CiToxLAB
Safety and Health Research Laboratories

TOXICOLOGY SERVICES

- General toxicology:
  - Rodents
  - Non-rodents: dogs, NHPs and minipigs
- Infusion
- Inhalation
- Dermal
- Ocular
- Immunotoxicology
- Reproductive toxicology including minipigs and NHPs
- Carcinogenicity studies also in rasH2 and p53+/- mice
- Genetic toxicology: ICH compliant package
- *In vitro* toxicology: BCOP, MUSST, DPRA, Photo 3T3, Episkin™
- Agrochemical / Chemical / REACH
- QSAR
- Physical chemistry
- Ecotoxicology: wide range of test species

SAFETY PHARMACOLOGY

- Integrated Safety Pharmacology in Toxicology Studies
  - CV (JET), BP
  - Respiratory (JET), plethysmography
  - CNS (FOB) and JET-EEG
- Safety pharmacology core battery
- Early safety pharmacology screening
  - hERG
  - Rodent and non-rodent LVP telemetry
  - Anesthetized models: ECG, ABP, LVP and QA

DMPK AND BIOMARKERS

- Radiolabelled DMPK: in all species
- Bioanalysis LC-MS/MS, GC-MS/MS, LC-ICP/MS, ELISA, RIA
- Toxicogenomics, miRNA: Affymetrix™ Accredited service provider, Next Generation Sequencing (Illumina®)
- Immunology: 10-color flow cytometer, Luminex, Mesoscale

SPECIALIZED EXPERTISE

- Juvenile studies including minipigs
- Fertility studies in rodents and NHPs
- Radiation safety and efficacy studies
- Tissue Cross Reactivity: human and animal tissue banks
- Gene therapy vector biodistribution via qPCR
- ES cell testing: devTOX™ and cardioTOX™ (with Stemina)
- Lead optimization and predictive toxicology services: Leadscreen™

CiToxLAB GROUP COMPANIES

CiToxLAB in France
+33 (0)2 32 29 26 26
contact.france@citoxlab.com
B.P. 563 - 27005 Evreux Cedex, France

CiToxLAB in North-America
+1 888 353 2240
contact.northamerica@citoxlab.com
445, Armand-Frappier Blvd,
Laval, Quebec, H7V 4B3, Canada

CiToxLAB in Denmark
+45 56 86 15 00
contact.scantox@citoxlab.com
Hestehavevej 36A, Ejby,
DK-4623 Lille Skensved, Denmark

CiToxLAB in Hungary
+ 36 88 545-300
contact.hungary@citoxlab.com
Veszprém, Szabadságpuszta,
8200, Hungary

Atlanbio
+33 (0)2 51 10 01 00
atlanbio@atlanbio.com
www.atlanbio.com
1 Rue Graham Bell - Z.I de Brais
B.P 40309,
44605 Saint Nazaire Cedex, France

ALSO REPRESENTED BY

Media Services Ltd
+81 3 3666 9915
citoxlab@mediaservices-jp.com
Fuji 16 Bldg 7F
1-11-2 Nihonbashi Kayabacho,
Chuo-ku, Tokyo 103-0025, Japan

Croen Research Inc.
+82 31 888 9390
customerservice@croen.co.kr
Advanced Institutes of Convergence
Technology - B-6th Fl., 864-1, lui-dong,
Yeongtong-gu, Suwon-si - Gyeonggi-do,
443-270, Korea

