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Evaluation of the immune response induced by intradermal vaccination by using a needle-less system in comparison with the intramuscular route in conventional pigs

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ABSTRACT

The immune response induced by intradermal vaccination using a needle-less device was evaluated in conventional pigs in comparison with the more conventional intramuscular vaccination; to this purpose, vaccination against Aujeszky's Disease (AD) was used as a model of antiviral immunity. Two groups of pigs (n = 10 each) were vaccinated 4 weeks apart respectively by the intramuscular (IM group) and intradermal route (ID group; needle-less I.D.A.L.® vaccinator) with an AD modified live virus. Ten pigs injected with the vaccine adjuvant only were kept as sham-vaccinated controls (C group).

On blood samples collected at 0, 2, 4, 5, 6 and 7 weeks post-vaccination (PV) ADV-specific virus neutralizing (VN) antibodies, IFN-γ secreting cells (SC), lymphocyte subsets and IFN-γ gene expression in PBMC were evaluated.

VN antibodies increased after the 1st vaccination and peaked after the 2nd vaccination in both vaccinated groups. Also IFN-γ SC reached maximum levels in both groups after administration of the booster dose. Pigs in the control group remained negative for both parameters throughout the study. Flow cytometry showed persistently higher levels of CD3−CD8α+ Natural Killer cells in both vaccinated pigs. The ID group showed an earlier and regulated activation characterized by an increase of cytotoxic CD8β+ T lymphocytes and CD25+ cells after the boosting dose. No statistically significant differences between treated and control groups were detected for memory CD4+CD8α+^{low} T cells. Upregulation of IFN-γ gene expression in PBMC was detected in ID and IM pigs after both vaccine administrations, although at a different extent. Overall, the results showed that the intradermal vaccine delivery by a needle-less device can prime a strong humoral and cellular immune response comparable to that obtained by the intramuscular vaccination.

1. Introduction

Progress in pig immunology has allowed better evaluation of the efficiency of the immune response to viral infections, leading to improved efficacy of conventional vaccines and development of new vaccination strategies for prevention, control and eradication of viral infections.

During viral infection, besides the role of antibodies, cell-mediated immunity plays a major task in the reduction of virus replication and disease prevention. Regulation of the antiviral response is generally due to differentiated and activated CD4+ T helper-1 lymphocytes that migrate into the sites of infection and secrete cytokines such as IFN-γ. This cytokine is involved in both triggering the innate responses and sustaining adaptive immunity. In particular, IFN-γ induces CD8+ cell differentiation into cytotoxic effector cells and promotes the killing of infected cells by Natural Killer (NK) cells and macrophages, as well as stimulates IgG synthesis (Zuckermann et al., 1998; Laval et al., 2002; van Rooij et al., 2004; Charerntantanakul and Roth, 2006).

A typical effector activity against viruses is mediated in swine by CD8+ MHC-I-restricted cytotoxic T lymphocytes (CTL), which secrete perforins and granzyme B, promote viral clearance and stimulate immunological memory (Harty et al., 2000; Seder and Hill, 2000; Suradhat et al., 2001; Woodland, 2003; Welsh et al., 2004). Also virus neutralizing (VN) antibodies mediate several responses such as blocking of virus-cell interaction and viral penetration. Antibody opsonisation can trigger virus clearance sustaining phagocytosis by macrophages, killing of infected cells by complement-mediated cytolysis, or antibody-dependent cellular

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cytotoxicity (ADCC) by NK or Lymphokine Activated Killer (LAK) cells (Mettenleiter, 1996; Welsh et al., 2004).

Several studies have been carried out to investigate the humoral immune response after vaccination/infection, but efficient immune cell activation is considered a fundamental parameter to evaluate the efficiency of vaccination protocols.

Furthermore, new routes of vaccine administration have also been studied, not only to improve the efficiency of vaccination, but also to increase the practical advantages of control programmes when mass vaccination is applied. Needle-less vaccination has been used as an advantageous method to cross the epidermal barrier and efficiently deliver antigen into the dermal layer (Giudice and Campbell, 2006). Some of the most important advantages of the intradermal administration by a needle-less device are that it is less invasive and painless, safe, as well as quick and easy; furthermore it needs a reduced volume of fluid as compared to the more conventional intramuscular route (Zhao et al., 2006; Goubier et al., 2008).

Some previous studies showed the importance of the route and device for vaccine administration in the improvement of protective immunity. Besides the traditional intramuscular (IM) route, the intradermal (ID) route, utilizing needle-less systems, has been proposed as a new tool for vaccination (van Rooij et al., 1998; Mikulska-Skupien et al., 2005; Martelli et al., 2007, 2009). It is well-established that skin is a complex multilayer compartment where immune cells and mediators act against antigens. The skin contains a heterogeneous pool of dendritic cells (DCs), macrophages, mast-cells, and also T cells able to react against non-self antigens. Particularly, dermal DCs are responsible for antigen capture and presentation to T lymphocytes in the skin-draining lymph nodes, triggering proliferation and activation (Bautista et al., 2002, 2005).

Since it is known that specialized antigen presenting cells (APCs) are essential for effective induction of cellular immunity (Fu et al., 1997), and that skin contains more specialized APCs (Bos, 1997) than muscle, the aim of this study was the characterization of primary and secondary activation of the immune response in conventional pigs under field conditions, after intradermal vaccination using a needle-less system (I.D.A.L.® vaccinator) in order to obtain information that could sustain the use of such vaccine delivery system in pig mass vaccination. To this purpose, vaccination against Aujeszky's Disease (AD) was used as a model of antiviral response, being characterized by a highly inducible Th1 response (Chinsakchai and Molitor, 1994; Mettenleiter, 1996; Gerdts et al., 1997; van Rooij et al., 2004), and intradermal vaccinated pigs were compared to pigs vaccinated by the more traditional intramuscular route.

