

A Sub Acute Immunotoxicity Study in Göttingen Minipigs® with the Immunosuppressive Compounds Cyclosporin A and Dexamethasone

Geertje JD van Mierlo^{1*}, C Frieke Kuper², Mary-Lène de Zeeuw-Brouwer², Marcel A Schijf³, Joost P Bruijntjes¹, Marlies Otto¹, Niels-Christian Ganderup⁴ and André H Penninks¹

¹Triskelion, Utrechtseweg 48, 3704 HE, Zeist, The Netherlands

²Utrechtseweg 48, 3704 HE, Zeist, The Netherlands

³Department of Immunology, Danone Research - Center for Specialised Nutrition, Bosrandweg 20, 6704 PH, Wageningen, The Netherlands

⁴Ellegaard Göttingen Minipigs A/S, Soroe Landevej 302 DK-4261 Dalmose, Denmark

Abstract

Introduction: There is a growing interest in the minipig as a non-rodent species in the safety assessment of (bio) pharmaceuticals and (agro) chemicals. For (bio) pharmaceuticals the need to evaluate the potential adverse effects on the immune system is an increasingly important aspect of safety evaluation. In the present study, cyclosporin A and Dexamethasone were used as model compounds to examine whether the regulatory endpoints requested for immunotoxicity testing can be tested in the Göttingen minipig.

Methods: Minipigs were treated with vehicle, 20 mg/kg/day cyclosporine A or 0.4 mg/kg/day dexamethasone for 39 (males) or 40 (females) consecutive days. Clinical signs, body weight, hematology, lymphocyte subset analysis in peripheral blood mononuclear cells, Natural Killer cell activity, primary and secondary antibody response and Delayed Type Hypersensitivity response against Keyhole Limpet Hemocyanin (KLH), *ex vivo* mitogen-induced lymphocyte proliferation, examination at necropsy for gross macroscopic changes, weights and histopathology of lymphoid organs were used as criteria for evaluation of immunotoxic effects.

Results: Most parameters measured were implemented successfully in the minipig. Cyclosporin A treatment of minipigs had a slight effect on thymus weight, white blood cell counts and Keyhole Limpet Hemocyanin specific IgM responses. Clear effects were observed on mitogen-induced proliferation, Keyhole Limpet Hemocyanin specific IgG responses and Delayed Type Hypersensitivity response. Dexamethasone treatment resulted in decreased body weight, white blood cell counts, proliferative response to mitogen stimulation, Natural Killer cell activity and thymus weight. Keyhole Limpet Hemocyanin specific antibody responses were unaffected or slightly increased after dexamethasone treatment.

Discussion: With few exceptions, results obtained with cyclosporine A and dexamethasone in the minipig was in accordance with those presented for other species in the literature. Overall, the results presented in this article indicate that the minipig has potential to serve as an alternative non-rodent species for immunotoxicity testing as part of the safety assessment of (bio) pharmaceuticals and chemicals.

Keywords: Cyclosporin A; Dexamethasone; Sub acute immunotoxicity study; KLH; Lymphoid organs; Minipig; TDAR; Toxicologic pathology

Abbreviations: BSA: Bovine Serum Albumin; CFSE: 5,6-Carboxy-Fluorescein Succinimidyl Ester; ConA: Concanavalin A; CsA: Cyclosporin A; DEX: Dexamethasone; DTH: Delayed Type Hypersensitivity; EDTA: Ethylenediaminetetraacetic Acid; E/T ratio: Effector-to-target Ratio; FITC: Fluorescein Isothiocyanate; KLH: Keyhole Limpet Hemocyanin; NK cell: Natural Killer cell; PBMC: Peripheral Blood Mononuclear Cells; PBS: Phosphate Buffered Saline; PE: Phycoerythrin; TDAR: T Cell Dependent Antibody Response; WBC: White Blood Cell(s)

Introduction

The ICH S8 guideline dealing with Immunotoxicity Studies for Human Pharmaceuticals [1] states that safety evaluation of human pharmaceuticals on the immune system, mainly focussed on immune suppression, should be incorporated into the standard drug development programme. This should enable the prediction of unintended immunosuppression. Suppression of the immune response can result in decreased host resistance to infectious agents or tumor cells.

The non-clinical safety studies to be performed to support the development of safe new drug products are outlined in ICH M3 [2]. These include general toxicology, reproductive toxicology, safety

pharmacology, local tolerance, genotoxicity, and immunotoxicology studies. To screen for these different toxicity endpoints both studies in rodents and non-rodent species are required. The selection of a non-rodent species is often restricted to dog, and non-human primates. Although, these species are usually selected by default, they do not always represent the best predictive animal species. Moreover, social and legislative resistance against the use of non-human primates is mounting. These are both important reasons why the attention is increasingly focussed on the potential use of minipigs, which are also likely to be a more acceptable test species to the public, as pigs are being used as a food animal [3].

As the pig closely resembles man in many features of its anatomy, physiology, biochemistry and lifestyle, the minipig model is considered

***Corresponding author:** Geertje JD van Mierlo, TNO Triskelion B.V. Utrechtseweg 48, 3704 HE, Zeist, The Netherlands, Tel: 31-888665011; E-mail: geertje.vanmierlo@tno.triskelion.nl

Received April 19, 2013; Accepted June 11, 2013; Published June 14, 2013

Citation: van Mierlo GJD, Kuper CF, de Zeeuw-Brouwer ML, Schijf MA, Bruijntjes JP, et al. (2013) A Sub Acute Immunotoxicity Study in Göttingen Minipigs® with the Immunosuppressive Compounds Cyclosporin A and Dexamethasone. Clin Exp Pharmacol S4: 006. doi:10.4172/2161-1459.S4-006

Copyright: © 2013 van Mierlo GJD, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

of relevance for both safety and efficacy testing. In particular, the digestive tract, the cardiovascular system, the skin, the urogenital system and metabolic aspects of the minipig are considered more similar to humans than those of other non-rodent species [4,5]. Because of these similarities, the toxic effects of test compounds in minipigs may resemble the potential effects in man even more closely than the effects observed in other rodents or non-rodents [4,6].

The immune system of the pig is better characterized than that of e.g. the dog [7]. Research on the immune system of minipigs revealed that the basic structure of the porcine immune system resembles that of other mammal species, although some differences have been observed. One discrepancy between minipigs and other species is the inverted lymph node structure. Furthermore, unlike rodents and humans, pigs possess quite a lot of $\gamma\delta$ -T cells [8]. Both pigs and monkeys possess high levels of CD4⁺CD8⁺ double positive T cells in the periphery [9] whereas this cell type in humans is found in pathological conditions only. On the other hand, normal hematology and clinical chemistry values of pigs are, with a few exceptions, in range with those of other test species [10].

For safety assessment of potential immunomodulatory effects, the minipig has not been explored sufficiently. Therefore, we performed an immunotoxicity study in minipigs with the aim to develop, implement and acquire experience in testing immune-toxicological endpoints in Göttingen Minipigs® using two immunomodulating compounds, Cyclosporin A (CsA) and Dexamethasone (DEX).

CsA is a drug that is extensively used in prevention of transplantation rejection, for avoiding lethal graft-versus-host disease, treatment of autoimmune disease and for controlling hypersensitivity disorders. Because of its selective action on lymphocyte function [11-13] it is used as a prototype immunotoxic compound.

DEX is a synthetic glucocorticosteroid which is used in the treatment of autoimmune and inflammatory diseases and in combination with other drugs also as anticancer chemotherapeutic. It is also used as a model compound to examine the impact of glucocorticoid hormones produced during stress and its subsequent effects on e.g. immune status. DEX is known to be immunosuppressive and causes significant changes in T cell development and function [14,15]. DEX regulates T cell repertoire by influencing thymocyte development [14,15]. DEX also targets IL-2 production resulting in a skewing towards Th1 cytokine production [14-16]. Furthermore it is shown that DEX can have an effect on B cell proliferation and apoptosis, dependent on stage of B cell maturation [17].

Minipigs were exposed to CsA and DEX for 39 or 40 consecutive days by oral administration. Criteria for identification of (immuno) toxicological effects in Göttingen Minipigs were comparable to those used in other test species as described in the ICH S8 guideline [1]: clinical signs, body weight, hematology, lymphocyte subset analysis (immunophenotyping) in Peripheral Blood Mononuclear Cells (PBMC; data not presented), Natural Killer (NK) cell activity in PBMC, the primary and secondary antibody response against Keyhole Limpet Hemocyanin (KLH), the Delayed Type Hypersensitivity (DTH) response to KLH upon an intradermal challenge, *ex vivo* mitogen and KLH-induced lymphocyte proliferation, examination at necropsy for gross macroscopic changes, lymphoid organ weights, and histopathology of the collected lymphoid organs.

