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C McMenamin; ... et. al

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γδ T Cells Down-Regulate Primary IgE Responses in Rats to Inhaled Soluble Protein Antigens[1]

Christine McMenamin,* Michelle McKersey,* Petra Kühnlein,† Thomas Hünig,† and Patrick G. Holt[2†]

*Division of Cell Biology, Institute for Child Health Research, West Perth, Western Australia; and †Institute for Virology & Immunobiology, University of Würzburg, Würzburg, Germany

The biologic role and repertoire of cells bearing the γδ T cell receptor has not been fully defined. However, their tropism for epithelial microenvironments is recognized and suggests an important role for these cells in immune defense at mucosal tissue surfaces. The study presented below utilizes an experimental model in which repeated exposure of Brown Norway rats to OVA by inhalation induces a state of Ag-specific, IgE isotype-specific "tolerance" via immune deviation. This process seems similar to oral tolerance in the gut. This form of tolerance was adoptively transferred to naive syngeneic recipients by i.p. injection of as few as 10^3 positively selected TCR-$\gamma\delta^+$ cells from OVA-exposed rats. These TCR-$\gamma\delta^+$ T-cells are demonstrated to produce high levels of INF-γ in response to OVA stimulation, and this provides a potential mechanism for the inhibition of Th2 cell proliferation, resulting in suppression of IgE production. The unique potency of these cells in selective suppression of IgE Ab production in response to natural "mucosal" Ag exposure suggests a potentially important role in protection against primary allergic sensitization in vivo. *The Journal of Immunology,* 1995, 154: 4390–4394.

Down-regulation of immune responses to inert nonpathogenic Ags is central to the maintenance of immunologic homeostasis at mucosal surfaces in the respiratory and gastrointestinal tracts, and failure of the underlying control mechanism(s) has been suggested as a key etiologic factor in allergic disease (1, 2).

An important component of this process is the selective suppression of Th2-dependent IgE response to inhaled or fed Ags, which is mediated by Ag-specific $CD8^+$ T cells (3). Recent experiments from our laboratory have demonstrated that the effector cells in this process are class I MHC-restricted $CD3^+$ $CD4^-$ $CD8^+$ T cells that secrete high levels of INF-γ in response to soluble protein Ags (4). The present experiments provide evidence that the in vivo immunoregulatory activity of this $CD8^+$ T cell population is attributable to a small subset of Ag-responsive $CD8^+$ $\gamma\delta^+$ T cells.

Materials and Methods

Animals

Inbred adult (10 to 16 wk of age) Brown Norway rats were used in all experiments. Male WAG or Sprague-Dawley animals were used as serum recipients for IgE estimations by passive cutaneous anaphylaxis (PCA)[3] reaction (see below). All animals were specific pathogen free and barrier housed.

Ag exposure and immunization

The animals were exposed to an aerosol derived from 1% OVA (w/v) in saline (OVA; Grade V, Sigma Chemical Co., St. Louis, MO) via the Tri-R Airborne Infection Apparatus (Tri-R Instruments, New York, NY) for 30 min daily, 5 days per wk for 2 wk as previously described (5). Intraperitoneal immunization employed 100 μg OVA in 10 mg aluminium hydroxide gel (AH).

Ab determinations

Anti-OVA and anti-Der p1 IgE titers were estimated by PCA and titers expressed as reciprocal $\log_2$ titer. Anti-OVA IgG levels were determined by haemagglutination of OVA-coupled SRBCs and titers expressed as reciprocal $\log_2$. Both methods have been described previously (5). Measurement of anaphylactic IgG_{2b} was conducted by a modified PCA method (6) as follows; IgE Abs were eliminated by heating of the serum to 56°C for 1 h; animals were then sensitized for 4 h before challenge with Ag.

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[1] This work was supported by the National Health and Medical Research Council of Australia.

[2] Address correspondence and reprint requests to Dr. P. G. Holt, Division of Cell Biology Institute for Child Health Research, P. O. Box 855, West Perth, Western Australia 6872.

