NIH-PA Author Manuscript

NIH Public Access
Author Manuscript
Nat Med. Author manuscript; available in PMC 2007 March 14.

Published in final edited form as:
Nat Med. 2003 February ; 9(2): 206–212.

Tolerance is dependent on complement C3 fragment iC3b binding to antigen-presenting cells

Jeong-Hyeon Sohn[1], **Puran S. Bora**[1], **Hye-Jung Suk**[1], **Hector Molina**[2], **Henry J. Kaplan**[1], and **Nalini S. Bora**[1]

1 *Department of Ophthalmology and Visual Sciences, Kentucky Lions Eye Center, University of Louisville, Louisville, Kentucky, USA*

2 *Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri, USA*

Abstract

Systemic tolerance can be induced by the introduction of antigen into an immune-privileged site. Here we investigated the role of complement in the induction of tolerance after intraocular injection. We found that the development of antigen-specific tolerance is dependent on a complement activation product. The ligation of the complement C3 activation product iC3b to complement receptor type 3 (the iC3b receptor) on antigen-presenting cells resulted in the sequential production of transforming growth factor-β2 and interleukin-10, which is essential for the induction of tolerance. These observations may extend to the development of both neonatal tolerance and other forms of acquired tolerance.

Complement is a major component of natural immunity. Well-known effector functions of the complement system include anaphylatoxic activity, opsonization for phagocytosis and lysis of cells or foreign particles[1,2]. However, in recent years it has become increasingly evident that complement is also involved in antigen-specific immune responses. These include antigen processing and presentation, T-cell proliferation and differentiation, and B-cell activation[3–7]. Systemic tolerance can be induced by the introduction of antigen into an immune-privileged site[8]. The present study was undertaken to determine the role of complement in systemic tolerance induced by the injection of antigen into the eye.

The anterior chamber (AC) of the eye is an immune-privileged site[8]. After the introduction of antigen into the AC, there is a deviant systemic immune response resulting in the generation of antigen-specific regulatory T cells and the suppression of delayed type-hypersensitivity (DTH)—a phenomenon initially described as F1 lymphocyte-immune deviation[9,10], and subsequently described as anterior chamber associated immune deviation (ACAID)[11]. The antigenic signal delivered to the AC is carried into the circulation and delivered to the spleen[11,12], where regulatory T cells are generated[13]. CD-1 reactive natural killer T cells are required for the inhibition of DTH after the AC injection of antigen[14]. DTH is a form of T cell-mediated immunity that plays an important role in protection against pathogens[9–14].

Inhibition of DTH (after intraocular injection) has been reproduced *in vitro*[15,16] and has revealed that several immunosuppressive factors are important in the induction of tolerance to antigen[17]. However, the role of complement in the induction of tolerance has not been explored. Suppression of DTH (identical to ACAID) can also be evoked by the intravenous

Correspondence should be addressed to N.S.B.; email: nsbora01@gwise.louisville.edu.

Competing interests statement
The authors declare they have no competing interests.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

injection of antigen-presenting cells (APCs) pulsed *in vitro* with soluble antigen in the presence of immunosuppressive factors present in ocular microenvironment[18].

Previously, we showed that a functionally active complement system is present within the AC of the normal eye[19]. In the present study we investigated whether the presence and activation of complement are necessary for the induction of antigen-specific systemic tolerance after AC injection.

Role of complement in the inhibition of DTH after AC injection

The role of complement in the inhibition of DTH was investigated using Lewis rats depleted of systemic complement by cobra venom factor (CVF). Our results (Fig. 1*a*) showed that the anterior chamber injection of ovalbumin (OVA) in normal Lewis rats resulted in inhibition of DTH (group 3). However, the *in vivo* depletion of systemic complement with CVF, before the injection of OVA, blocked ($P < 0.001$) the inhibition of DTH (group 4) but not OVA-specific DTH (group 2), which was comparable to the normal complement control (group 1). Both groups 2 and 4 received CVF, but the animals in group 4 only were given the AC injection of OVA. A CH_{50} hemolytic assay[20] confirmed the absence of functionally active complement from CVF-injected rats (data not shown) for 5 days[19]. Thus, complement seemed to be essential for the inhibition of DTH after the AC injection of antigen.

Role of C3 and iC3b in the inhibition of DTH after AC injection

Using C3-deficient mice, we next explored whether the inhibition of DTH required C3. AC inoculation of OVA induced inhibition of DTH in the wild-type control mouse, but not the C3-deficient mouse (Fig. 1*b*). Thus, C3 was required for suppression of DTH.

Using the *in vitro* and *in vivo* models of suppressed DTH (refs. 15,[16]), we next studied whether iC3b was required for the inhibition of DTH. In our initial experiments, both polymeric iC3b and iC3b-opsonized erythrocytes (EA-iC3b) were used.

Using the *in vitro* model of suppressed DTH, our results demonstrated that antigen-pulsed peritoneal exudate cells (PEC) exposed to piC3b inhibited ($P < 0.01$) the development of DTH, whereas in the absence of piC3b this effect was not observed (Fig. 2*a*). Similar results were obtained with iC3b opsonized sensitized erythrocytes (EA-iC3b; Fig. 2*a*). This *in vitro* model employs antigen, PECs and syngeneic splenocytes to induce regulatory T cells that inhibit the DTH response of antigen-specific sensitized T cells.

Similar results were obtained with the *in vivo* model of suppressed DTH (ref. 18). In this model suppression of DTH is evoked by the i.v. injection of APCs pulsed *in vitro* with soluble antigen in the presence of immunosuppressive factors present in the eye. Animals that received an intravenous injection of OVA-PEC treated with EA-iC3b or piC3b were unable to evoke OVA-specific DTH, whereas recipients of OVA pulsed PEC (OVA-PEC) incubated in the absence of EA-iC3b or piC3b developed a strong DTH response (Fig. 2*b*).

