Selective Expression of a Novel Surface Molecule by Human Th2 Cells In Vivo

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The search for reliable marker molecules discriminating between human Th1 and Th2 cells identified a gene encoding a novel member of the G protein-coupled leukocyte chemoattractant receptor family, which is selectively expressed in Th2 but not Th1 lineage cells, thereby named CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells). Studies with anti-CRTH2 mAbs demonstrated that CRTH2 was expressed in a small population (0.4–6.5%) of CD4+ T cells in fresh PBMCs of healthy adults, but no remarkable expression was seen in B cells and NK cells. In some cases, CD8+ T cells (~3.5%) expressed CRTH2. Phenotypes of CD4+ T cells expressing CRTH2 were CD45RA−, CD45RO+, and CD25+, similar to those of Ag-activated effector/memory T cells. Freshly isolated CRTH2+ CD4+ T cells produced Th2- but little or no Th1-type cytokines upon stimulation with PMA and ionomycin. In addition, an allergen-induced proliferative response in fresh PBMCs was significantly and selectively reduced by subtracting CRTH2+ cells. Together, these results indicate that CRTH2 is selectively expressed in vivo in an activated state of Th2 cells including allergen-responsive Th2 cells, suggesting its pivotal roles in ongoing Th2-type immune reactions. The Journal of Immunology, 1999, 162: 1278–1286.

C

D4+ effector Th cells can be divided into at least three distinct subsets termed Th1, Th2, and Th0 in both mice and humans, based on the profile of their cytokine production (1–4). In humans, Th1 cells typically secrete IFN-γ and predominantly promote cell-mediated immune responses such as delayed-type hypersensitivity reaction and macrophage activation. Th2 cells are characterized by their production of IL-4, IL-5, and IL-13, which promote strong humoral immunity including IgE production, and growth and differentiation of mast cells and eosinophils. Th0 cells secrete cytokines typical of both Th1 and Th2 cells, but Th0 function is not fully elucidated. Various pathological conditions, such as infection, autoimmune diseases, and allergy, exhibit the polarized Th1 and Th2 responses that are believed to be closely implicated in the onset and outcome of these diseases (4, 5).

Since the discovery of Th1 and Th2 subsets there has been intense interest in finding their cell-surface markers that would be useful not only in monitoring but also in manipulating the subsets in vivo. Several surface molecules have been reported to be differentially expressed between Th1 and Th2 cells. LAG-3 (6), active ligands for P- and E-selectin (7), IL-12Rβ2 subunit (8, 9), and CC chemokine receptor (CCR)3 (10) were shown to be dominantly expressed on Th1 cells, whereas expression of CD30 (11), IFN-γR β-chain (12), CCR3 (13), CCR4 (14), and ST2L (15) was reported to be preferential to Th2 cells. All of these molecules were originally identified with in vitro cell culture system. However, expression specificity of these molecules in human Th1 and Th2 cells in vivo has not yet been fully established. Identification of reliable markers in humans that are stably expressed in either Th1 or Th2 subsets in vivo would facilitate our understanding of functional involvement of the subsets in normal and disorderly condition.

In this study, we took advantage of the gene expression screen method (16, 17) to clone genes for molecules that are differentially expressed between human Th1 and Th2 cells. One clone was finally selected that encodes a novel G protein-coupled receptor, named CRTH2, selectively expressed in Th2 but not Th1 cells in vitro and in vivo. Our results suggested that CRTH2+ cells play central roles in allergen-induced immune responses. Collectively, CRTH2 could be a useful tool for the study of human Th2 in vivo as well as in vitro.

