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Original article

Cholera toxin promotes the generation of semi-mature porcine monocyte-derived dendritic cells that are unable to stimulate T cells

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Abstract – Cholera toxin (Ctx) is a powerful mucosal adjuvant with potential applications for oral vaccination of swine. Dendritic cells (DC) play a key role in the decision between immunity and tolerance, and are likely target cells for mediating Ctx functions in vivo. Therefore, we examined the capacity of Ctx to enhance stimulatory activity of porcine monocyte-derived DC (MoDC). Ctx promoted the development of a semi-mature DC phenotype, with decreased levels of MHC class II and CD40, but increased CD80/86 expression. These changes were associated with activation of extracellular signal-regulated kinase (ERK), but not NFκB or c-Jun N-terminal kinase (JNK). Functionally, Ctx-priming greatly diminished T cell stimulatory capacity both in antigen-specific and superantigen-induced proliferation assays. The lower proliferation rate was not due to increased apoptosis of either DC or T cells. Ctx suppressed TNFα secretion by MoDC, but induced IL-10 production. The observed effects on T cell proliferation could only be partially mimicked by IL-10 alone. However, addition of recombinant TNFα to co-cultures of Ctx-primed MoDC and lymphocytes restored lymphocyte proliferation in a concentration-dependent manner. Ctx-primed DC were not actively tolerogenic, since they could not suppress proliferative T cell reactions induced by untreated DC.

pig / dendritic cell / cholera toxin / adjuvant

1. INTRODUCTION

Oral vaccine delivery is a convenient mode of immunisation because of easy administration and good acceptability. It can also be used for bait vaccination of wildlife, e.g. immunisation of wild boar against classical swine fever [24, 33]. To prevent unnecessary immune reactions against harmless food proteins, the default reaction to orally applied, non-replicating antigens is the development of tolerance [45]. This has to be overriden by co-application of mucosal adjuvants.

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Cholera toxin (Ctx) is a potent mucosal adjuvant that has been used successfully in a large number of murine studies [10, 13, 14, 29]. Unfortunately, the high toxicity of native Ctx in humans precludes the clinical use of Ctx [32]. In the pig, studies investigating the potential of Ctx as a mucosal adjuvant yielded contradictory results: co-administration of Ctx together with keyhole limpet hemocyanin (KLH) did not evoke any detectable antibody response to KLH [16]. In contrast, Verdonck et al. [48] could demonstrate that co-application of Ctx with *Escherichia coli* F4 fimbriae improved both humoral and cellular response to F4, and reduced *E. coli* excretion in a challenge experiment.

The results from studies in humans and mice indicate that the adjuvanticity of Ctx may largely depend on its interaction with dendritic cells (DC), which mature in the presence of Ctx [18, 19, 22]. DC are professional antigen-presenting cells (APC) with the unique ability to activate naïve T cells [4]. Specific DC subsets and maturation stages of DC can direct the immune system towards either tolerance or immunity [7, 15]. In the pig, the effects of Ctx on alveolar macrophages have been analysed [17]. In vitro stimulation with Ctx caused an increased expression of inflammatory cytokines and co-stimulatory molecules, indicating macrophage activation, which could promote an active immune response against other antigens. However, the functional roles of alveolar macrophages as APC clearly differ from those of DC. Furthermore, Ctx can also confer immunomodulatory effects through DC [29].

To further analyse the adjuvant mechanism of Ctx in pigs, we focussed on DC as key antigen presenting cells. Although DC located in the intestinal lamina propria, Peyer's Patches and mesenteric lymph nodes are the most likely target cells for mucosal adjuvants, these cells are not easily accessible for in vitro studies. In contrast, DC generated from blood monocytes (monocyte-derived dendritic cells, MoDC) are a well established and stable model system in swine research [8, 23, 35, 37, 38]. Therefore porcine MoDC were used for an exploratory in vitro study to investigate the effects of Ctx on porcine DC. Our data show that, in contrast to previous observations with human MoDC, Ctx induces a unique semi-mature MoDC phenotype with impaired stimulatory capacity in the porcine system.

2. MATERIALS AND METHODS

2.1. Animals and blood samples

Blood samples were taken from specific pathogen-free Landrace pigs kept at the Institute of Virology and Immunoprophylaxis (IVI), Switzerland, using citrate as an anticoagulant. Alternatively, heparinised blood was obtained from Landrace pigs kept under standard conditions at the Institute of Medical Technology Magdeburg GmbH, Germany. To avoid stress reactions, pigs were sedated with Ketamine/Xylacine (standard dose) during sampling procedures in some instances. All animal experiments were in accordance with local animal welfare regulations, permission numbers 19/05 (Switzerland) and 203.6.1-42502/2-641 (Germany).

2.2. Isolation and culture of cells

PBMC were isolated from whole blood by Ficoll density gradient centrifugation (Pharmacia, Uppsala, Sweden). Monocytes were further enriched to > 95% purity by positive magnetic bead selection (MACS, Miltenyi-Biotec, Bergisch Gladbach, Germany) using an antibody directed against CD172a (mAb 74-22-15A) and goat anti-mouse IgG microbeads together with LS separation columns (MACS). Cells were then cultured at 39 °C, 5%

CO_2 with phenol-red free Dulbecco's modified Eagle's Medium (DMEM; Invitrogen, Basel, Switzerland) supplemented with porcine serum (10% v/v; Sigma, Buchs, Switzerland), recombinant porcine (rp) GM-CSF (150 ng/mL, kindly provided by Dr S. Inumaru, Institute for Animal Health, Ibaraki, Japan, or purchased from Biosource, Camarillo, CA, USA) and rp IL-4 (50 ng/mL; prepared at the IVI, or from Biosource) to generate MoDC as described previously [8, 49]. Cultures were supplemented with fresh cytokines after three days and further processed as indicated.

