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The Identification, Characterization, and Distribution of Guinea Pig CCR4 and Epitope Mapping of a Blocking Antibody*

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Louise A. Jopling‡, Ian Sabroe‡, David P. Andrew§, Tracey J. Mitchell‡, You Li§, Martin R. Hodge§, Timothy J. Williams‡, and James E. Pease‡¶

From the ‡Leukocyte Biology Section, Biomedical Sciences Division, Faculty of Medicine, Imperial College of Science, Technology and Medicine, London SW7 2AZ, United Kingdom and §Millennium Pharmaceuticals Inc., Cambridge, Massachusetts 02139

Th2 lymphocytes play a central role in the control and maintenance of allergic inflammation. The chemokine receptor CCR4 is preferentially expressed on the surface of Th2 lymphocytes polarised *in vitro*. However, CCR4 is found on the surface of a significant proportion of circulating memory T lymphocytes, some of which are capable of producing the Th1-associated cytokine interferon γ. To investigate the function of CCR4 on guinea pig (gp) T lymphocytes, we identified the open-reading frame of gpCCR4, which encodes a 361-amino acid protein with 88 and 81% amino acid identity to human and murine CCR4 sequences, respectively. Cells transfected with gpCCR4 migrated toward the human and murine orthologues of the CCR4 ligands, macrophage-derived chemokine and thymus and activation-regulated chemokine. Surface expression of CCR4, using an anti-human CCR4 monoclonal antibody, 10E4, was detected on ~12% of guinea pig peripheral blood T helper cells, and CCR4$^+$ guinea pig thymocytes were detected in low numbers. However, CCR4$^+$ T helper cells constituted ~9% of the T lymphocyte population within the normal guinea pig lung and 52% of the guinea pig bronchoalveolar lavage fluid, which is consistent with a role for CCR4 in T lymphocyte development and trafficking through normal tissues. Subsequent analysis of chimeric chemokine receptors indicated that 10E4, a functional inhibitor of gpCCR4 responses, recognized the amino terminus of CCR4.

Allergic inflammation of the lung in diseases such as asthma is largely controlled and maintained by the recruitment of antigen-specific T lymphocytes to the lung tissue (1). The Th2-type lymphocytes that predominate at sites of allergic inflammation secrete cytokines such as IL-4, IL-5 and IL-13,[1] which influence the actions of other leukocyte populations involved in the inflammatory response (2, 3). For example, IL-4 and IL-13 can induce B lymphocyte production of IgE (4, 5) and airway epithelial cell generation of eotaxin, which is central to eosinophil recruitment (6, 7), whereas IL-5 is a potent eosinophil maturation and survival factor (8, 9). Overexpression of IL-13 in the lung causes many of the histopathological and physiological features of asthma (10).

Increasing evidence suggests that chemokines are important regulators of the selective trafficking of leukocytes in homeostasis, hematopoiesis, and inflammation (11). Chemokines can be divided into four subfamilies dependent upon the position of their amino-terminal cysteine residues; the two main chemokine groups are the CXCL and the CCL subfamilies (12), which signal via CXCR and CCR receptors belonging to the G-protein coupled receptor superfamily (13, 14). The differential expression of chemokine receptors on the surface of leukocytes contributes to the selective recruitment of cells to specific sites, and, consequently, these receptors may provide useful targets for therapeutic intervention in the modulation of inflammatory disorders. Studies of T lymphocytes polarized *in vitro* have shown that Th1 cells preferentially express CCR5 and CXCR3 whereas Th2 cells preferentially express CCR3, CCR4, and CCR8 (15–17). However, recent data have shown that CCR4 is highly expressed on a significant proportion of circulating CD4$^+$ T lymphocytes, and this receptor can be coexpressed with both CXCR3 and CCR5 (18). In addition, some of the CCR4$^+$ memory T lymphocytes are also capable of producing interferon γ (18). Campbell *et al.* (19) showed that the majority of T lymphocytes expressing the cutaneous lymphocyte-associated antigen isolated from skin lesions of patients with Th1- and Th2-associated skin diseases express high levels of CCR4. Vestergaard *et al.* (20) further demonstrated that the NC/Nga strain of mice, capable of developing atopic dermatitis-like lesions when not maintained under specific pathogen-free conditions, showed increased TARC expression within these lesions. Together these data suggest that CCR4 and its ligands play a role in the specific recruitment of T lymphocytes to sites of cutaneous inflammation.

Evidence exists suggesting that CCR4 plays a role in T lymphocyte migration during allergic lung inflammation *in vivo*, however, this role remains controversial. Murine models of pulmonary inflammation have demonstrated that blockade of the CCR4 ligands MDC and TARC significantly reduced lung inflammation (21, 22) with the MDC·CCR4 interaction re-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF431971.

¶ To whom correspondence should be addressed: Leukocyte Biology Section, Biomedical Sciences Division, Sir Alexander Fleming Bldg., Faculty of Medicine, Imperial College of Science, Technology and Medicine, Exhibition Rd., London, SW7 2AZ, United Kingdom. Tel.: 44-207-594-3162; Fax: 44-207-594-3119; E-mail: j.pease@ic.ac.uk.

[1] The abbreviations used are: IL, interleukin; BALF, bronchoalveolar lavage fluid; ORF, open reading frame; MDC, macrophage-derived chemokine; TARC, thymus and activation-regulated chemokine; gp, guinea pig; RPE, Rhodamine phycoerythrin; HA, hemagglutinin; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; mAb, monoclonal antibody; hu, human; mu, murine; PBS, phosphate-buffered saline; PBMC, peripheral blood mononuclear cells.

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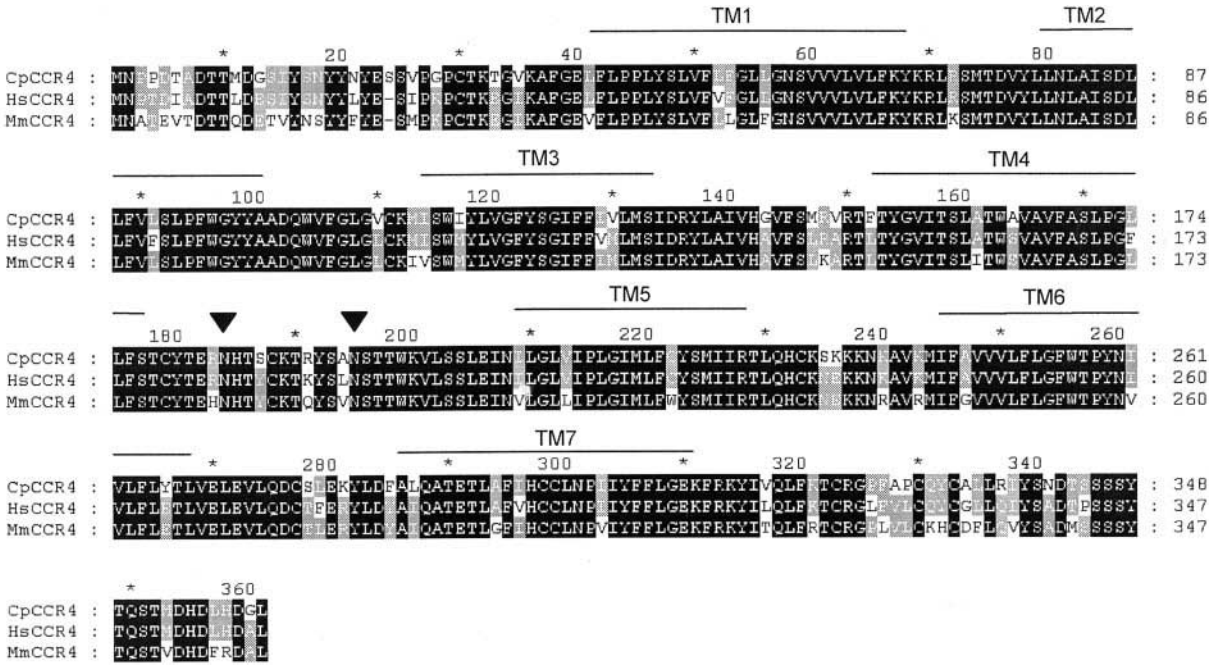

