Endogenous IRBP can be dispensable for generation of natural CD4+CD25+ regulatory T cells that protect from IRBP-induced retinal autoimmunity

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Susceptibility to experimental autoimmune uveitis (EAU), a model for human uveitis induced in mice with the retinal antigen interphotoreceptor retinoid–binding protein (IRBP), is controlled by "natural" CD4+CD25+ regulatory T (T reg) cells. To examine whether endogenous expression of IRBP is necessary to generate these T reg cells, we studied responses of IRBP knockout (KO) versus wild–type (WT) mice. Unexpectedly, not only WT but also IRBP KO mice immunized with a uveitogenic regimen of IRBP in complete Freund's adjuvant (CFA) exhibited CD25+ regulatory cells that could be depleted by PC61 treatment, which suppressed development of uveitogenic effector T cells and decreased immunological responses to IRBP. These EAU–relevant T reg cells were not IRBP specific, as their activity was not present in IRBP KO mice immunized with IRBP in incomplete Freund's adjuvant (IFA), lacking mycobacteria (whereas the same mice exhibited normal T reg cell activity to retinal arrestin in IFA). We propose that mycobacterial components in CFA activate T reg cells of other specificities to inhibit generation of IRBP–specific effector T cells in a bystander fashion, indicating that effective T reg cells can be antigen nonspecific. Our data also provide the first evidence that generation of specific T reg cells to a native autoantigen in a mouse with a diverse T cell repertoire requires a cognate interaction.

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Thymus–derived "natural" CD4+CD25+ regulatory T (T reg) cells have been shown to play a role in the control of disease in various models of autoimmunity, but many questions remain concerning their antigenic specificity as well as the qualitative and quantitative requirements of antigen expression in the thymus for their development (1, 2). Studies in double transgenic mice expressing a neo-antigen and a TCR specific for that antigen have suggested that antigen-specific CD4+CD25+ T reg cells are selected by interaction with the cognate self-antigen in the thymus (3, 4). Disease-relevant T reg cells in autoimmune diabetes and encephalomyelitis appear to be autoantigen specific (5, 6). However, the question whether endogenous expression of the target self-antigen is necessary to generate T reg cells capable of protecting from autoimmune disease in mice with a full T cell repertoire has not yet been examined.

Experimental autoimmune uveitis (EAU) in mice is a model of human posterior uveitis that in the United States alone affects 150,000 persons annually and accounts for about 10% of legal blindness (7, 8). EAU can be induced in many animal species by immunization with retinal antigens such as interphotoreceptor retinoid-binding protein (IRBP) or retinal arrestin (retinal soluble antigen [S-Ag]) in CFA. In immunologically normal mice, EAU is a cell-mediated, Th1-dependent disease that targets the neural retina where the target antigens are located, leading to an irreversible destruction of photoreceptor cells and loss of vision (9).

Despite the immune–privileged nature of retinal antigens, it is now known that susceptibility to EAU is controlled in large part by central (thymic) mechanisms (10, 11). Our recent study demonstrated that these mechanisms include elimination of uveitogenic effector cell clonotypes as well as generation of

thymic-derived CD4+CD25+ T reg cells (11). However, whereas that study demonstrated directly, by thymic transplantation between IRBP KO and WT mice, that IRBP in the thymus is needed to eliminate IRBP-specific effector cells, it did not address the question whether expression of IRBP is also needed to elicit the EAU-relevant T reg cells.

The present study addresses the question whether T reg cells that protect from IRBP-induced EAU require endogenous expression of IRBP. We reasoned that if this is the case, depletion of CD25+ cells from IRBP KO mice will not alter their responses to IRBP. We show here that contrary to this hypothesis, IRBP KO mice possess EAU-relevant CD4+CD25+ T reg cells that down-regulate responses to IRBP and limit generation of uveitogenic T cells. These T reg cells do not seem to be specific to IRBP. Rather, they seem to be T reg cells of other specificities that are activated by microbial components present in CFA and inhibit development of IRBP-specific effector T cells in a bystander fashion. This study also indicates that a cognate interaction is needed to generate antigen-specific T reg cells to a native autoantigen from a normal, diverse T cell repertoire.