2.3. Stimulation of MoDC cultures

Ctx or Ctx-B-subunit (Sigma, Taufkirchen, Germany) were added to MoDC cultures for 24 to 48 h on day 3 at final concentrations of 1 μg/mL, following previous studies with human and murine DC [2, 18, 19, 22]. To exclude the influence of LPS contamination of the toxin preparation, Ctx detoxified with Detoxi-Gel™ AffinityPak™ Columns (Perbio Science, Bonn, Germany) was used in some experiments. Negative controls were prepared by pre-incubation of Ctx with its soluble receptor, GM1 (10 μg/mL; Sigma). DC maturation was induced with different combinations of the following reagents: rp IFNα (1 000 U/mL), rp TNFα (10 ng/mL), or LPS (1 μg/mL, Sigma). To analyse the influence of IL-10 on MoDC, cultures were stimulated with 1-1 000 ng/mL of rp IL-10 (Biosource). IFNα was prepared at the IVI laboratory as described previously [3], and TNFα was kindly provided by Dr G. Bertoni, Bern [50] or purchased from NatuTec (Frankfurt, Germany).

2.4. Monoclonal antibodies (mAbs) and phenotyping

For phenotyping, mAbs against the following cell surface molecules were used: CD172a (mAb 74-22-15A; recognises SIRPα, SWC3a), MHC class II (mAb MSA3), CD40 (mAb G28-5, anti-human), CCR1 (mAb 145, anti human, R&D Systems, Abingdon, UK). A human CTLA-4 muIg fusion protein (Ancell, Bayport, MN, USA) was used to detect expression of CD80 and CD86. Hybridomas for the mAbs 74-11-10, MSA3, and 74-22-15A were donated by Prof. A. Saalmüller (University of Veterinary Medicine Vienna, Austria). Hybridoma supernatant for the mAb G28-5 was provided by T. Bedke (Medical School of Hanover, Germany).

For immunofluorescence labelling, the cells received the mAbs at pre-titrated saturating concentrations for 30 min at 4 °C, then the appropriate isotype-specific conjugates (goat anti-mouse antibodies, coupled to FITC or PE; Southern Biotechnology, Birmingham, AL, USA) for another 15 min at 4 °C. The cells were analysed with a FACSCalibur flow cytometer using CellQuest Pro® software (Becton Dickinson).

2.5. Antigen uptake

Uptake of soluble antigen was studied using DQ™ ovalbumin (OvaDQ, Molecular Probes, Leiden, Netherlands). MoDC were harvested from the plates and incubated for 30 min at 37 °C with 10 μg/mL of OvaDQ in culture medium. The cells were then analysed by flow cytometry.

2.6. MoDC proliferation assays

Superantigen-dependent stimulation was analysed in a staphylococcal enterotoxin B (SEB) presentation assay. Autologous lymphocytes were enriched by depletion of CD172a positive cells from PBMC using the MACS system. MoDC pre-treated with Ctx and/or other stimulants were harvested, washed, and

then incubated 1 h at 39 °C with SEB (1 μg/mL, Toxin Technology, Sarasota, FL, USA), followed by three washing steps. MoDC were then co-cultured at a ratio of 1:10 with 200 000 lymphocytes per well in triplicates. After three days of culture, the cells were pulsed with 1 μCi/well [^{3}H]methyl-thymidine (Moravek Biochemicals) for another 18 h. Plates were harvested onto filter mats and counted in a 1450 MicroBeta® Trilux counter (Wallac). Alternatively, cell proliferation was evaluated by BrdU-ELISA (Roche, Mannheim, Germany) according to the manufacturer's instructions, or by MTT-assay as described previously [11]. Proliferation index was calculated by dividing measured absorption of samples by absorption value of untreated lymphocytes.

Antigen-specific stimulation was assessed using lymphocytes from pigs primed with classical swine fever virus (CSFV) by infection with 10^3 $TCID_{50}$ of CSFV strain Alfort [41]. MoDC cultures were pulsed with the following antigens for 26 h, with Ctx added after 2 h if required: (1) CSFV strain Alfort (0.01 $TCID_{50}$/cell), (2) mock antigen prepared from uninfected SK6 cells used to propagate the virus [27], (3) E2-protein of CSFV, baculovirus-expressed (4 μg/mL) produced as described elsewhere [36], or (4) CSFV-non-structural protein 3 (NS3)-protein of CSFV, *E. coli*-expressed (2 μg/mL; kindly provided by Dr Hilde Revets, Free University Brussels, Belgium). MoDC were then co-cultured with autologous lymphocytes in the presence of the respective antigens for 3–4 days, and analysis of [^{3}H]methyl-thymidine incorporation was performed as described above.

To examine the role of TNFα in MoDC-lymphocyte co-cultures, pretreated MoDC were loaded with SEB as described above and cultured with autologous lymphocytes in the presence of rp TNFα (0.1-10 ng). Proliferation was determined by BrdU-ELISA. For these experiments, β-mercaptoethanol (50 μmol, PAN Biotech, Aidenbach, Germany) was added to the medium, which resulted in considerably higher proliferation indices.

2.7. Apoptosis assay

Induction of apoptosis and cell death by Ctx was assessed using Annexin V-FITC and propidiumiodide (Serotec, Oxford, UK). MoDC were stimulated with 1 μg of Ctx for 2, 6 or 24 h. Alternatively, co-cultures of pre-stimulated MoDC and lymphocytes were set up as described previously for the proliferation assays, and lymphocytes were analysed for apoptosis after 2, 6 or 24 h of co-culture. In positive control samples, apoptosis was induced by adding staurosporin (1 mM, Sigma). Samples were then stained with Annexin V-FITC following the manufacturer's protocol. After addition of propidiumiodide (5 μg/mL), the cells were analysed with a FACSCalibur flow cytometer using CellQuest Pro® software (Becton Dickinson).

