Population analysis of CD4+ T cell chemokine receptor transcript expression during in vivo type-1 (mycobacterial) and type-2 (schistosomal) immune responses

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Abstract: Chemokine receptor transcripts were defined among CD4+ T cells in lymph nodes of mice with type-1 and type-2 inflammation, respectively, elicited by mycobacterial and schistosomal Ag. CXCR3 and CCR6 transcripts were biased to type-1, and CCR4 transcripts increased in type-1 and type-2 populations. CCR3 and CCR5 signals were too weak to establish differences. CCR8 transcripts were not increased among unstimulated populations. Compared to naïve, type-1 and type-2 populations had reduced CCR7 and enhanced CXCR5 transcripts, consistent with a shift to memory cells. Subset depletion revealed that transcript expression was induced among CD44+ memory T cells. Surprisingly, CCR3 transcripts were enriched among CD44lo fractions. Ag stimulation augmented CXCR3, CCR4, and CCR8 but down-regulated CCR6 and CXCR5. CCR4 showed association with IFN-γ- and IL-4-producing cells, but other receptor transcripts were expressed among IFN-γ/IL-4 negative memory T cells. These studies provide several novel findings regarding Th cell chemokine receptor expression in vivo. *J. Leukoc. Biol.* 72: 363–372; 2002.

Key Words: *Th1 · Th2 · granulomas*

INTRODUCTION

Type-1 and type-2 helper T cell (Th1 and Th2, respectively) subpopulations display distinct patterns of chemokine receptor expression [1–6]. Chemokine receptor expression by T cells is considered to be flexible, changing with maturational and activation state [2, 7]. Generally, artificially generated Th cell clones or in vitro polarized, polyclonal Th cells have been used to define chemokine receptor expression patterns for Th1 and Th2 cells, but it is unclear if these patterns translate to the more complex in vivo environment. For example, the model initially developed by Sallusto et al. [8] based on the study of human Th clones assigned CXCR3 and CCR5 expression to Th1 memory cells and CCR3 and CCR4 to Th2 memory cells, but a recent study of human peripheral blood revealed that cells with Th1 and Th2 characteristics expressed CCR4 [9, 10]. Thus, it is becoming increasingly apparent that chemokine receptor expression among T cells in the in vivo environment is complex and subject to endogenous, tissue-derived, microenvironmental and exogenous, pathogen-derived regulatory signals. Therefore, efforts to exploit chemokine receptor dynamics for clinical benefit must involve detailed in vivo analyses.

To this end, we measured transcript levels for multiple chemokine receptors among CD4+ T cell populations isolated from draining lymph nodes in models of Th1 and Th2 cell-mediated, pulmonary, granulomatous inflammation, elicited, respectively, by antigens derived from *Mycobacteria bovis* and ova of the helminthic parasite, *Schistosoma mansoni* [11–13]. These models provide a unique means to directly compare chemokine receptor expression under highly polarized in vivo conditions. A broad spectrum of reliable antibodies (Ab) with specificity for mouse chemokine receptors is not available, but the recent advent of real-time reverse transcriptase-polymerase chain reaction (RT-PCR) technology has permitted highly sensitive and reproducible, quantitative detection of chemokine receptor transcripts. Using this approach, we herein demonstrate distinct shifts of receptor transcript expression among CD4+ Th cell populations generated in vivo during type-1 mycobacterial and type-2 schistosomal granulomatous responses. Our in vivo study provides a number of novel observations, some of which challenge certain aspects of paradigms derived from in vitro T cell clone analyses. Overall, the findings support the concept of flexible chemokine receptor expression by T cells and provide in vivo evidence, suggesting that sensitization with strongly polarizing antigens (Ag) leads to induction of common and restricted chemokine receptor expression among broad, polyclonal T memory cell populations.

MATERIALS AND METHODS

Animals

Female, CBA/J mice were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were maintained under specific pathogen-free conditions and were provided food and water ad libitum.

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Received January 25, 2002; revised March 25, 2002; accepted April 8, 2002.

In vivo generation Th1 and Th2 immune responses

Type-1 and type-2 secondary Ag-bead pulmonary, granulomatous responses were generated in CBA mice as previously described [12]. Briefly, mice were sensitized by subcutaneous injection of 20 μg *M. bovis* purified protein derivative (PPD; Department of Agriculture, Veterinary Division, Ames, IA) incorporated into 0.25 ml complete Freund's adjuvant (#F-5881, Sigma Chemical Co., St. Louis, MO) or 3000 *S. mansoni* eggs suspended in 0.5 ml phosphate-buffered saline (PBS). Fourteen to 16 days later, PPD and schistosome egg-sensitized mice were, respectively, challenged by tail vein with 6000 Sepharose 4B beads (in 0.5 ml PBS) covalently coupled to PPD or to *S. mansoni*-soluble schistosome egg antigens (SEA) obtained from the World Health Organization (Geneva, Switzerland). Draining mediastinal lymph nodes were collected from five mice on day 3 and were pooled for CD4+ T cell purification described below. Replicates consisted of separate studies using five mice per group in each study. To confirm polarization, culture supernates were analyzed by enzyme-linked immunosorbent assay (ELISA) for cytokine profiles as described [14].

To examine the effect of Ag stimulation on Th cell chemokine receptor expression, draining mediastinal lymph nodes from type-1 (PPD)- or type-2 (SEA)-sensitized and bead-challenged animals were dispersed and cultured for 48 h in media supplemented, respectively, with 5 μg/ml PPD or SEA as previously described [14]. Next, CD3+CD4+ T cells were isolated as described below and subjected to mRNA analysis.

Real-time RT-PCR analysis of mRNA

Poly(A) pure mRNA was isolated from cells using Poly(A)Pure mRNA isolation kits (Ambion, Austin, Texas). Each mRNA sample of approximately 1 μg was reverse-transcribed in a 20-μl reaction in a PCR reaction tube using reverse transcription system kits (Promega, Madison, WI). Four separate reactions were conducted to minimize variability between tubes. The products from each reaction tube were pooled to make one cDNA sample. ABI PRISM 7700 Sequence Detection System (Perkin-Elmer, Foster City, CA) was used for real-time PCR analysis. For the current study, a comparative method (see User Bulletin #2, Perkin-Elmer) was adopted with glyceraldehyde 3-phosphate dehydrogenase as endogenous reference, and TaqMan (Perkin-Elmer) predeveloped reaction kits for mouse chemokine receptors CXCR2, CXCR3, CXCR5, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, and CCR8 were used for amplification. In all cases, the thermal cycling condition was programmed according to the manufacturer's instructions. To verify purity of mRNA, real-time PCR was perfomed on samples before and after reverse transcription, and it was routinely demonstrated that 37 or more PCR cycles were required to detect any activity in nontranscribed samples, indicating very low DNA contamination. For each chemokine receptor, the mRNA was considered detectable only when the signal was tenfold more abundant than the contamination level. In addition, an undetectable receptor gene among our panel of multiple receptors indicated lack of DNA contamination. Among the CD4+ T cell mRNA, CXCR2 and CCR1 transcripts were consistently negative.

