Splenic B Cells Act as Antigen Presenting Cells for the Induction of Anterior Chamber-Associated Immune Deviation

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PURPOSE. To characterize the role of B cells in the induction of anterior chamber-associated immune deviation (ACAID).

METHODS. An in vitro model of the ACAID spleen was used to recapitulate the events that occur when antigen is introduced into the anterior chamber of the eye and culminates in the appearance of antigen-specific, CD8\(^+\) suppressor cells.

RESULTS. In vitro–generated suppressor cells mimicked those produced by anterior chamber injection of antigen, as shown by their antigen specificity, surface expression of CD8, and capacity to suppress DTH, which is mediated by previously immunized T cells. B cells were found to be necessary for suppressor cell development. The B cell receptor (BCR) was necessary for the induction of ACAID and conveyed antigen specificity to the suppressor T cells. Lysosomal acidification of internalized antigen was necessary for B cells to induce ACAID; however, transporter of antigen processing (TAP) was not required for the generation of ACAID.

CONCLUSIONS. The results suggest that B cells use the BCR to capture and internalize antigen from ACAID-inducing macrophages. Lysosomal acidification of the captured antigen is essential for the processing of the ACAID antigen before TAP-independent presentation to suppressor cells. (Invest Ophthalmol Vis Sci. 2003;44:5242-5251) DOI:10.1167/iovs.03-0768

The eye is endowed with a unique immune privilege that protects delicate ocular tissues from inflammatory damage that can lead to blindness. Passive features such as a blood–tissue barrier, deficiency in lymphatic drainage, and decreased expression of classic major histocompatibility complex (MHC) molecules on the corneal cells restrict the afferent arm of the tissue barrier, deficiency in lymphatic drainage, and decreased that can lead to blindness. Passive features such as a blood–tissue barrier, deficiency in lymphatic drainage, and decreased expression of classic major histocompatibility complex (MHC) molecules on the corneal cells restrict the afferent arm of the immune response, thereby inhibiting immunologic perception of antigens that enter the eye.\(^1\) Soluble factors within the aqueous humor, such as macrophage-migration inhibitory factor (MIF),\(^2\) transforming growth factor (TGF)-\(\beta\),\(^3\) and neu-ropetides,\(^4\) also contribute to the immune privilege of the eye through their immunosuppressive and anti-inflammatory effects. Constitutive expression of FasL on intraocular cells also provides protection by inducing apoptosis of activated lymphocytes and neutrophils that might damage ocular tissues that possess limited regenerative capacities.\(^5\)

A unique form of immune regulation, termed anterior chamber-associated immune deviation (ACAID), protects the eye from antigen-specific, immune-mediated injury inflicted by delayed-type hypersensitivity (DTH).\(^1\) ACAID is induced when pathogens or antigens enter the anterior chamber (AC) of the eye and results in systemic suppression of DTH. ACAID regulatory cells suppress both the induction and the expression of DTH in an antigen-specific manner. Studies have revealed that ACAID regulatory cells that suppress the expression of DTH (i.e., efferent suppressor cells) are CD8\(^+\) T cells.\(^6\)

There is compelling evidence that F4/80\(^+\) antigen-presenting cells (APCs) capture antigen in the eye and enter the blood stream within 24 hours of AC injection of antigen.\(^7\) These antigen-primed macrophages migrate from the eye and enter the spleen, where they induce the production of the ACAID suppressor cells.\(^7,8\) However, the cellular interactions that occur in the spleen and culminate in the generation of regulatory T cells are poorly understood. Results from several laboratories indicate that in addition to ACAID-inducing macrophages, several other cell populations participate in the generation of CD8\(^+\) regulatory T cells. These include: splenic NK T cells,\(^9\) B cells,\(^10\)–\(^13\) and \(\gamma\delta\) T-cell receptor (TCR)-bearing cells.\(^14\)–\(^17\)

B cells play a crucial role in other models of antigen-specific suppression by acting as tolerogenic APCs.\(^11\)–\(^13,18\) Results from our laboratory have shown that antigens introduced into the AC induce the formation of a B-cell population that can transfer ACAID to naive recipients.\(^15\) The hypothesis that B cells act as ancillary APCs in the generation of ACAID stems from previous findings that demonstrated that macrophages in other body sites, such as the lung and peritoneum, have the capacity to process antigen and regurgitate peptide fragments that can be captured by a variety of cells, which then present the peptides to T cells.\(^20\)–\(^22\) With this in mind, we entertained the hypothesis that ACAID-inducing macrophages release antigenic peptides that are captured by B cells and internalized through the B cell receptor (BCR) before presentation to regulatory T cells. We further examined the requirement of phagolysosomal acidification of the captured antigen before presentation to T cells.

