Role of natural interferon-producing cells and T lymphocytes in porcine monocyte-derived dendritic cell maturation

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Summary

Maturation of dendritic cells (DC) is a key immunological process regulating immune responses to pathogens and vaccines, as well as tolerance and autoimmune processes. Consequently, the regulation of DC maturation should reflect these multifaceted immunological processes. In the present study, we have defined the role of particular cytokines, Toll-like receptor (TLR) ligands and T lymphocytes in the porcine monocyte-derived DC (MoDC). Interferon-α (IFN-α) alone was a poor inducer of MoDC maturation, but in association with tumour necrosis factor-α (TNF-α), or TLR ligands such as lipopolysaccharide and polyinosinic-polycytidylic acid I:C, an up-regulation of major histocompatibility complex II and CD80/86 expression was noted, along with reduced endocytic activity. In contrast, TNF-α alone or in combination with the TLR ligands was a poor inducer of DC maturation, but co-operated with T-lymphocytes in the presence of antigen to induce DC maturation. Natural interferon producing cells (NIPC, or plasmacytoid DCs) represent a danger-recognition system of the immune defences, and can respond to viruses not otherwise recognized as posing a danger. Indeed, MoDC did not respond to transmissible gastroenteritis virus (TGEV), whereas NIPC produced high levels of IFN-α and TNF-α after TGEV stimulation. Moreover, supernatants from the stimulated NIPC induced maturation in MoDCs. These matured MoDCs displayed an enhanced ability to present antigen to and thus stimulate T cells. Taken together, the present work demonstrates that maturation of MoDC not only results from TLR signalling, but can require co-operation with various cell types – principally NIPC and activated T cells – which would reflect the particular immunological situation.

Keywords: plasmacytoid dendritic cells; interferon-α; tumour necrosis factor-α; Toll-like receptor ligands; monocyte-derived dendritic cells; porcine

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Introduction

Dendritic cells (DC) are professional antigen-presenting cells (APC) specialized in antigen capture, migration to secondary lymphoid organs and T-cell priming. DC maturation is essential for the generation of effective immune responses against most pathogens. This process can be induced by proinflammatory cytokines, microbial products, or cross-linking of CD40 molecules.[1–3] Mature DCs are most efficient at expressing peptide–major histocompatibility complexes (MHCs) on their surfaces to activate T cells, for which the high expression levels of MHC and costimulatory molecules – such as CD80, CD86 and CD40 – on the DC is essential. During this process, the DCs also acquire the ability to migrate into the inductive sites of the immune system, such as the lymph nodes, to initiate specific immune responses.

Although the recognition of pathogen-associated molecular patterns (PAMPs) by DC surface receptors will initiate cell maturation, the '*in vivo*' situation is more

complex because of the multifaceted nature of immune responses. For example, the stimulation of DC by bacterial or fungal PAMPs can result in autoimmune processes or immune responses against relatively harmless gut flora and food antigen. It can therefore be of benefit to the immune system that DCs involved in antigen presentation do not express all Toll-like receptors (TLR)s to recognize all PAMPS. With human immune defences, mainly the natural interferon producing cells (NIPC, or plasmacytoid DC) express TLR7 and TLR9, important receptors for microbial nucleic acid.[4] Of course, a result of this differential TLR expression may be that myeloid DC might not be fully equipped to recognize certain viruses.

Both human and porcine DC closely resemble each other, making the porcine system a valuable model for studies such as evaluation of vaccine adjuvants and identification of responsiveness to pathogens.[5–8] For such analyses, both monocyte-derived DC (MoDC)[9] and the NIPC[10] can be applied. As with the situation in humans, porcine NIPC are the only DC subset identified so far as responding to DNA containing CpG type A motifs by producing large quantities of interferon-α (IFN-α).[11] These CpG did not induce the maturation of MoDC, although recently, Raymond and Wilkie demonstrated the responsiveness of MoDC to another CpG sequence.[8]

The present work sought to characterize the consequences of interaction between myeloid DC and NIPC in the promotion of immune response development against viruses not recognized by myeloid DC. Transmissible gastroenteritis virus (TGEV) was used as a model based on its known stimulatory effects for porcine NIPC.[12]

Considering the known importance of interferon type I (IFN type I) for the maturation[3] it was of importance to determine the effect of IFN-α, tumour necrosis factor-α (TNF-α) alone or combined with 'classic' PAMPS such as TLR3 and TLR4 ligands, on porcine monocyte-derived DC maturation. Previous work has demonstrated the principle responsiveness of MoDC to TLR ligands[5,7,8,13] but no significant effect of lipopolysaccharide (LPS) on MHC II up-regulation, representing a key events of DC maturation, was reported.[7,8] Furthermore, a quantitative and comparative analysis of maturation markers is lacking.

Consequently, the objective of the present study was to characterize the interplay among myeloid DC, NIPC and lymphocytes and the relative role played by IFN-α, TNF-α and TLR ligands leading to DC maturation.

Materials and methods

Isolation and culture of cells

Citrated blood of a total of 10 different specific pathogen-free Swiss Landrace pigs was obtained. The animals were from two different litters, of mixed gender and 1–2 years of age. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (1·077 g/l, Amersham Pharmacia Biotech AG, Dubendorf, Switzerland) density centrifugation. Cells were cultured in phenol red-free Dulbecco's modified Eagle's minimal essential medium (DMEM; Invitrogen, Basel, Switzerland), supplemented with porcine serum (10% v/v, Sigma Chemicals, Buchs, Switzerland).

For enrichment of NIPC, the PBMC were separated into $CD172a^+$ ($SWC3^+$) cells using the Miltenyi magnetic-activated cell sorting (MACS) separation system with LD columns (Miltenyi Biotec GmBH, Bergish Gladblach, Germany) and the anti-CD172a mAb clone 74-22-15 (ATCC, Molsheim, France).[11] Using multicolour immunofluorescence cytometric analyses, NIPC were identified as $CD4^{high}$ $CD172a^{low}$ cells.[10,11]

Monocyte-derived DCs (MoDCs) were prepared as previously described.[9,13] Briefly, monocytes were purified using CD172a sorting as described above, but with LS columns. The cells were cultured in DMEM medium with porcine serum (10% v/v), recombinant porcine (rp) granulocyte–macrophage colony-stimulating factor (GM-CSF, 150 ng/ml) and rp interleukin-4 (IL-4, 100 U/ml). The IL-4 and GM-CSF were prepared in our laboratory as described previously.[9,10] After 3 days of culture, the non-adherent cells dominated by immature MoDC were harvested.

