Eosinophils are major effector cells implicated in a number of chronic inflammatory diseases in humans, particularly bronchial asthma and allergic rhinitis. The β-chemokine receptor C-C chemokine receptor 3 (CCR3) provides a mechanism for the selective recruitment of eosinophils into tissue and thus has recently become an attractive biological target for therapeutic intervention. In order to develop in vivo models of inflammatory diseases, it is essential to identify and characterize the homologues of human eotaxin (C-C chemokine ligand 11) and CCR3 from other species, such as non-human primates. Accordingly, we cloned the macaque eotaxin and CCR3 genes and revealed that they were 91 and 92% identical at the amino acid level to their human homologues, respectively. Macaque CCR3 expressed in the murine pre-B L1-2 cell line bound macaque eotaxin with high affinity ($K_d = 0.1 \text{ nM}$) and exhibited a robust eotaxin-induced Ca$^{2+}$ flux and chemotaxis. Characterization of β-chemokines on native macaque CCR3 on eosinophils was performed by means of eotaxin-induced shape change in whole blood using a novel signaling assay known as gated autofluorescence forward scatter. Additionally, mAbs were raised against macaque CCR3 using two different immunogens: a 30-amino acid synthetic peptide derived from the predicted NH$_2$ terminus of macaque CCR3 and intact macaque CCR3-transfected cells. These anti-macaque CCR3 monoclonal antibodies exhibited potent antigen activity in receptor binding and functional assays. The characterization of the macaque eotaxin/CCR3 axis and development of antagonistic anti-macaque CCR3 monoclonal antibodies will facilitate the development of CCR3 small molecule antagonists with the hope of ameliorating chronic inflammatory diseases in humans.

Bronchial asthma is a multifactorial disease characterized clinically by reversible bronchoconstriction leading to shortness of breath. In the pathophysiology of the disease, a chronic inflammatory condition persists in the airways of most patients, which involves a complex interplay between blood leukocytes, airway epithelial cells, and bronchial smooth muscle cells. One of the most striking aspects of asthma is the selective accumulation and activation of distinct subtypes of leukocytes into the airways, particularly eosinophils, and it is these cells that are postulated to play a key role in the pathophysiology of the disease (1, 2).

Inflammatory mediators, such as chemoattractants, generated at the involved sites, promote the migration of eosinophils from the vasculature into the tissue. Unlike eosinoloids and complement cleavage fragments, which display activities on a wide variety of cells, candidate molecules for the selective recruitment of eosinophils into the airways are a class of proteins called hematotactic cytokines or chemokines. Chemokines are a growing superfamily of >50 small molecular mass proteins (~8–10 kDa) and are characterized by their actions on distinct subtypes of leukocytes (3, 4). These proteins can be classified into two major subfamilies based on the arrangement of the first two conserved cysteines in the protein. In the α or C-X-C family, these two cysteines are separated by any amino acid, whereas in the β or C-C family, these two cysteines are adjacent to one another. Some members of this latter family have been discovered to possess strong eosinophil migratory and activating properties.

Chemokines exert their effects by binding to members of the G-protein-coupled receptor superfamily of receptors, which contain seven transmembrane domains. The discovery of a potent eosinophil-specific β-chemokine, eotaxin, isolated from the bronchoalveolar lavage (BAL)$^1$ fluid from ovalbumin-challenged guinea pigs (5), led to the identification of CCR3 (6–8), the third β-chemokine receptor characterized in an expanding family that numbers close to 20 to date (3). Although its expression was first thought to be limited to eosinophils, CCR3 is now known to be more widely expressed on cells involved in allergic inflammation, such as basophils (9), mast cells (10), airway epithelial cells (11), and potentially TH$_2$ T-lymphocytes (12). Chemokines that are selective for CCR3 include eotaxin, eotaxin-2, and eotaxin-3, whereas the potent eosinophil-activating β-chemokines RANTES, MCP-3, and MCP-4 bind to other chemokine receptors in addition to CCR3 (13).

CCR3-activating β-chemokines are elevated in human asthematics (14–16) and are markedly up-regulated upon allergen challenge in animal models (5, 17–21) and in humans (22–26).

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The relatively high expression of CCR3 on eosinophils, along with expression of specific CCR3-activating β-chemokines in the involved tissue offers a potent mechanism for the selective recruitment, activation, and retention of this cell type into tissue sites during allergic inflammation. It has been shown with a blocking monoconal antibody directed against human CCR3 that the actions of multiple β-chemokines on eosinophils are mediated through CCR3 (27), indicating that CCR3 is the major β-chemokine receptor expressed on these cells. For these reasons, CCR3 has recently emerged as a significant anti-inflammatory pharmacological target, and CCR3 antagonists are currently being developed for the treatment of asthma and other allergic disorders (28, 29).

A prominent non-human primate model of asthma was established over a decade ago in the cynomolgus macaque (30, 31), and its utility in the preclinical evaluation of therapeutic agents in humans has proven to be extremely valuable (31–40). To gain a better understanding of the role of CCR3 and its ligands in this important animal model of asthma, we have cloned and functionally expressed the macaque CCR3 in the murine pre-B L1-2 cell line. We have also cloned the macaque eotaxin and characterized chemically synthesized protein to functionally characterize the macaque CCR3. Moreover, we have generated murine monoclonal antibodies directed against the macaque CCR3 using two different immunizing antigens and have demonstrated that these mAbs are potent functional antagonists of this receptor through a series of receptor binding and signaling assays in vitro.

