Immunosafety evaluation in Juvenile Göttingen Minipigs

Linda Allais\textsuperscript{a}, Alicia Perbet\textsuperscript{a}, Fabienne Condevaux\textsuperscript{a}, Jean-Paul Briffaux\textsuperscript{a} and Marc Pallardy\textsuperscript{b}

\textsuperscript{a} Charles River Laboratories France Safety Assessment, Saint-Germain-Nuelles, France; \textsuperscript{b} Inserm, Inflammation, Microbiome, and Immunosurveillance, Université Paris-Saclay, Chatenay-Malabry, France

\textbf{ABSTRACT}

Although an extrapolation from the clinical experience in adults can often be considered to support the pediatric use for most pharmaceutical compounds, differences in safety profiles between adult and pediatric patients can be observed. The developing immune system may be affected due to exaggerated pharmacological or non-extrapolated effects of a new drug. Toxicology studies in juvenile animals could therefore be required to better evaluate the safety profile of any new pharmaceutical compound targeting the pediatric population. The Göttingen minipig is now considered a useful non-rodent species for non-clinical safety testing of human pharmaceuticals. However, knowledge on the developing immune system in juvenile minipigs is still limited. The objective of the work reported here was to evaluate across-age proportions of main immune cells circulating in blood or residing in lymphoid organs (thymus, spleen, lymph nodes) in Göttingen Minipigs. In parallel, the main immune cell populations from healthy and immunocompromised piglets were compared following treatment with cyclosporin A (CsA) at 10 mg/kg/day for 4 wk until weaning. The study also assessed functionality of immune responses using an in-vivo model after “Keyhole limpet hemocyanin” (KLH) immunization and an ex-vivo lymph proliferation assay after stimulation with Concanavalin A. The results demonstrated variations across age in circulating immune cell populations including CD21$^+$ B-cells, \( \alpha \beta \)-T- and \( \gamma \delta \)-T-cells, NK cells, and monocytes. CsA-induced changes in immune functions were only partially recovered by 5 mo after the end of treatment, whereas the immune cell populations affected by the treatment returned to normal levels in animals of the same age. Taken together, the study here shows that in this model, the immune function endpoints were more sensitive than the immunophenotyping endpoints.

\textbf{Introduction}

Toxicology studies in juvenile animals can be required to adequately evaluate the safety profile of any new pharmaceutical compound targeting pediatric populations (ICH S11 2020; EMEA/CHMP/SWP/169215/2005 2008; Cappon et al. 2009). Although an extrapolation from the clinical experience in adults is generally used to support the pediatric use for most pharmaceutical compounds, differences in safety profiles between adult and pediatric patients are often observed, especially on organs or systems going through different stages of maturation during childhood or early adolescence. These differences can expose children to a direct risk of under or overdosing and a delayed risk of long term adverse effects (Turner et al. 2014).

In view of the heterogeneity of new drugs developed these last decades, the immune system is an increasing target of adverse effects of new medicines, particularly when the immune system is still under development, and therefore more vulnerable to adverse effects, as it can be seen in children. Immune endpoints are therefore required in safety animal studies to anticipate any unwanted effect of a future medicine on the immune system (EMEA/CHMP/SWP 169215/2005, 2008; FDA 2006; ICH S11 2020). Although several adequate methods are available for the detection of immune modulation or developmental immunotoxicity, further investigation is still needed to better characterize the window of vulnerability of the developing immune system, which is much longer and less well-defined than that of other organ systems.

The species of choice for the conduct of a juvenile safety study is selected on the basis of scientific and technical criteria such as the pharmacological action of the tested compound, target organs previously identified in adult animals, pharmacokinetic profile, organ/system development stage, and technical feasibility. For the above-mentioned reasons, the most relevant rodent or non-rodent species previously used in standard adult toxicity studies is often selected for the conduct of the juvenile toxicity study.

The minipig is now considered a useful non-rodent species for safety testing of human pharmaceuticals (Rubic-Schneider et al. 2016; Descotes et al. 2018). Though non-rodent toxicity data are still prominent with dogs and cynomolgus macaques, historical control data in Göttingen minipigs is progressively increasing over the years in contract research organizations as a result of general toxicology, safety pharmacology, reproductive, and juvenile toxicity studies. The immune system of the adult pig has been studied, particularly in relation to different infectious diseases observed in pigs and to xenotransplantation. Still,
a clear understanding of the developing immune system in piglets is limited and should be further assessed to better anticipate the safety profile of new pharmaceuticals, especially those targeting pediatric populations (Penninks et al. 2012).

This work was performed to examine in Göttingen piglets the immunotoxicity of the well-known immunosuppressant cyclosporin (CsA) following daily oral gavage in the period from Postnatal Day 3 to Postnatal Week 4 (weaning). The principal objective was to analyze selected immune endpoints, such as immunophenotyping (B-cells, T-helper cells, cytotoxic-T cells, γδ-T cells, NK cells, monocytes, "dendritic-like" cells) in the blood, thymus, spleen, and lymph nodes, as well as to evaluate functional tests like ex-vivo lymphoproliferation and TDAR (T-Dependent Antibody Responses) in the 4-wk-old piglets treated with CsA. Lastly, in parallel, a comparative analysis was performed across age from the early postnatal phase to the adult age (6-mo-old) to see how immune cell proportions and response levels differed between juvenile and adult animals.

**Materials and methods**

**Test substance**

Cyclosporin (CsA, Neoral® 100 mg/mL solution) was obtained from Novartis Pharma (Rueil-Malmaison, France). The stock solution was diluted in water (Cooper, Melun, France) to concentrations of 5 mg/mL for oral administration to the piglets.