FIG. 1. **Amino acid alignment of human (*Hs*), murine (*Mm*), and guinea pig (*Cp*) CCR4 sequences.** Residues in *black boxes* are identical between all three species, those in *gray* are conserved between species, and those in *white* are unique to one or more species. *TM1–7* represent the deduced transmembrane spanning domains of CCR4. Putative *N*-linked glycosylation sites of guinea pig CCR4 are represented by a *closed triangle*. Human and murine CCR4 share 86% identity, human and guinea pig CCR4 share 88% identity, and murine and guinea pig CCR4 share 81% identity.

quired throughout later stages of allergic lung disease (23). In contrast to this, CCR4-deficient mice showed no significant difference in predisposition to allergic lung inflammation compared with wild-type mice (24) perhaps supporting the existence of an additional MDC receptor as other researchers have proposed (25). In humans, examination of chemokine receptor expression on T lymphocytes isolated from the bronchoalveolar fluid (BALF) of asthmatics or non-asthmatic individuals indicated that CCR4 is expressed at similar levels (5%) in each of these groups (26). However, Panina-Bordignon *et al.* (27) demonstrated that, following allergen challenge of asthmatics, significant up-regulation of CCR4, and to a lesser extent CCR8, was observed in the lung tissue, with a corresponding up-regulation of MDC and TARC expressed on the airway epithelium. Therefore, the development of further tools to unravel the precise role of CCR4 in allergic lung inflammation is required. We have identified, characterized, and investigated the functional expression of the guinea pig homologue of CCR4 for future use in a well established *in vivo* model of allergic lung inflammation.

EXPERIMENTAL PROCEDURES

Cloning the Open Reading Frame of the Guinea Pig CCR4 Gene—A 550-bp PCR fragment of gpCCR4 was amplified by the PCR from guinea pig genomic DNA using oligonucleotides based on highly conserved regions of human and murine CCR4 sequences (28, 29). The oligonucleotide sequences were as follows: sense gpCCR4, 5′-GCG CAT AAG CTT TTC CTG GAT GTA CTT GGT GGG CTT TT-3′; antisense gpCCR4, 5′-GCA TAT CTC GAG CAG AGT TTC TGT GGC CTG GAT GGC-3′. The resultant, partial gpCCR4 sequence was used to screen a guinea pig genomic DNA library in the vector EMBL 3 (CLONTECH, Palo Alto, CA) as previously described (30). Briefly, three rounds of screening were carried out to isolate a pure bacteriophage clone that hybridized with the gpCCR4 fragment probe. 1 μg of bacteriophage DNA was digested with 5 units of *Eco*RI for 4 h at 37 °C. DNA fragments were separated by gel electrophoresis and "shotgun" cloned into pUC19 (CLONTECH). Two fragments of ~2.2 and 1.5 kb were cloned and sequenced using an ABI 377 sequencer (Applied Biosystems, Foster City, CA). The minor fragment contained 150 bp of the open reading frame (ORF) of gpCCR4, which terminated at an *Eco*RI site, and the major fragment contained the remaining 933 bp of the gpCCR4 ORF. Based on these sequences, a 1.1-kb single-exon ORF was identified and primers were designed to incorporate a 5′ HA tag (31) plus *Hin*dIII restriction site and a 3′ *Xho*I restriction site to facilitate subcloning. PCR amplification of guinea pig genomic DNA yielded a complete gpCCR4 DNA sequence, which contained an *Eco*RI site at the predicted position. Products from duplicate PCR reactions were subcloned into pcDNA3 (Invitrogen, Groningen, The Netherlands), and sequencing of both products yielded an identical consensus sequence. Primer sequences: sense, 5′-ATA TAG CGC **AAG CTT** *GCC ACC* ATG <u>TAT CCA TAT GAT GTC CCA GAT TAT GCC</u> AAC CCC CCT GAC ACA GCA GAC-3′; antisense, 5′-ATA TCG CGA **CTC GAG** ATA TTA CAA CCC ATC ATG GAG ATC ATG-3′ (bases in boldface indicate restriction enzyme sequences, those underlined indicate the HA epitope-tag sequence, and those in italics indicate the Kozak sequence for enhanced translation of the gene).

Generation of a Cell Line Stably Transfected with gpCCR4—20 μg of the construct gpCCR4.pcDNA3 was linearized with *Pvu*I, and transfection of the L1.2 cells was carried out as previously described (30). Briefly, the L1.2 cells were electroporated in the presence of linearized DNA and subsequently cultured at 37 °C in RPMI supplemented with 10% fetal calf serum. After 48 h, geneticin (G418) was added at a final concentration of 0.8 mg/ml and the cells were then cultured for 2–3 weeks. Cells exhibiting a chemotactic response to 50 nM murine MDC using a Transwell chemotaxis assay were cloned out by limiting dilution and subsequently expanded.

Antibodies—A panel of unconjugated anti-human CCR4 antibodies was used in this study (hybridomas 2B10 (18), 4B10, 5E5, 6B11, 7G7, and 10E4). Mouse IgG1 and IgG2a control antibodies (clones MOPC-21 and UPC-10, respectively) were obtained from Sigma-Aldrich (Poole, UK). FITC-conjugated anti-guinea pig-CD8, -Pan T cell, and -T helper subset antibodies were obtained from Serotec (Oxford, UK). Rabbit anti-mouse $F(ab)'_2$-RPE secondary antibody was obtained from Dako (Ely, UK).

Chemotactic Migration of gpCCR4-transfected Cells—12–18 h prior to chemotaxis experiments, chemokine receptor transfectant L1.2 cells were stimulated with 10 mM *n*-butyric acid to stabilize receptor expression (32). The wells of 96-well ChemoTx plates with 5-μm pore size (Receptor Technologies, Adderbury, UK) were "blocked" with RPMI + 1% BSA for 30 min at 37 °C, to reduce the nonspecific binding of chemokines to the assay plate. The assays were carried out according to manufacturer's instructions and as described previously (33).

In studies to investigate the inhibition of chemotaxis by specific

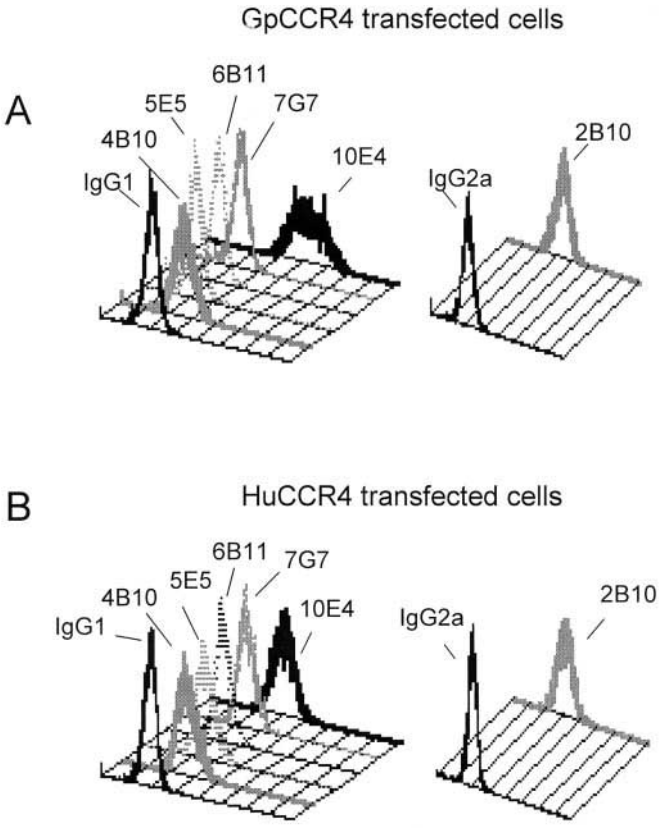

FIG. 2. **CCR4 expression of gpCCR4 and huCCR4 transfected L1.2 cells.** Flow cytometric analysis of CCR4 expression on (*A*) gpCCR4-transfected L1.2 cells or (*B*) huCCR4-transfected L1.2 cells. Surface CCR4 expression can be detected after staining transfected cells with 10 μg/ml of the anti-huCCR4 antibodies indicated compared with isotype control staining. Data shown are representative of six experiments.

mAbs, the Transwell chemotaxis assay system was used (30). 24-well tissue culture plates were blocked as before, and cells were resuspended at a density of 1×10^6/ml in assay buffer and preincubated for 10 min at room temperature with 20 μg/ml of either isotype control or the anti-CCR4 antibody 10E4, prior to placement within the Transwell. Antibodies were present throughout the duration of the assay. Chemokine dilutions were made up to 300 μl/well, and 200 μl of cell suspension was added to the Transwell and incubated at 37 °C for 5 h in the presence of 5% CO_2 under humidified conditions, after which time the number of cells migrating into the lower chamber was quantified by flow cytometry (30).

