Continuous large-scale production of the cytokine CXCL8 from a novel porcine cell line

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ABSTRACT. Cytokine production from two unstimulated porcine cell lines (SL-24 and SK-L) was examined using porcine cytokine detection ELISA kits and RT-PCR. Porcine IL-1α, IL-6, and CXCL8 were detected in all samples examined. In particular, the SL-24 cell line (derived from bone marrow cells of a malignant lymphoma-affected pig), produced large amounts of porcine CXCL8. Flow cytometer analysis showed the cell line to be strongly CD44 positive, and was therefore considered to be of monocyte or macrophage origin. Porcine CXCL8 production was greatest (83.86 ± 32.33 ng/mL) at six days post-cultivation. The SK-L cell line (derived from porcine kidney) also produced CXCL8, but production was less than 1.5 ng/mL. Porcine CXCL8 from the SL-24 cell line, induced chemotactic activity in porcine neutrophils, while the production of CXCL8 from the SL-24 cell line was inhibited by dexamethasone, which suggests that the mechanism of CXCL8 production is related to an NF-κB binding site. The production of CXCL8 from the SL-24 cell line was enhanced by the addition of recombinant porcine IL-15, which is the first reported observation of such CXCL8 production. Cloning of the SL-24 cell line by limited dilution revealed two types of cells present in the starting population. One cell type, designated as long-form cells (LC), produced large amounts of CXCL8, while the other, designated short-form cells (SC), produced small amounts of the cytokine. The LC cells were adapted to grow in serum-free medium in which they produced large amounts of CXCL8. The large-scale production of porcine CXCL8 from the SF-24 cell line will be of value in determining the mechanism of cytokine production and as a source of naturally produced porcine CXCL8.

Keywords: porcine CXCL8, production, porcine cell line

Cytokines are produced by many kinds of immune cells, but particularly by immunocytes, and have a variety of functions such as maintaining homeostasis and providing immunity against infectious diseases [1, 2]. The use of laboratory-maintained cell lines as a source of cytokines for experimental work is well established [3, 4]. While cytokine production is normally observed only after stimulation of cells with LPS (lipopolysaccharide), mitogens, lectins, viruses, or other cytokines [4-7], this was not the case with the porcine cell lines used in the present study, which produced large quantities of porcine CXCL8 without stimulation. This study also investigated the mechanism of porcine CXCL8 production and cellular characteristics of cloned cell lines.

MATERIALS AND METHODS

Cells

The cell line SL-24 was derived from the bone marrow of a sow with malignant lymphoma *(figure 1)*. The SL-24 cells have characteristics of seat cells and were able to be cultured in 5% fetal calf serum (FCS) containing RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). To establish the cell lines, 1 mL of culture medium containing 2×10^5 cells were placed in each well of a 24-well plastic plate (Sumitomo Co., Tokyo, Japan). An established cell line, SK-L, which was derived from porcine kidney cells from a normal pig [8], was included in this experiment as a control cell line. This control cell line was cultured in 5% FCS containing Eagle MEM medium (Nissui Pharmaceutical Co., Ltd.). Approximately 3×10^5 cells in 1 mL of medium were placed in each well of a 24-well plastic plate (Sumitomo Co.).

