Hyper Immunoglobulin E Response in Mice with Monoclonal Populations of B and T Lymphocytes✪

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Abstract

A key event in the pathogenesis of allergies is the production of antibodies of the immunoglobulin (Ig)E class. In normal individuals the levels of IgE are tightly regulated, as illustrated by the low serum IgE concentration. In addition, multiple immunizations are usually required to generate detectable IgE responses in normal experimental animals. To define the parameters that regulate IgE production in vivo, we generated mice bearing monoclonal populations of B and T lymphocytes specific for influenza virus hemagglutinin (HA) and chicken ovalbumin (OVA), respectively. A single immunization of the monoclonal mice with the cross-linked OVA-HA antigen led to serum IgE levels that reached 30–200 μg/ml. This unusually high IgE response was prevented by the infusion of regulatory α/β $CD4^+$ T cells belonging to both $CD25^+$ and $CD25^-$ subpopulations. The regulation by the infused T cells impeded the development of fully competent OVA-specific effector/memory Th2 lymphocytes without inhibiting the initial proliferative response of T cells or promoting activation-induced cell death. Our results indicate that hyper IgE responses do not occur in normal individuals due to the presence of regulatory T cells, and imply that the induction of regulatory $CD4^+$ T cells could be used for the prevention of atopy.

Key words: atopy • Th2 differentiation • immunoglobulin class switching • T lymphocytes, regulatory • mice, mutant strains

Introduction

High IgE antibody responses are a hallmark of atopic allergy and asthma (1), and have been described in other human diseases such as Omenn's syndrome (2, 3), hyper IgE syndrome (4), and AIDS (5). IgE production is tightly regulated, with serum concentrations about 1,000-fold lower than any other antibody class and plasma half-life measured in hours, not in days, as it is the case for IgG and IgM antibodies (6, 7). It is therefore possible that high IgE responses result from loss of immunoregulation (8). In fact, T lymphocyte abnormalities have been reported in a number of hyper IgE conditions. Omenn's syndrome, an immunodeficiency caused by defective recombinase activating genes (RAG)* (9), is characterized by oligoclonal T lymphocyte populations with a skewed Th2 phenotype, while a decrease in $CD4^+$ cell numbers is a hallmark of AIDS.

In vivo, IgE production is strongly dependent on T/B lymphocyte interactions (10). Studies of T cell class switching in vitro identified two essential signals for IgE class switching. A soluble signal is provided by the Th2 cytokine IL-4. Accordingly, IL-$4^{-/-}$ mice are highly deficient in IgE production (11). However, IL-$4^{-/-}$ mice can produce IgE upon infection with mouse acquired immunodeficiency syndrome (MAIDS) virus (12). In addition, IL-$4^{-/-}$ mice infected with *Plasmodium chabaudi* produced IgE, albeit at fivefold lower levels than IL-4^+ mice (13). Another Th2 cytokine that is relevant for IgE production is IL-13, whose receptor shares the IL-4Rα chain with the IL-4 receptor (14). In fact, IL-$13^{-/-}$ mice have been found to have a 3–5-fold lower basal levels of IgE than normal mice do (15).

IL-4 signaling induces germ line transcription of Cγ1 and Cε through the activation of signal transducer and acti-

✪The online version of this article contains supplemental material.

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**Abbreviations used in this paper:* CFSE, carboxyfluorescein diacetate; EAE, experimental autoimmune encephalomyelitis; ES, embryonic stem; HA, influenza virus hemagglutinin; IBD, inflammatory bowel disease; PNA, peanut agglutinin; RAG, recombinase activating gene.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2001/11/1349/11 $5.00
Volume 194, Number 9, November 5, 2001 1349–1359
http://www.jem.org/cgi/content/full/194/9/1349

vator of transcription (STAT)-6, a transcription factor that binds to cis-elements in the Cγ1 and Cε promoters (16). Unlike IgE, switching to IgG1 can still be induced, albeit at lower levels, during T cell–dependent responses in the absence of the IL-4/STAT6 pathway (17, 18).

During class switching to IgE, a second essential signal is delivered through cognate interaction between B lymphocytes and Th lymphocytes and is primarily mediated by the CD40 molecule expressed on B cells and its ligand, CD40L, expressed on activated T cells (19–21). CD40L and IL-4 synergize for the induction of germline Cγ1 and Cε transcripts (22). Mutations that disrupt CD40 or CD40L result in the failure of B lymphocytes to form germinal centers, to undergo class switching to IgG, IgE, and IgA, and to develop B cell memory (23–25). Both the IL-4 and CD40 mediated signals for IgE class switching can be provided by Th2 cells, with Th1 cells being unable to provide IL-4. Thus IgE responses in vivo are dependent on the polarization of the Th response.

Here we describe a model of immunization-induced hyper IgE in mice that carry monoclonal populations of T and B lymphocytes. We demonstrate that the monoclonality of the T cell repertoire is what determines the hyper IgE response, and that hyper IgE develops due to the absence of $CD4^+$ regulatory T lymphocytes. We also show that, in this model, regulatory T lymphocytes affect neither the initial proliferative expansion of antigen-specific T helper lymphocytes nor the subsequent apoptotic phase of the T helper response. Instead, regulatory lymphocytes block the differentiation of T helper cells into fully competent high IL-4–producing Th2 cells, causing a drastic reduction in class switching to IgE.

Materials and Methods

Mice. The rearranged V(D)J genes of the 17/9 antibody were cloned from the 17/9 hybridoma (26) and used to construct targeting vectors for the heavy and κ chain Ig loci. Targeting constructs containing a neo-loxP cassette were transfected into the J1 embryonic stem (ES) cell line and G418 resistant colonies carrying homologous integrations were identified by Southern analysis. Probes A, B, C, and D in Fig. 1 were used to characterize the 5′ and 3′ targeting sites. The neo-loxP cassette was removed by cre-mediated recombination. Mice carrying both rearranged receptor genes were generated by crosses between single targeted mice, and are referred to as 17/9 mice. All mice were backcrossed onto a BALB/c background and were housed under specific pathogen-free conditions at the Skirball Institute, New York University School of Medicine. All 17/9 DO11.10 $RAG^{-/-}$ animals in experiments that involved transfer of $CD4^+CD25^-$ splenocytes (Fig. 3 B), were given sulfamethoxazole (400 mg/liter)/trimethoprim (80 mg/liter) in the drinking water for the duration of the experiment. This treatment prevented wasting of mice transferred with $CD4^+CD25^-$ cells.

Immunizations. The influenza virus hemagglutinin (HA) peptide (YPYDVPDYASLRS) was synthesized by Research Genetics. A cross-linked OVA-HA antigen was prepared by glutaraldehyde cross-linking of the HA peptide to chicken OVA (Sigma-Aldrich). Mice were injected by intraperitoneal route with 100 μg of cross-linked OVA-HA adsorbed onto 1 mg of alum. A single immunization was performed in all experiments.

