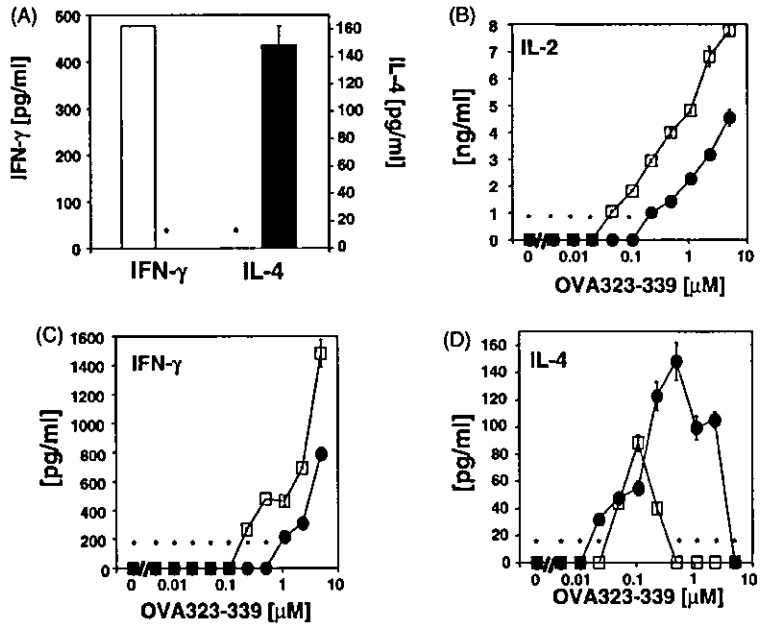

Fig. 2. A326V modified cytokine secretion profile of naive CD4$^+$ T cells induced by various concentrations of OVA323-339. (A) Naive CD4$^+$ T cells and APCs were cultured with 0.5 μM OVA323-339 in the absence (open bar) or presence (closed bar) of A326V at 50 μM. (B–D) Naive CD4$^+$ T cells were stimulated with various concentrations of OVA323-339 in the absence (open square) or presence (closed circle) of A326V at 50 μM. The levels of IL-2 (B), IFN-γ (A and C), and IL-4 (A and D) in each culture supernatant were determined by means of ELISA. Detection limits were 750 pg/ml for IL-2, 188 pg/ml for IFN-γ, and 31 pg/ml for IL-4. *Not detected. The data are shown as the average from triplicate cultures ± S.D. The results shown are representative of more than three independent experiments.

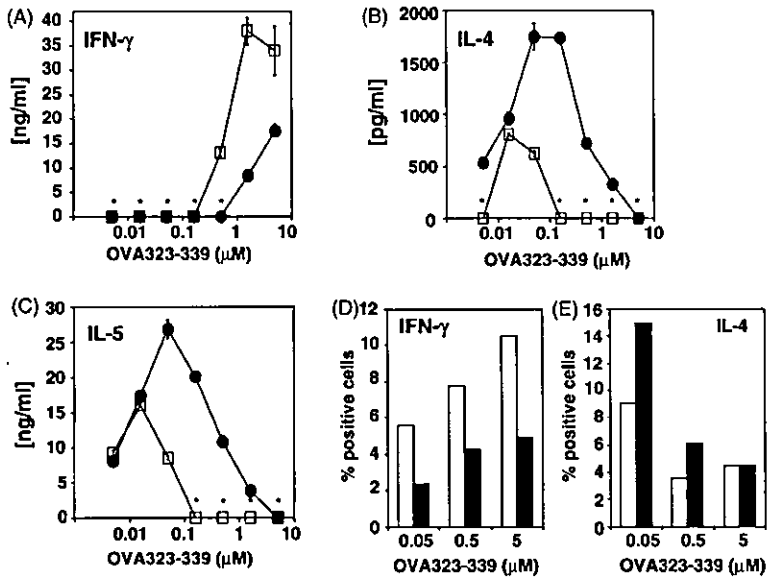

Fig. 3. A326V modified Th1/Th2 differentiation of naive CD4$^+$ T cells. (A–C) Naive CD4$^+$ T cells were primed with various concentrations of OVA323-339 plus APCs in the absence (open square) or presence (closed circle) of A326V at 50 μM. After 7 days of priming, viable cells were recovered and re-stimulated for 48 h with 5 μM OVA323-339 plus APCs. Supernatants were analyzed for levels of IFN-γ (A), IL-4 (B), and IL-5 (C) by means of ELISA. No cytokine was detected when cultured in the absence of OVA323-339. Detection limits were 11.3 ng/ml for IFN-γ, 225 pg/ml for IL-4, and 3.8 ng/ml for IL-5. *Not detected. The data are shown as the average from triplicate cultures ± S.D. The results shown are representative of more than three independent experiments. (D and E) Naive CD4$^+$ T cells were primed with OVA323-339 at 0.05, 0.5, or 5 μM in the absence (open bars) or presence (closed bars) of A326V at 50 μM. After 7 days, viable cells were stimulated with PMA plus ionomycin in the presence of monensin for 6 h. Cells were fixed, permeabilized, and stained for intracellular IL-4 and IFN-γ. The staining profile was analyzed by FACS and percentages of IL-4$^+$ (D) and IFN-γ$^+$ (E) CD4$^+$ T cells were shown. The results shown are representative of two independent experiments.

presence of A326V. These results show that the TCR antagonist induced not only a shift in the antigen-dose-dependent profile of Th1/Th2 cytokine secretion by naive CD4+ T cells, but also an increase of Th2-type cytokine responses.

Fig. 3 shows the secondary cytokine responses of differentiated T cells generated by priming with OVA323-339 plus A326V for 1 week. In the presence of A326V at 50 μM together with the indicated concentrations of OVA323-339 in 1-week primary cultures of naive CD4+ T cells, IFN-γ secretion induced by subsequent stimulation with OVA323-339 alone was decreased (Fig. 3A). As for Th2-type cytokines, IL-4 and IL-5, the dose of OVA323-339 eliciting the highest levels of cytokine secretion was shifted from 0.015 to 0.05 μM in the presence of A326V and the maximum levels of the cytokines secreted were significantly increased (Fig. 3B and C). We further investigated Th1/Th2 differentiation at the single cell level, by staining intracellular IL-4 and IFN-γ production. The presence of A326V in the primary culture decreased the development of IFN-γ-producing cells (Fig. 3D) but enhanced the differentiation of IL-4-producing cells (Fig. 3E). These results show that, as expected from the primary response of naive CD4+ T cells, the TCR antagonist could also affect the Th1/Th2 differentiation profile.

3.3. A326V modified expression of GATA-3 mRNA

GATA-3 is an important transcription factor in Th2 cells. We have previously demonstrated that the expression of GATA-3 mRNA was altered depending on the dose of OVA323-339 and corresponded to that of IL-4 mRNA [13]. Fig. 4 shows that A326V altered the profile of GATA-3 mRNA expression in naive CD4+ T cells stimulated with varying doses of OVA323-339 in a similar manner to that seen with IL-4 secretion by naive CD4+ T cells. These results suggest that A326V may modify Th2 response by altering the expression of GATA-3.

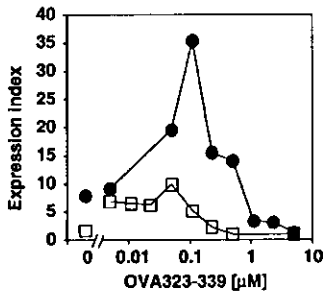

Fig. 4. A326V modified expression of GATA-3 mRNA by naive CD4+ T cells. Naive CD4+ T cells were cultured with 0.005–5 μM OVA323-339 plus APCs in the absence (open square) or presence (closed circle) of A326V at 50 μM. Cells were harvested 24 h following stimulation, and total RNA was prepared. Expression of the GATA-3 mRNA was analyzed by quantitative real-time RT-PCR, and values were normalized to the level of GAPDH mRNA. The results shown are representative of three independent experiments.

4. Discussion

In the present study, we demonstrate that a TCR antagonist has the potential to change the balance of Th1/Th2 cytokine secretion of naive CD4+ T cells in response to the original antigenic peptide. Furthermore, the TCR-antagonist peptide, which inhibited the proliferation of naive CD4+ T cells, significantly enhanced secretion of IL-4 by naive CD4+ T cells and their Th2 differentiation induced in the presence of a specific dose of the original peptide. These results suggest that, for naive CD4+ T cells, a TCR antagonist does not simply inhibit all of the T-cell responses as observed in assays using T-cell clones [18,19], but rather modifies the TCR-mediated signals so as to enhance at least one aspect of the T-cell responses.

To date, two potential mechanisms of TCR antagonism have been proposed. One hypothesis is that TCR antagonists directly interfere with the generation of a TCR-mediated signal by the original antigenic peptide by effectively preventing TCR oligomerization and functional TCR triggering [21]. Another possibility is generation of a negative signaling pathway by the antagonist that suppresses the activation pathway induced by the original antigenic peptide [22,23]. The latter hypothesis is supported by studies using T cells expressing two distinct TCRs having distinct antigenic specificity as a model system [24,25]. Using this model, it has been shown that a TCR antagonist for one TCR inhibited T-cell responses induced by signals mediated by the other TCR. However, opposing results have also been reported using similar experimental systems [26,27]. Thus, the mechanism of TCR antagonism is still controversial.