[3] Abbreviations used in this paper: PCA, passive cutaneous anaphylaxis; AH, aluminum hydroxide.

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Table I. *In vitro cytokine production by splenocyte subsets from animals after chronic exposure to 1% OVA*

In vivo Treatment	Cells	In vitro Treatment	INFγ (u/ml)	IL-2 (u/ml)
OVA aerosol	Unfractionated	100 μg/ml OVA	43 ± 0.7	3.2 ± 1.2
"	*αβ (4.9% $\gamma\delta^+$ cells)	"	38 ± 4.2	2.2 ± 0.8
"	*$\gamma\delta^-$ (39% $\alpha\beta^+$ cells)	"	2.1 ± 0.8	9.7 ± 1.7
"	$\gamma\delta^+$ only	"	0	0
"	$\gamma\delta^+$ & T cell-depleted splenocytes	"	31 ± 5.4	3.0 ± 1.4
"	T cell-depleted splenocytes	"	0	0

Data expressed as X ± SD of replicate 24-h culture supernatants. The cell preparations failed to respond to an irrelevent control Ag (BSA). Control cells from normal animals did not secrete detectable levels of cytokines in the presence of OVA.
* ≤1% contaminating $\alpha\beta^+$ or $\gamma\delta^+$ cells.

Abs

mAbs used were as follows: R73 (anti-TCR-αβ; (7)), V65 (anti-TCR-γδ; (8)), OX19 (CD5, pan T cell marker; (9)) OX8 (CD8; (9)). OX21 (anti-human C3b; (10)) was used as a negative isotype control and provided by Professor D. Mason (University of Oxford, Oxford, England). Fluorescent staining was conducted using PE-conjugated donkey $F(ab')_2$ anti-mouse IgG as a secondary Ab (Jackson ImmunoResearch, Westgrove, PA) and an OX19-FITC conjugate prepared in our laboratory.

Cell preparation

Splenocytes were prepared as previously described (3) and depleted of adherent cells by passage through nylon wool when positive selection of T cell subsets was conducted.

Isolation of lymphocyte subsets

Positive selection of $\gamma\delta^+$ T cells was conducted by fluorescence-activated cell sorting (Epics Elite, Coulter Electronics, Hialeah, FL), which routinely yielded preparations of >98.5% purity. Similarly, $\alpha\beta^-$ (γδ-enriched) or $\gamma\delta^-$ (αβ-enriched) lymphocyte subsets were purified by negative selection, gating out cells staining with either anti-αβ-PE or anti-γδ-PE; depleted cell preparations contained ≤2% and ≤0.2% stained cells respectively. Double staining of the $\gamma\delta^+$ T cells was conducted as previously described (12) using the V65 Ab, PE-conjugated donkey $F(ab')_2$ anti-mouse IgG and an OX8-FITC direct conjugate prepared in our laboratory. The accessory cell population (T cell-depleted splenocyte populations) used to generate the data in Table I were prepared by negative selection; i.e., gating out the OX19-FITC-positive population.

Adoptive transfer procedures

Variable numbers of cells (see text) were injected i.p. into naive recipients, who were concomitantly challenged with Ag and aluminum hydroxide and bled over the time course of the IgE/IgG response.

Cell cultures

Splenocytes were cultured at 4×10^5 per microplate well in RPMI containing 10^{-5} M 2-ME plus antibiotics supplemented with 5% normal rat serum and stimulated with 100 μg/ml OVA. Supernatants were harvested after 24 h and frozen at −20°C until assayed for cytokines (3).

IL-2 assay

The standard bioassay employing the CTLL-2 cell line was used to quantify IL-2 in culture supernatant; the standard curve was generated from dilutions of human rIL-2 (Cetus Corporation, Emeryville, CA).

INF-γ assay

INF-γ levels were determined by an ELISA method as detailed in (11), and standardized against rat rINF-γ (all reagents were kindly provided by Dr. P. Van der Meide, TNO, Rijswijk, The Netherlands).

