Molecular cloning of mXCR1, the murine SCM-1/lymphotactin receptor

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Abstract Single C motif-1 (SCM-1)/lymphotactin is a C-type member of the chemokine superfamily. Previously, we identified its specific receptor XCR1. Here we isolated the murine homologue of XCR1 (mXCR1). To demonstrate its biological activity, we produced recombinant mouse SCM-1 by the baculovirus expression system. B300-19 murine pre-B cells expressing mXCR1 responded to mSCM-1 in chemotactic and calcium-mobilization assays. mXCR1 mRNA was weakly expressed in spleen and lung of normal C57BL/6 mice. In spleen, $CD8^+$ cells and $NK1.1^+$ cells were found to express mXCR1. Identification of mXCR1 will now allow us to study the role of this unique cytokine system in the mouse models of inflammation and immunity.

Key words: SCM-1/lymphotactin receptor; XCR1 receptor; Mouse homologue

1. Introduction

Single C motif-1 (SCM-1)/lymphotactin/activation-induced, T cell-derived and chemokine-related molecule (ATAC) is a novel cytokine with structural homology to chemokines, especially to the CC subfamily, but lacking the first and third of the four canonical cysteine residues conserved in all other chemokines [1–4]. In humans, there are two highly homologous SCM-1 genes, *SCYC1* and *SCYC2*, which are localized close together on chromosome 1q23 and encode two SCM-1 proteins, SCM-1α and SCM-1β, with only two amino acid differences [5]. The biological functions as well as pathophysiological roles of SCM-1 are still mostly unknown. It was reported to be chemotactic for lymphocytes and NK cells [1,4,6,7]. However, its chemotactic activity is still controversial [8]. It is selectively expressed by activated $CD8^+$ T cells and double negative αβ-type thymocytes [1,3,4]. Furthermore, epidermal dendritic γδ-type T cells and intestinal intraepithelial γδ-type T cells were found to produce copious amounts of this cytokine upon activation [9]. Interleukin 2 (IL-2)-activated murine NK cells and human NK clones were also found to express its mRNA [7]. Thus, SCM-1/lymphotactin/ATAC is a unique cytokine whose expression is highly selective for certain subsets of T cells and NK cells.

Previously, we have identified a seven transmembrane G protein-coupled receptor specific for human SCM-1α and SCM-1β, and termed it XCR1 following the set rule of chemokine receptor nomenclature [10]. SCM-1 showed a high-affinity binding to XCR1 with a K_d of 10 nM and induced vigorous chemotaxis and calcium mobilization in XCR1-transfected murine L1.2 cells [10]. Northern blot analysis of various human tissues revealed that XCR1 mRNA was expressed strongly in placenta and weakly in spleen and thymus [10]. Hardly any signals were, however, detected in peripheral blood lymphocytes even after a long exposure. Thus, the cells expressing XCR1 appear to be associated with particular tissues but are rarely present in the blood. This makes identification of the cells expressing XCR1 in humans rather difficult. Thus, we decided to identify the mouse counterpart of XCR1 and to study the biological roles of this unique cytokine system in the murine models. Here, we report the identification and preliminary characterization of the murine homologue of XCR1 termed mXCR1.

2. Materials and methods

2.1. Cells and chemokines

The murine pre-B cell line B300-19 was kindly provided by Dr. H. Kawasaki at Tokyo University and maintained in RPMI 1640 supplemented with 10% fetal calf serum. Recombinant mouse SCM-1 was produced by using a baculovirus expression system and purified to homogeneity as described previously [10]. *N*-Glycanase and *O*-glycanase were purchased from Genzyme (Boston, MA, USA).

2.2. Southern blot analysis

This was carried out using DIG High Prime DNA Labeling and Detection Starter Kit II (Boehringer Mannheim). In brief, 25 μg of DNA from the liver of a C57BL/6 mouse was digested with *Hin*dIII. Digested DNA was fractionated by electrophoresis on 0.7% agarose gel and transferred onto a positively charged nylon membrane (Boehringer Mannheim) in alkaline conditions. Human XCR1 cDNA [10] was labeled with digoxigenin by the random priming method and used as a probe. Hybridization was carried out at 42°C for 15 h and hybridized bands were detected following the manufacturer's protocol.