FACS® Analysis. Single cell suspensions in staining buffer (PBS containing 2% fetal calf serum and 0.1% NaN_3), were incubated for 45 min at 4°C with the antibody cocktails. Samples were analyzed in a FACSCalibur™ instrument (Becton Dickinson). Peanut agglutinin (PNA)-FITC and PNA-biotin were purchased from Vector Laboratories. Antibodies were purchased from BD PharMingen or Caltag. FITC-labeled and biotin-labeled HA peptides were purchased from Research Genetics.

Carboxyfluorescein Diacetate Labeling, Cell Purification, and Transfer. Total splenocytes were labeled with carboxyfluorescein diacetate (CFSE; Molecular Probes) by incubating 10^7 cells/ml in PBS with 5 μM of CFSE resuspended in DMSO. Labeling was stopped with media containing 10% fetal calf serum. Depletion of $CD4^+$, $CD8^+$, $B220^+$, and $CD25^+$ lymphocytes, and enrichment of $CD25^+$ and $CD4^+$ from spleen cell suspensions was performed by magnetic sorting using a Miltenyi VarioMACS apparatus. Cell purity was checked by FACS® analysis: CD8 depletion was >98% effective, whereas CD4 and B220 depletion were both >99% effective. Purified lymphocyte populations were obtained from spleen of wild-type BALB/c mice or BALB/c $\mu MT^{-/-}$ mice. The $CD25^+CD4^+$ purified fraction contained no more than 3% $CD4^+CD25^-$ cells. Depletion of $CD25^+$ cells was >98% effective. Cells were resuspended in PBS and injected intravenously.

ELISA. Total or HA-specific serum antibodies were quantified by ELISA. Purified and biotinylated antibodies to all Ig isotypes, and standard purified immunoglobulin isotypes were purchased from BD PharMingen or Caltag. Serum titer was defined as the lowest dilution that rendered an optical density value (OD450) higher than 0.1 after background subtraction. For the determination of HA-specific IgE, serum samples were preincubated with Gamma-bind Plus Sepharose (Amersham Pharmacia Biotech) to remove IgG and then added to HA-coated plates. IgE levels were subsequently determined using biotinylated anti-IgE monoclonal antibodies. While the titer of HA-specific IgE in 17/9 DO11.10 $RAG^{-/-}$ mice reached 10,000 on the third week after immunization, IgE titers in 17/9 DO11.10 RAG^+, 17/9 DO11.10 $RAG^{-/-}$ transferred with normal splenocytes, or unimmunized mice, were lower than 100.

Cytokine Production Ex Vivo. Cytokine production was analyzed ex vivo by intracellular staining. Briefly, spleen cells were stimulated with 1 μM OVA 323–339 peptide for 4 h or with PMA (10 ng/ml) plus Ionomycin (500 ng/ml) for 4 h. Cytokine secretion was subsequently inhibited by the addition of Monensin (Sigma-Aldrich) for 2 h. Cells were then harvested, surface stained with FITC-KJ1–26 and PerCP–anti-CD4 antibodies, permeabilized, stained with PE-labeled anti–IL-4 and APC-labeled anti–IFN-γ antibodies (BD PharMingen), and analyzed in a FACSCalibur™ (Becton Dickinson).

Online Supplemental Materials. Supplemental figures can be accessed at http://www.jem.org/cgi/content/full/194/9/1349/DC1. The supplementary data section includes the analysis of all immunoglobulin isotypes after immunization, the regulation of IgG1 production, and a comparative analysis of T and B cell activation in spleen and LNs.

Results

A Mouse Model of Hyper IgE. To monitor the induction and regulation of the IgE response we used mice that pro-

duce a high frequency of T and B lymphocytes specific for defined antigens. A new mouse strain which carries V(D)J replacements for the heavy and light chain genes of the monoclonal antibody 17/9 was generated by gene targeting (Fig. 1). This mouse strain, referred to as 17/9, harbors a large number of mature B lymphocytes specific for HA of the influenza virus. 17/9 DO11.10 $RAG^{-/-}$ mice were generated by crossing the 17/9 mice with the DO11.10 anti-OVA T cell receptor transgenic mice (27), and subsequently incorporating a RAG1 null mutation (28). 17/9 DO11.10 $RAG^{-/-}$ mice produce monoclonal populations of anti-HA B lymphocytes and anti-OVA $CD4^+$ T lymphocytes. In contrast, 17/9 DO11.10 RAG^+ mice harbor, in addition to the HA and OVA-specific lymphocytes, other B and T lymphocytes expressing antigen receptors encoded by the endogenous loci (Fig. 2 A). Immunization of the 17/9 DO11.10 $RAG^{-/-}$ and 17/9 DO11.10 RAG^+ mice with a cross-linked OVA-HA antigen induced a T cell–dependent antibody response in both groups of mice. However, the outcome of the immunization was very different in both groups, resulting in a two order of magnitude increase in total IgE levels in the 17/9 DO11.10 $RAG^{-/-}$ but not in the 17/9 DO11.10 RAG^+ mice (Fig. 2 B). The possibility that the observed differences reflected an impaired clearance of serum Igs in the $RAG^{-/-}$ strain rather than increased class switching was ruled out by the measurement of Ig half-life (online supplemental Figure S1). The production of HA-specific antibodies of all IgG isotypes was stimulated in mice of both strains by the immunization (online supplemental Figure S2). However, serum titers for HA-specific IgGs were higher in the 17/9 DO11.10 $RAG^{-/-}$ strain than in the RAG^+ strain. Among HA-specific IgGs, the largest difference was in IgG1, which reached titers ~20 fold higher in 17/9 DO11.10 $RAG^{-/-}$ mice than in their RAG^+ littermates (Fig. 2 C).

Monoclonality of the T Cell Compartment Determines the Hyper IgE Response. The unusually high levels of IgE displayed by 17/9 DO11.10 $RAG^{-/-}$ mice suggested that these mice lack a normal mechanism for the downregulation of IgE responses. As 17/9 DO11.10 RAG^+ mice, which do not develop hyper IgE, harbor both T and B cells expressing endogenous receptors, it was possible that either one or both of these lymphocyte populations were important to prevent hyper IgE responses.

