Selective Expression of the Eotaxin Receptor CCR3 by Human T Helper 2 Cells

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There is growing evidence that T helper cell subsets (T_H1 and T_H2) can be differentially recruited to promote different types of inflammatory reactions. Murine T_H1 but not T_H2 cells are recruited through P- and E-selectin into inflamed tissues, where they induce delayed-type hypersensitivity reactions. The human eotaxin-receptor CCR3, originally described on eosinophils and basophils, was also found to be expressed by T_H2 cells. An antibody to CCR3 was used to isolate T cells from peripheral blood that give rise to T_H2-polarized cell lines and to identify T_H2 cells derived from naïve T cells in vitro. Eotaxin stimulated increases in intracellular calcium and chemotaxis of $CCR3^+$ T cells. The attraction of T_H2 cells by eotaxin could represent a key mechanism in allergic reactions, because it promotes the allergen-driven production of interleukin-4 and interleukin-5 necessary to activate basophils and eosinophils.

The regulation of leukocyte migration is a complex process involving the participation of adhesion molecules such as selectins and integrins (*1*) as well as chemokines and chemokine receptors (*2*). The combined action of adhesion molecules and chemokines is thought to provide an address code for leukocyte migration to different sites (*3*).

Effector T lymphocytes are heterogeneous in their functional capabilities (*4*). T_H1 cells produce interferon-γ (IFN-γ) and activate mononuclear phagocytes, thus protecting against intracellular microbes. In contrast, T_H2 cells produce interleukin-4 (IL-4) and IL-5 and are involved in responses dominated by immunoglobulin E (IgE), eosinophils, and basophils. Mouse T_H1 cells express the ligand for P- and E-selectin, which promotes their migration into inflamed tissues (*5*). T_H2 cells do not enter the same sites as T_H1, suggesting a distinct migratory capacity. Because tissues undergoing allergic reactions contain T_H2 cells together with eosinophils and basophils, it is conceivable that common factors may be responsible for the recruitment of these three cell types.

The CC-chemokine eotaxin, produced by epithelial and phagocytic cells, is a potent and selective chemoattractant for eosinophils and basophils (*6*, *7*). Eotaxin binds with high affinity and fidelity to a specific receptor, CCR3, which is selectively expressed on eosinophils and basophils (*8*).

We observed that an antibody to CCR3 (7B11) (*9*) stained a small proportion of T lymphocytes in the human adult peripheral blood of certain donors but not in cord blood (*10*). About 1% of peripheral blood T cells express CCR3 (Fig. 1). $CCR3^+$ and $CCR3^-$ lymphocytes were sorted, expanded as polyclonal lines by stimulation with phytohemagglutinin (PHA) and IL-2, and tested for expression of CCR3 and cytokine production (*11*). Lines derived from $CCR3^+$-sorted cells had an increased proportion of $CCR3^+$ cells (19%) and produced IL-4 and IL-5. In contrast, lines derived from unsorted or $CCR3^-$-sorted cells comprised only very few $CCR3^+$ cells (<1%) and produced little IL-4 and IL-5. Although IL-4 and IL-5 production correlated with the expression of CCR3, the production of IFN-γ did not, because all the lines produced it in comparable amounts.

To identify type 2 T cells unambiguously, cytokine production analysis was performed on single cells (Fig. 1, D and G) (*12*). The lines derived from $CCR3^+$-sorted cells comprised a high proportion (17%) of typical type 2 cells producing large amounts of IL-4 and no IFN-γ, whereas the lines derived from unsorted or $CCR3^-$-sorted cells comprised only small proportions of cells mostly producing intermediate amounts of IL-4 (<2%). A second round of sorting from partially enriched cell lines led to a further enrichment of type 2 cells (>50%) and depletion of type 1 cells (Fig. 1H) (*13*). The expression of CD4 or CD8 was assessed together with cytokine production in polyclonal cell lines by three-color immunofluorescence (*12*). Cells producing only IL-4 were found in both $CD4^+$ and $CD8^+$ subsets and were enriched in $CCR3^+$ and depleted in $CCR3^-$ lines, as compared to unsorted cells (*14*). We conclude that CCR3 expression identifies a small population of peripheral blood T cells that gives rise to type 2 cell lines in vitro and that most likely have acquired this phenotype in vivo.

