Histamine H4 receptor agonists have more activities than H4 agonism in antigen-specific human T-cell responses

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Summary

Histamine not only mediates immediate allergic reactions, it also regulates cellular immune responses. H4R is the most recently identified histamine receptor (HR). In the present study, we examined the *in vitro* effect of histamine and H4R agonists on the responses of human T cells to purified protein derivative from *Mycobacterium tuberculosis* (PPD) and to Cry j1, the major allergen of *Cryptomeria japonica* pollen. Dimaprit, clobenpropit and clozapine, which are H4R agonists, dose-dependently blocked both PPD-induced interferon-γ and Cry j1-induced interleukin-5 production by both peripheral blood mononuclear cells (PBMCs) and antigen-specific T-cell lines. However, the addition of thioperamide, an H3R/H4R antagonist, as well as a mixture of *d*-chlropheniramine, famotidine and thioperamide, did not reverse the inhibition. Pretreatment of PBMCs with SQ22536 and 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer, had varying abilities to reverse the inhibitory effects of H4R agonists, except for clobenpropit. Moreover, the addition of H4R agonists induced annexin-V expression on PBMCs, especially in $CD19^+$ and $CD4^+$ cells. cDNA microarray analysis revealed that, among 16 600 genes tested, increased expression following treatment with clozapine was seen in 0·8% of the genes, whereas decreased expression was seen in 3·0% of the genes. These results suggest that H4R agonists inhibit antigen-specific human T-cell responses, although H4R does not appear to be important for this effect. In addition, the present study indicated that there may be orphan receptors or HR subtypes which can bind dimaprit, clobenpropit and clozapine, and that can exert an inhibitory effect on antigen-specific cellular responses via a cAMP/cAMP-dependent protein kinase-dependent, apoptotic pathway.

Keywords: cytokine; histamine; H4R; human studies; T cell

Introduction

Histamine has numerous physiological effects, including the induction of allergic responses and gastric acid secretion. These effects are mediated by several histamine receptors (HRs). To date, four subtypes (H1R, H2R, H3R and H4R) of HRs have been identified and cloned. All belong to the seven-transmembrane domain G-protein-coupled receptor family, and coupled G proteins and subsequent activated intracellular signals have been characterized.[1] Of these, H4R was most recently identified, and this receptor is expressed at high levels in mast cells and leucocytes.[2,3]

Histamine not only mediates immediate airway hyperresponsiveness, but also regulates cellular immunity by

Abbreviations: Ag, antigen; APC, antigen-presenting cell; DC, dendritic cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HR, histamine receptor; IFN-γ, interferon-γ; IL, interleukin; 4-MH, 4-methylhistamine; α-MH, alpha-methylhistamine; PBMC, peripheral blood mononuclear cell; 2-PEA, 2-pyridylethylamine; PKA, protein kinase A; PPD, purified protein derivative of *Mycobacterium tuberculosis*; PMA, phorbol 12-myristate 13-acetate; RT–PCR, reverse transcription–polymerase chain reaction; SD, standard deviation; TCC, T-cell clone; TCL, T-cell line; Th1, T helper 1; Th2, T helper 2.

controlling the production of pro-inflammatory cytokines and chemokines, the expression of adhesion molecules, and the migration of inflammatory cells, such as eosinophils.[1,4,5] The maturation and activity of dendritic cells (DCs) is also affected by histamine.[6] In addition, histamine regulates T-cell function.[7,8] For example, histamine can enhance T helper 1 (Th1)-type responses by stimulating H1R, whereas both Th1- and T helper 2 (Th2)-type responses are negatively regulated by H2R.[7] At present, there is one report that $CD8^+$ T cells produce interleukin (IL)-16 in response to histamine activation of H4R.[9] However, despite the high expression of H4R on both antigen-presenting cells and T cells, it is not known whether signals through H4R affect antigen-specific human T-cell responses.[3] Therefore, in the present study, we investigated the roles of H4R agonists in antigen-specific human T-cell responses. The findings presented here may help to identify new therapeutic approaches for using HR agonists to treat allergic diseases.

Materials and methods

Antigen and reagents

The purified protein derivative of *Mycobacterium tuberculosis* (PPD) was purchased from Nihon BCG Seizo Co. (Tokyo, Japan). Cry j1 was purified from the crude extracts of *Cryptomeria japonica* pollen using a well-established procedure.[10] The protein concentration was determined using a bicinchoninic acid assay (Pierce, Rockford, IL), according to the manufacturer's instructions. Endotoxin contamination was considered to be negligible because of a negative Endospec™ ES test result (Seikagaku Kogyo Corporation, Tokyo, Japan). Histamine was purchased from Nacalai Tesque Inc. (Kyoto, Japan). 2-Pyridylethylamine (2-PEA; H1R agonist) and 4-methylhistamine (4-MH; H2R agonist) were provided by GSK (Welwyn, Garden City, UK). Dimaprit (H2R/H4R agonist), clobenpropit (H4R agonist/H3R antagonist) and thioperamide (H3R/H4R antagonist) were purchased from Tocris (Ellisville, MO). Clozapine (H4R agonist) was purchased from MP Biomedicals (Irvine, CA). α-Methylhistamine (α-MH; H3R agonist) was a gift from Professor J. C. Schwartz (INSERM, Paris, France). *d*-Chlropheniramine (H1R antagonist) and famotidine (H2R antagonist) were provided by Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan). SQ22536 was purchased from Sigma (St Louis, MO). RP-8-Br-cAMP was purchased from BIOLOG Life Science Institute (Bremen, Germany).

Isolation and culture of peripheral blood mononuclear cells (PBMCs)

All experiments were approved by the Institutional Review Board in the affiliated hospital of Okayama University Graduate School of Medicine. Informed consent was obtained from each volunteer. Twelve Japanese subjects (five men and seven women; age 20–52 years; mean age 39·5 years) with positive tuberculin skin tests, and 12 Japanese patients (seven men and five women; age 19–59 years; mean age 37·4 years) with Japanese cedar pollinosis showing a positive skin scratch test to Japanese cedar pollen, were examined. PBMCs were isolated and cultured as described previously.[11] The culture medium used throughout the study was RPMI-1640 (Sigma) supplemented with 10% human AB serum (ICN Biomedicals, Aurora, OH), 100 U/ml of penicillin, 100 μg/ml of streptomycin (Sigma) and 20 mM L-glutamine (Gibco BRL, Grand Island, NY). In brief, PBMCs (1×10^6/ml) were incubated in the presence or absence of 2 μg/ml of PPD or 10 μg/ml of Cry j1, together with histamine and/or HR-selective agonists or antagonists, for 72 hr in 24-well plates (Corning Inc., Corning, NY) at 37° in a 5% CO_2/air mixture. To determine the involvement of adenylate cyclase and cAMP-dependent protein kinase A (PKA) in the action of HR agonists, PBMCs were pretreated for 1 hr at 37° with 5 mM SQ22536 and 5 mM RP-8-Br-cAMP, respectively.[12] After this incubation, the cells were washed twice with culture medium and then cultured as described above.

Generation and culture of antigen-specific T-cell lines

The $CD4^+$ PPD^- and Cry j1-specific T-cell lines (TCLs) used were generated using a procedure described previously.[10] In flat-bottomed 96-well microtiter plates (Corning Inc.), 2×10^4 TCL were mixed with 1×10^5 irradiated autologous PBMCs (PBMCx) as antigen-presenting cells (APCs). Following this, the cells were cultured in the presence or absence of antigen (Ag), in 0·2 ml of culture medium, together with HR-selective agonists, for 65 hr.

To determine adenylate cyclase activity, TCLs and/or APCs were incubated with SQ22536 at 37° in 24-well plates for 1 hr. Following this, the cells were washed with culture medium three times, after which they were mixed and cultured in the same manner described above.

Measurement of cytokine production

The levels of interferon-γ (IFN-γ), IL-5 and IL-10 in culture supernatants were measured using Opt EIA™ sets (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. The detection limits for IFN-γ, IL-5 and IL-10 in these assays were 20, 20 and 1·9 pg/ml, respectively.

Analysis of apoptosis by annexin V staining

PBMCs (1×10^6/ml) were incubated with 100 μM histamine or HR-selective agonists for 24 or 72 hr. The cells were then harvested, and apoptotic cells were detected

using an Annexin V-FITC Apoptosis Detection KitI (BD Biosciences), according to the manufacturer's protocol. Stained cells were analysed using a fluorescence-activated cell sorter (FACScan) with CELLQUEST software (BD Biosciences).

cDNA microarray analysis

cDNA microarray analysis was performed using IntelliGene HS Human expression chips, containing about 16 600 probe sets (Takara, Tokyo, Japan). PBMC (1×10^6/ml) were incubated with 2 μg/ml of PPD, in the presence or absence of 100 μM clozapine, for 12 hr. Total cellular RNA was extracted by the RNeasy™ mini kit (Qiagen, Tokyo, Japan), according to the manufacturer's instructions. Four micrograms of total RNA from PBMCs treated with and without clozapine were labeled using the RNA Transcript SureLABEL Core Kit (Takara) with Cy-5 UTP and Cy-3 UTP (Amersham Bioscience Corp., Piscataway, NJ), respectively, in each paired case. Labeled samples were hybridized to IntelliGene HS Human expression chips, according to the manufacturer's instructions. After hybridization for 16 hr at 65°, the slides were washed and then scanned for Cy-5 and Cy-3 fluorescence using the Affymetrix 428 scanner (Affymetrix Japan, Tokyo, Japan). The signal intensity of hybridization was evaluated photometrically by the IMAGENE computer program (BioDiscovery K. K., Tokyo, Japan). A gene expression ratio (Cy-5/Cy-3) of > 2·0 and < 0·5 was considered significant.

Real-time quantitative polymerase chain reaction

PPD- and Cry j1-specific TCLs were immediately soaked in RNAlater™ RNA stabilization reagent (Qiagen) and stored at −30° until use. Total cellular RNA extraction, reverse transcription to generate cDNA, and real-time quantitative polymerase chain reaction (PCR) assays were performed as described previously.[10] In brief, the assays were performed using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) with QuantiTect SYBR Green PCR (Qiagen). The PCR primer sequences and product sizes were as follows: H1R, forward 5′-AAGT CACCATCCCAAACCCCCAAG-3′ and reverse 5′-TCAGG CCCTGCTCATCTGTCTTGA-3′ (195 bp); H2R, forward 5′-AGGAACGAGACCAGCAAGGGCAAT-3′ and reverse 5′-GGTGGCTGCCTTCCAGGAGCTAAT-3′ (197 bp); H3R, forward 5′-TGCAAGCTGTGGCTGGTAGTGGAC-3′ and reverse 5′-AGCTCAGGATGGCTGGTCCGTACA-3′ (202 bp); H4R, forward 5′-CCGTTTGGGTGCTGGCCTTCTTAG-3′ and reverse 5′-GATCACGCTTCCACAGGCTCCAAT-3′ (204 bp); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5′-ACCACAGTCCATGCCATCAC-3′ and reverse 5′-TCCACCACCCTGTTGCTGTA-3′ (452 bp).[9] The expression level of H1R, H2R, H3R and H4R was estimated by dividing each signal into the signal for GAPDH. As a positive control for H3R, the testicular cell line, NEC14, was used.[13]

Statistical analysis

Statistical comparisons were performed by the non-parametric Mann–Whitney *U*-test and Wilcoxon's signed-rank test. Differences were considered significantly different at a *P*-value of < 0·05. Values are given as means ± standard deviation (SD).

