Immunity 24, 827–838, June 2006 ©2006 Elsevier Inc. DOI 10.1016/j.immuni.2006.03.019

Maintenance and Polarization of Human T_H2 Central Memory T Cells by Thymic Stromal Lymphopoietin-Activated Dendritic Cells

Yui-Hsi Wang,[1,5] Tomoki Ito,[1,5] Yi-Hong Wang,[1] Bernhard Homey,[3] Norihiko Watanabe,[1] Rachel Martin,[4] Christopher J. Barnes,[2] Bradley W. McIntyre,[1] Michel Gilliet,[1] Rakesh Kumar,[2] Zhengbin Yao,[4] and Yong-Jun Liu[1,*]

[1] Department of Immunology
Center of Cancer Immunology Research
Graduate School of Biomedical Science
[2] Department of Molecular and Cellular Oncology
The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030
[3] Department of Dermatology
Heinrich-Heine-University
Düsseldorf 40225
Germany
[4] Tanox, Inc.
Houston, Texas 77025

Summary

The identity of T_H2 memory cells and the mechanism regulating their maintenance during allergic inflammation remain elusive. We report that circulated human $CD4^+$ T cells expressing the prostaglandin D2 receptor (CRTH2) are T_H2 central memory T cells, characterized by their phenotype, T_H2 cytokine production, gene-expression profile, and the ability to respond to allergens. Only dendritic cells (DCs) activated by thymic stromal lymphopoietin (TSLP) can induce a robust expansion of $CRTH2^+CD4^+$ T_H2 memory cells, while maintaining their central memory phenotype and T_H2 commitments. $CRTH2^+CD4^+$ T_H2 memory cells activated by TSLP-DCs undergo further T_H2 polarization and express cystatin A, Charcot-Leydon crystal protein, and prostaglandin D_2 synthase, implying their broader roles in allergic inflammation. Infiltrated $CRTH2^+CD4^+$ T_H2 effector memory T cells in skin lesion of atopic dermatitis were associated with activated DCs, suggesting that TSLP-DCs play important roles not only in T_H2 priming, but also in the maintenance and further polarization of T_H2 central memory cells in allergic diseases.

Introduction

The immunopathologic hallmark of allergic diseases is the infiltration and accumulation at the sites of inflammation of polarized $CD4^+$ T_H2 T cells, which produce large amounts of interleukin (IL)-4, IL-5, and IL-13 (Robert and Kupper, 1999; Kay, 2001; Shinkai et al., 2002). Recent studies have suggested that memory-like T_H2 T cells are the principal cell population responsible for the maintenance of chronic allergic inflammation and the rapid relapse of acute allergic inflammation upon re-exposure to allergens (Van Reijsen et al., 1997; Prescott et al., 1999; Mojtabavi et al., 2002). Since the discovery of the T_H1/T_H2 paradigm, much effort has been focused on studies of the molecular regulation of naive T cell differentiation into T_H1 or T_H2 cells (Ho and Glimcher, 2002), but our knowledge about T_H1 or T_H2 memory cells is rather limited.

In humans, $CD4^+$ memory T cells have been defined as $CD45RO^+CCR7^+CD62L^+$ central memory cells (T_{CM}) and $CD45RO^+CCR7^-CD62L^{+/-}$ effector memory cells (T_{EM}) by their distinct surface phenotype, homing capacity, and effector functions (Sallusto et al., 1999, 2004). While T_{CM} can undergo homeostatic proliferation in response to the cytokines IL-7 and IL-15 and differentiate into effectors, T_{EM} with a reduced proliferative capacity can elicit immediate effector functions (Geginat et al., 2001). Interestingly, T_{CM} and T_{EM} can be further divided into subsets that are prone to produce T_H1 or T_H2 cytokines according to their differential expression of chemokine and tissue-homing receptors (Campbell et al., 2001; Rivino et al., 2004; Sallusto et al., 2004), linking memory T cell subsets with polarized T_H1 or T_H2 function. Recently, a subpopulation of $CD4^+$ T cells expressing chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRTH2), a seven-transmembrane G protein-coupled receptor for prostaglandin D2, was found to produce IL-4, IL-5, and IL-13 but not interferon γ (IFN-γ) upon T cell receptor (TCR) triggering, suggesting that $CRTH2^+CD4^+$ T cells were committed T_H2 cells (Cosmi et al., 2000; Nagata et al., 1999). However, it is unclear whether circulated $CRTH2^+CD4^+$ T cells represent T_H2 effector or central memory cells.

Although immunological memory against microbial pathogens has been extensively studied (Sprent and Surh, 2001), little is known about the allergen-specific memory T_H2 cells and regulation of their maintenance. At the sites of allergic inflammation, dendritic cells (DCs) are in close contact with epithelial cells (Lambrecht and Hammad, 2003) and function not only in priming T_H2-mediated immune responses but also in sustaining the allergic inflammation by maintaining the allergen-specific T_H2 memory T cells (Jahnsen et al., 2001; Lambrecht et al., 2001; Julia et al., 2002). However, the type of DCs and the nature of stimuli that endows DCs with the ability to maintain T_H2 memory cells as well as the underlying molecular mechanisms are unknown. TSLP, an IL-7-like cytokine, was shown to be a master switch of allergic inflammation at the epithelial cell and DC interface leading to the pathogenesis of atopic dermatitis (AD) and asthma in humans and mice (Soumelis et al., 2002; Al Shami et al., 2005; Yoo et al., 2005; Zhou et al., 2005). TSLP strongly activates DCs, which prime naive T cell to differentiate into T_H2 cells (Soumelis et al., 2002; Watanabe et al., 2004). Here, we report that human $CRTH2^+CD4^+$ T cells represent a unique subset of central memory T cells with T_H2 functional attributes and the novel function of TSLP-DCs in the maintenance of human T_H2 memory cells, through an IL-7- and IL-15-independent mechanism. The findings of infiltrated $CRTH2^+CD4^+$ T_H2 effector memory T cells associated with activated DCs in atopic dermatitis,

*Correspondence: yjliu@mdanderson.org
[5] These authors contributed equally to this work.

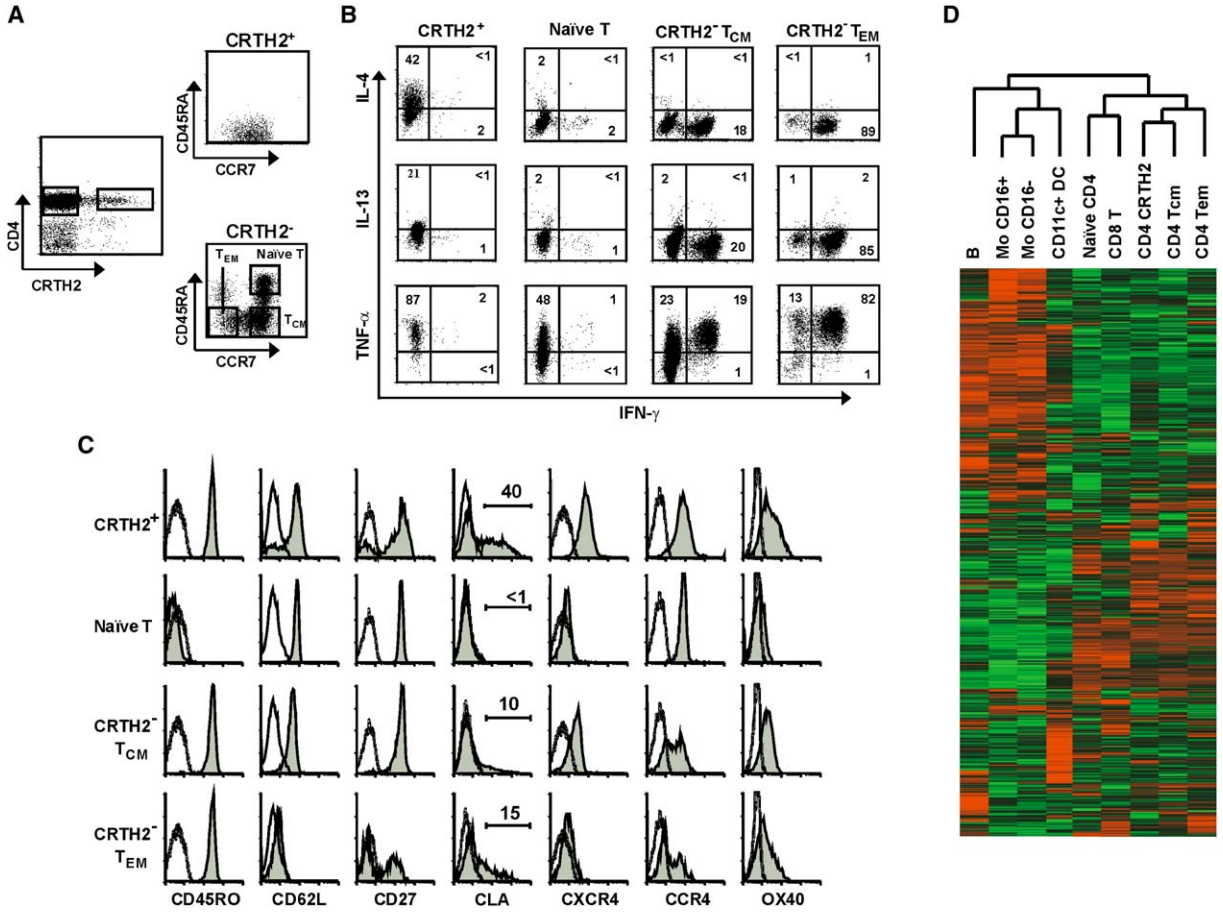

Figure 1. Phenotypic, Functional, and Molecular Characteristics of CRTH2$^+$CD4$^+$ T Cells

(A) Enriched CD4$^+$ T cells were divided into CRTH2^{hi} and CRTH2$^-$ subsets, and the CRTH2$^-$ subset was further sorted into naive T, T_{CM}, and T_{EM} subsets based on surface expression of CCR7 and CD45RA.

(B and C) Sorted fresh CRTH2$^+$CD4$^+$ T cells and other CD4$^+$ T cell subsets were stimulated with PMA and ionomycin for analysis of intracellular cytokine productions (B) or analyzed for the expression of other surface markers (C). Filled histograms represent the staining of indicated cell subset with markers shown below the histogram; open histograms represent isotype control.

