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9. ERK2, ERK2 K52A, ERK2 D319N, SAPKα (JNK2), SAPKβ (JNK3), and p38 MAP kinases were produced in *E. coli* as GST fusion proteins and purified by binding to glutathione-Sepharose. All MAP kinases were >90% pure. Bead-immobilized MAP kinases were used for binding MKP-3 (*13*); for phosphatase activation (*10*) MAP kinases were eluted with 50 mM tris (pH 8.0) containing 5 mM glutathione. For some experiments GST-ERK2 and GST–MKP-3 were cleaved from their GST fusion protein by incubation with thrombin and further purified by fast protein liquid chromatography with Mono Q Sepharose followed by dialysis against 20 mM tris (pH 7.5) containing 0.5 mM EGTA, 5 mM $MgCl_2$, and 2 mM dithiothreitol (DTT). This ERK2 was >95% pure. MAP kinase phosphorylation assays were done with [γ−$^{32}$P]ATP (adenosine 5′-triphosphate) as described (*6*, *8*).
10. MKP-3, catalytically inactive MKP-3 (C293S), MKP-3ΔN (amino acids 153 to 381), MKP-3ΔC (amino acids 1 to 221), and MKP-4 subcloned into pGEX 4T3 (*6*, *8*) were expressed in *E. coli* by induction with 100 μM isopropyl-β-D-thiogalactopyranoside and growth at 20°C. GST fusion proteins were purified with glutathione-Sepharose (Pharmacia LKB Biotechnology) and eluted in 50 mM tris (pH 8.0) containing 5 mM glutathione. His–MKP-3 was expressed under identical conditions and purified with Ni-agarose and eluted with 300 mM imidazole. All proteins were >90% pure. Phosphatase activity was measured in 96-well plates in 200 μl of 50 mM imidazole (pH 7.5) containing 5 mM DTT, 20 mM *p*-NPP, and the indicated concentrations of MKP-3 and various purified MAP kinases (*9*). Reaction rates were measured at 405 nm in a microplate reader (Molecular Devices).
11. M. Camps, C. Gillieron, S. Arkinstall, unpublished data.
12. His-tagged MKP-3 (0.1 μg) was incubated with MAP kinases immobilized on beads (3 μg) in 20 mM tris-acetate (pH 7.0) containing 1% Triton X-100, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 0.27 M sucrose, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, and 0.1% β-mercaptoethanol together with a cocktail of protease inhibitors overnight at 4°C with mixing. Beads were washed four times in 10 mM tris (pH 7.4), and bound MKP-3 was analyzed by protein immunoblotting with a polyclonal antibody directed to the peptide VVLYDENSSDWNENTGGE (amino acids 95 to 112). In some experiments COS-7 cells were transfected with pMT-SM-Myc-MKP-3 or pMT-SM-Myc-MKP-4 (*6*, *11*), and binding to immobilized MAP kinases (3 μg) was measured under identical conditions except that MKP-3 and MKP-4 protein was detected with monoclonal antibody to the Myc epitope (*8*). Abbreviations for the amino acid residues are as follows: D, Asp; E, Glu; G, Gly; L, Leu; N, Asn; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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16. Constitutively active rabbit MEK1 EE (S217E and S221E) was purified and used to activate ERK as described (*8*).
17. COS-7 cells were transfected with pEXV3-Myc-ERK2, pEXV3-Myc-ERK2 D319N, or pcDNA1-HA-ERK1 together with various concentrations of pMT-SM-MKP-3 or pMT-SM-MKP-3ΔN followed by EGF stimulation, MAP kinase immunoprecipitation, and immune complex assays were performed exactly as described (*6*, *8*). pMT-SM-MKP-3ΔN (amino acids 153 to 381) was constructed by digesting pMT-SM-MKP-3 (*6*) with Pst I–Xba I followed by ligation with a double-stranded oligonucleotide containing an ATG codon following a Kozak consensus.
18. We thank J. R. Woodgett (Ontario Cancer Institute, Canada) for pMT2-HA-SAPKβ (JNK3) and pGEX-SAPKα (JNK2); E. Bettini (Glaxo Wellcome, Verona, Italy) for pGEX-c-Jun-(1-79); S. Stimpson (Glaxo Wellcome, Research Triangle Park, NC) for pGEX-p38; and C. J. Marshall (Chester Beatty Labs, ICR, London, UK) for pGEX-2T/ERK2, pGEX-2T/ERK2 D319N (Sevenmaker), pGEX-2T/ERK2 K52A, pGEX-3X/MEK1 (S217E, S221E), pEXV3-Myc-ERK2, pEXV3-Myc-ERK2 D319N, and rabbit antibody 122 specific for ERK2.