RESULTS AND DISCUSSION
Both CD25–depleted WT and KO mice immunized with a uveitogenic regimen of IRBP/CFA show enhanced responses to IRBP and enhanced ability to generate uveitogenic effector cells

Our previous study demonstrated that thymic expression of IRBP is necessary to eliminate IRBP-specific uveitogenic effector T cells, but it did not address the question whether it is also necessary to induce EAU-relevant T reg cells. To answer this question, we used IRBP KO mice that lack the promoter region and 81% of the coding sequence of IRBP, and fail to express detectable IRBP in the eye or the thymus (11, 12). Although the IRBP KO mice are unable to develop EAU, having no target antigen in the retina, they develop robust antigen-specific responses and their primed cells can transfer EAU into IRBP-sufficient recipients (11). We hypothesized that if endogenous IRBP is needed to generate EAU-relevant T reg cells, depletion of CD25+ cells from IRBP-deficient mice will fail to alter their IRBP-specific immune responses or their ability to generate uveitogenic effector T cells.

IRBP KO and WT mice were depleted, or not, of T reg cells by two infusions of the PC61 mAb as described in Materials and methods, and were immunized with a uveitogenic regimen of IRBP in CFA. The results showed that CD25 depletion enhanced IRBP-specific delayed-type hypersensitivity (DTH) responses as well as proliferation of splenocytes to IRBP in IRBP KO mice at least as strongly or more than in WT mice (Fig. 1). The greater enhancement of proliferative response in the KO might be due to an expanded repertoire of effector T cells present in KO mice compared to WT (11), resulting in stronger responses after T reg cells are removed. In addition, after CD25 depletion in WT as well as in KO mice, proinflammatory cytokines (IFN-γ, TNF-α,
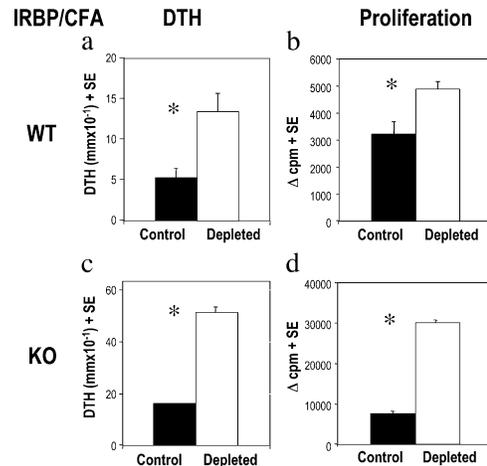

Figure 1. **CD25–depleted WT and KO mice immunized with IRBP/CFA show enhanced antigen–specific immunological responses.** Mice depleted of CD25+ cells by treatment with PC61 on days −10 and −7 were immunized with IRBP in CFA on day 0. Immunological responses were determined 12 d after immunization. *, P < 0.05. (a and c) DTH responses as specific increment in ear thickness; (b and d) specific proliferation to IRBP of splenocytes as counts per minute of [³H]thymidine after subtraction of background (between 1,000 and 3,000 counts, depending on group). Proliferation of lymph node cells was similarly increased compared with nondepleted control mice (not depicted). Data from a representative experiment with five mice per group are shown.

IL-2, and IL-6) were increased in supernatants of IRBP-stimulated cell cultures (not depicted). These results suggested that regulatory cells that could control the anti-IRBP response were present in IRBP KO donors.

We next examined the effect of CD25 depletion on EAU. CD25 depletion reproducibly enhanced EAU severity in WT mice (Fig 2, a and c; P < 0.009; reference 11). EAU development in CD25-depleted IRBP KO cannot be tested directly because these mice lack the target antigen in the retina. To be able to use clinical uveitis as readout, we performed an adoptive transfer of primed lymphoid cells from depleted or nondepleted KO mice into naive WT recipients that would provide the essential retinal target. Adoptively transferred T cells from IRBP KO donors, depleted of CD25+ cells and primed with IRBP, elicited higher disease than did parallel cells from nondepleted donors, again suggesting that an EAU-relevant regulatory cell had been removed (Fig. 2 b; P < 0.004).

Immunization in IFA reveals differences in responses to IRBP after CD25 depletion between WT and KO mice

