Cutting Edge: The Chemokine Receptor CCR8 Is Preferentially Expressed in Th2 But Not Th1 Cells[1,2]

Alessandra Zingoni,[3]* Hortensia Soto,[3†] Joseph A. Hedrick,[†] Antonella Stoppacciaro,[‡] Clelia T. Storlazzi,[§] Francesco Sinigaglia,[¶] Daniele D'Ambrosio,[¶] Anne O'Garra,[†] Douglas Robinson,[†] Mariano Rocchi,[§] Angela Santoni,*[†] Albert Zlotnik,[3,4†] and Monica Napolitano[3*‖]

In this paper we report on the cloning and characterization of mouse CCR8. Like its human homologue, it is predominantly expressed in the thymus. In the periphery, murine CCR8 mRNA was found most abundantly expressed in activated Th2-polarized cells and in NK1.1$^+$CD4$^+$ T cells. Human CCR8 is also preferentially expressed in human Th2-polarized cells and clones. This pattern of expression suggests that CCR8 is part of a Th2-specific gene expression program. The CCR8 ligands I-309 and its mouse homologue T cell activation gene 3 (TCA-3) are potent chemoattractants for Th2-polarized cells. Taken together, these observations strongly suggest that CCR8 plays a role in the control of Th2 responses, and may represent a potential target for treatment of allergic diseases. ***The Journal of Immunology,*** **1998, 161: 547–551.**

Chemokines are a superfamily of proteins involved in leukocyte recruitment and activation that have been divided into four classes, CC, CXC, C, and CX3C (1, 2). The biological effects of chemokines on target cells are mediated by interaction with 7-transmembrane G-protein-coupled receptors. To date several CC chemokine receptors have been cloned and characterized (3, 4).

We have previously reported the molecular cloning of human CCR8, a T cell specific G-protein-coupled, 7-transmembrane receptor. This human receptor, previously known as TER1 (5), ChemR1 (6), or CKR-L1 (7), and now as CCR8, is functionally activated by the CC chemokine I-309 (8, 9) whose mouse homologue is T cell activation gene 3 (TCA-3)[5]. Human CCR8 is expressed only in lymphoid organs and in particular in the thymus (5, 7).

Th lymphocytes have been functionally separated into type 1 (Th1) and type 2 (Th2) subsets based on their ability produce discrete sets of cytokines (10). Th1 subsets produce IL-2, IFN-γ, TNF-α, and lymphotoxin and are believed to participate in cell-mediated immunity. The Th2 subset produces IL-4, IL-5, IL-6, IL-10, and IL-13 and have been associated with allergy-related phenomena and favor humoral responses.

In this study we report the cloning of the murine CCR8 receptor (mCCR8). mCCR8 is also expressed mainly in the thymus. In the periphery, mCCR8 mRNA was found in significant amounts only in activated Th2 T cells. These cells respond in chemotaxis assays to known CCR8 ligands. These observations strongly suggest that mCCR8 is associated with Th2 responses and may represent a potential therapeutic target for intervention during allergic diseases.

Materials and Methods

Cloning and sequencing

The open reading frame (ORF) of the human CCR8 gene was used as probe to screen the murine 129/SV genomic library in the l/fix vector (Stratagene, La Jolla, CA); phages were plated and hybridized with the labeled human CCR8 cDNA, and positive genomic phage clones were isolated, subcloned, and sequenced as previously described (5).

*Laboratory of Pathophysiology, Regina Elena Cancer Institute, Rome, Italy; †Department of Immunobiology, DNAX Research Institute, Palo Alto, CA 94304; ‡Department of Experimental Medicine and Pathology, University of Rome, "La Sapienza", Rome, Italy; §Department of Genetics, University of Bari, Bari, Italy; ‖Laboratory of Vascular Pathology, Istituto Dermopatico dell'Immacolata, Rome, Italy; and ¶Roche Institute, Milan, Italy

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[1] Database deposition: The nucleotide sequence of mCCR8 has been deposited in GenBank under accession number AF001277.

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[3] These authors contributed equally to this work.

[4] Address correspondence and reprint requests to Dr. Albert Zlotnik, DNAX Research Institute, 901 California Ave., Palo Alto, CA 94304. E-mail address: zlotnik@dnax.org

[5] Abbreviations used in this paper: TCA-3, T cell activation gene 3; m, murine; ORF, open reading frame; HPRT, hypoxanthine phosphoribosyltransferase; MIP-1α, macrophage inflammatory protein-1α.