In this view, the study was focused on the comparison of the two different routes of vaccine administration strictly on an immunological basis, by evaluating both humoral (ADV-specific virus neutralizing antibodies) and cellular immunity (ADV-specific IFN-γ secreting cells, lymphocyte subsets and IFN-γ gene expression in PBMC) and not on the assessment of vaccination efficacy in terms of clinical protection against the Aujeszky's Disease.

2. Materials and methods

2.1. Pigs and experimental design

Sixty-day-old conventional pigs ($n = 30$) born to 10 vaccinated sows from an Aujeszky's Disease-free herd, were equally distributed into three groups. All pigs were bred using conventional management procedures in the farm of origin throughout the study, in accordance with the requirements of animal welfare.

Two groups (10 pigs each) were vaccinated twice at 60 and 88 days of age with a gE-/TK-attenuated vaccine (Porcilis® Begonia – Intervet) via the intramuscular route (IM group) and intradermally (ID group) by using a needle-less vaccinator (I.D.A.L.® – Intervet). The vaccine dose [$10^{5.5}$ $TCID_{50}$ of modified live virus (strain Begonia)] was suspended in 2 ml and 0.2 ml of an α-tocopherol acetate-containing aqueous adjuvant (Diluvac Forte®, Intervet) for intramuscular and intradermal administration respectively, based on field routine practice. Ten pigs were sham-vaccinated by the intramuscular route with the same adjuvant only and kept as control group (C). Vaccine (IM and ID routes) and adjuvant administration were performed in the right lateral neck area, which was daily monitored for local reactions in all groups for one week after administration.

At 0 (first dose), 2, 4 (booster dose), 5, 6 and 7 weeks post-vaccination (PV) blood samples were collected for evaluation of ADV-specific VN antibodies and IFN-γ secreting cells, peripheral lymphocyte subsets and IFN-γ gene expression in PBMC.

2.2. Serological investigations

2.2.1. ELISA for Aujeszky's Disease virus (ADV gE)

The presence of ADV-specific antibodies to the gE glycoprotein was evaluated in serum by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (HerdChek® PRV g1 (gE) test kit – IDEXX Laboratories) according to the manufacturer's directions.

2.2.2. Virus neutralization test for Aujeszky's Disease virus (ADV)

To perform the virus neutralization test, serial twofold serum dilutions were prepared in microtiter plates and mixed with an equal volume of ADV (250 $TCID_{50}$/50 μl).

After incubation at 37 °C for 24 h, VERO cells were added. The cells have been examined for the presence or absence of a typical cytopathic effect for at least 4 days.

AD virus neutralizing (VN) antibody titres were expressed as $\log_2$ of the reciprocal of the highest serum dilution at which all virus was neutralized (100% end-point).

2.3. IFN-γ ELISpot assay

An IFN-γ ELISpot assay was performed as described by Martelli et al. (2009) to evaluate the levels of IFN-γ secreting cells in PBMC. Briefly, MultiScreen®$_{HTS}$-IP plates (MSIPS4510 – Millipore) were coated with 10 μg/ml anti-pig IFN-γ mAb (P2G10 – BD). After overnight incubation at 4 °C, plates were washed with sterile PBS and blocked with RPMI-1640/10% FBS for 2 h at 37 °C, 5% CO_2. PBMC were isolated by density gradient using Histopaque-1077® (Sigma) and plated at 2×10^5 cells/well in RPMI-1640/10% FBS. The *ex vivo* recall response was stimulated by incubation for 20 h at 37 °C, 5% CO_2, with the ADV vaccine virus (strain Begonia) used for vaccination in RPMI-1640/10% FBS; the linear response was tested between 0.1 and 1 MOI. The cells were then removed by washings with PBS/0.05% Tween-20 and the plates incubated for 1 h at 37 °C with 0.5 μg/ml anti-pig IFN-γ biotin-labelled mAb (P2C11 – BD) in PBS/0.5% BSA. After washings, 1/750 anti-biotin alkaline phosphatase-conjugated mAb (SP3020 – Vector Labs) in PBS/0.5% BSA were added and the plates incubated for 1 h at 37 °C. Finally, a BCIP/NBT solution was added and plates were incubated for 5–7 min at room temperature (RT) in the dark. The frequency of MLV vaccine-specific IFN-γ secreting cells was determined by an AID® ELISpot Reader (AID® ELISpot software v.3.5). As a positive control, 1×10^5 PBMC/well were incubated with 10 μg/ml PHA; as a negative control, 2×10^5 PBMC were incubated with medium only. The background values were subtracted from the respective counts of the stimulated cells; the immune response was expressed as number of IFN-γ secreting cells per million of PBMC (IFN-γ SC/10^6 PBMC). The pigs in the vaccinated groups were also

Table 1
IFN-γ responsiveness categories in intradermally (ID)-vaccinated and intramuscularly (IM)-vaccinated pigs against Aujeszky's Disease. SC: secreting cells; PV: post-vaccination; n.d.: not detected.