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# Requirement for γδ T Cells in Allergic Airway Inflammation

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The factors that contribute to allergic asthma are unclear but the resulting condition is considered a consequence of a type-2 T helper ($T_H2$) cell response. In a model of pulmonary allergic inflammation, mice that lacked γδ T cells had decreases in specific immunoglobulin E (IgE) and IgG1 and pulmonary interleukin-5 (IL-5) release as well as in eosinophil and T cell infiltration compared with wild-type mice. These responses were restored by administration of IL-4 to γδ T cell-deficient mice during the primary immunization. Thus, γδ T cells are essential for inducing IL-4-dependent IgE and IgG1 responses and for $T_H2$-mediated airway inflammation to peptidic antigens.

Allergic asthma is a chronic inflammatory disease associated with a predominant $T_H2$ response, IgE synthesis, airway infiltration by inflammatory cells, particularly eosinophils, and bronchial hyperreactivity (*1*). Identification of the mechanisms involved in the in vivo commitment of naive T cells to a $T_H2$ phenotype will aid our understanding of the initiation and maintenance of tissue inflammation. IL-4 drives $T_H2$ responses and promotes IgE synthesis (*2*), but the nature of the cells that provide this cytokine after in vivo interactions among peptidic antigens, antigen-presenting cells, and $T_H$ cells remains largely elusive.

A subset of γδ T cells can produce $T_H2$-type cytokines (*3*, *4*) which suggests their possible participation in the development of $T_H2$ responses and, thus, in the onset of pulmonary allergic reactions. To address this question, we backcrossed 6-week-old mice that were genetically deficient in the δ chain of the T cell antigen receptor (TCR) and developed no γδ T cells (*5*) to BALB/c mice for 10 generations. These γδ T cell-deficient ($\gamma\delta^{-/-}$) and BALB/c wild-type ($\gamma\delta^{+/+}$) mice were repeatedly immunized intraperitoneally with soluble ovalbumin (OVA) and then challenged intranasally with OVA or saline (*6*). We analyzed airway infiltration by inflammatory cells (*7*), cytokine release in the bronchoalveolar lavage (BAL) fluid (*8*), bronchopulmonary hyperreactivity to inhaled methacholine (*9*), and OVA-specific IgE and IgG1 titers in the serum (*10*).

Repeated intranasal OVA challenges in immunized $\gamma\delta^{+/+}$ mice resulted in a significant increase in the number of eosinophils and of $CD4^+$ and $CD8^+$ T lymphocytes infiltrating the bronchial tissue (Fig. 1, A, D, and G). Eosinophils and T cells were located in the bronchial submucosa and around the blood vessels (Fig. 1, B, E, and H). Antigen-induced eosinophilia also occurred in the blood, BAL fluid, and bone marrow (*11*, *12*). Unlike $\gamma\delta^{+/+}$ mice, OVA-challenged $\gamma\delta^{-/-}$ mice showed only a moderate increase in the number of eosinophils in bronchial tissue, BAL fluid, blood, and bone marrow; no significant changes in T cell counts in lung tissue were observed (Fig. 1) (*12*).