Radiolabeled Ligand Binding to gpCCR4 Transfectants—Stable transfectants expressing gpCCR4 or huCCR4 were butyrate-treated overnight, as above. The cells were washed in RPMI + 0.5% BSA + 25 mM HEPES (pH 7.4) (binding buffer), counted, and resuspended in binding buffer at a density of 2×10^7 cells/ml. The assay was carried out as previously described (34). Briefly, cells were incubated with 0.1 nM ^{125}I-huMDC and increasing concentrations of unlabeled huMDC or muMDC for 60 min at room temperature. Following centrifugation through Nyosil oil (Nye, Fairhaven, MA), cell-bound chemokine was determined using a Canberra Packard Cobra 5010-counter (Canberra Packard, Pangebourne, UK). Approximately 28% of ^{125}I-huMDC was bound by huCCR4-transfected cells in the absence of competitor whereas 6% was bound by gpCCR4-transfected cells. Scatchard analysis of data was performed using MacLigand (35).

Isolation of Guinea Pig PBMCs—Naïve guinea pigs were killed by CO_2 inhalation, and blood was immediately taken via cardiac puncture into a 15-ml polypropylene tube containing EDTA at a final concentration of 10 mM. The blood was centrifuged at $300 \times g$ for 20 min, and the upper platelet-rich plasma was removed and discarded. The remaining buffy coat was mixed with 9% dextran (Amersham Biosciences, Inc., Uppsala, Sweden) in saline, and 0.9% saline, at a ratio of 3:1:4, respectively, and incubated at room temperature for 40 min. The upper leukocyte-rich layer was removed and centrifuged at $350 \times g$ for 5 min. The cell pellet was then resuspended in 2 ml of PBS (without Ca^{2+}/Mg^{2+}) and overlaid onto a two-layer 70%/80% Percoll/PBS gradient and centrifuged at $400 \times g$ for 25 min. The mononuclear cells were removed from the upper layer of the gradient, washed in PBS, and counted using a hemacytometer, prior to use.

Isolation of Mononuclear Cells from Guinea Pig Tissues—Naïve guinea pigs were killed by CO_2 inhalation prior to dissection of the thymus and spleen, or by intraperitoneal anesthetic overdose prior to dissection of the airways and lung tissue. To collect bronchoalveolar fluid (BALF), the trachea was cannulated and the lungs were lavaged with 3 × 10-ml fractions of PBS + 10 mM EDTA, which were subsequently pooled after collection. BALF cells were pelleted by centrifugation and resuspended in staining buffer (PBS + 0.25% BSA). To isolate tissue cells, samples were roughly chopped into 2- to 3-mm^3 pieces and disrupted by passage through a 50-μm pore size tissue disaggregator for 60 s (Medimachine, Dako, Ely, UK). The isolated cells were then washed into staining buffer prior to the analysis of surface chemokine receptor expression by flow cytometry.

Analysis of Chemokine Receptor Expression by Flow Cytometry—Human CCR4- or gpCCR4-transfected L1.2 cells were incubated with 10 μg/ml isotype control antibody or 10 μg/ml anti-CCR4 antibodies in staining buffer (PBS + 0.25% BSA) for 30 min on ice, washed by the addition of 1 ml of cold staining buffer, and centrifuged at $350 \times g$ for 5 min, then they were stained with goat anti-mouse FITC-conjugated $F(ab)'_2$ for 20 min on ice in the dark. Cells were then washed and resuspended in 500 μl of staining buffer for acquisition on a FACSCalibur flow cytometer (Becton Dickinson, Mountainview, CA).

To investigate surface expression of CCR4 in guinea pig blood, 50-μl aliquots of whole blood were washed in staining buffer and incubated on ice for 30 min with primary or isotype control antibody at a final concentration of 10 μg/ml. Samples were washed, and bound antibody was detected using RPE-conjugated $F(ab)'_2$ rabbit anti-mouse Ig, which had been pre-absorbed with 10% guinea pig serum (Sigma-Aldrich, Poole, UK) on ice for 15 min prior to use. Samples were washed and then incubated with an excess of mouse IgG (50 μg/ml) (Sigma-Aldrich, Poole, UK) for 5–10 min. Samples were then incubated with FITC-conjugated antibodies (mouse IgG1, anti-gp Pan T cell, anti-gp T helper subset, or anti-gp CD8 antibodies (all at 1:20 dilution)). A final wash step was carried out, followed by erythrocyte lysis using 1 ml of 1× FACSLyse (Becton Dickinson) per sample according to the manufacturer's instructions. Samples were analyzed on a FACSCalibur flow cytometer, using appropriate compensation settings between fluorochromes.

To investigate CCR4 expression on T lymphocyte subsets from various tissues, two-color flow cytometry was carried out on isolated thymocytes, splenocytes, lung tissue cells, and BAL cells as described above, with the omission of the final lysis step.

Down-regulation of gpCCR4 from the Surface of Stably Transfected L1.2 Cells and Guinea Pig PBMCs—Butyrate-treated gpCCR4 stably transfected L1.2 cells or purified gpPBMCs were washed in ice-cold PBS + 0.25% BSA (assay buffer) and resuspended at a density of 1×10^7cells/ml. 5×10^5 cells were pipetted into pre-chilled Eppendorf tubes and incubated with 50 μl of buffer or chemokines in duplicate, and then incubated at 37 °C for 60 min. Control samples were incubated with buffer or the highest concentration of chemokine for 60 min on ice. The cells were then immediately chilled and washed in assay buffer. The duplicate samples of each condition were stained either with the anti-human CCR4 antibody (10E4) or with the IgG1 isotype control antibody to determine if any nonspecific binding was altered under different stimulation conditions. Guinea pig PBMCs were dual stained with FITC-conjugated anti-gp T helper antibody. The samples were analyzed by flow cytometry, and levels of control staining were subtracted from the levels of CCR4 expression. In some experiments cells were preincubated at room temperature for 15 min with 20 μg/ml of either 10E4 or IgG1 and further incubated with the appropriate stimuli for 60 min to examine the ability of 10E4 to block gpCCR4 internalization.

Generation of Chimeric Chemokine Receptors by Overlap PCR Mutagenesis—Overlap PCR was carried out as described previously (36) using huCCR4 or huCCR8 (both HA-tagged) plasmid DNA to amplify the appropriate domains of the chemokine receptors. Chimera 1 comprised the amino terminus of huCCR8 ligated onto the backbone of huCCR4, whereas chimera 2 was the reciprocal construct, comprising the amino terminus of huCCR4 ligated onto the backbone of huCCR8. The amino termini of both receptors are shown in Fig. 8*A*. Fusion of each amino terminus was with a conserved leucine within the first transmembrane domain (leucine 42 of huCCR4 and leucine 38 of huCCR8). Sequence analysis verified the expected product. Oligonucleotide sequences were as follows: HA tag sense, 5′-GCG CAT AAG CTT GCC ACC ATG TAT CCA TAT GAG GTC CCA GAG TAT AAA GAA TTC-3′; chimera 1 sense, 5′-CTG CCC CCA CTG TAT TCC TTG

