action. The cDNAs were inserted into pBluescript−, and the DNAs of several clones were sequenced.
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Regulation of IgE Responses to Inhaled Antigen in Mice by Antigen-Specific γδ T Cells

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Indirect evidence implicates γδ T cells in the cross-regulation of CD4 αβ T cell responses. Adoptive transfer of small numbers of γδ T cells from ovalbumin (OVA)-tolerant mice selectively suppressed T_H2–dependent immunoglobulin E (IgE) antibody production without affecting parallel IgG responses. Challenge of these γδ T cells in vitro with specific antigen resulted in production of high levels of interferon γ. The effects of the γδ T cells may be mediated by direct inhibition of OVA-specific $CD4^+$ T_H2 cell proliferation or selection for specific CD4 T_H2 cells.

The "normal" immune response to nonpathogenic soluble protein antigens presented at the body's major mucosal surfaces is the selective suppression of antigen-specific delayed-type hypersensitivity (DTH) and IgE production. The process occurring in the gastrointestinal tract in response to food antigens has been termed oral tolerance (*1*). We have described an analogous tolerance process in the respiratory tract of the rat in response to inhalation of antigen (*2*, *3*) and demonstrated the capacity of purified $CD8^+$ T cells to mediate its adoptive transfer (*4*), a finding consistent with later reports on oral tolerance (*5*, *6*).

The $CD8^+$ T cells that mediate adoptive transfer of IgE-selective antigen-specific "tolerance" in the rat model are major histocompatibility complex (MHC) class I restricted and are responsive to soluble OVA antigen (*7*). Activation of these cells depends on interleukin-2 (IL-2) which, at least in vitro, is provided by MHC class II–restricted $CD4^+$ T cells responding to the same antigen (*8*). The early phase of the response occurs in the regional lymph nodes draining the conducting airways; it involves IgE production (*4*, *7*, *8*) as well as IL-4 and IL-2 secretion by OVA-specific $CD4^+$ T_H2 cells. This phase is halted by the emergence of antigen-specific $CD8^+$ T cells which release high levels of interferon γ (IFN-γ) when cultured with OVA (*8*). The OVA-responsive $CD8^+$ T cells express CD3, but surface αβ T cell receptors (TCRs) were not detected with the single TCR chain–specific antibody available for rat (*9*). Accordingly, the model was reestablished here in the mouse, for more detailed analysis.

IgE-selective "tolerance" to inhaled OVA has previously been demonstrated in several strains of mice (*7*, *10*), as has adoptive transfer of the phenomenon by splenic Thy 1.2^+ T cells (*7*). Thus, repeated exposure of C57Bl mice to aerosolized OVA ablated their capacity to mount primary IgE responses to parenteral challenge with OVA plus aluminum hydroxide (AH) adjuvant, without suppressing corresponding IgG responses (*7*). Adoptive transfer of 10^6 unfractionated splenocytes from such tolerized animals (*11*) inhibits IgE (but not IgG) antibody responses to OVA (anti-OVA) in the recipients (Fig. 1). Furthermore, the $CD8^+$ splenocyte subpopulation mediated inhibition of IgE (Fig. 1). The magnitude of the overall IgG anti-OVA response did not change significantly in aerosol-exposed mice (Fig. 1). However, analysis of individual IgG subclasses (*12*) indicated that suppression of the IgE response was accompanied by decreased IgG1 reactivity and a compensatory rise in IgG2a, whereas IgG2b and IgG3 responsiveness remained essentially unaltered.

The capacity of splenocytes to suppress the IgE response was lost by depletion of $\gamma\delta^+$ T cells (Fig. 2). In 10^6 splenocytes there are roughly 3×10^4 γδ T cells. When this number of γδ T cells (purified to ≥98.5% by positive selection) was transferred, suppression of IgE responses was comparable in magnitude to that seen in animals receiving 10^6 unfractionated cells.

