

Differentiation of Human T Cells Alters Their Repertoire of G Protein α -Subunits*[§]

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Because T cell differentiation leads to an expanded repertoire of chemokine receptors, a subgroup of G protein-coupled receptors, we hypothesized that the repertoire of G proteins might be altered in parallel. We analyzed the abundance of mRNA and/or protein of six G protein α -subunits in human CD4⁺ and CD8⁺ T cell subsets from blood. Although most G protein α -subunits were similarly expressed in all subsets, the abundance of $G\alpha_o$, a protein not previously described in hematopoietic cells, was much higher in memory *versus* naive cells. Consistent with these data, activation of naive CD4⁺ T cells *in vitro* significantly increased the abundance of $G\alpha_o$ in cells stimulated under non-polarizing or T_H17 (but not T_H1 or T_H2)-polarizing conditions. In functional studies, the use of a chimeric G protein α -subunit, $G\alpha_{o5}$, demonstrated that chemokine receptors could couple to $G\alpha_o$ -containing G proteins. We also found that $G\alpha_{i1}$, another α -subunit not described previously in leukocytes, was expressed in naive T cells but virtually absent from memory subsets. Corresponding to their patterns of expression, siRNA-mediated knockdown of $G\alpha_o$ in memory (but not naive) and $G\alpha_{i1}$ in naive (but not memory) CD4⁺ T cells inhibited chemokine-dependent migration. Moreover, although even in $G\alpha_o$ - and $G\alpha_{i1}$ -expressing cells mRNAs of these α -subunits were much less abundant than $G\alpha_{i2}$ or $G\alpha_{i3}$, knockdown of any of these subunits impaired chemokine receptor-mediated migration similarly. Together, our data reveal a change in the repertoire of $G\alpha_{i/o}$ subunits during T cell differentiation and suggest functional equivalence among $G\alpha_{i/o}$ subunits irrespective of their relative abundance.

Exposure of naive T cells to cognate antigens in the context of infection results in their rapid clonal expansion to produce large numbers of antigen-specific effector cells. After an infection has been cleared, most effector cells die, leaving behind a smaller population of long-lived memory cells that are able to mount a rapid response upon reinfection (1). Because it is often difficult to distinguish between effector and long-lived memory cells in blood or other sites by phenotypic markers alone, non-naive T cells are often described as “effector/memory” cells.

Unlike naive cells, which are generally homogeneous except for their antigen receptors, the effector/memory population is highly heterogeneous. For CD4⁺ or helper T (T_H)³ cells, separate pathways of effector/memory differentiation have been described, resulting in, for example, T_H1, T_H2, and T_H17 cells, which can be identified by their abilities to produce the signature cytokines, interferon- γ (IFN γ), interleukin 4 (IL-4), and IL-17, respectively. For CD8⁺ or cytotoxic T cells, progressive differentiation results in the production of proteins such as perforin and granzymes, which enhance the ability of these cells to kill infected cells (1–3).

A more general scheme has been proposed for understanding CD4⁺ and CD8⁺ effector/memory T cell differentiation, whereby naive cells give rise first to central memory T cells (T_{CM}), and these can in turn, either through homeostatic or antigen-driven proliferation, yield effector memory T cells (T_{EM}) (4) (not to be confused with effector/memory cells). T_{CM} express L-selectin (CD62L) and the chemokine receptor CCR7, which are necessary for trafficking to non-inflamed lymphoid tissues, whereas T_{EM} lose expression of CD62L, CCR7, or both along with the ability to enter non-inflamed lymphoid organs. T_{EM} possess the highest levels of effector functions, such as the production of effector cytokines or cytotoxicity (4). An expanded ability to migrate into tissue to combat infection is an essential component of effector/memory T cell differentiation. This latter capability is due in part to the expression of an expanded number of species of chemokine receptors (for example, from two on naive cells, to at least 15, in various combinations, on effector/memory T cells) and is associated with an intrinsic, enhanced response to chemokines in assays *in vitro* (5). In humans, chemokine receptors form a group of 19 seven-transmembrane domain, G protein-coupled receptors (GPCRs), which have ligands that form a corresponding group of more than 40 chemoattractant proteins.

We hypothesized that the expanded repertoire of receptors on memory T cells might be associated with gains and/or losses of downstream effector molecules, such as the heterotrimeric G proteins, which are the proximal transducers of chemokine receptor signals. Changes in the abundance of G protein α -subunits have functional consequences for GPCR-mediated signaling. In some cellular contexts, the abundance of certain G protein α -subunits is limiting, and increased responsiveness to various ligands is achieved through the increased expression of

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³ The abbreviations used are: T_H, helper T (cells); IL, interleukin; T_{CM}, central memory T cells; T_{EM}, effector memory T cells; GPCR, G protein-coupled receptor; NP, nonpolarized; TCR, T cell receptor; rIL, recombinant interleukin.

$G\alpha_o$ Expression in Human Memory T Cells

G protein α -subunits, as well as, or in place of, increased expression of a given GPCR (6–10). Regulation can be subtle, for example the result of selectively increasing or decreasing the abundance of individual $G\alpha_{i/o}$ family members while leaving others unchanged (7, 8), and changes in the abundance of various G proteins can result in functional differences by affecting the balance among different signaling pathways (6). Changes in the abundance of G_i -family G proteins might have particular relevance for chemokine receptor signaling, because these G proteins are critical for chemoattractant receptor signaling (11–13).

We compared naive and memory T cells for the expression of G protein α -subunits. In experiments described below, we found that effector/memory T cells from human peripheral blood, much more so than naive T cells, expressed the G_i family member $G\alpha_o$, which has not been described previously in hematopoietic cells. In functional studies, we found that despite being of relatively low abundance in the effector/memory population based on mRNA expression, $G\alpha_o$ contributed significantly to chemokine receptor signaling in these cells. We also found that $G\alpha_{i1}$ had a reciprocal pattern as compared with $G\alpha_o$; $G\alpha_{i1}$ was expressed and functioned with chemokine receptors in naive cells but was down-regulated after differentiation to effector/memory cells. Together, these data are the first description of the presence and function of $G\alpha_o$ (and $G\alpha_{i1}$) in leukocytes and suggest that the low abundance of some $G\alpha$ proteins belies their functional importance in GPCR signaling.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Cell culture media and salt solutions were obtained from Invitrogen, and fetal bovine serum (FBS) was purchased from Gemini Bio-Products (Woodland, CA). Ficoll-Hypaque was purchased from Amersham Pharmacia Biotech. All recombinant chemokines were purchased from Peprotech (Rocky Hill, NJ). The anti-CD3 mAb (OKT3) was obtained from Ortho Biotech (Raritan, NJ). The anti-CD28 mAb clone 9.3 was a kind gift from Carl June (University of Pennsylvania, Philadelphia). Anti-IL-4, anti-IL-12, and anti-IFN γ antibodies were obtained from BD Biosciences. rIL-2 was obtained from Hoffmann-La Roche. The cytokines IL-4, IL-12, IFN γ , and TGF- β were purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal antibodies against $G\alpha_{i2}$ (catalogue number sc-7276), $G\alpha_{i3}$ (sc-262), $G\alpha_o$ (sc-387), $G\alpha_{q/11}$ (sc-392), $G\alpha_{i3}$ (sc-410), goat polyclonal antibody against actin (sc-1615), and anti-rabbit and anti-goat preadsorbed HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A second rabbit polyclonal antibody against $G\alpha_o$ (catalogue number 3975) was purchased from Cell Signaling Technology (Danvers, MA). The metalloproteinase inhibitor TAPI-0 was obtained from Peptides International (Louisville, KY).

Plasmid DNAs—cDNA encoding human CXCR6 was amplified by polymerase chain reaction (PCR) using the previously described pCEP4: CXCR6 clone C3-9.1 as a template (14) and inserted in-frame into the pEYFP-N1 vector (Clontech Laboratories, Palo Alto, CA) to encode a CXCR6 C-terminal fusion protein with yellow fluorescent protein (YFP). DNAs were ver-

ified by sequencing. The construction of plasmids expressing G protein chimeras, in which the five C-terminal amino acid residues of $G\alpha_q$ were replaced with those of $G\alpha_{i2}$ (qi5 chimera), $G\alpha_o$ (qo5), or $G\alpha_s$ (qs5), has been described previously (15, 16); these were a kind gift of Bruce M. Conklin (Gladstone Institute of Cardiovascular Disease, University of California, San Francisco). All chimeras have an internal hemagglutinin (HA) epitope, allowing for recognition by an anti-HA antibody (clone 12CA5, Roche Diagnostics). The cDNA clones for human $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oA}$, $G\alpha_{oB}$, and $G\alpha_{i3}$ in pcDNA3.1(+), were obtained from the University of Missouri cDNA Resource Center.

Purification and Sorting of Leukocyte Subsets—Elutriated lymphocytes and monocytes were obtained from healthy donors by the Department of Transfusion Medicine, Clinical Center, National Institutes of Health (Bethesda, MD), under an Institutional Review Board-approved protocol. In initial experiments, CD4⁺ and CD8⁺ T lymphocytes were isolated from elutriated lymphocytes by negative selection with either the CD4⁺ T cell isolation kit II or the CD8⁺ T cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. These cells were further purified using CD45RA microbeads (Miltenyi Biotec) for positive and negative selection of naive and memory cells, respectively. CD45RA⁻CD4⁺ T cells (memory T cells) were further purified by incubation with CD62L microbeads for positive selection of central memory (CD62L⁺) and negative selection of effector memory (CD62L⁻) cells. For the isolation of other leukocyte subsets from elutriated cells, positive selection was performed using microbeads for the following cell type-specific markers (all from Miltenyi Biotec): CD3 (total T cells) and CD11b (monocytes). All selected leukocyte subsets were analyzed for phenotypic cell surface marker expression by antibody staining and flow cytometric analysis as described below. In later experiments (indicated in the legends to Figs. 2, 3, and 8), CD4⁺ and CD8⁺ T cell subsets were first purified by negative selection with either the RosetteSep CD4⁺ T cell or CD8⁺ T cell enrichment mixtures (StemCell Technologies, Vancouver, British Columbia, Canada) as described previously (17), and naive and effector/memory subsets were purified by sorting using a FACSAria flow cytometer (BD Biosciences) after staining with the following antibodies obtained from BD Biosciences: FITC-conjugated anti-CD8, phycoerythrin-conjugated anti-CD62L, phycoerythrin-Cy5-conjugated anti-CD3, allophycocyanin-conjugated anti-CD45RO, and allophycocyanin-Cy7-conjugated anti-CD4 as described previously (18). It is noteworthy that published experiments have demonstrated the cleavage of CD62L from the surface of lymphocytes through the activity of a zinc metalloproteinase (19, 20). In our sorting of CD8⁺ T cell subsets, we sometimes detected substantial amounts of CD62L mRNA in the "CD45RO⁻CD62L⁻" effector/memory population, presumably through contamination with naive cells in which CD62L had been cleaved. We found that treatment of CD8⁺ T cells during staining and sorting with TAPI-0 (50 μ g/ml), a specific inhibitor of the zinc metalloproteinase responsible for CD62L cleavage (21), resulted in very low amounts of CD62L mRNA in the effector/memory population (data not shown). The data presented here are from experi-

ments in which real-time RT-PCR analysis demonstrated little or no contamination of the CD8⁺CD62L⁻ subsets with cells expressing mRNA for CD62L (data not shown).

