FULL PAPER *Immunology*

Molecular Cloning, Chromosomal Location, and Biological Activity of Porcine Interleukin-21

Yoshihiro MUNETA[1)], Reiko KIKUMA[1)], Hirohide UENISHI[2)], Tomoaki HOSHINO[3)], Kazuhiro YOSHIHARA[1)], Maiko TANAKA[4)], Noriyuki HAMASHIMA[2)] and Yasuyuki MORI[1)]

[1)]National Institute of Animal Health, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, [2)]National Institute of Agrobiological Sciences, 2 Ikenodai, Tsukuba, Ibaraki 305-8602, [3)]Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011 and [4)]STAFF-Institute, 446-1 Ippaizuka, Kamiyokoba, Tsukuba, Ibaraki 305-0854, Japan

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ABSTRACT. A pig interleukin-21 (IL-21) cDNA was successfully cloned and sequenced from porcine peripheral blood lymphocytes (PBL) stimulated with 10 μg/m*l* concanavalin A (ConA), 10 μg/m*l* phytohemagglutinin P (PHA), 50 ng/m*l* phorbol 12-myristate 13-acetate (PMA), and 0.5 μg/m*l* anti-porcine CD3 antibody for 48 hr. The open reading frame of the porcine IL-21 cDNA is 459 base pairs in length and encodes 152 amino acids. The predicted amino acid sequence of the porcine IL-21 shows 86.2%, 77.7%, and 58.4% identi ty to the bovine, human, and murine IL-21, respectively. The porcine IL-21 gene was mapped to porcine chromosome 8 (8q22→q23) by means of fluorescence in situ hybridization and radiation hybrid mapping, where the porcine IL-2 gene had been mapped nearby. The recombinant porcine mature IL-21 expressed by *E. coli* induced dose-dependent proliferation and IFN-γ production from a human NK cell line, NK0. The porcine IL-21 identified in this study will be helpful for the enhancement of innate immune responses of pigs.
KEY WORDS: cloning, innate immunity, interleukin-21, NK cell, porcine.

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Interleukin-21 (IL-21) is a novel cytokine that regulates the proliferation of T and B cells, and involves the maturation and expansion of NK cells from bone marrow progenitor cells [20]. IL-21 is produced by activated T cells [20, 21], and is a four-helix-bundle type I cytokine with structural similarity to IL-2, IL-4, and IL-15 [20, 21]. IL-21 receptor also has significant amino acid similarities with IL-2, IL-4, and IL-15 [18], and shares the common γ chain for the subunit of the IL-21 receptor complex [2]. The common γ chain-containing receptor binded by its ligand such as IL-2 and IL-21 causes the activation of the Janus tyrosine kinase family 3 (JAK3) [6, 17], and transduce its signal to the downstream molecules. Recent studies have indicated that IL-21 has various biological activities that affect T and NK cell functions [20, 21], immunoglobulin production [19], antitumor activity [1], Th1 response [26], Th2 response [30], and growth and survival for myeloma cells [4]. Currently, IL-21 is thought to be a mediator cytokine, which promotes the transition from innate to adaptive immunity [9, 21].

One of the most interesting characteristics of IL-21 is its effect on NK cell maturation and expansion [20], because pigs have high NK cell populations in the peripheral blood [31], and many piglets suffer from diarrhea and respiratory diseases in their neonatal periods during which adaptive immunity is immature. We previously reported the cloning and expression of porcine IL-18 [16], and the IFN-γ induction by IL-18 from neonatal piglets [13]. Recently, Strengell *et al.* reported that IL-21 also enhances IFN-γ production from human NK and T cells in synergy with IL-18 [25]. Taken, together, we would like to isolate porcine IL-21, a recent cytokine that promotes the transition from innate to adaptive immunity [9, 21], in order to utilize this cytokine for the enhancement of neonatal immunity of pigs. We also reported the cloning, and the expression of bovine IL-21 using a baculovirus expression system [15].

In the present study, cDNA encoding porcine IL-21 was cloned and characterized, and the chromosomal location of the porcine IL-21 gene was investigated using a fluorescence in situ hybridization technique (FISH) and a radiation hybridization (RH) panel. In addition, the recombinant porcine IL-21 was expressed by *E. coli* and the biological activity was examined in regard to its utilization in the enhancement of porcine innate immunity, especially mediated by NK cells.