2.8. Detection of cytokine production

Cell-free culture supernatants of MoDC were collected for analysis of cytokines. To avoid a direct influence of the added stimulants on the PK15-15 cells in the TNFα bioassay, medium was exchanged twice after the first hour of incubation with the stimulants, and supernatants were collected after a total of 6, 24 or 48 h. IL-10 and IL-6 were quantified using commercial ELISA kits (IL-10: Biosource; IL-6: R&D Systems, Minneapolis, USA). Similarly, IL-12 levels were detected by ELISA as previously described [20]. TNFα production was analysed by bioassay as previously described by Bertoni et al. [6]. Briefly, PK15-15 cells (kindly provided by Dr G. Bertoni, Bern, Switzerland) grown

in DMEM (Invitrogen, Basel, Switzerland) supplemented with 7.5% FCS (Sigma) were seeded at a density of 2×10^5 in 96 well plates. After over night culture at 37 °C, 5% CO_2, the cells were sensitised to TNFα-induced cell death with 3 μg/mL actinomycin D (Sigma, Taufkirchen, Germany) for 2 h before adding supernatant samples. The number of viable cells was analysed after 16 h using MTT, as described above. TNFα concentrations were calculated from a standard curve generated with rp TNFα (NatuTec, Frankfurt, Germany).

2.9. SDS-PAGE and immunoblotting

MoDC were stimulated for 10–90 min with 10 μg/mL Ctx to ensure optimum loading of receptors during the short incubation time. The cells were then lysed, and proteins were subjected to SDS-PAGE and immunoblotting as described previously [34]. Immunoblots were developed using enhanced chemiluminescence (ECL, Amersham Biosciences) according to the manufacturer's instructions. The following antibodies were used to detect the respective proteins: anti-IκBα (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-IκBα (Ser32/36), anti-extracellular signal-regulated kinase (ERK), anti-phospho-ERK, and anti-phospho- c-Jun N-terminal kinase (JNK) (all Cell Signaling, Frankfurt, Germany).

2.10. Analysis of the effect of secreted cytokines on lymphocyte proliferation

To assess the direct effects of IL-10 on proliferation of porcine lymphocytes, 5–100 ng/mL of rp IL-10 (Biosource) were added to PBMC cultures (1×10^5/well) together with ConA (10 μg/mL; Sigma). Alternatively, to detect the influence of other factors secreted by MoDC, PBMC were incubated in the presence of cell-free culture supernatants collected 3–24 h after stimulation of the DC, and cell proliferation was measured by MTT-assay after 3 days of culture in DMEM supplemented with 10% of porcine serum at 39 °C, 5% CO_2.

2.11. Statistical analyses

Statistical analyses were performed with Microsoft® Excel 2000 software. The results are given as mean ± standard deviation. Differences between values were analysed for statistical significance with the Student's *t*-test and were considered significant at $P < 0.05$.

3. RESULTS

3.1. Influence of Ctx on expression of maturation markers by MoDC

In order to evaluate DC maturation status, we analysed surface expression of MHC class II (MHC-II), CD40, CD80/CD86, and chemokine receptor CCR1 by flow cytometry. Stimulation of MoDC with LPS, TNFα, LPS+TNFα, or IFNα+TNFα ("maturation factors"), caused an increase in MHC-II, CD40 and CD80/86 expression, and a decrease in CCR1 expression. These changes are typically associated with functional DC maturation. Representative histograms are shown in Figure 1. Surprisingly, addition of cholera toxin (Ctx) to MoDC cultures decreased surface expression of both MHC-II and CD40. This effect was consistently observed, with a geometric mean fluorescence intensity reduction of MHC class II by 28% ± 5% ($P < 0.001$, Student's *t*-test). The decreased expression of maturation markers became especially prominent in the presence of maturation factors, indicating inhibition of DC maturation by Ctx. In clear contrast to the effect