MoDC maturation

In order to induce maturation, the immature MoDC were cultured for an additional 24 hr in the presence of IFN-α (usually 1000 U/ml) and TNF-α (20 ng/μl), polyinosine-polycytodylic acid (pIC, 12·5 μg/ml; Sigma) or LPS (1 μg/ml; Sigma). IFN-α was prepared in our laboratory as described[14] and TNF-α (TNF) was prepared from L929 cells expressing porcine TNF-α (kindly provided by Dr G. Bertoni, Bern[15]). Alternatively, MoDCs were matured for 24 hr with a cocktail of proinflammatory cytokines – IL-1/IL-6/prostaglandin E_2 (PGE_2)/TNF-α – used for maturation of human MoDC.[16] This was composed of 20 ng/ml rpTNF-α, 10 ng/ml rpIL-1 (Pierce Endogen, Lausanne, Switzerland), 10 ng/ml rpIL-6 (Pierce Endogen) and 1 μM PGE_2 (Sigma).

Cell maturation was determined by measuring the cell surface expression of MHC II (MSA3 monoclonal antibody (mAb), kindly donated by A. Saalmüller), and CD80/86 detected using a human cytotoxic T-lymphocyte associated antigen (CTLA4)–mouse immunoglobulin fusion protein (Alexis Corporation, Lausen, Switzerland[9]). Reactivity was detected by using phycoerythrin-conjugated goat $F(ab')_2$ anti-mouse isotype-specific immunoglobulins (Southern Technology, Birmingham, UK). Cells only stained with the conjugate were used as negative controls. Endocytic activity of MoDCs was evaluated by incubation of the cells with ovalbumin (OVA)-dQ (10 μg/ml; Molecular Probes, Eugene, OR) for 1 hr at

37°.[10] OVA-dQ acquires fluorescence after dequenching through proteolytic enzyme cleavage, permitting the analyses of both antigen uptake and processing. The geometric mean fluorescence intensity (MFI) was quantified by flow cytometry with 25 000 events acquired, and calculated using the Flowjo program. This program was also used to determine the probability whether a test population is different from a control population. To this end, the probability binning (PB) algorithm was employed.[17] This algorithm calculates the T(X) value. $T(X) = 0$ means that the two datasets are indistinguishable ($P = 0{\cdot}5$) and $T(X) > 4$ implying that the two distributions are different ($P < 0{\cdot}01$). An empirical determination for the T(X) cutoff value suitable for the present labellings was determined by measuring the variability within 10 replicates of unstimulated cultures labelled for CD80/86, MHC II and OVA-dQ. Within these labellings the T(X) values varied between 0·1 and 15. Based on this, a T(X) cutoff value 20 was selected for our data in order to have a stringent cutoff.

T-cell stimulation assays

Lymphocytes were purified by depletion of $CD172a^+$ cells (containing monocytes and APCs[10]) using the MACS system. Lymphocytes were cultured at 2×10^5 cells/well. The microbial superantigen staphylococcal enterotoxin B (SEB; Alexis) was used to measure the potency of DCs at inducing T cell responses through MHC II–T-cell receptor cross linking.[18,19] MoDCs were incubated with SEB (100 ng/ml) for 1 hr at 39°, washed four times and titrated in 96-well plates as described. After 4 days, 1 μCi (37 kBq) [^{3}H]thymidine was added for another 18 hr to quantify the proliferation.

The processing and presentation of antigen was assessed in tetanus toxoid (TT) antigen-specific T-cell proliferation assays. To this end, lymphocytes and MoDCs isolated from two pigs vaccinated against TT by immunizing with human tetanus vaccine (kindly provided by the Netherlands Vaccine Institute, Bilthoven, Netherlands) followed by a booster immunization after 4 weeks. Immature and matured MoDCs were cultured with T cells (2×10^5 cells/well), to obtain T/DC ratios of 10 : 1, 30 : 1 and 90 : 1. TT or diphtheria toxoid (DT, used as a negative control antigen; Netherlands Vaccine Institute) were added at 1·2 Flocculation units/ml (Lf/ml). Lymphoproliferation was quantified in triplicate cultures after 5 days by measuring [^{3}H]thymidine uptake as described above. Only immunized pigs responded to TT antigen in a T-cell proliferation assay.

Cytokine responses

Enriched NIPC or MoDC were stimulated with TGEV or CpG oligodeoxynucleotides (CpG-ODN) (sequence D32 ggT GCG TCG ACG CAG ggg gg, 10 μg/ml[11]). The virus was prepared from infected ST cell lysates as described[10] and used a multiplicity of infection 2 $TCID_{50}$/cell. Lysate preparations from uninfected ST cells were used as mock controls. Intracellular cytokine detection was after 6 hr, analysis of secreted cytokines in cell supernatants after 24 hr.

Secreted IFN-α was detected by enzyme-linked immunosorbent assay (ELISA) using with anti-porcine IFN-α mAbs K9 and F17 (kindly provided by Dr Bernard Charley, INRA, Jouy-en-Josas, France) as described.[11] TNF-α was assayed with actinomycin D-treated PK15-15 cells.[20] IL-6 was analysed using a duoset ELISA kit from R & D systems (Oxford, UK).

After staining of cell surface molecules, the cells were fixed and permeabilized (Fix & Perm, Caltag, CA) and stained with anti-IFN-α mAbs F17 and K9 (10 μg/ml; R & D Systems) or anti-porcine TNF-α mAbs (clone 4F4, Perbioscience). For flow cytometry detection, isotype-specific FITC, R-PE (Southern Biotechnology Associates) and R-PE-Cy5 (Dako) conjugates were used as described.[11] Inhibition of IFN-α induced by TGEV was achieved by blocking anti-protein M mAb (clone 25-22), kindly provided by Dr Bernard Charley, INRA). For neutralization of IFN-α, samples were incubated for 30 min on ice with 1 μl of rabbit polyclonal antibody against porcine IFN-α (as indicated by the supplier (R & D systems). As control, rpIFN-α was neutralized by this polyclonal antibody (data not shown).