EXPERIMENTAL PROCEDURES

Southern Hybridization—Genomic DNA was isolated from venous blood of cynomolgus (Macaca fascicularis) and rhesus macaques (Macaca mulatta, respectively; Merck Research Laboratories, in-house colony) using the QIAamp DNA blood kit (Qiagen, Valencia, CA). Also, cynomolgus macaque DNA was purchased from Therion (Troy, NY), and rhesus macaque DNA was purchased from CLONTECH (Palo Alto, CA). Southern blot hybridization was performed using standard procedures (41). Briefly, genomic DNA (20 μg) was digested with a set of restriction enzymes for 7 h, followed by phenol/chloroform extraction, ethanol precipitation, and then separation on a 0.7% agarose gel. The gel was then saturated in 1.5M NaCl and 0.5 M ethanol precipitation, and then separation on a 0.7% agarose/Tris acetate-EDTA gel. The gel was then baked 42°C for 1 h in 6X SSC, 5X Denhardt’s solution, 0.5% SDS, 50% formamide, and 100 μg/ml salmon sperm DNA, followed by hybridization for 16 h in the identical solution containing 2X 10⁶ cpm/ml ³²P-labeled 1.1-kb human CCR3 DNA fragment (Ready-to-Go DNA labeling kit; Amersham Biosciences) comprising the open reading frame (7). The membrane was washed twice in 2X SSC, 0.1% SDS at room temperature for 15 min; twice in 0.1X SSC, 0.1% SDS at room temperature for 15 min; once at 55 °C for 15 min; and once at 65 °C for 15 min. The membrane was then exposed onto Eastman Kodak Co. X-OMAT film and developed.

Cloning of Cynomolgus and Rhesus CCR3—PCR was performed using cynomolgus and rhesus macaque genomic DNA as template with primers designed from the 5' and 3'-untranslated regions of the human CCR3 gene. The 5'-primer contained the sequence 23 to 3 bp upstream of the ATG initiation codon and a HindIII site (5’-GGCTT-AGC-TTC-TAT-CAC-AGG-GAG-AAG-TG-3’). The 3’-primer contained the sequence 11–29 bp downstream of the TAG termination codon and a NotI site (5’-CCT-CAT-CT-CCT-GGC-GCC-GCT-CCT-TAG-GCA-ATT-TTC-3’). PCR was performed for 30 cycles: 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 2 min in a PerkinElmer Life Sciences model 9600 DNA thermal cycler. The resultant PCR products from the cynomolgus and rhesus DNA were subcloned into expression vector pBJ-Neo (7) and pcDEF3 (42) (a generous gift from Dr. J. Langer, University of Medicine and Dentistry of New Jersey, respectively). 2

Cloning of Rhesus Macaque Eotaxin—Poly(A)⁺ mRNA was isolated by oligo(dT)-cellulose chromatography from rhesus macaque small intestine and used to generate first strand cDNA using a tagged oligo(dT) primer (3’-RACE kit; Invitrogen). The cDNA was then used as template for PCR performed with the following two primers: 5’-primer (5’-AAC-CAC-CTC-CTA-CGC-C-3’) and 3’-primer (5’-CAC-GCT-CTG- GTT-TGG-TTT-CAA-3’). PCR was performed for 30 cycles: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The resultant PCR product was subcloned into pcCR2.1-TOPO (Invitrogen). A consensus sequence for rhesus macaque eotaxin was identified by DNA sequencing of six independent clones. The rhesus macaque eotaxin protein was chemically synthesized (Glyphon Sciences, South San Francisco, CA) based on the predicted amino acid sequence.

Characterization and Blockade of Macaque CCR3 in the Murine Pre-B Cell Line, L1-2, and the Human Eosinophilic Cell Line, AML14.3D10—The murine pre-B L1-2 cell line was a generous gift from Dr. I. Weissman (Stanford University). Transfection of the L1-2 and AML14.3D10 cell lines were carried out as described (7, 8) with slight modifications. Briefly, 5 x 10⁶ cells were washed in Hanks’ balanced saline solution, mixed with 20 μg of pcDEF3-rhesus CCR3 or pcDEF3-human CCR3 (for L1-2 transfection), and pcBJ/Neo-cynomolgus CCR3 (for AML14.3D10 transfection) in a 0.4-cm electroporation cuvette, electroporated at 250 V and 860 μF microfarads, and then cultured in complete medium (RPMI 1640 with 10% fetal bovine serum). After 48 h, the cells were placed in medium containing 0.8 mg/ml G418 and plated onto 96-well plates at 18,000 cells/well. Clones were transferred into six-well plates, and positive clones were selected by their ability to bind ¹²⁵I-human eotaxin (Amersham Biosciences).

Chemokine Receptor Binding Assay—¹²⁵I-Human Eotaxin (2000 Ci/mmol) was obtained from PerkinElmer Life Sciences, and ¹²⁵I-macaque eotaxin (2000 Ci/mmol) was custom-radioiodinated at Amersham Biosciences. L1-2/macaque CCR3 cells (30,000 cells/assay for ¹²⁵I-macaque eotaxin and 200,000 cells/assay for ¹²⁵I-human eotaxin) were mixed with 30 pm (20,000 cpm) iodinated chemokine and varying concentrations of β-chemokines (PeproTech, Rocky Hill, NJ) in a binding buffer consisting of 50 mM Hepes, pH 7.2, 1 mM CaCl₂, 5 mM MgCl₂, 0.1% gelatin, and 0.05% Tween 20, at the following concentrations: 50 nm (0.5% maximum binding), 500 nm (50% maximum binding), 5 μM (90% maximum binding), and 30 μM (100% maximum binding). Radioactivity retained on polyethyleneimine-treated Whatman GF membranes after washing in binding buffer (using a PerkinElmer Life Sciences harvester) containing 0.5 mM NaCl was counted in a TopCount (PerkinElmer Life Sciences). Binding results were analyzed using KaleidaGraph (Synergy Software, Reading, PA) and LIGAND (43).