(D) Each column represents data from sorted CRTH2$^+$CD4$^+$ T_H2 memory T cells, other CD4$^+$ T cell subsets, and leukocytes with purity >99% as described in Experimental Procedures. Microarray data obtained from three (T cell subsets) or two (other cell lineages) independent samples were centered, and 1834 genes were grouped based on similarity in expression patterns by hierarchical clustering. Clustered image accompanies a dendrogram showing the relationships among tested samples. Each row represents relative hybridization intensities of a particular gene across different samples. Colors reflect the magnitude of the relative expression of a particular gene across samples.

not T_H1-mediated inflammatory diseases, suggest critical roles of T_H2 memory cells and TSLP-DCs in the maintenance of allergic inflammation.

Results

Circulated CRTH2$^+$CD4$^+$ T Cells Display Central Memory Phenotype

CRTH2 was found to be the most reliable marker for the detection of circulating human T_H2 cells. We have isolated CRTH2$^+$ T cells that represent 2%–6% of the total peripheral CD4$^+$ T cells from healthy donors by cell sorting (Figure 1A). CRTH2$^+$CD4$^+$ T cells produced T_H2 cytokines (IL-4, IL-5, and IL-13) and TNF-α, but not the T_H1 cytokine IFN-γ upon TCR triggering (Figure 1B), consistent with previous findings (Cosmi et al., 2000). In contrast, naive T cells produced neither T_H1 nor T_H2 cytokines, and CRTH2$^-$CCR7$^-$ T_{EM} and the CRTH2$^-$CCR7$^+$ T_{CM} produced T_H1 cytokine IFN-γ and TNF-α but not T_H2 cytokines (Figures 1A and 1B). These results confirm previous reports that CRTH2$^+$CD4$^+$ T cells represent the committed T_H2 cells in vivo.

To determine whether circulated CRTH2$^+$CD4$^+$ T cells from normal donors represent T_H2 effector memory or central memory cells, a comparative phenotypic analysis of sorted CRTH2^{hi}CD4$^+$ and CRTH2$^-$ CD45RA$^-$CD4$^+$CCR7$^+$ T_{CM} and CRTH2$^-$CD45RA$^-$CD4$^+$ CCR7$^-$ T_{EM} and naive CD4$^+$ T cells was performed. In contrast to naive T cells, CRTH2$^+$CD4$^+$ T cells were found to be CD45RA$^-$CD45RO$^+$ and majority of these cells express CCR7 (50%–80%), CD62L (>90%), and CD27 (>90%), suggesting their T_{CM} but not T_{EM} phenotype (Figures 1A and 1C; Sallusto et al., 1999). Moreover, a fraction of CRTH2$^+$CD4$^+$ T cells expressed skin-homing marker CLA (~15%–45%) and the gut-related homing receptor β7 integrin (5%–10%) in normal donors (Figure 1C and data not shown). Similar to T_{CM}, CRTH2$^+$ T cells expressed CXCR4 and CCR4 but not CCR1,

CCR3, or CCR5 (Figure 1C and data not shown). Interestingly, $CRTH2^+CD4^+$ T cells expressed the highest level of surface OX40 among all T cell subsets. The phenotypic analyses reveal that circulated $CRTH2^+CD4^+$ T cells from normal donors represent a distinctive subset of T_{CM}.

$CRTH2^+CD4^+$ T Cells Display the Molecular Signatures of T_{CM}

To further examine whether $CRTH2^+CD4^+$ T cells are central memory T_H2 cells, we performed global gene-expression profiling of sorted $CRTH2^+CD4^+$ T cells, $CD4^+$ naive T, T_{CM}, T_{EM}, and $CD8^+$ T cells, and other selected hematopoietic cell lineages (including B, $CD11c^+$ DC, and $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes) by using the Affymetrix microarray. Nine samples were organized on the basis of overall similarity in their gene-expression patterns by an unsupervised hierarchical clustering algorithm of variable genes (Koopman et al., 2003). A dendrogram, in which the pattern and length of the branches reflect the comparative differences in gene-expression profiles between cell lineage samples, was utilized to determine the similarity of molecular features of $CRTH2^+CD4^+$ T cells with other cell lineages. Out of 38,500 known genes, 1834 were differentially expressed among the nine samples. The comprehensive analyses revealed that gene-expression profiles of $CRTH2^+CD4^+$ T cells and T_{CM} are closely clustered together, suggesting that the molecular characteristics of $CRTH2^+CD4^+$ T cells are similar to those of T_{CM} (Figure 1D). These data were further confirmed by real-time PCR analyses of selected groups of genes (Table 1). $CRTH2^+CD4^+$ T cells preferentially express genes that are highly expressed by T_H2 cells or T_{CM} including *CRTH2, GATA-3*, *c-maf, IL17RB, OX40*, *Rank*, and *p21/Cip1* (Fort et al., 2001; Hoshino et al., 2003; Lawson et al., 2004; Mowen and Glimcher, 2004; Matsuoka et al., 2000), but did not express genes that are highly expressed by T_H1 cells or T_{EM} cells including *IFN-*γ and *IL-18Rap* (Neurath et al., 2002) or *CCR5* and *CD70* (Sallusto et al., 2004). Consistent with FACS analysis, $CRTH2^+CD4^+$ T cells expressed *CCR7* transcript that are highly expressed by T_{CM} and naive T cells but not by T_{EM}. Together, the composite molecular analyses demonstrate that circulated $CRTH2^+CD4^+$ T cells from normal donors are a subset of T_{CM} with T_H2 characteristics, distinct from naive and T_{EM} T cells.

TSLP-DCs Induce Robust Homeostatic Expansion of $CRTH2^+CD4^+$ T_H2 Memory Cells

To investigate the cellular and molecular mechanisms that regulate the proliferation and maintenance of $CRTH2^+CD4^+$ T_H2 memory cells, sorted $CRTH2^+CD4^+$ T_H2 memory cells were cultured for 7 days with autologous $CD11c^+$ DCs in medium (med-DCs) or DCs activated by TSLP, poly(I:C), CD40 ligand (CD40L), lipopolysaccharide (LPS), or R848 for 24 hr, or cytokine IL-7, IL-15, IL-15 plus IL-7, or anti-CD3/CD28. TSLP-DCs induced a robust expansion of $CRTH2^+CD4^+$ T_H2 memory cells with 12-fold increase in total T cell numbers (Figure 2A), whereas no significant proliferation driven by poly(I:C), CD40L, LPS, or R848-activated DCs or med-DCs was observed (Figure 2A). IL-15, IL-15 plus IL-7, or anti-CD3/CD28 could also induce a significant expansion of these cells with 5-, 7-, or 4-fold increase in total T cell numbers within 7 days of culture, respectively (Figure 2A). Their proliferative responses induced by these stimuli were visualized by tracing the cell divisions of $CRTH2^+CD4^+$ T_H2 memory cells labeled with the carboxyfluorescein diacetate succinimidyl diester (CFSE) via flow cytometry (see Figure S1A in the Supplemental Data available with this article online). Moreover, we found that unlike med-DCs, IL-7, IL-15, IL-15 plus IL-7, or anti-CD3/CD28, the majority of the expanded $CRTH2^+CD4^+$ T_H2 memory cells driven by TSLP-DCs still maintained their capability to proliferate after three rounds of stimulation, whereas their potential for proliferation after stimulation by IL-15 plus IL-7 or anti-CD3/CD28 was significantly reduced (Figure S1B), leading to more than 400-fold or 100-fold increase in total cell numbers within 3 weeks, respectively (Figure 2B).

Table 1. Real-Time PCR Analysis of Gene Expression in CD4 T Cell Subpopulations

Accession Number	Gene Product	Naive	CRTH2	T_{CM}	T_{EM}	
NM_002051	GATA3	2.0	8.0	1.8	1.6	T_H2
NM_004778	CRTH2	5.2	94	1.0	2.1	T_H2
NM_172234	IL17RB	1.0	1168	11	87	T_H2
NM_003839	RANK	1.0	22	1.9	1.3	T_H2
NM_001838	CCR7	28	12	19	1.0	Memory
NM_003327	OX40	1.0	36	17	16	Memory
NM_005360	C-MAF	1.0	3.6	5.4	5.7	Memory
NM_000389	P21/Cip1	1.0	33	21	30	Memory
NM_000579	CCR5	1.5	2.3	1.0	30	Effector
NM_001252	CD70	1.1	3.6	2.3	7.8	Effector
NM_000619	Interferon-γ	17	1.0	40	114	Effector
NM_003853	IL-18Rap	9.0	1.0	1.4	56	Effector

Relative gene expression in sorted T cell subsets as indicated in Figure 1A were measured by real-time PCR analysis. Data are from one of three independent experiments.

$CRTH2^+CD4^+$ T_H2 Memory and T_{CM} Cells Respond to Similar Stimuli

To compare proliferative responsiveness of $CRTH2^+CD4^+$ T_H2 memory cells with other T cell subsets, sorted $CRTH2^+CD4^+$ T_H2 memory, naive T, $CRTH2^-$ T_{CM}, and $CRTH2^-$ T_{EM} cells were cultured for 7 days with IL-15 plus IL-7 or with autologous $CD11c^+$ DCs activated by TSLP, poly(I:C), LPS, CD40L, or R848, or med-DCs. While both $CRTH2^+CD4^+$ T_H2 memory cells and $CRTH2^-$ T_{CM} underwent expansion in response to TSLP-DCs and IL-15 plus IL-7 induction, naive T cells expanded only in response to TSLP-DCs, but not IL-15 plus IL-7. $CRTH2^-$ T_{EM} failed to proliferate under these culture conditions (Figure 2C and data not shown). The results indicate that $CRTH2^+CD4^+$ T_H2 memory cells undergo expansion in response to the same stimuli known to drive self-renewal of T_{CM}, further supporting the notion that these cells represent a unique subset of T_{CM}.