MATERIALS AND METHODS

Cloning of porcine IL-21 cDNA: Porcine peripheral blood mononuclear cells (PBMC) were obtained by means of Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient separation of the peripheral blood of healthy pigs. Peripheral blood lymphocytes (PBL) were isolated and cultured as described previously [14]. The PBL were stimulated with 10 μg/m*l* ConA (Sigma Chemicals Co., St. Louis, Mo, U.S.A.), 10 μg/m*l* PHA (Difco Laboratories, Detroit, MI, U.S.A.), 50 *n*g/m*l* PMA (Sigma), and 0.5 μg/m*l* anti-porcine CD3 antibody (VMRD Inc., Pullman, WA, U.S.A.) for 48 hr. Then, total RNA was isolated using TRIZOL reagents (Life Technologies, Gaithersburg, MD, U.S.A.). The first strand cDNA was prepared from 1 μg of total RNA using oligo dT adaptor primer (Takara RNA PCR kit Ver.2.1, Takara Shuzo Co., Ltd., Osaka, Japan). The oligonucleotide primer pairs used for degenerate PCR were designed based on the bovine IL-21 (Genbank Accession No. AB073021) sequence as follows: sense primer, 5'-

ATGCGGTGGCCGGGGAACATGGAG-3', antisense primer, 5'-CTAGGACAGATGCTGATGAAT-3'. Amplification was performed in a thermalcycler (Geneamp 9600, Perkin Elmer Cetus, CA, U.S.A.) for 35 cycles at 94°C for 45 sec, at 48°C for 45 sec, and at 72°C for 60 sec. After agarose-gel electrophoresis of the PCR-amplified products, a single band at approximately 450 base pairs was purified from the gel. The purified PCR products were ligated into plasmid pCR 3.1 using a TA cloning Kit (Invitrogen Co., Carlsbad, CA, U.S.A.). The recombinant plasmid was used to transform *E. coli* INVαF' (Invitrogen). The positive clones were selected and verified by DNA sequencing. Two independent cDNA clones from different experiments were sequenced in order to ensure that no PCR-induced mutations had occurred. The sequence data was submitted to DDBJ/EMBL/GenBank and assigned an accession number AB073020.

Fluorescence in situ hybridization (FISH): The bacterial artificial chromosome (BAC) clones containing the porcine IL-21 gene were isolated from a swine BAC library [27], available in the DNA Bank of the National Institute of Agrobiological Sciences (Tsukuba, Japan). The primers used for isolation of porcine IL-21 genomic DNA were 5'-CGGGGAACATGGAGAAAATA-3' and 5'-CAAGTCATGAACATAATTTTTCAGC-3'. These primers were constructed within putative exon 1 of porcine IL-21 expected by human IL-21 genomic sequence (Genbank Accession No. NT-016354). FISH was performed as described previously [3, 29] with a slight modification. Briefly, the chromosome spreads were treated with RNase and then denatured with 2 × SSC containing 50% formamide. Five hundred *n*g of DNA of BAC clone 162F9 (The size of the insert was 150 kb confirmed by pulse field gel electrophoresis.), containing the swine IL-21 gene, was biotinylated with a biotin nick-translation labeling mix kit (Roche Diagnostics GmbH, Mannheim, Germany). The biotinylated probe DNA was dissolved in 10 μl of formamide and then mixed with 10 (l of 2 × hybridization buffer (4 × SSC, 100 mM phosphate buffer [pH 7.0], 20% dextran sulfate, 2 × Denhardt's solution, and 0.2% SDS) containing 5 μg of porcine Cot-5 DNA (repetitive sequence-enriched DNA). The mixture was heated at 90°C for 2 min and cooled gradually at room temperature. The chromosome spreads were then covered with the mixture and incubated at 37°C for 16 hr in a humidified chamber. Hybridization signals were detected using an FITC-conjugated streptavidin/biotinylated anti-streptavidin system (Vector Laboratories, Burlingame, CA, U.S.A.), and located on the specific chromosome according to the procedure described previously [12, 28]. The hybridization signals visualized under B2 excitation were captured by a cooled CCD camera (Hamamatsu Photonics Inc., Hamamatsu, Japan), and the R-band patterns under G excitation were superimposed on the image of the hybridization signals.

