UNIVERSITÀ DEGLI STUDI DI MILANO

Graduate School of Animal Health and Production:

Science, Technology and Biotechnologies

Department of Veterinary Science and Public Health

PhD Course in Biotechnologies Applied to Veterinary

and Animal Husbandry Sciences

(Cycle XXVI)

Doctoral Thesis

PIG TONSIL CELLS AS A MODEL TO EVALUATE ORAL, LOW-DOSE CYTOKINE TREATMENTS

(VET 05)

Elisabetta RAZZUOLI

Nr R09008

Tutor. Prof. Luigi BONIZZI and Dr. Massimo AMADORI

Coordinator: prof. Fulvio GANDOLFI

Accademic Year 2012-2013

ABSTRACT

Cytokines are low molecular weight mediators of the immune system. Because of their properties and biological roles, they have been used as diagnostic reagents for human and animal diseases, as well as for prognostic tests and prophylactic treatments. Concerning this kind of treatments, there is uncertainty as to how cytokines are effective after oral administration. They probably act on the oral lymphoid tissues (palatine and pharyngeal tonsils) and trigger a cascade of events leading to activation of the immune system and control of the inflammatory cascade in tissues and organs. Owing to above, the aim of our project was to develop a reliable *in vitro* model related to the crucial interactions between cytokines and oral lymphoid cells, in terms of homeostatic regulation of the inflammatory response and antibody production. In this project, we focused our attention on the IFN-α system. The study was divided into 4 steps: 1) Isolation and culture of pig tonsil lymphocytes; 2) evaluation of IPEC-J2 cells as a reporter system of the anti-inflammatory control actions of interferon-alpha; 3) A pig tonsil cell culture model for evaluating oral, low-dose IFN-α treatments; 4) Disease-dependent modulation of tonsil cell phenotypes. In the first steps we develop reliable procedures for isolation and culture of pig tonsil cells, which were validated for use in functional immunoassays. Our results indicate that pig tonsil cells can be employed within 2 months from freezing to maintain suitable conditions in terms of recovery, vitality and release of antibody *in vitro*. Tonsil mononuclear cells also showed the ability to secrete antimicrobial peptides and to respond *in vitro* to immunological stimuli. In the second part of the project, IPEC-J2 (a continuous cell line of porcine intestinal epithelial cells) was validated as reporter system of the biological properties of IFN-α. Three different experimental conditions (oxidative stress, inflammatory response, and amplification of lymphoid cell signals) were selected to evaluate the effects of porcine recombinant IFN-α1 (rIFN-α) and 2 natural porcine IFN-α preparations (nIFN-α). The IFNs under study showed significantly different control actions in IPEC-J2 cells. In particular, rIFN-α was shown to down-regulate interleukin (IL)-8, IL-1β, tumor necrosis factor (TNF)-α, and β-defensin 1 genes either directly, or indirectly through second messengers released by IFN-α-treated lymphoid cells. With regard to IL-6, only second messengers from IFN-α-treated lymphoid cells could regulate the expression of this cytokine. Our results indicate that IPEC-J2 cells can be a useful tool for investigating the regulatory actions of type I IFNs and second messengers thereof. In the third step we developed an *in vitro* model of interaction between different types of human and porcine IFNs-α at low / moderate concentrations and pig tonsil cells. The IFNs-α under study showed different properties with respect to three fundamental control actions: 1) IgA release in culture (up and down-regulation, respectively), 2) release of natural antimicrobial compounds, and 3) homeostatic regulation of the inflammatory response. Some IFNs-α caused a

3

significant inhibition of IL-8 (protein release and gene expression) and β-Defensin 1 (gene expression) through second messengers released by IFN α-treated tonsil cells. Interestingly, a human lymphoblastoid IFN-α under study caused the decrease of polyclonal IgA release by pig tonsil cells and significantly stimulated the *in vitro* recall antibody response of swine PBMC to Foot-and-Mouth Disease virus. The modulation of IgA and antibacterial compounds was accompanied by an anti-inflammatory control action at the same, low to moderate IFN-α concentrations (1 to 100 U / ml). This highlights the very foundation of the homeostatic control actions performed by Type I IFNs: to promote an effective host response to infectious and non-infectious stressors and to turn off noxious inflammatory responses associated to tissue damage and waste of metabolic energy. The described tonsil cell model *in vitro* can be conducive to a further development of oral cytokine treatments in human and animals in the "one health" conceptual framework.

INDEX

1. CYTOKINES

1.1 Cytokines: main features

Cytokines are proteins of low molecular weight involved in many biological processes like inflammation, immune response, stem cells differentiation and pathogenesis of important diseases like atherosclerosis and cancer (Daniello 2007). These compounds are mainly produced and secreted by monocytes and T-lymphocytes; however other cell types like fibroblasts can synthesize these molecules (Belardelli, 1995). Their production mainly occurs during the immune response; in effect, the majority of these molecules is not accumulated in the cell but produced *ex-novo* after the transcription of their genes. Cytokine mRNAs have a short life span, so that they are produced only when necessary. The mRNAs exert a negative feedback on protein production, so that cytokine release is a brief and self-limited event (Bianchi, 2007).The main feature of cytokines is pleiotropism, i.e. the ability of a particular cytokine to cause different effects on different cell types. Other properties are the possibility to start or inhibit the production and release of other cytokines (Bianchi, 2007). The effects of these molecules can be exerted (Figure 1) on the same cytokine-producing cells (autocrine action) or near of the production site (paracrine action, nearby cells). High doses of cytokine, by the systemic circulation, can act on distant sites (endocrine action). These molecules can act like polypeptide hormones and perform their actions by binding to specific, high-affinity membrane receptors, with dissociation constants of 10^{-10}-10^{-12}M. This implies that low concentrations of cytokines do have biological effects. Table 1 shows the main cytokines, their source and target cells (Bianchi, 2007).
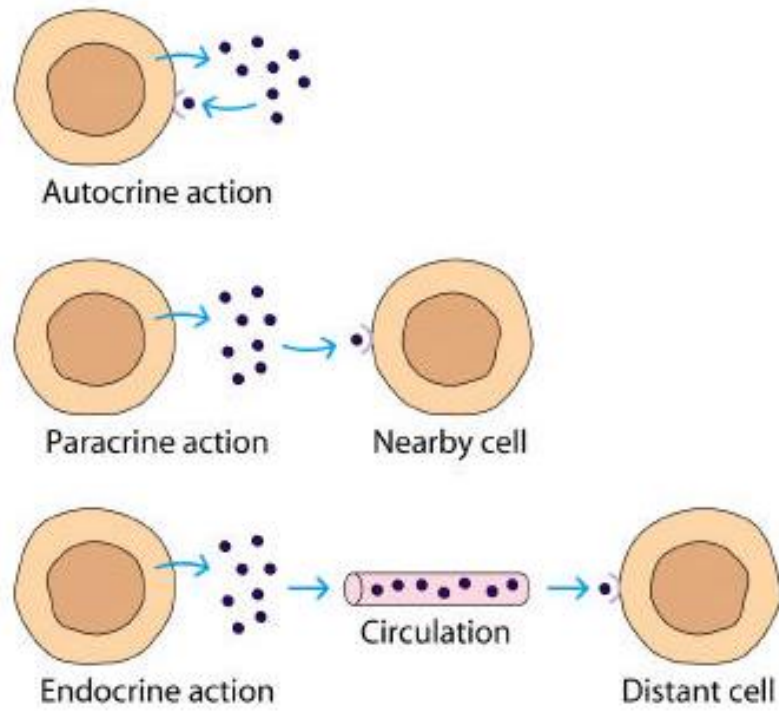
Figure 1.Cytokines: modes of action

Autocrine action

Paracrine action   Nearby cell

Endocrine action   Circulation   Distant cell

Table 1. Main cytokines, effects and cells target

CYTOKINE	CELL SOURCE	CELL TARGET	MAIN EFFECTS
IL-1	Monocytes, Macrophages, Fibroblast, epithelial cells, endothelial cells, Astrocytes	B and T lymphocytes endothelia cells Hypothalamus, Liver	Co-stimulation compound Pro-inflammatory fever, production of acute phase proteins
IL-2	T Lymphocytes NK Cells	T Lymphocytes B Lymphocytes Monocytes	Growth Activation
IL-3 and IL-7	T Lymphocytes	Bone marrow precursors	Growth and differentiation
IL-4	T Lymphocytes	B Lymphocytes, Eosynophils	Growth Activation
IL-5	T Lymphocytes	B Lymphocytes, Eosynophils	Co-stimulation compound
IL-6	T Lymphocytes	T Lymphocytes, B Lymphocytes, Liver	Co-stimulation compound, Growth, production of acute phase proteins
IL-8	T Lymphocytes	Neutrophils	Activation and chemotaxis
IFN-α	Macrophages	All cells	Antiviral NK activation
IFN-γ	T Lymphocytes NK Cells	Monocytes, endothelial cells, tissue cells, Macrophages	Activation, Expression of MCH I e II
TNF-α	T Lymphocytes, Macrophages	IL1-Like	IL1-Like
GM-CSF	T Lymphocytes, Macrophages, endothelial cells, fibroblasts	bone marrow precursors	Growth and differentiation

1.2 Cytokines: some applications

Because of their properties and biological roles, cytokines have been used as diagnostic reagents for human and animal diseases (Wood and Jones, 2001; Sanchez-Correa et al., 2013), as well as for prognostic use (Trevisi et al., 2011) and prophylactic treatments (Zecconi et al., 2009).

1.2.1. Cytokines: therapeutic and prognostic use in Humans

Clinical studies on recombinant cytokines showed that these compounds can exert antitumor activity in patients with metastatic cancer. In this respect, cytokines that have demonstrated the greatest usefulness are interleukin (IL)-2, IL-15 and interferon (IFN)-α. Regarding IL-2, parenteral high-doses of this cytokine stimulates immune effector cells bearing the IL-2R (in particular NK and T cells) and producing antitumor effects in 15% of patients with melanoma or kidney carcinoma. Moreover, some studies reported a low reduction of metastatic breast cancer (Carson and Liang, 2007).

In case of melanoma and kidney carcinoma, another cytokine that gives greater results in terms of tumor progression and/or recurrence is IFN-α; high-doses of this cytokine determine a clinical response in 5-10% of patients while no significant effects were shown in breast cancer (Lens and Dawes, 2002). Concerning the prognostic purposes, strong evidence supports the existence of cytokine dysregulation processes in many tumors. For example, in patients with acute myeloid leukemia (AML) there is an aberrant production of pro-inflammatory cytokines like IL-6 and TNF-α and of anti-inflammatory ones like IL-10. Low levels of IL-6 and high levels of IL-10 represent favorable prognostic factors for survival in AML patients. These results support the idea that cytokine dysregulation may be a useful marker for predicting clinical evolution in AML patients (Sanchez-Correa et al., 2013). Recent studies (Cho et al., 2013) implied that assessment of certain cytokines, such as IL-6 and IL-8, may contribute to prognosis of breast cancer in patients with particular tumor subtypes. In particular, the cited authors speculated that tumor subtype-specific approaches to regulate cytokine levels could be a therapeutic option for reducing the risk of tumor recurrence and improving the prognosis of breast cancer.

1.2.2 IL-6 and prognostic use in cows

Increased disease rates are commonly reported among high yielding dairy cows (HYDC) in the transition period, extending from 3 weeks before to 3 weeks after calving, and characterized by the occurrence of an inflammatory response in terms of both positive and negative acute phase proteins (APP+ and APP-, respectively). To determine the above inflammatory response, Trevisi and co-workers developed the Liver Functionality Index (LFI), which defines the above condition on the basis of some APP- responses (albumin, cholesterol *sensu stricto* + bilirubin) during the first month of

lactation (Trevisi et al., 2011); in this respect, low LFI values are associated with high inflammatory response and disease occurrence (Trevisi et al., 2012). The relationship between LFI and inflammatory cytokine response was further investigated from day -28 to day +28 with respect to calving in a cohort of 54 high-yielding dairy cows in two experimental dairy farms. Cows were divided into 3 groups of LFI: low (LOLFI), intermediate (INLFI) and high (HILFI) LFI values, which represent poor, intermediate and good predicted conditions, respectively, in terms of consequences of the inflammatory challenge around calving (Trevisi et al., 2011; Trevisi et al., 2012). As expected, the costs of drug treatments were 2-4-fold higher in LOLFI, compared with HILFI cows. Interestingly, the costs of antimicrobials (antibiotics and sulfamides) and other drugs demanding a withdrawal time after their use (mainly anti-inflammatory products) confirmed similar differences between the LFI groups. Moreover, IL-6 serum concentrations were always higher in LOLFI cows ($P < 0.05$ on day +28 vs HILFI cows), as previously reported by Trevisi and co-workers (2012), which investigated the fertility of the cows belonging to LO and HILFI groups. The greater IL-6 levels were correlated with higher ceruloplasmin (APP+) and lower lysozyme serum concentrations ($P < 0.05$ and < 0.1, respectively), from the dry period onwards. This implies that cows at risk for disease occurrence could be recognized in the non-lactating period. This way, they could be timely submitted after calving to proper cares. A possible preventive treatment could be based on anti-inflammatory drugs, which actually promotes improved animal health and welfare conditions on farm (Bertoni et al., 2004; Trevisi et al., 2005; Trevisi and Bertoni, 2008). In particular, early detection of the above parameters and early start of relevant pharmacological treatments can greatly improve the effectiveness of drugs and prevent the onset of chronic disease cases. The positive impact of early diagnosis was verified in the above trial (Trevisi et al., 2012), where diagnosis of disease conditions was always performed at a very early stage in one of the two herds under study. As a result, the course of the affections in that herd was quick and often solved by using treatments with galenic remedies only, or just stimulating appetite and rumination activity (e.g. by yeast and/or glucogenic supplements).

1.2.3 IL-2 and prophylactic treatments in cows

Zecconi and co-workers (2009) reported the results obtained by using IL-2 to prevent mastitis in dairy herds. The treatment consisted of a single, 800-picogram IL-2 dose injected subcutaneously into the skin region drained by the supramammary lymph node 3-5 days after calving. The study included 45 cows (23 treated and 22 controls) of three commercial dairy herds. The results showed that the treatment had no side effects and caused a significantly higher frequency of healthy udder quarters until day 17-19 after calving in the treated group, compared with the control one. Although these

results should be confirmed by further, large-scale field studies, they nevertheless provide important evidence as to how a targeted and site-specific modulation of the local immune response could be an efficient strategy for mastitis control in dairy cattle, leading to a lesser usage of antibiotics in dairy farms.

2   INTERFERONS

IFNs are a family of cytokines synthesized and secreted by most cell types that elicit pleiotropic biological effects. They are proteins named after their capacity to interfere with viral infections of animal cells (Isaacs and Lindenmann, 1957) and also endued with immunomodulatory and anti-proliferative activities. Three distinct classes of interferon molecules are known to date, i.e. Type I, Type II and Type III IFNs. Type II consists of one molecular species only (IFN-γ), whereas Type I IFNs are a heterogeneous group including several distinct families (IFN-α, IFN-β, IFN-ε, IFN-ω, IFN-k, IFN-δ and IFN-τ), with some of them (like IFN-α) consisting of different subtypes. Type III IFN includes 3 molecular species (IFNs λ1, λ2 and λ3, also named IL-29, IL-28A and IL-28B, respectively) showing similar biological properties (Flores et al., 1991; Kotenko, 2003; Donnelly and Kotenko, 2010).Within Type I IFNs, porcine IFN-β is a glycoprotein encoded by one gene only, whereas porcine IFN-α represents a multigene family with 17 functional genes (Sang et al., 2010) located on chromosome 1 (Yerle et al., 1986); this gene family shares 96–99.8% identity at the nucleotide level and 91.1–100% at the amino acid level (Cheng et al., 2006). Both constitutive and virus-induced production of IFN-α can be recognized in porcine PBMC, and IFN protein can be either released or maintained in intracellular stores (Amadori et al., 2009).

The biological effects of IFNs are mediated through IFN-regulated genes. Three major IFN-regulated pathways involving RNA-dependent protein kinase (PKR)/the eukaryotic initiation factor (eIF)- a system, 2-5A synthetase/RNase L system and Janus Kinase (JAK)/signal transducer and activator of transcription (STAT) system have been identified (Dafny, 1998). PKR/eIF2a system mainly mediates type I IFN effects and the protein synthesis factor eIF-2a functions as the effector of PKR-induced effects in this pathway (Dafny, 1998). The IFN system plays a pivotal role in the innate immune system and in the regulation of the adaptive immune response. In addition, recent evidence accumulated in humans, mice and farm animals points at type I IFN as a crucial homeostatic system, aimed at avoiding unnecessary tissue damage and waste of food energy because of a dysregulated inflammatory response (Cadorso et al., 1990).

2.1 Type I IFNs as homeostatic agents

Microbial infections induce the pro-inflammatory effector functions of IFN-α in the framework of the innate immune response. However, the low tissue concentrations of IFN-α (≤10 U/ml) in the late phases of microbial infections underlie a major shift to a potent anti-inflammatory activity, based on the transcriptional control of genes coding for inflammatory cytokines, pathogen-associated molecular pattern (PAMP) receptors (e.g. CD14) and, possibly, other undetected molecules (Amadori, 2007). This kind of regulation can be transmitted *in vivo* to distant sites by potent, unknown second messengers, as shown in several models of low-dose, oral IFN-α treatment (Tompkins, 1999). In this respect, a substantial down-regulation of inflammatory cytokine genes can be achieved *in vivo* following oral, low-dose IFN-α treatment in pigs at weaning (Amadori et al, 2012); the importance of this regulation is accounted for by the increased expression of these inflammatory cytokine genes after weaning in piglets (Pié et al., 2004). Overall, IFN-α is likely to play an important role in the homeostatic regulation of the inflammatory/stress response in co-operation with other cytokines like interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β) (Amadori, 2007). Interestingly, circumstantial evidence shows that inability to produce IFN-α is associated to recurrent inflammatory disorders, such as chronic vestibular inflammation in women (Gerber et al., 2002) and allergic asthma in children (Bufe et al., 2002). Such a role of IFN-α is also in line with an established model of physiological IFN response (Bocci et al., 1985), and with other studies showing an IFN-α response to stressful, non-infectious events like early weaning and transportation of healthy piglets (Artursson et al., 1989; Wattrang et al., 1998). Likewise, the brain can mount an IFN-α response to non-inflammatory as well as inflammatory stress, as also shown for other cytokines like IL-1. On the whole, the above data point at a major steady-state role of type I IFNs (α and probably β) as homeostatic agents, acting for a successful control of the inflammatory response to infectious and non-infectious noxae. This is not in conflict with the known functions of type I IFN in the innate immune system and would actually complement these in line with major, evolution-based needs of the host (Amadori M., 2007). Such a control by type I IFNs would be based on a subtle regulation of their tissue concentrations and, probably, of the relative proportions among different IFN-α subtypes. Glycosylation of IFN-α and the relative proportions between glycosylated and non-glycosylated molecules are other important, usually neglected features, underlying the final outcome of IFN-α effector functions.

2.2 Dose/response curve of IFN-α

The foundation of the above arguments can be found in the dose/response curve of IFN-α. In general, IFN-α shows a bell-shaped dose-response curve in the modulation of immune effectors functions: beyond a narrow concentration range in which the dose response is positive, response diminishes or even reverses as in the change from stimulation to suppression of the primary antibody response to Sheep Red Blood Cells in mice (Nagao et al., 1998). This is clearly at odds with the antiviral and anti-proliferative activities, which increase with increasing IFN-α concentrations. Accordingly, IFN-α often abides by the general rule of most cytokines: low dose priming, high dose suppression (Cummins et al., 1998). This is the crucial feature which dictates the very outcome of the IFN-α response: this may be dramatically different as a result of both timing and concentration of this cytokine in tissues and organs. Thus, the meaning and the main goals of the IFN-α response may vary to a large extent according to the above parameters and, probably, to the ratios among IFN-α subtypes during the response.

2.3 Parenteral IFN-α treatments

High parenteral IFN-α doses induce pro-inflammatory T helper (Th)1 responses, whereas the highest doses can directly trigger inflammatory and pyrectic responses in humans and cattle (Amadori., 2007). Interestingly, with regard to high-dose IFN-α trials, results may be quite different in heterologous species (different from the IFN-α species) or even in different organs of humans. Thus, regional, anti-inflammatory control actions may co-exist in humans with opposite systemic effects. For instance, high-dose, parenteral, human interferon alpha 2 treatments proved successful in patients with ulcerative colitis and Crohn's disease. In particular, there was continual clinical improvement and the inflammation of the colon mucosa was effectively cleared up (Ruther et al., 1998). A caveat should be expressed though about the dosage (6 million Units, subcutaneously, 3 times per week), which might be associated to a direct role against the human herpesviruses found in the inflamed mucosa (Amadori, 2007). Behcet's disease is a systemic vasculitis characterized by recurrent oral and genital ulcers and ocular inflammation. The disease may also involve the joints, skin, central nervous system, the lungs, and gastrointestinal tract. The cause of Behcet's disease is unknown, but autoimmune and genetic factors are considered important in its pathogenesis. In these patients, high-dose, parenteral IFN-α proved effective in reducing the number of oral and genital ulcers, cutaneous lesions and articular

signs, while keeping the above-mentioned side effects of high-dose treatment (O'Duffy et al., 1998). This is another example of the regional, organ-specific effects of IFN-α which may show concomitant aspects of outright pro and anti-inflammatory control activity. As for laboratory animals, human IFN-α can exert a direct inhibitory effect on acute inflammation in mice, reducing for example carrageenan-induced paw swelling (Mecs and Koltai, 1985). Systemic administration of IFN-α/β suppresses the local Shwartzman reaction in mice; intriguingly, the reaction can be elicited by all Gram-negative bacteria, except by Brucella, which is a potent inducer of circulating IFN-α/β (Amadori, 2007). In addition, protective effects of IFN-α were reported towards severe tissue injuries associated to acute inflammation and oxidative stress. Thus, IFN-α could significantly reduce the intestinal injury score and malonaldehyde levels in a model of experimental necrotizing enterocolitis in rats. Protection against oxidative stress was also demonstrated in rats in a model of carbon tetrachloride-induced nephrotoxicity (Amadori, 2007) and confirmed *in vitro* in stressed hepatocytes and isolated liver mitochondria: zinc and manganese-dependent superoxide dismutase as well as the enzyme activities of glutathione peroxidase were increased, while lipid peroxidation was inhibited (Lu et al., 2002).

2.4 Oral, low-dose, IFN-α treatments

Evidence of an important regulatory control of the inflammatory response came to light from *in vivo* experiments of low-dose, oral IFN-α treatment in many species (cattle, pigs, cats, etc.). Oral IFN-α was shown to be therapeutic for at least two kinds of clinical conditions: viral infections and immune-mediated diseases (Cummins et al., 2005). Viral infections included respiratory tract infections of cattle, transmissible gastroenteritis or rotavirus diarrhea in swine and a number of other viral infections in mice. Immune-mediated diseases were polymyositis, multiple sclerosis, and experimental autoimmune allergic encephalomyelitis in rats and mice. All of these diseases involve serious inflammatory responses in the host. As a further confirmation of this assumption, oral IFN-α treatment induces dramatic amelioration of chronic inflammatory conditions (uveitis, glossitis, tonsil hyperplasia, etc.) in Feline Leukemia Virus (FeLV) and Feline Immunodeficiency Virus (FIV)-infected cats, even without influence on viremia and virus replication (Cummins et al., 1998; Pedretti et al., 2006). Low-dose, oral IFN-α treatment was also shown to exert an anti-inflammatory control action in standard bred racehorses affected by inflammatory airway disease; in 8 to 15 days a significant reduction of neutrophils, macrophages and lymphocytes was observed in bronchoalveolar

16

lavage fluid in IFN-α–treated, as opposed to control animals. Ingested IFN-α given daily can decrease islet inflammation and suppress diabetes in non-obese mice; the same result can be obtained by adoptive transfer of splenocytes secreting IL-4 and IL-10 from IFN-α fed donors. Oral, low-dose IFN-α treatment proved effective in patients having active Sjogren's syndrome, i.e. an autoimmune disease characterized by inflammation of the exocrine glands and secretory failure; in particular, there was a significant improvement of the whole saliva flow, compared with subjects given placebo; improvements were also observed with regard to oral and ocular dryness symptoms (Amadori, 2007). Primary Sjogren's syndrome might be in fact the result of a dramatic, dysregulated activation of the type I interferon system in plasmacytoid dendritic cells after exposure to RNA-containing immune complexes. Again, elevated IL-10 levels are detectable in the saliva of Sjogren's syndrome patients and the IL-10 response correlates with the severity of the disease (Bave et al, 2005). Interestingly, a previous exposure of macrophages to IFN-α can turn IL-10 into a potent pro-inflammatory cytokine (Amadori, 2007). This is a clear example of the complex balance between pro and anti-inflammatory properties of IFN-α and of their often unpredictable outcome.

2.4.1 IFN-α treatment and Porcine Reproductive and Respiratory Syndrome virus (PRRSV)

The IFN system could be an important target of an immunomodulation strategy for PRRS, having in mind the negative regulatory actions of PRRSV on this crucial arm of the innate immune system (Mateu and Diaz,2008). PRRSV is susceptible to the direct antiviral mechanisms displayed by IFN-α (Albina et al., 1998) and IFN-γ (Bautista and Molitor, Arch Virol 1999, 144, 1191-1200). Most important, IFNs were shown to reverse distinct immunosuppressive functions of PRRSV. Thus, IFN-α can block *in vitro* the development of T regulatory (Treg) cells induced by co-culture of lymphocytes with PRRSV-infected dendritic cells (DCs) (Silva-Campa et al., 2009). *In vivo*, positive effects were observed in pigs injected with a non-replicating human adenovirus 5 vector expressing porcine IFN-α, in terms of lower febrile response, lesser involvement of lungs, delayed viremia and Ab response after challenge with PRRSV one day later (Brockmeier et al., 2009). Yet, the peak levels and duration of viremia were not significantly different between treated and control animals (Brockmeier et al., 2009), which points once again at the possible, profound discrepancies between parameters of clinical and virological protection. Moreover, in our experience oral low-dose IFN-α treatments (10 IU/Kg b.w./ daily) determine significant reduction of dead and "poor-doer" piglets (P <0.01). The treatment can also give rise to a significant increase of the daily mean weight gain from 22 to 86 days of age. The same treatment carried out in sows in farrowing cages led to a significant reduction of both abortions and mortality in suckling piglets. These interesting results on farm could not be reproduced in an

experimental PRRSV infection in isolation facilities. Under these conditions, the differences between treated and control piglets were minimal: there was a decrease of pyretic days and a transient decrease of circulating CD8+ T cells for a possible homing to PRRSV-infected tissues. In practice, there was a strong indication that the activity of IFN-α was related to the interactions between PRRSV / environmental bacteria / airborne LPS. This is substantiated by *in vitro* data (Begni B. et al., 2005): very low concentrations of human IFN-α (0.5 − 5 IU/ml) down-regulate CD14 expression in swine PBMC, as opposed to higher concentrations. This may dramatically affect signalling by LPS/LPS binding protein and the released CD14 may be a potent scavenger of LPS.

2.4.2 IFN-α treatments and non-infectious stressors

Owing to the above, the use of IFN-α at low dose can modulate some immune parameter and determine better environmental adaptation in pigs. For this purpose, six litters of Large White X Landrace (sow)/commercial Hypor hybrid (boar) were selected in a farm showing excellent figures of animal health, welfare and post-weaning growth. Animals were allocated to the experimental groups, weaned at the indicated ages (22 and 28 days of age) and given the same starter feed with antibiotic (amoxicillin 400 ppm + colistin 120 ppm) and ZnO (2000 ppm) supplements over the first 10 days. Litters 3 and 4 were treated with human lymphoblastoid IFN-α (TF-21, Guna, Milan) in drinking water at a dose of 1 IU/kg b.w. over 10 days on the basis of the expected growth rate and related water consumption. Litters 5 and 6 were weaned at 28 days of age in accordance with the official recommendations of Italian Legislative Decree 20/2/2004, n. 53.

In particular, no clinical difference was observed between subjects weaned at 22 and 28 days, respectively. On the contrary, in terms of daily weight gain (DWG), greater growth rates ($P < 0.001$) were shown by IFN-α-treated piglets (litters 3 and 4) compared with the control ones (litters 1 and 2). In practice, litters 3 and 4 showed the same final weight at 47–48 days of age as litters 5 and 6, despite the different age at weaning; the one-day difference between the weight checks (47 days of age for litters 5 and 6, as opposed to 48 days for litters 3 and 4; see Table 2) might imply though a residual growth advantage of the late weaning group (litters 5 and 6), in line with a generally increased DWG in piglets weaned at 28 days of age compared with those weaned at 21 days. The better growth performance of IFN-α-treated piglets could be the result of the slightly greater mean weight of piglets at weaning, since this is reputedly predictive of the following growth rate. However, the mean weights at weaning of all litters were by far beyond 10-lbs, a limit for which a delay of the final market weight was observed. The absence of a significant effect beyond the above limit is also indicated by very similar weights at 47 days of age of litters 5 and 6, weaned later. On closer examination, this

hypothesis can be also dismissed on the basis of the actual time-course of the weight gains. DWG data were similar in IFN-treated and control animals between days +1 and +6 (234 and 246 g in control and treated animals, respectively), and DWG was even lesser in the treated ones between days +6 and +12 (121 versus 166 g). The overt advantage in treated piglets was observed instead between days +12 and +26 (405 versus 361 g). This latter result is in agreement with the much higher concentrations of TNF-α on day +26 in control piglets weaned at 22 days of age, also showing a much greater prevalence of subjects with a high serum TNF-α concentration (300 pg/ml) (Chi-square, $P < 0.05$). The IL-6 titres showed a negative correlation with the TNF-α titres, which underlies a substantial nadir of IL-6 on day +26; the highest titres on the day of weaning were shown instead in groups 5 and 6. The reported values of both TNF-α and IL-6 were determined by bioassays; the absolute values are not usually comparable to those obtained by ELISA or other immuno-enzyme techniques, even though the relative differences would persist. In line with previous experiments and ours, an endogenous low-titered IFN-α response to the stress of weaning was observed by a bioassay in all the litters, despite the very good health and welfare conditions of pigs and the absence of detectable virus infections. The frequency of IFN-α responses in serum was significantly higher (Chi-square, $P < 0.01$) in pigs weaned at 22 days compared with those weaned at 28 days. IFN-α was unambiguously identified by means of a neutralization assay with a monoclonal antibody to porcine IFN-α.