Results

Effect of histamine on PPD-induced IFN-γ production

PBMCs from subjects with positive tuberculin skin tests produced IFN-γ in response to PPD, whereas there was negligible IFN-γ production by PBMCs in the absence of PPD. Histamine inhibited the PPD-induced production of IFN-γ in a dose-dependent manner. Typical results are shown in Fig. 1(a). Using 12 subjects, the mean concentration of IFN-γ induced by PPD was 10 479 ± 10 783 pg/ml. IFN-γ production was significantly inhibited to a mean of 4697 ± 4657 pg/ml (54·3 ± 35·8% inhibition; $P = 0{\cdot}010$ by Wilcoxon's signed-rank test) upon exposure of the cells to 100 μM histamine (Fig. 1b). PBMCs did not produce IL-10 in response to PPD, and the addition of histamine did not alter IL-10 production (data not shown).

Effect of HR-selective agonists on PPD-induced IFN-γ production

Next, we examined the effect of HR-selective agonists on PPD-induced IFN-γ production. H4R agonists, including

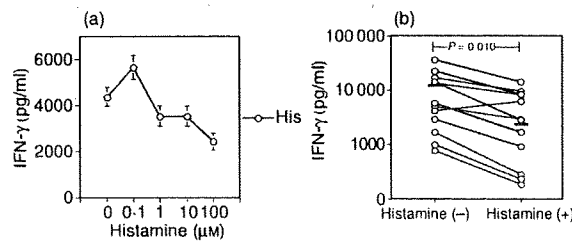

Figure 1. Inhibition of purified protein derivative of *Mycobacterium tuberculosis* (PPD)-induced interferon-γ (IFN-γ) production by histamine. (a) Peripheral blood mononuclear cells (PBMCs) were cultured with 2 μg/ml of PPD in the presence or absence of serial dilutions of histamine. Results are expressed as the mean concentrations ± standard deviation (SD) from triplicate cultures. Data are representative of four separate experiments. (b) PBMCs from 12 subjects with positive tuberculin skin tests were stimulated with 2 μg/ml of PPD in the presence or absence of 100 μM histamine. The *P*-value was obtained using Wilcoxon's signed-rank test. Vertical bars represent the mean concentrations for each group.

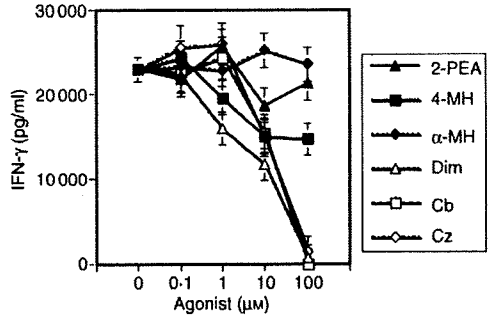

Figure 2. Inhibition of purified protein derivative of *Mycobacterium tuberculosis* (PPD)-induced interferon-γ (IFN-γ) production from peripheral blood mononuclear cells (PBMCs) by histamine receptor (HR)-selective agonists. PBMCs were cultured with 2 μg/ml of PPD in the presence or absence of serial dilutions of HR-selective agonists. Cb, clobenpropit; Cz, clozapine; Dim, dimaprit; 4-MH, 4-methylhistamine; α-MH, alpha-methylhistamine; 2-PEA, 2-pyridylethylamine. Results are expressed as the mean concentrations ± standard deviation (SD) from triplicate cultures. Data are representative of four separate experiments.

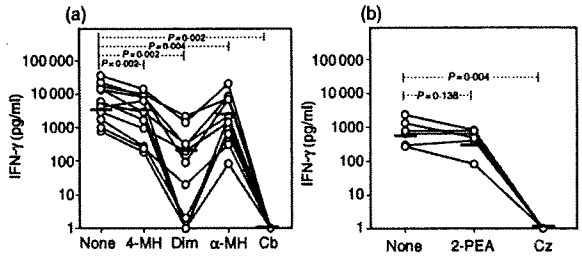

Figure 3. Inhibition of purified protein derivative of *Mycobacterium tuberculosis* (PPD)-induced interferon-γ (IFN-γ) production by histamine receptor (HR)-selective agonists. Peripheral blood mononuclear cells (PBMCs) were stimulated with PPD in the presence or absence of 100 μM HR-selective agonists. (a) Effect of 4-methylhistamine (4-MH), alpha-methylhistamine (α-MH), dimaprit (Dim) and clobenpropit (Cb) on PPD-induced IFN-γ production ($n = 12$). (b) Effect of 2-pyridylethylamine (2-PEA) and clozapine (Cz) on PPD-induced IFN-γ production ($n = 6$). The *P*-values were obtained using Wilcoxon's signed-rank test. Vertical bars represent the mean concentrations for each group.

dimaprit, clobenpropit and clozapine, inhibited PPD-induced IFN-γ production in a dose-dependent manner (Fig. 2). Complete inhibition of PPD-induced IFN-γ production was observed in the presence of 100 μM dimaprit (97·4 ± 3·8% inhibition; $P = 0·002$), clobenpropit (100% inhibition; $P = 0·002$), or clozapine (100% inhibition; $P = 0·004$). In contrast, 100 μM 2-PEA did not cause significant inhibition (33·4 ± 47·2% inhibition; $P = 0·138$) of PPD-induced IFN-γ production, and partial inhibition was observed using 100 μM 4-MH (63·6 ± 16·5% inhibition; $P = 0·002$) and α-MH (50·3 ± 35·0% inhibition; $P = 0·004$) (Fig. 3). None of the HR-related agonists

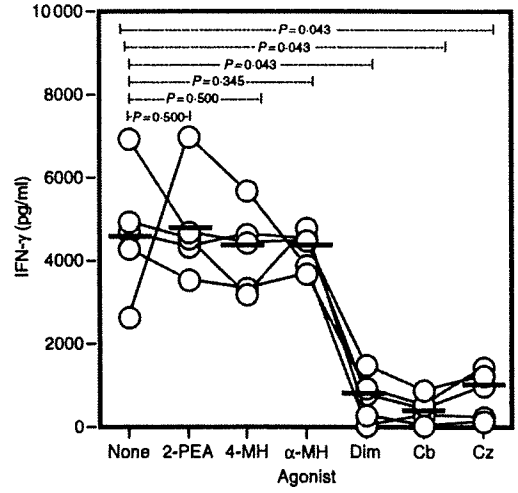

Figure 4. Histamine receptor (HR)-selective agonists-mediated inhibition of purified protein derivative of *Mycobacterium tuberculosis* (PPD)-induced interferon-γ (IFN-γ) production by T-cell lines (TCLs). Five PPD-specific TCLs were mixed with antigen-presenting cells (APCs) and cultured with 2 μg/ml of PPD for 65 hr in the presence of 100 μM 2-pyridylethylamine (2-PEA), 4-methylhistamine (4-MH), alpha-methylhistamine (α-MH), dimaprit (Dim), clobenpropit (Cb) and clozapine (Cz). Following incubation, supernatant was collected and the IFN-γ concentration of each sample was determined by enzyme-linked immunosorbent assay (ELISA). *P*-values were determined using Wilcoxon's signed-rank test. Data on each TCL are representative of two separate experiments.

induced IL-10 production in PPD-stimulated PBMCs (data not shown).

Similar results were seen when we used PPD-specific TCLs, the more purified cell populations. We generated five PPD-specific TCLs from five donors. Treatment with 2-PEA, 4-MH and α-MH did not affect the PPD-specific IFN-γ production. However, treatment with dimaprit, clozapine and clobenpropit strongly and dose-dependently inhibited these responses (Fig. 4).

Effect of HR-selective antagonists on the inhibition of IFN-γ production by H4R-selective agonists

To verify that the H4R-selective agonists inhibit PPD-specific IFN-γ production via H4R, we examined the influence of a panel of HR-specific antagonists. Addition of thioperamide, an antagonist of both H4R and H3R, did not reverse the inhibition of PPD-induced IFN-γ production by either histamine or H4R agonists (Fig. 5a). Furthermore, a mixture of *d*-chlorpheniramine, famotidine and thioperamide did not reverse the inhibition (Fig. 5b). These results were seen in both high responders (IFN-γ production > 10 000 pg/ml) and low responders (IFN-γ production < 10 000 pg/ml) for PPD-induced IFN-γ production (data not shown).

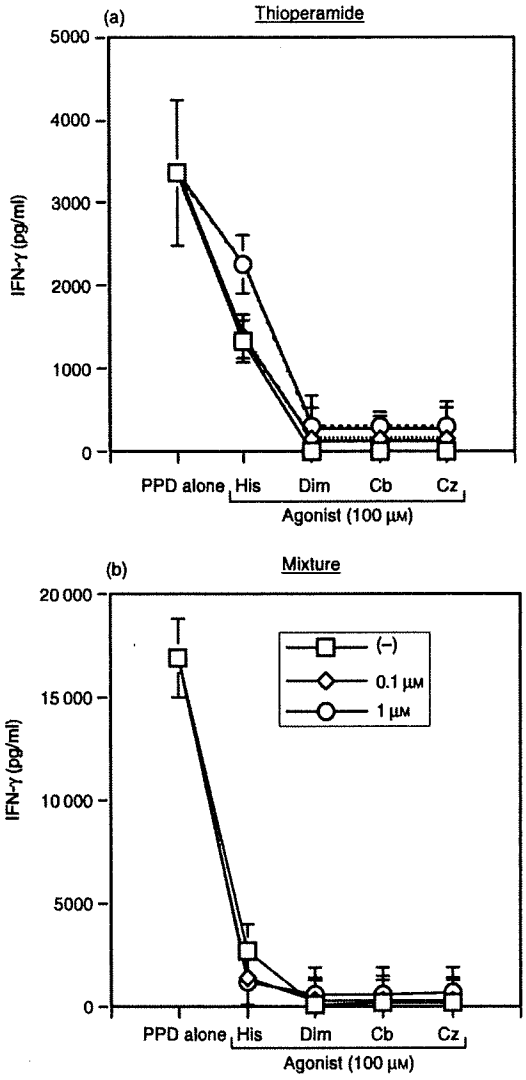

Figure 5. Effect of histamine receptor (HR)-selective antagonists on the inhibition of interferon-γ (IFN-γ) production induced by H4R-selective agonists. Peripheral blood mononuclear cells (PBMCs) were pretreated for 30 min with thioperamide (a) or with a mixture of antagonists (b) at the following concentrations: (a) pretreatment with 0 μM (square), 0·1 μM (diamond) or 1·0 μM (circle) thioperamide; (b) simultaneous pretreatment with each concentration of 0 μM (square), 0·1 μM (diamond) or 1·0 μM (circle) *d*-chlrophenriramine, famotidine and thioperamide. Then, cells were incubated with 2 μg/ml of purified protein derivative of *Mycobacterium tuberculosis* (PPD) and 100 μM histamine (His), dimaprit (Dim), clobenpropit (Cb), or clozapine (Cz). Results are presented as the mean concentrations ± standard deviation (SD) from triplicate cultures. Data are representative of three separate experiments.