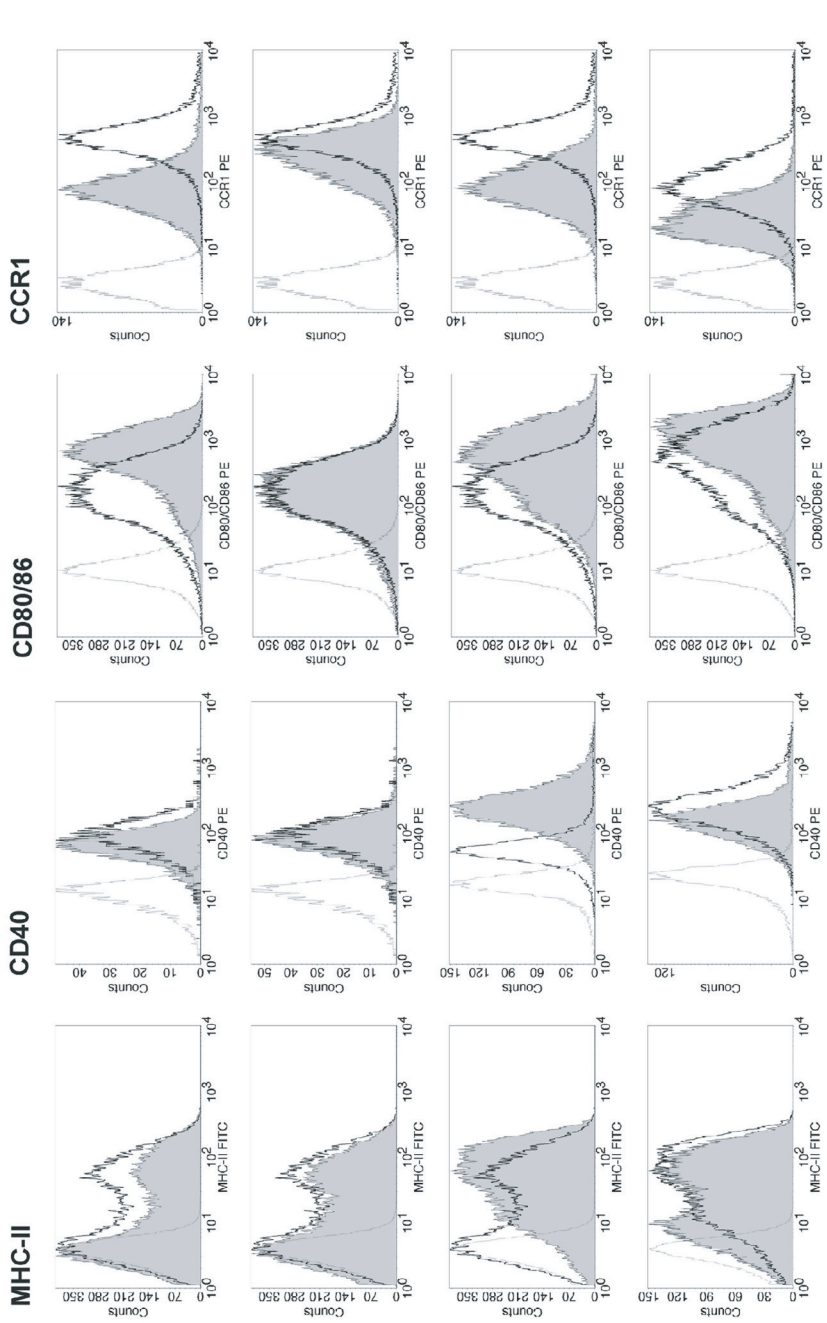

Figure 1. Influence of Ctx on surface expression of MoDC maturation markers. FACS analysis of MHC-II, CD40, CD80/86 and CCR1 expression on porcine MoDC. Three-day porcine MoDC were cultured for 48 h in the presence or absence of Ctx and/or maturation factors. GM1 was used to block Ctx binding to MoDC as a control. Black outline: upper three rows – untreated control MoDC, bottom row – MoDC + maturation factors; solid grey histogram: MoDC + stimulants as indicated on the left; dotted grey outline: conjugate control. Maturation factors were IFNα+TNFα for the MHC-II, CD80/86 and CCR1 histograms, and LPS+TNFα for the CD40 histograms. Representative results from more than five similar experiments are shown.

on MHC and CD40, Ctx caused an increased CD80/86 and decreased CCR1 expression, consistent with maturation. In the presence of IFNα+TNFα, Ctx had an additive effect on the modulation of CD80/86 and CCR1 expression. All effects were already seen after 24 h, but became more prominent after 48 h of stimulation. In general, Ctx-induced effects on maturation marker expression could be largely abrogated if soluble GM1 receptor was added to the cultures, which demonstrates that the observed changes were Ctx-specific. Similarly, Ctx retained its activity after removal of LPS using Detoxi-Gel™ AffinityPak™ columns (data not shown), confirming that the observed effects were not caused by LPS-contamination of the Ctx preparation.

3.2. Cholera toxin does not alter endocytic capacity of MoDC

Antigen uptake is a typical feature of immature DC, and is therefore reduced after maturation. We tested the endocytic capacity of porcine MoDC after 48 h of culture in the presence of different stimulants, using the protein conjugate DQ™ Ovalbumin (OvaDQ) as marker substances. As shown in Figure 2, Ctx alone had no obvious effect on OvaDQ uptake. Likewise, although the maturation factors LPS+TNFα caused the expected down-regulation of OvaDQ uptake, this was not further modified by the addition of Ctx to the cultures.

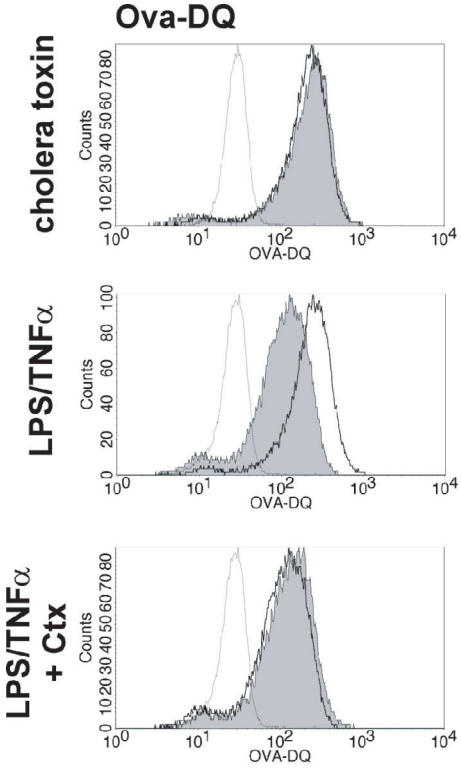

Figure 2. Influence of Ctx on endocytic capacity of MoDC. Uptake of DQ™ ovalbumin (OvaDQ) by MoDC after 30 min incubation at 39 °C was analysed by flow cytometry. DC maturation induced by LPS+TNFα caused a reduction in OvaDQ uptake. Dotted grey outline: control cell incubated at 4 °C; black outline: upper two histograms – untreated control MoDC; bottom histogram – MoDC+LPS+TNFα; solid grey histogram: MoDC + stimulants as indicated on the left. One representative experiment out of three is shown.