Statistical analyses

Statistical and graphical analyses were carried out with the SigmaStat and SigmaPlot software package (SPSS). If applicable parametric analysis of variance (ANOVA) combined with Student's *t*-test were used. A value of $P < 0{\cdot}05$ was considered statistically significant.

Results

Influence of TLR-ligands on MoDC maturation, compared with IFN-α/TNF-α

Following differentiation of purified $CD172a^+$ cells into immature MoDC – using GM-CSF and IL-4 for 3 days[9] – the effect of additional TNF-α, IFN-α and the TLR ligand pIC was determined. Alone, only the maturation signals IFN-α and LPS induced a weak but statistically significant up-regulation of CD80/86 (Fig. 1a). Although pIC and TNF-α alone had no statistically significant effect, TNF-α applied together with pIC was effective for induction of CD80/86 up-regulation (Fig. 1a). This was also seen with all combinations which included IFN-α. For MHC II, IFN-α was the only stimulus able to promote the expression of MHC II when applied alone (Fig. 1b). Combinations of IFN-α with any other stimuli, synergistically

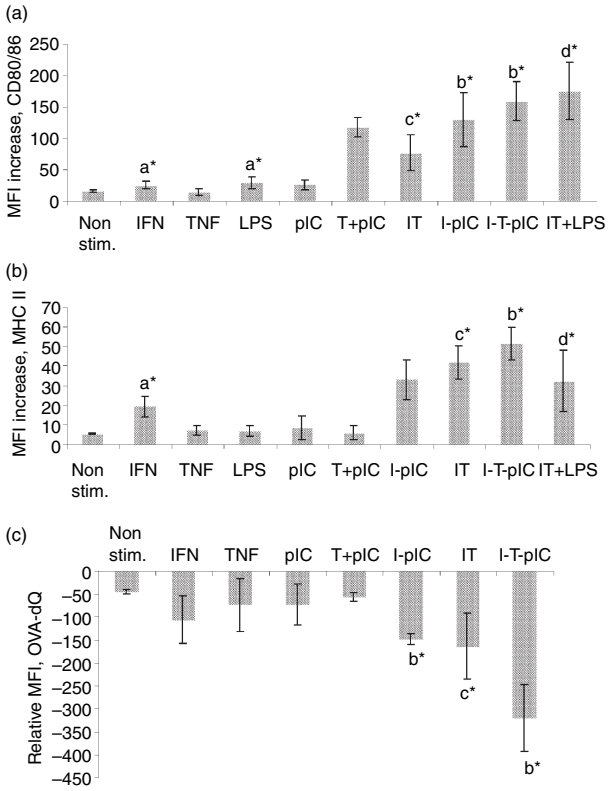

Figure 1. Synergistic effect of the IFN-α/TNF-α cocktail together with the TLR ligands LPS and pIC on MoDC maturation. Immature MoDC were stimulated with IFN-α (IFN, 1000 U/ml), TNF-α (TNF, 20 ng/ml), pIC (12·5 μg/ml), LPS (1 μg/ml), alone or in the combinations as indicated in the *x*-axis (IT: IFN-α + TNF-α, I-pIC: IFN-α + pIC, IT-pIC: IFN-α + TNF-α + pIC, IT + LPS: IFN-α + TNF-α + LPS). After 24 hr, the maturation of the MoDCs was determined in terms of the expression levels of CD80/86 (a), MHC II (b), as well as the internalisation/degradation of OVA-dQ (c) determined by flow cytometry. The relative increase or decrease in mean fluorescence intensity (MFI) was calculated by subtraction of the treated MFI from the unstimulated MFI value for the respective labelling of the experiment. The average value calculated from seven independent experiments with standard error of mean, obtained with three different animals, is shown. The statistically significant differences of the treated compared to controls is indicated by * and was calculated using the PB algorithm and statistical analysis using ANOVA on ranks. The following treatments induced a statistically significant difference: a, treated cells compared to untreated; b, cytokines + pIC compared to pIC alone; c, IT compared to IFN; d, IT + lPS compared to LPS alone.

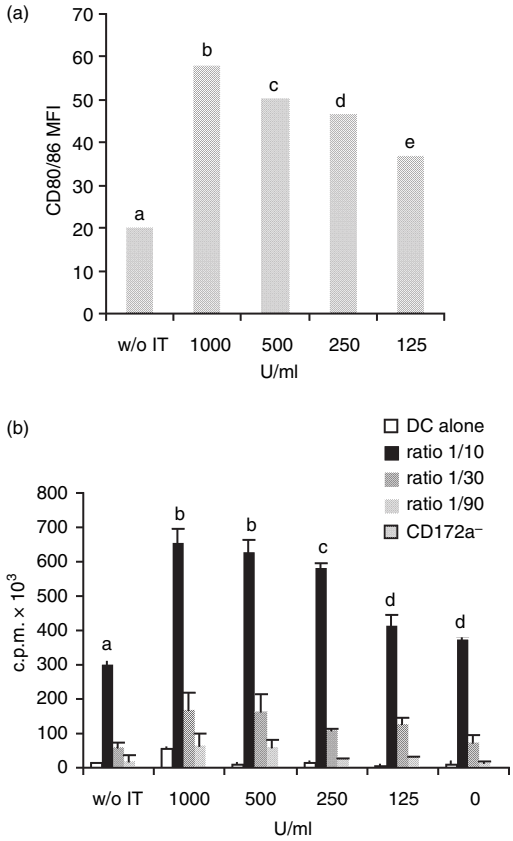

Figure 2. The dose dependency of IFN-α induced DC maturation. The effect of different concentrations of IFN-α (125–1000 U/ml) were tested in combination with a fixed concentration of TNF-α (20 ng/ml) or without IFN-α and TNF-α (w/o IT) on CD80/86 expression by MoDCs (a), and on the capacity of these MoDCs to promote a SEB-dependent T-cell proliferation (b). In (b), the DCs were matured and then loaded with SEB before coculture with lymphocytes at different ratios DC/T ($CD172a^-$) cells. The statistically significant differences was calculated using the PB algorithm (a) and statistical analysis using ANOVA on ranks (a, b). The letters a, b, c, d and e indicate the results of significance testing for differences between treatments. These results are representative of three independent experiments.

up-regulated expression of MHC II (Fig. 1b). The maturation-related phenotypic modulation was reflected in a modulated endocytic activity. Only the IFN-α alone or cocktails containing IFN-α together with TNF-α or pIC, decreased the uptake of OVA-dQ, again relating to DC maturation. Combining IFN-α, TNF-α and pIC had the most potent negative effect on endocytic/processing activity (Fig. 1c).