Materials and Methods

Minipigs

Male and female Göttingen minipigs (circa 3-3.5 months old, with a weight range of 7.2-9.4 kg at start of the study) were provided by Ellegaard Göttingen Minipigs (Dalmose, Denmark). The welfare of the animals was maintained in accordance with the general principles governing the use of animals in experiments of the European Communities (Directive 86/609/EEC) and Dutch legislation (Experiments on Animals Act, 1997).

Animals were group housed divided by treatment group (males and females separated) under conventional conditions. The experimental room was ventilated with approximately 10 air changes per hour and was maintained at a temperature of 20-24°C and a relative humidity of 40-70%. The animals were fed a commercial diet (SMP (E) SQC; SDS Special Diets Services, Whitham, England) and received a measured amount of food twice each day.

The animals were ad libitum provided with tap-water suitable for human consumption (quality guidelines according to Dutch legislation based on EC Council Directive 98/83/EC). The study was started after acclimatization to the laboratory conditions for 10 days.

Treatments

Animals (4/sex/group) were treated with either vehicle control (corn oil), CsA (Neoral®, Novartis Pharma BV, Arnhem, the Netherlands) or DEX (Vetranal®, Fluka, Sigma-Aldrich) for 39 or 40 consecutive days (from start of study, day 0, until sacrifice on days 39 [males] or 40 [females]). Minipigs received 20 mg CsA/kg/day or 0.4 mg DEX/kg/day, orally once a day mixed in a small amount of feed. Once per week, the dose volumes were adjusted to the latest recorded body weight for each animal, to maintain a constant dose-level in terms of the animal's body weight.

To determine the effects on the functionality of the T cell dependent antibody response (TDAR), all animals were immunized with KLH (mCKLH Inject®, Pierce, Rockford, USA) on study day 14 (primary immunization) and study day 28 (secondary immunization). KLH formulations were administered i.m. in the neck region at a constant dose volume of 200 μ l, containing 10 mg KLH per injection.

Blood sampling

Blood was sampled via the neck vein prior to administration of the study substances. Blood collected in heparin anticoagulant tubes was used (days -1, 13 and 27) for isolation of PBMC for determination of NK cell activity, T cell proliferation and lymphocyte subset analysis. These blood samples were diluted in PBS, after which PBMC were isolated using Ficoll-Paque. After separation, cells were washed twice in PBS and cell suspensions of 1.0×10^6 viable PBMC/ml were prepared in enriched RPMI-medium (containing 10% Fetal Calf Serum, 100 U/ml Penicillin, 100 μ g/ml streptomycin) using an automated hematology analyzer (K-800, Sysmex Toa, Kobe, Japan).

For hematological analysis, blood was sampled on days -1, 13 and 27 into EDTA tubes. For determination of anti-KLH antibodies blood was sampled in coagulation tubes on days -1, 19, 21, 24, 33, 35 and 38. These blood samples were allowed to clot at room temperature after which serum was isolated by centrifugation and stored at -18°C until analysis.

Hematology

The parameters determined for hematology were: haemoglobin,

packed cell volume, Red Blood Cells (RBC), reticulocytes, thrombocytes, White Blood Cells (WBC), differential WBC (absolute and relative) and prothrombin time. The values for mean corpuscular volume, mean corpuscular haemoglobin, and mean corpuscular haemoglobin concentration were calculated.

Natural Killer (NK) cell assay

The protocol used for determination of NK activity was based on the protocol described by Piriou et al. [18]. The target cells (K562, a human myeloid leukemia cell line) cultured in RPMI medium enriched with 10% foetal calf serum and penicillin/streptomycin were harvested and labeled with 5,6-Carboxy-Fluorescein Succinimidyl Ester (CFSE). FACS-tubes were filled with 100 µl of the target cell suspension containing 2.5×10^4 cells/ml. Of the effector cell suspension (PBMC isolated as described before), 100 µl was added such that effector-to-target (E/T) ratios of 100/1, 50/1, 25/1 and 12.5/1 were obtained. At each E/T ratio the NK activity was measured in the absence and in the presence of 5 ng/ml recombinant human IL-2 (R&D systems, Minneapolis, USA) in duplicate. Cell suspensions were mixed and spun down (2 minutes, 120 g). The cell pellets were incubated for 18-20 hours in an incubator (37°C, 5% CO₂), after which the tubes were put on ice and analyzed by flowcytometry using propidium iodide as a marker to differentiate between dead and living cells. Specific cytotoxicity was calculated from the mean of each set of triplicates using the formula:

$$\frac{\% \text{ dead target cells in test tube} - \% \text{ target cells that died spontaneously}}{100 - \% \text{ target cells that died spontaneously}} \times 100$$

Elisa for determination of Anti-klh Igm and Igg Antibodies

An ELISA for determination of anti-KLH IgM and IgG antibodies was developed before analysis of the study samples. Specificity of the assay was established by pre-incubation of positive serum samples with KLH, leading to background values for optical density. Flat bottom plates (NUNC Immuno MaxiSorp Plate, Roskilde, Denmark) were coated overnight at 2-10°C with KLH 1 µg/well (100 µl of a 10 µg/ml stock dilution) dissolved in carbonate-bicarbonate buffer (0.2M, pH 9.4; Pierce, Rockford, USA). After washing the plates three times (0.1% v/v Tween-20 solution in PBS; Sigma-Aldrich), they were blocked with 4% BSA in PBS/Tween-20 for at least 60 minutes at 37°C. After washing the plates three times (0.1% v/v Tween-20), serial dilutions of the respective test-sera (in PBS/1% BSA/0.1% Tween-20) were incubated in duplicate (60 minutes, 37°C). After washing the plates three times (0.1% v/v Tween-20), mouse-anti-pig IgG biotin (1:250 in PBS/1% BSA/0.1% Tween-20) or mouse-anti-pig IgM biotin (1:250, in PBS/1% BSA/0.1% Tween-20) was added for at least 1 hour at 37°C. After washing the plates three times (0.1% v/v Tween-20), streptavidin-HRP was added (1:1000 diluted in PBS/1% BSA/0.1% Tween-20). After incubation (20-30 minutes at 37°C) plates were washed 3 times (0.1% v/v Tween-20) and 100 µL one-step TMB was added. The reaction was subsequently stopped with 2N H₂SO₄. Optical density was measured at 450 nm using a Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories, Richmond, CA). Blank wells were included as controls on each plate. In addition, every plate contained a standard curve of positive control serum, obtained from a minipig in a pilot KLH study. The endpoint titre was calculated according to the standard curve. The highest standard was arbitrarily set to 100 units.

Delayed type hypersensitivity (DTH)

On day 31 of the study, each animal received three intradermal injections (50 µl each) in the flank at sufficient distance from each other. The different injections contained PBS only, 1 mg KLH or 2 mg KLH in PBS respectively. Injection sites were shaved before injection.

DTH skin reactions were measured 48 and 72 h later (days 33 and 34, respectively). The largest diameter was measured. A diameter of ≥ 5 mm was considered a positive result.

Ex vivo lymphocyte proliferation

Part of the PBMC population prepared as described was frozen in 10% DMSO/40% FCS/50% enriched RPMI medium and stored at <-80°C until use for the lymphocyte proliferation assay. After thawing, cells were washed and suspended in enriched RPMI medium to a viable cell concentration of 1.5×10^6 /ml PBMC. Of this dilution 100 µl/well was added to 96-well round-bottomed microplates. Cells were incubated with medium only, Concanavalin A (ConA; Sigma-Aldrich; 5 µg/ml) or KLH (50 µg/ml). All conditions were tested in triplicate. Plates were incubated at 37°C in a humidified, 5% carbon dioxide atmosphere for 5 days before being pulsed for 6-8 hours with 1 µCi methyl-³H-thymidine (Amersham Pharmacia Biotech, UK) per well. After this incubation, cells were harvested on a filtermat using a cell harvester. Filters were dried and put into a sample bag. Five ml of scintillation fluid (betaplate-scint; Perkin Elmer) was added. The counts per minute were determined using a MicroBeta counter (1450 Microbeta plus, Wallac). Nett tritium incorporation was calculated by deducting background counts (medium only).

Pathology

At necropsy on day 39 (males) or 40 (females), the animals were examined for external changes and grossly for pathological changes. Special attention was given to lymphoid organs and tissues, which were collected (thymus, spleen, Peyer's patches, mesenteric- and cervicalis superficialis lymph nodes), weighed (thymus and spleen) and preserved in a neutral aqueous phosphate-buffered 4% solution of formaldehyde. The organs and tissues were embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin for histopathological evaluation.