On the basis of our findings, we can postulate that an oral, low-dose IFN-α treatment at weaning could be an important side measure on farm, in line with major concerns about food safety 'from farm to fork'. Such a strategy can be adequately supported by a timely assessment of clinical immunology parameters. These can be seen as a practical and accurate reporter system of environmental adaptation, and suitable readout of interventions for better health and welfare conditions, in association with fundamental clinical and productive parameters of farmed pigs.(These data are reported in Amadori et al., 2012)

2.5 Aims

How is IFN-α effective after oral administration? It probably acts on the oral lymphoid tissues (palatine and pharyngeal tonsils) and triggers a cascade of events leading to activation of the immune system and control of the inflammatory cascade in tissues and organs. The signal provided by IFN-α and conveyed by a powerful amplification system is induced by very low oral doses (1 – 10 IU/kg body weight) and is lost at higher daily doses (Tompkins, 1999).Owing to the above, the central aim of our study was to develop a reliable *in vitro* model related to the crucial interactions between cytokines

and oral lymphoid cells, in terms of homeostatic regulation of the inflammatory response and antibody production. In our project, we focused our attention on the IFN-α system.

The study was divided into 4 steps:

1. Isolation and culture of pig tonsil lymphocytes;

2. IPEC-J2 cells as a reporter system of the anti-inflammatory control actions of IFN-α;

3. A pig tonsil cell culture model for evaluating oral, low-dose IFN-α treatments;

4. Disease-dependent modulation of tonsil cell phenotypes.

There are a few commercial IFNs-α; however, the best choice for the veterinary medicine is human IFN-α to the following reasons:

✓ The cost of supply.

✓ The availability.

✓ The biological activity on different animal species.

✓ Absence of problems due to drug residues in feed and food.

In our model we tested 4 different IFNs-α:

1. Human recombinant IFN-α (Humoferon, registered drug);

3. Human Lymphoblastoid IFN-α (natural IFN obtained in Namalwa cell line stimulated with Sendai virus, a paramyxovirus);

4. Recombinant porcine IFN-α1 (commercial product);

5. Porcine natural IFN-α (natural IFN secreted by PBMC stimulated with NDV-Lasota virus, a paramyxovirus).

Among farm animal species, pigs show interesting properties of their Type I IFN system in the response to environmental stressors (Razzuoli et al., 2010). In particular, several IFN-α subtypes are activated in a model of early weaning stress, which determines a notable increase of IFN-α positive

cells in peripheral blood mononuclear cells (PBMC), a low-titred IFN secretion and a transcriptional block of all IFN-α genes at day 6 after weaning (Razzuoli et al., 2011).

3   REFERENCES

1. Albina E., Carrat C., Charley B. 1998 "Interferon-alpha response to swine arterivirus (PoAV), the porcine reproductive and respiratorysyndrome virus." J Int Cyt Res. 18:485-90.

2. Amadori M, Razzuoli E and Nassuato C. 2012 "Issues and possible intervention strategies relating to early weaning of piglets." CAB Rev. 7: 1–15.

3. Amadori M. 2007 "The role of IFN-α as homeostatic agent in the infiammatory response: a balance between danger and response?." J. Interferon Citokyne Res. 27: 181-189.

4. Amadori M., Cristiano A., Ferrari M. 2009 "Constitutive expression of interferons in swine leukocytes." Res. Vet. Sci. Doi: 10.1016/j.rvsc.2009.05.011.

5. Artursson, K., Wallgren, P., Alm, G.V., 1989 "Appearance of interferon-α in serum and signs of reduced immune function in pigs after transport and installation in a fattening farm." Vet. Immunol. Immunopathol. 23: 345-353.

6. Bautista EM., Molitor TW. 1999 "IFN gamma inhibits porcine reproductive and respiratory syndrome virus replication in macrophages." Arch. Virol. 144: 1191-1200.

7. Bave U., Nordmark G., Lovgren T., Ronnelid J., Cajander S., Eloranta ML., Alm GV., Ronnblom L. 2005 "Activation of the type I interferon system in primary Sjogren's syndrome: a possible etiopathogenic mechanism." Arthritis Rheum. 52: 1185-1195.

8. Begni B., Amadori M., Ritelli M., Podavini, D. 2005 "Effects of interferon-α on the inflammatory response of swine leukocytes to bacterial endotoxin." J. Interferon Cytokine Res. 25: 202-208.

9. Belardelli F. 1995 "Role of interferons and other cytokines in the regulation of the immune response." APMIS. 103: 161-179.

10. Bertoni, G., Trevisi, E., Piccioli-Cappelli, F. 2004 "Effects of Acetyl-salycilate used in post calving of dairy cows." Vet Res Comm. 28: 217-219.

11. Bianchi I. 2007 "Concetti generali sulle citochine." In:Bianchi I. (Eds), Trattato di medicina fisiologica di regolazione. Nuova Ispa editore, Palermo. 1: 19-66.

12. Bocci V., Paulesu L., Muscettola M., Viti A.,1985 "The physiologic interferon response. VI. Interferon activity in human plasma after a meal and drinking." Lymphokine Res. 4: 151-158.

13. Brockmeier SL., Lager KM., Grubman MJ., Brough DE., Ettyreddy D., Sacco RE., Gauger PC., Loving CL., Vorwald AC., Kehrli ME., Lehmkuhl HD. 2009 "Adenovirus-mediated expression of interferon-alpha delays viral replication and reduces disease signs in swine challenged with porcine reproductive and respiratory syndrome virus." Viral Immunol. 22: 173-180.

14. Bufe A., Gehlhar K., Grage-Griebenow E., Ernst M. 2002 "Atopic phenotype in children is associated with decreased virus-induced interferon-alpha release." Int. Arch. Allergy Immunol. 127: 82-88.

15. Cardoso E., Arzt E., Coumroglon M., Andrada EC., Andrada JA. 1990 "Alpha interferon induces cortisol release by human adrenals in vitro." Int. Arch. Allergy Appl. Immunol. 93: 263-266.

16. Carson WE., Liang MI. 2007 "Current immunotherapeutic strategies in breast cancer" Surg Oncol Clin N Am.16:841-60.

17. Cheng G., Chen W., Li Z., Yan W., Zao X., Xie J., Liu M., Zhang H., Zhong Y., Zheng Z. 2006 "Characterization of the porcine alpha interferon multigene family." Gene. 382: 28-38.

18. Cho YA., Sung MK., Yeon JY., Ro J., Kim J. 2013 "Prognostic role of interleukin-6, interleukin-8, and leptin levels according to breast cancersubtype." Cancer Res. Treat. 45:210-9.

19. Cummins JM, Tompkins MB, Olsen RG, Tompkins WA, Lewis MG. 1998 "Oral use of human alpha interferon in cats." J. Biol. Response Mod. 7: 513-523.

20. Cummins JM., Krakowka GS., Thompson CG. 2005 "Systemic effects of interferons after oral administration in animals and humans." Am. J. Vet. Res. 66: 164-176.

21. Dafny N. 1998 "Is interferon-alpha a neuromodulator?" *Brain Res. Brain Res. Rev.* 26: 1-15.

22. Daniello AC. 2007 "Historical insights into cytokines." Eur. J. Immunol. 37: 34-45.

23. Donnelly RP., Kotenko SV. 2010 "Interferon-lambda: a new addition to an old family." J Interferon Cytokine Res. 30: 555–64.

24. Flores I., Mariano TM., Pestka S. 1991 "Human interferon-ω (omega) binds to the alpha/beta receptor." J. Biol. Chem. 266: 19875-19877.

25. Gerber S., Bongiovanni AM., Ledger WJ., Witkin SS. 2002 "A deficiency in interferon-alpha production in women with vulvar vestibulitis." Am. J. Obstet. Gynecol. 186: 361-364.

26. Isaacs A., Lindenmann I. 1957 "Virus interference." In; The interferon. Proc. R. Soc. (London) , Ser. B. 147: 258-273.

27. Kotenco SV. 2003 " IFN λs mediate antiviral protection trough a distintic class II cytokine receptor complex." Nat. Immunol. 4: 69-77.

28. Lens MB., Dawes M. 2002 "Interferon alfa therapy for malignant melanoma: a systematic review of randomized controlled trials." J Clin Oncol. 20:1818-25.

29. Lu G., Shimizu I., Cui X., Itonaga M., Tamaki K., Fukuno H., Inoue H., Honda H., Ito S. 2002 "Interferon-alpha enhances biological defense activities against oxidative stress in cultured rat hepatocytes and hepatic stellate cells." J. Med. Invest. 49: 172-181.

30. Mateu E., Diaz I 2008 ".The challenge of PRRS immunology." Vet J. 177:345-51.

31. Mecs I., Koltai M. 1985 "Direct evidence for the anti-inflammatory effect of human interpheron-alpha in CFLP mice." Brief report. Arch. Virol. 85: 151-155.

32. Nagao Y., Yamashiro K., Hara N., Horisawa Y., Kato K., Uemura A. 1998 "Oral-mucosal administration of IFN-alpha potentiates immune response in mice." J. Interferon Cytokine Res. 18: 661-666.

33. O'Duffy JD., Calamia K., Cohen S., Goronzy JJ., Herman D., Jorizzo J., Weyand C., Matteson E. 1998 "Interferon-alpha treatment of Behcet's disease." J. Rheumatol. 25: 1938-1944.

34. Pedretti E, Passeri B, Amadori M, Isola P, Di Pede P, Telera A, Vescovini R, Quintavalla F, Pistello M. 2006 "Low-dose interferon-α treatment for feline immunodeficiency virus infection." Vet. Immunol. Immunopathol. 109: 245-254.

35. Pié S., Lallès JP., Blazy F, Laffitte J, Sève B, Oswald IP. 2004 "Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets." J. Nutr. 134, 641-647.

36. Razzuoli E., Dotti S., Archetti IL., Amadori M. 2010 "Clinical chemistry parameters of piglets at weaning are modulated by an oral, low-dose interferon-alpha treatment." Vet Res Commun 34: 189–S192.

37. Razzuoli E., Villa R., Sossi E., Amadori M. 2011 "Characterization of the interferon-alpha response of pigs to the weaning stress." J. Interferon Cytokine Res. 31: 237–247.

38. Ruther U., Nunnensiek C., Muller HA., Bader H., May U., Jipp P. 1998 "Interferon alpha (IFN alpha 2a) therapy for herpes virus-associated inflammatory bowel disease (ulcerative colitis and Crohn's disease)." Hepatogastroenterology. 45: 691-699.

39. Sang Y., Rowland RR., Hesse RA., Blecha F. 2010 "Differential expression and activity of the porcine type I interferon family." Physiol Genomics. 42:248–58.

40. Silva-Campa E., Flores-Mendoza L., Resendiz M., Pinelli- Saavedra A., Mata-Haro V., Mwangi W., Hernandez J. 2009 "Induction of T helper 3 regulatory cells by dendritic cells infected with porcine reproductive and respiratory syndrome virus." Virology. 387: 373-379.

41. Tompkins WA. 1999. "Immunomodulation and therapeutic effects of the oral use of interferon-α: mechanism of action." J. Interferon Cytokine Res. 19: 817-828.

42. Trevisi E., Amadori M., Archetti I., Lacetera N., Bertoni G. 2011 "Inflammatory response and acute phase proteins in the transition period of high-yielding dairy cows." In: Veas, F. (Ed.), Acute Phase Protein as early non-specific biomarkers of human and veterinary disease, InTech Published, pp 355.

43. Trevisi E., Amadori M., Cogrossi S., Razzuoli E., Bertoni G. 2012 "Metabolic stress and inflammatory response in high-yielding, periparturient dairy cows." Res. Vet. Sci. 93: 695–704.

44. Trevisi E., Bertoni G. 2008 "Attenuation with acetylsalicylate treatments of inflammatory conditions in periparturient dairy cows." In "Aspirin and health research progress", Nova Science Publishers, Hauppauge, NY, USA. pp 23-37.

45. Trevisi E., Ferrari A., Archetti I, Bertoni G. 2005 "Anti-inflammatory treatments in calving dairy cows: effects on haematological and metabolic profiles." Ital. J. Ani Sci. 4: 203-205.

46. Wattrang E., Wallgren P., Lindberg A., Fossum, C. 1998 "Signs of infections and reduced immune functions at weaning of conventionally reared and specific pathogen free pigs." J. Vet. Med. B. 45: 7-17.

47. Wood PR., Jones SL. 2001 "BOVIGAM: an in vitro cellular diagnostic test for bovine tuberculosis." Tuberculosis. 81:147-55.

48. Yerle M., Gellin J., Echard G., Lefevre F., Gillois M. 1986 "Chromosomal localization of leukocyte interferon gene in the pig (Sus scrofa domestica L.) by in situ hybridization." Cytogenet Cell Genet. 42:129–32.

49. Zecconi A., Piccinini R., Fiorina S., Cabrini L., Daprà V., Amadori M. 2009 "Evaluation of interleukin-2 treatment for prevention of intramammary infections in cows after calving." Comp. Immunol. Microbiol. Infect. Dis. 32: 439-451.

CHAPTER 1

ISOLATION AND CULTURE OF PIG TONSIL LYMPHOCYTES.

Razzuoli E, Faggionato E, Dotti S, Villa R, Lombardo T, Boizza L, Ferrari M, Amadori M.*

Laboratory of Cellular Immunology, IZSLER, Brescia, Italy.

* Corresponding author. Tel. +39 030 2290248; fax +39 030 2290392

E-mail address: massimo.amadori@izsler.it (M. Amadori)

ABSTRACT

Tonsils are secondary lymphoid organs that play an important role in host defense. The aim of our study was to develop reliable procedures for isolation and culture of pig tonsil cells, and to validate their possible use in functional immunoassays. Using our isolation procedure, we recovered on average $238.7 \pm 107.1 \times 10^{(6)}$ cells per tonsil couple with a mean vitality of $89.8 \pm 2.7\%$. These values significantly decreased 8 months after freezing at -80°C along with the subsequent spontaneous release of both IgA and IgG in culture. These results suggest to use pig tonsil cells within 2 months from thawing to maintain suitable conditions in terms of recovery, vitality and release of antibody *in vitro*. Tonsil mononuclear cells also showed the ability to secrete antimicrobial peptides and to respond *in vitro* to immunological stimuli. On the whole, our study has defined operating conditions for tonsil processing, control of bacterial contaminations, time limits of storage at -80°C, as well as for evaluating polyclonal Ig production *in vitro*. Such procedures are likely to be of some importance in studies on regional immunity and in the development of large animal models for biomedical sciences.

Key word: Tonsil, Pig, Primary culture, B cells, Immunoglobulin

INTRODUCTION

Tonsils are secondary lymphoid organs which represent a major component of mucosa-associated lymphoid tissue (MALT). This consists of lymphoid cell aggregates widely distributed in the mucosal surfaces (e.g. nasopharynx, oropharynx and laryngopharynx). On the basis of the anatomical localization, MALT can be subdivided into regional structures, including the lymphoid tissues of the Waldeyer's ring (Horter et al., 2003; Liebler-Tenorio and Pabst, 2006). This is located at the opening of both digestive and respiratory tracts and consists of lymphoid structures named tonsils; in pigs are present five different tonsils, i.e. pharyngeal, paraepiglottic, lingual, tubal and soft palate tonsils. The latter are the most developed ones, consisting of bilateral oval plaques of lymphoid tissue with a size of approximately 5 cm length and 3 cm width in adult pigs. Many tonsilar fossules are visible on the surface, leading to crypts located in the center of tonsillar follicles, separated in turn by diffuse lymphoid tissue (Casteleyn et al., 2011). Tonsils can be infected by viral agents which sustain e.g. classical swine fever, pseudorabies, foot-and-mouth disease and porcine respiratory and reproductive syndrome, to cite a few (Alexandersen et al., 2001; Romero et al., 2003; Rowland et al., 2003). In addition to these, various nonpathogenic and pathogenic bacteria like Salmonella spp.,Yersinia enterocolitica, Pasteurella multocida, Haemophilus parasuis, Mycoplasma hyopneumoniae can be isolated from porcine tonsils (Lowe et al., 2011; O'Sullivan et al., 2011). Moreover, there is evidence of an important role in the host's defense played by tonsils, which often represent the first site of interaction between microbial agents and lymphoid tissues. This is the reason why the isolation and characterization of tonsil lymphoid and myeloid cells (Bourges et al., 2004) are pivotal for studies of pathogenesis, latency and immune response to viral and bacterial agents. Furthermore, there is evidence that oral lymphoid tissues are extremely susceptible *in vivo* to low-dose cytokine treatments (Cummins et al., 2005). These treatments were shown to be beneficial in a variety of infectious and non-infectious inflammatory and immuno-pathological conditions (Pedretti et al., 2006). In practice, oral lymphoid tissues could interact with orally administered cytokines and convey potent regulatory signals, as shown in a swine PBMC model (Begni et al., 2005). Owing to the above, it is important to culture viable tonsil cells to be used in functional assays of innate immunity and inflammatory response *in vitro* models of host/pathogen and cell/cytokine relationships. Therefore, the aim of our study was to develop reliable procedures for isolation and culture of pig tonsil cells, and to demonstrate their possible use in functional assays.

2. MATERIALS AND METHODS

2.1. Collection of pig tonsils

Pig tonsils were collected from ten, 9 to 10-month old, Landrace $\times$ Large White pigs, randomly selected in one farm classified as "healthy" because of the previous clinical findings, the low levels of environmental stressors and the excellent production figures at the beginning of the trial. Tonsils were collected at the slaughterhouse and immediately inserted into 50-ml, screw-capped vials containing 25 ml of Hank's balanced salt solution (HBSS) supplemented with an antibiotic mixture (penicillin, streptomycin, neomycin) for refrigerated transport to the laboratory. A few concentrations of antibiotics were tested to check bacterial contaminations: 5$\times$ (250 mg/ml penicillin, 250 mg/ml streptomycin, 50 mg/ml neomycin), 2$\times$ (100 mg/ml penicillin, 100 mg/ml streptomycin, 20 mg/ml neomycin), 1$\times$ (50 mg/ml penicillin, 50 mg/ml streptomycin, 10 mg/ml neomycin). On arrival at the laboratory tonsils were washed twice with the same type of transport medium, and the excess of connective tissue was discarded. Tonsils were then stored overnight at +4 $\circ$C in 25 ml of HBSS with the same antibiotic concentration used for transportation.

2.2. Isolation of tonsil mononuclear cells

Our protocol was developed after a previous procedure for culture of human lymphocytes from tonsils recovered from surgical patients (Johnston et al., 2009). Using sterile forceps, tonsils of one pig were placed in a Ø10-cm Petri dish, kept moistened with 2 ml of HBSS and then cut into 3 to 10-mm fragments with sterile scissors. Tissue fragments were placed onto a stainless steel sterile sieve inside another Petri dish with 2 ml of HBSS. Leukocytes were pushed through the mesh (0.5 mm $\times$0.5 mm) using the flat end of a 30-ml plastic syringe plunger. Fragments were rinsed three times with HBSS until the liquid was visually clear, and the resulting cell suspension was transferred to 50-ml centrifuge tubes. In this phase, two different concentrations of antibiotic mixture were evaluated: HBSS 1$\times$and HBSS 2$\times$. Mononuclear cells were separated by centrifugation on Histopaque 1.077 (Sigma–Aldrich, St. Louis, MO, USA) at 1100 $\times$g, 25 min, 20 $\circ$C and washed twice with RPMI 1640 medium before assessing cell concentration and vitality by a trypan blue assay adapted to an automated counter (Countess, InvitrogenTM, Oregon, USA). Viable cells were resuspended at 3 millions ml-1 and cultivated in RPMI 1640 medium + 2-mercaptoethanol (2-ME) (5 $\times10-4$ M) + 10% fetal calf serum (FCS). The cells in 12-well plates (Costar®, Corning incorporated, NY, USA) were incubated at 37 $\circ$C in 5% CO2; each culture was set up in duplicate wells, with and without the antibiotic supplement, respectively. Also, the supernatants were harvested 18 or 2 h later for cells grown with and without antibiotic supplement, respectively. Supernatants were stored at -80 $\circ$C. The remaining cells were

31

immediately divided into aliquots at 4×10^6 viable cells/ml in RPMI 1640 medium + 40% fetal calf serum (FCS) + 10% dimethyl sulfoxide (DMSO) and stored at −80 ◦C. Later on they were thawed and employed in flow cytometry and IgA ELISPOT assays.

2.3. Evaluation of residual bacterial contamination

After 18 h in culture, 1-ml aliquots of serial 10-fold dilutions of the above supernatants (range 10^{-2}–10^{-12}) were inoculated in triplicate into tubes containing Trypticase Soy Broth (TSB) medium. Inoculated and non-inoculated control tubes were incubated over 3 days at 37 ◦C, and 50% end-point titers were determined on the basis of turbidity using the formula by Reed and Muench (1938). The differences between two titers were checked for statistical significance by Pizzi's formula (Pizzi, 1950).

2.4. Cell vitality and functions

Concentrations and vitality of cells were checked at different times after freezing by the trypan blue assay using the aforementioned automated cell counter (InvitrogenTM). This assay was performed on fresh cells and at 1, 2, 5 and 8 months after freezing. The response of cells to natural porcine IFN-α was evaluated before freezing. The latter cytokine was obtained from virus-stimulated PBMC according to an established procedure (Razzuoli et al., 2011). The test was performed as follows: for each pig under study cultures were set up in two replicate wells at 3 million cells ml–1 (2 ml/well); cells were cultivated with or without 100 U/ml of natural porcine IFN-α for 18 h at 37 ◦C in 5% CO_2. Then, the supernatant was collected and IgA was measured by ELISA. The functional activity of tonsil B cells after thawing was assessed by an ELISPOT assay for IgA-producing cells adapted to swine B lymphocytes. Briefly, nitrocellulose plates (Millipore, Billerica, MA cat. 01821) were coated overnight at 4 ◦C with a polyclonal antibody (PAB) to swine IgA (Bethyl Laboratories, Inc., Montgomery, TX, USA) (5 μg/ml in PBS). Plates were washed four times with PBS + 0.25% Tween 20 and incubated for 2 h at 37 °C with blocking buffer (4% bovine serum albumin, BSA, in PBS).

During the blocking phase, tonsil cells were rapidly thawed at 38 ◦C, washed twice with RPMI 1640 medium, counted and resuspended at 1×10^6/ml in 1× culture medium (RPMI 1640 + 10% FCS). 10^5 cells/well were grown in the presence of lipopolysaccharide (LPS, Sigma–Aldrich, cat. L2630) and porcine recombinant interleukin-6 (IL-6, R&D System, Minneapolis, MN, cat. 686PI) (both at 1 ng/ml), for 24 h at 37 °C in 5% CO_2. Afterwards, plates were washed 4 times in PBS + 0.25% Tween 20 and reacted for 1 h at 26 °C with a goat anti-pig IgA HRP-conjugate (0.4 μg/ml) (Bethyl Laboratories) in PBS + 1% BSA + 0.05% Tween 20.

After 5 washings with PBS + 0.25% Tween 20, spots were visualized using 3-3-diaminobenzidine (Sigma–Aldrich, cat: MFCD00007725) for 20 min at room temperature in the dark according to the

manufacturer's recommendation. After two thorough washings with distilled water, plates were dried overnight and spots were identified and counted by a EL.VIS, ELI SCAN apparatus (Tema Ricerca, Milan). Ig isotype-specific kits (Bethyl Laboratories, cat: E100- 104 and E100-102) were used toinvestigate B cell functions on the basis of IgG and IgA release by fresh and frozen cells after 5 and 8 months of storage at −80 °C.

2.5. ELISA assays

Swine IgA and IgG were measured by commercial ELISA kits as suggested by the manufacturer (Bethyl Laboratories).Briefly, Maxi Sorp NUNC ELISA plates (NuncTM, Serving Life Science, Denmark) were adsorbed, respectively, with anti-swine IgA or IgG capture polyclonal antibody (PAB) at 4 μg/ml in 0.1 M carbonate/bicarbonate buffer pH 9.6 and incubated overnight at 4 ◦C or 1 h at 26 ◦C, respectively. After blocking with 1% BSA in 0.14 M NaCl, 150 mM Tris-buffered saline (assay buffer), plates were washed thrice with 0.2% Tween 20 in assay buffer (AB-Tween). Then, 100 μL/well of undiluted test samples or AB-Tween + 1% BSA (sample diluent, blank control) were added in duplicate; also, porcine IgA or IgG (Bethyl Laboratories) at 10 μg/ml and seven 3-fold dilutions thereof were added in duplicate, and plates were incubated at room temperature for 1 h. Then, plates were washed thrice with AB-Tween and 100 μL/well of horseradish peroxidase (HRP), anti-swine IgA or IgG antiserum at 0.1 μg/ml in sample diluent was added, and plates were further incubated for 1 h at room temperature. After 3 washings with AB-Tween, swine IgG and IgA were revealed by 50 μL/well of ortho-phenilenediamine (OPD, Sigma–Aldrich, cat. P9187) + 0.02% H_2O_2 as substrate of the color reaction. This was blocked after 15 min by adding 50 μL/well of 2N H_2SO_4. Plates were read spectrophotometrically at 492 nm. Antibody concentrations were determined from a standard curve created with the above dilutions of porcine IgG and IgA using software Prism 2.01 (Graph Pad Software, Inc. Avenida de la Playa, USA).

2.6. Flow cytometry

Staining of cells was carried out according to an established procedure (Amadori et al., 2009), with minor modifications. Briefly, frozen tonsil cells were rapidly thawed at 38 ◦C and washed twice with ice-cold FACS Buffer (2% FCS in PBS + 0.1% sodium azide). Then, they were counted, divided into aliquots (106 cells each) and reacted with mAb 8/1 (SwC1) (Saalmüller et al., 1994), 1G6 (antiswine Ig light chain), 2E8 (anti-swine IgM) (Archetti et al., 1993), a biotinylated anti-swine Ig antiserum reacting with all Ig isotypes (Vector Lab, Peterborough, UK, cat. BA9020), mAb anti-pig IgA (AdB Serotec, cat. MCA638), or FACS buffer only (control) for 30 min at 4 °C, respectively. Cells were washed, and again incubated for 30 min at 4 ◦C in FACS buffer containing either goat anti-mouse IgG-FITC (Invitrogen, Molecular Probes®, cat: A10683) for the aliquots reacted with mAb 8/1, 1G6,

33

IgA and 2E8, or Phycoerythrin-conjugated streptavidin (Southern Biotechnology Associates, cat. 7100) for the aliquot reacted with the biotinylated antiserum. After washing twice in FACS buffer, cells were resuspended in 300 μL of the same buffer. Samples were analyzed in a APOGEE A40 flow cytometer (Apogee Software, South Bascom AVE, Campbell, CA). The typical forward and side scatter, lymphocyte/monocyte gate was set to exclude dead cells from the analysis. The percentage of positive cells beyond the threshold fluorescence channel was assessed in each sample on 10,000 events and compared between mAb-treated and control cells. For each antibody, results were expressed in terms of net percentage of positive cells.

2.7. Antibacterial activity

Tonsil cell cultures were set up in antibiotic-free medium at 37 °C in 5% CO_2. Supernatants were collected after 2 h and antibacterial activity of supernatants was determined by a qualitative microbiological screening method based on reference bacterial strains (Okerman et al., 1998). Briefly, 1.9×10^5 Colony-Forming Units (CFU) of Bacillus subtilis (Merck, Whitehouse Station, NJ, USA. Cod 10649), 5×10^6 CFU of Bacillus cereus ATCC 11778 and B. cereus K 250 (resistant to tetracyclines), 50×10^6 CFU of Micrococcus luteus (Kocuria rhizophila) ATCC 9341 were seeded in Ø10-cm Petri dishes within melted agarized medium (Antibiotic medium 1, OXOID cat. CM 0327B).

2×10^5 CFU of Escherichia coli 14 strain were seeded into melted agarized Standard II medium (Bicrobiol, cat. 70.233). After medium solidification, 50 μL of each supernatant were put in duplicate into 10-mm holes made in the agar. After a 30-min period of diffusion, the Petri dishes were incubated at 30 °C for 12 h. Then, the diameters of growth check were measured in comparison with that of the supernatant of the 5th (last) washing of the cells before culture (possible antibacterial activity due to antibiotic residues).

2.8. Statistical analysis

Viability and concentration of thawed tonsil cells were assessed in triplicate at different times after freezing. Normality of data was checked by the Shapiro–Wilk test and expressed as mean ±1 standard deviation (SD). Differences between means were checked for significance by one-way analysis of variance (ANOVA). The results of the ELISPOT and IFN-α-driven IgA production assays were tested for significance using Student's t test. The threshold for significance was set at $P < 0.05$.