We investigated the role of T cells expressing endogenous receptors by using an adoptive transfer system. HA-specific monoclonal B cells from 17/9 $RAG^{-/-}$ mice, which lack T cells, were transferred into two groups of B cell-deficient recipients, DO11.10 $RAG^{-/-}$ mice and DO11.10 $\mu MT^{-/-}$ mice. DO11.10 $RAG^{-/-}$ mice generate exclusively OVA-specific T cells, whereas DO11.10 $\mu MT^{-/-}$ mice generate both OVA-specific T cells and other T cells expressing endogenous T cell receptors. Transfers of HA-specific B cells were performed on day 3 postimmunization to ensure that the initial T helper cell activation occurred in the absence of any B cells. Monoclonal HA-specific B cells were also transferred into immunized DO11.10 $RAG^+\mu MT^+$ mice to determine the effect of normal B cells on the IgE response. Fig. 2 D shows that only DO11.10 $RAG^{-/-}$ recipient mice developed hyper IgE, whereas IgE levels were 70- and 200-fold lower in DO11.10 $\mu MT^{-/-}$ and DO11.10 $RAG^+\mu MT^+$ mice re-

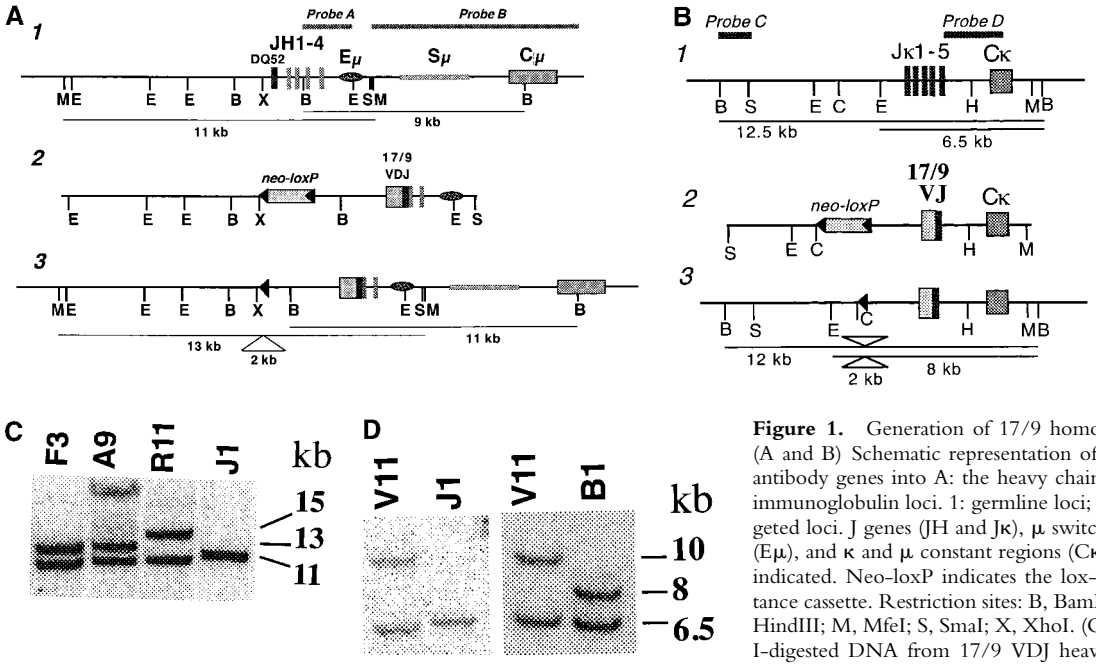

Figure 1. Generation of 17/9 homologous replacement mice. (A and B) Schematic representation of the targeting of the 17/9 antibody genes into A: the heavy chain, and B: the κ light chain immunoglobulin loci. 1: germline loci; 2: targeting vectors; 3: targeted loci. J genes (JH and Jκ), μ switch region (Sμ), μ enhancer (Eμ), and κ and μ constant regions (Cκ and Cμ, respectively) are indicated. Neo-loxP indicates the lox-P flanked neomycin resistance cassette. Restriction sites: B, BamHI; C, Cla I; E, EcoRI; H, HindIII; M, MfeI; S, SmaI; X, XhoI. (C) Southern analysis of Mfe I-digested DNA from 17/9 VDJ heavy chain gene targeted ES clones before (R11 clone) and after (F3 and A9 clones) cre-mediated deletion of the neo-loxP cassette. The filter was hybridized to probe A in panel A. DNA from the untargeted J1 ES cell line is also shown. (D) Southern analysis of VJ 17/9 κ light chain targeted ES clones. BamHI/EcoRI double-digested DNA was hybridized to probe D in B. Targeted clones before (V11) and after (B1) deletion of the neo-loxP cassette. J1: untargeted ES cell line.