Results and Discussion

We have shown previously that repeated exposure of rats to a 1% OVA aerosol results in the selective suppression of IgE responses in these animals when subsequently challenged i.p. with OVA/AH, while their capacity to respond to the Ag by IgG Ab production is preserved (1). Adoptive transfer studies have shown that the cells mediating immune deviation resulting in "tolerance" in the IgE isotype are $CD8^+$ T cells (3) which are MHC-class I restricted and responsive to soluble OVA (4). Moreover, preliminary evidence suggested that the $CD8^+$ cells in this system lacked αβ-chains in the TCR (12), but the negative selection technique employed (viz. depletion of TCR-$\alpha\beta^+$ T cells) was unable to definitively distinguish between the dual possibilities that effector cells were conventional $CD8^+$ T cells with down-modulated αβ-chains (via recent activation), vs γδ T-cells.

The experiments in Figure 1 address this issue, employing the recently developed antirat TCR-γδ Ab V65 (8). Thus, adoptive transfer of 10^7 unfractionated splenocytes mediates profound suppression of primary IgE responses in naive recipients (data column 2; see column 1), and this suppressive activity is restricted entirely to the $CD8^+$ subset (3, 4). γδ T cells normally constitute 1.5 to 4% of rat splenocytes in the Brown Norway strain. However, exclusion of γδ T cells from the adoptive transfer population by cell sorting (data column 7) ablates suppressive activity, and full suppressive activity is seen employing as few as 10^3 positively selected γδ T cells (column 5). Double staining of the positively selected γδ T cell population shows that they are all also CD8 positive (Fig. 2).

As demonstrated previously (4, 13), overall IgG-anti-OVA-titers were unaffected in adoptive recipients (Fig. 1); however, anaphylactic IgG titers in the order of 5.0 $\log_2$ units (putative IgG_{2b}; 14) were observed in controls, but were absent in recipients of γδ T cells (Fig. 1), suggesting coregulation of IgE and IgG_{2b} in rat analogous to that seen for IgE and IgG1 in the mouse (15).

Figure 3 illustrates the Ag specificity of this regulatory process. It can be seen that adoptively transferred unfractionated splenocytes or positively selected γδ T cells from OVA-tolerant donors suppress primary IgE responses to OVA, but not the irrelevant Ag Der p1; it should be noted