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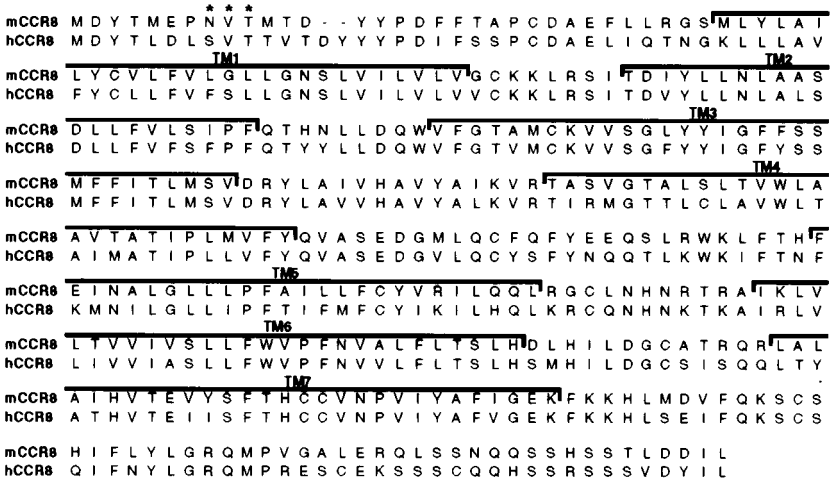

FIGURE 1. Amino acid alignment between the human and the murine CCR8 receptor. Putative transmembrane regions (TM1-7) are indicated. Identical amino acids are boxed. The asterisk represents an N-glycosylation site in the mCCR8 sequence.

Analysis of mCCR8 mRNA expression by RT-PCR and Northern blot

RNA from purified cells were prepared with RNeasy total RNA kit (Qiagen, Chatsworth, CA), following the manufacturer's instructions. mCCR8 expression was analyzed by RT-PCR using standard methods and ^{32}P-labeled mCCR8 and hypoxanthine phosphoribosyltransferase (HPRT) probes.

Poly(A)$^+$ RNA was extracted from cell lines using the FAST/TRACK method (Invitrogen, San Diego, CA) and from homogenized frozen tissues, from 4-wk-old BALB/c mice, using TRIZOL (Life Technologies, Gaithersburg, MD) followed by oligotex(dT) particles (Qiagen). Five micrograms of poly(A)$^+$ RNA was subjected to Northern blot analysis as described (5).

Analysis of mCCR8 mRNA expression by Southern blot of cDNA libraries

For Southern blot analysis of cDNA libraries, 5 μg of excised cDNA was analyzed from each of various cells and tissue cDNA libraries constructed at DNAX. A probe corresponding to the coding region of mCCR8 was nonisotopically labeled using the DIG high-prime kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions, and hybridization was conducted under high stringency (0.2× SSC at 65°C). The blot was then developed using chemiluminescence.

Separation of mouse T cell subsets

Adult thymocyte subsets were separated as described (11). Briefly, single cell suspensions of thymocytes were prepared from 6-wk-old BALB/c mice and stained with the following mAbs: anti-CD4 TriColor (Caltag, South San Francisco, CA), anti-CD8α (LyT2) (53-6.7) phycoerythrin and anti-CD3ϵ FITC (PharMingen, San Diego CA). Single positive cells were gated and then sorted for expression of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ as well as double positive CD4$^+$CD8$^+$ cells. All sorts yielded a purity of $>$ 99% upon reanalysis.

Mouse polarized Th1/Th2 cells

Polarized Th1 and Th2 cells were derived from naive CD4$^+$ T cells from DO-11.10 TCR transgenic mice with a TCR specific for the OVA peptide (OVA 323-339), as previously described (12). Their successful polarization was confirmed by analyzing their cytokine profile before use in other assays (data not shown). RNA was extracted from cell pellets using Qiagen RNeasy midi kits, following the manufacturers' directions. Cell groups to be used for chemotaxis assays were stimulated in vitro for 5 h before use in the chemotaxis assays.