Radiation hybrid (RH) mapping: The mapping procedure using a radiation hybrid panel of the porcine genome (SSRH) described previously [7] was used. Briefly, PCR was performed in duplicate in a 15-μl reaction mixture containing 1 μg of DNA from each hybrid, 1.5 mM $MgCl_2$, 1 × buffer (Applied Biosystems Japan, Tokyo, Japan), 0.5 U of AmpliTaq Gold, 20 μM dNTP, and each primer pair at 0.125 μM. The primers used for RH mapping were 5'-CACTGTGAGCAGTCAGCTTTTT-3' and 5'-GCCCATGTTTCTGTCTTCTCC-3'. The mixture was incubated at 94°C for 10 min, and then subjected to 35 cycles of PCR, with each cycle consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C, and 30 sec of extension at 72°C. An extra 5 min incubation at 72°C was performed at the end of the reaction. As controls, murine and porcine genomic DNA were used as templates for PCR. PCR without any template was also performed simultaneously as a control. The distances between the porcine IL-21 mapped in this study and the framework markers on the SSRH map were calculated using the program RHMAPPER [24] and its "Z-extension" [23], with vector data of the framework markers.

Expression of recombinant porcine IL-21 by E. coli: Recombinant porcine IL-21 was expressed by a QIAexpress™ *E. coli* expression system (QIAGEN Inc., Chatsworth, CA, U.S.A.). In brief, the gene encoding porcine mature IL-21 was amplified by PCR and sub-cloned into expression vector pQE70 (QIAGEN Inc.), and expression of the recombinant porcine IL-21 was performed following the manufacturer's instructions. Purified porcine IL-21 was obtained using a Hi-trap chelating sepharose column (Amersham Pharmacia Biotech) as described by the manufacturer's instructions.

Biological activity of porcine IL-21: Human NK cell line NK0 [10], kindly provided by Dr. Tomoaki Hoshino (Kurume University School of Medicine, Kurume, Japan), was cultured at 1×10^4 cells/well in a 96-well plate (Nunc, Denmark) and stimulated with 10-fold serially diluted porcine IL-21 and human IL-21 (R&D systems Inc., Minneapolis, MN, U.S.A.) for 96 hr. Cell proliferation was then assayed using a WST-1 cell counting kit (Dojindo, Kumamoto, Japan). At the same time, the culture supernatant was collected, and IFN-γ concentration was measured using a human IFN-γ specific ELISA (Endogen Inc., Woburn, MA, U.S.A.). In order to inhibit the JAK3 pathway, we used a specific inhibitor of JAK3 (4-(4'-hydroxyphenyl) amino-6, 7-dimethoxyquinazoline, CALBIOCHEM, La Jolla, CA, U.S.A.) in the above mentioned assay at 100 μM.

RESULTS

Characterization of porcine IL-21 cDNA: The cDNA sequence encoding the porcine IL-21, and the predicted amino acid sequence are shown in Fig. 1. The cDNA contains an open reading frame of 459 base pairs and encodes 152 amino acids. The nucleotide sequence of porcine IL-21 shows 91.5%, 85.4%, and 72.7% sequence identity with the bovine, human, and mouse IL-21, respectively. The predicted amino acid sequence of the porcine IL-21 shows 86.2%, 77.7% and 58.4% identity to those of the bovine, human, and mouse, respectively. The predicted molecular

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        10         20         30         40         50         60
ATGCGGTGGCCGGGGAACATGGAGAAAATAGTCATCTGCCTGATGGTCATCTTCTCAGGC
M  R  W  P  G  N  M  E  K  I  V  I  C  L  M  V  I  F  S  G

        70         80         90        100        110        120
ACAGTGGCCCATAAATCAAGCTTCCAAGGACAAGATCGCCTCTTGATTAGACTGCGTCAA
T  V  A  H  K  S  S  F  Q  G  Q  D  R  L  L  I  R  L  R  Q

       130        140        150        160        170        180
CTTATAGACACTGTTGATCAGCTGAAAAATTATGTTCATGACTTGGACCCTGAATTGCTG
L  I  D  T  V  D  Q  L  K  N  Y  V  H  D  L  D  P  E  L  L

       190        200        210        220        230        240
CCAGCTCCAGAAGATGTACAGAGACACTGTGAGCAGTCAGCTTTTTCATGTTTTCAGAAG
P  A  P  E  D  V  Q  R  H  C  E  Q  S  A  F  S  C  F  Q  K

