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Analysis of chemokine receptor CCR7 expression on porcine blood T lymphocytes using a CCL19-Fc fusion protein

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ABSTRACT

The chemokine receptor CCR7 has been a useful marker for the characterization of human and mouse T cell subsets. We have produced the porcine CCR7 ligand CCL19 fused to the human IgG1 Fc fragment, and used it to analyse CCR7 expression in swine. CCL19-Fc bound to and induced the migration of cells expressing porcine CCR7 but not of untransfected cells, corroborating its specificity. On blood lymphocytes, CCL19-Fc labelled the majority of $CD4^+$ T cells expressing the 2E3 marker, associated with a naïve phenotype, whereas the $2E3^-$ cells were mostly negative. Among $CD8^+$ T cells CCL19-Fc labelled two subsets: one, $CD8\beta^{hi}$ $CD11a^{lo}$ $CD45RA^+$, $perforin^{-/lo}$, which produced low amounts of IFN-γ after stimulation, which might correspond to naïve cells; and a second small population of $CD8\beta^{lo}$ cells which expressed high levels of CD11a, and were mostly $CD45RA^-$, a phenotype which resembles that of human central memory T cells.

1. Introduction

Chemokines are a family of small chemotatic proteins which regulate the traffic and effector functions of leukocytes both under homeostatic and inflammatory conditions, playing important roles in the development of protective immunity and in the establishment of immunological tolerance. Chemokines mediate their actions by binding and activating receptors of seven-transmembrane G protein-coupled type, present on the surface of cells of immune system (Zlotnik and Yoshie, 2000). These receptors are differentially expressed among leukocytes as a function of their lineage, development and activation state, and have been used in combination with other markers to discriminate leukocyte subsets in human and mouse (Moser et al., 2004).

The chemokine receptor CCR7 and its ligands CCL19 and CCL21 accomplish important roles in migration of T cell subsets towards secondary lymphoid organs, where these chemokines are constitutively expressed (Forster et al., 2008). So, mice deficient in CCL19, CCL21 or CCR7 display an altered structural organization of their secondary lymphoid organs (Forster et al., 1999; Gunn et al., 1999) and show profound defects in the development of antigen-specific immune responses, with a severe delay in the production of antibodies and the generation of cytotoxic T cells (Junt et al., 2004). CCR7, in combination with other markers, has been used to identify distinct mouse and human T cell populations that correspond to different stages of differentiation. Naïve T cells are characterized by a low expression of CD11a ($CD11a^{lo}$) and a high expression of CD45RA and the lymph node homing receptors CD62L and CCR7 (Okumura et al., 1993; Sanders et al., 1988; Wherry and Ahmed, 2004; Youseffi-Etemad and Axelsson, 1996). After antigen activation, a small proportion of the responding cells survive to give rise to Ag-specific memory T cells, which are typically $CD11a^{hi}CD45RO^+$. Some of these cells are $CCR7^{-/lo}$ (effector memory T cells), that express receptors for migration to inflamed tissues and display immediate effector functions. Others are $CCR7^{hi}$ (central memory T cells), that express lymph-node homing receptors and can undergo rapid clonal expansion in response to antigenic challenge and differentiate into $CCR7^-$ effector cells (Sallusto et al., 2004, 1999).

Although the sequences of genes encoding the porcine CCR7 and its ligand CCL19 have been reported, no studies on the distribution and function of these molecules in swine have been yet published. In this study, we have generated a chimeric molecule consisting in the porcine CCL19 fused in its C-terminal end to the Fc fragment of human IgG1, characterized its specificity and used it to monitor CCR7 cell surface expression by flow cytometry. Labelling PBMC with this fusion protein allowed us to discriminate several subsets of T cells, with features of naïve and memory cells.

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Table 1
Primer sequences used in this study.

Gen	Primer	Sequence	T_{AN} (°C)	bp
CCL19-GFP	CCL19-GFP-F	CGCTTGCTGAGAGTCTGCTCACCCTG	65	329
	CCL19-GFP-R	GCTAAGAGCAAGCACCACCGCAACC		
CCL19-Fc	XbaCCL19F	GCGCTCTAGACGCTTGCTGAGAGTCTGCTCACCCTG	68	326
	XbaCCL19R	GCGCTCTAGAGTTGCGGTGGTGCTTGCTCTTAGC		
CCR7-GFP	CCR7ATG-F	GCCAAGAGTGACATGGACCTGG	61	1141
	CCR7GFP-R	GGGGGGAGAAGGTGGTGG		

2. Materials and methods

2.1. Animals and cells

Blood samples were obtained from 6 to 12-month-old outbred Large-White pigs. The reported experiments have been executed in full compliance with guidelines by the ethical committee of the Institute.

Peripheral blood mononuclear cells (PBMC) were isolated on Percoll discontinuous gradients after blood sedimentation through dextran, as previously described (Gonzalez et al., 1990). Cells were resuspended in RPMI 1640 medium (Bio-Whittaker, Verviers, Belgium), containing 10% foetal calf serum (FCS, Bio-Whittaker), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol and 50 μg/mL gentamicin (Lonza, Walkersville, MD, USA).