Ligand-induced CCR3 Mobilization—Changes in intracellular calcium level were measured on a fluorescence imaging plate reader (FLIPR; excitation 488-nm argon laser line/emission 530-nm bandpass interference filter; Molecular Devices, Inc., Sunnyvale, CA) following the manufacturer’s instructions with modifications. L1-2/macaque CCR3 or L1-2/human CCR3 cells were washed in wash buffer (Hanks’ balanced saline solution containing 20 mM Hepes, pH 7.2, 0.1% bovine serum albumin) and labeled with fluo-3 (Molecular Probes, Inc., Eugene, OR) at 1.5 x 10⁶ cells/ml in labeling buffer at 37 °C for 1 h. Labeling buffer was prepared as follows. 50 μM of fluo-3 was dissolved in 44 μl of 10% pluronic F-127 in Me₂SO and then 11 ml of wash buffer. Following labeling with fluo-3, cells were washed twice in wash buffer. About 1.5 x 10⁶ cells in 135 μl of wash buffer were added to each well of a 96-well plate (black, clear bottom; Corning Costar, Cambridge, MA) and then centrifuged for 5 min (no brake). The plate was placed on FLIPR, 67.5-μl ligands (one-half of the cell volume) at various concentrations were added, and fluorescence changes were recorded. For blocking experiments, mAbs were preincubated with cells for 20 min prior to addition of 5 μM macaque eotaxin was added. Results were calculated using KaleidaGraph.

Ligand-induced Chemotaxis—L1-2/macaque CCR3 cells were labeled with 5 μM calcein (Molecular Probes), washed, and resuspended in chemotaxis buffer (RPMI 1640 plus 0.5% bovine serum albumin) at 5 x 10⁶ cells/ml. To the bottom chamber of a 96-well Neuroprobe ChemoTx plate (5-μm pore size; NeuroProbe, Inc., Gaithersburg, MD), increasing concentrations of chemokine were added in a volume of 29 μl of chemotaxis buffer. To the top chamber, 30 μl of cells (1.5 x 10⁶ total cells) were added, and chemotaxis was allowed to proceed at 37 °C for 1 h. Unmigrated cells were removed from membrane with a Kimwipe. The plate was then analyzed in a CytoFluor II fluorometer (excitation, 485 nm; emission, 530 nm; PerSeptive Biosystems, Framingham, MA). For blocking experiments, 5 μM macaque eotaxin was added to the bottom chamber. To the top chamber, 15 μl of cells (1.5 x 10⁵ total) was mixed together with 15 μl of mAb at various concentrations.
Characterization and Blockade of Macaque CCR3

**Generation of Mouse Anti-macaque CCR3 mAbs—**To immunize antibody-producing hybridomas, monoclonal antibodies against the macaque CCR3. The first approach used viable whole L1-2 cells stably transfected AML14.3D10 cells. The most potent serum blocked 125I-human eotaxin binding to L1-2/macaque CCR3 cells. Postimmune sera were most potent in flow cytometry and inhibition of 125I-human eotaxin binding by 50% at 1:1000 serum dilution and blocked 51% of the total cell surface CCR3 on L1-2/rhesus macaque CCR3 cells as immunogen. As above, the mouse that produced the most mAbs produced anti-macaque CCR3 mAbs, mouse IgG (ICN) (negative control), whose sera were most potent in flow cytometry and inhibition of 125I-human eotaxin binding to L1-2 cells at 1:1000 serum dilution and blocked 51% of the total cell surface CCR3.

**Transmission of Macaque CCR3**—To determine the complexity of the CCR3 genes from different non-human primate species, a Southern blot analysis was performed on both cynomolgus and rhesus macaque genomic DNAs. Southern blots hybridized with the 32P-labeled human CCR3 cDNA probe surprisingly revealed that the hybridization banding pattern was identical for both of these macaque species when the genomic DNAs were digested with BamHI, BgIII, EcoRI, and HindIII (Fig. 1). Although this banding pattern was different from that of human genomic DNA (8), a simple hybridization pattern was observed, indicating that CCR3 is encoded by a single copy gene in both the cynomolgus and rhesus macaque.

Given that human CCR3 lacked intervening sequences within the coding region (45), it was postulated that the coding region of the rhesus and cynomolgus macaque CCR3 genes would be intronless as well. Using PCR based on primers designed from the 5′- and 3′-untranslated regions of the human CCR3 gene, we independently cloned the rhesus and cynomolgus macaque CCR3 genes from genomic DNA, and the sequences we obtained were genetically distinct from those pre-

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3. T. Hansel, personal communication.
4. B. L. Daugherty, unpublished data.
Characterization and Blockade of Macaque CCR3

Since it has been previously shown that eotaxin is highly expressed in the gastrointestinal tract in humans (52), the rhesus macaque small intestine was chosen as a source of mRNA to clone the eotaxin cDNA by reverse transcriptase-PCR from this species. The two-loop structure of the predicted amino acid sequence of rhesus macaque eotaxin is shown (Fig. 3A) (hereafter referred to as macaque eotaxin throughout this report). The macaque eotaxin is 88 and 94% identical to the human eotaxin sequence at the amino acid and nucleotide level, respectively. As shown, there are only seven amino acid differences between the mature human and macaque eotaxins (six nonconservative, one conservative), with six of the differences occurring within the NH2-terminus-proximal 30 amino acids of the protein. Fig. 3B represents an amino acid lineup of the macaque eotaxin to all of the known eotaxin sequences to date, which include human, horse (66% identical at the amino acid level), bovine (59% identity), guinea pig (58% identity), rat (59% identity), and mouse (56% identity).