       250        260        270        280        290        300
GTCGAACTAAAGTCAGCAAATACGGGAGACAATGAAAAGATAATCAATGTATTAATAAAA
V  E  L  K  S  A  N  T  G  D  N  E  K  I  I  N  V  L  I  K

       310        320        330        340        350        360
CAGCTGAAGAGGAAACTACCTCCCACAAATGCAGGGAGAAGACAGAAACATGGGCTAACA
Q  L  K  R  K  L  P  P  T  N  A  G  R  R  Q  K  H  G  L  T

       370        380        390        400        410        420
TGTCCTACATGTGATTCGTATGAGAAAAAACCAATCAAAGAATTCCTAGAAAGACTGAAA
C  P  T  C  D  S  Y  E  K  K  P  I  K  E  F  L  E  R  L  K

       430        440        450        460
TCGCTCATCCAAAAGATGATTCATCAGCATCTGTCCTAG
S  L  I  Q  K  M  I  H  Q  H  L  S  *
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Fig. 1. Nucleotide and predicted amino acid sequence of porcine IL-21. The numbers with each line indicate the nucleotide position. The putative amino acid sequences of the signal peptide are underlined. Bold letters show the amino acid sequences of mature porcine IL-21. Stop codon is indicated by an asterisk. Nucleotide sequence data reported in this paper will appeared in the DDBJ, EMBL, and Genbank databases with the following accession number AB073020.

weight of porcine mature IL-21 is 14,224 dalton. Amino acid alignment (Fig. 2) of the porcine IL-21 with bovine, human, and mouse IL-21 shows that a potential cleavage site for processing to the mature IL-21 was conserved. Four cysteine residues to form proper protein structure were all conserved. In addition, a Gln residue (amino acid position 145), which has been shown to be involved in the binding to common γ chain [32], was also conserved in porcine IL-21.

Chromosomal location of porcine IL-21: To investigate chromosomal location of porcine IL-21 gene, we used FISH and RH mapping methods. By FISH analysis, when more than 50 chromosome spreads were examined after hybridization, the positive signals were consistently detected on chromosome 8. Integration of the R-band pattern with the image of the FISH signals indicated that the precise location of the IL-21 gene was on pig chromosome 8q22→q23 (Fig. 3). The RH mapping also showed that the porcine IL-21 gene was located between S0086 and S0069, which reside on pig chromosome 8 (Fig. 4).

Expression and biological activity of porcine IL-21: Porcine mature IL-21 fused with six histidine tags was expressed by *E. coli*, and purified using a Ni^{2+} chelating column (data not shown). The purified porcine mature IL-21 induced dose-dependent proliferation of the human NK cell line, NK0 cells (Fig. 5). In addition, porcine IL-21 induced IFN-γ production from NK0 cells in a dose-dependent fashion (Fig. 6). However, both cell proliferation and the IFN-γ production of NK0 cells by porcine IL-21 were inhibited by JAK-3 inhibitor (Figs. 5 and 6).

DISCUSSION

In this study, porcine IL-21 gene was cloned and characterized for the first time. Porcine IL-21 cDNA showed significant homology with bovine, human, and mouse IL-21, respectively. Amino acid alignment also indicated that potential cleavage sites to the mature IL-21 and four cysteine residues were all conserved. In addition, porcine IL-21 has a conserved Gln residue, which has been implicated in the interaction with IL-2 receptor γ chain. These results indicated that the porcine IL-21 identified in this study has a structure similar to those of IL-21 cloned in other species.

FISH and RH mapping results demonstrated that the porcine IL-21 gene mapped to chromosome 8 (8q22→q23), near the site where porcine IL-2 gene is located [5]. Human IL-2, IL-15, and IL-21 genes are located in the same region

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                          10          20          30          40          50
Po IL-21      1 MRW-PGNMEK IVICLMVIFS GTVAHKSSFQ GQDRLLIRLR QLIDTVDQLK    50
Bo IL-21      1 MRW-PGNMER IVICLMVIFS GTVAHKSSSQ GQDRLFIRLR QLIDIVDQLK    50
Hu IL-21      1 MRSSPGNMER IVICLMVIFL GTLVHKSSSQ GQDRHMIRMR QLIDIVDQLK    50
Mu IL-21      1 -------MER TLVCLVVIFL GTVAHKSSPQ GPDRLLIRLR HLIDIVEQLK    50
                        **     ** ***  **  **** * * **  ** *  *** * ***