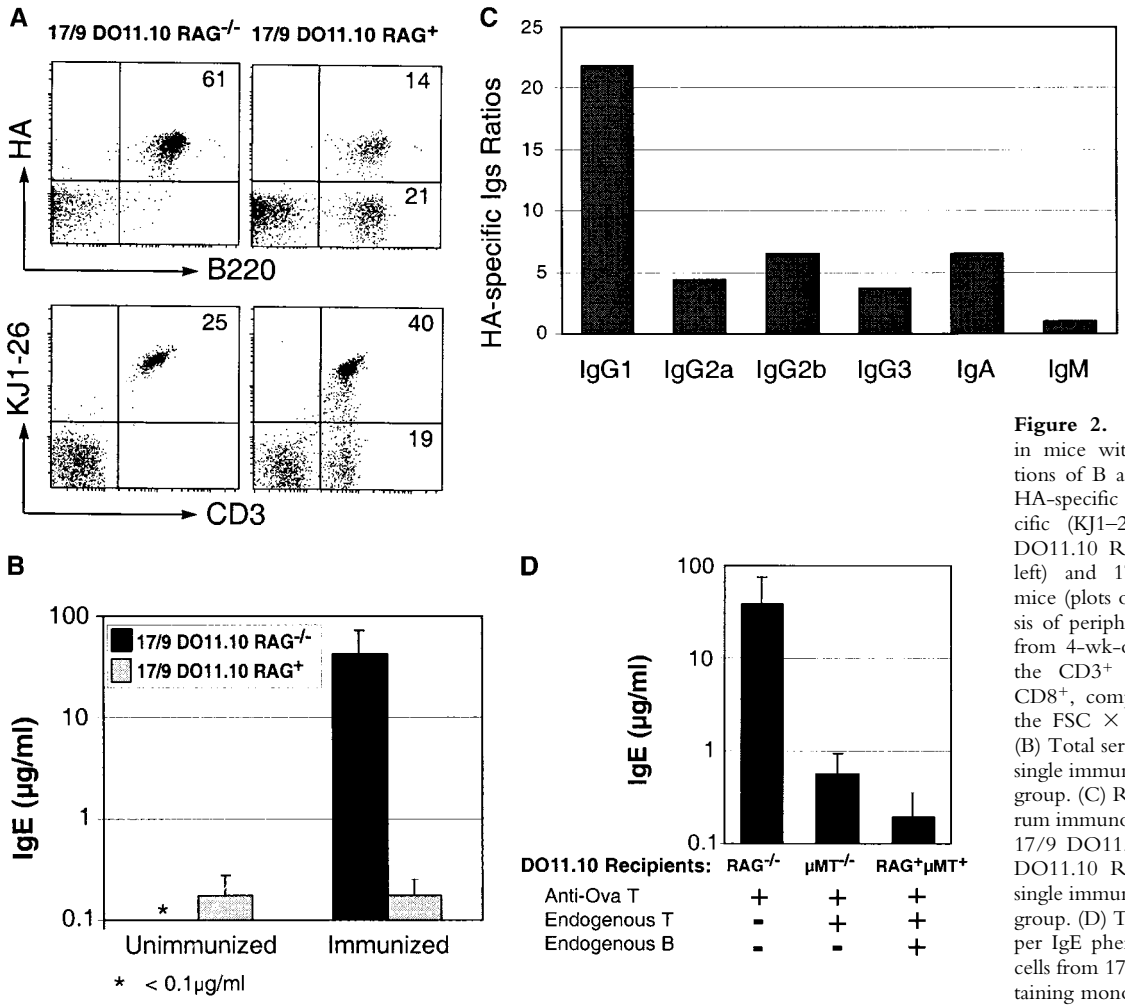

Figure 2. Hyper IgE production in mice with monoclonal populations of B and T lymphocytes. (A) HA-specific B cells and OVA-specific (KJ1–26$^+$) T cells in 17/9 DO11.10 RAG$^{-/-}$ mice (plots on left) and 17/9 DO11.10 RAG$^+$ mice (plots on right). FACS® analysis of peripheral blood lymphocytes from 4-wk-old mice. Only 10% of the CD3$^+$ KJ1–26$^-$ T cells are CD8$^+$, comprising less than 2% of the FSC × SSC lymphocyte gate. (B) Total serum IgE levels 14 d after single immunization. $n = 7$ mice per group. (C) Ratios of HA-specific serum immunoglobulin titers in sera of 17/9 DO11.10 RAG$^{-/-}$ and 17/9 DO11.10 RAG$^+$ mice 14 d after single immunization. $n = 7$ mice per group. (D) T cells determine the hyper IgE phenotype. 5×10^6 spleen cells from 17/9 RAG$^{-/-}$ mice (containing monoclonal anti-HA B cells) were transferred into DO11.10 RAG$^{-/-}$ mice (left bar), DO11.10 µMT$^{-/-}$ mice (middle bar), and DO11.10 RAG$^+$µMT$^+$ mice (right bar) 3 d after single immunization. The graphic shows total IgE levels in sera of mice on day 14 after immunization. $n = 3$ to 4 mice per group.

spectively. These results demonstrate that the repertoire of the T cell compartment has the major effect on the magnitude of the IgE response.

It has been shown that optimal levels of T cell priming by antigen-presenting B cells require the involvement of antigen-specific B cells (29). B cell receptor–mediated uptake of antigen leads to a rapid upregulation of the expression of B7–2, thereby enhancing T cell stimulation (30). Although the monoclonality of the T cell compartment determined the hyper IgE response, it is, however, likely that the high number of HA-specific B cells contributed to the stimulation of OVA-specific T cells induced by immunization with the cross-linked OVA-HA antigen.

CD4$^+$ α/β T Cells Downregulate the IgE Response. We subsequently tested whether lymphocytes from normal donors would also display regulatory activity upon transfer into 17/9 DO11.10 RAG$^{-/-}$ recipients. In this experiment, the responding OVA-specific T cells are identical in both transferred and nontransferred experimental groups. As the OVA-specific T cell populations of 17/9 DO11.10 RAG$^{-/-}$ and 17/9 DO11.10 RAG$^+$ mice may differ in number and/or TCR expression levels, the use of the transfer system overcame this potential problem. Total spleen cells from wild-type mice, TCR α$^{-/-}$ mice (which lack mature α/β T cells [31]), or normal splenocytes depleted of CD4$^+$, CD8$^+$, or B220$^+$ lymphocytes, were transferred into 17/9 DO11.10 RAG$^{-/-}$ mice 24 h before immunization. The analysis of the IgE response showed that the regulatory activity of normal splenocytes is absent in TCR α$^{-/-}$ spleen cells and in CD4$^+$ T cell–depleted normal spleen cells (Fig. 3 A). Thus, CD4$^+$ α/β T cells are essential for the control of hyper IgE by normal splenocytes. Neither CD8$^+$ T cells nor B cells were necessary for this regulation. Furthermore, by transferring cells from BALB/c IFN-γ$^{-/-}$ and BALB/c IL-4$^{-/-}$ mice, we were able to establish that the downregulation of the IgE response was only marginally dependent on IFN-γ and was independent of IL-4 (online supplemental Figure S3 c).

A subpopulation of CD4$^+$ T cells which expresses CD25 (the α chain of the IL-2 receptor) displays downregulatory activity in some autoimmune diseases and tumor rejection responses (32–34). To investigate whether