These results demonstrate that suppression of DTH is mediated by either piC3b or EA-iC3b; thus, it is iC3b dependent. Furthermore, the possibility that IgM, C1q and C4b—which are present on EA-iC3b—mediated suppression was eliminated by the absence of suppression using control EA (which also contain these molecules[21]). Thus, EA-iC3b was used in most of our subsequent experiments.

In the next series of experiments, the importance of iC3b at the time of antigen contact was studied. Suppression of DTH was observed in the animals that received an intravenous injection of OVA-PEC cultured with piC3b for only the first 12 hours, as well as for the entire 36 hours

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

(Fig. 2*c*). In contrast, the animals that received OVA-PEC cultured in the absence of piC3b for the first 12 hours, followed by culture in the presence of piC3b for the next 24 hours, elicited a strong DTH response to OVA (Fig. 2*c*). These findings indicate that the result of antigen contact with the APC is critically dependent on the presence of iC3b. Specifically, antigen contact with PEC in the presence of iC3b results in the inhibition of DTH, whereas the absence of iC3b during the early phase of antigen and PEC contact (that is, within 12 hours) elicits a vigorous DTH response.

The antigen specificity of iC3b-induced suppression of DTH was investigated using the *in vitro* model. Suppression of DTH was not observed when OVA-PEC exposed to iC3b was mixed with keyhole limpet hemocyanin (KLH)-primed regulatory T cells (Fig. 2*d*). These results indicated that iC3b-induced suppression of DTH was antigen specific.

Effect of OX-42 on suppression of DTH

The results of *in vitro* and *in vivo* experiments suggested that iC3b was necessary for suppression of DTH. We postulated that the binding of iC3b to PEC (via complement receptor type 3 (CR3)) was responsible for the induction of tolerance; thus, we used the monoclonal antibody OX-42, which prevents the binding of iC3b to CR3, to investigate this possibility. The binding of human iC3b to CR3 present on rat PEC was confirmed (data not shown) by rosette formation[22].

The *in vivo* effect of OX-42 on suppression of DTH induced by AC injection of antigen, as well as by the intravenous injection of APCs, was investigated. Blockade of CR3 by OX-42, 24 hours before the AC injection of OVA, reversed ($P < 0.01$) the inhibition of DTH in Lewis rats (Fig. 3*a*). In contrast, DTH was inhibited in control animals injected with normal mouse IgG2a (isotype control).

Additionally, blockade of CR3 by OX-42 reversed ($P < 0.01$) the suppression of DTH induced by the intravenous injection of OVA-pulsed PEC, cultured in the presence of immobilized iC3b, in Lewis rats (Fig. 3*b*). In contrast, iC3b-induced inhibition of DTH was observed in the animals injected with normal mouse IgG2a (Fig. 3*b*).

Similar results were obtained with the *in vitro* model of suppressed DTH. OVA-PEC incubated with EA-iC3b failed to induce suppression of DTH in the presence of OX-42, but not in the presence of isotype control mouse IgG2a (Fig. 3*c*). Thus, our *in vivo* and *in vitro* data using OX-42 confirmed that the binding of iC3b to its receptor, CR3, on PEC was central to the induction of tolerance.

Effect of iC3b on TGF-β2, IL-10 and IL-12 production by PEC

We studied the expression of interleukin (IL)-10, transforming growth factor (TGF)-β2 and IL-12 by RT-PCR (TGF-β2 and IL-10) and ELISA (IL-10 and IL-12) because of their important role in the development of the DTH response and suppression of DTH. At 24 hours, low levels of IL-10 (190 pg/ml) and extremely low levels of IL-12 (12 pg/ml) proteins were observed by ELISA. The presence of iC3b in the culture did not have any effect on these cytokines at this time point (data not shown). However, at 48 hours the secretion of IL-10 by PEC increased significantly ($P < 0.01$) when cultured in the presence of iC3b (Fig. 4*a*). In contrast, secretion of IL-12 by PEC was significantly ($P < 0.01$) reduced in the presence of iC3b (Fig. 4*b*).

We next examined the effect of OX-42 on the secretion of IL-10 and IL-12 by PEC exposed to both OVA and iC3b *in vitro* at 48 hours. IL-10 production was suppressed by receptor blocking, as treatment of PEC with OX-42, but not mouse IgG2a, resulted in significantly reduced ($P < 0.05$) secretion of IL-10 (Fig. 4*c*) and significantly higher ($P < 0.05$) IL-12 (Fig.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

4*d*). These results indicated that iC3b binding to CR3 provided the signal that upregulated IL-10 and downregulated IL-12 production by PEC.

Using RT–PCR, we detected constitutive low amounts of IL-10 (292 bp) and TGF-β2 (676 bp) mRNA in OVA-PEC at 0 hours. Amounts of IL-10 and TGF-β2 remained at the low constitutive level for between 3 and 48 hours in the absence of iC3b. In contrast, TGF-β2 transcripts increased at 3 hours in the presence of iC3b and remained at this maximal level for 48 hours (Fig. 5*a*). IL-10 remained at the constitutive level at 3 hours, increased at 6 hours and was maintained at that level for 48 hours (Fig. 5*a*) in the presence of iC3b. Thus, both TGF-β2 and IL-10 showed an increase in mRNA following incubation with iC3b. The difference in kinetics of IL-10 mRNA and protein production most probably represents the time required for translation, post-translational processing and secretion of the protein.

The sequence of cytokine production was determined by measuring the signal intensity of mRNA for TGF-β2 and IL-10 relative to that of a housekeeping gene, GAPDH. A strong band at 983 bp for GAPDH indicated equal amounts of RNA in each lane. No band was seen in the controls without RNA or reverse transcriptase (data not shown). However, in the presence of iC3b we observed upregulation of TGF-β2 message before that of IL-10 (Fig. 5*a*).