Our findings that a TCR antagonist increased the dose of the original antigenic peptide required to induce T-cell responses, equivalent to those induced in the absence of the antagonist (Fig. 2B–D), may be explained by the interference of generation of TCR-mediated signaling and/or the generation of negative signaling, leading to inhibition of all TCR-mediated signaling. In contrast, the notable increase in Th2 responses accompanied by the increase of GATA-3 mRNA expression in the presence of a TCR antagonist cannot be explained by these mechanisms, but seems to be explained by the generation of some negative or positive signaling, partially modifying TCR signaling. A recent study demonstrated that reduction of Erk activity led to a dramatic increase in IL-4 production of naive CD4+ T cells and Th2 generation [28]. Thus, it is possible that TCR-mediated signaling induced by the TCR antagonist specifically inhibited activation of Erk.

Several physiological roles of endogenous TCR antagonists have been proposed. TCR antagonists have been reported to be involved in positive selection in the thymus [29–32]. Furthermore, several studies have demonstrated an important role for TCR antagonists in supporting survival and homeostatic proliferation of mature T cells in the periphery [33–35]. Our findings suggest that endogenous peptides functioning as TCR antagonists for CD4+ T cells would af-

fect the Th1/Th2 response of these cells. Some self-peptides with TCR-antagonist activity for T cells specific for an allergen would have an influence on the onset and/or deterioration of the allergic reaction to the allergen by means of enhancement of Th2 responses.

In conclusion, we demonstrate here that a TCR antagonist for a naive $CD4^+$ T cell can affect its Th1/Th2 cytokine responses and functional differentiation and can enhance IL-4 secretion and Th2 differentiation. This indicates that, for naive $CD4^+$ T cells, TCR antagonists do not inhibit or down-regulate all responses to the original antigenic peptide and even enhance at least one aspect of the T-cell response. Analysis of the alteration of TCR-mediated signals in naive $CD4^+$ T cells induced in the presence of TCR antagonists would enhance our understanding of the mechanism of TCR antagonism.

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Ca^{2+} signaling down-regulates TGF-β1 gene expression in $CD4^+$ T cells

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Received 11 November 2004
Available online 15 December 2004

Abstract

In the immune system, TGF-β1 exerts two major functions, anti-inflammatory and immuno-suppressive effects. This work aims to investigate the molecular mechanisms involved in the regulation of the TGF-β1 gene expression in $CD4^+$ T cells. The TGF-β1 gene expresses three transcripts of 2.5, 1.9, and 1.4 kb. The 1.9 kb mRNA which has the highest translation activity was the major transcript. The relationship between T cell receptor (TCR) stimulation and the expression of the gene was investigated. TCR stimulation with a low dose of antigen peptide enhanced the gene expression, whereas a higher dose suppressed the expression. TCR stimulation activates PKC/MAPK and Ca^{2+} signaling pathways. PMA increased the gene expression, whereas ionomycin decreased the gene expression, markedly. The results indicate that Ca^{2+} signaling down-regulates TGF-β1 gene expression. The molecular regulation of TGF-β1 gene expression is unique when comparing to other cytokine genes which are generally activated by Ca^{2+} signaling.

Keywords: Anergic T cells; Ca^{2+} signaling; $CD4^+$ T cells; TGF-β1

Transforming growth factor-β1 (TGF-β1) is an immuno-inhibitory cytokine increasingly recognized as a key factor for immuno-regulation. In the immune system, TGF-β1 has been shown to have two major functions, anti-inflammatory and immuno-suppressive effects [1]. TGF-β1 producing T cells are limited to Th3 cells functioning in oral tolerance [2] and some regulatory T cells [3]. In addition, TGF-β1 has been implicated in participating in apoptosis [4,5]. Apoptotic T cells release both latent and active TGF-β1, so that tissues or cells near the apoptotic cells may be able to avert inflammation and tissue damage. It is thus of considerable interest to investigate the molecular mechanisms involved in TGF-β1 production from T cells for designing strategies to control unwanted immune responses such as autoimmunity, inflammation, and allergy.

The regulatory mechanisms governing the several steps from the transcription of the TGF-β1 gene to secretion of TGF-β1 from T cells are complicated and are poorly understood. Gene expression of T cell cytokines is, in general, induced by TCR-mediated signals together with signals dependent on co-stimulatory molecules [6] or exogenous cytokines [7]. In contrast, signals that induce the transcription of the TGF-β1 gene are not well documented. Stimulation through TCR activates protein kinase C (PKC), mitogen activated protein kinase (MAPK), and Ca^{2+} signaling pathways. Several reports suggest that activation of the PKC pathway enhances TGF-β1 secretion [8], however detailed molecular relationship between TCR signals and the expression of TGF-β1 mRNA is not well understood. Moreover, post-translational regulation involved in TGF-β1 production makes the mechanisms even more complicated. For example, some T cells may store TGF-β1 in the intracellular membrane-bound compartments (e.g.,

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doi:10.1016/j.bbrc.2004.12.029

mitochondria) and release the cytokine depending on changes in environments or outer stimuli [4].

The TGF-β1 gene lacking the TATA box is expressed in every leukocyte lineage. This nature of the gene makes the mode of the gene expression mechanism different from those of other cytokine genes such as IL-2, IL-4, IL-10, and IFN-γ. The reports using non-immuno-competent cells suggest that the TGF-β1 gene expresses three distinct transcripts of 2.5, 1.9, and 1.4 kb. The 2.5 kb mRNA that is the major transcript in many cells carries a highly GC rich 5′ untranslated region (UTR) of ~900 bases [9]. The UTR has been shown to be critical for translational regulation. The 1.4 kb transcript starts 25 bases upstream of the initiator codon AUG, whereas the 1.9 kb transcript has two start sites 366 and 401 bases upstream of the AUG codon [10]. The 2.5 and 1.4 kb transcripts appear to be poorly translated. In contrast, the 1.9 kb transcript is highly efficiently translated [10]. However, critical studies in terms of the relationship between immune response and TGF-β1 transcripts have not been reported.

In the present study, we focused on the relationship between TCR signals and the expression of TGF-β1 mRNA in $CD4^+$ T cells toward a better understanding of TGF-β1 expression in immune response. We present evidence that strong TCR stimulation down-regulates the expression of TGF-β1 mRNA and the activation of the Ca^{2+} signaling pathway causes a marked decrease in TGF-β1 gene expression.

Materials and methods

Mice. BALB/c mice were purchased from Japan CLEA (Tokyo, Japan). DO11.10 (DO) transgenic (Tg) mice carrying a TCR specific for ovalbumin peptide (OVAp) were kindly provided by M. Kubo (Research Institute for Biological Sciences, Tokyo University of Science, Chiba, Japan).

Cell culture, anergy induction, and cell stimulation. $CD4^+$ T cells were purified using CD4 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) as previously described [11]. The purity of $CD4^+$ T cells was routinely estimated to be 94–98%. In some experiments, these $CD4^+$ T cells were differentiated into Th1 cells by a standard protocol [12]. Anergy was induced by treatment of Th1 cells (1×10^6 cells/ml) with 1 μM ionomycin for 16 h. For the experiments in Fig. 1, a Th2 clone (68-41) was used. For the experiments in Fig. 2, naïve $CD4^+$ T cells from DO Tg mice were stimulated with 0–5 μM OVAp. For the experiments in Fig. 3, a thymoma $CD4^+$ T cell line (EL-4) or naïve $CD4^+$ T cells were stimulated with ionomycin (50 nM) or phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) alone, or with both of them. For the experiments in Fig. 4, anergy induced Th1 cells were stimulated with anti-CD3 and anti-CD28 mAbs.

T cell proliferation assay. The T cell proliferation assay was performed in 96-well flat bottomed plates. $CD4^+$ T cells (5×10^4/well) in a total volume of 200 μl were stimulated with 0–5 μM OVAp in the presence of irradiated syngeneic spleen cells (2×10^5/well). Cells were cultured for 54 h. Proliferation was assessed by measuring the incorporation of [^{3}H]thymidine (1 μCi/well) added for the final 18 h of culture.

Real-time RT-PCR. Cells were harvested at indicated time points, and the total RNA was prepared using ISOGEN (Nippon Gene). The total RNA was treated with deoxyribonuclease I (Invitrogen), and cDNA was synthesized using oligo(dT) primers and Superscripts polymerase (Wako). The Light Cycler PCR and real-time detection

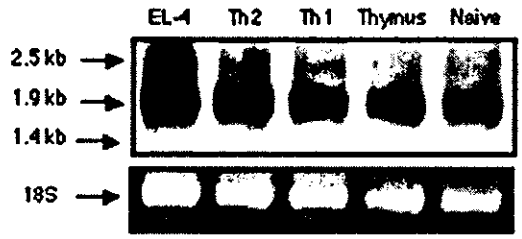

Fig. 1. Transcripts of the TGF-β1 gene. Total RNA was prepared from non-activated EL-4 cells, Th1 cells, the 68-41 Th2 clone, thymocytes, and naïve $CD4^+$T cells. The expression levels of TGF-β1 mRNA were determined by Northern blotting. The lower panel shows ethidium bromide (EtBr)-stained 18s rRNA as a control for equal loading.