Human Th1 and Th2 lines

Human neonatal leukocytes were purified from freshly collected, heparinized cord blood and Th cell lines generated as described (13). Cells were washed and restimulated with 50 ng/ml PMA (Sigma, St. Louis, MO) and 1 mg/ml ionomycin (Sigma) for 4 h. Brefeldin (10 mg/ml) was added for the last 2 h of culture. Cells were then fixed with 4% paraformaldeyde, permeabilized with saponin, and stained with FITC-labeled anti-IFN-γ (PharMingen), phycoerthrin-labeled anti-IL-4 (PharMingen), and Quantum red-labeled anti-CD4 (Sigma) Abs. Samples were analyzed by FACScan (Becton Dickinson).

Human Th1 and Th2 clones

The human Th1 and Th2 clones used here include the human Th1 clone ET 3.22 (specific for the hepatitis δ antigen) and the Th2 clone E 4.1 (specific for Lo1 p1 allergen) that have been described previously (14).

Chemotaxis assays

The chemotactic activity of highly polarized, mouse Th1/Th2 cells was assessed by microchemotaxis as described (1). The chemotactic index was calculated as the number of cells migrating in test well/number cells migrating in control well. Medium only (DMEM, no serum) was used as background control. Chemokinesis controls were included and were negative in all cases.

Ca^{2+} flux assay

Cells were washed and loaded with 2 mM Fluo-3AM for 30 min at 37°C (Molecular Probes, Eugene OR). Cells were washed and stained with quantum-red conjugated anti-CD4 Abs (Sigma). Cells were then analyzed in a FACStar^{Plus} (Becton Dickinson). Flow cytometric analysis was gated only to the CD4$^+$ cells monitoring emissions at 525 and 613 nm.

Results and Discussion

A mouse 129/SV genomic library was screened using the ORF of human TER1/CCR8 as probe (5). Seven positive phage genomic clones corresponded to the mouse homologue of the CCR8 gene. Sequence analysis revealed an ORF of 1059 nucleotides, predicting a 353-amino acid protein (Fig. 1). PCR was used to amplify cDNAs from the thymus. This cDNA does not have introns, a feature of most chemokine receptor genes (3), and is identical to the genomic sequence. The deduced amino acid sequence shows 71% homology to human CCR8 and 39% to 45% homology to murine CCR1, CCR3, CCR4, CCR5 and D6 (15). Alignment between the human and mouse CCR8 amino acid sequences is shown in Figure 1.

Restriction enzyme and Southern blot analyses of murine genomic DNA indicated a simple hybridization pattern, suggesting that CCR8 is present as a single copy in the genome (data not shown). By using fluorescent in situ hybridization (FISH), the mCCR8 gene was mapped to the telomeric f4 region of chromosome 9 in a region syntenic with human chromosome 3p21-24 where human CCR8 and other CC chemokine receptor genes are located (16).

Northern blot analysis using poly(A)$^+$ RNA prepared from several organs of a BALB/c mouse (Fig. 2*A*) indicated that mCCR8 is

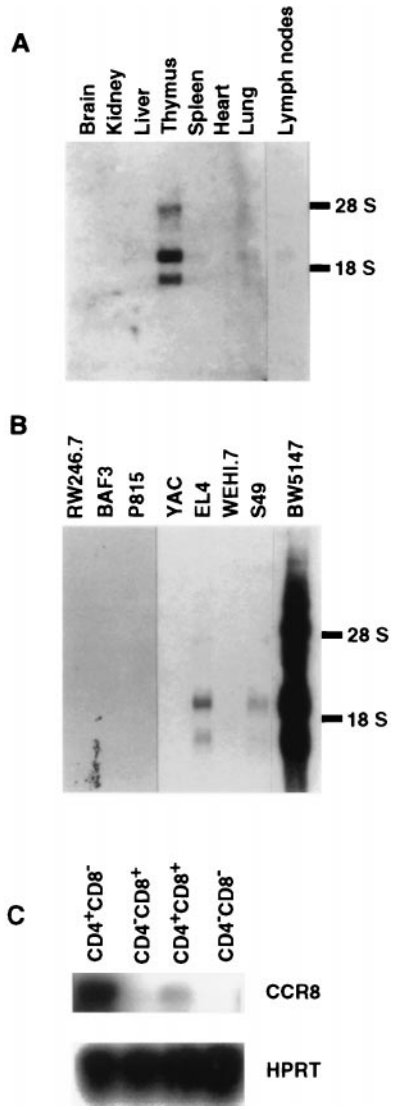

FIGURE 2. Northern blot analysis of mCCR8 mRNA in different mouse tissues and cell lines. *A,* Northern blot analysis of mCCR8 in various organs. *B,* Northern blot analysis of mCCR8 with mRNA from cell lines as indicated. *C,* Southern blot analysis of RT-PCR products from thymocyte subpopulations.