Expression of Macaque CCR3 in Murine Pre-B L1-2 Cells

Both the human and macaque CCR3 were subcloned into expression vector pCDEF3 (42), which uses the human elongation factor 1α promoter and has been proven to drive high level expression of proteins in heterologous cell lines. Given that the murine pre-B L1-2 cell line was successfully used for the heterologous expression of human CCR3 (8), we also chose to use this cell line for functional expression of macaque CCR3. Positive clones were picked by their ability to bind 125I-human eotaxin, and one, designated clone 19, was chosen for further analysis.

Competition Binding of Various β-Chemokines on L1-2/Macaque CCR3 Cells

Competition binding studies were performed with 125I-macaque eotaxin on clone 19 in order to characterize the pharma-
**A**

![Serpentine diagram of the macaque CCR3 receptor](image)

The four extracellular segments including the NH₂-terminus (OUT) and the four intracellular segments including the COOH-terminal tail (IN) are shown. The seven transmembrane-spanning domains are shown between the two horizontal lines. The four cysteines, one in each of the four extracellular segments, postulated to form two disulfide bridges, are indicated with a thick black line. Nonconservative amino acid changes between the macaque and the human CCR3 sequence are indicated in white letters. Conservative amino acid changes are encircled within a thick black line.

**B**

![Amino acid alignment of CCR3 sequences](image)

The figure shows the predicted CCR3 sequences for macaque (rhesus), human (7), African green monkey (AGM) (46), sheep (accession number AF266468), guinea pig (GP) (56), rat (83), murine SV/129 (84), and murine Balb/c. The positions of the seven putative transmembrane-spanning regions are designated with overlines. A minimum of four identical residues is indicated in the shaded region. The complete nucleotide sequence of the rhesus CCR3 (accession numbers AF405535–AF405537), cynomolgus CCR3 (accession numbers AF405533 and AF405534), and murine Balb/c CCR3 (accession number AY049018) are available from EMBL/GenBank/DBJ.
Fig. 3. A, amino acid sequence of the macaque β-chemokine eotaxin. The four cysteines that are known to form two disulfide bridges are indicated by thick bars to form a two-loop structure. Nonconservative amino acid changes between the rhesus and the human eotaxin sequence are indicated in white letters. Conservative amino acid changes are encircled within a thick black line. B, amino acid alignment of eotaxin sequences from various species. The downward arrow designates the predicted position of signal peptidase cleavage to generate the mature protein. The figure shows the predicted eotaxin amino acid sequences for macaque (rhesus), human (6, 52, 85), horse (86), bovine (accession number AJ132003), guinea pig (GP) (87), rat (88), and mouse (89). A minimum of four identical residues is indicated in the shaded region. The complete nucleotide sequence of the rhesus macaque eotaxin is available from EMBL/GenBank®/DDBJ under accession number AY049019.

Fig. 4. Equilibrium binding of β-chemokines to L1-2/macaque CCR3 cells. Increasing concentrations of unlabeled macaque eotaxin ( ), human eotaxin-2 ( ), human eotaxin-3 ( ), human MCP-4 ( ), and human RANTES ( ) were used to compete against a fixed concentration of 125I-rhesus macaque eotaxin (Rh-Eotaxin). All values are the averages of triplicate determinations. Typically, 3000–4000 cpm of iodinated ligand was bound in the absence of competitor, with signal/noise ratios exceeding 10. Results are representative single experiments. A Scatchard plot of the binding isotherm in A is shown (inset).

Characterization and Blockade of Macaque CCR3...
Characterization and Blockade of Macaque CCR3

TABLE I

Binding affinities of various chemokines comparing macaque and human CCR3 expressed in murine L1-2 cells

| Competitor               | K<sub>i</sub> (nM) | Macaque CCR3 | Human CCR3 |
|--------------------------|-------------------|-------------|------------|
| 125I-macaque eotaxin/CCL11 | 0.1 ± 0           | ND          | ND         |
| Murine eotaxin/CCL11      | 0.4 ± 0           | ND          | ND         |
| Eotaxin/CCL11             | 0.6 ± 0           | ND          | ND         |
| Eotaxin-2/CCL24           | 3.3 ± 0.2         | ND          | ND         |
| Eotaxin-3/CCL26           | 8.1 ± 3.9         | ND          | ND         |
| MCP-4/CCL13               | 1.4 ± 0.2         | ND          | ND         |
| MCP-3/CCL7                | 17.2 ± 5.1        | ND          | ND         |
| RANTES/CCL5               | 80 ± 22           | ND          | ND         |
| 125I-human eotaxin/CCL11  | 0.7 ± 0.4         | 0.3 ± 0.1   | 0.5 ± 0    |
| Eotaxin/CCL11             | 0.6 ± 0.1         | 0.5 ± 0     | 0.0 ± 0    |
| Eotaxin-2/CCL24           | 1.5 ± 1.0         | 0.6 ± 0     | 0.0 ± 0    |
| Eotaxin-3/CCL26           | 5.2 ± 3.0         | 0.9 ± 0.3   | 0.0 ± 0    |
| MCP-4/CCL13               | 1.1 ± 0.1         | 0.4 ± 0.2   | 0.0 ± 0    |
| MCP-3/CCL7                | 3.3 ± 0.1         | 3.4 ± 0.8   | 0.0 ± 0    |
| RANTES/CCL5               | 9.2 ± 4.0         | 1.5 ± 0.2   | 0.0 ± 0    |

<sup>a</sup> K<sub>i</sub> values were calculated using LIGAND (43).

affinity (K<sub>i</sub> = 5.2 nM). Likewise, human RANTES binds human CCR3 with 6-fold higher affinity (K<sub>i</sub> = 1.5 nM) than macaque CCR3 (K<sub>i</sub> = 9.2 nM).