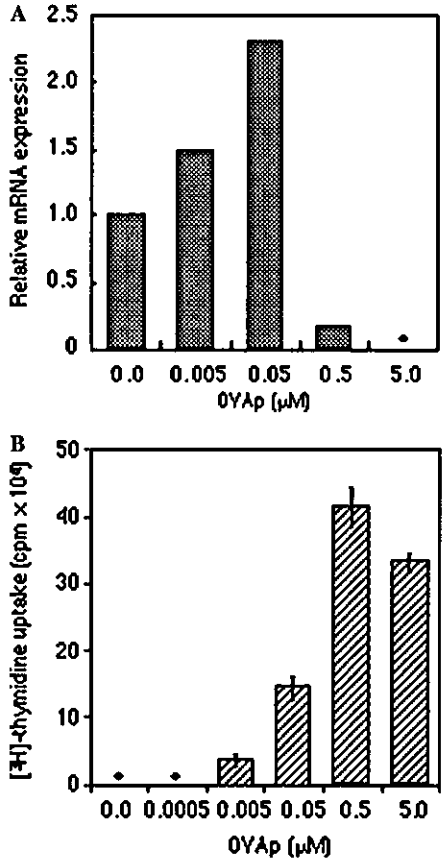

Fig. 2. Strong antigenic stimulation down-regulates TGF-β1 mRNA expression. (A) Naïve $CD4^+$ T cells derived from DO Tg mice were stimulated with varying dose of OVAp in the presence of antigen presenting cells (APCs) prepared from BALB/c mice spleen for 24 h. The expression levels of TGF-β1 mRNA were determined by real-time PCR. The results shown are representative of two independent experiments. (B) Naïve $CD4^+$ T cells derived from DO Tg mice were stimulated with varying dose of OVAp in the presence of APCs prepared as described above. Proliferative response was measured by [^{3}H]thymidine incorporation.

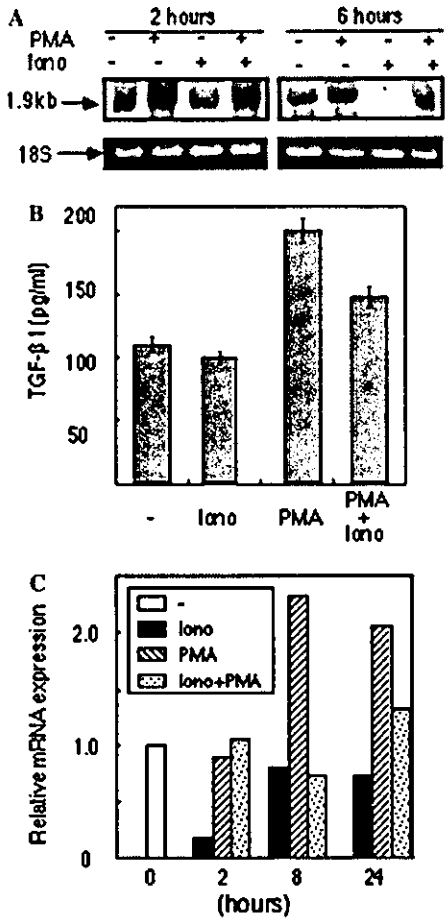

Fig. 3. Ca^{2+} signaling down-regulates TGF-β1 mRNA expression. (A) EL-4 cells were stimulated with ionomycin (50 nM) or PMA (100 ng/ml) alone, or with both of them, Cells were harvested after 2 and 6 h. The expression levels of TGF-β1 mRNA were determined by Northern blotting. The results shown are representative of three independent experiments. The lower panel shows EtBr-stained 18s rRNA as a control for equal loading. (B) EL-4 cells were stimulated as described above. Culture supernatants were recovered after 24 h and the concentration of TGF-β1 was measured. The data are shown as the average from triplicate cultures (±SD). (C) Naïve $CD4^+$ T cells were stimulated with ionomycin (200 nM) or PMA (50 ng/ml) alone, or with both of them. Cells were harvested after 2–24 h. Total RNA was prepared and the expression levels of TGF-β1 mRNA after 2, 8, and 24 h stimulation were determined by real-time PCR. The results shown are representative of three independent experiments.

system (Roche) was used for amplification and in-line quantification. The pairs of primers used were as follows: glyceraldehyde-3-phosphate dehydrogenenase (GAPDH) sense, 5′-TGAAACGGGAAGCTGG-3′; GAPDH antisense, 5′-TCCACCACCCTGTTTGGTGTA-3′; TGF-β1 sense, 5′-CCAAGGAGACGGAATACAGG-3′; TGF-β1 antisense, 5′-GTTCATGTCATGGATGGTGC-3′. The hybridization probe format was used to quantify the amplified fragment. Hybridization probes consisted of two different short oligonucleotides that hybridize close to each other in an internal sequence of the amplified fragments during the annealing phase of PCR cycles. One probe was labeled at the 5′-end with the Light Cycler Red 640 (LC640) fluorophore, and the other was labeled 3′-end with FITC. The pairs of hybridization probes used were as follows: GAPDH, 5′-CTGAGGACCAGGTTGT TGTCTCCTGCGA-FITC 3′ and 5′-Red 640-TTCAACAGCAACT

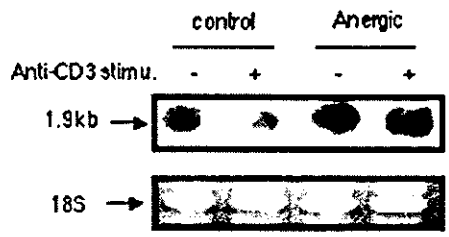

Fig. 4. Expression of TGF-β1 mRNA in anergic T cells after TCR stimulation. Th1 cells and anergy induced Th1 cells were stimulated with anti-CD3 and CD28 mAbs. Cells were harvested after 3 h to prepare the total RNA. The expression levels of TGF-β1 mRNA expression were determined by Northern blot analysis. The results shown are representative of two independent experiments. The lower panel shows EtBr-stained 18s rRNA as a control for equal loading.

CCCACTCTTCCACC-3′; TGF-β1, 5′-GTGACAGCAAAGATAA CAAACTCCAC-FITC 3′ and 5′-Red 640-TTCAACAGCAACTCCC ACTCTTCCACC-3′. Normalization was calculated by assessing the amounts of mRNA for GAPDH as an internal control for each sample.

Northern blot analysis. Cells were harvested at indicated time points, and the total RNA was prepared using ISOGEN (Nippon Gene). Equal amounts of RNA samples (3–5 μg) were fractionated on 1% agarose gels containing 2.4% formaldehyde and then transferred to positively charged nylon membranes. After fixation under calibrated ultraviolet irradiation, the membranes were hybridized with dioxigenin (DIG)-labeled riboprobes and visualized using alkaline phosphatase-labeled anti-DIG antibody following the manufacturer's instructions (Roche). For preparation of a TGF-β1 probe, an *Eco*RI fragment of mouse TGF-β1 cDNA was cloned into Bluescript (Statagene) as previously described. DIG-labeled riboprobe was synthesized using a DIG-RNA labeling kit (Roche), following the manufacturer's instructions.

Measurement of TGF-β1 concentration. EL-4 cells (1×10^6) in a total volume of 1 ml were stimulated with A23187 (100 nM) or PMA (50 ng) alone, or with both of them for 24 h. Concentration of TGF-β1 in culture supernatant was determined by ELISA using anti-TGF-β1 mAbs (A75-2.1) and biotin-A75-3.1 purchased from PharMingen (San Diego, CA).

Results

The 1.9 kb mRNA is the major transcript in T cells

Initially, we determined the molecular sizes of the TGF-β1 transcripts in non-activated T cells (EL-4, Th1 line, Th2 clone, thymocytes, and naïve $CD4^+$ T cells), since the gene transcription produces three different sizes of mRNA (1.4, 1.9, and 2.5 kb). The results suggest that the TGF-β1 gene is transcribed constitutively (Fig. 1). Interestingly, the expression levels of the 2.5 and 1.4 kb transcripts, in general, were marginal in these T cells, although EL-4 cells expressed a relatively stronger band of 2.5 kb. The 1.9 mRNA that is supposed to have a most efficient translation activity was the major transcript. Based on these results, we concentrated on the analyses of the 1.9 kb transcript in the following experiments.

Strong antigenic stimulation down-regulates the expression of TGF-β1 mRNA

The structure of the TGF-β1 gene promoter has been extensively characterized [13], however little is known about the molecular mechanisms regulating the expression of the gene transcripts in $CD4^+$ T cells. We measured the expression levels of TGF-β1 mRNA of OVA specific naïve T cells prepared from DO11.10 (DO) transgenic Tg mice after stimulation with various doses of OVAp by real-time PCR analysis. The addition of a relatively low dose of OVAp (up to 0.05 μM) enhanced its transcription levels, whereas a higher dose (more than 0.05 μM) suppressed the expression dramatically (Fig. 2A). Proliferation of T cells required a higher dose of OVAp (more than 0.05 μM) (Fig. 2B). Thus, the expression of TGF-β1 mRNA was inversely correlated with proliferation. The results suggest that TCR stimulation strong enough for clonal expansion of T cells down-regulates the expression of the TGF-β1 gene.

Activation of Ca^{2+} signaling pathway down-regulates TGF-β1 mRNA expression

TCR stimulation activates PKC/MAPK and Ca^{2+} signaling pathways [14,15]. Addition of PMA which is a PKC activator and ionomycin (a calcium ionophore) is able to mimic TCR stimulation. Weak or partial TCR stimulation is often associated with impaired Ca^{2+} signaling. These results prompted us to speculate that TGF-β1 mRNA down-regulation is accompanied by full activation in the Ca^{2+} signaling pathway. To this end, initially we utilized EL-4 cells due to the following reasons. It is known that EL-4 cells produce TGF-β1 that can be detected by ELISA [16]. Then, we will be able to investigate the relationship between the transcription of the TGF-β1 gene and the released TGF-β1 protein from the T cells with relative ease, unlike primary T cells.

EL-4 cells constitutively expressed the TGF-β1 transcripts and the addition of PMA enhanced the transcription levels in the time course assay (Fig. 3A). In contrast, ionomycin treatment resulted in a marked decrease in TGF-β1 mRNA expression after 6 h. In addition, the combination of PMA and ionomycin caused a decrease in the transcription levels after 6 h, but to a lesser extent compared to ionomycin alone. Next, we assessed the concentration of released TGF-β1 after 24 h of stimulation by ELISA (Fig. 3B). PMA enhanced the amount of secreted TGF-β1, whereas the addition of ionomycin plus PMA induced a decrease in the concentration of TGF-β1. Stimulation with ionomycin alone caused a decrease in the TGF-β1 level, but not to a great extent, suggesting that intracellular TGF-β1 was released as described by Chen et al. [5]. In general, the ELISA results were not contradictory to the Northern blot results.