Role of adenylate cyclase and apoptosis in histamine suppression of PPD-induced IFN-γ production

We examined whether the inhibition by histamine and H4R-selective agonists depends on the activity of adenylate cyclase. Pretreatment of PBMCs with SQ22536, an adenylate cyclase inhibitor, reversed the inhibition of PPD-induced IFN-γ production by histamine and dimaprit. Furthermore, pretreatment with RP-8-Br-cAMPS, a PKA type I inhibitor, completely reversed the inhibition by histamine and dimaprit (Fig. 6a,b). Pretreatment of PBMC with SQ22536 and RP-8-Br-cAMPS also reversed the inhibition by clozapine (Fig. 6d), but the effects were weaker than observed for the reversal of inhibition by histamine. However, a minimal effect of SQ22536 and RP-8-Br-cAMPS was seen on the effect of clopenpropit (Fig. 6c). These results were seen in both high and low responders for PPD-induced IFN-γ production (data not shown). The addition of SQ22536 or RP-8-Br-cAMPS had no effect on the IFN-γ production by PBMCs in response to PPD alone (data not shown).

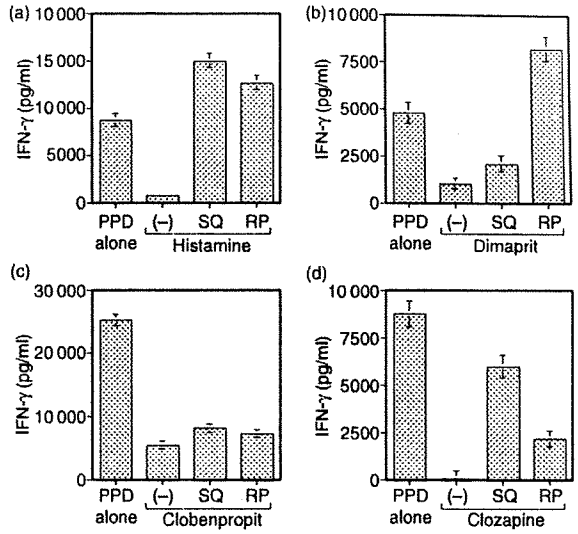

Figure 6. Roles of cAMP and protein kinase A (PKA) in the inhibition of purified protein derivative of *Mycobacterium tuberculosis* (PPD)-induced interferon-γ (IFN-γ) production by H4R-selective agonists. Peripheral blood mononuclear cells (PBMCs) were pretreated for 60 min with SQ22536 (SQ) or RP-8-Br-cAMPS (RP) and then incubated with 2 μg/ml of PPD and 100 μM histamine (a), dimaprit (b), clobenpropit (c), or clozapine (d). The results are expressed as the mean concentrations ± standard deviation (SD) from triplicate cultures. Data are representative of three separate experiments.

Pretreatment of PPD-specific TCLs alone with SQ22536, followed by coculture with intact APCs, partially suppressed inhibition of the PPD-specific IFN-γ production by dimaprit. Similar suppression was observed when APCs alone were pretreated with SQ22536, followed by the coculture with intact TCLs. However, pretreatment of both TCLs and APCs with SQ22536 markedly reversed the inhibitory effects of dimaprit on the PPD-specific response (Fig. 7).

The addition of 100 μM dimaprit, clobenpropit, or clozapine, but not of histamine, induced annexin-V expression on PBMCs (Fig. 8). $CD19^+$ cells were highly

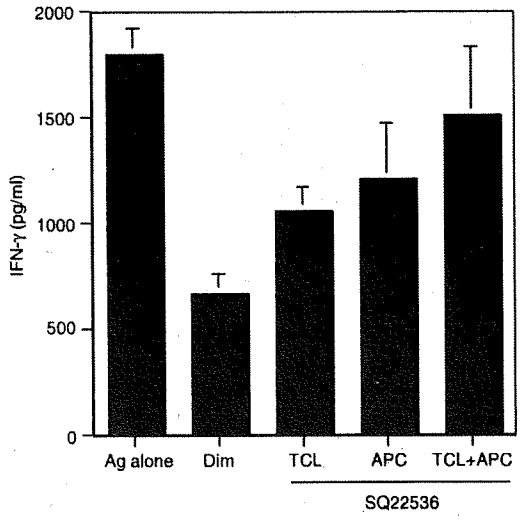

Figure 7. Reversal of H4R-selective agonist-induced inhibition of antigen (Ag)-specific T-cell responses with an adenylate cyclase inhibitor. Purified protein derivative of a *Mycobacterium tuberculosis* (PPD)-specific T-cell line (TCL) alone, antigen-presenting cells (APCs) alone, or both TCL and APC, were pretreated with SQ22536 at 37° for 1 hr. Following incubation, the cells were washed with culture medium three times, after which they were mixed and cultured with the PPD, in the presence of 100 μM dimaprit (Dim), for 65 hr. Data are representative of two separate experiments.

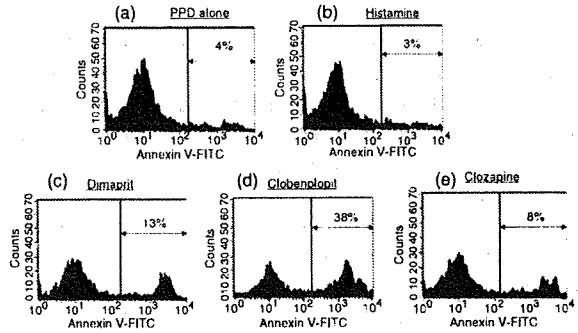

Figure 8. Expression of annexin-V by peripheral blood mononuclear cells (PBMCs) following treatment with H4R-selective agonists. PBMCs were incubated for 72 hr with purified protein derivative of *Mycobacterium tuberculosis* (PPD) (a) and histamine (b), dimaprit (c), clobenpropit (d), or clozapine (e), and the expression of annexin-V on $CD19^+$ cells was analysed by flow cytometry. Data are representative of six separate experiments.

susceptible to the induction of annexin-V expression following exposure to H4R-related agonists. H4R agonists also induced annexin-V expression in $CD4^+$ cells, but not in $CD8^+$ cells (data not shown).

cDNA miroarray analysis

We sought to compare the comprehensive expression of mRNA in PBMC following PPD stimulation in the presence or absence of clozapine. Among 16 600 genes tested, the amounts of mRNA were unchanged in 96·2% of the genes. However, the mRNA levels of 0·8% of the genes (such as melanocortin 1 receptor) were increased (> 200%) in the presence of clozapine. On the contrary, the mRNA levels of 3·0% of the genes, such as IFN-γ, were decreased (< 50%) (Table 1).

Effect of HR-selective agonists on Cry j1-induced IL-5 production

We examined the effect of HR-selective agonists on human Th2 responses. PBMCs from patients with Japanese cedar pollinosis produced a comparable amount of IL-5 in response to Cry j1. The addition of 100 μM 2-PEA, 4-MH or α-MH displayed no significant effect on Cry j1-induced IL-5 production. However, H4R agonists, including dimaprit, clobenpropit and clozapine, significantly inhibited Cry j1-induced IL-5 production (Fig. 9).

Expression of the four histamine receptors on Ag-specific TCLs

Finally, mRNA expression of the four histamine receptors was examined in five PPD- and five Cry j1-specific TCLs by reverse transcription–polymerase chain reaction (RT–PCR). H3R mRNA expression was completely undetectable in all TCLs. On the other hand, H1R, H2R and H4R mRNA were clearly detected in most TCLs (Fig. 10). Using real-time PCR analysis, relative expression levels of the four HRs were not observed to differ significantly among Cry j1- and PPD-specific TCLs (H1R, $P = 0{\cdot}117$; H2R, $P = 0{\cdot}245$; H4R, $P = 0{\cdot}344$; using the Mann–Whitney *U*-test). The expression levels of H4R in TCLs were lower than that of H1R; however, significantly increased expression of the H4R was observed as compared to H2R and H3R (Fig. 11).

Discussion

In the present study, we demonstrated that histamine inhibits PPD-induced IFN-γ production in PBMCs. Several studies have investigated the regulatory role of histamine on IFN-γ production by T cells.[7,8,14–16] Lagier *et al.* reported that histamine inhibits IFN-γ production by human Th1-like T-cell clones (TCCs) specific for *Dermatophagoides pteronyssinus*, whereas it did not have a significant inhibitory effect on T helper 0 (Th0)-like TCCs, and it had no effect on Th2-like TCCs stimulated with phorbol 12-myristate 13-acetate (PMA) and a calcium ionophore.[8] Krouwels *et al.* reported that histamine inhibited IFN-γ production in 21 out of 52 human TCCs (40%), whereas IFN-γ production was enhanced in eight of the TCCs (16%). Also, histamine did not affect IFN-γ production in 23 TCCs (44%) in response to plate-bound

Table 1. Ranked list of up-regulated and down-regulated transcripts in purified protein derivative of *Mycobacterium tuberculosis* (PPD)-stimulated peripheral blood mononuclear cells with clozapine

	Up-regulated transcripts				Down-regulated transcripts		
Rank	Gene name	GenBank acc. no.	Ratio[1]	Rank	Gene name	GenBank acc. no.	Ratio[1]
1	Melanocortin 1 receptor	NM_002386	8·50	1	Interferon-γ	NM_000619	0·02
2	LOC90271	XM_030445	5·59	2	Chemokine (C-C motif) ligand 5	NM_002981	0·02
3	ATP-binding cassette, subfamily G (WHITE) member 1, transcript variant 1	NM_004915	4·75	3	Matrix metalloproteinase 10	NM_002425	0·02
4	Actin-binding LIM protein 2	NM_032432	4·69	4	Chemokine (C-X-C motif) ligand 5	NM_002994	0·03
5	Liver-expressed antimicrobial peptide 2	NM_052971	4·32	5	KIAA1046 protein	NM_014928	0·03
6	Oviductal glycoprotein 1, 120 000 MW	NM_002557	4·30	6	Secreted phosphoprotein 1	NM_000582	0·03
7	Killer-specific secretory protein of 37 000 MW	NM_031950	3·96	7	Chemokine (C-X-C motif) ligand 1	NM_001511	0·03
8	LOC286006	XM_209854	3·83	8	Tumor necrosis factor (ligand) superfamily member 15	NM_005118	0·03
9	Myelin-basic protein	NM_002385	3·73	9	Similar to immune-responsive protein 1	XM_292184	0·04
10	Hypothetical protein LOC157562	XM_098779	3·71	10	Matrix metalloproteinase 7	NM_002423	0·04

[1]Ratio: with clozapine versus without clozapine.
GenBank acc. no., GenBank accession number; MW, molecular weight.

anti-CD3 mAb.[14] In contrast, pretreatment with histamine does enhance IFN-γ production in response to plate-bound anti-CD3 mAb in human Th1 cells.[7] Furthermore, Osna *et al.* showed that the effect of histamine on IFN-γ production was dependent on the stimulatory signals.[15]

We investigated the PPD-induced human T-cell responses to clarify whether histamine affects antigen-specific T-cell responses. Our results are consistent with previous reports. Osna *et al.* reported that histamine up-regulates IL-10 production in murine splenocytes and inhibits IFN-γ production.[16] In the current study, PBMCs did not produce IL-10 in response to PPD, and IL-10 production was not induced in the presence of histamine, suggesting that factors other than IL-10 may be involved in the inhibitory effect of histamine.