Data are expressed as arbitrary units (AU), which are calculated from the fluorescence amplication factor as measured by the real-time PCR, fluorescent detection unit. The original gene copy number (Co) is related to fluorescence of the generated signal as follows: $Co = F \times E^{-1} \times I \times 2^{-n}$, where F is an arbitrary conversion constant, E^{-1} is amplification efficiency constant (approximately 1 for manufacturer's real-time primers sets), I is the fluorescent-intensity reading, and n is the amplification cycle number. Hence, $F \times E^{-1} \times I \times 2^{-n}$ constitutes an arbitrary measure of original copy number that is directly related to the fluorescent product and inversely related to cycle number. As E is roughly equivalent for the various primer sets, the expression levels among genes are comparable at orders of magnitude, but because of possible minor variations in reaction conditions between assays, a single Y-scale cannot be applied to all data.

Flow cytometric analysis of cytoplasmic cytokines

Ten million Th cells were suspended in 3 ml culture media with anti-CD28 at 2 μg/ml and were restimulated on an anti-CD3-coated 35-mm culture dish. Alternatively, in some studies, lymph node cells (5×10^6/ml) were stimulated with specific Ag. One hour after the initial stimulation, 2 μL GolgiStop (Pharmingen, San Diego, CA) was added to the culture media. The cells were cultured for another 4 h in a 37°C 5% CO_2 humidified incubator and then collected in cold PBS. Fixation was done with 1 ml CytoFix/CytoPerm (Pharmingen). After blocking with 10% bovine serum albumin, the cells were stained with the following phycoerythrin (PE)-labeled antibodies: rat immunoglobulin G (IgG)1-PE (isotype control), rat anti-mouse-interferon-γ (IFN-γ)-PE or cytochrome-labeled antibodies, rat anti-mouse-interleukin (IL)-4-PE, rat IgG2a-Cy-chrome, and rat anti-mouse-CD4-Cy-chrome (all from Pharmingen). A FACScan flow cytometer with CellQuest software (Becton-Dickinson, San Jose, CA) was used for data acquisition and analysis. Ten thousand events per sample were analyzed. In vitro-generated Th1 cells were routinely IL-4 negative and greater than 95% IFN-γ producing, whereas in vitro-generated Th2 cells were IFN-γ negative, and 40–50% of the cells were IL-4-producing. For chemokine receptor analysis, Th2 cells were further enriched as described below.

T cell subpopulation enrichment and depletion

Single cell suspensions were prepared from freshly harvested lymph nodes. The red blood cells and dead cells were removed from the preparations by using density centrifugation on Lympholyte-M (Cedarlane, Hornby, Ontario). Mouse T cell CD4 subset column kits (R&D Systems, Minneapolis, MN) were used to enrich for $CD3^+CD4^+$ cells. The purity of the recovered cells was confirmed by flow cytometric analysis and was routinely greater than 95%. For CD3+ CD4+ CD25+ and CD3+ CD4+ CD44+ subpopulation depletion, biotinylated anti-CD25 or anti-CD44 antibodies (Pharmingen) were added into the CD3+ CD4+ cell enrichment cocktail (StemCell Technologies, Vancouver, BC, Canada). Cells were then incubated with the enrichment cocktails, and the unwanted cells were magnetically labeled and removed according to the manufacturer's protocol. CD25 depletion routinely removed 30 ± 14% and CD44 depletion, 50 ± 15% of draining lymph node CD4+ T cells.

A similar procedure was used for $CD4^+IFN\text{-}\gamma^-$ and $CD4^+IL\text{-}4^-$ cells enrichment. Cytokines from antigen-stimulated T cells were released and then captured on the cell surface by using cytokine secretion assay cell enrichment and detection kits (#130-054-101, Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Biotinylated anti-IFN-γ or anti-IL-4 antibodies (Pharmingen) were added into the CD3+ CD4+ cell enrichment cocktail (StemCell Technologies), and the target cells were magnetically removed. Treated and control cell fractions were analyzed for IFN-γ and IL-4 transcripts to confirm depletion. In addition, flow cytometric detection of cytoplasmic cytokine expression was further used to confirm depletion.

Cytokine measurement

Mouse IL-4 and IFN-γ were measured by ELISA using commercially available reagents (Pharmingen and R&D Systems); sensitivities ranged from 10 to 50 pg/ml. Commercially available, recombinant murine cytokines served as standards in all assays (Genzyme, Cambridge, MA; Preprotech Inc., Rocky Hill, NJ; and R&D Systems).

Statistics

Analysis of variance with Tukey or Bonferoni error protection was used for intergroup comparisons. Values of $P \geq 0.05$ were considered to indicate lack of significance.

RESULTS

Draining lymph node CD4+ Th cells display novel profiles of chemokine receptor transcripts during in vivo-polarized type-1 (mycobacterial) and type-2 (schistosomal) immune responses

To assess Th chemokine receptors under in vivo polarizing conditions, we examined CD4+ T cells using well-described in vivo models of type-1 and type-2 polarized immune responses

induced by protein Ag of *M. bovis* bacteria or *S. mansoni* eggs, respectively [12]. In these models, mice are sensitized with a PPD of *M. bovis* or soluble Ag from *S. mansoni* eggs (SEA) followed by an intravenous challenge of agarose beads covalently coupled to the respective sensitizing Ag. The Ag-coated beads embolize to the lung where they induce type-1 and type-2 cytokine-dependent, inflammatory responses with correspondingly highly polarized Th1 and Th2 cytokine profiles in draining lymph nodes [13, 14]. In the present study, draining lymph nodes from the mediastinum were collected and subjected to multiple analyses 3 days after Ag-bead challenge. **Figure 1A** compares levels of IFN-γ and IL-4 in the cultured lymph node cells and clearly demonstrates the expected polarized Th1 and Th2 cytokine production in PPD (type-1) and SEA (type-2) models. Figure 1B shows a typical flow cytometric analysis of cytoplasmic cytokine expression among CD4+ T cells in the Ag-stimulated lymph nodes. Approximately 3–4% of CD4+ T cells expressed IFN-γ in the type-1 cultures with no significant IL-4-positive cells detected. In type-2 cultures, about 2% of CD4+ T cells expressed IL-4 with no significant IFN-γ-positive cells detected. These findings further confirmed distinct cytokine polarization in these models.

