control mice. Seven days after reconstitution, mice were immunized SC with OVA emulsified in CFA. Seven days after the SC immunization, OVA-specific DTH responses were assessed. The results indicated that the inability to produce of either IFN-γ or IL-4 did not affect the ability of γδ T cells to reconstitute the generation of ACAID (**FIGURE 23**). On the other hand, γδ T cells from IL-10 KO donors were incapable of restoring ACAID in γδ T cell KO mice, indicating

that the production of IL-10 was crucial for the γδ T cell's contribution to the induction of ACAID (**FIGURE 23**).

A.


40
30
20
10
0
Ear Swelling (x10^{-4} In)
P=0.014
P=0.025
γδ T cells B6
γδ T cells IFN γ KO
None
POS. Control
NEG. Control
Reconstitution of γδ KO mice

B.


40
30
20
10
0
Ear Swelling (x10^{-4} In)
P=0.014
P=0.001
γδ T cells B6
γδ T cells IL-4 KO
None
POS. Control
NEG. Control
Reconstitution of γδ KO mice

C.

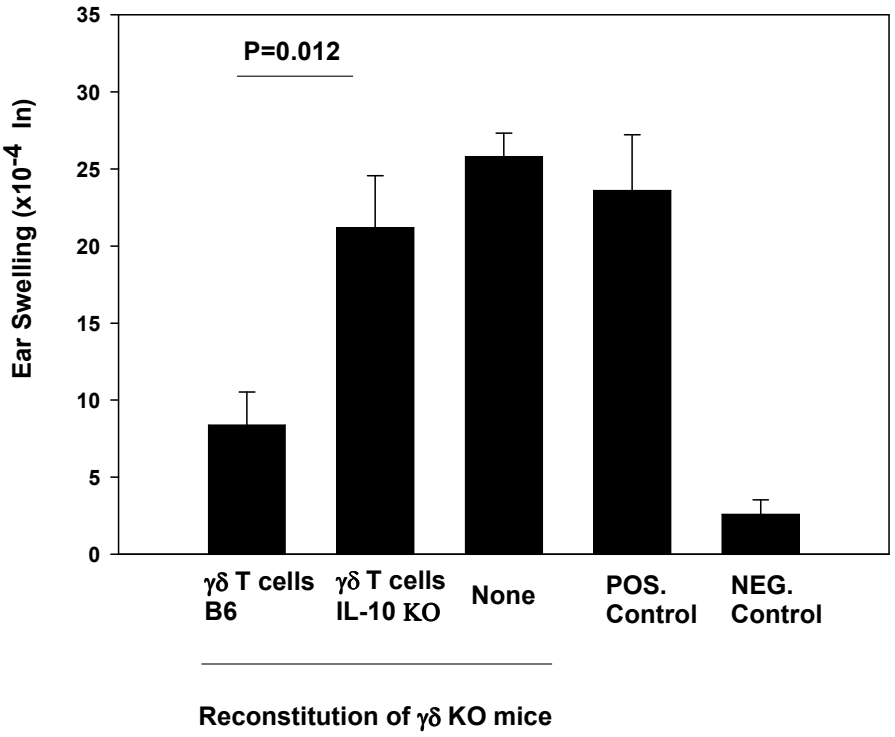

P=0.012
Ear Swelling (x10-4 In)
35
30
25
20
15
10
5
0
γδ T cells B6
γδ T cells IL-10 KO
None
POS. Control
NEG. Control
Reconstitution of γδ KO mice

FIGURE 23. γδ T cells need to secrete IL-10, not IFN-γ or IL-4, for ACAID induction

A) γδ T cell KO mice were reconstituted with spleen equivalents of γδ T cells from wild-type or IFN γ KO mice. OVA was injected into the AC 7 days after γδ T cell reconstitution. Mice were then immunized SC with OVA + CFA 7 days later. DTH responses to OVA were assessed 7 days after SC immunization using an ear swelling assay.
B) γδ T cell KO mice were reconstituted with spleen equivalents of γδ T cells from wild-type or IL-4 KO mice. OVA was injected into the AC 7 days after γδ T cell reconstitution. Mice were then immunized SC with OVA + CFA 7 days later. DTH responses to OVA were assessed 7 days after SC immunization using an ear swelling assay.
C) γδ T cell KO mice were reconstituted with spleen equivalents of γδ T cells from wild-type or IL-10 KO mice. OVA was injected into the AC 7 days after γδ T cell reconstitution. Mice were then immunized SC with OVA + CFA 7 days later. DTH responses to OVA were assessed 7 days after SC immunization using an ear swelling assay.

Recombinant mouse IL-10 can fully replace the function of γδ T cells in the induction of ACAID

The aforementioned ACAID spleen cell culture system was used to confirm the role of γδ T cell-derived IL-10 in the induction of ACAID. OVA-pulsed ACAID-inducing APC were added to spleen cell cultures from γδ T cell KO mice. Spleen cell cultures were then supplemented with rmIL-10 (10 ng/ml), rmIL-4 (10 ng/ml), or γδ T cells from wild-type mice. Five days later, the generation of ACAID Tregs was determined using the aforementioned LAT assay. The results indicated that ACAID was restored in spleen cell cultures from γδ T cell KO mice by the addition of rmIL-10 or the reconstitution with γδ T cells from wild-type mice (**FIGURE 24**). However, addition of IL-4 did not restore the generation of ACAID. The effect shown with rmIL-10 was not merely a non-specific effect of IL-10 on spleen cells, as no Tregs were detected unless OVA-pulsed APC were present in IL-10 supplemented culture medium (**FIGURE 24**). These results suggest that the major function of γδ T cells in the induction of ACAID is their secretion of IL-10.