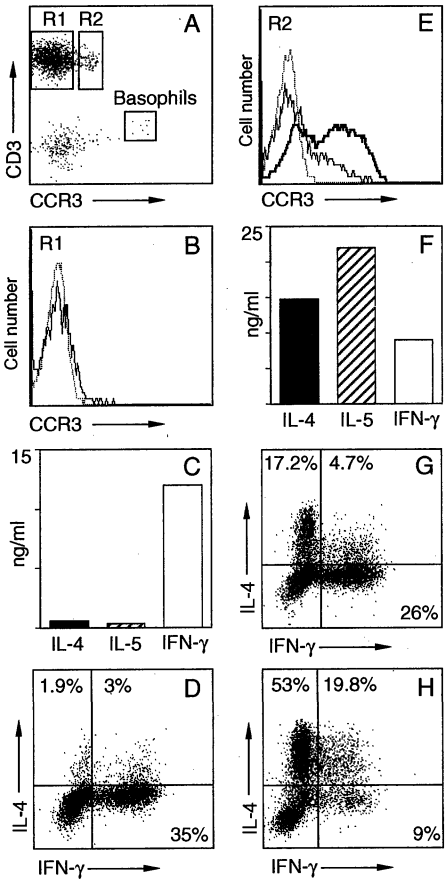

Fig. 1. CCR3 expression identified type 2 T cells in adult peripheral blood. (**A**) CCR3 expression on PBMC and (**B** and **E**) CCR3 expression on polyclonal T cell lines obtained from $CCR3^-$ (R1) and $CCR3^+$ (R2) cells are shown. Data are displayed on a three-decade logarithmic scale. Dotted line represents the staining with isotype-matched control antibody. (**C** and **F**) Production of IL-4, IL-5, and IFN-γ by the same T cell lines as measured by ELISA and (**D** and **G**) IFN-γ and IL-4 production at the single-cell level measured by intracellular staining (four-decade logarithmic scale) are shown. (**H**) IFN-γ and IL-4 production by $CCR3^+$ cells sorted from the CCR3-enriched cell line is shown. The CCR3 staining of this population is shown as a thick line in (E). The cells expressing high levels of CCR3 in peripheral blood were identified as basophils by sorting and Giemsa staining.

To identify the factors that induce CCR3 expression in T cells, we cultured cord blood naïve T cells in conditions that lead to either type 1 or type 2 polarization. Type 2 cell lines polarized in the presence of IL-4 plus anti-IL-12 (Fig. 2, A through D) (*15*) comprise up to 50% of $CCR3^+$ cells. When $CCR3^+$ and $CCR3^-$ cells were sorted and immediately analyzed for cytokine production, the $CCR3^+$ cells were almost completely of the type 2 phenotype, whereas the $CCR3^-$ cells showed a more heterogeneous pattern with few IFN-γ–producing cells. Conversely, type 1 cell

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lines polarized in the presence of IL-12 plus anti–IL-4 (Fig. 2, E through H) contained very few CCR3$^+$ cells. Interestingly, the rare CCR3$^+$ cells gave rise to clones that produced IL-4 and low or no IFN-γ. In contrast, all clones obtained from CCR3$^-$ cells produced large amounts of IFN-γ but no IL-4. Thus, CCR3 expression is induced in T cells under type 2–polarizing conditions and correlates with the capacity to produce IL-4 even in the rare cells that develop this phenotype under type 1–polarizing conditions.

Because both IFN-α (*16*) and transforming growth factor–β (TGF-β) (*17*) can interfere with T_H2 development, we tested whether they may also interfere with acquisition of CCR3 expression (Fig. 3). When added to the T_H2-polarizing condition, IFN-α dominantly induced generation of T_H1 cells, whereas TGF-β inhibited development of both IL-4– and IFN-γ–producing cells. In both situations, there was an almost complete inhibition of CCR3 expression, indicating that the expression of this receptor is linked to the program of T_H2 differentiation.

