Validation of three flow cytometry panels for blood cell subpopulation analyses in Göttingen Minipigs.

M. Carrière[1], M-L. Hamel[1], T. Rubic-Schneider[2]; P. Schiønning[3]; H.D. Pedersen[4], R. Forster[1]; P. Ancian[1]

1 INTRODUCTION

The Göttingen Minipig is generating growing interest as a non-rodent alternative animal model for the immunological safety evaluation of drug candidates. New specific immunological tools, such as immunophenotyping on peripheral blood, are thus required. For this purpose, three flow cytometry-based methods were developed and validated for evaluation of the following cell subpopulations: total, cytotoxic and helper T cells, CD4+/CD8+ double positive T cells, regulatory T cells and natural killer (NK) cells (+/- activation marker) (panel 1: CD3, CD4, CD8a, CD25, FoxP3, CD335), B cells (panel 2: CD3, CD21, CD79a, SLA-DR) as well as mature and immature monocytes (panel 3: CD14, CD172a, CD163, SLA-DR).

After antibody titration, samples were analyzed using the Miltenyi MACSQuant® Analyzer 10. Results were expressed both as relative and absolute counts, taking into account the hematology results determined using an ADVIA® 120 System. Precision (within- and between-run), sample stability before/after staining, and carry-over were evaluated.

Once the three methods had been successfully validated for their intended use, they were used to analyze samples from a toxicology study control group. We present herein the data from the validation study and from the four control-male Göttingen Minipigs used in the toxicology study.

2 MATERIALS AND METHODS

Based on a combination of specific markers, the following lymphocyte subpopulations were assessed by Flow Cytometry in whole blood of minipigs

- Panel 1: T and NK cells

Subpopulation	Markers
T Cells	CD3+
Cytotoxic T Cells	CD3+/CD8a+
Helper T Cells	CD3+/CD4+
Double positive T cells	CD3+/CD4+/CD8a+
Activated T cells	CD3+/CD4+/CD25+ CD3+/CD8a+/CD25+ CD3+/CD4+/CD8a+/CD25+
Regulatory T Cells	CD3+/CD4+/CD25+/FoxP3+
NK Cells	CD3-/CD335+

- Panel 2: B cells

Subpopulation	Markers
B Cells	CD3-/CD21+/CD79a+/SLA-Class II DR+

- Panel 3: mature and immature monocytes

Subpopulation	Markers
Myeloid cells	CD14+/ CD172a+
Immature monocytes	CD14+/ CD172a+/ CD163-/SLA-Class II DR+
Mature monocytes	CD14+/ CD172a+/ CD163+/SLA-Class II DR+

In order to validate the method before use, blood samples were collected from stock minipigs at the Danish site. They were received within 24 hours at the Evreux French site and used for immunophenotyping by flow cytometry and the determination of lymphocyte absolute counts (in G/L; 10^9 L) using the ADVIA 120 Hematology System.

Results were expressed as:
- *Relative count (%)*
- *absolute counts(cells/µL)*:
  % subset among lymphocytes x lymphocyte counts (G/L) x 1000

The following assay performance parameters were evaluated:

- **Within-run precision** (5 replicates / 3 animals),
- **Between-run precision** (1 replicate / 3 animals / 3 runs)
- **Sample Stability** (1 replicate / 3 animals): blood samples analyzed just after receipt represented the reference time (T0):
  Recovery = (% at x hours / % at Tref) x 100.
  - **pre-staining**: samples kept at room temperature (protected from light) during different storage periods before staining
  - **post-staining**: samples stained immediately upon receipt, and kept at +4° C (protected from light) during different storage periods before acquisition
- **Carry over** was evaluated by acquiring a PBS sample just after a stained blood sample.

After validation of the method, it was used for analysis of samples from Göttingen Minipigs in a toxicology study.

3 RESULTS

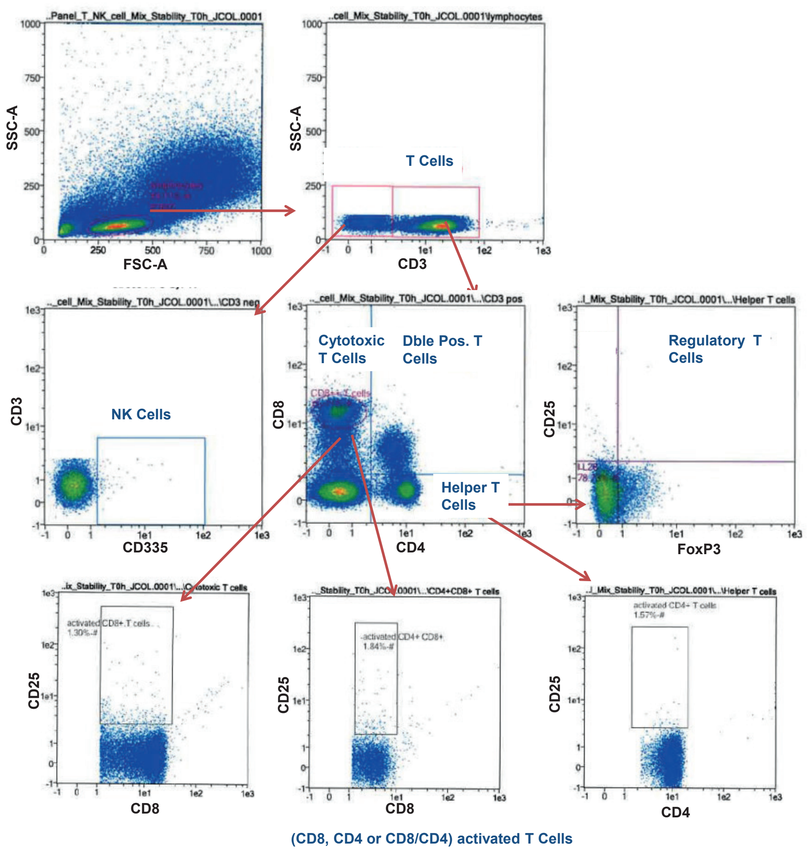

Figure 1. Representative example of plots for gating strategy of Panel 1: T-NK cells