Characterization of SL-24 cells

For the characterization of SL-24 cells, monoclonal antibodies against the porcine cluster of differentiation (CD) antigens were used and binding was analyzed by flow cytometry. The monoclonal antibodies used in this experiment were as follows: anti- porcine CD1, anti-porcine CD2, anti-porcine CD3, anti-porcine CD4, anti-porcine
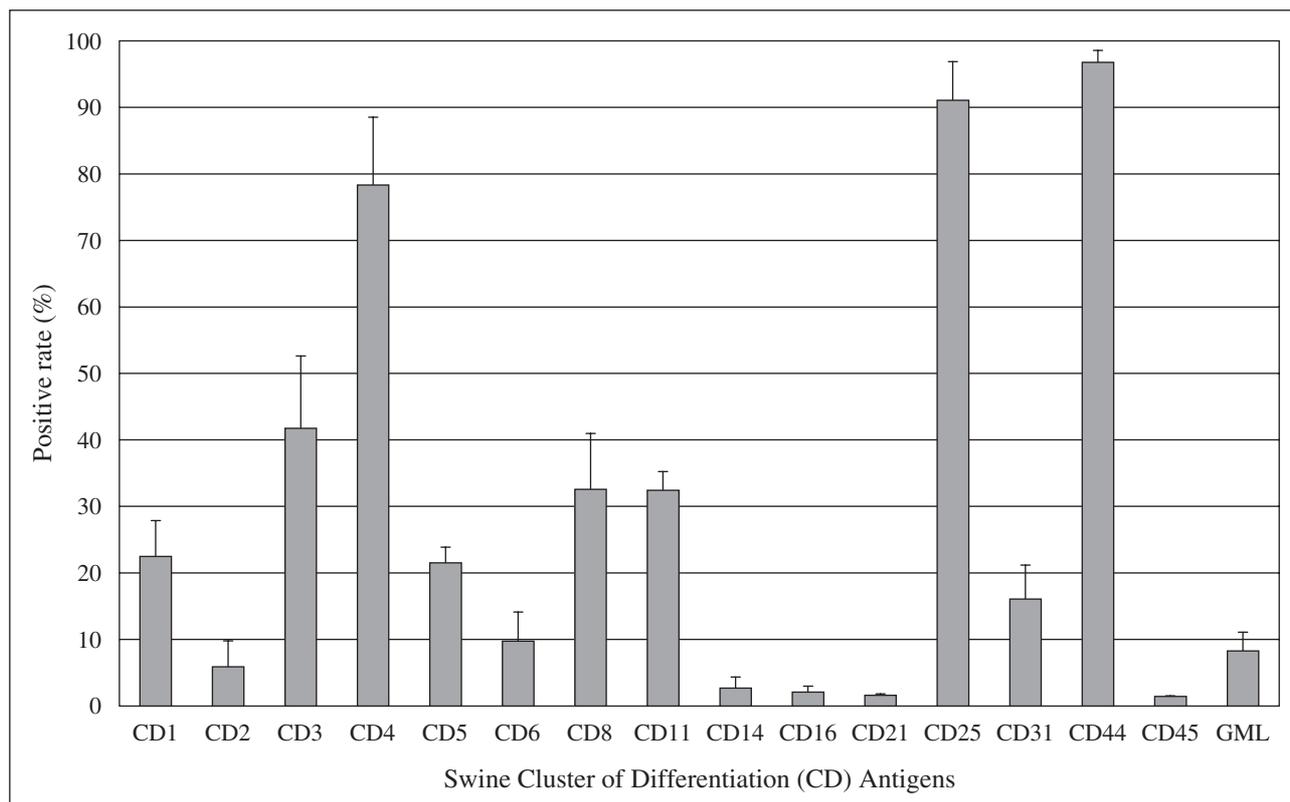
Figure 1
Flow cytometric analysis of cell surface marker staining of SL-24 cells. Percentage of cells stained with each antibody is expressed on the y-axis as the "positive rate".

CD5, anti-porcine CD6, anti-porcine CD8α, anti-porcine CD11a/18, anti-porcine CD25, and anti-porcine CD44. Monoclonals raised against porcine granulocyte, monocyte and lymphocyte sub-populations (GML) (VMRD Inc. Pullman, USA) were isolated from murine ascitic fluid. Anti-porcine CD14, anti-porcine CD45RA, and anti-porcine vimentin (Serotec Ltd., Oxford, UK) were derived from murine hybridoma culture supernatant fluid. Anti-porcine CD16 and anti-porcine CD21 (MONOSAN, Uden, Netherlands) were isolated from murine ascitic fluid. Anti-human and anti-bovine cytokeratin 18 (PROGEN, Heidelberg, Germany) were derived from murine hybridoma culture supernatants. For flow cytometry, monoclonal antibodies against CD1, CD2, CD3, CD4, CD5, CD6, CD8, CD11, CD14, CD16, CD21, CD25, CD44, and CD45 were used for cell surface staining as previously described [9]. Analysis was performed immediately after staining using a Beckman Coulter flow cytometer XLTM (Beckman Coulter K.K., Tokyo. Japan). For vimentin and cytokeratin detection, immunohistochemistry was performed using a Vectastain ABC kit (Vector Laboratories Ltd., Peterborough, UK).

Porcine cytokine detection

Porcine IFN-γ, IL-1β, IL-2, IL-4, CXCL8, IL-10 and TNF-α production in each cell culture were detected using commercial porcine cytokine ELISA kits (BioSource International Inc., CA, USA). The ELISA kit was a solid-phase sandwich ELISA. An antibody-specific or monoclonal antibody for each cytokine was coated onto the wells of microtiter strips. Samples, including standards of known porcine cytokine content, control specimens, and cell culture medium, were then added to the wells (for CXCL8 detection, cell culture supernatant was diluted x 8 or x 40 by dilution buffer of ELISA). After washing, 50 μL biotinylated antibody specific for each of the cytokines was added to specific wells. After removal of excess second antibody by washing, 100 μL streptavidin-peroxidase was added. Following a third incubation and washing to remove all unbound enzyme, 100 μL substrate solution were added. The intensity of color change was directly proportional to the concentration of cytokine present in the original specimen. The sensitivity of the kit for IFN-γ, IL-1β, IL-2, IL-4, CXCL8, IL-10 and TNF-α was over 2 pg/mL, 15 pg/mL, 5 pg/mL, 2 pg/mL, 10 pg/mL, 3 pg/mL and 6 pg/mL, respectively.