Airway eosinophilia in OVA-challenged $\gamma\delta^{+/+}$ mice paralleled IL-5, but not interferon-γ (IFN-γ), production in the BAL fluid (Fig. 2), a finding consistent with selective induction of a $T_H2$ response in the airways. In contrast, $\gamma\delta^{-/-}$ mice failed to release IL-5 in response to intranasal administration of OVA (Fig. 2A) and the amounts of IFN-γ remained very low in both saline- and OVA-challenged animals (Fig. 2B). High concentrations of IL-4 were detected in the BAL fluid of saline-challenged $\gamma\delta^{+/+}$ and $\gamma\delta^{-/-}$ mice (445.6 ± 98.9 and 280.8 ± 42.9 pg/ml, respectively). These quantities decreased slightly after intranasal OVA challenge in

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both $\gamma\delta^{+/+}$ and $\gamma\delta^{-/-}$ mice (210.6 ± 15.1 and 189.4 ± 28.5 pg/ml, respectively), which suggests that IL-4 release in the lung is not enhanced under these experimental conditions.

Despite the differences in OVA-induced airway eosinophilia and IL-5 release in the BAL fluid, $\gamma\delta^{+/+}$ and $\gamma\delta^{-/-}$ mice developed similar bronchial hyperreactivity to methacholine (*13*). This may be due to the comparable concentrations of serum IgE observed in both types of mice after intranasal OVA challenge (see below). The dissociation between eosinophil infiltration into the airways and bronchial hyperreactivity has been observed in other models (*14*).

To determine whether impaired pulmonary inflammation observed in $\gamma\delta^{-/-}$ mice resulted from an inefficient antigen priming at the periphery or from a defective response in the lung, we analyzed serum titers of OVA-specific IgE and IgG1 in OVA-immunized $\gamma\delta^{+/+}$ and $\gamma\delta^{-/-}$ mice that were challenged intranasally with either saline or OVA (Fig. 3). Repeated intraperitoneal injections of OVA into $\gamma\delta^{+/+}$ mice resulted in high titers of OVA-specific IgG1 and in production of low, but detectable, concentrations of OVA-specific IgE (Fig. 3, saline). This same protocol of immunization elicited only 1/100th of the production of OVA-specific IgG1 and undetectable concentrations of IgE in $\gamma\delta^{-/-}$ compared with wild-type mice (Fig. 3, saline). Intranasal OVA challenge boosted the specific response to comparable levels in both $\gamma\delta^{+/+}$ and $\gamma\delta^{-/-}$ mice (Fig. 3, OVA). Decreased specific IgE and IgG1 titers in OVA-immunized saline-challenged $\gamma\delta^{-/-}$ mice were not compensated by an increase in the number of OVA-specific antibodies of other isotypes (*15*). Thus, the peripheral immune response to soluble OVA that is generated as a consequence of the multiple intraperitoneal injections of antigen was impaired in $\gamma\delta^{-/-}$ mice. This may be the basis of the reduction in pulmonary inflammation after intranasal antigen challenge. The possibility that residual 129/Sv background genes present in the backcrossed $\gamma\delta^{-/-}$ mice could account for the differences presented here was minimized by analysis of OVA-induced peripheral and pulmonary responses in BALB/c, 129/Sv, and (BALB/c × 129/Sv) $F_1$ animals, which were found to be comparable (*16*). The possibility still exists, however, that a recessive 129/Sv gene interacts with a BALB/c gene to generate the observed phenotype. Experiments conducted with TCR$\gamma^{-/-}$ mice could further clarify this issue. However, the organization of the TCR$\gamma$ locus in the mouse, with four different C$\gamma$ regions spanning several megabases in the chromosome, prevents the production of these mice by currently available techniques.