METHODS

Animals

C57BL/6 (H-2\(b\)); C57BL/6 B-cell receptor transgenic (BCR-Tg) mice (H-2\(b\)); C57BL/6-Tg(Th11h-lg1-MD1+I-Cag); C57BL/6-J-Tap\(^{11001}\) (H-2\(b\)) (TAP knockout, KO); and B6.129S2-Igh-6tm1Cgn (H-2b) B cell-deficient (B-cell KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c (H-2\(d\)) mice were obtained from the mouse colony at the University of Texas Southwestern Medical Center at Dallas. 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 ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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Screening BCR-Tg Mice

BCR-Tg mice (C57BL/6-TgN(IGHMΔ4):CqG) are hemizygotic for the transgene encoding B cell receptor expression of Hen Egg Lysozyme (HEL). Over 90% of the splenic B cells are of the proper allotype, and 60% to 90% of these cells are capable of binding HEL. Overexpression of BCR transgene encoding B cell receptor expression of Hen Egg Lysozyme (HEL) in C57BL/6 mice, and offspring were screened for expression of the BCR transgene using PCR on DNA isolated from the mouse tail. PCR was performed on the DNA samples according to the protocol provided by The Jackson Laboratories. The PCR products were visualized on a 1.5% agarose gel containing ethidium bromide. PCR products from BCR-Tg-positive animals displayed a band of 430 bp.

Subcutaneous Immunization

Mice were immunized by subcutaneous (SC) injection of 250 μg of either ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO) in PBS or hen egg lysozyme (HEL; Sigma-Aldrich) in PBS. The soluble antigen was emulsified 1:1 in complete Freund’s adjuvant (CFA; Sigma-Aldrich). Each animal received a total volume of 200 μL.

Intracameral Injection

Mice were anesthetized with 0.133 mg/kg ketamine hydrochloride (Fort Dodge Laboratories, Fort Dodge, IA) and 0.006 mg/kg xylazine (Bayer Animal Health, Shawnee Mission, KS) given intraperitoneally (IP). A glass micropipette (approximately 80 μm diameter) was fitted onto a sterile infant feeding tube (no. 5 French; Professional Medical Products Inc., Greenwood, SC) and mounted onto a 0.1-mL syringe (Hamilton Co. Inc., Whittier, CA). An automatic dispensing apparatus (Hamilton) was used to inject 5 μL of antigen into the AC. A single intracameral injection of OVA was used for the in vivo induction of ACAID. Antigen used for these experiments was 20 mg/mL OVA (Sigma-Aldrich) in PBS (100 μg OVA).

DTH Assay

Both ear pinnae of experimental and control animals were measured with a Mitutoyo engineer’s micrometer (Mitutoyo, Aurora, IL) immediately before challenge. For OVA experiments, OVA (200 μg) in 20 μL PBS was injected intradermally into the left ear pinna. The right ear pinnae received 20 μL sterile PBS alone (negative control). Both ears were measured 24 hours later, and the difference in thickness was used as a measure of DTH. Results were expressed as: specific ear swelling = (24-hour measurement − 0-hour measurement) of the experimental ear − (24-hour measurement − 0-hour measurement) of the negative control ear.

Isolation of Peritoneal Exudate Cells and Generation of ACAID-Inducing Macrophages

ACAID-inducing macrophages were generated with a previously described protocol that has been used extensively for analyzing ACAID regulatory cells. Peritoneal exudate cells were collected from naive C57BL/6 mice and plated on Petri dishes (Primaria; BD Biosciences). The adherent macrophages were cultured overnight (2 × 10^6 cells/mL) in complete RPMI medium supplemented with 10 mg/mL OVA or 10 mg/mL HEL and 2 mg/mL human TGF-β2 (R&D Systems, Minneapolis, MN).

Generation of ACAID B Cells

B cells that are capable of inducing ACAID in naive recipients were generated as previously described. Putative suppressor cells were collected from the in vitro ACAID cultures or from spleen cell suspensions of AG-injected mice. The putative suppressor cells were suspended at 5 × 10^7 cells/mL in 10 mg/mL OVA or HEL in PBS. Immune cells were elicited by SC immunization of naive C57BL/6 mice on day 0 with 250 μg of either OVA or HEL in PBS and were emulsified in an equal volume of CFA. Immune splenocytes were collected on day 14 and were suspended at 5 × 10^7 cells/mL in 10 mg/mL of OVA or HEL. The immune and suppressor cell populations were then mixed 1:1 in the presence of antigen (10 mg/mL). Both ears of naive C57BL/6 mice were measured with a engineer’s micrometer immediately before challenge. The left