TSLP-DCs Maintain T_{CM} Phenotype and T_H2 Commitment

To examine whether $CRTH2^+CD4^+$ T_H2 memory cells expanded by TSLP-DCs, IL-15 plus IL-7, or anti-CD3/CD28 can maintain their memory phenotype and T_H2

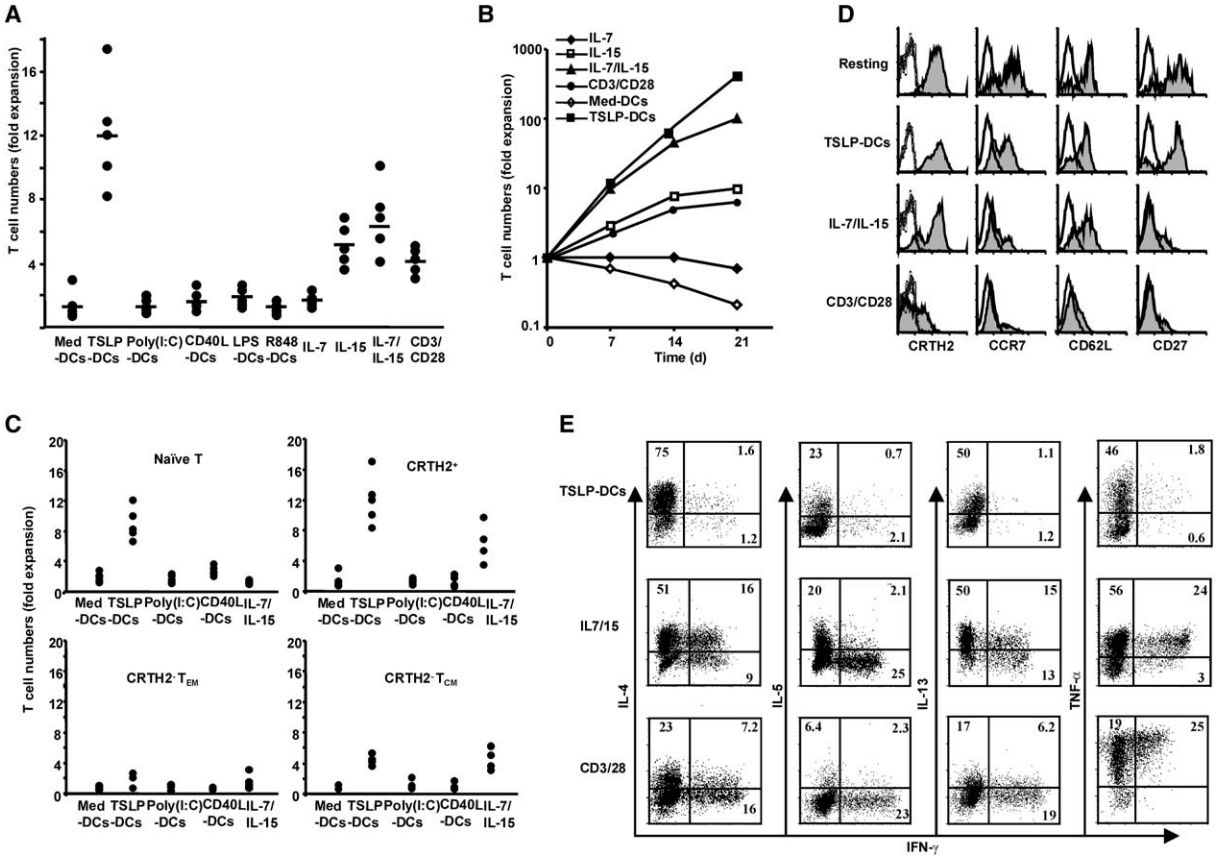

Figure 2. Homeostatic Expansion and Phenotypic and Functional Maintenance of CRTH2$^+$CD4$^+$ T_H2 Memory T Cells Mediated by Autologous TSLP-DCs

(A and B) Sorted CRTH2$^+$CD4$^+$ T_H2 memory T cells were cultured in the following conditions, including indicated cytokines, anti-CD3/CD28, or activated autologous CD11c$^+$ DCs. DCs and T cells were cultured at a ratio of 1:2 either for 7 days (A) or for three weeks with three rounds of stimulation (B). Filled circles represent the times of cell expansion by dividing the final cell number by the initial T cell number in five individual experiments (A) or by the number at the onset of each week's culture (B); horizontal bars indicate the median.

(C) Sorted CRTH2$^+$CD4$^+$ T_H2 memory T cells or other CD4$^+$ T cell subsets as indicated in Figure 1 were cultured with cytokines IL-15 plus IL-7 or autologous CD11c$^+$ DCs activated by various stimuli (horizontal axis) at a DC:T cell ratio of 1:2 for 7 days. Their proliferative responses were compared, and filled circles indicate the times of cell expansion as described in (A).

(D and E) For the characterization of expanded CRTH2$^+$CD4$^+$ T_H2 memory T cells driven by TSLP-DCs, IL-15 plus IL-7, or anti-CD3/CD28 for 7 days, the proliferated cells were collected and examined for phenotypic analysis by flow cytometry (D) or restimulated with PMA plus ionomycin for analysis of intracellular cytokine productions (E). Filled histograms represent staining of CRTH2$^+$CD4$^+$ T_H2 memory T cells with the markers indicated below histograms; open histograms represent the isotype control. Numbers within the quadrants indicate the percentage of expanded cells that stained positive for each respective cytokine. The results in each panel are from separate experiments. Data represent one of five experiments.

commitment, the proliferated cells were collected and analyzed by flow cytometry after 7 days in culture. While the majority of proliferated CRTH2$^+$CD4$^+$ T_H2 memory cells driven by TSLP-DCs maintained their expression of CRTH2/CCR7/CD27/CD62L, cells cultured with IL-15 plus IL-7 or anti-CD3/CD28 lost the expression of CCR7 and CD27, markers for T_{CM} (Figure 2D). The majority of expanded cells induced by TSLP-DCs were found to maintain the ability to produce classical T_H2 cytokines IL-4, IL-5, and IL-13, but not IFN-γ (Figure 2E). However, more than 20% of the CRTH2$^+$CD4$^+$ T_H2 memory cells expanded by IL-15 plus IL-7 or anti-CD3/CD28 were found to produce IFN-γ (Figure 2E). These data were further confirmed by ELISA analyses (Figure S1C). Interestingly, TSLP-DCs together with cytokine IL-15, IL-12, or IL-4 can induce the majority of these cells differentiating into effector memory cells, some of which become IFN-γ-producing cells (data not shown). These results suggest that only TSLP-DCs can maintain both the central memory phenotype and T_H2 commitment of proliferated CRTH2$^+$CD4$^+$ T_H2 memory cells, whereas anti-CD3/CD28, IL-15 plus IL-7, or TSLP-DCs plus cytokines can induce CRTH2$^+$CD4$^+$ T_H2 central memory cells to differentiate into effector memory cells.

T_H2 Progression of CRTH2$^+$CD4$^+$ T_H2 Memory Cells Induced by TSLP-DCs

To investigate why the expanded CRTH2$^+$CD4$^+$ T_H2 memory cells driven by TSLP-DCs possess distinctive T_H2 functional attributes, we performed global gene-expression analyses of naive T cells and of resting and expanded CRTH2$^+$CD4$^+$ T_H2 memory cells induced by TSLP-DCs, IL-15 plus IL-7, or anti-CD3/CD28. Upregulated genes induced by anti-CD3/CD28, IL-15 plus IL-7, or TSLP-DCs are clustered as group I, II, or, IV, respectively, whereas genes induced by all stimuli are in

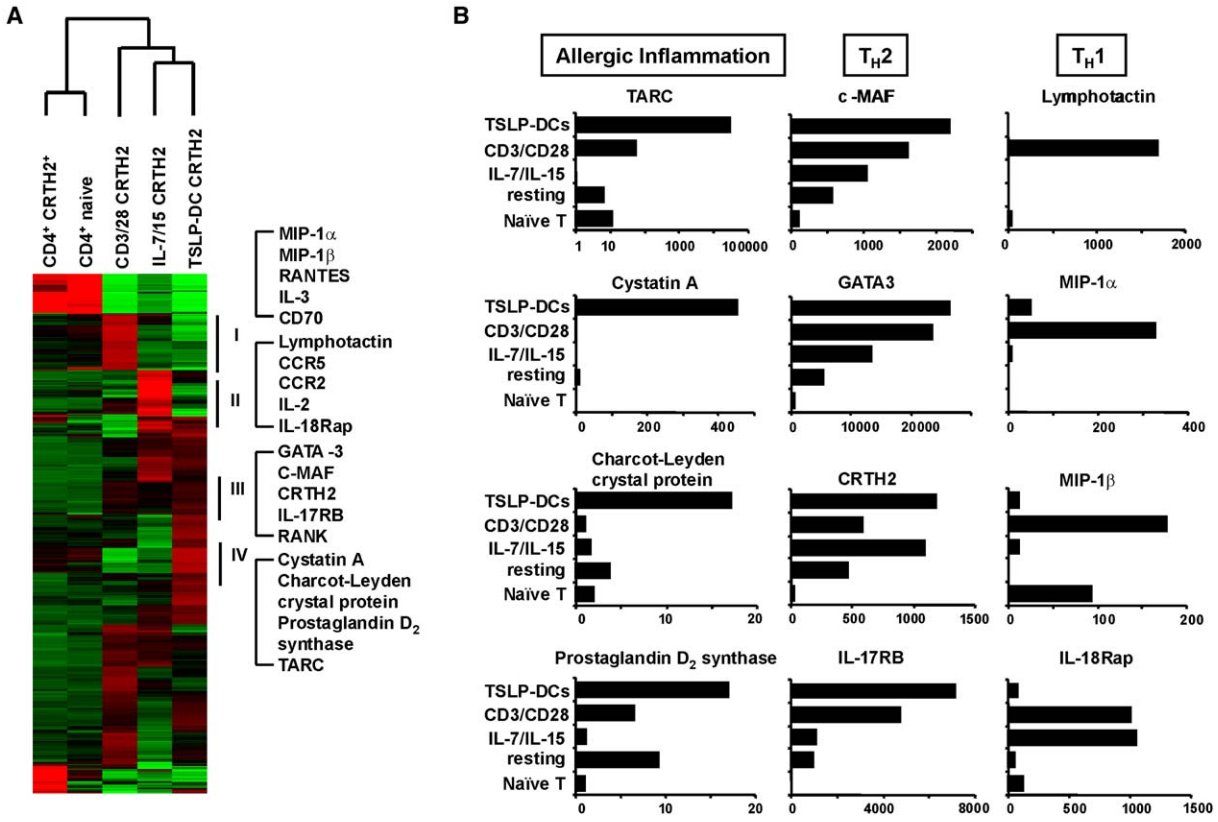

Figure 3. TSLP-DCs Induce CRTH2$^+$CD4$^+$ T_H2 Central Memory T Cells to Undergo further T_H2 Polarization and Express Allergy-Related Genes

Gene-expression profiles of the expanded CRTH2$^+$CD4$^+$ T_H2 memory T cells by autologous TSLP-DCs, IL-15 plus IL-7, or anti-CD3/CD28 and resting naive and CRTH2$^+$CD4$^+$ T_H2 memory T cells were assessed by DNA microarray (A) or real-time PCR (B). Of the differentially expressed genes, 517 were selected and subjected to hierarchical cluster analysis as described in Experimental Procedures. Relativity intensity of a particular gene across different samples is given by the color code in each row. Clusters I–IV and selected genes are shown. Genes with higher expression in the expanded CRTH2$^+$CD4$^+$ T_H2 memory T cells induced by anti-CD3/CD28, IL-15 plus IL-7, or TSLP-DCs are shown in clusters I, II, or IV, respectively. The genes upregulated in all of activated CRTH2$^+$CD4$^+$ T_H2 memory T cells are shown in cluster III (A). Expression level of indicated genes that are involved in the allergic inflammation, T_H1 or T_H2 differentiation was measured by real-time PCR as described in Experimental Procedures. Genes were selected according to the DNA microarray and hierarchical cluster analysis. Relative differences in expression level of indicated gene between samples marked in the left panel are indicated in horizontal axis (B). Data are from one of three independent experiments.