Gene expression of porcine IL-1α, IL-6, and IL-12 was measured by RT-PCR and nested PCR using previously described methods [6, 10, 11]. Expression of IL-1α was determined by RT-PCR, and IL-6 and IL-12 were measured by nested PCR.

Analysis of biological activity of CXCL8

The activity of CXCL8 produced by the SL-24 cells was measured using a chemotactic activity assay of porcine neutrophils. Chemotaxis was measured in collagen-coated 24-well plastic plates with plastic chemotaxis chambers [3]. Medium (10 mL) containing neutrophils was collected from 3-month-old Large White and 3-month-old Duroc pigs, and was purified using dextran 200,000 (MP Biomedicals Inc., CA, USA) and percoll gradients (MP Biomedicals Inc., CA, USA) [9]. The neutrophils were

maintained in RPMI medium at a concentration of 2 x 10^7/mL. Samples of CXCL8 were 10-fold serially diluted in RPMI medium and 650 µL of the sample was placed into wells of the collagen-coated plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). The plastic chemotaxis chambers (Corning Life Science Japan, Tokyo, Japan), containing 150 µL of neutrophil suspension, were placed into the wells and incubated for 2 hours at 37°C. The plastic chemotaxis chambers were removed and the 24-well plates were fixed using methanol, then stained using May-Grünwald Giemsa. The numbers of porcine neutrophils were counted using a compound microscope.

Mechanism analysis of CXCL8 production from SL-24 cells

The mechanism of CXCL8 production from SL-24 cells was examined using the known CXCL8 inhibitors FK506 (EMD Bioscience, Inc., CA, USA) [12] and dexamethasone (MP Biomedicals Inc., CA, USA) [12, 13]. These were placed in wells of 24-well plates containing 1-day-old cultures of SL-24 cells at concentrations of 1 ng/mL and 0.5 ng/mL, respectively. Supernatant medium was then collected between two and seven days of culture and used in a porcine CXCL8 ELISA kit.

Evaluation of porcine cytokines on CXCL8 production from SL-24 cells

The recombinant porcine cytokines IL-1β, IL-2, IL-4, IL-6, IL-10, IL-15, and IFNγ (BioSource International Inc., CA, USA) were placed into 1-day cultured SL-24 cells in 24-well plates at a concentration of 0.25 µg/mL each. Supernatant from two days to seven days post-cultivation was collected to measure the CXCL8 concentration by ELISA.

SL-24 cell cloning

The SL-24 cell line was subjected to continued 2-fold limited dilution on 96-well plates in RPMI medium containing 10% FCS. At four to seven days post-cultivation, those clones which had grown successfully were trypsinized and subjected to a further limiting dilution step until the cloned cell lines LC and SC were established.

Adaptation of SL-24 cell lines to serum-free medium

The cloned cell lines SL-24-LC and -SC were cultured in serum-free medium and examined for growth following at least 20 passages. Control cells were cultured in RPMI 1640 medium containing 5% FCS. The commercial, serum-free media Opti-Pro^{TM}SFM (GIBCO Invitrogen Japan KK, Tokyo, Japan), VP-SFM (GIBCO Invitrogen Japan KK, Tokyo, Japan), Hybridoma-SFM (GIBCO Invitrogen Japan KK, Tokyo, Japan), and NCTC135 (Dainippon Pharmaceutical Co., LTD. Tokyo, Japan) were evaluated on the success of SL-24 growth in culture.

RESULTS

Characterization of SL-24 cell type

Flow cytometry results of the SL-24 cell type are shown in *figure 1*. The percentage of cells staining positive for CD4, CD25, and CD44 were 78.17%, 90.93%, and 96.63%, respectively, and these markers were thus considered to be representative of SL-24 cells, particularly CD44. The percentage of cells staining positive for CD3, CD8, and CD11 were 41.6%, 32.43%, and 32.27%, respectively, and these markers were therefore considered to be weakly positive for SL-24 cells. Other CD markers tested were negative for SL-24 cells. Vimentin stained strongly positive on SL-24 cells, while cytokeratin staining was not observed.

Cytokine production from porcine cell lines

Results for cytokine production from the SL-24 and SK-L cell lines are shown in *table 1*. Detectable levels of porcine IL-1α, IL-6, and CXCL8 were detected from both cell lines. The level of CXCL8 production was significantly greater from the SL-24 cell line than from the SK-L cell line. The maximum concentration of CXCL8 produced from the SL-24 cells was 83.86 ± 32.33 ng/mL at six days post-cultivation *(figure 2)*. Chemotactic activity was also observed in culture fluid from the SL-24 cells at this time point *(figure 3)*. Porcine IFN-γ, IL-1β, IL-2, IL-4, IL-10, IL-12, and TNF-α were not detected in any samples examined. Production of detectable CXCL8 was also observed from SK-L cells, but at a concentration below 1.5 ng/mL.