Depletion and Enrichment of T Cell Populations

CD4 T cells or CD8 T cells were isolated by magnetic separation (M+ columns; Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer’s instructions. Briefly, splenocytes were isolated and erythrocytes lysed before the cells were incubated with rat anti-mouse B220 antibody–conjugated microbeads (10 μL beads/10^7 cells) in 0.5% FCS in PBS for 15 minutes in the refrigerator. The cells were washed with 0.5% BSA in PBS followed by magnetic separation (M+ columns; Miltenyi Biotec), as described by the manufacturer. The retained cells were eluted from the column and shown by flow cytometric analysis to be more than 95% B220-. B cells (4 × 10^7) were incubated with 2 × 10^6 OVA ACAID-inducing or OVA non-ACAID-inducing macrophages in a medium Petri dish (5 mL) for 48 hours to generate ACAID-inducing B cells or non-ACAID–inducing B cells.

Intracameral Injection

Mice were immunized by intracameral injection of OVA (200 μg) in a volume of 0.1 L of antigen into the AC. A single intracameral injection of OVA was used for the in vivo induction of ACAID. Antigen used for these experiments was 20 mg/mL OVA (Sigma-Aldrich) in PBS (100 μg OVA).

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CD4 T cells or CD8 T cells were isolated by magnetic separation (M+ columns; Miltenyi Biotec) according to the manufacturer’s instructions. Briefly, splenocytes were isolated and erythrocytes lysed before the cells were incubated with either CD4+ or CD8-specific microbeads (10 μL beads/10^7 cells) in 0.5% BSA in PBS for 15 minutes at 4°C. The cells were washed with 0.5% BSA in PBS followed by magnetic separation (M+; Miltenyi). The enriched populations were then incubated with either anti-CD8 (CD8-enriched cell suspension) or anti-CD4 antibody (CD4-enriched cell suspension) and incubated on ice for 30 minutes. The cells were washed three times with HBSS and resuspended in infant rabbit complement (1:10; Low-Tox; Accurate Chemical, Westbury, NY) for 30 minutes at 57°C in 5% CO₂. The CD4+ and CD8-enriched cell suspensions were examined by flow cytometry for expression of the respective surface determinant and were found to be more than 95% pure.

Induction of ACAID Regulatory Cells Using In Vitro Spleen Cell Cultures

An in vitro spleen cell culture system that mimics the spleen of an ACAID-induced animal was used as previously described. CD4+ T cells generated in this in vitro culture are capable of suppressing a DTH response of T cells isolated from previously immunized animals. Briefly, ACAID-inducing macrophages (5 × 10^6) were added to a large Petri dish (Falcon 3005; BD Biosciences Labware) containing 5 × 10^7 spleen cells, 5 × 10^7 CD4+ T cells, or 5 × 10^7 CD8+ T cells from naive C57BL/6, B-cell KO, or HEL BCR-Tg mice, respectively. In some experiments, cell populations were cultured in transwell chambers separated by a semipermeable membrane (Costar, Corning, NY; pore size 0.4 μm) that prevented cell migration between culture chambers, but allowed free movement of macromolecules. All spleen cell cultures were incubated at 37°C for 5 to 7 days before being tested for the presence of ACAID suppressor cells.

B-Cell Treatment with Chloroquine

B cells (5 × 10^7) were incubated in 10 mL of 400 μM chloroquine (Sigma-Aldrich) in complete RPMI for 30 minutes at room temperature. Cell viability was confirmed by trypan blue exclusion after the incubation.

Local Adoptive Transfer Assay

A local adoptive transfer (LAT) assay used to test for ACAID suppressor cells. Putative suppressor cells were collected from the in vitro ACAID cultures or from spleen cell suspensions of AG-injected mice. The putative suppressor cells were suspended at 5 × 10^7 cells/mL in 10 mg/mL OVA or HEL in PBS. Immune cells were elicited by SC immunization of naive C57BL/6 mice on day 0 with 250 μg of either OVA or HEL in PBS and were emulsified in an equal volume of CFA. Immune splenocytes were collected on day 14 and were suspended at 5 × 10^7 cells/mL in 10 mg/mL of OVA or HEL. The immune and suppressor cell populations were then mixed 1:1 in the presence of antigen (10 mg/mL). Both ears of naive C57BL/6 mice were measured with a engineer’s micrometer immediately before challenge. The left
ear pinnae of naïve C57BL/6 mice were injected with 20 μL (1 x 10⁶) of the mixed-cell population. The right ear pinnae were injected with 10 mg/mL of OVA or HEL as a negative control. Ear swelling was measured 24 hours later to measure DTH.