cluster III (Figure 3A). Real-time PCR analyses revealed that TSLP-DCs induce CRTH2$^+$CD4$^+$ T_H2 central memory cells undergoing further T_H2 polarization by upregulating the expression of genes involved in T_H2 differentiation, including *CRTH2, GATA-3*, *c-maf*, and *IL17RB*. By contrast, TSLP-DCs do not induce the expression of genes encoding chemokines lymphotactin, MIP-1α, and MIP-1β, which are produced during T_H1-polarized inflammation (Ritz et al., 2004; Muller et al., 2003), and IFN-γ, IL-18Rap, CCR5, and CD70, which are expressed by T_H1 or T_{EM} cells. Interestingly, only TSLP-DCs induced CRTH2$^+$CD4$^+$ T_H2 memory cells to express genes encoding cystatin A, Charcot-Leydon crystal protein, and prostaglandin D2 synthase, which are known to be selectively expressed by eosinophils and basophils (Golightly et al., 1992; Ackerman et al., 1993) and critical for allergic inflammation (John et al., 2000; Matsuoka et al., 2000), as well as chemokine TARC known to recruit T_H2 cells (Ritz et al., 2004) (Figure 3B). These results suggest that TSLP-DCs not only maintain the central memory phenotype but also further strengthen the "T_H2" properties of CRTH2$^+$CD4$^+$ T_H2 memory cells.

Oligoclonal Expansion of CRTH2$^+$CD4$^+$ T_H2 Memory Cells by Allergen-Pulsed TSLP-DCs

Human are exposed to different types of allergens constantly. To address whether CRTH2$^+$CD4$^+$ T_H2 memory cells are allergen-specific T_H2 memory T cells that may mount recall responses to common allergens, we first performed immunofluorescence analysis of TCRV$_\beta$ usage by these cells via a panel of mAbs against specific TCRV$_\beta$. Among 10 healthy donors examined, resting CRTH2$^+$CD4$^+$ T_H2 memory cells display polyclonal TCRV$_\beta$ repertoire similar to those of circulated naive T cells from the same donors (Figure 4A). The expanded CRTH2$^+$CD4$^+$ T_H2 memory cells by autologous TSLP-DCs also display polyclonal TCRV$_\beta$ repertoire similar to those of freshly isolated cells (Figure 4A), indicating that autologous TSLP-DCs can maintain the polyclonality of CRTH2$^+$CD4$^+$ T_H2 memory cells, while inducing their homeostatic proliferation.

To investigate whether only CRTH2$^+$CD4$^+$ T_H2 memory cells, but not other T cell subsets, could mount a recall response to common allergens, autologous TSLP-DCs loaded with or without allergens for 18 hr

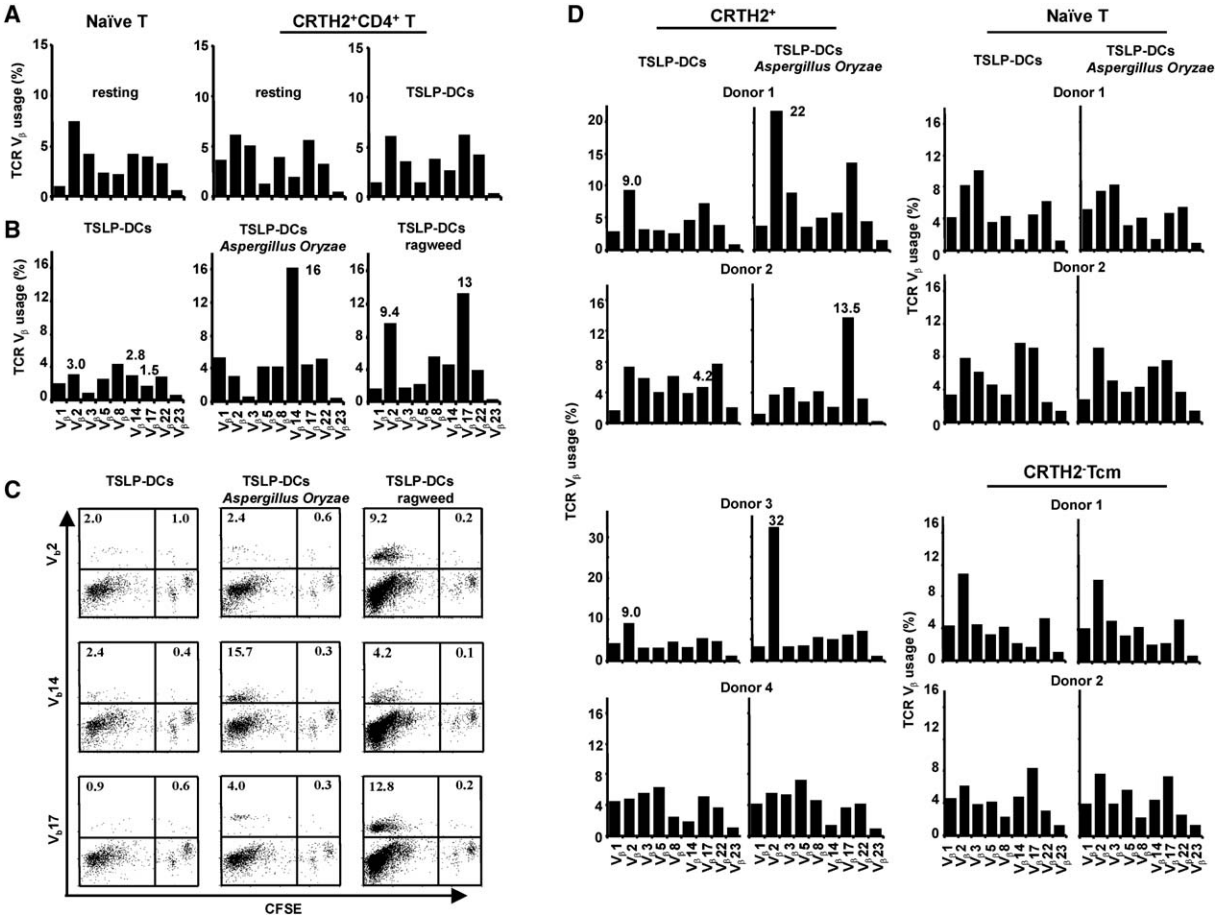

Figure 4. Allergen-Pulsed TSLP-DCs Induce Skewed TCR Repertoire Displayed by Expanded CRTH2$^+$CD4$^+$ T_H2 Memory T Cells

(A) Purified peripheral naive and CRTH2$^+$CD4$^+$ T_H2 memory T cells from the same donors or expanded CRTH2$^+$CD4$^+$ T_H2 memory T cells by autologous TSLP-DCs were analyzed for their TCRV_β usage by flow cytometry. Data represent one of the six responding normal donors.

(B–D) CRTH2$^+$CD4$^+$ T_H2 memory T cells labeled with CFSE (B, C) or naive, CRTH2$^+$CD4$^+$, and CRTH2$^-$CD4$^+$ T_{CM} cells isolated from the same donors (D) were cultured with autologous TSLP-DCs only, or loaded with *Aspergillus Oryzae* or ragweed pollen extracts for 7 days and collected for the analysis of TCRV_β usage by flow cytometry. Numbers within the quadrants indicate the percentage of the expanded cells labeled with diluted CFSE and specific anti-TCRV_β mAb (C).

were cocultured with CRTH2$^+$CD4$^+$ T_H2 memory cells or other T cell subsets for 7 days. Among 12 normal donors examined, six donors showed significantly skewed TCRV_β repertoire after being cultured with allergen-pulsed TSLP-DCs. In one of these six donors, TSLP-DCs pulsed with *Aspergillus Oryzae* or ragweed pollen extracts induced robust oligoclonal expansion of CRTH2$^+$CD4$^+$ T_H2 memory cells bearing TCRV_β14 (16%), or TCRV_β2 (9.4%) and TCRV_β17 (13%), respectively (Figure 4B). The expansion of these oligoclones could be visualized by the dilution of CFSE (Figure 4C). The skewed oligoclones within the CRTH2$^+$CD4$^+$ T_H2 memory cells induced by allergen-pulsed TSLP-DCs vary significantly among the responding donors (Figure 4D and data not shown). While TCRV_β2- or V_β17-expressing CRTH2$^+$CD4$^+$ T_H2 memory cells were selectively expanded in donors 1 and 3 or donor 2, respectively, donor 4 representing one of the other six donors displayed unaltered TCRV_β repertoire (Figure 4D). By contrast, naive and CRTH2$^-$$T_{CM}$ T cells isolated from all three donors did not respond to TSLP-DCs pulsed with either allergens, but only superantigen SEB, and underwent oligoclonal expansion (Figure 4D, Figure S2, and data not shown). Allergen-pulsed med-DCs or poly(I:C)-DCs could not induce cell proliferation; therefore, no significant skewed TCRV_β repertoire of any of the three T cells subsets were observed in the autologous DC-T coculture system (Figure S3). These results suggest that only allergen-pulsed TSLP-DCs can mount a recall response by inducing selective oligoclonal expansion of CRTH2$^+$CD4$^+$ T_H2 memory cells.