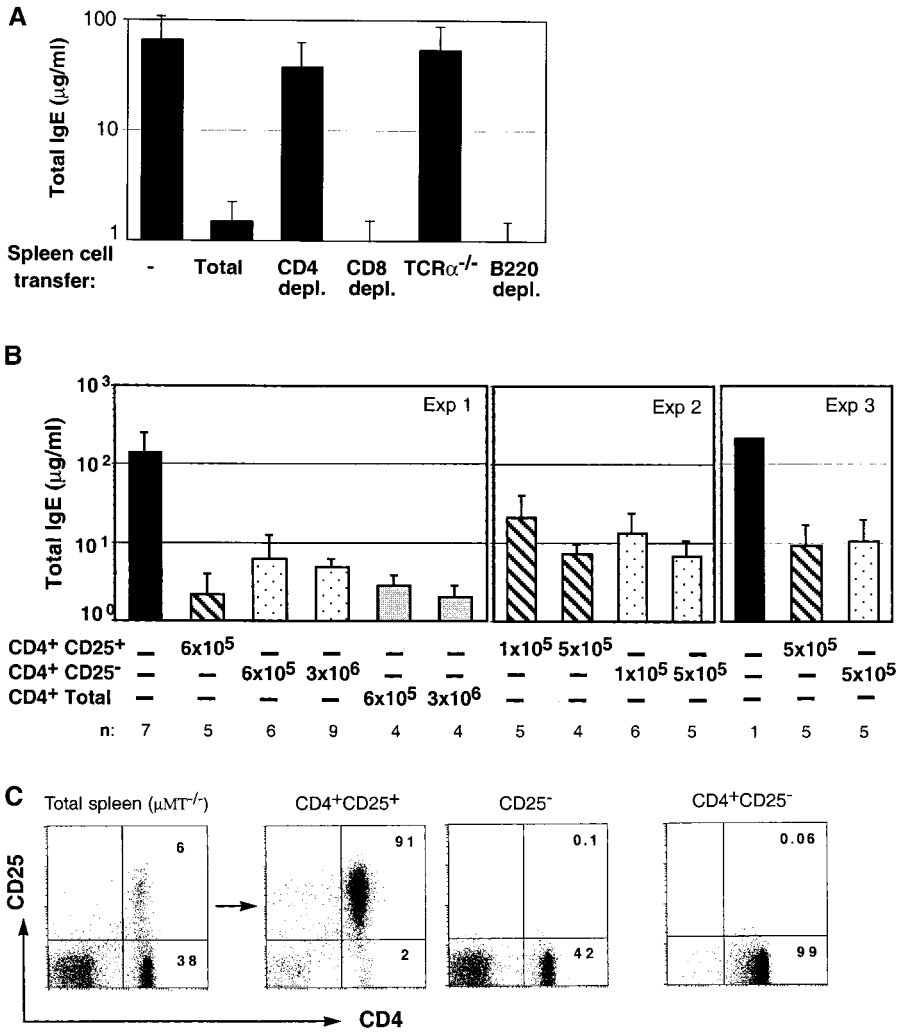

Figure 3. Development of hyper IgE can be prevented by normal TCR α/β^+CD4^+ splenocytes. (A) Total (2.5×10^7) or fractionated spleen cells (CD4-depleted, CD8-depleted, B220-depleted: 2×10^7) from wild-type BALB/c mice or total spleen cells from TCR $\alpha^{-/-}$ mice (2×10^7) were transferred into 17/9 DO11.10 $RAG^{-/-}$ recipients 1 d before immunization. Total IgE serum levels were determined 14 d after immunization. $n = 6$–8 mice per group. (B) Purified spleen cells populations obtained from wild-type BALB/c mice or BALB/c $\mu MT^{-/-}$ mice were transferred to 17/9 DO11.10 $RAG^{-/-}$ recipients one day before immunization. Serum IgE levels were determined 19 d after immunization. Exp 1: three cell populations were obtained from wild-type BALB/c donors and transferred: (1) purified $CD4^+CD25^+$ spleen cells (6×10^5); (2) spleen cells depleted of $B220^+$, $CD8^+$, and $CD25^+$ lymphocytes (6×10^5 or 3×10^6 $CD4^+CD25^-$ cells transferred); (3) spleen cells depleted of $B220^+$ and $CD8^+$ lymphocytes (6×10^5 or 3×10^6 total $CD4^+$ T cells transferred). Exp 2: purified $CD4^+CD25^+$ spleen cells, and spleen cells depleted of $CD25^+$ cells were obtained from BALB/c $\mu MT^{-/-}$ donors and transferred in doses of 5×10^5 or 10^5 $CD4^+$ T cells. Exp 3: $CD4^+CD25^+$ and $CD4^+CD25^-$ donor spleen cells were obtained from BALB/c $\mu MT^{-/-}$ donors. To purify $CD4^+CD25^-$ cells, spleen cells were first depleted of $CD25^+$ cells. Subsequently, $CD4^+$ cells were positively sorted. (C) Typical FACS® profiles of purified donor populations are shown. Dot plots contain the profiles of cells in a broad FSC/SSC lymphocyte gate. The three plots on the left show donor cells from Exp 2. The plot on the right shows purified $CD4^+CD25^-$ cells from Exp 3.

$CD4^+CD25^+$ T lymphocytes could reduce the hyper IgE response, splenocytes were harvested from wild-type BALB/c mice or BALB/c $\mu MT^{-/-}$ mice, and separated into fractions containing $CD4^+CD25^+$ T cells, $CD4^+$ $CD25^-$ T cells, or total $CD4^+$ T cells. Spleen cell fractions containing equal numbers of $CD4^+$ T cells were then transferred into 17/9 DO11.10 $RAG^{-/-}$ recipients one day before immunization. The results of three experiments (Fig. 3 B) demonstrated that the transfer of total $CD4^+$, $CD4^+CD25^+$, or $CD4^+CD25^-$ spleen populations prevented the development of hyper IgE in 17/9 DO11.10 $RAG^{-/-}$ recipient mice. Although there was some variation in IgE levels between experiments, it is clear that both $CD4^+CD25^+$ and $CD4^+CD25^-$ splenic populations contain T lymphocytes capable of downregulating the HA-specific IgE response following similar dose–response curves (Fig. 3 B).

In all experiments described, HA-specific IgG1 serum levels were analyzed and found to be regulated in the same way as IgE, however, the extent of IgG1 downregulation was less marked than the IgE downregulation found in the same mice (online supplemental Figure S3).

Regulation by T Cells Does Not Inhibit Early Antigen-specific T Cell Activation. Our next experiments aimed to determine which phase of the immune response was affected by regulatory lymphocytes. As T cells determine the hyper IgE phenotype (Figs. 2 and 3), and T cell activation precedes B cell activation in T cell–dependent responses (35, 36), we hypothesized that regulation would primarily affect the responding T helper lymphocytes. In T cell–dependent responses antigen-specific T lymphocytes undergo an initial proliferative burst that is followed by a dramatic decrease in cell numbers believed to represent activation-induced T cell death (AICD). Some T cells emerging from this initial burst migrate to the follicles, participate in the germinal center reaction, and develop into memory cells (35). We wished to determine whether the mechanism of action of regulatory lymphocytes involved the inhibition of the initial proliferative phase of the T helper response, the stimulation of the apoptotic phase of the Th response, and/or an impaired generation of effector/memory Th2 lymphocytes.

Early T cell activation and proliferation was assessed by the counting of OVA-specific T cells, the determination of cell size, the expression of early activation markers such as

CD25 and CD69, and the rate of cell division. Fig. 4 A shows a kinetic analysis of the numbers of splenic OVA-specific (KJ1–26$^+$CD4$^+$) T cells in immunized 17/9 DO11.10 RAG$^{-/-}$ mice, 17/9 DO11.10 RAG$^+$ and 17/9 DO11.10 RAG$^{-/-}$ mice transferred with normal splenocytes (cell numbers of unimmunized mice shown at time point zero). Expansion of the anti-OVA T cells was similar in the three groups, with cell numbers peaking around days 2 and 3 d after immunization and declining thereafter. Likewise, the kinetics of upregulation and downregulation of CD25 and CD69 expression in spleen cells from 17/9 DO11.10 RAG$^{-/-}$ mice and 17/9 DO11.10 RAG$^{-/-}$ mice transferred with normal splenocytes showed no differences. Most OVA-specific T cells upregulated CD25 and CD69 expression levels at 15 h after immunization in both groups (Fig. 4 B, and online supplemental Figure S4). Similar kinetics of expansion/contraction in the number of OVA-specific T cells and expression of activation markers were observed after analysis of lymph node cells (online supplemental Figure S5).

The analysis of cell division profiles of a trace population of CFSE-labeled OVA-specific T cells also did not reveal differences between 17/9 DO11.10 RAG$^{-/-}$ mice and 17/9 DO11.10 RAG$^{-/-}$ mice transferred with normal splenocytes (Fig. 4 C). Interestingly, the proportion of cells in the high CFSE peaks (low cell division number) increased from the 3rd to the 5th day after immunization suggesting that T cell death in the low CFSE (high cell division) peaks was taking place. As the transferred OVA-specific T cells could not be distinguished from the endogenous ones after they became CFSE negative, differences due to a small population of dividing cells at later time points could not be determined.

These results demonstrate that a massive activation of OVA-specific T cells takes place in mice that develop hyper IgE as well as in mice that efficiently control this response, indicating that the hyper IgE phenotype is not due to large differences in the magnitude of the initial T helper response. The fact that expansion was followed by a contraction of the antigen-specific T cell population in all immunized groups indicates in addition that the hyper IgE phenotype is not caused by a defect in AICD in the 17/9 DO11.10 RAG$^{-/-}$ mice.