Subsequent dose-response experiments (Fig. 3) demonstrated that as few as 5×10^2 positively selected γδ T cells are sufficient for suppression of the IgE component of the anti-OVA response. γδ T cells prepared by negative selection [that is, depletion of αβ T cells (*13*)] from OVA-tolerant donors yielded suppression of the IgE response comparable to that achieved with positively selected cells. We have shown previously that adoptive transfer of splenocytes depleted of $\alpha\beta^+$ T cells from OVA-tolerant rats was capable of mediating antigen-specific tolerance in the IgE isotype (*9*).

To test for the antigen specificity of γδ T

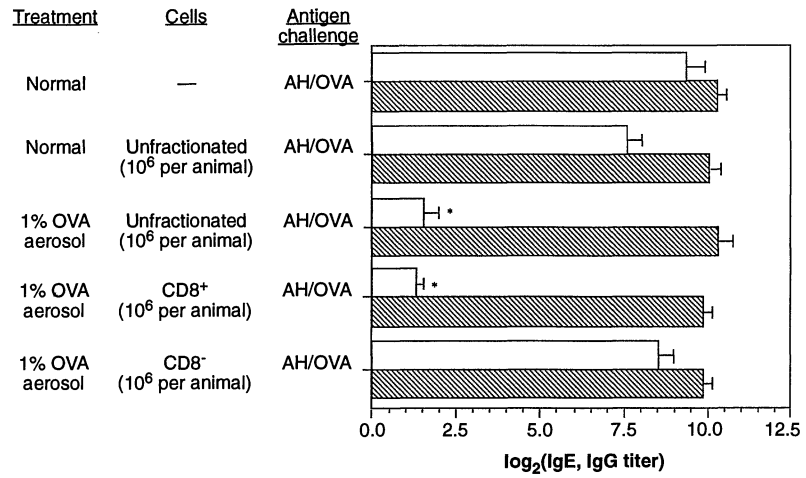

Fig. 1. Selective suppression of IgE anti-OVA responses by adoptive transfer of $CD8^+$ lymphocytes from C57Bl/6J mice rendered "tolerant" to OVA by repeated exposure (*11*). The $CD8^+$ population used here was >99.5% pure, and the $CD8^-$ population contained <0.4% of contaminating $CD8^+$ cells. Data shown are the mean ± SD (n = 5 to 10 per group) at day 21 (peak primary Ig response) and indicate reciprocal $\log_2$(IgE and IgG) anti-OVA titers as determined by standard methods (*8*). Data from day 14 did not alter interpretation of the results of these experiments. Open bars, IgE; hatched bars, IgG (asterisk, <controls; $P < 0.01$).

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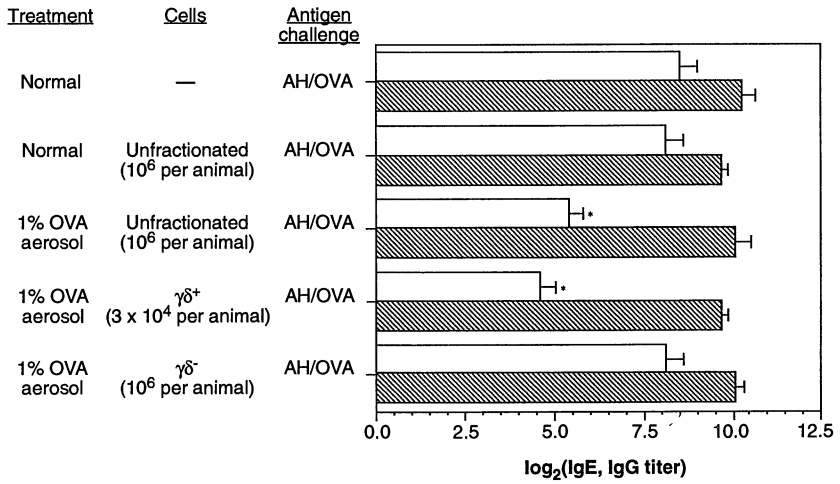

Fig. 2. Suppression of IgE response by γδ T cells. Spleen cell populations from tolerized animals were negatively depleted of γδ T cells with the antibody GL3 (*33*). GL3$^+$ (γδ$^+$) cells were prepared by positive selection. Adoptive transfer, intraperitoneal antigen challenge, and determination of primary IgE and IgG responses were as in Fig. 1. Open bars, IgE; hatched bars, IgG (asterisk, <controls; $P < 0.01$).