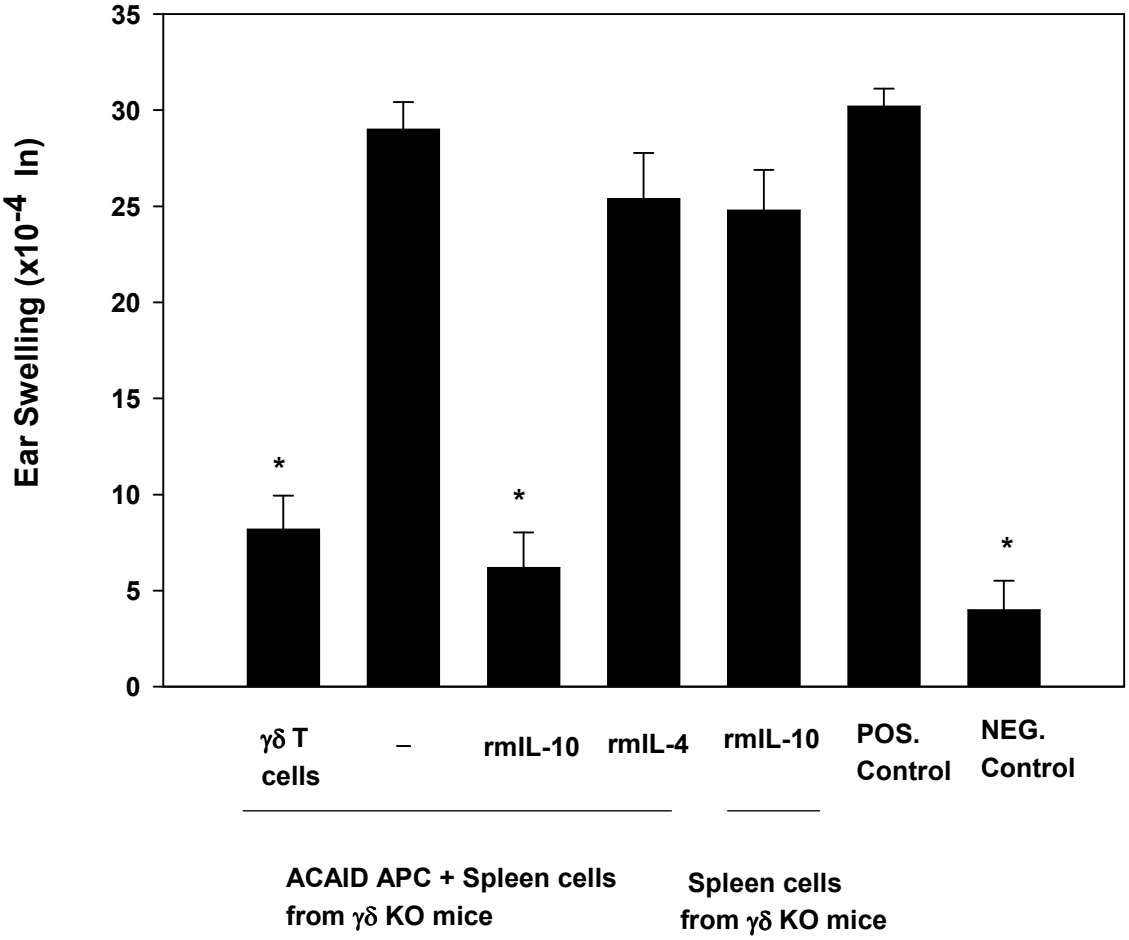


FIGURE 24. Recombinant mouse IL-10 fully replaces γδ T cell function in ACAID

ACAID-inducing APC were generated *in vitro*, incubated ($5x10^6$) with spleen cells ($5x10^7$) from γδ T cell KO mice with or without γδ T cells from B6 mice. One group contained rm IL-10 (10 ng/ml) but no γδ T cells. Another group contained rmIL-4 (10 ng/ml) but no γδ T cells. A control group contained rm IL-10 and spleen cells from γδ KO mice but no APC. After 5-7 days, the spleen cell suspensions were tested in a LAT assay for their capacity to suppress DTH responses to OVA. * $P<0.01$

CHAPTER FIVE

Conclusions and Recommendations

ACAID AS A COMPONENT OF IMMUNE PRIVILEGE

Clinical relevance of this study

The peripheral tolerance induced by AC injection of antigen is more than a curious artifact and has important clinical implications. Peripheral tolerance that is induced when antigen enters an immune privileged site, such as the eye, not only prevents immune-mediated responses directed at self antigens, but also regulates harmful DTH immune responses that can lead to irreparable injury to innocent bystander cells that are incapable of regeneration. Corneal endothelial cells and cells forming the retina are examples of terminally differentiated ocular cells that cannot undergo mitosis and regenerate. Injury to either of these cell populations can lead to blindness. Whether ACAID prevents the generation or the expression of autoimmune Th1 immune responses in the eye under normal physiological conditions remains to be established. However, it is noteworthy that inducing ACAID by AC injection of either retinal-specific autoantigens or corneal alloantigens results in the mitigation of ocular autoimmune diseases such as experimental autoimmune uveitits and the acceptance of cornal allografts respectively (8, 117, 118, 138-140). Orthotopic corneal grafts are in direct contact with the AC of the eye, and thus, can induce ACAID, which is intimately associated with corneal allograft survival (8, 138). Moreover, induction of ACAID by AC injection of donor-derived cells prior to corneal transplantation enhances corneal allograft survival, while maneuvers that prevent the corneal allograft's capacity to induce ACAID, such as splenectomy or thymectomy, result in increased

graft rejection (118, 140). In brief, ACAID is crucial for the normal function of an immune privileged organ such as the eye. The clinical significance of studying ACAID can be further emphasized by comparing it to other models of tolerance that has both similarities and difference from our model. The model of Nickel-induced tolerance is one of these models that parallels ACAID in many respects.

ACAID vs. Nickel-induced tolerance

ACAID shares many of the properties with orally induced immune tolerance to nickel, including the participation of B cells and NK T cells (13, 89, 98, 141-143). In addition, NK T cells have been shown to be crucial for the induction phase and not the effector suppression phase in both systems. Adoptive transfer of APC or B cells to mice lacking NK T cells in the nickel-induced model does not lead to induction of tolerance. Similar sorts of experiments need to be done in the ACAID model to delineate the site of action of NK T cells. Although both IL-4 and IL-10 are needed for nickel-induced tolerance, only IL-10 has been shown to be crucial for ACAID (63, 144). IFN-γ has been shown not to be required in both systems (63, 144). Expansion of B cells following ACAID induction is paralleled with the doubling of splenic NK T cells following antigen injection in nickel-induced tolerance. Finally, orally induced tolerance to nickel can be adoptively transferred with B cells; however, the B cells induce a form of infectious tolerance that can be transferred to third-generation hosts with either T cells or APC (143). It will be important to determine if a similar form of infectious tolerance occurs in ACAID. These similarities between both systems make it plausible to assume that this sort of tolerance is a general mechanism that manifests itself in different forms but with the same basic

components and general relay of tolerogneic signals between the different cell populations involved.

ACAID vs. BRAID

Another model that is similar to ACAID in several ways is a model that functions in the brain, which is another immune privileged site. Antigens introduced into the brain induce an immune deviation that bears a notable resemblance to ACAID and is therefore called brain-associated immune deviation (BRAID) (3, 25). In contrast to ACAID, an intact spleen is not required for BRAID, nor is BRAID adoptively transferable with spleen cells (3). BRAID has not been as thoroughly characterized as ACAID, but certainly merits serious investigation for its involvement in maintaining immune privilege in the CNS. For example, it will be important to determine if the sympathetic nervous system and the many lymphoid cell populations needed for ACAID are also crucial for BRAID.