IFN-α and TNF-α requirements for MoDC maturation

The capacity of IFN-α to induce MoDC maturation was further analysed in terms of the stimulatory concentrations required. Varying concentrations of IFN-α were tested with a fixed concentration of TNF-α (20 ng/ml). Figure 2(a) shows that the up-regulation of CD80/86 expression was dependent on the IFN-α dose (similar results were obtained with MHC II expression – data not shown). Maximum expression was obtained with 1000 U/ml of IFN-α (Fig. 2a); higher doses did not show any additional modulation (data not shown).

A dose-dependent effect of TNF-α on DC maturation was also determined, with respect to MHC II and CD80/

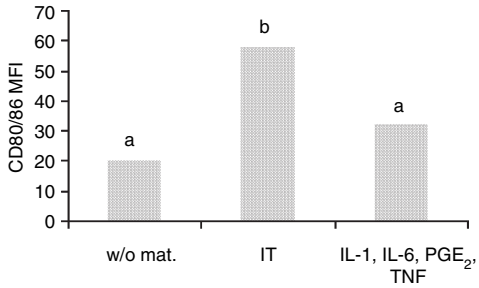

Figure 3. Comparison of MoDC maturation with a IFN-α/TNF (IT) or a IL-1/IL-6/PGE_2/TNF-α cocktail. Immature MoDC were stimulated as indicated, cultured for 24 hr and the expression levels of CD80/86 molecules were measured. The statistically significant difference was calculated using the PB algorithm and statistical analysis using ANOVA on ranks. The letters a, b indicate the results of significance testing for differences between treatments.

86 expression. In the presence of 1000 U/ml IFN-α, the TNF-α promoted phenotypic DC maturation particularly in the range of 10–100 ng/ml (data not shown).

Although the IFN-α/TNF-α cocktail up-regulated both CD80/86 and MHC II expression, it was necessary to determine if this related to a functional modulation of the DCs. Indeed, IFN-α/TNF-α treated MoDCs promoted a stronger SEB-dependent T lymphocyte proliferation (Fig. 2b). Again, this was dependent on the dose of IFN-α, with between 250 and 1000 U/ml of IFN-α relating to maximum responsiveness. The relative efficiency of the IFN-α/TNF-α cocktail at inducing DC maturation was estimated by comparing with an IL-1/IL-6/PGE_2/TNF-α cocktail, which has been demonstrated to be potent for maturation of human MoDC.[16] Interestingly, the IFN-α/TNF-α induced a higher expression of the maturation markers compared to the IL1/IL6/PGE_2/TNF-α cocktail (Fig. 3).

Maturation of DC influenced by virus-activated NIPC

DC maturation induced by cytokines such as a combination of IFN-α and TNF-α would be especially relevant when the immune system encounters pathogens not recognized as danger by myeloid DC. This situation can arise with viruses, particularly those not replicating in DC, under which circumstances the DC maturation requires a signal such as that given by the IFN-α/TNF-α cocktail described above. One prime source for these cytokines is the NIPC.[21] Attempted verification that this concept would result in DC maturation employed TGEV, a coronavirus that cannot replicate in MoDC (data not shown). The direct effect of the virus on MoDC was compared with its indirect effect mediated through NIPC.

TGEV was unable to induce IFN-α, TNF-α or IL-6 production by MoDC. In contrast, high levels of all three

Table 1. TGEV-induced cytokine responses in $CD172a^+$ cells and MoDCs

		IFN-α (U/ml)	TNF-α (U/ml)	IL-6 (pg/ml)
$CD172^+$ cells	Mock	<3	<5	<125
	TGEV	15045 ± 340	3528 ± 339	1481 ± 116
MoDC	Mock	<3	<5	<125
	TGEV	<3	<5	<125

The $CD172a^+$ PBMC and MoDCs were stimulated with TGEV (MOI 2 $TCID_{50}$/ml) or mock (uninfected ST cells lysate). Supernatants were tested for the presence of IFN-α, TNF-α and IL-6 by ELISA at 24 hr post-stimulation.

cytokines were detectable in the supernatant after TGEV stimulation of $CD172a^+$, which would contain NIPC as well as conventional blood DC and monocytes (Table 1). The only source of IFN-α production by PBMC or $CD172a^+$ cells after stimulation with TGEV are the NIPC[10,11] which represents between 2 and 5% of the total $CD172a^+$ cells. Intracellular cytokine staining confirmed that only the $CD4^+$ $CD172a^{low}$ cells – NIPC[10] – contained detectable IFN-α or TNF-α after stimulation with TGEV (Fig. 4). The dot-plot in Fig. 4(a) shows the identification of the NIPC as $CD4^+$ $CD172a^{low}$ cells (region R2). In Fig. 4(b), the intracellular staining of the cells in R2 is shown for IFN-α and TNF-α. In contrast to the $CD4^+$ $CD172a^{low}$ cells (R2), the remaining $CD172a^+$ cells, defined in R3 of Fig. 4(a), containing the $CD172a^+$ $CD4^-$ monocytes, the $CD172a^{low}$ $CD4^-$ conventional blood DC and an unknown $CD172a^+$ $CD4^+$ subset, did not produce detectable intracellular IFN-α and TNF-α after stimulation with TGEV (Fig. 4c).

