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Modulation of *in vitro* porcine natural killer cell activity by recombinant interleukin-1α, interleukin-2 and interleukin-4

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SUMMARY

In order to understand better how cytokines modulate porcine lymphocyte-mediated natural cytotoxicity and to develop a rapid and reliable colorimetric assay to study that activity in young pigs, we studied inherent and cytokine induced *in vitro* natural killer (NK) activity. The cytokines we studied were human recombinant interleukin-1α (IL-1α), IL-2, IL-4 and interferon-gamma (IFN-γ). Natural killer activity by peripheral blood mononuclear cells (PBMC), reported as per cent specific lysis (%SL), was determined by the colorimetric measurement of lactate dehydrogenase released from tumour cell targets, YAC-1 and K562. Inherent NK activity was low and remained relatively unchanged by alterations of assay length or effector cell concentration. Low NK activity was also observed in response to IL-4 and IFN-γ. IL-2 and, to a lesser extent, IL-1α induced significant NK activity with trends towards increasing %SL with increasing cytokine dose. Optimal IL-1α- and IL-2-induced NK activity could be observed at 18 hr, with significant activity stimulated by IL-2 as early as 4 hr. IL-2-induced NK activity was sensitive to effector cell concentration; %SL decreased as the effector to target ratio decreased. IL-1α- and IL-2-induced NK activities were decreased in the presence of IL-4. These results indicate porcine PBMC are sensitive to *in vitro* modulation by human recombinant IL-1α, IL-2 and IL-4. The ability of IL-1α and IL-2 to induce swine NK activity and the ability of IL-4 to inhibit that activity are similar to the actions of those cytokines in human NK systems.

INTRODUCTION

Natural killer (NK) cells are a subpopulation of lymphocytes involved in non-specific immune responses, especially cell-mediated cytotoxicity. In humans, these cells which are primarily large granular lymphocytes, function *in vitro* without previous exposure to foreign antigen to lyse tumour cells or infected target cells without major histocompatibility complex (MHC) class II restrictions. *In vitro* NK activity by peripheral blood lymphocytes (PBL) may be altered by several cytokines, including interleukin-1 (IL-1), -2, -4 and -7, interferon (IFN) and tumour necrosis factor (TNF).[1-6] Porcine NK effector cells are morphologically different from human NK effectors[7,8] in that they are small to medium sized, low density non-granular lymphocytes. However, porcine *in vitro* NK cell response to cytokines does parallel that of humans. Interferons have been shown to be potentiators of NK activity.[9,10] Likewise, IL-2 also augments pig NK activity. Either recombinant human[11-13] or supernatant-derived native[13,14] IL-2 will enhance cytotoxicity but usually requires a 1–3-day induction period. Takamatsu and Koide[14] found that monocytic supernatants thought to contain IL-1 could also enhance non-specific killing.

The study of NK cells in swine has been important from two perspectives. First, certain *in vitro* characteristics implicate NK cells as important *in vivo* components in an animal's immune response to infectious diseases. The study of *in vitro* NK activity against targets infected with Aujesky's disease virus[15] and transmissible gastroenteritis virus[16] has enabled researchers to characterize partially the host response against naturally occurring swine pathogens. Second, *in vitro* NK activity may be a reasonable indicator of an animal's changing immunological status. Richerson and Misfeldt[17] noted increased NK activity in swine chronically exposed to infectious and parasitic diseases. This supported earlier speculation that exposure to environmental pathogens could stimulate a cytokine response with resultant NK cell activation.[18] Kim[19] found *in vitro* augmentation of NK activity in specific pathogen-free piglets treated with a biological response modifier (BRM), OK432, a putative cytokine inducer. The possibility that increased *in vivo* cytokine levels may contribute to enhanced *in vitro* NK activity was partially substantiated when treatment of pigs with recombinant human IL-2 resulted in enhanced *in vitro* NK activity.[20] Thus an *in vitro* NK assay has the potential to be a convenient and rapid way to follow changes in the immunological status of swine as a result of disease or BRM administration.

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The objective of the current study was to evaluate the ability of porcine PBMC to perform NK activity. In addition, the ability of recombinant cytokines to alter the responses was examined.