Immune privilege of the eye

Early work done by the Dutch ophthalmologist, van Dooremaal, more than a century ago, has shed light on the unique immunological privilege characteristics that the eye possesses. Van Dooremaal observed a delayed rejection of mouse skin grafts placed in the AC of the dog eye (18). Medawar followed up on that work and noticed a prolonged survival of skin allografts placed into the AC of rabbit eyes (20). He was the first to coin the term “immune privilege” (20). Immune privilege referred to the fact that foreign grafts placed in the AC of the eye (or any

other immune privileged sites) survive for prolonged, often indefinite, intervals, whereas placement of such grafts at conventional non-privileged body sites leads to acute, irreversible immune rejection (145). Medawar believed that immune privilege was a result of "immunological ignorance" because of the existence of blood:tissue barriers and absence of lymphatic drainage routes (146). More than 20 years later, Medawar's view of "immunological ignorance" was refuted by experiments that strongly suggested that immune privilege is the product of a deviant systemic immune response to antigens in the eye (22, 23, 146).

Recent interest in studying immune privilege has broadened our understanding of the phenomenon and it has become clear that immune privilege is not just the result of an anatomical barrier that makes a particular organ "unrecognized" by the immune cells. In fact, there are multiple anatomical, physiological, and immunoregulatory mechanisms that function in concert. The outcome of all these mechanisms is the unique immune privilege that characterizes particular organs such as the testis, ovary, central nervous system, and the eye. It has therefore become clear that induction of peripheral tolerance , maintained by Tregs, is a crucial component of immune privilege (1-3, 25).

In fact, our definition of immune privilege over the past 3 decades has been extended to embrace, on the one hand, the induction of ACAID after AC injection of the antigen, and on the other hand, the ability of the ocular microenvironment (especially aqueous humor) to regulate intraocular inflammatory responses that threaten the integrity of the visual axis (145). Thus, ocular immune privilege is one of the forms of immune privilege that operates specifically in the eye with the purpose of safeguarding against intraocular inflammation. Ocular inflammation,

whether expressed within the cornea or within the uveal tract, is a major cause of blindness (4, 145). There are main features for ocular immune privilege that makes it a potent mechanism for preserving visual integrity in the eye. These include blood:ocular barriers, absence of blood vessels in the cornea, absence of patent lymph nodes draining the eye which insures that contents of AC are drained directly into the systemic circulation, presence of soluble immunoregulatory factors in the aqueous humor secreted by the ciliary body, the presence of tolerance-promoting APC in iris stroma, the expression of immunomodulatory ligands on the surface of ocular parenchymal cells (pigment epithelium that lines the posterior surface of the iris, and corneal endothelium which acts as a barrier between the AC and the corneal stroma), and ACAID (146).

Corneal allografts in humans have very high acceptance rates due to functional immunosuppressive mechanisms in the eye. Thanks to immune privilege of the eye, corneal allografts can still be accepted in murine models without the need for any immunosuppressive treatments (147). However, any compromise to ocular immune privilege of the recipient eye or of the donor graft seriously affects the graft acceptance rates rendering the eyes "high-risk eyes" that are more likely to reject the graft (148, 149). ACAID is an important, albeit one of many, factor that contributes to the immune privilege of corneal transplants. These factors include, Fas ligand which plays a critical role in protecting corneal allografts from alloimmune attack, the avascularity of the graft beds which protects the grafts from attack by $CD8^+$ CTL, and the presence of immunosuppressive factors in the aqueous humor that inhibit cell-mediated immunity and complement-dependent lysis (21)

Much of what was learned about ocular immune privilege has been gained through the study of the AC of the eye. However, most of the immunoregulatory mechanisms that function in the AC apply to other ocular compartments that display immune privilege, such as the vitreous cavity and the subretinal space (150, 151). Thus, the study of ACAID can help scientists recognize and examine different mechanisms and components of ocular immune regulation and ocular immune privilege. In addition, the suppression of systemic immune responses that follows injection of antigen into the AC of the eye is a good model to study induction of peripheral tolerance. Different T cell tolerance models may have similar mechanisms that regulate immune responses. For example, the B cells' role as APC in the induction of T cell tolerance has been reported elsewhere as in a model of T cell tolerance to aeroallergens (152, 153). In this study, we demonstrate a similar function for B cells in ACAID. Also, a regulatory role for γδ T cells mediated through their IL-10 production was demonstrated in studies with tumor models (111, 154). In our model, we report a similar finding where γδ T cells need to secrete IL-10 in order for ACAID to be generated.

B cells as crucial components for ACAID generation

Peripheral tolerance can be induced by simply introducing an antigen into the AC of the eye. This peripheral tolerance induced via the AC (i.e., ACAID) is characterized by a complex series of cellular interactions that involve the eye, thymus, spleen, as well as elements of the sympathetic nervous system (59, 62, 68, 69). Once the antigen is injected into the AC of the eye, $F4/80^+$ APC present in the anterior segment of the eye process the antigen , then emigrate to the thymus and to the spleen (5, 68, 155). After arriving into the spleen, the $F4/80^+$ ocular APC

initiate a complex series of cellular interactions that involve B cells (13, 14, 141), NK T cells (6, 97), γδ T cells (114, 156, 157), $CD4^+$ Tregs (5, 10, 11), and $CD8^+$ Tregs (11). Without the migration of the ocular APC to the spleen, none of these interactions would occur and Tregs would not be generated.

Studies have examined the function of ocular APC in relation to other splenic cells in ACAID, such as B cells. BCR on B cells is required for the antigen-specific generation of Tregs (14). Treatment with anti-Ig or irrelevant antigen prevents the generation of the antigen-specific suppression of DTH, which is characteristic of ACAID (14). B cells collected from mice primed in the AC with OVA will induce OVA-specific ACAID when adoptively transferred to naïve mice (13, 14). *In vitro* and *in vivo* studies suggest that $F4/80^+$ ocular APC release peptide fragments that are captured and internalized by splenic B cells through their BCR, which act as ancillary APC and induce the generation of $CD4^+$ Tregs and $CD8^+$ Tregs (14). Thus, the BCR is required to convey antigen specificity to ACAID Tregs (14). A similar model of antigen uptake by B cells has been previously reported (14, 90, 158). It is also known that the BCR has the capacity to concentrate small amounts of antigen and determine the epitopes to be presented to other cells, and that the antigen-presenting B cells can prime naïve CD4 T cells *both in vivo* and *in vitro* (159-164). In addition, T cell priming in the absence of B cells results in responses of reduced magnitude (165, 166). As a result, a critical role for B cells in the splenic phase of ACAID generation is strongly suggested.