Molecular Interactions between TSLP-DC and CRTH2$^+$CD4$^+$ T_H2 Memory Cells

To investigate the molecular mechanisms underlying the unique ability of TSLP-DCs to induce robust homeostatic expansion of autologous CRTH2$^+$CD4$^+$ T_H2 memory cells, we first analyzed the function of peptide-MHC complexes, costimulatory molecules, and cytokines. We found that anti-HLA-DR, CD80, or CD86, but not anti-IL-7 or anti-IL-15, efficiently abrogated the proliferation of the CRTH2$^+$CD4$^+$ T_H2 memory induced by autologous TSLP-DC in a dose-dependent manner (Figure 5A). To address why only TSLP-DCs, but not other

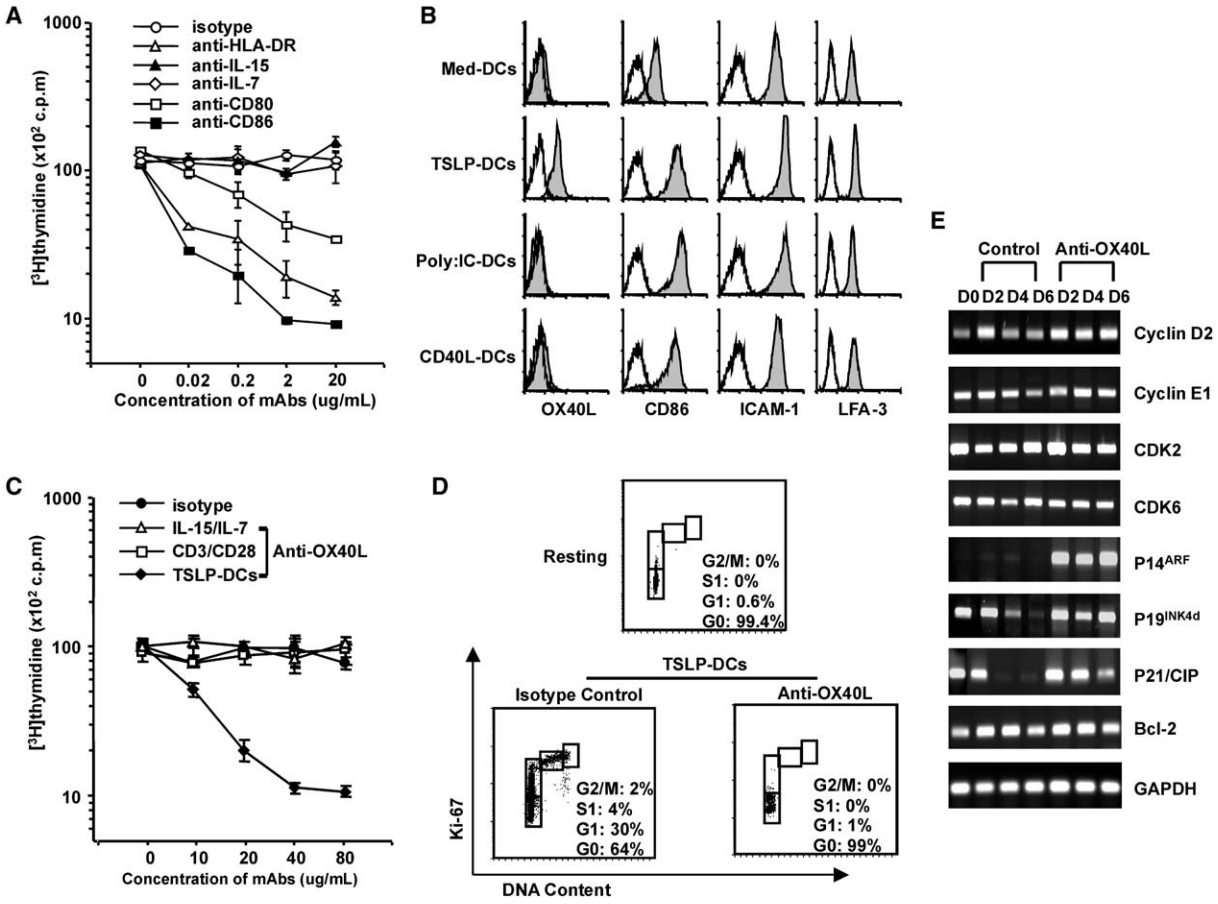

Figure 5. Mechanisms Underlying the Homeostatic Expansion of CRTH2$^+$CD4$^+$ T_H2 Memory T Cells Driven by TSLP-DCs

(A and C) Proliferation assays for CRTH2$^+$CD4$^+$ T_H2 memory T cells cultured with autologous TSLP-DCs at a DC:T cell ratio of 1:2 (A, C), IL-15 plus IL-7 (C), or anti-CD3/CD28 (C) for 7 days were assessed by [^{3}H]thymidine incorporation. Neutralizing mAbs to HLA-DR, IL-7, IL-15, CD80, and CD86 (A) or OX40L (C) or isotype control were used at various concentrations (horizontal axis). Data represent the mean (± SD) of three experiments.

(B) Surface phenotype of CD11c$^+$ DCs activated by various stimuli or cultured with medium for 24 hr was analyzed by flow cytometry. Filled histograms represent staining of CD11c$^+$ DCs with the markers indicated below histograms; open histograms represent the isotype control.

(D) Cell-cycle status of in vivo derived CRTH2$^+$CD4$^+$ T_H2 memory T cells was evaluated immediately after sorting (top: resting). Expanded CRTH2$^+$CD4$^+$ T_H2 memory T cells induced by TSLP-DCs at a DC:T cell ratio of 1:2 in the presence of anti-OX40L (40 μg/mL) or isotype control mAb were cultured for 7 days before the examination of cell-cycle status.

(E) RNA samples from CRTH2$^+$CD4$^+$ T_H2 memory T cells cultured with TSLP-DCs at 0, 2, 4, or 6 days in the presence or absence of OX40L mAb (20 μg/mL) were isolated and reverse transcribed and used as the templates to examine the expression of indicated genes involved in the regulation of cell cycle by PCR analysis by means of primer sequences as described (Fink and LeBien, 2001).

activated DCs, are capable of inducing homeostatic expansion of autologous CRTH2$^+$CD4$^+$ T_H2 memory cells, we examined surface expression of molecules critical for DC-T cell interactions. DCs activated by TSLP, poly(I:C), or CD40L express similar level of surface MHCII, ICAM-1/CD54, LFA3/CD58, B7.1/CD80, and B7.2/CD86; however, only TSLP-DCs express distinctive level of surface OX40L (Figure 5B and data not shown; Soumelis et al., 2002). Since CRTH2$^+$CD4$^+$ T_H2 memory cells express high level of OX40 (Figure 1C) and OX40/OX40L interaction has been implicated in the maintenance of memory T cell expansion (Salek-Ardakani et al., 2003), we next investigated whether OX40L expressed by TSLP-DCs play an important role in driving the homeostatic proliferation of CRTH2$^+$CD4$^+$ T_H2 memory cells. Indeed, we found that anti-OX40L mAb could efficiently abrogate the expansion of CRTH2$^+$CD4$^+$ T_H2 memory cells induced by TSLP-DCs in a dose-dependent manner, but not the expansion driven by anti-CD3/CD28 or by IL-15 plus IL-7 (Figure 5C).

OX40/OX40L Interactions Contribute to Prolonged DC-T Cell Cognate Formation

Prior study showed that emigrated DCs and memory T cells could form distinctive T cell-DC conjugates in a human skin organ culture (Pope et al., 1995), and others demonstrated that OX40/OX40L play critical roles in cell-cell adhesion (Imura et al., 1996). To investigate whether OX40/OX40L interactions are involved in the conjugate formation between TSLP-DCs and CRTH2$^+$CD4$^+$ T_H2 memory cells, we first compared DC-T cell cognate formation between CRTH2$^+$CD4$^+$ T_H2 memory cells and DCs activated by different stimuli. When CRTH2$^+$CD4$^+$ T_H2 memory cells were cocultured with TSLP-DCs, CD3$^+$CD11c$^+$ T cell-DC conjugates represent 16% or 12% of total viable cells at 2 days or

4 days of culture. By contrast, only a few conjugates between CRTH2$^+$CD4$^+$ T_H2 memory cells and poly(I:C)-DCs (6%), CD40L-DCs (8%), or med-DCs (6%) could be observed at day 2, and these conjugates were rapidly lost after day 4 (Figure S4A). Interestingly, anti-OX40L mAb could abrogate the formation of T cell-DC conjugates at day 2 and 4 of culture similar to the effect by anti-LFA-1 mAb, and the combination of both mAbs completely blocked the T cell-DC conjugate formation at day 4 of culture (Figure S4B).

Blockade of OX40/OX40L Interactions Induces the Expression of Cyclin Inhibitors

To further investigate why the loss of OX40 signaling resulted in the failure of cell expansion, cell-cycle status of CRTH2$^+$CD4$^+$ T_H2 memory cells cocultured with TSLP-DCs in the presence or absence of anti-OX40L mAbs after 7 days were examined. We found that the presence of anti-OX40L mAb in culture resulted in CRTH2$^+$CD4$^+$ T_H2 memory cells arresting at G_0 phase and remaining viable as indicated by the lack of subdiploid cells (Figure 5D). Detailed analysis of genes involved in cell cycling revealed that all cultured cells express similar level of cyclin E1, E2, D2, cyclin-dependent kinase (CDK) 2, 4, 6, and Bcl-2, regardless of the loss of OX40 signaling during culture, indicating that these cells were viable and capable of undergoing active cell cycling (Figure 5E, and data not shown). However, the blockade of OX40/OX40L interactions induced the expression of p14^{ARF} (inhibitor of CDK4) known to induce G1 and G2 check point arrest and maintain the expression of other CDK inhibitors, P21/CIP1, p19^{INK4d}, and p27^{KIP1}. By contrast, the expanded cells driven by TSLP-DCs gradually shut down the expression of P21/CIP, p19^{INK4d}, and p27^{KIP1} transcripts and did not express p14^{ARF} gene (Figure 5E, and data not shown; Carnero et al., 2000). The results demonstrate that OX40 signaling is required for the activation and maintenance of CRTH2$^+$CD4$^+$ T_H2 memory cells driven by TSLP-DCs and that the loss of OX40 signaling during T cell-DC interaction induces the expression of CDK inhibitors in CRTH2$^+$CD4$^+$ T_H2 memory cells particularly, p14^{ARF}, thereby resulting in cell-cycle arrest.

CRTH2$^+$CD4$^+$ T_H2 Cells Associate with DCs in Atopic Dermatitis

To investigate whether the occurrence of CLA$^+$CRTH2$^+$ CD4$^+$ T_H2 memory cell infiltration was associated with T_H2-type allergic inflammation but not T_H1-mediated inflammatory diseases in vivo, we examined skin sections from normal skin, nonlesional, and lesional skins of AD by immunohistology. Strong TSLP expression was found in keratinocytes of the apical layers of the epidermis in AD, but not in normal or nonlesional skin as previously reported (Soumelis et al., 2002). Large numbers of CD3$^+$ T cells were found to infiltrate the dermis of lesional skin of AD and in close association with the DC-lysosome-associated membrane protein$^+$ (DC-LAMP) activated DCs (four patients, Figure 6A, i, ii), whereas few CD3$^+$ T cells or DC-LAMP$^+$ DCs were scattered in nonlesional skin from AD samples (three patients, Figure 6A, iii, iv) or normal skin (three samples, data not shown). Staining of an adjacent section via isotype-matched control gave negative results (data not shown). Interestingly, the majority of accumulated CD3$^+$ T cells surrounded by the CD11c$^+$ DCs within the clusters were found to express T_H2 memory cell marker, CRTH2 in AD (Figure 6B, i–iv), whereas these T cells did not express CRTH2 in the skin section obtained from T_H1-mediated inflammatory diseases via laser-scanning confocal microscopy (Figure 6C, i–iv). These results provide the in vivo evidence suggesting that CRTH2$^+$CD4$^+$ T_H2 memory cells may play a critical role in the maintenance of T_H2-mediated allergic skin diseases, but not T_H1-mediated inflammatory skin diseases.