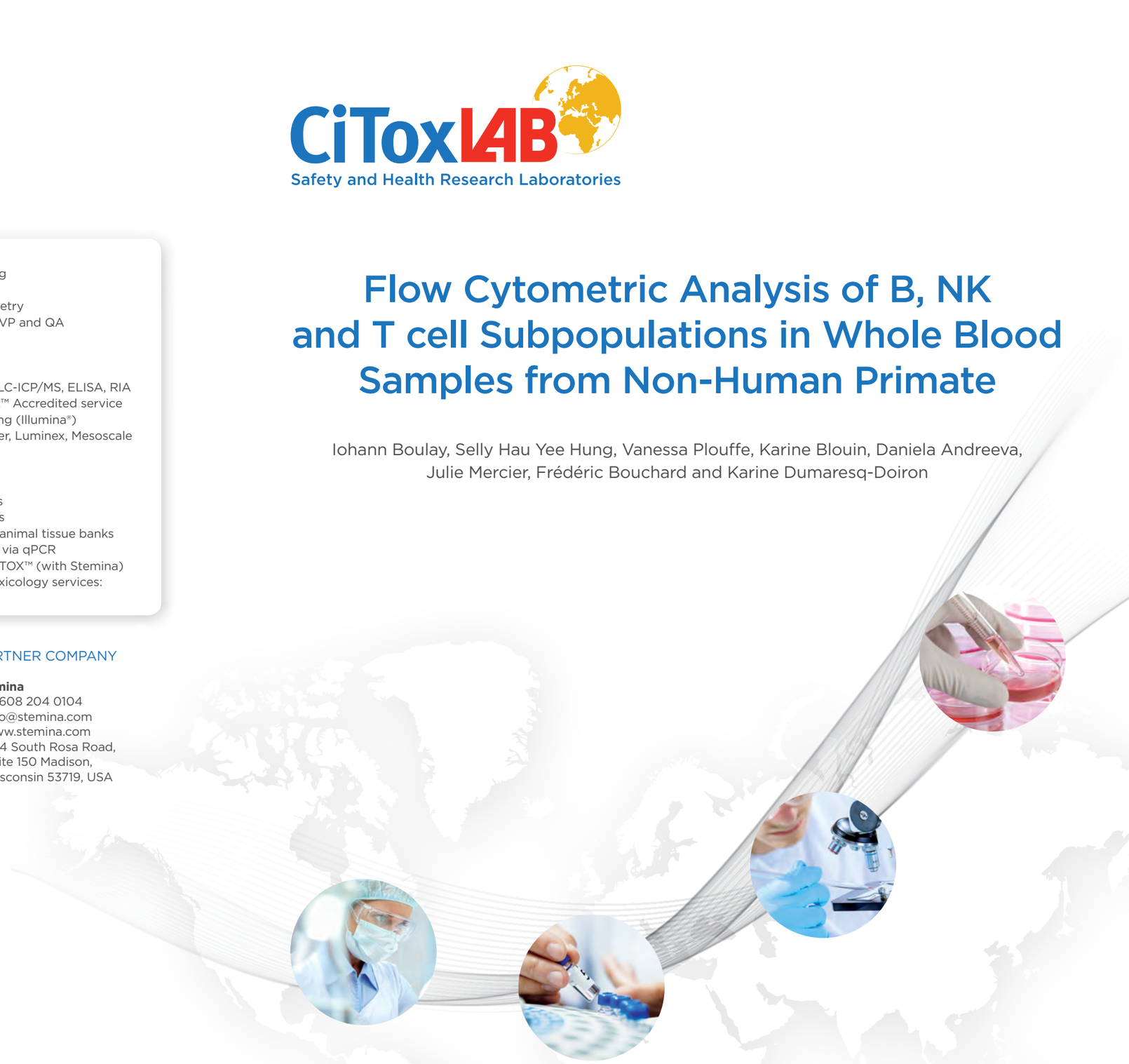
PARTNER COMPANY

Stemina
+1 608 204 0104
info@stemina.com
www.stemina.com
504 South Rosa Road,
Suite 150 Madison,
Wisconsin 53719, USA

CiToxLAB
Safety and Health Research Laboratories

Flow Cytometric Analysis of B, NK and T cell Subpopulations in Whole Blood Samples from Non-Human Primate