We considered several explanations for the presence of EAU-relevant T reg cells in IRBP KO mice. Residual expression of an IRBP fragment in IRBP KO mice, which are deleted for the promoter region and 81% of the IRBP sequence, has largely been excluded. Neither we nor others were able to demonstrate IRBP protein in IRBP KO mice using potent polyclonal antibodies, nor do they develop signs of ocular
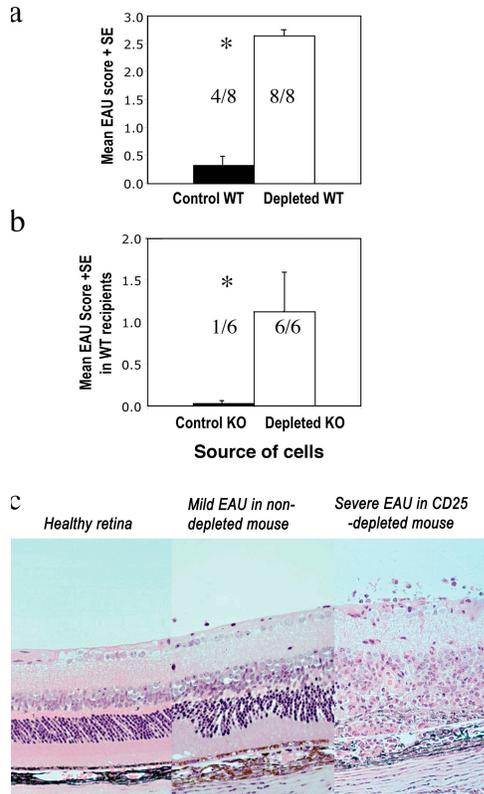
Figure 2. IRBP KO mice have CD25⁺ EAU–relevant T reg cells.
(a) EAU in WT mice is enhanced by depletion of CD25⁺ cells. CD25 depletion and IRBP immunization were as described in the legend to Fig. 1. Disease was scored by funduscopy 21 d after immunization. Incidence is shown as positive/total above each column. EAU was significantly enhanced by CD25 depletion *, P < 0.0002. Data pooled from two repeat experiments are shown. (b) EAU is enhanced in recipients of primed T cells from CD25-depleted IRBP KO mice. Naive WT mice were infused with 10 × 10⁶ antigen–activated lymphoid cells from IRBP KO mice that had been depleted or not of CD25⁺ cells and immunized with IRBP in CFA. EAU in recipients was scored by funduscopy 14 d after transfer. Incidence is shown as positive/total. Disease was significantly enhanced by CD25 depletion of donors (P < 0.004). Data pooled from two repeat experiments are shown. (c) Representative retinal histology of mice from panel a. Note much more severe destruction of the retinal architecture in CD25-depleted mice.

Figure 3. CD25–depleted IRBP KO mice immunized in IFA fail to up–regulate responses to IRBP, but do up–regulate responses to S–Ag. Depletion and immunization were as described in the legend to Fig. 1, except that IRBP (a–d) and S-Ag (e and f) were injected in IFA. (a and c) Specific DTH to IRBP as increment in ear thickness; (b and d) lymphocyte proliferation to IRBP after subtraction of background (500 to 2,500 counts, depending on group); (e and f) DTH and lymphocyte proliferation to retinal S-Ag. All graphs are from a representative experiments with five mice per group. n.s., not significant.

inflammation after immunization with IRBP at any dose (11, 12). To date, no cross-reactive antigen to IRBP has been identified. Although there are other retinoid-binding proteins in the body, they are structurally distinct from IRBP and are not known to be immunologically cross-reactive. A more plausible possibility appeared to be that T reg cells relevant to EAU might be elicited by component(s) of CFA, which is used to stimulate innate responses and promote the Th1 adaptive immunity needed for EAU induction, either through a mimic antigen or nonspecifically. We therefore examined whether T reg cells able to inhibit immunological and immunopathogenic responses to IRBP can be demonstrated in IRBP KO mice in the absence of CFA.
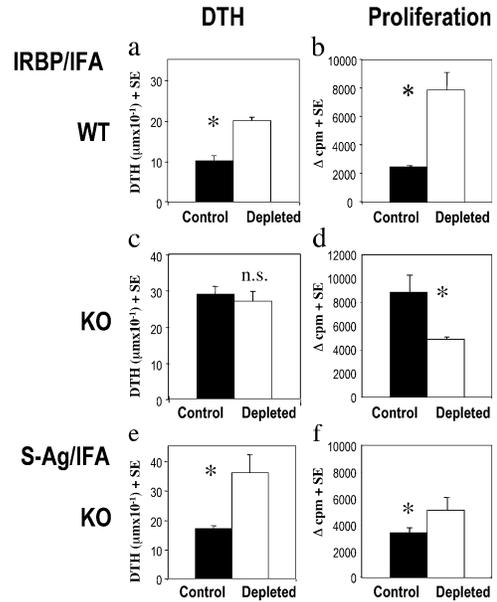
IRBP KO and WT mice were immunized with 150 μg IRBP in IFA as described in Materials and methods. This immunization regimen elicits robust immunological responses to IRBP but is not uveitogenic. When immunized with IRBP/IFA, only the depleted WT mice exhibited enhanced DTH and lymphocyte proliferative responses to IRBP, whereas the depleted KO mice had unchanged DTH and their proliferative response to IRBP, if anything, was even decreased (Fig. 3). These results suggested that IRBP-specific T reg cells were present only in WT mice. We interpret the reduced proliferative response to IRBP in CD25-depleted KO mice as elimination of IRBP-specific effector cells, which could be observed only in the absence of T reg cells, whose depletion would normally be dominant in effect and mask some parallel depletion of effector activity. Importantly, the defect in T reg cell generation appeared to be restricted only to IRBP, as IRBP KO mice generated antigen-specific T reg cells to retinal S-Ag (arrestin) (Fig. 3, e and f).

