NOTE *Immunology*

Molecular Cloning of the Swine Interleukin-23 Subunit p19 and of its Receptor Components Interleukin-23Rα and -12Rβ1

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ABSTRACT. Interleukin (IL)-12 and IL-23 play central roles in the regulation of distinct helper T-cell subsets, *i.e.* Th1 and Th17, respectively. Although IL-12 and IL-23 have been well studied in human and rodent systems, little is known about their significance in other animals, including livestock mammals such as cattle and pigs. In this study, we performed molecular cloning and genetic characterization of a small component of swine IL-23, *i.e.,* IL-23p19; in addition, we identified and performed chromosomal assignment of the genes encoding its receptor (R) subunits IL-23Rα and IL-12Rβ1. These results provide genetic information about both swine IL-23/IL-23R and IL-12/IL-12R systems, which allows for better understanding of IL-12/IL-23 systems involved in pig immunity.

KEY WORDS: cytokine, cytokine receptors, interleukin-12, interleukin-23, swine.

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Interleukin (IL)-12 and IL-23 play significant roles in the onset of immune reactions mediated by distinct T-cell subsets, *i.e.* Th1 and Th17, respectively [11, 17]. IL-12 was initially identified as a stimulating factor for NK cells [20] and cytotoxic T lymphocytes (CTLs) [8] and is currently recognized as a key cytokine for promoting Th1-type cell-mediated immune responses in host defense. IL-23, a newly identified member of the IL-12 family, has been recognized as a growth factor for a recently discovered subset of helper T cells, *i.e.* Th17, which is named after its unique ability to secrete the proinflammatory cytokine IL-17 [1, 2]. Although the physiological role of the subset and the immune functions of regulatory (IL-23) and effector cytokines (IL-17) in host defense still remain unclear, these T cells have been reported to enhance inflammatory reactions in autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) [22] and inflammatory bowel disease (IBD) [16].

Moreover, growing knowledge about the association of the polymorphisms in the IL-23 receptor locus with Crohn's disease [4] as well as with other autoimmune inflammatory diseases, *i.e.*, ulcerative colitis [5] and multiple sclerosis [3], suggests the significant role of the IL-23/IL-23R system in the development of inflammatory tissue lesions in immunological disorders.

In spite of the difference in target population, IL-12 and IL-23 are structurally related with each other and share the same subunit protein, *i.e.* IL-12p40, as a common component [12, 20]. In the active form of IL-12, IL-12p40 is associated with the IL-12 specific subunit IL-12p35; in contrast, during the assembly of IL-23, it covalently links to the IL-23 specific component IL-23p19. Moreover, it has been shown that IL-12 and IL-23 bind to the structurally related heterodimeric receptors IL-12R and IL-23R, respectively. Both receptors share the same 80 kd subunit, *i.e.* IL-12Rβ1, as a common component [13, 14]. This molecule, which is thought to be an affinity-converting molecule with Tyk2 kinase-binding activity [18], lacks an intracellular tyrosine phosphorylation site; it forms a functional receptor for IL-12 in combination with a possible signaling subunit IL-12Rβ2 or for IL-23 with IL-23Rα.

Many *in vitro* and *in vivo* studies have been conduced to investigate the biology of IL-12 and IL-23. However, most of the studies were performed using human and rodent systems, the findings which are not always applicable to cytokine functions in other species such as livestock mammalian species. For instance, Foss and Murtaugh, who first reported the complete primary structures of the swine IL-12p35 and IL-12p40 subunits [6], have shown that, unlike human cells, phytohemagglutinin (PHA)-activated swine peripheral blood mononuclear cells (PBMCs) do not proliferate in response to IL-12. Their results, together with other findings, indicate that human and rodent model-based observations may not be sufficient to understand the immune systems of other mammalian species.