Iohann Boulay, Selly Hau Yee Hung, Vanessa Plouffe, Karine Blouin, Daniela Andreeva, Julie Mercier, Frédéric Bouchard and Karine Dumaresq-Doiron



aaalac INTERNATIONAL

GLP CERTIFIED

atlanbio BIOANALYSIS EXPERTISE

Stemina BIOMARKER DISCOVERY

www.citoxlab.com

M1058

Flow Cytometric Analysis of B, NK and T cell Subpopulations in Whole Blood Samples from Non-Human Primate

Iohann Boulay, Selly Hau Yee Hung, Vanessa Plouffe, Karine Blouin, Daniela Andreeva, Julie Mercier, Frédéric Bouchard and Karine Dumaresq-Doiron

Immunology Department, CiToxLAB North America, Laval, Quebec, Canada

PURPOSE

While comprehensive assessment of the immune system health often involves the evaluation of major lymphocyte cell lineages (B, NK and T cells), the advent of modern flow cytometry now also allows more in-depth analysis of these cell subsets. In support of toxicology studies, two staining panels were developed and qualified to monitor naïve (TN), effector memory (TEM) and central memory (TCM) T cytotoxic and helper cells, as well as B and NK cells, in Non-Human Primate peripheral whole blood.

MATERIAL AND METHODS

Table 1. Material and Methods

	Cynomolgus Monkey Panel	Rhesus Monkey Panel
Procedure	Whole blood samples stained with the Lyse/Wash procedure	
Anticoagulant	Sodium Heparin	Lithium Heparin
Antibodies	Anti-CD3 PE-Cy7	Anti-CD3 Alexa Fluor 488
	Anti-CD4 PerCP-Cy5.5	Anti-CD4 APC-eFluor 780
	Anti-CD8 APC	Anti-CD8 APC
	Anti-CD16 PE	Anti-CD16 PE
	Anti-CD28 BV421	Anti-CD28 V450
	Anti-CD95 BB515	Anti-CD95 PE-Cy7
	Anti-CD20 BV510	Anti-CD45 BV510
Viability Stain	Fixable Viability Dye eFluor 780	Annexin V PerCP-Cy5.5
Acquisition Setting	Acquisition Stop set at 20 000 lymphocytes	
Instrument	BD Biosciences FACSVerse with integrated flow sensor	

Fluorochrome selection was based on instrument configuration as well as surface marker expression to minimize spillover and maximize resolution. The use of the built-in flow sensor allowed the establishment of absolute cell counts in a simplified single-platform manner, avoiding the use of counting beads with the samples.

Table 2. Lymphocyte Subsets Phenotype

Lymphocyte Subset	Abbreviation	Phenotype
B cells (Cynomolgus monkey only)	B	**CD20+** CD3-
NK cells	NK	**CD16+** CD3-
NK CD8+ cells	CD8+ NK	**CD16+** CD3- **CD8+**
T cells	T	**CD3+** CD16-
Cytotoxic T cells	Tc	**CD3+** CD16- **CD8+** CD4-
Naïve Cytotoxic T cells	Tc N	**CD3+** CD16- **CD8+** CD4- **CD28+** CD95-
Central Memory Cytotoxic T cells	Tc CM	**CD3+** CD16- **CD8+** CD4- **CD28+ CD95+**
Effector Memory Cytotoxic T cells	Tc EM	**CD3+** CD16- **CD8+** CD4- CD28- **CD95$^{low/neg}$**
Helper T cells	Th	**CD3+** CD16- **CD4+** CD8-
Naïve Helper T cells	Th N	**CD3+** CD16- **CD4+** CD8- **CD28+** CD95-
Central Memory Helper T cells	Th CM	**CD3+** CD16- **CD4+** CD8- **CD28+ CD95+**
Effector Memory Helper T cells	Th EM	**CD3+** CD16- **CD4+** CD8- CD28- **CD95$^{low/neg}$**

Lymphocytes were identified by their size and granularity. Dead cells and doublets were excluded from analysis. When used in the combo, CD45 negative events were also excluded from analysis.