	0	2	4	5	6	7	Weeks PV
Responsiveness categories (IM) (IFN-γ SC/10^6 PBMC)							
100–300	n.d.	5/10	10/10	4/10	4/10	2/10	
305–600	n.d.	4/10	n.d.	3/10	3/10	5/10	
605–1000	n.d.	1/10	n.d.	3/10	n.d.	n.d.	
1005–2000	n.d.	n.d.	n.d.	n.d.	3/10	3/10	
Responsiveness categories (ID) (IFN-γ SC/10^6 PBMC)							
100–300	n.d.	8/10	7/10	3/10	5/10	4/10	
305–600	n.d.	1/10	3/10	3/10	4/10	2/10	
605–1000	n.d.	1/10	n.d.	3/10	1/10	4/10	
1005–2000	n.d.	n.d.	n.d.	1/10	n.d.	n.d.	

classified according to arbitrarily defined responsiveness categories based on IFN-γ SC numbers (Table 1).

2.4. Flow cytometry analysis of peripheral blood lymphocytes

Phenotyping of peripheral blood lymphocyte subsets was performed by flow cytometry as described by Borghetti et al. (2006). Briefly, 50 μl of heparinized blood were mixed with 5 μl of the specific monoclonal antibody (mAb) for each surface CD antigen at the appropriate dilution (according to manufacturer's recommendations). After 15 min incubation in the dark, at RT, cells were washed with PBS/1% FBS and centrifuged for 5 min at 420*g*. In the case of unlabelled primary antibodies, cells were further mixed with the corresponding goat anti-mouse IgG_1 or anti-mouse IgG_{2a} antibody and washed. The contaminating red cells were lysed using an NH_4Cl solution, pH 7.2, for 15 min at RT, in the dark. The cell suspension was washed with PBS/1% FBS, centrifuged for 5 min at 420*g*, suspended in PBS/1% FBS and then analyzed by flow cytometry (Epics® XL-MCL, Beckman-Coulter). Double staining was performed by using anti-CD4α-RPE (clone 74–12–4 – Southern Biotech) and anti-CD8α-FITC (clone 76–2–11 – Southern Biotech), anti-CD3ε-RPE (clone PPT3 – Southern Biotech) and anti-CD8α-FITC, anti-CD4α-RPE and unlabelled anti-CD25 (clone K231.3B2, IgG_1 – Serotec) mAbs; single staining was performed by using anti-CD16-FITC (FcγIII receptor, clone G7 – Serotec) mAb and primary unlabelled anti-CD8β mAb (clone PG164A, IgG_{2a} – VMRD). Unlabelled CD25 and CD8β antigens were detected by using respectively a goat anti-mouse IgG_1 (M32001) and a goat anti-mouse IgG_{2a} (M32201) FITC-labelled secondary antibody (Caltag Labs). The results are based on lymphocyte gating on a forward scatter vs. side scatter graph after acquisition of at least 20.000 cell events. Relevant immune cell subsets were considered according to literature (Yang and Parkhouse, 1996; Zuckermann and Husmann, 1996; Saalmuller et al., 1999, 2002; de Bruin et al., 2000; Sinkora et al., 2001; Käser et al., 2008).

2.5. IFN-γ gene expression

2.5.1. Total RNA extraction and cDNA synthesis

Swine PBMC were isolated by density gradient using Histopaque-1077® (Sigma) and total RNA was extracted by TRIreagent® (Ambion) according to manufacturer's instructions; purity and concentration were assessed by UV spectrophotometry. Total RNA (1 μg) was reverse transcripted using a Ready-To-Go™ You-Prime First-Strand Beads kit (Amersham Biosciences) and oligo dT primer (Ambion). The cDNA (5 μl) was used as template for Real-Time PCR.

2.5.2. IFN-γ Real-Time PCR

Real-Time PCR was performed using a 36/72-well Rotor-Gene 3000™ (Corbett Research, Rotor-Gene 3000™ software v.5.0). The cDNA (30 ng/20 μl) was amplified in duplicate with Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) along with primers (0.5 μM) for pig IFN-γ (sense 23-mer: 5′-TGGTAGCTCTGGGAAACT GAATG-3′; anti-sense 18-mer: 5′-GGCTTTGCGCTGGATCTG-3′) and pig GAPDH (sense 20-mer: 5′-GGTGAAGGTCGGAGTGAACG-3′; anti-sense 21-mer: 5′-GCCAGAGTTAAAAGCAGCCCT-3′). The primers were designed based on published gene sequences (GenBank) and synthesized by MWG; the housekeeping gene GAPDH was selected as endogenous control according to Fisher et al. (2006). After an initial step at 50 °C for 2 min, samples were kept at 95 °C for 2 min to allow denaturation of secondary structures. Each of the 45–50 cycles consisted of a denaturation step at 95 °C for 15 s, an annealing step at 58 °C for 30 s and an extension step at 72 °C for 30 s. Fluorescence due to SYBR® Green incorporation was acquired at the end of the extension step. A no-template and a no-reverse transcriptase control were included in each experiment. A melting curve analysis for specific amplification control was performed (from 55 to 95 °C) at the end of the amplification cycles.

Data were analyzed according to the Relative Standard Curve Method (Bustin, 2000). Expression levels were calculated by normalizing the quantified IFN-γ cDNA to the GAPDH cDNA amount and expressed as arbitrary units (IFN-γ/GAPDH A.U.).

2.6. Statistical analysis

The correlation between the different parameters was estimated by the Pearson's correlation coefficient. Data were analyzed by the Analysis of Variance (ANOVA) test for repeated measurements, using a linear mixed model and accounting for the correlation structure of the data (i.e. repeated measurements on subjects). The pre-vaccination value was included as covariate in the model. The significance of the difference between the treatments (ID vs. IM, ID vs. C and IM vs. C) after administration of the primary and booster doses was derived from linear contrasts for each system. The variance–covariance structure of the random effects in the model was selected using the Likelihood Ratio test. The level of significance was set at 0.05 and tests were two-sided. Statistical analysis was performed using SAS software v.9.1 (SAS® Institute Inc., Cary, NC, USA).