Discussion

Long-lived CD4$^+$ T_H2 memory cells have been postulated as the central player at the sites of inflammation during type 2 immune responses (Holt et al., 1999; Prescott et al., 1999). However, detailed characterization of this cell subset has been hindered by the lack of specific markers and their paucity in blood. Here, we report that a unique subset of CD4$^+$ T cells expressing CRTH2 was characterized as the circulated T_H2 memory cells, based on their distinctive features of central memory phenotype, gene-expression profile, capability of eliciting immediate T_H2 cytokines, and proliferative responsiveness to cytokine IL-15 and TSLP-DCs. CRTH2$^+$CD4$^+$ T_H2 memory cells express CCR4 and CXCR4, CLA, or β7 integrin, suggesting their readiness to migrate into non-lymphoid tissues upon exposure to allergens.

TSLP-DCs can induce sustained expansion of CRTH2$^+$CD4$^+$ T_H2 memory cells after multiple rounds of stimulation, while maintaining their central memory phenotype and T_H2 commitment. By contrast, IL-15 plus IL-7 and in particular anti-CD3/CD28 induced these cells to differentiate into effector memory cells with limited proliferative potential and caused a substantial fraction of them to produce T_H1 cytokines. Interestingly, the combinations of TSLP-DCs and cytokine IL-15, IL-4, or IL-12 can induce a significant fraction of these cells differentiating into effector memory cells, some of which become IFN-γ-producing cells (data not shown), suggesting that the ability of TSLP-DCs to maintain central memory phenotype and T_H2 commitment of the proliferated CRTH2$^+$CD4$^+$ T_H2 memory cells can be regulated by cytokines present in the inflammatory microenviroment. While TSLP-DCs can also induce limited proliferation of CRTH2$^-$CD4$^+$ T_H2 central memory cells, a significant fraction of these expanded cells can differentiate into CRTH2$^+$CD4$^+$ T_H2 central memory cells when cultured with TSLP-DCs in the T_H2, but not T_H1, conditions (data not shown), implicating that CRTH2$^+$CD4$^+$ T_H2 central memory cells can be induced from central memory cell pool by TSLP-DCs.

A recent study demonstrated that the maintenance of gut-specific memory T cells required the existence of anatomically corresponded DCs (Mora et al., 2003), emphasizing the importance of specialized DCs for the maintenance of heterogeneous T cell memory pools. Elevated expression of TSLP in inflamed skins led to AD in patients (Soumelis et al., 2002) who also have significantly increased frequency of circulated CRTH2$^+$CD4$^+$ T_H2 memory cells in blood (Cosmi et al., 2000). Although we found that the majority of CRTH2$^+$CD4$^+$ T_H2

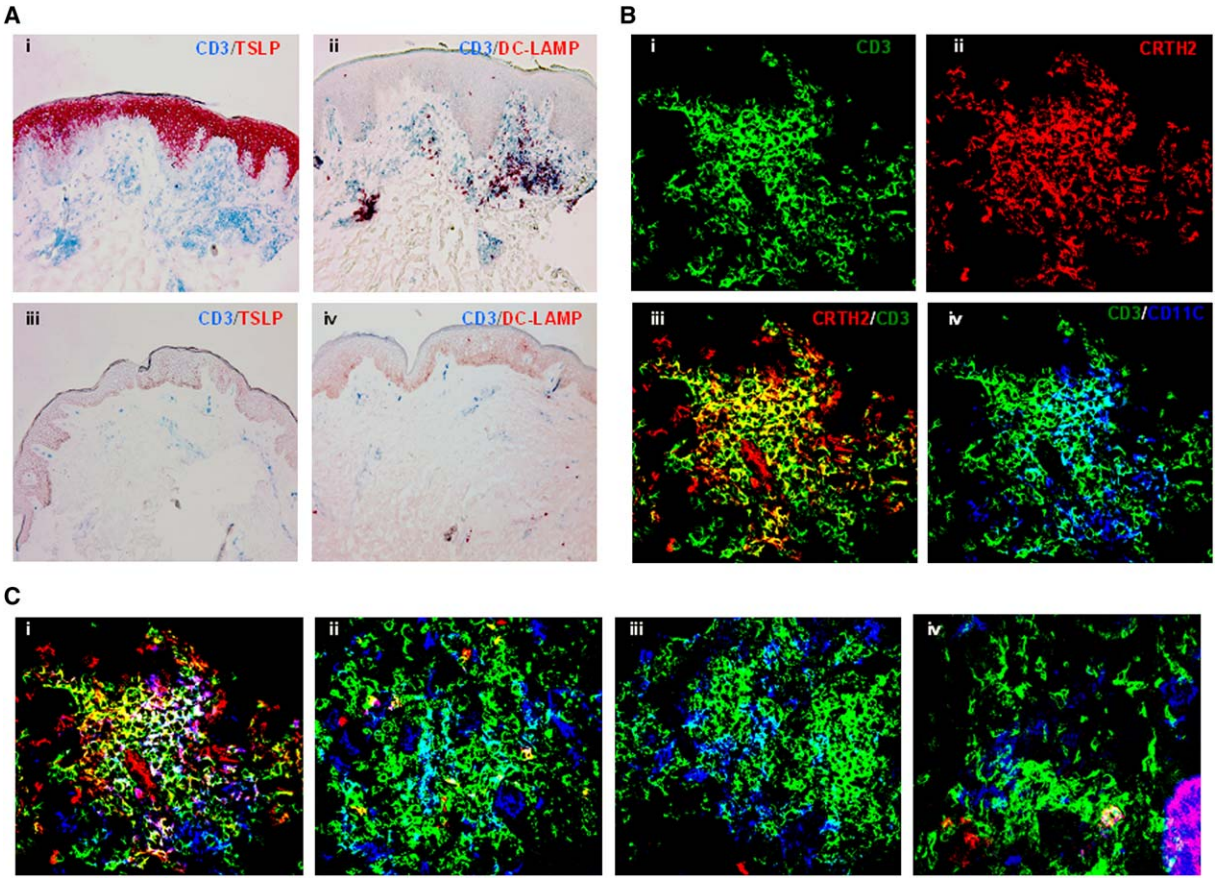

Figure 6. Colocalization of the Accumulated CRTH2$^+$ T Cells and Activated DCs at the Inflammatory Sites of AD

(A) Double staining of TSLP (red) and CD3 (blue) showed that T cells accumulation within the dermis is associated with the strong TSLP expression in the epidermis of lesional skin (i), but not of nonlesional skin (iii) from AD. Double staining of CD3 (blue) and DC-LAMP (red) on the same section of lesional biopsies showed that accumulated T cells were found to colocalize with many activated DCs within the dermis of lesional skin (ii), and only few scattered T cells, or activated DCs existed in the dermis of nonlesional skin (iv).

(B) Tricolor immunofluorescence staining with indicated Abs performed on the same section of lesional skin from AD showed that the majority of CD3$^+$ (green) T cells (i) coexpressed CRTH2 (red) (ii) and these accumulated CRTH2$^+$ T cells (yellow) (iii) were surrounded by CD11c$^+$ DCs (blue) (iv).

(C) Colocalization of infiltrated CRTH2$^+$ T cells and activated DCs could be observed within the clusters (white) of AD sample (i). Infiltrated CD3$^+$ T cells surrounded by CD11c$^+$ DCs do not express detectable CRTH2 in the epidermis of lesional skin from T_H1-mediated inflammatory diseases, including psoriasis vulgaris (ii), cutaneous lupus erythematosus (LE) (discoid type) (iii), and lichen planus (iv).

memory cells isolated from normal donors are resting central memory cells, most of these cells in patients with ongoing allergic diseases may be activated and display effector memory phenotype. Our findings demonstrate that accumulated CRTH2$^+$CD4$^+$ T_H2 memory cells are closely associated with DC-LAMP$^+$ DCs within dermis of lesional skin of AD, but not T_H1-mediated inflammatory skin diseases, suggesting that TSLP-DCs may interact with CRTH2$^+$CD4$^+$ T_H2 effector memory cells and induce their further T_H2 polarization at the inflammatory sites, thereby contributing to the maintenance and relapse of T_H2-mediated allergic diseases. These in vivo observations were further substantiated by the findings that signals from TSLP-DCs are the most effective on upregulation of genes involved in T_H2 polarization, such as *CRTH2, GATA-3*, *c-MAF*, and *IL17RB* (Fort et al., 2001; Matsuoka et al., 2000), as well as genes encoding the proteins that are found in epithelial cells, eosinophils, basophils, or antigen-presenting cells during allergic inflammation, including the T_H2-attracting chemokine TARC, cystatin A (John et al., 2000), Charcot-Leydon crystal protein (Golightly et al., 1992; Ackerman et al., 1993), and prostaglandin D2 synthase. Our findings broaden the understanding of heterogeneous T_H2 cells that are not only the principal producers of T_H2 cytokines but also capable of producing other effective molecules to trigger allergic inflammation.

Circulated CRTH2$^+$CD4$^+$ T_H2 memory cells isolated from normal donors display polyclonal TCR repertoire, which can be maintained by autologous TSLP-DCs after their expansion. Interestingly, allergen-pulsed TSLP-DCs, but not med-DCs or poly(I:C)-DCs, can selectively induce oligoclonal expansion of these cells among responding normal donors, which resembles the relapse of allergen-specific memory responses. By contrast, allergen-pulsed TSLP-DCs cannot induce significant skewed TCRV$_\beta$ repertoire of naive and CRTH2$^-$ T_{CM} T cell subsets from same donors. These observations suggest that only allergen-pulsed TSLP-DCs can mount a recall response of CRTH2$^+$CD4$^+$ T_H2 memory cells, but

not other T cell subsets, and that their skewed TCRV$_\beta$ repertoire varies significantly between individuals.