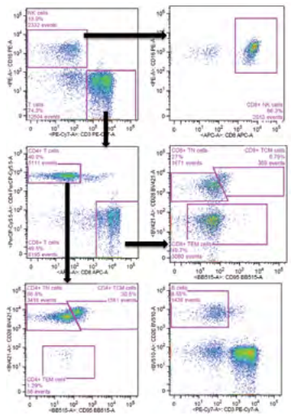


Figure 1: Gating Strategy – Cynomolgus Monkey Panel

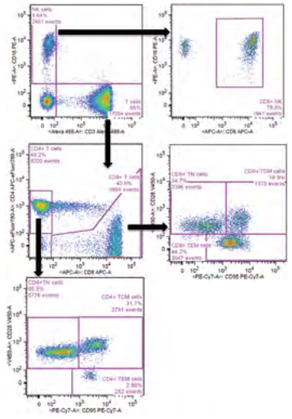


Figure 2: Gating Strategy – Rhesus Monkey Panel

ASSAY QUALIFICATION

Interaction Between Reagents: Because the selected reagents are used in combination, verification that an antibody recognizes the same cells, in the same proportion, whether it is used alone or in combination was performed.

Table 3. Cynomolgus Monkey Panel: Interaction Between Reagents

		Proportion in Lymphocytes (%gated in lymphocytes)									
		Ind. 1								Ind. 2	Ind. 3
		CD16+	CD4+	CD3+	CD8+	FVD-	CD28+	CD20+	CD95+		
Alone	Mean (n=2)	33.7	29.7	59.6	56.3	98.0	46.8	3.08	16.0	21.5	19.5
Combination	Mean (n=5)	36.6	27.4	58.1	58.7	96.7	45.8	3.39	13.7	20.3	16.4
	% Difference	**8.4**	**-7.7**	**-2.4**	**4.3**	**-1.4**	**-2.1**	**9.6**	**-14.9**	**-5.7**	**-17.3**
		Absolute Cell Count (x10^6 cells/L)									
		Ind. 1								Ind. 2	Ind. 3
		CD16+	CD4+	CD3+	CD8+	FVD-	CD28+	CD20+	CD95+		
Alone	Mean (n=2)	1425	962	2335	2241	3509	1918	114.9	644.4	816.3	871.6
Combination	Mean (n=5)	1341	1003	2127	2149	3537	1678	124.5	503.5	766.7	702.3
	% Difference	**-6.1**	**4.3**	**-9.3**	**-4.2**	**0.8**	**-13.4**	**8.0**	**-24.6**	**-6.3**	**-21.5**

Table 4. Rhesus Monkey Panel: Interaction Between Reagents

		Proportion in Lymphocytes (%gated in lymphocytes)							
		Ind. 1							
		CD16+	CD4+	CD3+	CD8+	Annexin V-	CD28+	CD45+	CD95+
Alone	Mean (n=2)	10.9	43.5	72.6	44.9	98.9	63.2	99.8	73.8
Combination	Mean (n=5)	10.5	44.4	73.3	44.3	97.7	64.1	99.7	76.6
	% Difference	**-3.4**	**2.0**	**1.0**	**-1.3**	**-1.2**	**1.4**	**-0.1**	**3.8**
		Absolute Cell Count (x10^6 cells/L)							
		Ind. 1							
		CD16+	CD4+	CD3+	CD8+	FVD-	CD28+	CD20+	CD95+
Alone	Mean (n=2)	620.7	2615	4126	2685	5591	3791	5548	4155
Combination	Mean (n=5)	532.2	2236	3697	2231	4924	3231	5024	3861
	% Difference	**-15.3**	**-15.6**	**-11.0**	**-18.5**	**-12.7**	**-15.9**	**-9.9**	**-7.3**

Minimal interaction between the different antibodies and the viability dyes were detected. The CD95+ cell counts in the Cynomolgus Monkey panel is slightly underestimated in the combo staining yet, not in all individuals tested.

Precision: To evaluate precision, 5 independent multicolor staining replicates were performed and the closeness of the individual measures was evaluated.

