

Web Only Data Supplement

This data supplement contains additional information on materials and methods employed in this study. In addition, further figures are presented describing the amino acid sequence homology of cynomolgus monkey CCR8 and characteristics of the test article ML604086.

MATERIALS AND METHODS

Cynomolgus monkey CCR8 cloning

The full length cDNA of *Macaca fascicularis* (*M fascicularis*, cynomolgus monkey) CCR8 (GenBank accession number: JX431563) was cloned using genomic DNA isolated from *M fascicularis* peripheral blood mononuclear cells (PBMCs) and by a combination of cross species PCR with primers designed from the *Macaca mulatta* (*M mulatta*) CCR8 gene (Genbank accession number: AF100205) using rapid amplification of cDNA ends (RACE).

Anesthesia and antigen challenge

Animals were initially tranquilized with ketamine HCl (approximately 10 mg/kg, IM) and maintained under general anesthesia by intravenous infusion of propofol (approximately 0.2 mg/kg/min,). The animals were intubated and maintained on a ventilator for challenge procedures. While each animal was anesthetized, a single dose of *Ascaris suum* (*A suum*) antigen (Greer Laboratories, Lenoir, NC) was administered on day 1 (15 h post initiation of ML604086 administration) and day 2 (29 h post initiation of ML604086 administration), respectively, via intermittent positive pressure breathing with a ventilator and in-line nebulizer. Animals were monitored for signs of distress throughout the challenge period.

Sample collection and processing

Bronchoalveolar lavage (BAL) fluid: Animals were tranquilized and BAL was performed by guiding a pediatric fiberoptic bronchoscope past the carina to wedge in a major bronchus. At each time point of 4 time points: 0 h for baseline measurement on Day -3, 6 h post 1st-Ag challenge on Day 1, 24 h post 1st-Ag challenge on Day 2 (immediately before 2nd-Ag challenge), and 48 h post 1st-Ag challenge on Day 3 (24 h after 2nd-Ag challenge), three 20 ml aliquots of sterile saline were injected into lung to lavage different lung fields and gently aspirated, and then pooled together. The BAL samples were analyzed for total and differential leukocytes counts. The cells were removed by centrifugation and supernatant was reserved for cytokine analysis.

Blood: Blood was collected from a peripheral vessel at 9 time points: pre-dosing on Day 0; prior to, 1 h post and 6 h post 1st-Ag challenge on Day 1 (15 h, 16 h, and 21 h after dosing start); prior to tranquilization, prior to and 1 h post 2nd-Ag challenge on Day 2 (38 h, 40 h and 41 h after dosing start); and prior to tranquilization and prior to MCh dose response on day 3 (63 h and 65 h after dosing start) respectively. A 0.6 mL volume of whole blood was collected into sodium heparin tubes and stored at 4°C for pharmacodynamic assay. A 1.4 ml volume of blood was collected into K2EDTA tubes and the plasma was processed by centrifugation for assessment of plasma drug concentrations.

Analysis of cytokine and mucin in BAL fluid

The levels of IFN γ , IL-4, IL-5, IL-13 and mucin in BAL fluid were analyzed using monkey specific ELISA kits, according to the manufacturer's instructions (Cell Sciences, Canton, MA), and assayed by triplicates. The limit of detections was 2-4 pg/ml. The level of IFN γ was

measured by Pierce Biotechnology Inc. (Woburn, MA) using a cyno specific ELISA. BAL total protein (mg/ml) was determined using DC protein assay reagent (Bio-rad, Hercules, CA).

Cytokine levels in BAL were normalized to pg/mg of total protein. BAL levels of mucin were quantified as follows: 50 μ l of BAL fluid diluted in coating buffer (0.1M sodium bicarbonate, PH 8.2) or 50 μ l of standard solution ranging from 500 to 7.8125 ng/ml mucin type 1 from bovine submaxillary glands (SIGMA, St. Louis, MO) were placed into 96-well immunosorb plates (VWR international, West Chester, PA) in triplicate and incubated at 4°C over night. Plates were washed 5 times with PBST (PBS containing 0.01% Tween-20) and blocked with 200 μ L of 3% I-block (Applied Biosystems, Bedford, MA) in PBS at 37°C for 2 h. After washing, mucin was detected by using 50 μ L of 4 μ g/mL biotinylated jacalin in PBS (VECTOR International, Burlingane, CA) and incubated at 37°C for 1 h, followed by 5 washes. 50 μ L/well of 2.5 μ g/ml avidin-Peroxidase (SIGMA, St. Louis, MO) in PBS was added and incubated at room temperature for 30 min. After the plates were washed 5 times, 100 μ L of mixed substrate solution (equal volume of ABTS peroxidase substrate and peroxidase solution B obtained from KPL Inc., Gaithersburg, MD) was added into each well, and absorbance at 405 nM was determined using a SpectraMax plate reader (Molecular Devices). Cytokine and mucin concentrations were calculated using standard curves and the SoftMax Pro software (Molecular Devices).