                          60          70          80          90          100
Po IL-21     51 NYVHDLDPEL LPAPEDVQRH CEQSAFSCFQ KVELKSANTG DNEKIINVLI   100
Bo IL-21     51 NYVNDLDPEF LPAPEDVKRH CERSAFSCFQ KVQLKSANNG DNEKIINILT   100
Hu IL-21     51 NYVNDLVPEF LPAPEDVETN CEWSAFSCFQ KAQLKSANTG NNERIINVSI   100
Mu IL-21     51 IYENDLDPEL LSAPQDVKGH CEHAAFACFQ KAKLKPSNPG NNKTFIIDLV   100
                   ** **  * ** **    **  ** *** *  **  * *  *   *

                         110         120         130         140         150
Po IL-21    101 KQLKRKLPPT NAGRRQKHGL TCPTCDSYEK KPIKEFLERL KSLIQKMIHQ   150
Bo IL-21    101 KQLKRKLPAT NTGRRQKHEV TCPSCDSYEK KPPKEYLERL KSLIQKMIHQ   150
Hu IL-21    101 KKLKRKPPST NAGRRQKHRL TCPSCDSYEK KPPKEFLERF KSLLQKMIHQ   150
Mu IL-21    101 AQLRRRLPAR RGGKKQKHIA KCPSCDSYEK RTPKEFLERL KWLLQKMIHQ   150
                  * *  *     *  ***    ** ******    ** ***  * * ******

                         160
Po IL-21    151 HLS------- --      152
Bo IL-21    151 HLS------- --      152
Hu IL-21    151 HLSSRTHGSE DS      162
Mu IL-21    151 HLS------- --      146
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Fig. 2. Amino acid comparison of porcine (Po) IL-21 with bovine (Bo), human (Hu) and mouse (Mu) IL-21. Numbers with each line indicate the amino acid position. Identical amino acid residues of four species are indicated by asterisks. The putative amino acid sequences of the signal peptide are underlined. The conserved cysteine residues are shown by bold letters. The conserved Gln res idues, which are involved in the binding with the common γ chain, are boxed.

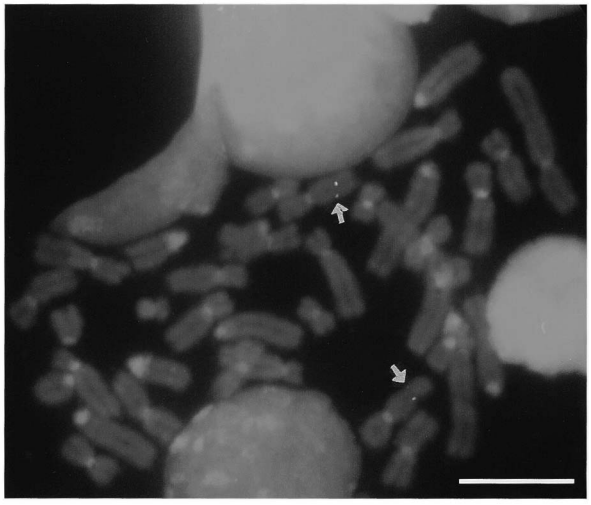

Fig. 3. Assignment of IL-21 to swine chromosomes by FISH using swine genomic BAC DNA containing the porcine IL-21 gene as a probe. White arrows indicate the position of IL-21 on pig chromosome 8 (8q22→23). White bar shows 10 μm.

of human chromosome 4 [11, 20, 22], which shows synteny with porcine chromosome 8 [5]. These results suggested that these highly related genes necessary to NK cell functions, IL-2, IL-15, and IL-21, also form gene clusters on pig chromosome 8.

Recombinant porcine mature IL-21 was successfully expressed using an *E. coli* expression system. In order to confirm the biological activity of the recombinant porcine IL-21, we used a human NK cell line, NK0, because no cell line for NK cells was available in pigs, and no cell surface marker expressed only on NK cells in order to isolate NK cells has been established in pigs. Using NK0 cells, recombinant porcine mature IL-21 induced dose-dependent proliferation of NK0 cells, and induced IFN-γ production from NK0 cells. These results indicate that the porcine IL-21 obtained in this study was biologically active, and effective for the enhancement of NK cell functions. In addition, the JAK3-specific inhibitor inhibited both cell proliferation and IFN-γ production from NK0 cells by porcine IL-21 stimulation. The common γ chain was shown to interact with JAK3 in the cytoplasmic domain and transduce intracellular signals by phosphorylation of JAK3 [6, 17]. Recently, the common porcine γ chain gene has also been reported [8]. The results of this study also showed that porcine IL-21 stimulates the JAK3 signaling pathway for the NK cell proliferation and its IFN-γ production.