3. RESULTS AND DISCUSSION

Regarding the control of bacterial growth, the 5× transport medium gave significantly better results (Table 1); in particular, contaminations were not visually detected in the corresponding cell cultures. There was no significant difference in terms of residual contamination between the results provided by 1× and 2× washing medium during and after cell separation (data not shown). On the whole, the adopted procedure with 5×transport and 2× washing medium enabled us to successfully culture lymphocytes from tonsils, the amount of cells per couple of tonsils ranging between 170 and 400 $\times 10^6$, with a mean value of 238.7 ± 107.1 $\times 10^6$. Using the described isolation procedure, cell vitality ranged between 88 and 95% with a mean value of 89.8 ± 2.7%. This significantly decreased (P < 0.05)at 5 and 8 months after freezing; also, the number of cells recovered at thawing decreased significantly (P < 0.05) at 8 months with respect to the values observed at 1 and 2 months after freezing (Fig. 1). Lymphoid tonsil cells proved competent for the spontaneous release of IgA and IgG in culture, the activity decreasing significant (P < 0.05) after freezing. In fact, the concentration of IgG and IgA in fresh cell culture supernatants amounted to 258.1 ± 106.1 ng/ml and 229.5 ±98.8 ng/ml, respectively; these values decreased at 8 months to 42 ± 15 and 102 ±50 ng/ml, respectively.

These results suggest to use the cells within 2 months from freezing to have the best conditions in terms of recovery, vitality and release of antibody *in vitro*. With regard to the phenotype of the mononuclear cells under study, flow cytometry tests provided the following prevalence results: total swine surface (s) Ig: 73.7 ± 14.1%; SwC1: 11.1 ±7.1%; sIg light chain λ: 38.2 ± 8.04%; sIgM: 11.0 ± 8.1%; sIgA: 2.0 ± 0.6%. Beyond methodological issues, tonsil B lymphocytes showed in our study a prevalence of surface Ig isotypes by far different from that of peripheral blood mononuclear cells and mesenteric lymph nodes. As opposed to peripheral blood and mesenteric lymph nodes, there was a substantial balance between _ and allegedly _ light chain-positive cells, and sIgM was shown to be expressed in a minority of tonsil B cells (Archetti et al., 1993). Also, the prevalence of sIgA positive cells was very limited. Interestingly, the sum of SwC1+ (non-B) and sIg+ B cells closely approached 100% in some pigs, whereas discrepancies were revealed in other cases (data not shown). A different prevalence of activated T cells down-regulating the expression of SwC1 (Saalmüller et al., 1987) probably accounts for the observed differences. Finally, as a caveat, the above set of data refers to 9 or 10-month old animals; important differences in the prevalence of cell phenotypes may be obviously possible with data sets obtained from younger pigs. Because of the importance of tonsils as a primary site of interaction between bacteria and lymphoid cells, we tested the ability of tonsil cells to spontaneously release peptides with antibacterial properties. After 2 h of culture the tonsil cells of 6 out of 10 different pigs released this type of compounds (see Table 2). The assay for antibacterial

activity was intended as a rapid screening procedure to reveal peptides released by lymphocytes, to be submitted to further identification and characterization steps. Since granulocytes were absent in our tonsil cell cultures, the released antibacterial peptides were probably part of the defensin, and not of the cathelicidin family (Sang and Blecha, 2009). Concerning fresh cells, untreated and IFN-α treated cell cultures released 109 ± 40 and 177 ± 88 ng/ml of IgA, respectively; this difference resulted significant($P < 0.05$).The ELISPOT assay showed the cells' capacity to respond to the combined stimuli of LPS and IL-6 after thawing. The tests revealed on average 40 ± 28 and 41 ± 20 IgA producing cells/10^5 at 1 month and 2 months after freezing, respectively. 2 ± 1 IgA-producing cells/10^5were revealed in the control wells (Fig. 2). The reduced number of IgA secreting cells (about 40 out of 10^5) with respect to the number of sIgA+ B cells (about 2000 out of 10^5) may be related to the freezing conditions. Therefore, a two-month storage at -80 °C is a reasonable time limit for polyclonal Ig production. On the whole, our study has defined operating conditions for tonsil processing, control of bacterial contaminations, time limits of storage at -80 °C, as well as for evaluating polyclonal IgA production *in vitro*. Such procedures are also of some importance in the framework of large animal models, in which the pig is in a favorable position, gaining substantial momentum in immunological science (Rothkotter et al., 2002; Meurens et al., 2012).

Thus, our methodological approach was intended for a contribution to the above areas of investigation, on the basis of robust and user-friendly test procedures, not demanding specialized logistics and infrastructure.

REFERENCES

1. Alexandersen, S., Oleksiewicz, M.B., Donaldson, A.I., 2001. The early pathogenesis of foot-and-mouth disease in pigs infected by contact: a quantitative time-course study using TaqMan RT-PCR. J. Gen. Virol. 82, 747–755.

2. Amadori, M., Farinacci, M., Begni, B., Faita, R., Podavini, D., Colitti, M., 2009. Effects of interferon-alpha on the inflammatory response of swine peripheral blood mononuclear cells. J. Interferon Cytokine Res. 29, 241–247.

3. Archetti, I.L., Capucci, L., Saalmüller, A., Verardi, R., Konig, M., Amadori, M., 1993. Production and characterization of monoclonal antibodies differentiating subpopulations of porcine B lymphocytes in blood and lymphoid tissues. Zentralbl. Veterinarmed. B 40, 485–493.

4. Begni, B., Amadori, M., Ritelli, M., Podavini, D., 2005. Effects of IFN-alpha on the inflammatory response of swine leukocytes to bacterial endotoxin. J. Interferon Cytokine Res. 25, 202–208.

5. Bourges, D., Wang, C.H., Chevaleyre, C., Salmon, H., 2004. T and IgA B lymphocytes of the pharyngeal and palatine tonsils: differential expression of adhesion molecules and chemokines. Scand. J. Immunol. 60, 338–350.

6. Casteleyn, C., Breugelmans, S., Simoens, P., Van den Broeck, W., 2011. Thetonsils revisited: review of the anatomical localization and histological characteristics of the tonsils of domestic and laboratory animals. Clin. Dev. Immunol. 2011, http://dx.doi.org/10.1155/2011/472460.

7. Cummins, J.M., Krakowka, G.S., Thompson, C.G., 2005. Systemic effects of interferons after oral administration in animals and humans. Am. J. Vet. Res. 66, 164–176.

8. Horter, D.C., Yoon, K.J., Zimmerman, J.J., 2003. A review of porcine tonsils in immunity and disease. Anim. Health Res. Rev. 4, 143–155.

9. Johnston, A., Sigurdardottir, S.L., Ryon, J.J., 2009. Isolation of mononuclears cells from tonsillar tissue. In: Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M., Strober, W. (Eds.), Current Protocols in Immunology. John Wiley & Sons, Inc., USA, pp. 7.8.1–7.8.4.

10. Liebler-Tenorio, E.M., Pabst, R., 2006. MALT structure and function in farm animals. Vet. Res. 37, 257–280.

11. Lowe, B.A., Marsh, T.L., Isaacs-Cosgrove, N., Kirkwood, R.N., Kiupel, M., Mulks, M.H., 2011. Microbial communities in the tonsils of healthy pigs. Vet. Microbiol. 147, 346–357.

12. Meurens, F., Summerfield, A., Nauwynck, H., Saif, L., Gerdts, V., 2012. The pig: a model for human infectious diseases. Trends Microbiol. 20, 50–57.

13. Okerman, L., De Wasch, K., Van Hoof, J., 1998. Detection of antibiotics in muscle tissue with microbiological inhibition tests: effects of the matrix. Analyst 123, 2361–2365.

14. O'Sullivan, T., Friendship, R., Blackwell, T., Pearl, D., McEwen, B., Carman, S.,Slavic, D., Dewey, C., 2011. Microbiological identification and analysis of swine tonsils collected from carcasses at slaughter. Can. J. Vet. Res. 75, 106–111.

15. Pedretti, E., Passeri, B., Amadori, M., Isola, P., Di Pede, P., Telera, A., Vescovini, R., Quintavalla, F., Pistello, M., 2006. Low-dose interferon-alpha treatment for feline immunodeficiency virus infection. Vet. Immunol. Immunopathol. 109, 245–254.

16. Pizzi, M., 1950. Sampling variation of the fifty percent end-point, determined by the Reed-Muench (Behrens) method. Hum. Biol. 22, 151–190.

17. Razzuoli, E., Villa, R., Sossi, E., Amadori, M., 2011. Characterization of the interferon-alpha response of pigs to the weaning stress. J. Interferon Cytokine Res. 31, 237–247.

18. Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27, 493–497.

19. Romero, C.H., Meade, P.N., Homer, B.L., Shultz, J.E., Lollis, G., 2003. Potential sites of virus latency associated with indigenous pseudorabies viruses in feral swine. J. Wildl. Dis. 39, 567–575.

20. Rothkotter, H.J., Sowa, E., Pabst, R., 2002. The pig as a model of developmental immunology. Hum. Exp. Toxicol. 21, 533–536.

21. Rowland, R.R., Lawson, S., Rossow, K., Benfield, D.A., 2003. Lymphoid tissue tropism of porcine reproductive and respiratory syndrome virus replication during persistent infection of pigs originally exposed to virus in utero. Vet. Microbiol. 96, 219–235.

22. Saalmüller, A., Aasted, B., Canals, A., Dominguez, J., Goldman, T., Lunney, J.K., Pauly, T., Pescovitz, M.D., Pospisil, R., Salmon, H., 1994. Analysis of mAb reactive with the porcine SWC1. Vet. Immunol. Immunopathol. 43, 255–258.

23. Saalmüller, A., Jonjic, S., Buhring, H.J., Reddehase, M.J., Koszinowski, U.H., 1987. Monoclonal antibodies reactive with swine lymphocytes. II. Detection of an antigen on resting T cells down-regulated after activation. J. Immunol. 138, 1852–1857.

24. Sang, Y., Blecha, F., 2009. Porcine host defense peptides: expanding repertoire and functions. Dev. Comp. Immunol. 33, 334–343.
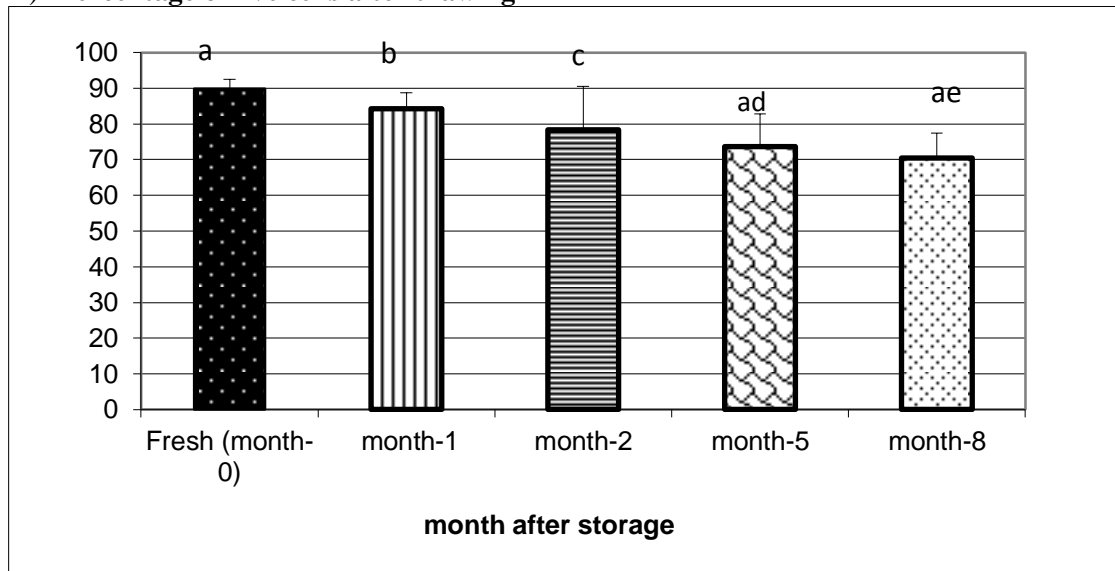
Figure 1

**Percent recovery of cells (live and dead) after thawing**

A) Pig
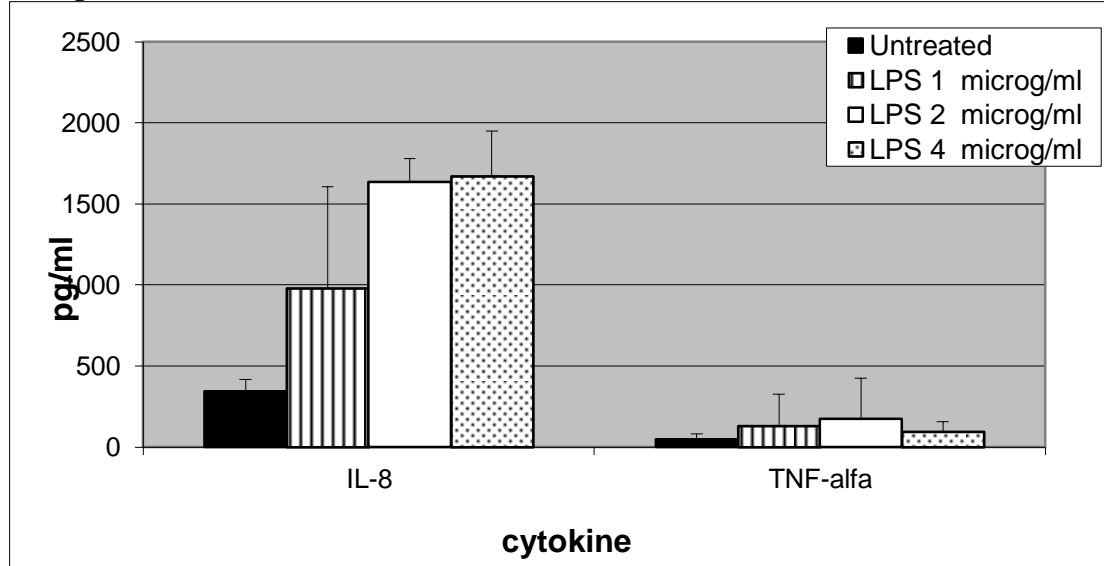
Results are shown in terms of % recovery with respect to time 0 (mean and standard deviation). The same superscripts on the bars indicate significant differences in one-way ANOVA for repeated measures.

B) **Percentage of live cells after thawing**

Results are reported as mean and standard deviation of 10 pig tonsils. The same superscripts on the bar indicate significant differences in one-way ANOVA for repeated measures.

Figure 2
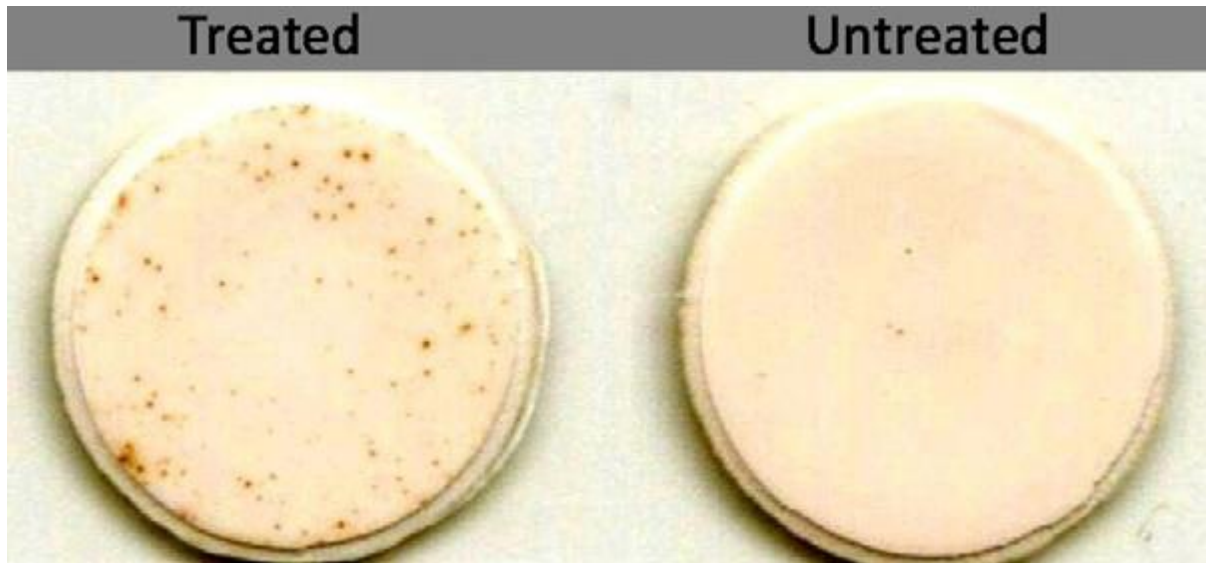
ELISPOT assay for IgA-secreting cells in pig tonsils. IgA-secreting cells were measured by an ELISPOT assay on pig tonsil lymphocytes stimulated by LPS and IL-6 (left, both at 1 ng/ml) and on control, unstimulated cells (right).

Table 1

Evaluation of residual bacterial contamination in tonsil cell cultures after transport in medium with different concentrations of an antibiotic mixture

Transport medium	Dilution of cell culture supernatant					
	10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-10}	10^{-12}
Antibiotic 1x supplement	+	+	+	-	-	-
Antibiotic 2x supplement	+	+	-	-	-	-
Antibiotic 5x supplement	-	-	-	-	-	-

Tonsil cell cultures were set up after transport of organs in the presence of different concentrations of antibiotics. The 1x mixture corresponds to 50 μg/ml penicillin, 50 μg/ml streptomycin, 10 μg/ml neomycin, and the 2x and 5x to proportionally greater concentrations. After 18 hours in culture, 1-ml aliquots of serial 10-fold dilutions of the cell supernatants (range $10^2 - 10^{12}$) were inoculated in triplicate in tubes containing Trypticase Soy Broth (TSB) medium. Inoculated and non-inoculated control tubes were incubated over 3 days at 37 °C, and 50% end-point titres were determined on the basis of turbidity using the formula by Reed and Muench. The titer differences were checked for statistical significance by Pizzi's formula. The titers obtained with the 5x mixture ($<10^{-2}$) were significantly different from the other ones.

Table 2

Evaluation of the antibacterial activity of tonsil cell cultures

Bacterial strains	Cultures showing antibacterial activity	Diameters of bacterial growth inhibition
B. cereus ATCC	0/10	ND
B. cereus K250	1/10	15
B. subtilis	6/10	14±0.9
M. luteus	5/10	15.8±2.5
E. Coli 14	0/10	ND

Antibacterial activity of cell-free supernatants of ten tonsil cell cultures in antibiotic-free medium at 2 hours was determined by a qualitative microbiological screening method based on reference bacterial strains included in an agarized medium (Okerman et al. 1998). The antibacterial activity was evaluated in terms of diameter of the inhibition haloes around the test wells, the threshold for significance being a 2-mm difference from the negative control; the latter was the medium of the 5th (last) washing of the tonsil cells, intended for removal of the antibiotic mixture. Diameters are shown in terms of mm, as mean and standard deviation.

ND: not detected

CHAPTER 2

IPEC-J2 CELLS AS REPORTER SYSTEM OF THE ANTI-INFLAMMATORY CONTROL ACTIONS OF INTERFERON-ALPHA.

Razzuoli E[a], Villa R[b], Amadori M[b].

a S.S Genova, Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Piazza Borgo Pila 39-24,16129-Genova.

b Laboratory of Cellular Immunology, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, via A. Bianchi 9, 25124 Brescia.

* Corresponding author. Tel. +39 030 2290248; fax +39 030 2290392

E-mail address: massimo.amadori@izsler.it (M. Amadori)

ABSTRACT

Interferon-alpha (IFN-α) shows potent immunomodulatory properties, which underlies its use for low-dose oral treatments of diverse viral infections and immunopathological conditions. The studies on oral administration have been hampered by the lack of recognized *in vitro* models, reproducing the *in vivo* control action of IFN-α over inflammatory cytokine responses. Owing to these reasons, the aim of our study was to validate IPEC-J2 (a continuous cell line of porcine intestinal epithelial cells) as a reporter system of the properties of IFN-α. Three different experimental conditions (oxidative stress, inflammatory response, and amplification of lymphoid cell signals) were selected to evaluate the effects of porcine recombinant IFN-α1 (rIFN-α) and 2 natural porcine IFN-α preparations (nIFN-α). The IFNs under study showed significantly different control actions in IPEC-J2 cells. In particular, rIFN-α was shown to down-regulate interleukin (IL)-8, IL-1β, tumor necrosis factor (TNF)-α, and β-defensin 1 genes either directly, or indirectly through second messengers released by IFN-α-treated lymphoid cells. With regard to IL-6, only second messengers from IFN-α-treated lymphoid cells could regulate the expression of this cytokine. Our results suggest that IPEC-J2 cells can be a useful tool for investigating the regulatory actions of type I IFNs and the second messengers thereof. The results provided by this model could be conveniently exploited in studies on enteric diseases sustained by infectious or noninfectious stressors.

INTRODUCTION

Interferons (IFNs) are proteins named after their capacity to interfere with viral infections of animal cells and are also endowed with immunomodulatory, anti-proliferative, and anti-inflammatory activities (Amadori 2008; Wang and Fish 2012). Three distinct classes of IFN molecules are known to date: Type I, Type II, and Type III IFNs. Type I IFNs are a heterogeneous group including several distinct families: IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ο, IFN-δ, and IFN-τ. These may be associated with distinct profiles of antiviral and antitumor activities, as well as of regulation of the T-helper 1/Thelper 2 ratio (Garcia-Sastre 2011; Gajewski 2012). The IFN system plays a pivotal role in the innate immune system as well as in the regulation of the adaptive immune response (Gonzalez-Navajas and others 2012). In addition, recent evidence accumulated in humans, mice, and farm animals points at type I IFN as a crucial homeostatic system that is aimed at avoiding unnecessary tissue damage and waste of food energy due to a dysregulated inflammatory response (Amadori 2007; Trevisi and others 2011). Among farm animal species, pigs show interesting properties of their Type I IFN system in their response to environmental stressors. In particular, the constitutive expression of several IFN-α subtypes was shown to be modulated in a model of early weaning stress on which IFN-α can exert a regulatory role (Razzuoli and others 2010). Such a regulation is badly needed, as the stress associated to weaning leads to mast cell activation and low feed intake, both of which play a pivotal role in the loss of barrier function of gut (Wijtten and others 2011). In this scenario, a low-dose IFN-α treatment at weaning was shown to be effective, the results being probably due to an anti-inflammatory control action of this cytokine (Amadori and others 2009). Understanding the direct and indirect control actions of oral IFN-α in pigs is difficult, because there are no recognized *in vitro* models that evaluate the biological effects mentioned earlier after oral administration (Peters and others 2011). For this reason, the objective of this study was to demonstrate the suitability of IPEC-J2 (a continuous line of porcine intestinal epithelial cells) as an *in vitro* reporter system of the anti-inflammatory control action of IFN-α at different concentrations.

2 MATERIALS AND METHODS

2.1 Cells and IFNs

IPEC-J2 cells (porcine intestinal epithelial cells, IZSLER Cell Bank code BS CL 205) were grown in Minimum Essential Medium (MEM) enriched with Fetal Calf Serum (FCS) (10% v/v), 2mM glutamine, and antibiotics (50 mg/mL penicillin, 50 mg/mL streptomycin, and 10 mg/mL neomycin). These cells show a spontaneous secretion of interleukin (IL)-8 and were previously employed in studies on the inflammatory response (Sargeant and others 2011). They have a typical epithelial morphology and are permissive for commensal and pathogenic bacteria; their profile of cytokine and chemokine expression makes them suited for studies on innate immunity. Cells were seeded into 12-well tissue culture plates (2 mL per well, $2X10^5$ cells/mL) and incubated at 37°C in 5% CO_2 until confluence (about 24 h). Porcine recombinant IFN-α_1 (rIFN-α was purchased from PBL Biomedical Laboratories (cat. 17100-1). Its concentration is expressed in terms of U/mL with regard to the international reference standard for human leukocyte IFN (Ga-902- 530) provided by National Institutes of Health (Bethesda, MD). Natural porcine IFN-α (nIFN-α) was obtained from Paramyxovirus-stimulated peripheral blood mononuclear cells (PBMC) of 2 different pigs as described in our previous paper (Razzuoli and others 2011a). The concentration of the 3 IFNs under study was measured by ELISA (Razzuoli and others 2011a) and a bioassay on MDBK cells, calibrated with rIFN-α (Meager 1987).

2.2 Flow cytometry

Staining of cells for IL-1β was carried out according to established procedures (Schuerwegh and others 2001; Walravens and others 2002), with minor modifications. Samples were analyzed by the A40 Apogee Flow System (Enterprise House, Hertfordshire, United Kingdom). The percentage of positive cells beyond the threshold FL2 fluorescence channel was assessed in each sample on 20,000 events and compared between mAb-treated and control cells using Fischer exact test (threshold for significance set at $P < 0.05$).

2.3 Gene expression

The expression of porcine IFN-β, IL-8, IL-6, bD1, bD2, IL-1β, and tumor necrosis factor (TNF)-α was investigated using primer sets described in previous studies (Amadori and others 2009; Veldhuizen and others 2009; Collado-Romero and others 2010; Razzuoli and others 2011b). Porcine β2-microglobulin (B2M) was used as a housekeeping control gene (Table 1). EVA Green Real-time PCR amplification was performed in a CFX96-Real-time System (Bio-Rad, Milan, Italy) after the reverse transcription step as previously described (Razzuoli and others 2011b). In each sample of IPEC-J2

cells, the relative expression of the selected genes was calculated using the formula $\Delta Ct = Ct$ (target gene)–Ct (housekeeping), where Ct (cycle of threshold) values are the mean of 3 test replicates – 1 standard deviation. Negative samples were given a Ct 40 fictitious value for further statistical examination. The average intensity of expression (mean DCt sample-DCt negative control) of the genes under study was compared by one-way analysis of variance (ANOVA). The threshold for significance was set at $P < 0.05$. Assays for cytokine concentrations Swine IL-8 and IL-1β were measured by commercial ELISA kits as suggested by the manufacturer (R&D system, DUOset cat. DY535 and cat. DY681). Plates were read spectrophotometrically at 492 nm. Cytokine concentrations were calculated from a standard curve that had been created using seven 3-fold dilutions of porcine recombinant IL-8 and IL-1β.

Data were analyzed by software Prism 2.01, (Graph Pad Software; Avenida de la Playa, La Jolla, CA); the LOQ (limits of quantification) corresponded to 10 and 5 pg/mL for swine IL-8 and IL-1β, respectively. TNF-α and IL-6 were measured by 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT)-based biological-assays, as previously described (Grenett and others1991; Asai and others 1993). Cytokine concentrations were calculated from a standard curve that had been created with reference preparations of porcine recombinant TNF-α and IL-6 (Pierce Endogen, Rockford, IL). Depending on the number of cell passages in culture, the LOQ in both tests varied between5 and 50 pg/mL.

2.4 Treatments of IPEC-J2 cells

Each of the following experiments was performed thrice (see Table 2):

Oxidative stress. IPEC-J2 cells were seeded and incubated in 12-well tissue culture plates until confluence as described earlier. Six wells were used as an unstimulated control. The others were pretreated with rIFN-α and 2 nIFN-α at 100, 25, 6, and 1U/mL. After 2 h at 37_C in 5% CO_2, cells were washed once with MEM medium and stimulated with 1 mM H_2O_2 and 2 ng/mL of porcine Tumor Necrosis Factor-α (TNFα, R&D system, cat. 690-PT) for 18 h. Three out of 6 control wells were treated with MEM only (K-), whereas the 3 wells left (K +) were stimulatedwithH_2O_2 and TNF-α as described earlier. After this phase, cells were used for a colorimetric apoptosis test (Titer Tacs, R&D system, cat. TA600) and supernatants for a measurement of IL-1β and IL-8 release.

Inflammatory response. IPEC-J2 cells grown in 96- or 12- well plates were stimulated with lipopolysaccharide (LPS) only (from Escherichia coli O111:B4, Sigma–Aldrich, cat. L4391) at 1, 2, and 4 μg/mL, or LPS (at the same concentrations) + rIFN-α at 1 and 100 U/mL. Untreated cells were

used as a negative control. After 18 h of incubation at 37°C in 5% CO_2, supernatants were harvested and stored at - 80°C for ELISA analyses (IL-1β, IL-8) and bioassays (TNF-α, IL-6). RNA from cells grown in 12-well plates was extracted to evaluate cytokine gene expression. Cells from 96-well plates (treated with LPS only at 0, 1, 2, and 4 μg/mL) were tested for caspase-1 activity (Colorimetric Assay Kit; BIOVISION, San Francisco, CA, cat. K111-100).

Amplification of lymphoid cell signals. Pig tonsils were collected at the slaughterhouse from 5 healthy, 9- to 10- month-old, Landrace x Large White pigs, and processed as previously described (Razzuoli and others 2012). Viable cells were resuspended at 3 million mL–1 and cultivated in RPMI 1640 medium + 2-mercaptoethanol (2-ME) + 10% FCS. Tonsil cells were treated with rIFN-α at 0, 1, and 100 U/ mL, and then incubated at 37°C in 5% CO_2. Supernatants were harvested 24 h later and stored under aseptic conditions in aliquots at–80°C. IPEC-J2 cells at confluence were washed once with MEM and treated with 1:4 diluted tonsil supernatants for 18 h at 37°C in 5% CO_2. IL-1β and IL-8 were measured in supernatants of IPEC-J2 cells; their total RNA was extracted to evaluate the expression of β2 and β1-defensins (bD1 and bD2), IL-1β, TNF-α, IL- 8, and IL-6 genes.

2.5 Statistical analysis

Differences in terms of protein release and gene expression after the treatments with different IFN-α preparations were evaluated by one-way ANOVA for repeated measures. The threshold for significance was set at $P < 0.05$.

3 RESULTS

3.1 Flow cytometry

IPEC-J2 cells were shown to produce IL-1β. Under our test conditions, there were 42.5% − 6.5% positive cells (difference between mAb-treated and control cell) for intracellular IL-1β (data not shown).