Statistical analyses

Statistical analyses were performed using Proventus version 6.5 (Instem) or SAS (release 8.02 TS). One-way analysis of variance (ANOVA) was performed for body weight data, NK cell activity, proliferation assay data, anti-KLH antibody titers, hematology data and organ weights. When variances were not homogeneous or data were not normally distributed, the data were stepwise log or rank transformed prior to the ANOVA or nonparametric test was performed (Kruskal-Wallis). If the ANOVA yielded a significant effect ($p < 0.05$), intergroup comparisons with the control group were made by Dunnett's multiple comparison test. If Kruskal-Wallis yielded a significant effect, intergroup comparisons with the control group were made by Dunn's test. Fisher's exact probability test was performed for incidences of histopathological changes. Tests were performed as two-sided tests with results taken as significant where the probability of the results was $p < 0.05$.

Results

Clinical observations and body weights

No treatment related clinical signs were observed. Although male minipigs were not significantly affected in their growth (expressed as body weight) by the CsA or DEX treatments a tendency towards a slightly reduced bodyweight gain of DEX-treated males from day 14 onwards was observed, leading to approximately 10% lower body weight at day 39 (Figure 1). Female animals treated with DEX

experienced a statistically significant setback in weight gain compared to vehicle control animals (Figure 1). From day 21 onwards, the body weights differed significantly between the DEX-treated females and the vehicle treated females, ranging from 15% at day 21 to 18% at day 40. It should be noted that before start of treatment, mean body weight of females in the DEX group already were 7% lower than in the vehicle group.

Hematology

The results of hematological analysis are summarized in Figure 2. With a few exceptions (e.g. % monocytes, thrombocyte numbers and % basophils), most measured hematological parameters in the vehicle controls were within historical data of Ellegaard Göttingen Minipigs [19]. On day 13 no significant effects were observed on the number of WBC and the differentiated WBC subsets both in the CsA- and DEX-treated male and female minipigs except for the increased absolute (not shown) and relative (Figure 2) neutrophil numbers and the decreased relative lymphocyte numbers (percentage of total WBC count) in CsA treated males compared to the vehicle controls. On day 27 there was a slight decrease in the number of WBC in male and female minipigs of both the CsA- and DEX treatment groups. The reduction in WBC is mainly caused by a diminished relative number of lymphocytes in both CsA- and DEX-treated minipigs. The absolute and relative numbers of eosinophils in the blood were significantly raised in only the DEX-treated male animals, whereas the absolute and relative numbers of neutrophils were significantly increased in only the DEX-treated females. No effects were observed on the red blood cell parameters (RBC, Hb, PCV, MCV and MCHC) and thrombocyte numbers in the CsA- and DEX-treated animals (data not shown). The percentage of reticulocytes is statistically significantly reduced in CsA- and DEX treated male animals at day 27. A temporary drop in reticulocytes is observed in DEX-treated females at day 13 which is absent on day 27.

Natural killer cell activity assay

In vitro NK cell activity is specifically triggered upon addition of IL-2 to the assay conditions. In the present study NK cell activity was observed when IL-2 was added to the cultures and was negligible in the absence of IL-2 (specific target cell lysis below 10% in most cultures, data not shown). Cultures with E/T ratio's 25/1 and 12.5/1 did not show detectable NK activity in the presence of IL-2 (not shown). Data of NK cell activity assay with an E/T ratio 100/1 in the presence of IL-2 are depicted in figure 3. NK cells of female animals showed less target cell lysis than NK cells from male animals. This difference is obvious

in all groups. A statistically significant difference between male and female animals ($P < 0.05$) was found when data of all animals on day -1 (all groups together) were used. Mean values of target cell lysis for females were $21.7 \pm 15.6\%$ compared to $40.3 \pm 20.1\%$ for males. CsA treatment left NK cell activity more or less unaffected. At an E/T ratio of 100, there certainly was a trend towards lower target cell lysis by NK cells of DEX-treated animals compared to vehicle control animals. Although this trend is obvious in both male and female animals and on both day 13 and day 27, this trend is not statistically significant due to high variability of data.

Proliferation assay

In vitro cultivation with medium only did, as expected, not reveal high proliferative responses of PBMC (data not shown). After restimulation with KLH (Figure 4a), both in males and in females, a significantly higher proliferative response was observed with PBMC isolated on day 27 from CsA-treated animals compared to those from vehicle control and DEX-treated animals. A higher proliferation was observed after *in vitro* stimulation with ConA (Figure 4b). As a result of large standard deviations and small group sizes, statistically significant differences in proliferative responses were only observed between vehicle- and DEX treated female animals on day 27. A combination of data of male and female animals (covariance analysis with sex as a covariate) revealed a significant decreased proliferation of PBMC of both CsA and DEX treated animals compared to PBMC of vehicle control animals in response to *in vitro* Con A stimulation.

Anti-KLH antibody responses (TDAR)

As is depicted in Figure 5a, the peak of the primary KLH-specific IgM response was observed between day 19 and day 24, with a peak 7 days after primary immunization. The secondary IgM response after the second KLH immunization on day 28 remained low as measured on days 33, 35 and 38 (data not shown). The primary KLH-specific IgG response was absent or very low as measured on days -1, 19, 21 and 24 (data not shown), with only some animals showing marginal IgG responses on day 24. The peak of the secondary KLH-specific IgG response was observed between days 33 and 38 (Figure 5b). The peak response was measured 7 days after secondary immunization. No statistically significant differences between groups were observed in both primary IgM and secondary IgG response (area under the curve of the peak response i.e. days 19-24 for IgM and days 33-38 for IgG). It was remarkable to see that in the vehicle control group (both males and females) only 2 out of 4 animals (female animals number 3 and number 5 and male animals #2 and #8) showed a substantial KLH-specific IgM

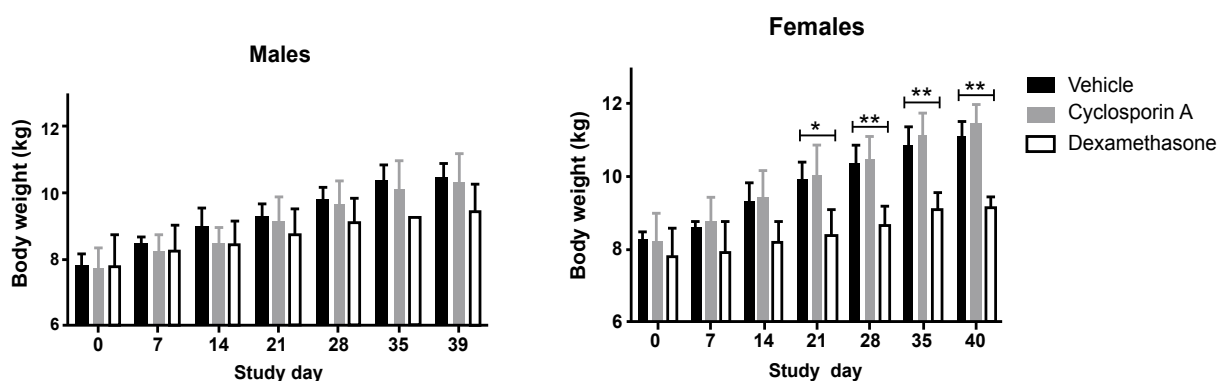


Figure 1: Mean body weights during exposure to CsA and DEX. The body weight of each animal was recorded once weekly and on day of sacrifice. Mean \pm SD is depicted for each group. * $P < 0.05$; ** $P < 0.01$.