In addition to the aforementioned observations, ACAID-inducing B cells can be generated *in vitro* by co-culturing splenic B cells with OVA-pulsed ACAID APC (13, 14). This *in vitro*

system permits us to better dissect interactions of B cells with other cell populations in ACAID. Using this *in vitro* model, Skelsey *et al* have demonstrated that acidification of the endosome is required for B cells to be capable of inducing ACAID Tregs (14). It is important to note that ACAID B cells, whether generated *in vivo* (by AC injection of the antigen) or *in vitro* (by co-incubation with ACAID APC), do not function as regulatory cells, but instead, induce the development of Tregs by promoting the generation of $CD4^+$ Tregs and $CD8^+$ Tregs (13, 14). Thus, the weight of evidence suggests that B cells act as ancillary APC for the induction of ACAID. This is not unusual, as it was reported in other models that B cells acted as APC for inducing T cells tolerance (152, 153, 167). In one of these models, naive antigen-specific T cells efficiently bound naive antigen-specific B cells in the context of a normal immune repertoire and an unperturbed microenvironment (152).

Ocular APC are required to deliver the ACAID-inducing signal to B cells

The existence of a camero-splenic axis that links the ocular environment to the systemic circulation and the spleen has been previously demonstrated (4, 146). Blood withdrawn from mice 48 hrs after AC injection of antigen contained $F4/80^+$ cells that could induce ACAID in naïve recipients. These recipients of the blood-borne $F4/80^+$ cells developed Tregs that were antigen-specific without having received any antigenic injections in their AC. Blood analysis has demonstrated that the antigen-bearing $F4/80^+$ APC was the ACAID-inducing signal present in the transferred blood (146). Indigenous $F4/80^+$ APC are present within the eye adjacent to the pigment epithelium of the iris, the ciliary body, and the retina (168, 169). As stated above, $F4/80^+$ APC harvested 24 hrs after AC injection of the antigen can induce ACAID following

adoptive transfer into naïve mice unless the recipient mice were splenectomized prior to the adoptive transfer (73). These results suggest that $F4/80^+$ APC carry AC-injected antigens through the blood stream (through the trabecular meshwork) into the spleen. In the marginal zone of the spleen $F4/80^+$ APC interact with B cells, naïve $CD4^+$ and $CD8^+$ T cells, NK T cells and perhaps γδ T cells (8, 146).

Results presented in this dissertation extend previous findings and shed light on our understanding of the function of B cells in ACAID. These results indicate that ocular APC deliver a signal to B cells that renders them tolerogenic, which means that B cells will now be able, by themselves, to induce immune regulation in the periphery. As shown in the Results section, ocular APC must process the antigen in order for B cells to acquire the capacity to induce ACAID. B cells exposed to OVA alone cannot induce ACAID due to the lack of the ocular tolerogenic APC. B cells exposed to OVA-pulsed normal APC cannot induce ACAID due to the lack of APC with tolerogenic capacity. B cells exposed to untreated ACAID APC (ACAID APC not exposed to antigen) do not induce ACAID because the ACAID APC need an antigenic signal to set the process in motion. These results show the importance of both ocular APC and antigen to render B cells tolerogenic. However, cell contact between antigen-pulsed ACAID APC and B cells is not required and tolerogenic B cells can be generated even if the two cell populations are separated by a semi-permeable membrane. This interaction between ACAID APC and B cells involves the induction of a form of infectious tolerance where the tolerance-inducing capacity has been transmitted from the ACAID APC to the B cells. It is possible that the nature of antigenic interaction with B cells determines if the B cells will act in an immunogenic or a tolerogenic manner. This is similar to the findings in a model of respiratory

exposure to antigen in which antigen introduced intranasally drives the B cells to present antigen to T cells in a tolerogenic manner (153).

Expansion of B cells in ACAID

In vitro expansion

Intrigued by the observation that at least one million tolerogenic B cells need to be adoptively transferred for ACAID to be generated as compared to only twenty $F4/80^{+}$ ocular APC (73), it seemed plausible to hypothesize that there is an amplification of the tolerogenic response manifested by B cell expansion in ACAID. *In vitro* and *in vivo* experiments proved this hypothesis. *In vitro* proliferation assays showed a marked difference in proliferation kinetics of B cells incubated with antigen-pulsed ACAID APC and B cells incubated with antigen-pulsed non-ACAID APC. After two days of incubation, there was a surge in proliferation of B cells incubated in the *in vitro* ACAID setting, which was significantly different from B cells incubated with regular APC. This difference in kinetics is consistent with our hypothesis. We noticed, however, that the surge in proliferation of ACAID B cells did not remain as high after 5 days of incubation. This interesting difference in kinetics *in vitro* is at present hard to explain.

In vivo expansion

To better monitor B cell proliferation, different antigens were injected into the eyes of HEL BCR mice. An antigen-specific marked expansion in the numbers of B cells was noticed.

This presumably increased the number of antigen-specific B cells that are available to present antigen to T cells for the induction of ACAID. Flow cytometric analysis of HEL BCR transgenic mice primed in the AC with HEL, OVA, or PBS indicated that splenic B cell proliferation is restricted to B cells that express the BCR that recognizes the antigen injected into the AC. This means that this expansion that we noticed was not merely a non-specific response to any antigenic challenge to the eye.

Inhibition of B cell expansion

Results shown above indicate that ACAID B cells treated with either γ-irradiation or mitomycin-C are not able to adoptively transfer ACAID, suggesting that clonal expansion of B cells is necessary for the induction of ACAID. Another possibility is that B cells got recruited to the spleen as a result of antigen introduction in the AC. It can be argued that γ-irradiation or mitomycin-C can alter protein synthesis and compromise cell viability. However, we believe that using both techniques for inhibiting cell proliferation in different experimental settings still gives considerable weight to support the hypothesis that B cell proliferation is needed for ACAID.

Antigen presentation by B cells in ACAID

The two main techniques that have been used for characterizing the role of B cells in the induction of ACAID are: AC injection of antigen and the adoptive transfer of splenic B cells from mice primed in the AC with antigen to naïve recipients (13, 14). In both cases, antigen-

specific $CD4^+$ Tregs and $CD8^+$ Tregs are induced, which leads to the question as to how B cells can simultaneously induce two populations of Tregs that are MHC class II- restricted and MHC class I-restricted respectively. Results from several *in vivo* and *in vitro* experiments, shown in this dissertation, indicate that a defect in either the assembly or expression of either MHC class I or MHC class II molecules on B cells prevents B cells from inducing ACAID. The defect in MHC class II antigen presentation can be bypassed, though, by supplying exogenous rmIL-10. In addition, if both MHC class I KO B cell population and MHC class II KO B cell population are combined, they are capable of restoring ACAID in B cell KO mice, suggesting that B cells present antigen on both MHC class I and II molecules. This also suggests that antigen presentation on B cells need not to be on the same B cell. This model of antigen presentation in the spleen is one example of how B are key factors in inducing T cell tolerance and is in line with observations of how B cells can affect the function of T cells in other models (170, 171).