The low levels of IL-10 and TGF-β2 in the absence of iC3b can be attributed to the thioglycollate derivation of the PEC. It has been reported that several cytokine mRNAs were detectable in murine thioglycollate elicited PEC, but not in resident macrophages[23].

OX-42 was used to determine if the binding of iC3b to CR3 (on the PEC) is related to the upregulation of TGF-β2 and IL-10 transcripts. At 6 hours we observed a decline in both TGF-β2 and IL-10 to constitutive levels (Fig. 5*b*). Our results indicated that the binding of iC3b to CR3 induced PEC to upregulate the secretion of TGF-β2, followed by the upregulation of IL-10.

Effect of antibodies against IL-10 and TGF-β2

We then tested the ability of antibodies against IL-10 and TGF-β2 to modify iC3b induced suppression of DTH using the *in vitro* model. PEC incubated with OVA, iC3b and goat IgG inhibited DTH. However, Lewis rats injected with OVA-pulsed PEC incubated with iC3b and antibodies against either IL-10 or TGF-β2 showed a positive DTH ($P < 0.05$; Fig. 5*c*). Thus, antibodies against both IL-10 and TGF-β2 reversed the effect of iC3b on the suppression of DTH.

Discussion

We previously showed that the complement system is chronically active in the normal rat eye[19]. The present studies were undertaken to determine if complement is involved in the development of antigen-specific systemic tolerance after intra-ocular injection.

First, we demonstrated that complement was essential for the development of suppressed DTH in the rodent after the AC injection of antigen (that is, ocular tolerance or ACAID)[11–13]. Lewis rats depleted of complement with CVF (ref. 24) did not display inhibited DTH. As C3, the third component of complement, has been demonstrated to have an important role in both T- and B-cell–mediated immune responses[5–7], we investigated if it might be required for the inhibition of DTH. We report here, for the first time, that C3-deficient mice[25] were unable to suppress the DTH response to OVA after the AC injection of antigen.

We then focused our attention on iC3b because we noticed that C3 cleavage fragments, including iC3b, are present in the normal rat eye[19]. Using the *in vivo* and *in vitro* models of

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

suppressed DTH, we observed that iC3b was important for the inhibition of DTH. The role of iC3b was further probed both *in vitro* and *in vivo* using monoclonal antibodies. A monoclonal antibody (OX-42)[26] against CR3, the receptor for iC3b (refs. 27, 28), inhibited both *in vivo* and *in vitro* models of suppressed DTH. Our data demonstrated the new finding that iC3b bound to CR3 on APC (that is, PEC) is central to the induction of tolerance after the AC injection of antigen.

Immune privilege in the eye is attributed to several factors such as the lack of lymphatic drainage[17], soluble immunosuppressive factors[29–31], low expression of MHC molecules[32], and constitutive expression of Fas ligand[33]. The induction of ACAID depends on the presence of TGF-β2 within the anterior chamber[15]. Although many soluble factors in addition to TGF-β2 have been shown to have an important role in ACAID, the possible role of an intraocular complement had not been addressed. Our data suggested that complement is essential for the development of inhibited DTH after the AC injection of antigen and, presumably, for immune privilege within the eye.

Several reports have indicated that C3 and its activation products (C3b and iC3b) are important in the development of T-cell responses. C3b bound to antigen has been shown to enhance T-cell responses, and C3 deposition on APC enhances the proliferation of antigen-specific T cells[5,7]. Ultraviolet irradiation induces suppression to a contact sensitizer 2, 4-dinitro-1-fluorobenzene (DNFB) in mice and the complement system and iC3b have been implicated in this local immunosuppression[34,35]. Similar to our observations, *in vivo* administration of antibodies against CR3 inhibited ultraviolet (UV)-induced immunosuppression and restored the ability to induce contact sensitivity to DNFB (ref. 34). It has also been reported that locally activated complement was involved in the early events of contact sensitivity to picryl chloride[36].

As IL-10 has been shown to be important in the development of ACAID (ref. 37), we asked whether iC3b bound to CR3 could alter IL-10 production by PEC. We observed that in the presence of iC3b, PEC increased secretion of IL-10 protein and decreased secretion of IL-12 protein, within 48 hours. When PEC were incubated with OX-42, which blocks iC3b binding to CR3, this effect was reversed. Our data indicated that iC3b binding to CR3 transduced a signal that regulates IL-10 and IL-12 production and secretion from APC.

Our observations are consistent with those reported by others on the effect of complement C3 fragments on IL-12 and IL-10 production. It was previously reported that incubation with dimerized C3b caused the downregulation of IL-12 by human monocytes in culture[38]. Additionally, iC3b deposited in UV-exposed human skin has been shown to induce the secretion of IL-10 and downregulation of IL-12 by monocytes[39].

D’Orazio and Niederkorn reported that deletion of IL-10 by either mAb or the use of IL-10 deficient mice abolished OVA-induced ACAID (ref. 37). They concluded that TGF-β2 may have an important role in the induction of ACAID through the enhanced secretion of IL-10 by ocular APC. We observed that in the *in vitro* model of inhibited DTH, antibody against either TGF-β2 or IL-10 reversed iC3b-dependent suppression. Thus, our results are in agreement with the conclusions reached by D’Orazio and Niederkorn[37] and suggest that IL-10 and TGF-β2 play an important role in iC3b induced suppression of DTH.

TGF-β2 has been documented in multiple immune-privileged sites, including the aqueous humor and vitreous humor[15], and has been implicated as an important factor in the development of tolerance, including ocular and oral tolerance[37,40,41]. It is a known inducer of IL-10, although the mechanism of TGF-β–induced IL-10 secretion is unknown. The amount of TGF-β mRNA has been shown to be altered during complement activation[42].