The H4R agonists dimaprit, clobenpropit and clozapine all eliminated PPD-induced proliferation and IFN-γ production in PBMCs. H4R is selectively expressed in cells of haematopoietic lineage, including mast cells, eosinophils and lymphocytes.[3,17] Physiological roles of H4R in mast cells, eosinophils, neutrophils and dendritic cells have been implied in recent years, but whether signals through H4R can affect T-cell functions has not been determined.[6,17–19] One report demonstrated that histamine induces IL-16 production by human $CD8^+$ T cells through H2R and H4R.[9] Our results, using HR-related agonists, suggest that signals through H4R may exert an inhibitory role on antigen-specific T-cell responses. However, thioperamide, an H3R and H4R antagonist, did not reverse the inhibition of PPD-induced IFN-γ production by either histamine or H4R agonists. Thioperamide, *d*-chlorpheniramine and famotidine, the HR antagonists used in the present study, were confirmed to be functional in previous studies.[20,21] This suggests that the inhibitory effect of histamine and H4R agonists is not mediated by H4R. In addition, a mixture of *d*-chlorpheniramine, famotidine and thioperamide did not reverse the inhibition by histamine, suggesting that the effect is independent of H1R, H2R and H3R/H4R.

2-PEA, an H1R-selective agonist, did not affect PPD-induced IFN-γ production. On the other hand, 4-MH, an H2R-selective agonist, partially inhibited its production. It is known that histamine regulates cytokine production, via H2R, on T cells.[8,14,15,22] In addition, histamine inhibits IL-12 production by monocytes via H2R.[23] Our finding that dimaprit, an H2R and H4R agonist, inhibited PPD-induced IFN-γ production, suggests that signals through H2R may be involved in the inhibition. However, the inhibitory effect of either histamine or dimaprit

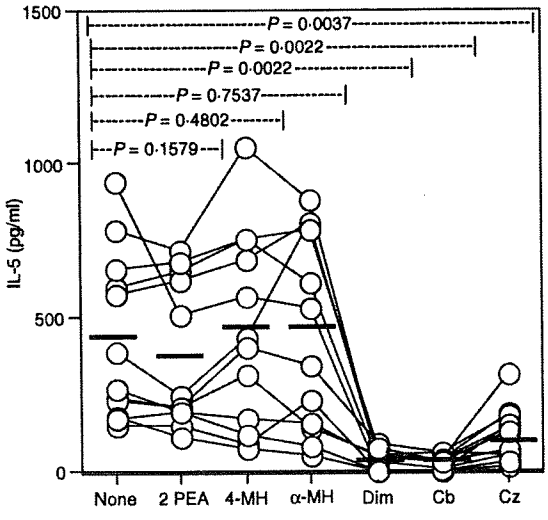

Figure 9. Histamine receptor (HR)-selective agonists-mediated inhibition of Cry j1-specific interleukin (IL)-5 production by peripheral blood mononuclear cells (PBMCs). PBMCs from 12 patients with Japanese cedar pollinosis were cultured with 10 μg/ml of Cry j1, in the presence or absence of 2-pyridylethylamine (2-PEA), 4-methylhistamine (4-MH), alpha-methylhistamine (α-MH), dimaprit (Dim), clobenpropit (Cb) or clozapine (Cz), at 100 μM, for 72 hr. Following incubation, supernatant was collected and the concentrations of IL-5 were determined in each sample using an enzyme-linked immunosorbent assay (ELISA). *P*-values were obtained using Wilcoxon's signed-rank test.

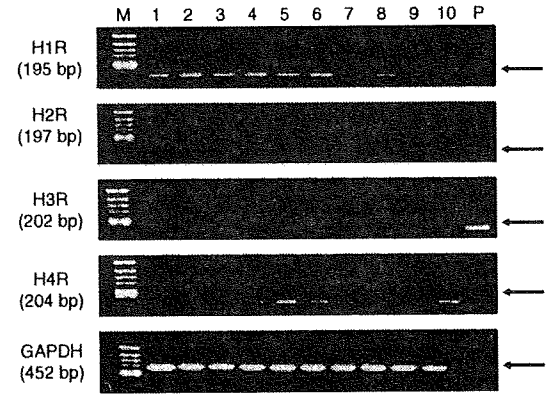

Figure 10. Expression of four histamine receptors (HRs) by human T-cell lines (TCLs). mRNA was extracted from five Cry j1-specific TCLs (lanes 1–5) and from five purified protein derivative of *Mycobacterium tuberculosis* (PPD)-specific TCLs (lanes 6–10), after which the levels of H1R, H2R, H3R, H4R and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were detected by reverse transcription–polymerase chain reaction (RT–PCR), as described in the Materials and methods. M, molecular marker; P, positive control (NEC14).

was not reversed by famotidine, suggesting that H2R signalling had a negligible role in the inhibition of PPD-induced IFN-γ production.

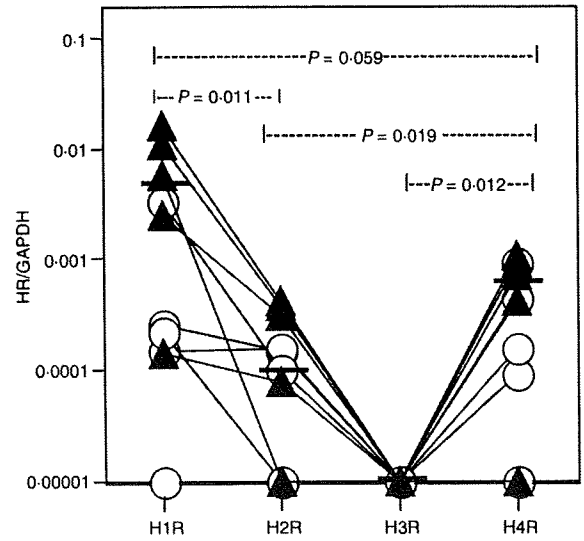

Figure 11. Comparison of the amounts of four histamine receptors (HRs) among T-cell lines (TCLs). The expression levels of four HRs were determined in five Cry j1-specific TCLs (closed triangle) and in five purified protein derivative of *Mycobacterium tuberculosis* (PPD)-specific TCLs (open circle) using real-time reverse transcription–polymerase chain reaction (RT–PCR). Each bar represents the median expression level of each messenger. *P*-values were obtained using Wilcoxon's signed-rank test.

The pretreatment of PBMCs with SQ22536, an adenylate cyclase inhibitor, and with RP-8-Br-cAMPS, a PKA type 1 inhibitor, reversed the inhibition of PPD-induced IFN-γ production by histamine and H4R agonists. Previous reports demonstrated that 5 mM SQ22536 and 5 mM RP-8-Br-cAMPS reversed, almost completely, the inhibitory effect of several compounds on human PBMC responses.[12] These results suggest that H4R agonists, except for clobenpropit, inhibit PPD-induced IFN-γ production by elevating intracellular cAMP levels and activating PKA. Moreover, results using PPD-specific TCLs and APCs suggest that H4R agonists may influence PPD-specific cellular responses at both the T-cell and APC level. It is known that H2R activation causes an elevation of the intracellular cAMP level.[24] In addition, PKA plays a pivotal role in histamine-mediated regulation of IFN-γ production, especially via the stimulation of T-cell receptors.[15] Although dimaprit can act as an H2R agonist, clozapine and clobenpropit cannot.[25] H4R is coupled to $G_{i/o}$, which leads to the inhibition of cAMP formation.[26] This further supports the possibility that the inhibitory effect of H4R agonists is not associated with typical H4R signaling. Rather, signals similar to those mediated by H2R may participate in the inhibition. However, we also observed that the inhibitory effect of either histamine or dimaprit was not reversed by famotidine, suggesting that H2R signaling had a negligible role in the inhibition of PPD-induced IFN-γ production. In addition, the varied effect of SQ22536 and RP-8-Br-cAMPS on IFN-γ

production induced by different agonists may suggest the presence of unidentified pleural receptors in the action of agonists.

$CD19^+$ and $CD4^+$ cells express annexin-V following exposure to H4R agonists, suggesting that these compounds induce apoptosis in these cells. On the other hand, cDNA microarray analysis revealed that the changes of mRNA levels were seen in 0·8% of the genes tested, such as melanocortin 1 receptor, following the stimulation with H4R agonist (Table 1), indicating that the inhibitory effects of H4R agonists were not non-specific or solely the result of an apoptotic effect. For example, melanocortin 1 receptor has signal transducer activity and it is involved in immunosuppression.[27] Thus, the suppressive role of H4R agonists may be associated with the activation of melanocortin 1 receptor. In addition, histamine did not induce the expression of annexin-V. These results suggest that the inhibitory effect of H4R agonists is not associated with the binding to classical HRs.

Concentrations of histamine and H4R agonists ranging from 10^{-5} to 10^{-4} M displayed a dose-dependent inhibition of PPD-induced IFN-γ production by PBMCs. Although it was difficult to define precisely the histamine concentration in the target organ, concentrations of histamine from 10^{-6} to 10^{-4} M have been reported to be comparable to those measured in tissues after mast cell degranulation.[28]

In conclusion, we examined the effect of histamine and H4R agonists on antigen-specific human T-cell responses. H4R signaling is important for functions of other immune cells, such as mast cell chemotaxis, eosinophil chemotaxis and suppression of IL-12 production by dendritic cells.[3,29] H4R agonists inhibit Ag-specific cytokine production; however, our investigations, using antagonists of H4R and inhibitors of adenylate cyclase or PKA, revealed that H4R plays a negligible role in the inhibition. These results indicate that there may be a previously unidentified HR or receptor subtype that can bind to dimaprit, clobenpropit and clozapine and that can mediate the inhibition of antigen-induced cellular responses via a cAMP/PKA-dependent, apoptotic pathway. More recently, Lim *et al.* have reclassified 4-MH as the most selective H4R agonist so far at the H4R.[30] This seems to be relevant for understanding the present results, suggesting a new orphan receptor. Furthermore, our observations may provide the basis for novel therapeutic approaches in the management of allergic and autoimmune diseases.

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IMMUNOLOGY ORIGINAL ARTICLE

Differential effects of tumour necrosis factor-α and interleukin-12 on isopentenyl pyrophosphate-stimulated interferon-γ production by cord blood Vγ9 T cells

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Summary

Lower numbers of Vγ9Vδ2 T cells in cord blood (CB) than in adult peripheral blood (PB), as well as their impaired ability to produce interferon-γ (IFN-γ) in response to stimulation, are associated with functional deficiency in the immune system in newborns. In this study, we stimulated CB Vγ9 T cells with their T-cell receptor-specific ligand, isopentenyl pyrophosphate (IPP), plus exogenous costimulatory cytokines such as interleukin-2 (IL-2), IL-12 and tumour necrosis factor-α (TNF-α), which are known to play important roles in the activation of PB γδ T cells. Our data show that CB Vγ9 T cells are able to produce IFN-γ at levels comparable to PB Vγ9 T cells by the addition of TNF-α in the presence of IPP and IL-2; however, under the same culture conditions, IL-12 does not efficiently activate CB Vγ9 T cells to produce IFN-γ. The frequency of TNF-α receptor II-positive Vγ9T cells and the expression levels of TNF-α receptor II are similar in CB and PB; in contrast, the frequency of IL-12 receptor βI (IL-12Rβ1) -positive Vγ9T cells and expression levels of IL-12Rβ1 are significantly lower in CB than PB. TNF-α but not IL-12 increases the expression of IL-2Rβ on CB Vγ9 T cells. These results provide new insights into the role of TNF-α in the activation of CB Vγ9 T cells.