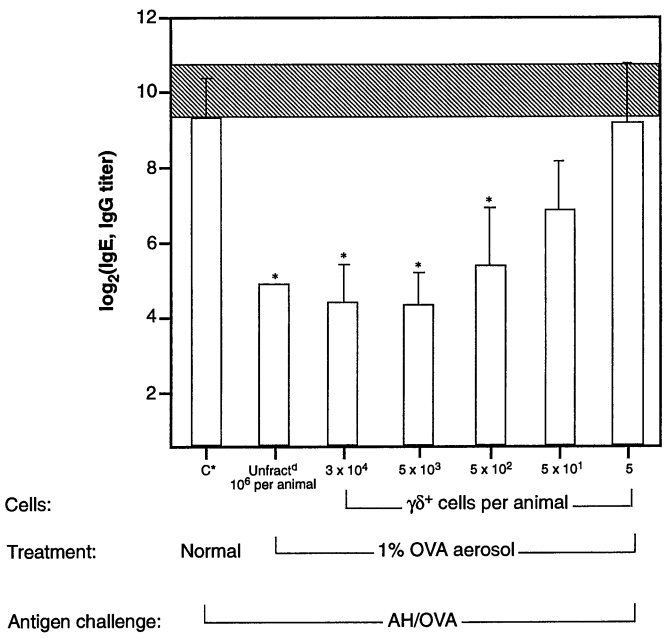

Fig. 3. Dose-response analysis of adoptive transfer of OVA-specific tolerance by positively selected γδ T cells from OVA-tolerant mice. Data shown are IgE titers from individual mice; the shaded area represents the 95% confidence limits for the peak primary IgG response in normal animals, and IgG titers in all animals in the experiment fell within this area. C*, comparable results obtained with untouched controls or recipients of splenocytes from naïve animals. Asterisk, <controls; $P < 0.01$.

Table 1. In vitro cytokine responses by splenocyte subsets from OVA-tolerant mice. Samples of splenocytes from OVA-tolerant animals described in Fig. 1 were depleted of >99.5% CD4$^+$, CD8$^+$, αβ$^+$ TCR or γδ$^+$ TCR cells and challenged in vitro with OVA (100 μg/ml). Data shown are the mean ± SD of replicate 24-hour culture supernatants. IL-2 secretion was determined by the standard CTLL assay as described in (*8*), and IFN-γ and TGF-β1 were determined by ELISA with the detection system supplied by Pharmingen and Genzyme, respectively. The cell preparations failed to respond to an irrelevant (control) antigen. Control cells from normal animals did not secrete detectable levels of cytokines in the presence of OVA. ND, not determined.

Cells	Cytokine secretion		
	IFN-γ (ng/ml)	IL-2 (U/ml)	TGF-β1 (ng/ml)
Unfractionated	226.5 ± 7.8	2.7 ± 0.3	1.59 ± 0.15
CD4$^-$	245.0 ± 28.3	1.8 ± 1.1	1.84 ± 0.73
CD8$^-$	63.5 ± 12.0	9.3 ± 0.7	2.01 ± 0.57
αβ$^-$	147.5 ± 21.9	2.6 ± 1.9	1.65 ± 0.39
γδ$^-$	90.0 ± 2.5	8.7 ± 1.2	1.98 ± 0.24
Non–T cells (CD3$^-$)	ND	ND	0.45 ± 0.09

cell–mediated suppression, we transferred unfractionated splenocytes or purified γδ T cells from OVA-tolerant mice to groups of syngeneic recipients, which were then challenged with OVA or a second antigen, Der p1, from the house dust mite. The transferred cells suppressed primary anti-OVA responses but did not affect corresponding anti–Der p1 responses (Fig. 4). Antigen specificity was still observed in this system at 50-fold higher cell dosages. Selective suppression of specific IgE in rodents in response to repeated inhalation of ragweed allergen and Der p1 has been reported previously (*14*, *15*).