Regulatory Cells Inhibit the Differentiation of Th2 Lymphocytes. We next investigated whether the formation of effector/memory T helper cells was affected by regulatory lymphocytes. As effector/memory T helper cells are characterized by a fast and intense production of cytokines after stimulation (37), we analyzed the production of IL-4 and IFN-γ by OVA-specific T cells from immunized 17/9 DO11.10 mice ex vivo by intracellular staining. As shown in Fig. 5 A, IL-4$^+$IFN-γ$^-$ T cells were virtually undetectable in 17/9 DO11.10 RAG$^{-/-}$ unimmunized mice, were present in low numbers 3 d after immunization and

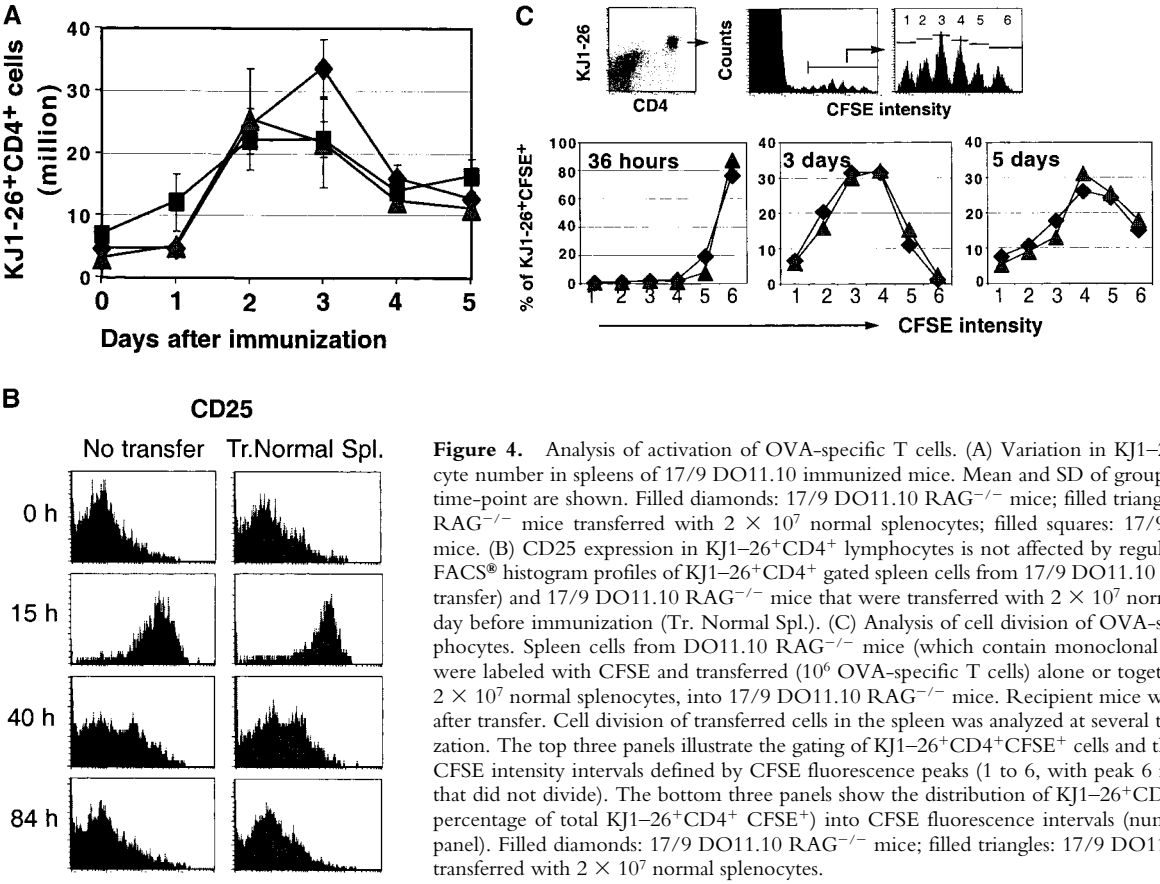

Figure 4. Analysis of activation of OVA-specific T cells. (A) Variation in KJ1–26$^+$CD4$^+$ lymphocyte number in spleens of 17/9 DO11.10 immunized mice. Mean and SD of groups of three mice per time-point are shown. Filled diamonds: 17/9 DO11.10 RAG$^{-/-}$ mice; filled triangles: 17/9 DO11.10 RAG$^{-/-}$ mice transferred with 2×10^7 normal splenocytes; filled squares: 17/9 DO11.10 RAG$^+$ mice. (B) CD25 expression in KJ1–26$^+$CD4$^+$ lymphocytes is not affected by regulatory lymphocytes. FACS® histogram profiles of KJ1–26$^+$CD4$^+$ gated spleen cells from 17/9 DO11.10 RAG$^{-/-}$ mice (No transfer) and 17/9 DO11.10 RAG$^{-/-}$ mice that were transferred with 2×10^7 normal spleen cells one day before immunization (Tr. Normal Spl.). (C) Analysis of cell division of OVA-specific CD4$^+$ lymphocytes. Spleen cells from DO11.10 RAG$^{-/-}$ mice (which contain monoclonal anti-OVA T cells) were labeled with CFSE and transferred (10^6 OVA-specific T cells) alone or together with unlabeled 2×10^7 normal splenocytes, into 17/9 DO11.10 RAG$^{-/-}$ mice. Recipient mice were immunized 1 d after transfer. Cell division of transferred cells in the spleen was analyzed at several times after immunization. The top three panels illustrate the gating of KJ1–26$^+$CD4$^+$CFSE$^+$ cells and the determination of CFSE intensity intervals defined by CFSE fluorescence peaks (1 to 6, with peak 6 representing T cells that did not divide). The bottom three panels show the distribution of KJ1–26$^+$CD4$^+$ CFSE$^+$ cells (as percentage of total KJ1–26$^+$CD4$^+$ CFSE$^+$) into CFSE fluorescence intervals (numbers 1 to 6 in top panel). Filled diamonds: 17/9 DO11.10 RAG$^{-/-}$ mice; filled triangles: 17/9 DO11.10 RAG$^{-/-}$ mice transferred with 2×10^7 normal splenocytes.