Functional Coupling of Macaque CCR3 in L1-2 Cells

Ligand-induced Ca<sup>2+</sup> Mobilization—To investigate the ability of β-chemokines to activate signal transduction in L1-2/macaque CCR3 cells, agonist-induced calcium mobilization was measured (Table II). The amplitude at which intracellular calcium reaches its highest level in L1-2 cells occurs within 10 s of ligand addition. The rank order of potency obtained by binding affinity via competition of β-chemokines on macaque CCR3 with 125I-macaque eotaxin was comparable with the agonist potency obtained by calcium mobilization (see Tables I and II); however, the functional EC<sub>50</sub> values were 10-fold lower than the binding K<sub>i</sub> values for most of the chemokine ligands. Interestingly, macaque eotaxin was shown to be equipotent as an agonist on the human and macaque CCR3. Furthermore, human RANTES was unable to trigger a calcium flux even at a concentration of 100 nM.

Ligand-induced Chemotaxis—Since heterologous chemokine receptors are functionally coupled to the chemotaxis pathway in murine pre-B L1-2 cells, chemotaxis assays were carried out with various β-chemokines on L1-2/macaque CCR3 cells. The rank order of potency of the various β-chemokine ligands was as follows: macaque eotaxin > human eotaxin > human MCP-4 > human eotaxin-2 > human MCP-3 > human eotaxin-3 > human RANTES (Fig. 5, b and d; Table III). Similar to human CCR3, no response was observed with human MIP-1α. This rank order of potency was generally consistent with radiolabeled binding and calcium flux data, except for human MCP-3, which induces chemotaxis with a higher relative potency than it does in agonist-induced calcium flux. Unlike the other chemotactic β-chemokines that are active on L1-2/macaque CCR3, human eotaxin-2 is only a partial agonist, exhibited by a chemotactic index of less than 50% of the amplitude observed with the other eotaxins.

Identification of Monoclonal Antibodies against Macaque CCR3

Two approaches were taken to produce mouse mAbs against macaque CCR3 (see “Experimental Procedures”). The first approach used the L1-2/macaque CCR3 cell line as the immunizing antigen, and one clone, designated mAb 5B9 (IgG2a/c), was identified by flow cytometry (Fig. 6a). The second approach used a 30-amino acid NH<sub>2</sub>-terminal macaque CCR3 synthetic peptide as the immunizing antigen, and six positive clones were identified by enzyme-linked immunosorbent assay. Only two of these clones, designated mAb 51 (IgG1/c) and mAb 52 (IgG2b/c), were positive by flow cytometry on L1-2/macaque CCR3 cells (Fig. 6, b and c). All three of these anti-macaque CCR3 mAbs were negative on the parental L1-2 cell line by flow cytometry (Fig. 6, a–c), indicating specificity for macaque CCR3. Only mAb 52 exhibited some specificity for human CCR3 by flow cytometry, whereas none of these three anti-macaque CCR3 mAbs were found to be positive for murine CCR3 (data not shown).

Activation of Native Macaque CCR3: Ligand-induced Shape Change of Rhesus Macaque Eosinophils

Activation of native macaque CCR3 was assessed by β-chemokine-induced shape change of rhesus macaque eosinophils in whole blood by GAFS assay (44). Eosinophils, like all leukocytes, undergo cytoskeletal rearrangement (via actin polymerization) upon chemokine receptor activation, resulting in a change of cellular morphology. These shape changes can be readily monitored via increases in forward scatter by flow cytometry. Moreover, eosinophils are highly autofluorescent and therefore can be gated out in whole blood using flow cytometry without separation of the different leukocytes. For the GAFS assay, blood from a rhesus macaque that contained a high circulating level of eosinophils (>15%) was used. The rank order of potency for the various β-chemokine ligands in the rhesus eosinophil GAFS assay was as follows: macaque eotaxin > human eotaxin − human MCP-4 > human MCP-3 > human eotaxin-2 > human eotaxin-3 > human RANTES (Fig. 5, a and c; Table III). This rank order was very similar, but not identical, to that from the chemotaxis assay. Most notably, macaque eotaxin was more potent than human eotaxin, and human eotaxin-2 did not appear to be acting as a partial agonist in the GAFS assay. Unlike human eosinophils, which express CCR1 (7, 44), the rhesus macaque eosinophils did not respond to human MIP-1α in the GAFS assay, most likely reflecting the absence of CCR1 on these cells or species differences for activity of human MIP-1α between human and macaque CCR1.