3.3. Effects of Ctx on ERK and NF-κB activation

We investigated the impact of Ctx on the inducible activation of ERK, JNK and NFκB in MoDC. As shown in Figure 3, phosphorylation of ERK was induced within 20 min and increased steadily within the indicated periods of time. In contrast to ERK activation, we could not recognise phosphorylation of the NF-κB inhibitor IκBα in Ctx-treated porcine DC. Additionally, we observed no IκBα degradation in response to Ctx. Further, we observed no phosphorylation of JNK, a kinase that regulates the activity of c-Jun within the transcription factor complex AP-1, in Ctx-treated cells (data not shown).

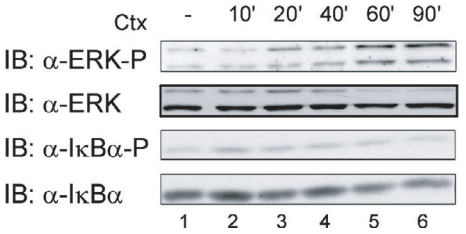

Figure 3. ERK activation in Ctx-treated DC. Monocyte-derived dendritic cells were either left untreated or stimulated with 10 μg/mL Ctx for 10–90 min. The protein extracts were subjected to SDS-PAGE and analysed in an immunoblot with antibodies against phospho-ERK, ERK (loading control), phospho-IκBα and IκBα. The IκBα protein was not degraded in response to Ctx. Data are representative of two independent experiments.

Therefore, we conclude that Ctx induces the signalling cascades leading to ERK activation, but has no effect on the regulation of the immediate early response transcription factors NF-κB and AP-1.

3.4. Ctx-primed DC inhibit T-cell proliferation

To further analyse the functional effects of Ctx on MoDC, the capacity of Ctx-primed DC to stimulate T-cells was studied in a staphylococcal enterotoxin B (SEB) proliferation assay. As shown in Figure 4A, pre-treatment of MoDC with Ctx for 24 h significantly inhibited their T-cell stimulatory capacity. This effect was more prominent if DC were matured by adding LPS+TNFα and could be averted by adding GM1 to the cultures.

Antigen-specific proliferation assays with CSFV-antigens and lymphocytes of CSFV-primed donors revealed a similar suppression of stimulatory capacity of MoDC by Ctx. This effect was observed when co-cultures were infected with CSFV-virus (Fig. 4B) or mock, and also when the E2 (Fig. 4C) or the CSFV-non-structural protein 3 (NS3) proteins of CSFV (Fig. 4D) were used as antigens.

3.5. Ctx does not induce apoptosis of DC or lymphocytes

To analyse whether the reduced T cell proliferation induced by Ctx-primed MoDC was due to increased cell death, we performed Annexin-V FITC assays to detect apoptosis of MoDC and co-cultured lymphocytes. Figure 5A demonstrates that neither Ctx or LPS+TNFα alone nor in combination caused an increased rate of MoDC apoptosis after 2, 6 or 24 h. In contrast, > 80% of apoptotic cells were detected after 24 h of incubation with staurosporin.

Although Ctx did not directly cause apoptosis of MoDC, we hypothesised that Ctx-primed DC might mediate increased T cell death. Therefore, the percentage of apoptotic lymphocytes was measured in DC-lymphocyte co-cultures. As shown in Figure 5B, 24 h priming of MoDC with up to 10 μg/mL of Ctx did not result in a functional state of the DC that induced lymphocyte apoptosis. The presence of maturation factors did not alter the outcome of this experiment. Similarly, PI staining signifying necrosis and later stages of apoptosis was not influenced by Ctx (data not shown). Thus, apoptosis is unlikely to be the cause for the reduced proliferation rates.

3.6. Ctx induces IL-10 and inhibits TNFα expression by porcine MoDC

Since the reduced lymphocyte proliferation caused by Ctx-treatment of MoDC was not due to increased lymphocyte apoptosis, we examined whether pro- or anti-inflammatory cytokines might play a role. The amounts of IL-10, IL-6 and IL-12 in culture supernatants of MoDC were studied by ELISA. As shown in Figure 6A,