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

We have observed a novel pathway for iC3b-dependent production of TGF-β2 and IL-10 in the *in vitro* model of suppressed DTH. Using RT–PCR, we noticed the temporal expression of TGF-β2 and IL-10 transcripts by PEC cultured in the presence of iC3b, with TGF-β2 upregulated earlier than IL-10. Finally, in the presence of OX-42, mRNA production for TGF-β2 and IL-10 was inhibited, indicating that the binding of iC3b to CR3 was required for the induction of these cytokines in APC.

Our findings describe a novel role for complement in the induction of systemic tolerance to antigen injected into the AC of the eye. Most importantly, this study provides evidence that the presence of a complement activation product, iC3b, during the early phase (within 12 hours) of antigen and APC contact is essential for the development of tolerance. The binding of iC3b to CR3 on APC induced the production and secretion of TGF-β2, followed by upregulation of IL-10, which has been previously shown to mediate the development of suppressed DTH (ref. 37). It is plausible to assume that these observations can be extended to the development of both neonatal tolerance and other forms of acquired tolerance (for example, oral tolerance).

The complement system provides a unique pathway for immune protection of the eye against infection. It seems to perform two important functions. First, the complement system is continuously activated at a low level in the normal eye[19]. We postulate that this chronic low level of complement activation serves as a primary defense mechanism of the eye against pathogenic infection and is finely regulated by the soluble and membrane-bound intraocular complement regulatory proteins. This enables destruction of the putative pathogen without inadvertent damage of ocular tissue, which is vital for the maintenance of vision. Second, our results in this manuscript demonstrate that iC3b (generated as a result of the low level of complement activation) results in the selective suppression of DTH after the intraocular injection of antigen. In this role iC3b protects the eye from innocent bystander destruction associated with the DTH response to the offending pathogen. This selective suppression of DTH by iC3b does not make the eye vulnerable to pathogenic infection because low-level complement activation serves to clear pathogenic organisms. Thus, in the eye, complement functions as a double-edged sword—it provides innate immunity against pathogens, while simultaneously instructing the adaptive immune response to suppress any harmful, antigen-specific DTH. It is likely that the complement system has a similar role in other immune-privileged sites in which inadvertent tissue damage would be detrimental to organ and host survival—for example, in the central nervous system.

Methods

Animals

Lewis rats were from Harlan Sprague-Dawley (Indianapolis, Indiana) and wild-type control (for C3-deficient mice), 129 × C57BL/6 F1 mice were from The Jackson Laboratory (Bar Harbor, Maine). This study was approved by the Institutional Animal Care and Use Committee (IACUC), University of Louisville.

Culture medium

We used serum-free RPMI 1640 supplemented with ITS^+ culture supplement (Becton Dickinson, Bedford, Massachusetts).

Preparation of EA-iC3b and polymeric iC3b (piC3b)

EA-iC3b and piC3b were prepared following standard methods[21,43–45]. EA-iC3b or control EA re-suspended in RPMI containing OVA (5 mg/ml, Sigma, St. Louis, Missouri) or piC3b (10 μg/ml) was added to PEC at the 'preparation of APC' step of *in vitro* suppression of DTH

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

(refs. 14–16). The erythrocytes were removed by washing before addition of the splenocytes. PEC incubated with PBS served as the control for piC3b.

Induction of *in vivo* suppression of DTH by AC injection

Rats and mice received AC injection of 100 and 60 μg of OVA, respectively[11–14]. Controls received a similar injection of sterile PBS. In two additional groups of Lewis rats, complement was depleted using CVF (ref. 19), 24 h before the AC injection. DTH specific to OVA was measured[11–14].

Induction of *in vivo* suppression of DTH by intravenous injection

PEC prepared as described below were cultured with OVA in the presence and absence of piC3b or EA-iC3b. After a 12-h incubation, PEC (5×10^4) were injected intravenously[18] into naive Lewis rats and OVA-specific DTH was measured[11–14].

To determine the importance of iC3b during the contact of antigen and APC, OVA and PEC cultured with piC3b for 12 h were washed and cultured for an additional 24 h, with or without freshly added piC3b. OVA and PEC were also cultured in the absence of piC3b for 12 h followed by culture with or without iC3b for an additional 24 h. These OVA-PEC (5×10^4) were injected intravenously[18] into naive rats and OVA-specific DTH was measured[11–14].

Induction of *in vitro* suppression of DTH

APC were prepared following standard methods[14–16]. Plastic adherent PEC were cultured overnight (12 h) with OVA (5 mg/ml) and were then co-cultured with naive syngeneic splenocytes from naive Lewis rats for 5 d. Non-adherent cells were collected, resuspended in RPMI containing OVA (10 mg/ml) and mixed with equivalent numbers of OVA-primed syngeneic splenocytes. This cell mixture (2×10^6 cells) was injected into the foot-pad, and foot-pad swelling was measured at 24 h (refs. 12–16). Splenocytes from naive rats served as the negative control, while the positive control consisted of primed splenocytes alone. Regulatory T cells generated by OVA-APC exposed to iC3b-and KLH-primed syngeneic splenocytes were used to determine the antigen specificity of iC3b-induced suppression of DTH.

In vivo antibody treatment

The IgG fraction (0.5 mg) of mouse anti-rat-CR3 (clone OX-42, mouse IgG2a, BD Biosciences, San Jose, California) was administered intraperitoneally in Lewis rats, once only, 24 h before the AC injection of OVA. Control animals received a similar injection of normal mouse IgG2a.

For intravenous injection, the IgG fraction of anti-rat CR3 (4.0 μg/ml) was added to OVA and PEC and the reaction was allowed for 1 h before incubation with EA-iC3b. OVA-PEC cultured with normal mouse IgG2a was used as the control.