Having confirmed that the TGEV would induce IFN-α/TNF-α production by NIPC, the influence of this stimulation on DC maturation was determined. As a control, TGEV was shown not to influence directly MHC II or CD80/86 expression on MoDCs (Fig. 5a, b). This was found with both the UV-inactivated and live virus (data not shown). MoDC responsiveness was controlled through the use of the IFN-α/TNF-α cocktail to induce high expression of MHC II and CD80/86. The role of the NIPC cytokines in the indirect maturation of MoDCs was then tested. Enriched NIPC (from $CD172a^+$ sorted cells) were stimulated overnight with TGEV, supernatants were then harvested and added to MoDC at 10% v/v. Following a further 24 hr incubation, analyses of the maturation status of the MoDCs, demonstrated that similar to the IFN-α/TNF-α cocktail, supernatants from TGEV- or CpG-stimulated NIPC down-regulated OVA-dQ uptake and enhanced MHC II as well as CD80/86 expression (Fig. 5c). MoDC in an advanced maturation stage were identified as cells with high levels of MHC II or CD80/86 and reduced endocytic activity for the OVA (Fig. 5d,

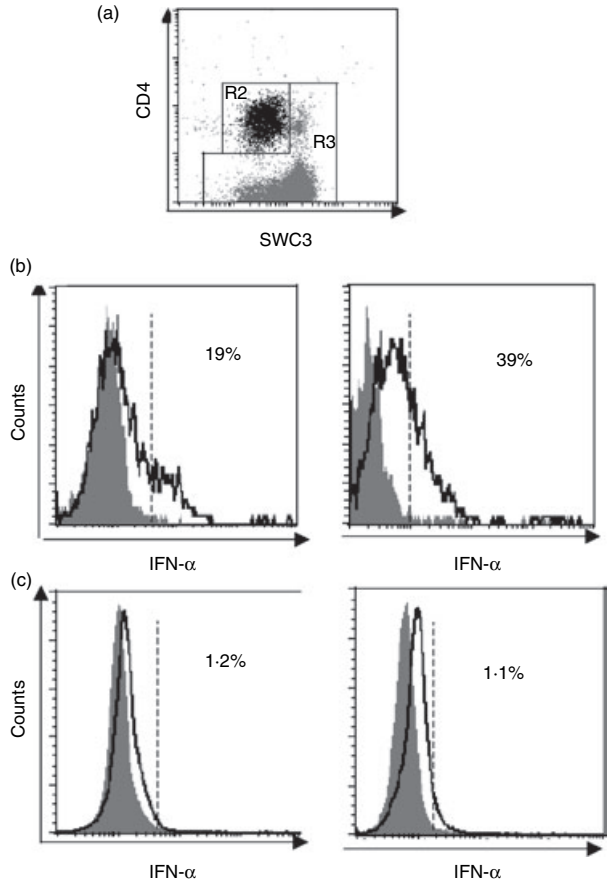

Figure 4. Detection of IFN-α and TNF-α in NIPCs after stimulation by TGEV. (a) CD172a$^+$-sorted cells were labelled for coexpression of CD4 and electronically gated to show CD4$^+$ CD172a^{low} NIPC (R2, black) and other CD172a$^+$ population (R3, grey). (b) Intracellular detection of IFN-α and TNF-α in CD172a$^+$-sorted PBMC gated on the NIPC (R2) as shown in (b) and on the other CD172a$^+$ cells (R3) is shown in (c) after stimulation with TGEV for 6 hr. The relative quantity of IFN-α and TNF-α detected by intracellular staining of the cells is shown as bold line histograms. The filled histograms are the untreated mock controls corresponding to ST cell lines. A representative experiment out of five is shown.

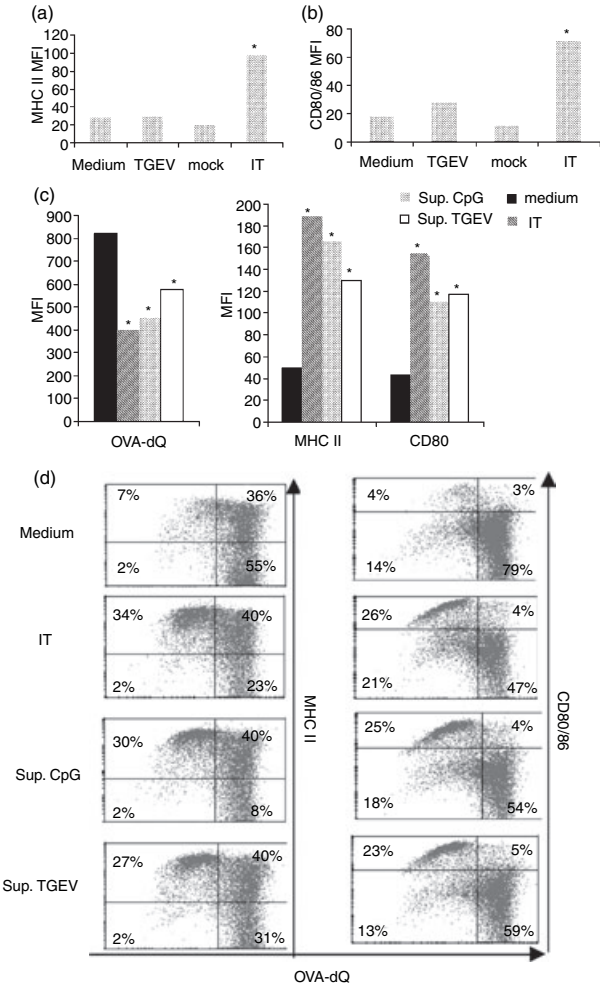

Figure 5. Indirect maturation of MoDC induced by TGEV. (a, b) Expression (represented by mean fluorescence intensity, MFI) of MHC II (a) and CD80/86 (b) on MoDCs at 24 hr after culture with TGEV, a mock control of ST cell culture supernatant (mock) or the IFN-α/TNF-α cocktail (IT). One of two representative experiments is shown. (c, d) MoDC were cultured alone or with a 1 : 10 dilution of supernatants (Sup) from CD172a$^+$ cells stimulated for 16 hr with CpG-ODN D32 or TGEV (Sup CpG and Sup TGEV, respectively). As a positive control, MoDC were stimulated with the IFN-α/TNF-α cocktail (IT). After 24 hr, the uptake and degradation of ovalbumin (OVA-dQ), MHC II and CD80/86 were determined. In c, the MFI values and in d, OVA-dQ/MHC II and OVA-dQ/CD80/86 dot-plots of a representative experiment out of three different is shown. For a–c, the statistically significant difference of the treatments compared to controls is indicated by *, and is calculated using the PB algorithm and statistical analysis using ANOVA on ranks.

upper left quadrants of all plots). The percentage of this population was promoted by the supernatant of TGEV- or CpG-stimulated NIPC, comparable to the effects of the IFN-α/TNF-α cocktail.