Gamma Delta T cells in ACAID

Location

As mentioned before, the induction of ACAID involves a complex series of events and the participation of at least four organs: the eye, thymus, spleen, and sympathetic nervous system, and at least six different cell populations: ocular APC (4), B cells (14, 126), γδ T cells (114, 136, 157), NK T cells (96, 97), $CD4^+$ T cells (5, 10), and $CD8^+$ T cells (84). After capturing antigen in the AC of the eye, $F4/80^+$ ocular APC migrate to the thymus (9) and spleen (172). In the spleen, the $F4/80^+$ ocular APC interact with NK T cells, $CD4^+$ T cells, and B cells (97, 98).

Splenic B cells use their BCR to capture antigenic peptides released by the F4/80$^+$ ocular APC and present these antigens to both CD4$^+$ and CD8$^+$ T cells, leading to the generation of efferent Tregs (126). As shown in the previous sections, cell-cell contact between ocular APC and B cells is not necessary for the generation of ACAID. The full detail of interactions of ACAID B cells with NK T cells, γδ T cells, and T cells will need more experimentation to be fully explained. As primary steps toward understanding this, some of the experiments presented in this dissertation address the site where γδ T cells function in the induction of ACAID. The results indicate that the γδ T cell acts downstream from the ACAID B cell, as adoptive transfer of ACAID B cells into γδ T cell KO mice or γδ T cell-depleted C57BL/6 mice fails to induce ACAID.

Functions

There are several strategic points in the induction of ACAID where γδ T cells might function. To dissect the functions of γδ T cells in ACAID generation, we considered the obvious explanation that γδ T cells acted as efferent Treg cells that can directly inhibit the expression of DTH, as γδ T cells are known to secrete immunosuppressive and anti-inflammatory molecules (136). Moreover, some γδ T cell populations have been shown to express the CD8 molecule, which is also found on ACAID efferent Tregs (104). Our findings demonstrate, however, that depletion of γδ T cells from ACAID CD8$^+$ Treg suspensions does not abolish CD8$^+$ T cell-mediated suppression of DTH. This clearly proves that γδ T cells do not function as ACAID efferent Tregs.

The next hypothesis was that γδ T cells act as ancillary APC in the induction of ACAID. This was based on a recent report indicating that γδ T cells can function as antigen presenting cells (128). Chloroquine treatment prevents antigen presentation and the induction of ACAID by F4/80^{+} ACAID APC and by splenic B cells (14, 173). Therefore, γδ T cells were subjected to a similar chloroquine treatment protocol to ascertain if γδ T cells acted as APC in the induction of ACAID. However we noticed that chloroquine treatment, which is sufficient to inhibit the antigen presenting function of γδ T cells (128), yet does not affect the capacity of γδ T cells to contribute to the generation of ACAID, did not affect the function of γδ T cells in ACAID. In addition, the induction of ACAID, which requires simultaneous presentation of antigens on both MHC class I and class II molecules (126), was not adversely affected when γδ T cells from mice deficient in the expression of either MHC class I or II molecules were used. In other words, γδ T cells that are not capable of antigen presentation can still contribute to the induction of ACAID.

We were also attracted to the hypothesis that γδ T cells act as ancillary producers of IL-10, which is known to be crucial for the induction of ACAID and for other forms of tolerance (63). This proposition is supported by the finding that γδ T cells from wild-type mice, IL-4 KO mice, or IFN-γ KO mice can restore ACAID in γδ T cell KO mice, while γδ T cells from IL-10 KO donors cannot. Moreover, the contribution of γδ T cells in the induction of ACAID could be replaced by simply adding exogenous rmIL-10 cytokine to ACAID spleen cell cultures lacking γδ T cells. These results fit well with data showing a cytokine secretion function for γδ T cells in other forms of T cell tolerance (131-136, 174). These results are also in accordance with data from tumor models showing a critical regulatory role for IL-10 production through γδ T cells in

inhibiting immune elimination of tumors (111, 154). Future suggested experiments can include different transwell assays. For instance, an assay with a transwell plate separating ACAID APC-pulsed spleen cells from normal mice (top chamber) and spleen cells from γδ T KO mice (bottom chamber). The presence of γδ T cells in the top chamber should lead to IL-10 secretion that will induce the generation of Tregs in the bottom chamber even in the absence of γδ T cells in the bottom chamber. The advantage of this experiment is that IL-10 levels will be more relevant than using an arbitray concentration of exogenous rm IL-10.

There still remains many unanswered questions pertaining to the role of γδ T cells in ACAID. The segregation of γδ T cells into functionally specialized cell populations in correlation with TCR variable gene expression (175) raises an interesting, yet challenging question, as to which of these subpopulations of γδ T cells is involved in ACAID and what kind of interaction it has with other γδ T cells and other immune cells. Since γδ T cells are known to interact with cells of the innate system at many levels (175), unraveling these interactions in the immunoregulatory setting of ACAID is also important. Finally, the details of the antigen recognition process by γδ T cells in ACAID need to be thoroughly investigated.

Role of IL-10 in ACAID

Other questions pertaining to the role of IL-10 in ACAID need to be examined. IL-10 has been shown to be secreted by multiple cell populations in ACAID. ACAID APC, $CD4^+$ T cells, $CD8^+$ T cells, γδ T cells, and NK T cell have all been shown to secrete IL-10 (63, 119). IL-10 might function as a soluble mediator that transmits the tolerogenic signal between

different cell populations in the spleen, probably by spreading infectious tolerance between the tolerogenic APC and one another (176). Alternatively, the regulatory function of IL-10 can be attributed to its down-regulatory effect on APC signals (MHC class II and costimulatory molecules), incomplete maturation of APC loaded with antigen, and generation of regulatory T cell subtypes (177). All of these are relevant to ACAID. For example, IL-10 may downregulate the expression of MHC Class II molecules on ocular APC to prevent them from competing with B cells, which present antigens in a tolerogenic manner through MHC Class II molecules. IL-10 may also induce the generation of Tregs in the periphery, possibly through downregulation of proinflammatory genes on T cells in the periphery. Skelsey *et al* have shown that $CD4^+$ T cells secrete IL-10 and that this secretion of IL-10 does not interfere with the flow of the tolerogenic signal or with antigen specificity of the process (10). Furthermore, results presented here further emphasize the role of $CD4^+$ T cells in secretion of IL-10 and show that the requirement of antigen presentation by B cells to $CD4^+$ T cells can be bypassed by supplying rmIL-10 in culture. Another area that needs to be examined is the role of NK T cell-derived IL-10 on B cell presentation of antigens. The effect of IL-10 on the regulatory role of $CD8^+$ efferent regulatory cells also needs to be examined . The autocrine effect of IL-10 secretion on cells that produce it may be a way of amplifying the tolerogenic signal. All of these areas need further investigation.