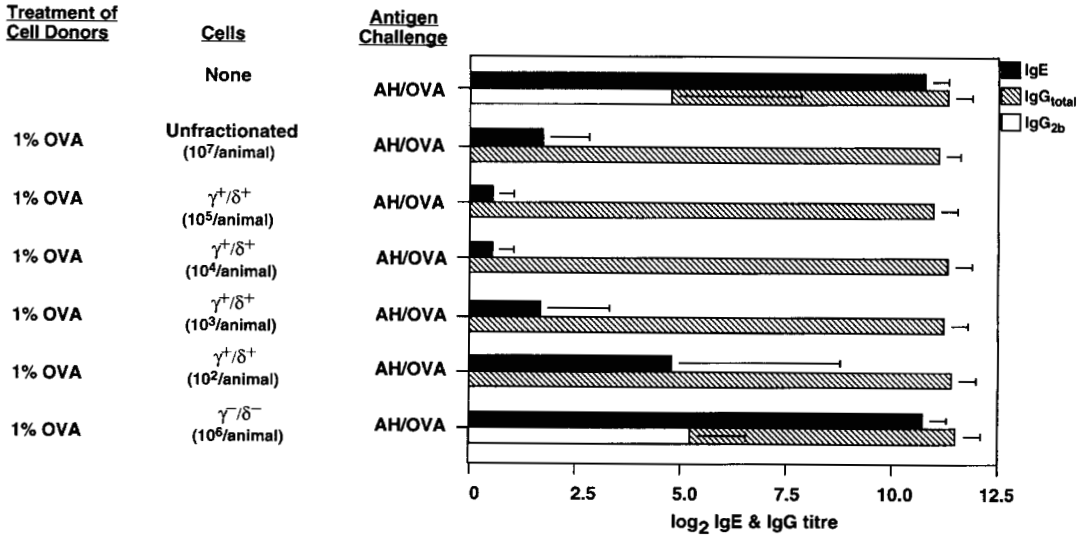

FIGURE 1. IgE and IgG responses to OVA following adoptive transfer of γδ T cells. Dose-response analysis of adoptive transfer of OVA-specific IgE and IgG responses by positively selected γδ T cells from OVA-tolerant rats to naive rats with concomitant OVA/AH challenge. Data shown are X ± SD (n = 5 to 10 per group) at day 14 (peak primary IgE response) and day 21 (peak primary IgG response) post-challenge and indicate reciprocal $\log_2$ IgE, IgG (total), and IgG_{2b} anti-OVA titers determined by PCA, HA, and modified PCA respectively. * < controls; $p < 0.01$.

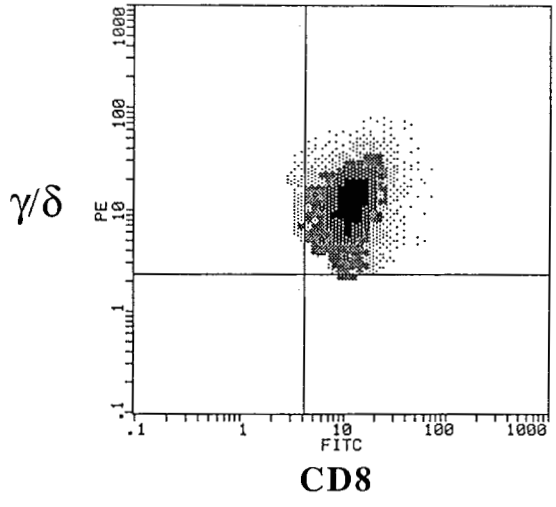

FIGURE 2. Double staining profile of post-sorted γδ T cells. Nylon wool prepared splenocytes were double stained with anti-γδ (PE) and anti-CD8 (FITC) Abs. Cells were selected by positive selection using forward- and side-scatter lymphocyte gates, as well as γδ staining. The post-sorted γδ T cells' staining profile for CD8 and γδ is shown as a dot plot.

that the number of γδ T cells transferred here represented >100-fold the threshold level (see Fig. 1) required for suppression of the anti-OVA response.

Recent experiments employing this model indicated that the predominant cytokine released in vitro by OVA-tolerant (unfractionated) splenocytes in response to OVA challenge was IFN-γ, and further, that the low-level IL-2 response of these cells was boosted by depletion of endogenous $CD8^+$ cells (4). Comparable data are shown in Table I. Thus, depletion of γδ T-cells from the splenocyte population on the one hand ablates the strong OVA-specific IFN-γ response, while simultaneously unmasking underlying capacity for a vigorous OVA-specific IL-2 response from cell(s) within the αβ subset, which was apparently suppressed by the presence of γδ T-cells. We have also shown previously that depletion of the "suppressor" cell in this system unmasks capacity for proliferation in response to OVA (4). Collectively, these findings seem to be consistent with a recent report suggesting an important role for γδ T cells in limiting αβ T cell proliferation and IL-2 production (16).

It has recently been reported that γδ T cell receptors are capable of recognizing Ag directly, in the absence of Ag processing (17). However, in the system described herein, highly purified γδ T cells require the presence of exogenous APCs to respond to soluble Ag in vitro (Table I).

An increasing number of reports point to potential immunoregulatory role(s) for γδ T cells in αβ T cell-dependent immune responses, including provision of "help" for secretory IgA responses in the gut (18), and similar activities in relation to adoptive transfer of contact hypersensitivity (19) and in in vitro αβ T cell responses to microbial Ags (20); additionally, γδ T cells have been shown to produce a wide range of cytokines after stimulation (21), in particular IFN-γ (22, 23). The latter activity (illustrated in Table I) provides a plausible mechanism for selective IgE suppression in this model via $CD8^+$ γδ cell-mediated inhibition of the growth of IL-4 secreting Th2 cells (24) during the response to inhaled OVA.