TABLE II

Agonist potency via calcium mobilization of various chemokines comparing macaque and human CCR3 expressed in murine L1-2 cells

| Chemokine      | Macaque CCR3 | Human CCR3 |
|----------------|-------------|------------|
| Macaque eotaxin/CCL11 | 1 nM       | 1 nM       |
| Eotaxin/CCL11  | 8 nM       | 3 nM       |
| Eotaxin-2/CCL24 | 30 nM      | 10 nM      |
| Eotaxin-3/CCL26 | 33 nM      | 9 nM       |
| MCP-4/CCL13    | 10 nM      | 2 nM       |
| MCP-3/CCL7     | 50 nM      | >1000 nM   |
| RANTES/CCL5    | >1000 nM   | 9 nM       |

All results are the averages of a minimum of two experiments.
Anti-macaque CCR3 mAbs Recognize Native CCR3 on Rhesus Macaque Eosinophils

The anti-macaque CCR3 mAbs were assessed by flow cytometry on primary rhesus eosinophils to determine binding to native CCR3. Partial purification of the rhesus eosinophils yielded a final mixture that contained 24% eosinophils, 1% lymphocytes, and 75% neutrophils by differential staining. Of the three anti-macaque CCR3 mAbs, only two of these (mAb 5B9 and mAb 51) bound to a subset of the rhesus leukocyte cell suspension (Fig. 6, a and b), the exact proportion by differential staining representative of eosinophils (Fig. 6, e and f), whereas mAb 52 did not react with this subset at all (Fig. 6, g). An anti-integrin mAb (anti-CD11b) was used as a positive control, which stained the entire population of cells in the rhesus leukocyte mixture (Fig. 6, h). In contrast to data observed with human T-lymphocytes (12), rhesus macaque T-lymphocytes were not shown to express CCR3 via double staining the rhesus macaque PBMC mixture with anti-CD3 and anti-macaque CCR3 (5B9) mAbs, followed by flow cytometry (data not shown).

Anti-macaque CCR3 mAbs Block Eotaxin Binding to L1-2/Macaque CCR3 Cells

The anti-macaque CCR3 mAbs were next tested for inhibition of eotaxin binding. As shown in Fig. 7a, mAbs 5B9 and 51 exhibited highly potent inhibition of $^{125}$I-human eotaxin binding to L1-2/macaque CCR3 cells, whereas mAb 52 did not inhibit binding at all (Fig. 7, a and b). An anti-integrin mAb (anti-CD11b) was used as a positive control, which inhibited eotaxin binding almost completely (Fig. 7, c). The functional potency of the various $^{125}$I-chemokines to induce a shape change in rhesus eosinophils and chemotaxis in L1-2/macaque CCR3 cells is listed in Table III.

| Chemokine          | EC50 Shape change | Chemotaxis |
|-------------------|-------------------|------------|
| Macaque Eotaxin/CCL11 | 2 0.3             |            |
| Eotaxin/CCL11      | 5 0.5             |            |
| Eotaxin-2/CCL24    | 70 5              |            |
| Eotaxin-3/CCL26    | 300 60            |            |
| MCP-4/CCL13        | 7 1               |            |
| MCP-3/CCL7         | 30 6              |            |
| RANTES/CCL5        | >300 150          |            |

Fig. 5. Ligand-induced shape change of rhesus eosinophils (a and c) and chemotaxis of L1-2/rhesus-CCR3 cells (b and d). Increase in eosinophil shape change in rhesus macaque whole blood was determined by GAFS as described under “Experimental Procedures.” Chemotactic index was determined by the fluorescence reading obtained with ligand divided by that obtained with buffer alone. a and c, results are representative single experiments. Chemokine ligands are designated by the following symbols: macaque eotaxin (●), human eotaxin (○), human eotaxin-2 (▲), human eotaxin-3 (□), human MCP-4 (□), human MCP-3 (■), human RANTES (▲), and human MIP-1α (▼).

Fig. 6. Flow cytometry of L1-2/macaque CCR3 cells (a–d) and partially purified rhesus eosinophils (e–h) is indicated by a thick line using antibodies as designated: anti-macaque CCR3 mAb 5B9 (a and e); anti-macaque CCR3 mAb 51 (b and f); anti-macaque CCR3 mAb 52 (c and g); mouse IgG control (d); anti-human CD11b mAb (h). Flow cytometry of untransfected (parent) L1-2 cells with anti-macaque CCR3 mAbs is indicated by a thin line (a–c).
ing to L1-2/macaque CCR3 cells with IC\textsubscript{50} values of 0.027 and 0.051 μg/ml, respectively. Also shown in Fig. 7a, mAb 52 or mouse IgG control did not demonstrate any inhibition of eotaxin binding at concentrations up to 100 μg/ml. None of the three anti-macaque CCR3 mAbs inhibited \textsuperscript{125}I-human eotaxin binding to L1-2/human CCR3 cells (data not shown).

Anti-macaque CCR3 mAbs Display Potent Functional Antagonism

The anti-macaque CCR3 mAbs were analyzed in a series of functional assays to determine antagonist activity. As shown in Fig. 7b, pretreatment of L1-2/macaque CCR3 cells with mAbs 5B9 and 51 inhibited macaque eotaxin-induced calcium flux with IC\textsubscript{50} values of 0.5 and 1.6 μg/ml, respectively. Additionally, none of the anti-macaque CCR3 mAbs blocked human eotaxin-induced calcium flux in L1-2/human CCR3 cells (data not shown). Moreover, the mAbs were also evaluated in both the rhesus macaque whole blood GAFS assay and in chemotaxis of L1-2/macaque CCR3 cells. As shown in Fig. 7c, mAbs 5B9 and 51 antagonized macaque eotaxin-induced shape change of rhesus eosinophils with IC\textsubscript{50} values of 0.088 μg/ml and 2.1 μg/ml, respectively. Finally, mAbs 5B9 and 51 blocked macaque eotaxin-induced chemotaxis of the L1-2/macaque CCR3 cell line with IC\textsubscript{50} values of 0.05 and 0.3 μg/ml, respectively (Fig. 7d). These two anti-macaque CCR3 antagonist mAbs also inhibited human MCP-4-induced chemotaxis of L1-2/macaque CCR3 cells with a potency comparable with that of macaque eotaxin (data not shown). Consistent with the binding studies, mAb 52 or control mouse IgG did not exhibit any inhibition in either the calcium flux assay (at concentrations up to 1 mg/ml) or the GAFS and chemotaxis assays (at concentrations up to 100 μg/ml for both) (data not shown).