2.3. Isolation of the mXCR1 genomic clone

A Lambda Fix II 129SVJ mouse liver genomic phage library (Stratagene) was plated on *Escherichia coli* strain XL1-Blue at a density of 4×10^4 plaques/plate. Filter lifts from 20 plates were screened with ^{32}P-labeled human XCR1 cDNA [10]. DNA from a positive clone was digested with *Eco*RI, subcloned into pBluescript II (Stratagene), and sequenced on both strands. The nucleotide sequence has been deposited in DDBJ/EMBL/GenBank with accession number AB028459.

2.4. Construction of the receptor expression plasmid

The coding region of mXCR1 was amplified from the mouse genomic clone by polymerase chain reaction (PCR) using specific primers (+ 5′-CTCGAGGCCATGGAGTCCTCTACAG-3′ and − 5′-TTGCGGCCGCTCCTCTCAGTAGAAGGAGGG-3′) and subcloned into pCRIITA vector. After confirmation of the sequence, the coding region was recloned into a retrovirus vector (pMX-I/E)

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to generate pMX-I/E-mXCR1. pMX-I/E was generated from pMX [11] by inserting the internal ribosome entry site (IRES) (Clontech) and the coding sequence for enhanced green fluorescent protein (EGFP) (Clontech).

2.5. Stable expression of mXCR1

BOSC23 cells obtained from ATCC were seeded into 60 mm dishes (2×10^6 cells/dish) 1 day before transfection. The cells were transfected with pMX-I/E-mXCR1 or pMX-I/E using Lipofectamine (Gibco-BRL) and cultured for 48 h. The supernatants containing the recombinant retroviruses were then used to infect B300 cells to isolate cells stably expressing mXCR1. In brief, 1.1×10^5 B300 cells were incubated with 3 ml of virus stock in the presence of 10 mg/ml of polybrene for 8 h. Cells expressing EGFP were counted under a fluorescent microscope before use.

2.6. Chemotaxis assay

Migration of control and mXCR1-expressing B300 cells was assessed in Transwell plates (Costar) with 3 μm pores. After incubation at 37°C for 4 h, cells migrating into lower wells were counted as described previously [10].

2.7. Calcium mobilization assay

This was carried out as described previously [10]. In brief, cells loaded with Fura-2/AM (Molecular Probes, Eugene, OR, USA) were placed in a cuvette (1×10^6 cells/300 μl) and kept at 37°C with constant stirring on a spectrofluorimeter (F-2500, Hitachi, Japan). Following addition of mSCM-1, the fluorescence intensity ratio ($R_{340/380}$) was monitored by measuring emission fluorescence at 510 nm upon excitation at 340 nm and 380 nm with a time resolution of 5 points/s.

2.8. Northern blot analysis

This was carried out as described previously [10]. A multi-tissue Northern blot filter was purchased from Clontech. The mXCR1 probe of 1.2 kb was generated by PCR using specific primers described above.

2.9. Cell fractionation

Female C57BL/6 mice were purchased from Clea Japan (Osaka) and used at about 7 weeks of age. Spleen cells were mechanically dispersed and cells with indicated surface markers were purified by magnetic cell sorting using MACS (Miltenyi Biotech, Germany). In brief, $CD4^+$ cells, $CD8^+$ cells, and $B220^+$ cell were positively selected on a VS separation column (Miltenyi) after labeling with anti-CD4 microbeads, anti-CD8 microbeads, and anti-B220 microbeads, respectively. For isolation of macrophages, plastic adherent cells were resuspended with EDTA treatment and vigorous pipetting, stained with FITC-conjugated F4/80 (Pharmingen), and positively selected on a VS column after labeling with anti-FITC microbeads (Miltenyi). For isolation of NK cells, spleen cells were double-stained with biotinylated anti-CD3 and FITC-conjugated anti-NK1.1. $CD3^+$ cells were negatively selected on a BS depletion column (Miltenyi) after reacting with streptavidin microbeads. Subsequently, $NK1.1^+$ cells were positively selected on a VS column after reacting with anti-FITC microbeads. The purity of each cell fraction was examined on a flow cytometer after staining with FITC-labeled antibodies to respective cell surface markers.