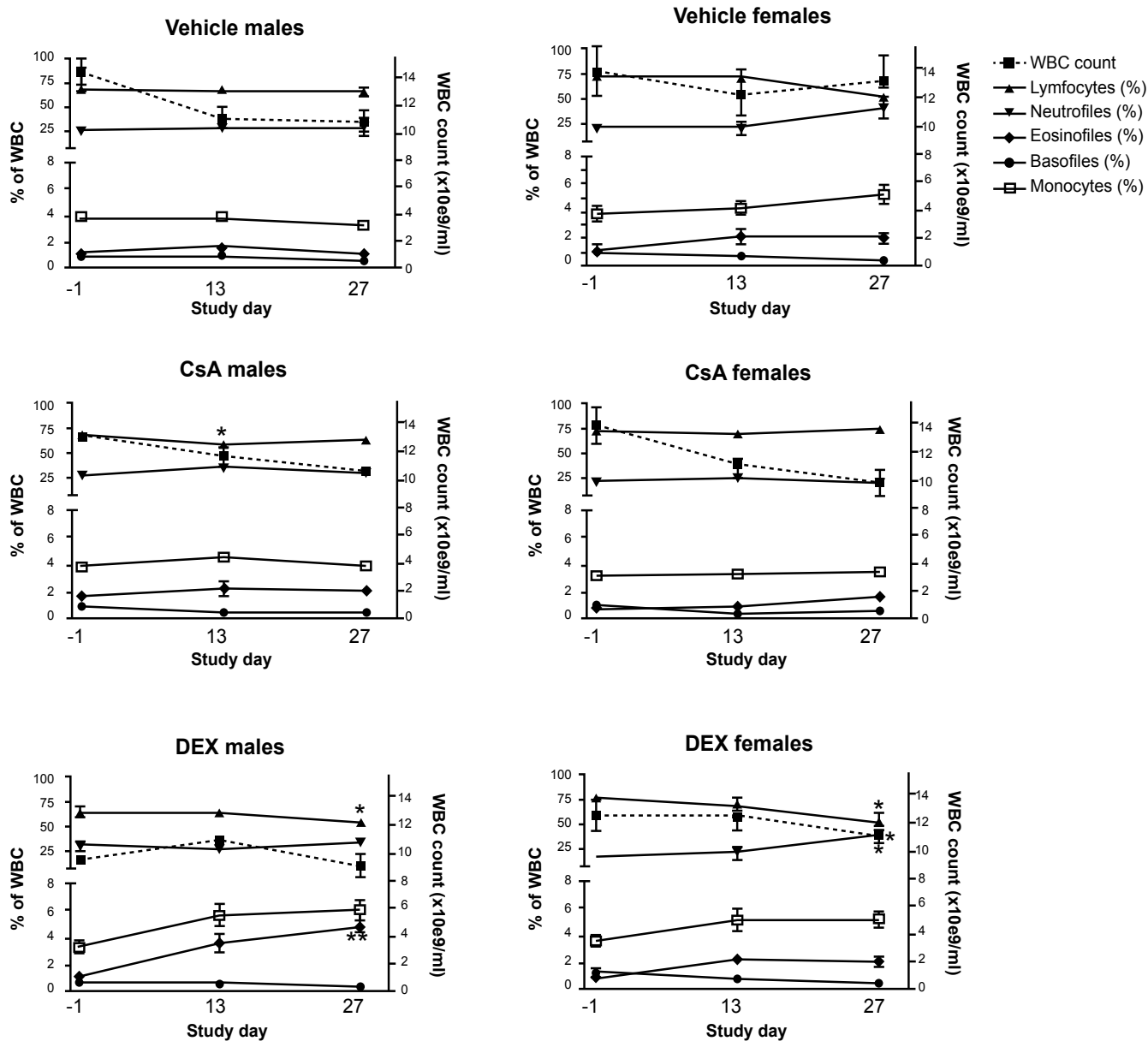


Figure 2: Effects of CsA and DEX treatment on white blood cell counts and differentiation. WBC counts (absolute numbers, right axis) and differentiation (as a percentage of total WBC counts, left axis) in blood of minipigs collected on days -1, 13 and 27 of the study. Mean \pm SD is depicted. * $P < 0.05$; ** $P < 0.01$ compared to vehicle control group on the same day for the same sex.

and IgG response. For CsA-treatment, in both sexes the primary KLH-specific IgM response was reduced compared to the vehicle controls. Upon DEX-exposure the mean primary IgM response was higher (males) or comparable (males) to the mean IgM levels observed in the vehicle controls. Also, the secondary KLH-specific IgG response was reduced in females and to a lesser extent in males treated with CsA when compared with the vehicle controls. For the DEX-treated animals the mean secondary KLH-specific IgG levels were almost comparable to (females) or even slightly increased (males) when compared to the vehicle controls. Due to high variability in the KLH-specific IgM and IgG responses, particularly in the vehicle control animals, it is difficult to draw firm conclusions with respect to the effects observed in the CsA- and DEX-treated minipigs. Even after merging male and female

data, no significant decrease by CsA or DEX treatment in TDAR was observed.

Delayed type hypersensitivity response

The group results for the DTH responses upon intradermal KLH-injection are presented in Table 1 (data of injections with 1 mg KLH not shown). Responses to PBS were negative in the three treatment groups at 48 and 72 hours, except for a slight and unexpected reaction in the CsA treated females at 72 hours after intradermal PBS injection (a response of one animal only). At 48 hours after intradermal injection of 2 mg KLH no clear differences could be observed between groups, whereas at 72 hours a clear reduced DTH response could be observed in the CsA-treated animals. The DTH responses in DEX-treated male

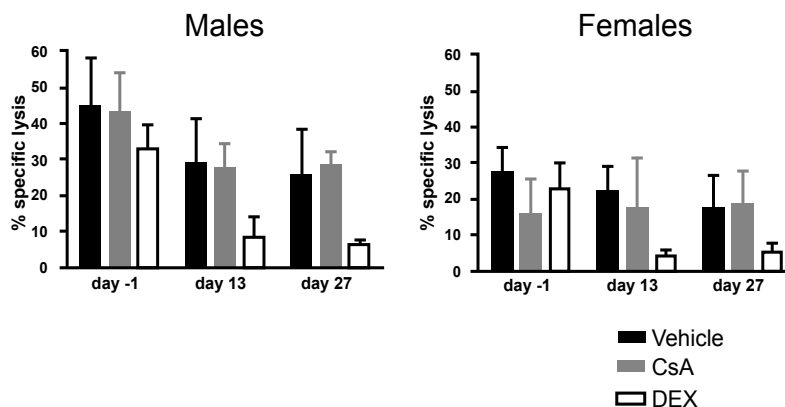


Figure 3: Effects of CsA and DEX treatment on NK activity of PBMC. Mean specific lysis of NK sensitive K562 cells by PBMC isolated from minipigs on study days -1, 13 or 27 in the presence of rHL-2 at an Effector-to-Target ratio of 100:1. Mean+SD is depicted for males and females separately.

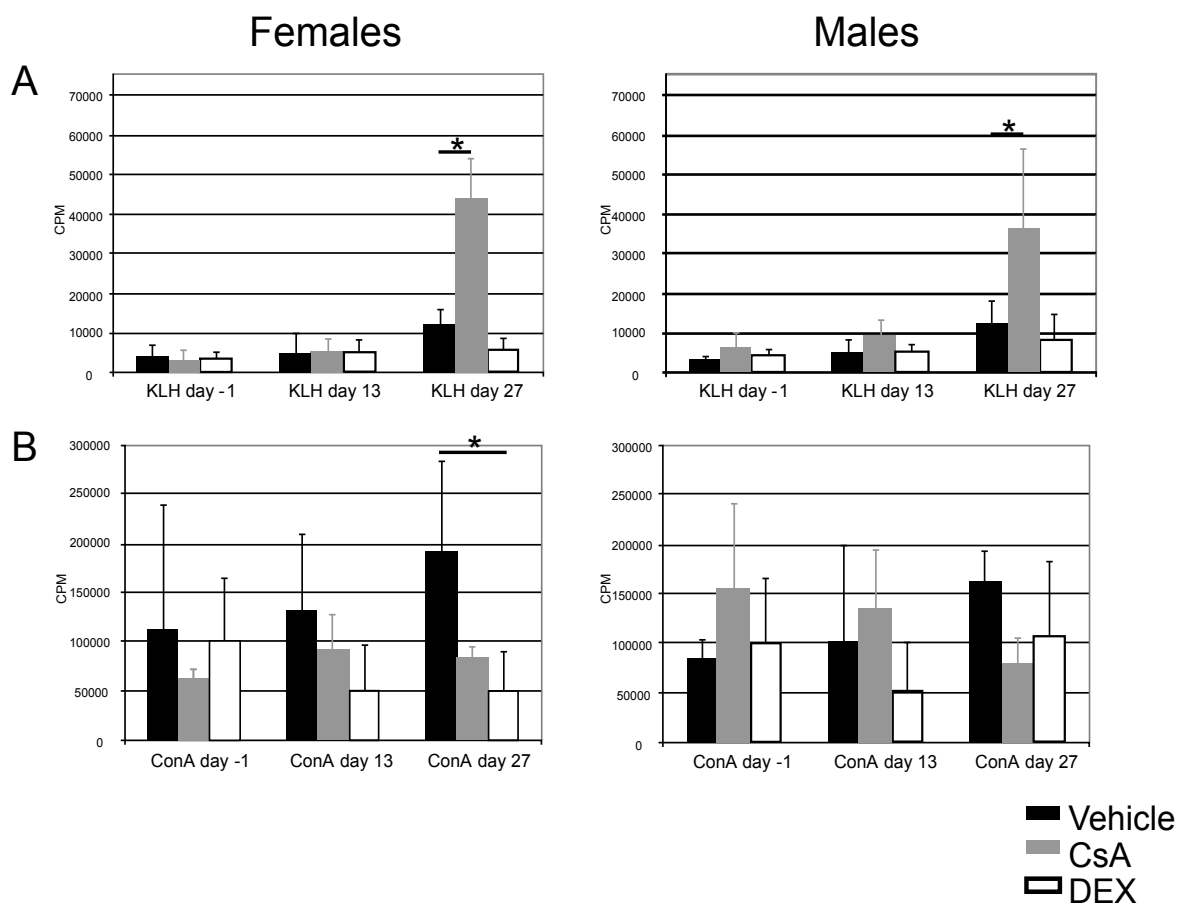


Figure 4: Effects of CsA and DEX treatment on proliferation after *in vitro* stimulation with KLH (A) or ConA (B). 150,000 viable PBMC were incubated for five days with A) 50 µg/ml KLH or B) 5 µg/ml ConA at 37 °C before being pulsed for 6-8 hours with 1 µCi methyl-³H-thymidine per well. Proliferation was determined based on ³H-thymidine incorporation. Mean + SD is depicted. Black bars: vehicle control; Grey bars: CsA treated; white bars: DEX treated. * P<0.05.

and female animals were comparable to those observed in vehicle treated animals.