3. Results

3.1. Clinical examination

None of the IM-vaccinated, ID-vaccinated and sham-vaccinated control (C) pigs showed any clinical sign related to Aujeszky's Disease as well as to other diseases at any time and no pig died during the whole experimental period. No relevant local reactions were observed in the neck area, where injections were performed.

3.2. Serological investigations

ELISA antibodies against the ADV gE glycoprotein were not detected at any time. ADV-specific virus neutralizing (VN) antibody levels are shown in Fig. 1. All the pigs under study still had maternally-derived VN antibodies against Aujeszky's Disease virus at vaccination (60 days).

Although a higher level was detected at 4 weeks after the 1st dose in the IM group ($P < 0.05$), overall, no significant differences between the two vaccinated groups were observed. VN antibody levels increased after the 1st vaccine administration in both the ID- and IM-vaccinated pigs and showed the strongest increase after the booster dose, reaching and maintaining comparable titres. All sham-vaccinated pigs showed the vanishing of maternally-derived

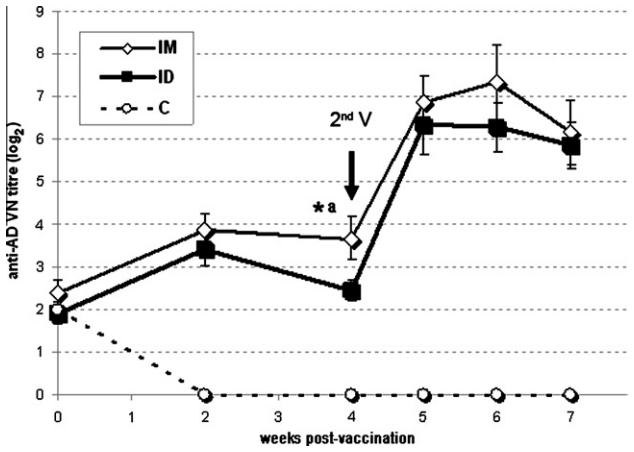

Fig. 1. Post-vaccination levels of AD virus neutralizing (VN) antibodies in serum samples of intradermally (ID)-vaccinated, intramuscularly (IM)-vaccinated and sham-vaccinated control (C) pigs. Titres are expressed as $\log_2$ of the reciprocal of the highest serum dilution at which all virus was neutralized (100% end-point). Each value represents the mean response of 10 pigs ± standard error of the mean (s.e.m.). (^{*a}) $P < 0.05$, ID vs. IM and C. Except for values at 0 weeks PV, all mean values in both vaccinated pigs are significantly different from those in the C group ($P < 0.05$). 2nd V: booster vaccination.

antibodies (i.e. negative at the VN test) at 2 weeks after adjuvant administration and remained negative throughout the experiment.

3.3. ADV-specific IFN-γ ELISpot assay

The cell-mediated immune response evaluated as levels of ADV-specific IFN-γ secreting cells (SC) in PBMC showed a comparable trend between the ID and IM group. No ADV-induced secretion was detected before vaccination (60 days); the IFN-γ-specific response arose in both vaccinated groups after the 1st vaccination and showed a strong booster effect after the 2nd vaccination. Particularly, at 1 week after the booster dose, both vaccinated groups showed the highest mean values (Fig. 2). Taking into account individual responsiveness of the animals (Table 1), the majority of pigs showed values between 100 and 300 SC/10^6 PBMC after the 1st vaccination. After the booster vaccination, the number of pigs belonging to the 605–1000 and 1005–2000 SC/10^6 PBMC categories increased. During the last 2 weeks, the response was still characterized by high responders (>305 SC/10^6 PBMC) in both groups. Furthermore, large spots were detected throughout the study in all vaccinated pigs (Fig. 3), testifying high IFN-γ productivity per cell (high amount of IFN-γ secreted).

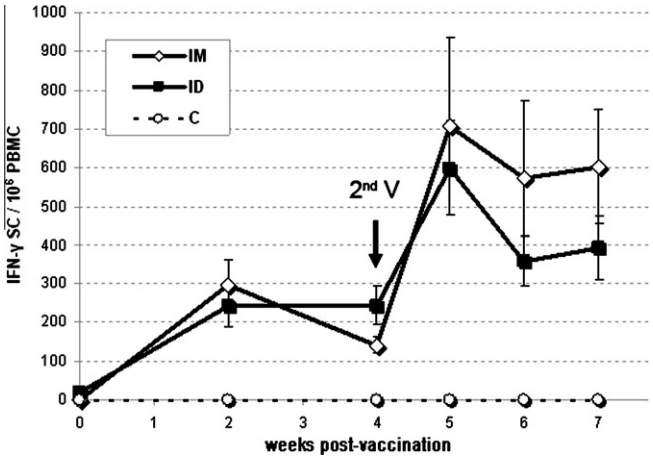

Fig. 2. Post-vaccination levels of ADV-specific IFN-γ secreting cells (SC) in PBMC of intradermally (ID)-vaccinated, intramuscularly (IM)-vaccinated and sham-vaccinated control (C) pigs. The *ex vivo* recall response was stimulated by the addition of ADV vaccine, strain Begonia (0.5 MOI, 20 h). The data are expressed as number of IFN-γ secreting cells (SC) per million of PBMC (IFN-γ SC/10^6 PBMC) and each value represents the mean response of 10 pigs ± standard error of the mean (s.e.m.). Except for values at 0 weeks post-vaccination, all mean values in both vaccinated pigs are significantly different from those in the C group ($P < 0.05$). 2nd V: booster vaccination.