Next, CD4+ T cells purified from freshly harvested lymph nodes from naïve mice or those draining the bead-challenged lungs of sensitized mice and were subjected to chemokine receptor transcript analysis by real-time RT-PCR. As shown in Figure 1C, CD4+ T cell populations from type-1 (PPD) and type-2 (SEA) lymph nodes displayed detectable shifts in chemokine receptor transcript profiles as compared with naïve CD4+ T cells. As demonstrated for in vitro-generated Th1 cells, CXCR3 transcripts were strongly enhanced in the type-1 (PPD) response. The CCR3 transcript signals were too weak to define clear differences. Similarly, draining lymph node CD4+ T cells showed weak CCR5 transcript expression without evidence of polarization to the Th1 response as demonstrated for

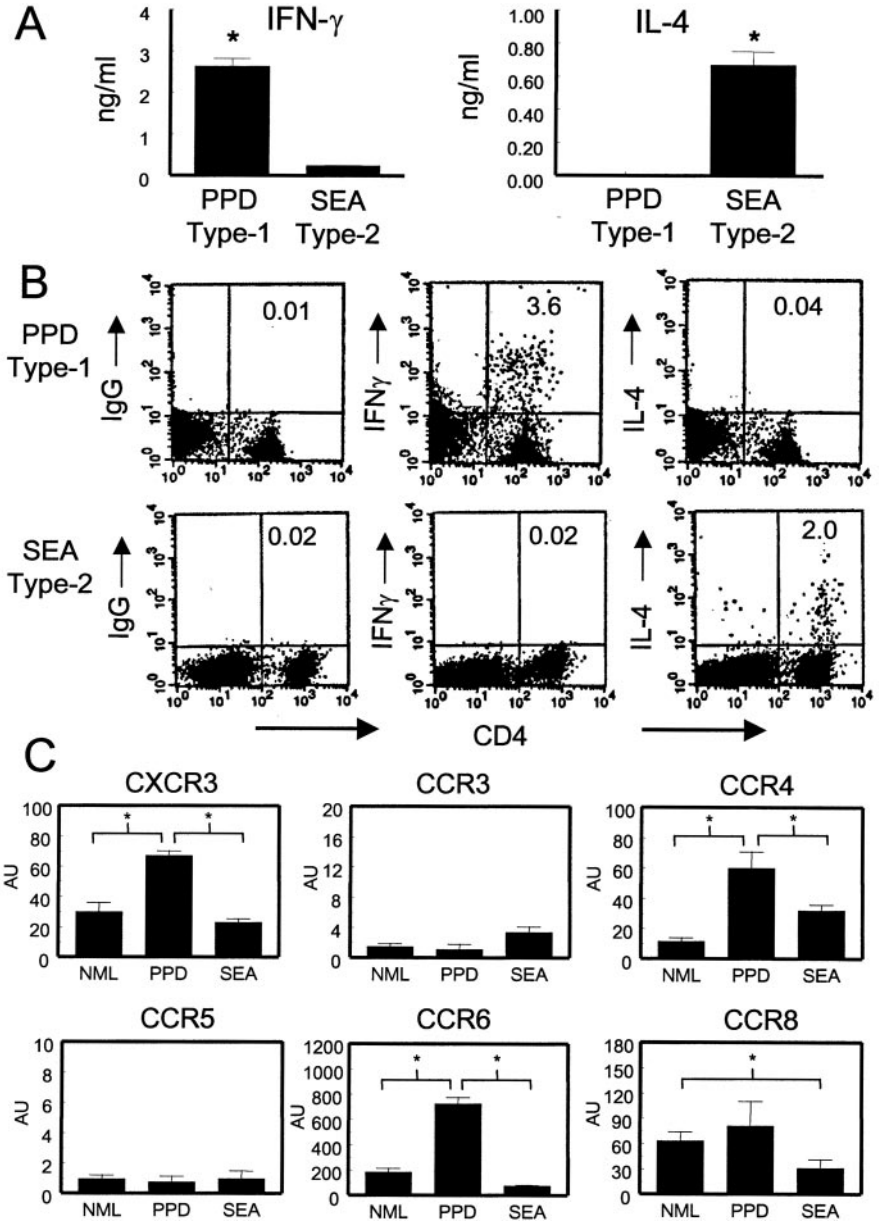

Fig. 1. Chemokine receptor transcript expression by draining lymph node CD4+ T cells from mice with type-1 (PPD) and type-2 (SEA) T cell-mediated granuloma formation. (A) IFN-γ and IL-4 production by Ag-stimulated, draining type-1 (PPD) and type-2 (SEA) lymph node cultures demonstrating polarized cytokine expression. (B) Flow cytometric analysis of cytoplasmic IFN-γ and IL-4 expression by type-1 (PPD) and type-2 (SEA) Ag-stimulated, draining lymph node CD4+ T cells. Numbers in upper-right quadrants indicate percentage of specific Ag reactive, CD4+ T cells expressing cytokine. Scattergrams are representative of three experiments. (C) Chemokine receptor transcript expression by freshly isolated lymph node CD4+ T cells from naïve mice (NML) and mice with type-1 (PPD) or type-2 (SEA) pulmonary granulomatous inflammation. Bars are mean AU ± SD derived from three separate experiments. *, $P < 0.05$.

in vitro-generated cells [15, 16]. It should also be noted that levels of CXCR2, CCR1, and CCR2 transcripts among CD4+ lymph node preparations were likewise consistently weak (data not shown).

A distinctly novel finding of this study was that the CCR6 message was strongly expressed by and biased to type-1 (PPD) CD4+ T cells (Fig. 1C). CCR4 was induced among type-1 (PPD) and type-2 (SEA) populations with a trend to greater expression among the former. Specifically, among type-1 CD4+ T cells, CCR4 transcripts were sixfold above, and type-2 CD4+ T cells were threefold above the naïve CD4+ T cells. Levels of CCR8 transcripts were comparable with naïve cells among type-1 preparations and were significantly reduced among type-2 CD4+ T cells. These findings regarding CCR4 and CCR8 transcript expression were unexpected, as artificially generated Th2 cells express these receptors [17, 18].

The above findings indicated induction of restricted and shared chemokine receptor transcripts among type-1 and type-2 CD4+ T cell populations. Type-1 populations showed induction of CXCR3, CCR4, and CCR6, whereas type-2 preparations showed enhancement of only CCR4 transcripts. As lymph node CD4+ T cells likely represent a mix of naïve and memory T cells as well as quiescent and activated cells, further subpopulation analysis was needed for better interpretation.