OVA-induced pulmonary responses largely depend on early production of IL-4, because airway eosinophilia and local IL-5 release are prevented by administration of antibodies to IL-4 during the peritoneal immunization period (*17*). Because some $\gamma\delta$ T cells secrete IL-4 (*3*, *4*), we postulated that the impaired immune response and allergic airway inflammation observed in OVA-immunized $\gamma\delta^{-/-}$ mice could result from a lack of early IL-4 production. To verify this hypothesis, we injected $\gamma\delta^{-/-}$ mice intraperitoneally during the immunization period with a complex of active IL-4 and a monoclonal antibody (mAb) to IL-4 to increase half-life and prolong IL-4 activity in vivo (*18*). Seven days after the last OVA immunization, IL-4-treated $\gamma\delta^{-/-}$ mice had OVA-specific serum IgE (Fig. 4A) and IgG1 (Fig. 4B) concentrations similar to those of $\gamma\delta^{+/+}$ mice (compare Fig. 4, A and B, with Fig. 3). IL-4 administration also rendered $\gamma\delta^{-/-}$ mice prone to respond to

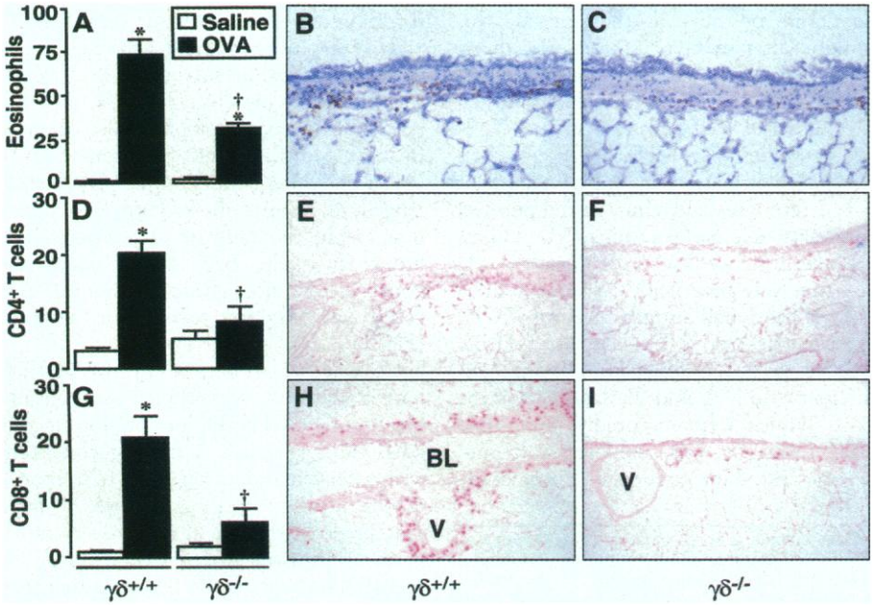


**Fig. 1.** Inflammatory cell distribution in the bronchial tissue of antigen-challenged $\gamma\delta^{+/+}$ and $\gamma\delta^{-/-}$ mice. Statistical data from six tissue sections per mouse and six mice per group are plotted for eosinophils (**A**), CD4+ T cells (**D**), and CD8+ T cells (**G**). Cryostat lung sections from $\gamma\delta^{+/+}$ (**B**, **E**, and **H**) or $\gamma\delta^{-/-}$ (**C**, **F**, and **I**) mice were processed for immunohistochemical analysis (*7*). Original magnifications, ×200 (B and C) and ×100 (E, F, H, and I). *$P < 0.05$; †$P < 0.05$ [one-way analysis of variance (ANOVA) followed by Student's $t$ test for unpaired values] compared with saline- or OVA-challenged $\gamma\delta^{+/+}$ mice, respectively. BL, bronchial lumen; V, vessel.