To investigate whether the CCR3 expressed on T cells is functional, we measured the increase in intracellular Ca^{2+} in response to eotaxin (Fig. 4) (*18*). A CCR3$^+$ but not a CCR3$^-$ T cell clone responded with a characteristic rapid rise in intracellular Ca^{2+} to stimulation with appropriate concentrations of eotaxin. CCR3$^+$ T cell clones were also tested for their chemotactic response to eotaxin by use of a standard chemotaxis assay. CCR3$^+$ but not CCR3$^-$ T cells responded to eotaxin with a similar efficiency as to other T cell chemoattractants, such as monocyte chemotactic protein–1 (MCP-1) and IFN-inducible protein–10 (IP-10) (*19*).

We show that CCR3 is acquired as a consequence of type 2 polarization both in vivo and in vitro, and is a stable marker of type 2 T cells, which allows them to respond to eotaxin. Together with the report that murine T_H1 cells selectively express ligands for P- and E-selectin (*5*), our results on human T_H2 cells support the concept that the T cell differentiation program goes beyond the cytokine production pattern and involves migratory capacity as well (*20*).

The expression of the eotaxin receptor on eosinophils, basophils, and T_H2 cells shows that these three cells can respond similarly to eotaxin as well as other agonistic chemokines (*21*). Eotaxin expression is up-regulated in tissues known to be sites of allergic reactions such as the airways (*7*) and is important for the attraction of eosinophils leading to lung eosinophilia (*6*). However, it is also known that the generation and maintenance of an allergic reaction requires antigen-specific T_H2 cells as a source of IL-4 and IL-5 that serve as growth and stimulation factors for basophils and eosinophils (*22*). The presence of CCR3 on T_H2 cells and on T cells that colocalize with eosinophils in diseased tissues (*23*) reveals a possible pathogenetic mechanism for T cell recruitment in the airways (*24*) and provides a target for therapeutic intervention. Thus, the distribution of eotaxin and of its receptor could coordinately and simultaneously at-

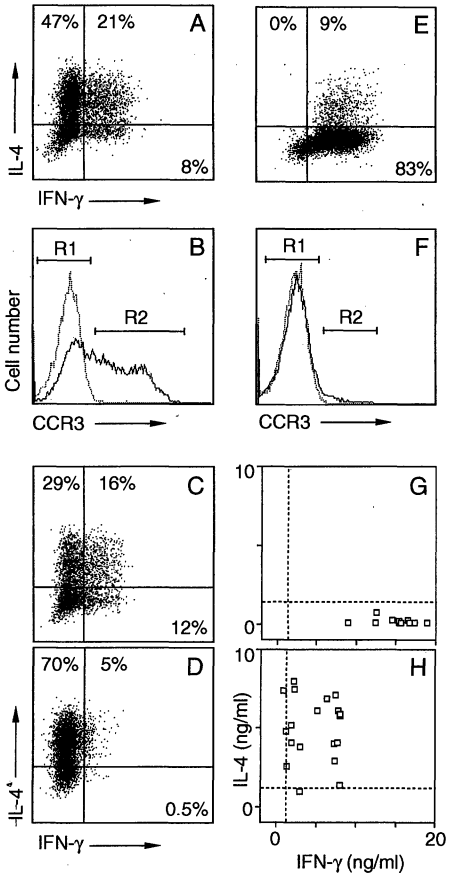

Fig. 2. CCR3 expression was induced under type 2 polarizing conditions and correlates with IL-4 production. Cytokine production pattern (four-decade logarithmic scale) and CCR3 expression (three-decade logarithmic scale) are shown for cell lines obtained from cord blood T cells under type 2– (**A** and **B**) or type 1– (**E** and **F**) polarizing conditions. From the type 2–polarized cell line, CCR3$^-$ (R1) and CCR3$^+$ (R2) cells were sorted and immediately analyzed for cytokine production (**C** and **D**). From the type 1–polarized cell line, CCR3$^-$ (R1) and the rare CCR3$^+$ (R2) cells were sorted, cloned by limiting dilution, and the clones analyzed for cytokine production by ELISA (**G** and **H**). Dashed lines represent the end of the assay. Similar results were obtained with six different cord blood samples.