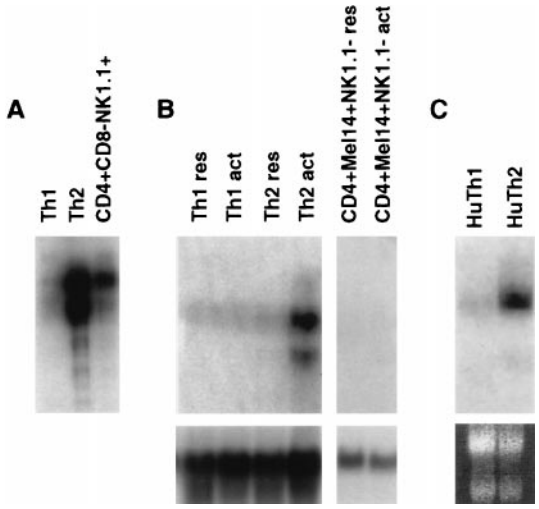

FIGURE 3. Expression of CCR8 in T cell subsets. *A,* Southern blot analysis of mCCR8 on activated mouse Th1 and Th2 polarized cell and in activated mouse $CD4^+NK1.1^+$ T cell cDNA libraries. *B,* Northern blot analysis of mCCR8 expression in mRNA from 3-wk polarized resting or activated polarized Th1 or Th2 cells. Also, expression of mCCR8 mRNA in $CD4^+Mel14^+NK1.1^-$ resting or activated mouse spleen T cells. *C,* Expression of human CCR8 in human activated polarized Th1 or Th2 cells. The *lower panels* of *B* and *C* show α-actin hybridization and ethidium staining, respectively.

highly expressed in the thymus. Three mRNA species of about 4, 3, and 2 kb were identified for mCCR8, suggesting the existence of different transcription initiation and/or polyadenylation sites. Goya et al. (17) have recently reported similar observations.

We analyzed the pattern of mCCR8 expression in a number of hematopoietic tumor cell lines and found mCCR8 mRNA only in some thymomas. In particular, BW5147 exhibited high levels of mCCR8 message (Fig. 2*B*). Interestingly, I-309 has been reported to prevent apoptosis in BW5147 (18). This finding suggests that the anti-apoptotic activity of I-309 may be mediated through mCCR8.

The distribution of mCCR8 among different thymic subpopulations was monitored by RT-PCR followed by Southern blot analysis with mCCR8 or HPRT (control) probes (Fig. 2*C*) on single positive (SP) $CD4^+CD8^-CD3^+$, $CD4^-CD8^+CD3^+$, double positive (DP) $CD4^+CD8^+$, or triple negative (TN) $CD4^-CD8^-CD3^-$ thymocytes. mCCR8 message is abundant in $CD4^+$ SP cells (*lane 1*) and detectable in DP cells (*lane 3*) but not detectable in $CD8^+$ SP (*lane 2*) or TN cells (*lane 4*). This result indicates that CCR8 mRNA expression is associated with the $CD4^+$ lineage. The low expression in DP thymocytes may represent cells committed to the $CD4^+$ lineage. This, along with the potential anti-apoptotic effects discussed above, suggest that CCR8 signaling may be involved in positive selection of thymocytes.

The distribution of mCCR8 mRNA in thymus sections was also analyzed by in situ hybridization. Expression was observed in both cortical and medullary thymocytes (data not shown). In the mouse spleen very few positive cells were observed in the T cell region of the white pulp (data not shown).

These results point to a T cell-specific expression pattern. Since only the thymus expressed significant mCCR8 mRNA (Fig. 2*A*), we probed 28 mouse peripheral lymphoid tissues or hemopoietic cell line cDNA libraries with mCCR8, including lymph nodes, spleen, T and B cell populations, monocytes, and dendritic cells. Only a cDNA library from activated, Th2-polarized cells expressed abundant mCCR8 (data not shown). To confirm this, we analyzed various mouse T cell populations. As shown in Figure 3*A*, activated Th2 cells strongly expressed mCCR8, and is also present in a cDNA library from activated $NK1.1^+CD4^+$ T cells, which are known to express several Th2-specific genes (19) and may participate in Th2 differentiation through their ability to produce IL-4. In contrast, mCCR8 mRNA was rare in an activated, polarized Th1 cell cDNA library (Fig. 3*A*).