RESULTS

In Vivo and In Vitro Production of ACAID Suppressor Cells

The LAT assay has been used by many laboratories to demonstrate that spleen cells from AC-injected animals contain antigen-specific suppressor cells that are capable of inhibiting DTH responses by preimmune T cells (i.e., efferent-acting suppressor cells). The putative suppressor cells and immune cells were combined and transferred in the presence of antigen to the ear pinnae of naïve animals. The ear-swelling responses of mice that received immune cells combined with naïve, non-ACAID spleen cells displayed significant ear swelling indicative of DTH (positive control). However, this ear-swelling response induced by immune cells was significantly reduced when spleen cells from ACAID-affected animals were coinjected with the immune cells. The results of a typical experiment are shown in Figure 1A. The suppressed DTH indicates the presence of suppressor cells within the spleen of animals with induced ACAID.

The LAT assay was used to demonstrate the efferent suppressive properties of ACAID regulatory cells generated in vitro and those that appear in the spleen after AC priming. The results demonstrated that the in vitro–generated ACAID regulatory cells inhibited the expression of DTH responses as effectively as ACAID regulatory cells isolated from the spleens of AC primed donors (Fig. 1B).

An additional experiment was performed to confirm that the in vitro model of ACAID generates efferent suppressor cells that are antigen-specific and express the CD8 surface determinant. Therefore, in vitro ACAID spleen cell cultures were prepared with OVA-specific ACAID-inducing macrophages cocultured with spleen cells from naïve donors. Five days later, the spleen cell cultures were treated with either anti-CD4 or anti-CD8 antibody plus complement and tested for the presence of OVA-specific suppressor cells using the aforementioned LAT assay. The in vitro–generated ACAID suppressor cells were coinjected into the ears with either OVA and spleen cells from OVA-immunized donors or HEL and spleen cells from HEL-immunized donors. The results demonstrated that suppression was present in ACAID cultures containing CD8⁺ spleen cells, but not in those depleted of CD8⁺ spleen cells (Fig. 2). The in vitro generated suppressor cells were antigen-specific, as they suppressed OVA responses, but failed to inhibit DTH in response to HEL (Fig. 2).

Thus, the in vitro ACAID spleen cell culture system mimicks the cellular events that occur in the spleens of ACAID mice and is a facile method for analyzing the generation ACAID-inducing suppressor cells.

Absence of Direct Contact of Macrophages with Spleen Cells for the Generation of Suppressor Cells

It has been hypothesized that B cells function as APCs for the development of ACAID suppressor cells. ACAID-inducing macrophages are thought to transport antigen from the eye to the spleen, where they directly present antigen to T cells. It is possible that ACAID-inducing macrophages release antigenic peptides for subsequent acquisition by B cells. The B cells could then present antigen in a manner that produces ACAID suppressor cells. To address this hypothesis, ACAID-inducing macrophages were tested to determine whether they required direct contact with the spleen cells to induce ACAID suppressor cells. The in vitro ACAID spleen cell culture was modified with a plate consisting of a semipermeable membrane separat-
Sing ACAID-inducing macrophages from spleen cells that ultimately produce the ACAID suppressor cells (Transwell, costar; Corning Inc., Corning, NY). Therefore, cell contact was prevented and only macromolecules could pass through the membrane between the two chambers of the plates. OVA-primed ACAID-inducing macrophages were added to the top chamber of the culture plate, and naïve C57BL/6 spleen cells were added to the lower chamber. This modified in vitro culture was incubated for 5 days before testing the bottom chamber for the presence of ACAID suppressors using the LAT assay. The results showed that direct contact between ACAID-inducing macrophages and spleen cells was not needed for the generation of suppressor cells (Fig. 3).

**Figure 2.** In vitro-generated ACAID suppressor cells were CD8<sup>+</sup> and antigen-specific. In vitro ACAID spleen cultures were prepared using OVA-specific ACAID-inducing macrophages cocultured with normal spleen cells. Spleen cells were isolated after the 5-day culture period and treated with either anti-CD4 or anti-CD8 monoclonal antibody plus complement and tested for suppressor cell activity in the LAT assay using either OVA-immune or HEL-immune spleen cells. All results are reported as mean ear swelling ± SD. There were at least five animals per group. *P > 0.05 compared with untreated OVA ACAID group. **P = 0.001 compared to OVA ACAID group; P > 0.05 compared with the positive control group; ***P > 0.05 compared with the HEL immune group.