2.10. RT-PCR

Cells were lysed with Trizol (Gibco-BRL) and total RNA was purified by using RNeasy (Qiagen). Total RNA from each cell fraction (290 ng) was reverse transcribed by using 6 pmol of oligo$(dT)_{18}$ primer and SuperScript II reverse transcriptase (Gibco-BRL). The resulting first-strand DNA (15 ng of total RNA equivalent) and original total RNA (15 ng) were subjected to PCR amplification in a final volume of 20 μl containing 10 pmol of each primer and 1 unit of Ex-Taq polymerase (TaKaRa, Japan). Primers used were: + 5′-ATACCTGTCTGTAGTGAGC-3′ and − 5′-AAAGCACTGGGTTGAAACA-3′ for mXCR1; + 5′-ATGGATGACGATATCGCT-3′ and − 5′-ATGAGGTAGTCTGTCAGGT-3′ for mouse β-actin. PCR was carried out by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Amplification products (6 μl each) were separated by 2% agarose and stained with ethidium bromide.

3. Results and discussion

3.1. Identification of mouse XCR1

The genomic DNA derived from a C57BL/6 mouse was digested with *Hin*dIII and subjected to Southern blot analysis using the human XCR1 cDNA [10] as a probe. A 2 kb band was detected even by hybridization at high stringency conditions (data not shown). This suggested the presence of a gene in the mouse genome that was highly homologous to human XCR1. We therefore screened a mouse liver genomic library using XCR1 cDNA as a probe. A positive clone was isolated and the 11 kb insert was sequenced. The clone contains a long open reading frame without introns encoding a putative seven transmembrane G protein-coupled receptor. There are two potential initiation codons with an in-frame stop codon in the upstream region. Since the second ATG, but not the first ATG, conforms to Kozak's rule [12] and also corresponds to the first ATG of the human sequence, we postulate that the translation starts from the second ATG. The encoded protein of 322 amino acids has seven transmembrane regions and an acidic N-terminus, the features common to other chemokine receptors (Fig. 1). The predicted amino acid sequence shows the highest homology with human XCR1 with 71% identity. The murine sequence has no *N*-glycosylation site in the putative extracellular domains as the human sequence. Furthermore, the DRYLAIV motif in the second intracellular domain, which is highly conserved in other chemokine receptors and is involved in G protein docking [13], is altered

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                                                                    TM1
mXCR1   MES-----STAF--YDYHDKLSLLCENNVIFFSTISTIVL
        :::     ::: :  ::      :::     : : :  : ::
hXCR1   MESSGNPESTTFFYYDLQSQ---PCENQAWVFATLATTVL
                                                                    TM2
        ____________________________________________________________
mXCR1   YSLVFLLSLVGNSLVLWVLVKYENLESLTNIFILNLCLSD
        : :::::::::::::::::::::::::: ::::::::::::::::
hXCR1   YCLVFLLSLVGNSLVLWVLVKYESLESLTNIFILNLCLSD
                                                                    TM3
        ____________________________________________________________
mXCR1   LMFSCLLPVLISAQW-SWFLGDFFCKFFNMIFGISLYSSI
        : : ::::: ::    : :::: ::  :::: :::::::
hXCR1   LVFACLLPVWISPYHWGWVLGDFLCKLLNMIFSISLYSSI
                                                                    TM4
        ____________________________________________________________
mXCR1   FFLTIMTIHRYLSVVSPISTLGIHTLRCRVLVTSCVWAAS
        ::::::::::::::::: :::   :::::::::  :: ::
hXCR1   FFLTIMTIHRYLSVVSPLSTLRVPTLRCRVLVTMAVWVAS
                                                                    TM5
        ____________________________________________________________
mXCR1   ILFSIPDAVFHKVISLNCKYSEHHGFLASVYQHNIFFLLS
        :: :: :  :::: :  : ::     : ::::: :::::
hXCR1   ILSSILDTIFHKVLSSGCDYSELTWYLTSVYQHNLFFLLS
                                                                    TM6
        ____________________________________________________________
mXCR1   MGIILFCYVQILRTLFRTRSRQRHRTVRLIFTVVVAYFLS
          :::::::: :::::::: ::  ::::: ::: :::::::
hXCR1   LGIILFCYVEILRTLFRSRSKRRHRTVKLIFAIVVAYFLS
                                                                    TM7
        ____________________________________________________________
mXCR1   WAPYNLTLFLKTGIIQQ---SCESLQQLDIAMIICRHLAF
        : ::: :::: :    :   ::: ::: :  ::: :::
hXCR1   WGPYNFTLFLQTLFRTQIIRSCEAKQQLEYALLICRNLAF

