In vivo induction of interferon-α in pig by non-infectious coronavirus: tissue localization and *in situ* phenotypic characterization of interferon-α-producing cells

Sabine Riffault, Charles Carrat, Lydia Besnardeau, Claude La Bonnardière and Bernard Charley

Unité de Virologie et Immunologie Moléculaires, INRA, 78352 Jouy-en-Josas cedex, France

A low frequency peripheral blood mononuclear cell (PBMC) subpopulation, referred to as natural interferon-producing (NIP) cells, is described as producing interferon-α (IFN-α) following contact with non-infectious viral structures, namely viral glycoproteins. These cells are characterized *in vitro* as non-T, non-B, MHC class II and CD4 cells. In this study, NIP cells were analysed *in vivo* after an intravenous injection of UV-inactivated transmissible gastroenteritis virus in newborn piglets, which resulted in strong serum IFN-α production. Splenocytes, but not PBMC, were the IFN-α producers *in vivo*. Using double immunohistochemical labelling for both IFN-α and leukocyte markers, we established that splenic NIP cells were not T or B cells. The majority were MHC class II and only a minority expressed a macrophage marker. NIP cells were localized in contact with MHC class II-expressing cells and T cells, which suggested that NIP cells might modulate the antiviral immune response.

Production of type I interferon (IFN), IFN-α/β, constitutes one of the earliest host responses to viruses. In addition to their well-established antiviral effects (Van den Broek *et al.*, 1995), type I IFN can also modulate antiviral immune responses (Belardelli & Gresser, 1996). Two main populations of IFN-α-secreting cells (IFN-α SC) have been characterized in *in vitro* studies using virus-induced blood leukocyte preparations. Monocytes/macrophages produce low IFN-α levels following viral infection (Gobl *et al.*, 1988). In contrast, an infrequent but highly productive leukocyte subpopulation, referred to as natural IFN-α-producing (NIP) cells, is able to produce IFN-α following brief contact with non-infectious viral structures (Fitzgerald-Bocarsly, 1993). *In vitro* studies performed on human and pig blood leukocytes have shown that NIP cells are non-phagocytic, non-adherent cells that lack T, B and natural killer cell lineage specific markers, but express CD4, CD36 and MHC class II molecules (Sandberg *et al.*, 1991; Nowacki & Charley, 1993). A recent study on purified, *in vitro* herpes simplex virus (HSV)-induced human NIP cells indicates that these cells share several phenotypic and functional properties with dendritic cells, including their ability to present viral antigens to T cells (Svensson *et al.*, 1996).

A few studies have been carried out that describe IFN-α SC *in vivo*. Thus, when IFN-α/β is detected in the blood during lymphocytic choriomeningitis virus infection in mice, IFN-α mRNA-expressing cells occur mainly at the lymphoid organ level (Sandberg *et al.*, 1994). IFN-α SC were accordingly detected in the spleen or the draining lymph node following intravenous (i.v.) or subcutaneous injection of UV-inactivated virus, respectively (Artursson *et al.*, 1995; Eloranta *et al.*, 1996; Riffault *et al.*, 1996).

In our previous studies on IFN-α induction, we took advantage of several features of transmissible gastroenteritis virus (TGEV) in order to analyse the role played by external viral glycoproteins in inducing leukocytes to secrete IFN-α in this virus model. TGEV is an enveloped RNA coronavirus which induces acute and often fatal diarrhoea and high IFN-α production in infected newborn piglets (La Bonnardière & Laude, 1981). It has only three external proteins, named M, S and sM, and the only antibodies able to block IFN-α induction *in vitro* are directed to the external domain of glycoprotein M (gM) (Charley & Laude, 1988; Riffault *et al.*, 1997). In addition, a TGEV mutant named dm49–4, selected on the basis of its defective ability to induce IFN-α production *in vitro*, is characterized by a point mutation in the N-terminal domain of gM (Laude *et al.*, 1992).

In order to analyse *in vivo* the mechanism of IFN-α induction by non-infectious TGEV and to identify the origin of IFN-α SC, colostrum-deprived, 24-h-old piglets were injected i.v. with UV-inactivated TGEV ($1{\cdot}5 \times 10^8$ p.f.u. before UV inactivation). Two TGEV strains, the wild Purdue 115 strain and the mutant virus dm49–4 (Laude *et al.*, 1992), were used as virus sources. Blood was collected at 0, 7 and 30 h post-injection (p.i.). The kinetics of serum IFN-α production was

Author for correspondence: Sabine Riffault.
Fax +33 1 34 65 26 21. e-mail Riffault@biotec.jouy.inra.fr

0001-4767

Table 1. Frequency of IFN-α SC in blood and lymphoid organs, 7 h after inactivated TGEV i.v. injection

Cells were either assayed directly after their isolation from tissue (*'ex vivo'*), or following *in vitro* restimulation with UV-inactivated TGEV (*'in vitro'*).