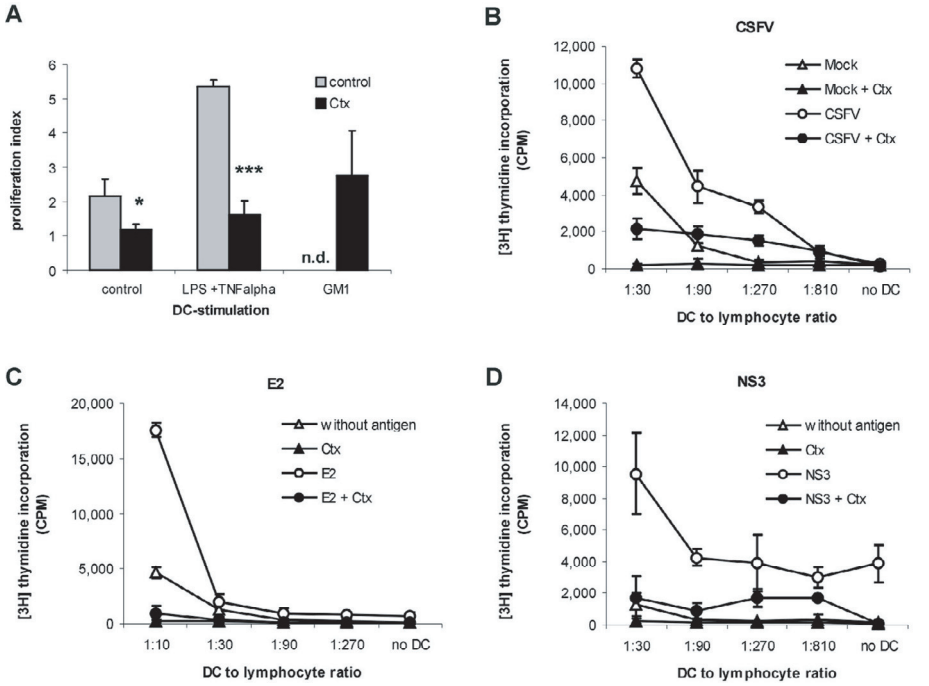

Figure 4. Inhibition of T-cell proliferation by Ctx-primed MoDC. (A) SEB-dependent proliferation assay. Porcine MoDC were left untreated or stimulated for 24 h with LPS+TNFα or GM1 in the presence or absence of Ctx. These cells were then loaded with SEB. The proliferative response of CD172a-depleted PBMC to these cells at a 1:10 ratio was determined after 4 days by BrdU-ELISA and is shown as the proliferation index. Mean ± SD of triplicate wells from one representative out of five experiments are shown. n.d. = not done, * $P < 0.05$, *** $P < 0.001$. (B – D) Antigen-specific proliferation assays. After a total incubation time of 26 h, antigen-pulsed MoDC with or without Ctx treatment were co-cultured with autologous lymphocytes in the presence of (B) CSFV (0.01 $TCID_{50}$) or mock antigen; (C) E2-antigen (4 μg/mL), or (D) NS3-antigen (2 μg/mL). Proliferative responses were determined after 4 days by [H^3] thymidine incorporation and are shown as counts per minute (CPM).

Ctx alone or in combination with either LPS, TNFα, or IFNα+TNFα induced the secretion of IL-10 after 24 h. IL-10 levels were the highest in cultures where Ctx was used in combination with LPS. LPS alone consistently induced low but detectable amounts of IL-10, whereas no IL-10 was found in cultures treated with TNFα, IFNα+TNFα, or in control cultures. In contrast to IL-10, neither IL-6 nor IL-12 secretion could be induced by addition of maturation factors to MoDC cultures at any of the time points analysed (6 h, 24 h, and 48 h), even after stimulation with LPS (data not shown). This was not significantly altered by addition of Ctx.

As shown in Figure 6B, Ctx had a significant effect on TNFα secretion. Maturation factors LPS+TNFα induced a release of TNFα after 6 h. If Ctx was present in the MoDC cultures in addition to the maturation factors, TNFα secretion was significantly reduced ($P < 0.001$).

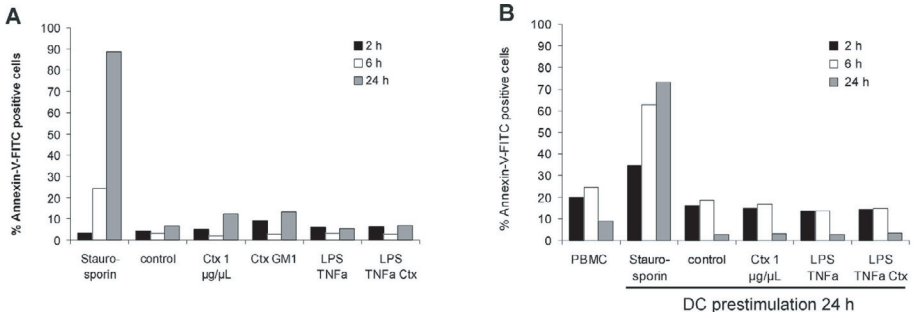

Figure 5. Ctx treatment of MoDC does not cause apoptosis of DC or of co-cultured lymphocytes. (A) Three-day MoDC cultures were stimulated with Ctx (1 μg/mL), Ctx + GM1 (10 μg/mL), LPS (1 μg/mL) + TNFα (10 ng/mL), or Ctx + LPS + TNFα for 2, 6 or 24 h. (B) Three-day MoDC cultures were stimulated with Ctx (1 μg/mL or 10 μg/mL) and/or maturation factors for 24 h (1 μg/mL Ctx ± 1 μg/mL LPS + 10 ng/mL TNFα). Cells were then harvested, treated with SEB and co-cultured with lymphocytes according to the protocol used for antigen-unspecific proliferation assays. Apoptosis of (A) MoDC and (B) lymphocytes was analysed by Annexin-V FITC-binding. Graphs show the percentage of apoptotic cells determined by FACS after the indicated periods of time. As a positive control, apoptosis was induced by addition of staurosporin (1 μM).

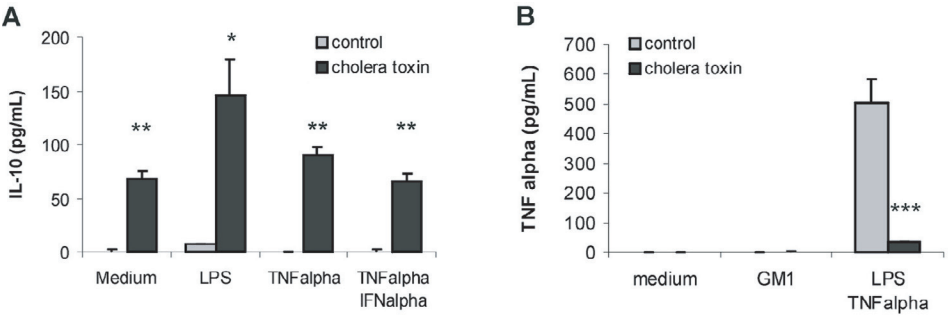

Figure 6. Cytokine production by Ctx-primed MoDC. (A) IL-10 levels in culture supernatants of MoDC stimulated with maturation factors (LPS, TNFα, or IFNα+TNFα) in the presence or absence of Ctx were determined by ELISA. Duplicate supernatants of three independent cultures were analysed after 24 h. (B) TNFα production by MoDC cultures stimulated with Ctx, Ctx + GM1, LPS+TNFα, or Ctx+LPS+TNFα. Medium was exchanged twice after the first hour of incubation with the stimulants, and culture supernatants sampled after a total of 6 h were analysed by bioassay using PK15-15 cells. The results from one out of four independent experiments are shown. Differences between Ctx-treated and control cultures were tested for significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