Although immunization with IRBP in IFA does not elicit EAU, and primed T cells from IRBP/IFA–immunized donors do not transfer disease, addition of recombinant IL-12 to the in vitro culture with IRBP can convert IRBP/IFA–primed T cells to a Th1-polarized, uveitogenic phenotype (13). This provided an approach to examine whether CD25 depletion permits enhanced generation of EAU-relevant effector T cells in IRBP KO mice in the absence of CFA.
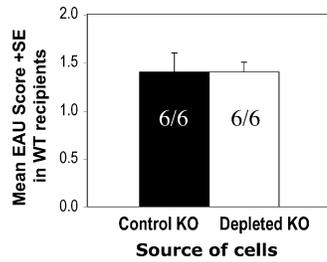
Figure 4. CD25 depletion does not enhance generation of IRBP–specific uveitogenic precursors in IRBP KO mice immunized with IRBP/IFA. Naive WT mice were infused with 5×10^6 activated lymphoid cells from IRBP KO mice that had been depleted or not of CD25$^+$ cells and immunized with IRBP/IFA. Donor cells were cultured before transfer with IRBP and 50 ng/ml IL-12 for 72 h. Funduscopy scores + standard error of WT recipients 14 d after adoptive transfer are shown. Combined data from two repeat experiments are shown.

We cultured spleen and lymph node cells from IRBP/IFA-immunized, CD25-depleted or nondepleted IRBP KO mice with IRBP and IL-12, and infused them into naive WT recipients. In contrast to the situation after IRBP/CFA immunization, the recipients did not develop enhanced EAU, again indicating that EAU-relevant T reg cells are not generated by IRBP KO mice in the absence of CFA (Fig. 4).

Only WT mice have transferable T reg cells that suppress development of IRBP-specific effector responses in IRBP KO recipients

To directly confirm absence of an IRBP-specific T reg cell population in IRBP KO mice, we infused IRBP KO recipients with T reg cells purified from either naive WT or naive KO donors, and immunized the recipients with IRBP in IFA. As predicted, only WT T reg cells, but not KO T reg cells, were able to significantly reduce the response to IRBP of recipient KO mice (Fig. 5, a and c). Lymphoid cells from these primary recipients were then transferred to WT secondary recipients (after in vitro activation with IRBP plus IL-12) to examine generation of EAU-relevant effector T cells. The IRBP-stimulated cells from primary recipients of WT T reg cells induced significantly less disease in the secondary recipients than did cells from primary recipients of KO T reg cells, or from KO donors that did not receive any T reg cells, indicating that WT T reg cells, but not KO T reg cells, were able to inhibit effector generation in the primary recipients (Fig. 5, b and d).

In the aggregate, these results support the notion that the EAU-relevant T reg cells generated by IRBP KO mice immunized in the presence of CFA are not IRBP specific. We consider the presence of a mimic antigen in CFA as unlikely because immunization of WT mice with CFA alone does not result in EAU (unpublished data). Our data do not distinguish whether the T reg cells in question recognize mycobacterial antigens or whether these are T reg cells of undetermined specificity directly activated through Toll-like receptors (which is our preferred interpretation). T reg cells that con-
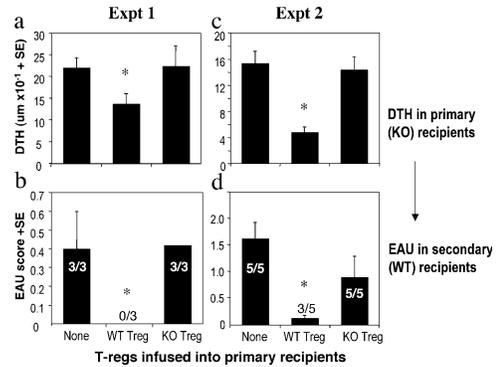