Materials and Methods

Cell lines

Human cell lines used in this study were: T cell lines Jurkat, Hut102, Hut78, MT-2, TL-Mor, CCRF-CEM, and Molt-4; B cell lines Daudi, BJAB, and an EBV-transformed lymphoblastoid cell line LCL-Nag; an erythroid line HEL; a monocyte/macrophage line THP-1; a cervix carcinoma line HeLa; a hepatoma line Hep-G2; and an adenovirus-transformed embryonic kidney line 293 obtained from the American Type Culture Collection (Manassas, VA). A mouse T cell line BW5147, mouse myeloma line SP2/O-Ag8, rat T cell line TART-1, monkey kidney line COS7, and an EBV-transformed lymphoblastoid cell line BJAB, and an EBV-transformed lymphoblastoid cell line LCL-Nag; an erythroid line HEL; a monocyte/macrophage THP-1; a cervix carcinoma line HeLa; a hepatoma line Hep-G2; and an adenovirus-transformed embryonic kidney line 293 obtained from the American Type Culture Collection (Manassas, VA). A mouse T cell line BW5147, mouse myeloma line SP2/O-Ag8, rat T cell line TART-1, monkey kidney line COS7, and an EBV-transformed lymphoblastoid cell line BJAB, and an EBV-transformed lymphoblastoid cell line LCL-Nag; an erythroid line HEL; a monocyte/macrophage line THP-1; a cervix carcinoma line HeLa; a hepatoma line Hep-G2; and an adenovirus-transformed embryonic kidney line 293 obtained from the American Type Culture Collection (Manassas, VA). A mouse T cell line BW5147, mouse myeloma line SP2/O-Ag8, rat T cell line TART-1, monkey kidney line COS7, and an EBV-transformed lymphoblastoid cell line BJAB, and an EBV-transformed lymphoblastoid cell line LCL-Nag; an erythroid line HEL; a monocyte/macrophage line THP-1; a cervix carcinoma line HeLa; a hepatoma line Hep-G2; and an adenovirus-transformed embryonic kidney line 293 obtained from the American Type Culture Collection (Manassas, VA). A mouse T cell line BW5147, mouse myeloma line SP2/O-Ag8, rat T cell line TART-1, monkey kidney line COS7, and an EBV-transformed lymphoblastoid cell line BJAB, and an EBV-transformed lymphoblastoid cell line LCL-Nag; an erythroid line HEL; a monocyte/macrophage line THP-1; a cervix carcinoma line HeLa; a hepatoma line Hep-G2; and an adenovirus-transformed embryonic kidney line 293 obtained from the American Type Culture Collection (Manassas, VA). A mouse T cell line BW5147, mouse myeloma line SP2/O-Ag8, rat T cell line TART-1, monkey kidney line COS7, and an EBV-transformed lymphoblastoid cell line BJAB, and an EBV-transformed lymphoblastoid cell line LCL-Nag; an erythroid line HEL; a monocyte/macrophage line THP-1; a cervix carcinoma line HeLa; a hepatoma line Hep-G2; and an adenovirus-transformed embryonic kidney line 293 obtained from the American Type Culture Collection (Manassas, VA). A mouse T cell line BW5147, mouse myeloma line SP2/O-Ag8, rat T cell line TART-1, monkey kidney line COS7, and an EBV-transformed lymphoblastoid cell line BJAB, and an EBV-transformed lymphoblastoid cell line LCL-Nag; an erythroid line HEL; a monocyte/macrophage line THP-1; a cervix carcinoma line HeLa; a hepatoma line Hep-G2; and an adenovirus-transformed embryonic kidney line 293 obtained from the American Type Culture Collection (Manassas, VA).
cells, which were cultured in DMEM with the same addition) and supplemented with 10% FCS and antibiotics at 37°C under 5% CO2 in air.

**Generation of Th1 and Th2 lines and clones from PBMC**

PBMCs were isolated from heparinized blood by density gradient centrifugation using Ficoll–Paque (Pharmacia Biotech, Uppsala, Sweden) and cultured in RPMI 1640 medium containing 10% FCS and antibiotics with appropriate additives at 37°C under 5% CO2 in air. Th1 cells were induced from PBMCs of healthy volunteers by stimulation with 1 µg/ml of PHA (PHA-P; Wako Pure Chemical, Osaka, Japan) or 5 µg/ml of a purified protein derivative of Mycobacterium tuberculosis (PPD) (Japan BCG Laboratory, Tokyo, Japan) in the presence of 5 ng/ml of human rIL-2 (R&D Systems, Minneapolis, MN) and 100 ng/ml of human rIFN-γ (Genzyme, Cambridge, MA) (Th1 line). Th2 cells were induced from PBMCs from normal adults by stimulation with PHA or an extract of Dermatophagoides pteronyssinus (Dynal, Lake Success, NY) and plated in a 96-well round-bottom plate at 0.5–10 cells per well in the presence of PHA (1 µg/ml) and IL-2 (100 U/ml) with 1–5×105 TCL-Nag cells/well, which had been previously treated with 50 µg/ml of mitomycin C (Kyowa Hakko Kogyo, Tokyo, Japan) at 37°C for 30 min. One half of the medium was replaced with fresh medium containing IL-2 (100 U/ml) twice a week. Clones were examined for their production of cytokines to specify their property.