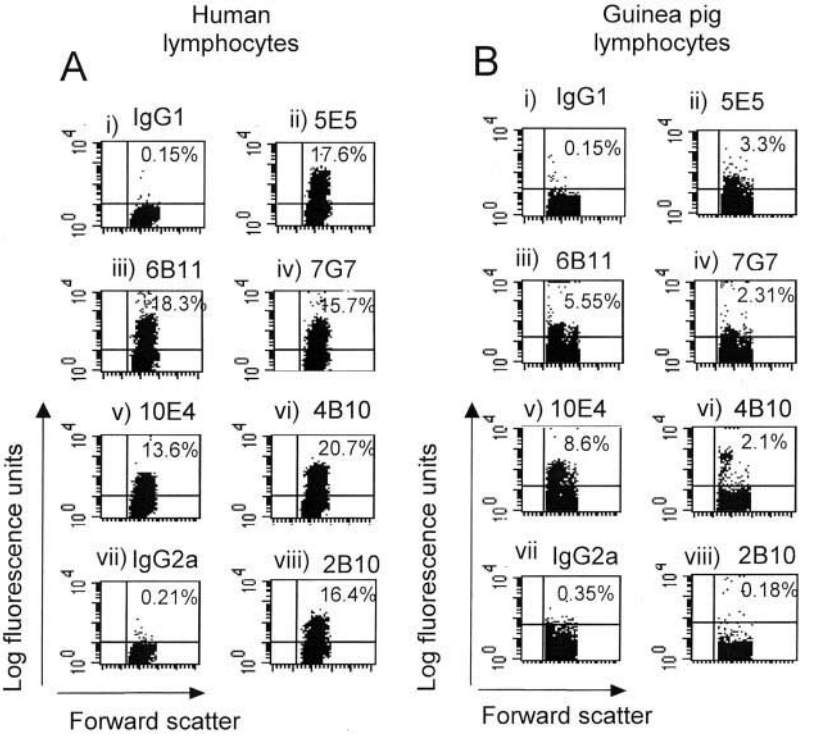

FIG. 3. **Staining profiles of anti-human CCR4 monoclonal antibodies on human and guinea pig circulating lymphocytes.** *A*, CCR4 was detected on human lymphocytes. *Dot plots* illustrate typical data and are representative of three experiments. Percent staining positive for CCR4 is indicated in the *upper right quadrant*. *B*, CCR4 was detected on guinea pig lymphocytes. *Dot plots* illustrate typical data and are representative of three experiments. Percent staining positive for CCR4 is indicated in the *upper right quadrant*.

GTT T-3′; chimera 1 antisense, 5′-AAA CAA AGG ACA GTG GGG GCA GGA GCA ACT TGC CAT TTG TCT-3′; chimera 2 sense, 5′-CTT GCT GTC TTT TAT TGC CTC CTG T-3′; chimera 2 antisense, 5′-ACA GGA GGC AAT AAA AGA CAG CAA GGA AGA GCT CCC CAA ATG CC-3′; huCCR4 antisense, 5′-ATA TCT CTC GAG CTA CAG AGC ATC ATG AAG ATC AT-3′; huCCR8 antisense, 5′-ATA TCT CTC GAG CCT CAC AAA ATG TAG TCT ACG CTG-3′.

Generation of Transiently Transfected L1.2 Cells Expressing Chimeric Chemokine Receptors—20 μg of chimeric chemokine receptor.pcDNA3 construct was transfected into L1.2 cells as previously described (30). Briefly, the L1.2 cells were electroporated in the presence of plasmid DNA and subsequently cultured at 37 °C in RPMI supplemented with 10% fetal calf serum. After 6 h cells were treated with *n*-butyric acid (final concentration of 10 mM) to stabilize receptor expression and cultured overnight at 37 °C + 5% CO_2 in humidified conditions, prior to flow cytometric analysis.

Statistical Analyses—Data were analyzed using one-way analysis of variance with a Bonferroni post-test using Prism 3.0 software (GraphPad Software Inc. San Diego, CA).

RESULTS

Identification of the Open Reading Frame of Guinea Pig CCR4—The 550-bp PCR product amplified from guinea pig genomic DNA using oligonucleotides based on known CCR4 sequences encoded an ORF that was 83% identical, at the nucleotide level, to the human and murine CCR4 sequences. This PCR product therefore provided a specific probe for the subsequent screening of a guinea pig genomic DNA bacteriophage library. A bacteriophage clone with homology to the partial gpCCR4 probe was isolated and digested with *Eco*RI, and the resulting fragments were shotgun-cloned into pUC19. Based on the hybridization to the gpCCR4 probe and predicted arrangement of the digested fragments, two *Eco*RI fragments were cloned and sequenced. The smaller fragment contained a 150-bp portion of the deduced ORF; the second fragment contained a 933-bp portion of the deduced ORF. Primers based on these combined sequences amplified a 1.1-kb ORF from guinea pig genomic DNA that contained an *Eco*RI site at the predicted location. Fig. 1 shows the amino acid comparisons of the full open reading frames of human, murine, and guinea pig CCR4. Guinea pig CCR4 shares 81% identity with murine CCR4 and shares 88% amino acid identity with human CCR4. Surpris-

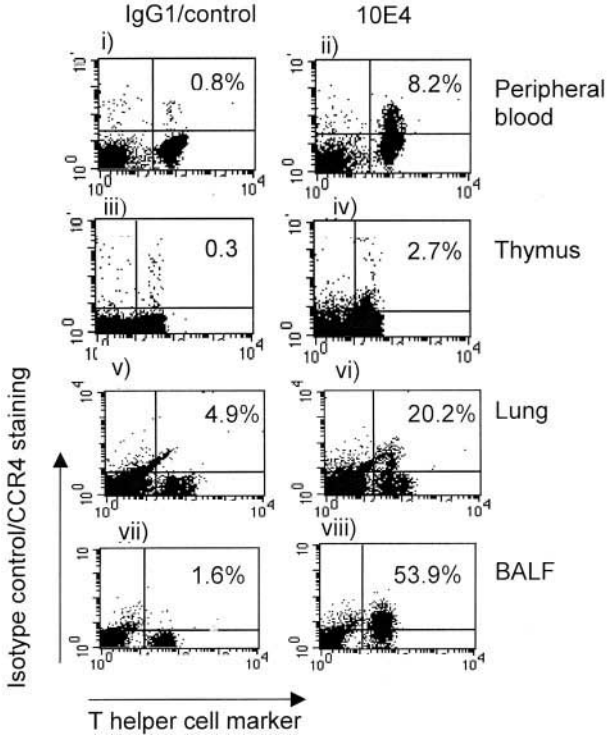

FIG. 4. **Guinea pig CCR4 expression levels in tissue.** Cells isolated from tissues of naive guinea pigs were stained using 10 μg/ml of unconjugated primary antibody (IgG1 control or anti-CCR4, 10E4), detected using secondary rabbit anti-mouse Ig-RPE (pre-absorbed with 10% guinea pig serum), and dual stained with anti-gp T helper-FITC-conjugated antibody. Dual staining of guinea pig peripheral blood T helper cells demonstrated CCR4 positivity (*ii* compared with control staining in *i*). *Dot plots* illustrate typical data and are representative of four experiments. Percent of T helper-positive cells staining for CCR4/control is indicated in the *upper right quadrant*. 2.4% of T helper$^+$ gp thymocytes are CCR4$^+$ (*iv* compared with control staining *iii*). 15.1% of T helper$^+$ cells isolated from guinea pig lung tissue were CCR4$^+$ (*vi versus v*). 52% of T helper cells within guinea pig BAL fluid were CCR4$^+$ (*viii versus vii*). Data shown are from a single experiment and are representative of four to six experiments.

ingly, guinea pig CCR4 possesses an additional serine at position 24 from the amino terminus, compared with human and murine forms of CCR4. The sequence was deposited in the GenBank™ data base (accession number AF431971).

Generation of gpCCR4 Stable Transfectants—L1.2 cells transfected with gpCCR4 were enriched by chemotaxis toward 50 nM murine MDC, isolated by limiting dilution cloning, and subsequently expanded. We investigated the ability of a panel of anti-human CCR4 monoclonal antibodies to detect surface expression of gpCCR4 on these transfected cells by flow cytometry. Each of these antibodies showed some degree of cross-reactivity with guinea pig CCR4. Fig. 2 (*A* and *B*) show the staining profiles of butyrate-treated gpCCR4-transfected and huCCR4-transfected L1.2 cells, respectively, with the panel of anti-human CCR4 antibodies. Of these antibodies, 10E4 gave high levels of staining of both human and gpCCR4 transfected cells but did not bind wild-type L1.2 cells (data not shown).

Analysis of Surface CCR4 Expression on Guinea Pig T Lymphocytes by Flow Cytometry—We investigated the staining profiles of the anti-huCCR4 antibodies for guinea pig and human lymphocytes using whole blood flow cytometric analysis. Single color flow cytometry revealed that a proportion of cells within the lymphocyte region (as determined by forward/side scatter profiles) bound these antibodies, compared with isotype control. Fig. 3*A* shows the staining profiles of human lymphocytes, where between 14 and 20% of lymphocytes were recognized by the anti-huCCR4 antibodies. In contrast, Fig. 3*B* shows the staining profiles of guinea pig whole blood staining with the same antibodies. The antibody 2B10 has previously been used to examine CCR4 expression levels on human T lymphocytes (18, 19) and, in this study, detected 16% of human lymphocytes but failed to stain guinea pig lymphocytes, while the remaining antibodies recognized between ~2 and 9% of guinea pig lymphocytes. The mAb 10E4 gave the most consistent staining of gpCCR4-transfected cells and native guinea pig T lymphocytes compared with the other antibodies.