MATERIALS AND METHODS

Effectors

Donor animals were 6–10-week-old cross-bred pigs. Whole blood obtained by jugular venipuncture was collected into tubes containing acid-citrate dextrose. Following centrifugation at 100 ***g*** for 20 min, mononuclear cells were removed and layered onto Percoll (Sigma P1644; Sigma, St Louis, MO) adjusted to a specific gravity of 1·077. Following centrifugation at 400 ***g*** for 20 min, the cells at the Percoll interface were removed. Red blood cells were then removed by hypo-osmotic lysis. The remaining mononuclear cells were washed once with phosphate-buffered saline (pH 7·4) and resuspended in 25 ml serum-free RPMI-1640 (Gibco-380-2400 AJ; Gibco, Grand Island, NY). Adherent cells were removed by adding this suspension to a 75 cm^2 plastic tissue culture flask and incubating for 90 min at 37°, 100% humidity and 5% CO_2. Non-adherent cells were collected and resuspended in assay medium composed of RPMI-1640, 50 μg/ml gentamicin and 1 mg/ml bovine serum albumin (Sigma A4503).

Targets

YAC-1 and K562 (ATCC, Rockville, MD) were subcultured in RPMI-1640, gentamicin (50 μg/ml), 2-mercaptoethanol (50 μm) and 10% foetal bovine serum (Hyclone, characterized; Hyclone, Logan, UT). Prior to use in cytolytic assays, the cells were pelleted by centrifugation at 400 ***g*** for 5 min and resuspended in assay medium.

Cytokines

Human recombinant cytokines obtained from Genzyme (Cambridge, MA) [IL-1α (10^8 U/mg), IL-2 ($2·5 \times 10^6$ U/mg, IL-4 (10^8 U/mg) and IFN-γ ($2·5 \times 10^7$ U/mg)] were diluted to four times the desired final concentration in assay medium prior to use and added at 25 μl/well.

Cytolytic assay

The basic assay for colorimetric determination of natural cytotoxicity was adopted from Korzeniewski and Callewaert.[21] Various numbers of effectors (50 μl) and targets (25 μl) were added to duplicate wells of 96-well flat-bottom microtitre plates (Costar, Cambridge, MA) containing 25 μl of assay medium with or without cytokine. The plates were agitated briefly and centrifuged (3 min, 200 ***g***) to facilitate contact between effectors and targets. Plates were incubated from 4 to 20 hr at 37°, 100% humidity and 5% CO_2. To harvest the assay, 25 μl of cold 0·4% saponin in Earle's balanced salt solution (EBSS) was added to wells containing targets alone, all other wells received 25 μl cold EBSS. The microplate was agitated for 30 min (600 rpm on Sarstedt TPM-2 shaker) to ensure total lysis of targets for determining maximum lactate dehydrogenase (LDH) release. An indicator substrate solution (100 μl) consisting of $5·4 \times 10^{-2}$ M L-lactic acid (Sigma L1750), $6·6 \times 10^4$ M *p*-iodonitrotetrazolium violet (Sigma I8377), $2·8 \times 10^4$ M phenazine methosulphate (Sigma P9625), and $1·3 \times 10^{-3}$ M nicotinamide adenine dinucleotide (Sigma N3014) in 0·2 M Tris (Sigma T1503), pH 8·2, was added to all wells. The optical density (OD) at 490 nm was determined at 12-second intervals for 2 min on a V_{max}® multiwell plate reader (Molecular Devices, Palo Alto, CA). The rate of change in OD over time (mOD/min, ΔOD) is directly proportional to LDH released during cell death. The quantity of LDH released into the supernatant is directly proportional to lysis. The per cent specific lysis (%SL) was calculated as follows:

$$[(\Delta OD_{E+T} - \Delta OD_{E} - \Delta OD_{T\text{-spon}})/(\Delta OD_{T\text{-total}} - \Delta OD_{T\text{-EBSS}})] \times 100$$

where: E = effectors; T = targets; T-total = targets with 0·4% saponin; T-EBSS = targets with EBSS; T-spon = T-EBSS minus ΔOD of medium control wells.

Differences between treatment means were analysed by ANOVA.