Overall, a positive correlation between AD VN antibody and IFN-γ SC levels ($\rho = 0.61$; $P < 0.05$) was found in the treated pigs. Sham-vaccinated animals did not show any specific cellular response to *ex vivo* ADV re-stimulation throughout the PV period.

3.4. Time-related changes of peripheral blood lymphocytes

In both vaccinated groups, the levels of double positive (DP) CD4+CD8α+^{low} lymphocytes (subset containing memory T cells) increased at 4 weeks after the 1st vaccine dose and promptly decreased at 1 week after the booster dose. Only in the ID-vaccinated pigs a peak was detected at 6 weeks PV (Fig. 4). The high individual variability strongly influenced group comparisons so that no statistically significant differences were detected between the ADV-vaccinated and sham-vaccinated pigs. Cell levels in the sham-vaccinated pigs did not show any changes except for a transient, but not significant, increase at 1 week after the booster administration.

The course of CD8β+ cells, mostly representing cytotoxic T effector lymphocytes (Fig. 5), showed significantly higher cell numbers in both treated groups at 4 weeks after the 1st vaccination compared to the C group. Two weeks after the booster vaccination, ID pigs showed higher levels of CD8β+ cells compared to both the IM and C group ($P < 0.05$).

A significant increase of CD4+CD25+ and CD4−CD25+ cells was observed in the ID-vaccinated pigs at 2 weeks after the booster dose ($P < 0.05$; Figs. 6 and 7); both CD25+ subpopulations peaked one week later in the IM-vaccinated pigs. Control pigs did not show any relevant changes.

The levels of CD3−CD8α+ (Fig. 8) and CD16+ (data not shown) cells showed a similar course in both vaccinated groups; they significantly increased after the 1st vaccination and maintained higher levels compared to the C group throughout the study ($P < 0.05$).

3.5. IFN-γ gene expression in PBMC

Real-Time PCR analysis showed a significant increase of IFN-γ gene expression in PBMC at 2 weeks PV in both vaccinated groups with regards to sham-vaccinated animals (Fig. 9). In the IM group, higher IFN-γ gene expression levels were evident up to the 4th week post-vaccination; they significantly decreased up to 3 weeks after the second vaccine administration ($P < 0.05$). On the contrary, IFN-γ gene expression after the booster vaccination exhibited a further increase in the ID group, showing higher but not significant levels as compared to the IM-vaccinated pigs. Levels in both vaccinated groups declined to basal levels 3 weeks after boosting. In the C group, no significant changes of IFN-γ expression were detected throughout the study.

4. Discussion and conclusions

In this study humoral and cellular immune responses triggered by intradermal vaccination in conventional pigs were evaluated in comparison with the more conventional intramuscular vaccination. The comparison mainly focused on the efficiency of intradermal delivery by using a needle-less device, which appears to be more practically advantageous. To this purpose, vaccination

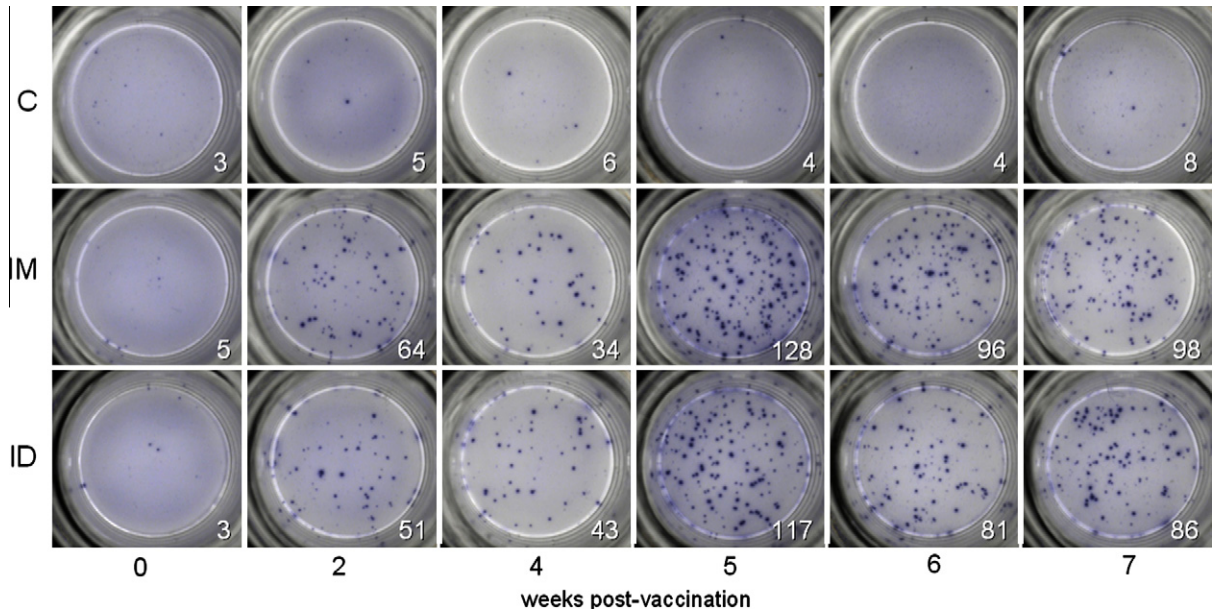

Fig. 3. ADV-specific IFN-γ ELISpot response in a representative intradermally (ID)-vaccinated, intramuscularly (IM)-vaccinated and sham-vaccinated control (C) pig over time. The number of spots shown corresponds to the number of IFN-γ secreting cells/2×10^5 PBMC.