3.2 Effects of IFN-α in an oxidative stress model

The treatment of IPEC-J2 cells with H_2O_2 and TNF-α caused a significant ($P < 0.05$) increase (39 − 2.8 mOD in our colorimetric test) in cell apoptosis with regard to untreated wells. The cells spontaneously released 405 − 64 pg/mL of IL- 8; after treatment with H_2O_2 and TNF-α, IL-8 release increased to 825 − 124 pg/mL (+ 420 pg/mL with regard to the spontaneous release, $P < 0.05$). The

IFN-α treatments partly restored the control condition (Fig. 1). Differences in terms of regulatory action on IL-8 release were also shown among different types of IFNs; in particular, 25 U/mL of rIFN-α completely reversed the agonist effect of the applied oxidative stress on the secretion of IL-8 (Fig. 1). Stimulation with TNF-α and H_2O_2 did not cause any increase of IL-1β release; this occurred after treatment with one of the 2 nIFN-α under study at 100 U/mL, whereas 1U/Ml of rIFN-α and of the second nIFN-α significantly decreased IL-1β release (both - 31 pg/mL, Fig. 1).

3.3 Effect of IFN-α on the LPS-driven inflammatory response

LPS treatments (2 and 4 μg/mL) gave rise to significant increases (P < 0.01) of IL-8 release by IPEC-J2 cells (+ 1,290 and 1,330 pg/mL respectively, see Fig. 2). TNF-α release was also increased but not significantly (Fig. 2), nor were there effects in terms of IL-6 and IL-1β secretion (data not shown).

There was instead an increase, albeit not significant, of caspase-1 activity, as shown by our colorimetric test. In particular 1, 2, and 4 μg/mL of LPS caused increases of 11, 15, and 23 mOD in our caspase-1 colorimetric test (no significant differences, see Fig. 2). As for gene expression analyses, the LPS treatments significantly increased IL-8 and TNF-α expression (P <0.01) at all concentrations used (Fig. 3). 1 μg/mL of LPS resulted in a significant (P < 0.05) decrease of IL-1β expression (Fig. 3), while the other concentrations of LPS under study showed no effects. IL-6 expression was not modulated by any concentration of LPS (Fig. 3), whereas IFN-β gene expression(data not shown) slightly increased after treatment with 2 and 4 μg/mL of LPS (no significant). With the exception of IL-6, treatment with 100 U/mL of rIFN-α significantly decreased (Fig. 4) pro-inflammatory cytokine gene expression at all tested LPS concentrations (IL-1β P < 0.001, IL-8 P < 0.001, and TNF-α P < 0.001). TNF-α and IL- 8 expression was significantly up-regulated (P <0.01) after IFN-α treatment at a much lower concentration (1 U/mL). In addition, IFN-β was significantly (P <0.05) modulated (- 1.24 ΔΔCt) in cells treated with 4 μg/mL of LPS and 1U/mL of IFN-α but not at other LPS/IFN combinations (data not shown).

3.4 IPEC-J2 as reporter system of signals generated by lymphoid cells

In this experiment, IPEC-J2 cells did not release IL-1β. Treatment of IPEC-J2 with tonsil cell supernatants obtained after 24 h in culture resulted in a significant increase of IL-8 release (P < 0.05). The same effect was not observed using supernatants of tonsil lymphocytes treated with rIFN-α (Table 3). No significant effect was shown in terms of TNF-α and IFN-β gene expression after treatment with control tonsil cell supernatant (Fig. 5). With regard to IL-8 and IL-6, stimulation with control tonsil cell supernatants significantly increased cytokine gene expression (P < 0.01 and P <0.05 respectively) with regard to untreated IPEC-J2 cells. The treatment with supernatant of tonsil cells stimulated with

100U/mL of rIFN-α restored the initial expression levels of IL-8 and IL-6 in IPEC-J2 cells, whereas no effect was observed with supernatants of tonsil cells treated with 1U/mL of rIFN-α. With regard to IL-1β, treatment with control tonsil supernatant non significantly decreased the expression of this cytokine; the value of untreated IPEC-J2 cells was observed after stimulation with tonsil supernatant treated with 1U/ mL of rIFN-α (P < 0.05, Fig. 5); no significant effect was instead observed with supernatant of tonsil cells treated with 100 U/mL of rIFN-α.

3.5 Antimicrobial peptide expression: direct and indirect modulation

With regard to β1 and 2 defensin gene expressions, there were no significant effects of LPS on IPEC-J2 cells (data not shown). After IFN-α stimulation, direct or indirect effects onb2-defensin gene expression were not shown (Table 4). On the contrary, the b1-defensin gene was significantly down regulated with regard to control IPEC-J2 cells by direct treatment with 100 or 1U/mL of rIFN-α, and also by supernatants of tonsil cells treated with 100U/mL of rIFN-α (Table 4).

4 DISCUSSION

Type I IFNs represent a crucial link between innate and adaptive immune responses, which has stimulated many investigations into their role in established disease models (Gonzalez-Navajas and others 2012). These cytokines cause concentration-dependent effects: high doses of IFN-α (≥ 100 U/mL) usually show anti-proliferative, antiviral, and pro-inflammatory activities; whereas low doses (< 10 U/mL) show preferential immuno-modulatory and anti-inflammatory activities (Amadori 2007). In this scenario, Type I IFNs act as important homeostatic agents in the control of environmental, non infectious stressors such as early weaning in pigs, which causes transient anorexia and up-regulation of inflammatory cytokine genes in both proximal and distant tracts of the small intestine (McCracken and others 1999; Pie and others 2004). In this model, the control circuits of the inflammatory response can be adequately targeted by Type I IFNs to prevent its excessive amplification at weaning and the related disease losses (Amadori and others 2012). These findings prompted us to develop an *in vitro* model depicting the critical interaction between type I IFNs and intestinal epithelial cells and the relevant regulation of the inflammatory response. The IPEC-J2 line was developed from the jejunal epithelial cells of a newborn, no fed piglet, and it represents a good model to investigate the pathogenesis of microbial intestinal infections (Schierack and others 2006; Liu and others 2010). In a comprehensive study, the complete morphological and functional characterization of these cells was presented, and their suitability for microbiological studies was demonstrated (Brosnahan and Brown 2012); in addition, the expression of mRNAs for pro-inflammatory cytokines and chemokines such as

51

IL-6, IL-8, and TNF-α was confirmed (Mariani and others 2009). This profile was further characterized in our study, which demonstrated the ability of this cell line to produce IL-1β and to express both IL-1β and IFN-β genes. These features, along with the production of other pro-inflammatory cytokines, characterize IPEC-J2 cells as a good model for studies on gut inflammation and enteric disease (Zhou and others 2012). In our work, this cell line was used to investigate IFN-α in a model based on LPS and oxidative stress-driven changes of both expression and secretion of inflammatory cytokines and chemokines (IL-8); the choice of an oxidative stress approach is relevant to the present lean-type pig phenotypes, characterized by high plasma concentrations of Reactive Oxygen Metabolites under resting conditions, as a result of a major imbalance between cardio-circulatory system and development of the muscular mass (Brambilla and others 2002). In this regard, previous studies had shown that IPEC-J2 cells secrete cytokines and chemokines as a response to oxidative stress (Paszti-Gere and others 2012). These results were confirmed in our study, whereby an oxidative stress caused a significant increase of IL-8 release. Interestingly, pretreatment of IPEC-J2 cells with different types of IFN-α could significantly decrease such a response. With regard to natural, non-purified IFNs, the different results obtained in terms of inflammatory response could be related to the presence of contaminating cytokines or to different ratios among IFN-α subtypes. This assumption is in line with our previous study, where we demonstrated *in vitro* the expression of different porcine IFN-α subtypes after PBMC stimulation with Newcastle Disease Virus (NDV) LaSota strain (Razzuoli and others 2011a). These subtypes can show wide differences in terms of antiviral activities (Sang and others 2010), but at the moment, there is no precise information about their different anti-inflammatory potentials. For this reason, we decided to use only rIFN-α for the subsequent experiments. Treatment with different concentrations of LPS caused an inflammatory response in terms of IL-8 gene expression and protein secretion. Interestingly, no significant effect was detected in terms of TNF-α, IL-1β, and IL-6 protein release. In addition, caspase-1 activity was not significantly increased by LPS stimulation. It should be stressed that inflammatory cytokines are regulated by both gene expression and mRNA stability in the cytoplasm; an increase of IL-1βb and TNF-α also causes a compensatory release of IL-6 (Myers and Murtaugh 1995). Accordingly, in our study, LPS stimulation caused a significant increase of TNF-α gene expression but not of cytokine release, nor was LPS stimulation associated to an increased expression of IL-6 and IL-1b genes. These results differ from the data obtained in human macrophages and monocytes, where LPS causes an increase of all the cytokines mentioned earlier through TLR4/NF-KB signaling; remarkably, these latter components are expressed in IPECJ2 cells as well (Myers and Murtaugh 1995; Mariani and others 2009). The absence of IL-1β and IL-6 responses to LPS in IPEC-J2 cells could represent a form of endotoxin tolerance that is, a physiological condition of intestinal epithelial cells that are refractory to inflammatory signals of commensal bacteria under healthy gut conditions (Lotz and others 2006).

On the basis of the findings cited earlier, we investigated the activity of IFN-α in the same experimental model. Our results highlight the ability of a moderate concentration of IFN-α (100 U/mL) to regulate the LPS-driven inflammatory response in terms of pro-inflammatory cytokine gene expression.1U/mL of rIFN-α showed an opposite regulation in terms of IL-8 and TNF-α gene expression, whereas no control action was exerted on IL-1b. In a global view, the lack of a control action on IL-1β at low IFN-α concentrations would not be of concern because of further biological checkpoints; among these, the availability of Caspase-1 through the inflammasome reaction is likely to play a major role (Rathinam and others 2012).The observed control action of IFN-α on inflammatory cytokines could be performed through different, no mutually exclusive dose-dependent pathways: mRNA stability control by tristetraprolin induction (Anderson and others 2004), TAM receptor-mediated activation of SOCS proteins through IFNAR I signaling (Lemke and Rothlin2008), and down-regulation of CD14 expression (Begni and others 2005).With regard to the indirect effects of IFN-α, supernatants of untreated tonsil cells caused a moderate inflammatory response in IPEC-J2 cells, as opposed to supernatants of tonsil cells treated with rIFN-α. In particular, untreated tonsil cell supernatants up-regulated IL-8 and IL-6 gene expression. This might be related to IL-6 inducing cytokines such as TNF-α or IL-1β released by tonsil cells (Murtaugh 1994). IFN-α could inhibit their expression, or induce anti-inflammatory components such as IL-10 (Ouyang and others 2011). Interestingly, supernatants of tonsil cells treated with 1U/mL of IFN-α kept IL-1β expression at the levels observed in untreatedIPEC-J2 cells (Fig. 5).β-defensins were included in our study because of their involvement in the regulation of the inflammatory response(Yang and others 2002). In addition, a previous study(Mariani and others 2009) showed that IPEC-J2 cells constitutively express porcine bD1 and (to a lesser extent) bD2; this latter defensin is also expressed in the small intestine (Sang and others 2006), which justifies the use of IPEC-J2 cells as a useful investigation model of β-defensin expression. Treatment of IPEC-J2 cells with LPS caused no effects in terms of β-defensin expression; this finding is in agreement with the results by Zhang and co-workers (Zhang and others 1999),who demonstrated that porcine bD1 activity was not inducible by an inflammatory stimulation (LPS, TNF-a, IL-1);this is probably due to the lack of consensus binding sites in the defensin promoter region for both NF-kB and NF-IL6(Yang and others 2002). We also demonstrated the ability of IFN-α to down-regulate bD1 expression (Table 3); our findings are compatible with both a direct regulation on the bD1gene and an indirect action of IFN-α through second messengers secreted by tonsil cells, IPEC-J2 cells, or both (experiments B and C, see Materials and Methods).In conclusion, our results indicate that IPEC-J2 cells can be employed as a model for *in vitro* studies on the regulation of the intestinal inflammatory response, the onset and the course of enteric disease. Moreover, our results show different control actions of nIFN-α and rIFN-α, as well as different effects of nIFN-α depending on the PBMC cultures employed for IFN induction. In addition, our results show that a substantial regulation

of the inflammatory response is exerted at moderate concentrations of IFN-α (100 U/mL),which may be found at the initial stages of a microbial infection. In a global view, this might imply that pro and anti-inflammatory control actions of Type I IFNs take place at the same time on different cellular targets. Among these, intestinal epithelial cells need a very stringent control of their response to enteric infections and/or dysbiosis of intestinal microbiota; this can be accounted for by the state of controlled gut inflammation in the presence of commensal bacteria.

ACKNOWLEDGMENTS

AUTHOR DISCLOSURE STATEMENT

The authors have no conflicts of interest to disclose.

REFERENCES

1. Amadori M. 2007. The role of IFN-alpha as homeostatic agent in the inflammatory response: a balance between danger and response? J Interferon Cytokine Res 27(3):181–189.

2. Amadori M. 2008. Physiological response and constitutive expression of interferons. Role and functions. In: Durand M, Morel C, eds. New research on innate immunity. New York: Nova Science Publisher. pp 1–11.

3. Amadori M, Farinacci M, Begni B, Faita R, Podavini D, Colitti M. 2009. Effects of interferon-alpha on the inflammatory response of swine peripheral blood mononuclear cells. J Interferon Cytokine Res 29(4):241–247.

4. Amadori M, Razzuoli E and Nassuato C. 2012. Issues and possible intervention strategies relating to early weaning of piglets. CAB Rev 7 (046):1–15.

5. Anderson P, Phillips K, Stoecklin G, Kedersha N. 2004. Posttranscriptional regulation of pro-inflammatory proteins. J Leukoc Biol 76(1):42–47.

6. Asai T, Okada M, Ono M, Irisawa T, Mori Y, Yokomizo Y, Sato S. 1993. Increased levels of tumor necrosis factor and interleukin 1 in bronchoalveolar lavage fluids from pigs infected with mycoplasma hyopneumoniae. Vet Immunol Immunopathol 38(3–4):253–260.

7. Begni B, Amadori M, Ritelli M, Podavini D. 2005. Effects of IFNalpha on the inflammatory response of swine leukocytes to bacterial endotoxin. J Interferon Cytokine Res 25(4):202–208.

8. Brambilla G, Civitareale C, Ballerini A, Fiori M, Amadori M, Archetti LI, Regini M, Betti M. 2002. Response to oxidative stress as a welfare parameter in swine. Redox Rep 7(3): 159–163.

9. Brosnahan AJ, Brown DR. 2012. Porcine IPEC-J2 intestinal epithelial cells in microbiological investigations. Vet Microbiol 156(3–4):229–237.

10. Collado-Romero M, Arce C, Ramirez-Boo M, Carvajal A, Garrido JJ. 2010. Quantitative analysis of the immune response up on salmonella typhimurium infection along the porcine intestinal gut. Vet Res 41(2):23.

11. Gajewski TF. 2012. Cancer immunotherapy. Mol Oncol 6(2): 242–250.

12. Garcia-Sastre A. 2011. Induction and evasion of type I interferon responses by influenza viruses. Virus Res 162(1–2):12–18.

13. Gonzalez-Navajas JM, Lee J, David M, Raz E. 2012. Immunomodulatory functions of type I interferons. Nat Rev Immunol 12(2):125–135.

14. Grenett HE, Danley DE, Strick CA, James LC, Otterness IG, Fuentes N, Nesbitt JE, Fuller GM. 1991. Isolation and characterization of biologically active murine interleukin-6 produced in escherichia coli. Gene 101(2):267–271.

15. Lemke G, Rothlin CV. 2008. Immunobiology of the TAM receptors. Nat Rev Immunol 8(5):327–336.

16. Liu F, Li G, Wen K, Bui T, Cao D, Zhang Y, Yuan L. 2010. Porcine small intestinal epithelial cell line (IPEC-J2) of rotavirus infection as a new model for the study of innate immune responses to rotaviruses and probiotics. Viral Immunol 23(2):135–149.

17. Lotz M, Gutle D, Walther S, Menard S, Bogdan C, Hornef MW. 2006. Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. J Exp Med 203(4):973–984.

18. Mariani V, Palermo S, Fiorentini S, Lanubile A, Giuffra E. 2009. Gene expression study of two widely used pig intestinal epithelial cell lines: IPEC-J2 and IPI-2I. Vet Immunol Immunopathol. 131(3–4):278–284.

19. McCracken BA, Spurlock ME, Roos MA, Zuckermann FA, Gaskins HR. 1999. Weaning anorexia may contribute to local inflammation in the piglet small intestine. J Nutr 129(3):613–619.

20. Meager A. 1987. Quantification of interferons by anti-viral assays and their standardization. In: Clemens MJ, Morris AG, Gearing AJH, eds. Lymphokines and interferons, a practical approach. Oxford: IRL Press. pp 129–147.

21. Murtaugh MP. 1994. Porcine cytokines. Vet Immunol Immunopathol 43(1–3):37–44.

22. Myers MJ, Murtaugh MP. 1995. Biology of tumor necrosis factor. In: Myers MJ, Murtaugh MP, eds. Cytokines in animal health and disease. New York: Marcel Dekker, Inc. pp 121–151.

23. Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. 2011. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. Annu Rev Immunol 29:71–109.

24. Paszti-Gere E, Csibrik-Nemeth E, Szeker K, Csizinszky R, Jakab C, Galfi P. 2012. Acute oxidative stress affects IL-8 and TNF-alpha expression in IPEC-J2 porcine epithelial cells. Inflammation 35(3):994–1004.

25. Peters SM, Yancy H, Bremer E, Monroe J, Paul D, Stubbs JT,3rd, Myers MJ. 2011. In vitro identification and verification of inflammatory biomarkers in swine. Vet Immunol Immunopathol139(1):67–72.

26. Pie S, Lalles JP, Blazy F, Laffitte J, Seve B, Oswald IP. 2004. Weaning is associated with an up-regulation of expression of inflammatory cytokines in the intestine of piglets. J Nutr 134(3):641–647.

27. Rathinam VA, Vanaja SK, Fitzgerald KA. 2012. Regulation of inflammasome signaling. Nat Immunol 13(4):333–332.

28. Razzuoli E, Villa R, Sossi E, Amadori M. 2011a. Characterization of the interferon-alpha response of pigs to the weaning stress. J Interferon Cytokine Res 31(2):237–247.

29. Razzuoli E, Villa R, Sossi E, Amadori M. 2011b. Reverse transcription real-time PCR for detection of porcine interferon alpha and beta genes. Scand J Immunol 74(4):412–418.

30. Razzuoli E, Dotti S, Archetti IL, Amadori M. 2010. Clinical chemistry parameters of piglets at weaning are modulated by an oral, low-dose interferon-alpha treatment. Vet Res Commun 34 Suppl 1:S189–S192.

31. Razzuoli E, Faggionato E, Dotti S, Villa R, Lombardo T, Boizza L, Ferrari M, Amadori M. 2012. Isolation and culture of pig tonsil lymphocytes. Vet Immunol Immunopathol 148(3–4): 320–325.

32. Sang Y, Rowland RR, Hesse RA, Blecha F. 2010. Differential expression and activity of the porcine type I interferon family. Physiol Genomics 42(2):248–258.

33. Sang Y, Patil AA, Zhang G, Ross CR, Blecha F. 2006. Bioinformatic and expression analysis of novel porcine beta-defensins. Mamm Genome 17(4):332–339.

34. Sargeant HR, Miller HM, Shaw MA. 2011. Inflammatory response of porcine epithelial IPEC J2 cells to enterotoxigenic E. coli infection is modulated by zinc supplementation. Mol Immunol 48(15–16):2113–2121.

35. Schierack P, Nordhoff M, Pollmann M, Weyrauch KD, Amasheh S, Lodemann U, Jores J, Tachu B, Kleta S, Blikslager A, et al. 2006. Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. Histochem Cell Biol 125(3):293–305.

36. Schuerwegh AJ, Stevens WJ, Bridts CH, De Clerck LS. 2001.Evaluation of monensin and brefeldin A for flow cytometric determination of interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha in monocytes. Cytometry 46(3):172–176.

37. Trevisi E, Amadori M, Archetti I, Lacetera,N., Bertoni, G. 2011. Inflammatory response and acute phase proteins in the transition period of high-yielding dairy cows. In: Veas F, editor. Acute phase proteins as early non-specific biomarkers of human and veterinary disease. Rijeka: InTech Publisher,pp 355–379.

38. Veldhuizen EJ, Koomen I, Ultee T, van Dijk A, Haagsman HP.2009. Salmonella serovar specific up-regulation of porcine defensins 1 and 2 in a jejunal epithelial cell line. Vet Microbiol 136(1–2):69–75.

39. Walravens K, Wellemans V, Weynants V, Boelaert F, de Bergeyck V, Letesson JJ, Huygen K, Godfroid J. 2002. Analysis of the antigen-specific IFN-gamma producing T-cell subsets in cattle experimentally infected with mycobacterium bovis. Vet Immunol Immunopathol 84(1–2):29–41.

40. Wang BX and Fish EN. 2012. The yin and yang of viruses and interferons. Trends Immunol 33(4):190–197.

41. Wijtten PJ, van der Meulen J, Verstegen MW. 2011. Intestinal barrier function and absorption in pigs after weaning: A review. Br J Nutr 105(7):967–981.

42. Yang D, Biragyn A, Kwak LW, Oppenheim JJ. 2002. Mammalian defensins in immunity: More than just microbicidal. Trends Immunol 23(6):291–296.

43. Zhang G, Hiraiwa H, Yasue H, Wu H, Ross CR, Troyer D, Blecha F. 1999. Cloning and characterization of the gene for a new epithelial beta-defensin. genomic structure, chromosomal localization, and evidence for its constitutive expression. J Biol Chem 274(34):24031–24037.

44. Zhou C, Liu Z, Jiang J, Yu Y, Zhang Q. 2012. Differential gene expression profiling of porcine epithelial cells infected with three enterotoxigenic escherichia coli strains. BMC Genomics 13(1):330.
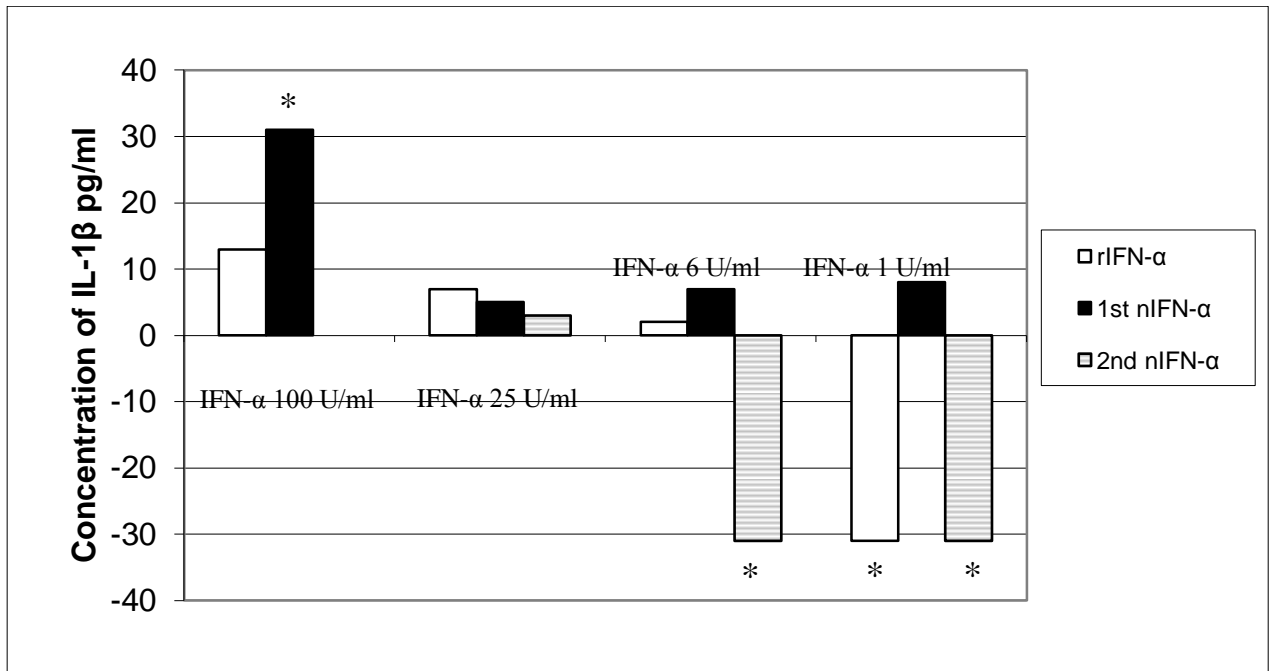
Figure 1

Effect of IFN-α treatments on IPEC-J2 cells stimulated with H2O2 and TNF-α

A) IL-8 release
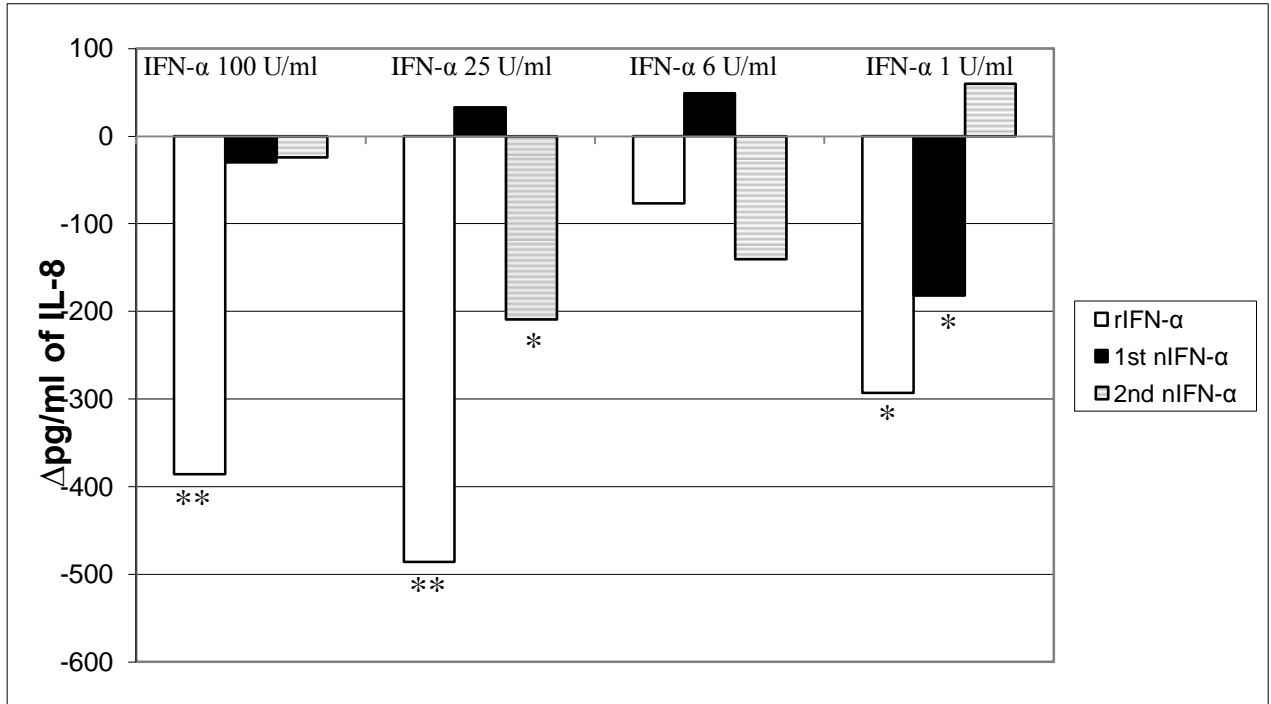
B) IL-1β release

IPEC-J2 cells were treated with recombinant (r), or natural (n) IFN-α (2 preparations) at different concentrations (100, 25, 6, 1 U/ml) or kept as untreated control. Then, they were submitted to an oxidative stress by adding TNF-α and H_2O_2 (2 ng/ml and 1μM, respectively); supernatants were harvested 18 hours later. Results are express as difference between release of IL-8 and IL-1β by IFN α-treated and control wells.