**Test system**

Six pregnant Göttingen minipig sows (Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark) were delivered to Charles River Lyon Safety Assessment Facility (Charles River, Lyon, France) at least 3 weeks before the estimated farrowing date. During the gestation and pre-weaning phases, each sow, with its litter (where applicable), was housed in double pens (2 × 2 m²) in a conventional animal unit at the AAALAC-accredited facility maintained at 19–25 °C, with a minimum relative humidity 35%, and a 12-hr light-dark cycle. The facilities underwent 10 air changes/hr. After separation from the mother, the 5-wk-old piglets were group-housed. Sawdust bedding, toys, chewing chains, sleeping baskets, and dry or fresh fruits were provided to the sows and piglets during the whole in-life phase of the study. In addition, a heating lamp was used to maintain the comfort of the piglets during their first days of life. The piglets were also given 1 mL of iron intramuscularly within 24 days after birth. The sows and piglets (after weaning) were fed pelleted complete commercial diet (SDS SMP MOD(E) SQC), with an incomplete pelleted diet; then both were combined progressively until completion of the weaning period.

At birth, piglets were pre-selected from the six litters on the basis of body weight and physical/functional development parameters. Piglets in poor clinical condition were immediately euthanized. Spare healthy animals were retained at the disposal of the test facility for other investigational purposes. Eighteen selected piglets (9/sex) from three litters were given CsA at a dose of 10 mg/kg/d (2 mL/kg/day of 5 mg/mL CsA) by gavage from Day 3 (Postnatal Day 3 [PN3]) to Week 4 (Postnatal Week 4 [PNW4]) of age. Eighteen selected piglets (9/sex) from three other litters were given water at the same administration volume for the same treatment period. Controls were treated under the same experimental conditions as the piglets treated with CsA. All piglets received two subcutaneous injections (1 mL/animal) of a solution of 2.5 mg KLH/mL (mcKLH Imject®, Pierce, Rockford, IL) 24 and 10 days before each scheduled termination. At the end of the treatment period at Week 4, a subset of piglets (3/sex) from the water and CsA groups was humanely euthanized by intravenous sodium pentobarbital injection and necropsied. A second and third subset in each post-weaning group (3/sex/group) was kept after Week 4 for a 1-mo and a 5-mo recovery period (Postnatal Month 2 [PNM2] and Postnatal Month 6 [PNM6], respectively). During their in-life phase period, all piglets were examined daily and were weighed at least twice weekly during the pre-weaning phase and once weekly after weaning. The study design was reviewed and approved by the Charles River’s Ethics Committee based upon animal health and welfare guidelines [(Directive 2010/63/EU; Decree n° 2001–464; Decree n° 2001–486)].

At each selected occasion (Week 2 and/or 4, PNM2, PNM6), blood was collected from each piglet or adult via the neck vein into K3-EDTA anticoagulant tubes for immunophenotyping analysis, into lithium-heparin tubes for ex-vivo T-cell proliferation assays, and into tubes without anticoagulant for determination of anti-KLH IgG titers in serum (TDAR assay).

At each occasion (PNW4, PNM2, PNM6), each selected animal was pre-anesthetized with ketamine-xylazine before being transferred to the necropsy room and humanely euthanized by intravenous sodium pentobarbital injection. Immediately, the thymus, spleen, and iliac lymph nodes were collected for immunophenotyping analysis. Samples from each site were immediately placed in cell culture medium (RPMI 1640 medium [Lonza, Walkersville, MD] and 10% fetal bovine serum [FBS, Biowest, Riverside, MO]) held at 2–8 °C. The tissues were then manually dissociated and then filtered through 40-μm pore nylon filters, and the separated cells were then processed for FACS staining (see below).

**Immunophenotyping by flow cytometry analysis**

Details about the primary and secondary antibodies used in both studies are presented in Supplementary Table A1. In brief, a volume of 100 μL of whole blood or 10⁶ cells/mL of thymus, spleen, or lymph node suspension was incubated for 20 min at 2–8 °C in the dark with primary antibodies followed by secondary antibodies, where applicable. Cell viability was assessed using a LIVE/DEAD™ Near-IR Dead Cell Stain Kit (ThermoFisher, Eugene, OR). After labeling, the samples were fixed and analyzed. Washing steps were performed using Ca²⁺, Mg²⁺-free Dulbecco’s phosphate buffered saline (DPBS, Sigma, St. Louis, MO).

The phenotype analysis was performed in a Fortessa flow cytometry system (BD Biosciences, San Jose, CA). Live cells were gated; after doublet discrimination, the following identifications were performed: CD21⁺ B-cells (CD45⁺CD21⁺), naïve B-cells (CD21⁺CD45RA⁻), T-cells (CD45⁺CD3⁺), αβ-T-cells (CD45⁺CD3⁺ γδ-TCR⁺) and subsequent αβ-T subsets (CD8⁺ cytotoxic T-cells, CD4⁺ helper T-cells, and double-positive CD4⁺CD8⁺), γδ-T-cells (CD45⁺CD3⁻ γδ-TCR⁺) and subsequent γδ-T subsets (CD2D2⁺, CD2⁺, CD2⁺, and CD2CD8⁺) (Supplementary Figure S1), NK cells (CD45⁺CD3⁻CD8⁻) and subsequent subsets (CD16low/higNKp46⁻/+ ) (Supplementary Figure S2), monocytes using high forward and side-scatter gating and subsequent subsets (CD14⁺CD16⁻, CD14⁺CD16⁻, CD14⁺CD16⁻, CD14⁺CD16⁻).
CD14⁻CD16⁺) (Supplementary Figure S2), "dendritic like" cells (MHCI⁻ gating followed by subsequent subsets using CD172a, CD163, CADM1, and/or CD207 surface markers), and DC-sign cells (CD209⁺ cells) (Supplementary Figure S3). In all cases, a minimum of 20,000 events/sample was acquired. All data was analyzed using FACSDiva™ software (BD Biosciences).