        ____________________________________________________________
mXCR1   SHCCFNPVLYVFVGIKFRRHLKHLFQQVWLCR---KTSST
        :::::::::::::::: ::: ::::   : : ::
hXCR1   SHCCFNPVLYVFVGVKFRTHLKHVLRQFWFCRLQAPSPAS

mXCR1   VPCSPGTFTYEGPSFY
          : ::: : ::: :::
hXCR1   IPHSPGAFAYEGASFY
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Fig. 1. Alignment of the amino acid sequences of mXCR1 and human XCR1. The amino acid sequence of mXCR1 is deduced from the coding sequence of the mXCR1 genomic clone. Gaps are marked by dashes. Identical residues are indicated by colons. TM, transmembrane regions. The sequence corresponding to the DRY motif is boxed.

to HRYLSVV in both human and murine XCR1. This probably affects their coupling with G protein partners. From these and other results described below, we concluded that the sequence represents the murine counterpart of XCR1 (mXCR1).

3.2. Production of recombinant mouse SCM-1

In order to test the biological activity of mXCR1, we generated recombinant murine SCM-1 (mSCM-1) by the baculovirus expression system. Insect High Five cells were infected with a recombinant baculovirus encoding mSCM-1 under the control of the polyhedrin promoter. Secreted mSCM-1 in the culture supernatants was purified by cation exchange chromatography, heparin affinity chromatography and reverse phase high performance liquid chromatography (HPLC). As shown in Fig. 2A, mSCM-1 was eluted as a single peak from HPLC. The purified mSCM-1 migrated as a broad band of 15 kDa on a gradient SDS-PAGE (Fig. 2B). The N-terminal amino acid sequence analysis revealed that the mature mSCM-1 started at valine 22 like human recombinant SCM-1 and natural ATAC [8,10]. Treatment with *O*-glycanase but not with *N*-glycanase

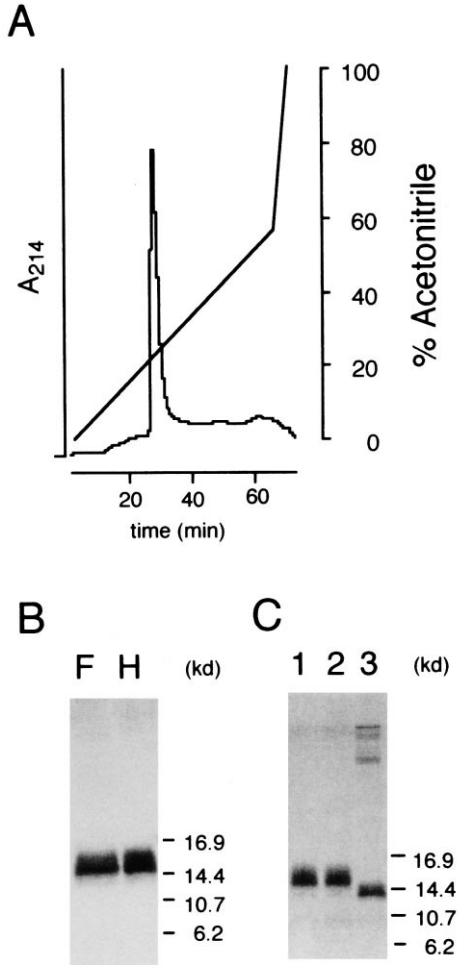

Fig. 2. Purification of recombinant mSCM-1. A: Elution profile of mSCM-1 on reverse phase HPLC. B: Silver staining of purified mSCM-1 on SDS-PAGE. F, the peak fraction eluted from Hitrap heparin; H, the peak fraction eluted from reverse phase HPLC. C: Deglycosylation of mSCM-1. Purified mSCM-1 was treated without or with *N*-glycanase or *O*-glycanase. After SDS-PAGE, silver staining was done. 1, untreated; 2, treated with *N*-glycanase; 3, treated with *O*-glycanase. The molecular weight markers are shown on the right (kDa).

