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# Determination of Normal: Flow Cytometry Analysis of Major Immune Cell Populations in Peripheral Blood of Naïve Cynomolgus Monkeys

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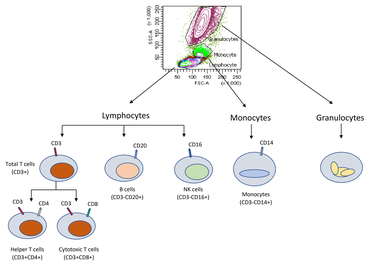
## ABSTRACT

Changes in immune cell populations in the peripheral blood of cynomolgus monkeys, as determined by flow cytometry, are routinely used as an immunological endpoint in toxicology studies. To determine what constitutes a "normal" value for immune cell populations in the peripheral blood, we analyzed large cohorts of naïve adolescent (males: n=84, females: n=84) and infant (n=33) cynomolgus monkeys. We determined relative percentage and absolute counts of immune cell populations, such as CD3+ total T cells, CD3+CD4+ T cells, CD3+CD8+ T cells, CD3-CD20+ B cells, CD3-CD16+ NK cells, and CD3-CD14+ monocytes. The number of total T cells were 3.06±1.25, 3.24±1.35, 2.88±1.12, 4.97±1.55 x 106 per mL, number of B cells were 1.34±0.75, 1.45±0.90, 1.23±0.56, 1.85±1.01x 106 per mL, the number of NK cells were 7.59±4.24, 8.56±4.44, 6.63±3.81, 4.48±1.94 x 105 per mL, and the number of monocytes were 2.48±1.09, 2.46±1.22, 2.50±0.94, 4.44±2.08 x 105 per mL for total adolescent both sexes combined, adolescent male, adolescent female, and infant cynomolgus monkeys, respectively. Our results indicated significant differences based on age and sex. Infants had higher counts of total lymphocytes, monocytes, total T cells, CD4+ T cells, and B cells compared to adolescents (p<0.005, Mann Whitney test) and lower absolute counts of NK cells (p<0.0001, Mann Whitney test). We also observed sex-dependent differences where females had lower counts of CD3-CD16+ NK cells compared to males (p<0.001, Mann Whitney test). Overall, these reported values serve as a guideline for improved understanding of the various toxicology related changes introduced by therapeutic treatments during drug development.

## INTRODUCTION

Major immune cell populations in whole blood consist of lymphocytes, monocytes, and granulocytes. Significant changes in these populations in response to test article administration can signal a serious safety issue. As a result, these populations and their subpopulations are frequently identified and monitored during preclinical toxicology testing using flow cytometry. Subpopulations of lymphocytes include T cells, helper T cells, cytotoxic T cells, B cells, and NK cells. T cells can be identified by presence of CD3 receptor on the cell membrane. Helper T cells are a subset of the T cell population, responsible for activating the humoral immune response. They are identified by the presence of CD4 and CD3 receptors on the cell surface. Cytotoxic T cells, identified by expression of CD8 and CD3 on the cell surface, carry out targeted destruction of tumor and infected cells. NK cells, identified by the presence of CD16 and absence of CD3, are part of the innate immune response responsible for nonspecific clearance of tumor and infected cells. B cells, identified by the presence of CD20 and absence of CD3 on the cell surface, produce antibodies against foreign pathogens. CD14-expressing monocytes carry out phagocytosis, antigen presentation, and cytokine production. Despite their routine monitoring during safety assessment, little information is available on what constitutes a normal baseline reading for these populations.

Here, we leverage a large number of datasets collected from 168 adolescent and 33 infant cynomolgus monkeys animals to compile a baseline level of major immune cell populations in the whole blood. We also report various gender and age-specific differences observed in these populations.




**Figures 1.** Overview of major immune cell populations in peripheral blood of cynomolgus monkeys detected using flow cytometry.

Three major immune populations of peripheral blood, lymphocytes, monocytes, and granulocytes can be detected using flow cytometry, due to the differences in size and granularity. These populations can be further divided into subpopulations of expression cell-specific markers that can be detected using flow cytometry. T cells express CD3; helper T cells express both CD3 and CD4; and cytotoxic T cells express CD3 and CD8. B cells and NK cells express CD20 and CD16 respectively, while lacking CD3 expression. Monocytes can be identified based on CD14 expression.

## METHODS

**Specimens and Reagents**

- 168 adolescent cynomolgus monkeys (84 males, 84 females), naïve, aged between 2 to 5 years
- 33 infant cynomolgus monkeys, naïve, aged between 26 to 30 postnatal days
- Anticoagulant: K2 EDTA

**Assay Procedure**

Whole blood (0.1 mL) from cynomolgus monkeys was mixed and added to the immunophenotyping antibodies against CD3, CD4, CD8, CD20, CD16, and CD14. The samples were then incubated in the dark at ambient temperature (AT) for 15 to 20 minutes. Leukocytes were isolated by whole blood lysis using 1X BD FACS lysing solution. Samples were incubated at AT for 5 to 12 minutes followed by centrifugation (500 x g). Cells were washed once by adding 1X calcium- and magnesium-free phosphate buffered saline (1X PBS), resuspended in 1X PBS, and analyzed on the cytometer.