Table 1. NK reactivity: specific lysis as a function of effector cell concentration. Mean ($n = 10$) %SL ± SEM of YAC-1 (4×10^4/well) or K562 (1×10^4/well) by non-adherent PBL at varying E/T ratios. Data are from a single 4-hr assay and are representative of three other experiments

YAC-1		K562	
E/T ratio	%SL	E/T ratio	%SL
10	0±0	40	1·6±1·0
5	0·3±0·2	20	5·7±1·1
2·5	0·7±0·4	10	4·8±0·9
1·2	1·3±0·4	5	5·5±0·8

Table 2. NK reactivity: kinetics of lysis. Mean ($n = 12$) %SL ± SEM of YAC-1 (E/T = 10:1) or K562 (E/T = 40:1) by non-adherent PBL (4×10^5/well) at 4, 8, 12 and 20 hr. Results are from duplicate assays with six animals each

Time (hr)	YAC-1	K562
4	1·4±0·7	5·8±1·9
8	0·1±0·1	5·0±1·3
12	0±0	4·5±2·4
20	0±0	1·3±1·2

RESULTS

NK activity

The effects of incubation time and effector concentration on NK activity were examined. Specific lysis by non-adherent PBL against K562 or YAC-1 target cells was low. Lysis of either target after 4 hr of culture varied from 0 to 6%SL over the range of effector to target (E/T) ratios tested (Table 1). This activity remained relatively constant at 4, 8, 12 or 20 hr (Table 2). NK

Table 3. Effect of E/T ratio on IL-2-induced NK activity. Mean ($n=9$) %SL±SEM* of YAC-1 (4×10^4/well) or K562 (10^4/well) by non-adherent PBL at varying E/T ratios in the presence of 125 U/ml IL-2 in duplicate 18-hr assays

YAC-1		K562	
E/T ratio	%SL	E/T ratio	%SL
10	36±4·7	40	46±1·8
5	40±6·3	20	44±3·0
2·5	14±3·7[a]	10	21±2·7[a]
1·2	2·4±1·9[a]	5	7·3±0·8[a]
0·6	0[b]	2·5	2·0±0·7[c]
0·3	0·2±0·2[b]	1·2	1·2±0·5[d]

* Significance levels for these data are $P<0·01$.
[a] Different from all higher E/T ratios; [b] different from E/T ratios of 2·5:1 and higher; [c] different from E/T ratios of 10:1 and higher; [d] different from E/T ratios of 5:1 and higher.

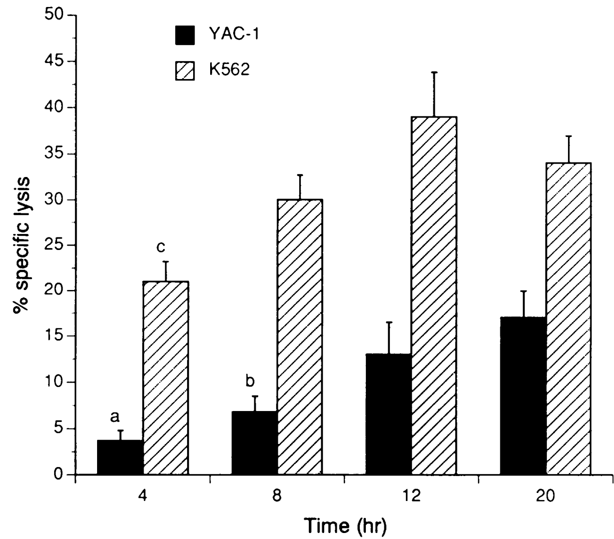

Figure 1. Effect of assay length on IL-2-augmented NK activity. Mean %SL±SEM (significant at $P<0·01$) of YAC-1 (E/T=10:1) or K562 (E/T=40:1) by non-adherent PBL (4×10^5/well) at the indicated times in the presence of 25 U/ml IL-2. Results are pooled from three experiments of six animals each ($n=18$) except for time=12 hr ($n=12$). [a] Different from 12- and 20-hr kinetics with YAC-1 cells; [b] different from 20-hr kinetics with YAC-1 cells; [c] different from 8-, 12- and 20-hr kinetics with K562 cells.

activity did not vary as a function of age under the conditions tested; %SL in 6-week-old pigs was equivalent to that in 10-week-old animals.

Effect of cytokines on NK activity

The effects of assay length and E/T ratio on IL-2-augmented NK activity were also determined. IL-2-enhanced cytotoxicity was sensitive to effector cell concentration; %SL of both targets in 18-hr assays decreased significantly ($P<0·01$) with decreasing E/T ratio (Table 3). The onset of lytic activity in the presence of IL-2 against K562 cells was rapid. Significant ($P<0·01$) lysis above background NK levels occurred after 4 hr and was maximal at 12 hr (Fig. 1). Significant ($P<0·01$) lysis of YAC-1 above background first occurred at 12 hr and peaked at 20 hr. Lysis of either target at 20 hr was not statistically different from that at 12 hr. In subsequent cytokine studies, specific lysis was measured at 18 hr.