Keywords: cord blood; interferon-γ; interleukin-12; tumour necrosis factor-α; Vγ9 T cells

Introduction

Vγ9Vδ2 T cells are the major subset of the circulating γδ T cells in adult peripheral blood mononuclear cells (PBMC), and are known to participate in the defence against microbial pathogens and to have anti-tumour activity.[1,2] This subset is activated by a wide range of non-peptide antigens, including phosphoantigens,[3,4] alkylamines[5] and bisphosphonates.[6,7] Such T cells proliferate rapidly upon exposure to these antigens during infections or following immunizations, causing an expansion from 2 to 50% in circulating blood T cells,[8,9] in parallel with rapid elevations in tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) secretion.[10] The unconventional specificity for self and non-self signals bridges innate and acquired immunity and places Vγ9Vδ2 T cells at a key regulatory and effector position in the immune system.

Studies show that newborns are more susceptible to infectious diseases than adults and present more prolonged and severe symptoms when infected.[11] Newborns are generally considered to have an immature and hyporesponsive cellular immune system compared to that of adults. This is also true for Vγ9Vδ2 T cells whose functional immaturity as well as low numbers in cord blood has been observed in comparison with adults.[12–15] This functional hyporesponsiveness includes a reduction in the expression of T helper type 1 (Th1) or Th2 cytokines or cytotoxic effector molecules;[12] moreover, under stimulation of cord blood mononuclear cells (CBMC) with the Vγ9 T-cell receptor (TCR) -specific ligand, isopentenyl pyrophosphate (IPP), neonatal Vγ9Vδ2 T cells are unable to express IFN-γ,[12] which is an important cytokine for the regulation of immune responses.[16,17]

Costimulatory factors, such as the cytokines interleukin-2 (IL-2), IL-12 and TNF-α, play important roles in the activation of adult γδ T cells. Exogenous IL-2 has been used to allow the selective proliferation of γδ T cells responding to non-peptide ligands.[18,19] It has also been

reported that IL-2 can efficiently stimulate the cellular expansion of IPP-activated Vγ9 T cells.[20] Interleukin-12 has been shown to activate adult γδ T cells to express CD25 without other stimuli,[21] and also to contribute to the cellular expansion of IPP-reactive human Vγ9 T cells.[20] Tumour necrosis factor-α is known for its wide range of activities extending from regulating cell homeostasis to its antibacterial properties.[22,23] In addition, exogenous TNF-α added to a culture, augments IL-12-induced CD25 expression on adult γδ T cells, suggesting that TNF-α may play a role in IL-12-induced activation on such cells.[21]

So far, there have been very few studies on the activation mechanisms of Vγ9Vδ2 T cells in newborns, especially as related to IFN-γ production; therefore, in this work, we expanded upon previous investigations in terms of quantity and quality, as well as evaluating the TCR-stimulated production of IFN-γ on CB Vγ9Vδ2 T cells in comparison with that in adult PB, especially upon the stimulation with IL-2, IL-12 and TNF-α.

Materials and methods

Lymphocyte sample collection

Cord blood samples were collected following healthy full-term deliveries by venepuncture of the umbilical vein immediately after delivery. Women donating CB were human immunodeficiency virus (HIV)-negative. Exclusion criteria were clinical symptoms of infectious disease during pregnancy and preterm deliveries. Samples were collected at Kawatetsu Hospital, in Chiba city, Japan. Heparinized peripheral blood samples were obtained from healthy adult volunteers at Chiba University, their ages ranged from 30 to 50 years. It was also confirmed that adult volunteers were HIV-negative. Approval was received from the Ethics Committee of Chiba University and informed consent forms were obtained from all women donating cord blood.

CBMC/PBMC cultures

All blood samples were processed within 24 hr of collection. The CBMC and PBMC were isolated by density centrifugation over Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. For stimulation, cells were cultured at a concentration of $0{\cdot}5 \times 10^6$ in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated pooled human AB serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. The IPP (16 μg/ml) (Sigma-Aldrich, St. Louis, MO) was added to the cultures for 24 hr. Human recombinant IL-2 20 IU/ml (R&D Systems, Minneapolis, MN), human recombinant IL-12 100 pg/ml (Sigma-Aldrich) and human recombinant TNF-α 100 ng/ml (Sigma-Aldrich) were added to the culture. Brefeldin A 10 μg/ml (Sigma-Aldrich) was added for the last 6 hr of incubation. Cells were incubated for 24 hr at 37° under 5% CO_2.

Flow cytometric analysis

For intracellular staining, cells were harvested, washed once with phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA) with 1% fetal bovine serum, and then surface stained at 4° for 30 min. Cells were washed twice with PBS/1% fetal bovine serum and then fixed with 4% paraformaldehyde in PBS for 15 min at 4°; washed twice and permeabilized with fluorescence-activated cell sorting (FACS) permeabilizing solution 2 (Becton Dickinson, San Jose, CA) for 10 min at 4°. Three-colour intracellular staining was performed, and 1×10^5 cells were acquired on a FACSCalibur flow cytometer (Becton Dickinson) and gated on the basis of forward and side scatter profiles. Gates were set in accordance with the profiles of the antibody isotype control. For surface staining, resting cells were harvested on ice, and subjected to three-colour surface staining with monoclonal antibodies (mAbs) for 30 min. The cells were washed twice, and 1×10^5 lymphocytes were acquired for each sample by a FACSCalibur flow cytometer. The results were analysed using CELLQUEST software (Becton Dickinson).

Antibodies

The following mAbs were used: fluorescein isothiocyanate-conjugated (FITC-) anti-Vγ9 mAb, FITC-anti-Vδ2 mAb, phycoerythrin-conjugated (PE-) anti-IFN-γ mAb, PE-anti Vγ9 mAb, PE-anti-CD122 (IL-2 receptor β chain) mAb, PE-anti-CD132 (IL-2 receptor γ chain) mAb, PE-anti-CD25 (IL-2 receptor α chain) mAb, PE-anti-IL-12 receptor βI (IL-12Rβ1) and -Rβ2, peridinin chlorophyll protein-conjugated anti CD3 mAb (all Becton Dickinson). PE-anti-TNF-αRI and RII (R&D Systems). The respective isotype controls were included.

Statistical analyses

Statistical analyses were performed using the software program GRAPHPAD PRISM version 4.0 (GraphPad Software Inc., La Jolla, CA). Comparisons between CBMC and PBMC samples were evaluated using the non-parametric Mann–Whitney test. The differences in the paired comparison of cord blood samples were evaluated by the Wilcoxon matched pairs test. Values of $P < 0{\cdot}05$ were considered significant.

Results

In this study, CBMC and PBMC were compared in terms of their frequencies and TCR expression levels of Vγ9Vδ2

T cells; the production of IFN-γ by CB Vγ9 T cells stimulated with IPP and the cytokines IL-2, TNF-α and IL-12, which are known to be effective stimulants of adult γδ T cells, was also evaluated. In addition, the expression levels of IL-2, TNF-α and IL-12 receptors on Vγ9 T cells were investigated.

The frequency of Vγ9Vδ2 T cells was low in CB compared with PB (Fig. 1a); whereas the mean fluorescence intensity (MFI) expression levels of TCR on Vγ9Vδ2 T cells were comparable in both groups (Fig. 1b). Since the number of Vγ9Vδ2 T cells is very low in CB and the production of IFN-γ by stimulation with IPP is difficult to measure using enzyme-linked immunosorbent assay, intracellular IFN-γ production in CB Vγ9 T cells was examined by flow cytometry.

In evaluating the response of Vγ9 T cells to exogenous stimuli, it was noted that the addition of 16 μg/ml IPP did not cause CB Vγ9 T cells to produce IFN-γ (Fig. 2a); furthermore, the addition of 20 IU/ml exogenous IL-2 resulted in a minimal increase in the production of IFN-γ, which was still very low in comparison with PB Vγ9 T cells (Fig. 2b). To investigate why CB Vγ9 T cells respond so poorly to IL-2 stimulation, the IL-2Rα, β and γ chains were studied. It was found that the minimal

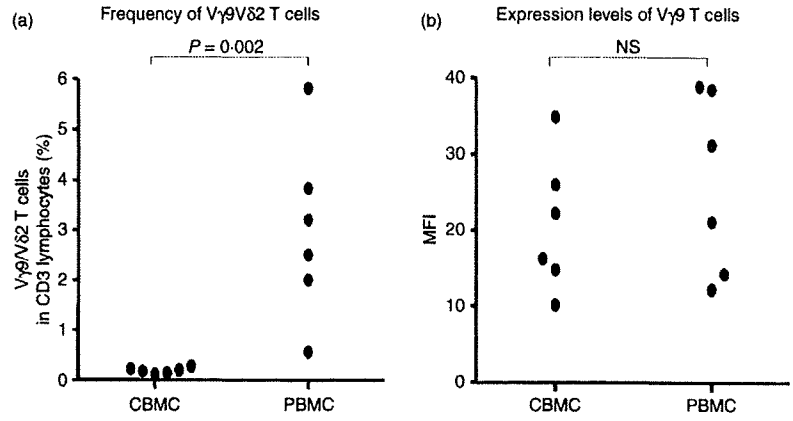

Figure 1. Frequency of Vγ9Vδ2 T cells among CD3 lymphocytes in cord blood and peripheral blood mononuclear cells (CBMC and PBMC) (a) and mean fluorescence intensity (MFI) expression levels of Vγ9 T-cell receptor (TCR) on CBMC versus PBMC (b). The data in these figures are representative of six cord blood samples and six adult peripheral blood samples. Individual values are shown for each sample set. Non-parametric Mann–Whitney test was used to calculate the *P*-values between groups, NS, not significant.

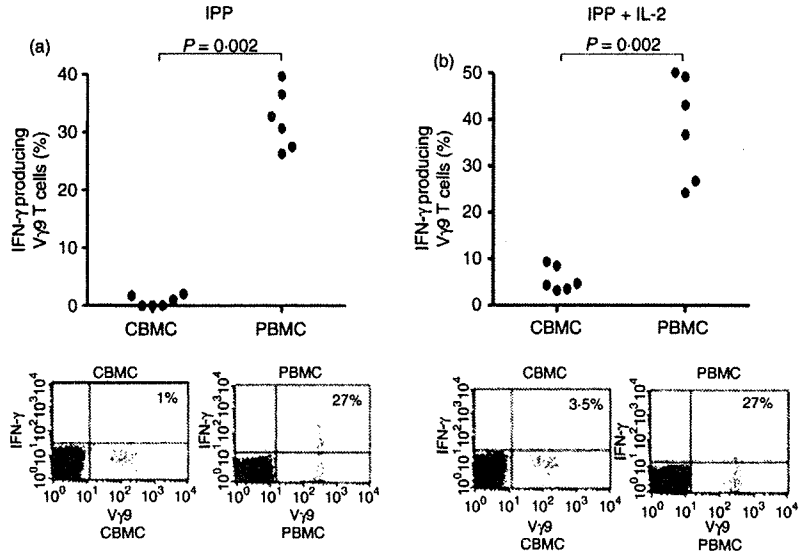

Figure 2. Percentage of Vγ9 T cells in cord blood and peripheral blood mononuclear cells (CBMC and PBMC) producing intracellular interferon-γ (IFN-γ) following stimulation with 16 μg/ml isopentenyl pyrophosphate (IPP) for 24 hr in the presence of 10 μg/ml brefeldin A (BFA) (a) and percentage of Vγ9 T cells in CBMC and PBMC producing intracellular IFN-γ following stimulation with 16 μg/ml IPP plus 20 IU/ml interleukin-2 (IL-2) for 24 hr in the presence of 10 μg/ml BFA (b). The data in these figures are representative of six cord blood samples and six adult peripheral blood samples. Individual values are shown for each sample set. Non-parametric Mann–Whitney test was used to calculate the *P*-values. Flow-cytometry panels show a representative result, and the numbers indicate the percentage of positive IFN-γ-producing Vγ9 T cells within the total Vγ9 T-cell population.