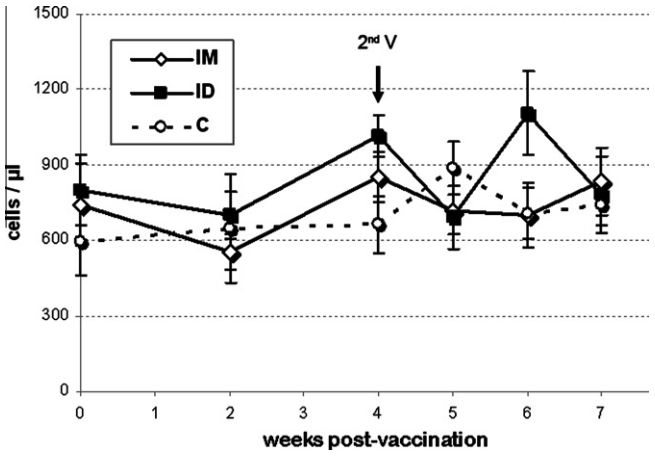

Fig. 4. Time-related changes in peripheral CD4+CD8α+^{low} T lymphocyte levels of AD MLV intradermally (ID)-vaccinated, intramuscularly (IM)-vaccinated and sham-vaccinated control (C) pigs; data are expressed as cells/µl. Each value represents the mean response of 10 pigs ± standard error of the mean (s.e.m.). 2nd V: booster vaccination.

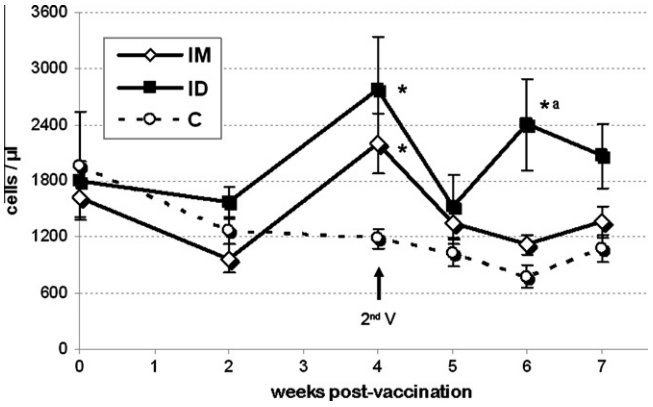

Fig. 5. Time-related changes in peripheral CD8β+ T lymphocyte levels of AD MLV intradermally (ID)-vaccinated, intramuscularly (IM)-vaccinated and sham-vaccinated control (C) pigs; data are expressed as cells/µl. Each value represents the mean response of 10 pigs ± standard error of the mean (s.e.m.). (*) $P < 0.05$, ID and IM vs. C; (*a) $P < 0.05$, ID vs. IM and C. 2nd V: booster vaccination.

against Aujeszky's Disease (AD) was used as a classical model of antiviral response, being characterized by highly inducible humoral and cellular immune responses.

In the present study a challenge infection was not considered since efficacy of both intramuscular and intradermal ADV vaccination in terms of clinical protection has been already demonstrated (Vannier and Cariolet, 1991; Visser, 1997).

The specific humoral immune response, measured by VN antibody titres, was not influenced by the route (ID or IM) of vaccine administration. Differently from Mikulska-Skupien et al. (2005), both routes induced a strong anamnestic response so that comparable levels were observed after the 2nd vaccination. These authors however investigated ADV-vaccinated animals under different background and vaccination conditions since pigs were born to unvaccinated sows and ID-vaccinated pigs were inoculated with 1/10 of vaccine amount. The fact that they detected higher titres of VN antibodies in the IM-vaccinated pigs after both the first and booster vaccine dose may have been strongly influenced by the lower vaccine amount. Furthermore, the VN antibodies in the ID-vaccinated animals reached comparable, if not higher, levels after challenge. This underlines that the extent of humoral immunity after vaccination is not strictly predictive of the response after challenge related to immune protection, supporting the importance of evaluating also the cellular immune compartment, which activation can be fundamental.

It is known that achieving an appropriate stimulation of both the humoral and cellular immune response in maternally immune piglets is a major concern since residual antibodies can interfere with vaccine efficacy (van Rooij et al., 2006). In our study, VN antibody titres in the ID-vaccinated animals were comparable to those observed in the IM group and testify an intense post-vaccination humoral immune stimulation in accordance with other studies (Vanderpooten et al., 1997; Meier et al., 2003; van Rooij et al., 1998), despite the presence of maternal immunity. Although the importance of post-vaccinal VN antibodies or anti-ADV-specific immunoglobulins for the clearance of challenge virus was pointed

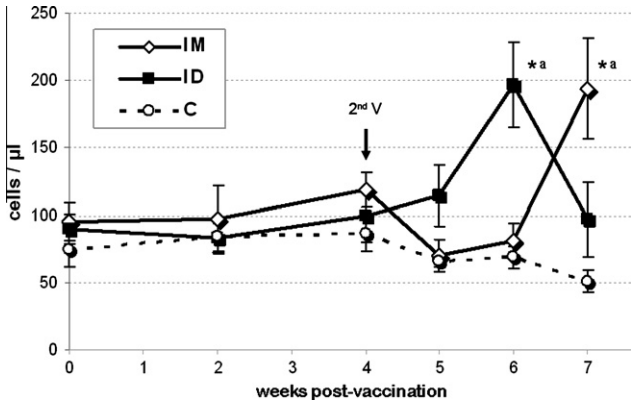

Fig. 6. Time-related changes in peripheral CD4+CD25+ T lymphocyte levels of AD MLV intradermally (ID)-vaccinated, intramuscularly (IM)-vaccinated and sham-vaccinated control (C) pigs; data are expressed as cells/µl. Each value represents the mean response of 10 pigs ± standard error of the mean (s.e.m.). (*a) $P < 0.05$, ID vs. IM and C (6 weeks PV) and $P < 0.05$, IM vs. ID and C (7 weeks PV). 2nd V: booster vaccination.