**DISCUSSION**

In this paper, we describe the molecular cloning and functional expression of the macaque CCR3 β-chemokine receptor. Previous studies investigating macaque CCR3 have principally focused on AIDS research exploring the mechanisms of human immunodeficiency virus type 1 and 2 co-receptor usage (46) and AIDS-associated neuropathogenesis in the macaque (53–55). In addition, two sequence notes have previously been reported (47, 48). This is the first report describing full pharmacological characterization of macaque CCR3 with a variety of β-chemokine ligands in a series of radiolabeled binding and signal transduction assays. The macaque CCR3 sequences described in this report are genetically distinct from those previously reported, most likely reflecting genetic polymorphisms in the macaque CCR3 gene. We have also cloned the macaque β-chemokine eotaxin and were able to employ chemically synthesized protein in the biochemical and functional assays described in this report.

In addition, we describe the generation of monoclonal antibodies directed against the macaque CCR3 and have demonstrated that these mAbs are functional antagonists in a series of *in vitro* assays. The *in vitro* potency of the anti-macaque CCR3 mAbs reported here compares favorably with other antagonistic murine anti-CCR3 mAbs raised against CCR3 from other species, which include human CCR3 (mAb 7B11) (27) and guinea pig CCR3 (mAb 2A8) (56). In addition, *in vivo* studies demonstrated that the anti-guinea pig CCR3 mAb 2A8 was able to block eotaxin-induced eosinophil recruitment into the guinea pig skin. Furthermore, a rat mAb was generated against murine CCR3 (57). Although this antibody was non-neutralizing, it was very effective at depleting eosinophils in the blood, lung, and BAL fluid in a *Nippostrongylus brasiliensis*-infected model of eosinophil accumulation in mice. This is the first report describing the development of blocking mAbs specifically raised against a macaque chemokine receptor.

The anti-macaque CCR3 mAbs were generated by two different immunizing antigens, intact macaque CCR3-transfected cells, and a macaque CCR3 NH\textsubscript{2}-terminal synthetic peptide of 30 amino acids in length. The method utilizing heterologous stably transfected cell lines as immunogens has proven most successful at producing antagonist mAbs against chemokine receptors (27, 56, 58–65). We were able to produce two antagonist mAbs against macaque CCR3 (5B9 and 51), one from each of the two methods; however, the more potent mAb was the one employing intact macaque CCR3-transfected L1-2 cells as the immunogen (mAb 5B9). This mAb (5B9) was 2-fold more potent in blocking radiolabeled eotaxin binding and, in functional
assays, 3-, 24-, and 6-fold more potent in eotaxin-induced calcium flux, shape change, and chemotaxis, respectively, than mAb 51 (see Fig. 7).

Antagonistic monoclonal antibodies raised against chemokine receptors have been invaluable tools for deciphering ligand binding domains on these receptors. These mAbs essentially fall into two main categories, those that bind to the NH$_2$-terminal region and those that recognize the second extracellular loop, although some mAbs generated against human CCR5 have been mapped to multidomain conformational epitopes (63). Besides the anti-macaque CCR3 mAb 51 described in this report, neutralizing mAbs that have been shown to bind to the NH$_2$ terminus of chemokine receptors include those that were raised against human CXCR1 and CXCR2 (58, 59, 61), human CXCR3 (66), human CCR4 (67), and human CCR5 (63). Neutralizing mAbs that recognize the second extracellular loop of chemokine receptors include human CCR2 (68), CCR5 (62, 63), and CXCR4 (69). Although beyond the scope of this report, it would be interesting to compare the neutralizing epitopes on CCR3 of the anti-macaque CCR3 mAb 5B9 as described here with that of the anti-human CCR3 mAb 7B11 (27), given the close homology between these two sequences and that both of these mAbs were generated with identical strategies using intact L1-2 transfectants as immunogens in mice.

Whereas we were able to generate a total of three mAbs that reacted with recombinant macaque CCR3 expressed on either L1-2 or AML14.3D10 cells, only two of these were able to recognize native CCR3 on primary rhesus macaque eosinophils by flow cytometry (mAbs 5B9 and 51; see Fig. 6). The inability of mAb 52 to recognize macaque CCR3 on the rhesus eosinophils has several plausible explanations. The epitope recognized by this mAb is masked by physical association with some other molecular entity on the cell surface (i.e. endogenous surface proteins, sugar moieties, etc.). Alternatively, this antigenic determinant on macaque CCR3 expressed on recombinant cell lines is in a conformation that is distinct from that on native rhesus eosinophils, resulting in the lack of reactivity with mAb 52. In support of this concept, the principle human immuno-deficiency virus type 1 co-receptors CCR5 and CXCR4 have been shown to exist in multiple conformational states on the cell surface using a panel of specific neutralizing mAbs (63, 70, 71). Interestingly, mAb 52 was the only one of the three anti-macaque CCR3 mAbs that recognized recombinant human CCR3.