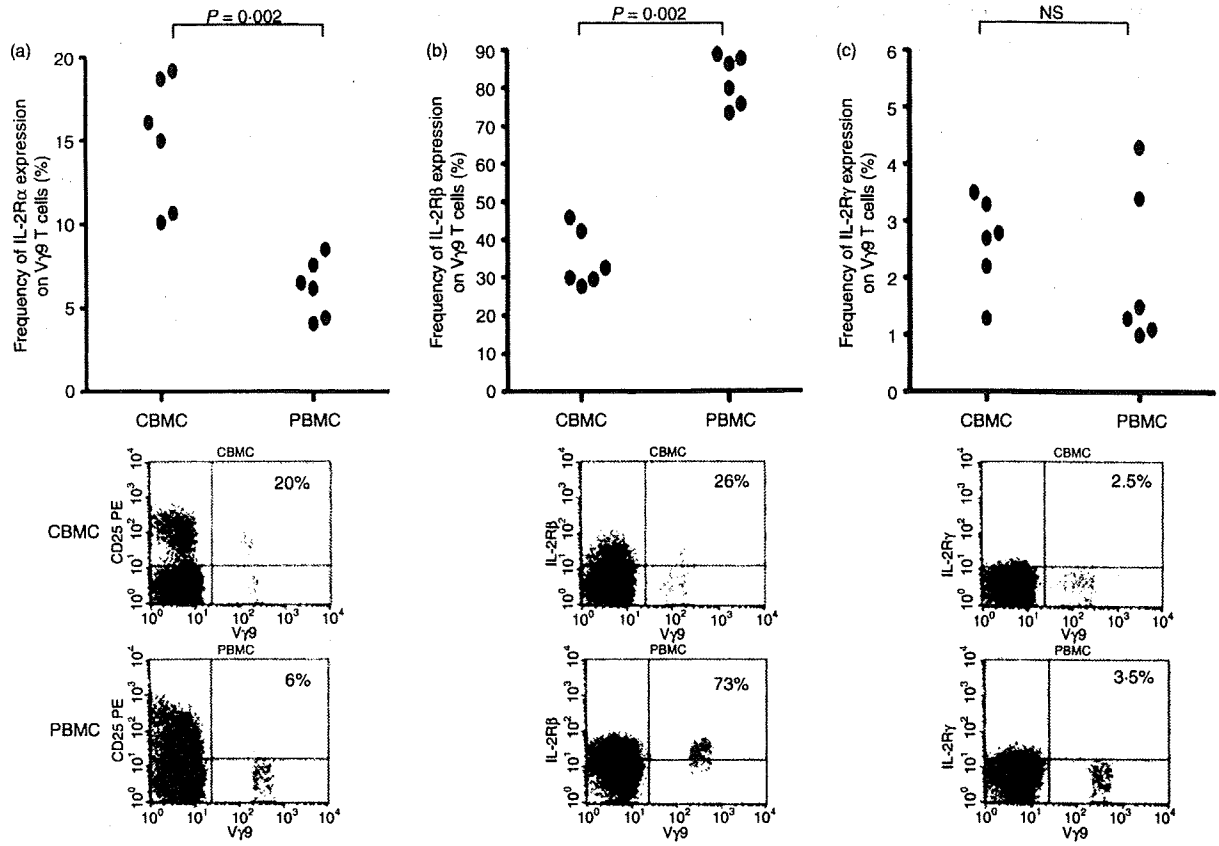

Figure 3. Percentage of resting cord blood and peripheral blood mononuclear cells (CBMC and PBMC) Vγ9 T cells expressing the interleukin-2 receptor α (IL-2Rα) chain (a), IL-2Rβ chain (b), and IL-2Rγ chain (c). The data in these figures are representative of six cord blood samples and six adult peripheral blood samples. Individual values are shown for each sample set. Non-parametric Mann–Whitney test was used to calculate the *P*-values. Panels show a representative result. Numbers indicate the frequency of IL-2Rα chain, β chain and γ chain on positive Vγ9 T cells within the total Vγ9 T-cell population.

response to IL-2 stimulation is associated with lower frequencies of high-affinity IL-2Rβ-chain-positive Vγ9 T cells in CB compared with PB (Fig. 3b). Nevertheless, the frequencies of IL-2Rα-chain-positive Vγ9 T cells are higher in CB than in PB (Fig. 3a), whereas those of IL-2Rγ-chain-positive Vγ9 T cells are similar in both groups (Fig. 3c).

Stimulation with TNF-α and IL-12 alone did not induce IFN-γ production by Vγ9 T cells in newborns or adults without simultaneous IPP stimulation. The addition of 100 ng/ml TNF-α, but not 100 pg/ml IL-12, had a strong effect on IFN-γ production by Vγ9 T cells in CB in the presence of IPP and IL-2 (Fig 4a and b). It was found that the expression of the IL-2Rβ chain on CB Vγ9 T cells increased more following TNF-α stimulation than IL-12 stimulation in 12 hr cultures (Fig. 5), while the expressions of the IL-2Rα and γ chains did not change (data not shown). To determine the reason for the different effects of TNF-α and IL-12 on IL-2Rβ chain expression, we measured the frequencies of TNF-αR- and IL-12R-positive Vγ9 T cells and the expression levels of TNF-αR and IL-12R on Vγ9 T cells. The frequencies of TNF-αRII-positive Vγ9 T cells and the MFI of TNF-αRII on Vγ9 T cells were found to be similar in CB and PB; in contrast, the frequencies of IL-12RβI-positive Vγ9 T cells and the MFI of IL-12RβI on Vγ9 T cells were significantly lower in CB than PB (Table 1). No expression of IL-12RβII or TNF-αRI was found on Vγ9 T cells in either group.

Discussion

Our data show that CB Vγ9 T cells are able to produce IFN-γ at levels comparable to those in PB Vγ9 T cells by stimulation with TNF-α for 24 hr followed by stimulation with IPP in the presence of IL-2; however, under the same culture conditions, IL-12 stimulation does not efficiently activate CB Vγ9 T cells to produce IFN-γ.

The numbers of Vγ9 T cells in CB are much lower than in PB, as previously shown.[14,15] However, the expression

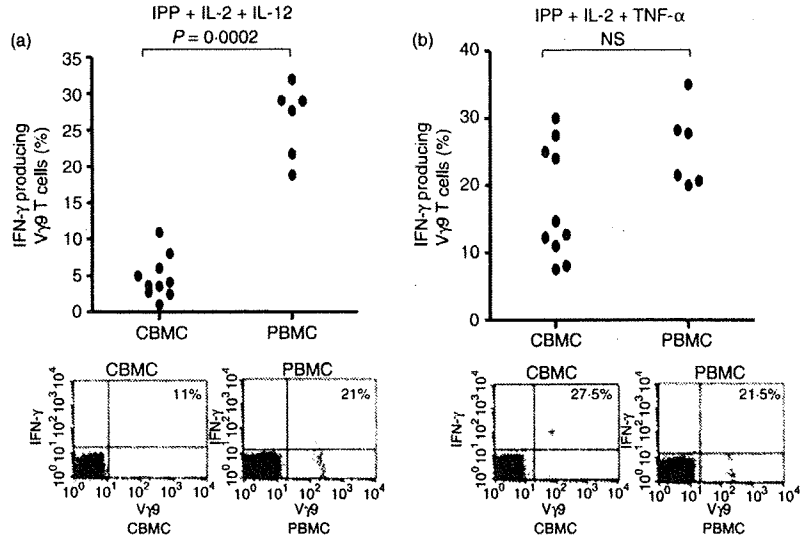

Figure 4. Intracellular interferon-γ (IFN-γ) production by Vγ9 T cells in cord blood and peripheral blood mononuclear cells (CBMC and PBMC) stimulated with 16 μg/ml isopentenyl pyrophosphate (IPP) plus 20 IU/ml interleukin-2 (IL-2) and 100 pg/ml IL-12 for 24 hr in the presence of 10 μg/ml brefeldin A (BFA) (a) and frequency of intracellular IFN-γ production by Vγ9 T cells in CBMC and PBMC stimulated with 16 μg/ml IPP plus 20 IU/ml IL-2 and 100 ng/ml tumour necrosis factor-α (TNF-α) for 24 hr in the presence of 10 μg/ml BFA (b). The data in these figures are representative of 10 cord blood samples and six adult peripheral blood samples. Individual values are shown for each sample set. Non-parametric Mann–Whitney test was used to calculate the *P*-values. Panels show a representative result, and the numbers indicate the percentage of positive IFN-γ-producing Vγ9 T cells within the total Vγ9 T-cell population.

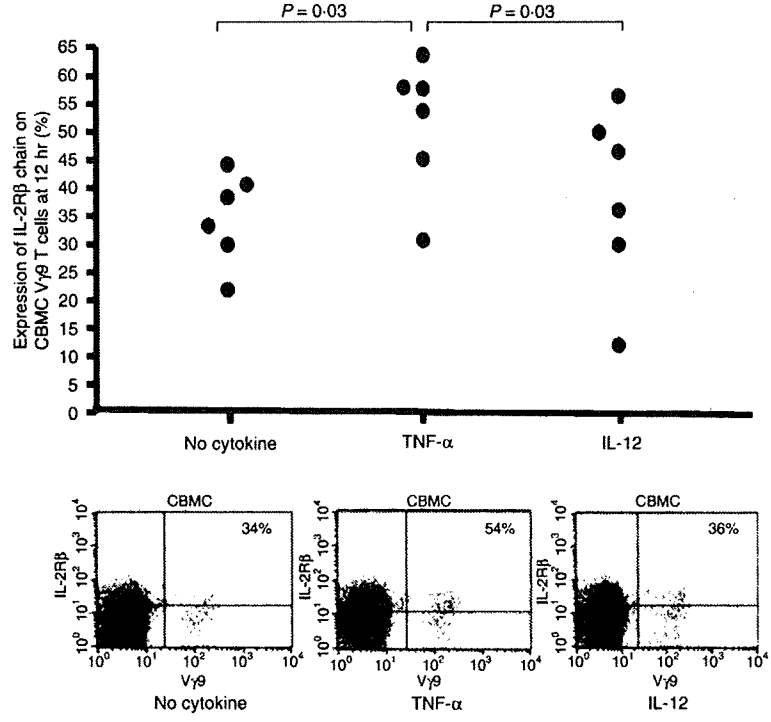

Figure 5. Percentage of cord blood mononuclear cell (CBMC) Vγ9 T cells expressing the interleukin-2 receptor β (IL-2Rβ) chain following stimulation with 100 ng/ml tumour necrosis factor-α (TNF-α) and 100 pg/ml IL-12 for 12 hr. Medium (No Cytokine) was used as a control. The data in these figures are representative of six cord blood matched pair samples. Wilcoxon matched pairs test was used to evaluate the significance of differences in the paired comparison. Panels show a representative result, and the numbers indicate the frequency of the expression of IL-2Rβ chain on CB Vγ9 T cells within the total Vγ9 T-cell population.