Figure 5. CD25$^+$ cells from WT mice, but not IRBP KO mice, suppress IRBP–specific immune responses in KO recipients immunized with IRBP/IFA. IRBP KO mice were infused with 2–2.5×10^6 CD4$^+$CD25$^+$ T reg cells from WT or KO donors and were immunized with IRBP/IFA on day 0. Immunological assays were performed as described in Materials and methods. Two repeat experiments are shown. (a and c) DTH responses; (b and d) EAU in secondary (WT) recipients of IRBP-stimulated cells from mice in panels a and c, respectively (funduscopy scores + standard error 14 d after adoptive transfer). *, P < 0.05.

trol responses to microbes as well as direct activation of T reg cells through Toll-like receptors have been demonstrated in other models (14, 15), so both mechanisms may be at play.

Although our study does not directly address the site of action of the T reg cells, we hypothesize that the CFA-elicited T reg cells, which lack their corresponding stimulus in the eye, might act primarily in the periphery to limit generation of effector T cells elicited by immunization. In contrast, IRBP-specific T reg cells (and perhaps T reg cells specific to other self-tissue components), which have a corresponding antigenic trigger in the eye, might also act to inhibit their action in the eye. These questions will be addressed in a separate study.

We speculate that our data could also help to explain the well-known but poorly understood protective effects of CFA and pertussis administered before induction of autoimmunity (16). It is conceivable that pretreatment with these bacterial components generates regulatory cells, which, upon reexposure to the same microbial stimulus in emulsion with antigen, inhibit generation of autopathogenic effector cells in a bystander fashion.

It is also important to emphasize that our data provide the first direct evidence of a requirement for the cognate antigen to generate specific T reg cells in a natural system comprising a native autoantigen and a diverse T cell repertoire. Thus far, evidence for this was wholly based on experiments in double transgenic mice expressing a T cell receptor and a neo–self-antigen, where both the effector and the regulatory cells bear the same T cell receptor (which is not what is believed to occur in the genetically unmanipulated animal) (17).

Conclusions
This study provides evidence that generation of T reg cells to IRBP, a native autoantigen in a normal mouse with a

diverse T cell repertoire, requires a cognate interaction. This contrasts with a lack of requirement for expression of this antigen to positively select effector T cells (11). Nevertheless, although endogenous expression of IRBP is needed to generate IRBP-specific T reg cells, EAU–relevant T reg cells do not have to be IRBP specific. We propose that these are T reg cells activated by microbial components present in CFA that inhibit development of IRBP-specific effector T cells in an antigen-nonspecific bystander fashion. This kind of mechanism could be relevant not only to autoimmune uveitis, but also to other autoimmune diseases in humans, which often are thought to be triggered by microbial infections, possibly combining a mimic antigen and an innate stimulus promoting tissue-destructive cellular immunity. Our data show that (a) pathogenic autoimmunity can be controlled in a bystander fashion by T reg cells that do not recognize the target antigen, and (b) that the same adjuvant effect needed to elicit EAU also activates T reg cells that help control the disease. Therefore, we propose to extend the concept of CD4$^+$CD25$^+$ T reg cells as a protective shield against autoimmunity that incorporates a broad basis, including self- as well as microbial components.

MATERIALS AND METHODS

Animals. B10.RIII mice (H-2^r) were purchased from The Jackson Laboratory. IRBP KO mice lacking the promotor region and 81% of the IRBP coding sequence were generated as described previously (12) and backcrossed onto the B10.RIII background for 10 generations. Mice were used between 8 and 26 wk of age. All experiments were approved by an Institutional Review Board.

Antigens and reagents. Native bovine IRBP was prepared as described previously (18–20). CFA was purchased from Difco and supplemented with additional *Mycobacterium tuberculosis* H37RA to 2.5 mg/ml. The monoclonal anti–CD25 antibody 7D4 (FITC-labeled) and anti–CD4 (PE-labeled) for flow cytometry were from BD Biosciences. Monoclonal PC61 anti–CD25 antibodies for in vivo use were produced in-house by collecting ascites from nude mice injected with the hybridoma cells, which were purchased from American Type Culture Collection. Bovine retinal arrestin (S-Ag) was provided by P. Hargrave and H. McDowell (University of Florida, Gainesville, FL).