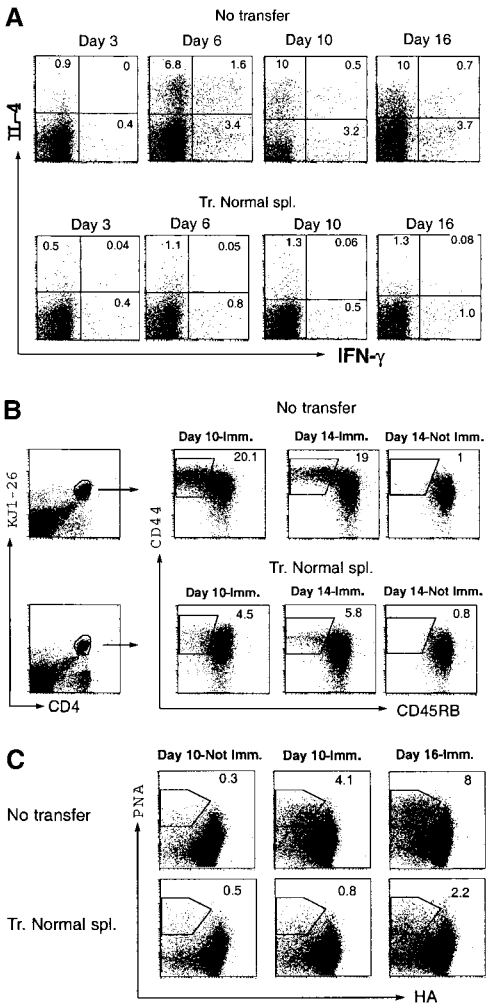

Figure 5. Effector/memory Th2 development and germinal center formation are inhibited by regulatory lymphocytes. (A) Cytokine production by OVA-specific T cells after immunization. Spleen cells from 17/9 DO11.10 $RAG^{-/-}$ immunized mice (No transfer) or 17/9 DO11.10 $RAG^{-/-}$ mice that were transferred with 2×10^7 normal spleen cells one day before immunization (Tr. Normal Spl.) were analyzed for the production of IL-4 and IFN-γ by intracellular staining. Cells were stimulated in vitro with OVA peptide and processed as described in Materials and Methods. The graphic shows anti–IL-4 and anti–IFN-γ plots of $KJ1\text{–}26^+CD4^+$ gated spleen cells. Unimmunized mice display less than 0.1% of $KJ1\text{–}26^+CD4^+$ positive cells with either anti–IL-4 or anti–IFN-γ antibodies and no detectable population of $IL\text{-}4^+IFN\text{-}\gamma^+$ double-positive cells. (B) CD44/CD45RB staining of $KJ1\text{–}26^+CD4^+$ gated spleen cells from 17/9 DO11.10 $RAG^{-/-}$ immunized mice (No transfer) or 17/9 DO11.10 $RAG^{-/-}$ mice that were transferred with 2×10^7 normal spleen cells one day before immunization (Tr. Normal Spl.). Unimmunized controls analyzed simultaneously as day 14 samples are also shown. (C) Decreased number of PNA^+ cells in 17/9D011.10 $RAG^{-/-}$ mice transferred with normal splenocytes. PNA/HA staining is shown for $B220^+$ gated splenocytes from 17/9 DO11.10 $RAG^{-/-}$ mice (No transfer) and from 17/9 DO11.10 $RAG^{-/-}$ mice that were transferred with 2×10^7 normal spleen cells (Tr. Normal Spl.). Unimmunized controls analyzed simultaneously with the 10-d immunization samples are also shown. As expected, germinal center B cells showed downregulation of the B cell receptor.

reached up to 10% of the OVA-specific ($KJ1\text{–}26^+$) $CD4^+$ splenic cells by day 10 after immunization. $IFN\text{-}\gamma^+IL\text{-}4^-$ cells also increased but to a lesser degree (Fig. 5 A). Strikingly, cells from 17/9 DO11.10 $RAG^{-/-}$ mice transferred with normal splenocytes never developed into high cytokine producers, indicating that the mechanism of regulation does not involve immune deviation to a Th1 phenotype. The intracellular staining data was confirmed by ELISA as well as quantitative competitive RT-PCR (online supplemental Figure S6).

The production of IL-4 and IFN-γ by donor-derived T cells in 17/9 DO11.10 $RAG^{-/-}$ mice transferred with wild-type splenocytes was analyzed by intracellular staining. The percentage of $IL\text{-}4^+IFN\text{-}\gamma^-$ cells in the $KJ1\text{–}26^-$ T cell population was below 0.5% at all time points analyzed (days 3, 6, and 10 after immunization), whereas the percentage of $IFN\text{-}\gamma^+IL\text{-}4^-$ cells in the same population was below 1.1%. The frequency of $IL\text{-}4^+IFN\text{-}\gamma^+$ double positive cells was always below 0.1% (online supplemental Figure S7).

The large increase in the production of cytokines, particularly IL-4, by OVA-specific T cells from 17/9 DO11.10 $RAG^{-/-}$ mice took place after the initial proliferative-apoptotic phases of the response, and coincided with the appearance of substantial numbers of OVA-specific T cells expressing high levels of the surface molecule CD44 and low levels of CD45RB (Fig. 5 B). Upregulation of CD44 and downregulation of CD45RB have been described in cells with effector/memory phenotype (37). Another example documenting that differentiation to high cytokine-producing cells is related to changes in T cell marker expression has been shown for CD45RB (38). Accordingly, the $CD44^{hi}CD45RB^{lo}$ population was considerably reduced in 17/9 DO11.10 $RAG^{-/-}$ mice transferred with normal splenocytes (Fig. 5 B). Taken together, the results of the experiments shown in Fig. 5 demonstrate that the differentiation of effector/memory T helper lymphocytes is greatly impaired in the presence of regulatory T lymphocytes.

Regulatory Cells Inhibit Germinal Center Formation. A substantial proportion of T cell–dependent Ig class switching occurs in specialized lymphoid follicles named germinal centers (39, 40). To monitor germinal center formation, we stained spleen cells with PNA, a lectin that binds to B cells located exclusively in germinal centers (41). Fig. 5 C shows that while the presence of PNA^+ B lymphocytes was evident in 17/9 DO11.10 $RAG^{-/-}$ mice 10 d after immunization, the number of PNA^+ B cells in 17/9 DO11.10 $RAG^{-/-}$ mice transferred with normal splenocytes was significantly lower. The reduction in germinal center formation was probably secondary to the inhibition of Th2 differentiation, as it has been shown that Th2 cells are necessary to mediate the germinal center reaction (42).

Discussion

We described here that mice displaying a monoclonal T and B cell repertoire developed a very high antigen-specific IgE response, with serum levels in the range of 30–200 μg/ml upon a single immunization with the cognate antigen. In this model, the genetic background of the mice, as well

as the adjuvant and route of immunization favored the differentiation of Th2 cells. In addition, the genetic ablation of regulatory T cells in the 17/9 DO11.10 $RAG^{-/-}$ mice provided an unobstructed environment for T helper cell differentiation to occur. Cognate help from differentiated Th2 cells in turn allows a high rate of B lymphocyte class switching to IgE. We also showed here that regulation by T cells affects the development of fully competent effector/memory Th2 lymphocytes without inhibiting the initial proliferative response of T cells and without acceleration of activation-induced cell death.