Flow Cytometry—For phenotypic analysis of leukocyte subsets based on the expression of cell surface markers, 100 μ l of cells at 1×10^7 cells/ml in PBS, 1% FBS, 10 mM HEPES was incubated with dye-conjugated primary antibodies (1 μ g) for 15 min on ice. Cells were then washed twice with PBS/FBS/HEPES and fixed in 1% paraformaldehyde. Antibody-staining data were collected on a FACSCalibur (BD Biosciences) flow cytometer. Flow cytometry data files were analyzed with FlowJo software (Tree Star Inc., San Carlos, CA).

In Vitro Activation of Naive Cord Blood CD4⁺ T Cells—Human cord blood was obtained from term placentas following the delivery of healthy newborns at Shady Grove Adventist Hospital (Gaithersburg, MD) as approved by that institution's review board. Naive CD4⁺ T cells were isolated from cord blood with the RosetteSep/human CD4⁺ T cell reagent (StemCell Technologies). Stimulation of the CD4⁺ T cells was performed as described previously (17). Cells were cultured at 1×10^6 cells/ml in 24-well plates in RPMI 1640 medium/10% FBS with gentamicin (20 μ g/ml; Invitrogen) and stimulated with plate-bound anti-CD3 (OKT3, 10 μ g/ml), soluble anti-CD28 (1 μ g/ml), and rIL-2 (200 IU/ml). In addition, these cells were treated under one of four regimens: anti-IL-4 (0.4 μ g/ml), anti-IL-12 (2 μ g/ml), and anti-IFN γ (8 μ g/ml) with rTGF- β (10 ng/ml) for nonpolarizing (NP) conditions; rIL-12 (2 ng/ml) and anti-IL-4 (0.4 μ g/ml) for T_H1-polarizing conditions; rIL-4 (4 ng/ml), anti-IL-12 (2 μ g/ml), and anti-IFN γ (8 μ g/ml) for T_H2-polarizing conditions; and with no cytokines (other than rIL-2) and no antibodies as a control. On day 3 or 4, cells were harvested, washed, and resuspended in fresh medium at 1×10^6 cells/ml containing rIL-2 and the appropriate cytokine and antibody combinations. In some experiments, to produce T_H17 cells, CD4⁺ T cells from cord blood were activated with anti-CD3 and anti-CD28 as above (without rIL-2) with rIL-23 (100 ng/ml), rIL-6 (20 ng/ml), rIL-1 β (10 ng/ml), rTNF α (10 ng/ml), and rTGF- β (either 2 or 10 ng/ml) as well as anti-IL-4 (0.4 μ g/ml), anti-IL-12 (2 μ g/ml), and anti-IFN γ (8 μ g/ml) antibodies.

Isolation of RNA, Synthesis of cDNA, and Semiquantitative Real-time RT-PCR—In early experiments, total cellular RNA was isolated with the TRIzol reagent (Invitrogen), and first-strand cDNA synthesis was performed with the SuperScript first-strand synthesis system for RT-PCR (Invitrogen) as described by the manufacturer. In experiments involving cells purified by cell sorting, RT-PCR was performed with the Platinum quantitative inventoried RT-PCR ThermoScript one-step system (Invitrogen) as described by the manufacturer. Inventoried primer and probe sets (FAM/MGB-labeled) for human G α _{i1}, G α _{i2}, G α _{i3}, G α _o, G α ₁₃, CD3, CD62L, and GAPDH and for mouse G α _{i2}, G α _o, and β ₂-microglobulin were purchased from Applied Biosystems (Foster City, CA). Primers and probes specific for human G α _{oA} and G α _{oB} were designed with Primer Express v2.0 (Applied Biosystems) with probes overlapping variant-specific splice junctions. Primer/probe sequences are available upon request. Real-time PCR analysis was performed on samples in duplicate with either an ABI 7700 or 7900 se-

quencer system (Applied Biosystems), and data were analyzed by the $2^{-\Delta\Delta C_T}$ method as described (22). Except as noted, ΔC_T values for each target mRNA compared with GAPDH (or β ₂-microglobulin) were determined; $\Delta\Delta C_T$ values were calculated by subtracting the highest ΔC_T value from the other ΔC_T values. Samples with the highest ΔC_T values for a given mRNA were given the value of 1, and mRNA levels in other samples were expressed as the -fold difference in mRNA (through calculation of $2^{-\Delta\Delta C_T}$).

Cell Transfections—HEK293:hCCR6 cells (14) or HEK293T cells were harvested by trypsinization, washed, counted, and transfected with the Amaxa Nucleofector device as described by the manufacturer (Amaxa Inc., Gaithersburg, MD). Cells were resuspended at 5×10^7 cells/ml in Nucleofector solution V (Amaxa Inc.), and 100 μ l of cells together with plasmid DNAs (total 5 μ g) were treated according to program Q-001. Transfected cells were resuspended in 5 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and split into two wells of a 6-well plate. Transfected cells were incubated at 37 °C, 5% CO₂ for 48 h before being harvested.

Knockdown of G Protein α -Subunits by Short Interfering RNA (siRNA)—SmartPool control siRNAs (catalogue number D-001810-10-0005), siRNAs specific for G α _{i1} (L-010404-00-0005), G α _{i2} (L-003897-00-0005), G α _{i3} (L-005184-00-0005), G α _o (L-009486-00-0005), and G α _t (L-009827), and a separate custom siRNA for G α _o as described by Krumins and Gilman (23) along with a non-targeting control were obtained from Dharmacon (Lafayette, CO). Each siRNA sample was resuspended in 250 μ l of buffer as per the manufacturer's instructions to give 20 μ M stock solutions. Transfections were performed with the Amaxa Nucleofector device. Jurkat cells were transfected in solution V with the program S-018, whereas sorted subsets of primary CD4⁺ T cells were transfected with the T cell Nucleofector solution and program V-024. In all cases, cells were resuspended at 5×10^7 cells/ml in Nucleofector solution, and 100 μ l of cells together with 15 or 20 μ l of siRNA solution were treated with the appropriate program. Transfected cells were resuspended in 5 ml of DMEM containing 10% FBS and split into two wells of a 6-well plate. Transfected cells were incubated at 37 °C, 5% CO₂ for 72 h before being harvested.

Measurement of Calcium Flux—Assays of calcium flux in transfected cells were performed on an RF-M2001 ratio fluorescence spectrometer (Photon Technology International, South Brunswick, NJ) essentially as described (24). Briefly, transfected cells were harvested by trypsinization, washed twice with PBS, and resuspended at 10^7 cells/ml in Hanks' balanced salt solution (HBSS) containing calcium and magnesium, 10 mM HEPES, and 1% FBS (HBSS/FBS) and loaded with 2 μ M fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) for 40 min at 30 °C followed by two washes in HBSS/FBS buffer. Measurements of calcium flux were made in cuvettes containing 2 ml of cells at 0.5×10^6 cells/ml. Excitation was done alternately at 340 and 380 nm, and emission was measured at 510 nm. The integration time was 0.5 s, and the ratios of the signals obtained at each excitation wavelength were plotted as a function of time.

G_{α_o} Expression in Human Memory T Cells

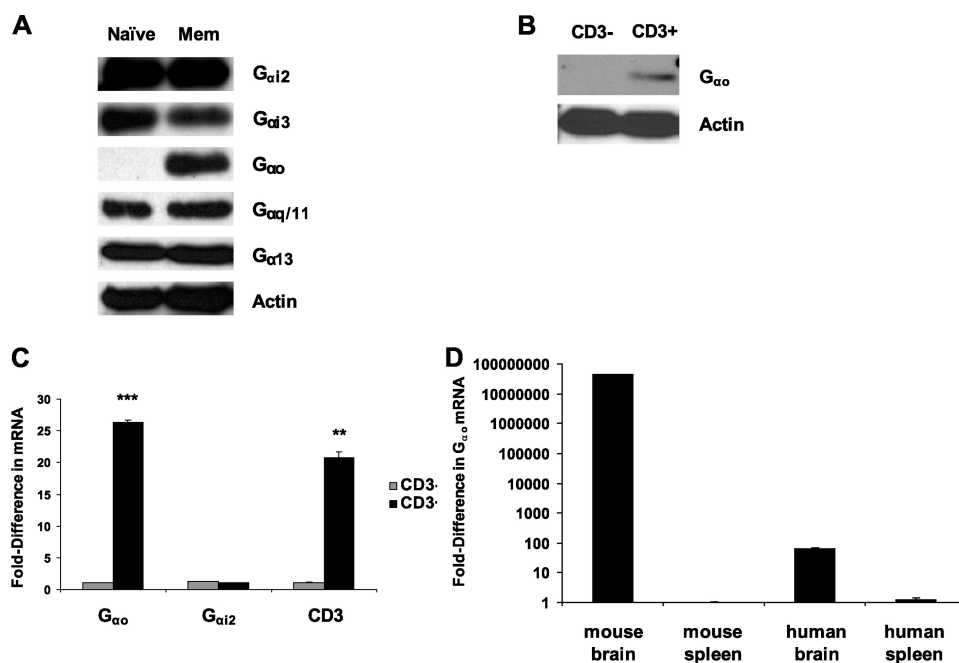


FIGURE 1. G_{α_o} is expressed in peripheral blood T cells, preferentially in the memory subset. A, CD4⁺ T cells were isolated from peripheral blood by negative selection and then further purified into naive and effector/memory subsets as described under "Experimental Procedures." Whole-cell lysates (100 μ g of protein) were analyzed by Western blotting with rabbit polyclonal anti- G_{α} or anti-actin antibodies as indicated. Results are for naive and effector/memory CD4⁺ T cells from one donor and are representative of three donors. Representative full-length Western blots that show molecular mass markers and positive controls can be seen in supplemental Fig. S1. B, CD3⁺ and CD3⁻ cells were purified from peripheral blood with magnetic beads for CD3. Whole-cell lysates (100 μ g of protein) were then analyzed by Western blotting with anti- G_{α_o} and anti-actin antibodies. Results are for cells from one donor and are representative of two donors. C, CD3⁺ and CD3⁻ cells were isolated as described for B, total RNA was isolated from each subset, and samples were then analyzed by semiquantitative real-time RT-PCR for the expression of mRNAs for G_{α_o} , $G_{\alpha_{i2}}$, CD3, and as a control, GAPDH. The -fold differences in abundance of the indicated mRNAs, each normalized to GAPDH expression, between CD3⁻ (gray bars) and CD3⁺ cells (black bars) were calculated according to the method of $\Delta\Delta C_T$. For each mRNA of interest, the lowest level of expression versus GAPDH from a single well for both CD3⁺ and CD3⁻ samples was set at one. Values cannot be compared between mRNA species. The data shown are the mean -fold differences \pm S.E. in expression for the indicated mRNAs from one donor from assays performed in duplicate and are representative of three donors, separate from those analyzed in B. **, $p < 0.005$; and ***, $p < 0.0005$, when comparing CD3⁻ and CD3⁺ cells. D, poly(A)⁺ mRNA from mouse and human brain and spleen samples were analyzed by semiquantitative real-time RT-PCR for the expression of mRNAs for G_{α_o} and either β_2 -microglobulin in the case of mouse samples or GAPDH for human samples. Data shown are the mean -fold differences \pm S.E. in G_{α_o} mRNA for each sample within each species, assayed in triplicate, from one experiment.

