Immunotoxicology Evaluation in the Juvenile Göttingen Minipig

Linda Allais, Fabienne Condevaux, Alicia Perbet, Alan Hoberman
Charles River Lyon - Safety Assessment, St Germain Nuelles, France

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1 INTRODUCTION

Safety evaluation of new pediatric medicines is performed by the conduct of toxicology studies using juvenile animals. The Göttingen minipig is now considered as a useful alternative non-rodent species for safety testing of pharmaceuticals. Human parallels in many features of its anatomy, physiology and biochemistry make the minipig a good model for man. For use in juvenile toxicology studies, the development of main organs or systems of the minipig still requires further characterization, the immune system being one of the main areas to be explored. Although the immune system of the adult pig has been studied, particularly in relation to different infectious diseases in pigs and xenotransplantations, the development of the immune system in juvenile pigs is still unknown or was evaluated with very limited immune developmental endpoints. There is a real need to better understand the immune system organization and response in minipig to better evaluate the toxicological effect of new pharmaceuticals in development in this species.

2 MATERIALS AND METHODS

Thirty six piglets, 18 males and 18 females, were born in our animal facilities from Göttingen sows provided by Ellegaard (Denmark) and were raised with their respective mother under optimal housing conditions until weaning, i.e. 4 weeks after birth. During the 4-week preweaning phase, 9 male and 9 female piglets were treated with Cyclosporin A (Neoral® Novartis) at the dose level of 10 mg/kg/day by the oral route (gavage) from PND (Post-Natal Day) 3 to PND 28 (figure 1). Another group of 9 males and 9 females was treated with water as negative control under the same experimental conditions as for the group treated with Cyclosporin A (CsA).

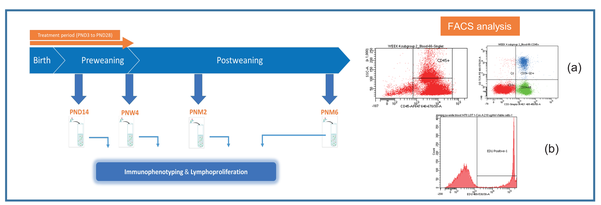

Figure 1: Experimental design: Göttingen piglets (9/sex/group) are treated by the oral route (gavage) with water or with CsA at 10 mg/kg/day from PND 3 to PND 28. Blood immunophenotyping (a) and lymphoproliferation (b) analyses were performed after the first 2 weeks of dosing (PND 14), at the end of the 4-week dosing period (PNW 4), 4 weeks after the end of the dosing period (PNM2) and 5 months after the end of the dosing period (PNM6).

After weaning, the animals were group-housed. The study design was approved by the Animal Ethical Committee and conducted in compliance with the European Animal Welfare Guidelines in an AAALAC accredited Test Facility.
On each occasion, i.e. PND 14, PNW (Post-Natal Week) 4, PNM (Post-Natal Month) 2 and PNM 6, blood samples were collected in K2-EDTA tubes for immunophenotyping or in lithium heparin tubes for the lymphoproliferation test, and then analyzed by Flow Cytometry (Fortessa, BD Biosciences). In addition, total and differential White Blood Cell (WBC) counts were analyzed for each animal (ADVIA 120).

Blood Immunophenotyping by Flow Cytometry Analysis (FACS):

Details on primary and secondary antibodies used in this study are presented in Table 1. A volume of 100 µL of whole blood was incubated for 20 minutes at +2-8°C in the dark with primary antibodies followed by secondary antibodies, where applicable. Cell Viability was assessed using the LIVE/DEAD™ Near-IR Dead Cell Stain Kit (Thermo Fisher). Following labeling, erythrocytes were lyzed and the samples were fixed before being analyzed. Washing steps were performed using Dulbecco's phosphate buffered saline (DPBS, Sigma) without calcium nor magnesium.