Inhibition of CXCL8 production by FK506 and dexamethasone

The inhibition of CXCL8 production from the SL-24 cell line was examined by adding FK506 (1 ng/mL) and dexamethasone (0.5 ng/mL) to the culture medium *(figure 4)*. Inhibition of CXCL8 production was observed following addition of dexamethasone, but not FK506.

Effect of porcine cytokines on CXCL8 production from SL-24 cells

Porcine IL-15 enhanced production of CXCL8, but no effect was observed using IL-1β, IL-2, IL-4, IL-6, IL-10, or IFN-γ *(figure 5)*.

CXCL8 production from cloned SL-24 cell lines

Two cloned lines were examined; the first was characterized by long-form cells (LC: long form clone) *(figure 6A)* and the other by short-form cells (SC: short form clone) *(figure 6B)*. The production of CXCL8 by LC was higher than from SC *(figure 7)*. The concentration of CXCL8 production from LC was 71.02 ± 1.84 ng/mL at seven days post-cultivation, with 7.25 ± 3.78 ng/mL from SC. The rate of cell growth in the LC cell line was also slightly higher than that of the SC line from three days post-cultivation *(figure 7)*.

Adaptation of SL-24 cloned cell lines to serum-free medium

The LC cell line was successfully adapted to culture in VP-SFM serum-free medium, but not in any other serum-free preparations. The SC cell line failed to adapt to any kinds of serum-free media used in this experiment. The production of CXCL8 from the serum-free adapted LC cell line (NS-LC) was similar to that of the LC cell line cultured with RPMI 1640 containing 5% FCS, with a concentration of 64.94 ± 5.71 ng/mL at seven days post-cultivation.

Table 1
Production of cytokines by porcine cell lines

Cytokine Days after start of cultivation								
Cell line	1	2	3	4	5	6	7	8
SL-24 CXCL8	1.73[a]	2.0	4.51	16.53	32.28	83.86	74.03	13.14
IL-1β	< 5.0[b]	< 15.0	< 15.0	< 15.0	< 15.0	< 15.0	< 15.0	< 15.0
IL-2	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0
IL-4	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
IL-10	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0
IFN-γ	2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
TNF-α	< 6.0	< 6.0	< 6.0	< 6.0	< 6.0	< 6.0	< 6.0	< 6.0
IL-1α	+[c]	+	+	+	+	+	+	+
IL-6	+[d]	+	+	+	+	+	+	+
IL-12	-[d]	-	-	-	-	-	-	-
SK-L CXCL8	0.089[a]	0.174	0.168	0.287	0.659	0.871	1.275	1.435
IL-1β	< 15.0[b]	< 15.0	< 15.0	< 15.0	< 15.0	< 15.0	< 15.0	< 15.0
IL-2	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0
IL-4	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
IL-10	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0
IFN-γ	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
TNF-α	< 6.0	< 6.0	< 6.0	< 6.0	< 6.0	< 6.0	< 6.0	< 6.0
IL-1α	+[c]	+	+	+	+	+	+	+
IL-6	+[d]	+	+	+	+	+	+	+
IL-12	nd	nd	nd	nd	nd	nd	nd	nd

nd: not done.
[a] Cytokine detection by ELISA: ng/mL. Data shown in Table are means of triplicate wells.
[b] Except for CXCL8, other cytokines detection by ELISA: pg/mL.
[c] Cytokine detection by RT-PCR.
[d] Cytokine detection by Nested-PCR.
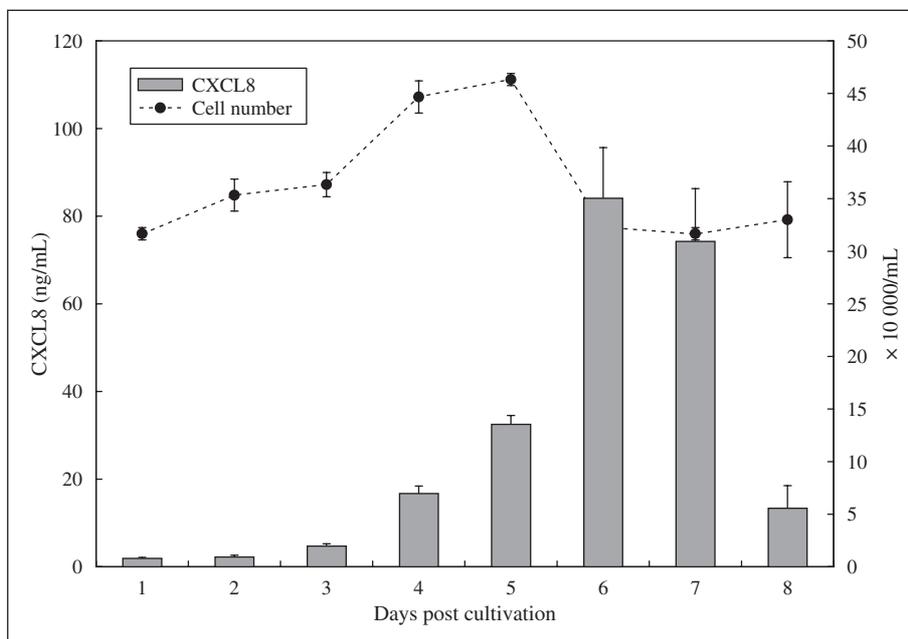
Figure 2
CXCL8 production in SL-24 cells at 1, 2, 3, 4, 5, 6, 7, and 8 days post cultivation with RPMI 1640 medium containing 5% FCS.
CXCL8 was measured by ELISA.