Chemotaxis Assays—Chemotaxis assays were performed according to standard protocols. Cultured cells were harvested, washed twice in PBS, counted, and resuspended in chemotaxis medium (RPMI 1640 containing 25 mM HEPES, pH 8, and 1% BSA) at 10^7 cells/ml. Chemotaxis assays were performed with Transwell plates containing 5- μ m pores. Cells in 100 μ l of chemotaxis medium were added to inserts that were preincubated at 37 $^{\circ}$ C for 30 min before being placed in wells that contained either medium alone or medium with 100 ng/ml CXCL12 or CCL20 and incubated for 2 h at 37 $^{\circ}$ C, 5% CO₂. Cells that had migrated into the lower wells were harvested and counted with a Vi-CELL analyzer (Beckman Coulter). Dose-response experiments showed that 100 ng/ml was the optimal concentration for both CXCL12 and CCL20 in these chemotaxis assays (data not shown).

Western Blotting—For analysis of leukocyte populations for the expression of individual G protein α -subunits, total protein was measured using either the Bio-Rad assay or Pierce Coomassie Plus reagent (Pierce Biotechnology, Inc.). Cell samples containing equal amounts of protein were boiled in 2 \times Laemmli

buffer and resolved by 10% SDS-PAGE. We used gels of both 8.6 \times 6.8 cm and 16 \times 16 cm. Gels were transferred to Immobilon-P PVDF membranes (Millipore Corp., Bedford, MA) overnight in the cold in a Transblot apparatus (Bio-Rad Laboratories) at 50 V in 25 mM Tris, 190 mM glycine, 20% methanol. Blots were washed in Tris-buffered saline, pH 7.4, containing 0.01% Tween-20 (TBST) and then blocked in TBST containing 2% (w/v) nonfat dry milk (Bio-Rad Laboratories) for 1 h at room temperature. All subsequent incubations and washes were done in TBST, 2% milk. Blots were incubated with the appropriate primary antibodies (at 1:200 dilution, or 1 μ g/ml) for 2 h followed by two 10-min washes and then incubated in a 1:2000 dilution of the appropriate secondary antibody (HRP-conjugated goat anti-rabbit, or donkey anti-goat) for 30 min followed by two washes. For visualizing bands, blots were treated with SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology) and exposed to X-OMAT XAR film (Eastman Kodak Co.). Quantification of Western blots was performed with Adobe Photoshop (San Jose, CA) and ImageJ software (National Institutes of Health) as described previously (25).

Statistical Analysis—Unpaired, two-tail t tests were performed using InStat version 3 software from GraphPad Software Inc. (San Diego, CA). Statistical significance was indicated by a two-tailed p value of <0.05 . Where multiple comparisons were required, simple one-way ANOVA was performed followed by running the Bonferroni post-hoc test.

RESULTS

G_{α_o} Is Expressed in Human Peripheral Blood T Cells—To detect differences in the expression of G proteins between naive and effector/memory T cells, we analyzed purified human peripheral blood CD4⁺ T cells by Western blotting with subtype-specific antibodies against a number of G protein α -subunits. The major G_i family members found in lymphocytes, $G_{\alpha_{i2}}$ and $G_{\alpha_{i3}}$ (26, 27), were equivalently abundant in naive and memory cells, as were $G_{\alpha_{q/11}}$, and $G_{\alpha_{i3}}$ (Fig. 1A). Because the G_i proteins are crucial to chemokine-induced functions in leukocytes (28), we investigated the expression of G_{α_o} , a G_i family member for which the expression and function in leukocytes had not been described previously. (In the absence of a reliable specific antibody, we were unable to assess the expression of

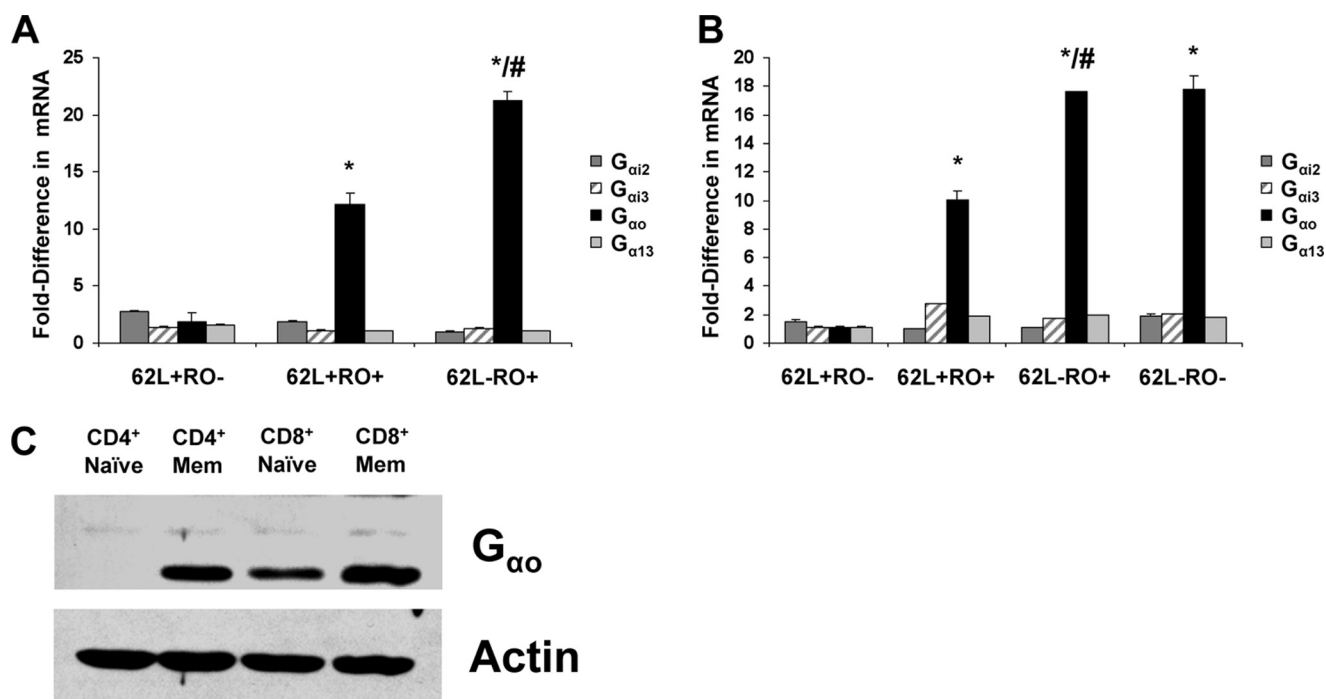


FIGURE 2. $G\alpha_o$ mRNA and protein expression is highest in the most differentiated subsets of human $CD4^+$ and $CD8^+$ T cells. $CD4^+$ (A) and $CD8^+$ (B) T cells from peripheral blood were sorted into naive ($CD62L^+CD45RO^-$), T_{CM} ($CD62L^+CD45RO^+$), and T_{EM} ($CD62L^-CD45RO^+$; $CD62L^-CD45RO^-$) subsets as described under "Experimental Procedures." Each subset was analyzed by semiquantitative real-time RT-PCR for mRNAs for $G_{\alpha_{i2}}$, $G_{\alpha_{i3}}$, G_{α_o} , and $G_{\alpha_{i13}}$. -Fold differences in the abundance of the indicated mRNAs, each normalized to that of GAPDH mRNA, between each subset of $CD4^+$ (A) or $CD8^+$ (B) T cells were calculated for each mRNA species as described in the legend for Fig. 1. For each mRNA of interest, the lowest level of expression versus GAPDH from a single well was set at 1. Values cannot be compared between mRNA species. The data shown are the mean -fold differences \pm S.E. in expression for the indicated mRNAs from assays performed in duplicate from separate donors for $CD4^+$ and $CD8^+$ T cells and are representative of five and three donors for $CD4^+$ and $CD8^+$ T cells, respectively. *, $p < 0.05$, when compared with mRNA in naive subsets; #, $p < 0.05$ when comparing mRNA content of the $CD62L^-CD45RO^+$ subset with that of the $CD62L^+CD45RO^+$ subset. C, whole-cell lysates (40 μ g of protein) of purified subsets of $CD4^+$ and $CD8^+$ T cells, isolated as described under "Experimental Procedures," were analyzed by Western blotting with anti- $G\alpha_o$ and anti-actin antibodies. Results are for naive and combined memory subsets ($CD4^+$ Mem, $CD8^+$ Mem) of $CD4^+$ or $CD8^+$ T cells. Data are from the same donor for both $CD4^+$ and $CD8^+$ T cell subsets and are representative of three donors.

$G\alpha_{i1}$ protein.) We could easily detect $G\alpha_o$ protein but only in effector/memory cells (Fig. 1A). Representative full-length Western blots that show molecular mass markers and positive controls can be seen in supplemental Fig. S1A. In supplemental Fig. S1B, using transfected cells, we provide additional evidence that the anti- $G\alpha_{i/o}$ antibodies recognized the appropriate proteins. We also confirmed our finding that the $G\alpha_o$ protein was expressed in effector/memory $CD4^+$ T cells in separate experiments using a second commercially available anti- $G\alpha_o$ antibody (supplemental Fig. S2).

This finding led us to examine other leukocyte populations for the expression of $G\alpha_o$. Analysis of Western blots of lysates of cells purified from peripheral blood on the basis of CD3 revealed that $G\alpha_o$ was found in $CD3^+$ cells (consisting mainly of $CD4^+$ and $CD8^+$ α/β T cells) but not in $CD3^-$ cells (Fig. 1B). This difference was confirmed at the level of mRNA by real-time RT-PCR analysis of the same cell types, where the relative difference in the expression of $G\alpha_o$ mRNA between the purified $CD3^-$ and $CD3^+$ samples was similar to that of CD3 itself (Fig. 1C). The abundance of $G_{\alpha_{i2}}$ mRNA was not significantly different between the groups (Fig. 1C).