Validation parameter	*Acceptance criteria*	*Results Panel 1: T-NK cells*	*Results Panel 2: B cells*	*Results Panel 3: mature and immature monocytes*
Blood sample reception	-	Within 24-h post collection	Within 24h post-collection	Within 24h post-collection
Within run precision	*% CV <15%*	% CV = **[0.57%; 10.94%]** in relative counts	% CV = **2.04%** in relative counts	% CV = **[7.28%; 17.54%]** in relative counts
Between run precision	*% CV <30%*	% CV = **[0.62%; 21.28%]** in relative counts	% CV = **3.30%** in relative counts	% CV= **[8.70%; 9.56%]** in relative counts
		% CV = **[4.65%; 15.86%]** in absolute counts	% CV = **4.17%** in absolute counts	% CV = **[9.50%; 15.09%]** in absolute counts
Carry over	*<1%*	No significant inter-sample contamination (mean of 0.011%)	No significant inter-sample contamination (mean of 0.008%)	No inter-sample contamination (mean of 0.063%)
Pre-staining stability	*%recovery = [70%; 130%]*	Up to **9-h at room temperature** *(with a particular attention for activated cytotoxic T cells; activated CD4+CD8+ T cells and Regulatory T cells in absolute counts)*	Up to **22h at room temperature**	Up to **4h at room temperature**
Post-staining stability		No storage after processing	Up to **22h at +4°C**	Up to **22h at +4°C**
ADVIA 120 stability		Up to **22h at room temperature**	Up to **22h at room temperature**	Up to **22h at room temperature**

Figure 2. Validation results summary (Panel 1; 2 & 3)

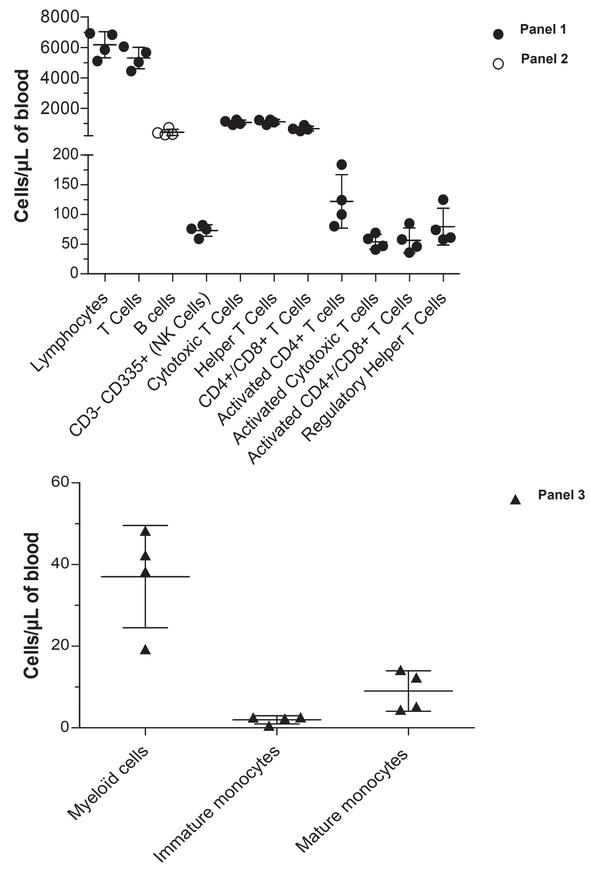

Figure 3. Inter-individual variability: Mean and SD of absolute counts: Results from 4 male Göttingen SPF minipigs from a toxicology study control group. The animals were 15 weeks old on arrival and weighed 6 to 8 kg. They were kept for a 3-week acclimation period before use. Each individual dot represents the mean of 4 different time points (Days 6 to 14) in the same animal.

4 CONCLUSION

The results obtained in the validation experiments demonstrate that the method used for the three immunophenotyping panels is valid for the intended use of the assay in whole blood from Göttingen Minipigs.

- Within- and between-run coefficient of variation (CV%) ranged from 0.57% to 17.54% and from 0.62% to 21.28%, respectively.
- Blood samples for stability assessment can be kept for up to:
  - 9, 22 and 4 hours at room temperature and protected from light after receipt (within 24h post-collection) before staining for panels 1, 2 and 3, respectively. For panel 1, particular attention should be paid to the interpretation of activated cytotoxic T cells; activated CD4+CD8+ T cells and Regulatory T cells in absolute counts.
  - 0, 22 and 22 hours at +4C and protected from light post-staining and before acquisition for panels 1, 2 and 3, respectively.
- No inter-sample contamination was found using this method.

Specific worksheets describing the method step-by-step were written at the end of this validation study to constitute the operating procedure of this method, which was then used to analyze samples from male Göttingen Minipigs in a toxicology study. The analysis results from the control group in the toxicology study provided us with initial data for the future determination of reference ranges for the different subsets of interest in Göttingen Minipigs

1 Charles River, Evreux (Citoxlab France)
2 Novartis AG, Switzerland
3 Charles River, Lille Skensved (Citoxlab Denmark)
4 Ellegaard Göttingen Minipigs A/S, Denmark