**Figure 3.** ACAID-inducing macrophages did not require direct contact with normal spleen cells for the generation of ACAID suppressor cells. A modified in vitro ACAID culture system was established using a double-chambered culture plate with a semipermeable membrane insert. ACAID-inducing macrophages were generated with OVA and placed in the upper chamber and naïve C57BL/6 spleen cells were placed in the lower chamber. Spleen cells were evaluated for regulatory cell activity using the LAT assay. All results are reported as mean swelling ± SD. *P = 0.001 for positive versus negative controls. **P = 0.0001 for positive control versus cultured macrophages (top chamber) and naïve spleen (bottom chamber). Each group contained at least five animals.

**B-Cell Induction of ACAID Suppressor Cells In Vivo**

Experiments were performed to establish the validity of the in vitro ACAID culture system and confirm that B cells are required for the generation of ACAID suppressor cells in vivo. In the first experiment, spleen cell suspensions from B-cell KO mice were used to determine whether B cells are necessary for the generation of ACAID suppressor cells in vitro. ACAID cultures combined OVA-primed, ACAID-inducing macrophages with spleen cells from either B-cell KO mice or normal C57BL/6 mice. The cell cultures were tested for ACAID suppressor cells in the LAT assay, and the results demonstrated that spleen cells from B-cell KO mice did not promote the development of ACAID suppressor cells (Fig. 4A). By contrast, ACAID spleen cell cultures containing normal B cells did support the production of ACAID regulatory cells and thus established the validity of the spleen culture system as an in vitro correlate of ACAID.

To ensure that the inability to produce suppressor cells was due to a lack of B cells and not because of ancillary defects that can sometimes occur in KO mice, the experiment was repeated using spleen cell suspensions from normal mice that were depleted of B cells. OVA-primed, ACAID-inducing macrophages were combined with spleen cell suspensions depleted of B cells by a traditional panning technique. The B-cell-depleted spleen cell cultures were then tested for the generation of ACAID suppressor cells using an LAT assay. The results confirmed that when splenic B cells were depleted by panning, the in vitro ACAID culture could not produce suppressor cells capable of downregulating DTH (Fig. 4B). The ability to produce the suppressive activity was restored when B-cell-depleted spleen cell cultures were reconstituted with normal B cells.

An additional experiment was performed to confirm that ACAID-inducing macrophages transferred antigen-specific signals to ACAID B cells, which in turn induced the development of ACAID effector suppressor cells. ACAID-inducing B cells were generated by coculturing B cells with OVA ACAID-inducing macrophages for 48 hours in vitro, as described earlier. Nonadherent B cells were removed by rigorous washing with culture medium and were then treated with either anti-B220 antibody plus complement or anti-F4/80 antibody plus complement before adding them to spleen cell cultures. Five days later, the spleen cell cultures were tested for the presence of OVA-specific suppressor cells using the aforementioned LAT
assay. The in vitro-generated ACAID suppressor cells were coinjected into the ears of naïve mice with either OVA and spleen cells from OVA-immunized donors or HEL and spleen cells from HEL-immunized donors. The results confirmed the role of B cells in the in vitro generation of ACAID, as depletion of B cells prevented the development of efferent suppressor cells (Fig. 5). The results also demonstrate that the B cells delivered an antigen-specific signal for the generation of ACAID (Fig. 5).

Effect of In Vitro–Generated ACAID B Cells on Suppressor Cells In Vivo

The rationale for developing the in vitro model of ACAID was to dissect the role of the splenic B cell in the generation of ACAID suppressor cells. Accordingly, ACAID spleen cell cultures were used to test the hypothesis that B cells act as ancillary APCs by acquiring antigen released by ACAID-inducing macrophages. OVA-specific, ACAID-inducing macrophages were generated in vitro, washed three times in HBSS, and coincubated with B-cell suspensions to produce ACAID B cells. The ACAID B cells were removed from the plastic-adherent macrophages by vigorous pipetting followed by treatment with anti-Fc/80 antibody plus complement to eliminate any contaminating macrophages. Some of the B cells were also treated with anti-B220 antibody plus complement. All the B-cell suspensions were injected intravenously (4 × 10⁶ cells/mouse) into naïve C57BL/6 mice. Mice were immunized SC with OVA and CFA 7 days later. On day 14, spleens were collected from the adoptive cell transfer recipients and tested for the presence of ACAID suppressor cells using the LAT assay. The results of a typical experiment demonstrate that in vitro–generated ACAID B cells induced ACAID after adoptive transfer to normal recipients (Fig. 6).