**Ex-vivo T-lymphocyte proliferation assay**

Each blood sample was diluted 1:10 in culture medium (RPMI and 10% FBS), placed into 48-well plates (1 mL/well), treated with Concanavalin A (10 µg ConA/mL; as mitogen to stimulate lymphocyte proliferation) or culture medium (negative control), and incubated at 37°C for 72 hr. Twenty-four hr before the end of incubation, 1 mM Click-IT EdU (5-ethyl-2'-deoxyuridine, thymidine analog; ThermoFisher) was added to each well to permit measures of newly-synthesized DNA. On the day of analysis, the cells were collected from each well, fixed, permeabilized by addition of Click-IT® saponin, treated with Click-IT® reaction cocktail, and then incubated for 30 min at room temperature. Detection of the new DNA was based on a Click reaction which is a copper-catalyzed covalent reaction between an azide (the EdU) and an alkyne (Alexa Fluor® dye). Cell viability was also assessed in this test using the LIVE/DEAD™ Near-IR Dead Cell Stain Kit (ThermoFisher).

**TDAR (T-cell dependent antibody response) test**

Flat-bottom plates (NUNC Immuno MaxiSorp Plate, Roskilde, Denmark) were coated with 1 µg/well (100 µL of 10 µg/mL stock dilution) of KLH dissolved in 0.2 M carbonate-bicarbonate buffer pH 9.4 (Pierce) overnight at 2–8°C. The plates were then washed three times with 0.1% [v/v] Tween-20/PBS solution (Sigma) and nonspecific binding sites blocked for at least 15 min at ambient temperature with buffer (ThermoFisher). Thereafter, 100 µL serum from each animal (diluted 1:5000 in 0.3% Tween-20/PBS; Sigma) was added to duplicate wells and the plates then incubated for 2 hr at ambient temperature. The plates were then washed three times with 0.1% Tween-20/PBS before each well received 100 µL of streptavidin-horseradish peroxidase-conjugated mouse-anti-pig IgG biotin (diluted 1:100,000 in 0.3% Tween-20/phosphate-buffered saline [PBS]) and was incubated at 37°C for at least 1 hr. After washing the plates five times (using 0.1% Tween-20/PBS), 100 µL TMB substrate solution (3,3',5,5'-tetramethyl-benzidine; ThermoFisher) was added to each well. The reaction in each well was stopped after 20 min by addition of 100 µL 1 N HCl (Sigma). Optical density was then measured at 450 nm in a Benchmark Plus Microplate Spectrophotometer (BioRad, 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. Anti-KLH titers for each serum sample were calculated by extrapolation from the standard curve generated in parallel.

**Statistical analysis**

Prism 6.0 software (GraphPad, La Jolla, CA) was used for analysis of data sets. Statistical tests applied to each dataset are indicated in the relevant figure legend. Statistical analysis was performed by a one-way analysis of variance (ANOVA) followed by a Dunnett’s multiple comparison test. Statistical significance was defined as *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001.

**Results**

A preliminary analysis showed that all the data obtained in this experiment were comparable between males and females for all ages (data not shown). The results obtained from both genders were therefore combined to augment the power of our interpretations and of statistical analyses.

**B-lymphocytes**

Though specific antibodies to B-cells are not available for swine, anti-CD21 antibodies can be used to target some B-cell populations in this species, i.e. these are mainly naïve and activated B-cells (Butler et al. 2009, 2017; Sinkora and Butler 2009, 2016; Blanc et al. 2020) CD2, CD25, CD172a et CD79a have also been used as potential surface markers for some B-cells, mostly in the first phases of differentiation (Butler et al. 2009; Saalmüller and Gerner 2016; Dawson and Lunney 2018; Blanc et al. 2020).

In this experiment, the percentage of circulating CD21⁺ B-cells decreased with age (mean ± SEM: 8.1 [± 1.2], 6.2 [± 0.5], and 2.6 ± 0.2 % in the 4-wk, 2-mo, and 6-mo-old minipigs, respectively) (Figure 1). A significantly lower (p < 0.001) percentage of circulating CD21⁺ B-cells was also noted in 4-wk-old minipigs treated with CsA compared with in age-matched control animals (2.7 ± 0.6% CsA vs. 8.1 [± 1.2]% control). The immunosuppressive effect of CsA on the proportion of CD21⁺ B-cells was no longer observed after the 4-wk (PNM2) or 5-mo (PNM6) observation period (Figure 1).

CD21⁺ B-cells were also analyzed in the spleen. No relevant differences in percentages were noted between ages or groups (individual values ranging from 14.5 to 45.7%). CD45RA, a surface marker for naïve cells, was expressed on average by 90% of CD21⁺ B-cells in the blood and by 60% of CD21⁺ B-cells in the spleen (data not shown).

**T-lymphocytes**

The mean percentage of circulating T-lymphocytes (CD3⁺ cells) was slightly higher in 4-wk-old minipigs (41.1 [± 2.2]%) than in older minipigs (24.1 [± 5.0] and 26.9 [± 2.4]% in 2-mo and 6-mo-olds, respectively) (Figure 1). A trend to higher percentages of circulating γδ-T-cells was noted in the 6-mo-old minipigs (39.6 [± 6.4%] compared to in younger animals (23.5 [± 3.7]% and 20.9 [± 4.1]% in 4-wk and 2-mo-olds, respectively), despite evidence of a higher inter-individual variation in older animals (individual values ranging from 23.1 to 52.0%).