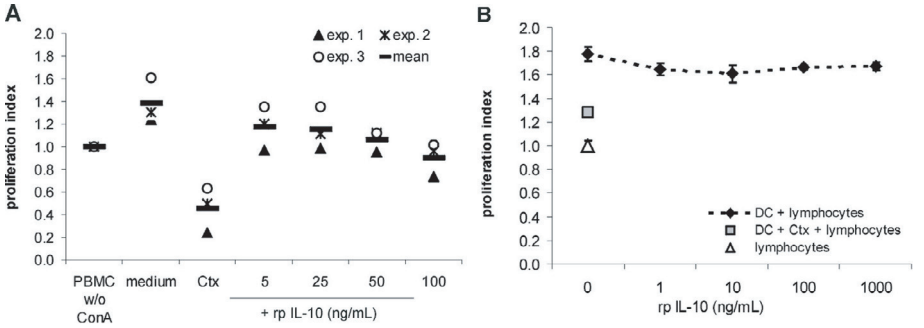

Figure 7. Influence of IL-10 in Ctx-induced inhibition of lymphocyte proliferation. (A) IL-10 induced inhibition of mitogen-driven lymphocyte proliferation. PBMC were cultured for 3 days in the presence of ConA and various concentrations of rp IL-10. Ctx (1 μg/mL) was used as positive control to suppress proliferation. Proliferation was determined by MTT-assay. Mean (bar) and data from individual experiments (exp., other symbols) of three independent experiments are shown. (B) IL-10 has no influence on stimulatory capacity of porcine MoDC. Porcine MoDC were stimulated for 24 h with Ctx or different concentrations of rp IL-10 and then loaded with SEB. The proliferative response of CD172a-depleted PBMC to the DC at a 1:10 ratio was determined after 3 days using an MTT-assay. One representative of three independent experiments is shown. The proliferation index was calculated by dividing absorption measured for the individual samples by absorption of unstimulated PBMC cultures.

3.7. Altered cytokine secretion profiles contribute to the inhibitory effects of Ctx-primed MoDC on lymphocyte proliferation

Since we observed increased IL-10 secretion by MoDC after Ctx stimulation, and IL-10 has been associated with a tolerogenic function of DC, we hypothesised that IL-10 might mediate the decreased T cell proliferation induced by Ctx-priming of MoDC. Therefore, we analysed whether IL-10 can directly inhibit proliferation of porcine lymphocytes. As shown in Figure 7A, the addition of IL-10 to mitogen-stimulated PBMC cultures caused a reduction of proliferation in a concentration-dependent manner. However, this effect was relatively weak, despite the high concentrations of IL-10 used in the assays. In addition, culture supernatants taken 6 or 24 h after MoDC stimulation with Ctx had no significant effect on PBMC proliferation (data not shown).

DC were stimulated with different IL-10 concentrations before performing SEB-proliferation assays in order to investigate possible autocrine effects of secreted IL-10 on the MoDC. As shown in Figure 7B, IL-10 concentrations between 1 ng/mL and 1 μg/mL did not alter the stimulatory capacity of the DC.

We hypothesised that the decreased TNFα secretion by Ctx-primed MoDC contributed to the reduced lymphocyte proliferation. Therefore rp TNFα was added to co-cultures of Ctx-primed DC and lymphocytes during the SEB-proliferation assays. Figure 8 demonstrates that the presence of TNFα in the co-cultures could fully restore proliferation in a concentration dependent manner.

3.8. Ctx-primed MoDC cannot inhibit lymphocyte proliferation that is induced by other MoDC

To investigate whether Ctx-primed MoDC have direct suppressor effects on

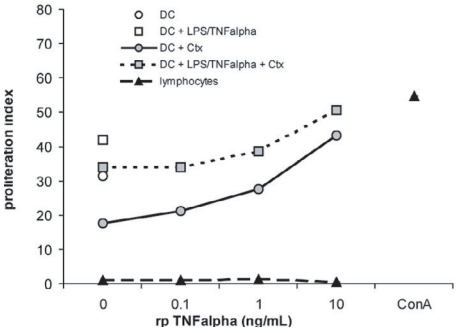

Figure 8. TNFα can restore lymphocyte proliferation in co-cultures with Ctx-primed MoDC. MoDC were treated with rp TNFα (10 ng/mL) and LPS (1 μg/mL) to induce maturation or left untreated. Simultaneously, Ctx was added to some cultures as indicated. After 24 h, DC were loaded with SEB and then co-cultured with lymphocytes at a 1:10 ratio in the presence of different concentrations of rpTNFα. Proliferation was determined by BrdU-ELISA after 4 days and is given as a proliferation index. As controls, lymphocytes alone were stimulated with TNFα or ConA (5 μg/mL). Mean from one of two similar experiments is shown.