FIGURE 25 lists different cells involved in IL-10 secretion in ACAID.

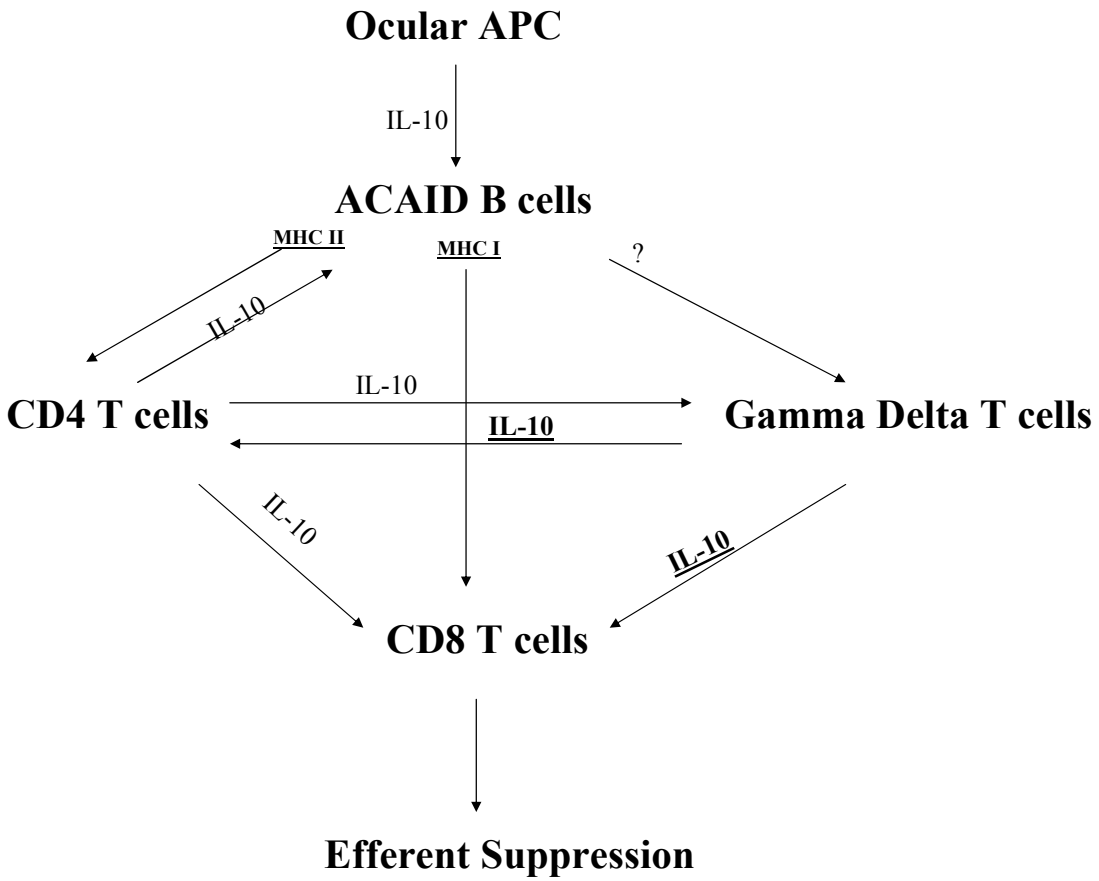


FIGURE 25. IL-10 is a main immunosuppressive cytokine in ACAID.

Although NK T cells secrete IL-10 as well, they have been removed from this cartoon for simplicity. The secretion of IL-10 by γδ T cells and the requirement of both MHC I and MHC II expression on B cells are underlined since these results were first demonstrated by work described in this dissertation.

Mode of action of $CD8^+$ Tregs

We now know that ACAID B cells and γδ T cells do not directly suppress DTH (14). An exciting future area of research will be to determine how $CD8^+$ Tregs regulate DTH responses. There might be soluble factor(s) that mainly mediate this function. Potential candidates for this role are IL-10 and TGF-β, as they both have been implicated as key cytokines at different levels of the induction of ACAID and express potent anti-inflammatory activity (63). However, treating efferent ACAID regulatory cells with anti-TGFβ2 does not abolish their capacity to inhibit DTH in a LAT assay (93). Thus, TGFβ2 can be excluded as a candidate cytokine responsible for efferent regulation of DTH by $CD4^+$ Tregs. Since immune T cells from CD95 deficient mice can still be suppressed by Tregs, apoptosis of immune cells through a Fas-FasL interaction is also excluded as a potential mechanism for action of $CD8^+$ Tregs (94). $CD8^+$ Tregs might also condition APC to become suppressive APC that inhibit $CD8^+$ T cells from mediating DTH responses, possibly through IL-10 secretion, as was suggested by several scientists (143, 176). In a very recent paper, Cone and colleagues have shown that the suppression of DTH by $CD8^+$ Tregs requires IFN-γ (178). They have also demonstrated that although the expression of FasL by $CD8^+$ Tregs was dispensable, IFN-γ secretion was not (178). Finally, they demonstrated that, whereas the generation of these $CD8^+$ Tregs was independent of IFN-γ, their regulatory function required IFN-γ receptor stimulation. This area still merits further analysis, as it will have implications on how $CD8^+$ Tregs might be acting in other models of immune tolerance.

ACAID as a Th2-like response

It is known that two of the hallmarks of ACAID are the down-regulation of DTH on one hand and the up-regulation of IgG1 non-complement fixing antibodies on the other hand (177). IL-10 KO mice fail to acquire ACAID which further suggests that ACAID is a Th2 immune response (63). Upregulation of GATA-3 transcription factor is additional evidence suggesting a Th2 response in ACAID (179, 180). Collectively, these results may lead one to think that ACAID is just another type of a Th2 immune response that is elicited through an unusual route of antigen delivery which is the AC of the eye. However, the discovery that IL-4 is not required for the induction of ACAID has challenged this concept (63). Furthermore, activated Th1 or Th2 immune cells cannot be found in the spleens of mice injected in the AC with OVA unless the mice receive a subsequent immunization with OVA with CFA (88). However, cells resembling Th2 cells are detected in the spleens and draining lymph nodes (88). This finding supports the idea that ACAID is a Th2-like phenomenon that bears resemblance to Th2 immune responses, but it is still unique. Using a murine model of OVA-specific, Th2-dependent pulmonary inflammation, Katagiri *et al* have demonstrated that antigens injected into the AC of the eye impaired both Th1 and Th2 immune responses (177). This finding argues against the concept of ACAID being a Th-2 like phenomenon. That is, if ACAID were to function in a Th-2 like manner, one would not expect it to downregulate other Th-2 immune responses. ACAID may be indeed a unique form of immune tolerance that shares some properties with Th2 responses but it is quite distinct from it. From a therapeutic point of view ACAID is a major component of the immune privilege of the eye which can regulate inflammation produced by either Th1- and Th2-type immune responses. Th2-based inflammation can be severe and damage the integrity of

many organs, not only the eye. For example, the granulomas that produce severe disease in schistosomiasis are Th-2 mediated (181). Also, the remodeling and fibrosis that occur in allergic asthma are Th2-mediated (182, 183).