When comparing the T-lymphocyte populations in the blood, thymus, spleen, and lymph nodes, the highest proportion of CD3⁺ T-cells was observed in the spleen and blood in 4-wk-old piglets (51.8 [± 4.0], 41.1 [± 2.2], 25.7 [± 4.0], and 24.0 [± 1.9]% in spleen, blood, lymph nodes, and thymus, respectively). In adult minipigs, the highest proportion was observed in the lymph nodes and spleen (57.3 [± 2.4], 55.4 [± 3.9], 26.9 [± 2.4], and 22.6 [± 1.9]% in the lymph nodes, spleen, blood and thymus, respectively) (Figure 2). The highest proportion of γδ-T-cells was noted in the blood for both juvenile (23.5 [± 3.7]% in blood vs. 14.2 [± 1.7], 13.8 [± 2.8], and 9.1 ± 1.5% in lymph nodes, spleen, and thymus, respectively) and adult (39.6 [±6.4]% in blood vs. 19.7 [± 1.0], 16.5 [± 1.2], and 7.2 [± 0.7]% in spleen,
thymus and lymph nodes, respectively) animals. Consequently, the putative αβ-T cell population (negative gate for CD3+ population) represented the lowest proportion in blood by comparison with the same population in the lymphoid organs (Figure 2).

From the putative αβ-T-population (CD3+ γδ-TCR+) gating analysis, helper-T (CD4+) and cytotoxic-T (CD8a+) cell subsets were compared across ages. The proportion of the major subset, represented by circulating helper T-cells, decreased with age (59.4 ± 3.2, 35.8 ± 8.0, 37.5 ± 3.1% in 4-wk-, 2-mo-, and 6-mo-old minipigs, respectively). Percentages of cytotoxic T-cells were not affected by age (23.3 ± 1.8, 23.8 ± 3.2, 28.1 ± 2.8% in 4-wk-, 2-mo-, and 6-mo-olds, respectively). The circulating αβ-T-population was also composed of a small proportion of double CD4+CD8a+ T-cell subsets, which was twice as higher in the 2- and 6-mo-old minipigs (7.3 ± 2.0% at PNM2 and 7.1 ± 0.9% at PNM6) compared to in 4-wk-old piglets (3.2 ± 0.6% at PNW4) (Figure 3). The double-positive CD4+CD8a+ subset was also analyzed in the thymus and, as expected, represented the major population of thymocytes; however, there was no consistent trend across age (43 ± 2, 74 ± 2, and 49 ± 5% in 4-wk-, 2-mo-, and 6-mo-old minipigs, respectively).

From the γδ-T-population (CD3+ γδ-TCR+) gating analysis, three subsets of CD2+CD8+, CD2+CD8−, and CD2−CD8− cells previously identified in swine (Stepanova and Sinkora 2013) were also analyzed in the blood. The data revealed no relevant differences across age in the proportions of CD2+CD8+ cells, the major γδ-T subset (94 ± 1, 91 ± 2, and 88 ± 1% in 4-wk-, 2-mo-, and 6-mo-olds, respectively) or in the other two minor subsets CD2+CD8− and CD2−CD8− (each <6%).

Both αβ-T and γδ-T populations were unaffected, in terms of proportions, by treatment of the piglets with 10 mg CsA/kg/day by oral gavage for 4 weeks (Figure 1). However, the percentage of circulating cytotoxic T-cells was 1.6-fold lower (p < 0.05) in
CsA-treated piglets (14.7 [± 1.7]%) than in controls (23.3 [± 1.8]%) at PNW4 (Figure 3). This difference between control and CsA-treated groups was still present after the 1-mo observation period (PNM2) but not after 5-mo (PNM6).

**NK cells**

Natural Killer (NK) cell identification in swine is not as clear-cut as in other species due to a lack of a lineage marker available for the pig. The NK cell phenotype in human and other species is commonly identified as CD3–CD8a–CD56+CD16+ Perforin1 NKp46+ (Mair et al. 2013, 2016). NKp46 (CD335), a member of the natural cytotoxicity receptor (NCR), is considered as a lineage marker for NK cells across mammalian species (Walzer et al. 2007); however, in swine, NKp46 is expressed only by a subset of NK cells (Mair et al. 2012, 2013; Talker et al. 2013). Another peculiarity for porcine NK cells concerns their morphology, i.e. they have a smaller size and a cytoplasm poorer in granules than their counterpart cells in other species (Denyer et al. 2006).

Here, a putative NK cell population was identified by the phenotype CD3–CD8a+; these were further divided into four subsets according to expression (low/high) of CD16 and "on/off" expression of NKp46, i.e. these were CD16lowNKP46+; CD16lowNKP46+, CD16+lowNKP46+ and CD16+highNKP46+ (Figure 4). Each of the above-mentioned NK cell subset was analyzed in blood as well as in lymph nodes collected from 4-wk-, 2-mo-, and 6-mo-old minipigs.

The percentage of CD3+CD8+ cells in blood was comparable with the one analyzed in lymph nodes of 4-wk- and 2-mo-old minipigs (individual percentage values not exceeding 17% for blood and lymph node populations). In 6-mo-old minipigs, the individual percentages of CD3+CD8+ cells were increased with age (3–44% in blood whereas they remained <17% in the lymph nodes of the same animals).

Amongst the CD3+CD8+ population, the percentage of CD16high cells was comparable between blood and lymph nodes in adult animals (47 [± 3]% in blood and 42 [± 7]% in lymph nodes at PNM6) (Figure 4). In young piglets, however, the percentage of CD16high cells in the lymph nodes was three times lower than in the adults (12 [± 4]% at PNW4), while it was still comparable to the adult level in blood (60 [± 12]% at PNW4). The percentage of CD3+CD8+CD16low was 1.6–1.9-times higher in lymph nodes than in blood for young piglets (60 [± 13]% in lymph nodes compared to 39 [± 12]% in blood at PNW4) and for adult animals (56 [± 7]% in lymph nodes compared to 29 [± 2]% in blood at PNM6). There were no relevant differences between ages with regard to either circulating or lymph node CD16low relative proportions (Figure 4).