**Southern and Northern blot analyses**

Total RNAs were extracted from cells with Trizol reagent (Life Technologies, Gaithersburg, MD). Southern and Northern blot analyses were conducted with Hybond N+ nylon membranes (Amersham, Buckinghamshire, U.K.) under stringent hybridization condition according to the manufacturer’s instructions (Version 2). 32P-labeling of probes was performed with a random primer DNA-labeling kit (Takara Shuzo, Shiga, Japan). A 246-bp RT-PCR product of a random primer DNA-labeling kit (Takara Shuzo, Shiga, Japan). A 246-bp RT-PCR product of a random primer DNA-labeling kit (Takara Shuzo, Shiga, Japan). A 246-bp RT-PCR product of a random primer DNA-labeling kit (Takara Shuzo, Shiga, Japan). A 246-bp RT-PCR product of a random primer DNA-labeling kit (Takara Shuzo, Shiga, Japan). A 246-bp RT-PCR product of a random primer DNA-labeling kit (Takara Shuzo, Shiga, Japan). A 246-bp RT-PCR product of a random primer DNA-labeling kit (Takara Shuzo, Shiga, Japan). A 246-bp RT-PCR product of a random primer DNA-labeling kit (Takara Shuzo, Shiga, Japan).

**Preparation of a subtracted Th2 cDNA library and isolation of Th2-specific cDNA fragments**

A subtracted Th2 cDNA library was prepared by the gene expression screen method (16, 17) using total RNAs from a typical Th1 clone 2P15 and a typical Th2 clone 2P26, both of which were obtained from the same donor (K.T.). Three rounds of subtractive hybridization were performed between 2P15 and 2P26 cDNA fragments as described (17). The final products were used as subtracted Th1 and Th2 probes. A portion of the subtracted Th2 probe was digested with XbaI and cloned into the XhoI site of pBlueScript SK+ (Stratagene, La Jolla, CA), generating a subtracted Th2 cDNA library. The subtracted Th2 cDNA library was screened by differential hybridization with the 32P-labeled subtracted Th1 and Th2 probes. Clones that were selectively hybridized with the subtracted Th2 probes were selected. DNA sequences were determined by the dye-terminator cycle sequencing method on ABI 377 automated sequencer (Perkin-Elmer Japan, Chiba, Japan).

**Cloning of full-length cDNA**

cDNA was synthesized from the 2P26 poly(A)+ RNA with a SuperScript Choice System (Life Technologies) and cloned into the EcoRI site of Lambda ZAP II phage vector (Stratagene). The resultant phage library was screened by plaque hybridization with the selected Th2-specific cDNA fragment B (see text).

**Expression of CRTH2 in mammalian cell lines**

The full-length CRTH2 cDNA (clone B19) was subcloned into three mammalian expression vectors: pCXN2 (19), pRe/CMV (Innogenetix, San Diego, CA), and pcDL-SRa296 (20). In the cDNA, the cDNA insert was excised separately at the EcoRI site in the cloning adapter, HindIII/XhoI sites in the multicloning site of the phagemid vector, or EcoRI site in 5′-side adapter and EcoRV site within the cDNA (nucleotide 1365), then subcloned into the EcoRI site of pCXN2, HindIII/XhoI sites of pRe/CMV, or EcoRI/HindIII (blunted) sites of pcDL-SRa296, respectively, generating pCXN2/B19, pRe/B19, and pcDL-SRa/B19. These expression plasmids were introduced in various cell lines by electroporation, and stable transfectants were selected in the presence of geneticin (Sigma, St. Louis, MO). Transfectants used in this study were pCXN2/B19-transfected TART-1 (TART/B19-12.10) and BW5147 (BW/B19-3.4), pRe/B19-introduced 293 (293/B19-1), and pcDL-SRa/B19-transfected Jurkat (Jurkat/B19-1).

**Generation of mAbs and anti-peptide Abs**

mAbs were generated by i.p. immunization of Wistar rats with 107 TART/B19-12.10 cells once a cell. Three days after the fifth immunization, sensitized spleen cells were fused with 2P2O/Ag8 cells. Culture supernatants of the hybridomas were screened by indirect membrane immunofluorescence method using TART-1, TART/B19-12.10, and phycoerythrin (PE)-conjugated goat anti-rat IgG (BioSource International, Camarillo, CA). Anti-peptide Abs to CRTH2 were generated by immunization of New Zealand White rabbits with peptide corresponding to the deduced sequence of the first (MSANATLKLCPFEQMSRQLQSSHSTNISRYIDH), the third (RDTTSRLDGRIMCNYVINLNPDPDRDATNSQ), or the fourth (PYHVSTLSLEARAHANPGLR) extracellular domain of CRTH2, which had been conjugated to keyhole limpet hemocyanin with m-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce, Rockford, IL) (21). The anti-peptide Ab was affinity-purified using corresponding peptide coupled to an Affi-Gel10 (Bio-Rad Laboratories, Richmond, CA).