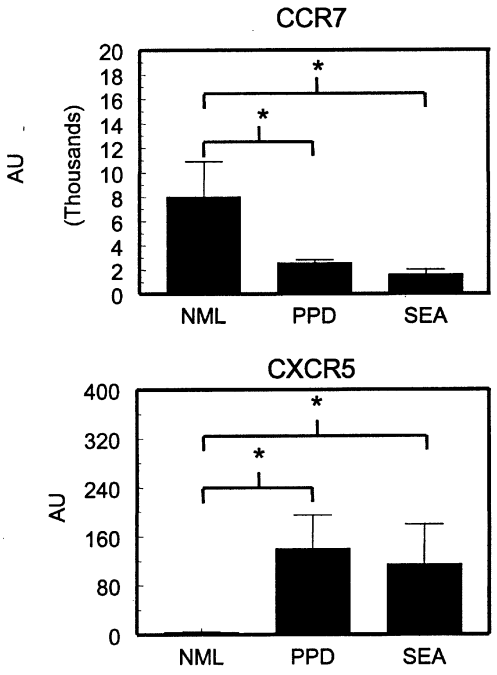

Fig. 2. CCR7 and CXCR5 transcript expression by draining lymph node CD4+ T cells from mice with type-1 (PPD) and type-2 (SEA) T cell-mediated granuloma formation. Transcripts expression was measured in freshly isolated lymph node CD4+ T cells from NML and mice with type-1 (PPD) or type-2 (SEA) pulmonary granulomatous inflammation. Bars are mean AU ± SD derived from three separate experiments. *, $P < 0.05$.

CD44+ memory T cells primarily express induced chemokine receptor transcripts among CD4+ T cells during type-1 (mycobacterial) and type-2 (schistosomal) immune responses

Type-1 (PPD) and type-2 (SEA) draining lymph node CD4+ T cells were next analyzed for CCR7 and CXCR5 transcripts, chemokine receptors reportedly respectively, associated with naïve and Ag-primed memory T cells [19, 20]. As shown in **Figure 2**, freshly isolated CD4+ T cells from PPD- and SEA-sensitized and -challenged mice displayed a significant decrease in CCR7 expression and increase in CXCR5 expression when compared to those from lymph nodes of age-matched NML mice. This result suggested a decrease in naïve T cells and expansion of Ag-primed, CD4+ memory T cells. Moreover, similar shifts from naïve to CXCR5+ memory populations occurred in type-1 and type-2 responses, an observation not previously reported.

We next analyzed chemokine receptor mRNA in type-1 (PPD) and type-2 (SEA) draining node CD4+ T cells depleted of CD44+ (memory) cells or CD25+ (recently activated) cells. The latter marker was used because activation alters chemokine receptor expression [8]. As shown in **Figure 3A**, CCR7 was enriched by three- to fourfold among type-1 and type-2 CD4+ T cells after depletion of CD44+ cells. Conversely, CXCR5, which is expressed by memory T cells, was largely eliminated by CD44+ cell depletion (Fig. 3B). This argued that the method was indeed depleting the memory CD44hi, CCR7- T cell population. Interestingly, depletion of CD25+ cells significantly augmented CXCR5 transcript levels, suggesting the presence of a CD25+ population with low or absent CXCR5 transcript expression, which was partially diluting the mRNA pool. Figure 3C demonstrates that the enhanced expression of CXCR3 in the type-1 response was also associated with CD25−, CD44+ memory T cells but not CD25+ cells. As before, CCR3 transcript expression remained weak, but depletion of CD44+ cells consistently augmented levels among type-2 preparations, indicating that CCR3 was expressed by a CD44lo population, and a similar trend was observed in type-1 preparations (Fig. 3D). The possibility of a novel CCR3+, naïve CD4+ population will require further investigation. CCR4 transcripts were also associated with memory T cells in type-1 and type-2 responses (Fig. 3E). CCR5 mRNA again remained low, and significant depletion-related effects were not demonstrable (Fig. 3F). As noted above, CCR6 transcripts were strongly biased to the type-1 response and were clearly associated with memory T cells. It is interesting that CCR8 transcripts showed reductions following CD25+ cell depletion as well as 80–90% reductions with depletion of CD44+ cells in both types of responses. This finding suggested the presence of a CD25+, CCR8+ T memory cell population. Taken together, these results indicated that CD44+ memory T cells were responsible for much of the induced chemokine receptor transcripts following adaptive sensitization to PPD and SEA, whereas the naïve CD44− population expressed mainly CCR7.

To summarize, in the type-1 (PPD) response, CD4+, CD44+ memory cells showed enhanced CXCR5, CXCR3, CCR4, and CCR6 mRNA, and in the type-2 (SEA) response, CD4+ T cells had a more limited repertoire, showing increases only of CXCR5 and CCR4 when compared with nonimmune lymph node CD4+ T cells. In addition, subpopulations of CD25+ (presumably activated), CD4+ T cells were detectable, which were CCR8+.

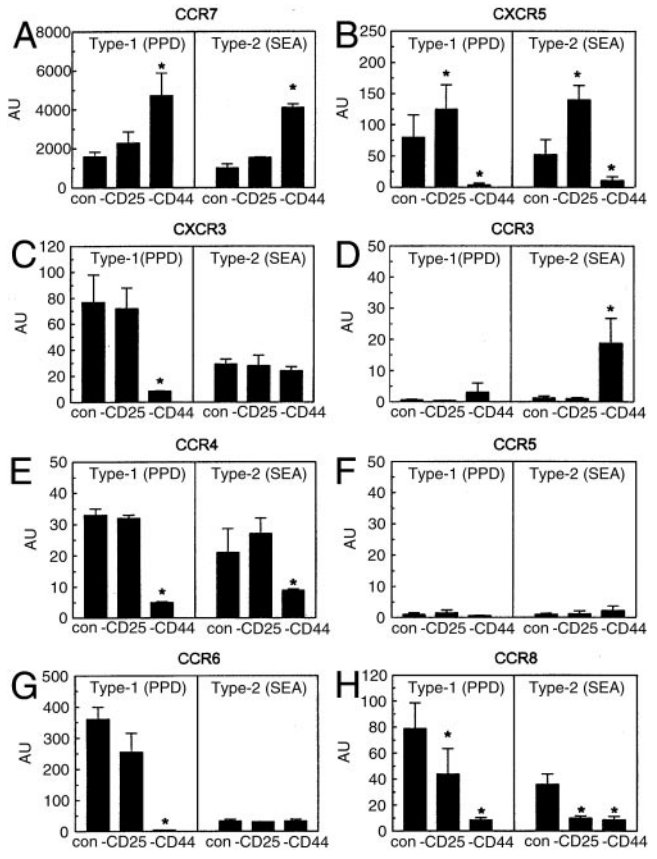

Fig. 3. Chemokine receptor transcript association with CD44+ memory and CD25+ activated CD4+ T cells from draining lymph node of mice with type-1 (PPD) and type-2 (SEA) T cell-mediated granuloma formation. Freshly isolated lymph node CD4+ T cells were subjected to chemokine transcript analysis following column depletion of CD25+ or CD44+ cells as described in Materials and Methods. Bars are mean AU ± SD derived from two separate experiments. *, $P < 0.05$ as compared with unfractionated control cells (CON).