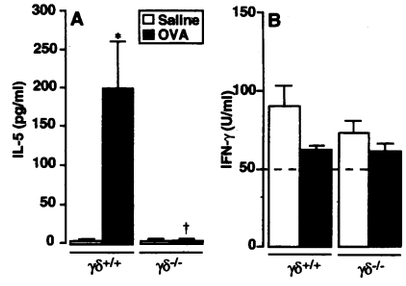


**Fig. 2.** Concentrations of IL-5 (**A**) and IFN-γ (**B**) in the BAL fluid of OVA-immunized $\gamma\delta^{+/+}$ and $\gamma\delta^{-/-}$ mice. Seventy-two hours after the final intranasal saline or OVA challenge, cell-free supernatants from BAL fluids were harvested and assayed for IL-5 and IFN-γ production by enzyme-immunometric assay and ELISA, respectively (*8*). Results are means ± SEM (vertical bars) of five or six mice per group. *$P < 0.05$; †$P < 0.05$ (one-way ANOVA followed by Student's $t$ test for unpaired values) compared with saline- or OVA-challenged $\gamma\delta^{+/+}$ mice, respectively. Dotted line in (B) indicates the sensitivity of the assay.

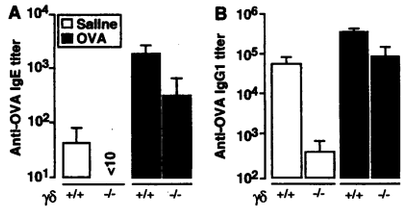


**Fig. 3.** OVA-specific IgE and IgG1 titers in the sera of immunized $\gamma\delta^{+/+}$ and $\gamma\delta^{-/-}$ mice. All animals were immunized with OVA and challenged with either saline or OVA (*6*). Blood was harvested from post vena cava 72 hours after the final intranasal saline or OVA challenge and sera were tested for the presence of IgE (**A**) and IgG1 (**B**) by ELISA (*10*). Results are means ± SD (vertical bars) of five animals per group. One experiment representative of three is shown.

OVA stimulation with eosinophil accumulation and IL-5 release into the BAL fluid (Fig. 4, C and D). Thus, γδ T cells were essential for initial IL-4 production, early IgE and IgG1 synthesis, and development of a $T_H2$ response in the airways.

Mast cells, $CD8^+$ TCRαβ cells and $NK1.1^+$ TCRαβ T cells also secrete IL-4 (*19*), which suggests that all these cell types have the capacity to initiate a $T_H2$ response in vivo. However, mast cell-deficient mice have reduced eosinophil accumulation in the BAL fluid but no changes in OVA-specific IgE and IgG1 (*20*), indicating that factors originating from mast cells participate in the onset of pulmonary eosinophilia but not in development of the humoral response. The analysis of OVA-driven specific IgE response, BAL eosinophilia, and the expression of $T_H2$-type cytokines in bronchial lymph nodes of $\beta_2$-microglobulin ($\beta_2M$)-deficient mice, which lack both $CD8^+$ TCRαβ and $NK1.1^+$ TCRαβ T cells, showed that these cells are not required for $T_H2$ cell-mediated in vivo pulmonary allergic reactions (*21*). Together, these observations suggest that the source of IL-4 early in this response may be the γδ T cells themselves. IL-4-producing γδ T cells are present in mutant mice lacking $\beta_2M$, because they are selected independently from $\beta_2M$-associated class I molecule expression (*4*). By releasing IL-4, such TCRγδ cells may actively participate in the initiation of $T_H2$ immune responses, as shown for IgE and IgG1 production in αβ T cell-deficient mice (*22*). Further characterization of the subset of peripheral γδ T cells directly involved in IL-4 production will represent an important step for understanding and modulating the development of $T_H2$ responses, particularly in the context of allergic diseases such as bronchial asthma.