**Immunoblotting and endoglycosidase F (endo F) treatment**

Cells (~5×106) were labeled with an anti-CRTH2 mAb (10–20 µg/ml) at 4°C for 30 min, washed, and lysed in 1 ml of lysis buffer (1% sucrose monolaurate (Dojindo Laboratories, Kumamoto, Japan), 25 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and protease inhibitors) at 4°C for 1 h. The cell lysate was clarified by centrifugation, and the immune complex was immunoprecipitated with 107 anti-rat IgG-coupled magnetic beads (Dynal). The precipitates were washed five times with the lysis buffer, then were subjected to SDS-PAGE or endo F treatment followed by SDS-PAGE as previously described (22). The proteins in the polyacrylamide gel were electrically transferred onto a BA-S85 nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), and CRTH2 was visualized with a peroxidase anti-CRTH2 mAb (5 µg/ml) and a horseradish peroxidase-labeled goat anti-rabbit IgG (Zymed Laboratories, San Francisco, CA) followed by chemiluminescent detection using Western blot chemiluminescence reagent (New England Nuclear, Boston, MA).

**Flow cytometry**

The following materials were obtained from Becton Dickinson (San Jose, CA): FITC-conjugated mAbs to CD3 (clone Leu-4), CD4 (Leu-3a), CD8 (Leu-2a), CD19 (Leu-12), HLA-DR (L243), and IFN-γ (25723.11); PE-conjugated mAbs to IL-4 (3010.211) and IFN-γ; peridinin chlorophyll protein (PerCP)-conjugated mAb to CD4; and appropriate isotype-matched controls. FITC-conjugated mAbs to CD16 (3G8), CD45RO (UCHL-1), and CD45RA (HI100); PE-conjugated mAbs to CD25 (M-A251), IL-5 (TRFK5), and IL-13 (JES10-52A); and control conjugates were purchased from PharMingen (La Jolla, CA). PE- and RED670-labeled streptavidin, FITC-conjugated streptavidin, and FITC-labeled anti-CD62L mAb (Dreg 56) were obtained from Life Technologies, Biomeida (Foster City, CA), and Immunotech (Marseille Cedex, France), respectively. Control rat IgG2a was purchased from Zymed. To biotinylate mAbs, a long-arm N-hydroxysuccinimidyl-biotin (Vector Laboratories, Burlingame, CA) was used. Staining of cell surface Ags was performed in accordance with the manufacturer’s instructions. Intracellular cytokines were stained according to the method of Picker et al. (23). The stained cells were analyzed on FACScan flow cytometer using CellQuest software (both from Becton Dickinson).

**RT-PCR**

Total RNA was treated with RNase-free DNasel (Promega, Madison, WI) and was reverse transcribed in 20 µl of reaction mixture using Superscript II (Life Technologies) according to the manufacturer’s recommended method. A portion of the reaction product was subjected to PCR using an AmpliTaq DNA polymerase (Perkin-Elmer). Sense and antisense primers used in this study were as follows: for CRTH2, 5′-CCTCCTGTGGGACGCCCCACGATGTCCGC and 5′-CAGGCAGAAAGAATAGGTGAAGAAG; for β-actin, 5′-TGAAGTCTGAGTGGACATC and 5′-ACTCTGATACTTCCGTGTG (24). For each PCR reaction (25 µl), the sample was first denatured at 95°C for 2 min and amplified by 30 cycles of PCR (94°C, 1 min; 67°C, 1 min; and 72°C, 2 min) for CRTH2 or by 25 cycles of PCR (94°C, 1 min; 65°C, 1 min; and 72°C, 2 min) for β-actin.
Fractionation of fresh PBMC and cultured cells

For purification of CRTH2⁺ or CRTH2⁻ cells, fresh PBMCs or cultured cells were heavily labeled with BM16 at 50 μg/ml for 30 min at 4°C in the presence of 10% normal human serum. After extensive washing, Ab-labeled cells were either positively isolated by labeling with anti-rat IgG-coupled microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) followed by two rounds of purification on MS⁺ positive selection column (Miltenyi) attached with 26-gauge needle for flow regulation, or they were removed from unlabeled cells with excess amounts of anti-rat IgG-coupled magnetic beads (Dynal).