We evaluated the effect of a cytokine dose on NK activity. IL-4 at doses ranging from 20 to 2×10^3 U/ml and IFN-γ at doses ranging from 10 to 10^4 U/ml failed to augment cytotoxicity (Figs. 2a,b). Specific lysis of K562 ($P<0·01$) and YAC-1 induced by IL-2 tended to increase as the cytokine dose was increased from 6·2 to 100 U/ml (Fig. 2c). IL-1α (Fig. 2d), at 500 U/ml (the highest dose tested), induced significant ($P<0·01$) NK activity against both target cell lines. There was a trend toward increased %SL with increasing IL-1α dose, yet there were no statistical differences among doses below 500 U/ml when compared with background NK activity. The onset of IL-1α induced LAK activity was delayed compared to that of IL-2. Thus, lysis of K562 ($P<0·01$) and YAC-1 first appeared at 12 and 20 hr, respectively, times which also coincided with maximal activity against each target (data not shown). Overall, IL-2 generated a more potent NK response than IL-1α.

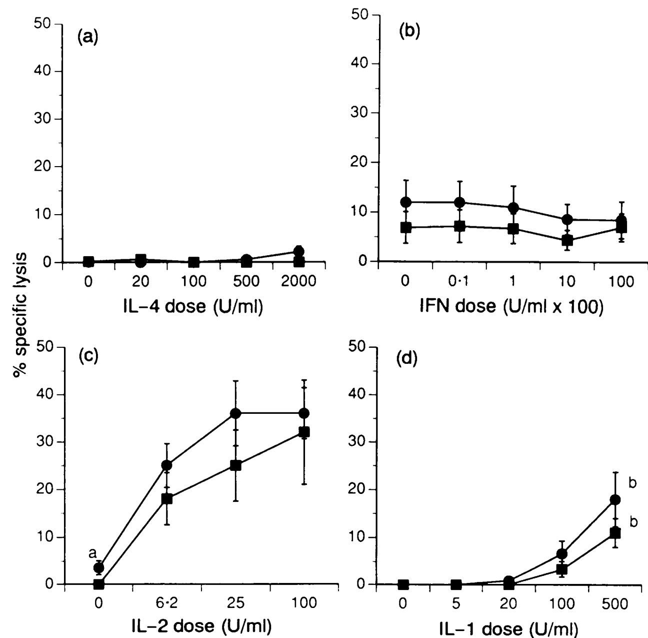

Figure 2. Effect of IL-4 ($n=10$) (a), IFN-γ ($n=10$) (b), IL-2 ($n=4$) (c), and IL-1α ($n=5$) (d) dose on NK activity. Mean %SL±SEM of YAC-1 (■), E/T=10:1, or K562 (●), E/T=40:1, by non-adherent PBL (4×10^5/well) in the presence of human recombinant cytokines at the indicated concentrations. Assay length was 18 hr. [a] Different ($P<0·01$) from all IL-2 doses with K562 cells; [b] different ($P<0·01$) from all lower IL-1 doses and background NK activity against both targets.

Effect of cytokine combinations on NK activity

Interactions between cytokine pairs are summarized in Table 4. The addition of IFN-γ to either IL-1α or IL-2 did not

Table 4. Effect of paired cytokines on NK activity. Mean ($n=11$) %SL±SEM* of YAC-1 (E/T = 10:1) or K562 (E/T = 40:1) by non-adherent PBL (4×10^5/well) in the presence of combinations of IL-1α (500 U/ml), IL-2 (25 U/ml), IL-4 (500 U/ml) and IFN-γ (10^3 U/ml). Results are from two 18-hr assays with five or six animals per study. Human recombinant IL-2 (125 U/ml) was run for comparison

Cytokines	YAC-1	K562
IL-2+IL-1	27±3·3	46±7·5
IL-2+IFN	23±3·7	42±5·0
IL-2+IL-4	10±2·1[a]	31±4·6
IL-2 only	21±3·5[b]	40±8·1
IL-1+IFN	9·5±2·7	19±4·2[e]
IL-1+IL-4	2·5±1·4[b]	13±4·5[e]
IL-1 only	12±3·4	22±4·1[e]
None	0·7±0·6[c]	0·6±0·6[d]
IL-2 (125 U/ml)	34±4·8	48±6·2

* Significance levels for these data are $P<0·01$.