Within the CD3+CD8+ population, the percentage of cells expressing NKp46 in blood was low for both young and adult minipigs (mean values <10%), with no significant differences between age—but with a high interindividual variability. In contrast, in the lymph nodes, >80% of the NK cell population expressed NKp46 in 4-wk-old piglets, for both CD16low and CD16high subsets; these values significantly decreased with age (dropped to 8 [±3]% CD16lowNKP46+ and 30 [±3]% CD16highNKP46+ at PNM6).

Both circulating and lymph node NK cell populations did not seem affected by host CsA treatment for 4 weeks.

**Monocytes**

As in humans, porcine monocytes express CD14 and/or CD16 surface markers, leading to identification of three main subsets: CD14+CD16+ (traditional), CD14−CD16+ (intermediate), and CD14low−CD16+ (non-traditional). However, the relative proportion of monocytes expressing CD16 corresponds to the major population (traditional subset) in the pig whereas it represents only a minor population in humans. Figure 5 presents the relative proportion of these three monocyte subsets, in addition to a double-negative subset, analyzed from the blood collected from 4-wk- and 6-mo-old minipigs. As expected, the major subset in the gated monocyte population was CD14+CD16+, with no relevant differences due to host age (61 [± 4] and 71 [± 7]% at PNW4 and PNM6, respectively). In contrast, the double-positive CD14+CD16+ population increased with age (3 [±1] and 20 [± 3]% at PNW4 and PNM6, respectively), whereas the CD14+CD16− population in 4-wk-old piglets (6.1 [± 0.2]%) was no longer detectable in the blood of 6-mo-old minipigs.

None of the three monocyte subsets were affected by the host treatment with CsA for 4 weeks (data not shown).

"Dendritic-like" and myeloid/macrophage cells

Dendritic cells (DC) originate mostly from the bone marrow; a very small proportion is present in blood and a larger proportion is found in various lymphoid and non-lymphoid tissues. DC exist as different sub-populations with a phenotypic
heterogeneity depending on maturation status, localization in an organism, and their main functions. Conventional DC, plasmacytoid DC, and inflammatory DC are the most common forms studied in human and animal hosts, including the pig (Summerfield and McCullough 2009; Marquet et al. 2011; Meurens et al. 2012; Mair et al. 2014; Niederwerder 2017; Franzoni et al. 2019). The conventional DC are divided into two subsets, i.e. cDC1 (porcine phenotype MHCII$^+$CD172alowCADM1$^+/high$) and cDC2 (porcine phenotype MHCII$^+$CD172ahighCD163intCADM1$^+/low$).

In this experiment, a combination of multiple surface markers, MHC-II, CD172a, CD163, CADM1 and CD207, was used to identify "dendritic-like" cell subsets in the piglet/pig blood and lymph nodes. As a first step, the MHC-II$^+$ population was gated, among which another sub-population was identified by targeting CD172a (SIRP$\alpha$). CD172a is expressed at variable levels by several immune cells, including conventional and plasmacytoid dendritic cells, and has been proven to be highly expressed by both Human and porcine cDC2 cells. The proportion of MHC-II$^+$ cells was relatively higher and more variable in adult pigs (6-mo-old) than in 4-wk-old piglets; this age difference was particularly notable in the circulating population (Figure 6). Treatment with CsA was associated with a significant decrease in proportions of circulating MHCII$^+$ populations in 4-wk-old piglets (11.8 ± 0.8% in control, 7.2 ± 0.5% in CsA group, $p < 0.05$). There was no relevant effect of the CsA immunosuppressant on the MHC-II$^+$ populations resident in lymph nodes of the 4-wk- or 6-mo-old minipigs.

It should be noted that reliable data for the MHC-II$^+$ population could not be obtained for the 2-mo-old animals due to a technical issue with the MHC-II labeling procedure. The following cell subsets were therefore compared between 4-wk- and 6-mo-old minipigs. The MHCII$^+$CD172a$^+$ subset identified in both matrices was comparable between both age groups (PNW4 and PNM6) and was not affected by CsA treatment (Figure 6). It should be noted that the intensity of expression of CD172a was variable between individuals; therefore, CD172a$^+$ cells included both low and high expression of the protein. Approximately 31–42% of MHC-II$^+$ cells co-expressed CADM1 in the blood and lymph nodes. cDC2 cells could not be specifically identified in blood or lymph nodes due variable expression between individuals.

Among the MHCII$^+$CD172a$^+$ population, two other subsets were analyzed using CADM1 and CD207 surface markers (Figure 7) and CD163 (Figure 8). CADM1 (SynCAM: Synaptic Cell Adhesion Molecule) has been proven to be highly expressed by both human and porcine cDC1 cells, especially by DC residing in tissues. In the current study, > 60% of MHCII$^+$CD172a$^+$ cells in both the blood and lymph nodes were found to co-express CADM1 in the 6-mo-old minipigs whereas < 10% of the same population was found to be CADM1$^+$ in 4-wk-old piglets.
Relative proportion of MHC-II$^+$ cells and MHCII$^+$CD172a$^+$ cells in blood and iliac lymph nodes (ILN) from 4-wk-old (PNW4) and 6-mo-old (PNM6) minipigs. Comparative analysis between piglets treated by oral gavage with water (Control; $n = 6$) or CsA ($n = 6$) during first 4 wk after birth. Box and Whiskers plots demonstrate relative proportion of each defined populations within singlets or MHC-II$^+$ gates. Whiskers represent minimal and maximal values for each population. Statistical analysis performed using one-way ANOVA followed by a Dunnett’s multiple comparison test. Significance defined as $^*p < 0.05$.