For biological analysis of IL-12 and IL-23 in pigs, genetic information about the molecules involved in the IL-12 and IL-23 systems—*i.e.,* ligands and receptors—is required as an analytical tool. However, sufficient information is not available in this regard, and no data are available on the primary structures of the IL-23p19, IL-23Rα, and IL-12Rβ1 subunits. In this study, we aimed to clone entire open read-

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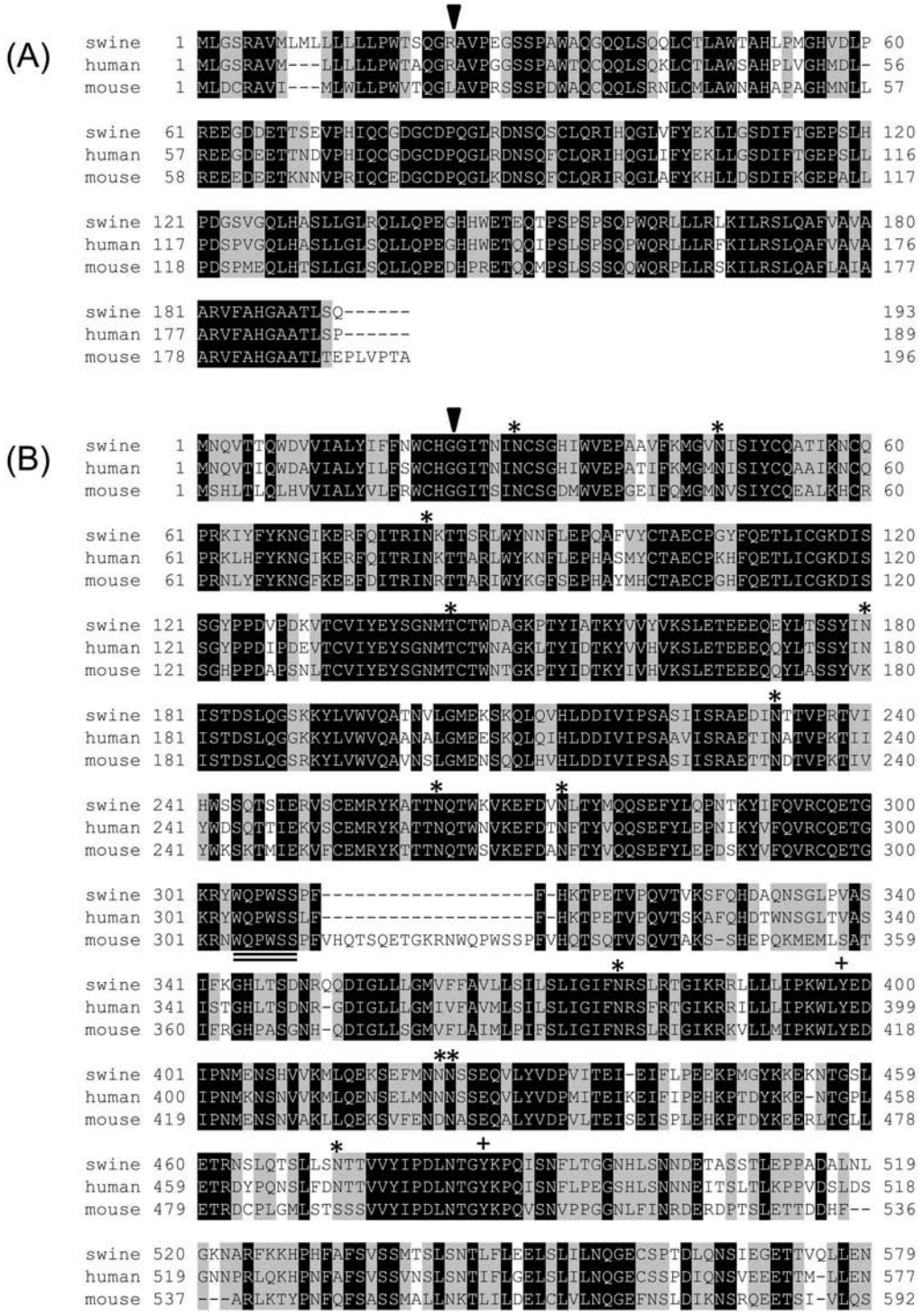

ing frame (ORF) sequences encoding these molecules and genetically characterized them to obtain fundamental information regarding the role of IL-12/IL-23 systems in pig immunity.