These findings were confirmed by Northern blot analysis. Purified populations of mouse splenic T cells were analyzed for expression of CCR8 mRNA. As shown in Figure 3*B*, freshly isolated $CD4^+NK1.1^-Mel14^+$ cells (representing Th precursor cells), either resting or activated, do not express mCCR8. Three-week polarized Th1 and Th2 were also tested. Only activated, polarized Th2 cells (but not Th1) expressed mCCR8 strongly. Mouse $CD8^+$ splenocytes do not express mCCR8 (data not shown). Preliminary experiments indicate that the difference in CCR8 expression between Th1 and Th2 cells is detectable following polarization for relatively short periods of time (72 h). In similar experiments, we

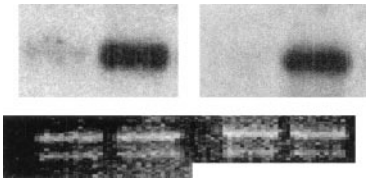

FIGURE 4. Expression of CCR4 (*left panel*) and CCR8 (*right panel*) in the human Th1 clone ET3.22 (*lanes 1* and *3*) and the human Th2 clone E4.1 (*lanes 2* and *4*) monitored by Northern blot analysis. Also shown are the 28S and 18S ribosomal subunits. *Lanes 1* and *2* were probed with a CCR4 ORF probe, whereas *lanes 3* and *4* were probed with a CCR8 ORF probe.

have observed that IL-4 alone does not mediate the CCR8 polarization observed. Instead, it appears to require T cell activation at some point during the polarization protocol (data not shown).

We then produced highly polarized activated human Th1 or Th2 cells and performed Northern blot analysis for human CCR8. As shown in Figure 3*C*, activated human Th2-polarized cells showed strong expression of human CCR8 whereas activated human Th1-polarized cells did not. CCR4 has been recently reported to be preferentially expressed in Th2 cells (14). As shown in Figure 4, we also observed strong CCR8 and CCR4 mRNA expression in the human Th2 clone E 4.1, but not in a human Th1 clone. These results suggest that both CCR4 and CCR8 are potential markers for the differentiation of human Th2 cells.

Thus, CCR8 may be part of a Th2-specific genetic program, part of which is shared with CD4 thymocytes. IFN-γ inhibits the proliferation of both Th2 cells (20) and CD4 thymocytes (21). This finding may represent a mechanism through which Th1 cells can control the development of Th2 responses, and is due to the differential expression of IFN-γ receptor in Th1 vs Th2 cells (22). Thus, CD4 thymocytes share some characteristics with Th2 cells.

These data predict that Th2 cells should respond to CCR8 ligands. As shown in Figure 5*A*, I-309 and TCA-3 are potent chemoattractants for activated Th2-polarized T cells. Although activated Th1 T cells also respond to TCA-3, their response required 1000-fold higher ligand concentration than Th2 cells. No chemotaxis was observed by resting Th1, whereas only slight chemotaxis (chemotactic index = 2.5) was observed with resting Th2 T cells (not shown). Control experiments indicated that the responses observed were due to chemotaxis, not chemokinesis (not shown). To our knowledge, this is the first report of chemotactic activity of I-309/TCA-3 for T cells, a result that reflects the high specificity of CCR8 expression in activated Th2 T cells. Recently, CCR5 has been reported to be preferentially expressed by Th1 cells (14, 23). In agreement with this result, we observed that Th1-polarized mouse cells respond to macrophage inflammatory protein-1α (MIP-1α) better than to TCA-3 (Fig. 5*B*).

Finally, as shown in Figure 6, we detected a calcium flux in response to I-309 in Th2 but not Th1 human polarized cells. Taken together, these results indicate that the expression of CCR8 by Th2 cells has functional consequences.

Recently, CCR3 has been reported to be associated with Th2 differentiation (24). However, other studies found low frequency of CCR3$^+$ T cell clones among IL-4 secreting clones (25), and low expression of CCR3 mRNA and low chemotactic responsiveness of bulk Th2 cultures in response to the CCR3 ligand eotaxin (14), suggesting that CCR3 expression may be confined to a subset of Th2 cells. CCR4 has also been reported to be associated with the Th2 phenotype (14). As shown in Figure 4, both CCR4 and CCR8 mRNA are strongly expressed by Th2 cells, suggesting that both of these receptors may be potential markers for human Th2 cells.