The experimental advantages of using mice led us to investigate whether $G\alpha_o$ was found in mouse lymphocytes, where published data suggested that $G\alpha_o$ was not expressed (29). To compare relative levels of $G\alpha_o$ mRNA, we analyzed the abundance of $G\alpha_o$ mRNA in spleen and brain for both BALB/c

mouse and human tissues. In the human samples, real-time RT-PCR analysis showed a $\Delta\Delta C_T$ value of between 6 and 7 when comparing brain and spleen RNAs, indicating an ~90-fold higher abundance of $G\alpha_o$ mRNA in brain compared with spleen (Fig. 1D). However, $G\alpha_o$ mRNA, although highly expressed in mouse brain, was undetectable in spleen, resulting in a $\Delta\Delta C_T$ of at least 25 (Fig. 1D), consistent with published data from Northern blot analysis (29). Together, the data demonstrate that although $G\alpha_o$ can be detected in human T cells, this is not the case in mice.

$G\alpha_o$ Is Most Abundant in Differentiated $CD4^+$ and $CD8^+$ T Cells—Because we found that $G\alpha_o$ protein was found in $CD3^+$ T cells and that it was more abundant in memory than in naive $CD4^+$ T cells, we looked more carefully at $G\alpha_o$ mRNA and protein in specific subsets of $CD4^+$ and $CD8^+$ T cells, which were sorted on the basis of the surface markers CD62L and CD45RO to define naive ($CD62L^+CD45RO^-$), central memory ($CD62L^+CD45RO^+$), and effector memory ($CD62L^-CD45RO^+$; $CD62L^-CD45RO^-$) cells (5, 18). CD45RO is a protein-tyrosine phosphatase involved in T cell activation for which alternative mRNA splicing yields isoforms differentially expressed on naive cells compared with effector/memory cells (30). Substantial numbers of $CD62L^-CD45RO^-$ cells are present only within the $CD8^+$ population; we needed to use the metalloproteinase inhibitor TAPI-0 to obtain pure populations of these cells, which otherwise were contaminated

$G\alpha_o$ Expression in Human Memory T Cells

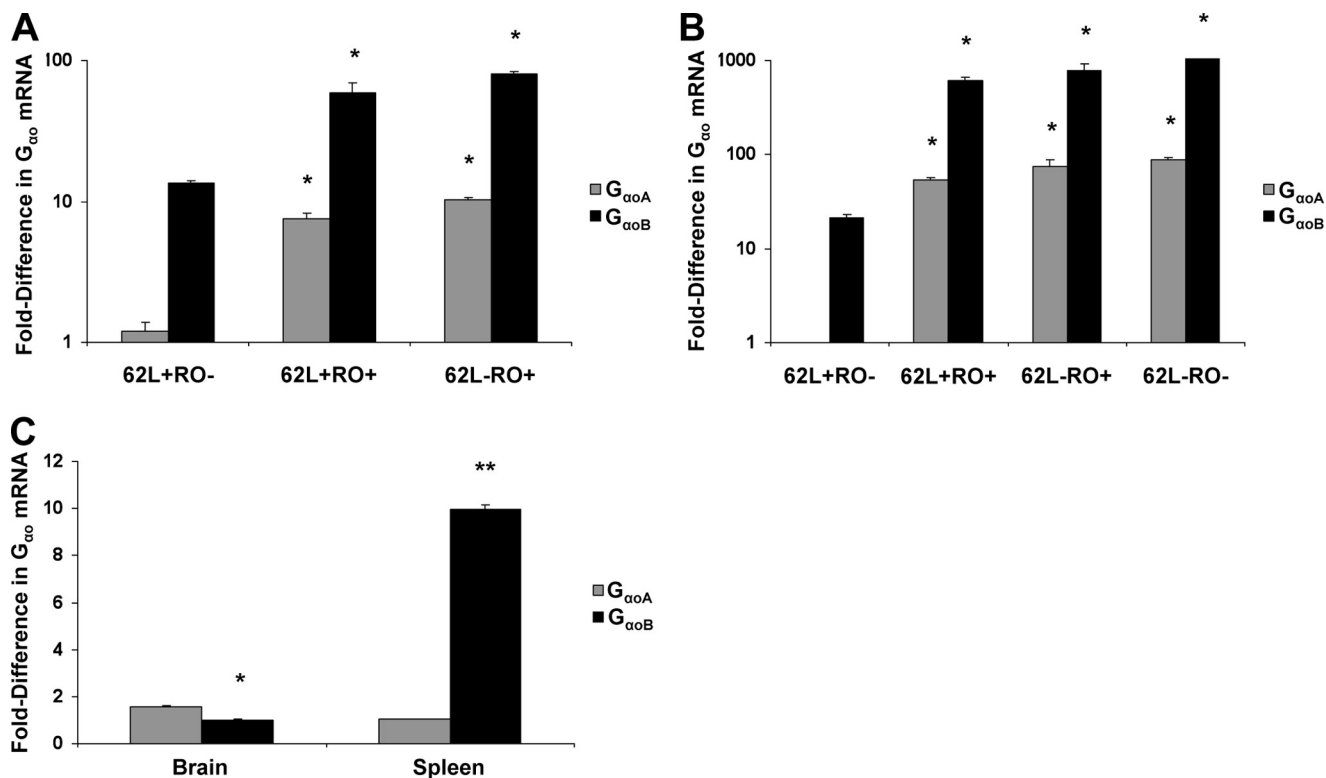


FIGURE 3. $G\alpha_{oB}$ mRNA is expressed more abundantly than $G\alpha_{oA}$ mRNA in $CD4^+$ and $CD8^+$ T cells. $CD4^+$ (A) and $CD8^+$ (B) T cells from peripheral blood were sorted into naïve ($CD62L^+CD45RO^-$), T_{CM} ($CD62L^+CD45RO^+$), and T_{EM} ($CD62L^-CD45RO^+$; $CD62L^-CD45RO^-$) subsets, and each subset was analyzed by semiquantitative real-time RT-PCR for mRNAs for $G\alpha_{oA}$ and $G\alpha_{oB}$. -Fold differences in the expression of the indicated mRNAs, each normalized to GAPDH expression, for each subset of $CD4^+$ (A) or $CD8^+$ (B) T cells were calculated as described in the legend for Fig. 1, relative to $G\alpha_{oA}$ mRNA abundance in the naïve subset. Data shown in A and B are from one donor each for $CD4^+$ and $CD8^+$ T cells and are representative of three donors. *, $p < 0.05$ for abundance of each species of mRNA when compared with amounts in naïve subsets. Within each $CD4^+$ and $CD8^+$ subset, differences between $G\alpha_{oA}$ and $G\alpha_{oB}$ mRNA levels were also significant. C, poly(A)⁺ mRNA from human brain and spleen were analyzed by semiquantitative real-time RT-PCR for the expression of mRNAs for $G\alpha_{oA}$, $G\alpha_{oB}$, and GAPDH as described earlier. Data shown are the mean fold-differences in $G\alpha_{oA}$ and $G\alpha_{oB}$ mRNAs in spleen relative to the amount of $G\alpha_{oA}$ mRNA and in brain relative to the amount of $G\alpha_{oB}$ mRNA, assayed in triplicate, from one experiment. *, $p < 0.05$; **, $p < 0.005$ when comparing $G\alpha_{oA}$ and $G\alpha_{oB}$ mRNA levels within each tissue.

with naïve ($CD45RO^-$) cells that had lost surface CD62L during processing (see “Experimental Procedures”). The three subsets of $CD4^+$ and four subsets of $CD8^+$ T cells were sorted by flow cytometry, and real-time RT-PCR was performed as described previously. Analysis of $CD4^+$ T cell subsets revealed that, as before, $G\alpha_o$ mRNA was expressed at low abundance in naïve cells ($CD62L^+CD45RO^-$) but was progressively more abundant in T_{CM} ($CD62L^+CD45RO^+$) followed by T_{EM} ($CD62L^-CD45RO^+$), (Fig. 2A). Moreover, none of the mRNAs for $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{i3}$ were significantly different in their abundance among the $CD4^+$ T cell subsets.

A similar pattern of $G\alpha_o$ mRNA expression was observed when sorted subsets of $CD8^+$ T cells were analyzed (Fig. 2B). Naïve $CD8^+$ T cells expressed the lowest amount of $G\alpha_o$ mRNA, whereas the most differentiated T_{EM} ($CD62L^-CD45RO^+/CD45RO^-$) generally had the highest abundance of $G\alpha_o$ mRNA, with no significant differences among the subsets for the other G protein α -subunits analyzed (Fig. 2B). We analyzed purified subsets of $CD8^+$ T cells and, for the purposes of comparison, $CD4^+$ T cells from the same donor by Western blotting. Memory cells were pooled for Western blotting because of limitations in the numbers of cells within memory subsets from individual donors. Consistent with the above patterns of $G\alpha_o$ mRNA expression, $G\alpha_o$ protein was more abundant in memory $CD8^+$ T cells than in naïve cells (Fig. 2C). Both naïve and mem-

ory subsets of $CD8^+$ T cells had higher amounts of $G\alpha_o$ protein than did their $CD4^+$ T cell counterparts (Fig. 2C), although $G\alpha_o$ could not be detected in naïve $CD8^+$ T cells from all donors (data not shown).

$G\alpha_{oB}$ mRNA Is More Abundant Than $G\alpha_{oA}$ mRNA in Differentiated T Cells—Alternative splicing of $G\alpha_o$ mRNA gives rise to two protein isoforms, $G\alpha_{oA}$ and $G\alpha_{oB}$, which differ in their C-terminal sequences. The $G\alpha_{oA}$ isoform is more abundant than the $G\alpha_{oB}$ isoform in mouse brain and heart (29, 31, 32). We used real-time RT-PCR to examine the relative expression of $G\alpha_{oA}$ and $G\alpha_{oB}$ mRNAs in T cell subsets. We first used plasmid DNAs encoding $G\alpha_{oA}$ or $G\alpha_{oB}$ sequences to demonstrate the specificities and equal efficiencies for the primer/probe sets that we had designed (data not shown). We then analyzed sorted subsets of $CD4^+$ and $CD8^+$ T cells for the expression of $G\alpha_{oA}$ versus $G\alpha_{oB}$ mRNAs and found that both $G\alpha_o$ isoforms were more highly expressed in T_{EM} subsets compared with naïve subsets (Fig. 3, A and B). Comparison of the ΔC_T values revealed that, unexpectedly, $G\alpha_{oB}$ mRNA was ~12-fold more abundant than $G\alpha_{oA}$ mRNA (Fig. 3, A and B). Real-time RT-PCR analysis of $G\alpha_o$ isoform mRNA expression in human brain revealed that, as expected, $G\alpha_{oA}$ mRNA was ~2-fold more abundant than that of $G\alpha_{oB}$ (Fig. 3C). By contrast, and consistent with our data from peripheral blood lymphocytes,

$G\alpha_{oB}$ mRNA was ~10-fold more abundant than $G\alpha_{oA}$ mRNA in human spleen (Fig. 3C).