The nucleotide sequences encoding the entire ORFs for IL-23p19, IL-23Rα and IL-12Rβ1 genes were isolated using a reverse transcription polymerase chain reaction (RT PCR)-based strategy, followed by rapid amplification of cDNA ends (RACE) analysis. Nucleotide primers for each target gene were designed on the basis of motifs conserved among previously known homologues of mammalian origin that were registered in the public database. Next, we per-

(continued)

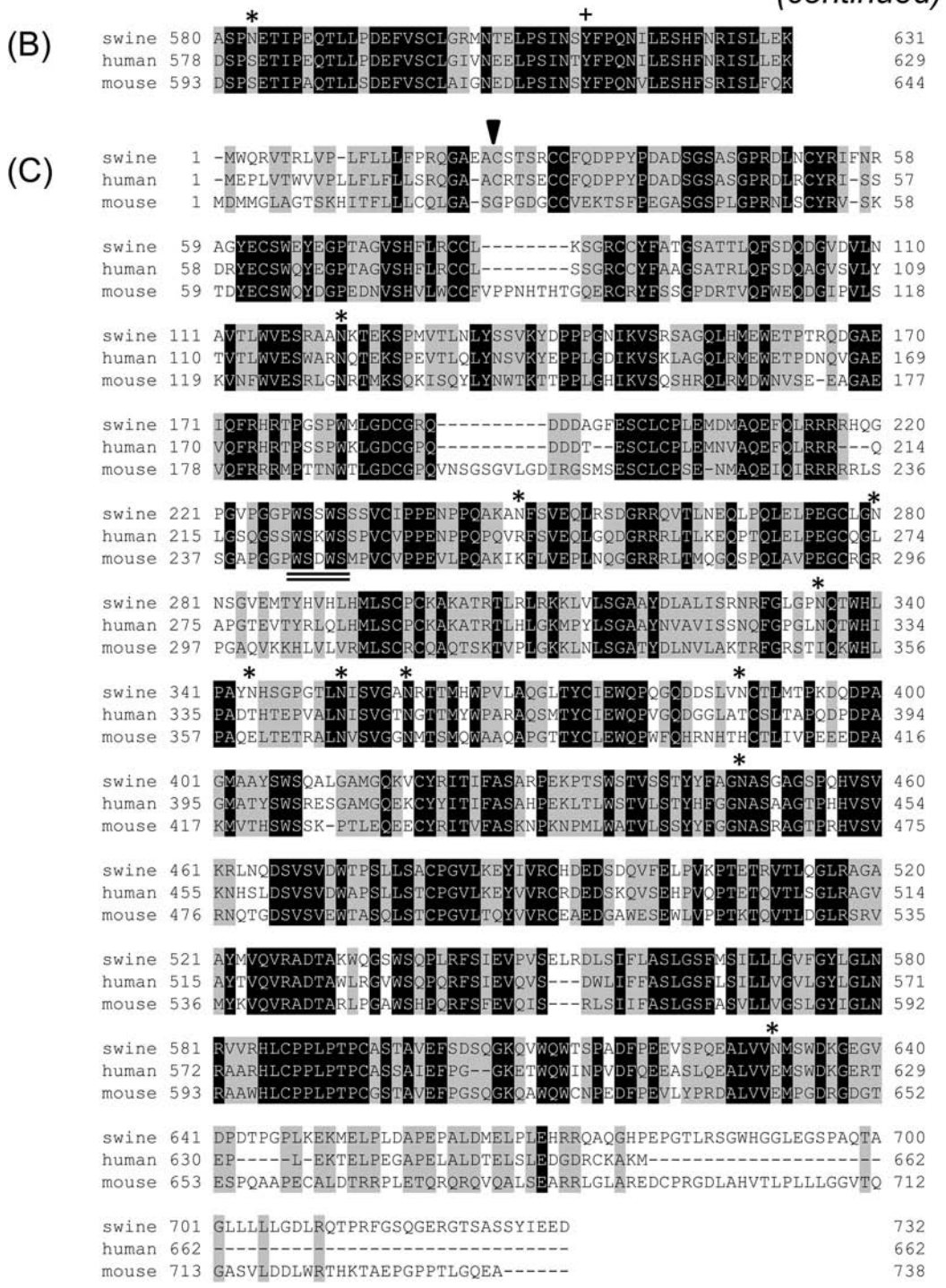

Fig. 1. Comparative analysis of IL-23p19 (A), IL-23Rα (B), and IL-12Rβ1 peptide sequences (C) of swine, human and mouse origin. Consensus between identical residues for all 3 mammals is shown by a solid box, and semiconserved substitutions are indicated by a gray box. The predicted signal sequences and *N*-glycosylation sites are shown by arrowheads and asterisks, respectively. Conserved WS/QXWS motifs have been double underlined. Common putative tyrosine phosphorylation sites at 398, 485, and 613 positions in the swine IL-12Rβ1 sequence are also indicated as (+). The GenBank accession numbers are as follows: IL-23p19: AB521204 (swine), AB030000 (human), and NM_031252 (mouse); IL-23Rα: AY948114 (swine), NM_144701 (human), and AF461423 (mouse); IL-12Rβ1: AB490071 (swine), U03187 (human), and NP_032379 (mouse).

formed reverse transcription of total RNA samples prepared from PHA-activated swine PBMCs, as described in a previous study [9]. Briefly, 1% PHA-P (BD, Franklin Lakes, NJ, U.S.A.) was added into a cell culture in a 25-cm^2 flask at the density of 10×10^6 cells/m*l*. The cells were then incubated for 4 days at 37°C and in 5% CO_2 and were subjected to total RNA extraction with ISOGEN reagent (Nippon Gene, Tokyo, Japan), according to the manufacturer's protocol. First strand DNA synthesis was performed using an RNA PCR kit (Takara Bio Inc., Tokyo, Japan) with