Results

Cloning of a novel G protein-coupled receptor from a Th2 cDNA library

After screening of 3 × 10⁶ cDNA fragments from the subtracted Th2 cDNA library, we obtained 45 independent cDNA fragments that were differentially hybridized with the subtracted Th2 (2P26-derived) but not Th1 (2P15-derived) probe. Northern blot analyses using total RNAs of 2P15 and 2P26 cells showed that 13 of the 45 cDNA fragments were actually expressed at detectable levels in a 2P26-specific manner. Search of the EMBL, GenBank, and DDBJ databases revealed that 6 of the 13 cDNA fragments were novel, whereas the others are known genes. After analyzing mRNA levels using a large panel of Th1 and Th2 cells, we selected a cDNA fragment called fragment B as a candidate for the Th2-specific gene.

The cDNA fragment B (272 bp) was hybridized with a 3.0-kbp mRNA species that was selectively expressed in all Th2 clones and lines but not in any Th1 clones and lines (Fig. 1). The significant expression of this gene was not observed in human cell lines derived from various tissues in Northern blot analysis.

After screening of a 5 × 10⁶ phage Th2 (2P26) cDNA library with the cDNA fragment B as a probe, we selected two cDNA clones, B6 (2884 bp) and B19 (2911 bp). The DNA sequencing revealed that their nucleotide sequences overlap completely. The DNA sequencing data of cDNA clone B19 will appear in the GenBank database (accession number AB008535). The cDNA fragment B corresponds to nucleotide 1908–2179 in cDNA clone B19.

The longest open reading frame of 1185 nucleotides of cDNA clone B19 starts from the first ATG (nucleotide 113), which roughly conforms to the Kozak rule (25), and encodes a protein of 395 amino acids with a calculated molecular mass of 43 kDa. Fig. 2 shows the deduced amino acid sequence of the protein encoded by B19. A hydropathy analysis indicated seven putative transmembrane domains, which is a characteristic feature of the G protein-coupled seven transmembrane receptor (STR) superfamily. In-
reported to be expressed on T cells (29, 32), we carefully examined the possible cross-reactivity of anti-CRTH2 mAb BM16 with known chemokine receptors. But no positive reaction was observed with transfectants stably expressing CCR1, CCR2B, CCR3, CCR4, CCR5, CCR6, CCR7, CXCR1, or CXC4 in flow cytometric analysis (data not shown).

Characterization of CRTH2 protein

The Far-Western blotting method was performed to analyze the CRTH2 protein. As shown in Fig. 3B, BM16 specifically precipitated a 55- to 70-kDa protein, which was recognized by a rabbit anti-CRTH2 peptide (the fourth extracellular domain) Ab from cell lysates of CRTH2-transfected Jurkat and a Th2 clone, 6L21. This protein band was further confirmed to be CRTH2 itself by using two other independent anti-peptide Abs against the first and third extracellular domains of CRTH2. Specificity of the binding of these anti-peptide Abs was corroborated by blocking tests with each peptide. As a result of the treatment with endo F, the CRTH2 decreased its molecular mass from 55–70 kDa to 35–40 kDa (Fig. 3B, lanes 7 and 9), indicating the presence of N-linked sugar on the CRTH2 as predicted from amino acid sequence.

Expression of CRTH2 on the cultured Th clones and lines

Using mAb BM16, CRTH2 expression in Th clones was examined at the single cell level. As shown in Fig. 3C, most cells of each Th2 clone expressed significant levels of CRTH2. All eight typical Th2 clones showed similar results. In contrast, all five typical Th1 clones expressed little or no visible CRTH2. Th2-selective expression of CRTH2 was also confirmed in polyclonal Th2 lines (Fig. 3C).

To further verify the Th2-specificity of CRTH2 expression, we examined the cytokine profile of CRTH2+ cells in polyclonal PBMC cultures in which both Th1 and Th2 cells were growing concurrently. As shown in Fig. 4, isolated CRTH2+ cells produced typical Th2 cytokines IL-4, IL-5, and IL-13, but they produced little or no Th1 cytokine IFN-γ. These results clearly indicate that CRTH2 is selectively expressed in Th2 but not Th1 cells. The results also indicate that most Th0 cells do not express CRTH2. On the other hand, a significant number of Th2 cells remained in CRTH2-depleted cell fraction (Fig. 4), suggesting CRTH2 was expressed in not all but a large population of Th2 cells in such mixed Th cultures.