$G\alpha_o$ mRNA Expression Is Increased in $CD4^+$ T Cells Activated in Vitro under Nonpolarizing or T_H17 -polarizing Conditions—The increased expression of $G\alpha_o$ mRNA in T_{EM} compared with naive cells suggested that $G\alpha_o$ expression was induced during T cell activation and differentiation. We investigated this with an *in vitro* system for activation and differentiation of human T cells. Cord blood naive $CD4^+$ T cells were activated in the presence of cytokines and anti-cytokine antibodies to yield T_H1 , T_H2 , or NP cells, and cells were also activated in the presence of IL-2 alone, *i.e.* under “neutral” conditions without other added cytokines or anti-cytokine antibodies. Consistent with our previous data from adult peripheral blood (Figs. 1A and 2C), naive $CD4^+$ T cells from cord blood had no detectable $G\alpha_o$ by Western blotting (Fig. 4A). Activation of these cells for 4 days led to an increase in the abundance of $G\alpha_o$ protein in cells that were not polarized but not in cells activated under T_H1 - or T_H2 -polarizing conditions (Fig. 4A). Analysis of $G\alpha_o$ mRNA by real-time RT-PCR supported the Western blotting data (Fig. 4B). By contrast with $G\alpha_o$, the amounts of mRNAs for $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{13}$ were not affected by activation under these conditions (Fig. 4D).

In an effort to examine the stability of $G\alpha_o$ expression in nonpolarized cells, we activated naive cord blood cells as described above, and after 6 days removed the cells from activation and cultured them for 3 days in IL-2 alone. A sample of nonpolarized cells was also moved to media with polarizing cytokines, either IL-12 or IL-4. After the 9 days, $G\alpha_o$ protein was still confined to the nonpolarized cells and was not detectable in cells activated initially under either T_H1 - or T_H2 -polarizing conditions (Fig. 4C, *Day 9 Activated*). Treatment of nonpolarized, $G\alpha_o$ -high cells with IL-12 for 3 days substantially decreased the abundance of $G\alpha_o$, whereas treatment with IL-4 had no such effect (Fig. 4C, *3d cytokines*). In investigating non- T_H1 and non- T_H2 subsets; we next analyzed cord blood cells cultured under conditions that have been described as driving mouse $CD4^+$ T cells to differentiate to produce IL-17 (33). As noted above, T_H17 cells are a recently recognized separate lineage of non- T_H1 , non- T_H2 memory/effector $CD4^+$ T cells thought to be critical in autoimmune disease (34). Cells activated under T_H17 conditions showed significant induction of $G\alpha_o$ mRNA, comparable to that seen in the nonpolarized cells (Fig. 4D).

Chemokine Receptors CCR6, CXCR6, and CXCR4 Couple to $G\alpha_o$ -containing G Proteins—We next investigated whether the presence of $G\alpha_o$ in memory/effector T cells might have functional relevance for the many chemokine receptors found on these cells. We measured intracellular Ca^{2+} mobilization in cells transfected with plasmids encoding chimeric G protein α -subunits, in which the last five amino acid residues of $G\alpha_q$ have been replaced with those of $G\alpha_o$, $G\alpha_{i2}$, or $G\alpha_s$ to create the chimeras $G\alpha_{qo5}$, $G\alpha_{qi5}$ and $G\alpha_{qs5}$, respectively (15, 16, 35). Because the last five amino acid residues of the α -subunit are critical for the coupling of a G protein to a given GPCR, $G\alpha_{qo5}$, $G\alpha_{qi5}$, and $G\alpha_{qs5}$ allow GPCRs coupled to $G\alpha_o$, $G\alpha_{i2}$, and $G\alpha_s$ -containing G proteins, respectively, to activate $G\alpha_q$ -dependent pathways such as phospholipase $C\beta$ -mediated intracellular Ca^{2+} mobilization (15, 16, 35). Because the last five amino acid resi-

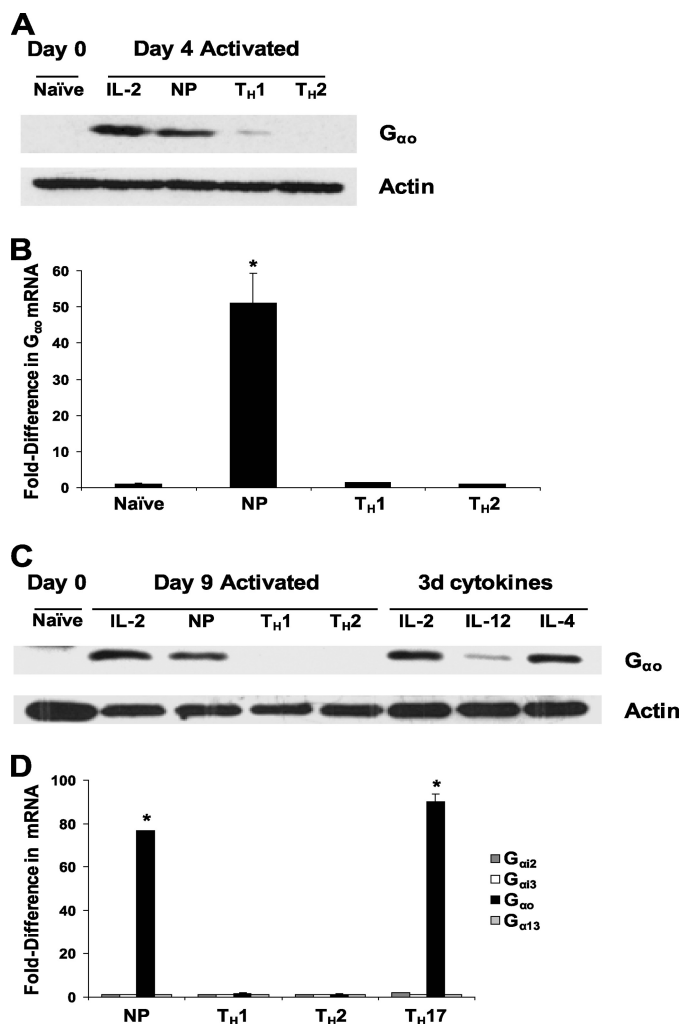


FIGURE 4. $G\alpha_o$ mRNA and protein are expressed in $CD4^+$ T cells activated *in vitro* under NP or T_H17 -polarizing conditions. Naive $CD4^+$ T cells were isolated from cord blood by negative selection and then activated for 6 days with anti-CD3 and anti-CD28 in the presence of rIL-2 alone (IL-2); rIL-2, rTGF- β and antibodies against IL-12, IFN γ , and IL-4 (NP); rIL-2 with rIL-12 and anti-IL-4 (T_H1); or rIL-2 with rIL-4 and anti-IL-12 and anti-IFN γ (T_H2) as described under “Experimental Procedures.” A, whole-cell lysates (100 μ g of protein) from each condition harvested after 4 days of activation were analyzed by Western blotting with anti- $G\alpha_o$ and anti-actin antibodies. The data shown are from one donor and are representative of three donors. B, in separate experiments, cells were activated for 6 days, as described in A, after which each cell type was analyzed by semiquantitative real-time RT-PCR for the expression of mRNAs for $G\alpha_o$ and, as a control, for GAPDH, as described in the legend for Fig. 1. The data shown are the mean -fold differences \pm S.E. in expression for $G\alpha_o$ mRNA for assays performed in duplicate for one donor and are representative of three donors. *, $p < 0.05$ when compared with mRNA abundance in the naive subset. C, naive $CD4^+$ T cells were isolated from cord blood by negative selection and activated for 6 days as described in A. Subsequently, cells were cultured for an additional 3 days under NP or polarizing conditions as indicated. In addition, on day 6, cells that had been activated under NP conditions were left untreated (IL-2) or were treated with rIL-12 (IL-12) or rIL-4 (IL-4) until day 9. Whole-cell lysates (100 μ g of protein) from each condition were analyzed by Western blotting with anti- $G\alpha_o$ and anti-actin antibodies. Results are from one donor and are representative of three donors. D, naive $CD4^+$ T cells from cord blood were activated for 6 days with anti-CD3 and anti-CD28 under NP, T_H1 , T_H2 , or T_H17 -polarizing conditions as described under “Experimental Procedures.” -Fold differences among the samples in the expression of $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, and $G\alpha_{13}$ mRNAs, normalized to GAPDH mRNA, were calculated as described in the legend for Fig. 1. For each mRNA of interest, the lowest level of expression *versus* GAPDH from a single well was set at 1. Values cannot be compared between mRNA species. The data shown are the mean -fold differences \pm S.E. in the abundance of the indicated mRNAs assayed in duplicate for one donor and are representative of two donors. *, $p < 0.05$ when compared with $G\alpha_o$ mRNA abundance in T_H2 -polarized cells, which showed the lowest $G\alpha_o$ expression.