oligo-dT primers. To isolate the target genes, we performed PCR-based cloning by using primer sets for IL-23p19 (forward primer: 5'-agggactcagggacaacagtcag-3', reverse primer: 5'-gcgaaggatcttgaggcggagaa-3'), IL-23Rα (forward primer: 5'-gaattacaaatataaactgctctggccaca-3', reverse primer: 5'-cgagctgttgttttattaatccttgtga-3') and IL-12Rβ1 genes (forward primer: 5'-ccctgtgtgcgttccccctg-3', reverse primer: 5'-gtgtcggcaggaatgtgcca-3'); we analyzed the amplicons with a PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

To obtain complete ORF sequences, the 5'-and 3'-RACE technique was performed using a SMART RACE cDNA Amplification kit (Takara), according to the manufacturer's protocol. RACE products encoding sequences upstream or downstream of the target genes were cloned into the pCR2.1-TOPO vector (Invitrogen, San Diego, CA, U.S.A.) and were then subjected to nucleotide sequence analysis. The entire ORFs of IL-23p19, IL-23Rα and IL-12Rβ1 genes were 582 bp, 1896 bp, and 2199 bp in length and showed up to 87.5%, 86.8%, and 76.7% of homology, respectively, at the nucleotide level and up to 87.5%, 82.1%, and 70.8% of identity, respectively, at the amino acid level with equivalent molecules from other mammalian species (Fig. 1). The calculated MWs of IL-23p19, IL-23Rα and IL-12Rβ1, which possess 0, 13, and 10 respectively sites for *N*-glycosylation, are 21.1 kd, 80.6 kd, and 72.0 kd, respectively. Additionally, IL-23Rα was found to share similar cytokine receptor motifs such as the WS/QXWS motif (X=anonymous) in the extracellular region and 3 possible tyrosine phosphorylation sites (^{398}Tyr, ^{485}Tyr, and ^{613}Tyr; [19]) in the intracellular domain. The nucleotide and amino acid sequences of swine IL-23p19, IL-23Rα, and IL-12Rβ1 subunits were deposited in GenBank under the accession numbers AB521204, AY948114, and AB490071, respectively.