Tissue of origin of cells	IFN-α SC per 10^5 cells*	
	ex vivo	*in vitro*
Spleen	1·95 ± 0·33 ($n = 11$)	10·6 ± 2·4 ($n = 11$)
Blood	0 ($n = 12$)	19·2 ± 4·2 ($n = 15$)
Mesenteric lymph node	0 ($n = 8$)	0·10 ± 0·10 ($n = 10$)
Liver	0·25 ± 0·25 ($n = 4$)	0·30 ± 0·19 ($n = 7$)

* IFN-α SC were detected using a specific ELISPOT assay. The number of nucleated cells in each cell suspension used in the ELISPOT assay was counted to calculate the frequency of IFN-α SC and results were expressed as the number of spots per 10^5 cells. The figures are mean ± SEM.
n, No. of animals assayed in three independent experiments.

studied by means of a specific ELISA for porcine IFN-α (Diaz de Arce *et al.*, 1992). After wild TGEV i.v. injection, early and very significant IFN-α production was detected in the blood with highest titres (1308 ± 512 U/ml, $n = 9$) observed at 6–8 h p.i.; residual IFN-α titres (121 ± 43 U/ml, $n = 9$) were still present at 30 h p.i. Similar IFN-α production kinetics are described after i.v. injection of UV-inactivated HSV in C57Bl/6 mice (Bhuiya *et al.*, 1994; Eloranta *et al.*, 1996). Furthermore, the rapid IFN-α production described above was similar to the IFN-α response of pigs experimentally infected with TGEV (La Bonnardière & Laude, 1981).

In contrast, no circulating IFN-α could be detected after i.v. injection of the dm49–4 mutant virus ($n = 5$) or after injection of the control medium (MEM) ($n = 3$). The absence of IFN-α in dm49–4-injected animals clearly indicated that the N-terminal domain of gM is essential for IFN-α induction by TGEV *in vivo* in accordance with the mechanism previously described *in vitro*. The present experimental model was therefore appropriate for the *in vivo* study of IFN-α induction by the external proteins of non-infectious viruses.

To identify the cellular origin of IFN-α produced in serum after i.v. injection of UV-inactivated wild TGEV, animals were sacrificed at 7 h p.i. by a lethal anaesthetic injection, and heparinized blood, spleen, mesenteric lymph nodes and liver were collected. Mononuclear cell suspensions from these tissues were prepared and depleted of plastic-adherent cells as previously described (Nowacki & Charley, 1993). Cells were then assayed for IFN-α secretion, either with or without additional *in vitro* incubation with UV-inactivated TGEV, using the ELISPOT technique previously described (Nowacki *et al.*, 1993). Results of three independent experiments (Table 1) show that no IFN-α SC could be detected in PBMC of IFN-α-producing animals, although addition of the virus to the same cells *in vitro* did induce IFN-α secretion. In lymph node and liver cells, very few or no IFN-α SC were detected, either with or without *in vitro* incubation with TGEV (Table 1). A significant number of IFN-α SC was, however, found in spleen mononuclear cells prepared from virus-injected animals (Table 1). The frequency of *in vivo*-induced spleen IFN-α SC (around two positive cells per 10^5 non-adherent mononuclear cells), although five times lower than the frequency obtained when the same cells were incubated *in vitro* with TGEV (Table 1), was in agreement with the characteristic low frequency of NIP cells *in vitro* (Cederblad & Alm, 1990; Nowacki *et al.*, 1993). This last finding and the fact that *in vivo* induction of IFN-α SC was mediated by non-infectious TGEV, possibly by its gM, strongly suggested that the IFN-α SC described in the present study were the *in vivo* counterpart of the NIP cells previously described *in vitro*.