Then, we examined the effects of PMA and ionomycin on the expression of the TGF-β1 transcripts in primary T cells to confirm the EL-4 results (Fig. 3C). Naïve $CD4^+$ T cells were stimulated with PMA or ionomycin alone, or with both of them. The expression levels were assessed by a real-time PCR method. PMA increased the expression of TGF-β1 mRNA more than twofold after 8 h. Ionomycin caused a significant decrease in TGF-β1 mRNA expression in the 2 h assay, but recovered to its original level in 8 h. Addition of PMA and ionomycin caused a significant decrease compared to the addition of PMA alone after 8 and 24 h. Thus, the naïve $CD4^+$ T cell results were reminiscent of the EL-4 results.

Expression of TGF-β1 mRNA is not down-regulated in anergic T cells

Anergic T cells often demonstrate impaired Ca^{2+} signaling [17,18]. Thus, treatment of primary Th1 cells with ionomycin is a standard method to tolerize T cells. Rao et al. [18] reported that T cells anergized by this method demonstrate a strong impaired Ca^{2+} mobilization. The transcription of the TGF-β1 gene was examined in T cells anergized by pre-treatment of ionomycin after 24 h (Fig. 4). Proliferative response of these T cells diminished as described by Macian et al. [19] (data not shown). Stimulation of primary T cells with anti-CD3 mAb suppressed the expression of TGF-β1 mRNA. In contrast, TCR stimulation with anti-CD3 mAb did not diminish TGF-β1 mRNA expression in anergic T cells either. The amount of TGF-β1 produced from these primary T cells was below the detection level by ELISA.

Discussion

TGF-β1 is a pleiotropic growth factor exerting different functions on cell growth and differentiation depending on cell type [1]. In the immune system, TGF-β1 is one of the most important immuno-suppressive cytokines produced from T cells, however its behavior in several T cell responses is not well understood. Therefore, it is of interest to investigate the molecular mechanisms involved in TGF-β1 gene expression. In the current study, we carried out a series of experiments to clarify the relationship between TGF-β1 expression and T cell responses including Ca^{2+} metabolism, proliferation, and anergy. The TGF-β1 gene was constitutively transcribed from naïve T cells and the major transcript was 1.9 kb which has the most efficient translation activity. In our experiments, the expression levels of the 2.5 and 1.4 kb transcripts were marginal, however other reports on non-immunological cells demonstrate that the 2.5 kb mRNA is the major transcript. The reason for this discrepancy is not clear.

TGF-β1 mRNA expression was accelerated with an antigen dosage that was too low for T cell proliferation, whereas a higher antigen dosage inducing T cell proliferation caused a marked suppression in the gene expression. The gene expression profile contrasts with those of other cytokine genes involved in Th1/Th2 cells. In addition, we and other investigators have demonstrated that antigen peptides with weak binding affinity or altered peptide ligands (APLs) are able to induce the expression of the TGF-β1 gene in T cells [20–23]. The involvement of Ca^{2+} signaling in the regulation of TGF-β1 gene expression has been reported. Stimulation of T cells with peptides with weak binding affinity or APLs induces a decrease in proliferation and impaired Ca^{2+} signaling [20,24,25]. T cells generated in oral tolerance produce TGF-β1, and generally the proliferative response of these T cells is low [26]. Recently, we reported that the level of Ca^{2+} influx of T cells derived from orally tolerized mice was diminished [17], suggesting that the level of Ca^{2+} signaling is low in TGF-β1 producing T cells. These results suggest that the expression of the TGF-β1 gene is enhanced in T cells with the impaired Ca^{2+} signaling mechanism. Strong TCR stimulation with anti-CD3 mAb to in vitro anergized T cells did not cause a decrease in TGF-β1 gene expression. As described above, the anergic T cells carry an impaired Ca^{2+} mobilization mechanism. It is plausible that the regulatory mechanism of the TGF-β1 gene is not functioning in anergic T cells due to the impaired Ca^{2+} signaling.

The Ca^{2+} ionophore ionomycin decreased the expression of the TGF-β1 gene in EL-4 cells. However, the amount of TGF-β1 secreted from EL-4 cells did not show a concomitant reduction, suggesting that TGF-β1 stored inside the cell was released. T cells carry a certain amount of the cytokine in mitochondria and on the cell surface [4]. After some stimulation, TGF-β1 is released. It is also known that $CD4^+$ T cells release intracellular TGF-β1 by apoptotic signaling, so that apoptotic T cells could avoid an inflammatory response in the nearby environment [4,5]. Thus, it is difficult to judge the production levels of TGF-β1 from its gene transcription levels. Treatment of EL-4 cells with PMA and ionomycin induced a decrease in TGF-β1 concentration compared with PMA alone. The decreased amount of the released TGF-β1 protein reflected the decreased transcription level of the TGF-β1 gene. The TGF-β1 levels produced in T cells stimulated with PMA alone exceeded the production levels stimulated with PMA and ionomycin. The results suggest that the down-regulation of TGF-β1 transcription is one of the mechanisms involved in the observed decrease in TGF-β1 production.

What are the underlying biochemical reasons involved in the regulation of TGF-β1 gene expression by Ca^{2+} signaling? The transcription of the TGF-β1 gene is active constitutively in resting T cells. As well, it could be beneficial for T cells that a TCR signal inducing full activation of T cells elicits a decrease in TGF-β1 gene expression. The immuno-suppressive factor, TGF-β1, produced from T cells may quench the activation of the T cells. The production of the immuno-suppressive cytokine may also contribute to induction and maintenance of an anergic state. Our finding that a low dose stimulation of TCR by a specific antigen enhances the gene expression may support the results of the activation of TGF-β1 gene expression by partial or weak TCR stimulation.

PMA stimulation alone is able to accelerate TGF-β1 gene expression. This may suggest that the PKC signaling pathway regulates the gene expression positively. It is clear that impaired/weak Ca^{2+} signaling is associated with positive regulation of TGF-β1 gene expression. The possible involvement of Ca^{2+} signaling in the positive regulation of TGF-β1 gene expression requires further investigation. Mechanisms responsible for the down-regulation of the TGF-β1 gene are not well understood. One possibility is that the Ca^{2+} signaling pathway directly controls its promoter activity. This possibility is supported by the finding that several calcium responding elements exist in the promoter region of the TGF-β1 gene [13]. Another possibility is that the Ca^{2+} pathway controls the stability of the TGF-β1 mRNA. Previous reports on the effects of calcium metabolism on mRNA stability may support this hypothesis [27,28].

In summary, we have demonstrated that Ca^{2+} signaling down-regulates the expression of the TGF-β1 gene. TGF-β1 is one of the most important immuno-suppressive cytokines. Further elucidation of the molecular mechanisms involved in the positive and negative regulation of TGF-β1 gene expression will aid in the control of immune responses.

Acknowledgment

This work was supported in part by grants from the Japan Health Sciences Foundation and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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BioFactors 21 (2004) 159–162
IOS Press

Oral administration of food antigen induces T cell mediated intestinal inflammation: A model using TCR-transgenic mice

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Abstract. To investigate the mechanisms inducing food-sensitive intestinal inflammation, we focused on the OVA23-3 mouse, a transgenic mouse strain expressing a T cell receptor that recognizes ovalbumin (OVA). Mice administered an egg-white (EW) diet containing OVA showed a trend of loose feces and significant weight loss. Histology of the jejunum showed severe inflammation with villous atrophy. Thus, we studied the role of T cells and intestinal microflora in the development of the inflammation. Severe villous disruption was observed in sections of the jejunum from OVA23-3 mice and RAG-2 gene-deficient OVA23-3 mice fed with EW-diet. Further, a larger number of T cells was found in the lamina propria of the jejunum of EW-diet fed OVA23-3 mice, RAG-2 gene-deficient mice and germfree OVA23-3 mice compared with those of control-diet fed mice. However, severe inflammation was not detected in the jejunum of germfree OVA23-3 mice. $CD4^+$ T cells from the MLN of EW-diet fed OVA23-3 mice showed a Th2 cytokine secretion profile. These observations have thus clarified that antigen-specific Th2 cells play important roles in the development of intestinal inflammation. Although the presence of indigenous bacteria was not essential for the inflammation, T cells could mediate a more severe inflammatory response in their presence.

Keywords: Food allergy, oral administration of antigen, intestinal inflammation antigen-specific T cells, Th2 type $CD4^+$ T cells, probiotics, intestinal flora

1. Introduction

The mechanisms inducing food-sensitive intestinal inflammation remain to be clarified due to the lack of excellent animal models [2,3]. In an animal model of food allergy, the sole administration of antigens orally should induce 1) an increase of antigen specific IgE in the serum and 2) gastrointestinal inflammation and other symptoms. Previous studies in our laboratory showed that feeding egg-white (EW) diet to OVA23-3 mice, a transgenic mouse strain expressing an OVA-specific T cell receptor, elicited an OVA-specific serum IgE response [5]. Further, we found that OVA23-3 mice fed with EW-diet demonstrated intestinal inflammation accompanied with wasting diseases and diarrhea which are

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similar to clinical symptoms of food allergy patients (H, Nakajima-Adachi; submitted). Thus, this mouse could serve as a unique model to investigate the mechanisms of food-sensitive intestinal inflammation. In this study, we firstly analyzed the roles of T cells in this inflammation and secondly we investigated the effect of intestinal flora on intestinal inflammation.