To determine the CCR4 expression profiles of guinea pig T lymphocyte phenotypes, two-color flow cytometric analysis was carried out using antibodies against guinea pig T cell markers and the anti-huCCR4 antibody 10E4. Fig. 4*ii* illustrates expression of CCR4 in normal guinea pig whole blood, with 7.4% of circulating guinea pig T helper cells expressing CCR4 after subtracting nonspecific isotype control staining (Fig. 4*i*). In the guinea pig thymus, 2.4% of T helper$^+$ cells were labeled with 10E4 compared with isotype control antibody (illustrated in Fig. 4, *iii* and *iv*). Fig. 4*vi* illustrates that 15.1% of T helper$^+$ cells in the lung tissue are CCR4$^+$ after subtracting the isotype control antibody staining (Fig. 4*v*). Furthermore, although ~3% of BAL cells express the guinea pig T helper phenotype (not shown), Fig. 4*viii* demonstrates that ~52% of T helper$^+$ cells in the normal guinea pig BALF are CCR4$^+$ compared with the isotype control antibody (Fig. 4*vii*).

Chemotactic Migration of gpCCR4-transfected Cells—To investigate the ability of gpCCR4-transfected L1.2 cells to migrate to human and murine CCR4 ligands, butyrate-treated gpCCR4-transfected L1.2 cells were studied in assays of chemotactic migration. Fig. 5*A* shows that gpCCR4-transfected L1.2 cells migrated toward both murine and human forms of both MDC and TARC. Of these ligands, the order of potency was muMDC > muTARC > huTARC > huMDC. No significant migration toward either a truncated form of human MDC-(3–69) (previously reported as a CCR4 antagonist (37)), gp eotaxin, or huMig was observed in these assays. Wild-type L1.2 cells did not migrate toward MDC or TARC (data not shown). Fig. 5*B* demonstrates that the migration of gpCCR4-transfected L1.2 cells toward 10 nM murine MDC was significantly blocked by preincubation of the cells with the anti-human CCR4 antibody, 10E4, but not by an isotype control antibody. Similarly, 10E4 was also able to block the chemotactic migration of huCCR4-transfected L1.2 cells toward huMDC (data not shown).

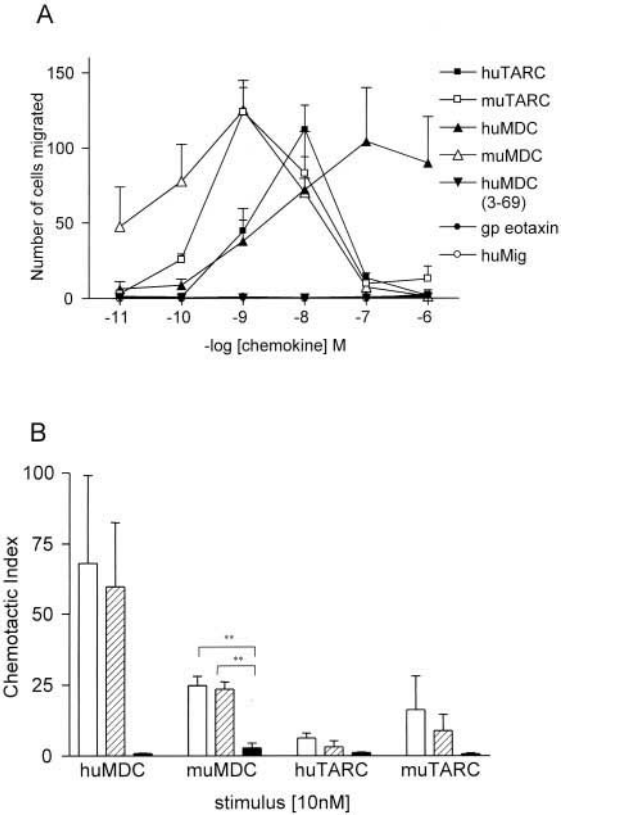

FIG. 5. **Specific chemotactic responses toward the murine and human CCR4 ligands, MDC and TARC, can be induced via gpCCR4.** *A*, murine and human homologues of MDC and TARC induced chemotaxis of gpCCR4-transfected cells *in vitro*, whereas the CCR4 antagonist human MDC-(3–69), and the chemokines, gp eotaxin and human Mig, did not induce migration of this cell line. muMDC and muTARC both had maximal chemotactic effects via gpCCR4 at 1 nM concentration, while huTARC and huMDC responses were maximal at 10 and 100 nM, respectively, in the 96-well assay. *B*, chemotactic migration toward 10 nM of the murine and human CCR4 ligands was blocked by preincubation of gpCCR4-transfected cells with an anti-human CCR4 antibody (10E4) (*black bars*). Preincubation of cells with isotype control antibody (*hatched bars*) or buffer alone (*empty bars*) did not affect migration. Data is expressed as mean chemotactic index (±S.E.), and each experiment was carried out in duplicate (n = 4 for muMDC and n = 3 for other ligands). Statistical analysis was performed on muMDC data alone.

Radiolabeled Ligand Binding to gpCCR4 Transfectants—To determine the binding affinities of human and murine CCR4 ligands to huCCR4 and gpCCR4-transfected cells, ^{125}I-huMDC was displaced from both cell lines in the presence of increasing concentrations of the human and murine forms of MDC. Fig. 6 (*A–D*) shows displacement curves of ^{125}I-huMDC from huCCR4- and gpCCR4-transfected cells by increasing concentrations of unlabeled huMDC (Fig. 6, *A* and *C*) and muMDC (Fig. 6, *B* and *D*). Calculation of dissociation constants by Scatchard analysis showed that ^{125}I-huMDC had a high affinity for huCCR4 with a dissociation constant (K_d) for huMDC with a mean value of 0.5 nM (±S.E. 0.24 nM) but with a lower affinity for gpCCR4 with a mean K_d value of huMDC = 15.3 nM (±S.E. 2.8 nM). The murine homologue of MDC was able to displace ^{125}I-huMDC from both huCCR4 and gpCCR4 transfected cells with mean inhibition constant (K_i) values of 0.39 nM (±S.E. 0.07 nM) and 6 nM (±S.E. 1 nM), respectively.