* indicates a significant differences between control and IFN-α treatment (one-way ANOVA for repeated measures, $P < 0.05$)
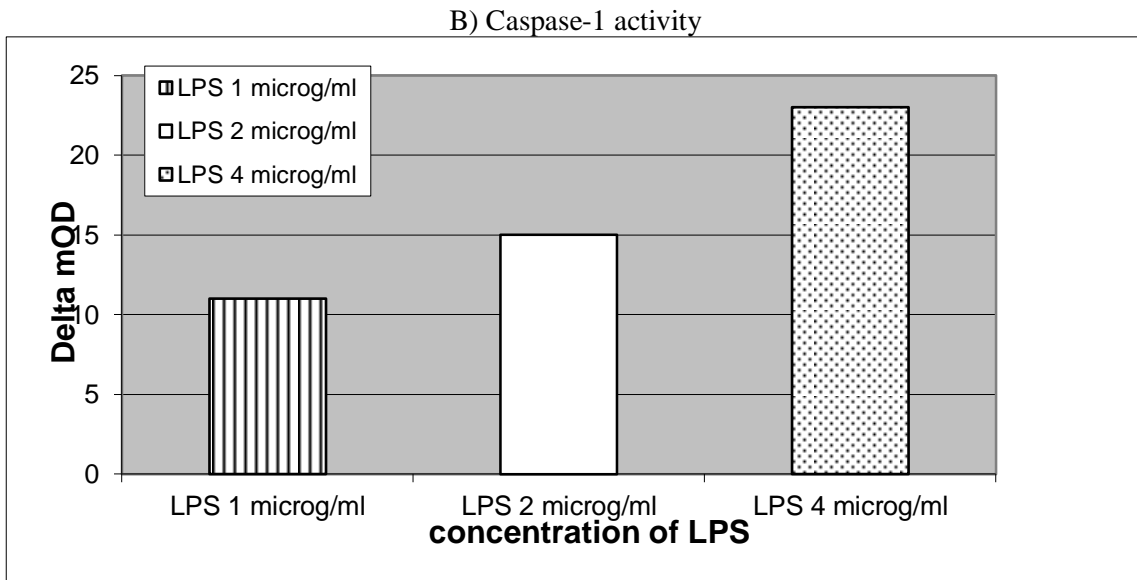
** $P < 0.01$

Figure 2

Inflammatory response to LPS of IPEC J2 cells

A) IL-8 and TNF-alpha
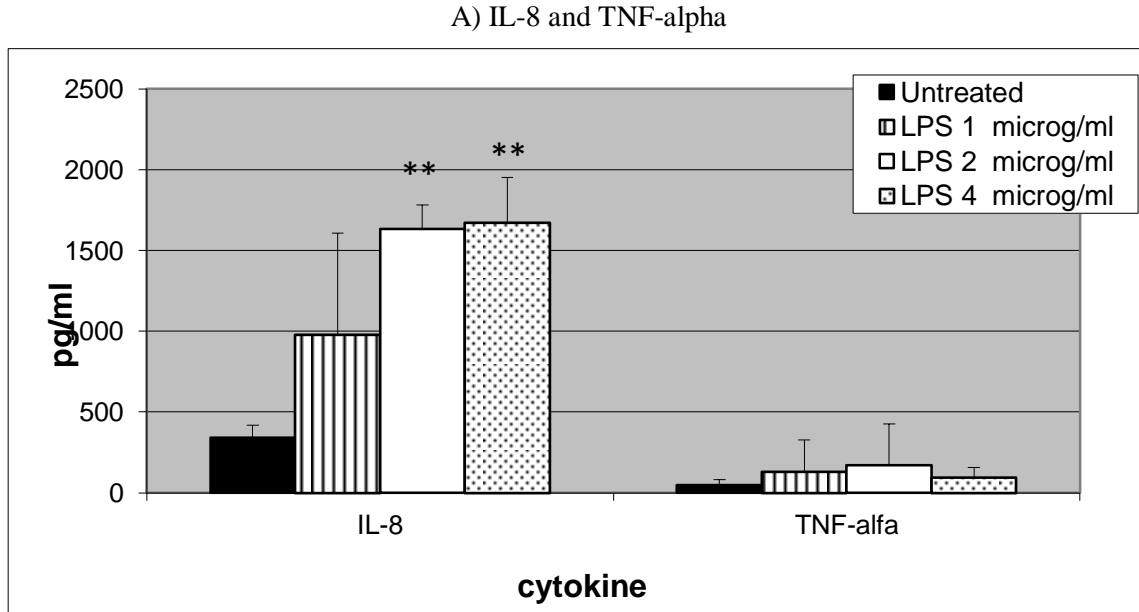
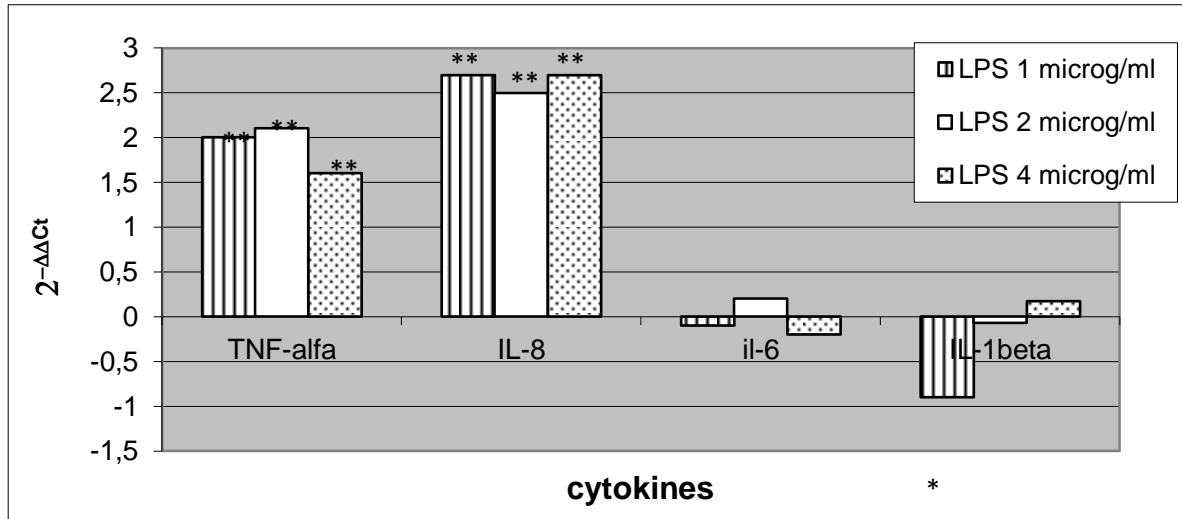One-way ANOVA for repeated measures. **P<0.01

B) Caspase-1 activity

Data are express as difference between LPS-stimulated and untreated wells (Delta mOD)

IPEC-J2 cells were stimulated with LPS only at 1, 2 and 4 µg/ml; untreated cells were used as negative control.

Figure 3

Effects of LPS on expression of cytokine genes in IPEC-J2 cells
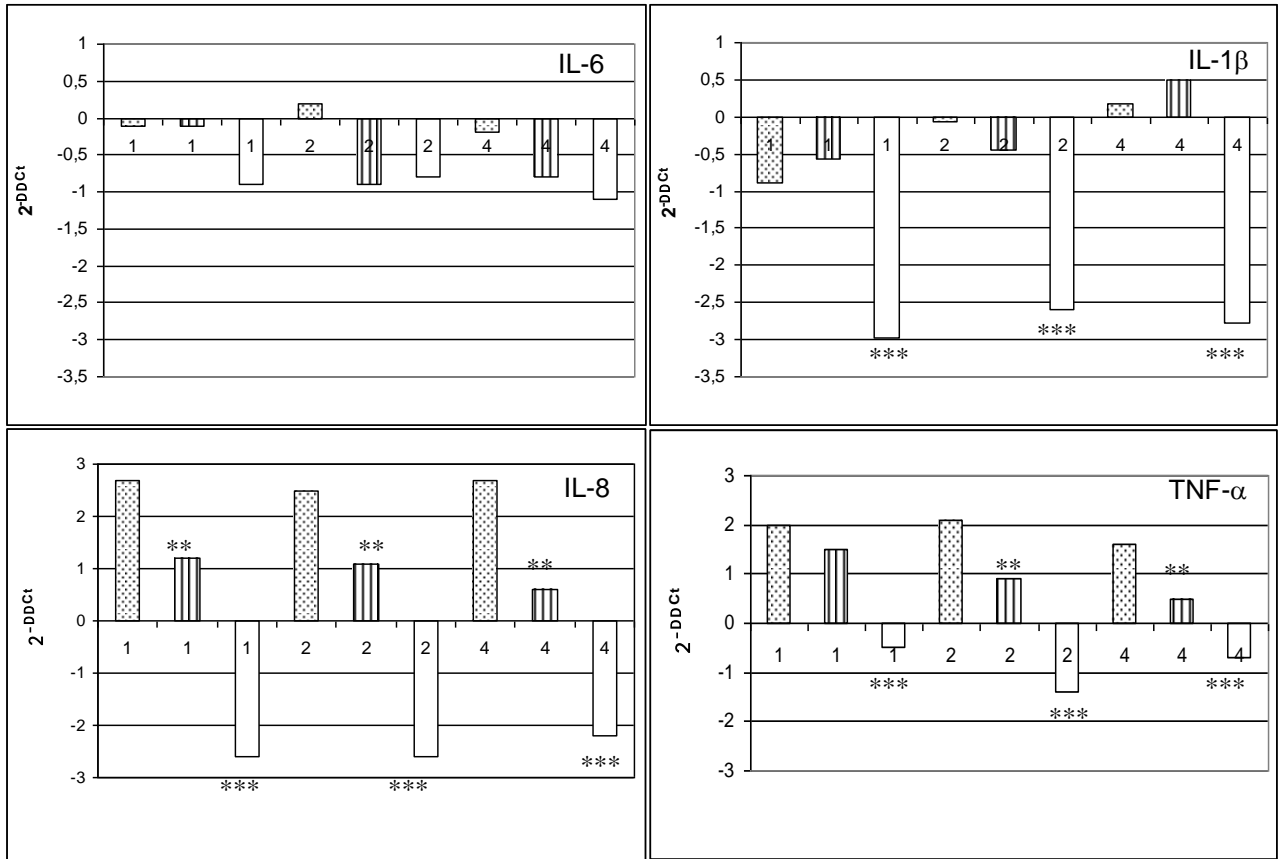

IPEC-J2 were stimulated with LPS only at 1, 2 and 4 μg/ml or LPS (at the same concentration) + rIFN-α at 1 and 100 U/ml. Untreated cells were used as negative control. After 18 hours of incubation, RNA from cells was extracted to evaluate cytokine gene expression Results are express as ΔΔCt (ΔCt cell control - ΔCt treatment).

* indicates a significant difference between control and LPS treatment (one-way ANOVA for repeated measures). The threshold for significance was set at $P < 0.05$.

*P<0.05

**P<0.01

Figure 4
IFN-α effects on cytokine gene expression in IPEC-J2 cells

Legend:

1: 1 μg/ml of LPS
2: 2 μg/ml of LPS
4: 4 μg/ml of LPS

▨ Untreated cells
⊞ Cells treated with rIFN-α, 1 U/ml
☐ Cells treated with rIFN-α, 100 U/ml

⊠ Untreated cells

☐ Cells treated with rIFN-α1 U/ml

☐ Cells treated with rIFN-α100 U/ml

IPEC-J2 cells were stimulated with LPS only at 1, 2 and 4 μg/ml or LPS (at the same concentration) + rIFN-α at 1 and 100 U/ml. Untreated cells were used as negative control. After 18 hours of incubation, RNA from cells was extracted to evaluate cytokine gene expression.

Numbers under the *x* axis indicate the concentration of LPS expressed as μg/ml; different filling signs of bars indicate different concentrations of IFN-α (see legend).
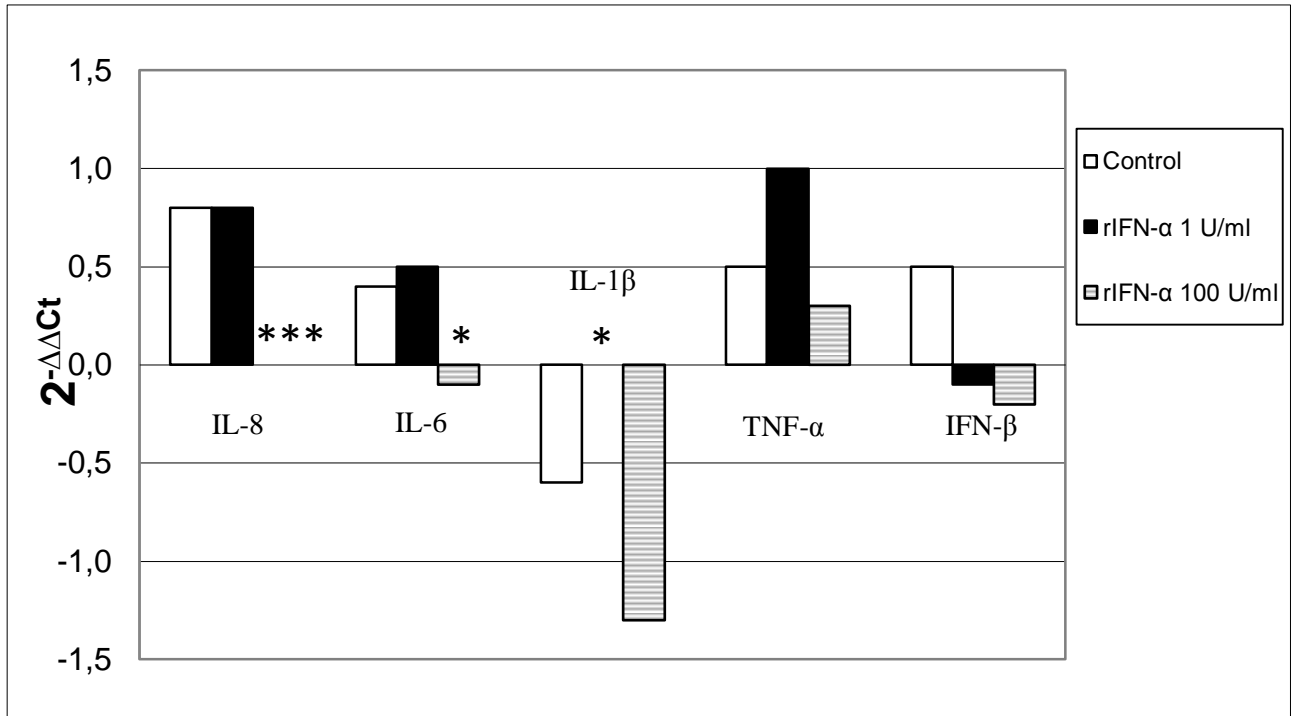
Data are express as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (\Delta Ct$ of untreated cells$)- (\Delta Ct$ of treated cells$)$. The stars indicate significant differences between control and IFN-α treatments (one way ANOVA).

** P<0.01
***P<0.001

Figure 5

Indirect effects of IFN-α on cytokine gene expression in IPEC-J2 cells

Pig tonsil cells were treated with rIFN-α at 0, 1 and 100 U/ml, and then incubated at 37 °C in 5% CO2. Supernatants were harvested 18 hours later and stored under aseptic conditions in aliquots at − 80°C. IPEC-J2 cells at confluence were washed once with MEM medium and treated with 1:4 diluted tonsil supernatants for 18 h at 37 °C in 5% CO2. RNA of IPEC-J2 was extracted to evaluate the expression of IL-1β, TNF-α, IL-8, IL-6 and IFN-β genes.

Data are express as $2-\Delta\Delta Ct$, where $\Delta\Delta Ct$ = (ΔCt of untreated cells)- (ΔCt of treated cells). The stars indicate significant differences between control and IFN α-treated tonsil supernatants (one-way ANOVA).

*P<0.05

***P<0.001

Table 1

Oligonucleotide primer sequences for EvaGreen qRT Real-time PCR amplification of porcine genes

GENE	PROTEIN	PRIMERS	GeneBank gi-number
IL-8	porcine IL-8	F : 5'-CTGTACAACCTTCTGCACCCA-3' R : 5'-TTCGATGCCAGTGCATAAATA-3'	M86923
IL-6	porcine IL-6	F: 5'-CAGAGATTTTGCCGAGGATG-3' R: 5'-TGGCTACTGCCTTCCCTACC-'3	NM_214399
IL-1β	porcine IL-1β	F: 5'-AATTCGAGTCTGCCCTGTACCC-3' R: 5'-GCCAAGATATAACCGACTTCACCA	NM_001005149
TNF-α	porcine TNF-α	F: 5'-TGCCTACTGCACTTCGAGGTTATC-3' R: 5'CAGATAAGCCCGTCGCCCAC-3'	NM_214022
βD1	porcine Defensine-1β	F:5'- TGCCACAGGTGCTCT-3' R:5' CTGTTAGCTGCTTAAGGAATAAAGGC-3'	NM_213838
βD2	porcine Defensine-2β	F: 5'-CCAGAGGTCCGACCACTA-3' R: 5'-GGTCCCTTCAATCCTGTT-3	NM_214442
B2M	Sus scrofa, beta-2-microglobulin	F: 5'CGCCCCAGATTGAAATTGATTTGC 3' R: 5'GCTATACTGATCCACAGCGTTAGG 3'	397033
IFNB	porcine IFN-β	F: 5-AGTTGCCTGGGACTCCTCAA-3 R: 5-CCTCAGGGACCTCAAAGTTCAT-3	NM_21455

F: forward primer

R: reverse primer

Table 2

Effects of tonsil cell supernatants on spontaneous IL-8 release by IPEC J2 cells

TREATMENT	IL-8 (pg/ml ± 1 D.S.)
IPEC Untreated wells	565 ± 207
IPEC + tonsil supernatant 24 h	1055 ± 338*
IPEC + tonsil supernatant + IFN-α 100	820 ± 244
IPEC + tonsil supernatant + IFN-α 1	820 ± 348

Results are shown in terms of pg/ml ± 1 standard deviation. The asterisk indicates a significant difference with respect to untreated control wells (one-way ANOVA for repeated measures, $P<0.05$).

Pig tonsils were collected at the slaughterhouse and processed to obtained viable cells. These were resuspended at 3 million ml–1 and cultivated in RPMI 1640 medium + 2-mercaptoethanol (2-ME) + 10% fetal calf serum (FCS). Tonsil cells were treated with rIFN-α at 0, 1 and 100 U/ml, and then incubated at 37 °C in 5% CO_2. Supernatants were harvested 18 hours later and stored under aseptic conditions in aliquots at – 80°C. IPEC-J2 at confluence were washed once with MEM and treated with tonsil supernatants (from 5 pigs) diluted 1:4 for 18 h at 37 °C in 5% CO_2. IL-8 were measured in IPEC supernatants.

Table 3

Direct and indirect effects of IFN-alpha on anti-microbial peptide expression

TREATMENT	β1-Defensins	β2-Defensins
IPEC Untreated wells	6.3 ± 0.3	13.9 ± 1.5
TREATMENT / INDIRECT EFFECTS		
IPEC + tonsil supernatant 24 h	6.4 ± 0.15	13.5 ± 1.2
IPEC + tonsil supernatant + IFN-α 100 UI/ml	7.5 ± 0.05***	13.8 ± 1.3
IPEC + tonsil supernatant + IFN-α 1 UI/ml	6.6± 0.2	14.1 ± 1.5
TREATMENT / DIRECT EFFECTS		
IPEC + rIFN-α 100 UI/ml + LPS 1 µg/ml	10.5± 0.4 ***	15.3±1.9
IPEC + rIFN-α 1 UI/ml + LPS 1 µg/ml	9.1 ± 0.5 **	15.2± 0.6
IPEC + rIFN-α 100 UI/ml + LPS 2 µg/ml	11± 0.2***	16.4±1.7
IPEC + rIFN-α 1 UI/ml + LPS 2 µg/ml	8.6± 0.07*	16±0.2
IPEC + rIFN-α 100 UI/ml + LPS 4 µg/ml	10.8± 0.01***	16.3±1.2
IPEC + rIFN-α 1 UI/ml + LPS 4 µg/ml	8.7±0.07 *	15.5±0.7

Results are shown in terms of ΔCt ± standard deviation.

Asterisks indicate significant differences with respect to untreated wells (one-way ANOVA for repeated measures).

***$P<0.001$

**$P<0.01$

*$P<0.05$

CHAPTER 3

A PIG TONSIL CELL CULTURE MODEL
FOR EVALUATING ORAL, LOW-DOSE IFN-α TREATMENTS

Elisabetta Razzuoli[a], Riccardo Villa[b], Angelo Ferrari[a], and Massimo Amadori[b]*

a S.S Genova, Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Piazza Borgo Pila 39-24,16129-Genova.

b Laboratory of Cellular Immunology, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, via A. Bianchi 9, 25124 Brescia.

* Corresponding author. Tel. +39 030 2290248; fax +39 030 2290392

E-mail address: massimo.amadori@izsler.it (M. Amadori)

Article submitted

ABSTRACT

Oral, low-dose IFN-α treatments proved effective in several models of viral infections and immunopathological conditions. Also, they do not give rise to the serious side effects observed after parenteral inoculation of high doses (10^5 U / kg b.w. and higher). There is convincing evidence that such treatments work through an early, effective interaction with oral lymphoid tissues before the IFN-α molecules are rapidly destroyed by gut enzymes. Yet, the paucity of detailed information about these crucial interactions and the lack of recognized *in vitro* models hamper the development of proper administration protocols. On the basis of a previous study (Razzuoli et al., 2013), we developed an *in vitro* model of interaction between different types of human and porcine IFNs-α at low / moderate concentrations and pig tonsil cells. The IFNs-α under study showed different properties with respect to three fundamental control actions: 1) IgA release in culture (up and down-regulation, respectively), 2) release of natural antimicrobial compounds, and 3) homeostatic regulation of the inflammatory response. This was checked in pig intestinal epithelial cells (IPEC-J2 cell line) treated with supernatants of control and IFN α-treated tonsil cell cultures, respectively, in terms of inflammatory cytokine and chemokine responses. Some IFNs-α caused a significant inhibition of IL-8 (protein release and gene expression) and beta Defensin1 (gene expression) through second messengers released by IFN α-treated tonsil cells. Interestingly, a human lymphoblastoid IFN-α under study caused the decrease of polyclonal IgA release by pig tonsil cells and significantly stimulated the *in vitro* recall antibody response of swine PBMC to Foot-and-Mouth Disease virus. The modulation of IgA and antibacterial compounds was accompanied by an anti-inflammatory control action at the same, low to moderate IFN-α concentrations (1 to 100 U / ml). This highlights the very foundation of the homeostatic control actions performed by Type I IFNs: to promote an effective host response to infectious and non-infectious stressors and to turn off noxious inflammatory responses associated to tissue damage and waste of metabolic energy. The described tonsil cell model *in vitro* can be conducive to a further development of oral cytokine treatments in human and animals in the "one health" conceptual framework.

Keywords: pig, interferon-α, tonsil, cytokines, antibody, IPEC-J2 cells

INTRODUCTION

Cytokines are proteins of low molecular weight involved in many biological processes like inflammation, immune responses, atherosclerosis and cancer, to name a few (Dinarello, 2007). For this reason, cytokines have been used as diagnostic reagents for human and animal diseases (Wood and Jones, 2001; Sanchez-Correa et al., 2013), as well as for prognostic use (Trevisi et al., 2011) and prophylactic treatments (Zecconi et al., 2009). Regarding this latter aspect, some cytokines like Interleukin (IL)-2, IL-15 and Type I Interferons (IFNs) have been widely used for high and low-dose treatments in both humans and farm animals (Amadori, 2007). As for type I IFNs, high parenteral doses (2-5 M unit) are employed e.g. for melanoma therapy (Tarhini et al., 2012), even if such treatments cause serious side effects. These are not observed after oral, low-dose treatments, as shown e.g. in pigs and cats in the early weaning and Feline AIDS models, respectively (Pedretti et al., 2006; Razzuoli et al., 2010). In this respect, the effector mechanisms of orally administered IFN-alpha (-α) are still ill-defined. Only 1% of this cytokine is probably adsorbed by oral tissues and the rest is inactivated by intestinal enzymes (Bocci, 1999). Therefore, type I IFNs and other orally administered cytokines are likely to interact with oral lymphoid tissues. These could mount two distinct types of response: 1) the release of secondary messengers exerting systemic regulations (indirect effects), as shown e.g. in swine peripheral blood mononuclear cells (PBMC) (Amadori et al., 2009); 2) the local modulation of innate immunity and/or mucosal antibody production (direct effects). On the whole, the lack of recognized *in vitro* models for evaluating oral cytokine treatments thwarts the development of proper administration protocols. For this reason, the central aim of our study was to develop a reliable *in vitro* model related to the crucial interactions between cytokines and oral lymphoid cells, in terms of homeostatic regulation of the inflammatory response and antibody production. This working hypothesis was actually suggested by our previous study, in which swine intestinal epithelial cells were shown to react to secondary messengers released by IFN α-treated lymphoid tissues (Razzuoli et al., 2013). These results prompted us to prove the consistency of our results on a wider array of tested IFNs and to further develop a coherent, robust, *in vitro* model concerning the direct and indirect effects of orally administered cytokines on the basis of tonsil cell cultures.

2. MATERIALS AND METHODS

2.1 Interferons

Porcine recombinant IFN-α (rIFN-α) was purchased from PBL Biomedical Laboratories (cat. 17100-1). The units of this preparation are determined with respect to the international reference standard for human leukocyte interferon (Ga-902-530) provided by National Institutes of Health (Bethesda, Maryland, USA). Natural porcine IFN-α (nIFN-α was obtained from Paramyxovirus-stimulated PBMC of two different pigs as described in a previous paper (Razzuoli et al., 2011). Human lymphoblastoid IFN-α (hl IFN-α) was obtained from Namalwa cells as previously described (Shuttleworth et al., 1983). Lymphoblastoid Humoferon® (hum IFN-α licensed for human use is produced by Sigma-Tau, Pomezia, Italy. The concentration of the IFNs under study was determined by a cpe inhibition assay of Vesicular Stomatitis virus on bovine MDBK cells, as previously described (Meager A, 1987). The assay was calibrated with rIFN-α

2.2 Tonsil cell cultures

Palatine tonsils were collected from 10 healthy pigs (9 to 10-month old, Landrace x Large White pigs) at the slaughterhouse and processed as previously described (Razzuoli et al., 2012). Viable cells were resuspended at 3 millions/ml and cultivated in RPMI 1640 medium + 2-mercaptoethanol (2-ME) (5×10^{-4} M) + 10% fetal calf serum (FCS) + antibiotics in 12-well microtiter plates. Tonsil cells were treated with the aforementioned IFNs-α at 1 and 100 U/ ml for 18 hours at 37 °C in 5% CO_2. For each pig tonsil, 6 control wells (untreated cells) were set up as well. After 18 hours of incubation at 37 °C in 5% CO_2 , 200 μl of tonsil cell suspension (about 6×10^5 cells) from wells treated with 1 U/ ml of IFNs (rIFN-α, hl IFN-α, nIFN-α and hum IFN-α) were transferred into tonsil control wells for a further incubation at 37 °C in 5% CO_2 for 24 hour. Then, cell-free supernatants were harvested and stored at -80°C for the IPEC-J2 cell stimulation assays and total IgA ELISA (see hereunder).

2.3 Evaluation of IFN-α action on residual bacterial contamination

The activity of IFNs on residual bacterial contaminations was investigated after overnight incubation of tonsil cell cultures. 1-ml aliquots of serial 10-fold dilutions of tonsil cell culture supernatants (range 10^{-2} – 10^{-12}) were inoculated in triplicate into tubes containing Trypticase Soy Broth (TSB) medium for bacteriological examinations. Inoculated and non-inoculated TSB tubes were incubated over 3 days at 37 °C, and 50% end-point titres were determined as previously described (Reed and Muench, 1938) on the basis of turbidity. Also, tonsil cell supernatants were analysed for lysozyme by the lyso-plate-assay (Osserman and Lawlor, 1966).

2.4 IPEC-J2 cells

IPEC-J2 cells (an established line of porcine intestinal epithelial cells, IZSLER Cell Bank code BS CL 205) were grown in Minimum Essential Medium (MEM) enriched with FCS (10% v/v), 2 mM glutamine and antibiotics (50 μg/ml penicillin, 50 μg/ml streptomycin, 10 μg/ ml neomycin).

These cells show a spontaneous secretion of IL-8 and were previously employed in studies on the inflammatory response (Schmidt et al., 2008). Cells were seeded into 12-well tissue culture plates (2 ml per well, 2x105 cells/ml) and incubated at 37 °C in 5% CO2 until confluence (20-24 h). Then, they were stimulated with 1:4-diluted tonsil supernatants and incubated at 37 °C in 5% CO2 for 18 hours. The resulting IPEC-J2 cell supernatants were stored at –80°C and used in an ELISA assay for spontaneous IL-8 release. Total RNA was extracted from IPEC-J2 cells to evaluate the expression of the IL-1β, IL-6, TNF-α, bD1, bD2, IFN-α and IL-8 genes (see section 2.7).

2.5 IgA ELISA

Swine IgA was measured by a commercial ELISA kit as suggested by the manufacturer (Bethyl Laboratories, Montgomery). Briefly, Maxi Sorp NUNC ELISA plates (Nunc™, Serving Life Science, Denmark) were adsorbed with anti-swine IgA capture polyclonal antibody (PAB) at 4 μg/ml in 0.1M carbonate/bicarbonate buffer pH 9.6 and incubated for 1h at 26 °C. After blocking with 1% BSA in 0.14 M NaCl, 150mM Tris-buffered saline (AB, assay buffer), plates were washed thrice with 0.2% Tween 20 in assay buffer (AB-Tween). Then, 100 μL/well of undiluted test samples or AB-Tween + 1% BSA (sample diluent, blank control) were added in duplicate; also, porcine IgA (Bethyl Laboratories) at 10 μg/ml and seven 3-fold dilutions thereof were added in duplicate, and plates were incubated at room temperature for 1 h. Then, plates were washed thrice with AB-Tween, and 100 μL/well of horseradish peroxidase (HRP) anti-swine IgA antiserum at 0.1μg/ml in sample diluent was added; plates were further incubated for 1 h at room temperature. After 3 washings with AB-Tween, swine IgA antibody was revealed by adding 50 μL/well of ortho-phenilenediamine (OPD, Sigma-Aldrich, cat. P9187) + 0.02% H_2O_2 as substrate of the color reaction. This was blocked after 15 min by adding 50 μL/well of 2N H2SO4. Plates were read spectrophotometrically at 492 nm. Antibody concentrations were determined from a standard curve created with the above dilutions of swine IgA using software Prism 2.01, (Graph Pad Software, Inc. Avenida de la Playa, USA).

2.6 IL-8 ELISA

Porcine IL-8 was measured by a commercial ELISA kit as suggested by the manufacturer (R&D system, DUOset cat. DY535). Briefly, Maxi Sorp NUNC ELISA plates (Nunc™, Serving Life Science, Denmark) were adsorbed with anti-swine IL-8 capture polyclonal antibody (PAB) at 2 μg/ml in buffered saline (PBS) and incubated overnight at 26°C. Then, the plate was blocked with 1% BSA in PBS and washed

with PBS + 0.05% Tween 20. 100 μL/well of undiluted test samples or sample diluent (blank control) were added in duplicate; also, recombinant porcine IL-8 at 8 ng/ml and seven 3-fold dilutions thereof were added in duplicate, and plates were incubated at room temperature for 2 h. Then, plates were washed and 100 μL/well of biotinylated anti-swine IL-8 at 0.05 μg/ml in sample diluent was added. Plates were further incubated for 2 h at room temperature; then, after 3 washings, streptavidin-HRP was added and plates were incubated for a further 20 minutes at room temperature. After 3 washings with PBS-Tween, swine IL-8 was determined from a standard curve created with the above dilutions of IL-8 using software Prism 2.01 (Graph Pad Software, Inc. Avenida de la Playa, USA).