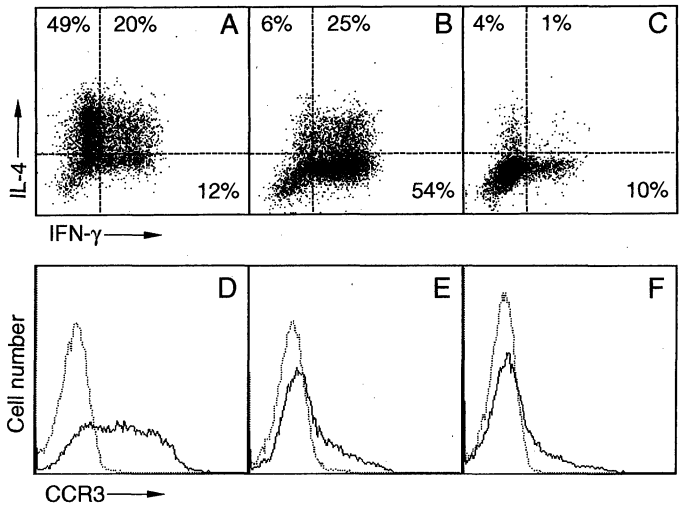

Fig. 3. IFN-α and TGF-β inhibit polarization to T_H2 and acquisition of CCR3. Cytokine production pattern and CCR3 expression are shown for cord blood T cell lines cultured with IL-4 plus anti–IL-12 either alone (**A** and **D**), or together with IFN-α (**B** and **E**) or TGF-β (**C** and **F**).

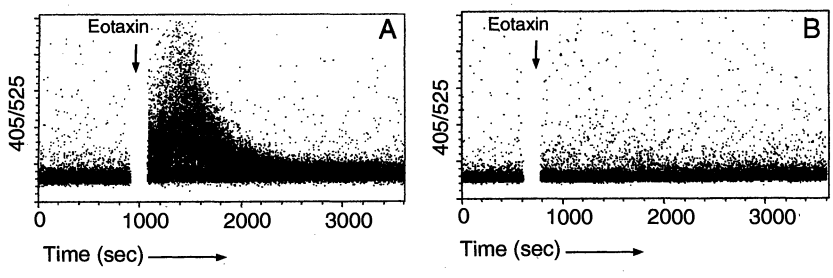

Fig. 4. Induction of intracellular Ca^{2+} increase in CCR3$^+$ T cells by eotaxin. Intracellular Ca^{2+} was recorded following stimulation with 10 nM eotaxin in a CCR3$^+$ (**A**) or a CCR3$^-$ (**B**) T cell clone. Both clones responded similarly when challenged with 10 nM RANTES (*25*).

tract to particular sites the three cell types that cooperate to generate an inflammatory allergic response.