Phenotype	Specificity	Host Isotype	Tag	Dilution	Clone	Supplier
Primary Antibodies						
CD45	Swine Pan leucocytes	Mouse IgG1	AF647	1:10	K252.1E4	Bio-Rad
CD3ε	Swine thymocytes and T cells	Mouse IgG2b	Biotin	1:20	BB23-8E6	SouthernBiotech
LT γδ-TCR	Swine LT γδ-TCR	Rat IgG2a,κ	PE	1:20	MAC320	BD Biosciences
CD4a	Swine LT-helper	Mouse IgG2b,k	PeCy7	1:20	74-12-4	BD Biosciences
CD8a	Swine LT-cytotoxic, NK	Mouse IgG2a,k	FITC	1:20	76-2-11	BD Biosciences
CD8a	Swine LT-cytotoxic, NK	Mouse IgG2a,k	-	1:20	MIL12	Bio-Rad
CD21	Swine Mature B cells	Mouse IgG1	PE	1:10	BB6-11C9.6	SouthernBiotech
CD16	Swine neutrophils and NK cells	Mouse IgG1	PE	1:20	G7	Bio-Rad
CD335 (NKp46)	Swine NKpK6 NK cells	Mouse IgG1	APC	1:20	VIV-KM1	Bio-Rad
Secondary Antibodies						
Anti-mouse IgG2a	-	Rat	BV605	1:100	-	BD Biosciences
Streptavidine	-	-	BV421	1:100	-	Biolegend

Table 1: Primary and secondary (where applicable) antibodies used to identify immune cell subsets by FACS analysis (3 separate panels).

Lymphoproliferation test by Flow Cytometry Analysis (FACS):

All blood samples were 1/10-diluted in culture medium and incubated at 37°C for 72 hours in 48-well plates with Concanavalin A (ConA, Sigma) at a final concentration of 10 µg/mL, or with culture medium (negative control). ConA is used as a mitogen to stimulate lymphocyte proliferation. Twenty four hours before the end of incubation, 1 mM EdU (5-ethynyl-2'-deoxyuridine, thymidine analog) was added to each well to measure the newly synthesized DNA. On the day of analysis, the cells were fixed, permeabilized and incubated with the Click-iT® reaction cocktail (InVitrogen) for 30 minutes. Detection is based on a click reaction, a copper-catalyzed covalent reaction between an azide (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 (Thermo Fisher).

3 RESULTS

3.1 Immunophenotyping data – Table 2 and Figure 2

- A trend towards higher circulating mature B (CD21⁺) cells is noted in 6-month old (PNM6) minipigs when compared with 4-week old (PNW4) or 2-month old (PNM2) minipigs. On the contrary, the proportion of helper-T (CD4+) cells seems to decrease with age.
- The proportion of circulating total, differential white blood cell counts, cytotoxic-T (CD8+) cells, double CD4+CD8+ positive T-cells (memory cells), NK cells and γδ-TCR cells is generally comparable from PNW4 to PNM6 Göttingen minipigs. There were no gender differences at any age. The increase in mean cytotoxic-T cell counts noted in PNM2 control animals was influenced by two high individual values.
- Treatment with CsA at 10 mg/kg/day over 4 weeks induced lower WBC counts, associated with reduced lymphocyte and monocyte counts. These changes were no longer seen 4 weeks or 5 months after the end of the treatment period.

Total and Differential WBC count (10^9/mL)	PNW4		PNM2		PNM6	
	0 mg/kg/day Water	10 mg/kg/day CsA	0 mg/kg/day Water	10 mg/kg/day CsA	0 mg/kg/day Water	10 mg/kg/day CsA
Total WBC	7,64 ± 1,80	**4,77 ± 0,82****	9,67 ± 2,72	8,87 ± 1,25	8.65 ± 1.11	11.71 ± 7.35
Lymphocytes	5,35 ± 1,46	**3,09 ± 0,73***	7,52 ± 2,46	5,21 ± 0,98	5.80 ± 1.02	4.68 ± 0.73
Monocytes	0,25 ± 0,06	**0,14 ± 0,05***	0,23 ± 0,05	0,23 ± 0,08	0.39 ± 0.09	0.27 ± 0.07
Neutrophils	1,74 ± 0,55	1,40 ± 0,55	1,80 ± 0,56	**3,15 ± 0,24*****	2.07 ± 0.37	**6.40 ± 7.31**
Eosinophils	0,18 ± 0,14	0,08 ± 0,03	0,12 ± 0,04	0,18 ± 0,06	0.21 ± 0.12	0.23 ± 0.14
Basophils	0,07 ± 0,05	0,02 ± 0,01*	0,05 ± 0,02	0,03 ± 0,01	0.04 ± 0.01	0.04 ± 0.03

Table 2: Total and differential White Blood Cell (WBC) counts. N= 6/group/occasion.
Two-tailed t-student test (*** $p \leq 0,0001$ ** $p \leq 0,01$; * $p \leq 0,05$).