CRTH2 expression in fresh PBMCs

We next examined the CRTH2 expression in fresh PBMCs from several healthy adults. The representative results are presented in Fig. 5. For all donors, gradual but distinct expression of CRTH2 was observed in a small population (0.4 – 6.5%) of CD4 memory T cells. CRTH2-specificity of the staining with mAb BM16 was confirmed by the dominant expression of CRTH2 mRNA in the sorted BM16-bound cells as compared with BM16-unbound cells (Fig. 5C).

Cytokine production in CRTH2+ PBMCs

It was of great interest to ask whether CRTH2 is also selectively expressed in Th2 cells in vivo. To ascertain this, we first compared
the cytokine patterns of unfractionated, CRTH2-depleted, and CRTH2-enriched cell fractions derived from the same fresh PBMCs of several healthy adults. As shown in Fig. 6, Th2 cells having the ability to produce IL-4, IL-5, or IL-13, but not IFN-γ, were greatly purified by the positive selection with BM16. Contrarily, a significant reduction of Th2 cells was observed in CRTH2^1 cell-depleted fraction as compared with unfractionated cell population. On the other hand, the proportion of Th1 cells was not significantly affected by the negative selection. However, the isolation procedure employed in this study seemed to somewhat affect the total ability of T cells to produce cytokines. Hence, we next stimulated cells in a whole blood to minimize cell damage according to instructions of the FastImmune cytokine system (Becton Dickinson), then directly observed for a correlation between the expression levels of CRTH2 and intracellular cytokines. The
multiple staining revealed that most CRTH2\(^{+}\) CD4\(^{+}\) cells (85%) produced at least one of three typical Th2 cytokines, IL-4, IL-5, and IL-13, but they produced little IFN-\(\gamma\) (Fig. 7). Together, these results indicate that CRTH2 is selectively expressed in Th2 but not Th1 cells, even in vivo. Similar results were obtained in all donors examined, although significant down-regulation of CRTH2 by T cell activation was observed in some cases.

CRTH2 expression in allergen-responsive T cells

Most allergen-responsive Th cells were shown to exhibit Th2 phenotype (5, 11). Therefore, we next examined whether CRTH2 is also expressed in such cells. PBMCs from adults susceptible to pollen allergens were examined for their proliferative responses against the allergens with cell populations negatively selected with BM16 or control IgG2a. As shown in Table I, proliferative responses against the pollen allergens were markedly reduced by subtracting CRTH2\(^{+}\) cells, whereas those against a typical Th1-type Ag PPD (2) were not significantly affected by the negative selection. When purified CD4\(^{+}\) cells were used as responder cells, nearly complete depletion of the allergen-specific response was observed (Table I, Expt. 2), which consequently suggested that the majority of CD4\(^{+}\) cells responsive to the pollen allergens also expressed CRTH2.

Effect of cytokines on the expression of CRTH2

The most potent environmental factors that influence Th1 and Th2 differentiation are known to be cytokines IL-12 and IL-4, respectively (3, 33, 34). Therefore, we next investigated the effect of these cytokines on the expression of CRTH2. In PHA-stimulated primary PBMC cultures, addition of IL-4 or IL-12 resulted in marked enhancement or complete repression of the development of CRTH2\(^{+}\) cells, respectively. However, in these cultures, it was difficult to verify the direct effect of IL-4 or IL-12 upon CRTH2 expression because proportions of Th2 cells in the cultures also considerably changed in response to these cytokines; so, we examined this with Th2 clones. Among five Th2 clones used, two clones (TKD21 and 6L21) showed slight enhancement in CRTH2 expression levels (26–28% increase in relative mean fluorescence intensity) in response to IL-4, while three clones (TKD23, TKD24, and 6L21) indicated reduced CRTH2 expression (22–50% reduction) after treatment with IL-12. Table II shows results with one of the responder clones, 6L21. These changes in CRTH2 expression were considered to be directly induced by the cytokines because the proportion of Th2 cells in the culture was not significantly affected by these treatments (Table II). When both cytokines were simultaneously added, the effect of IL-12 was dominant over that of IL-4. This inhibitory effect of IL-12 on CRTH2 expression was nearly completely cancelled by an anti-IL-12 p40 subunit mAb (clone C8.6; Genzyme).
Our main goal in this study was to isolate surface molecules by which we can easily distinguish Th1 or Th2 cells from other subsets of CD4+ Th cells in humans in vivo. In the present study, we isolated CRTH2 as a candidate for the Th2-specific gene from a subtracted Th2 library. Studies with a specific mAb clearly demonstrated that CRTH2 is selectively expressed on the surface of Th2 cells in vivo as well as in vitro. Little or no CRTH2 expression was observed on naive T cells, Th1 cells, and most Th0 cells in normal peripheral bloods. Surface phenotype (CD45RA−/CD25−/CD45RO+) of CRTH2+ cells was 5.1–6.5.