Model of cellular interactions in ACAID

The results described previously in this dissertation have shed light on some functional aspects of B cells and γδ T cells in ACAID. Although there is much to be learned about functional aspects and cross-talk between different cell populations in ACAID, such an intricate system can be divided into three phases; the ocular phase, the thymic phase, and the splenic phase. As mentioned previously, many studies on the ocular phase of ACAID have already demonstrated that the initial carrier of the tolerogenic signal is the ocular APC that expresses the $F4/80^{+}$ surface marker (184). Studies on the thymic phase have shown that recent thymic emigrants of NK T cells migrate from the thymus to the spleen (68). This means that all the cellular components of ACAID ultimately converge in the splenic phase. The ocular APC released antigen is captured by splenic B cells through their BCR. In theory, the ocular APC can directly present to the $CD8^{+}$ T cells and/or the $CD4^{+}$ T cells leading to the generation of Tregs. However, work done by D'Orazio *et al*, Skelsey *et al*, and the work presented in this thesis argue against that (14, 90). Based on this work, a revised model was proposed (126). In this model, ocular APC release antigenic peptides that are captured by B cells via their BCR. The peptides are then internalized, processed, and presented to T cells on both MHC I and MHC II molecules. This scenario is in accordance with the data presented in this thesis and with previous work on ACAID.

In spite of a large body of work on ACAID, the different splenic cellular interactions between the antigen-presenting B cells and other splenic cells such as γδ T cells and NK T cells are poorly understood. Our results demonstrate that γδ T cells act downstream of B cells. There is a weak possibility that γδ T cells, inspite of having receptors of limited antigen recognition capacity, respond to the antigen being presented on tolerogenic antigen-presenting B cells by secreting IL-10. A stronger possibility is that γδ T cells produce IL-10 constitutively and not in response to APC or antigens. Although the secretion of IL-10 from a small population of splenic γδ T cells might seem insignificant, the location of γδ T cells *in vivo* may be as crucial in inducing other cell components of the tolerogenic pathway. This is because it appears that the induction of ACAID versus conventional immune responses occurs at different locations within the spleen (185). For instance, γδ T cells may get recruited to the marginal zone of the spleen to interact with NK T cells, B cells and T cells that are known to interact in this location during the induction of ACAID (186). In this case, the IL-10 secreted by γδ T cells will be highly significant (even though it is secreted from a minor population of T cells in the spleen) due to its close proximity to other cells crucial for the induction of ACAID. MIP-2 has been shown to recruit NK T cells to the marginal zone (186). Thus, cytokine cues and environmental signals are extremely important in the induction of ACAID. Xu *et al* have shown that γδ T cells expand when ACAID is induced (156). This implies that the IL-10 signal delivered by γδ T cells will be amplified and thus might be a mechanism to ensure that the tolerogenic signal will ultimately impact the $CD8^+$ T cells that differentiate into efferent Tregs.

Although we showed that γδ T cells acted downstream of B cells, it does not exclude the

possibility that they may still influence B cells by secreting cytokines, such as IL-10. Some studies have shown that IL-10 can lead to B cell expansion (187). This fits well with the observation that B cells expand during the induction of ACAID (126). If the previous assumption is true, one could envision B cells proliferate in the marginal zone of the spleen after capturing antigen released from ocular APC. Once in the splenic marginal zone, γδ T cells might be activated, secrete IL-10 and induce more B cell expansion. This loop would proceed in a cascade-like manner to ensure the generation of antigen-specific tolerance.

The response of γδ T cells to antigens might be a non-specific response since these cells have limited diversity of their T cell receptors (188) . Although γδ T cells have limited antigen specificity, they can contribute to the generation of antigen-specific Tregs. In fact, ACAID is highly antigen specific as it leads to the generation of antigen-specific $CD8^+$ Tregs (186). However, this does not necessarily mean that every cell type that participates in the induction of ACAID has to be antigen specific. Antigen specificity can be determined at the level of B cell interaction with NK T cells and/or B cell interaction with Tregs rather than at the level of γδ T cell interaction with any of these cells. One of the crucial factors for the flow of the tolerogenic signal that comes initially from ocular APC could be the *in vivo* localization of γδ T cells in relation to B cells, NK T cells, $CD4^+$ T cells, and $CD8^+$ T cells. IL-10 secreted from γδ T cells can induce a particular function in B cells, NK T cells, or T cells (such as proliferation, cytokine secretion, antigen presentation, or active suppression)

Determining where the NK T cells fit in this whole cellular cross-talk and interplay requires insights as to the site of action of NK T cells and their specific interactions with B cells, γδ T cells, $CD4^+$ T cells, and $CD8^+$ T cells during the induction of ACAID. Whether B cells act

upstream, downstream, or both upstream and downstream of NK T cells in ACAID needs to be addressed. This is feasible as CD1d KO mice lacking NK T cells are commercially available.

Since NK T cells secrete IL-10 (96), it would be important to learn how NK T-cell derived IL-10 is any different from the IL-10 secreted by γδ T cells or $CD4^+$ T regs. It is entirely possible that NK T cell derived IL-10 serves to maintain B cell proliferation as was suggested before with the γδ T cell-derived IL-10. If NK T were to act downstream of B cells, then it will be plausible to suggest that one of their functions will be to recognize particular antigens such as hydrophobic peptides presented on B cells through the CD1d molecule. In this scenario, they will still affect the upstream cells by secreting IL-10.

It is noteworthy that the NK T cells required for the induction of ACAID belong to a subset that expresses the invariant Vα14 Jα18 TCR that is associated with a limited number of Vβ chains (Vβ 8, 8.2, or 7) (189-191). The specificity of the NK T cell invariant TCR is the CD1d molecule (191). A similar subset of CD1d-reactive NK T cells expressing Vα24JQα exists in humans (192). This implies that NK T cells in humans respond in a similar fashion to those in mice. Although CD1d is known to present lipids and hydrophobic molecules to T cells, this presentation is poorly understood (193). Synthetic glycolipids have been shown to be presented by CD1d to invariant NK T cells (194). Other reports suggest that CD1d-restricted TCR is stimulated regardless of the contents of the CD1d molecule (190, 195-197). There is a possibility that these class I-like molecules are presenting endogenous self peptides or glycolipids during immune or tolerogenic responses *in vivo.* This may play an unexplained role in tolerance to self molecules.