Ex vivo antigen stimulation differentially regulates chemokine receptor transcript expression by CD4+ T cells

As T cell receptor (TCR)-mediated activation regulates chemokine receptor expression for up to 72 h of in vitro culture [8], we examined the effect of ex vivo culture and specific Ag-mediated activation on chemokine transcripts. To this end, chemokine receptor transcripts were analyzed in CD4+ T cells from draining lymph nodes of mice with type-1 (PPD) and type-2 (SEA) granulomatous responses that were freshly isolated or cultured for 48 h in the presence or absence of specific Ag. The findings revealed that culture and antigen stimulation had differing effects on chemokine receptor transcripts. As illustrated in **Figure 4A**, Ag stimulation further reduced levels of CCR7 transcripts in type-1 and type-2 cultures. Levels of CXCR5 mRNA did not appear to be stable to culture since among type-1 CD4+ T cells, culture with or without Ag also caused about a 90% reduction of CXCR5 (Fig. 4B). A similar trend occurred in type-2 cells, but this did not reach significance in unstimulated cultures; however, Ag stimulation did cause an 80% reduction in CXCR5 mRNA expression. Activation-mediated down-regulation of CXCR5 expression would be commensurate with the observed enrichment of CXCR5 transcript levels after depletion of CD25+ cells (Fig. 3B).

Ag stimulation also affected other chemokine transcripts. As shown in Figure 4C, CXCR3 receptor transcripts levels were stable to culture without Ag but increased twofold among type-1 (PPD) CD4+ T cells when cultured with Ag. Type-2 cells displayed lower expression than naïve cells after Ag stimulation, possibly indicating negative regulation. CCR3 transcripts among type-2 (SEA) CD4+ T cells were low with no consistent changes demonstrable (Fig. 4D). CCR4 transcripts were stable to culture, and Ag stimulation enhanced expression among type-1 and type-2 CD4+ T cells by three- and twofold, respectively. (Fig. 4E). As above, CCR5 transcript levels remained low with no clear changes (Fig. 4F). The biased expression of CCR6 transcripts by type-1 (PPD) CD4+ T cells was stable to culture without Ag but decreased by 90% following Ag stimulation (Fig. 4G). Interestingly, the low CCR6 signal in the type-2 response (only 10–15% of that in the type-1 response) also showed a further decline with Ag stimulation. Finally, Ag stimulation strongly enhanced CCR8 transcripts above naïve levels and by three- and fourfold above freshly isolated type-1 and type-2 CD4+ T cells, respectively.

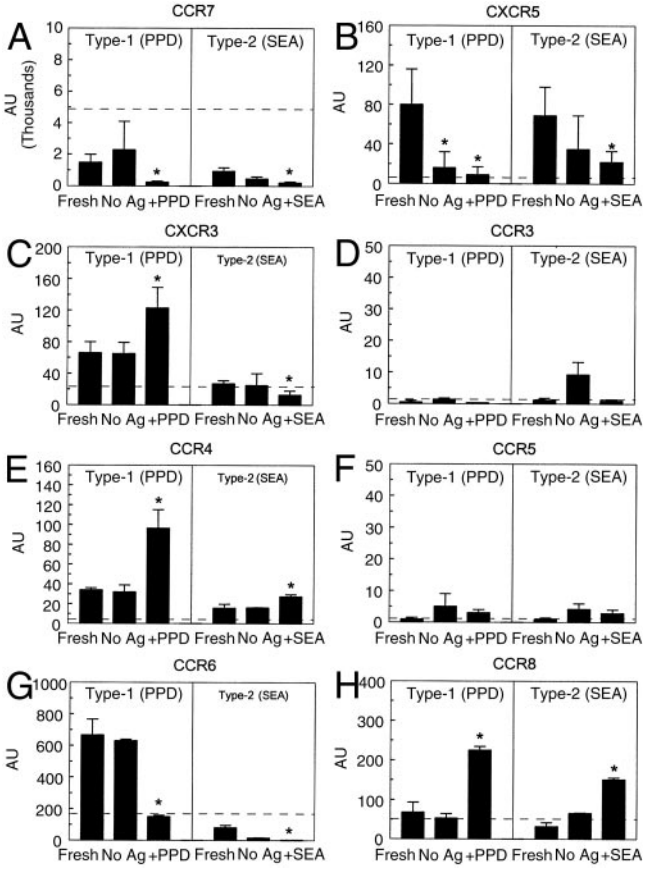

Fig. 4. Effect of specific antigen stimulation on chemokine receptor transcript expression by draining lymph node CD4+ T cells from mice with type-1 (PPD) and type-2 (SEA) T cell-mediated granuloma formation. Transcript levels were analyzed in isolated CD4+ T cells by real-time RT-PCR after culture for 48 h in the absence or presence of 5 μg/ml PPD or SEA. Bars are mean AU ± SD and are derived from two separate experiments. *, $P < 0.05$ as compared with freshly isolated CD4+ T cells. Dashed lines indicate level of expression by naïve CD4+ T cells.

This stimulation-mediated enhancement was consistent with our observation that CCR8 expression was associated with cells bearing the CD25 activation marker.

Although these observations may not reflect changes in Ag-specific CD4+ T cell subpopulations, they indicate that chemokine receptor transcript levels are independently regulated by Ag stimulation. The receptors could be divided into two main groups: those enhanced by Ag and those down-regulated by Ag stimulation. Among type-1 cells, CCR6, an apparent marker of type-1 memory cells, was profoundly down-regulated following Ag stimulation, and CXCR3, CCR4, and CCR8 were enhanced. Among the type-2 cells, CCR4 and CCR8 transcripts were also enhanced by Ag stimulation, and CCR3 showed no clear changes. Finally, CXCR5 transcripts were decreased in type-1 and type-2 cultures following culture and Ag stimulation.