**Discussion**

Our main goal in this study was to isolate surface molecules by which we can easily distinguish Th1 or Th2 cells from other subsets of CD4+ Th cells in humans in vivo. In the present study, we isolated CRTH2 as a candidate for the Th2-specific gene from a subtracted Th2 library. Studies with a specific mAb clearly demonstrated that CRTH2 is selectively expressed on the surface of Th2 cells in vivo as well as in vitro. Little or no CRTH2 expression was observed on naive T cells, Th1 cells, and most Th0 cells in normal peripheral bloods. Surface phenotype (CD45RA−/CD25−/CD45RO+) of CRTH2+ cells was 5.1–6.5.

The present study also showed that the expression of CRTH2 in PBMCs is highly restricted to an activated state (CD25+) of T cells (35). This is consistent with our observation that the majority of Th2 cells in PBMCs exhibited low to intermediate levels of CD25 Ag (data not shown). However, we also observed a small number of CRTH2+ cells in CD4+ lymphocytes. Thus, as yet, CRTH2 appears to be one of the most possible markers for human Th2 cells in vivo.

### Table I. Selective elimination of allergen-responsive cells from PBMCs by subtracting CRTH2+ cells

<table>
<thead>
<tr>
<th>Donor</th>
<th>Ab Used for Subtraction</th>
<th>% of CRTH2+ Cells</th>
<th>Mean (±SD)</th>
<th>[3H]Thymidine Uptake</th>
<th>Stimulation Index</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(cpm × 10^3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt. 1a</td>
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<tr>
<td>T.Y.</td>
<td>Rat IgG2a</td>
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<td>–</td>
<td>39.5 ± 4.7</td>
<td>3.8</td>
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<tr>
<td></td>
<td>Pollen</td>
<td>–</td>
<td>150.0 ± 10.4</td>
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<td></td>
<td>PPD</td>
<td>–</td>
<td>732.1 ± 101.5</td>
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<td></td>
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<tr>
<td></td>
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<td>56.4 ± 16.2</td>
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<tr>
<td></td>
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<td>534.1 ± 27.9</td>
<td>18.2</td>
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<tr>
<td></td>
<td>K.H.</td>
<td>Rat IgG2a</td>
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<td>–</td>
<td>4.6</td>
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<td></td>
<td>Pollen</td>
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<td>113.6 ± 19.2</td>
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<tr>
<td></td>
<td>PPD</td>
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<td>–</td>
<td>48.1 ± 19.2</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PPD</td>
<td>–</td>
<td>872.7 ± 60.1</td>
<td>32.9</td>
<td></td>
</tr>
<tr>
<td>Expt. 2b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K.H.</td>
<td>Rat IgG2a</td>
<td>2.9</td>
<td>–</td>
<td>3.6 ± 1.1</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>Pollen</td>
<td>–</td>
<td>41.2 ± 9.1</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PPD</td>
<td>–</td>
<td>159.3 ± 58.2</td>
<td>44.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BM16</td>
<td>0.2</td>
<td>–</td>
<td>3.4 ± 0.5</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Pollen</td>
<td>–</td>
<td>5.9 ± 2.8</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PPD</td>
<td>–</td>
<td>112.4 ± 26.1</td>
<td>33.1</td>
<td></td>
</tr>
</tbody>
</table>

a Percent of CRTH2+ cells in CD4+ lymphocytes.
b An extract of pollen of Japanese cedar Cryptomeria japonica (Torii), 1% (v/v).
c A total of 5 µg/ml.
d Mean cpm in Ag (+) cultures/mean cpm in Ag (−) cultures.

d **Table II. Effect of cytokines on the expression of CRTH2 in established Th2 cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Expression of CRTH2</th>
<th>Cytokine Production (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of CRTH2+</td>
<td>RMFIb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Th1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Th2</td>
</tr>
<tr>
<td>IL-4</td>
<td>99.4 ± 0.1</td>
<td>117.8 ± 7.0</td>
</tr>
<tr>
<td>IL-12</td>
<td>99.4 ± 0.1</td>
<td>150.9 ± 7.0</td>
</tr>
<tr>
<td>IL-4 + IL-12</td>
<td>93.4 ± 0.3</td>
<td>55.4 ± 0.8</td>
</tr>
</tbody>
</table>