By *in silico* investigation of a pig genomic library, bacterial artificial chromosomes (BACs) encoding the swine IL-23p19, IL-23Rα and IL-12Rβ1 genes were identified, and their loci were assigned to the corresponding swine chromosomes. In the human genome, the IL-23Rα gene is mapped at the proximal upstream of the IL-12Rβ2 locus on chromosome 1 (1p31.2-31.3) [7, 21]. Similarly, in the swine genome, the IL-23Rα and IL-12Rβ2 loci comprise a syntenic group in this order and are located within 50 cM (approximately 60 kbp) of each other in on chromosome 6 (6q31-35; Fig. 2A). In contrast, the swine IL-12Rβ1 locus was assigned to the chromosome 2 (2q14.1), and swine IL-23p19 locus, for which detailed location data is unavailable, was assigned to chromosome 5.

The IL-12Rβ1 subunit may associate with either the IL-12Rβ2 or IL-23Rα subunit and form a functional receptor for IL-12 or IL-23. The IL-12 and IL-23 systems are essential for the terminal differentiation of CD4+ immature helper T cells into the Th1 and Th17 subsets, respectively. Therefore, the regulation of these receptor component genes in immature T cells is of great interest. In this study, we compared the expression profiles of the IL-12R and IL-23R components with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; accession no. AF017079) in PHA-treated swine PBMCs by RT-PCR performed using an appropriate prime set for GAPDH (forward primer, 5'-gggcatgaaccatgagaagt-3'; reverse primer, 5'-aagcagggatgatgttctgg-3'), IL-12Rβ2 (accession no. AF330213 [9]; forward primer, 5'-gacaacataaaaccctacatctgttatgaa-3'; reverse primer, 5'-atgtcactcggggctgcaggctgtttattg-3'), IL-12Rβ1 (provided earlier in the paper), and IL-23Rα genes (provided earlier in the paper) at 94°C for 2 min, followed by 37 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. The swine IL-12Rβ1 gene was stably expressed in both resting and PHA-activated PBMCs, whereas the IL-12Rβ2 and IL-23Rα gene expression levels were enhanced in response to PHA (Fig. 2B). The IL-23Rα and IL-12Rβ2 genes showed distinct expression patterns wherein IL-23Rα gene expression was found to increase more quickly than IL-12Rβ2 gene expression and exhibited maximal levels immediately on activation; this suggested the presence of distinct mechanisms for regulating receptor components within the same synthenic group. Because both loci lack a TATA-box like motif in the proximal upstream region, their transcription is presumably controlled by TATA-independent machinery.

Foss and Murtaugh have reported that swine PBMCs, unlike human PBMCs, are not responsible to IL-12 when activated with mitogenic reagents *in vitro* [6]. Although we did not determine the number of IL-12 binding sites on the surface of PHA-activated swine lymphocytes, our observations suggest that such species-dependent responsiveness to the exogenous IL-12 is not attributable to the difference in receptor gene expression.