G_{α_o} Expression in Human Memory T Cells

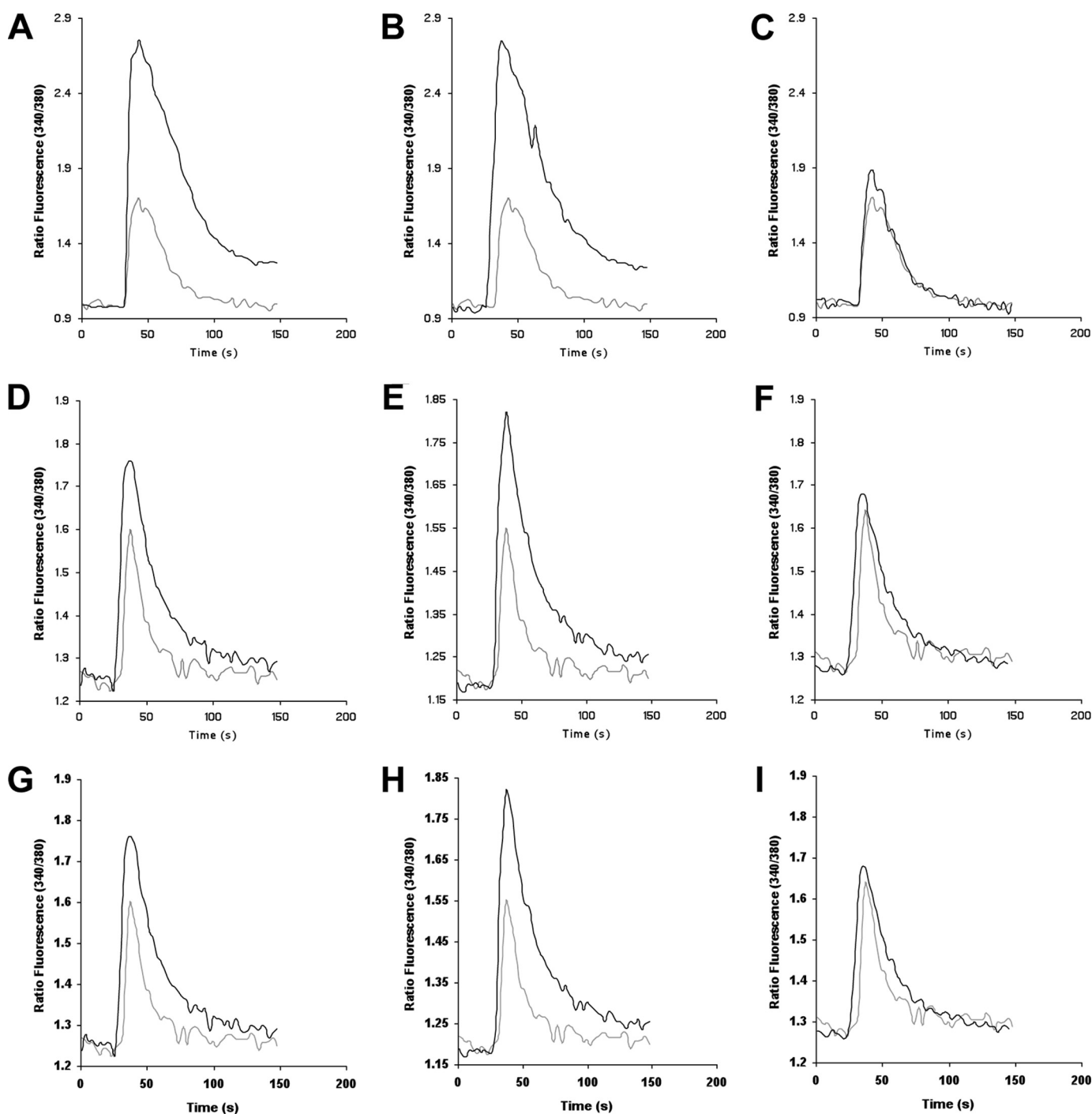


FIGURE 5. The human chemokine receptors, CCR6, CXCR6, and CXCR4 can couple to G proteins containing the chimeric $G_{\alpha_{qo5}}$. A–C, HEK293:CCR6 cells were transfected with plasmids encoding each of the chimeric G protein α -subunits, $G_{\alpha_{qo5}}$ (A), $G_{\alpha_{qi5}}$ (B), and $G_{\alpha_{qs5}}$ (C), and as a control, the empty vector, pcDNA3.1(+), as described under “Experimental Procedures.” D–F, in separate experiments, HEK293T cells were co-transfected with plasmids encoding CXCR6-YFP, each of the chimeric G protein α -subunits, $G_{\alpha_{qo5}}$ (D), $G_{\alpha_{qi5}}$ (E), and $G_{\alpha_{qs5}}$ (F), and as a control, the empty vector, pcDNA3.1(+). G–I, Jurkat T cells, which express CXCR4 endogenously, were transfected with plasmids encoding each of the chimeric G protein α -subunits, $G_{\alpha_{qo5}}$ (G), $G_{\alpha_{qi5}}$ (H), and $G_{\alpha_{qs5}}$ (I), and as a control, the empty vector, pcDNA3.1(+). After 48 h, cells were harvested, loaded with fura-2/AM, and assayed for intracellular calcium mobilization in response to treatment with the chemokine CCL20 (for CCR6), CXCL16 (for CXCR6), or CXCL12 (for CXCR4) at 1 μ g/ml, added at \sim 25 s. Fluorescence measurements were made as described under “Experimental Procedures.” Individual panels show intracellular calcium mobilization for cells transfected with a plasmid encoding the G protein chimera (black trace) compared with pcDNA3.1(+)-transfected control cells (gray trace) from one experiment each for HEK293:CCR6 cells (A–C), HEK293T cells (D–F), and Jurkat cells (G–I) and are representative of three experiments.

dues of $G_{\alpha_{oA}}$ and $G_{\alpha_{oB}}$ are identical (29), the $G_{\alpha_{qo5}}$ chimera serves to analyze responses common to both isoforms.

Transfection of an HEK293 cell line stably expressing CCR6 with a plasmid encoding $G_{\alpha_{qo5}}$ resulted in an increase in the

mobilization of intracellular Ca^{2+} in response to the CCR6 ligand, CCL20, when compared with cells transfected with empty vector (Fig. 5A), similar to the increase following transfection with a plasmid encoding $G_{\alpha_{qi5}}$ (Fig. 5B). CCR6 is a

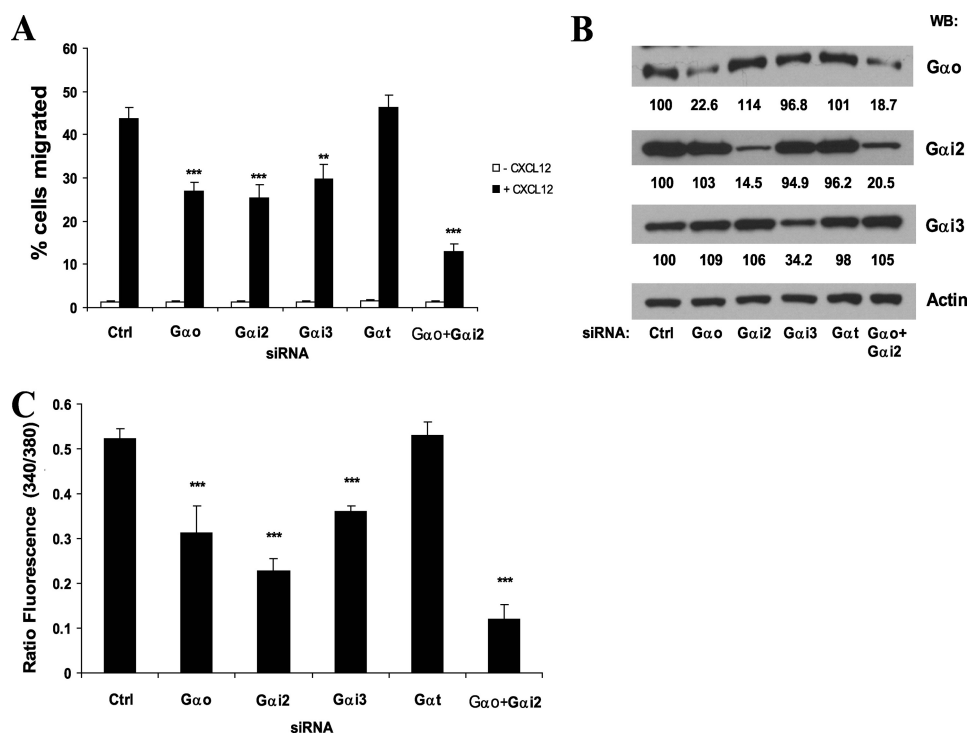


FIGURE 6. Knockdown of G α_o inhibits CXCL12-mediated chemotaxis and Ca $^{2+}$ flux in Jurkat cells. *A*, Jurkat cells transfected with the indicated SmartPool siRNAs were harvested 72 h later and used in duplicate in Transwell chemotaxis assays. Data shown are the mean percentage \pm S.E. of starting cells that migrated to either medium alone or CXCL12 (100 ng/ml) and are combined from six experiments. ***, $p < 0.001$; **, $p < 0.01$ when compared with CXCL12-dependent migration of cells transfected with control siRNA. *B*, lysates (15 μ g) of the cells used in the chemotaxis assays in *A* were resolved by SDS-PAGE and analyzed by Western blotting (WB) for the indicated G protein α -subunits and for actin as a loading control. Data shown are from a single experiment and are representative of three experiments. Actin is shown for only one of the three blots. For each blot, bands were analyzed by densitometry; the quantitative data, normalized to actin and then to control (Ctrl), which was set to 100, are shown below each lane. *C*, in parallel to chemotaxis assays, siRNA-transfected cells were analyzed for their ability to mobilize intracellular Ca $^{2+}$ in response to CXCL12 (1 μ g/ml) as described under "Experimental Procedures." Data shown are the mean ratio of fluorescence (340 nm:380 nm) \pm S.E. from three independent experiments. ***, $p < 0.001$ when compared with the CXCL12-dependent Ca $^{2+}$ flux of cells transfected with control siRNA.

receptor found on effector/memory T cells (36) and, in particular, on all cells of the T $_H$ 17 lineage (37–39). As expected, given that chemokine receptors do not couple to G $_s$ proteins (28), transfection with G α_{q5} had no effect on Ca $^{2+}$ mobilization (Fig. 5C). Western blots of cell lysates showed equivalent abundance of all three HA-tagged chimeric G proteins (data not shown). We performed similar experiments in 293T cells co-transfected with a plasmid encoding YFP-tagged CXCR6, a second chemokine receptor found on effector/memory T cells (40), and either control empty vector or plasmids encoding the chimeric G protein α -subunits as described above and treated with the CXCR6 ligand, CXCL16. As for CCR6, CXCR6 coupled to G α_{q5} (and G α_{q15})- but not G α_{q5} -containing G proteins (Fig. 5, D–F). Analogous experiments were performed in transfected Jurkat T cells, a human CD4 $^+$ T cell line, in which we found that the endogenously expressed chemokine receptor CXCR4, which is expressed on both naive and memory T cells, could also couple to both G α_{q15} - and G α_{q5} -containing G proteins to mobilize intracellular calcium (Fig. 5, G–I). The effects of expressing G α_{q5} or G α_{q15} on signaling through CXCR6 and CXCR4 were smaller than for CCR6, possibly because co-transfections were used to express CXCR6 and because of the lower efficiency of transfection for Jurkat versus HEK293T cells.

Knockdown of G α_o Inhibits CXCL12-dependent Chemotaxis and Ca $^{2+}$ Flux—To determine whether the presence of G α_o in T cell subsets was physiologically relevant, we used siRNA to knock down G α_o in Jurkat cells and examined these cells for their ability to migrate or mobilize intracellular Ca $^{2+}$ in response to CXCL12. Knockdown of G α_o resulted in a \sim 40% decrease in the number of cells that migrated to CXCL12 compared with that of cells transfected with either control siRNA or siRNA specific for G α_t , which is not expressed in these cells (Fig. 6A). We confirmed these data by knocking down G α_o with an independent G α_o -specific siRNA (23) (supplemental Fig. S3). Surprisingly, the effect of knockdown of G α_o on chemotaxis was similar to that of knockdown of either G α_{i2} or G α_{i3} (Fig. 6A). Chemotaxis of cells transfected with siRNAs against both G α_o and G α_{i2} was even more significantly inhibited than that of cells depleted of either protein alone. The efficiencies and specificities of these siRNAs in knocking down their targets were verified by Western blotting (Fig. 6B). We also examined the effect of knockdown of these G α_i subunits on CXCL12-

dependent Ca $^{2+}$ mobilization, which is a much more rapid functional response to stimulation of chemokine receptors than is chemotaxis. Knockdown of G α_o , G α_{i2} , or G α_{i3} had similar effects on the inhibition of CXCL12-induced Ca $^{2+}$ flux as compared with cells transfected with either control or G α_t -specific siRNAs (Fig. 6C). Consistent with our chemotaxis data (Fig. 6A), knockdown of both G α_o and G α_{i2} had a more dramatic effect on Ca $^{2+}$ flux than that observed in cells transfected with either siRNA alone (Fig. 6C).