In vitro antibody treatment

The IgG fraction of anti-rat CR3 (4.0 μg/ml) was added to OVA and PEC and the reaction was allowed for 1 h before incubation with EA-iC3b using *in vitro* suppression of DTH. The IgG fraction of goat antibodies against rat IL-10 (10.0 μg/ml) or porcine TGF-β2 (0.3 μg/ml) was added at the same time as addition of EA-iC3b. Antibodies against TGF-β2 and IL-10 were from R&D Systems (Minneapolis, Minnesota). Normal mouse IgG2a was the control for OX-42, whereas normal goat IgG was the control for antibodies against IL10 and TGF-β2.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

ELISA

OVA-PEC were incubated overnight under the following conditions: OVA-PEC with iC3b; OVA-PEC without iC3b; OVA-PEC with iC3b and OX-42; and OVA-PEC with iC3b and control IgG. The next day, erythrocytes were removed, fresh medium was added and the cells were cultured for an additional 24 and 48 h. Supernatants collected at 24 and 48 h were assayed for IL-10 and IL-12 proteins using ELISA kits from Endogen, Inc. (Woburn, Massachusetts) and Biosource International (Camarillo, California), respectively.

RT-PCR analysis

Total RNA (0.1 μg) from OVA-PEC cultured for 3, 6, 12, 24 and 48 h in the presence or absence of iC3b as well OVA-PEC cultured for 6 h in the presence of OX-42 and iC3b was used to detect the mRNA levels of GAPDH, IL-10 and TGF-β2 by RT–PCR using the RNA-PCR kit (Perkin-Elmer Corporation, Boston, Massachusetts). The negative controls consisted of omission of RNA or reverse transcriptase from the reaction mixture. PCR products were quantitated by densitometery, using the Molecular Analyst/PC program (Bio-Rad, Hercules, California). RT–PCR was carried out using the following primers: GAPDH (F-5′-TGAAGGTCGGTGT-CAACGGATTTGGC-3′, R-5′-CATGTAGGCCATGAGGTCCACCAC-3′); IL-10 (F-5′-AAGGACCAGCTGGACAACAT-3′, R-5′-AGACACCTTTGTCTTGGAGC-TTA-3′); TGF-β2 (F-5′-CTCCTGCATCTGGTCCCGGT-3′, R-5′-GCACG-GCGTCTGTCACGTCG-3′).

Statistics

Each group consisted of 6 animals and experiments were repeated 5 times with similar results. Differences between groups were evaluated by Student’s *t*-test.

Acknowledgements

We thank J.P. Atkinson for critical review of the data and Y. Wang for help with RT–PCR analysis. This work was supported in part by EY09730, EY10543 and EY 13335, Commonwealth of Kentucky Research Challenge Trust Fund and Research to Prevent Blindness Inc., NY.

References

1. Frank MM, Fries LF. The role of complement in inflammation and phagocytosis. Immunol Today 1991;12:322–326. [PubMed: 1755943]
2. Thomlinson S. Complement defense mechanisms. Curr Opin Immunol 1993;5:83–89. [PubMed: 8452679]
3. Carroll MC. The role of complement and complement receptors in induction and regulation of immunity. Annu Rev Immunol 1998;16:545–568. [PubMed: 9597141]
4. Nielsen CH, Fischer EM, Leslie RGQ. The role of complement in the acquired immune response. Immunology 2000;100:4–12. [PubMed: 10809953]
5. Arvieux J, Yssel H, Colomb MG. Antigen-bound C3b and C4b enhance antigen-presenting cell function in activation of human T-cell clones. Immunology 1988;65:229–235. [PubMed: 2973431]
6. Dempsey PW, Allison MED, Akkaraju S, Goodnow CC, Fearon DT. C3d of complement as a molecular adjuvant: Bridging innate and acquired immunity. Science 1996;271:348–350. [PubMed: 8553069]
7. Jacquier-Sarlin MR, Gabert FM, Villiers MB, Colomb MG. Modulation of antigen processing and presentation by covalently linked complement C3b fragment. Immunology 1995;84:164–170. [PubMed: 7890301]
8. Streilein JW. Immunologic privilege of the eye. Springer Semin Immunopathol 1999;21:95–111. [PubMed: 10457584]
9. Kaplan HJ, Streilein JW. Immune response to immunization via the anterior chamber of the eye. I F1 lymphocyte-induced immune deviation. J Immunol 1977;118:809–814. [PubMed: 321682]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