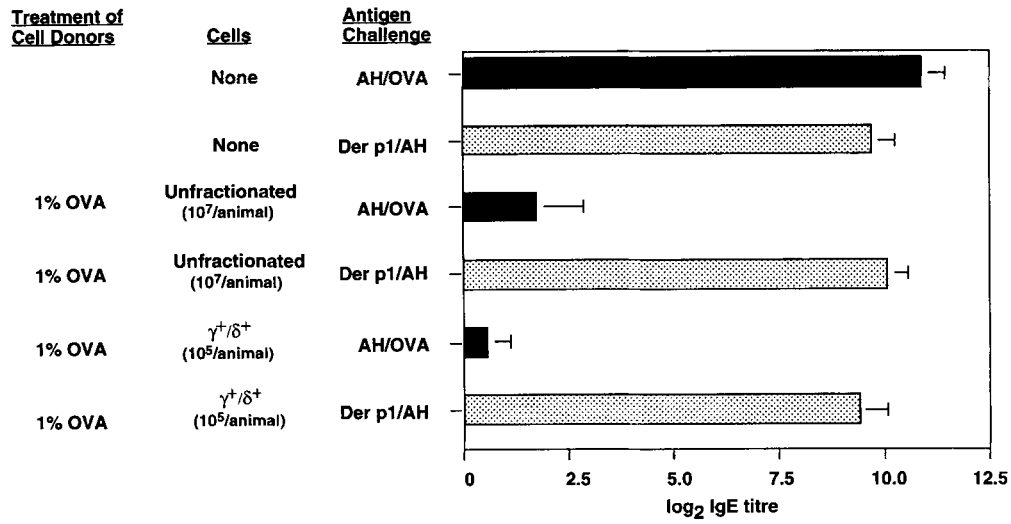

FIGURE 3. Ag specificity of γδ T cells in suppression of primary IgE responses. Unfractionated or positively selected γδ T cells from OVA-tolerant rats were adoptively transferred, and recipients challenged with OVA or the irrelevant Ag Der p1 (kindly supplied by Dr. W. Thomas, Institute for Child Health Research, Perth, Western Australia). Data shown are X ± SD (n = 5 to 10 per group) at day 14 (peak primary IgE response) post-challenge and indicate reciprocal $\log_2$ IgE anti-OVA or anti-Der p1 titers determined by PCA. * < controls; $p < 0.01$.

However, it is unclear how this potent suppression is achieved in vivo with such low numbers of adoptively transferred $CD8^+$ γδ T cells. Possible mechanisms include rapid expansion in adoptive recipients following challenge, but this appears unlikely on the basis of recent studies indicating the limited growth potential of $CD8^+$ γδ T cells after activation and differentiation (25); an alternative possibility (yet to be tested) is that they function via recruitment of more numerous secondary effectors. It is also not known whether these γδ T cells are derived from the small population found in lymphoid organs, or from the more numerous mucosal γδ population found in rodents. However, recent experiments employing the V65 Ab to immunostain γδ T cells in situ have detected a significant population in the bronchial-associated lymphoid tissue of rats, but failed to detect γδ T cells in the airway mucosa (our unpublished observations). In this context, our earlier studies indicate that the initial activation of Ag-specific $CD8^+$ T cells during the immune response to inhaled OVA occurs in the regional lymph nodes draining the upper respiratory tract, followed by later dissemination to other lymphoid organs (3, 4). Further experiments are required to clarify the relationship between $CD8^+$ γδ T cell populations at these different sites. Future studies will also address the issue of possible interspecies variation in the functions of these cells. Recent experiments performed here in C57 Black mice (26) indicate the operation of a comparable mechanism in this species, but no data are currently available on the role of $CD8^+$ γδ T cells in regulation of allergen-specific IgE responses in humans.

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