We extended our findings by examining the effect of knockdown of G protein α -subunits in primary cells. We sorted naive and memory subsets of human CD4 $^+$ T cells from peripheral blood and transfected them with the appropriate siRNAs. Transfection of naive CD4 $^+$ T cells with siRNA against G α_o had no effect on their chemotaxis to CXCL12 (Fig. 7A), consistent with their lack of expression of G α_o . However, knockdown of G α_{i2} reduced CXCL12-mediated chemotaxis by \sim 35% compared with that of control cells (Fig. 7A). In contrast, knockdown of G α_o in memory CD4 $^+$ T cells had a similar effect to that of G α_{i2} knockdown on chemotaxis to CXCL12 (Fig. 7A). As before, the specificities of the siRNAs were verified by Western blot analysis (Fig. 7B). These data suggest that the contribution

$G\alpha_o$ Expression in Human Memory T Cells

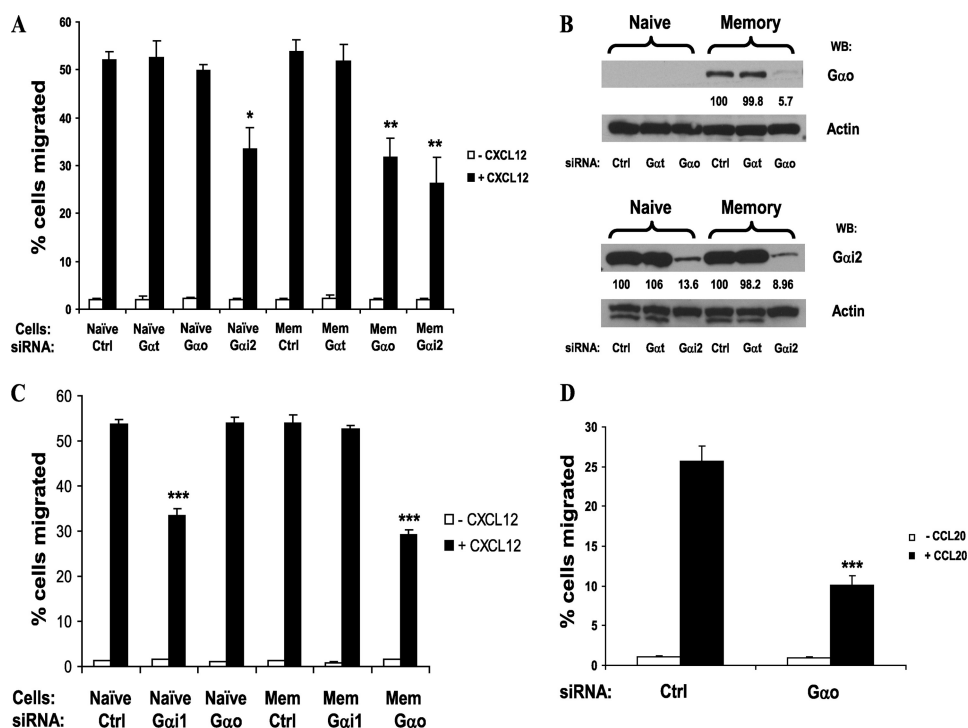


FIGURE 7. $G\alpha_o$ and $G\alpha_{i1}$ contribute, respectively, to chemotaxis of effector/memory and naive $CD4^+$ T cells. *A*, naive and memory $CD4^+$ T cells purified by cell sorting from peripheral blood were transfected with the indicated SmartPool siRNAs and harvested after 72 h. Chemotaxis assays were performed as described in the legend for Fig. 6. Data shown are the mean percentage \pm S.E. of starting cells that migrated to either medium alone or CXCL12 (100 ng/ml) and are combined from three experiments. *, $p < 0.05$ when compared with control (Ctrl) siRNA-transfected naive cells; **, $p < 0.01$ when compared with control siRNA-transfected memory cells. *B*, lysates (15 μ g) of siRNA-transfected naive and memory $CD4^+$ T cells were analyzed by Western blotting (WB) for the indicated G protein α -subunits and for actin as a loading control. Data shown are from one of two experiments. For each blot, bands were analyzed by densitometry and the quantitative data, normalized to actin and then to the matching Ctrl, which was set to 100, are shown below each lane having a detectable band. *C*, naive and memory $CD4^+$ T cells were purified, transfected, and analyzed in chemotaxis assays as in *A*. Data shown are the mean percentage \pm S.E. of starting cells that migrated to either medium alone or CXCL12 (100 ng/ml) from duplicate wells of one experiment and are representative of three experiments. ***, $p < 0.001$ when compared with the CXCL12-mediated migration of the matching control siRNA-transfected naive or memory cells. *D*, cord blood naive $CD4^+$ T cells were cultured under T_H17 -polarizing conditions after which CCR6-expressing cells were purified with magnetic beads. The purified cells were transfected with the indicated siRNAs and, after 72 h, analyzed in chemotaxis assays as described in *A* using the CCR6 ligand, CCL20. Data shown are the mean percentage \pm S.E. of starting cells that migrated to either medium alone or CCL20 (100 ng/ml) from experiments with three donors. ***, $p < 0.001$ when compared with the CCL20-mediated migration of the control siRNA-transfected cells.

of $G\alpha_o$ to chemokine-dependent migration of human memory $CD4^+$ T cells is equivalent to that of the abundant $G\alpha_{i2}$.

In separate experiments to test the effect of knockdown of $G\alpha_o$ on primary human memory $CD4^+$ T cells, we used siRNA specific for $G\alpha_{i1}$ as a negative control because $G\alpha_{i1}$ had not been previously detected in lymphocytes (26, 27). We were therefore surprised to find that knockdown of $G\alpha_{i1}$ in purified naive $CD4^+$ T cells led to a $\sim 35\%$ decrease in CXCL12-dependent chemotaxis compared with that of control naive cells, whereas knockdown of $G\alpha_{i1}$ in memory $CD4^+$ T cells had no effect (Fig. 7C). In agreement with our previous data, siRNA-mediated knockdown of $G\alpha_o$ inhibited CXCL12-dependent chemotaxis of memory, but not naive, $CD4^+$ T cells (Fig. 7C). Because we could not identify a $G\alpha_{i1}$ band on Western blots of naive $CD4^+$ T cells using commercial antibodies (data not shown), we verified the specificity of siRNA-mediated knockdown of the appropriate G protein α -subunits by real-time RT-PCR (data not shown). Together, these data suggest that, analogous to the role of

$G\alpha_o$ in memory $CD4^+$ T cell subsets, the minor G protein α -subunit $G\alpha_{i1}$ makes a substantial contribution to chemotactic responses in naive $CD4^+$ T cells.

To extend our findings for $G\alpha_o$ in primary cells, we cultured cord blood naive $CD4^+$ T cells under T_H17 -polarizing conditions and then used magnetic beads to purify cells that expressed CCR6, which is expressed on all T_H17 cells (39). Purified cells were transfected with control or $G\alpha_o$ -specific siRNAs and subjected to chemotaxis assays with the CCR6 ligand, CCL20. Knockdown of $G\alpha_o$ resulted in a $\sim 60\%$ reduction in migration (Fig. 7D), indicating that in addition to coupling to CXCR4, $G\alpha_o$ -containing G proteins also couple to CCR6 to mediate chemotaxis in primary $CD4^+$ T cells.

Comparative Analysis of the Expression of G Protein α -Subunit mRNAs Reveals the Enrichment of $G\alpha_o$ and the Decline of $G\alpha_{i1}$ in Memory T Cell Subsets—To make direct comparisons among the different G protein α -subunit mRNAs by RT-PCR, we first established that the corresponding sets of primers and probes showed equal efficiencies of amplification from plasmids containing each of the $G\alpha$ cDNA sequences (data not shown). We then used these sets of primers and probes to analyze samples from sorted populations of T cells. Our data are summarized in Fig. 8. $G\alpha_{i2}$

mRNA was the most abundant G protein α -subunit mRNA in all subsets analyzed. $G\alpha_o$ mRNA abundance in $CD4^+$ effector/memory cells was $\sim 2\%$ of that of $G\alpha_{i2}$ mRNA. As mentioned previously, the abundance of $G\alpha_o$ mRNA was generally higher in naive $CD8^+$ T cells than in their $CD4^+$ T cell counterparts, and similar (or sometimes higher) -fold increases in the abundance of $G\alpha_o$ mRNA were observed during $CD8^+$ T cell differentiation compared with that of $CD4^+$ T cells. These findings are reflected in $G\alpha_o$ mRNA being found at a higher percentage of that of $G\alpha_{i2}$ mRNA in $CD8^+$ T cells as compared with $CD4^+$ T cells (Fig. 8, C and D). Of interest, $G\alpha_{i1}$ mRNA was not detectable in most of the subsets analyzed but was expressed at low abundance in naive subsets of $CD4^+$ and $CD8^+$ T cells (Fig. 8, A and B), which is consistent with the effect of $G\alpha_{i1}$ -specific siRNA on the migration of naive, but not memory, $CD4^+$ T cells (Fig. 7C). The amount of $G\alpha_{i1}$ mRNA was substantially lower in the more differentiated cells, sometimes below the limit of