10. Kaplan HJ, Streilein JW. Immune response to immunization via the anterior chamber of the eye. II F1 lymphocyte-induced immune deviation. J Immunol 1978;120:689–693. [PubMed: 632581]
11. Streilein JW, Niederkorn JY. Induction of anterior chamber-associated immune deviation requires an intact, functional spleen. J Exp Med 1981;153:1058–1067. [PubMed: 6788883]
12. Wilbanks GA, Streilein JW. Macrophages capable of inducing anterior chamber associated immune deviation demonstrate spleen-seeking migratory properties. Reg Immunol 1992;4:130–137. [PubMed: 1303096]
13. Wilbanks GA, Streilein JW. Characterization of suppressor cells in anterior chamber-associated immune deviation (ACAID) induced by soluble antigen. Evidence of two functionally and phenotypically distinct T-suppressor cell populations. Immunology 1990;71:383–389. [PubMed: 1702748]
14. Sonoda KH, Exley M, Snapper S, Balk SP, Stein-Streilein J. CD1-reactive natural killer T cells are required for development of systemic tolerance through an immune-privileged site. J Exp Med 1999;190:1215–1225. [PubMed: 10544194]
15. Wilbanks GA, Mammolenti M, Streilein JW. Studies on the induction of anterior chamber-associated immune deviation (ACAID). III Induction of ACAID depends upon intraocular transforming growth factor-β. Eur J Immunol 1992;22:165–173. [PubMed: 1530916]
16. D'orazio TJ, Niederkorn JY. Splenic B cells are required for tolerogenic antigen presentation in the induction of anterior chamber-associated immune deviation (ACAID). Immunology 1998;95:47–55. [PubMed: 9767456]
17. Streilein JW. Immune privilege as the result of local tissue barriers and immunosuppressive microenvironments. Curr Opin Immunol 1993;5:428–432. [PubMed: 8347303]
18. Hara Y, Okamoto S, Rouse B, Streilein JW. Evidence that peritoneal exudates cells cultured with eye-derived fluids are the proximate antigen-presenting cells in immune deviation of the ocular type. J Immunol 1993;151:5162–5170. [PubMed: 8228216]
19. Sohn JH, Kaplan HJ, Suk HJ, Bora PS, Bora NS. Chronic low level complement activation within the eye is controlled by intraocular complement regulatory proteins. Invest Ophthalmol Vis Sci 2000;41:3492–3502. [PubMed: 11006244]
20. Kabat, EA.; Mayer, MM. Experimental Immunohistochemistry. Thomas, CC., editor. Springfield, IL: 1961. p. 133-239.
21. Diamond MS, Garcia-Aguilar J, Bickford JK, Corbi AL, Springer TA. The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. J Cell Biol 1993;120:1031–1043. [PubMed: 7679388]
22. Foris G, Fust G, Medgyesi GA. The effect of oligopeptides on the C3b receptor-mediated functions of rat macrophages. Immunol Lett 1983;6:7–11. [PubMed: 6220967]
23. Simpson AECM, Tomkins PT, Cooper KL. An investigation of the temporal induction of cytokine mRNAs in LPS-challenged thioglycollate-elicited murine peritoneal macrophages using the reverse transcription polymerase chain reaction. Inflamm Res 1997;46:65–71. [PubMed: 9085146]
24. Fritzinger DC, Bredehorst R, Vogel CW. Molecular cloning and derived primary structure of cobra venom factor. Proc Natl Acad Sci USA 1994;91:12775–12779. [PubMed: 7809120]
25. Wessels MR, et al. Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. Proc Natl Acad Sci USA 1995;92:11490–11494. [PubMed: 8524789]
26. Robinson AP, White TM, Mason DW. Macrophage heterogeneity in the rat as delineated by two monoclonal antibodies MRC OX-41 and MRC OX-42, the latter recognizing complement receptor type 3. Immunology 1986;57:239–247. [PubMed: 3512425]
27. Ross GD, Lambris JD. Identification of a C3bi-specific membrane complement receptor that is expressed on lymphocytes, monocytes, neutrophils, and erythrocytes. J Exp Med 1982;155:96–110. [PubMed: 6915077]
28. Ross GD, Vetvicka V. CR3 (CD11b, CD18): A phagocyte and NK cell membrane receptor with multiple ligand specificities and functions. Clin Exp Immunol 1993;92:181–184. [PubMed: 8485905]
29. Taylor AW, Streilein JW, Cousins SW. Identification of α-melanocyte stimulating hormone as a potential immunosuppressive factor in aqueous humor. Curr Eye Res 1992;11:1199–1206. [PubMed: 1490338]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

30. Taylor AW, Streilein JW, Cousins SW. Immunoreactive vasoactive intestinal peptide contributes to the immunosuppressive activity of normal aqueous humor. J Immunol 1994;153:1080–1086. [PubMed: 8027541]
31. Cousins SW, McCabe MM, Danielpour D, Streilein JW. Identification of transforming growth factor-β as an immunosuppressive factor in aqueous humor. Invest Ophthalmol Vis Sci 1991;32:2201–2211. [PubMed: 2071334]
32. Streilein JW. Unraveling immune privilege. Science 1995;270:1158–1159. [PubMed: 7502038]
33. Griffith TS, Brunner T, Fletcher SM, Green DR, Ferguson TA. Fas ligand-induced apoptosis as a mechanism of immune privilege. Science 1995;270:1189–1192. [PubMed: 7502042]
34. Hammerberg C, Duraiswamy N, Cooper KD. Reversal of immunosuppression inducible through ultraviolet-exposed skin by in vivo anti-CD11b treatment. J Immunol 1996;157:5254–5262. [PubMed: 8955170]
35. Hammerberg C, Katiyar SK, Carroll MC, Cooper KD. Activated complement component 3 (C3) is required for ultraviolet induction of immunosuppression and antigenic tolerance. J Exp Med 1998;187:1133–1138. [PubMed: 9529329]
36. Tsuji RF, et al. Requried early complement activation in contact sensitivity with generation of local C5-dependent chemotactic activity, and late T cell interferonγ: A possible initiating role of B cells. J Exp Med 1997;186:1015–1026. [PubMed: 9314551]
37. D'orazio TJ, Niederkorn JY. A novel role for TGF-β and IL-10 in the induction of immune privilege. J Immunol 1998;160:2089–2098. [PubMed: 9498745]
38. Karp CL, et al. Mechanism of suppression of cell-mediated immunity by measles virus. Science 1996;273:228–231. [PubMed: 8662504]
39. Yoshida Y, et al. Monocyte induction of IL-10 and down-regulation of IL-12 by iC3b deposited in ultraviolet-exposed human skin. J Immunol 1998;161:5873–5979. [PubMed: 9834066]
40. Kosiewicz M, Alard P, Streilein JW. Alteration in cytokine production following intraocular injection of soluble protein antigen: impairment in IFN-γ and induction of TGF-β and IL-4 production. J Immunol 1998;161:5382–5390. [PubMed: 9820512]
41. Miller A, Lider O, Roberts AB, Sporn MB, Weiner HL. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor β after antigen-specific triggering. Proc Natl Acad Sci USA 1992;89:421–425. [PubMed: 1370356]
42. Shankland SJ, et al. Differential expression of transforming growth factor-β isoforms and receptors in experimental membranous nephropathy. Kidney Int 1996;50:116–124. [PubMed: 8807580]
43. Kalli KR, Ahearn JM, Fearon DT. Interaction of iC3b with recombinant isotypic and chimeric forms of CR2. J Immunol 1991;147:590–594. [PubMed: 1830068]
44. Ross GD. Analysis of the different types of leukocyte membrane complement receptors and their interaction with the complement system. J Immunol Methods 1980;37:197–211. [PubMed: 7005344]
45. McQuillen DP, et al. Complement processing and immunoglobulin binding to *Neisseria gonorrhoeae* determined in vitro simulates in vivo effects. J Infect Dis 1995;179:124–135. [PubMed: 9841831]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

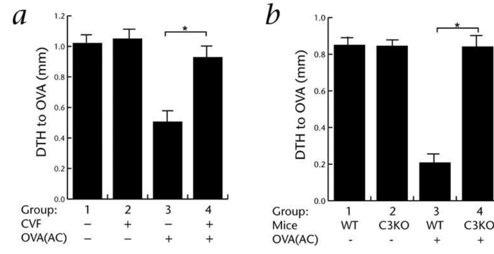

Fig. 1.