In addition to ligands of costimulatory molecules and peptide-MHC complexes, we found that the key molecule expressed by TSLP-DCs contributing to the maintenance of T_H2 memory cells is OX40L. The interactions between OX40 and OX40L contribute to strong and prolonged formation of T cell-DC conjugates. Loss of OX40 signaling during T cell-DC interaction induces the expression of $p14^{ARF}$ and other CDK inhibitors in CRTH2$^+$CD4$^+$ T_H2 memory cells, which results in cell-cycle arrest or cell senescence (Carnero et al., 2000). Triggering OX40 signaling in T_H2 memory cells by their ligands on TSLP-DCs is indispensable for the reactivation and homeostatic maintenance of the T_H2 memory pool by controlling their entrance into cell cycle, thereby linking the critical role of OX40L in the pathogenesis of allergic inflammation in animal models (Salek-Ardakani et al., 2003).

In conclusion, we demonstrated that human circulated CRTH2$^+$CD4$^+$ T cells are T_H2 central memory T cells. TSLP, a cytokine produced by epithelial cells during allergic inflammation, may activate DCs to migrate into the draining lymph node, where they trigger T_H2 differentiation of naive CD4$^+$ T cells as well as the homeostatic expansion of T_H2 central memory cells. TSLP-DCs may also play a critical role in inducing further T_H2 polarization of infiltrated CRTH2$^+$CD4$^+$ T cells to become effector memory cells at the inflammatory sites. Detailed characterization of CRTH2$^+$CD4$^+$ T_H2 memory T cells and their interaction with TSLP-DC during allergic inflammation may facilitate the development of new approaches to eliminate the pathogenic T_H2 memory cells for the prevention and treatment of human allergic diseases.

Experimental Procedures

Purification and Culture of Myeloid DC

This study was approved by the institutional review board for human research at The University of Texas M.D. Anderson Cancer Center (Houston, TX). CD11c$^+$lineage$^-$ DCs were isolated from blood of healthy donors (Gulf Coast Regional Blood Center, Houston, TX) by means of FACSAria as previously described (Soumelis et al., 2002). Sorted CD11c$^+$ DCs with a purity >99% were cultured in Yssel's medium containing 2% human AB serum (Gemini Bio-Products) or stimulated with 15 ng/mL TSLP, 10 μg/mL Poly(I:C), 1 μg/mL LPS, 1 μg/mL R848 (InvivoGen, San Diego, CA), or anti-CD40 mAb (2 μg/mL, G28.5) for 24 hr. For DCs pulsed with allergen experiments, cultured CD11c$^+$ DCs were added with 100 ng/mL *Aspergillus Oryzae* or ragweed pollen extracts after stimulation for 4 hr (Sigma, St. Louis, MO).

Naive, Central, Effector, and CRTH2$^+$ Memory CD4$^+$ T Cell Subset Purification

Autologous CD4$^+$ T cells were enriched by means of the CD4 T cell isolation kit (Miltney Biotech) according to manufacturer's instructions. Enriched CD4$^+$ T cells were stained with FITC-labeled lineage cocktail (CD14, CD15, CD19, CD25, CD56, and γδ-TCR), APC-CD45RO, APCcy7-CD4 (BD Bioscience), PE-CRTH2 (Milteney Biotec), biotin-CD45RA or PE-CD45RA, and biotin-CRTH2, and then washed, revealed with strepavidn-percpcy5.5. Stained cells were sorted into fractions of CD4$^+$CD45RA$^+$ naive T, CD4$^+$CD45RO$^+$CRTH2$^+$ T_H2 memory, and CD4$^+$CD45RO$^+$ CRTH2$^-$ memory T cells. Sorted CRTH2$^-$ memory T cells were further incubated with purified mouse anti-CCR7 and then revealed with FITC-labeled goat anti-mouse IgM for the isolation of CD4$^+$CD45RO$^+$CRTH2$^-$ CCR7$^+$ central and CD4$^+$CD45RO$^+$CRTH2$^-$CCR7$^-$ effector memory subsets with purity >99%.

Analysis of Cell-Surface Markers and TCRV$_\beta$ Repertoire

Freshly purified CD4$^+$CD45RO$^+$CRTH2$^+$, CD4$^+$CD45RO$^+$CRTH2$^-$ memory T cell subsets and naive T cells were stained with PE-labeled anti-CD27, CD62L, LFA-1 (CD11a), CLA, integrinβ7, CCR4, CXCR4, CCR1, CCR3, CXCR3, or CCR5 (all from BD Bioscience), washed, and analyzed with FACSCalibur (BD Bioscience). TCRV$_\beta$ repertoire of tested cells was stained as described (Watanabe et al., 2004). For examination of cell-surface markers of activated DCs, cells were blocked with FcR-blocking reagent (Miltenyi Biotech) and stained with FITC-anti-CD80, CD86, ICAM-1/CD54, LFA-3/CD58, MHCII (all from BD Bioscience), or PE-OX40L (Ancell).

DC-T Cell Coculture

After 24 hr of culture, stimulated CD11c$^+$ DCs were collected, washed, and cocultured with 1.0 × 10^4 autologous CD4$^+$ memory T cell subsets in triplicate at a DC:T cell ratio of 1:2. In some experiments, CD4$^+$CD45RO$^+$CRTH2$^+$ T_H2 memory cells were cultured with immobilized anti-CD3 (10 μg/mL, OKT3) and soluble anti-CD28 (1 μg/mL, L293.1) and IL-7 (20 ng/mL), IL-15 (10 ng/mL) (R&D Systems), or IL-12 (10 ng/mL). The following reagents were used in some assays: anti-HLA-DR, anti-IL-7 (BD Bioscience), anti-IL-15, anti-CD80, anti-CD86 (R&D Systems), and anti-OX40L (ik-5, a generous gift from Dr. Hori Toshiyuki).

T Cell Proliferation Assay

After 6 days, cells were pulsed for 16 hr with 1 μCi [^{3}H]thymidine (Amersham Biosciences) before being collected and assessed for radioactivity. Alternatively, cells were collected in an EDTA-containing medium and counted with trypan blue exclusion of dead cells at day 7. In some experiments, expanded CD4$^+$ T cells were counted at the end of each week's culture (day 7, 14, and 21) and 1.0 × 10^4 CD4$^+$ T cells were restimulated by IL-7, IL-15, IL-15 plus IL-7, anti-CD3/CD28, or autologous TSLP-DCs or med-DCs.

Analysis of Cytokine Production

In primary culture, CRTH2$^+$CD4$^+$ T_H2 memory cells were cocultured with autologous TSLP-DCs, soluble anti-CD3/CD28, or IL-15 plus IL-7 for 7 days. Expanded T cells were washed and restimulated with immobilized anti-CD3 (OKT3, 10 μg/mL) and soluble anti-CD28 (L293.1, 1 μg/mL) for 24 hr. Culture supernatants were assessed by ELISA for IL-4, IL-5, IL-13, IFN-γ, and TNF-α (all from R&D Systems). Intracellular cytokine staining was performed with PE-labeled IL-4, IL-5, and IL-13, FITC-TNF-α, and APC-IFN-γ (all from BD Bioscience).

RNA Isolation and Real-Time Quantitative RT-PCR

Total RNA samples of sorted cells were isolated by RNeasy kit (Qiagen). The cDNA templates were synthesized with SuperScript II (Life Technologies). Oligonucleotide primers were selected with Primer Express 2.0 (Applied Biosystems, Inc.). Real-time quantitative PCR was performed with ABI Prism 7900 detection system according to manufacture's instructions. Equal amounts of RNAs from samples were used as templates in reactions to obtain the threshold cycle (C_t), and the C_t was normalized with the known C_t from 18S RNAs to obtain ΔC_t. To compare the relative levels of gene expression in different cells, $\Delta\Delta C_t$ values were calculated with the ΔC_t values associated with the lowest expression levels as the bases. The $\Delta\Delta C_t$ was then transformed to the real value of increase in expression by $2^{\Delta\Delta Ct}$.

Microarray Analysis and Bioinformatics

Sample preparations and microarray analysis were performed as described (Ito et al., 2005). Samples were organized on the basis of overall similarity in their gene-expression patterns by an unsupervised hierarchical clustering algorithm of variable genes. Genes with variable expression levels across the nine samples were selected based on the following criteria: genes should be expressed (have presence calls) in at least one of the nine samples, and the σ_i/μ_i ratio should be >1, where σ_i and μ_i are the standard deviation and mean of the hybridization intensity values of each particular gene across all samples, respectively (Koopman et al., 2003). An unsupervised

hierarchical clustering algorithm by the software Cluster (Eisen et al., 1998) was applied to the group of nine samples shown in Figure 1D or five samples shown in Figure 3A based on the similarity of the expression profiles of the selected genes. For genes represented by multiple probe sets, results for only one representative probe set are shown.

Skin Biopsy Samples

After obtaining informed consent from patients, 3–6 mm punch biopsies were taken from either lesional or nonlesional skin from AD, psoriasis vulgaris, LE (tumidus, discoid), lichen rubber, or from normal healthy individuals. Skin samples were immediately frozen in liquid nitrogen and stored at −80ºC. The study was approved by the local ethics committees of the Department of Dermatology of the Heinrich-Heine-University, Dusseldorf, Germany.

Immunohistology

Immunohistology analysis on acetone-fixed cryostat sections (8 μm) of human skin were performed as described (Soumelis et al., 2002). For immunoflurescence staining, the slides were incubated with rat anti-CRTH2 (BD Bioscience) at room temperature for 1 hr, followed by biotinylated $F(ab')_2$ fragment rabbit anti-rat IgG antibody (Jackson ImmunoResearch Laboratories). After washing, anti-CRTH2-dependent tissue deposition of biotin was increased via a tyramide signal biotin amplification system and visualized by Alexa 549-conjugated streptavidin (Molecular Probes). The slides were then stained with CD3-FITC and CD11C-APC (BD Bioscience) for 30 min. For imaging, a laser-scanning confocal device (Olympus Fluoview 300) equipped with a 15 mW krypton/argon laser and attached to an inverted microscope (Olympus 1X81) was used.

Supplemental Data

The four Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/24/6/827/DC1/.

Acknowledgments

We thank Dr. Li Zhang's suggestions for microarray data analysis and Karen Ramirez, Zhiwei He, and Eric Wieder for cell sorting and support. We thank Dr. Chen Dong and Pornpimon Angkasekwinai for critical reading and suggestions. Yui-Hsi Wang received a postdoctoral fellowship sponsored by Tanox, Inc. This work was supported by the Sandler Foundation and UT M.D. Anderson Research Foundation to Y.-J.L.