2.7 RT Real-time PCR

IPEC-J2 cell monolayers were dispersed by trypsin-EDTA. Total RNA was extracted from the cells of each well of the culture plates using RNeasy Mini Kit (Qiagen, Milan, Italy) by the Qiacube System (Qiagen, Milan, Italy) in accordance with the manufacturer's instructions. cDNA synthesis was performed in the presence of Random Hexamers as previously described (Razzuoli et al., 2011, 237-247). The expression of porcine IFN-β, IL-8, IL-6, bD1, bD2, IL-1β and TNF-α genes was determined using the primer sets shown in Table 1. Porcine β2-microglobulin (B2M) was used as housekeeping control gene (Table 1). EVA Green Real-Time PCR amplification was performed in a CFX96™ Real-Time System (Bio-Rad, Milan, Italy) after the reverse transcription step as previously described (Razzuoli et al., 2011). In each sample of IPEC-J2 cells, the relative expression of the selected genes was calculated using the formula ΔCt=Ct (target gene) − Ct (housekeeping), where Ct (cycle of threshold) values were the mean of three test replicates ± 1 standard deviation. Negative samples were given a Ct 40 fictitious value for further statistical examination. The average intensity of expression (mean ΔCt sample-ΔCt negative control) of the genes under study was compared by one-way ANOVA. The threshold for significance was set at P< 0.05.

2.8 *In vitro* antibody responses to Foot-and-Mouth Disease Virus (FMDV)

Heparinized blood samples were collected from three sows vaccinated one year before with an O1 Manisa FMD vaccine. PBMC were separated by centrifugation on Ficoll 1,077 (1100 g, 25 min, 20°C) and grown at 37 °C, 5% CO2, in 6-well microtiter plates at $2x10^6$ / ml in RPMI 1640 medium + 5% FCS + 5x10-4 M 2-ME in medium only (control), or medium + sucrose-purified, inactivated, O1 Manisa FMDV antigen (1 μg / ml final), or medium + FMDV antigen and hl IFN-α at 2 / 20 / 200 U / ml final. 12 days later, the supernatants were collected and tested in a neutralization assay of O1 Manisa FMDV on IBRS-2 cells, as prescribed by the OIE manual (Anonimus, 2012, http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.05_FMD.pdf).

The same animals were again injected 3 months later with an oil-in-water (Montanide ISA 25, Seppic, France) O1 Manisa FMD vaccine. 7 days after the booster vaccine injection, PBMC were separated and grown under the same conditions in the presence or absence of the following components:

- O1 Manisa FMDV antigen (1 µg / ml final).
- hl IFN-α at 5 U / ml final.
- Porcine recombinant IL-10, 20 ng/ml final.

Seven days later, 1.7 ml / well of supernatant was collected, clarified and stored at − 20°C. 2 ml / well of medium supplemented with the same components except FMDV antigen was added, and plates were incubated for a further 5 days. Neutralizing antibody to FMDV O1 Manisa was measured in supernatants of PBMC cultures at days 7 and 12 according to the aforementioned OIE procedure.

2.9 Statistical analyses

Differences between means (Ct in Real-time PCR and protein concentrations in ELISA) were checked for significance by one-way analysis of variance (ANOVA). In quantal tests (bacterial end-points and assay of neutralizing Ab to FMDV), titers were assessed as previously described (Reed and Muench, 1938). The difference between two titers was checked for statistical significance by Pizzi's formula (Pizzi, 1950). In all statistical analyses, the threshold for significance was set at $P < 0.05$.

3. RESULTS

3.1 Effects of IFN-α on residual bacterial loads in tonsil cell cultures

Results are shown in Table 2. The different types of IFN-α at 1 U/ml did not show any effect on the residual bacterial loads in tonsil cell cultures with respect to untreated wells (P> 0.05). On the contrary, hum IFN-α at 100 U/ml significantly decreased the bacterial load with respect to untreated cells; rIFN-α at 100 U/ml caused a slower bacterial growth, but on day 3 of incubation there was no significant difference between rIFN-α treated and untreated cells (titers: 106/ml and 106.5/ml, respectively) (see Table 2). All samples tested negative for lysozyme (data not shown).

3.2 IgA release from tonsil cells

Treatments with rIFN-α caused no significant change of IgA release *in vitro* (Figure 1), as opposed to 1 U/ml of hl IFN-α and hum IFN-α (P < 0.01 and < 0.05, respectively). In particular, hum IFN-α increased (+ 70 ng/ml, Figure 1) and hl IFN-α decreased (- 81 ng/ml) IgA release with respect to untreated wells (342 ± 110 ng/ml) (Figure 2). At 100 U/ ml only nIFN-α significantly (P< 0.05) increased IgA secretion (+ 60 ng/ml) (Figure 2).

Concerning the effects of IFN α-treated tonsil cells, only nIFN-al showed a significant effect (P< 0.01) in terms of IgA secretion (+ 65 ng/ml) with respect to untreated control cultures at 48 hours (134 ± 52 ng/ml, Figure 2).

3.3 IL-8 release from IPEC-J2 cells

In agreement with our previous study on porcine rIFN-α (Razzuoli et al., 2013), a treatment with untreated tonsil supernatants significantly (P< 0.001) increased the spontaneous IL-8 release from IPEC-J2 cells. The effect was significantly enhanced by using tonsil cell supernatants after 48 hours of incubation (Figure 3). As shown for rIFN-α, also hum IFN-α and hl IFN-α at 100 U / ml in culture medium of donor tonsil cells significantly decreased IL-8 release from IPEC-J2 cells (both P< 0.05) (Figure 3). The same activity was not shown by nIFN-α and 1 U/ml of the different types of IFNs-α (Figure 4).

Also, the up-regulation of IL-8 was partly suppressed (from 1,056±299 to 740±294 pg/ml) if tonsil cell cultures were treated with the supernatant of other hl IFN α-treated tonsil cell cultures at 1 U/ml (P< 0.001). The other types of IFN caused no significant changes (Figure 4).

3.4 Expression of cytokine genes

Supernatants of untreated tonsil cell cultures significantly increase the expression of the IL-8 and IL-6 genes in IPEC-J2 cells, and this effect can be antagonized by rIFN-α (Razzuoli et al., 2013). On the basis of these results, different types of IFN-α were characterized for this peculiar regulation. Treatment of cultured tonsil cells with different types of IFN-α at 100 U/ml restored the usual levels of IL-8 expression in IPEC-J2 cells (Figure 5); on the contrary, 1 U/ml of IFNs caused no such effects (data not shown). Table 3 summarizes the expression of other cytokine genes in IPEC-J2 cells after stimulation with supernatants of control and IFN α-treated tonsil cells. Only IL-6 was significantly up-regulated by hl IFN-α at 100 U / ml. Regarding bD1, all the IFNs under study at 100 U/ml significantly down-regulated (P< 0.001, see Figure 6) gene expression. The treatment with tonsil supernatants at 48 hours caused no significant effect in terms of cytokine gene expression (data not shown).

3.5 *In vitro* antibody responses to FMDV

Lymphoblastoid hl IFN-α was further investigated in a model of *in vitro* recall antibody response to FMDV. This was actually boosted by hl IFN-α at 20 and 200 U / ml (see Table 4). In a subsequent experiment, the same effect was also observed in PBMC cultures of the same sows supplemented with 5 U / ml of hl IFN-α. This activity was antagonized by porcine recombinant IL-10 at 20 ng / ml (Figure 7).

4. DISCUSSION

Type I IFNs act as important mediators in the innate immune system and as homeostatic agents in the control of environmental, non-infectious stressors (Amadori, 2007; Amadori et al., 2012). Also, accumulated evidence shows that they represent a crucial link between innate and adaptive immune response, which has stimulated many investigations into established disease models (Farkas and Kemeny, 2012; Tarhini et al., 2012). Also, the negative repercussions of defective IFN responses on disease occurrence were characterized in human models like allergic asthma in children and vaginal vestibulitis in women (Bufe et al., 2002; Gerber et al., 2002). Therefore, the recognition of an important role of Type I IFNs as homeostatic agents vis-à-vis infectious and non-infectious stressors has led to several studies on the administration of these cytokines for prophylactic and therapeutical purposes (George et al., 2012). In particular, there is strong evidence that oral, low-dose IFN-α can improve the clinical conditions in several models of virus infections and immunopathological conditions (Cummins et al., 2005), even though the relevant effector mechanisms underlying the reported clinical results are still elusive. This is reflected by the lack of validated *in vitro* models, predictive of useful *in vivo* regulatory actions of type I IFNs. These models would be conducive to the development of correct protocols of use in human and veterinary medicine.

Owing to the above, we had developed ad hoc assays in IPEC-J2 cells (swine intestinal epithelial cell line) for an assessment of the anti-inflammatory control actions of porcine recombinant IFN-α at low and moderate concentrations. Also, we had highlighted potent regulatory activities of IFN α-treated tonsil cells, i.e. of the immediate targets of oral IFN-α (Razzuoli et al., 2013). On the basis of these results, we decided to widen the scope of our experimental procedures to a larger set of type I IFNs, and to develop a general model for assessing the suitability of cytokines for oral use based on cultured tonsil cells. This model was focused onto three main regulatory actions of orally-administered cytokines:

- Modulation of mucosal immunity in terms of IgA release.
- Modulation of natural antimicrobial compounds.
- Homeostatic regulation of the inflammatory response.

Concerning the first point, our results highlighted different activities of various IFNs. In particular, Hum IFN-α and nIFN-α caused an increase of IgA release, which was also observed when nIFN-α treated-tonsil cells were transferred to untreated cultures. This activity of IFNs and IFN-treated tonsil cells is probably related to a downstream activation of DCs, which release B lymphocyte stimulator proteins BLys (also called BAFF) and APRIL (Litinskiy et al., 2002). Also, there is an important role of IL-6 for polyclonal IgA release (Kishimoto, 2003), as highlighted in IgA ELISPOT assays on cultured pig tonsil cells (Razzuoli et al., 2012) and in the clinical model of IgA-related nephropathy in humans (Wardle, 2004). The inverse outcome of the hl IFN-α treatment in terms of *in vitro* IgA release was in agreement with a previous study (Naylor et al., 1999) that demonstrated the ability of human natural IFN-α to induces a transient decline of immunoglobulin release, probably mediated by IL-5. Instead, hl IFN-α could boost *in vitro* the recall antibody response to FMDV of swine PBMC. This effect can be related to the ability of IFN-α to improve Ag presentation in DCs and macrophages and, in particular, to induce BAFF in pig monocytic cells (Bergamin et al., 2007). These results are in line with an increased antibody production *in vivo* of IFN α-treated subjects and the use of IFN-α as vaccine adjuvant (Bracci et al., 2008). The suppression of APC functions and the generation of suppressive DCs (Ding et al., 2003) probably underlie the antagonist action of IL-10 on *in vitro* antibody production to FMDV. On the whole, our results indicate that different regulatory actions on Ig production can be exerted by closely similar IFNs-α, and that *in vitro* tests must be carefully planned in view of the desired outcome of *in vivo* treatments.

Regarding the second topic under study, our bacteriological tests showed the ability of some type I IFNs to modulate the release of antibacterial compounds in tonsil cell cultures. In particular, rIFN-α retarded bacterial growth, whereas hum IFN-α caused a significant decrease of bacterial load. The inhibition of bacterial growth was probably related to antimicrobial peptides other than lysozyme. This result is in agreement with our previous findings, whereby supernatants of pig tonsil cells cause a significant growth inhibition of B. cereus, B. subtilis and M. luteus in agar-plate diffusion assays (Razzuoli et al., 2012). This is not surprising, since beta-defensins are constitutively expressed in airway and oral mucosal surfaces of pigs (Oswald, 2006). In particular, beta-defensin-1 (bD1) is constitutively expressed in tongue epithelia (Sang and

Blecha, 2009), while bD2 and bD3 are primarily detected in lymphoid tissues. These are the likely targets of the regulatory actions of type I IFNs, underlying the observed inhibition of bacterial growth.

The role of IFN-α in the control of the inflammatory response was the 3rd topic of our study. This role had been demonstrated in terms of IL-8 release from IPEC-J2 cells (reporter system), following stimulation with rIFN-α and supernatants of rIFN α-treated tonsil cells (Razzuoli et al., 2013). Other IFNs-α were shown in this study to exert the same indirect regulation of the inflammatory response. In particular, all the IFNs-α under study at 100 U/ml, with the exception of nIFN-α, determined a decrease of IL-8 gene expression, which can be accounted for by an induction of anti-inflammatory mediators like IL-10 (Ouyang et al., 2011). Also, a sustained IL-8 response can be mounted if the gene promoter is derepressed, NF-kB and JNK pathways are activated, and the resulting mRNA is stabilized through the p38 MAPK pathway (Hoffmann et al., 2002). IFNs-α can antagonize this latter pathway by inducing negative regulators of mRNA stability like tristetrapolin, targeting AU-rich elements at the 3' untranslated region of mRNA (Sauer et al., 2006). It is conceivable that similar regulatory functions could be also exerted by second messengers released by IFN α-treated tonsil cells. As for the down-regulation of bD1 in IPEC-J2 cells, also observed in our previous study on porcine rIFN-α (Razzuoli et al., 2013), this should be also viewed in the framework of the global regulation of the inflammatory response. Antimicrobial peptides are potent mediators of inflammation, stimulating epithelial and inflammatory cells, cell proliferation, cytokine/chemokine production and chemotaxis (Niyonsaba and Ogawa, 2005). In particular, some beta defensins such as HBD3 can enhance the inflammatory response by complexing PAMPs like CpG DNA. The i.v. administration of HBD3/CpG complexes induce proinflammatory cytokines like IL-12, IFN-γ, IL-6 in serum, indicating a potential pathophysiological role for alarmin/DNA complexes in contributing to inflammation (Tewary et al., 2013). This highlights the very foundation of the homeostatic control actions performed by Type I IFNs: to promote an effective host response to infectious and non-infectious stressors and to turn off noxious inflammatory responses associated to tissue damage and waste of metabolic energy. Therefore, these two features could go along even in the acute phase of the response, characterized by moderate concentrations of Type I IFNs (around 100 U / ml).

Interestingly, the supernatants of untreated tonsil cell cultures did not up-regulate the IL-6 gene in IPEC-J2 cells, as opposed to the previous study on rIFN-α. The different herds of the pigs under study, the related differences of the oral bacterial cell populations and the different batch of IPEC-J2 cells employed in this study can reasonably account for the observed discrepancy. Vice versa, the up-regulation of the IL-8 gene by supernatants of untreated tonsil cell cultures was again observed in this study, which defines IL-8 as a suitable, robust reporter system in our *in vitro* model.

The above findings confirm that IFNs-α exert different regulatory actions, which can be related to different ratios among IFN-α subtypes, as well as to the presence of contaminating cytokines in non-purified IFNs (nIFN and hl IFN). Our results are in agreement with those of Sang and co-workers demonstrating a fundamental diversity of biological activities among subtypes of IFN-α in swine (Sang et al., 2010). This is also in line with another study of ours, where we demonstrated *in vitro* the constitutive and virus-induced

expression of different porcine IFN-α genes after stimulation of swine PBMC with Newcastle Disease Virus (NDV) Lasota strain (Razzuoli et al., 2011).

5. CONCLUSION

Our results indicate that tonsil cell cultures are a suitable *in vitro* model to evaluate the effects of oral, low-dose cytokine treatments. Therefore, this model could be adopted to develop protocols of oral administration of cytokines. Such a large animal model could be actually more predictive than others based on laboratory rodents (Meurens et al., 2012), in view of possible clinical applications in humans. Beyond the assessment of these comparative models, our results in pigs highlight the possible use of Type I IFNs to modulate the innate immune response toward greater disease resistance of farm animals, decreased drug usage and a consequently greater safety of the food chains.

ACKONWLEDGEMENTS.

REFERENCES

1. Amadori, M., 2007. The role of IFN-alpha as homeostatic agent in the inflammatory response: a balance between danger and response?. J. Interferon Cytokine Res. 27, 181-189.

2. Amadori, M., Farinacci, M., Begni, B., Faita, R., Podavini, D., Colitti, M., 2009. Effects of interferon-alpha on the inflammatory response of swine peripheral blood mononuclear cells. J. Interferon Cytokine Res. 29, 241-247.

3. Amadori, M., Razzuoli, E., Nassuato, C., 2012. Issues and possible intervention strategies relating to early weaning of piglets. CAB Reviews 7, 1-15.

4. Anonimus, 2012. Foot-and-Mouth Disease. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2.1.5. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.05_FMD.pdf.

5. Bergamin, F., Vincent, I.E., Summerfield, A., McCullough, K.C., 2007. Essential role of antigen-presenting cell-derived BAFF for antibody responses. Eur. J. Immunol. 37, 3122-3130.

6. Bocci, V., 1999. The oropharyngeal delivery of interferons: where are we and where do we need to go? J. Interferon Cytokine Res. 19, 859-861.

7. Bracci, L., La Sorsa, V., Belardelli, F., Proietti, E., 2008. Type I interferons as vaccine adjuvants against infectious diseases and cancer. Expert Rev. Vaccines 7, 373-381.

8. Bufe, A., Gehlhar, K., Grage-Griebenow, E., Ernst, M., 2002. Atopic phenotype in children is associated with decreased virus-induced interferon-alpha release. Int. Arch. Allergy Immunol. 127, 82-88.

9. Cummins, J.M., Krakowka, G.S., Thompson, C.G., 2005. Systemic effects of interferons after oral administration in animals and humans. Am. J. Vet. Res. 66, 164-176.

10. Dinarello, C.A., 2007. Historical insights into cytokines. Eur. J. Immunol. 37 Suppl 1, S34-45.

11. Ding, Y.Z., Fu, S., Zamarin, D., Bromberg, J., 2003. Interleukin-10. In: Thomson, A.G., Lotze, M.T. (Eds.), The Cytokine Handbook, 4th ed. Academic Press, London, pp. 603-625.

12. Farkas, A., Kemeny, L., 2012. Monocyte-derived interferon-alpha primed dendritic cells in the pathogenesis of psoriasis: new pieces in the puzzle. Int. Immunopharmacol. 13, 215-218.

13. George, P.M., Badiger, R., Alazawi, W., Foster, G.R., Mitchell, J.A., 2012. Pharmacology and therapeutic potential of interferons. Pharmacol. Ther. 135, 44-53.

14. Gerber, S., Bongiovanni, A.M., Ledger, W.J., Witkin, S.S., 2002. A deficiency in interferon-alpha production in women with vulvar vestibulitis. Am. J. Obstet. Gynecol. 186, 361-364.

15. Hoffmann, E., Dittrich-Breiholz, O., Holtmann, H., Kracht, M., 2002. Multiple control of interleukin-8 gene expression. J. Leukoc. Biol. 72, 847-855.

16. Kishimoto, T., 2003. Interleukin-6 (IL-6). In: Thomson, A.G., Lotze, M.T. (Eds.), The Cytokine Handbook, 4th ed. Academic Press, London, pp. 281-304.

17. Litinskiy, M.B., Nardelli, B., Hilbert, D.M., He, B., Schaffer, A., Casali, P., Cerutti, A., 2002. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. Nat. Immunol. 3, 822-829.

18. Meager A, 1987. Quantification of interferons by anti-viral assays and their standardization. In: Lymphokines and interferons, a practical approach, Clemens MJ, Morris AG, Gearing AJH., IRL Press Limited, Oxford, UK, pp. 129-147.

19. Meurens, F., Summerfield, A., Nauwynck, H., Saif, L., Gerdts, V., 2012. The pig: a model for human infectious diseases. Trends Microbiol. 20, 50-57.

20. Naylor, P.H., Naylor, C.W., Hendrix, S., Leveque, F.G., 1999. Oral administration of interferon-alpha induces a transient decline in oral mucosal immunoglobulins and an increase in interleukin-5. J. Interferon Cytokine Res. 19, 953-959.

21. Niyonsaba, F., Ogawa, H., 2005. Protective roles of the skin against infection: implication of naturally occurring human antimicrobial agents beta-defensins, cathelicidin LL-37 and lysozyme. J. Dermatol. Sci. 40, 157-168.

22. Osserman, E.F., Lawlor, D.P., 1966. Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. J. Exp. Med. 124, 921-952.

23. Oswald, I.P., 2006. Role of intestinal epithelial cells in the innate immune defence of the pig intestine. Vet. Res. 37, 359-368.

24. Ouyang, W., Rutz, S., Crellin, N.K., Valdez, P.A., Hymowitz, S.G., 2011. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. Annu. Rev. Immunol. 29, 71-109.

25. Pedretti, E., Passeri, B., Amadori, M., Isola, P., Di Pede, P., Telera, A., Vescovini, R., Quintavalla, F., Pistello, M., 2006. Low-dose interferon-alpha treatment for feline immunodeficiency virus infection. Vet. Immunol. Immunopathol. 109, 245-254.

26. Pizzi, M., 1950. Sampling variation of the fifty percent end-point, determined by the Reed-Muench (Behrens) method. Hum. Biol. 22, 151-190.

27. Razzuoli, E., Dotti, S., Archetti, I.L., Amadori, M., 2010. Clinical chemistry parameters of piglets at weaning are modulated by an oral, low-dose interferon-alpha treatment. Vet. Res. Commun. 34 Suppl 1, S189-92.

28. Razzuoli, E., Faggionato, E., Dotti, S., Villa, R., Lombardo, T., Boizza, L., Ferrari, M., Amadori, M., 2012. Isolation and culture of pig tonsil lymphocytes. Vet. Immunol. Immunopathol. 148, 320-325.

29. Razzuoli, E., Villa, R., Amadori, M., 2013. IPEC-J2 Cells as Reporter System of the Anti-Inflammatory Control Actions of Interferon-Alpha. J. Interferon Cytokine Res. 33, 597-605.

30. Razzuoli, E., Villa, R., Sossi, E., Amadori, M., 2011. Characterization of the interferon-alpha response of pigs to the weaning stress. J. Interferon Cytokine Res. 31, 237-247.

31. Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. Am J Hyg 27, 493-497.

32. Sanchez-Correa, B., Bergua, J.M., Campos, C., Gayoso, I., Arcos, M.J., Banas, H., Morgado, S., Casado, J.G., Solana, R., Tarazona, R., 2013. Cytokine profiles in acute myeloid leukemia patients at diagnosis: Survival is inversely correlated with IL-6 and directly correlated with IL-10 levels. Cytokine. doi: 10.1016/j.cyto.2012.12.023; 10.1016/j.cyto.2012.12.023

33. Sang, Y., Blecha, F., 2009. Porcine host defense peptides: expanding repertoire and functions. Dev. Comp. Immunol. 33, 334-343.

34. Sang, Y., Rowland, R.R., Hesse, R.A., Blecha, F., 2010. Differential expression and activity of the porcine type I interferon family. Physiol. Genomics 42, 248-258.

35. Sauer, I., Schaljo, B., Vogl, C., Gattermeier, I., Kolbe, T., Muller, M., Blackshear, P.J., Kovarik, P., 2006. Interferons limit inflammatory responses by induction of tristetraprolin. Blood 107, 4790-4797.

36. Schmidt, L.D., Kohrt, L.J., Brown, D.R., 2008. Comparison of growth phase on Salmonella enterica serovar Typhimurium invasion in an epithelial cell line (IPEC J2) and mucosal explants from porcine small intestine. Comp. Immunol. Microbiol. Infect. Dis. 31, 63-69.

37. Shuttleworth, J., Morser, J., Burke, D.C., 1983. Expression of interferon-alpha and interferon-beta genes in human lymphoblastoid (Namalwa) cells. Eur. J. Biochem. 133, 399-404.

38. Tarhini, A.A., Gogas, H., Kirkwood, J.M., 2012. IFN-alpha in the treatment of melanoma. J. Immunol. 189, 3789-3793.

39. Tewary, P., de la Rosa, G., Sharma, N., Rodriguez, L.G., Tarasov, S.G., Howard, O.M., Shirota, H., Steinhagen, F., Klinman, D.M., Yang, D., Oppenheim, J.J., 2013. beta-Defensin 2 and 3 promote the uptake of self or CpG DNA, enhance IFN-alpha production by human plasmacytoid dendritic cells, and promote inflammation. J. Immunol. 191, 865-874.

40. Trevisi, E., Amadori, M., Archetti, I., Lacetera, N., Bertoni, G., 2011. Inflammatory Response and Acute Phase Proteins in the Transition Period of High-Yielding Dairy Cows. In: Veas, F. (Ed.), Acute phase proteins as early non-specific biomarkers of human and veterinary disease, InTech, Published, pp. 355.

41. Wardle, E.N., 2004. Is IgA nephropathy induced by hyperproduction of interferon-alpha?. Med. Hypotheses 62, 625-628.

42. Wood, P.R., Jones, S.L., 2001. BOVIGAM: an in vitro cellular diagnostic test for bovine tuberculosis. Tuberculosis (Edinb) 81, 147-155.

43. Zecconi, A., Piccinini, R., Fiorina, S., Cabrini, L., Dapra, V., Amadori, M., 2009. Evaluation of interleukin-2 treatment for prevention of intramammary infections in cows after calving. Comp. Immunol. Microbiol. Infect. Dis. 32, 439-451.

Table 1

Oligonucleotide primer sequences for EvaGreen qRT Real-time PCR

amplification of porcine genes

GENE	PROTEIN	PRIMERS	GeneBank gi-number
IL-8	porcine IL-8	F : 5'- CTGTACAACCTTCTGCACCCA-3' R : 5'-TTCGATGCCAGTGCATAAATA-3'	M86923
IL-6	porcine IL-6	F: 5'-CAGAGATTTTGCCGAGGATG-3' R: 5'-TGGCTACTGCCTTCCCTACC-'3	NM_214399
IL-1β	porcine IL-1β	F: 5'-AATTCGAGTCTGCCCTGTACCC-3' R: 5'-GCCAAGATATAACCGACTTCACCA	NM_001005149
TNF-α	porcine TNF-α	F: 5'-TGCCTACTGCACTTCGAGGTTATC-3' R: 5'CAGATAAGCCCGTCGCCCAC-3'	NM_214022
βD1	porcine Defensine-1β	F:5'- TGCCACAGGTGCTCT-3' R:5' CTGTTAGCTGCTTAAGGAATAAAGGC-3'	NM_213838
βD2	porcine Defensine-2β	F: 5'-CCAGAGGTCCGACCACTA-3' R: 5'-GGTCCCTTCAATCCTGTT-3	NM_214442
B2M	Sus scrofa, beta-2-microglobulin	F: 5'CGCCCCAGATTGAAATTGATTTGC 3' R: 5'GCTATACTGATCCACAGCGTTAGG 3'	397033
IFNB	porcine IFN-β	F: 5-AGTTGCCTGGGACTCCTCAA-3 R: 5-CCTCAGGGACCTCAAAGTTCAT-3	NM_21455

F: forward primer

R: reverse primer

Table 2

Evaluation of residual bacterial contaminations in tonsil cell cultures after 24 h of incubation

Treatment	Dilution of cell culture supernatant					
	10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-10}	10^{-12}
Untreated cells	+	+	+	-	-	-
hum IFN-α 100 U/ml	+	-	-	-	-	-
rIFN-α 100 U/ml	+	+	+/-	-	-	-
nIFN-α 100 U/ml	+	+	+	-	-	-
hl IFN-α 100 U/ml	+	+	+	-	-	-
hum IFN-α 1 U/ml	+	+	+	-	-	-
rIFN-α 1 U/ml	+	+	+	-	-	-
nIFN-α 1 U/ml	+	+	+	-	-	-
hl IFN-α 1U/ml	+	+	+	-	-	-

After 18 hours, 1-ml aliquots of serial 10-fold dilutions of tonsil cell supernatants (range: 10^{-2} – 10^{-12}) were inoculated in triplicate in tubes containing Trypticase Soy Broth (TSB) medium. Inoculated and non-inoculated control tubes were incubated over 3 days at 37 °C, and 50% end-point titres were determined on the basis of turbidity using the formula by Reed and Muench (Reed and Muench, 1938). The titer differences were checked for statistical significance by Pizzi's formula (Pizzi, 1950). The titers obtained with hum IFN-α at 100 U/ml (10^{-2}) were significantly different from the other ones.

Table 3

Evaluation of gene expression in IPEC-J2 cells after treatment with different types of tonsil cell supernatant at 24 h of incubation

Treatment	U/ml	IL-6	IL-1β	TNF-α	bD2	IFN-α
rIFN	100	6.2±0.4	12.8±2.8	15.4±1.8	14.6±2.0	15.2±2.2
	1	5.9±0.5	11.8±2.6	14.2±2.0	13.4±2.9	14.5±2.6
hl IFN	100	**5.7±0.3***	11.6±2.1	15.3±1.0	14.3±1.0	13.9±1.0
	1	6.1±0.2	11.5±2.0	14.3±0.8	14.4±1.3	14.0±1.3
hum IFN	100	5.9±0.5	12.8±2.7	15.2±1.6	15.1±2.1	14.4±1.3
	1	6.2±0.5	11.4±2.7	14.4±1.7	14.5±2.9	15.0±0.7
nIFN	100	6.5±0.5	11.8±1.2	14.6±0.8	15.3±1.4	14.3±1.4
	1	5.9±0.5	11.2±1.3	15.0±1.2	15.3±0.7	15.0±1.6
Control		6.2±0.3	12.0±2.8	14.6±2.1	14.1±1.9	14.3±2.1

Results are shown in terms of ΔCt.± 1 standard deviation.

* indicates a significant difference with respect to treatment of IPEC-J2 cells with tonsil supernatant after 24h of incubation data are express as.

Control: IPEC-J2 cells exposed to untreated tonsil supernatants.

$*P<0.05$.