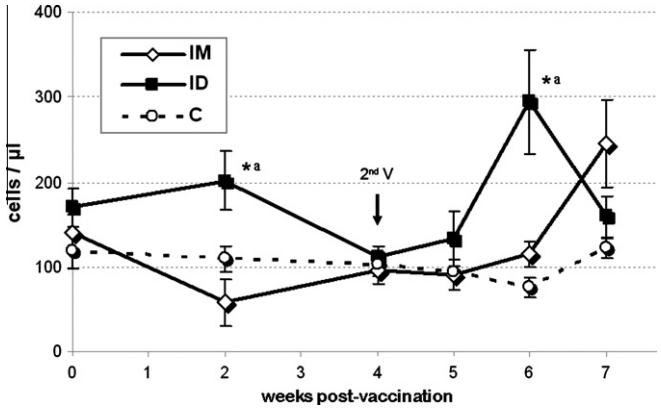

Fig. 7. Time-related changes in peripheral CD4−CD25+ T lymphocyte levels of AD MLV intradermally (ID)-vaccinated, intramuscularly (IM)-vaccinated and sham-vaccinated control (C) pigs; data are expressed as cells/µl. Each value represents the mean response of 10 pigs ± standard error of the mean (s.e.m.). (*a) $P < 0.05$, ID vs. IM and C. 2nd V: booster vaccination.

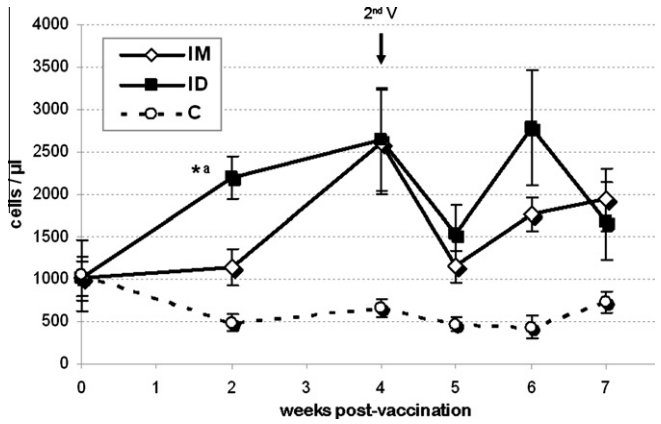

Fig. 8. Time-related changes in peripheral CD3−CD8α+ T lymphocyte levels of AD MLV intradermally (ID)-vaccinated, intramuscularly (IM)-vaccinated and sham-vaccinated control (C) pigs; data are expressed as cells/µl. (*a) $P < 0.05$, ID vs. IM and C. Except for values at 0 weeks post-vaccination, all mean values in both vaccinated pigs are significantly different from those in the C group ($P < 0.05$). 2nd V: booster vaccination.

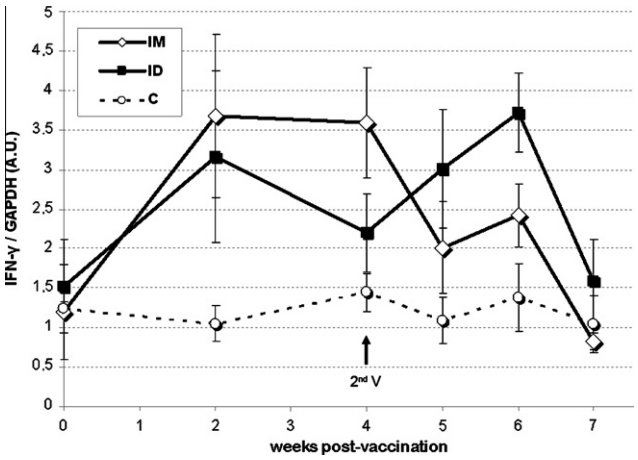

Fig. 9. IFN-γ gene expression levels in PBMC of AD MLV intradermally (ID)-vaccinated, intramuscularly (IM)-vaccinated and sham-vaccinated control (C) pigs; data are shown as relative values (arbitrary units, A.U.) with regard to GAPDH gene expression levels. Each value represents the mean response of 10 pigs ± standard error of the mean (s.e.m.). Except for values at 0 and 7 weeks post-vaccination, all mean values in both vaccinated pigs are significantly different from those in the C group ($P < 0.05$). 2nd V: booster vaccination.

out (Pensaert et al., 1990; Vannier et al., 1991, 1995; Lin et al., 2005), some authors reported no or poor correlation between the humoral response and clinical protection (Vannier and Cariolet, 1991; Vanderpooten et al., 1997). Besides a variable correlation between VN antibody titres and the severity of disease or the reduction of virus excretion (Kimman et al., 1995; Zuckermann et al., 1998; van Rooij et al., 2004), the cell-mediated immune response seems to be a more consistent and crucial parameter, correlated with early protection against challenge in pigs vaccinated by a modified live virus (MLV) (Laval et al., 2002; van Rooij et al., 2004). A rapid proliferation of ADV-specific T lymphocytes, followed by the influx into the site of infection and the production of IFN-γ, has been correlated with clinical protection and reduction of virus shedding soon after challenge (Kimman et al., 1995; van Rooij et al., 2004).