Organ weights and pathology

Macroscopy: Gross examination at necropsy revealed that thymuses, cervical lymph nodes and Peyer’s patches, were markedly

reduced in size in the DEX-treated animals. No treatment-related effects were observed in the CsA-treated animals.

Organ weights: Spleen and thymus weights are depicted in table 2. No significant differences were observed in absolute and relative spleen weights between treatment groups. Thymus weight in male animals after CsA treatment was decreased compared to vehicle treated male

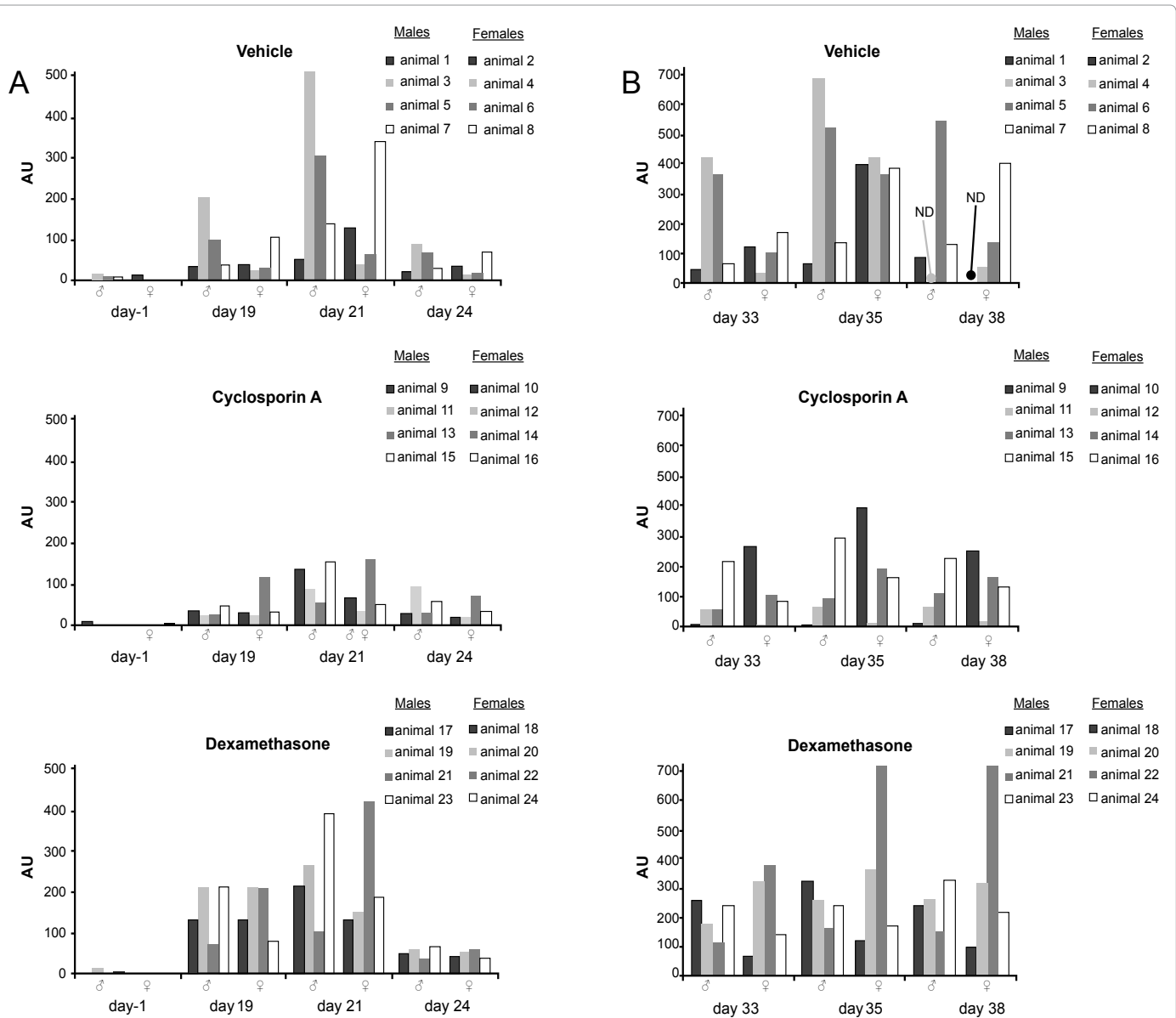


Figure 5: Effects of CsA and DEX treatment on primary IgM and secondary IgG responses against KLH. Animals were challenged with 10 mg KLH i.m. on study days 14 and 28. Anti-KLH antibodies were measured in serum of the animals on days -1, 19, 21, 24, 33, 35 and 38. A) Primary IgM responses; B) Secondary IgG responses. ND: not determined

animals, albeit not statistically significant. In females, CsA treatment resulted in a statistically significantly lower relative thymus weight compared to vehicle treated females. DEX-treated male and female animals showed a significant reduction in absolute and relative thymus weights compared to the vehicle control animals.

Microscopy: The morphology of lymphoid organs in the vehicle control minipigs shows more interindividual variability than in other test animals like dogs and rodents. The cortex medulla ratio in the thymus is generally rather low in the minipig. The spleen resembles the spleen of man with regards to an inconspicuous marginal zone and Periarteriolar Lymphoid Sheath (PALS). The ellipsoid structures in the spleen, mainly in the marginal zone, are characteristic for the pig. They are vessels with perivascular concentrically arranged macrophages. The lymph nodes are also different from dogs and rodents, with the

follicles in the more central part of the lymph nodes, and the efferent vessels in the periphery. The mesenteric lymph nodes demonstrated extended areas with large cells, presumably macrophages, with foamy, slightly eosinophilic cytoplasm. The morphology of the Peyer's patches does not differ from that of Peyer's patches in other laboratory species. In the CsA treated minipigs no distinct effects were observed in the lymphoid organs and tissues that could be ascribed to the treatment with CsA. Microscopic evaluation of the DEX-exposed thymuses showed the well-known distinct reduction in the cortex-medulla ratio due to a severe reduction in the number of cortical thymocytes. In one exposed animal this reduction was to such an extent that thymus tissue could not be retrieved properly. In addition, the Peyer's patches in most animals exhibited decreased cellularity in the interfollicular areas and small germinal centres in the mesenteric lymph nodes. The latter was observed in all DEX-treated males versus two out of four females.