Depletion and analysis of CD25$^+$ cells. To deplete CD25$^+$ cells, mice were given two i.p. injections, 3 d apart, of 1.0 mg of the anti–CD25 mAb PC61 (21). 7 d after the second injection, depletion was determined by flow cytometry in splenocytes of representative mice. At this time the mice were also immunized for EAU. Depletion reduced the CD4$^+$CD25$^+$ T cells in the spleen from ∼10% to well under 2% on gated CD4$^+$ cells with anti–CD25 mAb 7D4, which binds to a different epitope than PC61 on the IL-2 receptor (22).

Isolation and adoptive transfer of CD25$^+$ cells. CD25$^+$ T reg cells were isolated from lymph nodes (inguinal, iliac, axillary, mesenteric, and submandibular) of either naive WT or IRBP KO mice using anti–CD25–PE (clone 7D4) and anti–PE MicroBeads on an AutoMACS instrument (Miltenyi Biotec) according to manufacturer's instructions. The purity of isolated CD4$^+$CD25$^+$ T cells was >90% as determined by flow cytometry. Naive IRBP KO recipients were infused through the tail vein with 2–2.5 × 10^6 T reg cells from either naive WT or KO donors, or they received medium alone (no T reg cells). The recipients were then immunized with 150 μg IRBP emulsified in IFA. DTH was performed on day 12, and lymphoid cells were collected for adoptive transfer into WT secondary recipients as described below.

Immunizations, EAU induction, and EAU scoring. Groups consisted of three to five mice, depending on the availability of the IRBP KO animals. To elicit responses to IRBP, 150 μg bovine IRBP was emulsified in IFA 1:1 vol/vol for a total of 200 μl of emulsion injected subcutaneously, divided among both thighs and the base of the tail. This regimen elicits immunological responses but is not uveitogenic. To elicit responses to S-Ag, mice were immunized with 200 μg bovine S-Ag in IFA. To induce EAU, mice were immunized with 5 μg bovine IRBP emulsified in CFA supplemented with *M. tuberculosis* H37RA to 2.5 mg/ml. This IRBP dose was determined in prior experiments to elicit mild to moderate EAU scores that permitted disease enhancement after CD25 depletion in B10.RIII mice. Alternatively, EAU was induced by adoptive transfer of cells taken from IRBP-primed donors and cultured in vitro with IRBP as described previously (13). In brief, spleen and lymph node cells from IRBP-immunized donors were collected 12 d after immunization and stimulated in vitro with IRBP for 72 h. 5–10 million cultured cells were infused intraperitoneally into naive WT recipients. Disease scores were read in a masked fashion by funduscopy and confirmed by histopathology on eyes harvested 14 d after adoptive transfer, or 21 d after active immunization (approximately 9 d after onset). Scores were assigned on a scale from 0 (no inflammation) to 4 (complete destruction of the retina) in half-point increments as described previously (8).

Determination of immunological responses. IRBP- or S-Ag–specific DTH was determined by an ear swelling assay 12 d after immunization as described previously (23). In brief, 10 μg IRBP or 10 μg S-Ag in 10 μl PBS was injected into the right ear pinna, and 10 μl PBS alone was injected into the left ear pinna. After 48 h, the antigen-specific response was calculated by determining the difference in thickness (μm) between the antigen- and the PBS-injected ears using a spring-loaded micrometer (Mitutoya). For lymphocyte proliferation assay, spleens and draining lymph nodes (inguinal and iliac) were collected 12 d after immunization and pooled within the group. Proliferation was measured by [^{3}H]thymidine uptake during the last 16 h of a 72-h culture on triplicate cultures of 0.2 ml essentially as described previously (23). Lymphocytes of animals immunized with IRBP/CFA were stimulated with 10–30 μg IRBP, lymphocytes of mice immunized with IRBP/IFA were stimulated with 100 μg IRBP, and lymphocytes of animals immunized with S-Ag were stimulated with 30 μg S-Ag. Cytokine responses were determined using multiplex SearchLight Arrays technology (Endogen Chemical Co.; reference 24 and http://www.endogen.com/services).

Statistical analysis. All experiments were performed at least twice and were highly reproducible. Figures show data from representative experiments or from combined experiments as indicated. Statistical analysis of EAU scores was performed using the Snedecor and Cochran z test for linear trend in proportions (25). This is a nonparametric, frequency-based test that takes into account both disease severity and incidence. DTH, lymphocyte proliferation, and cytokine data were analyzed using an independent *t* test. Probability values of ≤0.05 were considered to be significant and are depicted as asterisks in the Figures.

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