Supplement figure 1. Amino acid alignment of cynomolgus monkey, human, and mouse CCR8 using ClustalW(1.74). Cyno CCR8 shares 94% and 72% amino acid homology with human and mouse CCR8, respectively.

Wang, et al. Supplemental Figure 1

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CynoCCR8_analysis      MDYTLDPSTMTMTDYYPDSLSSPCDGLIQRNDKLLAVFYCLLFVFSLLGNSLVILVL
HumanCCR8_analysis      MDYTLDLSTVTVDYYPDIFFSPCDAELIQNGKLLAVFYCLLFVFSLLGNSLVILVL
MouseCCR8_analysis      MDYTMEPNVT-MTD-YYPDFFTAPCDAEFLLRGSMLYLAILYCVLFVLGLLGNLSLVILVL
                        *****:..*:** *****:::***.*:.. * **::*:***:*****
CynoCCR8_analysis      VVCKKLRNITDIYLLNLALSDLLFVFSFPFQTYQLDQWVFGTVMCKVVSIFYIIFYSS
HumanCCR8_analysis      VVCKKLRNITDIYLLNLALSDLLFVFSFPFQTYQLDQWVFGTVMCKVVSIFYIIFYSS
MouseCCR8_analysis      VGCKKLRNITDIYLLNLALSDLLFVFSIPFQTHNLLDQWVFGTAMCKVVSIFYIIFYSS
                        * *****.**:***** *****:*.****: *****.******:****:*
CynoCCR8_analysis      MFFITLMSVDRYLAVVHAVYAIKVRTIRMGTTLCLAVVWLTAIMATIPLLVFYQVASEDGV
HumanCCR8_analysis      MFFITLMSVDRYLAVVHAVYAIKVRTIRMGTTLCLAVVWLTAIMATIPLLVFYQVASEDGV
MouseCCR8_analysis      MFFITLMSVDRYLAVVHAVYAIKVRTASVGTALSLTVWLAATVATIPLMVFYQVASEDGM
                        *****.******.******:***.*.*.***.*: *****.******:
CynoCCR8_analysis      LQCYSFYNQQTLEKWKIFTNFMNLLGLLIPFTIFMFCYIKILHQLKRCQNHNKTKAIRLV
HumanCCR8_analysis      LQCYSFYNQQTLEKWKIFTNFMNLLGLLIPFTIFMFCYIKILHQLKRCQNHNKTKAIRLV
MouseCCR8_analysis      LQCFQFYEEQSIRWKLFTHEINALGLLIPFALLFCYVRILQQLRGCLNHNRTAIRKLV
                        ***:.***::*.***:***:***:***:***:***:***:***:***:***:***:***:
CynoCCR8_analysis      LIVVIASLLFWVPFNVLFLTSLHSMHILDGCSISQQLNYATHVTEIISFTHCCVNPVIY
HumanCCR8_analysis      LIVVIASLLFWVPFNVLFLTSLHSMHILDGCSISQQLNYATHVTEIISFTHCCVNPVIY
MouseCCR8_analysis      LTVVIVSLLFWVPFNVALFLTSLHDLHILDGCATRQRLALAIHVTEVISFTHCCVNPVIY
                        * ***.******.******:*****: *.* * ****:*****
CynoCCR8_analysis      AFVGEKFKKHLSEIFQKSCSHIFLYLGRQMPRESCEKSSSCQHSFRSSSIDYIL
HumanCCR8_analysis      AFVGEKFKKHLSEIFQKSCSQIFNYLGRQMPRESCEKSSSCQHSRSSSDYIL
MouseCCR8_analysis      AFIGEKFKKHLMDVFQKSCSHIFLYLGRQMPVGALERQLSSNQSSHSSTLDLIL
                        **:*****:*****:*** *****:*. *.*.*:***:***:

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Supplement figure 2. CCR8 antagonist ML604086. A) Structure of ML604086. B) ML604086 inhibits the binding of F-CCL1 to CCR8⁺CD4⁺ T-cells in cyno whole blood with an IC₅₀ of 1.0 μM. The degree of CCR8 inhibition by ML604086 was expressed relative to the control (inhibition of 100 nM unlabeled CCL1). Error bars denote SEM from 2 independent experiments.

Wang, *et al.* Supplemental Figure 2