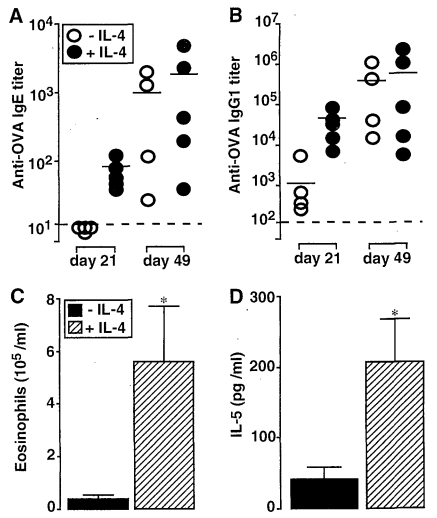


**Fig. 4.** In vivo IL-4 administration restored $T_H2$ responses in OVA-immunized $\gamma\delta^{-/-}$ mice. Mice were untreated or were injected intraperitoneally with a mixture of 5 μg of recombinant murine IL-4 and 50 μg of rat mAb to murine IL-4 (11B11) (*18*) at the time of the first OVA immunization (day 0) and on days 6 and 12. Blood was collected from the retroorbital plexus on day 21 (7 days after the last intraperitoneal OVA immunization) and from post vena cava 72 hours after the final intranasal antigen challenge (day 49) and serum concentrations of OVA-specific IgE (**A**) and IgG1 (**B**) were quantitated (*10*). The same animals were used for eosinophil (**C**) and IL-5 (**D**) determination in BAL fluid [legend to Fig. 2; see (*8, 11*)]. Results are means ± SEM (vertical bars) of four or five mice per group. $^*P < 0.05$ compared with IL-4-untreated mice (one-way ANOVA followed by Student's *t* test for unpaired values).