DISCUSSION

The cytokine CXCL8 is a macrophage-derived, neutrophil chemotactic factor that plays an important role in the recruitment of neutrophils to inflammatory loci [14-16].

This cytokine is often detected in cases of porcine pneumonia, following macrophage-stimulation by LPS [5] and can be induced *in vitro* following stimulation of macrophages with LPS, mitogens, lectins, viruses, and other cytokines [1, 4-6, 17]. In this experiment, CXCL8 production
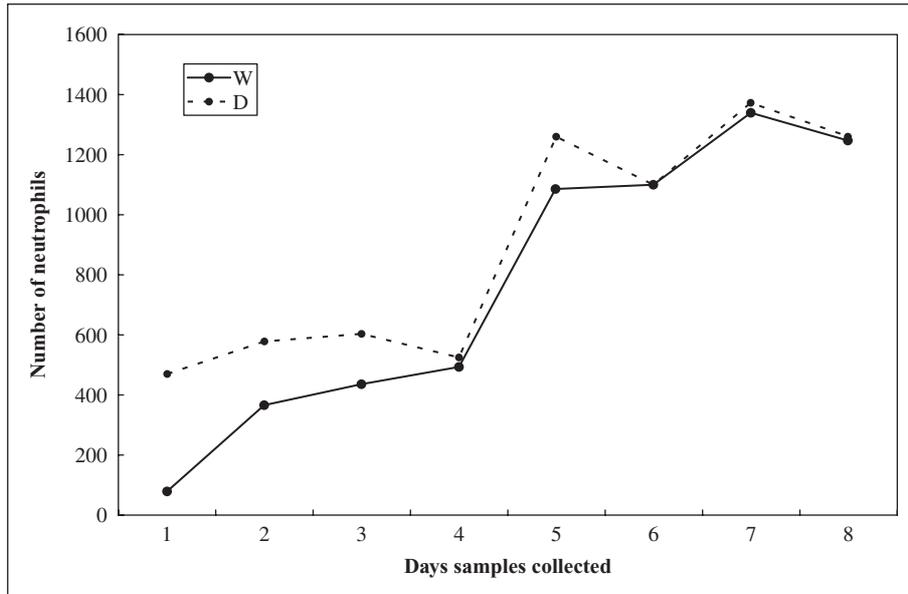
Figure 3
Chemotactic activity of porcine neutrophils in samples of SL-24 cells derived from Large White (W) and Duroc (D) pigs.
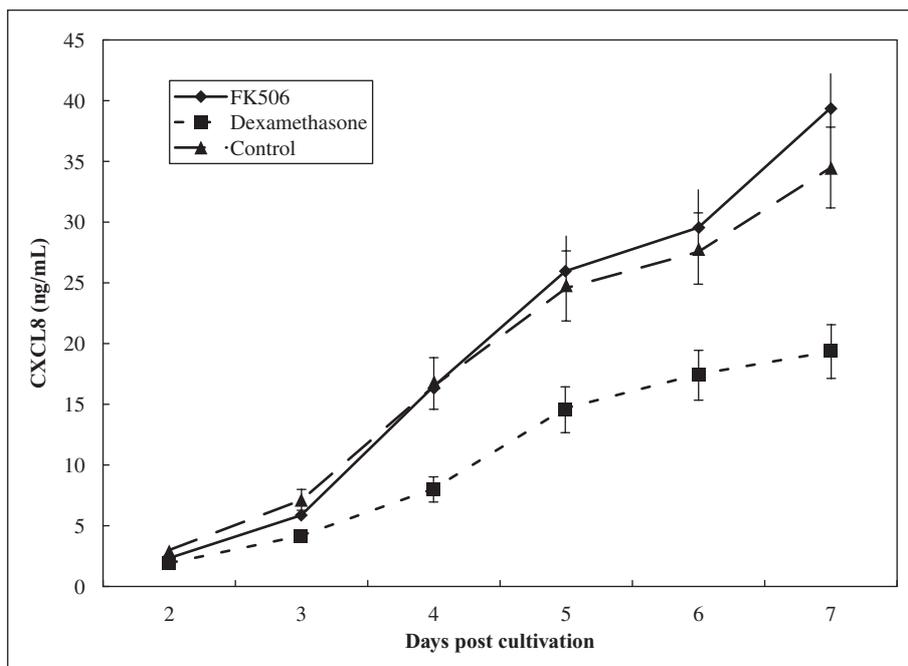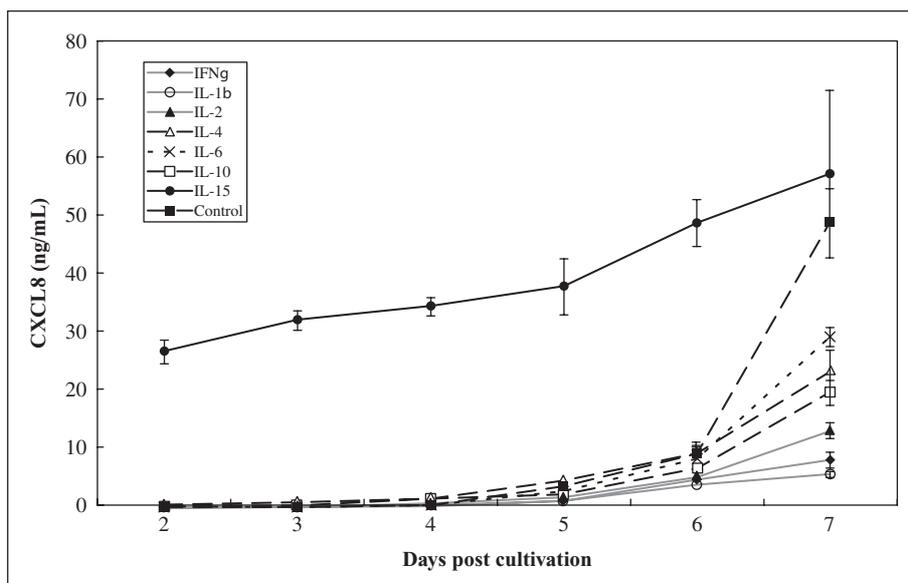