Induced chemokine receptor transcripts are not limited to Th1 (IFN-γ) or Th2 (IL-4) cytokine-producing T cells

As PPD and SEA Ag-specific, cytokine-producing cells represent only 2–5% of the type-1 and type-2 CD4+ T cell populations, it was surprising that distinct patterns of induced chemokine receptor expression could be so easily detected on whole population analysis. To determine the contribution of IFN-γ- and IL-4-producing T cells to the observed levels of chemokine receptor transcripts, we analyzed populations after depletion of Ag-elicited, cytokine-producing cells. Cytokine-producing cells were eliminated after 24 h of Ag stimulation using commercially available depletion kits. **Figure 5** shows the levels of IFN-γ and IL-4 transcripts in control and depleted preparations. Control populations again showed distinct polarization by transcript analysis as they did by flow cytometric and ELISA analyses (Fig. 1). Upon depletion, IFN-γ transcripts were reduced by greater than 60% among type-1 and IL-4 transcripts, by greater than 80% among type-2 CD4+ cells, indicating that the method was reasonably effective in reducing target, cytokine-producing cells. The cytokine transcripts were also shown to be limited to the CD44+ fraction (Fig. 5). **Figure 6** shows the normalized chemokine receptor analysis of these preparations expressed as percent change from the control. In addition, parallel analyses of memory cell (CD44+)-depleted, Ag-stimulated cultures are shown. Of the analyzed receptors, CCR4 showed the greatest association with the IFN-γ- or IL-4-producing cells, and to a lesser extent, CXCR3 showed some association with IFN-γ+ cells. Changes in CCR4 transcripts were greater in the treated type-2 populations, but this may have been because of the better depletion efficiency in these preparations. In memory cell-depleted cultures, there was again enrichment of naïve T cell-associated CCR7 and virtually complete elimination of CCR4, CCR8, and CXCR3 transcripts. As we observed for fresh, unstimulated CD4+ T cell preparations, CCR3 segregated to the nonmemory (CD44lo) cell fraction. As transcripts for CCR6 were down-regulated on CD44+ cells following stimulation, no segregation was detected (data not shown).

These results indicated that the bulk of the Ag-induced, chemokine receptor transcript expression was among populations not secreting IFN-γ and IL-4, but CCR4 did show a significant association with cytokine-producing Th1 and Th2 cells. Moreover, this induction was restricted to the memory T cell fraction.

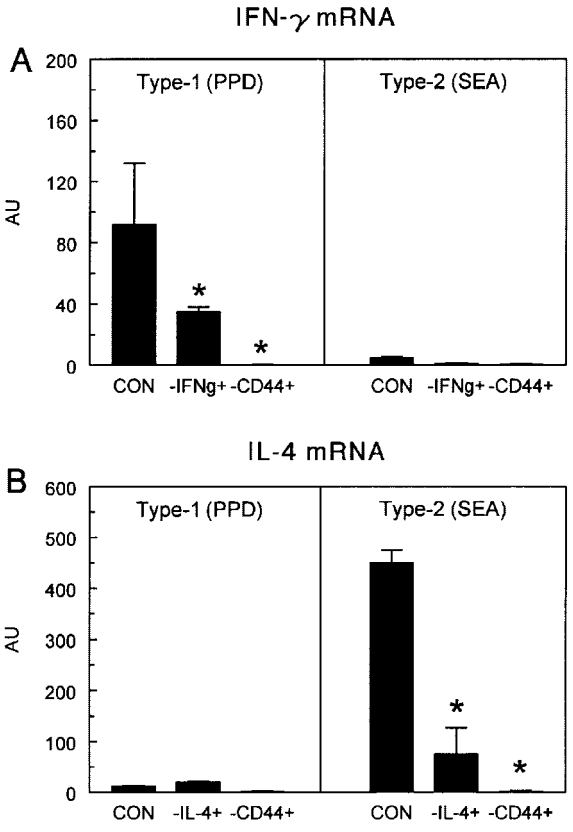

Fig. 5. Cytokine transcript expression following targeted depletion of IFN-γ-, IL-4-, and CD44-positive T cells from mice with type-1 (PPD) and type-2 (SEA) T cell-mediated granuloma formation. Type-1 or type-2 draining lymph node cells were, respectively, stimulated for 24 h with PPD or SEA Ag, and then specific depletions were performed as described in Materials and Methods. (A) IFN-γ mRNA in control and depleted fractions. (B) IL-4 mRNA in control and depleted fractions. Bars are mean AU ± SD and derived from five mice. *, $P < 0.05$ as compared with control preparations cultured and treated in parallel.

DISCUSSION

Chemokine receptors are likely important determinants of T cell functional behavior in adaptive immune responses, and in vitro analyses have provided important insights into the dynamic nature of T cell chemokine receptor expression [3, 4, 7, 21–23]. These findings have led to a paradigm that purports a flexible program of chemokine receptor expression that changes as T cells mature from early thymic stages to effector/helper cells. In the periphery, as naïve CD4+ T cells mature to Th1 and Th2 cells, they are considered to acquire distinct patterns of chemokine receptor expression. This paradigm is being tested in human disease conditions and animal models.

In the present study, we examined CD4+ T cell chemokine receptor transcript expression using in vivo models of type-1 and type-2 T cell-mediated inflammation elicited by Ag of *M. bovis* bacteria (PPD) and *S. mansoni* eggs (SEA), respectively.