Relative proportion of CADM1$^+$ and CD207$^+$ cells in blood and iliac lymph nodes (ILN) from 4-wk-old (PNW4) and 6-mo-old (PNM6) minipigs. Comparative analysis between piglets treated by oral gavage with water (Control; $n = 6$) or CsA ($n = 6$) during first 4 wk after birth. Box and Whiskers plots demonstrate relative proportion of each defined population within MHC-II$^+$CD172a$^+$ gates. Whiskers represent minimal and maximal values for each population. Statistical analysis performed using one-way ANOVA followed by a Dunnett’s multiple comparison test. No significant differences were noted between groups.
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4 wk after birth. Box and Whiskers plots demonstrate relative proportion of each defined populations within singlets or MHC-II evaluated if this subset was also CD163

Figure 8. Relative proportion of "dendritic-like" (MHC-II^CD172a^-CD163^+) and inflammatory (DC-sign) cells in blood and iliac lymph nodes (ILN) from 4-wk-old minipigs (PNW4) (data not available in older animals). Comparative analysis between piglets treated by oral gavage with water (Control; n = 6) or CsA (n = 6) during first 4 wk after birth. Box and Whiskers plots demonstrate relative proportion of each defined populations within singlets or MHC-II^+ gates. Whiskers represent minimal and maximal values for each population. Statistical analysis performed using one-way ANOVA followed by a Dunnett’s multiple comparison test. No significant differences were noted between groups.

CD163 (scavenger receptor) is known to be expressed by blood DC, but is also expressed by other immune cells like monocytes and macrophages. In this study, a focus was on the MHCII^CD172a^- subset identified in both blood and lymph node cell suspensions collected from the 4-wk-old minipigs and evaluated if this subset was also CD163^+. It was found that CD163 was co-expressed by 35–70% of MHCII^CD172a^- cells, regardless of matrix analyzed (Figure 8). An additional subpopulation was also investigated in the blood and lymph node cell suspension using CD209 (DC-Sign: Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin). CD209 is a C-type lectin receptor present on the surface of myeloid DC and macrophages. Here, the proportion of CD209^+ cells was found to be extremely low in both matrices (not exceeding 3% of the viable cell suspension) in cells collected from the 4-wk-old minipigs (Figure 8).

All of the above-mentioned "dendritic-like" cell and inflammatory DC cell subsets were not affected by the CsA treatment (Figures 7 and 8).

Ex vivo lymphoproliferation test

After ex-vivo ConA stimulation for 72 hr, lymphocyte cell proliferation rates were recorded as the percentage of EdU^+ cells present. This value increased to ≈ 50–60% with cells from water-treated (control) 4-wk- to 6-mo-old minipigs. As expected, CsA induced a severe reduction in this proliferation rate during the treatment period (0.9 ± 0.3% and 8.2 ± 4.5%) in 2-wk-old and 4-wk-old CsA-treated animals, respectively (Figure 9).

Immunosuppressive effects of CsA were less pronounced but still present 1 mo after end of treatment (34 ± 16% with cells from previously CsA-treated hosts compared to 53 ± 3% with cells from control PN2 animals). At 5 mo after the end of the treatment, proliferation rates were comparable between cells from the control (55 ± 2%) and previously CsA-treated (50 ± 6%) animals.

T-dependent antibody response (TDAR)

Anti-KLH IgG titers measured in adult control animals were nearly 3-times higher than those measured in young piglets (482 ± 70, 423 ± 107, and 1238 ± 110 ng/mL at PNW4, PN2, and PN6, respectively) (Figure 10). The IgG response was severely inhibited by CsA in 4-wk-old treated piglets (67 ± 7 ng/mL at PNW4). Though less pronounced, the suppressive effect of CsA was still evident in older minipigs even after a 5-month washout period (242 ± 51 and 838 ± 85 ng/mL in previously-CsA-treated animals at PN2 and PN6, respectively).

Discussion

The present study investigated the effect of an immunosuppressive treatment on the developing immune system of Göttingen minipigs. To achieve this objective, major immune cell populations in peripheral blood and in selected lymphoid organs (thymus, spleen and lymph nodes) together with an assessment of functional immune responses in piglets were analyzed. The dose level of CsA selected in the juvenile toxicology study corresponded to half of the adult dose level already tested in Göttingen minipigs (van Mierlo et al. 2013). The daily dose of 10 mg/kg/d given to the piglets by oral gavage from Postnatal Day 3 to Postnatal Week 4 induced a significant immunosuppression, but without nonspecific toxicological effects that could affect nonspecifically the immune status of the animals. Here, there were no CsA-related clinical observations and no growth retardation recorded in any of the treated piglets (data not shown). It should also be noted that the spleen of a 3-day-old piglet is in its developing stage, when the white pulp is observed along with hematopoietic activity in the red pulp. This hematopoietic tissue persists during the first few weeks after birth and then gradually disappears after weaning (Penninks et al. 2012).

The immunotoxicological endpoints selected in the juvenile minipig study here were those commonly employed and validated in other species, like the rat and cynomolgus monkey, as outlined in the "Immunotoxicity studies for Human Pharmaceuticals" ICH S8 guideline (ICH S88 2005). The recently...
finalized ICH S11 guideline "Nonclinical Safety Testing in Support of Development of Pediatric Medicines" (ICH S11 2020) does not provide any further or detailed information on developmental immune endpoints to be assessed in juvenile toxicology studies and also refers to the S8 ICH guideline issued more than 15 years ago.

Immunophenotyping by flow cytometry analysis is technically an easy endpoint that can be included in juvenile toxicology studies and also refers to the S8 ICH guideline issued more than 15 years ago.