2. Materials and methods

2.1. Mice

Conventional OVA23-3 TCR-transgenic mice (CV OVA23-3 mice), transgenic for OVA 323–339 specific and I-A^d restricted TCR-$\alpha\beta$, on a BALB/c genetic background were kindly provided by Professor Sonoko Habu of Tokai University and housed under Specific pathogen free (SPF) conditions. OVA23-3 mice were crossed to RAG-2 gene-deficient mice to produce RAG-2 gene-deficient (RAG-KO) OVA23-3 mice. Germfree (GF) OVA23-3 mice, which lack intestinal flora, were obtained from CV OVA23-3 mice by hysterectomy and foster nursed by lactating GF BALB/c mice in an isolator.

2.2. Administration of OVA

OVA23-3 mice were fed a pelleted diet containing egg-white protein at a concentration of 20% for 4 weeks. The EW diet contains approximately 10.4% OVA. A Casein (CN) diet was used as dietary control.

2.3. Histology and immunohistochemistry

Specimens were obtained from the jejunum, ileum and colon on week 4. Intestinal samples were fixed in 4% phosphate-buffered formaldehyde (pH7.2). Paraffin sections (thickness 3 μm) were stained with hematoxylin and eosin for identification of inflammatory cells, or with toluidine blue (pH4.1) for identification of mast cells. Intestinal specimens for immunohistochemistry were placed into molds and frozen in liquid nitrogen. Before staining, slides were fixed in acetone at $-20°C$ for 10 minutes. Blocked sections were incubated with biotinylated anti-mouse antibodies and then with streptavidin-HRP. DAB (Sigma) was used to stain $CD3^+$, $CD8^+$ cells.

2.4. Preparation of the mesenteric lymph nodes (MLN) cells and $CD4^+$ T cells.

$CD4^+$ T cells were purified from the MLN of transgenic mice by means of magnetic cell sorting (Miltenyi Biotech GmbH, Bergish Gladbach, Germany) with anti-CD4 beads.

2.5. Cell culture and cytokine assay with ELISA

Purified $CD4^+$ T cells (1×10^5 cells) were cultured with 0 or 1.0 mg/ml OVA in flat-bottom 96-well plates and 4×10^5 mitomycin C treated BALB/c splenocytes as antigen presenting cells (APCs) in RPMI 1640 media containing 10% heat-inactivated FCS (Sigma). For cytokine assays, supernatant samples were collected at 48 hours. IL-4 and IFN-γ cytokine production was measured by ELISA.

Fig. 1. Histology of jejunum from OVA23-3 mice fed with EW-diet. Samples were obtained on day 28 and stained with hematoxylin and eosin. A: OVA23-3 mice fed with EW-diet, B: RAG-KO OVA23-3 mice fed with EW-diet, C: GF OVA23-3 mice fed with EW-diet, D: OVA23-3 mice fed with CN (control) diet. (Original magnification: A, B, C and D 20×).

3. Results and discussion

3.1. Histologic analysis of the jejunum from OVA23-3 mice, RAG-KO OVA23-3 mice and GF OVA23-3 mice

Histologic features such as villous atrophy, crypt hyperplasia, goblet cell hyperplasia and infiltration of inflammatory cells, were detected in the jejunum of OVA23-3 mice fed with EW-diet (Fig. 1(A)). Mice fed with control (CN)-diet showed normal villous architecture (Fig. 1(D)). Severe inflammation was also detected in the jejunum of RAG-KO OVA23-3 mice fed with EW-diet (Fig. 1(B)). All T cells of RAG-KO OVA23-3 mice express the OVA-specific TCR, and these mice cannot produce antibodies due to lack of mature B cells. Thus, these observations suggest that OVA-specific T cells mediate intestinal inflammation, even in the absence of B cells. Severe inflammation was not detected in the duodenum and upper jejunum from GF OVA23-3 mice fed with EW-diet (Fig. 1(C)).

3.2. The number of $CD3^+$ T cells detected in the jejunum of OVA23-3 mice, RAG-2 gene KO OVA23-3 mice and GF OVA23-3 mice

The number of T cells detected in the jejunum of EW-diet fed OVA23-3 mice was significantly increased compared with CN-diet fed OVA23-3 mice (Table 1). Significant aggregates of T cells were also detected in EW-diet fed RAG-KO OVA23-3 mice and GF OVA23-3 mice. This immunohistochemical observation of the jejunum further supports the important role of antigen-specific T cells in inflammation. Indeed, a previous study of patients with food-sensitive enteropathy suggested that activated $CD4^+$ cells in the lamina propria may contribute to mucosal damage [4].

3.3. Cytokine production by $CD4^+$ T cells in the MLN

$CD4^+$ T cells from the MLN were cultured with OVA and cytokine secretion was examined by ELISA. High levels of IL-4 were secreted by $CD4^+$ T cells from OVA23-3 mice fed with EW-diet compared with mice fed with CN-diet (Table 2). $CD4^+$ T cells from mice fed with CN-diet did not secrete detectable levels of IL-4. Conversely, higher levels of IFN-γ were secreted by $CD4^+$ T cells from the MLN of CN-diet fed mice than EW-diet fed mice. We also found that a much higher level of IL-4 (524 pg/mL) was secreted by $CD4^+$ T cells from the MLN after 7 days administration of EW-diet compared with 28 days. These data showed that dysregulated Th2 cells play important roles in the pathological features of inflammation in the small intestine [1].

Table 1
The number of $CD3^+$ cells detected in the jejunum

Mouse groups	Diet	$CD3^+$ cells (N/villi) (mean ± SD)
SPF OVA23-3	EW ($n = 5$)	$35.7 \pm 10.1^{\alpha}$
	CN ($n = 4$)	8.4 ± 5.5
RAG-2 gene-KO OVA23-3	EW ($n = 4$)	$43.6 \pm 12.5^{\alpha}$
	CN ($n = 4$)	4.9 ± 0.9
GF OVA23-3	EW ($n = 5$)	$14.6 \pm 6.3^{\beta}$
	CN ($n = 6$)	4.3 ± 0.4
GF BALB/c	EW ($n = 4$)	3.4 ± 0.9

$^{\alpha}$Significantly increased in comparison to controls ($p < 0.05$).
$^{\beta}$Significantly increased in comparison to controls and EW-diet fed GF BALB/c ($p < 0.05$).

Table 2
Cytokine production by $CD4^+$ T cells harvested from the MLN of SPF OVA23-3 mice

Mouse groups (diet) and OVA concentration	IL-4 (pg/mL)	IFN-γ (ng/mL)
EW (OVA 1.0 mg/mL)	96.3	3.6
EW (OVA 0 mg/mL)	< 5	3.8
CN (OVA 1.0 mg/mL)	< 5	64.9
CN (OVA 0 mg/mL)	< 5	6.7

$CD4^+$ T cells harvested from the MLN of SPF OVA23-3 mice were cultured with OVA (1.0 or 0 mg/mL) and APC. Culture supernatants were assessed for Th1 and Th2 cytokines.

4. Conclusion

Analysis of inflammation in OVA23-3 mice and RAG-2 gene KO mice strongly suggests that antigen-specific Th2 type T cells and inflammatory cells primed by Th2 type responses play important roles in the development of the disease. Analysis of GF OVA23-3 mice indicated that intestinal T cells could directly respond to dietary antigens and cause inflammation without intestinal microflora. However, in the presence of indigenous bacteria, T cells could mediate a more severe inflammatory response. Our TCR-Tg system should be an informative system to evaluate the effect of probiotics, prebiotics, and other food factors on antigen-specific T cell responses.

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CD4$^-$c-kit$^-$CD3ε$^-$IL-2Rα$^+$ Peyer's patch cells are a novel cell subset which secrete IL-5 in response to IL-2: implications for their role in IgA production

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In this study, we examined which cell population contributes to IL-5 production by Peyer's patch (PP) cells. Thy1.2$^-$ fraction of PP cells, but not those of splenocytes, secreted IL-5 in response to IL-2. We found that CD3ε$^-$IL-2Rα$^+$ cells purified from the Thy1.2$^-$B220$^-$ fraction of PP cells secreted IL-5 when stimulated with IL-2. CD3ε$^-$IL-2Rα$^+$ cells were subdivided into CD4$^+$ and CD4$^-$ populations or c-kit$^+$ and c-kit$^-$ populations, and only the CD4$^-$ and c-kit$^-$ CD3ε$^-$IL-2Rα$^+$ cells secreted IL-5 in response to IL-2. CD3ε$^-$IL-2Rα$^+$ cells did not express NK cell-markers and exhibited a lymphoid morphology. We have therefore identified CD3ε$^-$IL-2Rα$^+$ cells as a unique lymphoid population that are not classified into conventional IL-5-producing cell populations, such as T cells, mast cells and NK cells. Depletion of CD3ε$^-$IL-2Rα$^+$ cells from PP resulted in reduced IL-5 production. Furthermore, IgA secretion by B cells was increased when PP B cells were cocultured with CD3ε$^-$IL-2Rα$^+$ cells. Taken together, these results suggest that the novel subset of CD4$^-$c-kit$^-$CD3ε$^-$IL-2Rα$^+$ PP cells are capable of secreting a high level of IL-5 in response to IL-2, contribute markedly to IL-5 production and help IgA secretion by B cells.