- The decrease in lymphocyte counts noted in the CsA-treated group correlated with lower mature B cells, helper-T and cytotoxic-T cells. The changes in immune cell subsets were no longer seen or were no longer statistically significant between the previously treated water- and CsA-groups, 4 weeks or 5 months after the end of the treatment period.
- NK and γδ-TCR cell counts were not affected by treatment with CsA at the dose level of 10 mg/kg/day in juvenile Göttingen minipigs.
- Inter-individual variations were particularly noted for neutrophils and CD8+ and γδ-TCR lymphocytes.

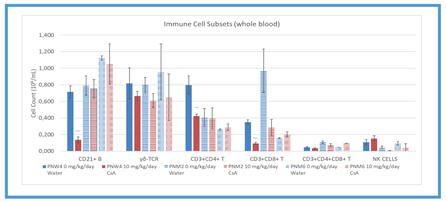

Figure 2: CD21+ cells (mature B cells), γδ-TCR cells, CD4⁺ cells (T-helper cell), CD8⁺ (T-cytotoxic cells), double C4⁺CD8⁺ T cells (memory T cells) and NK cells. N= 6/group/occasion.
Two-tailed t-student test (*** $p \leq 0,0001$ ** $p \leq 0,01$).

3.2 Lymphoproliferation test – Figure 3

- A marked reduction ($p \leq 0.001$) in proliferative responses to Con A stimulation was observed in juvenile Göttingen minipigs treated orally with CsA at 10 mg/kg/day over 2 and 4 weeks. There was no evidence of gender difference in lymphocyte proliferation in either group.
- The reduction in proliferative response was still statistically significant ($p \leq 0.05$) four weeks after the end of the treatment period (PNM2) but was no longer evident after a further four months (PNM6 minipigs).

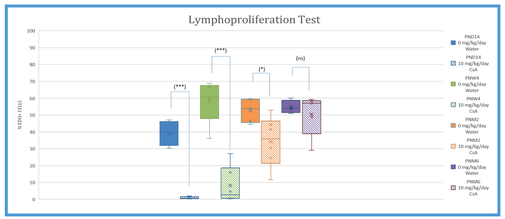

Figure 3: Proliferative response to ex-vivo ConA stimulation (10 µg/mL) after 72-hour incubation at 37°C. N= 6/group/occasion.
Two-tailed t-student test (*** $p \leq 0,0001$; * $p \leq 0,05$).

4 CONCLUSIONS

A trend towards higher peripheral mature B cell counts was observed in 6-month old minipigs when compared with younger animals, whereas circulating helper-T cell proportion was lower in 2- and 6-month old minipigs than in 4-week old piglets. The proportion of other main peripheral immune cell populations and the lymphoproliferation response (to ConA stimulation) were comparable between age (2-week, 4-week, 2-month and 6 month old minipigs) and between males and females.

The immunosuppressive effect of cyclosporin A (CsA) was demonstrated in 2-week and 4-week old juvenile Göttingen minipigs, as shown by reduced white blood cell counts, associated mainly with lower mature B cells, helper-T and cytotoxic-T cells, as well as a significant reduction in proliferative response to ConA stimulation.

CsA-related changes in immune cell counts were no longer seen or were no longer statistically significant, 4 weeks or 5 months after the end of the treatment period (i.e. 2- and 6-month old minipigs). The proliferative response to ConA stimulation was still reduced in previously treated CsA-minipigs despite the 4-week treatment-free period, although much less pronounced than during treatment.

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The authors have no conflict of interest to declare.