Figure 4
Inhibition of porcine CXCL8 production in SL-24 cells by FK506 and dexamethasone. CXCL8 production was inhibited by dexamethasone from 4 days post cultivation. The level of CXCL8 in dexamethasone-treated samples was lower than in control and FK506-treated samples at 7 days post cultivation.

was observed in the unstimulated porcine cell line SL-24. CXCL8 was produced in large amounts by this cell line and had strong chemotactic activity for porcine neutrophils. Flow cytometric analysis using monoclonal antibodies against several porcine CD markers characterized the SL-24 cells as macrophage–like, as did a previous study of human bone marrow-derived macrophage cell lines [18]. The SL-24 cells were particularly highly stained for CD44, which strongly indicated a macrophage lineage [19, 20]. Unlike previous studies on macrophage surface markers [18-20], results of staining with other CD markers such as CD11a, CD4, and CD25 did not support the conclusion

that the SL-24 cells are macrophages. However, we considered the cells to be of a macrophage origin due to the high production of CXCL8. While the SL-24 cells stained positive for CD3 and CD8, it was unlikely they were of thymic origin due to their adherent nature, even though phagocytic activity was not observed in SL-24 cells (data not shown). Flow cytometric analysis of cloned SL-24 cell lines supported the results obtained with the parent cell line. Differential production of CXCL8 was observed between the LC and SC cloned cell lines, but interestingly, the immunological characteristics of the clones were similar. The ability of CXCL8 production was therefore con-