Role of complement in the *in vivo* suppression of DTH. ***a***, Effect of CVF on OVA-induced suppression of DTH in Lewis rats. Pretreatment of animals receiving anterior chamber injection of OVA with CVF (group 4) significantly ($*P < 0.001$) blocked the suppression of DTH to OVA (group 3). CVF injection alone (group 2) did not affect OVA-specific DTH (group 1) in the absence of anterior chamber inoculation of OVA. ***b***, Absence of suppression of DTH in C3-deficient mice. Anterior chamber inoculation of soluble OVA did not suppress DTH to OVA in C3-deficient mice (group 4), while OVA-specific DTH was suppressed in age-matched, wild-type controls (group 3). The OVA-specific DTH response of C3-deficient mice (group 2) was similar to wild-type controls (group 1) in the absence of anterior chamber injection. $*P < 0.001$.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

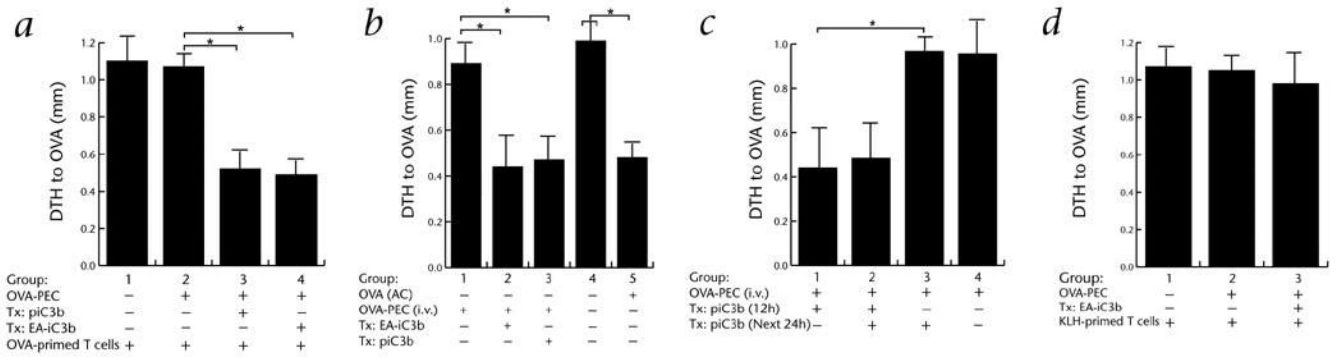

Fig. 2.

Role of iC3b in suppression of DTH. ***a***, *In vitro* model. Antigen-pulsed PEC exposed to either polymeric iC3b (piC3b, group 3) or EA-iC3b (group 4) significantly (**P* < 0.01) inhibited the development of OVA-specific DTH compared with antigen-pulsed PEC cultured in the absence of iC3b (group 2). Group 1 (positive control) represents DTH to OVA-sensitized splenocytes only. ***b***, *In vivo* model. Intravenous injection (i.v.) of OVA-PEC cultured with either EA-iC3b (group 2) or piC3b (group 3) significantly (**P* < 0.01) blocked the development of OVA-specific DTH (group 1) in Lewis rats and was similar to the suppression of DTH induced by intraocular OVA injection (group 5). Group 4 represents OVA-specific DTH in the absence of anterior chamber inoculation of OVA. ***c***, *In vivo* model. Suppression of DTH was observed in the animals injected intravenously with OVA-PEC cultured with piC3b for 12 h (group 1), as well as OVA-PEC cultured with piC3b for 12 h followed by an additional 24 h culture with freshly added piC3b (group 2). Removal of piC3b from the first 12 h culture (group 3) eliminated the suppression of OVA-specific DTH (group 4). ***d***, Antigen specificity (*in vitro* model). OVA-pulsed PEC exposed to iC3b (group 3) did not inhibit the development of KLH-specific DTH (group 2). Group 1 (positive control) represents DTH to KLH-sensitized splenocytes only. Tx, treatment of the OVA and PEC culture.

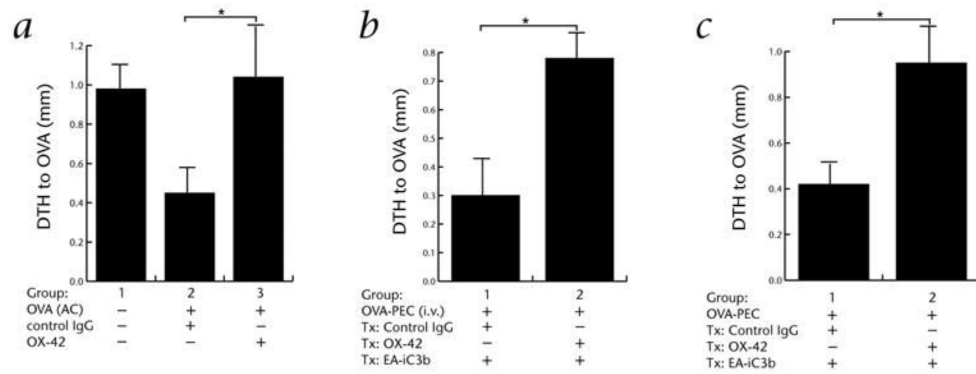

Fig. 3.