Table 1. Frequency and expression levels (MFI ratio) of TNF-αRII and IL-12Rβ1 on resting Vγ9 T cells

	Cord blood(%) $n = 6$	Adult PB (%) $n = 6$	P-value[2]
Frequency of TNF-αRII$^+$ Vγ9 T cells among total Vγ9 T cells	40·8 (33·6–60·4)[1]	47 (36·6–57·1)	0·70
Frequency of IL-12Rβ1$^+$ Vγ9 T cells among total Vγ9 T cells	27·4 (17·5–41·2)	53·2 (46·1–62·3)	0·004
Expression of TNF-αRII on Vγ9 T cells (MFI ratio)	32·1 (26·5–47·2)	43·7 (32·9–62)	0·17
Expression of IL-12Rβ1 on Vγ9 T cells (MFI ratio)	27·7 (25·8–36·8)	51·8 (28·4–61·4)	0·04

CBMC, cord blood mononuclear cells; IL-12Rβ1, interleukin-12 receptor β 1; MFI, mean fluorescence intensity; TNF-αRII, tumour necrosis factor-α receptor II.

No expression of IL-12Rβ11 and TNF-αRI on Vγ9 T cells in CBMC/PBMC.

[1]Values represent medians (25–75th quantiles) of six independent experiments on different donors.

[2]Mann–Whitney test (CBMC versus adult PBMC).

levels of TCR on Vγ9 T cells are similar in CB and PB. The finding that IPP stimulation does not induce IFN-γ production in CB Vγ9 T cells is consistent with a previous report.[12] We increased the amount of IPP to 30 μg/ml; nevertheless, there was still no IFN-γ production from CB Vγ9 T cells (data not shown), indicating a functional defect in TCR-mediated activation in such Vγ9 T cells.

Lower expression levels of the resting IL-2 Rβ chain, which is necessary for the formation of high-affinity IL-2R,[24,25] were found. Consistent with our findings, a lower expression of IL-2Rβ on CB leucocytes has been reported,[26,27] and the relative inability of CB leucocytes to produce IFN-γ has been hypothesized to be the result of a restriction in the early transduction of IL-2R ligand-induced signalling.[26] Our data are somewhat different from the findings of Zola *et al.*, who showed lower expression levels of the IL-2Rα and γ chains on CD4 and CD8 lymphocytes in CB,[27] and those by Hodge *et al.*, who showed no differences in the IL-2 Rα and γ chains on CD3$^+$ T cells between CB and PB.[26] Since these authors did not compare IL-2R chains on αβ T cells and γδ T cells between CB and PB, it remains possible that a difference exists in the expression of the IL-2 Rα and γ chains between γδ T cells and αβ T cells, although the reason for the difference from our results is not clear.

So far, there have been several reports related to the effective activation of IPP-stimulated PB Vγ9Vδ2 T cells by costimulants, such as TNF-α or IL-12 alone [28,29] or in synergy.[21] In those studies, the activation of Vγ9Vδ2 T cells is strongly dependent on IL-2 stimulation.[21,28,29] This suggests that the interaction between IL-2 and IL-2R is crucial for the activation of IPP-stimulated PB Vγ9Vδ2 T cells, and it seems one of the mechanisms involves the upregulation of IL-2R.[21] In this study, we for the first time found that TNF-α, but not IL-12, enhances IFN-γ production by CB Vγ9 T cells. The increase in IL-2Rβ produced by stimulation with TNF-α but not by IL-12 might be one mechanism for this phenomenon. It seems to be two clusters in terms of IFN-γ-producing Vγ9 T cells in CB (Fig. 4b), suggesting the presence of heterogeneous groups in response to TNF-α. It is not clear whether this is a real phenomenon because the number of CB samples is still limited, and therefore needs to be clarified in a future study.

Our findings are supported by similar levels of TNF-αR but lower levels of IL-12Rβ expression observed on CB Vγ9 T cells in comparison with PB. Low IL-12 production by CB cells has been reported,[30,31] results that may be related with the downregulation in the synthesis of IFN-γ by CB γδ T cells. Our findings also suggest that the impaired ability of CB Vγ9 T cells to produce IFN-γ under IL-12 exogenous stimulation may be explained for the low frequency and expression levels of IL-12 receptors on neonatal Vγ9 T cells.

Our study provides new insight into the role of TNF-α on CB Vγ9 T cells, although the specific mechanisms for the activation of CB Vγ9 T cells by TNF-α remain unknown. The activation of Vγ9 T cells in newborns by TNF-α could produce IFN-γ, which is involved in protective immunity against microbes and allergens. The importance of our findings should encourage further studies to elucidate the mechanisms of the activation of CB Vγ9 T cells by costimulants such as TNF-α.

Acknowledgements

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JEM

ARTICLE

Bmi1 regulates memory CD4 T cell survival via repression of the *Noxa* gene

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The maintenance of memory T cells is central to the establishment of immunological memory, although molecular details of the process are poorly understood. In the absence of the polycomb group (PcG) gene *Bmi1*, the number of memory CD4⁺ T helper (Th)1/Th2 cells was reduced significantly. Enhanced cell death of *Bmi1*$^{-/-}$ memory Th2 cells was observed both in vivo and in vitro. Among various proapoptotic genes that are regulated by Bmi1, the expression of proapoptotic BH3-only protein Noxa was increased in *Bmi1*$^{-/-}$ effector Th1/Th2 cells. The generation of memory Th2 cells was restored by the deletion of *Noxa*, but not by *Ink4a* and *Arf*. Direct binding of Bmi1 to the *Noxa* gene locus was accompanied by histone H3-K27 methylation. The recruitment of other PcG gene products and Dnmt1 to the *Noxa* gene was highly dependent on the expression of Bmi1. In addition, Bmi1 was required for DNA CpG methylation of the *Noxa* gene. Moreover, memory Th2-dependent airway inflammation was attenuated substantially in the absence of Bmi1. Thus, Bmi1 controls memory CD4⁺ Th1/Th2 cell survival and function through the direct repression of the *Noxa* gene.

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Abbreviations used: 5-Aza, 5-Aza-2'-deoxycytidine; BAL, bronchioalveolar lavage; ChIP, chromatin immunoprecipitation; MeDIP, methylated DNA immunoprecipitation; PcG, polycomb group; PRC, polycomb repressive complex; RL, lung resistance; Tg, transgenic.

The quality of adaptive immune responses depends on the size of the antigen-specific memory T cell pool, which is regulated through specific homeostatic mechanisms controlling both cell survival and proliferation. Upon antigen recognition, naive CD4 T cells undergo a rapid clonal expansion, followed by the differentiation into functionally distinct Th cell subsets, such as effector Th1 and Th2 cells (1–3). These effector T cells undergo a dramatic contraction in numbers after antigen clearance, with 90–95% succumbing to apoptosis within weeks (4–6). However, some of the effector T cells are maintained as memory T cells for long periods in vivo (7, 8).

In contrast to CD8 memory T cells, CD4 memory T cells may not require the signals through common cytokine receptor γ chain (9, 10). However, critical regulatory roles of IL-7 in the generation and survival of memory CD4 T cells were reported recently (11, 12). In addition, the homeostasis of memory CD4 T cells is dependent on the signals through the TCR as well as the IL-7 receptor (13). Bcl-2 and Mcl-1 are reported to be the downstream targets of the IL-7 receptor and promote T cell survival (14–16).

Several properties that distinguish memory T cells from naive T cells have been described, such as increased longevity and enhanced capacity for recall response to cognate antigen (17). Memory T cells have several features associated with stem cells (18), and the similarity of the gene expression pattern between memory T/B cells and long-term hematopoietic stem cells was reported (19). Similar to hematopoietic stem cells, memory T cells appear to possess the ability to proliferate in response to homeostatic signals. The homeostatic signals may drive self-renewal, whereas antigenic signals drive effector cell differentiation and function.

The *polycomb* group (PcG) gene *Bmi1* has recently been implicated in the maintenance of hematopoietic (20, 21), neural (22), and cancer stem cells (23). PcG gene products form multimeric complexes and maintain the early determined gene expression patterns of key developmental regulators, such as homeobox genes both in invertebrates and vertebrates (24, 25). Bmi1, Mel-18, M33, Pc2, Rae-28/Mph1,

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Mph2, and Ring1A/B are constituents of a multimeric protein complex similar to the *polycomb* repressive complex (PRC)1 identified in *Drosophila*. Another PcG complex, which contains Eed, Suz12, Ezh1, and Ezh2, is PRC2. PRC2 possesses an intrinsic histone H3-K27 methyltransferase activity (26–29), which implicates a likely mechanism for PcG-mediated gene silencing. Lack of individual components of PRC1 results in apoptosis and the loss of proliferative responses of immature lymphoid cells (30, 31). In mature lymphocytes, PcG gene products play several roles in differentiation and cell fate. Mel-18 controls Th2 cell differentiation through the regulation of GATA3 expression (32), and Bmi1 controls the stability of GTAT3 protein in developing Th2 cells (33). EzH2 is involved in B cell development and controls IgH V(D)J rearrangement (34).

We herein investigated the role of Bmi1 in the generation and maintenance of memory CD4$^+$ Th1/Th2 cells. In the absence of Bmi1, the generation of both Th1 and Th2 memory cells was impaired as a consequence of increased Noxa expression. Because our previous study indicated that the expression of Bmi1 is higher in Th2 cells than Th1 cells, we sought to investigate the molecular mechanisms underlying the Bmi1/Noxa-mediated regulation of memory T cell generation by focusing primarily on memory Th2 cells. Our results indicate that Bmi1 controls memory CD4 T cell survival and function through the direct repression of the *Noxa* gene.

RESULTS

Generation of memory Th1/Th2 cells was impaired in the absence of Bmi1

In *Bmi1*$^{-/-}$ mice, although a reduction of cell numbers in the lymphoid organs was seen, substantial numbers of CD4 and CD8 T cells developed with normal expression of developmental cell surface antigens (not depicted). After in vitro antigenic stimulation, *Bmi1*$^{-/-}$ CD4 T cells were shown to proliferate well (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20072000/DC1) and differentiate normally into IL-4–producing effector Th2 cells under Th2 conditions or IFN-γ–producing effector Th1 cells under Th1 conditions (33). Under IL-4–limiting conditions, Th2 cell differentiation was impaired significantly in the absence of Bmi1, and the Bmi1-mediated regulation of the stability of GATA3 protein was demonstrated (33).