Because *in vivo*-induced IFN-α SC were almost exclusively detected among spleen mononuclear cells by ELISPOT, their localization in spleen was studied using immunohistochemical staining. Pieces of spleen were fixed in 10% formol in PBS, dehydrated and finally embedded in paraffin (54–56 °C). Rabbit IgGs raised against purified recombinant porcine IFN-α MPA1 (Lefèvre *et al.*, 1990) were used to stain IFN-α SC in paraffin spleen sections in Tris buffer (50 mM, pH 7·4) with 0·02% saponin, 0·2% $CaCl_2$ and 1% heat-inactivated normal porcine serum (NPS). Sections were then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma), and the IFN-α SC were visualized using the Fast Red substrate (Fast Red TR/Naphthol AS-MX, Sigma). A few clearly positive IFN-α SC, mainly located in the periarteriolar lymphatic sheath, were detected in spleen sections from UV-inactivated-TGEV-injected animals (Fig. 1*a*). The IFN-α SC appeared as large cells whose cytoplasmic extensions were sometimes visible (Fig. 1*b*). No staining was observed with non-immune rabbit IgG or with sections from MEM-injected animals (not shown). In

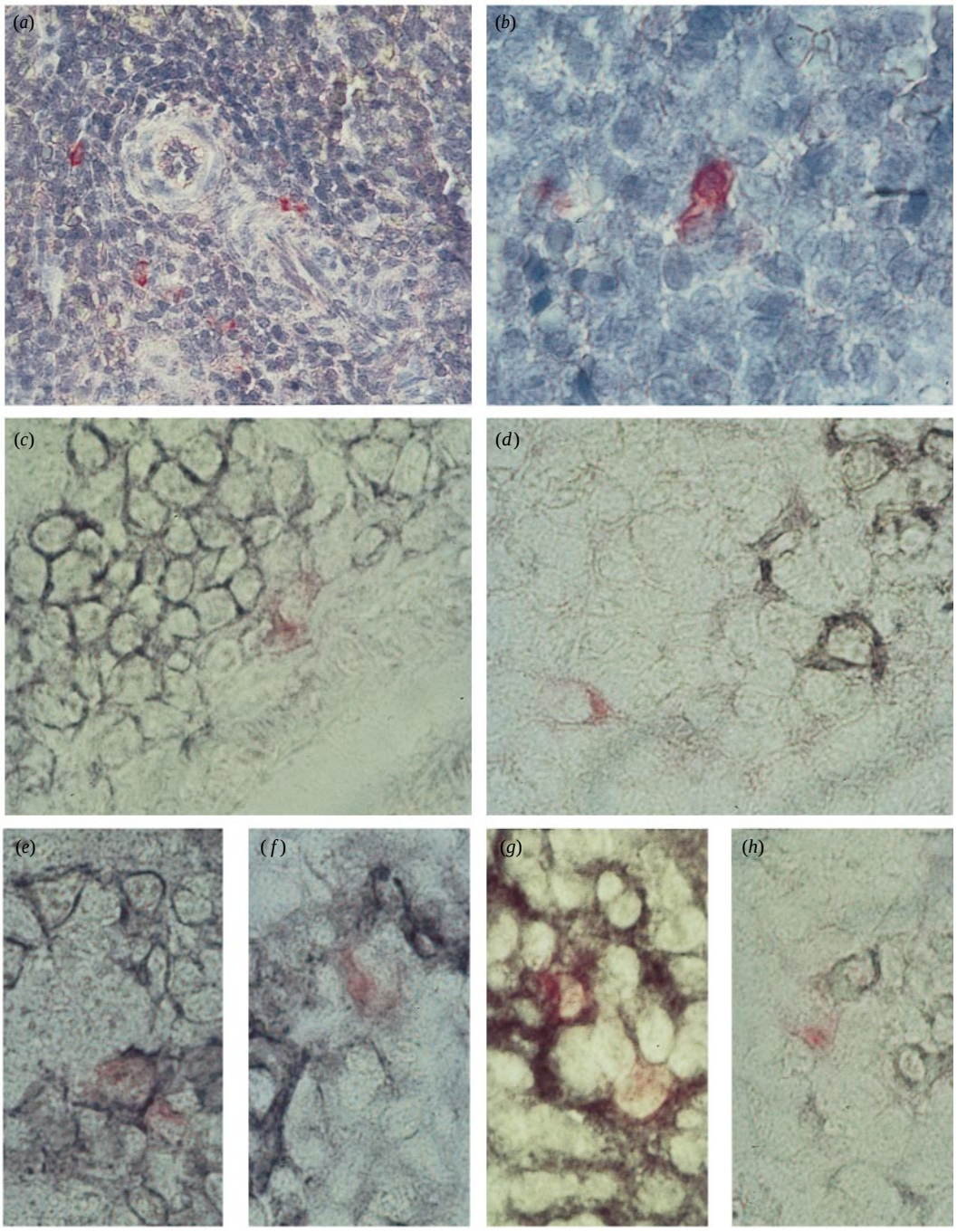

Fig. 1. Localization and phenotype of IFN-α SC in spleen 7 h after i.v. injection of UV-inactivated TGEV. Immunohistochemical staining using anti-polFN-α antibodies and paraffin sections showed IFN-α SC in the periarteriolar lymphatic sheath (*a*) with abundant cytoplasm and cytoplasmic extensions (*b*). Double staining of spleen cryosections with anti-polFN-α antibodies (red) and anti-leukocyte antibodies (dark blue) showed that IFN-α SC are distinct from CD3 expressing cells (*c*), and from Ig light chain-expressing cells (*d*). Some IFN-α SC did express MHC class II molecules (*e*) or a macrophage marker SWC3a (*g*). Conversely, MHC class II molecules (*f*) or a macrophage marker SWC3a (*h*) were not detectable on some IFN-α SC. Original magnification: ×132 for (*a*) and ×330 for (*b*)–(*h*).

contrast to the location of porcine IFN-α SC, the murine splenic IFN-α SC, induced by i.v. injection of UV-inactivated HSV, are exclusively found in the marginal zones (Eloranta *et al.*, 1996). This discrepancy may reflect the fact that the spleen structure of newborn piglets is not fully differentiated.

The phenotype of NIP cells has, until now, only been

Table 2. Specificity of mouse MAbs directed against swine leukocytes used in this study

MAb	Specificity	Isotype	Origin