a A Th2 clone, 6L21, was cultured in the presence of IL-2 alone (−) or IL-2 plus indicated cytokine(s), which were used at 100 ng/ml (IL-4) and 10 ng/ml (IL-12). After 4 days, CRTH2 expression and cytokine production were examined as described in Fig. 3. A mean (±SD) value of triplicate cultures (expression of CRTH2) and a value in a 1:1 mixture of cells from the triplicate cultures (cytokine production) are presented. * p < 0.01 (Student’s t test).
b Relative mean fluorescence intensity (RMFI) of total cells. RMFIs of cells stained with control IgG2a were 5.1–6.5.
of Th2 cells in the CD25− cell population. Therefore, CRTH2 might not be expressed in some populations of Th2 cells in vivo, such as CD25−-resting Th2 cells. Alternatively, the stimulation with PMA and ionomycin, which is widely used to determine Th phenotypes at a single cell level, is so strong and artificial that we might overestimate the number of genuine Th2 cells (23). In any case, it is most likely that the majority of circulating Th2 cells express CRTH2. The results of depletion experiments (as shown in Fig. 6 and Table I) strongly support this notion.

CCR3 has also been shown to be preferentially expressed on the surface of human Th2 cells by in vitro and in vivo studies (13). Although Th2-specificity of CCR3 in fresh PBMCs was not directly evidenced owing to its rapid down-regulation by T cell activation, reported percentages of CCR3+ T cells in peripheral bloods (<0.2−8%) are nearly comparable to those of CRTH2+ cells in CD4+ lymphocytes (0.4−6.5%). This agreement might imply that these two proteins are simultaneously expressed in the same Th2 cell population among PBMCs.

The involvement of CRTH2 in the function of Th2 cells is now uncertain. It is clear that CRTH2 expression itself is not essential for Th2 cytokine production because production of Th2 cytokines was also seen in CRTH2− CD4+ PBMCs that may consist of Th0 cells, naive T cells, and possibly some population of Th2 cells (Fig. 7). However, production levels of Th2 cytokines in each cell were apparently higher in CRTH2+ cells than in CRTH2− cells on average, suggesting some implication of CRTH2 in the mechanisms for cytokine production. Originally, chemotactranscripts and their receptors on leukocytes have been shown to be involved in their tissue-specific migration (29, 32, 36, 37). Recent evidence indicates that Th1 and Th2 cells are also differentially recruited to the sites of different types of inflammatory reaction (7, 38). Actually, CCR3 and CCR4 have been demonstrated to be functionally expressed on Th2 cells as receptors for chemotactrant eotaxin and macrophage-derived chemokine, respectively, and are suggested to be implicated in their tissue-specific migration (13, 14).

Similarly, CRTH2 might confer an additional property on Th2 migration. On the other hand, aside from chemotactic activity, chemotactranscripts are also shown to be involved in a number of biological responses such as angiogenesis, cellular adhesion, cytotoxicity, degranulation, and T cell activation (29, 39). CC chemokines macrophage inflammatory protein-1α and monocyte chemotactic protein-1 have been reported to differentially enhance the development of Th1 and Th2 cells respectively (40). Thus, it is also possible that CRTH2 and its ligand play important roles in some steps of Th2 development and function.

A phylogenetic analysis by unweighted pair-group method with arithmetic means shows the highest relation of CRTH2 to members of the N-formyl peptide receptor (FPR) subfamily such as FMLP receptor and C5α receptor. Members of FPR subfamily have been reported to be on chromosome 19 in a cluster (29). However, a computer search revealed that a fragment (266 bp) of untranslated region of CRTH2 cDNA is registered as cDNA clone IB1021 under accession number T15367 in expressed sequence tags database, and this gene is present on chromosome 11 (41). Therefore, CRTH2 may form another subfamily, suggesting that it utilizes an as yet unknown ligand. Indeed, in preliminary experiments using Fra-2 AM (Dojindo)-loaded 293/B19-1 cells, no calcium mobilization was induced by several known chemotactranscripts including FMLP, C5α, IL-8, RANTES, monocyte chemotactic protein-1, eotaxin, platelet-activating factor, and angiotensin II. To elucidate the functional implication of CRTH2, identification of corresponding ligand and detailed examination of its tissue distribution, including inflamed sites, are required.

In conclusion, we have cloned a novel surface molecule by which we can easily distinguish Th2 cells from naïve T cells, Th1 cells, and most Th0 cells among CD4+ lymphocytes of peripheral blood. The protein enables us to highly purify or remove rare Th2 cells from PBMCs or cultured Th cells. Thus, this protein will be useful for Th2 study and may also be a possible target for therapeutic intervention.

Acknowledgments

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References