Use of the B-Cell Receptor by ACAID B Cells in Generating Suppressor Cells

The results shown in Figure 6 are consistent with the hypothesis that splenic B cells acquire antigen that is released by ACAID-inducing macrophages and subsequently induce the production of ACAID suppressor cells. Experiments used transgenic mice whose BCR is specific for HEL to test the hypothesis that the BCR is required for the generation of ACAID suppressor cells. First, it was important to test whether the BCR-transgenic mice could possess ACAID suppressor cells for their specific antigen, HEL. ACAID-inducing macrophages were generated in vitro using HEL as the antigen. The ACAID-inducing macrophages were then added to spleen cell cultures prepared from HEL BCR-Tg mice. The in vitro ACAID cultures were incubated for 5 days, and an LAT assay was used to test for the presence of ACAID suppressor cells specific for HEL. As shown in Figure 7A, spleen cells from HEL BCR-Tg C57BL/6 mice induced ACAID as effectively as spleen cells from wild-type mice.

To address whether the antigen-specific BCR is needed for the B cell to contribute to the production of ACAID suppressor cells, B cells expressing HEL-specific BCRs were used in spleen cell cultures using OVA-pulsed, ACAID-inducing macrophages. The cultured cells were then tested in an LAT assay for the presence of ACAID suppressor cells that specifically inhibited DTH responses to OVA. The results shown in Figure 6 are consistent with the hypothesis that splenic B cells acquire antigen that is released by ACAID-inducing macrophages and subsequently induce the production of ACAID suppressor cells. Accordingly, ACAID spleen cell cultures were then tested for the presence of anti-Ig antibody. As shown in Figure 7B, the BCR is required for the B cells to induce ACAID suppressor cells.

It was necessary to confirm that the restrictive BCR expression in the Tg mice is the sole factor responsible for the inability to produce ACAID suppressor cells. Surface immunoglobulin (Ig) is known to act as a ligand to facilitate internalization of antigen by B cells. However, anti-Ig antibody treatment of B cells prevents antigen capture and presentation to T cells. Accordingly, experiments used antibodies specific for murine Ig to block the BCR and confirm its role in the induction of ACAID suppressor cells. OVA-specific, ACAID-inducing macrophages were incubated with naïve spleen cell cultures alone or in the presence of anti-Ig antibody. The spleen cell cultures were then tested for the presence of suppressor cells using the LAT assay. The presence of anti-Ig antibody prevented the generation of ACAID suppressor cells,
which suggests that the BCR is crucial for B cells to participate in the generation of ACAID (Fig. 8).

**B Cells Processing of Antigen for the Generation of Suppressor Cells**

Because the BCR appears to be necessary for the development of ACAID suppressor cells, it is possible that the B cells use the BCR to capture and internalize the antigen before presenting it to responder cells, as has been proposed in other models of antigen presentation by B cells.32–34 Treatment with chloroquine inhibits intracellular antigen processing.35 Therefore, B cells were treated with chloroquine and examined for their ability to promote the development of ACAID suppressor cells. ACAID spleen cell cultures were prepared in which ACAID-inducing macrophages were incubated with B-cell–depleted spleen cells that were reconstituted with either normal B cells or chloroquine-treated B cells. The in vitro ACAID cultures were then tested for the presence of ACAID suppressor cells. Although chloroquine treatment did not affect viability of the treated B cells (data not shown), it eliminated the B cells’ ability to promote the generation ACAID suppressor cells (Fig. 9).

B cells were also tested to determine whether defects in TAP expression inhibit production of ACAID suppressor cells. Based on the BCR and chloroquine data, we anticipated that B cells capture and internalize antigen fragments released by OVA-pulsed, ACAID-inducing macrophages through the exogenous, MHC class II–dependent pathway. Alternatively, it has been reported that exogenous antigens are sometimes processed through the endogenous, TAP-dependent pathway. To determine whether B cells use the TAP-dependent pathway for antigen processing, ACAID cultures were prepared with spleen cell suspensions that were depleted of B cells and reconstituted with B cells from TAP KO mice. The results showed that B cells from TAP KO mice promoted the production of ACAID suppressor cells (Fig. 10).