In the study here, when compared with adult (6-mo-old) minipigs, the following main changes in immune cell proportions were noted in the peripheral blood of pre-weaned piglets (4-wk-old). CD21⁺ B-cells have been investigated in previous works from farm and miniature piglets (Solano-Aguilar et al. 2001; Sinkora and Butler 2009; Blanc et al. 2020) and were found to represent < 20% of a typical peripheral blood mononuclear cell (PBMC) suspension. Comparable percentages of CD21⁺ B-cells were seen here when using a whole blood cell suspension for flow cytometric analysis. An age-related decrease in circulating CD21⁺ B-cells has also been reported in farm pigs and in miniature pigs (Solano-Aguilar et al. 2001; Sinkora and Butler 2009; Blanc et al. 2020). In swine, CD21⁺ cells correspond to a naïve (non-stimulated) portion of B-cells, as described in an ontogeny analysis (Sinkora and Butler 2009), also in correlation with the co-expression of CD45RA for most CD21⁺ cells noted in the present study.

T-Cell numbers have been reported to account for 40% of PBMC suspensions in young piglets and to increase with age (up to ≥ 70% in adult Göttingen minipigs with inter-individual and inter-assay variations (Penninks and van Mierlo 2012; Penninks 2016). In toxicology, few immunophenotyping data are available for minipigs, including Göttingen Minipigs (Penninks and van Mierlo 2012; van Mierlo et al. 2013); such endpoints have been even more rarely evaluated in piglets (Penninks et al. 2012).
et al. 2012; van Mierlo et al. 2013). In the study here, the percentage of T-cells (CD3⁺) in whole blood was found to be 40–50% in the 4-wk-old piglets and, on the contrary, decreased to 20–40% as the animals aged. Moreover, the proportion of circulating helper T-cells (CD3⁺CD4⁺) was found to be 1.4–2.6-times higher than circulating cytotoxic T-cells (CD3⁺CD8⁺) in both piglets and adult minipigs. The difference between both populations was particularly important in 4-wk-old piglets before weaning, due to a high percentage of CD4⁺ cells (≈ 60% at PNW4 compared to < 40% at PNM2 and PNM6). A higher proportion of circulating CD4⁺ in pre-weaned piglets was also reported in pre-weaned 12-d- and 18-d-old miniature piglets (Solano-Aguilar et al. 2001) and in pre-weaned 1-d- to 4-wk-old farm piglets (Stepanova et al. 2007).

In Göttingen minipigs, the only published study available to our knowledge, did not show any relevant difference between 4-wk-old piglets and adult minipigs in their proportions of circulating CD4⁺ cells (Penninks et al. 2012). In that same study, there was no evidence of an age-related increase in circulating double-positive CD4⁺CD8⁺ population (CD4⁺CD8α⁻) in Göttingen Minipigs, though it has been reported in farm and miniature pigs in other publications (Solano-Aguilar et al. 2001; Stepanova et al. 2007; Blanc et al. 2020); this was also confirmed in the study here of Göttingen Minipigs. The proportion of peripheral CD4⁺CD8⁺ population is known to be higher in pigs than in other species (Rubic-Schneider et al. 2016). The peripheral double-positive CD4⁺CD8⁺ population is considered to represent the effector/memory T-cell pool in swine and is not related to the transitional double positive stages occurring intra-thymically for the development of T-helper and T-cytotoxic cells.

The distribution of T-cell phenotypes by flow cytometry analysis in lymphoid swine tissues is still a matter of debate. Few publications are available in pigs and in miniature pigs (Zuckermann and Gaskins 1996; Solano-Aguilar et al. 2001; Stepanova et al. 2007). In the Stepanova study, levels of CD3⁺ cells analyzed from mesenteric, tracheobronchial, and popliteal lymph nodes were comparable from birth through to adulthood. In contrast, in the present study, an age-related increased proportion of CD3⁺ cells was noted in the iliac lymph nodes. Stepanova et al. also reported an age-related increase in CD3⁺ in PBMC and spleen; this was not observed here. The discrepancies between these studies could be explained by differences in cell isolation techniques (such as lymphoid cell filtration, PBMC vs. whole blood) but also could be due to different lymph node sites and/or origin of pigs used in both studies. The percentages of CD3⁺ among the lymphoid cells and blood/PBMC suspensions were greater in the Stepanova miniature pigs (56–94%) than in the Göttingen minipigs here (26–57%). Nevertheless, mean proportions of γδ-T-cells among the CD3⁺ cells recorded in the spleen and lymph nodes in the current study were comparable with those in the Stepanova study (generally < 20%). The peripheral γδ-T-cell proportion was also comparable between both studies (< 40%) except for the adult miniature pigs in the Stepanova study that had much higher γδ-T-cell percentages (69 ± 21% from PBMC) than the Göttingen adult minipigs here (39 ± 6% from whole blood).

The NK cell populations here were characterized first by gating CD3⁻CD8⁻ and NKp46⁺ phenotypes. In contrast to other species, NKp46 was shown to divide porcine CD3⁻CD8α⁻CD16⁺ NK cells into two subsets, i.e. NKp46⁺ and NKp46⁻. NKp46⁻ NK cells show phenotypic and functional properties of NK cells although they produce lower levels of IFNγ than NKp46⁺ NK cells (Mair et al. 2012, 2013). In the current study, the proportion of CD3⁻CD8⁻ population in blood was seen to increase with age, and only a few cells co-expressed NKp46 regardless of age. In the lymph nodes, the proportion of CD3⁺CD8⁻ remained unchanged between juvenile and adult animals and the majority of this population co-expressed NKp46, particularly the 4-wk-old piglets. The percentages of circulating NK cells highly-expressing CD16 were comparable between piglets and adults, but the same subset in lymph nodes was found to be in a small proportion in the 4-wk-olds. To our knowledge, there is no published data available on NK cells in juvenile Göttingen minipigs using this phenotyping combination.