[a] Different from IL-2 alone with YAC-1 cells; [b] different from IL-1 alone with YAC-1 cells; [c] different from all cytokine combinations except IL-1/IL-4 with YAC-1 cells; [d] different from all cytokine combinations with K562 cells; [e] different from IL-2-induced activity against K562 cells.

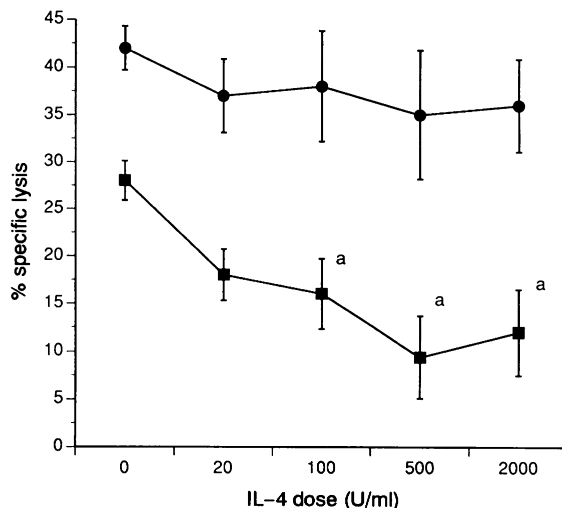

Figure 3. Effect of IL-4 dose on IL-2-induced NK activity. Mean ($n=6$) %SL±SEM of YAC-1 (■), E/T = 10:1, or K562 (●), E/T = 40:1, by non-adherent PBL (4×10^5/well) in the presence of IL-4 at the indicated concentrations and 25 U/ml IL-2 in a single 20-hr assay. [a] Different ($P<0·01$) from IL-2-induced NK activity in the absence of IL-4.

appreciably alter effector function against either target when compared to the activity of either cytokine alone. IL-1α and IL-2 did not work synergistically to increase NK activity against either target, as there was no statistical difference in activity between the IL-1α/IL-2 pair and the activity of IL-2 alone. Noticeable changes in activities occurred when IL-4 was paired with either IL-1α or IL-2. Interleukin-4 abrogated IL-1α induced LAK activity against YAC-1 ($P \leq 0·01$) and caused a 40% reduction in lysis of K562. When IL-4 was added simultaneously with IL-2, lysis of YAC-1 ($P<0·01$) and K562 were reduced 50 and 22%, respectively. Because of the inhibitory activity of IL-4, we examined the effect of various IL-4 doses on IL-2-induced lysis of both target cells (Fig. 3). The three higher doses of IL-4 significantly ($P<0·01$) inhibited lysis of YAC-1. There was no statistically significant ($P>0·05$) inhibition of specific lysis against K562, but the reduction in activity, 16% at 500 U/ml of IL-4, was comparable to the 22% decrease in the previous experiment (Table 3).

DISCUSSION

Our results suggest endogenous NK activity in non-adherent PBL from cross-bred swine is low. Moderate natural cytotoxicity against K562 targets has been reported elsewhere in young pigs, but the magnitude of lysis varies among laboratories. For example, Kim *et al.*[22] observed ~45%SL by PBL from 6-week-old germ-free and specific-pathogen-free pigs. Charley *et al.*[9] reported ~15%SL in 6-week-old pigs and 22%SL in 3–4-month-old pigs.[13] Yang and Schultz,[23] however, found that unfractionated PBL from 3-4 month old pigs induced 46%SL. Martin and Wardley[15] and Pinto and Ferguson[7] have seen NK activity at 18 hr but not at 4 hr. Pinto and Ferguson[7] suggested that disparities in NK activity among laboratories may be due to genetic differences among pigs. Richerson and Misfeldt[17] saw only modest NK activity (12%SL) in 6–10-week-old pigs which they attributed to the disease-free status of the animals compared to a second experimental group (43%SL) naturally exposed to normal swine pathogens. They also noted even lower NK activity (<5%SL) was observed in 'conventionally housed adult domestic swine'. Other researchers whose main intent was to explore the effect of IL-2 on porcine NK activity tended to observe low background NK activity (<10%SL) in the absence of IL-2.[11,14] Therefore the low NK activity we observed may be attributed to a number of factors which may affect inherent porcine *in vitro* NK activity; these factors include the methodology used to isolate the effector cells, the animal's age, its breed and its immunological status prior to and during testing.