**DISCUSSION**

A simple explanation for the development of ACAID suppressor cells is that ACAID-inducing macrophages migrate from the eye to the spleen where they directly present antigen to CD8⁺ T cells and elicit ACAID suppressor cells. Although appealing in its simplicity, this hypothesis is flawed, because other T-cell populations (γδ T cells and NK T cells) are required for the appearance of suppressor cells.10,15,17 Evidence also exists that splenic B cells are involved in ACAID suppressor cell develop-

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**FIGURE 5.** ACAID-inducing macrophages delivered an antigen-specific signal to B cells. OVA-specific, ACAID-inducing macrophages were cocultured with purified suspensions of B cells for 48 hours. All B-cell suspensions were isolated from plastic-adherent macrophages and treated with anti-F4/80 antibody plus complement. Some B-cell suspensions were also treated with anti-B220 plus complement. B cells were added to spleen cultures, and 5 days later spleen cell cultures were tested for OVA-specific ACAID using the LAT assay. Ears were challenged with OVA ACAID suppressor cells and either HEL immune spleen cells or OVA immune spleen cells. All results are reported as mean ear swelling ± SD. *P = 0.0001 compared with ACAID B cells treated with anti-F4/80. Each group contained at least five animals.

**FIGURE 6.** In vitro generated B cells induced ACAID suppressor cells in vivo. OVA ACAID-inducing macrophages or OVA non–ACAID-inducing macrophages were cocultured with B cell suspensions for 48 hours. B cells were removed from plastic-adherent macrophages by vigorous pipetting after treatment with anti-F4/80 antibody plus complement followed by treatment with anti-B220 antibody plus complement or complement alone. The B-cell suspensions were injected intravenously (4 × 10⁶ cells/mouse) into normal C57BL/6 mice. Mice were immunized SC with OVA. Fourteen days later, the mice were killed and their spleens tested for the presence of ACAID suppressor cells, using the LAT assay. *P = 0.05 for positive versus negative controls. **P = 0.02 for negative control versus non-ACAID. ***P = 0.01 for non-ACAID B cells versus ACAID B cells. All results are reported as mean swelling ± SD. Each group contained at least five animals.
11–13 Animals treated in vivo with anti- \( H9262 \) antibody do not generate ACAID, nor do B-cell-deficient mice.11–13 Moreover, B-cell-depleted spleen cell cultures do not produce ACAID suppressor cells in vitro. The participation of B cells in the induction of ACAID is not surprising, considering that B cells have a role in other forms of immune tolerance.36–39

Until now, the exact contributions of the B cells in the induction of ACAID were a mystery; however, the data presented herein suggest that B cells act as ancillary APCs for the generation of CD8\(^+\) suppressor T cells. Previous results have demonstrated that B cells from \( 2m \) KO mice cannot contribute to the generation of ACAID suppressor cells,13 suggesting that MHC class I is involved in the induction of suppressor cells. More specifically, it has been suggested that the nonclassic class I (class Ib) MHC Qa-1 is required for the B cells to promote the induction of ACAID.13 A tolerance model pro-
posed by Noble et al. 19 also indicates a role for Qa-1-restricted B cells in eliciting CD8+ suppressor cells. This is reminiscent of the Qa-1-restricted B cells that are necessary for the development of ACAID suppressor cells. 15 Moreover, Wang et al. 20 have described a Qa-1 receptor molecule on the surface of CD8+ T cells. Therefore, an attractive hypothesis is that ACAID B cells acquire antigen released from ocular APCs in the spleen and after capturing the regurgitated antigen, the B cells process and present ACAID-inducing peptides on Qa-1 molecules to CD8+ T cells.

In order for B cells to participate in the generation of ACAID, they must first acquire antigen from ocular APCs. The ACAID-inducing macrophages that carry antigen from the eye to the spleen are potential sources of antigen for the B cells. It is known that antigen can be released from mouse peritoneal macrophages in the form of either whole protein or degraded peptides, which are subsequently processed by secondary APCs. 22 The present experiments showing that B cells acquire the capacity to induce ACAID after exposure to ACAID-inducing macrophages further support this hypothesis.

A plausible mechanism for the transmission of antigen to B cells is through the BCR. Lanzavecchia 32,34 and D'Orazio et al. 18 have proposed the "vacuum cleaner" model of antigen uptake by B cells. It has been demonstrated that B cells are 1000 times more effective than conventional APCs in capturing and presenting antigens to T cells. 32 Both Igs and -chains of the BCR are essential for internalization and subsequent presentation of antigen by the B cell. 47 When a BCR binds antigen, the BCR-antigen complex is internalized and processed before subsequent antigen presentation. 32,45

To test whether the BCR is required for antigen acquisition, transgenic mice carrying the BCR transgene for HEL were used. The BCR transgenic mice were generated by Goodnow et al. 23 and Hartley et al. 24 who introduced rearranged Ig genes specific for HEL. More than 90% of the splenic B cells are of the proper allotype, and 60% to 90% of these cells are capable of binding the specific antigen HEL. 23 Aside from their specific BCR expression, these B cells were normal. The results showed that, in the transgenic mice, suppressor cells specific for OVA did not develop, and thus, ACAID did not develop. This experiment was supported by data showing that when the BCR is blocked with anti-Ig antibody, the B cells are unable to contribute to ACAID suppressor cell formation. This reinforces the proposition that B cells acquire regurgitated antigen through their BCR during the course of inducing ACAID.