Males

| Group | PBS 48h | 2 mg KLH | |
|---------|---------|----------|------|
| | | 48h | 72h |
| Vehicle | 0 | 9.0 | 11.4 |
| CsA | 0 | 5.5 | 2.2 |
| Dex | 0 | 10.2 | 9.2 |

Females

| Group | PBS 48h | 2 mg KLH | |
|---------|---------|----------|-----|
| | | 48h | 72h |
| Vehicle | 0 | 8.8 | 9.1 |
| CsA | 0 | 9.8 | 2.1 |
| Dex | 0 | 11.5 | 9.0 |

Table 1: DTH responses. Three days after the second KLH immunization, animals were injected intradermally with PBS or KLH. The DTH response was measured 48 and 72 hours after intradermal injection. Depicted is the group means of the largest diameter in mm.

| Group | | Spleen (g) | Thymus (g) | Spleen relative (g/kg BW) | Thymus relative (g/kg BW) |
|----------------|------|------------|------------|---------------------------|---------------------------|
| | | Vehicle | Mean | 16.79 | 12.32 |
| | S.D. | 2.25 | 4.21 | 0.24 | 0.42 |
| CsA | Mean | 20.01 | 8.65 | 1.94 | 0.83 |
| | S.D. | 3.19 | 2.03 | 0.27 | 0.14 |
| DEX | Mean | 18.28 | 3.10** | 1.96 | 0.33** |
| | S.D. | 3.23 | 1.00 | 0.40 | 0.08 |
| Females | | | | | |
| Group | | Spleen (g) | Thymus (g) | Spleen relative (g/kg BW) | Thymus relative (g/kg BW) |
| | | Vehicle | Mean | 20.14 | 19.18 |
| | S.D. | 1.98 | 4.00 | 0.25 | 0.33 |
| CsA | Mean | 18.15 | 14.13 | 1.58 | 1.24* |
| | S.D. | 3.16 | 1.20 | 0.19 | 0.16 |
| DEX | Mean | 16.24 | 2.95** | 1.78 | 0.33** |
| | S.D. | 4.43 | 1.00 | 0.47 | 0.11 |

Table 2: Absolute and relative organ weights on day of sacrifice (day 39 for males, day 40 for females). * Statistical analysis: P<0.05. ** Statistical analysis: P<0.01

Discussion

There is growing interest in the use of minipigs as a non-rodent species for non-clinical safety evaluation of (bio) pharmaceuticals and chemicals [3-5,7,20-22]. Here we report a first effort to assess the feasibility of using minipigs as an alternative non-rodent species for studying immunotoxicity of pharmaceuticals. Various quantitative and qualitative (functional) immunotoxicity endpoints [1] were investigated using the immunosuppressive compounds, CsA (20 mg/kg/day) and DEX (0.4 mg/kg/day). The doses of the test substances were chosen such that immune suppression was expected (based on pharmacological use in veterinary practice), such that there was only a small chance of losing animals due to the general side-effects of the test substances and such that indirect effects on the immune system due to stress were avoided.

The selection of the oral route of exposure in this study might be responsible for part of the inter-animal variation in all assays. It has been shown that by this route the systemic availability might vary substantially [23]. Possibly the use of direct gavage instead of mixture of test substances in the food, might slightly reduce the variability in dose exposure. Systemic exposure of CsA and DEX after oral administration is not confirmed in this study, but oral bioavailability has been shown

for DEX [24] and for CsA [25] in humans and pigs respectively. The differences observed in CsA and DEX effects between the various species considered may not only be due to differences in dose and route of exposure, but may also be attributed to differences in the kinetics and metabolism of CsA and DEX in the different species compared. The differential analysis of blood leukocytes (hematology), the assessment of a TDAR using KLH as antigen, the DTH response against KLH, the NK cell activity, the ex vivo lymphocyte proliferation and histopathology of the lymphoid organs were successfully implemented in the minipig. Lymphocyte subset analysis in blood or lymphoid organs has, as a non-functional assay, a high predictive value for the immunotoxic potential of test compounds [26]. However, although lymphocyte subset analysis in PBMC was performed in this study, the data of various lymphocyte subsets were highly variable between the days analysed. As they were considered not to be completely reliable they are not presented in this manuscript. Further improvement of the selected test protocol is initiated. The data presented support that the minipig has potential to be used in immunotoxicity testing, but like studies with other non-rodent species more results will become more solid by increasing the number of minipigs per group.

Hematology

In minipigs a slightly decreased number of WBC was observed in both sexes after CsA and DEX-treatment, mainly due to a diminished number of lymphocytes. Decreased WBC and lymphocyte counts are observed in rodents after CsA treatment also, dependent on the dose [27-31]. Rhesus monkeys showed a transient decrease of leukocyte counts at higher doses of CsA (≥ 60 mg/kg/day) and in dogs at 45 mg CsA/kg/day a slight decrease in leukocyte counts was observed as well [32]. A reduced lymphocyte number as observed in DEX treated minipigs has also been observed in a study with healthy volunteers [33]. From the latter study it is clear that the effects on WBC subtypes differs in time, indicating that the timing of the blood sampling can have an important influence on the effects observed. The number of neutrophils was increased after DEX-exposure of the minipigs. Increased neutrophil count is a well known stress-related effect associated with increased corticosteroid levels [34] that is also evident upon DEX exposure in other animal species both rodents and non-rodents [28,30,31,35,36].

NK cell activity assay

In the minipig, NK cell numbers can make up to 60% of total PBMC [37]. Changes in cellular distribution might have influenced the outcome of the analysis in the present study [31]. However, possible changes in NK cell percentages were not taken into consideration during the present study as any immunological tools are available yet to assess the number of NK cells. NK activity in female animals was significantly lower compared to male animals. Part of this difference can be explained by the lack of NK cell activity measured on all three measurement days in three female animals (2 CsA treated and 1 DEX treated female) whereas only one male animal (DEX-treated) lacked NK cell activity. These four animals showed specific target cell lysis <8% on all measurements. CsA-treatment showed only minor effects on NK cell activity of PBMC isolated from minipigs, which is in line with the lack of effect on NK cell activity in a placebo controlled study in human subjects with insulin-dependent diabetes mellitus type I [38]. In several observations made in rodents treated with CsA, either no effect [27,31,39,40] or a tendency to increased NK activity was observed [29,30,41]. Interestingly, in studies of Blot et al. [27], it was observed that in splenic cells of mice but not of rats NK cell activity was

dosedependently decreased upon exposure to comparable dose levels of CsA even though the blood concentrations of CsA were higher in the rat than in the mouse. A clear but not significant decrease in NK activity was observed after DEX treatment in minipigs. Comparable, DEX exposure of Sprague Dawley rats was associated with decreased NK cell activity in spleen cell suspensions [42,43]. Furthermore, an *in vitro* study by Piccolella et al. [44] using human PBMC showed an inhibitory effect of DEX on NK cell activity.

Proliferation assay

Proliferative responses to *in vitro* restimulation with KLH was significantly higher for PBMC isolated on day 27 from CsA-treated animals compared to those from vehicle control and DEX-treated animals. This difference was unexpected and remains inexplicable. It may be related to differential effects of CsA on specific subsets of T lymphocytes. Statistically significant decreased proliferative responses of PBMC to ConA stimulation were observed for both the CsA- and DEX-treated minipigs on day 27. Published studies in rodents treated with CsA demonstrate non-consistent results either showing no effect [45] or a reduced [27,30,31,39] splenocyte proliferation after ConA stimulation. In humans treated with CsA, either no effect [38] or a decreased [46] mitogeninduced proliferation has been observed. In a mouse study of Munson et al. [36] the ConA stimulated proliferation of spleen cells was found to be increased upon DEX exposure. Decreased PHA induced proliferation was observed in human PBMC in the presence of DEX or CsA [47].

TDAR

In the TDAR, only two out of four vehicle-treated control animals showed a substantial KLH-specific IgM and IgG response. The existence of non-responders to KLH in the minipig is not surprising, because similar observations are made in rodents [45,48]. Due to the high variability in the KLH-specific IgM and IgG responses firm conclusions cannot be drawn in respect to the effects observed in the CsA- or DEX-treated minipigs. Primary IgG responses in the minipig were, in contrary to responses in rats [48,49], dogs [50] and monkeys [51], too low to make comparisons between treatment groups. The absence of a (significant) influence of CsA treatment on the antigen-specific IgM response in minipigs is in agreement with observations in dogs [50], cynomolgus monkeys [51] and humans with uveitis [52]. However, inhibition by CsA of the KLH-specific primary IgM response was observed in various rodent studies using different treatment schedules and routes of KLH immunization [30,48,49,53]. The KLH specific secondary IgG response in CsA treated minipigs was reduced in females and to a lesser extent also in males. In the IILP study (unpublished results) oral CsA exposure of rats up to levels of 25 mg/kg/day for 33 days resulted in a dose dependent decrease in secondary KLH-specific IgG responses. In dogs after treatment with 25 mg/kg CsA, a clear decrease of the secondary IgG response was observed [50]. At the level of 50 mg CsA/kg/day secondary KLH-specific IgG responses were decreased in Cynomolgus monkeys [51] albeit not statistically significant as was also observed in the present minipig study. DEX-treated animals showed on the average equal (females) or slightly higher (males) KLH-specific IgM and IgG responses when compared to vehicle treated minipigs. Effects of DEX on KLH-specific IgM responses vary between species, from a slight increase in a study in Sprague-Dawley rats [54], to no effect in a mouse study [53], to a decreased IgM response in a dog 28-day immunotoxicity study after oral exposure to a slightly higher dose of DEX (0.5 mg/kg/day) [35]. Secondary anti-KLH IgG responses were decreased in a 28-day oral

immunotoxicity study in dogs [35]. The discrepancy in some of the results obtained upon KLH immunization between the minipig and other species can most likely be explained by a low mean response in the vehicle control group as a result of the occurrence of two non-responders in this group.