Received	28/10/03
Revised	29/4/04
Accepted	11/5/04

Key words: IL-5 / Peyer's patch / IgA

1 Introduction

IL-5 is mainly produced by Th2 cells and was originally recognized by its activity as a B cell growth factor [1], an IgA-enhancing factor [1–3], and as a differentiation or an activation factor for eosinophils [4–6]. IL-5 also induces growth and IgA and IgM production by B1 cells [7]. Analyses of IL-5 transgenic mice, IL-5 gene deficient (IL-5$^{-/-}$) and IL-5Rα$^{-/-}$ mice show that IL-5 is critically involved in the development and activation of B cells and eosinophils [8–12].

Several reports showed that IL-5 production from certain cells was also induced by IL-2 stimulation [13–16]. Recent studies showed that a human allergic CD4$^+$ T cell clone produced IL-5 in response to IL-2 [16] and that human NK cells can be induced to produce IL-5 following costimulation with gamma-irradiated melanoma cells and IL-2 [17]. These studies strongly suggest that IL-2 induces both T cells and non-T cells to produce IL-5.

Large numbers of IgA-producing cells, which are the first line of defense against luminal pathogens and bacteria, exist in the gut mucosa. Further, in allergic conditions, eosinophils infiltrate into these sites. These observations suggest that IL-5 is an important cytokine in regulating immunologic and allergic reactions in the gut mucosa. One of the representative organs of the gut-associated lymphoreticular tissue is the Peyer's patch (PP), which is an organized peripheral lymphoid tissue distributed along the intestinal tract and thought to be a major inductive site of IgA Ab [18, 19].

In the present study, we first examined the effect of exogenous IL-2 on IL-5 production by PP cells and found that PP cells produce IL-5 in response to IL-2 in a dose-dependent manner. We then isolated various cell types from PP and stimulated each to identify which population produces IL-5 in response to IL-2. Here we show that CD4$^-$CD3ε$^-$IL-2Rα$^+$ cells isolated from PP, which are unique and not previously classified as IL-5-

[DOI 10.1002/eji.200324696]

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Abbreviations: PP: Peyer's patch **SPL:** Spleen

producing cells such as CD4⁺ T cells or NK cells, secrete IL-5 when stimulated with PMA+A23187 or IL-2. We also suggest that CD4⁻CD3ε⁻IL-2Rα⁺ cells help B cells to secrete IgA.

2 Results

2.1 PP cells produce IL-5 in response to IL-2 in a dose-dependent manner

We have observed that freshly isolated PP cells derived from OVA-specific TCR-transgenic mice produced higher levels of IL-5 than splenocytes when stimulated with OVA in primary cultures (manuscript in preparation). Many studies have been published on the role and source of IL-5 in allergic conditions; however, few have discussed IL-5 production in the initiation of immune responses, though it has been thought to be an important cytokine for the mucosal immunity [20]. Since PP is thought to be a major inductive site for immune responses against luminal Ag, we chose to study IL-5 production by PP cells to identify which cell population may be responsible for IL-5 production at the beginning of immune responses.

It has been reported that IL-5 gene transcription and protein synthesis were also induced upon stimulation with exogenous IL-2 in human PBMC or in T cell clones from allergic patients [13–16]. We therefore speculated that exogenous IL-2 may also induce PP cells and splenocytes to produce IL-5 in mice, and hence we initially analyzed whether IL-2 might affect the IL-5 production by these cells. As shown in Fig. 1, PP cells from BALB/c mice produced IL-5 in response to IL-2 in a dose-dependent manner, but no IL-5 production was observed in splenocytes stimulated with IL-2. Both PP cells and splenocytes also produced IFN-γ and IL-6 in response to IL-2 (Fig. 1).

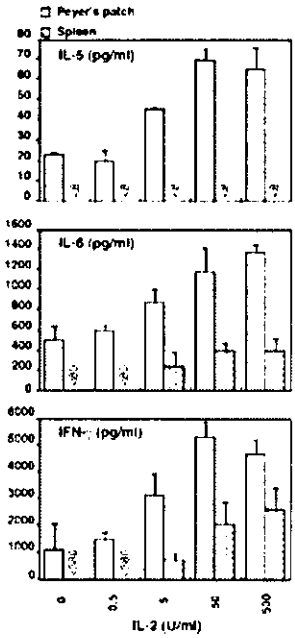

Fig. 1. PP cells produce IL-5 in response to IL-2 in a dose-dependent manner. PP cells and splenocytes were prepared by dispase treatment and cultured in a 96-well plate (1×10^5 cells/well) with variable concentrations of IL-2. Culture supernatants were collected at 48 h and examined for secreted cytokines using the respective cytokine-specific ELISA. The reults are expressed as the mean of triplicate cultures ± SD. Data are representative of four independent experiments.

Since Mori et al. [16] reported that human T cell clones produced IL-5 in the presence of IL-2, we tested whether naive PP CD4⁺ T cells could produce IL-5 by the stimulation with IL-2. Analysis of CD4⁺ T cells showed that stimulation with immobilized anti-CD3 and anti-CD28 mAb induced IL-5 production by naive PP CD4⁺ T cells, whereas IL-2 alone failed to induce IL-5 production (Fig. 2A). Similar results were obtained from analyses of naive spleen (SPL) CD4⁺ T cells (data not shown). We hypothesized that non-T cells in PP may be responsible for producing IL-5 in response to IL-2 and therefore we depleted Thy1.2⁺ cells from PP cells by negative immunomagnetic selection with MACS and examined the Thy1.2⁻ fraction for its response to IL-2. Fig. 2B shows that the Thy1.2⁻ fraction of PP cells produced IL-5 in response to IL-2 in a dose-dependent manner. The Thy1.2⁻ fraction of PP cells produced IL-6 and both the Thy1.2⁻ fraction of PP cells and splenocytes also produced IFN-γ in response to IL-2 (Fig. 2B). These results suggested that non-T cells that produced IL-5 in response to IL-2 exist in PP, but not in SPL.

2.2 CD3ε⁻IL-2Rα⁺ cells produce IL-5

To identify which cell population may produce IL-5 we separated the components of PP cells and stimulated each population. In another experiment we found that IL-5 production by the Thy1.2⁻ fraction of PP cells was strongly induced by PMA+A23187 compared to IL-2 (data not shown). In contrast, we could not detect IL-5 production by the Thy1.2⁻ fraction of splenocytes even though they were stimulated with PMA+A23187 (data not shown). We therefore adopted PMA+A23187 as the cell-stimulation method to observe IL-5 production by PP cells. Since the Thy1.2⁻ fraction of PP cells contains B cells, Mϕ and DC, we separated and stimulated each

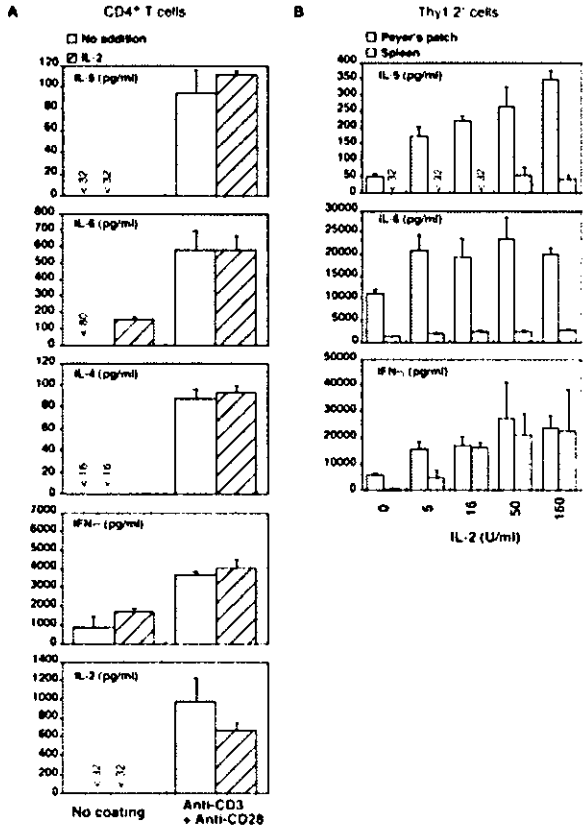

Fig. 2. Non-T cells from PP produce IL-5 in response to IL-2. CD4+ T cells were isolated from PP or SPL by MACS, cultured in a 96-well plate (4×10^5 cells/well) and stimulated by immobilized anti-CD3 (3 μg/ml) and anti-CD28 Ab (5 μg/ml). In some cultures IL-2 (50 U/ml) was added (A). Thy1.2$^-$ cells were isolated from PP or SPL by negative selection of MACS and cultured with variable concentrations of IL-2 (B). Culture supernatants were collected at 48 h and examined for secreted cytokines using the respective cytokine-specific ELISA. The results are expressed as the mean of triplicate cultures ± SD. Similar results were obtained from more than three independent experiments.