Table 4

Effects of IFN-α on the *in vitro* recall antibody response to FMDV

	Sow 1 Ab titer	Sow 2 Ab titer	Sow 3 Ab titer	Sow 4 Ab titer
FMDV Ag	1:93	1:93	1:93	1:93
FMDV Ag + IFN-α 2 U/ml	1:162	1:31	1:93	1:31
FMDV Ag + IFN-α 20 U/ml	1:840	1:93	1:280	1:54
FMDV Ag + IFN-α 200 U/ml	1:840	1:162	1:280	1:93
Medium only, control	< 1:2	< 1:2	1:10	1:3

PBMC of O_1 Manisa FMD-vaccinated sows were re-stimulated under different conditions . Results are expressed in terms of neutralizing Ab titers to FMDV O_1 Manisa, measured in the supernatants collected 12 days later.

Figure 1

Effects of porcine recombinant IFN-α and Humoferon IFN-α on IgA release
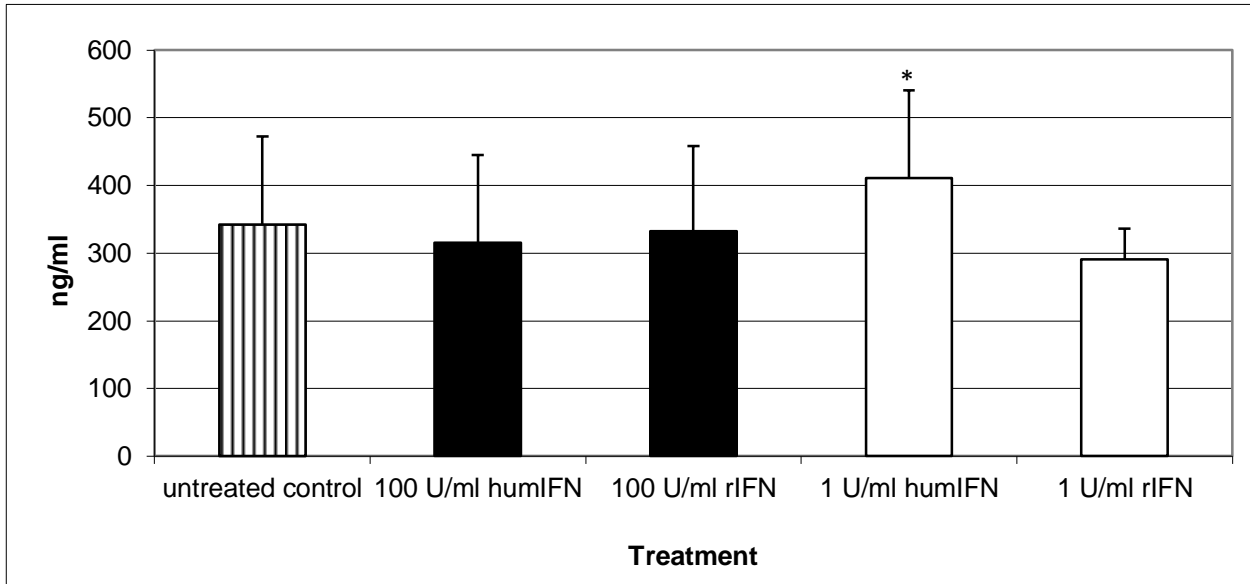
Tonsil cells obtained from 10 pigs were treated with two different types of IFN-α: recombinant porcine IFN-α_1 and Humoferon® at 1 and 100 UI/ml, and then incubated for 18 hours at 37 °C in 5% CO_2. IgA was then measured in cell supernatants. Differences between means were checked for significance by one-way analysis of variance (ANOVA). Data were expressed as mean ± standard deviation. The threshold for significance was set at $P<0.05$.

*indicates significant differences with respect to untreated control wells.

Figure 2

Effect of IFNs treatment on IgA release

A) Effects of nIFN-α
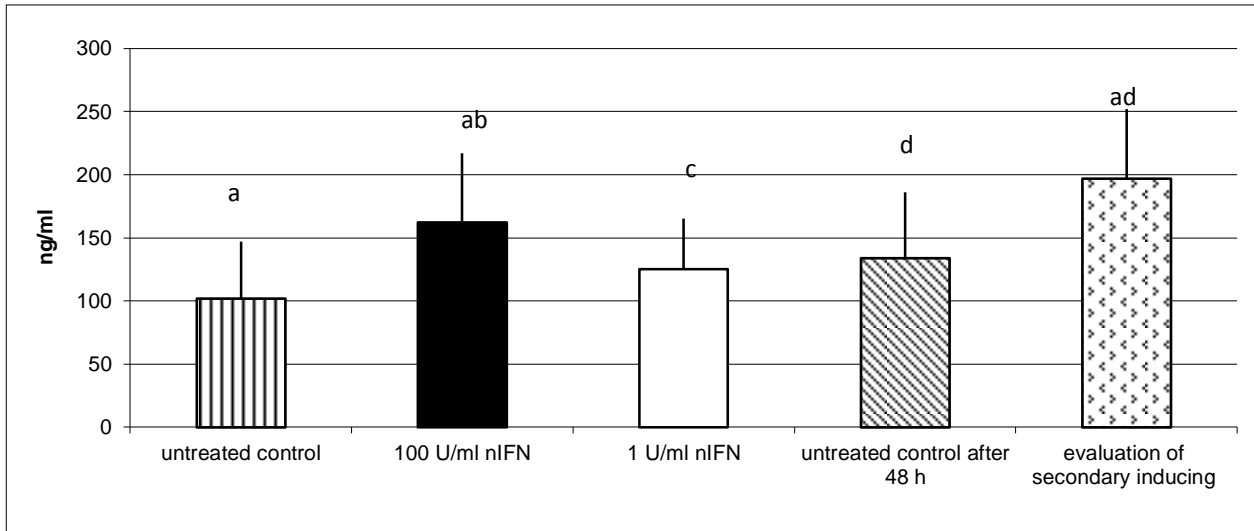
B) Effects of nl IFN-α
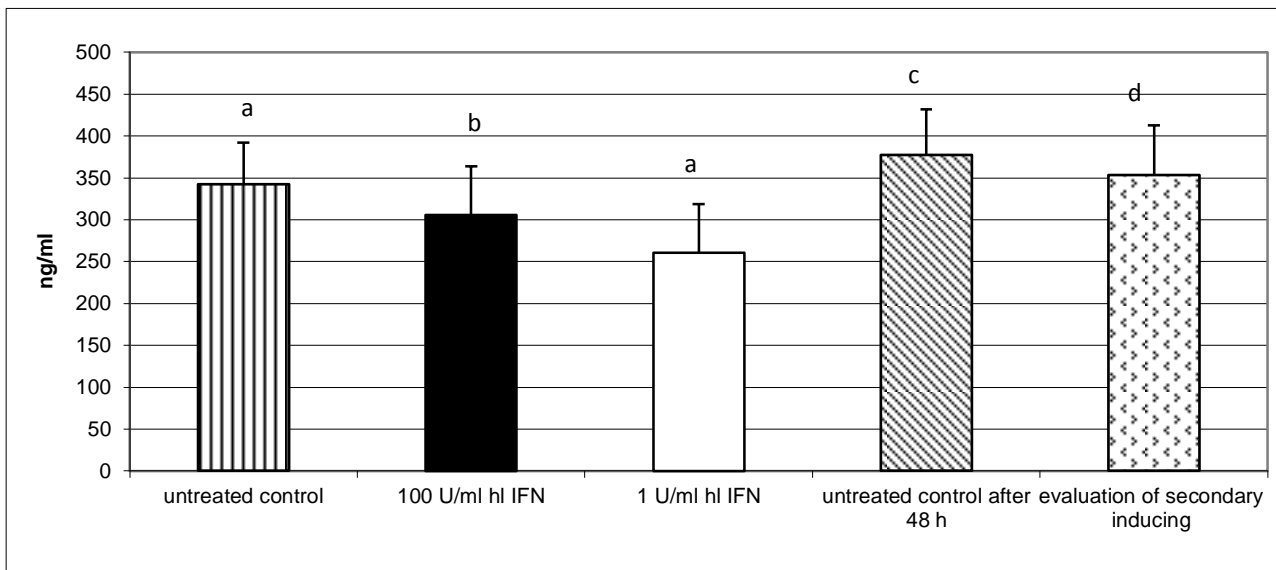
Cells obtained from 10 pigs were treated with IFN-α. 5 tonsil with porcine natural IFN-α and 5 with human lymphoblastoid IFN-α at 1 and 100 UI/ml than incubated 18 hours at 37 °C in 5% CO2. After 18 hours of incubation 200 μl of tonsil supernatant (about 6×10^5 cells) from wells treated with 1 U/ml of IFNs (hl IFN-α and nIFN-α) were collected and transferred into tonsil control wells, and incubated at 37 °C in 5% CO_2 for another 24 hours. Swine IgA was measured in the cell supernatant. Differences between means were checked for significance by one-way analysis of variance (ANOVA). The threshold for significance was set at P<0.05. Same superscripts indicate significant differences.

Figure 3

A) IL-8 release by IPEC-J2 treated with tonsil supernatants obtained after 24 or 48 hours of incubation
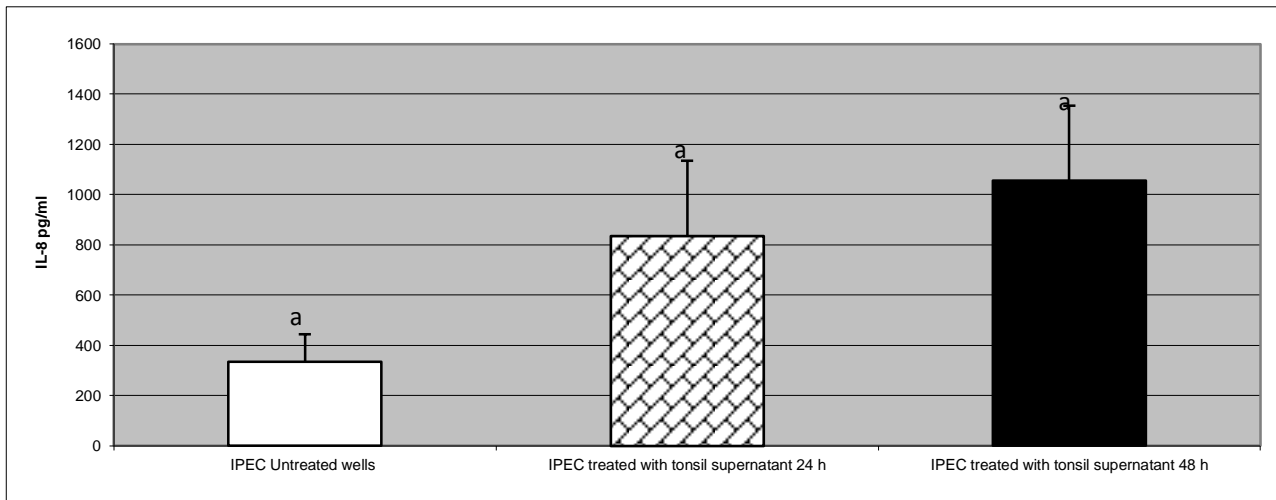
Results are shown in terms of pg/ml ± standard deviation. The same superscripts on the bars indicate significant differences (P<0.05) in one-way ANOVA for repeated measures.

B) IL-8 release by IPEC-J2 cells incubated with tonsil supernatant after treatment with 100 U/ml of different IFN-α
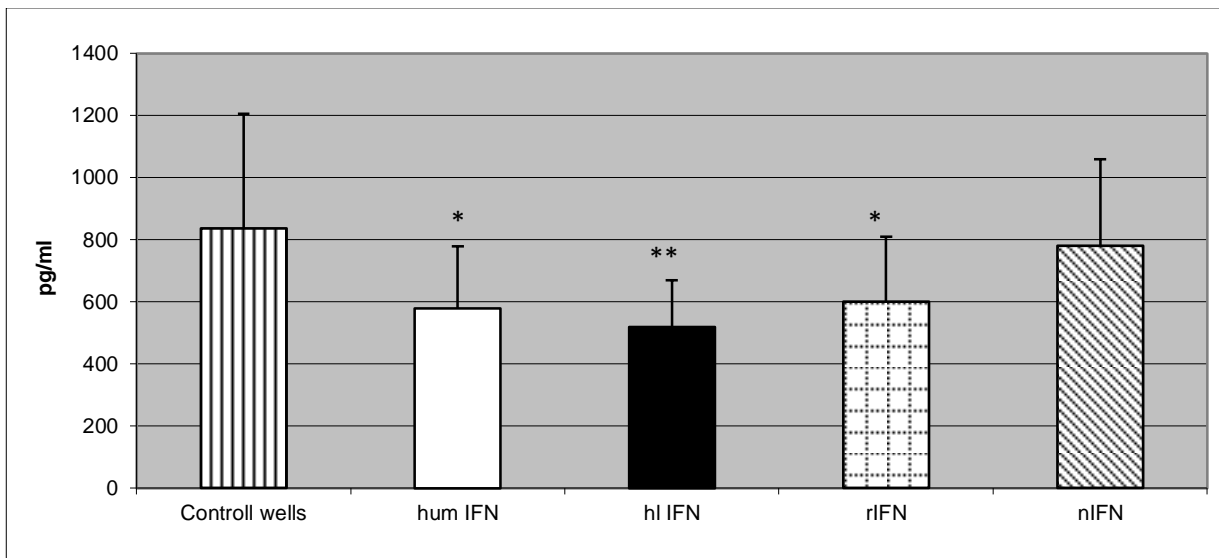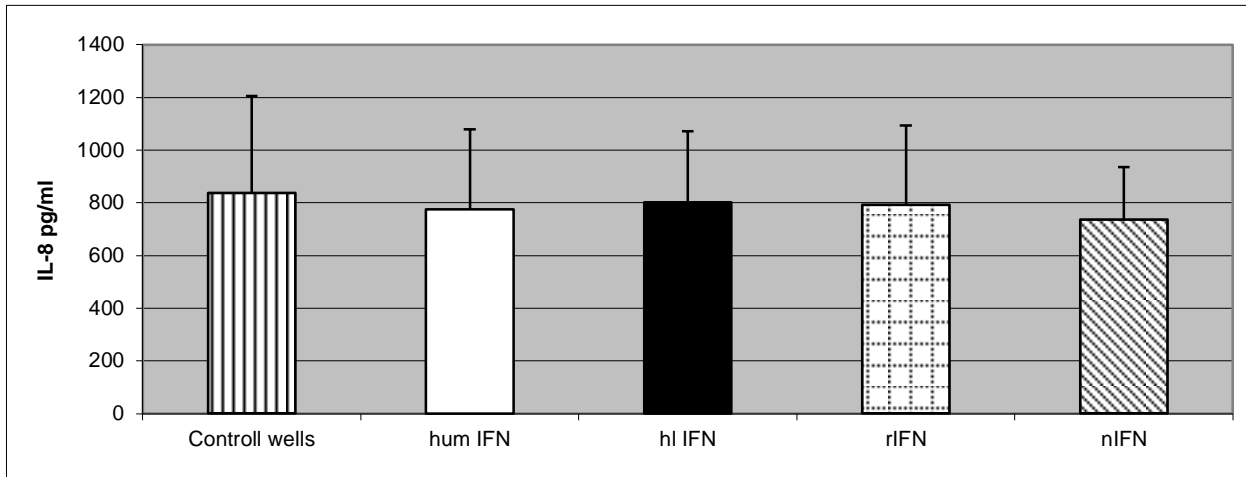

Tonsil cells from 10 pigs were treated with four different types of IFN-α: natural IFN-α (nIFN-α), recombinant porcine IFN-α (rIFN-α), human lymphoblastoid (hl IFN-α) and humoferon (hum IFN-α) at 100 U/ml. Cells were incubated for 18 hours at 37 °C in 5% CO_2. Then, samples of cell supernatants were harvested and stored at -80°C for an IPEC-J2 stimulation test. Five tonsil cell cultures were treated with rIFN-α, hum IFN-α and hl IFN-α; the other five with rIFN-α, hum IFN-α and nIFN-α, respectively. IPEC-J2 cells were stimulated with tonsil supernatants diluted 1:4. Results are shown in terms of pg/ml of IL-8 ± 1 standard deviation. Differences were checked for significance by one-way ANOVA for repeated measures. *P<0.05   **P<0.001.
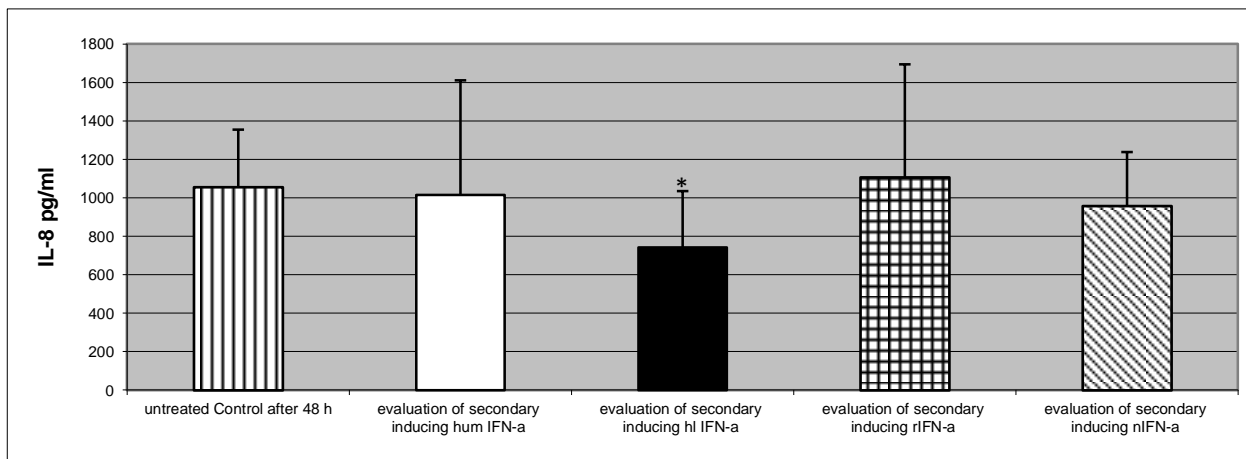
Figure 4

Effects of 1 U/ml of different IFNs-α on IL-8 secretion by IPEC-J2 cells

A)

B)

Pig tonsil cells were treated with four different types of IFN-α: natural IFN-α (nIFN-α), recombinant porcine IFN-α (rIFN-α), human lymphoblastoid (hl IFN-α) and humoferon (hum IFN-α) at 1 U/ml for 18 hours at 37 °C in 5% CO_2. For every tonsil cell culture 6 control wells remained untreated. After 18 hours of incubation, 200 μl of tonsil cell suspension (about 6×10^5 cells) from wells treated with IFNs were collected and transferred into tonsil control wells for a further 24 hours. Then, samples of cell-free supernatants were harvested and stored at -80°C for an IPEC-J2 stimulation test. Five tonsil cell cultures were treated with rIFN-α, hum IFN-α and hl IFN-α; the other five with rIFN-α, hum IFN-α and nIFN-α, respectively. IPEC-J2 were stimulated with tonsil supernatants diluted 1:4. Results are shown in terms of pg/ml of IL-8 ± 1 standard deviation. Differences were checked for significance (P<0.05) by one-way ANOVA for repeated measures.

Figure 5

Effects of tonsil supernatant on gene expression in IPEC-J2 cells
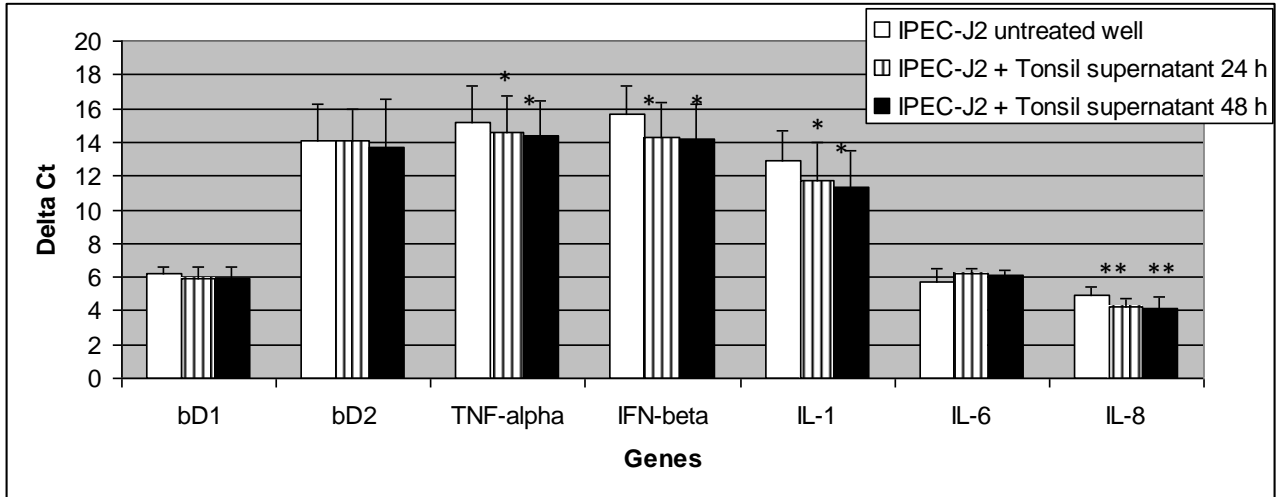
Pig tonsil cells were incubated at 37 °C in 5% CO_2. Supernatants were harvested 24 or 48 hours later and stored under aseptic conditions in aliquots at − 80°C. IPEC-J2 cells at confluence were washed once with MEM medium and treated with 1:4 diluted tonsil supernatants for 18 h at 37 °C in 5% CO_2. RNA of tonsil cells was extracted to evaluate the expression of bD1, bD2, IL-1β, TNF-α, IL-8 and IL-6 genes.

Data are express as ΔCt . The results were analyzed by one-way ANOVA. The stars indicate significant differences between untreated and tonsil supernatant-treated cells.
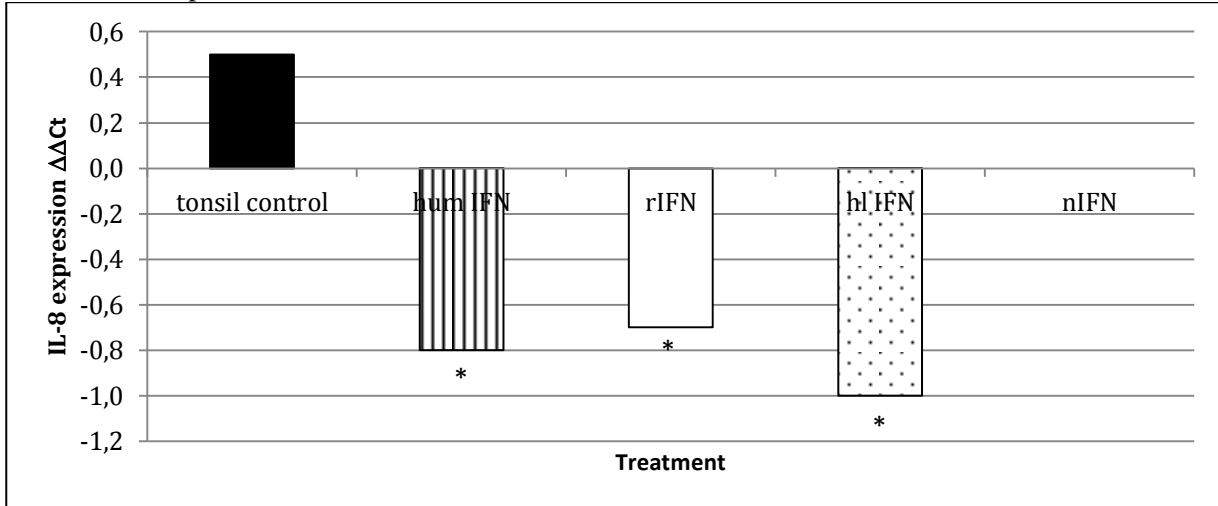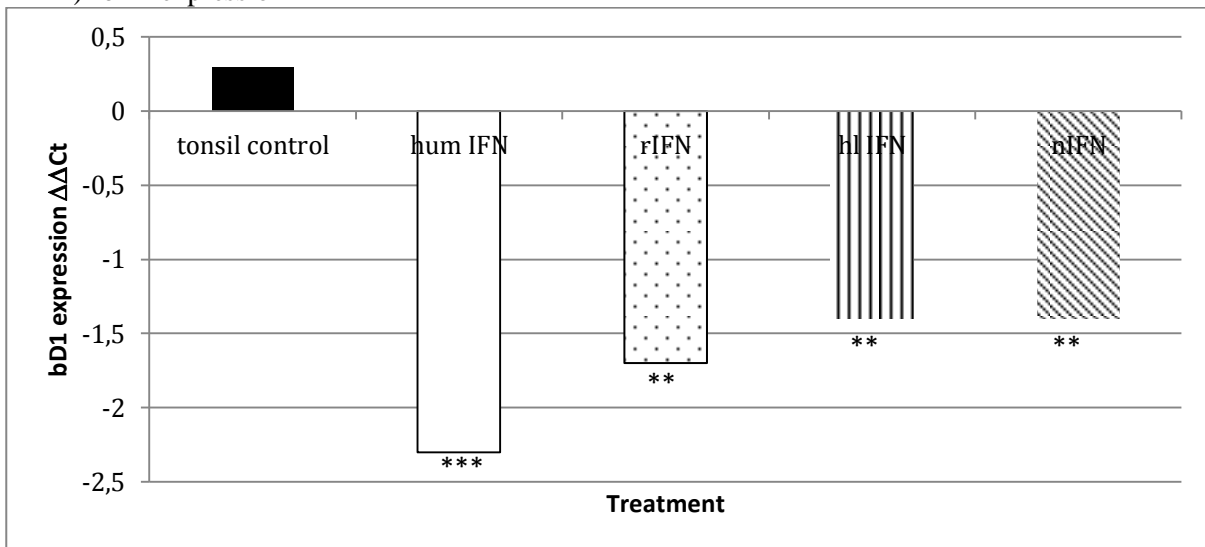
**$p < 0.01$

*$p < 0.05$

Figure 6

Effects of different types of IFN-α on gene expression in IPEC-J2 cells

A) IL-8 expression

B) bD1 expression

Pig tonsil cells were treated with 100 U/ml of different IFNs-α or kept as untreated controls. Supernatants were harvested 24 hours later and stored under aseptic conditions in aliquots at − 80°C. IPEC-J2 cells at confluence were washed once with MEM medium and treated with 1:4-diluted tonsil supernatants for 18 h at 37 °C in 5% CO_2. RNA of IPEC-J2 was extracted to evaluate gene expression in RT Real-time PCR. Data are express as ΔΔCt. Samples were analyzed by one-way ANOVA. The stars indicate significant differences between IPEC-J2 cells exposed to supernatants of control and IFN-treated tonsil cells. *$P<0.05$ **$P<0.01$ ***$P<0.001$.
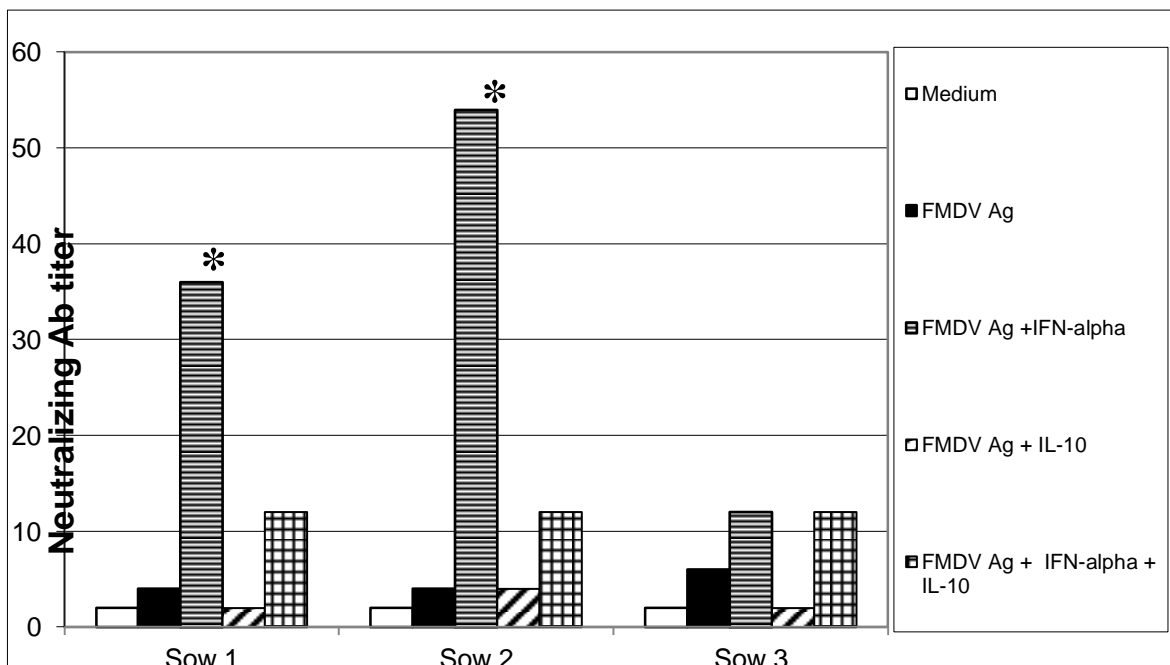
Figure 7

***In vitro* recall Ab response of swine PBMC to FMDV**

A) Day 7 in culture
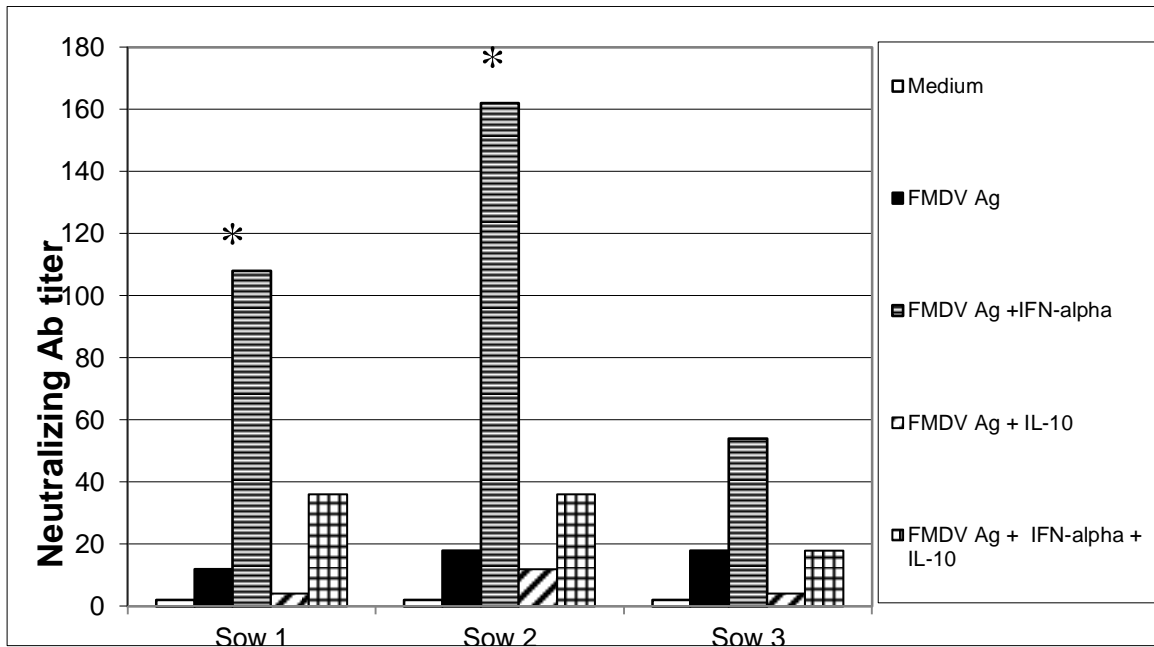
B) Day 12 in culture

PBMC of FMD-vaccinated sows were separated and grown in the presence or absence of inactivated FMDV antigen (1 μg / ml final), hl IFN-α at 5 U / ml final, porcine recombinant IL-10 (20 ng/ml final). Neutralizing antibody to FMDV O_1 Manisa was measured in supernatants of PBMC cultures at days 7 and 12.