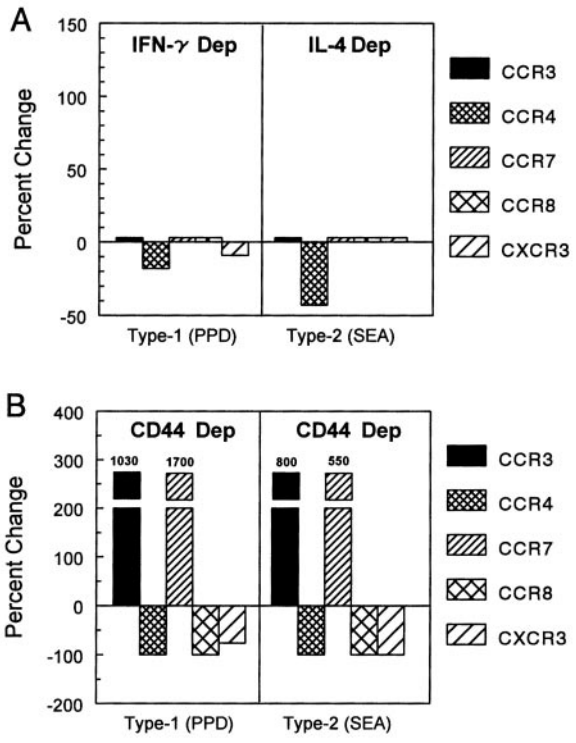

Fig. 6. Net change in chemokine receptor transcript expression following targeted depletion (Dep) of IFN-γ-, IL-4-, and CD44-positive T cells from mice with type-1 (PPD) and type-2 (SEA) T cell-mediated granuloma formation. Cultures were prepared and treated as in Figure 5 and then analyzed for chemokine receptor transcripts. (A) Effect of IFN-γ+ and IL-4+ cell depletion in respective type-1 and type-2 CD4+ T cell populations. (B) Effect of CD44+ cell depletion in type-1 and type-2 populations. Bars are mean percent change from control preparations cultured and treated in parallel. Normalized data are presented as a result of different signal intensity among receptor types. A negative 100% change is maximum elimination of signal. Positive changes indicate enrichment over control.

These models are well characterized and involve highly polarized Th1 and Th2 cytokine participation [11–13]. This study represents one of the most extensive analyses of T cell chemokine receptor transcript expression performed using in vivo models of polarized Th cell responses. We used real-time RT-PCR for transcript analysis, which provided a powerful tool to detect changes in cell subpopulations that could not be studied directly as a result of limited cell numbers and lack of reliable, specific, anti-mouse, chemokine receptor Ab. The method is of course limited in that transcript levels may not reflect levels of protein expression and biologic activity; nevertheless, transcript analysis has, to date, been the dominant parameter used for chemokine receptor studies in humans and animals. It is likely that transcript levels reflect at least some degree of protein expression, as in our initial verification, studies of artificially polarized mouse Th cell chemokine receptor transcripts correlated well with chemotactic responses and matched expression patterns demonstrated by others (data not shown).

Remarkably, our analysis detected patterns of biased chemokine receptor transcript expression among polyclonal CD4+ T cell populations isolated from lymph node-draining lungs with ongoing type-1 (PPD) and type-2 (SEA) granulomatous inflammation. With respect to CXCR3, the biased expression pattern matched that shown for artificially generated Th1 and Th2 cells [1]. Like artificially generated Th1 cells, type-1 PPD, Ag-elicited CD4+ T cells were associated with dominant expression of CXCR3, whereas type-2 T cells showed no induction over naïve populations. Thus, we provide further support for CXCR3 as a marker of type-1 polarization at least within the lymph node environment. With regard to the other receptor transcripts, we observed novel expression patterns that differed from those that would be predicted from artificially polarized cells. These findings are discussed below.

Unlike reports for in vitro-generated Th2 clones [17, 24], CCR3 and CCR4 did not polarize to type-2 CD4+ populations. Selective CCR3 expression by Th2 cells has been subject to controversy and has not been consistently detected in type-2-related, human disease conditions [5, 25]. We noted weak baseline signal in our populations, but to our surprise subsequent studies consistently showed enhanced expression in enriched CD44lo fractions. It is unknown if this reflects expression by naïve cells or the presence of a novel, specialized CD4+, CD44lo, CCR3+ population. Further studies will be required to determine the significance of this finding. CCR4 transcripts were induced in both responses and tended to be greater in the type-1 than in type-2 populations. Enhanced CCR4 has been shown among T cells in Th2-related human disease, but this was not directly compared with a Th1-related disease [5, 25]. Our results appear to agree with a recent study of human blood T cells demonstrating CCR4 expression by memory Th1 and Th2 cells and implicating CCR4 as a major trafficking receptor for Th cells in general [10].

Also contrasting with in vitro Th cell patterns was CCR5, which is associated with Th1 clones [2, 15], but provided only a weak signal and no distinct polarization among in vivo type-1 lymph node CD4+ T cell populations. Using flow cytometric analysis with available antimurine CCR5 Ab, we have observed weak expression and lack of polarization among freshly isolated blood and lymph node CD4+ T cells in our models; however, greater expression was detected among T cells isolated from PPD- but not SEA-bead granulomas (unpublished results). In accord with our findings, Iwasaki et al. [26] demonstrated that mouse T cells lack CCR5 transcript expression until exposed to IL-12. Thus, Th cells may acquire CCR5 expression upon contact with sites of local IL-12 production as with the type-1 (PPD) granuloma [27].

Our analysis of CCR6 transcript expression provided the novel observation that it was strongly biased to type-1, PPD-elicited CD4+ T cell preparations. Like the other induced receptors, it was associated with CD44+ memory T cells. The CCR6 receptor is a unique β-chemokine receptor that is expressed mainly in lymphoid tissues [28, 29]. Although memory CD4+ T cells are known to express CCR6 [30, 31], selective expression by Th1 subsets has not been shown. We also demonstrated Ag-mediated regulation of this receptor, which is discussed in more detail below.

Another surprising finding was that type-2 CD4+ T cells displayed reduced transcripts for CCR8, a receptor usually associated with in vitro Th2 cells [17, 18, 32]. Not only did we fail to observe CCR8 polarization among type-2 populations, baseline transcript levels were below that of naïve CD4+ T cell

preparations. However, CCR8 transcripts were associated with CD44+ memory cells and appeared to be expressed at least to some extent by CD25+ T cells. In fact, among the chemokine receptors we examined, CCR8 demonstrated the greatest positive association with CD4+ CD25+ T cells. These cells appeared to be Th memory cells, as they were associated with the CD44+ component, unlike the recently described CD4+ CD25+ "professional suppressor" cells that lack memory phenotype [33]. These findings invoke the possibility that CCR8 displays stage-specific or activation-related expression.

Another novel finding was our demonstration that sensitization caused comparable inductions of CXCR5 and decreases in CCR7 expression in type-1 (PPD) and type-2 (SEA) T cells. This was fully consistent with a shift to T memory cells, and indeed the CXCR5-expressing population correlated positively to CD44+ and negatively with the CD25 activation marker upon fractionation analysis. That these populations were so easily detected by transcript analysis suggests that our sensitization protocol caused a considerable expansion of the memory cell population.