There have been numerous reports describing the functionality of the $\beta$-chemokine RANTES as a very potent activator of human eosinophils. These biological effects include calcium mobilization, chemotaxis, integrin up-regulation, degranulation, and transendothelial migration in vitro (72–77). In addition, intradermal injection of human RANTES into allergic human subjects was shown to cause a rich eosinophil recruitment into the skin in vivo (78). In another human study, intranasal administration of RANTES into allergic rhinitis patients resulted in an inflammatory infiltrate into the nasal mucosa that was rich in eosinophils (79). Furthermore, we and others have demonstrated that this $\beta$-chemokine binds to CCR3 on human eosinophils and stimulates signal transduction through CCR3 with high potency (7, 8, 27). In contrast, human RANTES competed with low potency against radiolabeled macaque eotaxin ($K_d = 80$ nM, Table I; Fig. 4) and was ineffective at stimulating a calcium flux in macaque CCR3-transfected L1-2 cells (Table II). RANTES was able only at very high concentrations to trigger a shape change in rhesus macaque eosinophils and chemotaxis in L1-2/macaque CCR3 cells (Table III; Fig. 5). Additional evidence for the lack of human RANTES to activate macaque CCR3 was attained from studies in which human RANTES was injected intradermally into cynomolgus macaques in vivo. In these studies, a complete absence of eosinophil recruitment was observed, whereas the other human CCR3-active $\beta$-chemokines eotaxin, MCP-4, and MCP-3 stimulated a robust eosinophil accumulation into the skin of this species (80). Taken together, these results would indicate that human RANTES is not an efficacious ligand for macaque CCR3 due to species differences. It is unknown whether native macaque RANTES is a functional agonist for macaque CCR3.

We have been able to assess functionality of native macaque CCR3 on rhesus eosinophils through a novel signaling assay known as GAFS (44). This assay proved to be straightforward, efficient, and extremely rapid. The GAFS assay is based on the fact that leukocytes undergo a rapid shape change through actin polymerization upon activation by chemokine receptor agonists, and this signal transduction pathway is necessary for directional migration. This increase in shape change can readily be measured by a shift in forward scatter using flow cytometry. The GAFS assay was initially developed on partially purified human leukocytes (PBMCs versus granulocytes). We were able to adapt this assay to rhesus leukocytes, and the ability of the flow cytometer to gate out eosinophils in rhesus whole blood based on their high autofluorescence was extremely effective. Agonist properties using GAFS of an assortment of $\beta$-chemokines on rhesus eosinophils in whole blood were very analogous to those in chemotaxis assays using the transfected macaque CCR3 in the murine pre-B L1-2 cell line. Furthermore, the GAFS assay readily determined antagonistic properties of the anti-macaque CCR3 mAbs on eosinophils in rhesus whole blood, an assay condition not suitable for agonist-induced calcium flux and chemotaxis.

The most well established model of asthma in the non-human primate is the cynomolgus macaque (M. fascicularis) model (30, 31). These animals demonstrate a naturally occurring and reproducible airway sensitivity to Ascaris suum extract via inhalation. In the model, animals are exposed to single or multiple challenges of A. suum antigen, resulting in a prominent BAL eosinophilia. Indeed, eotaxin has been observed in the BAL rapidly within 6 h of after allergen challenge and remained high for 24 h in this cynomolgus macaque asthma model (17). Moreover, these non-human primates exhibit an early and late phase bronchoconstriction as well as a prominent airway hyperresponsiveness (AHR). The degree of AHR has been shown to be directly correlated with the level of eosinophil-derived major basic protein recovered from the BAL fluid, implying that eosinophil activation products may mediate the development and maintenance of AHR (30). Further support of eosinophil involvement in this primate asthma model has resulted from studies in which direct instillation of purified major basic protein into the airways caused the AHR (81) observed in these animals.

The use of the cynomolgus macaque model has proven crucial in the preclinical evaluation of therapeutic agents for the treatment of asthma in humans. Inhibition of airway eosinophilia and AHR in this non-human primate model was observed with several classes of compounds with disparate mechanisms of action. They include Rolipram, a specific inhibitor of phosphodiesterase IV (32), the corticosteroid dexamethasone (31, 33), the leukotriene D$_4$ receptor antagonist, ICI 198,615 (34), and mAbs directed against intercellular adhesion molecule-1 (35) and interleukin-5 (TRFK-5) (36). Other therapeutic agents that have shown efficacy in AHR include the long acting $\beta_2$-agonist, Salmeterol (33), and the leukotriene B$_4$ antagonist, CP-105,696 (37). Furthermore, agents that have shown efficacy in late phase bronchoconstriction include the platelet-activating factor...
receptor antagonist WEB 2170 (38), the 5-lipoxygenase inhibitor, BI-L-239 (39), and a monoclonal antibody directed against endothelial leukocyte adhesion molecule-1 (40), although it is unknown what effect these compounds have on eosinophil recruitment into the airways. In a related non-human primate asthma model, the leukotriene D$_4$ receptor antagonist Montelukast sodium (Singular), also known as MK-0476 (82), inhibited early and late phase bronchoconstriction in an A. suum challenge model in squirrel monkeys; however, the effect on BAL eosinophils in this model are unknown.

In summary, we have cloned and pharmacologically characterized the recombinant macaque CCR3 expressed in the murine pre-B cell line L1-2. We have also generated blocking monoclonal antibodies against macaque CCR3 using two different immunogens in mice. The mAbs were very potent in the inhibition of radiolabeled eotaxin binding to macaque CCR3, in addition to being potent CCR3 receptor antagonists in a variety of in vitro functional assays. These signaling assays include eotaxin-induced calcium mobilization and chemotaxis on recombinant macaque CCR3 and shape change on rhesus macaque eosinophils in whole blood. It is hoped that the blocking anti-macaque CCR3 mAbs reported here will be important in the macaque in proof-of-principle experiments to validate the CCR3 hypothesis preclinically as a potential therapeutic for asthma and other allergic diseases in humans.

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Functional Expression and Characterization of Macaque C-C Chemokine Receptor 3 (CCR3) and Generation of Potent Antagonistic Anti-macaque CCR3 Monoclonal Antibodies

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