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10. The percent of $CCR3^+$ T cells in peripheral blood varied in different donors from <0.2% to 8%. Three-color immunofluorescence analysis showed that a variable fraction (10 to 60%) of the $CCR3^+$ T cells express CD69. In all cases, however, the $CCR3^+$ cells account for only a fraction of all $CD69^+$ cells (*25*).
11. Peripheral blood mononuclear cells (PBMCs) were stained with monoclonal antibody 7B11 (anti-CCR3; IgG2a) (*9*). Positive and negative cells were sorted and expanded as polyclonal lines, using PHA (1 μg/ml; Wellcome, Buckingham, UK), human recombinant IL-2 (rIL-2; 500 U/ml) (*26*), and irradiated PBMC. After 14 days, the cells were washed and stimulated with plastic-bound anti-CD3 (monoclonal antibody TR66; IgG1) plus 10^{-7} M phorbol 12-myristate 13-acetate (PMA). Cytokine production was measured in the 24-hour culture supernatant by enzyme-linked immunosorbent assay (ELISA), using matched pairs of antibodies specific for IL-2, IL-4, IL-5, IL-10, tumor necrosis factor–α (TNF-α), TNF-β, and IFN-γ (PharMingen, San Diego, CA).
12. T cells were stimulated with 10^{-7} M PMA plus ionomycin (1 μg/ml) for 4 hours. Brefeldin A (10 μg/ml) was added during the last 2 hours. Cells were fixed with 2% paraformaldehyde, permeabilized with phosphate-buffered saline containing fetal bovine serum (1%) and saponin (0.5%) and stained with fluorescein isothiocyanate (FITC)–labeled anti–IFN-γ (IgG1) and phycoerythrin (PE)-labeled anti–IL-4 (IgG2b) monoclonal antibodies (Becton Dickinson, Mountain View, CA). In some experiments, the cells were stained, before permeabilization, with anti-CD4 (BL4, IgG2a, Immunotech, Marseille, France) or anti-CD8 (OKT8, IgG2a, American Type Culture Collection), then with biotin-labeled goat anti-mouse IgG2a (Southern Biotechnology, Birmingham, AL) and streptavidin-tricolor (Molecular Probes, Eugene, OR). Because T cell activation results in a rapid down-regulation of CCR3, it is not possible to assess CCR3 expression and intracellular cytokines simultaneously.
13. The difficulty in obtaining pure $CCR3^+$ T cell lines by direct sorting from peripheral blood may arise from the low level of CCR3 expression and the consequently high contamination by negative cells. A second sorting from partially enriched cell lines resulted in all cases in a substantial enrichment in $CCR3^+$ cells. Results comparable to those in Fig. 1 were obtained in sorted cell lines obtained from two healthy and three atopic individuals. In three out of five cases the CCR3-depleted cells produced lower concentrations of IL-4 and IL-5 than the unsorted cells. No clear differences were found in the production of IL-10, IFN-γ, IL-2, TNF-α, or TNF-β, or between healthy and atopic donors. A correlation between CCR3 expression and production of IL-4 but not IFN-γ was observed in 13 antigen-specific T cell clones (*14*).
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Fluorescence-Based Isolation of Bacterial Genes Expressed Within Host Cells

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A selection strategy was devised to identify bacterial genes preferentially expressed when a bacterium associates with its host cell. Fourteen *Salmonella typhimurium* genes, which were under the control of at least four independent regulatory circuits, were identified to be selectively induced in host macrophages. Four genes encode virulence factors, including a component of a type III secretory apparatus. This selection methodology should be generally applicable to the identification of genes from pathogenic organisms that are induced upon association with host cells or tissues.

Many bacterial pathogens survive in professional phagocytes by coordinately regulating the expression of a wide spectrum of genes (*1*). Because a microbe's ability to survive killing by phagocytes correlates with its ability to cause disease (*2*, *3*), the identification of genes that are preferentially transcribed in the intracellular environment of the host is central to our understanding of how pathogenic organisms mount a successful infection. So far, selections for in vivo–expressed genes (*4*-*6*) have been limited to bacterial pathogens with tractable genetic systems, because of the requirement for high frequencies of homologous recombination and extensive strain manipulation before gene selection (*4*, *6*).

We have developed a selection methodology, on the basis of differential fluorescence induction (DFI) (*7*), for the rapid identification of bacterial genes induced upon association with host cells that would work independently of drug susceptibility and nutritional requirements. Green fluorescent protein (GFP) (*8*) was used as a selectable marker in conjuction with fluorescence-activated cell sorting. Host cells infected with a bacterium bearing a transcriptionally active *gfp* gene fusion were separated by a fluorescence-activated cell sorter (FACS) and lysed, and the bacteria recovered were then grown under ex vivo conditions. Bacteria were then isolated by FACS on the basis of low to no fluorescence in the absence of host cells. Because all screening and selection steps are per-

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