Received: December 16, 2005
Revised: February 17, 2006
Accepted: March 14, 2006
Published: June 20, 2006

References

Ackerman, S.J., Corrette, S.E., Rosenberg, H.F., Bennett, J.C., Mastrianni, D.M., Nicholson-Weller, A., Weller, P.F., Chin, D.T., and Tenen, D.G. (1993). Molecular cloning and characterization of human eosinophil Charcot-Leyden crystal protein (lysophospholipase). Similarities to IgE binding proteins and the S-type animal lectin superfamily. J. Immunol. *150*, 456–468.

Al Shami, A., Spolski, R., Kelly, J., Keane-Myers, A., and Leonard, W.J. (2005). A role for TSLP in the development of inflammation in an asthma model. J. Exp. Med. *202*, 829–839.

Campbell, J.J., Murphy, K.E., Kunkel, E.J., Brightling, C.E., Soler, D., Shen, Z., Boisvert, J., Greenberg, H.B., Vierra, M.A., Goodman, S.B., et al. (2001). CCR7 expression and memory T cell diversity in humans. J. Immunol. *166*, 877–884.

Carnero, A., Hudson, J.D., Price, C.M., and Beach, D.H. (2000). p16INK4A and p19ARF act in overlapping pathways in cellular immortalization. Nat. Cell Biol. *2*, 148–155.

Cosmi, L., Annunziato, F., Iwasaki, M., Galli, G., Manetti, R., Maggi, E., Nagata, K., and Romagnani, S. (2000). CRTH2 is the most reliable marker for the detection of circulating human type 2 Th and type 2 T cytotoxic cells in health and disease. Eur. J. Immunol. *30*, 2972–2979.

Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA *95*, 14863–14868.

Fink, J.R., and LeBien, T.W. (2001). Novel expression of cyclin-dependent kinase inhibitors in human B-cell precursors. Exp. Hematol. *29*, 490–498.

Fort, M.M., Cheung, J., Yen, D., Li, J., Zurawski, S.M., Lo, S., Menon, S., Clifford, T., Hunte, B., Lesley, R., et al. (2001). IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. Immunity *15*, 985–995.

Geginat, J., Sallusto, F., and Lanzavecchia, A. (2001). Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. J. Exp. Med. *194*, 1711–1719.

Golightly, L.M., Thomas, L.L., Dvorak, A.M., and Ackerman, S.J. (1992). Charcot-Leyden crystal protein in the degranulation and recovery of activated basophils. J. Leukoc. Biol. *51*, 386–392.

Ho, I.C., and Glimcher, L.H. (2002). Transcription: tantalizing times for T cells. Cell Suppl. *109*, S109–S120.

Holt, P.G., Macaubas, C., Stumbles, P.A., and Sly, P.D. (1999). The role of allergy in the development of asthma. Nature *402*, B12–B17.

Hoshino, A., Tanaka, Y., Akiba, H., Asakura, Y., Mita, Y., Sakurai, T., Takaoka, A., Nakaike, S., Ishii, N., Sugamura, K., et al. (2003). Critical role for OX40 ligand in the development of pathogenic Th2 cells in a murine model of asthma. Eur. J. Immunol. *33*, 861–869.

Imura, A., Hori, T., Imada, K., Ishikawa, T., Tanaka, Y., Maeda, M., Imamura, S., and Uchiyama, T. (1996). The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. J. Exp. Med. *183*, 2185–2195.

Ito, T., Wang, Y.H., Duramad, O., Hori, T., Delespesse, G.J., Watanabe, N., Qin, F.X., Yao, Z., Cao, W., and Liu, Y.J. (2005). TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. J. Exp. Med. *202*, 1213–1223.

Jahnsen, F.L., Moloney, E.D., Hogan, T., Upham, J.W., Burke, C.M., and Holt, P.G. (2001). Rapid dendritic cell recruitment to the bronchial mucosa of patients with atopic asthma in response to local allergen challenge. Thorax *56*, 823–826.

John, R.J., Rusznak, C., Ramjee, M., Lamont, A.G., Abrahamson, M., and Hewitt, E.L. (2000). Functional effects of the inhibition of the cysteine protease activity of the major house dust mite allergen Der p 1 by a novel peptide-based inhibitor. Clin. Exp. Allergy *30*, 784–793.

Julia, V., Hessel, E.M., Malherbe, L., Glaichenhaus, N., O'Garra, A., and Coffman, R.L. (2002). A restricted subset of dendritic cells captures airborne antigens and remains able to activate specific T cells long after antigen exposure. Immunity *16*, 271–283.

Kay, A.B. (2001). Allergy and allergic diseases. First of two parts. N. Engl. J. Med. *344*, 30–37.

Koopman, L.A., Kopcow, H.D., Rybalov, B., Boyson, J.E., Orange, J.S., Schatz, F., Masch, R., Lockwood, C.J., Schachter, A.D., Park, P.J., and Strominger, J.L. (2003). Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. J. Exp. Med. *198*, 1201–1212.

Lambrecht, B.N., and Hammad, H. (2003). Taking our breath away: dendritic cells in the pathogenesis of asthma. Nat. Rev. Immunol. *3*, 994–1003.

Lambrecht, B.N., Hoogsteden, H.C., and Pauwels, R.A. (2001). Dendritic cells as regulators of the immune response to inhaled allergen: recent findings in animal models of asthma. Int. Arch. Allergy Immunol. *124*, 432–446.

Lawson, B.K., Baccala, R., Song, J.X., Croft, M., Kono, D.H., and Theofilopoulos, A.N. (2004). Deficiency of the cyclin kinase inhibitor p21(WAF-1/CIP-1) promotes apoptosis of activated/memory T cells and inhibits spontaneous systemic autoirnmunity. J. Exp. Med. *199*, 547–557.

Matsuoka, T., Hirata, M., Tanaka, H., Takahashi, Y., Murata, T., Kabashima, K., Sugimoto, Y., Kobayashi, T., Ushikubi, F., Aze, Y., et al. (2000). Prostaglandin D2 as a mediator of allergic asthma. Science *287*, 2013–2017.

Mojtabavi, N., Dekan, G., Stingl, G., and Epstein, M.M. (2002). Long-lived Th2 memory in experimental allergic asthma. J. Immunol. *169*, 4788–4796.

Mora, J.R., Bono, M.R., Manjunath, N., Weninger, W., Cavanagh, L.L., Rosemblatt, M., and von Andrian, U.H. (2003). Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. Nature *424*, 88–93.

Mowen, K.A., and Glimcher, L.H. (2004). Signaling pathways in Th2 development. Immunol. Rev. *202*, 203–222.

Muller, K., Bischof, S., Sommer, F., Lohoff, M., Solbach, W., and Laskay, T. (2003). Differential production of macrophage inflammatory protein 1gamma (MIP-1gamma), lymphotactin, and MIP-2 by CD4(+) Th subsets polarized in vitro and in vivo. Infect. Immun. *71*, 6178–6183.

Nagata, K., Tanaka, K., Ogawa, K., Kemmotsu, K., Imai, T., Yoshie, O., Abe, H., Tada, K., Nakamura, M., Sugamura, K., and Takano, S. (1999). Selective expression of a novel surface molecule by human Th2 cells in vivo. J. Immunol. *162*, 1278–1286.

Neurath, M.F., Finotto, S., and Glimcher, L.H. (2002). The role of Th1/Th2 polarization in mucosal immunity. Nat. Med. *8*, 567–573.

Pope, M., Betjes, M.G., Hirmand, H., Hoffman, L., and Steinman, R.M. (1995). Both dendritic cells and memory T lymphocytes emigrate from organ cultures of human skin and form distinctive dendritic-T-cell conjugates. J. Invest. Dermatol. *104*, 11–17.

Prescott, S.L., Macaubas, C., Smallacombe, T., Holt, B.J., Sly, P.D., and Holt, P.G. (1999). Development of allergen-specific T-cell memory in atopic and normal children. Lancet *353*, 196–200.

Ritz, S.A., Cundall, M.J., Gajewska, B.U., Swirski, F.K., Wiley, R.E., Alvarez, D., Coyle, A.J., Stampfli, M.R., and Jordana, M. (2004). The lung cytokine microenvironment influences molecular events in the lymph nodes during Th1 and Th2 respiratory mucosal sensitization to antigen in vivo. Clin. Exp. Immunol. *138*, 213–220.

Rivino, L., Messi, M., Jarrossay, D., Lanzavecchia, A., Sallusto, F., and Geginat, J. (2004). Chemokine receptor expression identifies Pre-T helper (Th)1, Pre-Th2, and nonpolarized cells among human CD4+ central memory T cells. J. Exp. Med. *200*, 725–735.

Robert, C., and Kupper, T.S. (1999). Inflammatory skin diseases, T cells, and immune surveillance. N. Engl. J. Med. *341*, 1817–1828.

Salek-Ardakani, S., Song, S.X., Halteman, B.S., Jember, A.G.H., Akiba, H., Yagita, H., and Croft, M. (2003). OX40 (CD134) controls memory T helper 2 cells that drive lung inflammation. J. Exp. Med. *198*, 315–324.

Sallusto, F., Geginat, J., and Lanzavecchia, A. (2004). Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu. Rev. Immunol. *22*, 745–763.

Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature *401*, 708–712.

Shinkai, K., Mohrs, M., and Locksley, R.M. (2002). Helper T cells regulate type-2 innate immunity in vivo. Nature *420*, 825–829.

Soumelis, V., Reche, P.A., Kanzler, H., Yuan, W., Edward, G., Homey, B., Gilliet, M., Ho, S., Antonenko, S., Lauerma, A., et al. (2002). Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat. Immunol. *3*, 673–680.

Sprent, J., and Surh, C.D. (2001). Generation and maintenance of memory T cells. Curr. Opin. Immunol. *13*, 248–254.

Van Reijsen, F.C., Bruijnzeel-Koomen, C.A., de Weger, R.A., and Mudde, G.C. (1997). Retention of long-lived, allergen-specific T cells in atopic dermatitis skin. J. Invest. Dermatol. *108*, 530.

Watanabe, N., Hanabuchi, S., Soumelis, V., Yuan, W., Ho, S., de Waal, M.R., and Liu, Y.J. (2004). Human thymic stromal lymphopoietin promotes dendritic cell-mediated CD4+ T cell homeostatic expansion. Nat. Immunol. *5*, 426–434.

Yoo, J., Omori, M., Gyarmati, D., Zhou, B., Aye, T., Brewer, A., Comeau, M.R., Campbell, D.J., and Ziegler, S.F. (2005). Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. J. Exp. Med. *202*, 541–549.

Zhou, B., Comeau, M.R., De Smedt, T., Liggitt, H.D., Dahl, M.E., Lewis, D.B., Gyarmati, D., Aye, T., Campbell, D.J., and Ziegler, S.F. (2005). Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. Nat. Immunol. *6*, 1047–1053.