In conclusion, the porcine IL-21 gene was isolated and mapped to chromosome 8, and the recombinant porcine mature IL-21 was produced with biological activity in this

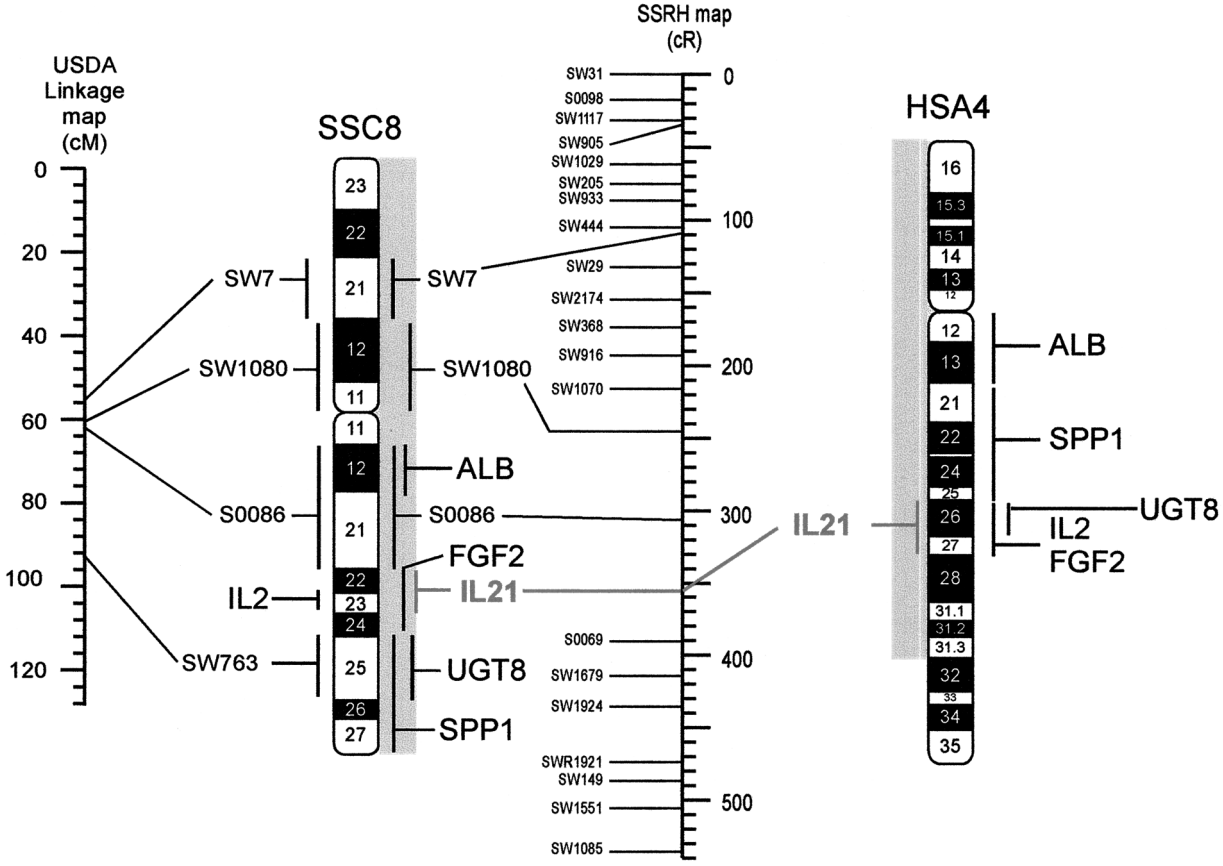

Fig. 4. RH panel mapping for porcine IL-21. The distances between the porcine IL-21 mapped in this study and the framework markers on the SSRH map were calculated as described in Materials and Methods. The position of the IL-21 gene is indicated by grey letters. The images of swine chromosome 8 (SSC8) and human chromosome 4 (HSA4) downloaded from a website (*www.toulouse.inra.fr/lgc/pig/compare/SSCHTML/SSC8B.HTL*) are shown beside the RH map for comparison. The known markers in pig and human chromosomes revealed by FISH analysis are also illustrated.