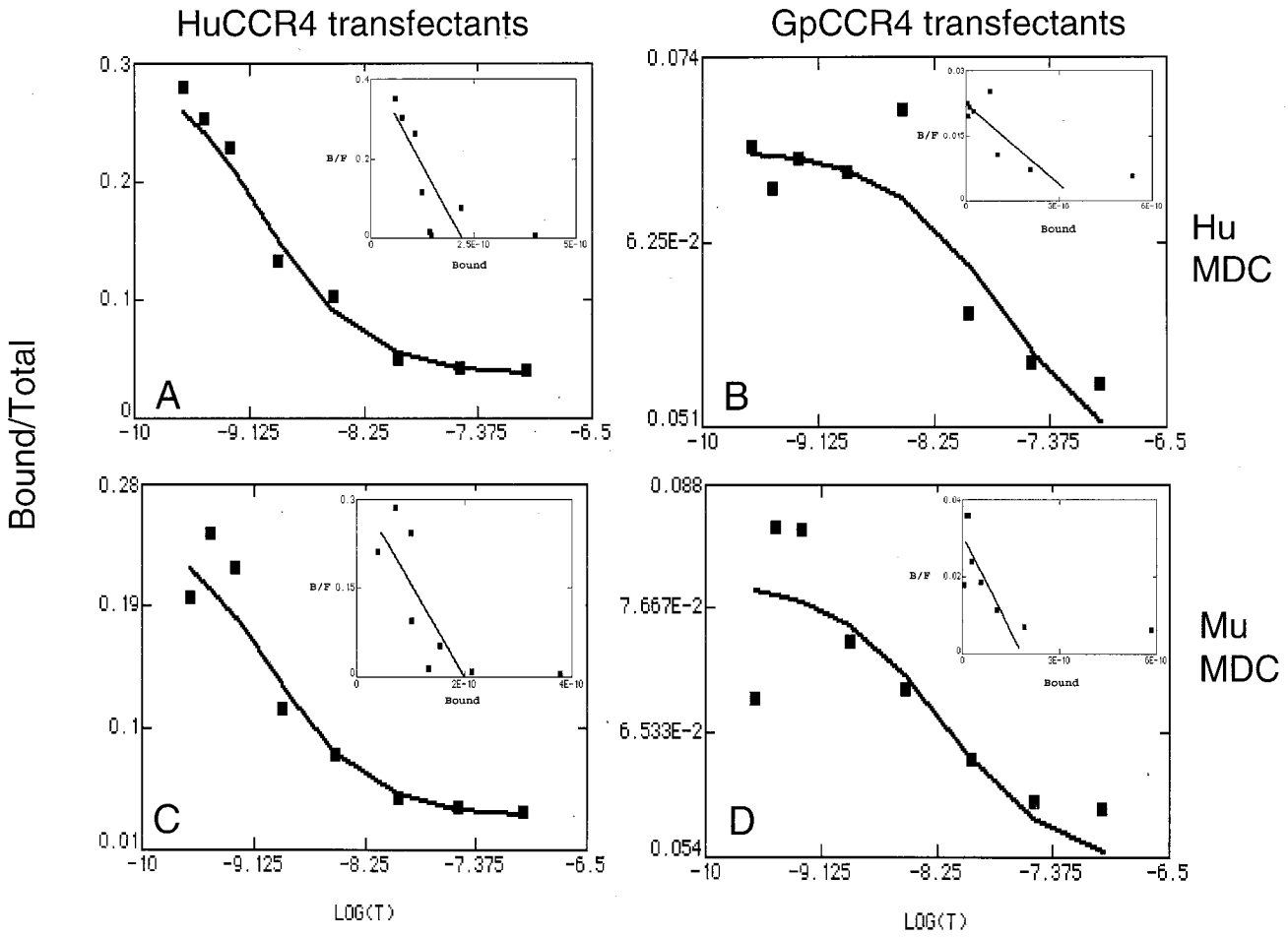

FIG. 6. **Radioligand binding studies using gpCCR4-transfected cells.** ^{125}I-huMDC (0.1 nM) was competed from either human CCR4-transfected (*A* and *C*) or gpCCR4-transfected cells (*B* and *D*) using increasing concentrations of unlabeled huMDC (*A* and *B*) or muMDC (*C* and *D*). Mean K_d values for the displacement of ^{125}I-huMDC by unlabeled huMDC are 0.5 nM (huCCR4) (±S.E. 0.24 nM) and 15.3 nM (gpCCR4) (±S.E. 2.8 nM), whereas mean K_i values for the displacement of ^{125}I-huMDC by unlabeled muMDC were 0.39 nM (huCCR4) (±S.E. 0.07 nM) and 6 nM (gpCCR4) (±S.E. 1 nM). Data are expressed as mean of three experiments, each carried out in triplicate.

Blockade of Ligand-induced Receptor Down-regulation from gpCCR4-transfected L1.2 Cells—Binding of chemokines to their receptors generally induces receptor internalization (38, 39). We incubated gpCCR4-transfected cells with increasing concentrations of human and murine CCR4 ligands in addition to control chemokines huMDC (3–69) and human SDF-1a and measured CCR4 expression levels by flow cytometry. Fig. 7*A* shows that 60 min after stimulation with 10 nM of the human-(1–69) and murine forms of MDC, surface gpCCR4 levels were reduced by ~50%. Similarly, gpCCR4 expression was reduced by ~70%, 60 min after stimulation with 100 nM of these chemokines, compared with control samples. Sixty minutes after stimulation of gpCCR4 transfectants with 10 nM of the human and murine forms of TARC, gpCCR4 expression levels were reduced by ~15–20%; gpCCR4 expression levels were reduced by ~45% after stimulation with 100 nM chemokine doses. In contrast, human MDC-(3–69) had no significant effect on surface gpCCR4 expression levels under the same conditions. Cells incubated on ice with any of the chemokines showed no significant reduction of surface gpCCR4 expression after 60 min, showing that the washing steps incorporated in the protocol avoided any competition between the ligand and detecting mAb that would give a false impression of receptor internalization. The murine L1.2 cells constitutively express CXCR4 (40); therefore, human SDF-1α, which also signals via murine CXCR4 (41), was included in the assay to determine whether CXCR4 down-regulation would affect surface CCR4 expression levels (heterologous desensitization). Stimulation of gpCCR4-transfected cells with human SDF-1α had no effect on surface CCR4 expression levels (Fig. 7*A*).

To examine the antagonist properties of the anti-human CCR4 mAb 10E4, gpCCR4-transfected L1.2 cells were preincubated with 20 μg/ml 10E4 or IgG1 control antibody, prior to stimulation with 10 nM or 100 nM ligand. Fig. 7*B* shows that preincubation with 10E4 significantly reduced internalization of gpCCR4 from the surface of transfected cells, compared with isotype control-treated or unstimulated cells.

Blockade of Ligand-induced Receptor Down-regulation from Guinea Pig T Lymphocytes—To confirm the above results using native T lymphocytes, guinea pig PBMCs were stimulated with murine MDC, stained with 10E4, and typed by staining with the anti-gp T helper antibody. Fig. 7*C* illustrates that stimulation of guinea pig PBMCs with 10 nM muMDC reduced the percentage of guinea pig T helper cells expressing CCR4 by 35–45% (untreated or IgG1 preincubated), compared with unstimulated cells. Preincubation of gpPBMCs with 10E4 caused a significant reduction of gpCCR4 down-regulation in response to 10 nM muMDC, compared with buffer treated cells.

Analysis of the Extracellular Domains of Human CCR4 Involved in 10E4 Recognition—To analyze the huCCR4 domain recognized by 10E4, chimeric chemokine receptors were generated by exchanging the amino termini of huCCR4 with huCCR8 and vice versa, using overlap PCR (36). Each construct encoded a 5′ HA tag to enable detection of surface re-

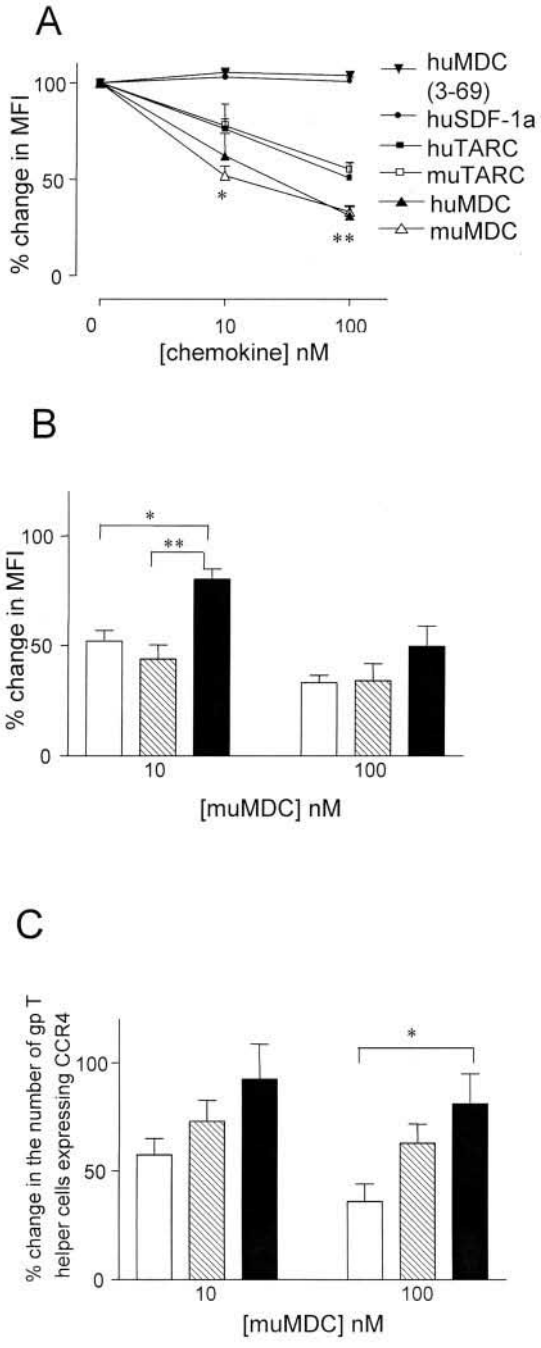

FIG. 7. **GpCCR4 is down-regulated by stimulation with the human and murine CCR4 ligands, MDC and TARC, as determined by flow cytometry.** *A*, gpCCR4-transfected cells were stimulated for 60 min at 37 °C with human and murine MDC and TARC, huMDC-(3–69) and huSDF-1a. (**, $p < 0.01$ for muMDC induced down-regulation of gpCCR4 where $n = 4$, all other ligands $n = 3$). *B*, preincubation of gpCCR4-transfected cells with a blocking CCR4 antibody, 10E4 (*black bars*) could significantly reduce muMDC-induced down-regulation compared with preincubation of the cells with isotype control antibody (*hatched bars*) or buffer alone (*empty bars*) (*, $p < 0.05$; **, $p < 0.01$). *C*, CCR4 down-regulation was also induced by muMDC in guinea pig peripheral blood T helper cells, and this could also be blocked with preincubation of the native cells with 10E4 (*black bars*) compared with preincubation of the cells with isotype control antibody (*hatched bars*) or buffer alone (*empty bars*). Data in all three figures are expressed as mean (±S.E.) ($n = 4$ unless otherwise stated) (*, $p < 0.05$).