DTH responses

CsA treatment in the minipig led to lower DTH responses upon intradermal KLH injection compared to vehicle-treated animals. A reduced DTH response was also observed in humans with chronic uveitis treated with CsA [52] and in our lab in a comparable immunotoxicity study using beagle dogs [55]. DEX treatment in minipigs did not affect the DTH response upon intradermal KLH injection, like was observed in the immunotoxicity study in beagle dogs performed in our lab [55]. Also, in Sprague Dawley rats no effect of DEX on DTH response against KLH was observed [54] although the DTH was measured relatively late after the last DEX exposure in the latter study. In contrary, Exon et al. [42,43] using different DEX treatment schedules in male Sprague Dawley rats, observed a decreased DTH response to BSA. Also in a mice study of Munson et al. [36] the DTH response to SRBC was found to be reduced upon DEX exposure levels up to 0.49 mg/kg in the drinking water for 90 days.

Effects on growth, organ weights and histopathology

Body weight and spleen weight were unaffected by CsA-treatment in minipigs. Similar results of unaffected body weight were observed in several rodent studies with CsA [27-30,56]. However, in other studies, reduction in body weight was observed, varying between laboratories and varying with sex [29,31,39]. In a 13-week oral toxicity study with CsA in Rhesus monkeys, body weight gain was decreased at 200-300 mg/kg/day but not at doses up to 60 mg/kg/day [32]. Lack of effect on spleen weight has been observed upon CsA exposure up to 25 mg/kg/day in rats [27,29-31,56] and up to 50 mg/kg/day in cynomolgus monkeys [51]. A significant increase in spleen weight was observed in female B6C3F1 mice after a 14-day exposure period with 25 mg CsA/kg/day [27], a slightly higher dose than used in the present minipig study. CsA-treatment resulted in a lower relative but not absolute thymus weight in minipigs. Most studies with rodents show no effect on thymus weight [28,30,31,56]. Also, in a 21-day oral immunotoxicity study in Cynomolgus monkeys with 0, 10 or 50 mg/kg/day, thymus weight was unaffected [51]. However, reduced thymus weight at an oral dose of 25 mg/kg for 4 weeks in rats has also been reported [39]. Overall, in most studies with rodents and monkeys and in the present study with minipigs, limited or no effects of CsA on thymus weight were observed. DEX treatment resulted in a slightly reduced body weight gain in minipigs. Similar results have been found in rodent studies [36,42]. DEX did not bring about an effect on spleen weights in the minipig. In contrast, reduced spleen weights were observed in rodents after DEX exposure [36,42,43], but treatment lasted longer or other treatment routes and dose levels were used making direct comparison difficult. Thymus weights of the DEX-treated minipigs were significantly reduced in both sexes, which is in line with observations made in rodents [36,42,43]. Thus, in general the effects of CsA and DEX on body and organ weights and histopathology of lymphoid organs in the minipig are in general in agreement with those observed in other species (both rodents and non-rodents).

General conclusion

The immunotoxicity methods in general have been implemented successfully in minipigs, with the exception of the lymphocyte subset

analysis. Further assessments will ultimately result in the selection of the best predicting tools to be used for immunotoxicity evaluation in minipigs. Overall, the effects of CsA and DEX obtained in the presented minipig study are in agreement with those found in other species. From the results obtained in this immunotoxicity study, it is concluded that the minipig has potential to serve as an alternative non-rodent species for immunotoxicological evaluation of new compounds.

Acknowledgement

The authors wish to thank all other colleagues of TNO that contributed to this study. Part of the results shown in this manuscript were published in [57] before.

Copyright

This article was modified and updated from Immunotoxicology: Studies in the Minipig by André H. Penninks and Geertje J. D. van Mierlo in *The Minipig in Biomedical Research*, edited by Peter A. McAnulty, Anthony D. Dayan, Niels-Christian Ganderup, and Kenneth L. Hastings. Copyright 2012 Taylor & Francis Group, LLC. Reprinted with permission.

References

1. ICH Harmonized Tripartite Guideline (S8) (2005) Immunotoxicity Studies for Human Pharmaceuticals. Step 4 document EMEA/CHMP/167235/2004 - ICH.
2. ICH Harmonized Tripartite Guideline M3(R2) (2009) Guidance on non-clinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals.
3. Forster R, Bode G, Ellegaard L, van der Laan JW; Steering Group of the RETHINK Project (2010) The RETHINK project—minipigs as models for the toxicity testing of new medicines and chemicals: an impact assessment. *J Pharmacol Toxicol Methods* 62: 158-159.
4. Bode G, Clausing P, Gervais F, Loegsted J, Luft J, et al. (2010) The utility of the minipig as an animal model in regulatory toxicology. *J Pharmacol Toxicol Methods* 62: 196-220.
5. van der Laan JW, Brightwell J, McAnulty P, Ratky J, Stark C; Steering Group of the RETHINK Project (2010) Regulatory acceptability of the minipig in the development of pharmaceuticals, chemicals and other products. *J Pharmacol Toxicol Methods* 62: 184-195.
6. Ganderup NC (2012) Adverse responses to drugs in man: critical comparison of reported toxicological findings in minipigs and humans. In: *The Minipig in Biomedical Research*. CRC Press/Taylor and Francis, New York, USA 573-594.
7. Forster R, Bode G, Ellegaard L, van der Laan JW (2010) The RETHINK project on minipigs in the toxicity testing of new medicines and chemicals: conclusions and recommendations. *J Pharmacol Toxicol Methods* 62: 236-242.
8. Sinkora M, Sinkorová J, Holtmeier W (2005) Development of gammadelta thymocyte subsets during prenatal and postnatal ontogeny. *Immunology* 115: 544-555.
9. Zuckermann FA, Gaskins HR (1996) Distribution of porcine CD4/CD8 double-positive T lymphocytes in mucosa-associated lymphoid tissues. *Immunology* 87: 493-499.
10. <http://www.ahc.umn.edu/rar/refvalues.html>
11. Borel JF (1990) Pharmacology of cyclosporine (sandimmune). IV. Pharmacological properties in vivo. *Pharmacol Rev* 41: 259-371.
12. Borel JF, Feurer C, Gubler HU, Stähelin H (1976) Biological effects of cyclosporin A: a new antilymphocytic agent. *Agents Actions* 6: 468-475.
13. Di Padova FE (1990) Pharmacology of cyclosporine (sandimmune). V. Pharmacological effects on immune function: in vitro studies. *Pharmacol Rev* 41: 373-405.
14. Ashwell JD, Lu FW, Vacchio MS (2000) Glucocorticoids in T cell development and function*. *Annu Rev Immunol* 18: 309-345.
15. Sigal NH, Dumont FJ (1992) Cyclosporin A, FK-506, and rapamycin: pharmacologic probes of lymphocyte signal transduction. *Annu Rev Immunol* 10: 519-560.
16. Ramírez F, Fowell DJ, Puklavac M, Simmonds S, Mason D (1996) Glucocorticoids promote a TH2 cytokine response by CD4+ T cells in vitro. *J Immunol* 156: 2406-2412.
17. Andréau K, Lemaire C, Souvannavong V, Adam A (1998) Induction of apoptosis by dexamethasone in the B cell lineage. *Immunopharmacology* 40: 67-76.
18. Piriou L, Chilmonec S, Genetet N, Albina E (2000) Design of a flow cytometric assay for the determination of natural killer and cytotoxic T-lymphocyte activity in human and in different animal species. *Cytometry* 41: 289-297.
19. <http://www.minipigs.dk/smardedit/upload/hematology.pdf>
20. Curtis, M. J. (2010). The work and conclusions of the RETHINK project. *J. Pharmacol Toxicol Methods* 62:157.
21. Ellegaard L, Cunningham A, Edwards S, Grand N, Nevalainen T, et al. (2010) Welfare of the minipig with special reference to use in regulatory toxicology studies. *J Pharmacol Toxicol Methods* 62: 167-183.
22. Forster R, Ancian P, Fredholm M, Simianer H, Whitelaw B; Steering Group of the RETHINK Project (2010) The minipig as a platform for new technologies in toxicology. *J Pharmacol Toxicol Methods* 62: 227-235.
23. Frey BM, Sieber M, Mettler D, Gänger H, Frey FJ (1988) Marked interspecies differences between humans and pigs in cyclosporine and prednisolone disposition. *Drug Metab Dispos* 16: 285-289.
24. Duggan DE, Yeh KC, Matalia N, Ditzler CA, McMahon FG (1975) Bioavailability of oral dexamethasone. *Clin Pharmacol Ther* 18: 205-209.
25. Hsiu SL, Hou YC, Wang YH, Tsao CW, Su SF, et al. (2002) Quercetin significantly decreased cyclosporin oral bioavailability in pigs and rats. *Life Sci* 72: 227-235.
26. Luster MI, Portier C, Pait DG, White KL Jr, Gennings C, et al. (1992) Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests. *Fundam Appl Toxicol* 18: 200-210.
27. Blot C, Lebrec H, Roger R, Bohuon R, Pallardy M (1994) Immune parameters are affected differently after cyclosporine A exposure in Fischer 344 rats and B6C3F1 mice: implications for immunotoxicology. *Toxicology* 94: 231-245.
28. De Waal EJ, Timmerman HH, Dortant PM, Kranjc MA, Van Loveren H (1995) Investigation of a screening battery for immunotoxicity of pharmaceuticals within a 28-day oral toxicity study using azathioprine and cyclosporin A as model compounds. *Regul Toxicol Pharmacol* 21: 327-338.
29. Schulte A, Althoff J, Ewe S, Richter-Reichhelm HB; BGVV Group Investigators (2002) Two immunotoxicity ring studies according to OECD TG 407-comparison of data on cyclosporin A and hexachlorobenzene. *Regul Toxicol Pharmacol* 36: 12-21.
30. Smith HW, Winstead CJ, Stank KK, Halstead BW, Wierda D (2003) A predictive F344 rat immunotoxicology model: cellular parameters combined with humoral response to NP-CgammaG and KLH. *Toxicology* 194: 129-145.
31. The ICICIS Group Investigators (1998) Report of validation study of assessment of direct immunotoxicity in the rat. *International Collaborative Immunotoxicity Study*. *Toxicology* 125: 183-201.
32. Ryffel B, Donatsch P, Madörin M, Matter BE, Rüttimann G, et al. (1983) Toxicological evaluation of cyclosporin A. *Arch Toxicol* 53: 107-141.
33. Schuld A, Birkmann S, Beitingner P, Haack M, Kraus T, et al. (2006) Low doses of dexamethasone affect immune parameters in the absence of immunological stimulation. *Exp Clin Endocrinol Diabetes* 114: 322-328.
34. Schwab CL, Fan R, Zheng Q, Myers LP, Hébert P, et al. (2005) Modeling and predicting stress-induced immunosuppression in mice using blood parameters. *Toxicol Sci* 83: 101-113.
35. Descotes J, Horand F, Ravel G (2007) Humoral response to KLH and lymphocyte subset analysis in dogs treated with Dexamethasone. *The Toxicologist* 96: 357.
36. Munson AE, Sanders VM, Douglas KA, Sain LE, Kauffmann BM, et al. (1982) In vivo assessment of immunotoxicity. *Environ Health Perspect* 43: 41-52.
37. Haverson K, Bailey M, Stokes CR, Simon A, LeFlufy L, et al. (2001) Monoclonal antibodies raised to human cells—specificity for pig leukocytes. *Vet Immunol Immunopathol* 80: 175-186.
38. Müller C, Zielinski CC, Kalinowski W, Wolf H, Mannhalter JW, et al. (1989) Effects of cyclosporin A upon humoral and cellular immune parameters in insulin-dependent diabetes mellitus type I: a long-term follow-up study. *J Endocrinol* 121: 177-183.
39. Richter-Reichhelm HB, Schulte AE (1998) Results of a cyclosporin A ringstudy. *Toxicology* 129: 91-94.