The study on myeloid immune cells, monocytes/macrophages and particularly the dendritic cells, induced over the last fifteen years a substantial increasing interest in swine vaccination development as well as its translational relevance to human vaccine research (McCullough and Summerfield 2009; Fairbairn et al. 2011; Marquet et al. 2011; Deloisy et al. 2016; Rubic-Schneider et al. 2016; Bernelin-Cottet et al. 2019). Combinations of multiple surface markers, including MHC-II, CD172a, CD163, CADM1, CD207, and CD209 were tested here as a preliminary comparative analysis across age for the “dendritic-like” cell populations in the blood and lymph nodes of the Göttingen minipigs. Swine conventional DC have been characterized in blood and tissues and compared with mouse and human counterparts (Summerfield and McCullough 2009; Maisonnasse et al. 2016; Saalmüller and Gerner 2016; Franzoni et al. 2019). As in those two species, there are two subsets of conventional DC in swine, i.e. cDC1 and cDC2. Some dermal and lymph node DC subsets also express CD207 (Bursch et al. 2007; Merad et al. 2008; Bigley et al. 2015; Clausen and Stoitzner 2015). Plasmacytoid dendritic cells (pDC) are MHC-II⁺CD172a⁺CD4⁺ (Bertho et al. 2011; Marquet et al. 2011). Another pig DC subset which is considered to correspond to a human CD14 DC was also identified as MHC-II⁺CD172a⁺CD163High (Marquet et al. 2014; Bernelin-Cottet et al. 2019). Unfortunately, the above-mentioned markers are known to be also expressed by a large panel of immune or inflammatory cells (including B-cells, neutrophils, monocytes, macrophages). In addition, in the current study, CD172a expression was variable between individuals. Therefore, specific DC types could not be distinctly defined here.

In the current study, circulating monocytes were divided into four subsets using CD14 and CD16 markers, following exclusion of lymphocyte and granulocyte populations by targeting the monocyte gate. Swine monocytes are well-characterized (Chamorro et al. 2000, 2004; Moreno et al. 2010; Blanc et al. 2020) and can be identified based upon their expression of multiple markers, including CD14 and CD16, but also specifically CD163. The swine CD163⁺ monocyte subset would resemble more mature cells such as macrophages and has been reported to share phenotypic and functional features with human CD14⁺CD16⁺ monocytes. In addition, it was shown that in vitro stimulation of monocyte differentiation into macrophages was associated with a higher expression of CD163 and a lower expression of CD14 (Chamorro et al. 2000). Here, CD163⁺ monocytes could not be specifically distinguished from CD163⁺ DC due to the variable level of expression of CD172a as mentioned above.

Oral gavage dosing of CsA at 10 mg/kg/day for 4 wk (starting at host being 3-d old) was associated with decreased proportions of CD21⁺ B-cells, MHC-II⁺ cells, and cytotoxic-T cells in the peripheral blood of 4-wk-old piglets. These CsA-related changes totally recovered 1- or 5-mo after end of the treatment, and were
not observed in the spleen, thymus or lymph nodes. The immunosuppressive effects of CsA were clearly demonstrated by the two functional tests, i.e. ex vivo lymphocyte proliferation test and TDAR test, with evidence of a severe inhibition of the mitogen-induced cell proliferation rate and of the less severely decreased anti-KLH IgG levels in CsA-treated piglets. The severe inhibition of the ex vivo lymphocyte proliferation and reduction in absolute lymphocyte counts (−42% of control values; data not shown) were noted in 4-wk-old piglets treated with CsA, whereas the mean percentage of circulating T-lymphocytes (CD3+ cells) was unaffected after CsA treatment. The reduced mean percentage of circulating CD21+ B-cells (which is limited to a subset of naïve B-cells) observed in CsA-treated piglets may be associated with the selective suppressive action of CsA on an early step of B-cell activation. Indeed, it has been described that CsA can alter naïve B-cell proliferation in humans (de Bruyne et al. 2015). This hypothesis would, however, require further investigative work to understand the mechanism of action.

The immunosuppression was still observed in both tests, though much less pronounced, 1-mo after the end of the treatment period, and in the TDAR test for the adult pigs despite a 5-mo off-treatment period. The only published immunotoxicology study performed in Göttingen minipigs (van Mierlo et al. 2013) also included ex vivo lymphocyte proliferation and TDAR tests among other functional tests. One group of 3.0–3.5-mo-old animals was treated with CsA at 20 mg/kg/day for 39/40 days. The CsA-related changes noted in that study (in young adult minipigs) were not as severe as those seen in the pre-weaned piglets here treated for 4 weeks at only half of the adult dose level. This disparity in outcomes re-emphasizes the importance of appropriate dose level selection when conducting juvenile animal toxicity studies.

Conclusions

When compared with in adult (6-mo-old) minipigs, several peripheral immune cells, such as CD21+ B-cells, αβ-T-cells, γδ-T-cells, NK cells, and monocytes were found at different proportions in 4-wk-old piglets. Expression levels of membrane phenotype markers also varied with age. Oral gavage dosing of CsA at 10 mg/kg/day for 4 wk was associated with decreased percentages of CD21+ B-cells, MHC-Ⅱ+ cells, and cytotoxic-T cells (CD8+) in the peripheral blood of 4-wk-old piglets. The immunosuppressive effect of CsA was still observed 5 mo after the end of treatment, at least in the TDAR test, whereas the immune populations affected during treatment returned to normal levels after the 4-wk (PNM2) or 5-mo (PNM6) observation period. The results from these studies emphasize the need for standard functional tests to be included in juvenile animal toxicology studies when immune endpoints need to be evaluated. These data also indicate that the minipig could be used as a relevant species for the need of translation of immune findings from animals to humans.

Acknowledgments

The Authors would like to thank Ellegaard Göttingen Minipigs A/S, Dalmose (Denmark) for their help in providing the pregnant sows allowing us to successfully conduct these studies.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The author(s) reported there is no funding associated with the work featured in this article.

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