CHAPTER 4

DISEASE-DEPENDENT MODULATION OF TONSIL CELLPHENOTYPE

E. Razzuoli§, M. Pesciaroli[2], N. Martinelli[1], J. Ruggeri[1], S. Dotti[1], R. Villa[1], M. Ferrari[1], S. Amendola[3], A. Battistoni[3], L. Alborali[1], P. Pasquali[2], G. Pezzoni[1], E. Pegoiani[1], L. Stercoli[1], A. Ferrari[1], M. Amadori[1]

[1]Laboratory of Cellular Immunology, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna , Brescia, Italy

[2]Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità, Roma, Italy.

[3] Department of Biology, Università di Roma Tor Vergata, Roma, Italy.

§Laboratory of Diagnostics, S.S. Genova, IZSPLV, Genova, Italy.

* Corresponding author. Tel. +39 030 2290248; fax +39 030 2290392

E-mail address: massimo.amadori@izsler.it (M. Amadori)

ABSTRACT

Tonsils are secondary lymphoid organs which, often represent the first site of interaction between microbial agents and lymphoid tissues. In this project we wondered if homeostasis of tonsil lymphocytes could be affected by two important disease agents: PRRSV and *Salmonella thyphimurium*. Our results demonstrate that PRRSV can modulate B cell populations in tonsils, giving rise to an increased prevalence of λ light chain-positive B cells. This type of modulation and the ability of this virus to modulate the IFN system suggest that PRRSV can alter the results obtained with our *in vitro* model if tonsils from infected animals are employed to this purpose. The same conclusion can be drawn in case of salmonella infection that caused modulation of tonsil lymphocyte populations. In conclusion, our experiment outlines the need to use healthy animals to set up our *in vitro* model. This could be conveniently employed not only to investigate cytokine-driven effects, but also the possible impact of viral and bacterial infections on innate and adaptive immunity.

Key word: Tonsil, lymphocytes, phenotypes, PRRSV, Salmonellosis

1.INTRODUCTION

Tonsils are secondary lymphoid organs which represent a major component of mucosa-associated lymphoid tissues (MALT). They can be infected in swine by viral agents which sustain e.g. classical swine fever, pseudorabies, foot-and-mouth disease and porcine respiratory and reproductive syndrome (PRRSV), to cite a few (Alexandersen et al., 2001; Romero et al., 2003; Rowland et al., 2003). In addition to these, various non-pathogenic and pathogenic bacteria like *Salmonella spp., Yersinia enterocolitica, Pasteurella multocida, Haemophilus parasuis, Mycoplasma hyopneumoniae* can be isolated from porcine tonsils (Lowe et al., 2011; O'Sullivan et al., 2011). Moreover, there is evidence of an important role in the host defense played by tonsils, which often represent the first site of interaction between microbial agents and lymphoid tissues. Owing to the above, we wondered if homeostasis of tonsil lymphocytes could be affected by microbial disease agents. To resolve this issue, we based on our *in vitro* model based on pig tonsil cells. This was adopted to evaluate the effects of two important disease agents: PRRSV and *Salmonella thyphimurium*.

2. MATERIALS AND METHODS

2.1 Animal experiments

All animal experiments were conducted at IZSLER, Brescia, in compliance with the Ethical Committee for Animal Experimentation of the Institution. The treatment, housing and husbandry conditions conformed to the European Union Guidelines (Directive 2010/63/EU on the protection of animals used for scientific purposes). Animal care and procedures were in accordance with the guidelines of the Good Laboratory Practices (GLP).

Experiment 1: We included in our study nine, 60 day-old Landrace x Large White pigs,. Animals were randomly divided into two groups: Group 1 (6 animals) and Group 2 (3 animals). After 1 week of acclimatization animals of group 1 were infected with a PRRSV strain (European , type I); for each pig 2 ml of viral suspension/nostril (104.5 TCID50/ml) were administered. Pigs of group 2 served as controls.
Clinical inspections and blood samplings were carried out by an appointed veterinary officer under the control of the Animal Health and Welfare Division of the Italian National Veterinary Services. Blood samples were collected at days post infection (p.i.)0 (T0 = exposure day) and 14. At day120 p.i. animals were sacrificed and tonsils were collected as previously described (Razzuoli et al., 2012). Cells were frozen at -80 °C for flow cytometry tests.

Experiment 2: We included in our study twenty-seven, 30 day-old piglets sero-negative for Salmonella spp. These were randomly allocated to three groups of 9 animals each, and treated as follows:

- (C) uninfected controls.

- (AT) oral administration of 5x109 CFU of attenuated (Pesciaroli et al., 2013) Salmonella enterica serovar typhimurium ΔznuABC.

- (WT) oral administration of $5x10^9$ CFU wild-type S. typhimurium.

On a daily basis, piglets were monitored for clinical conditions and feces were collected for bacteriological analyses. In addition, three animals per group were sacrificed at days p.i. 1, 3 and 7 for post-mortem examination, bacteriological tests on organs and immunological analyses.

2.2 Blood and fecal samples

Blood samples without anticoagulant were collected from animals of experiment 1. These were kept at room temperature for 2 h, refrigerated for 30 min, and then centrifuged at 4°C for 15 min at 2,000 rpm; serum was separated and stored in aliquots at 80°C for ELISA tests. The antibody response of pigs was evaluated by a commercial kit (Herdchek Porcine Reproductive and Respiratory Syndrome Antibody test Kit, IDEXX Laboratories) according to the manufacturer's directions. The threshold for low-positive sera was set at a sample to positive (s/p) ratio of 0.4 according to the following formula:

(Sample : PRRSV) – (Sample : NHC) / (Positive Control:PRRS) – (Positive Control : NHC), where NHC stands for normal host cell antigen.

Fecal samples of each pig were collected from animals of experiment 2 to assess fecal shedding of bacteria. The microbiological analysis was conducted according to the ISO 6579:2002/Amendment 1:2007 protocol.

2.3 Flow cytometry

Staining of cells was carried out according to an established procedure (Amadori et al., 2009), with minor modifications. Briefly, frozen tonsil cells were rapidly thawed at 38 ∘C and washed twice with ice-cold FACS-Buffer (2% FCS in PBS + 0.1% sodium azide). Then, they were counted, divided into aliquots (106 cells each) and reacted with mAb 8/1 (SwC1) (Saalmüller et al., 1994), 1G6 (anti-swine Ig light chain), 2E8 (anti-swine IgM) (Archetti et al., 1993), a biotinylated anti-swine Ig antiserum react-ing with all Ig isotypes (Vector Lab, Peterborough, UK, cat. BA9020), mAb anti-pig IgA (AdB Serotec, cat. MCA638), mAb CD3 (Talker et al., 2013), mAb CD21 (Southern Biotech, cat. 4530-02), Mil2 (AbCAM, cat. 23919-1), PMN (AbD Serotec, cat. MCA2599F) or FACS buffer only (control) for 30 min at 4 ∘C, respectively. Cells were washed, and again incubated for 30 min at 4 ∘C in FACS buffer containing either goat anti-mouse IgG-FITC (Invitrogen, Molecular Probes®, cat: A10683) for the aliquots reacted with mAb 8/1, 1G6, IgA and 2E8, Mild2, CD3 or Phycoerythrin-conjugated streptavidin (Southern Biotechnology Associates, cat. 7100) for the aliquot reacted with the biotinylated antiserum. After washing twice in FACS buffer, cells were resuspended

in 100 μL of the same buffer and 1:4 diluted. Samples were analyzed in a GUAVA MILLIPORE flow cytometer (Millipore Software). The typical forward and side scatter, lymphocyte/monocyte gate was set to exclude dead cells from the analysis. The percentage of positive cells beyond the threshold fluorescence channel was assessed in each sample on 10,000 events and compared between mAb-treated and control cells. For each antibody, results were expressed in terms of net percentage of positive cells.

2.4 Antibacterial activity of tonsil cell supernatants in experiment 2

Tonsil cell cultures were set up in antibiotic-free medium at 37 °C in 5% CO2. Supernatants were collected after 2 h and antibacterial activity of supernatants was determined by a qualitative microbiological screening method based on reference bacterial strains (Okerman et al., 1998). Briefly, 1.9×105 Colony-Forming Units (CFU) of Bacillus subtilis (Merck, Whitehouse Station, NJ, USA. cod 10649) were seeded in 10-cm Petri dishes within melted agarized medium (Antibiotic medium 1, OXOID cat. CM 0327B). 2×105 CFU of Escherichia coli 14 strain were seeded into melted agarized Standard II medium (Bicrobiol, cat. 70.233). After medium solidification, 50 μL of each tonsil cell supernatant were put in duplicate into 10-mm holes made in the agar. After a 30-min period of diffusion, the Petri dishes were incubated at 30 °C for 12 h. Then, the diameters of growth check were measured in comparison with that of the supernatant of the 5th (last) washing of the cells before culture (possible antibacterial activity due to antibiotic residues).

3. RESULTS

The choice of pathogens in our experiments was justified by the peculiar features of PRRSV and salmonella. In the first experiment, animals of group 2(controls) did not show any sign of disease throughout the observation period. The animals of group 1 showed anorexia, prostration at day 2 and 3 p.i., Serum samples were positive for PRRSV-specific antibody at day 7 p.i. The flow cytometry tests revealed a peculiar modulation of B-lymphocyte populations; in particular, we showed in animals of group 1 an increased prevalence of light chain λ- positive B cells with respect to animals of group 2 (43,4 ±2.1% vs. 36.3 ±2.5%, Figure 1) and a peculiar up-regulation of sIgM-positive B cells (39.9 ±1.8% in group 1 and 5.1 ±2.1% in group 2). The results obtained on group 2 are in agreement with our previous study (Razzuoli et al., 2012) and suggest that PRRSV infection modulates the homeostasis of pig tonsil lymphocytes.

Regarding the second experiment, the main results obtained can be summarized as follows. Piglets of group C did not show any sign of disease throughout the observation period. Piglets of group WT showed prostration, anorexia, reluctance to move, a transient increase of body temperature and diarrhea. During post mortem examination, both ileum and proximal part of the colon resulted slightly congested. Overall, the effect of the infection was more evident at days 1 and 3 p.i. Piglets of group AT showed the same clinical condition as piglets of group WT but to a lower extent. SIg+ B cells decreased at days 1 and 3 p.i. in the WT group (Figure2), whereas no major changes were observed in the prevalence of CD14+ cells (mainly monocytes). With respect to ileocolic lymph nodes, an opposite time-course of 2B2+ myeloid cells was observed in tonsils of WT-infected animals (decrease as early as PID 3). Concerning antimicrobial compounds, these were released by cultured tonsil cells of the 3 groups at days 1 and 3 p.i. (Table 1). Activity against E. coli was maintained at day 3 p.i. in cultures of control animals, only.

4 DISCUSSION AND CONCLUSION

In this study we evaluated the influence of two important disease agents on our cell model: PRRSV and Salmonella thyphimurium. Regarding PRRSV, there is strong evidence that this viral agent suppresses T-cell recognition of infected macrophages (Xiao et al., 2004), which can be eased by the poor accessory features of porcine alveolar macrophages (Basta et al., 2000). Also, distinct signs of immunosuppression are frequently observed in PRRS virus-infected pigs, which can be conducive to an increased incidence and severity of secondary bacterial infections (Thanawongnuwech et al., 2000; Allende et al., 2000), and contribute to the "Porcine Respiratory Disease Complex". PRRSV is also capable of potentiating the effects of a second viral infection (Van Reeth, 1997).The main immunosuppressive features of PRRSV have been actively investigated to reveal the structural and non-structural virus components exerting such activities. As for the down-regulation of the type I IFN response, a main role was evidenced of PRRSV non-structural proteins (NSPs) 1α, 1β, 2, 4, 11, with effector mechanisms related to inactivation and nuclear translocation of Interferon Response Factor (IRF) 3, ISG factor 3 (ISFG3), STAT 1, as well as processing of ISG15 and

IKKα (IFN response and NF-kB signaling, respectively). In practice, there is evidence that multiple suppressive functions are exerted by NSPs, whereas further suppressive activities could be related to IL-10 induced by the nucleocapsid (N) protein (Yoo et al., 2010). In our experiment we just evaluated the effects on tonsil lymphocytes in terms of cellular homeostasis. Our results demonstrate that PRRSV can modulate B cell populations in tonsils, giving rise to an increased prevalence of λ light chain-positive B cells. This type of modulation and the ability of this virus to modulate the IFN system suggest that PRRSV can alter the results obtained with our *in vitro* model if tonsils from infected animals are employed to this purpose.

The same conclusion can be drawn from experiment two, since also a salmonella infection caused modulation of tonsil lymphocyte populations. Moreover a previous study of ours had outlined the ability of Salmonella thyphimurium to modulate antimicrobial peptide expression in IPEC-J2 model; in this study we could confirm this effect on tonsil lymphocytes.

In conclusion, our experiment outlines the need to use healthy animals to set up our *in vitro* model. This could be conveniently employed not only to investigate cytokine-driven effects, but also the possible impact of viral and bacterial infections on innate and adaptive immunity.

Figure 1

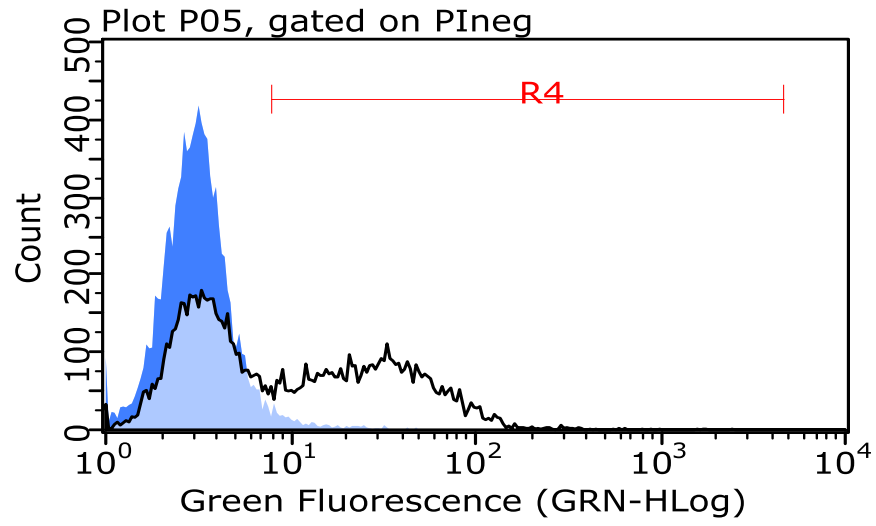**Expression of light chain λ on tonsil B-lymphocytes after PRRSV infection**

Figure 2

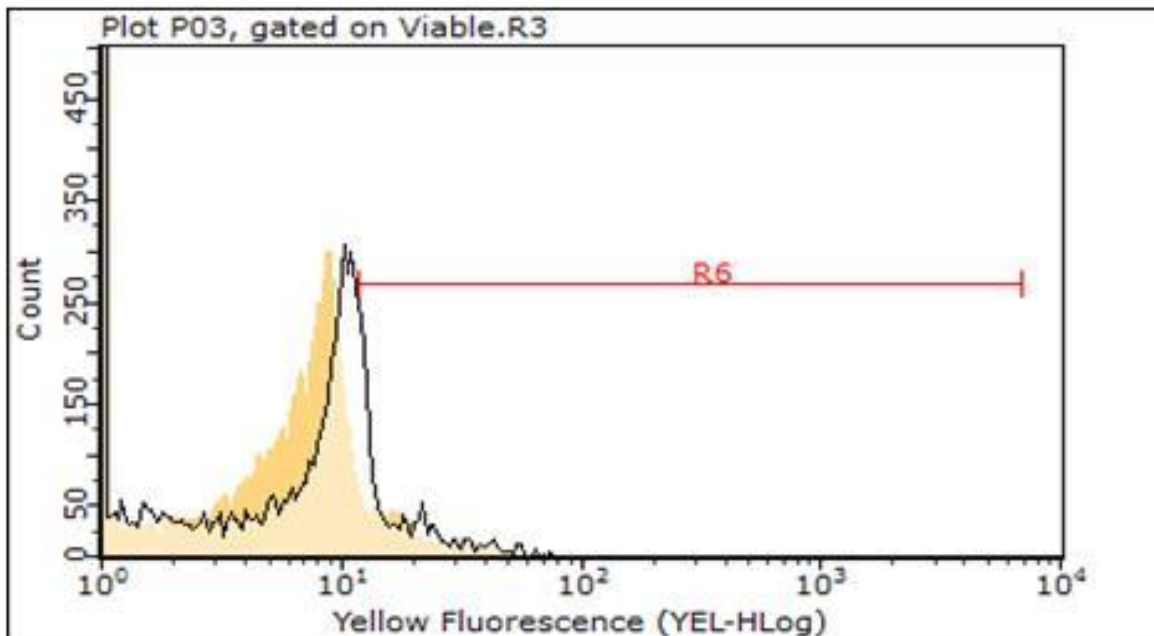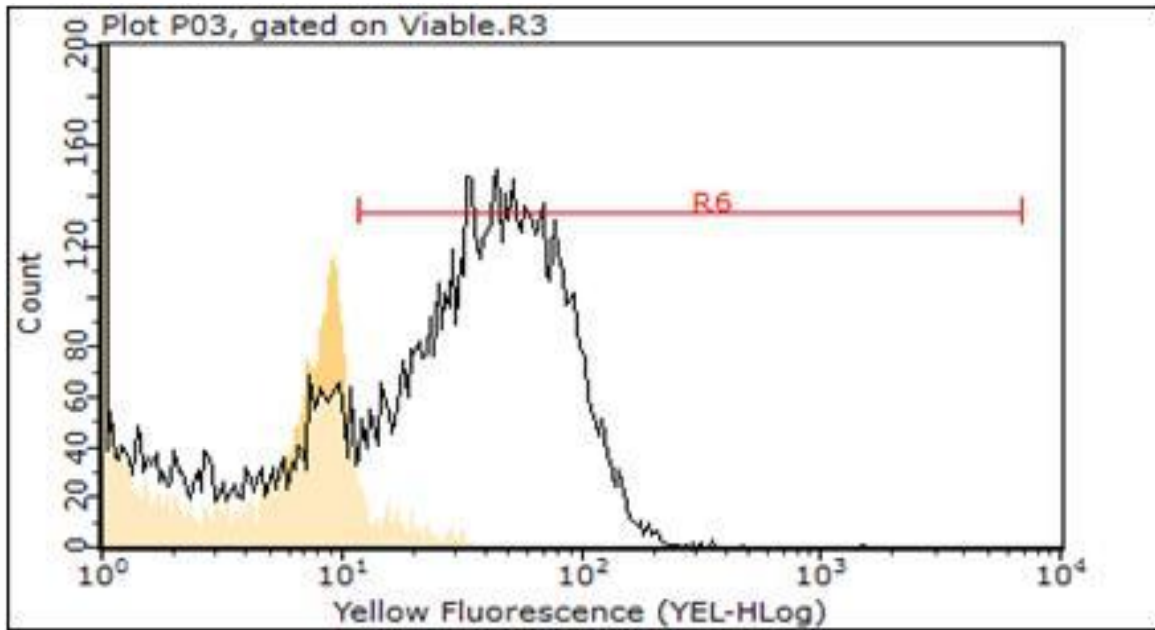**Down-regulation of sIg+ B cells in tonsils of WT Salmonella-infected pigs on PID 3**

Table 1

Release of antimicrobial compounds at days 1 and 3 p.i.

Cultured Tonsil Cells	Control Group		AT Group		AW Group	
	B. subtilis	*E. coli*	*B. subtilis*	***E. coli***	*B. subtilis*	***E. coli***
PID 1	3,8	4,3	3,9	3,8	4,4	4
PID 3	2,8	5	3	**0**	3,4	**0**

Data are expressed as mm of inhibition haloes.

REFERENCES

1. Alexandersen, S., Oleksiewicz, M.B., Donaldson, A.I., 2001. The early pathogenesis of foot-and-mouth disease in pigs infected by contact: a quantitative time-course study using TaqMan RT-PCR. J. Gen. Virol. 82, 747–755.

2. Allende, R., Laegreid, W.W., Kutish, G.F., Galeota, J.A., Wills, R.W., Osorio, F.A., 2000 Porcine reproductive and respiratory syndrome virus: description of persistence in individual pigs upon experimental infection. J Virol. 74:10834-10837.

3. Archetti, I.L., Capucci, L., Saalmüller, A., Verardi, R., Konig, M., Amadori, M., 1993. Production and characterization of monoclonal antibodies differentiating subpopulations of porcine B lymphocytes in blood and lymphoid tissues. Zentralbl. Veterinarmed. B 40, 485–493.

4. Basta, S., Carrasco, C.P., Knoetig, S.M., Rigden, R.C., Gerber, H., Summerfield, A., McCullough, K.C., 2000. Porcine alveolar macrophages: poor accessory or effective suppressor cells for T-lymphocytes. Vet Immunol Immunopathol. 29;177-190.

5. Lowe, B.A., Marsh, T.L., Isaacs-Cosgrove, N., Kirkwood, R.N., Kiupel, M., Mulks, M.H., 2011. Microbial communities in the tonsils of healthy pigs. Vet. Microbiol. 147, 346–357.

6. O'Sullivan, T., Friendship, R., Blackwell, T., Pearl, D., McEwen, B., Carman, S, Slavic, D., Dewey, C., 2011. Microbiological identification and analysis of swine tonsils collected from carcasses at slaughter. Can. J. Vet. Res. 75, 106–111.

7. Okerman, L., De Wasch, K., Van Hoof, J., 1998. Detection of antibiotics in muscle tissue with microbiological inhibition tests: effects of the matrix. Analyst 123, 2361–2365.

8. Pesciaroli, M., Gradassi, M., Martinelli, N., Ruggeri, J., Pistoia, C., Raffatellu, M., Magistrali, C.F., Battistoni, A., Pasquali, P., Alborali, G.L., 2013. Salmonella Typhimurium lacking the Znuabc transporter is attenuated and immunogenic in pigs. Vaccine. 12, 2868-2873.

9. Razzuoli, E., Faggionato, E., Dotti, S., Villa, R., Lombardo, T., Boizza, L., Ferrari, M., Amadori, M. 2012. Isolation and culture of pig tonsil lymphocytes. Vet Immunol Immunopathol 148(3–4): 320–325.

10. Romero, C.H., Meade, P.N., Homer, B.L., Shultz, J.E., Lollis, G., 2003. Potential sites of virus latency associated with indigenous pseudorabies viruses in feral swine. J. Wildl. Dis. 39, 567–575.

11. Rowland, R.R., Lawson, S., Rossow, K., Benfield, D.A., 2003. Lymphoid tissue tropism of porcine reproductive and respiratory syndrome virus replication during persistent infection of pigs originally exposed to virus in utero. Vet. Microbiol. 96, 219–235.

12. Saalmüller, A., Aasted, B., Canals, A., Dominguez, J., Goldman, T., Lunney, J.K., Pauly, T., Pescovitz, M.D., Pospisil, R., Salmon, H., 1994. Analysis of mAb reactive with the porcine SWC1. Vet. Immunol. Immunopathol. 43, 255–258.

13. Talker, S.C., Käser, T., Reutner, K., Sedlak, C., Mair, K.H., Koinig, H., Graage, R., Viehmann, M., Klingler, E., Ladinig, A., Ritzmann, M., Saalmüller, A., Gerner, W. 2013. Phenotypic maturation of porcine NK- and T-cell subsets. Dev Comp Immunol. 40:51-68.

14. Thanawongnuwech, R., Brown, G.B., Halbur, P.G., Roth, J.A., Royer, R.L., Thacker, B.J., 2000. Pathogenesis of porcine reproductive and respiratory syndrome virus-induced increase in susceptibility to Streptococcus suis infection. Vet Pathol. 37:143-152.

15. Van Reeth, K., 1997. Pathogenesis and clinical aspects of a respiratory porcine reproductive and respiratory syndrome virus infection. Vet Microbiol. 55: 223-230.

16. Xiao, Z., Trincado, C.A., Murtaugh, M.P., 2004. Beta-glucan enhancement of T cell IFN-gamma response in swine. Vet Immunol Immunopathol. 102 :315-320.

17. Yoo, D., Song, C., Sun, Y., Du, Y., Kim, O., Liu, H.C., 2010. Modulation of host cell responses and evasion strategies for porcine reproductive and respiratory syndrome virus. Virus Res. 154, 48-60.

CONCLUSIONS

There is uncertainty as to how cytokines are effective after oral administration. They probably act on the oral lymphoid tissues (palatine and pharyngeal tonsils) and trigger a cascade of events leading to activation of the immune system and control of the inflammatory cascade in tissues and organs. In this project, we developed n *in vitro* methods to evaluate some effects of cytokines on pig tonsil lymphocytes. The project was divided as follows: 1) Isolation and culture of pig tonsil lymphocytes; 2) IPEC-J2 cells as reporter system of the anti-inflammatory control actions of interferon-alpha; 3) A pig tonsil cell culture model for evaluating oral, low-dose IFN-α treatments; 4) Disease-dependent modulation of tonsil cell phenotypes.

In the first step, we defined operating conditions for tonsil processing, control of bacterial contaminations, time limits of cell storage at -80°C, as well as for evaluating polyclonal Ig production *in vitro*. Such procedures are likely to be of some importance in studies on regional immunity and in the development of large animal models for biomedical sciences.

The results obtained in the second and third steps indicate that tonsil cell cultures are a suitable *in vitro* model to evaluate the effects of oral, low-dose cytokine treatments. Therefore, this model could be adopted to develop protocols of oral administration of cytokines. Such a large animal model could be actually more predictive than others based on laboratory rodents, in view of possible clinical applications in humans. Moreover , in according to 3 Rs principle, our test procedures could be suitable alternatives to animal testing in according to directive EC/63/2010.

Beyond the development of these models, our results in pigs highlight the possible use of Type I IFNs to modulate the innate immune response toward greater disease resistance of farm animals, decreased drug usage and a consequently greater safety of the food chains. Regarding this tenet, the recent OIE/IABS international conference on "Alternatives to Antibiotics" (OIE / IABS, 2012) provided evidence that a reduction of antibiotic usage in farm animals can be achieved by a proper combination of natural antibacterial peptides, biological response modifiers, pre and probiotics, as well as by a correct development of the gut microbiome. Regarding antimicrobial peptides, our models could be employed to evaluate the ability of some cytokines or other compounds to modulate the release of antimicrobial peptides .

Concerning the 4th step of our project, our experiments outline the need to use healthy animals to set up our *in vitro* model. This could be conveniently employed not only to investigate cytokine-driven effects, but also the possible impact of viral and bacterial infections on innate and adaptive immunity. Furthermore, this model could be employed to evaluate the effects of non-infectious stressors (e.g. mycotoxins) and outright endocrine destroyers (heavy metals, dioxins) on the immune system.

ACKNOWLEDGMENTS

I would like to acknowledge my PhD tutors, Professor Luigi Bonizzi and doctor Massimo Amadori for taking me as their graduate student and letting me study animal immunology. Massimo was always helpful and encouraging and his experience and guidance let me to go on in this program and let me grow as a researcher and individual.

Special thanks to Cinzia Mantovani, who has supported me in these years and performed cellular test with me.

I would also like to acknowledge all the people that have get part to the research project for the helpful suggestions and their support to perform the experiments.

Thank you to my parents, my sisters and my husband for their love and support. Special thanks to my nephew Matteo, him made me smiling in the bad periods.