It has been amply documented that antigen-specific IgE responses can be downregulated by transfer of T cells (43–48). The main difference between our data and the previously published data is that in all of the referred manuscripts, antigen priming of the donor animals (or cells) was absolutely essential for the downregulation of IgE response, whereas in our case highly effective downregulation of IgE response was attained by T cells obtained from unimmunized wild-type donors. This discrepancy probably reflects the different phases of the T cell–dependent B cell response at which the different types of regulatory cells act. The regulatory activity that we described here displays its effect relatively early in the response, by drastically impairing helper T cell differentiation. This type of regulation was present in the recipients of antigen-primed regulatory T cells described by the other authors, since the recipient mice had a normal $CD4^+$ compartment. Thus, no additional regulation (no effect on the IgE response) was observed when unprimed T cells were transferred. Different aspects of a multilayer T cell regulation of IgE responses are, therefore, uncovered in different experimental models. In 17/9 DO11.10 $RAG^{-/-}$ mice, there are no regulatory T cells of any kind, and this is likely the reason why the IgE response reaches hyper IgE levels after a single immunization.

Our results clearly show that regulatory T cells do not prevent hyper IgE production by inducing immune deviation of the naive OVA-specific T cells to a Th1 phenotype, as OVA-specific T cells in mice transferred with wild-type splenocytes did not become IFN-γ producers. Moreover, both Th1 and Th2 responses were decreased by the infused regulatory cells (Fig. 5). In view of the abundance of data indicating the role of TGF-β (49–55) and IL-10 (48, 56–61) in the control of both Th1 and Th2 responses, it is likely that these cytokines are also involved in the downregulation of hyper IgE responses in our experimental system.

We described in this manuscript that regulatory α/β $CD4^+$ T cells displaying both $CD25^+$ and $CD25^-$ phenotype can prevent the hyper IgE response upon transfer into 17/9 DO11.10 $RAG^{-/-}$ mice. A population of T cells which coexpresses CD4 and CD25 has been shown to prevent the development of several autoimmune diseases and inflammatory bowel disease (IBD) (32, 33, 58, 62, 63). While the $CD4^+CD25^-$ population was initially considered to be devoid of regulatory cells, more recent work has challenged this perception. Thus, it was mentioned that $CD25^-$ T cells confer protection against experimental autoimmune encephalomyelitis (EAE) (64), and it was shown that $CD4^+CD25^-$ T cells can prevent diabetes (34) and IBD (58).

In our hyper IgE model, $CD25^+$ T cells were clearly protective. To assess the regulatory potential of $CD25^-$ T cells, recipient mice were kept under wide spectrum antibacterial/antifungal prophylaxis to prevent wasting disease. Under these conditions, both $CD25^+$ and $CD25^-$ T cells effected similar regulatory activity on the hyper IgE response. The downregulation of the IgE response by total splenocytes is, therefore, the result of combined effects of the $CD25^+$ and $CD25^-$ regulatory T cells. The differences between experimental systems may be explained in the following way. The $CD4^+CD25^-$ population contains Th precursors which are effective in causing IBD and related inflammatory diseases without the need for immunization, but does not contain a sizable number of OVA-specific Th cell precursors capable of driving a hyper IgE response upon a single immunization of the recipient mice. Thus, the contribution of the $CD4^+CD25^-$ donor-derived population to the B cell help is minimal in comparison to the large number of OVA-specific T cells present in 17/9 DO11.10 $RAG^{-/-}$ mice. In situations where the $CD25^-$ cells do not play a major effector role, such as in the 17/9 DO11.10 $RAG^{-/-}$ mice, the regulatory capacity of the $CD4^+CD25^-$ population can be easily appreciated. A similar argument can be put forward to explain the protective effect of $CD4^+CD25^-$ T cells in a TCR transgenic model of spontaneous EAE (64). Moreover, in the other publications in which $CD4^+CD25^-$ T cells were shown to display regulatory activity, the number of effector cell precursors present in the $CD25^-$ population was reduced either by depletion of recent thymic emigrants (34) or by using $CD4^+CD25^-$ cells from the $CD45RB^{lo}$ population (58).

The antigen specificity of the regulatory T cells that prevent hyper IgE responses remains a major unresolved issue. The relationship between the regulatory T cells which prevent hyper IgE and the regulatory T cells which prevent autoimmune diseases and graft rejection is also unclear. There are, however, remarkable similarities between the hyper IgE system described here and the spontaneous autoimmunity system that we described some years ago (65). In the latter system, autoimmune encephalomyelitis develops spontaneously in mice harboring a monoclonal self-specific T cell repertoire (TCR transgenic $RAG^{-/-}$), but not in TCR transgenic RAG^+ mice. In addition, a small number of polyclonal $CD4^+$ T cells (both $CD25^+$ or $CD25^-$) can suppress the development of spontaneous disease (64–66). There are also important differences, such as the need for immunization in order to obtain a hyper IgE response, the genetic background (C57BL versus BALB/c), and the nature of the response (Th1 in EAE and Th2 in the hyper IgE).

Hyper IgE is often associated with human immunodeficiencies such as the hyper IgE syndrome (4), Omenn's syndrome (3), and AIDS (5). Abnormalities in the T cell compartment have been described in all these diseases. Given the data shown herein, it is possible that the hyper IgE observed in patients afflicted with these immunodeficiencies is

caused by a marked deficiency in the regulatory T cell compartment. Similarly, high and sustained IgE responses have been observed in rats immunized after sublethal irradiation, treatment which causes a temporary lymphopenia (67). Our results may also help explain why the levels of autoantibodies belonging to T cell–dependent classes, in particular IgG1 and IgE, are increased in TCR $\alpha^{-/-}$ mice (68). While some T helper functions in these mice can be performed by γ/δ T cells (69) or $CD4^+$ T cells expressing the TCR β chain (70), regulatory T cells appear to be strictly α/β T cells and are thus absent in TCR $\alpha^{-/-}$ mice.

Atopy is a human condition of multigenic origin which is characterized by the predisposition to produce IgE against environmental, noninfectious antigens. The MHC, the IL-4/IL-13/IL-5/IL-9 cytokine cluster, IL-4 receptor, and the FcϵRI-β chain have all been implicated in the susceptibility to atopy and asthma in a variety of human or mouse studies (71). Based upon our results, we speculate that another group of susceptibility genes, perhaps shared between individuals afflicted with atopic or autoimmune diseases, may affect the downregulation of immune responses mediated by regulatory T cells. Interestingly, a negative correlation between atopic condition and the incidence/severity of certain cancers has been reported (72–75). As the experimental depletion of $CD25^+$ regulatory T cells in mice inoculated with syngeneic tumors leads to increased killer activity, decreased tumor size and reduced cancer-related death (76, 77), it is possible that the level of regulatory T cell activity of each individual is one of the determinants of the ease in which T helper–dependent responses occur. Low regulatory T cell activity may therefore result in exaggerated T helper differentiation during responses to self or environmental antigens, leading to autoimmune disease or atopy.

We thank Dr. Ian Wilson for providing the 17/9 hybridoma, Dr. Dan R. Littman for his support with ES cell injections, and Drs. G. Jeanette Thorbecke, Ed Skolnik, and Dan R. Littman for critically reading the manuscript.

Supported by the National Institutes of Health, Asthma and Allergy Foundation of America, the Bernard B. Levine Investigatorship in Allergy and Immunology, and the Hirschl-Caulier Trust.

Submitted: 6 July 2001
Revised: 4 September 2001
Accepted: 20 September 2001

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