As described before[12] mAb 25-22 directed against the viral transmembrane glycoprotein M can block the IFN-α-inducing capacity of TGEV viral particles. In order to demonstrate the role of cytokines in the MoDC maturation, TGEV was incubated with the anti-TGEV mAb before stimulation of CD172a$^+$ cells. MoDCs stimulated only with supernatants obtained from NIPC cultures without addition of the TGEV mAb, displayed phenotypic maturation measured in terms of increase of CD80/86 and MHC II (Fig. 6a, b). This related to the concentration of cytokines in the NIPC supernatants: without mAb 25-22 blocking 4451 U/ml IFN-α and 2006 U/ml of TNF-α were quantified whereas the anti-TGEV mAb completely abrogated these responses. The important role IFN-α was also confirmed by blocking experiments using neutralizing antibodies against the cytokine. Their addi-

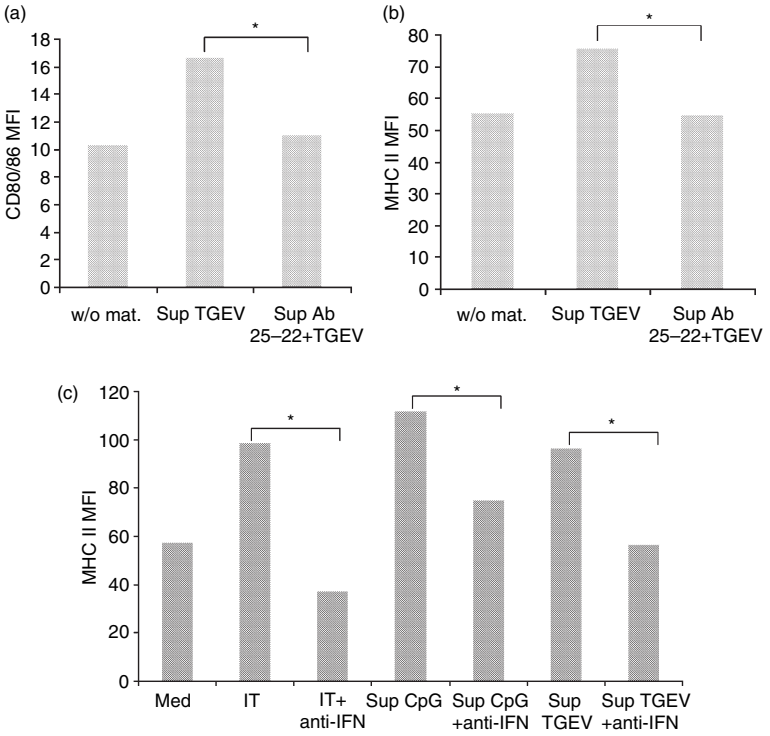

Figure 6. Inhibition of MoDC maturation with blocking antibodies against TGEV (a, b) and against IFN-α (c). CD172a$^+$ cells were stimulated with TGEV alone or with TGEV incubated with anti-protein M antibodies (25-22 mAb). After 24 hr of incubation, supernatant of cell culture were harvested, and added to immature MoDCs. Expressions of CD80/86 (a) and MHC II (b) were measured on MoDCs at 24 hr after culture. (c) Expression of MHC II was analysed after neutralization of IFN-α present in supernatant from TGEV-stimulated NIPC (Sup TGEV), from CpG-stimulated NIPC (Sup CpG) or in the IFN-α/TNF-α cocktail (IT) with polyclonal anti-IFN-α antibodies 30 min in ice before culture with MoDCs. The statistically significant differences of the effects of antibody treatments is indicated by *, and were calculated using the PB algorithm and statistical analysis using ANOVA on ranks. This result is representative of two independent experiments.

tion to the IFN-α/TNF-α cocktail or to supernatants from TGEV-stimulated NIPC completely abrogated their capacity to induce up-regulation of MHC II (Fig. 6c). Also with the supernatant of CpG-stimulated NIPC a clear reduction of the positive effect on MHC II expression was observed.

The above results demonstrated that MoDCs treated with the supernatants from stimulated NIPC had the characteristics of mature DCs.

The results from Fig. 2 implied that such MoDCs should possess enhanced T-cell stimulatory capacity. This was verified in an SEB-dependent T-cell proliferation assay (Fig. 7), for which the MoDC were matured using supernatant from TGEV-stimulated NIPC or the IFN-α/TNF-α cocktail for 16 hr before pulsing with SEB and adding to the T lymphocyte cultures. The MoDC matured with virus-activated NIPC supernatant were similar to those matured with the IFN-α/TNF-α cocktail in terms of stimulating T-cell proliferation (Fig. 7). These results confirmed the importance of NIPC for the indirect maturation of MoDC in response to viral stimuli.

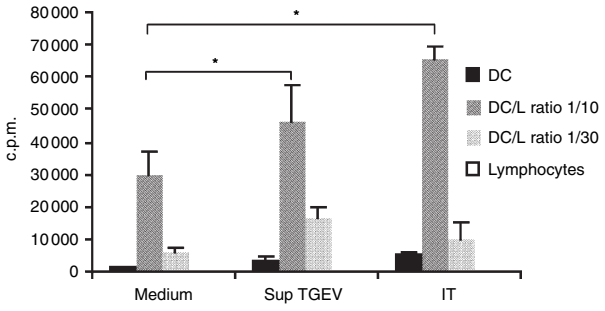

Figure 7. T-cell stimulation capacity of MoDC matured by supernatant from TGEV-stimulated NIPC cultures. MoDC were treated with a 1 : 2 dilution of supernatant from TGEV-stimulated NIPC (Sup TGEV), or the IFN-α/TNF-α cocktail (IT). After 16 hr, MoDCs were pulsed with SEB, washed and cocultured with T lymphocytes at different DC : lymphocyte (DC/l) ratios. Statistically significant differences are indicated by * and were calculated using using ANOVA on ranks. The proliferation results shown are representative of two independent experiments from one animal.