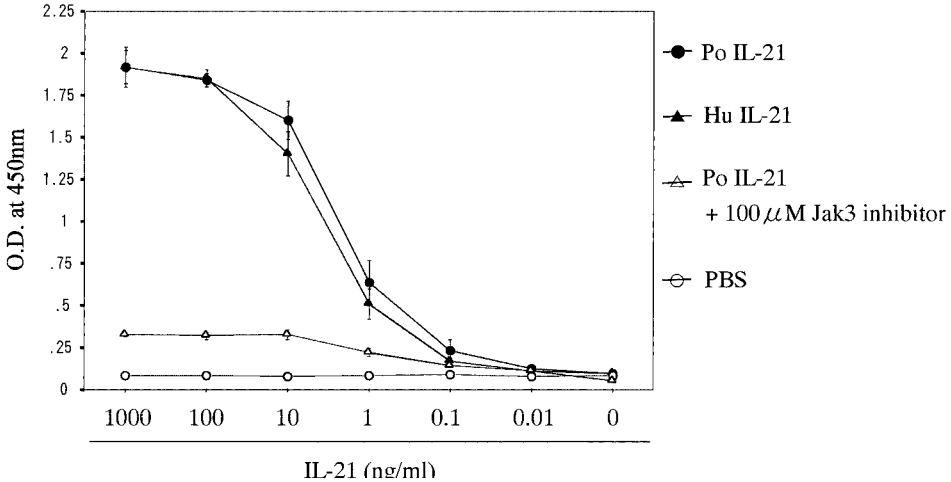

Fig. 5. Porcine mature IL-21 expressed by *E. coli* stimulates the proliferation of the human NK cell line, NK0. NK0 cells were cultured with the recombinant porcine IL-21 for 96 hr. Human recombinant IL-21 was used as a positive control. Phosphate buffered saline (PBS) was used as a negative control. In order to inhibit the JAK3 pathway, a specific inhibitor of JAK3 was used at 100 μM. Cell proliferation was assayed using a WST-1 cell counting kit.

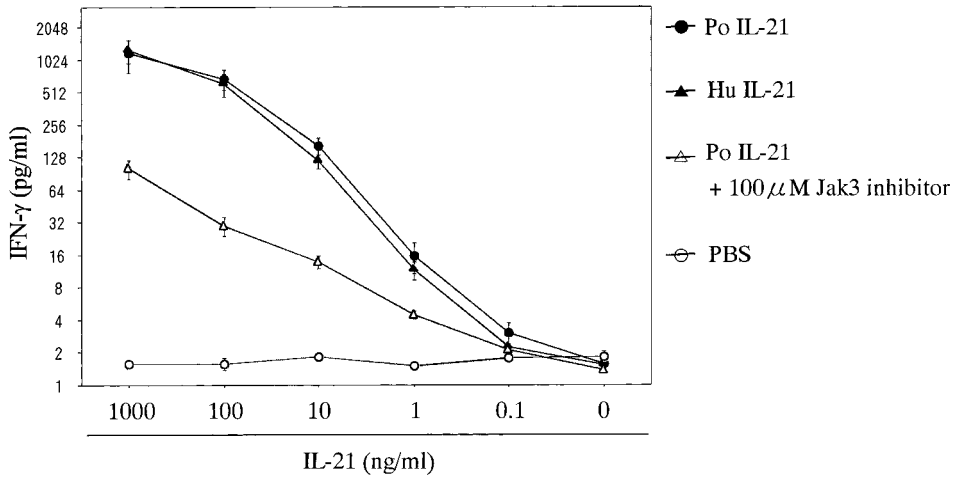

Fig. 6. Porcine IL-21 induces IFN-γ production from NK0 cells. Human NK cell line, NK0 cells, was cultured with the recombinant porcine IL-21 for 96 hr. Human recombinant IL-21 was used as a positive control. PBS was used as a negative control. To inhibit the JAK3 pathway, a specific inhibitor of JAK3 was used at 100 μM. The IFN-γ concentration in the culture supernatant was determined using a human IFN-γ-specific ELISA.

study. NK cells are one of the target cells for immunostimulation in neonatal animals. Further studies are needed to utilize this novel cytokine for the enhancement of innate immune responses in pigs.

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