Because macrophages are known to release both native protein and processed peptide, 22 it is possible that the BCR recognizes either processed peptide or native protein. However, it is not known which form of antigen is captured by the ACAID-inducing B cell. Further studies were performed to determine whether antigen processing was required for B cells to contribute to the development of suppressor cells. After antigen is internalized by the BCR, it is thought to enter into early endosomes that then fuse with lysosomes where antigen processing occurs through acid proteases. 32,44 Chloroquine prevents the acidification of lysosomes, thereby blocking the action of acid proteases necessary for antigen processing. 44 If B cells require phagolyosomal acidification, treatment with chloroquine should inhibit the B cells' ability to induce ACAID. B cells treated with chloroquine were unable to contribute to the generation of suppressor cells, suggesting that B cells must internalize and process antigenic moieties released from ACAID-inducing macrophages.

Internalized proteins are typically processed in endosomal compartments and associate with MHC class II for subsequent presentation to CD4+ T cells. 44 It might be assumed that ACAID B cells process exogenous regurgitated peptide in a manner that culminates in presentation on MHC class II molecules. However, previous findings suggest that the ACAID B cells present antigen in the context of a nonclassic class I molecule Qa-1. 15 MHC class Ia molecules are known to present endogenous antigen. After intracellular transport into the endoplasmic reticulum (ER), antigen is loaded onto class Ia molecules before traveling to the cell surface for presentation. 44 However, there are examples in which exogenous antigens can be presented by MHC class I molecules. 45 More significantly, it has been demonstrated that exogenous antigen can be internalized and processed for presentation on the nonclassic class Ib molecule Qa-1, through a TAP-independent pathway. 19,46 Tompkins et al. 46 described the internalization and

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**Figure 10.** B cells induce ACAID by a TAP-independent mechanism. C57BL/6 spleen cells and either OVA ACAID-inducing macrophages or OVA non-ACAID-inducing macrophages were used in an in vitro spleen culture to generate ACAID suppressor cells. Spleen cell cultures were depleted of B cells by panning and reconstituting with B cells (treated with anti-F4/80 antibody plus complement) from either normal or TAP KO donors. OVA-specific, ACAID-inducing macrophages were then added to the spleen cell cultures, which were incubated for 7 days. Spleen cell cultures were evaluated for ACAID regulatory cells, using the LAT assay. All results are reported as mean ear swelling ± SD. *P = 0.0003 for normal B cell versus positive control; P > 0.05 for normal cells versus TAP KO B cell. **P = 0.0001 for TAP KO B cell versus positive control. There were five animals in each group.
processing of exogenous pork insulin before Qa-1 presenta-
tion. Similar to the ACAID system, this report showed that
treatment with chloroquine abrogated the ability of conven-
tional APCs to process and present insulin antigen on Qa-1
molecules. Also, similar to results reported here, this antigen
processing and presentation was independent of a functional
TAP molecule. Therefore, it is plausible to conclude that
ACAID-inducing antigenic peptides, such as those from OVA,
are acquired passively and presented on the B cell through
nonclassic class Ib (Qa-1) molecules.46 The capacity of ACAID
B cells to directly induce ACAID regulation when adoptively
转移 to naïve recipients or by co-incubation with T cells in
vitro is consistent with the hypothesis that B cells use
the induction of ACAID. These data are consistent with the
B cell acts as an ancillary splenic APC that is necessary for
the B cell to present CD1d-associated antigen to NK T
independently present antigens to at least three T-cell popula-
ions of DTH.

It is important to bear in mind that induction of ACAID also
involves the coordinated participation of two other T-cell pop-
ulations: NK T cells10 and γδ T cells.8,9 Sonoda and Stein-
Streilein17 have recently provided evidence suggesting that
marginal zone B cells present CD1d-associated antigen to NK T
cells during the induction of ACAID. Our studies did not
specifically examine which T-cell population interacted with
the ACAID B cells. Thus, it is possible that ACAID B cells
independently present antigens to at least three T-cell popula-
ions: NK T cells, γδ T cells, and αβ T cells. These findings
underline the complexity of the cellular interactions in the
induction of ACAID and remind us that there must be much
more exploration before we can fully understand the events
that transpire in the induction and maintenance of immune
privilege in the eye.

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