Table 5. Cynomolgus Monkey Panel: Inter-tube Precision Results

	Proportion in Lymphocytes (%gated in lymphocytes)												
	Live	B	NK	CD8+ NK	T	Tc	Tc N	Tc CM	Tc EM	Th	Th N	Th CM	Th EM
Mean (n=5)	96.4	2.14	35.2	25.4	54.7	22.9	10.1	2.35	6.59	25.3	11.4	9.79	0.348
St. Dev.	1.6	0.36	0.7	0.6	1.4	0.9	0.8	0.09	0.56	0.6	0.9	0.30	0.058
% CV	**1.6**	**17.0**	**2.0**	**2.4**	**2.5**	**3.8**	**8.1**	**3.9**	**8.6**	**2.3**	**8.2**	**3.1**	**16.7**
	Absolute Cell Count (x10^6 cells/L)												
	Live	B	NK	CD8+ NK	T	Tc	Tc N	Tc CM	Tc EM	Th	Th N	Th CM	Th EM
Mean (n=5)	3583	79.85	1310	944.5	2034	849.3	373.7	87.33	244.8	942.4	424.4	364.0	12.90
St. Dev.	172	16.97	71	57.0	91	36.9	25.1	5.78	15.8	49.6	32.0	20.4	2.20
% CV	**4.8**	**21.2**	**5.4**	**6.0**	**4.5**	**4.3**	**6.7**	**6.6**	**6.5**	**5.3**	**7.5**	**5.6**	**16.9**

Table 6. Rhesus Monkey Panel: Inter-tube Precision Results

	Proportion in Lymphocytes (%gated in lymphocytes)												
	Live	CD45+	NK	CD8+ NK	T	Tc	Tc N	Tc CM	Tc EM	Th	Th N	Th CM	Th EM
Mean (n=5)	96.8	99.6	8.97	8.84	73.5	26.7	13.1	4.93	8.55	41.7	34.3	7.13	0.169
St. Dev.	0.5	0.1	0.41	0.40	0.5	0.2	0.3	0.14	0.19	0.4	0.5	0.18	0.016
% CV	**0.5**	**0.1**	**4.5**	**4.5**	**0.7**	**0.6**	**2.0**	**2.9**	**2.2**	**1.0**	**1.4**	**2.5**	**9.5**
	Absolute Cell Count (x10^6 cells/L)												
	Live	B	NK	CD8+ NK	T	Tc	Tc N	Tc CM	Tc EM	Th	Th N	Th CM	Th EM
Mean (n=5)	5216	4833	434.8	427.9	3564	1297	635.4	238.8	414.6	2023	1666	354.7	8.20
St. Dev.	401	223	10.3	10.8	178	57.6	28.9	12.0	22.5	108	94	32.9	0.80
% CV	**7.7**	**4.6**	**2.4**	**2.5**	**5.0**	**4.4**	**4.6**	**5.0**	**5.4**	**5.3**	**5.6**	**9.3**	**9.8**

The method presented excellent precision except for some cell populations of small size. The variation between the measurements for B cells and Th EM cells were slightly higher in the Cynomolgus Monkey panel, yet acceptable.

Sample Stability: Sample stability was assessed by subjecting whole blood samples to various storage time period. Upon collection, whole blood samples were stored at room temperature with orbital shaking. Upon staining procedure, stained samples were stored at room temperature and protected from light. Acceptable sample stability was observed as follow.

Condition	Cynomolgous Monkey Panel (n=3)	Rhesus Monkey Panel (n=3)
Upon Blood Collection	Up to 1 hour	Up to 24 hours
Upon Staining Procedure	Up to 3 hours	Up to 1 hour

Upon storage, the recovery of the CD4+ TEM cells was relatively high compared to the fresh sample, yet considered acceptable due to its small population size.

REFERENCES

1. Pitcher C.J. et al. *J of Immunology*, 2002; 128: 29-43 - 2. Nadazdin O. et al. *Am J Transplant*. 2010; 10(6): 1375-1384 - 3. Mothy M. *Leukemia*. 2007; 21: 1387-1394.