Determination of *ex vivo* Th1 cytokine production, particularly IFN-γ secretion (e.g. levels of IFN-γ secreting cells), has been used as a suitable parameter to monitor the efficiency of the immune response to ADV vaccination and infection (Meier et al., 2003; Hoegen et al., 2004; Zuckermann et al., 1998); in other pig vaccination/infection models such parameter has been also defined as a good indicator of the cell-mediated immune response (Suradhat et al., 2001; Piriou et al., 2003; Zuckermann et al., 2007; Martelli et al., 2009). Furthermore it was emphasized how the magnitude of the IFN-γ response may correlate with immune protection by the ADV-specific IgG response (van Rooij et al., 2004).

Some authors underlined how residual immunity is able to exert negative effects on several cellular responses such as virus-specific lymphoproliferation and IFN-γ secretion (Bouma et al., 1998; Van Nes et al., 2001; van Rooij et al., 2006). Conversely, under the conditions of this field study, induction of high ADV-specific IFN-γ secreting cell levels was detected soon after the 1st vaccination. This demonstrates that maternal immunity did not hamper the onset of such specific cellular immune response, which appeared strictly comparable in the ID- and IM-vaccinated pigs. Again, high IFN-γ productivity per cell (i.e. high IFN-γ secretion) detected throughout the study testifies an intense and lasting *ex vivo* PBMC responsiveness upon ADV re-stimulation. The ADV-specific IFN-γ secreting cell levels after both vaccinations were comparable to those reported by Meier et al. (2003). The identification of responsiveness categories clearly showed that the IFN-γ response was in-

tense in all vaccinated pigs soon after the 1st vaccination, even in the presence of residual maternally-derived immunity, and that the booster vaccine dose was effective in inducing further cell activation, characterized by very high IFN-γ responder pigs.

Upregulation of IFN-γ gene expression in PBMC of vaccinated pigs testifies *in vivo* activation of circulating lymphocytes after both the 1st and 2nd vaccine administration in comparison with the basal levels observed in control animals. Future studies will be addressed to phenotypically characterize virus-specific IFN-γ SC in the blood (e.g. CD8αβ+ lymphocytes). Preliminary data from intracellular IFN-γ staining showed how a large proportion of CD8α+IFNγ+ cells can be identified in AD MLV vaccinated pigs after the 1st vaccination and increases strongly after the booster administration; this is in agreement with the results observed using the IFN-γ ELISpot assay (Ferrari et al., 2010).

The time-related changes in the peripheral T lymphocyte subsets, detected especially after the booster dose, appeared to be related to the biphasic antigenic stimulation and can be interpreted as an effect of *in vivo* activation of the immune response triggered by the MLV vaccine.

Several authors demonstrated that CD8+ MHC-I-restricted cytotoxic cells play a crucial role as effector cells in antiviral immunity and that virus-specific CD8+ T cell proliferation can be correlated with immune protection (Seder and Hill, 2000; Woodland, 2003; Welsh et al., 2004). This has also been shown during MLV vaccination and ADV infection (Zuckermann et al., 1990; Saalmuller et al., 1999; de Bruin et al., 2000; Laval et al., 2002; van Rooij et al., 2004, 2006).

The major changes in cytotoxic CD8β+ T cell levels in the blood were observed prior to and after the booster dose; such course is justified by the involvement of this subset in IFN-γ production. Also CD3−CD8α+ and CD16+ (data not shown) subsets, containing NK cells able to secrete IFN-γ, showed higher numbers in vaccinated pigs.

The increase of CD8β+ cells in the ID-vaccinated pigs could indicate earlier and more efficient activation of peripheral cytotoxic T lymphocytes. Our results are partially in accordance with those by van Rooij et al. (1998) that showed how the intradermal and intramuscular routes are comparable in inducing lymphocyte proliferation. The inability of detecting significant differences of CD4+CD8+^{low} DP memory cells in vaccinated pigs could have been influenced by the higher individual variability associated with the low frequencies of this subpopulation in PBMC.

CD25 antigen (IL-2 receptor alpha chain) was investigated as marker of activation and proliferation on CD8+ lymphocytes (Saalmuller et al., 1994; Zuckermann et al., 1998; Sinkora et al., 2001) as well as of regulatory activity in circulating CD4+CD25+ T cells that comprise T regulatory lymphocytes (Treg), recently indentified in swine (Warnecke et al., 2006; Käser et al., 2008). The course of both CD4+CD25+ and CD4−CD25+ T cells in the ID-vaccinated pigs, peaking concomitantly with the transient increase of CD8β+ T cells suggests an activation state of this latter subset. Single and double staining did not allow us to identify specific subsets simultaneously expressing CD8β, CD4 and CD25 but it is well-established that CD25+ cells comprise regulatory and also activated effector cells. Particularly, the CD4−CD25+ cell pool does contain CD8+ cells. Furthermore, taking into account that CD4+CD25+ T cells have regulatory functions, this subset's trend could be involved in immune modulation to control cell activation and proliferation, as well as the consequent IFN-γ production. The transient increase of this subset only after the 2nd vaccination is in favour of a positive regulation of the boosted immune response.

In conclusion, the study suggests on a more extended immunobiological basis that intradermal vaccination in maternally immune pigs can prime and sustain a consistent stimulation of both humoral and cellular immunity. Under the conditions of this study, the intradermal vaccination by using a needle-less vaccinator elicited an immune response against Aujeszky's Disease virus strictly comparable to the more conventional intramuscular administration both in terms of VN antibody and virus-specific IFN-γ-mediated responses. Although further data are needed, our results sustain the potential use of the needle-less ID application in ADV mass vaccination programs.

Conflict of interest statement

None of the authors of this paper has a financial relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

The employees of Intervet/Schering-Plough Animal Health provided the vaccine, the I.D.A.L.® system and contributed to blood sampling.

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