MSA3	SLA-DR (MHC class II)	IgG2a	Hammerberg & Schurig (1986)
K139 3E1	Surface Ig light chain	IgG2a	Dr Stevens (University of Bristol, UK); see Kaeffer *et al.* (1991)
PPT3	CD3	IgG1	Yang & Parkhouse (1996)
74-22-15	SWC3a (monocytes, granulocytes)	IgG1	Pescovitz *et al.* (1984)

studied with PBMC *in vitro* (Fitzgerald-Bocarsly, 1993). As our present data have demonstrated that splenocytes and not PBMC were the source of NIP cells *in vivo*, the phenotypic characterization of NIP cells based on *in vitro* studies with PBMC may not be valid for *in vivo*-induced NIP cells. In the present study, we have therefore established the phenotype of NIP cells *in situ* by immunohistochemical methods, for both IFN-α and leukocyte markers. IFN-α SC were detected on spleen cryosections fixed with acetone using the antibodies described above diluted in PBS buffer with 1% NPS. After IFN-α SC staining, the sections were incubated with the different anti-leukocyte MAbs listed in Table 2, then with biotin-conjugated goat anti-mouse IgG (Biosys) and finally with peroxidase-conjugated streptavidin (Pierce). The labelled leukocytes were visualized with 4-chloro-1-naphthol (Sigma). Double staining of IFN-α SC and of different leukocyte subpopulations showed that the IFN-α SC were in close proximity to SLA-DR$^+$ cells (Fig. 1*e*, *f*) and CD3$^+$ cells (Fig. 1*c*), but distant from surface Ig (sIg) light chain$^+$ cells (Fig. 1*d*). The CD3 and sIg light chain antigens were not detectable on IFN-α SC (Fig. 1*c*, *d*). These findings are in agreement with previous *in vitro* studies demonstrating that NIP cells are distinct from T and B cells (Sandberg *et al.*, 1991; Nowacki & Charley, 1993). The SLA-DR antigen was expressed by two-thirds of IFN-α SC (Fig. 1*e*) but was not detectable on one-third of IFN-α SC (Fig. 1*f*). Conversely, the SWC3a antigen (macrophage/granulocyte marker) was expressed by one-third of the IFN-α SC (Fig. 1*g*), but was not detectable on two-thirds of IFN-α SC (Fig. 1*h*). This suggested that IFN-α SC might not constitute a homogeneous cell population. A precise estimation of the percentages of SLA-DR$^+$ IFN-α SC and SWC3a$^+$ IFN-α SC was complicated by their location in areas strongly positive for either SLA-DR or SWC3a antigens. No unspecific staining was observed on sections labelled without anti-IFN-α IgG, anti-leukocyte IgG or both or with non-immune murine IgG (not shown).