To investigate the role of *Bmi1* in the generation and maintenance of memory Th1/Th2 cells, we used a "memory Th1/Th2 mouse" system (35, 36) in which OVA-specific αβTCR transgenic (Tg) CD4 T cells from *Bmi1*$^{+/+}$, *Bmi1*$^{+/-}$, or *Bmi1*$^{-/-}$ mice were stimulated with antigenic OVA peptide and wild-type antigen-presenting cells under Th2 or Th1 culture conditions in vitro for 6 d, and these effector Th cells were then adoptively transferred into syngeneic BALB/c *nu/nu* mice (memory Th1/Th2 mice). 5 wk after cell transfer, the numbers of donor-derived KJ1$^+$ memory Th1/Th2 cells were assessed in various organs. As shown in Fig. 1 A, a *Bmi1* gene dose-dependent affect on the numbers of memory Th2 cells was observed in all tissues tested (spleen, liver, lung, and PBMCs). It is worth noting that even heterozygous *Bmi1*$^{+/-}$ groups showed marked effects. Although the expression levels of IL-7Rα and IL-2Rβ were slightly lower on *Bmi1*$^{-/-}$ memory Th2 cells as compared with wild-type cells, other surface marker antigens were expressed normally (Fig. S1 B). Next, KJ1$^+$ effector Th1 cells were transferred into BALB/c *nu/nu* mice. The memory Th1 cell generation from *Bmi1*$^{-/-}$ effector Th1 cells was also impaired in all tissues tested (Fig. 1 B). The reduction in the *Bmi*$^{+/-}$ group was not obvious. A similar loss of memory Th cell generation was observed in both Th2 (Fig. 1 C) and Th1 cells (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20072000/DC1) when *Bmi1*$^{-/-}$ effector cells were transferred into nonlymphopenic normal BALB/c mice. These results indicate that the generation of memory Th1/Th2 cells was impaired in the absence of Bmi1.

A kinetics study showed that the decrease in *Bmi1*$^{-/-}$ Th2 cells was observed from 2 d after cell transfer (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20072000/DC1). Consequently, we assessed the semi-acute survival of *Bmi1*$^{+/-}$ effector Th2 cells by a competition analysis using *Bmi1*$^{+/+}$ (Thy1.1) and *Bmi1*$^{+/-}$ (Thy1.2) C57BL/6 Ly5.2 effector Th2 cells and Ly5.1 host animals with a C57BL/6 background. Typical staining patterns of mixed cells for cell transfer and of transferred donor-derived cells (Ly5.2) from the indicated tissues are shown in Fig. S4. Interestingly, a significant decrease in the ratio of *Bmi1*$^{+/-}$/*Bmi1*$^{+/+}$ (Thy1.2/Thy1.1) cells was already observed 2 d after cell transfer, and this decline continues through days 7–21 (Fig. 1 D). The kinetics of the preferential reduction of *Bmi1*$^{+/-}$ cells was similar in all tissues tested. The defect in semi-acute survival in *Bmi1*$^{-/-}$ Th2 cells was also observed under lymphopenic conditions (Fig. S5).

The homeostatic proliferation as measured by BrdU incorporation in vivo was decreased slightly in *Bmi1*$^{-/-}$ memory Th2 cells as compared with wild type (14.3 ± 0.4% vs. 9.1 ± 1.5%) (Fig. 1 E). Next, we assessed the extent of ongoing apoptotic cell death in vivo by a TUNEL assay. In *Bmi1*$^{-/-}$ memory Th2 cells, ongoing apoptosis was enhanced dramatically as compared with wild type (8.2 vs. 57.1%) (Fig. 1 F). In addition, increased annexin V$^+$PI$^+$ cells were detected in *Bmi1*$^{-/-}$ memory Th2 cells after restimulation in vitro, indicating that these memory cells are highly prone to cell death (Fig. S1 C).

Deletion of *p16*^{ink4a} and *p19*^{arf} genes failed to restore the decreased memory Th2 cell generation in the absence of Bmi1

Bmi1 has been reported to promote cell proliferation, cell survival, and stem cell self-renewal by repressing the *Ink4a/Arf* locus (37). This locus codes for two proteins, p16^{ink4a} and p19^{arf} (Ink4a and Arf), through the use of alternative reading frames. Ink4a is a cyclin D–dependent kinase inhibitor that promotes cell cycle arrest after Rb activation. Arf induces p53 activation and p53-mediated cell death (38). In the *Bmi1*$^{-/-}$ effector Th2 cells, we confirmed the increased expression of

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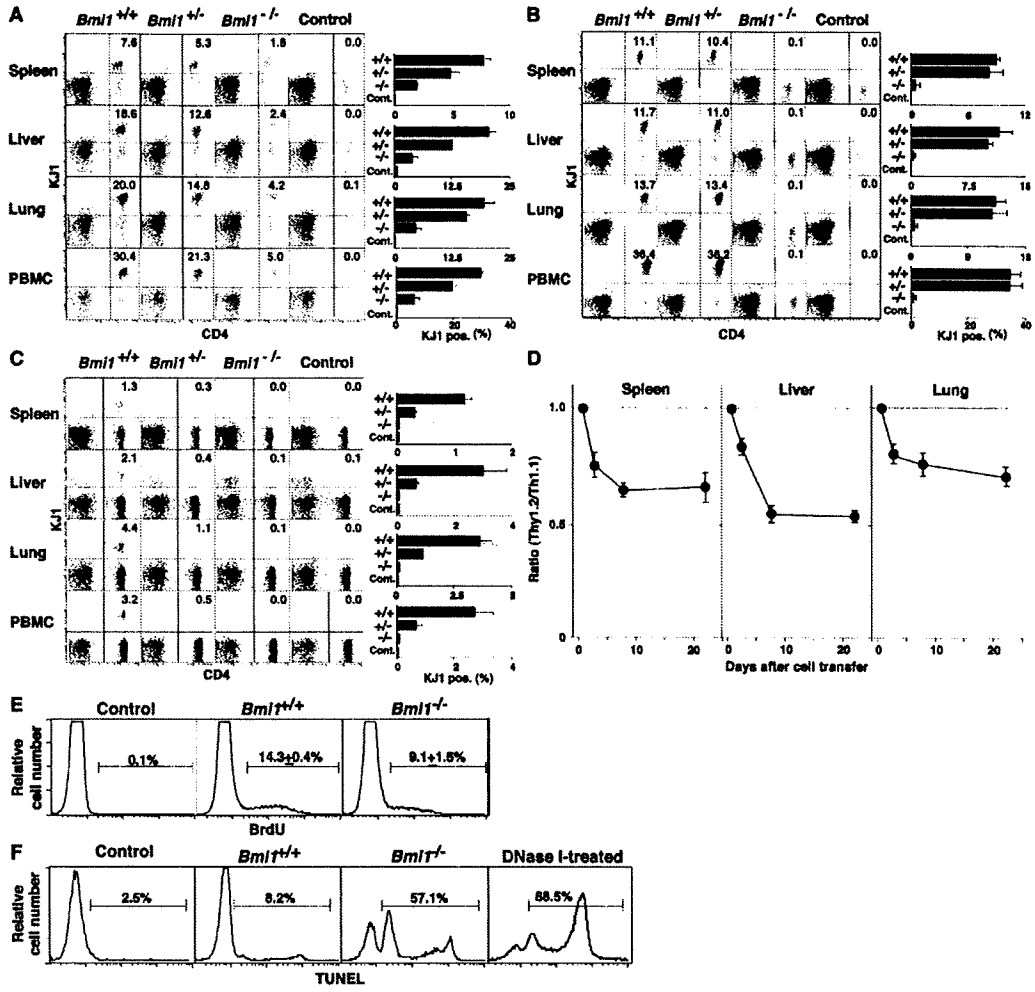

Figure 1. Impaired generation of memory Th1/Th2 cells from *Bmi1*$^{-/-}$ effector Th2 cells. (A and B) *Bmi1*$^{+/+}$, *Bmi1*$^{+/-}$, or *Bmi1*$^{-/-}$ effector Th2 (A) or Th1 (B) cells with DO11.10 Tg background were intravenously transferred into BALB/c *nu/nu* mice ($n = 5$). 5 wk later, the number of KJ1$^+$ memory Th2 cells was determined by flow cytometry. Typical staining patterns of KJ1/CD4 and percentages of KJ1$^+$ cells are shown. Four independent experiments were performed with similar results. (C) *Bmi1*$^{+/+}$, *Bmi1*$^{+/-}$, or *Bmi1*$^{-/-}$ effector Th2 cells with DO11. 10 Tg background were intravenously transferred into BALB/c mice ($n = 5$). (D) Impaired semi-acute survival of *Bmi1*$^{+/-}$ Th2 cells. Thy1.1 *Bmi1*$^{+/+}$ (Ly5.2 background) and Thy1.2 *Bmi1*$^{+/-}$ (Ly5.2 background) effector Th2 cells were mixed (1:1) and transferred (3×10^7 cells/mouse) into syngeneic C57BL/6 mice with a Ly5.1 background. The ratio of Thy1.2$^+$/Thy1.1$^+$ cells in Ly5.2$^+$ cells is shown ($n = 3$). (E) Homeostatic proliferation of the splenic memory Th2 cells was assessed by the BrdU incorporation in vivo. Representative BrdU staining profiles of KJ1$^+$ memory Th2 cells prepared 5 wk after cell transfer are shown. The percentages of BrdU$^+$ cells with standard deviation are shown ($n = 5$). Three independent experiments were performed with similar results. (F) Apoptotic cell death of freshly prepared memory Th2 cells from the spleen was measured by a TUNEL assay. As a positive control, cells were treated with DNase I. Representative TUNEL profiles are shown with percentages of TUNEL$^+$ cells. Two independent experiments were performed with similar results.

the *Ink4a/Arf* gene after anti-TCR stimulation (Fig. 2 A). An increased mRNA expression of both *Ink4a* and *Arf* was confirmed (not depicted). In addition, various proapoptotic genes, which are known to be targets for p53, such as *Bax*, *Puma*, *Noxa*, *Bim*, *Bad*, *Fas*, and *Fas ligand*, were increased in *Bmi1*$^{-/-}$ effector Th2 cells (Fig. 2 A). In *Bmi1*$^{-/-}$ memory Th2 cells, increased expression of mRNA was observed in the *Ink4a/Arf*, *Bax*, *Puma*, *Noxa*, *Bad*, and *Fas* genes, whereas expression of antiapoptotic genes *Bcl2*, *BclxL*, and *Mcl1* was unchanged (Fig. 2 B). To examine whether the increased expression of *Ink4a/Arf* in *Bmi1*$^{-/-}$ Th2 cells plays a role in the Bmi1-mediated maintenance of memory Th2 cells, *Bmi1*$^{-/-}$ mice were crossed with *Ink4a*$^{-/-}$/*Arf*$^{-/-}$ mice, and effector Th2 cells generated from *Bmi1*$^{-/-}$*Ink4a*$^{-/-}$*Arf*$^{-/-}$ with Ly5.2 background were transferred into Ly5.1 mice. 5 wk later, the number of Ly5.2$^+$ memory Th2 cells was assessed. Depletion of *Ink4a* and *Arf* genes itself had no effect on the generation of memory T cells (Fig. 2 C, panels 1 and 3) and failed to