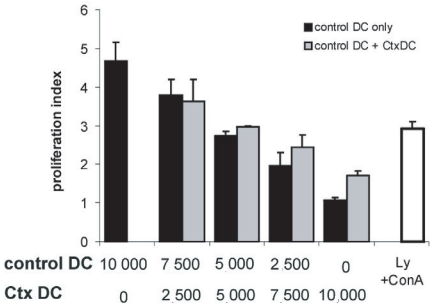

Figure 9. Ctx-primed MoDC do not suppress stimulatory capacity of untreated DC. Porcine MoDC were stimulated for 24 h with LPS+TNFα+Ctx (Ctx DC) or LPS+TNFα only (control DC). MoDC were then loaded with SEB, and the indicated numbers of control DC alone, or both control DC and Ctx DC were co-cultured with 100 000 lymphocytes. Lymphocytes (Ly) stimulated with ConA (10 μg/mL) served as positive control. Proliferation was determined after four days using a BrdU-proliferation assay. One representative experiment out of six is shown.

the function of other DC or lymphocytes and thus act as regulatory cells, lymphocyte proliferation in response to untreated MoDC in the presence of Ctx-treated MoDC was analysed. Stimulatory activity of untreated MoDC was not altered by the presence of Ctx-primed DC in the cultures, nor was the responsiveness of the T cells negatively modulated (Fig. 9). Thus, we could not obtain any evidence for an active suppressor function of Ctx-primed porcine MoDC.

4. DISCUSSION

In this study, the effects of cholera toxin (Ctx) on porcine monocyte-derived dendritic cells (MoDC) were analysed to explore the potential contribution of DC to the mucosal adjuvanticity of Ctx in the pig. Our main finding was a semi-mature, non-stimulatory phenotype of MoDC after in vitro Ctx-stimulation.

The phenotypic maturation of DC is typically associated with upregulation of molecules involved in the formation of the immunological synapse (e.g. MHC-I/II, CD40, CD80, CD86) [5]. After Ctx-stimulation, porcine MoDC acquired some features of mature DC, but retained others of immature DC, consistent with a "semi-mature" phenotype [31, 42]. Thus, expression of CD80/86 was increased, whereas expression of MHC-II and CD40 was decreased. Also, Ctx treatment caused a reduced expression of CCR1, a receptor for inflammatory chemokines which is typically down-regulated upon maturation to enable DC exit from inflamed tissue [44]. The endocytic capacity of DC, which decreases during the maturation process [43], was not modulated by Ctx stimulation.

It has previously been shown that Ctx confers stimulatory effects through DC in some experimental settings [18, 19, 26], and tolerogenic effects in others [28, 29]. This is possibly related to the activation and maturation stage of the DC at the time they encounter the Ctx. In our hands, Ctx induced the development of DC that were neither stimulatory nor actively suppressive. Ctx-primed porcine MoDC could neither induce T cell proliferation nor inhibit the proliferation of T cells activated by other DC present in the culture.

The observed phenotype of the DC in response to Ctx may largely be linked to an increase in intracellular cAMP. Ctx causes increased plasma levels of cAMP through the ADP-ribosyltransferase activity of the A-subunit after binding of the B-subunit to the cell surface via the ubiquitous GM1 ganglioside [2, 9, 10, 46]. Consistent with our observations for Ctx, cAMP-elevating agents can directly increase IL-10 production, inhibit TNFα secretion and MHC-II and CD40 expression, and reduce stimulatory capacity in murine DC [25]. Although Ctx-primed porcine MoDC secreted higher amounts of IL-10, IL-10 alone had only weak effects on the proliferation of porcine T cells. Also, direct application of IL-10 to DC did not induce a non-stimulatory functional state. It is thus unlikely that the observed effects were primarily due to IL-10.

In porcine MoDC cultures, TNFα cooperates with IFNα or T cells to induce DC maturation [21]. Recently, it was shown that blocking TNFα in human DC results in the generation of semi-mature DC [47]. Indeed, the stimulatory capacity of Ctx-primed MoDC seems to be largely due to a lack of TNFα, since lymphocyte proliferation could be fully restored by adding rp TNFα to the co-cultures in SEB-proliferation assays. This would also explain why Ctx-primed DC were not actively suppressive.

Activation of MAP kinases and other signal transduction pathways are important upstream events for the induction of DC maturation, migration or cytokine secretion [1, 30]. Analysis of key intracellular signalling molecules in Ctx-stimulated MoDC revealed ERK1/2-activation, but no regulation of JNK or NFκB activity. The observed ERK1/2-phosphorylation might relate to the increase in intracellular cAMP in response to Ctx. In other cells, e.g. PC12 cells, melanocytes, gastric and intestinal epithelial cells, elevation of cAMP results in ERK1/2-activation via a B-RAF – MEK1/2 pathway [12, 40, 51], which may also be responsible for Ctx-induced ERK activation in porcine MoDC. Functionally, ERK1/2 activation has been associated with high levels of IL-10 secretion by DC [52], which is consistent with our findings. It has been previously demonstrated that functional DC maturation involves NFκB activation [39]. Kawamura et al. observed nuclear translocation of NFκB together with upregulation of maturation markers in Ctx-stimulated murine BM-DC [26]. The absence of NFκB activation in Ctx-stimulated porcine MoDC corroborates our observations of incomplete maturation and absence of T cell stimulatory function.

Relating to the question of Ctx in pig vaccination, our observations could explain the failure of Ctx to work as an oral adjuvant in pigs as described by Foss and Murtaugh [16]. However, if the non-stimulatory phenotype of Ctx-primed DC was largely caused by lack of TNFα, it seems possible that other cells such as macrophages or epithelial cells would supply pro-inflammatory cytokines in an in vivo situation [10]. Notably, the F4-fimbriae co-administered with Ctx by Verdonck et al. [48] also directly act as mucosal antigens with the potential capacity to modulate DC phenotype. Further studies on intestinal DC or on co-cultures of DC

and intestinal epithelial cells are necessary to analyse this aspect.

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