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6. All mice were immunized intraperitoneally with 10 μg of OVA in 0.1 ml of sterile saline every 2 days for 2 weeks [D. I. Blyth, M. S. Pedrick, T. J. Savage, E. M. Hessel, D. Fattah, *Am. J. Respir. Cell Mol. Biol.* **14**, 425 (1996); Y. Chvatchko, M. H. Kosko-Vilbois, S. Herren, J. Lefort, J. Y. Bonnefoy, *J. Exp. Med.* **184**, 2353 (1996)]. Forty days after the beginning of immunization, mice were challenged three times, each 3 days apart, with 20 μg of OVA in 50 μl of saline delivered intranasally. Control animals were similarly immunized intraperitoneally with OVA and challenged intranasally three times with 50 μl of saline. Mice were used 3 days after the last intranasal saline or OVA challenge.
7. Seventy-two hours after the final intranasal saline or antigen challenge, animals were sacrificed and lungs were inflated by injecting into the trachea a 1-ml solution of optimum cutter temperature compound (OCT; BDH, Poole, UK) in distilled water (1:1). The dissected lobes were covered by OCT, placed in Eppendorf vials, frozen in liquid nitrogen, and kept at −80°C until use. Six-micrometer sections alongside the main intrapulmonary bronchus were cut in a cryostat and collected on glass slides previously coated with γ-methacryloxy propyltrimethoxysilane (Sigma) and fixed in acetone for 10 min. Cyanide-resistant eosinophil peroxidase activity, using potassium cyanide, diaminobenzidine, and hydrogen peroxide, was used to stain the eosinophils [C. Zuany-Amorim *et al.*, *J. Clin. Invest.* **95**, 2644 (1995)]. For the anti-CD4 and anti-CD8 antibodies (Tebu; Le Perray-en-Yvelines, France), an alkaline phosphatase–anti-alkaline phosphatase staining procedure was performed. Positive cells were enumerated in 1 μm of bronchial mucosa and results were expressed as numbers of cells per millimeter of bronchial epithelium.
8. IL-4, IL-5, and IFN-γ were measured in the supernatant of BAL fluids by either specific enzyme-immunometric assay (IL-5) [C. Zuany-Amorim *et al.*, *J. Immunol.* **157**, 377, 1996] or enzyme-linked immunosorbent assay (ELISA) (IL-4 and IFN-γ). Sensitivities were 50 pg/ml (IL-4), 5 pg/ml (IL-5), and 50 units/ml (IFN-γ). Recombinant murine IL-4, IL-5 (Immugenex; Los Angeles, CA), and IFN-γ (Sigma) were used to generate standard curves.
9. Unrestrained conscious mice were placed in a whole-body plethysmographic chamber (BUXCO Electronics; Sharon, CT), which analyzed the respiratory wave forms. After stabilization for a few minutes, an aerosol of methacholine (30 mM in the aerosolator) was delivered for 20 s. The airways resistance was expressed as Penh = 0.67 × [(expiratory time/40% of relaxation time) − 1] (peak expiratory flow/peak inspiratory flow) [S. Y. Eum, S. Hailé, J. Lefort, M. Huerre, B. B. Vargaftig, *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 12290 (1995)].
10. OVA-specific IgE and IgG1 in the serum samples were measured by ELISA, using biotinylated rat IgE antibody to mouse (Pharmingen; San Diego, CA) and alkaline phosphatase-conjugated goat IgG1 antibody to mouse (Southern Biotechnology; Birmingham, AL). To detect OVA-specific IgE, 25 μl of each serum was incubated twice with 50 μl of a 50% slurry of protein G–Sepharose (Pharmacia) in phosphate-buffered saline before the ELISA. This treatment allows the removal of about 95% of total IgG1 without altering the concentration of IgE.
11. Mice were anesthetized by an intraperitoneal injection of 1.4 g of ethylcarbamate per kilogram of body weight and BAL cells were harvested by injecting and recovering 1.5 ml of sterile saline through a tracheal cannula. Blood samples were also collected from the tail vein and total leukocytes and BAL cells were counted. Peripheral and BAL eosinophils were identified and counted after cytocentrifugation and staining with Diff-Quik stain (Baxter Dade; Duedingen, Switzerland). Bone marrow cells were collected from femurs in RPMI 1640 medium supplemented with 10% fetal calf serum, and eosinophils were enumerated after cytocentrifugation and peroxidase staining (*7*).
12. Eosinophil numbers in blood were 2.38 ± 0.48 × $10^3$ cells per microliter and 0.86 ± 0.46 × $10^3$ cells per microliter in OVA-challenged $\gamma\delta^{+/+}$ and $\gamma\delta^{-/-}$ mice, respectively ($n = 5$, $P = 0.01$). Eosinophil counts in BAL fluid were 3.37 ± 0.72 and 0.56 ± 0.2 × $10^5$ cells per milliliter in OVA-challenged $\gamma\delta^{+/+}$ and $\gamma\delta^{-/-}$ mice, respectively ($n = 6$, $P = 0.001$). Peroxidase-positive cells in the bone marrow were 7.13% ± 0.69% and 3.98% ± 0.37 % in OVA-challenged $\gamma\delta^{+/+}$ and $\gamma\delta^{-/-}$ mice, respectively ($n = 10$, $P = 0.001$).
13. Penh values in response to inhaled methacholine were 1.24 ± 0.09 and 1.83 ± 0.13 in saline- and OVA-challenged $\gamma\delta^{+/+}$ mice, respectively ($P < 0.001$, $n = 14$) and 1.38 ± 0.11 and 1.84 ± 0.14 in saline- and OVA-challenged $\gamma\delta^{-/-}$ mice, respectively ($P = 0.018$, $n = 17$).
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16. BALB/c, 129/Sv, and (BALB/c × 129/Sv) $F_1$ mice (5 to 12 mice per group) were immunized and challenged with OVA (*6*). No significant differences in the numbers of eosinophils (*11*) and in the release of IL-5 (*8*) in the BAL fluid, as well as in OVA-specific IgE and IgG1 serum titers (*10*), were noted among these strains.
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23. We thank S. Tonegawa for providing the TCR $\gamma\delta^{-/-}$ mice, E. Kilchherr and C. Heusser for the gift of IL-4, D. Joseph for eosinophil determinations in the bone marrow, G. Langsley for reading the manuscript, and J. M. Panaud for help in preparing photographic prints. Supported in part by grant 6969 from the Association pour la Recherche sur le Cancer.

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