The observed heterogeneity of MHC class II and macrophage marker expression could explain why the studies concerning the nature of human NIP cells are still controversial (Fitzgerald-Bocarsly, 1993). The MHC class II$^+$ or macrophage marker$^-$ phenotypes together with a morphology characterized by some cytoplasmic extensions could concord with the recent hypothesis of a dendritic lineage for NIP cells (Ferbas *et al.*, 1994; Svensson *et al.*, 1996), whereas the MHC class II$^-$ or macrophage marker$^+$ phenotypes might well be compatible with the hypothesis of a monocytic lineage (Francis & Meltzer, 1993; Grage-Griebenow *et al.*, 1996).

Previous studies have shown that IFN-α SC are mainly detected in the lymphoid tissues such as the spleen or regional lymph nodes following non-infectious virus injection (Artursson *et al.*, 1995; Splichal *et al.*, 1995; Eloranta *et al.*, 1996; Riffault *et al.*, 1996). However, this is the first demonstration that circulating PBMC did not produce a detectable amount of IFN-α *in vivo*. One reasonable explanation might be the rapid trapping of virions in the spleen after UV-inactivated virus i.v. injection.

Combined staining of NIP cells and leukocyte subpopulations showed that NIP cells were in contact with MHC class II$^+$ cells, T cells and occasionally macrophages. It is therefore conceivable that NIP cells could act as accessory cells at the time of antigen presentation to naive T cells. Indeed, IFN-α is involved in the promotion of T cell differentiation towards the Th1 phenotype (Belardelli & Gresser, 1996). NIP cells might thus contribute both to innate and specific antiviral defence.

In the case of TGEV, natural infection takes place primarily in the intestinal tract and is characterized by high IFN-α production both in serum and intestinal fluids (La Bonnardière & Laude, 1981). It would therefore be interesting to look for NIP cells in gut-associated lymphoid tissue, especially Peyer's patches.

The NIP cells described above that produce high quantities of IFN-α inside lymphoid tissues, in close contact with T cells and antigen-presenting cells, could participate in and modulate the initiation of the immune response towards viral pathogens. Experiments are currently in progress to determine to what extent IFN-α production by lymphoid tissue NIP cells can affect the antiviral immune response.

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References

Artursson, K., Lindersson, M., Varela, N., Scheynius, A. & Alm, G. V. (1995). Interferon-α production and tissue localization of interferon-α/β producing cells after intradermal administration of Aujeszky's disease virus-infected cells in pigs. *Scandinavian Journal of Immunology* **41**, 121–129.

Belardelli, F. & Gresser, I. (1996). The neglected role of type I interferon in the T-cell response: implications for its clinical use. *Immunology Today* **17**, 369–372.

Bhuiya, T. A., Shodell, M., Fitzgerald-Bocarsly, P. A., Murasko, D., Shah, K., Drake, D. & Siegal, F. P. (1994). Interferon-α generation in mice responding to challenge with UV-inactivated herpes simplex virus. *Journal of Interferon Research* **14**, 17–24.

Cederblad, B. & Alm, G. V. (1990). Infrequent but highly efficient interferon-α producing human mononuclear leukocytes induced by herpes simplex virus in vitro studied by immuno-plaque and limiting dilution assays. *Journal of Interferon Research* **10**, 65–73.

Charley, B. & Laude, H. (1988). Induction of alpha interferon by transmissible gastroenteritis coronavirus: role of a transmembrane glycoprotein E1. *Journal of Virology* **62**, 8–11.

Diaz de Arce, H., Artursson, K., L'Haridon, R., Perers, A., La Bonnardière, C. & Alm, G. V. (1992). A sensitive immunoassay for porcine interferon-α. *Veterinary Immunology and Immunopathology* **30**, 319–327.

Eloranta, M.-L., Sandberg, K. & Alm, G. V. (1996). The interferon-α/β responses of mice to herpes simplex virus studied at the blood and tissue level in vitro and in vivo. *Scandinavian Journal of Immunology* **43**, 355–360.

Ferbas, J. J., Toso, J. F., Logar, A. J., Navratil, J. S. & Rinaldo, C. R., Jr (1994). CD4+ blood dendritic cells are potent producers of IFN-α in response to in vitro HIV-1 infection. *Journal of Immunology* **152**, 4649–4662.

Fitzgerald-Bocarsly, P. (1993). Human natural interferon-α producing cells. *Pharmacology and Therapeutics* **60**, 39–62.

Francis, M. L. & Meltzer, M. S. (1993). Induction of IFN-α by HIV-1 in monocyte-enriched PBMC requires gp120-CD4 interaction but not virus replication. *Journal of Immunology* **151**, 2208–2216.