Our findings regarding the effect of Ag activation on chemokine receptor transcript expression by type-1 (PPD) and type-2 (SEA) CD4+ T cell preparations agrees in most respects with transcript analyses of Sallusto et al. [8] using TCR cross-linked human Th cells. Like that study, we observed down-regulated CCR6 and enhanced CCR4 and CCR8 transcripts following stimulation, but unlike that study, we observed abrogation of CXCR5. We also observed that removing T cells from the lymph node environment and culturing them ex vivo in the absence of a stimulus tended to decrease CXCR5 receptor transcript expression among type-1 and type-2 CD4+ T cells. This finding suggested that certain receptors may be unstable in the ex vivo environment. In addition, CXCR5 showed an inverse correlation with CD25 (IL-2 receptor) expression and was further reduced with Ag stimulation expression. Our observation is consistent with activation-mediated down-regulation of CXCR5, as demonstrated in human tonsil T cells [34, 35]. The effect could be mediated by IL-2, which down-regulates CXCR5 expression [8]. CXCR5 is thought to confer B cell follicle homing capacity and has been associated with "follicular B helper T cells" [34, 35]. Thus, loss of CXCR5 expression during culture may impair the capacity of Th cells to home to B cell zones [20, 36], a finding with potential implications for T cell homing studies.

As for other receptor transcripts, CXCR3, CCR4, and CCR8 displayed two- to fourfold enhancements among the type-1 CD4+ T cell populations, presumably providing potential responsiveness to several different chemokines. Enhanced expression of chemokine receptors by Ag stimulation is thought to promote post-activation functions, such as emigration through interstitial spaces to sites of inflammation or regulation of secretory events [7]. Unlike the type-1, type-2 CD4+ T cell preparations had a more limited repertoire, with CCR8 being enhanced by fourfold and CCR4 by only twofold. Indeed, CCR8 emerged as a relatively dominant signal among type-2 preparations after stimulation. This finding has particular relevance to our recent report showing deficits of the Th2 but not the Th1 response in CCR8 knockout mice [37]. Those findings were initially thought to reflect biased expression of CCR8 by Th2 cells, but as discussed below, our current study would indicate that memory CD4+ T cells other than those producing IL-4 can express CCR8. Consequently, effects of CCR8 knockout on other effector or regulatory populations must be considered.

Finally, the profound abrogation of CCR6 mRNA among type-1 T cells following stimulation would argue that it serves a preactivation function, such as attracting Th1 cells to lymphoid tissues or sites of likely Ag encounter. CCR6 is strongly expressed by dendritic cells and B cells [29–31, 38–40], and the only known ligand for CCR6 is CCL20 (liver and activation-regulated chemokine/Exodus/macrophage inflammatory protein-3α), which appears to be expressed primarily by epithelial cells, including those of liver, intestine, pancreas, and skin [29, 41–43]. Thus, CCR6 has been implicated in immune responses involving epithelial surfaces and the epithelial localization of dendritic cells [39, 44]. Recently, Cook et al. [45] demonstrated impairment of mucosal immunity in CCR6 knockout mice, and in an independent study, Varona et al. [46] noted abrogated T cell-mediated, delayed-type responses to cutaneous challenge with allogeneic cells following adoptive sensitization with T cells from CCR6 knockout mice. Relevant to these studies, Fitzhugh et al. [47] demonstrated that CCR6 promotes adherence to dermal endothelium, and Homey et al. [48] showed that CCR6 is highly expressed by skin-homing memory T cells in psoriatic lesions. These reports, taken together with our present findings, would support the notion that CCR6 promotes localization of memory Th1 cells to epithelial-rich organs or epithelial-associated lymphoid tissues.

Our analysis of Ag-stimulated type-1 and type-2 CD4+ T cell populations, respectively, depleted of IFN-γ- and IL-4-producing cells was particularly revealing. Of the receptor transcripts enhanced by Ag stimulation, CCR4 showed the greatest association with these cytokine-producing populations. As these populations represent only 2–5% of the overall CD4+ T cell populations, our ability to detect this association by transcript analysis would indicate strong expression in this fraction. The other receptor transcripts for CXCR3 and CCR8 remained in large part with the remaining memory T cell fraction, indicating expression by populations other than IFN-γ- and IL-4-positive cells. Thus, CCR4 appears to be an important effector cell-expressed chemokine receptor, and it should be noted that an important ligand of CCR4, CCL17—i.e., thymus activation-related chemokine—is induced locally in lungs with type-1 (PPD) and type-2 (SEA) granuloma formation and has been shown to support CCR4+ T cell recruitment to *Propionibacterium acnes* granulomas [49, 50]. The relatively minor association of CXCR3 with an IFN-γ-producing fraction was surprising, but several possible explanations can be offered. First, we could only deplete Ag-specific cells with the greatest IFN-γ-producing capacity, and we suspect there may be other Th1 clones remaining with weaker IFN-γ-producing capacity that may express CXCR3; this would explain our inabilty to deplete no more than 60% of the IFN-γ transcripts. Second, and as recently demonstrated by Kim et al. [51], Tnp (IFN-γ IL-4) memory cells can express CXCR3. Hence, a significant component of noncytokine-producing CD4+ T cells may express CXCR3. Finally, it is unclear to what extent the Th1 cytokine environment may induce CXCR3

expression on bystander T cell clones that are unreactive to PPD antigen stimulation.

In conclusion, our study supports the notion of flexible programs of T cell chemokine receptor expression and shows that distinct profiles of chemokine receptor transcripts can be detected among draining lymph node CD4+ T cell populations derived from in vivo models of polarized type-1 (PPD) and type-2 (SEA) immune responses. The findings emphasize the complexity of T cell chemokine receptor expression dynamics in the in vivo environment and point to new avenues of investigation. They show that strongly polarizing Ag can influence net chemokine receptor expression in broad populations of memory CD4+ T cells. Several findings were obtained and are summarized as follows: CXCR3 and CCR6 were highly biased to type-1 memory CD4+ T cells; post-sensitization-induced chemokine transcripts were distinctly associated with CD44+ memory T cells; CCR4 and CCR8 were enhanced following Ag stimulation and were expressed by type-1 and type-2 CD4+ T memory populations; CCR3 transcripts were associated with nonmemory CD4+ T cell fractions; and CCR4 transcripts showed the strongest association with IFN-γ- and IL-4-producing cells. It should be emphasized that our findings describe events within reactive lymph nodes, but additional modifications of chemokine receptors may occur in other compartments such as blood and inflammatory sites. For example, Kim et al. [51] demonstrated CCR7 expression by circulating blood memory Th cells, possibly allowing for lymphoid tissue recirculation. Further understanding of chemokine receptor dynamics will require more detailed subpopulation and compartment analyses as well as the use of murine chemokine receptor-specific Ab as they become available.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health-National Institute of Allergy and Infectious Disease (NIH-NIAID) grant AI43460 and in part by the Department of Veterans Affairs. Schistosome life stages or materials for this work were supplied through NIH-NIAID contract NO1-AI-55270.

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