Effect of OX-42 (monoclonal antibody against CR3) on suppression of DTH. ***a***, *In vivo*. Systemic pre-treatment of Lewis rats with OX-42, but not the isotype IgG control, inhibited the suppression of DTH to OVA after anterior chamber injection of OVA. The positive control represents OVA-specific DTH without anterior chamber inoculation of OVA. ***b***, *In vivo*. Intravenous injection of OVA-PEC cultured in the presence of iC3b and OX-42 (group 2) reversed the suppression of DTH to OVA induced by OVA-PEC cultured with iC3b and control IgG (group 1). ***c***, *In vitro*. OVA-PEC cultured with iC3b induced suppression of DTH to OVA when cultured with control IgG (group 1), but did not induce suppression of DTH in the presence of OX-42 (group 2). $^*P < 0.01$. Tx, treatment of the OVA and PEC culture.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

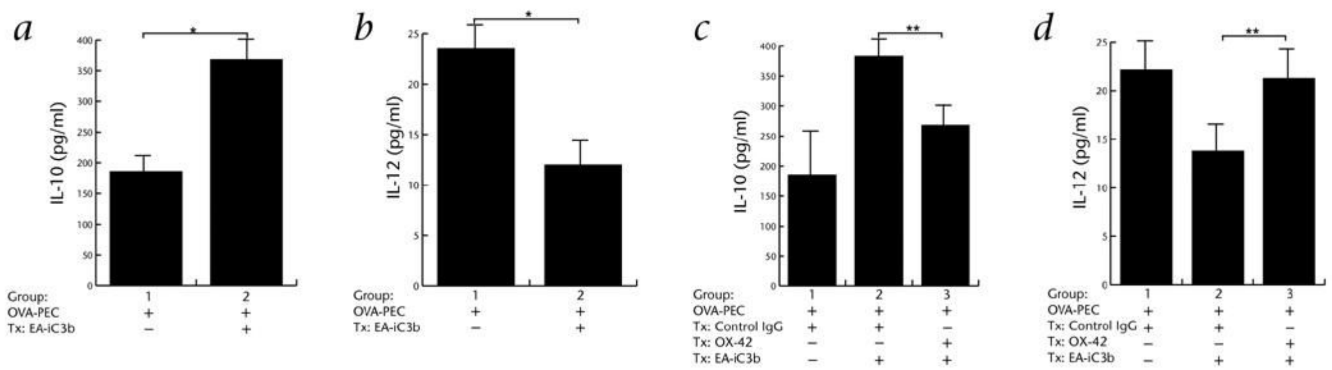

Fig. 4.

Effect of iC3b-CR3 ligation on IL-10 and IL-12. The binding of iC3b to OVA-PEC induced IL-10 secretion (group 2, ***a***), but downregulated the secretion of IL-12 (group 2, ***b***). ***c***, The iC3b-induced secretion of IL-10 (group 2) was suppressed by OX-42, an antibody against CR3 (group 3). ***d***, The iC3b-induced downregulation of IL-12 production (group 2) was reversed in the presence of OX-42 (group 3). Control IgG alone did not have any affect on IL-10 (group 1, *c*) or IL-12 (group 1, *d*) secretion by OVA-PEC in the absence of iC3b. *$P < 0.01$. **$P < 0.05$. Tx, treatment of the OVA and PEC culture.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

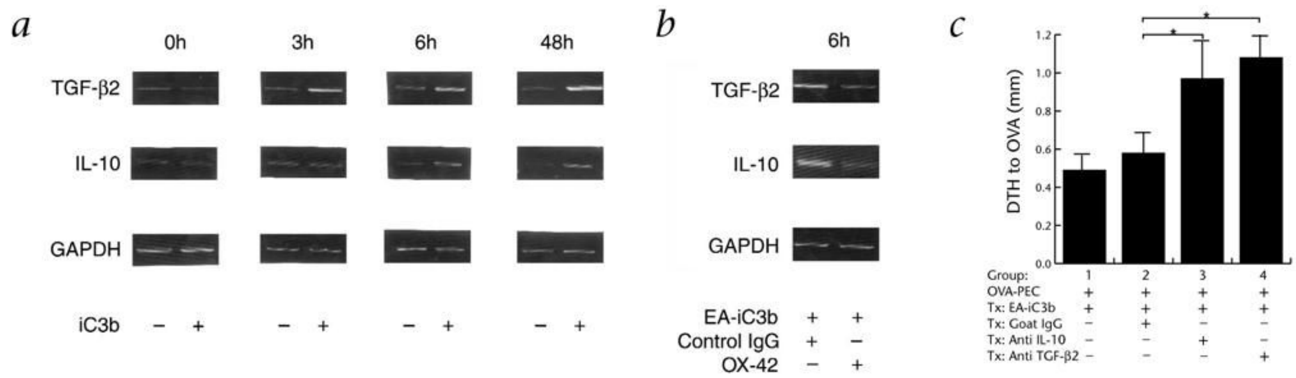

Fig. 5.

Analysis of IL-10 and TGF-β2 mRNA expression in OVA-PEC following incubation with iC3b. The figure shows ethidium-bromide–stained bands for PCR product after UV exposure. ***a***, TGF-β2 transcripts increased at 3 h in the presence of iC3b and remained at that level for 48 h. IL-10 mRNA increased at 6 h and was maintained at that level for 48 h. ***b***, OX-42 down-regulated both IL-10 and TGF-β2 mRNA production at 6 h. ***c***, Antibodies against both IL-10 (group 3) and TGF-β2 (group 4) alone reversed iC3b-induced suppression of OVA-specific DTH (group 1). Purified normal goat IgG (group 2) did not have any significant effect. *$P < 0.05$. Tx, treatment of the OVA and PEC culture.