The tolerance-inducing CD1d-reactive NK T cells in ACAID appear to function differently than other CD1d-reactive NK T cells activated during viral infections, immune inflammation, or after treatment with alpha-galactosylceramide (190, 198-200). If splenic B cells need to express CD1d for efficient generation of Tregs, then the CD1d reactive NK T cells may in fact facilitate the splenic B cell antigen presentation activity leading to the generation of Tregs. Another important function of NK T cells in ACAID is to help in the trafficking of cells within the marginal zone of the spleen. RANTES, which is produced by NK T cells as a result of CD1d signaling, is a known potent chemo-attractor for APC and T cells (98). This helps to pull together different cell populations required for the generation of Tregs together with the T cells to facilitate induction of T cells to become Tregs. The observation that these T cells are induced in the periphery to become Tregs is supported by studies by Keino *et al* who showed that induction of eye-derived tolerance does not depend on naturally occurring $CD4^{+}CD25^{+}$ T regs (201).

Therapeutic implications

It will be extremely important to draw on the findings in murine models of ACAID to move on to the more translational side of ACAID research. A very recent study has shown that by treatment with TGF-β2, adherent monocytes isolated from human samples have down-regulated IL-12 production and CD 40 molecule expression in a manner similar to what we know about murine ACAID APC (193). This suggests that adherent human monocytes acquire the property to induce ACAID by treatment with TGF-β2 (193). These similar properties exhibited by TGF-β2-treated human monocytes and TGF-β2-treated murine APC may help us draw conclusions from murine models that are relevant to human ACAID, especially that it is possible to generate

functional human APC from adherent peripheral blood monocytes (202).

ACAID and immune privilege have both positive and negative sides. On the positive side, ACAID helps prolong the survival rates of corneal allografts, usually permitting them to persist indefinitely. On the negative side, ACAID can promote the growth of tumor cells injected into the AC of the eye. This can ultimately lead to death as a result of the tumor extension into the brain. It is important to have the correct balance of immune privilege to avoid harboring tumors on one hand and to avoid destructive ocular inflammations on the other hand. Furthermore, suppression of DTH, as a result of ACAID, could carry significant risk to the well-being of the host as the resistance to many pathogens relies mainly on DTH-dependent Th1 responses (203).

ACAID is a form of tolerance that is dependent on the presence of the CD1d-reactive NK T cell population. Defective NK T cells has been associated with a variety of autoimmune diseases in mice and humans (204). For example, diabetes, myasthenia gravis, and multiple sclerosis are associated with NK T cell deficiencies (205, 206). In a murine model, adoptive transfer of NK T cells prevents diabetes in NOD mice (207, 208). This requirement of NK T cells to regulate immune responses to self-antigens in these autoimmune models is similar to the requirement of NK T cells for generation of Tregs in ACAID. Understanding how NK T cells contribute to immune regulation in ACAID can help us understand how NK T cells regulate immune responses in other models of autoimmune diseases.

Both Th1 and Th2 cells can produce immune-mediated inflammation that cause tissue destruction (209, 210). The target specificities of the Th cells that get activated in an

inflammatory setting can be tissue-restricted autoantigens as in the case of autoimmune diseases such as rheumatoid arthritis (211, 212). The target specificities of the activated Th cells can also be exogenous antigens in organs vulnerable to attack by activated T effector cells as in the case of immunopathogenic diseases such as Type 1 diabetes mellitus where beta-cells of the pancreatic islet are selectively eliminated (213, 214). Patients who have autoimmune or immunopathogenic diseases have T cells already sensitized for the disease-associated antigens such as citrullinated autoantigens in the case of rheumatoid arthritis (212). As mentioned above, ACAID has been used to suppress both Th1 and Th2 antigen-specific immune inflammation, even after the generation of the immune response (215). In other words, the $CD8^+$ Tregs in ACAID can inhibit the expression of the cell-mediated immune responses in an antigen-specific manner even after the immune T cells get sensitized for this antigen (146). For therapeutic purposes, this unique ability of ACAID can be exploited. For example, an ACAID-based therapy for autoimmune and immunopathogenic diseases can rely on the generation of antigen-specific ACAID-inducing APC *in vitro* by obtaining conventional monocytes from peripheral blood of the patient, treating these cells with TGF-β2 followed by pulsing with the antigen associated with the disease. Based on experiments done with murine and human cells (216, 217), one would predict that these cells would suppress antigen-specific autoimmunity and/or immunopathogenesis when re-injected i.v. In a broader sense, ACAID can be made to induce tolerogenic immunotherapy in humans in the case of Th1 and/or Th2-induced inflammation.

An approach similar to the previous one can still be used even if the target antigen is unknown. An example of this would be the case of some autoimmune and/or immunopathogenic diseases of Wegener's Granulomatosis in which there is an exaggerated hypersensitivity reaction to

an unknown antigen that enters through the respiratory tract (218). This approach relies on the immunosuppressive factors present in the aqueous humor that can convert the patients' T cells into Tregs which are antigen specific. By obtaining the patient's peripheral blood T cells, one would then have obtained the subpopulation of sensitized effector cells against this unknown antigen. If these T cells are incubated *in vitro* with aqueous humor, one would expect antigen-specific Tregs to be generated as was shown before in animal models (219). Results from animal models have convincingly demonstrated that α-MSH with TGF-β2-induced Treg cells can inhibit a tissue-specific autoimmune response (219). This led the authors to propose using these immunosuppressive cytokines to induce antigen-specific Tregs in order to prevent and suppress autoimmune diseases (219).

ACAID has been proven to inhibit the incidence and development of experimental autoimmune uveoretinitis (EAU) (220). It has also been shown to promote corneal grafts survival (221). Based on that, a future idea is that graft recipients could be pretreated with their own conventional APC that have been converted into ACAID-inducing APC by incubating them with TGF-β2 together with the donor alloantigens *in vitro*. If treatment strategies based on immune privilege mechanisms evolve, they can result in reduced toxicity and side effects when compared with some present therapies. However, an ACAID-based therapy like the one described might not be useful for all autoimmune diseases especially when there are multiple factors involved in pathogenesis. With *in vitro* generated ACAID Tregs, suppression might not be maintained for long enough periods of time in humans to counteract a sustaining immune response towards self-antigens. Adjustment of doses might not be easy since too much suppression may affect the quality of all of the body's immune responses.

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VITAE

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