ceptor expression using an anti-HA antibody. The design of the chimeras is shown in Fig. 8*A*. Fig. 8 illustrates that the anti-HA antibody could not detect chimera 1, suggesting that this receptor could not be expressed on the L1.2 cells. However, the remaining receptors (huCCR4, huCCR8, and chimera 2) were all expressed on these cells. Using 10E4 to detect CCR4 expression, we have demonstrated that chimera 2, consisting of the amino terminus of huCCR4 ligated to the backbone of huCCR8, was detected by 10E4, suggesting that this antibody recognizes an epitope within the amino terminus of huCCR4 (Fig. 8). In contrast, 10E4 was unable to detect expression of huCCR8, even though the HA tag demonstrated that this receptor was expressed on the cell surface.

DISCUSSION

Effective therapeutic strategies aimed at blocking chemokine receptor function *in vivo* require extensive analysis *in vitro* to determine the consequences of receptor blockade. CCR4 has previously been described as a putative Th2-specific chemokine receptor in studies using T cell clones that were polarized *in vitro* (15, 16, 42). This observation suggests that CCR4 may play a fundamental role in T lymphocyte recruitment to sites of Th2-dominated inflammatory reactions, such as the lung tissue in asthmatic individuals. Murine models of allergic lung inflammation data demonstrated that neutralization of the CCR4 ligands MDC and TARC significantly decreased eosinophilia of the lung tissue and BALF and reduced airway hyper-reactivity compared with control mice (21–23). However, in contrast to this, mice-deficient in the *CCR4* gene did not show a significant difference in predisposition to the development of allergic lung inflammation compared with wild-type mice (24). Furthermore, studies examining the expression of chemokine receptors on T lymphocytes isolated from the BALF of asthmatic and non-asthmatic individuals showed that CCR4 expression on T lymphocytes at these sites was not significantly different between these groups (26). However, CCR4 has been shown to be significantly up-regulated within the lung tissue of asthmatics following allergen challenge (27). Therefore, to examine the potential role of CCR4 in allergic lung inflammation and the feasibility of receptor blockade we aim to use a guinea pig model of disease which is well characterized (43).

CCR4 sequence homology between species is very highly conserved with human and murine forms of this receptor sharing 86% amino acid identity (28, 29). We identified the homologue of CCR4 from a guinea pig genomic DNA bacteriophage library, yielding a cDNA encoding an intronless, 361-amino acid open reading frame with 81 and 88% amino acid identity to murine and human CCR4 sequences, respectively, which is subsequently referred to as gpCCR4 (Fig. 1).

We generated stable transfectants expressing cell surface gpCCR4 to determine whether murine and human CCR4 ligands would induce functional responses in these cells using *in vitro* assays. GpCCR4-transfected cells bound anti-huCCR4 mAbs in assays of flow cytometry (Fig. 2*A*) and the antibody cross-reactivity of 2B10, 4B10, and 10E4 was in keeping with the high level of sequence homology between the human and guinea pig forms of CCR4. *In vitro* assays of chemotaxis showed that gpCCR4-transfected cells were functional and capable of specific migration toward the human and murine forms of the CCR4 ligands MDC and TARC (44, 45), further emphasizing the high levels of conservation of this receptor between species.

It has previously been demonstrated that CCR4 expression predominates on the surface of $CD4^+$ peripheral blood T lymphocytes with up to 30% of $CD4^+$ T lymphocytes expressing CCR4 whereas less than 5% $CD8^+$ T lymphocytes express CCR4 (18). Therefore, we examined the expression patterns of CCR4 on the surface of guinea pig T lymphocyte subpopulations by flow cytometry, using 10E4 and two-color flow cytometric analysis using commercially available T lymphocyte markers. A mean value of 12.95% (±S.E. 3.6%, $n = 4$) of T helper lymphocytes in guinea pig

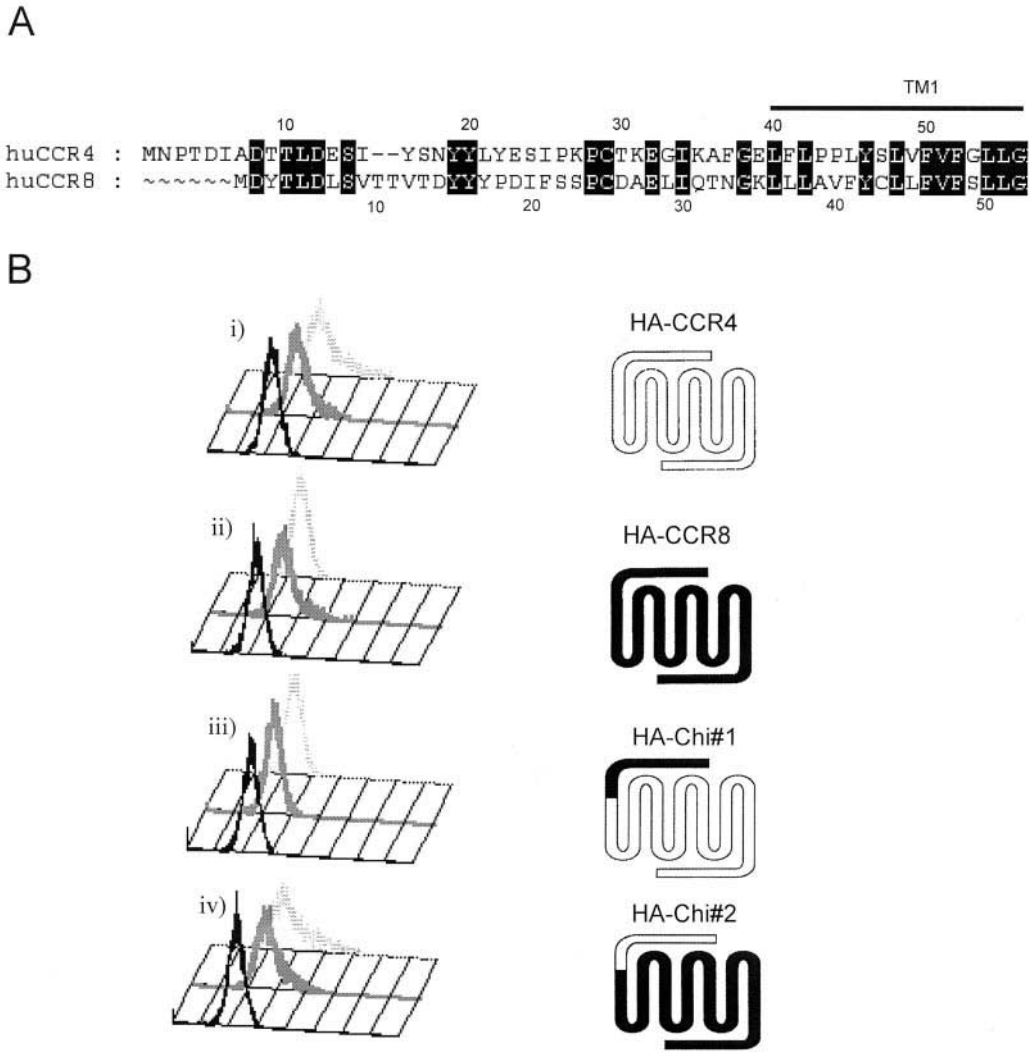

FIG. 8. **Determination of the huCCR4 epitope recognized by 10E4.** *A*, amino acid sequences of the human CCR4 and CCR8 amino termini. Chimeras 1 and 2 had their respective amino termini exchanged at the boundary of the first transmembrane domain, depicted as *TM1*. Identical residues conserved between both receptors are contained within *black boxes*. *B*, flow cytometric analysis of huCCR4/huCCR8 chimeric chemokine receptors transiently transfected into L1.2 cells, demonstrated surface expression of all receptors except chimera 1 (as detected by anti-HA antibody staining, *dark gray line*). 10E4 staining was observed for huCCR4 and chimera 2 only (*light gray line*). The *black line* shows IgG1 isotype control staining. Histograms shown are representative of four experiments.

peripheral blood expressed CCR4 similar to the staining profiles we observed when using 10E4 to detect CCR4 expression on human lymphocytes in peripheral blood. In contrast, CCR4 was expressed on only 2% of guinea pig circulating $CD8^+$ T lymphocytes (data not shown). Therefore, the T helper and $CD8^+$ T lymphocytes in both human and guinea pig contain a similar proportion of $CCR4^+$ T lymphocytes, suggesting that CCR4 expression is highly conserved and probably serves similar functions across species.