Gobl, A. E., Funa, K. & Alm, G. V. (1988). Different induction patterns of mRNA for IFN-α and IFN-β in human mononuclear leukocytes after in vitro stimulation with herpes simplex virus-infected fibroblasts and Sendai virus. *Journal of Immunology* **140**, 3605–3609.

Grage-Griebenow, E., Flad, H.-D. & Ernst, M. (1996). Fcγ receptor I (CD64)-negative human monocytes are potent accessory cells in viral antigen-induced T cell activation and exhibit high IFN-α-producing capacity. *Journal of Leukocyte Biology* **60**, 389–396.

Hammerberg, C. & Schurig, G. G. (1986). Characterization of monoclonal antibodies directed against swine leucocytes. *Veterinary Immunology and Immunopathology* **11**, 107–121.

Kaeffer, B., Bottreau, E., Marcon, D., Olivier, M., Lantier, I. & Salmon, H. (1991). Histocompatible miniature pig (d/d haplotype): generation of hybridomas secreting A or M monoclonal antibody. *Hybridoma* **10**, 731–744.

La Bonnardière, C. & Laude, H. (1981). High interferon titer in newborn pig intestine during experimentally induced viral enteritis. *Infection and Immunity* **32**, 28–31.

Laude, H., Gelfi, J., Lavenant, L. & Charley, B. (1992). Single amino acid changes in the viral glycoprotein M affect induction of alpha interferon by the coronavirus transmissible gastroenteritis virus. *Journal of Virology* **66**, 743–749.

Lefèvre, F., L'Haridon, R., Borras-Cuesta, F. & La Bonnardière, C. (1990). Production, purification and biological properties of an *Escherichia coli*-derived recombinant porcine alpha interferon. *Journal of General Virology* **71**, 1057–1063.

Nowacki, W. & Charley, B. (1993). Enrichment of coronavirus-induced interferon-producing blood leukocytes increases the interferon yield per cell: a study with pig leukocytes. *Research in Immunology* **144**, 111–120.

Nowacki, W., Cederblad, B., Renard, C., La Bonnardière, C. & Charley, B. (1993). Age-related increase of porcine natural interferon α producing cell frequency and of interferon yield per cell. *Veterinary Immunology and Immunopathology* **37**, 113–122.

Pescovitz, M. D., Lunney, J. K. & Sachs, D. H. (1984). Preparation and characterization of monoclonal antibodies reactive with porcine PBL. *Journal of Immunology* **133**, 368–375.

Riffault, S., Eloranta, M.-L., Carrat, C., Sandberg, K., Charley, B. & Alm, G. (1996). Herpes simplex virus induces appearance of interferon-α/β-producing cells and partially interferon-α/β-dependent accumulation of leukocytes in murine regional lymph nodes. *Journal of Interferon and Cytokine Research* **16**, 1007–1014.

Riffault, S., Grosclaude, J., Vayssier, M., Laude, H. & Charley, B. (1997). Reconstituted coronavirus TGEV virosomes lose the virus ability to induce porcine interferon-alpha production. *Veterinary Research* **28**, 105–114.

Sandberg, K., Eloranta, M. L., Johannisson, A. & Alm, G. V. (1991). Flow cytometric analysis of natural interferon-α producing cells. *Scandinavian Journal of Immunology* **34**, 565–576.

Sandberg, K., Eloranta, M.-L. & Campbell, I. L. (1994). Expression of alpha/beta interferons (IFN-α/β) and their relationship to IFN-α/β-induced genes in lymphocytic choriomeningitis. *Journal of Virology* **68**, 7358–7366.

Splichal, I., Rehakova, Z., Sinkora, J., Charley, B. & Sinkora, M. (1995). Interferon alpha secreting cells in hematopoietic organs of pig fetuses after in vivo stimulation by coronavirus TGEV. *Scandinavian Journal of Immunology* **41**, 642.

Svensson, H., Johannisson, A., Nikkilä, T., Alm, G. V. & Cederblad, B. (1996). The cell surface phenotype of human natural IFN-α producing cells as determined by flow cytometry. *Scandinavian Journal of Immunology* **44**, 164–172.

Van den Broek, M. F., Müller, U., Huang, S., Zinkernagel, R. M. & Aguet, M. (1995). Immune defence in mice lacking type I and/or type II interferon receptors. *Immunological Reviews* **148**, 5–18.

Yang, H. & Parkhouse, R. M. E. (1996). Phenotypic classification of porcine lymphocyte subpopulations in blood and lymphoid tissues. *Immunology* **89**, 76–83.

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