Splenocytes from mice tolerized to OVA were challenged in vitro with OVA (100 μg/ml), and supernatants were harvested at 24 hours for assessment of cytokine production (*16*). Unfractionated splenocytes from tolerant animals secreted high levels of IFN-γ in response to specific antigen, and this secretory response was markedly reduced by depletion of CD8$^+$, but not CD4$^+$, cells; depletion of CD8$^+$ cells markedly enhanced OVA-specific IL-2 responses (Table 1). This is identical to the cytokine profile recently reported by us for the effector cell mediating tolerance in the equivalent rat model (*8*). Depletion of γδ T cells mimicked the effect of CD8$^+$ depletion on IFN-γ and IL-2 secretion (Table 1).

Transforming growth factor–β1 (TGF-β1) has been suggested to play an important role in CD8 T cell–mediated tolerance to fed antigens (*17*) and was accordingly examined here. However, TGF-β1 was produced in similar amounts by all T cell subsets from tolerant animals after antigenic stimulation, regardless of their potency in transfer of tolerance (Table 1), which argues against a central role for this cytokine in the process.

A number of experiments also examined the capacity of splenic T cells from aerosol-exposed mice to respond in vitro to OVA by proliferation (Table 2). A moderate proliferative response to antigen was consistently seen in unfractionated splenocytes, which was abrogated by depletion of αβ T cells and enhanced by depletion of the γδ subset; the latter procedure was also accompanied by a large increase in IL-2 production (Table 1). This suggests inhibition of αβ T cell proliferation by the γδ population, which is consistent with the reported effects of in vivo γδ T cell depletion (*18*).

We conclude that the effector cells mediating selective suppression of IgE responses in this murine model are CD4$^-$CD8$^+$ γδ$^+$ T cells, which have specificity for OVA. From the data of Table 1, it appears that these cells secrete IFN-γ in response to OVA, a finding in keeping with recent studies on γδ cell responses to stimulation with microbial antigens (*19*). In addition, they may also trigger IFN-γ release from other cell populations that are CD4$^-$, such as natural killer

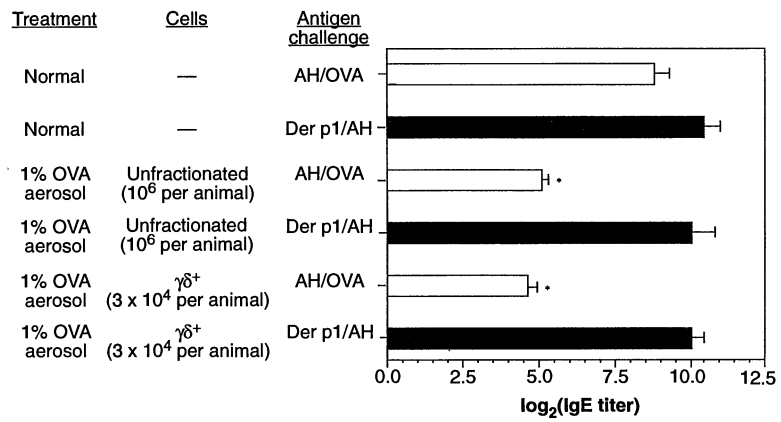

Fig. 4. Antigen specificity of γδ T cells in suppression of primary IgE responses. Unfractionated or positively selected γδ T cells from OVA-tolerant rats were transferred as in Table 2, and recipients were challenged with OVA or the irrelevant antigen Der p1 (supplied by W. Thomas, Institute for Child Health Research, Perth, Western Australia). Asterisk, <controls; $P < 0.01$.