40. Yabu K, Warty VS, Gorelik E, Shinozuka H (1991) Cyclosporine promotes the induction of thymic lymphomas in C57BL/6 mice initiated by a single dose of gamma-radiation. *Carcinogenesis* 12: 43-46.
41. Dean JH, Hincks JR, Remandet B (1998) Immunotoxicology assessment in the pharmaceutical industry. *Toxicol Lett* 102-103: 247-55.
42. Exon JH, Bussiere JL, Mather GG (1990) Immunotoxicity testing in the rat: an improved multiple assay model. *Int J Immunopharmacol* 12: 699-701.
43. Exon JH, Koller LD, Talcott PA, O'Reilly CA, Henningsen GM (1986) Immunotoxicity testing: an economical multiple-assay approach. *Fundam Appl Toxicol* 7: 387-397.
44. Piccolella E, Lombardi G, Vismara D, Del Gallo F, Colizzi V, et al. (1986) Effects of dexamethasone on human natural killer cell cytotoxicity, interferon production, and interleukin-2 receptor expression induced by microbial antigens. *Infect Immun* 51: 712-714.
45. Sovcikova A, Tulinska J, Kubova J, Liskova A, Syrova D, et al. (2002) Effect of cyclosporin A in Lewis rats in vivo and HeLa cells in vitro. *J Appl Toxicol* 22: 153-160.
46. Assan R, Feutren G, Debray-Sachs M, Quiniou-Debrie MC, Laborie C, et al. (1985) Metabolic and immunological effects of cyclosporin in recently diagnosed type 1 diabetes mellitus. *Lancet* 1: 67-71.
47. Reed JC, Abidi AH, Alpers JD, Hoover RG, Robb RJ, et al. (1986) Effect of cyclosporin A and dexamethasone on interleukin 2 receptor gene expression. *J Immunol* 137: 150-154.
48. Herzyk DJ, Gore ER (2004) Adequate immunotoxicity testing in drug development. *Toxicol Lett* 149: 115-122.
49. Gore ER, Gower J, Kurali E, Sui JL, Bynum J, et al. (2004) Primary antibody response to keyhole limpet hemocyanin in rat as a model for immunotoxicity evaluation. *Toxicology* 197: 23-35.
50. Finco-Kent D, Kawabata TT (2005) Development and validation of a canine T-cell-dependent antibody response model for immunotoxicity evaluation. *J Immunotoxicol* 2: 197-201.
51. Piccotti JR, Alvey JD, Reindel JF, Guzman RE (2005) T-cell-dependent antibody response: assay development in cynomolgus monkeys. *J Immunotoxicol* 2: 191-196.
52. Palestine AG, Roberge F, Charous BL, Lane HC, Fauci AS, et al. (1985) The effect of cyclosporine on immunization with tetanus and keyhole limpet hemocyanin (KLH) in humans. *J Clin Immunol* 5: 115-121.
53. White KL Jr, Sheth CM, Peachee VL (2007) Comparison of primary immune responses to SRBC and KLH in rodents. *J Immunotoxicol* 4: 153-158.
54. Dietert RR, Lee JE, Olsen J, Fitch K, Marsh JA (2003) Developmental immunotoxicity of dexamethasone: comparison of fetal versus adult exposures. *Toxicology* 194: 163-176.
55. EUROTOX (2008) Abstracts of the 45th Congress of the European Societies of Toxicology, Rhodes, Greece. *Toxicol Lett* 180 Suppl: S1-S245.
56. Kuper CF, Van Zijverden M, Klaassen C, Tegelenbosch-Schouten M, Wolterbeek AP (2007) Effects of cyclosporin A and cyclophosphamide on Peyer's patches in rat, exposed in utero and neonatally or during adult age. *Toxicol Pathol* 35: 226-232.
57. Penninks AH, van Mierlo GJ (2012) Immunotoxicity Studies in Minipigs. In: *The Minipig in Biomedical Research*. CRC Press/Taylor and Francis, New York, USA: 397-411.

Citation: van Mierlo GJD, Kuper CF, de Zeeuw-Brouwer ML, Schijf MA, Bruijntjes JP, et al. (2013) A Sub Acute Immunotoxicity Study in Göttingen Minipigs® with the Immunosuppressive Compounds Cyclosporin A and Dexamethasone. *Clin Exp Pharmacol S4*: 006. doi:[10.4172/2161-1459.S4-006](https://doi.org/10.4172/2161-1459.S4-006)

This article was originally published in a special issue, **Novel Developments in Immunopharmacology and Immunotoxicology** handled by Editor(s). Dr. Yuliang Ma, Weill Cornell Medical College, USA

Submit your next manuscript and get advantages of OMICS Group submissions

Unique features:

User friendly/feasible website-translation of your paper to 50 world's leading languages
Audio Version of published paper
Digital articles to share and explore

Special features:

250 Open Access Journals
20,000 editorial team
21 days rapid review process
Quality and quick editorial, review and publication processing
Indexing at PubMed (partial), Scopus, EBSCO, Index Copernicus and Google Scholar etc
Sharing Option: Social Networking Enabled
Authors, Reviewers and Editors rewarded with online Scientific Credits
Better discount for your subsequent articles

Submit your manuscript at: <http://www.omicsonline.org/submission>