Maturation of MoDC in cocultures with lymphocytes

During immune response development, DCs do not mature in isolation, but in a reciprocal interaction with T lymphocytes. For example, molecules such as CD40L expressed by activated T cells can promote DC maturation.[22] DCs can be induced into 'terminal' maturation during this interaction.[23] Consequently, the influence of coculturing MoDCs with autologous T lymphocytes was analysed during the induction of DC maturation with TNF-α alone or with an IFN-α/TNF-α cocktail. This coculture was in the presence or absence of antigen (DT control antigen or TT antigen, for which the T cells were specific) for 3 days. The IFN-α/TNF-α treatment of DCs was more efficient than TNF-α alone at up-regulating MHC II expression in the absence of T cells or with T cells in the absence of TT antigen (Fig. 8a, no antigen,

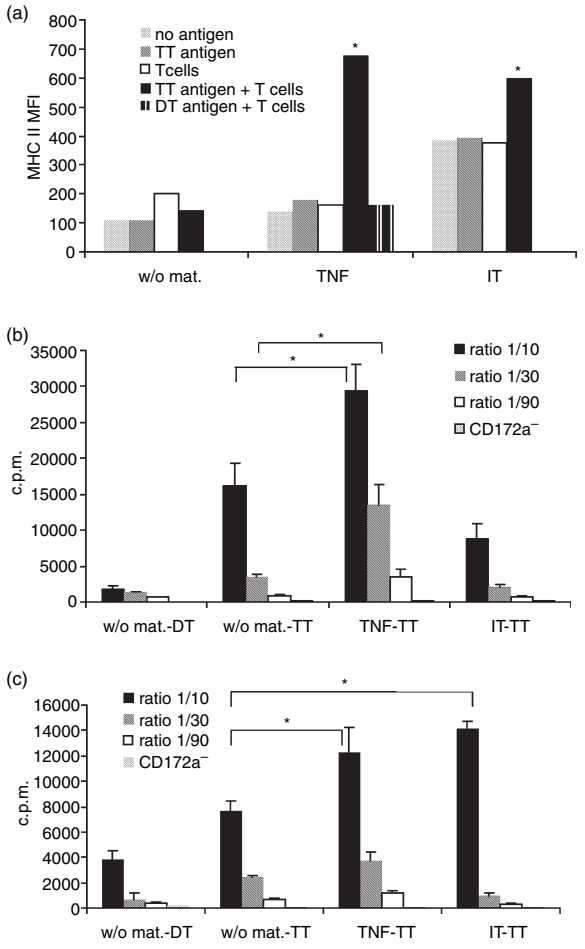

Figure 8. Terminal maturation of MoDCs during coculture with antigen-specific T lymphocytes and TT antigen. MoDCs were matured for 24 hr as shown on the *x*-axis of the graphs (without maturation, w/o mat; with TNF-α, TNF; or with IFN-α and TNF-α, IT), and then cocultured with autologous lymphocytes isolated from a pig immunized against TT antigen, together with the antigen (diphtheria toxin, DT antigen was used as a negative control). (a) MHC II expression on MoDC harvested after 3 days of coculture with the lymphocytes ($CD172a^-$ cells). (b) Antigen-specific lymphoproliferation in which the antigen was added to the MoDC were pretreated with the maturation-inducing cytokines before addition of the antigen and T cells ($CD172a^-$ cells). (c) Antigen-specific lymphoproliferation induced by MoDCs, which received the antigen prior to the addition of the maturation signals. w/o mat.-DT, no maturation of MoDCs, restimulation with DT antigens; w/o mat.-TT, no maturation of MoDCs, restimulation with TT antigens; TNF.-TT, TNF-α maturation of MoDCs, restimulation with TT antigens; IT.-TT, IFN-α/TNF-α maturation of MoDCs, restimulation with TT antigens. The statistically significant differences of the treated compared to controls (w/o) are indicated by *, and were calculated using the PB algorithm (a) and statistical analysis using ANOVA on ranks from triplicate with one animal (a, b, c). The results are representative of two independent experiments.

TT antigen, T cells, DT antigen + T cells). When the TT antigen was applied, there was a clear increase in the cytokine-induced MHC II up-regulation (Fig. 8a, TT antigen + T cells). Interestingly, the enhanced up-regulation was noted when either TNF-α alone or the IFN-α/TNF-α cocktail was applied. This would relate to the reported 'terminal' maturation effect of T lymphocytes.[23] Clearly, it was dependent on the presence of antigen for which the T cells were specific. Yet, in contrast to the observation shown in Fig. 8(a) for MHC II expression, no additional influence of the T lymphocytes was noted with CD80/86 expression (data not shown), implying that the IFN-α/TNF-α cocktail had already induced their maximal expression.

In order to confirm that the above results were reflecting increase DC functionality, as occurs with DC maturation, the cocultures were tested for TT antigen-specific T-cell proliferative activity. To this end, the DC were first matured with the cytokines for 24 hr, and then cocultured with T lymphocytes plus antigen (DT as control antigen, TT as specific antigen). The MoDC prematured with TNF-α induced a higher TT-specific lymphocyte proliferation compared with unmatured DCs and IFN-α/TNF-α prematured cells (Fig. 8b), particularly clear at DC : T cell ratios of 1 : 30 and 1 : 90.