Figure 5
Effect of porcine cytokines on CXCL8 production from SL-24 cells. Recombinant IL-15 enhanced the production of CXCL8 from 2 day post cultivation. Other cytokines had no effect on CXCL8 production.
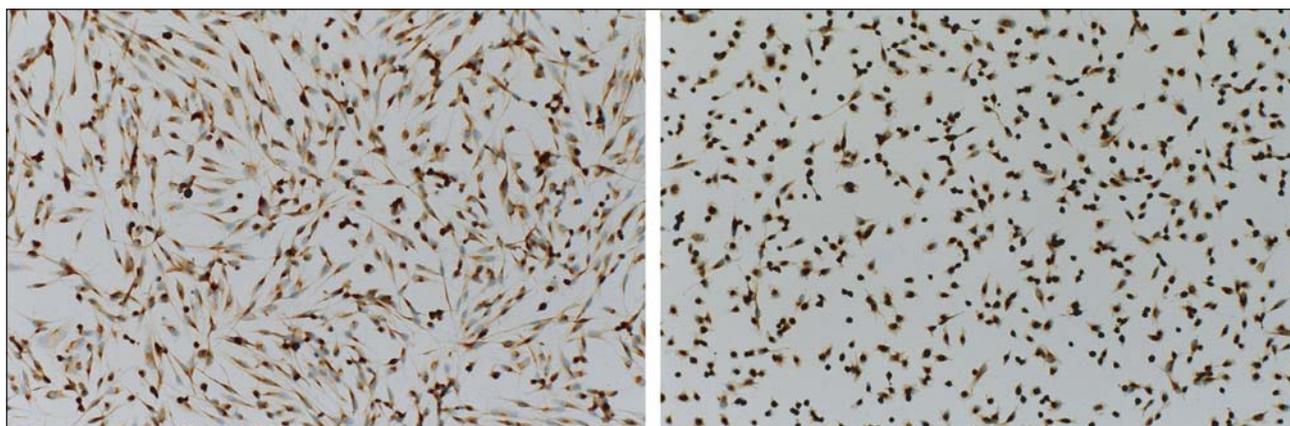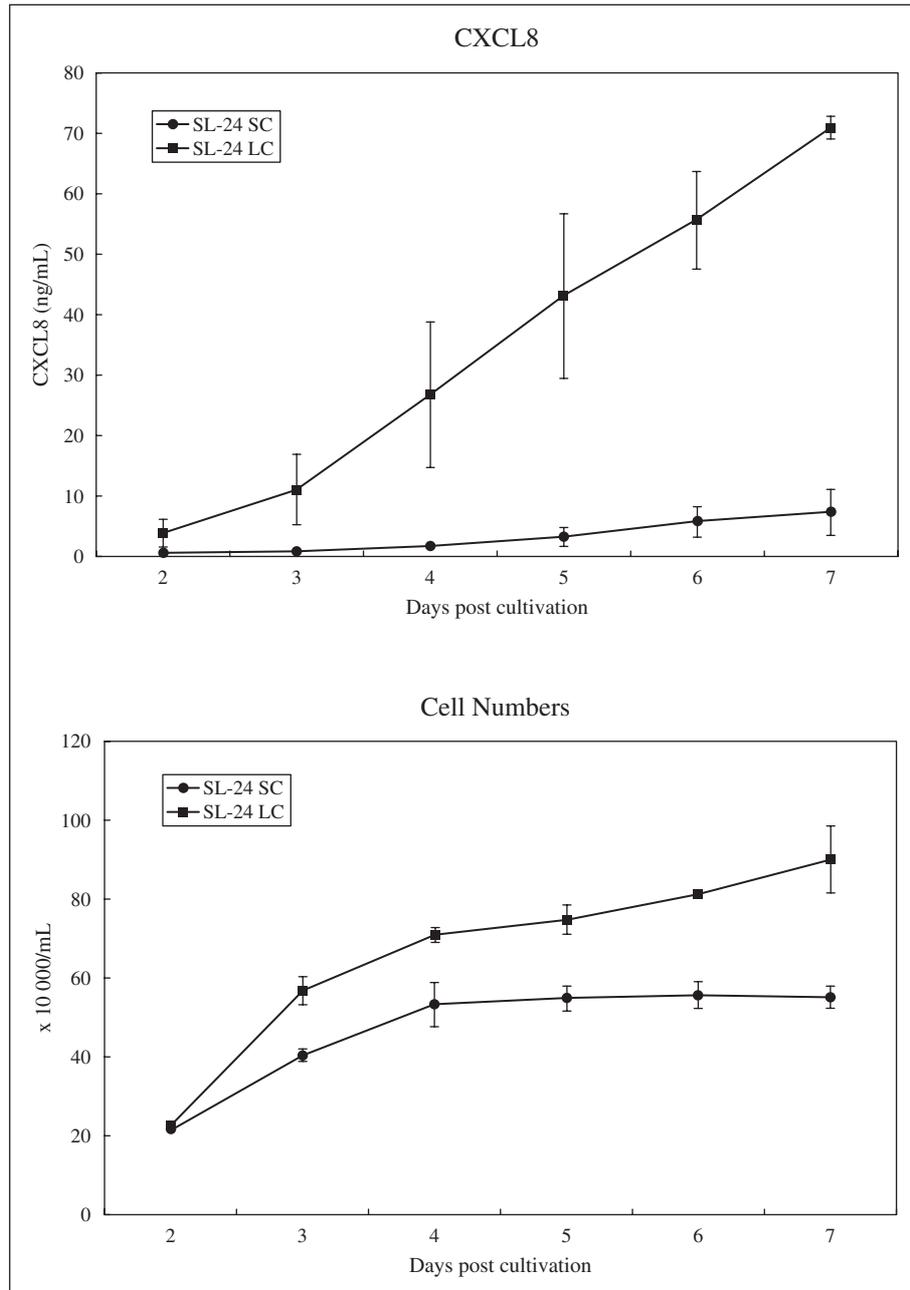