Analyses of human and murine thymus samples have shown that the CCR4 ligand MDC is highly expressed in the cortico-medullary junction of the thymus (46). Chantry *et al.* (46) have shown that $CCR4^+$ thymocytes are of the $CD3^+CD4^+CD8^{low}$ phenotype and constitute a small (1–2%) but relatively mature thymocyte subpopulation found in the cortico-medullary junction of the thymus. Flow cytometric analysis of guinea pig thymocytes revealed that a mean value of 2.3% (±S.E. 0.9%, $n = 3$) of total guinea pig thymocytes were of the $CCR4^+$ T $helper^+$ phenotype, whereas 1.3% (±S.E. 0.3%) of guinea pig thymocytes were of the $CCR4^+$ $CD8^+$ (data not shown). Therefore, we hypothesize that the small percentage of one or more $CCR4^+$ populations that we have detected in guinea pig total thymocyte preparations are of a similar phenotype to those previously characterized in human and mouse (46).

We have also shown that, although T helper cells constitute ~3% of total cells within the guinea pig lung, a mean of 9% (±S.E. 2.3%, $n = 6$) of guinea pig Th^+ cells found within the lung tissue of naïve guinea pigs express surface CCR4 (representative plot in Fig. 4*vi*). A higher level of nonspecific binding was apparent when staining lung samples. However, in all samples tested, a clear $CCR4^+Th^+$ population was seen in the lung, which corresponded with the lymphocyte forward scatter/side scatter gate determined from peripheral blood staining, demonstrating that CCR4 is expressed on a proportion of lung T lymphocytes. Interestingly, Th^+ cells constitute ~3% of cells within the BALF of naïve guinea pigs; however, a mean value of 46.8% (±S.E. 7.7%) of these Th^+ cells were also $CCR4^+$. Recently, it has been reported that ~5% of $CD4^+$ T lymphocytes isolated from the BALF of either asthmatic patients or non-asthmatic individuals express CCR4, with no significant differences in CCR4 expression on these cells between the two groups (26). Low levels of CCR4 expression were detected on the surface of T lymphocytes isolated from lung tissue (26). However, another study has shown a role for CCR4 in the

recruitment of T lymphocytes in human allergic asthma. CCR4 and the ligands MDC and TARC were significantly up-regulated after allergen challenge of asthmatic individuals (27). The recent evidence of a role for CCR4 in human allergic inflammation and the suggested high level of functional conservation between human and guinea pig suggest that CCR4 may also have a role in basal airway surveillance in humans, although further studies are required to confirm this. Studies in progress to analyze CCR4 expression patterns on T lymphocytes isolated from the tissues of control and allergen-challenged guinea pigs should give an insight into the role of CCR4 in the selective recruitment of T lymphocytes in this model.

To date, guinea pig CCR4 ligands have not been identified; however, Fig. 5*A* shows that the murine CCR4 ligands induced a greater chemotactic response of gpCCR4-transfected cells than the corresponding human CCR4 ligands. This suggests that guinea pig CCR4 ligands may be more likely to bear sequence resemblance to those of the mouse compared with the human CCR4 ligands. However, the human and murine homologues of MDC and TARC are relatively conserved between species. Human and murine homologues of MDC bear 75% sequence identity at the nucleotide level (47, 48), whereas human and murine homologues of TARC bear 72% nucleotide sequence identity (49, 50). A panel of anti-human CCR4 monoclonal antibodies was characterized with respect to functional antagonism of gpCCR4 using *in vitro* assays of chemotactic responsiveness. Chemotactic migration of gpCCR4-transfected cells toward murine and human forms of MDC and TARC could be significantly reduced by preincubation of these cells with the anti-human CCR4 antibody 10E4, whereas migration of cells preincubated with isotype control antibody had no significant effect on the chemotactic response (Fig. 5*B*).

Radioligand binding assays revealed that ^{125}I-huMDC had a high affinity for huCCR4 with subnanomolar K_d values for both huMDC (mean K_d = 0.5 nM) and muMDC (mean K_i = 0.4 nM). In contrast, huMDC had a lower affinity for gpCCR4 and was displaced more readily by muMDC (mean K_i = 6 nM) compared with huMDC (mean K_d = 15.3 nM). These data are in keeping with chemotaxis assays, which showed that muMDC was much more potent than huMDC at gpCCR4 (Fig. 3*A*). We were unable to calculate the K_d value for ^{125}I-muMDC, because the radiolabeled chemokine is not commercially available at present, and the ability of muMDC to bind to gpCCR4 and huCCR4 was abolished by in-house radiolabeling (data not shown).

It has been demonstrated that neutrophil binding of the chemokine IL-8 to its cognate receptors CXCR1 and/or CXCR2 induces receptor desensitization, signaling, and internalization (39, 51). Therefore, because this phenomenon has not been examined for guinea pig leukocytes, we determined whether gpCCR4 down-regulation from the cell surface could be induced by *in vitro* stimulation with the CCR4 ligands MDC and TARC. After stimulation of either gpCCR4-transfected cells or guinea pig T lymphocytes with increasing concentrations of human and murine CCR4 ligands, there was a significant reduction of surface CCR4 levels (Fig. 7, *A* and *C*). Chemokine receptor internalization is held to be critical for effective chemotactic migration of cells. Our data suggest that CCR4 is a functional receptor on guinea pig T lymphocytes. GpCCR4 down-regulation, from the surface of both transfected cells and peripheral blood T helper cells in response to stimulation with muMDC, could be significantly blocked by preincubation with 10E4.

Using chimeric chemokine receptors we have demonstrated that 10E4 recognizes the amino terminus of huCCR4 (Fig. 8). The amino termini of chemokine receptors such as CXCR1, CXCR2, CCR2, and CCR3 have been shown to be important in ligand-binding (52–54); however, the ligand binding domains of CCR4 have not been examined. This study has employed chimeric chemokine receptors and subsequently demonstrated that the blocking antibody 10E4 recognizes the amino terminus of huCCR4 (Fig. 8). Furthermore, 10E4 has been shown to block gpCCR4 function on both transfected cells and native guinea pig T lymphocytes using *in vitro* assays of chemotactic migration or receptor down-regulation. This suggests that the murine and human homologues of MDC and TARC also bind to a motif that includes the amino terminus of gpCCR4.

In summary, we have identified the guinea pig homologue of CCR4 and characterized this receptor using a blocking monoclonal antibody, and we have determined the extracellular domain of huCCR4 that this antibody recognizes. Further analysis of the role of CCR4 in disease models of cutaneous inflammation, septic shock, and allergic lung inflammation will determine whether this chemokine receptor provides a feasible therapeutic target in the treatment of these conditions.

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Note Added in Proof—While the manuscript was in proof, it came to our attention that the anti-human CCR4 monoclonal antibody 10E4 was of the isotype IgG2a and not IgG1 as was originally concluded during its production. The hybridoma line from which 10E4 came took several months and many rounds of single cell subcloning to stabilize, so we speculate that during the subcloning, the mAb switched subclasses. As a precaution, we have repeated some of the crucial experiments in our manuscript and shown that the 10E4 staining pattern of guinea pig tissue cells is identical when compared to an IgG1 isotype or two different IgG2a isotype negative controls. In experiments re-examining the ability of 10E4 to block guinea pig CCR4 stable transfectant chemotaxis to murine MDC and TARC, the results obtained using an IgG2a negative control were comparable with those obtained with an IgG1 negative control. Thus we believe that this mis-assignment of the antibody isotype does not invalidate any aspects of the data shown in the paper, although we regret any confusion caused.

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