Table 2. In vitro proliferation response of splenocyte subsets from OVA-tolerant mice. Negative selection of subsets was as described in (*13*) and proliferation was assessed after stimulation with OVA (100 μg/ml). Data shown are the mean ± SD of replicate cultures measured after 96 hours as incorporation of [^{3}H]thymidine into DNA. Subsets contained ≤0.5% contaminants. Cells from OVA-exposed animals did not proliferate in response to an irrelevant (control) antigen, and normal cells did not proliferate in the presence of OVA.

Cell population	Proliferation ([^{3}H]DNA synthesis)
Undepleted	5,232 ± 75
αβ$^-$	699 ± 32
γδ$^-$	13,255 ± 563

cells or (as suggested by the effects of αβ depletion) CD8$^+$ αβ T cells, both of which can be potent sources of IFN-γ (*19*, *20*). Thus, the T cell response to inhaled OVA in "tolerized" mice displays a T_H1–like profile, which is consistent with the pattern of selective suppression of specific IgE and IgG1 production and concomitantly enhanced IgG2a secretion (*21*) that was observed in these animals.

There is a growing body of evidence suggesting a wide range of immunoregulatory functions for murine γδ T cells, including the suppression of graft rejection (*22*), enhancement of specific IgA responses to fed antigens (*23*), provision of "help" for αβ T cells in adoptive transfer of contact sensitivity (*24*), and cooperation with αβ T cells in in vitro responses to microbial antigens (*25*). Additionally, challenge of the lungs in mice with mycobacterial antigen or live virus leads to expansion of local γδ T cell populations (*26*, *27*), and these cells are also prominent during the late phase of murine influenza (*28*).

The potency of the γδ T cells in regulating CD4$^+$ T cell–dependent immunity demonstrated in this model is unrivaled in the previous literature on "infectious suppression," with as few as 500 (perhaps as low as 50) adoptively transferred γδ T cells being sufficient to modulate in vivo CD4$^+$ responses to their specific antigen. In other published systems adoptive transfer of suppression typically requires several million cells, but in at least one of these, CD8$^+$ T cell suppression of allograft rejection, the rate-limiting "regulator" cell makes up less than 0.1% of the inoculum used to adoptively transfer suppression (*29*). The key to the potency of the latter regulator cell population was its capacity to expand rapidly after in vivo transfer. Experiments are in progress to test this possibility in the present system and to ascertain whether the γδ T cells may alternatively recruit secondary effector cells such as IFN-γ–secreting CD8$^+$ αβ T cells.

These experiments suggest an important role for antigen-specific γδ T cells in the maintenance of immunological homeostasis in the lung and airways by selective suppression of potentially pathogenic T_H2–dependent IgE responses, while preserving the host's capacity to produce specific IgG antibody. They may thus play an important role in protection against primary allergic sensitization to environmental antigens associated with immunoinflammatory diseases such as allergic rhinitis and asthma, assuming that similar mechanisms operate in humans.