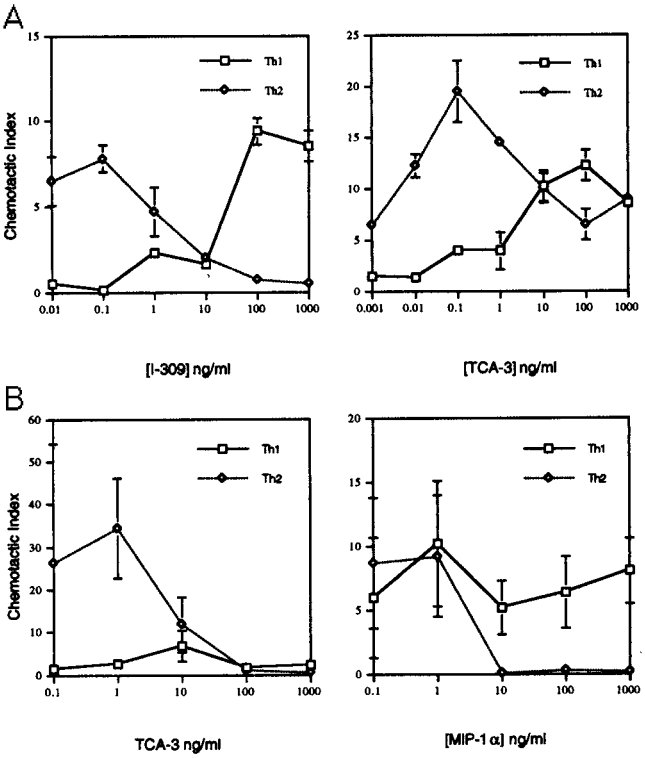

FIGURE 5. *A*, Th2 cells respond to CCR8 ligands. Chemotaxis of activated (three times polarized) Th1 (open squares) or Th2 cells (open diamonds) in response to human I-309 or mouse TCA-3 (as indicated) was measured in vitro. Results are expressed as chemotactic index vs chemokine concentration and are representative of three separate experiments. *B*, Comparison of the response of in vitro-polarized Th1 and Th2 mouse cells to MIP-1α and to TCA-3, showing that Th1-polarized cells respond to MIP-1α whereas Th2-polarized cells respond to TCA-3.

Although the role of cytokines such as IL-4, IFN-γ, and IL-12 in Th1 and Th2 development has been well documented (26), the role of chemokines and their receptors in Th cell polarization and recruitment remains poorly defined. The expression of chemokine receptors in Th1 and Th2 subsets may provide insights into the

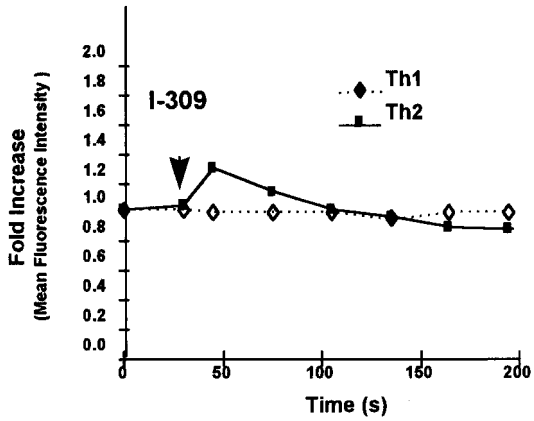

FIGURE 6. Calcium mobilization in response to I-309 in human Th1 and Th2 cells. Cells were loaded with Fluo-3AM and the Ca^{2+} flux was monitored by flow cytometry by gating in CD4$^+$ cells. Two million cells in 0.5 ml of buffer were exposed to 200 ng/ml I-309, and 3000 events were monitored at 0, 15 and 30 s. The response shown is the mean increase in fluorescence intensity relative to time 0. The mean fluorescence intensity was 5.1 and 7.2 for untreated Th1 and Th2 cells, respectively.

effects of these molecules in Th responses. Both Th1 and Th2 cells can produce TCA-3 (27), indicating that they can affect Th2 migratory patterns. The TCA-3/I-309-CCR8 interaction may also influence Th2 differentiation and/or may have anti-apoptotic effects on these cells as well (18). These observations have implications for therapy in allergic diseases and point the way for future research.

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