IFN-γ, which is known to induce lytic activity in swine[10] and humans,[1,24] did not augment NK activity under the conditions tested. In this respect, our data agree with Kim and Chung[10] who reported that IFN augmented pre-existing NK activity. It is possible that since the animals we tested lacked significant endogenous NK activity, IFN-γ augmentation of that response did not occur. Other possibilities for this lack of activity include poor cross-reactivity between human IFN-γ and porcine PBL or that effectors required a longer incubation with IFN-γ before becoming activated.

Whereas background and IFN-γ-induced NK activities were minimal, our data indicate specific lysis was dramatically increased by exogenous IL-2. Human recombinant IL-2 is known to augment porcine NK activity.[12] Bhagyam *et al.*[11] showed that PBL activated for several days with IL-2 had both increased tritiated thymidine uptake and NK activity. This

ability to respond to IL-2 is not found in newborn pigs, but develops over time.[13] Results of human studies indicate IL-2 may stimulate IFN-γ production; IFN-γ then induces non-specific cytolytic activity.[24,25] A similar scheme has been proposed for cattle by Jensen and Schultz[26] who reported that the onset of IFN-γ-induced NK activity preceded that of IL-2. Thus, they surmised that IL-2 induced endogenous IFN production which was then responsible for enhanced NK activity. Herein, we have shown that, after only 4 hr of culture, IL-2 augmented NK activity against K562. Since endogenous IFN-γ production, stimulated by IL-2, would most likely not have occurred within the 4-hr assay period,[27] our data suggest that IL-2 alone is capable of directly augmenting a porcine NK response.

We have also demonstrated that IL-1α was capable of triggering modest augmentation of NK activity. This possibility in pigs was addressed by Takamatsu and Koide[14] who described monocytic cell supernatants with IL-1 activity capable of inducing a NK response. The mechanism of such a response may involve the ability of IL-1 to increase IL-2 receptor expression on NK cells,[28,29] in addition to its ability to stimulate T cells to produce IL-2.[30,31] Thus IL-1α and IL-2 may synergistically amplify NK activity.[2] We found no synergy when IL-1α and IL-2 were paired possibly because the IL-2 concentration employed may have been to high to observe this effect. We did find that IL-4 inhibited both IL-1α and IL-2 NK responses; an observation suggesting that in pigs, IL-1α and IL-2 may work by a common pathway to augment NK activity.

T-cell-derived IL-4 is a potent immunoregulatory cytokine whose complex action on human NK cell function has only recently been explored. The effect of IL-4 on human PBL *in vitro* depended on the timings of its appearance when used in conjunction with IL-2. For example, IL-4 augmented NK activity by PBL preactivated with IL-2.[3] However, IL-4 added early in culture induced antigen-specific T-lymphocyte cytotoxicity but not non-specific NK activity.[32] Not only did IL-4 fail to induce NK activity, but PBL cultured together with IL-4 and IL-2 had diminished NK effector function compared to those treated with IL-2 alone.[4] We have found that IL-4 alone did not stimulate lysis and that when added simultaneously with IL-2 or IL-1α significantly ($P < 0{\cdot}01$) reduced NK activity against YAC-1. The IL-4 inhibition of IL-2-enhanced NK activity against K562 was less pronounced since this target was generally more susceptible to lysis than YAC-1 and therefore would not be as sensitive to subtle changes in effector function.[33] Thus, we may speculate, in pigs, if IL-1α elicits IL-2 production and IL-2 receptor expression, then we might expect the inhibitory effects of IL-4 to be more pronounced when added with IL-1α than with IL-2. When added with IL-2, IL-4 may work to block only partially the induction of NK activity over which IL-2 exerts its control since both cytokines are presented to the effectors simultaneously. However when added with IL-1α, the presence of IL-4 precedes the putative production of endogenous IL-2 and therefore successfully blocks the induction of effector function.

We have described assay parameters which have allowed us to study the action of the recombinant human cytokines IL-1α, IL-2, IL-4 and IFN-γ on porcine natural cytotoxicity. The ability of IL-1α and IL-2 to augment swine NK activity and the ability of IL-4 to inhibit that activity are similar to the actions of these cytokines on human NK responses.

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