To confirm the biological activity of the IL-23p19 subunit, we generated recombinant swine IL-23 in combination with the other component of the cytokine, *i.e.* IL-12p40, by using the baculoviral expression system. The swine IL-12p40 recombinant baculovirus was prepared with the Bac-to-Bac baculovirus expression system (Invitorgen), as described in a previous paper [10]. The swine IL-23p19 recombinant baculovirus was prepared in a similar method by substituting the coding sequence with the IL-23p19 ORF. To detect recombinant IL-23p19, we replaced the stop codon (taa) of the gene with a histidine ×6 tag coding sequence. Then, we grew *Trichoplusia ni*-derived BTI-TN-5B1-4 (TN5) cells in EX-CELL 405 medium (SAFC, Lenexa, KS, U.S.A.) containing IL-12p40 and IL-23p19 recombinant baculoviruses at a multiplicity of infection of 2.0

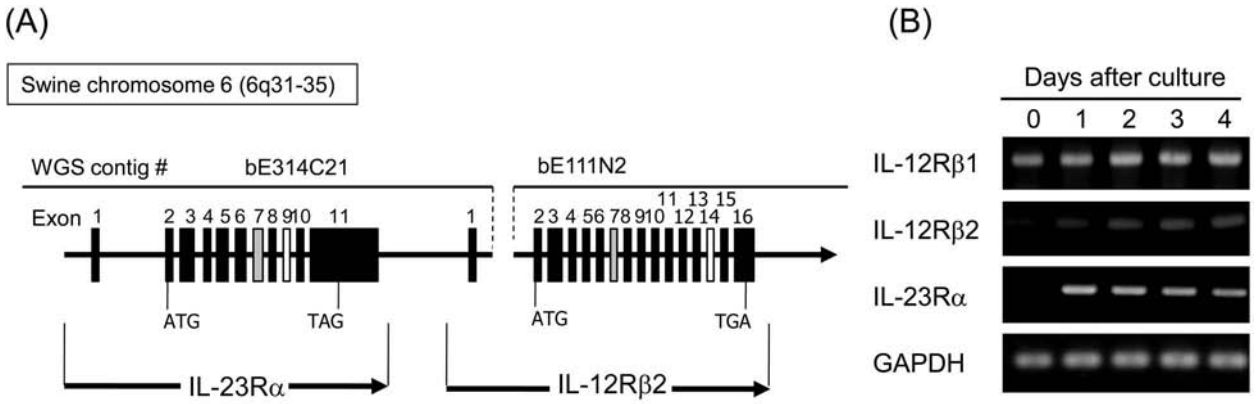

Fig. 2. Schematic structure of the *il23rα-il12rβ2* locus (A) and expression analysis of cytokine receptor genes in PHA-activated swine PBMCs (B). (A) By screening the public database of the swine genome (blastn), the flanking whole genome shotgun (WGS) contigs bE314C21 and bE111N2 were mapped to swine chromosome 6 (6q31–35). The IL-23Rα and IL-12R*β2* genes were found to contain 11 and 16 exons (including the untranslated first exon), respectively. The open box indicates a sequence encoding a possible transmembrane region, and the gray box indicates WS/QXWS coding exons. (B) After 35 cycles of PCR performed at an annealing temperature of 55°C with primers for IL-12R*β1*, IL-12R*β2*, IL-23Rα, or GAPDH (primer sequences provided in the text), the expression of the target genes (product sizes: 241 bp, 322 bp, 264 bp, and 230 bp, respectively) in 1% PHA-treated PBMCs was detected by 1% agarose/TAE gel electrophoresis.