Considering that the MoDC prematured with the IFN-α/TNF-α cocktail generally induced a low TT-specific proliferation, a possible explanation would be that these DCs had a reduced capacity to endocytose the TT, relating to the results shown in Fig. 1(c). This would imply that the TNF-α was not inducing maturation of the DCs before the T cells plus specific antigen were present. Consequently, in a second set of experiments the TT antigen was added to MoDC 24 hr before addition of the cytokines. After a further 24 hr, the MoDC were harvested and cocultured with autologous immune T lymphocytes. In this situation, both the TNF-α and the IFN-α/TNF-α cocktail promoted an increased TT-specific proliferation of T cells (Fig. 8c). Clearly, although TNF-α does not apparently induce phenotypic DC maturation, it does prime the cells for T-cell induced maturation, proving to be as effective as the IFN-α/TNF-α cocktail. This process was dependent on the combined effects of the cytokines and the presence of T cells responding to presented antigen – antigen alone had no such effect (Figs 8a,c).

Discussion

Innate immune defences are activated by exposure to PAMPs expressed by a diverse group of infectious microorganisms. These are known to induce pro-inflammatory cytokines and the maturation of DC required for initiation of specific immune responses. An imbalanced trigger of these responses has been associated with tolerance to antigens, or alternatively with autoimmune diseases. Consequently, DC maturation should be subject to a complex regulation, to avoid such processes. Knowledge of the components involved, and their role in DC matur-

ation, is required for both an understanding of disease pathogenesis and the development of improved immunotherapies with appropriate targeting. With these goals in mind, the present work sought to characterize the cellular interactions at play during the regulation of DC maturation in an *in vitro* model using porcine MoDC.

Although the TLR3 ligand pIC, and the TLR4 ligand LPS have been reported to activated MoDC[8,9,19] the present results demonstrate that the individual application of IFN-α, TNF-α, pIC, and LPS have only a relatively weak capacity to induce DC maturation. In contrast, IFN-α together with TNF-α, as well as IFN-α together with pIC or LPS, were particularly effective at inducing DC maturation. The additional presence of TNF-α in the latter two combinations further promoted DC maturation in a synergistic manner. The observation that pIC was not efficient at inducing DC maturation alone contrasted with previously published work.[8] This could result from different experimental procedures or source of cells.

During inflammation or infection *in vivo*, proinflammatory mediators such as IFN-α and TNF-α may cooperate with microbial stimuli or viral infection to induce rapid DC maturation. Interestingly, in the present assay system, the IFN-α/TNF-α cytokine cocktail was more potent at inducing DC maturation than the IL-1/IL-6/TNF-α/PGE_2 cocktail reported to induce complete maturation of human DCs.[16,24–26] Nevertheless, both cytokine cocktails have the potential to induce DC maturation. The refractory behaviour of porcine MoDC noted in response to the TLR ligands LPS and pIC in the present manuscript was surprising considering the literature on mouse and human DC, and could represent an *in vitro* phenomenon found with this type of culture. A possible explanation could be a down-regulation of certain TLR during monocyte-to-DC differentiation under the influence of IL-4.

In accordance with the concept proposed by Kitajima *et al.*[23] T lymphocytes were also shown to play an important role in the terminal maturation of MoDC. This would support a two-step process of DC maturation. First, pro-inflammatory cytokines would prime the DCs, inducing the initial increase in the expression of costimulatory molecules such as CD80 and CD86, increased expression of MHC II may also be observed. The second signal would be delivered during the interaction of the DC with the T lymphocytes, resulting particularly in further up-regulation of the MHC II molecules on the DC. Such a mechanism has been demonstrated *in vivo* during response against bacterial superantigens[27] as well as in severe combined immunodeficient and $RAG2^{-/-}$ mice.[28] In the present work, it was interesting to note that not only the IFN-α/TNF-α cocktail, but also TNF-α alone possessed the capacity to prime the DCs for 'terminal' maturation by T lymphocytes. In fact, the TNF-α alone was superior in this context. It should be stressed that the model used a secondary immune response, and it can be expected that the process of 'terminal' maturation would be dependent on the level of T-cell activation. Clearly, the process would employ a number of molecules – cytokines and cell surface molecules, especially those involved in the formation of the immunological synapse.

The main aim of the present work was to identify the mechanisms behind the priming of DCs for maturation and efficient interaction with the T lymphocytes against viral pathogens for which the cells are not equipped. This can be the case for single stranded RNA viruses, which do not replicate in DC, and thus cannot trigger receptors for double stranded RNA. In this situation their maturation can only occur indirectly through cytokines produced by other cell types, in particular NIPC. When exposed to viruses such as TGEV, as well as to CpG-ODN type A, these cells are particularly potent producers of IFN-α and TNF-α,[11] representing the cytokine cocktail effective at inducing the first stage of DC maturation. Furthermore, porcine and human myeloid DCs do not produce IFN-α in response to CpG type A motifs. Nevertheless, it has been recently described that porcine MoDC express some levels of TLR9 enabling them to respond to a CpG in terms of an up-regulation of MHC II and CD80/86 as well as mRNA of certain TLRs, IL-12 p35 and IFN-γ.[8] However, in our hands, no response of MoDC in terms of the induction of maturation and the induction of TNF-α/IFN-α protein was detected.[11] Again, this discrepancy might have been caused by different experimental procedures, readouts, source of cells or CpG sequence used.

Based on the present studies and relating to what as been described for human NIPC[21] it is in fact the NIPC which play a major role in responding to viral and ODN stimuli by producing the cytokine response required for myeloid DC maturation.[10,11] Stimulation of the NIPC by CpG-ODN Type A did induce levels of IFN-α/TNF-α which resulted in an indirect maturation of MoDCs. The importance of this functional link between the NIPC and myeloid DC has recently been reported by Fonteneau *et al.*[29] demonstrating that human immunodeficiency virus-activated plasmacytoid DCs (NIPC) mediate an indirect maturation of $CD11c^{+}$ DCs (myeloid DCs). Together, these two sets of observations confirm the concept that NIPC represent a crucial cell type in the immune system recognizing certain viruses and nucleic acid sequences to produce high levels of IFNα and TNF-α, which promote myeloid DC maturation. The *in vitro* model of porcine MoDC and NIPC employed in this study is proving of particular value in elucidating the processes involved, and its application in future studies investigating the innate immune response to pathogens should help identify optimised formulations for novel vaccine development and screening.

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