2.2. Monoclonal antibodies

Monoclonal antibodies (mAb) to porcine CD3 (BB23-8E6, IgG2b) and CD4 (74-12-4, IgG2b) were kindly provided by Pescovitz (Indiana University, Indianapolis, IN, USA) and Lunney (USDA, Beltsville, MD, USA), respectively. Anti-IFN-γ mAb G47 was a generous gift from Lefevre (INRA, Jouy-en-Josas, France).

mAb to CD21 (B-Ly4, IgG1) and phycoerythrin (PE)-conjugated mAb to human perforin (δG9, IgG2b) were purchased from BD Biosciences (Erembodegem, Belgium). mAb to CD8β (PG164A, IgG2a) was purchased from VMRD (Pullman, WA, USDA). mAbs to porcine CD45RA (3C3/9, IgG1), CD11a (BL1H8, IgG2b) and 2E3 (IgM) were developed and produced in our laboratory.

For multi-colour immunofluorescence assays, anti-CD45RA mAb 3C3/9 and anti-IFN-γ mAb G47 were purified by affinity chromatography on Protein G-Sepharose CL-4B (GE Healthcare, Uppsala, Sweden) and labelled with biotin or Alexa 488 (Molecular Probes, Eugene, OR, USA), respectively.

2.3. Cloning and expression of recombinant proteins

Porcine CCL19 (NM 006274) and CCR7 (AB090872) cDNAs, in plasmid pCMVFL3 were from cDNA libraries reported by Uenishi et al. (2007). To obtain CCL19 and CCR7 fused to GFP sequence, whole coding sequences were PCR amplified using oligonucleotide primers shown in Table 1, and DyNAzyme II DNA polymerase; and subcloned into pcDNA3.1/CT-GFP-TOPO, following manufacturer protocol. Integrity, fidelity and orientation were confirmed by wholly sequencing selected cDNA clones.

To produce the porcine CCL19 fused to the Fc of human IgG1 (pCCL19-Fc), CCL19 cDNA sequence was PCR amplified using oligonucleotide primers containing XbaI restriction sequences (Table 1). The sequence of Fc of human IgG1, was PCR amplified from the plasmid phCCL1-hFc, a gift of Dr. Leonor Kremer (Gutierrez et al., 2004), using oligonucleotide primers containing XbaI and EcoRI restriction sequences (Table 1), and ligated, after digestion with both restriction enzymes, into the pcDNA3.1 plasmid equally digested with XbaI and EcoRI restriction enzymes. Integrity, fidelity and orientation of cDNA sequences in selected clones were confirmed by sequencing.

2.4. Production of recombinant fusion proteins

CHO cells were transfected with plasmid coding pCCL19-Fc, by using the Lipofectamine Plus Reagent (Invitrogen, San Diego, CA, USA). The day prior to transfection 3×10^5 CHO cells were seeded in 6-well plates (Nunc, Rochester, USA) and grown in Dulbecco's modified Eagle's minimal medium (DMEM) (BioWhittaker, Verviers, Belgium) supplemented with 50 μg/mL gentamicin, 2 mM L-glutamine and 5% FCS. Immediately before transfection, cells were rinsed with medium without serum or antibiotics (transfection medium, TM). For each well, 500 ng of plasmid DNA and 6 μL of PLUS reagent were mixed, diluted in 100 μL of TM and incubated for 15 min at room temperature. After that, 4 μL of lipofectamine diluted in 900 μL of TM were added to the transfection mixture, and after 15 min of incubation at room temperature, the complexes were added to the cells and incubated at 37 °C in a 5% CO_2 atmosphere for 4 h. Then, 1 mL of fresh growth medium was added and cells cultured for 24 h. For analysis of expression, monensin (2 μg/mL, Sigma, St. Louis, MO, USA), was added 4 h before harvesting. Cells were washed, permeabilised with cold methanol, 10 min at −20 °C, and incubated for 30 min at 4 °C with FITC-conjugated goat anti-human IgG (Bio-Rad Laboratories, Hercules, CA, USA). After three washes, cells were analysed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Non-transfected cells and cells incubated with lipofectamine but with no cDNA were used as negative controls.

For selection of cells expressing the pCCL19-Fc construct, geneticin (Gibco G-418, Grand Island, NY, USA) at a concentration of 800 μg/mL was added to the cultures. After the initial selection period of 10 days, two rounds of limiting dilution cloning were performed. Isolated clones were expanded and screened by flow cytometry and Western analysis for the production of the fusion protein.

Similarly, CHO cells were transfected with plasmid encoding porcine CCR7-GFP fusion protein. After lipofectamine transfection and geneticin selection, positive clones were screened by direct flow cytometry detection of GFP.

2.5. Western blot analyses

Transfected cells expressing the pCCL19-Fc fusion protein (2×10^5) were treated for 4 h with 2 μg/mL monensin (Sigma, St. Louis, MO, USA), washed with PBS and solubilized in 0.2 mL of lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 10 μg/mL of aprotinin and 1 mM PMSF). After centrifugation, the lysate was mixed with electrophoresis sample buffer, boiled and run on a 15% SDS–PAGE gel under reducing conditions. Separated proteins were transferred to nitrocellulose and analysed by Western blotting with a biotin-conjugated goat anti-hIgG (Rockland Inmunochemicals, Gilbertsville, PA, USA) and streptavidin-HRP (Thermo Fisher Scientific, Rockford, IL, USA). Peroxidase activ-