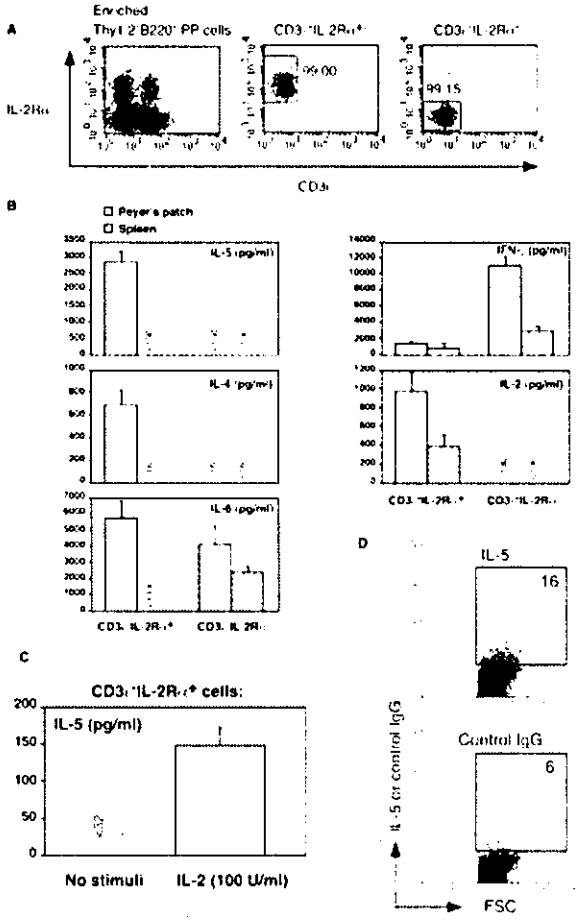

Fig. 3. CD3ε$^-$IL-2Rα$^+$ cells from PP produce IL-5. Thy1.2$^-$B220$^-$ cells were isolated from PP or SPL by negative selection of MACS and stained with anti-CD3ε and anti-IL-2Rα mAb. Then CD3ε$^-$IL-2Rα$^+$ cells and CD3ε$^-$IL-2Rα$^-$ cells were isolated by FACS sorting (A). These cells were cultured in a 96-well plate (5×10^4 cells/well) with 50 ng/ml of PMA plus 250 ng/ml of calcium ionophore A23187 (B). PP CD3ε$^-$IL-2Rα$^+$ cells were cultured in the presence or absence of 100 U/ml of IL-2 (C). Culture supernatants were collected at 48 h and examined for secreted cytokines using the respective cytokine-specific ELISA. The results are expressed as the mean of triplicate cultures ± SD. Purified PP CD3ε$^-$IL-2Rα$^+$ cells were stimulated with PMA and ionomycin and intra-cellular IL-5 was stained (D). Cells were gated on FSC and SSC to define the lymphoid population. Similar results were obtained from more than three independent experiments.

with PMA+A23187; however, we could not detect IL-5 production among these cells (data not shown), even though the Thy1.2$^-$B220$^-$ fraction of PP cells from which Mϕ or DC were separated produced IL-5 when stimulated with PMA+A23187 (data not shown). These results suggested that another cell population with an ability to produce IL-5 might exist in PP.

We hypothesized that because IL-2 activated cells to produce IL-5 they might express the IL-2R α chain (IL-2Rα), which forms a high affinity IL-2R with β and γ chain [21]. We therefore examined the expression of IL-2Rα on the Thy1.2$^-$B220$^-$ fraction from both PP cells and splenocytes and found about 10% of PP Thy1.2$^-$B220$^-$ cells and 5% of SPL Thy1.2$^-$B220$^-$ cells expressed IL-2Rα (Fig. 3A and data not shown). Next we purified CD3ε$^-$IL-2Rα$^+$ cells or CD3ε$^-$IL-2Rα$^-$ cells from the Thy1.2$^-$B220$^-$ fraction of PP cells or splenocytes and cultured each in the presence or absence of PMA+A23187. PP CD3ε$^-$IL-2Rα$^+$ cells produced IL-5, IL-4, IL-6, IFN-γ and IL-2 when

they were stimulated with PMA+A23187 (Fig. 3B). In contrast, we could not detect IL-5, IL-4 or IL-6 production by SPL CD3ε⁻IL-2Rα⁺ cells stimulated with PMA+A23187 (Fig. 3B). These results suggested that CD3ε⁻IL-2Rα⁺ cells existed in both PP and in SPL, however, only PP CD3ε⁻IL-2Rα⁺ cells could produce IL-5 in response to PMA+A23187 stimulation. Further analysis revealed that PP CD3ε⁻IL-2Rα⁺ cells produced IL-5 in response to exogenous IL-2 (Fig. 3C). Thus we defined CD3ε⁻IL-2Rα⁺ cells as the IL-2-responsive IL-5-producing population in PP. Cytostaining analysis revealed that approximately 10% of the PP CD3ε⁻IL-2Rα⁺ cells expressed IL-5 (Fig. 3D).

2.3 CD3ε⁻IL-2Rα⁺ cells are a unique subset of cells that secrete IL-5

It has been reported that Th2 cells, Tc2 cells, γδ T cells, mast cells, NK cells [17], NKT cells [22] or eosinophils [23] may produce IL-5. To identify the lineage of PP CD3ε⁻IL-2Rα⁺ cells, expression of surface molecules on PP CD3ε⁻IL-2Rα⁺ cells were analyzed by FCM. As shown in Fig. 4, CD3ε⁻IL-2Rα⁺ cells do not express TCRαβ, TCRγδ or Pan-NK cells marker DX5 [24]. Furthermore, we analyzed NK1.1 expression on PP CD3ε⁻IL-2Rα⁺ cells from C57BL/6N mice after confirming that PP CD3ε⁻IL-2Rα⁺ cells from C57BL/6N mice produced IL-5 when stimulated with PMA+A23187 (data not shown). CD3ε⁻IL-2Rα⁺ cells were found not to express NK1.1 molecules (Fig. 4). We therefore concluded that CD3ε⁻IL-2Rα⁺ cells are not T cells, NK cells or NKT cells.

To examine whether or not CD3ε⁻IL-2Rα⁺ cells are eosinophils, immunohistochemical analysis was carried out. This analysis revealed that CD3ε⁻IL-2Rα⁺ cells exhibit a lymphoid morphology, are small in size and have a relatively large nucleus to cytoplasm ratio (Fig. 5), thus indicating they differ from eosinophils, DC and Mϕ.

It was also revealed that almost all CD3ε⁻IL-2Rα⁺ cells from BALB/c mice express IL-7Rα and lack expression of CD8, B220, CD11b and CD23 (Fig. 4). The expression patterns of these and other cell surface molecules are shown in Table 1. We obtained similar results for the expression of surface molecules on CD3ε⁻IL-2Rα⁺ cells when we prepared PP cells by crushing PP mechanically (by non-enzymatic technique). CD3ε⁻IL-2Rα⁺ cells were further divided into c-kit⁺ and c-kit⁻ populations, or CD4⁺ and CD4⁻ populations (Fig. 4). To identify which subset of CD3ε⁻IL-2Rα⁺ cells produce IL-5, each subset was isolated and stimulated with PMA+A23187 or IL-2. As a result we found that c-kit⁻CD3ε⁻IL-2Rα⁺ cells and CD4⁻CD3ε⁻IL-2Rα⁺ cells produced IL-5 when stimulated with PMA+A23187 (Fig. 6A, B). Furthermore, the c-

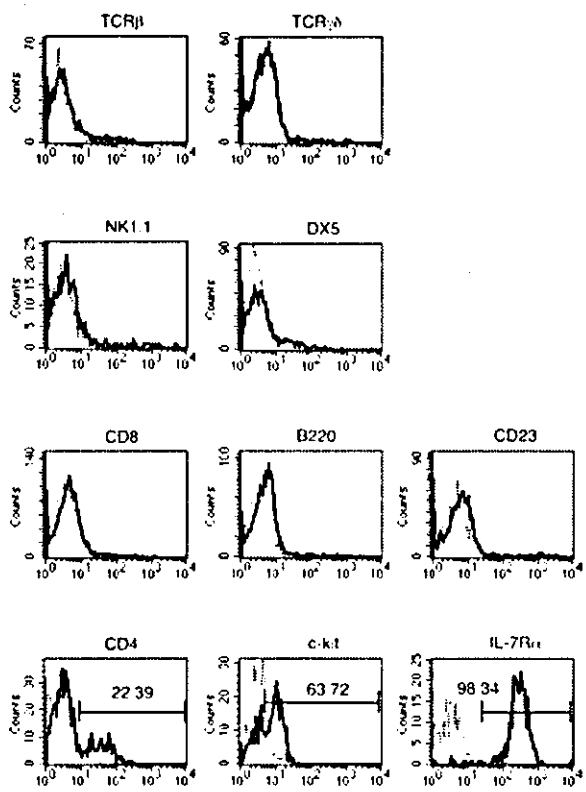

Fig. 4. Surface phenotypes of PP CD3ε⁻IL-2Rα⁺ cells. Thy1.2⁻B220⁻ fraction of PP cells were isolated from BALB/c mice or C57BL/6N mice by negative selection of MACS and stained with anti-CD3ε, anti-IL-2Rα and various mAb against surface molecules as indicated. Expression of TCRβ and TCRγδ, DX5, NK1.1, CD23, CD4, CD8, B220, c-kit, IL-7Rα (filled line) and isotype-matched mAb (solid line) of gated PP CD3ε⁻IL-2Rα⁺ cells are presented. Similar results were obtained from more than three independent experiments.

kit⁻CD3ε⁻IL-2Rα⁺ cells and CD4⁻CD3ε⁻IL-2Rα⁺ cells produced IL-5 in the presence of IL-2 (Fig. 6C). Conversely we could not observe IL-5 production by c-kit⁺CD3ε⁻IL-2Rα⁺ cells or CD4⁺CD3ε⁻IL-2Rα⁺ cells. Since CD3ε⁻IL-2Rα⁺ cells that have a potential to produce IL-5 do not express c-kit, these cells are distinguishable from mast cells. Taken together, these results strongly suggest that CD3ε⁻IL-2Rα⁺ cells are not classified with Th2 cells, Tc2 cells, γδ T cells, mast cells, NK cells, NKT cells or eosinophils and that CD3ε⁻IL-2Rα⁺ cells are a unique subset of cells that produce IL-5.

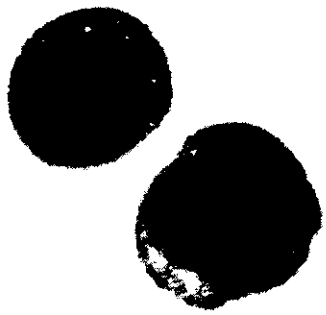

Fig. 5. Morphology of CD3ε⁻IL-2Rα⁺ cells. Purified PP CD3ε⁻IL-2Rα⁺ cells were stained with Diff-Quik.