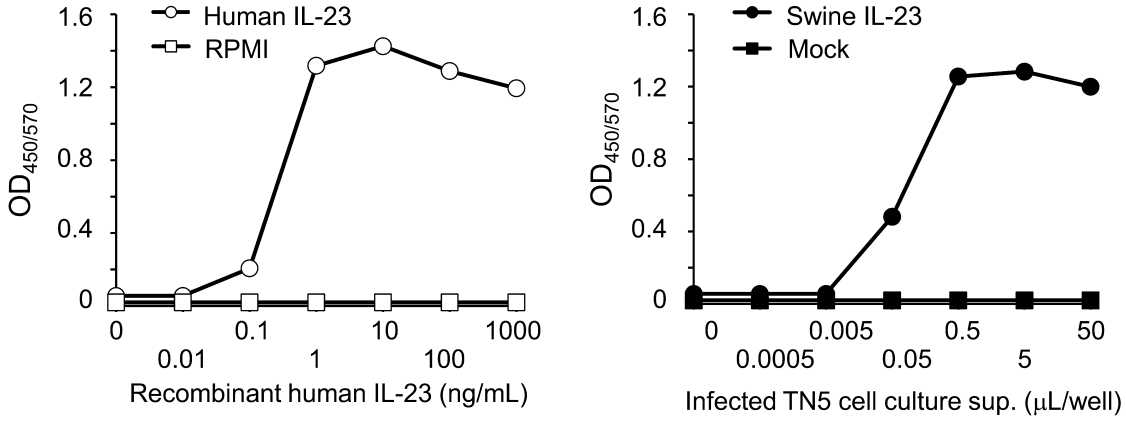

Fig. 3. Bioassay for recombinant swine IL-23. Mouse spleen cells (4.0×10^5 cells/well) were cultured with the indicated doses of recombinant human (R&D Systems Inc.; left panel, open circle) or swine IL-23 containing TN5 cell culture supernatant (right panel, solid circle), in the presence of recombinant mouse IL-2 (100 units/m*l*) for 6 days at 37°C, in the presence of 5% CO_2. Then, 50 μl of the culture supernatant from each well was collected and subjected to mouse IL-17 ELISA (R&D Systems Inc.), according to the manufacturer's protocol. The results are indicated by average optical density (OD) measured at the wave length 450 nm with a reference at 570 nm as a reference wave length. The OD values of buffer alone (open square) and of mock-infected TN5 cells (solid square), which were used as negative controls for the human and swine IL-23 assays, respectively, were undetectable in the IL-17 ELISA.

each. The culture supernatants were harvested at 96 hr after culture, clarified by low-speed centrifugation, and stored at –70°C until use. We confirmed the expression of both recombinant subunits by western blotting with the monoclonal antibody for human IL-12p40 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), or with the rabbit serum against histidine tag (Abcam, Cambridge, U.K.) as a primary antibody (data not shown). IL-23 bioassay was conducted as described in a previous paper [1]. Briefly, 4.0×10^5 spleen cells from C57BL/6 mice were cultured in a well of a 96-well plastic plate in the presence of 100 units/m*l* mouse recombinant IL-2 (Sigma, St. Louis, MO, U.S.A.) and various doses of recombinant human or swine IL-23 for 6 days at 37°C, in the presence of 5% CO_2. The culture supernatants were collected and used for mouse IL-17 ELISA (R&D Systems Inc., Minneapolis, MN, U.S.A.), according to the manufacturer's protocol. The histidine-tagged recombinant swine IL-23 as well as recombinant human IL-23 (R&D Systems Inc.) strongly induced IL-17 secretion from mouse spleen cells in a dose-dependent manner, whereas the culture supernatants of TN5 cells infected with the same titer of wild-type baculovirus did not increase IL-17 production (Fig. 3). This result clearly indicates the activity of recombinant swine IL-23.

In the preliminary experiment, we performed a similar bioassay using pig spleen cells as a responder. These cells produced IL-17A in response to IL-23 in the presence of IL-2 (data not shown), suggesting the activity of the recombinant cytokine on pig immune cells. This observation also supports that these cells are potent to express functional IL-23 receptor subunits, IL-12Rβ1 and IL-23Rα.

Our current data—taken together with the previous results of our group and those of other groups [6, 9]—provide a complete set of genetic data on the molecules related to the IL-12 and IL-23 cytokine networks of pig. It is important to understand the immune functions of the cytokine network in a given species; therefore, our findings would be helpful for better understanding of host defense mechanisms in pigs, an economically important species. In addition, these results may provide useful tools for analyzing certain T cell populations in infected and healthy pigs.

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