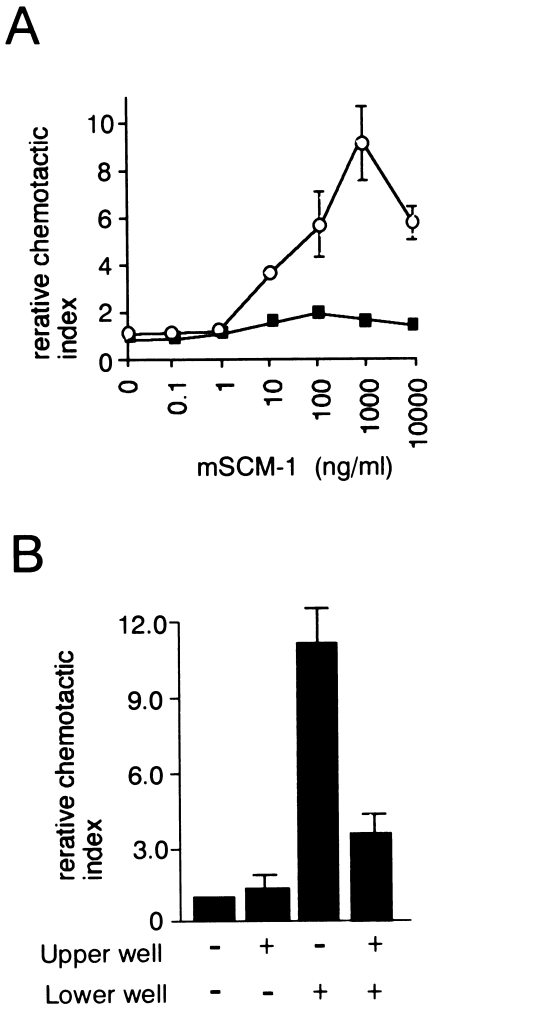

Fig. 3. Chemotactic response of mXCR1-expressing murine B300-19 pre-B cells. A: Dose-response experiments. Vector-transfected (closed squares) or mXCR1-transfected (open circles) B300-19 cells were placed in the upper wells of Transwell plates. mSCM-1 was added to the bottom wells at indicated concentrations. After incubation at 37°C for 4 h, the cells that had migrated into bottom wells were counted under the microscope. The assays were done in triplicate. Each point represents mean ± S.E.M. from three separate experiments. B: A checkerboard-type analysis. In the chemotaxis assays using mXCR1-expressing B300 cells, mSCM-1 was added to the upper and/or lower wells at 100 ng/ml as indicated. The assays were done in triplicate. Each point represents mean ± S.E.M. from three separate experiments.

reduced the molecular mass by about 1 kDa (Fig. 2C). This showed that mSCM-1 was *O*-glycosylated as reported for human natural ATAC [10].

3.3. Induction of chemotaxis and calcium mobilization by mSCM-1 via mXCR1

To determine the ability of mSCM-1 to signal via mXCR1, we stably expressed mXCR1 in the murine pre-B cell line B300-19 by using a retrovirus vector encoding mXCR-1 and GFP. Control cells were infected with a retrovirus vector encoding GFP only. After infection, GFP-expressing cells were found to be about 20% and 40%, respectively. As shown in Fig. 3A, mSCM-1 induced a vigorous chemotactic response in mXCR-1-expressing cells but not in control cells with a typical bimodal dose-response curve and a maximal response at 100 ng/ml (10 nM). A checkerboard-type analysis confirmed