RESULTS

To further characterize the performance of the assay, whole blood samples were obtained from immunocompromised (Thymoglobulin (ATG)-treated) Cynomolgus monkeys or immunocompromised and myeloablated Rhesus monkeys. The various cell populations were monitored to assess the extent of immunosuppression and recovery profiles.

The Immunosuppresive Model in Cynomolgus Monkey: While a single ATG administration did not have a profound effect on the various cell populations, T cell populations, especially the Th EM and Tc EM populations, seemed to be most affected by the ATG treatment. All T cell populations were able to recover to baseline population size during the course of the study.

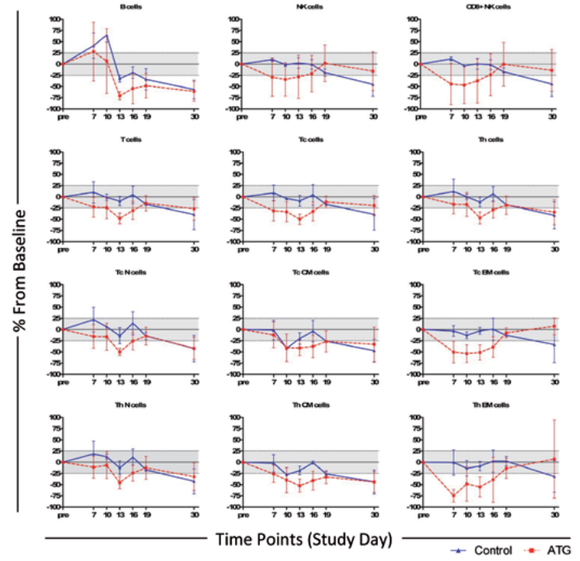


Figure 3: Population Kinetics – Cynomolgus Monkey

The Immunosuppresive and Myeloablative Model in Rhesus Monkey: Extensive decrease was observed in the size of the various populations after myeloablation. The dual treatment of the myeloablation and immunosuppression further exacerbated the decrease. Only T cells were able to recover fully from the myeloablative treatment even after an observation period of 30 days. The recovery observed in T cell population was mainly driven by the over-recovery of Tc cells, more specifically Tc EM cells. Yet, it is also interesting to note the important recovery of the central memory T cells, in contrast to the naïve T cells compartment.

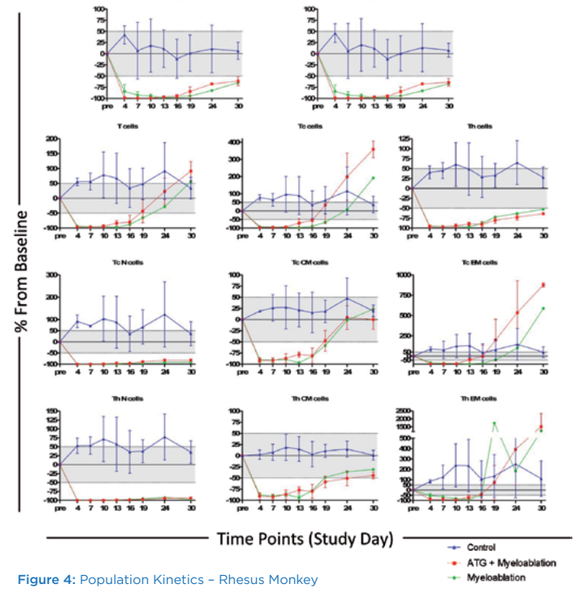


Figure 4: Population Kinetics – Rhesus Monkey

CONCLUSION

Overall, the flow cytometry methodology described herein was found to be suitable for the exploration of the population kinetics of various T cell subsets, as well as NK and B cells. The current panels allow characterization of the major contributors in the depletion/recovery phase of the lymphocyte population in more depth. The methodology is a single-platform without the need of counting beads. Furthermore, it possesses potential precision improvements over other single-platform methods requiring the spiking of counting beads and two-platform methods requiring a second analysis of the sample to obtain absolute count measurements.