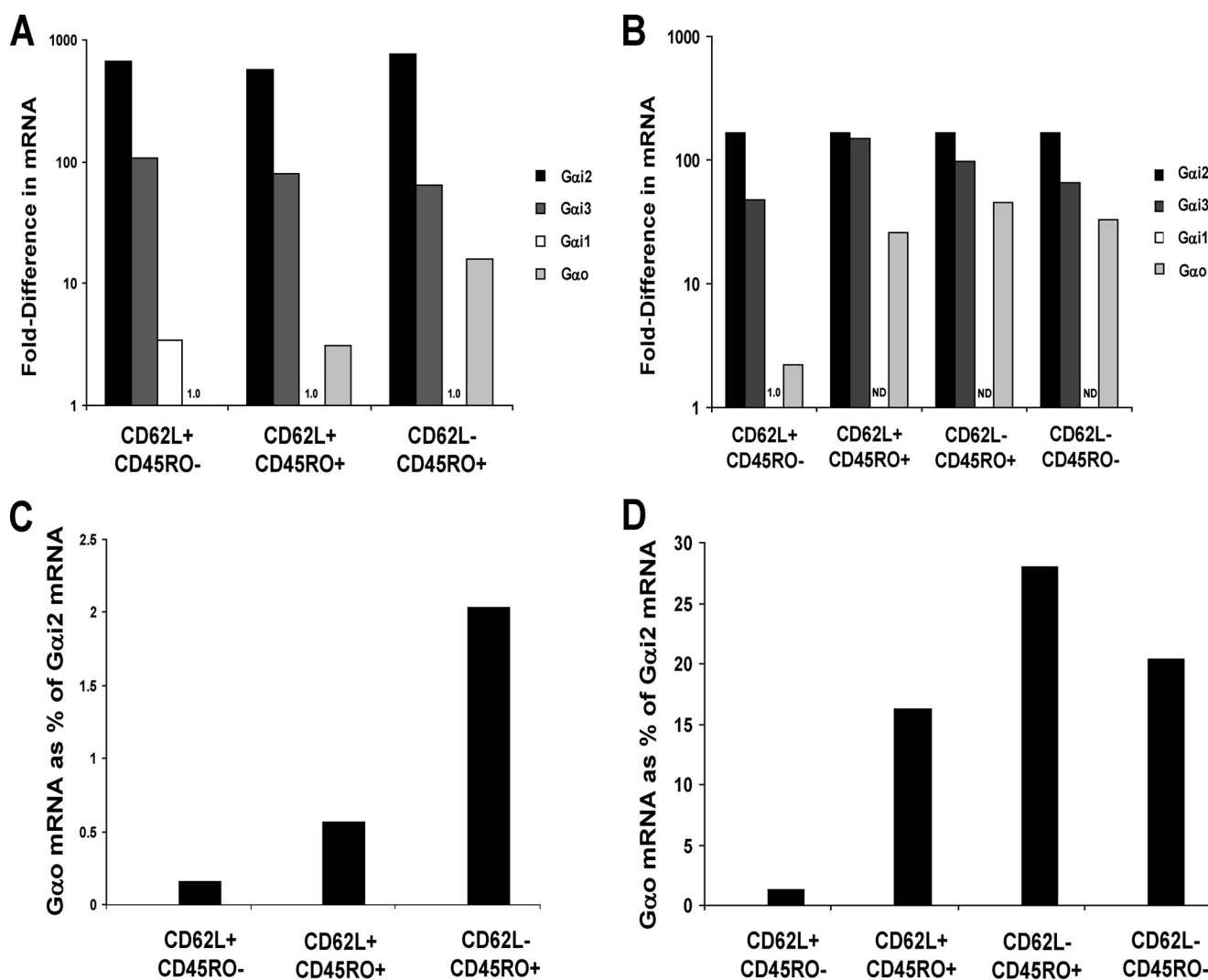


FIGURE 8. Relative amounts of $G\alpha$ subunit mRNAs. $CD4^+$ and $CD8^+$ T cell subsets were purified by cell sorting from peripheral blood and analyzed by semiquantitative real-time RT-PCR for the expression of mRNAs for the indicated G protein α -subunits and GAPDH for determining ΔC_T values. *A*, -fold differences in mRNAs in $CD4^+$ T cell subsets. Within each subset of cells, the -fold differences in expression were calculated versus the mRNA with the lowest expression (value set to 1), as shown above the positions for $G\alpha_o$ in the $CD62L^+CD45RO^-$ cells and $G\alpha_{i1}$ in the other subsets. *B*, -fold differences in mRNAs in $CD8^+$ T cell subsets. Within the $CD62L^+CD45RO^-$ subset, the -fold differences were calculated versus the mRNA for $G\alpha_{i1}$. For the other subsets, because the mRNA for $G\alpha_{i1}$ was not detectable (ND), the values for $G\alpha_{i3}$ and $G\alpha_o$ were normalized to the value for $G\alpha_{i2}$, which was set in each case equal to the $G\alpha_{i2}$ value in the $CD62L^+CD45RO^-$ subset. *C*, $G\alpha_o$ mRNA as a percentage of $G\alpha_{i2}$ mRNA in $CD4^+$ T cell subsets. *D*, $G\alpha_o$ mRNA as a percentage of $G\alpha_{i2}$ mRNA in $CD8^+$ T cell subsets. Data shown are for one donor and are representative of three donors for each group of $CD4^+$ or $CD8^+$ T cell subsets.

detection, especially in $CD8^+$ T cells. Despite our inability to detect $G\alpha_{i1}$ in Western blots of T cell protein, these findings raise the interesting possibility of a reciprocal relationship between $G\alpha_o$ and $G\alpha_{i1}$ mRNA expression in human T cell subsets.

DISCUSSION

As far as we are aware, ours is the first description of the expression of $G\alpha_o$ in hematopoietic cells and the first description of differences in the content of $G\alpha$ subunits in memory cells as compared with naive T cells. We have shown that among the major subsets of leukocytes in human peripheral blood, expression of $G\alpha_o$ was limited to T cells, and that, in contradistinction to other $G\alpha$ subunits, expression of $G\alpha_o$ was correlated with increasingly differentiated phenotypes, with the most dramatic increment occurring in

the naive to T_{CM} transition. Our mRNA data and knock-down experiments also suggest that expression of $G\alpha_{i1}$ shows a reciprocal profile. Beyond the immune system, we believe that these are the first examples reported of changes in the repertoire of $G\alpha_{i/o}$ subunits as part of pathways of cellular differentiation in the adult.

$G\alpha_o$ is highly abundant in the central nervous system, so much so that it constitutes ~ 0.5 – 1.0% of the plasma membrane protein of the brain (41, 42). Its expression has been reported to be restricted to the central nervous system, the endocrine system, and the heart, and $G\alpha_o$ cannot be detected in mouse spleen or thymus (29, 43). Two main forms of $G\alpha_o$ are expressed, $G\alpha_{oA}$ and $G\alpha_{oB}$, resulting from alternative splicing of mRNA, which produces differences in the C-terminal third of each protein (29, 31, 32, 44). In addition, there are two further isoforms of $G\alpha_o$, $G\alpha_{oC}$ and $G\alpha_{oD}$, which are

$G\alpha_o$ Expression in Human Memory T Cells

derived from posttranslational modifications of $G\alpha_{oA}$ and $G\alpha_{oB}$, respectively (45).

Within the nervous system, $G\alpha_o$ activates neuronal Ca^{2+} and K^+ channels (46–50). Mice defective in $G\alpha_o$ show abnormalities that include tremors, impaired olfactory behavior, and decreased survival of neurons in the basal vomeronasal organ, as well as defective ion channel regulation in the heart (43, 51–53). In contrast to $G\alpha_o$, $G\alpha_{i1}$ is more widely distributed (54), although it also has not been detected previously in mouse lymphocytes (26) or, to our knowledge, in human lymphocytes. Apparently, mice deficient in $G\alpha_{i1}$ exhibit no abnormalities (55). $G\alpha_{i2}$ and $G\alpha_{i3}$ are considered the major $G\alpha_i$ subunits in hematopoietic and immune cells given their expression in mouse T cells (56) and the immunological, proinflammatory phenotype of $G\alpha_{i2}$ -deficient mice (57, 58).

We found that by contrast to brain (and other tissues (29)), human T cells contained more $G\alpha_{oB}$ than $G\alpha_{oA}$ mRNA; these are the first cells, to our knowledge, described as showing this pattern of differential splicing. We recapitulated differentiation-associated induction of $G\alpha_o$ in T cells by activating naive cord blood cells *in vitro* through the T cell receptor (TCR), and have shown that, just as for many aspects of T cell differentiation, induction of $G\alpha_o$ depended both on activation and on the cytokine environment. Of particular interest, we found preferential expression of $G\alpha_o$ under conditions inducing T_H17 cells, which have recently been recognized as a separate lineage of effector/memory $CD4^+$ T cells important for autoimmune disease (34). We have provided the first evidence that $G\alpha_o$ -containing G proteins can couple to chemokine receptors that are expressed on T cells, including the T_H17 -associated receptor, CCR6 (37–39). Finally, we have shown that siRNA-mediated depletion of $G\alpha_{i1}$ in primary naive $CD4^+$ T cells and of $G\alpha_o$ in memory $CD4^+$ T cell subsets inhibited CXCR4-mediated migration to a similar extent to knocking down the major $G\alpha$ subunit, $G\alpha_{i2}$. These data suggest that CXCR4, and perhaps other GPCRs, switch their $G\alpha$ subunit usage from $G\alpha_{i1}$ in naive $CD4^+$ T cells to $G\alpha_o$ in memory $CD4^+$ T cell subsets and that the contribution of these $G\alpha$ subunits to GPCR-mediated functions belies their relatively low abundance in these cells. The discordance between functional importance and relative abundance was also reported for $G\alpha_q$ as compared with $G\alpha_{i1}$ in HeLa cells (23). It is notable that in those experiments, knocking down some species of $G\alpha_{i/o}$ subunits resulted in “compensatory” increases in others (23). We did not observe such changes among the limited number of $G\alpha$ proteins that we analyzed, perhaps because we harvested cells at only 3 days after transfection with siRNA and not the 5 days used in the previous study and/or because of cell type-specific differences.

We presume that $G\alpha_o$ and $G\alpha_{i1}$ have roles in T cells beyond chemokine receptor signaling. One possible role for $G\alpha_o$ would be in signaling from GPCRs such as species of opioid, muscarinic acetylcholine (mAChR), and somatostatin receptors, which are expressed in the central nervous system, have been shown to use $G\alpha_o$ (59–61), and can be expressed by T cells (62–69).

The published data on downstream targets of activated $G\alpha_o$ raise some additional, interesting possibilities as to specific roles for $G\alpha_o$ in memory T cells. For example, channels with

characteristics of L-type Ca^{2+} channels are found in T cells, and inhibitor studies have shown that these channels play a role in mediating TCR-induced lymphocyte activation and proliferation both *in vitro* and *in vivo* (70, 71). Given the documented, specific role for $G\alpha_o$ in regulating these channels in the heart (43), it is possible that $G\alpha_o$ contributes to the differences in Ca^{2+} signals, and other consequences of TCR activation that distinguish memory from naive T cells (72). Connections between chemokine receptors, G proteins, and the TCR are suggested by reports that CXCL12 acts as a costimulator for the activation of $CD4^+$ T cells (73) and that CXCR4 activates ζ chain-associated protein kinase of 70 kDa (Zap70) (74) and associates directly with the TCR (75). In addition, $G\alpha_{i2}$ mediates inhibition of TCR-induced Ca^{2+} signals and cytokine production (27).

Finally, $G\alpha_o$ is thought to mediate the activities of Wnt receptors (frizzled proteins) in mouse cells (76) and in *Drosophila* (77, 78), and the effectors of the Wnt pathway, β -catenin and lymphoid-enhancing factor/T cell factor proteins, are important, respectively, for thymocyte survival (79) and T cell differentiation (80). A possible role for ($G\alpha_o$ -mediated) Wnt signaling in the survival, proliferation, or both of memory T cells is intriguing but as yet unexplored. However, the accumulating data that chemokine receptors are involved in stimulating cell growth and proliferation (81, 82), and that GPCRs other than frizzled proteins can activate components of the Wnt pathway (83) raise the possibility that chemokine receptor signaling through $G\alpha_o$ to the β -catenin pathway in memory T cells could contribute to their proliferation. However, given the lack of $G\alpha_o$ protein in mouse lymphocytes, any of the possible roles that we have suggested for $G\alpha_o$ would by necessity be specific to human T cells, representing a clear difference between the immune system in humans and mice.

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$G\alpha_o$ Expression in Human Memory T Cells

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