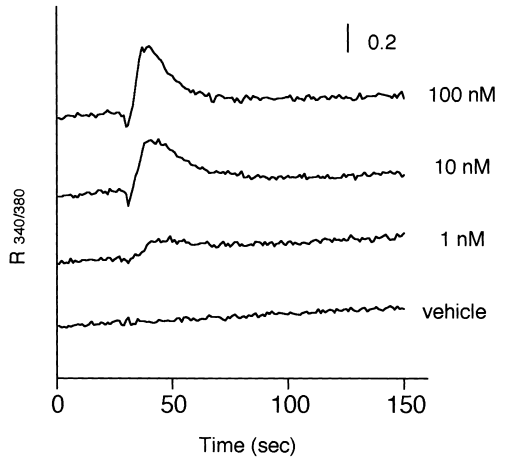

Fig. 4. Calcium mobilization in mXCR1-expressing B300-19 cells by mSCM-1. mXCR1-expressing B300-19 murine pre-B cells were loaded with Fura-2/AM and stimulated with mSCM-1 as indicated. The data shown are representative of three separate experiments. The scale bar (0.2) is shown on the right.

that the migration of mXCR1-expressing B300-19 cells toward mSCM-1 was mostly chemotactic (Fig. 3B). As shown in Fig. 4, mSCM-1 also induced calcium mobilization in mXCR1-expressing B300-19 cells in a dose-dependent manner. B300-19 cells expressing only GFP alone did not show any such response to mSCM-1 (data not shown). These results clearly demonstrate that mXCR1 is a functional receptor for mSCM-1.

3.4. Expression of mXCR1 in tissues and cells

Expression of mXCR1 in various mouse tissues was studied by Northern blot analysis. As shown in Fig. 5, weak signals of a 2.4 kb band were detected in spleen and lung after a long exposure. To further define the types of cells expressing mXCR1, we fractionated spleen cells obtained from C57BL/6 mice by using various surface markers. The expression of mXCR1 was analyzed by RT-PCR. As shown in Fig. 6, mXCR1 expression was detected in cells with CD8 marker and also weakly in NK cells ($NK1.1^+CD3^-$). No signals were detected without reverse transcription (not shown), excluding contamination of genomic DNA. Thus, $CD8^+$ cells and NK cells appear to express mXCR1.

The expression of mXCR1 in $CD8^+$ cells and $NK1.1^+$ cells is rather striking because these types of cells are also considered to be the main producers of SCM-1 [1,3,4,7,9]. However, self-recruitment through secretion of self-reactive chemokines appears to be rather common among various leukocytes [13,14]. The weak signals obtained from the fractionated cells even by RT-PCR might also suggest that only a subset of cells with these markers do express mXCR1. Generation of antibodies to mXCR1, which is now in progress in our laboratory, will allow us to identify the exact types of cells that express mXCR1 and respond to mSCM-1.

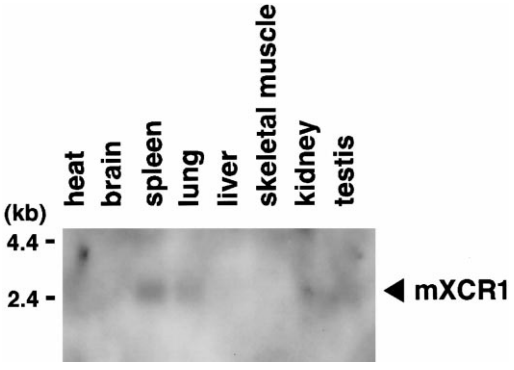

Fig. 5. Expression of mXCR1 mRNA in various mouse tissues. A filter blotted with 2 μg/lane of poly(A)$^+$ RNA from indicated mouse tissues (Clontech) was hybridized with the ^{32}P-labeled cDNA probe for mXCR1. For autoradiography, exposure was done at −80°C for 7 days with an intensifying screen.

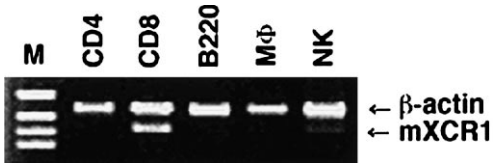

Fig. 6. RT-PCR analysis of mXCR1 expression in fractionated spleen cells. Total RNA was isolated from the indicated cell fractions, reverse transcribed and subjected to PCR amplification using mXCR1 specific primers. Simultaneously, amplifications of β-actin were performed as an internal control. Amplification products were run on 2% agarose gel and stained with ethidium bromide. Representative results from two separate experiments are shown. The purity of each cell fraction used was as follows: $CD4^+$, 95.1%; $CD8^+$, 94.5%; $B220^+$, 97.1%; macrophages, 81.6%; $NK1.1^+CD3^-$ cells, 88%.

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