REFERENCES AND NOTES

1. A. M. Mowat, *Immunol. Today* **8**, 93 (1987).
2. P. G. Holt and J. D. Sedgwick, *ibid.*, p. 14.
3. P. G. Holt and C. McMenamin, *Clin. Exp. Allergy* **19**, 255 (1989).
4. J. D. Sedgwick and P. G. Holt, *Cell. Immunol.* **94**, 182 (1985).
5. R. B. Nussenblatt *et al.*, *J. Immunol.* **144**, 1689 (1990).
6. A. Miller, O. Lider, H. L. Weiner, *J. Exp. Med.* **174**, 791 (1991).
7. P. G. Holt, J. E. Batty, K. J. Turner, *Immunology* **42**, 409 (1981).
8. C. McMenamin and P. G. Holt, *J. Exp. Med.* **178**, 889 (1993).
9. C. McMenamin *et al.*, *Immunology* **74**, 234 (1991).
10. P. G. Holt, D. Britten, J. D. Sedgwick, *ibid.* **60**, 97 (1987).
11. C57Bl/6J mice were exposed daily for 10 days to aerosolized OVA in phosphate-buffered saline and once weekly thereafter until used as described in (*4*, *8*). Intraperitoneal (ip) challenge of a subgroup of these animals with 10 μg of OVA in 4.0 mg of AH adjuvant revealed normal primary IgG responses but virtually complete suppression of parallel IgE responses, as demonstrated in earlier studies (*7*). Splenocytes were prepared from other (unchallenged) "tolerant" animals and divided into three samples. The first sample was left unfractionated, the second was negatively depleted of CD8$^+$ cells by cytometry, and CD8$^+$ cells were purified from the third by positive selection by cytometry (Epics Elite, Coulter Electronics); the CD8$^+$ antibody used was from the 53-6.72 clone (*30*) and the cytometry methodology used is described in (*8*). Immediately after ip injection of these cell populations, animals were immunized ip with 10 μg of OVA in 4.0 mg of AH adjuvant and bled at day 14 and 21.
12. IgG subclasses were measured by an in-house enzyme-linked immunosorbent assay (ELISA) with anti-IgG subclass antibodies (Southern Biotechnology).
13. Splenocytes were prepared as previously described and passed through nylon wool to remove adherent cells (*4*), thus yielding ~85% T cells. Negative selection of αβ T cells was done by flow cytometry with H57-597.19 (anti–αβ TCR) (*31*). γδ T cells constitute ~30% of the remaining cells; hence 1×10^5 splenocytes will contain 3×10^4 γδ T cells.
14. P. C. Fox and R. P. Siraganian, *Immunology* **43**, 227 (1981).
15. G. A. Stewart, P. G. Holt, *Int. Arch. Allergy Appl. Immunol.* **83**, 44 (1987).
16. For isolation of lymphocyte subsets for assessment of in vitro cytokine production and proliferation, αβ T cells were purified by negative selection by flow cytometry. Cells were cultured at 2×10^5 per microplate well in RPMI containing 10^{-5} M 2-ME plus antibiotics supplemented with 1 to 10% fetal calf serum and stimulated with OVA (100 μg/ml). Supernatants were harvested after 24 hours and frozen at −20°C until assayed. The antibody to CD4 used in this study was from the GK1.5 clone (*32*).
17. A. Miller, O. Lider, A. B. Roberts, M. B. Sporn, H. L. Weiner, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 421 (1992).
18. S. H. E. Kaufmann, C. Blum, S. Yamamoto, *ibid.* **90**, 9620 (1993).
19. S. Yamamoto, F. Russ, H. Teixeira, P. Conradt, S. H. E. Kaufmann, *Infect. Immun.* **61**, 2154 (1993).
20. S. Romagnani, *Immunol. Today* **13**, 379 (1992).
21. F. D. Finkelman *et al.*, *Immunology* **8**, 303 (1990).
22. R. M. Gorczynski, *ibid.* **81**, 27 (1994).
23. K. Fujihashi *et al.*, *J. Exp. Med.* **175**, 695 (1992).
24. W. Ptak and P. W. Askenase, *J. Immunol.* **149**, 3503 (1992).
25. M. J. Skeen and H. K. Ziegler, *J. Exp. Med.* **178**, 985 (1993).
26. A. Augustin, R. T. Kubo, G.-K. Sim, *Nature* **340**, 239 (1989).
27. S. R. Carding *et al.*, *J. Exp. Med.* **172**, 1225 (1990).
28. P. C. Doherty, W. Allan, M. Eichelberger, S. R. Carding, *Annu. Rev. Immunol.* **10**, 123 (1992).
29. D. W. Mason and S. J. Simmonds, *Immunology* **65**, 249 (1988).
30. J. Ledbetter, R. Rouse, S. Micklem, L. Herzenberg, *J. Exp. Med.* **152**, 280 (1980).
31. R. Kubo, W. Born, P. Kappler, M. Pigeon, *J. Immunol.* **142**, 2236 (1989).
32. D. P. Dialynas *et al.*, *ibid.* **131**, 2445 (1983).

33. T. Goodman and L. Lefrançois, *Nature* **333**, 855 (1988).
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