Table 1. Surface expression of molecules on PP CD3ε⁻IL-2Rα⁺ cells

Molecules	Expression
Thy1.2	Low to –
sIgD	–
sIgM	–
Mac-1	–
CD11c	–
Syndecan-1	–
B7-1	–
B7-2	–
CD40	–
CD44	+
LFA-1	+
CD62L	–
CD69	+
Fas	–
Integrin α4	Low to –
Integrin β7	+

2.4 CD3ε⁻IL-2Rα⁺ cells are a major source of IL-5 production in PP

To assess the influence of the CD3ε⁻IL-2Rα⁺ cells upon IL-5 production by PP cells we compared cytokine production by PP cells depleted of CD3ε⁻IL-2Rα⁺ cells with control PP cells upon stimuli. We prepared non-treated PP cells and Ab-stained PP cells as controls which were treated with the mAb described in Sect. 4.3. The latter control was included since it was considered possible that T cells and other cells might be activated by anti-CD3 mAb when depleting CD3ε⁻IL-2Rα⁺ cells. Since IL-5-producing CD3ε⁻IL-2Rα⁺ cells lack B220 (Fig. 4), we therefore depleted B220⁻CD3ε⁻IL-2Rα⁺ cells to observe the effect of these cells upon cytokine production by PP cells. Using this method, the proportion of B220⁻CD3ε⁻IL-2Rα⁺ cells in PP is diminished from 1.5% to 0.35%. IL-5, but not other cytokine production, was significantly reduced by the depletion of the CD3ε⁻IL-2Rα⁺ cells when stimulated with IL-2 or anti-CD3 mAb (Fig. 7A). IL-5 and IL-6 production by CD3ε⁻IL-2Rα⁺ depleted PP cells was also significantly reduced when stimulated with PMA+A23187 (Fig. 7B). In this case IL-5 production by CD3ε⁻IL-2Rα⁺ depleted PP cells was reduced by one-third (1,500 pg/ml to 530 pg/ml) compared with Ab-stained control PP cells. We also tested whether FACS sorting might affect cytokine production by PP cells but we did not find any effect of this procedure (data not shown). These results suggest that PP CD3ε⁻IL-2Rα⁺ cells contribute markedly to production of IL-5 and minimally to IL-6, but do not contribute to the production of IFN-γ and IL-2.

2.5 CD3ε⁻IL-2Rα⁺ cells enhance IgA production by PP B cells

Finally, we examined whether CD3ε⁻IL-2Rα⁺ cells might affect Ig-production by B cells. When B cells isolated from PP were cocultured with c-kit⁻CD3ε⁻IL-2Rα⁺ cells in the presence of LPS and IL-2, IgA, but not IgM, IgG1 and IgG2a, concentration in culture supernatants were significantly increased (Fig. 8). These observations indicate that CD3ε⁻IL-2Rα⁺ cells secrete IL-5 and help B cells to differentiate into IgA-producing plasma cells.

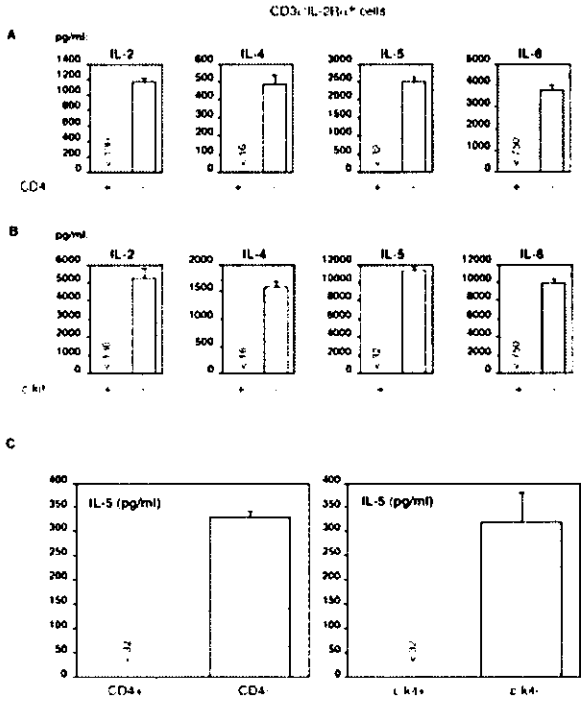

Fig. 6. CD4⁻CD3ε⁻IL-2Rα⁺ cells and c-kit⁻CD3ε⁻IL-2Rα⁺ cells produce IL-5 in response to IL-2. CD3ε⁻IL-2Rα⁺ cells were further divided into CD4⁺ and CD4⁻ populations or c-kit⁺ and c-kit⁻ populations by FACS sorting. CD4⁺ or CD4⁻ CD3ε⁻IL-2Rα⁺ cells (4×10^4 cells/well, A), c-kit⁺ or c-kit⁻ CD3ε⁻IL-2Rα⁺ cells (2×10^4 cells/well, B) were cultured in a 96-well plate. These cells were stimulated with PMA (50 ng/ml) plus A23187 (250 ng/ml, A, B) or IL-2 (250 U/ml, C). Culture supernatants were collected at 48 h and examined for secreted cytokines using the respective cytokine-specific ELISA. The results are expressed as the mean of triplicate cultures ± SD. Similar results were obtained from more than two independent experiments.

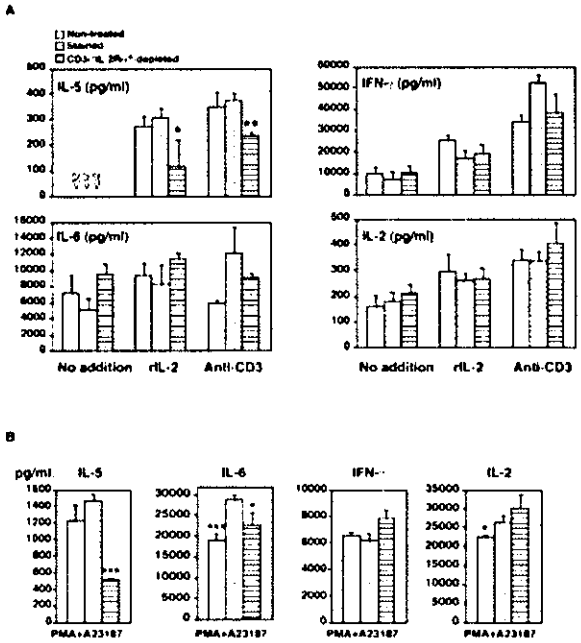

Fig. 7. CD3ε⁻IL-2Rα⁺ cells contribute to IL-5 production by PP cells. PP cells were stained with FITC-conjugated anti-B220, PE-conjugated anti-CD3ε and biotinylated anti-IL-2Rα Ab followed by streptavidin-conjugated RED 670. The B220⁻CD3ε⁻IL-2Rα⁺ cells were depleted from PP cells by FACS sorting. Non-treated PP cells, Ab-stained PP cells and B220⁻CD3ε⁻IL-2Rα⁺ depleted PP cells were cultured in a 96-well plate (1×10^6 cells/well) with or without IL-2 (50 U/ml), anti-CD3 Ab (1 μg/ml) (A), or PMA (50 ng/ml) plus A23187 (250 ng/ml) (B). Culture supernatants were collected at 48 h and analyzed for secreted cytokines using the respective cytokine-specific ELISA. The results are expressed as the mean of triplicate cultures ± SD. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ when compared with Ab-stained control PP cells. Similar results were obtained from three independent experiments.

3 Discussion

In this study we focused on IL-5 production by PP cells, and have shown that PP CD4⁻c-kit⁻CD3ε⁻IL-2Rα⁺ cells produce IL-5 in response to IL-2. Our *in vitro* analysis to assess an effect of CD4⁻c-kit⁻CD3ε⁻IL-2Rα⁺ cells on IL-5 production by PP cells showed that IL-5 production by PP cells was markedly reduced when CD3ε⁻IL-2Rα⁺ cells were depleted, even though normal numbers of naive CD4⁺ T cells or other immunocytes were present. Based on our analyses, when we reduced CD3ε⁻IL-2Rα⁺ cells from PP from 1.5% to 0.35% there was a decrease in IL-5 production from 1,500 pg/ml to 530 pg/ml. This indicates that approximately 80% of the total IL-5 produced by PP cells stimulated with PMA+A23187 is derived from CD3ε⁻IL-2Rα⁺ cells. Furthermore, comparing the ability of different cell types to produce IL-5 has shown that 4×10^5 naive PP CD4⁺ T cells produced 95 pg/ml of IL-5 (4.75×10^{-5} pg/cell) when stimulated with immobilized anti-CD3 and anti-CD28 mAb (Fig. 2A), whereas 2×10^4 PP c-kit⁻CD3ε⁻IL-2Rα⁺ cells produced 320 pg/ml of IL-5 (3.2×10^{-3} pg/cell) when stimulated with IL-2 (Fig. 6C). Though the stimulation methods differ between CD4⁺ T cells and c-kit⁻CD3ε⁻IL-2Rα⁺ cells, it remains a significant observation that CD3ε⁻IL-2Rα⁺ cells produce approximately 50 times more IL-5 than naive CD4⁺ T cells. Thus it is considerable interest that CD3ε⁻IL-2Rα⁺ cells are a major source for IL-5 production in Peyer's patches.

It is thought that IL-5 is produced by Th2 cells, Tc2 cells, γδ T cells, mast cells, NK cells [17], NKT cells [22], eosin-