Figure 6
Two types of cloned cell derived from the SL-24 cell line.
LC cells are long form (SL-24 LC) (**A**) while SC cells are short form (SL-24 SC) (**B**). Both cell lines were cultured for 3 days and stained strongly positive for vimentin using anti-porcine vimentin monoclonal antibody.

sidered to be the result of mechanical differences. The cloned SC and LC cell lines stained strongly with anti-vimentin antibody, as shown in *figure 6*, but were not stained with anti-keratin antibody. This suggests the cells are not epidermal in origin. A possible explanation for these results is that cell surface markers have changed following continuous passage of the cell lines.

As the addition of dexamethasone inhibited the production of CXCL8 from SL-24, it is possible that CXCL8 production from the cells is regulated by NF-κB [13, 21]. This is because the promoters of CXCL8 production are normally regarded as AP-1 binding sites (-126bp~-120bp), C/EBP/NF-IL6 (-94bp~-81bp), and NF-κB (-80bp~-71bp) in humans [12, 13, 21-23]. Production of CXCL8 from the human fibrosarcoma cell line 8387 [21] was dependent on C/EBP/NF-IL-6 and NF-κB, while CXCL8 production from the human gastric cancer cell line MKN-45 [4] and the T-cell line Jurkat [23] was promoted by the AP-1 binding site and NF-κB. Also, CXCL8 production from the human glioblastoma cell line T98G [13]

was from the AP-1 binding site, C/EBP/NF-IL-6, and NF-κB. Dexamethasone inhibits only NF-κB [13], while FK506 inhibits both NF-κB and the AP-1 binding site [12]. As our results demonstrated that FK506 did not inhibit the production of CXCL8 from the SL-24 cell line, it is suggested that the AP-1 binding site is not related to the production of CXCL8 from the SL-24 cell line. Further studies, using a wider range of glucocorticoids, are required to confirm this hypothesis. Results for CXCL8 production on SL-24 cells do not explain why these cells can continuously produce CXCL8. However, we consider that SL-24 cells have a mechanism of CXCL8 production related to the κB-like site, the same as for the human fibrosarcoma cell line 8387 [21], and that this mechanism is continuously active, thus enabling continuous CXCL8 production by SL-24 cells. In this study, the production of CXCL8 was measured by ELISA; however, measurement of gene expression using RT-PCR will be used in future studies to reveal the mechanism of CXCL8 production from the SL-24 cell line.

Figure 7
CXCL8 production from the LC and the SC cell lines. Solid lines indicate the production of CXCL8 from the LC cell line while the dotted line indicates the production of CXCL8 from the SC cell line. Production of CXCL8 from the LC cell line was clearly higher than that from the SC cell line. Growth in cell numbers of the LC cell line was slightly greater than that of the SC cell line from 3 days post cultivation.

CXCL8 is produced from macrophages following stimulation with IL-1β or TNFα [22, 24]. When we examined whether a range of porcine cytokines had a similar effect in the SL-24 cells, only recombinant porcine IL-15 stimulated the production of CXCL8 from the SL-24 cells. IL-10 has been reported to inhibit the production of CXCL8 [25], but this was not observed in the present study. As addition of TNFα destroyed the SL-24 cells during the CXCL8 inhibition test, the effect of TNFα could not be determined. The cytokine IL-15 was first described in 1994, and was characterized as a T-cell growth factor with similar activity to IL-2. IL-15 is produced by monocytes, epithelial cells and fibroblasts and plays a role in the differentiation and maintenance of NK, NKT, and γδT cells [14, 26]. However, there was no reported role for IL-15 in the production

of CXCL8 until now. It will be of interest if this result also occurs *in vivo*, as IL-15 is a cytokine with a key role in innate immunity. It is possible, therefore, that the involvement of IL-15 in the early stages of immunity may involve the stimulation of CXCL8 production and the recruitment of neutrophils.

Cloning of the SL-24 cell line showed that the parent line contained at least two kinds of cell, one of which, the LC, produced more than 20 times as much CXCL8 as the other, the SC. It is seems reasonable therefore, that cloning is necessary to ensure the continuous stable production of CXCL8. The ability to grow the LC cells in serum-free medium is also important as the serum used in cell culture has the potential to result in contamination.

The results of this study showed that porcine CXCL8 was easily produced by the SL-24 cell line and by the cloned LC cell line. These cell lines will be a valuable resource for studying the mechanism of production of CXCL8, while large-scale production of CXCL8 may prove useful in the treatment of chronic infectious diseases in pigs [22].
The SL-24 and the LC cloned cell lines are patented as number 3876386 in Japan.

UNCITED REFERENCE

[27, 28].

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