The following antibody panel was used:

| Tube Number | FITC | PE | APC | PerCP-Cy5.5 | PE-Cy7 | APC-Cy7 |
|---|---|---|---|---|---|---|
| 1 | $IgG_{2a}$ | $IgG_{2a}$ | $IgG_1$ | $IgG_{2b}$ | $IgG_1$ | CD3 |
| 2 | CD4 | CD14 | CD16 | CD20 | CD8 | CD3 |

**Data Acquisition**

Data acquisition was performed using a BD FACSCanto™ II cytometer equipped with BD FACSDiva™ (version 6) acquisition software. Appropriate filter sets and mirrors were used to capture FITC, PE, APC, PerCP-Cy5.5, Pe-CY7, and APC-Cy7 fluorescence. Flow cytometer qualification was performed prior to each analysis using cytometer setup and tracking beads obtained from BD Biosciences. Fluorescence compensation was conducted automatically using stained eBioscience UltraComp™ beads and FACSDiva™ software.

**Data Analysis**

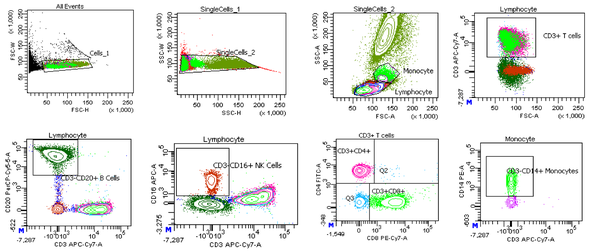
Data collected on the cytometer was analyzed using BD FACSDiva™ software (version 6) to measure relative percentages from the lymphocyte and monocyte gate. Relative percentages of T cells, CD4+ T cells, CD8+ T cells, B cells, NK Cells, and monocytes were obtained from the percent values present in FACSDiva™ statistics table. Total numbers of T cells, CD4+ T cells, CD8+ T cells, B cells, NK Cells, and monocytes were calculated using relative percent values and lymphocytes and monocyte counts obtained from Advia-180 hematology analyzer.

**Statistical Analysis**

Statistical analysis to test for significance was performed using Mann Whitney test in Prism GraphPad. Mean and standard deviation calculations were performed in Microsoft Excel.

## RESULTS

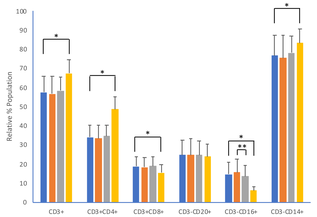
**Identification of T, Helper T, Cytotoxic T, B, NK, and Monocyte Cell Populations**




**Figures 2.** Gating strategy for detection of T, CD4+ T, CD8+ T, B, NK, and monocyte populations

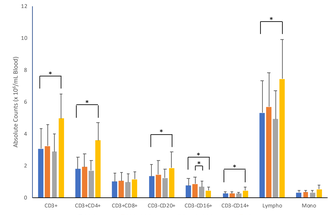
Data collected using flow cytometer was gated to identify the populations of interest as shown. The top two panels to the left show gating of single cells using FSC and SSC parameters. Top right panel shows gating on lymphocyte and monocyte populations. Center left panel is for gating on CD3+ total T cell population out of lymphocytes. Center middle panel is gated on B cell population of lymphocytes. Center right panel is gated on NK cells out of lymphocytes. Bottom left panel shows gating on CD4+ and CD8+ T cells out of CD3+ T cell population. The bottom right panel is gated on CD14+ monocyte population out of the monocyte gate.

**Analysis of Relative Percentage and Absolute Counts of T, CD4+ T, CD8+ T, B, NK, and Monocyte Populations in Peripheral Blood of Cynomolgus Monkeys**




**Figures 3.** Relative percentages of T, CD4+ T, CD8+ T, B, NK, and monocyte populations in peripheral blood of adolescent and infant cynomolgus monkeys.

Each bar represents mean and standard deviation of relative percentages (n=33 for infants, n=168 for adolescents; n=84 for males, n=84 for females) obtained using flow cytometry. Significant differences observed within values are shown with an asterisk (Mann Whitney test, *= p<0.005, **=p<0.05).




**Figures 4.** Absolute counts of T, CD4+ T, CD8+ T, B, NK, and monocyte populations in peripheral blood of adolescent and infant cynomolgus monkeys.

Each bar represents mean and standard deviation of absolute counts x 106 per mL of peripheral blood (n=33 for infants, n=168 for adolescents; n=84 for males, n=84 for females) calculated using relative percentages from flow cytometry and absolute counts of lymphocytes and monocytes obtained using hematology analyzer. Significant differences observed within values are shown with an asterisk (Mann Whitney test, *= p<0.005).

**Table 1.** Data Compilation of Relative Percentages and Absolute Counts of T, CD4+ T, CD8+ T, B, NK, and Monocyte Populations in Peripheral Blood of Adolescent and Infant Cynomolgus Monkeys.

Compiled mean and standard deviation values for **A)** relative percentages and **B)**, absolute counts (x 106 mL of T, Helper T, Cytotoxic T, B, NK, and monocyte cell populations in peripheral blood of cynomolgus monkeys categorized based on gender and age.

**A.**

| | CD3+ | CD3+CD4+ | CD3+CD8+ | CD3-CD20+ | CD3-CD16+ | CD3-CD14+ |
|---|---|---|---|---|---|---|
| Adolescents | 57.6 ± 8.4 | 34.1 ± 6.6 | 18.8 ± 5 | 24.8 ± 8 | 14.7 ± 6.5 | 76.9 ± 10.4 |
| Male Adolescents | 56.7 ± 9.5 | 33.4 ± 7.2 | 18.5 ± 5.3 | 24.8 ± 8.7 | 15.8 ± 7.0 | 75.6 ± 11.7 |
| Female Adolescents | 58.4 ± 7.2 | 34.7 ± 5.9 | 19.1 ± 4.8 | 24.8 ± 7.2 | 13.6 ± 5.9 | 78.2 ± 8.8 |
| Infants | 67.5 ± 7.3 | 48.7 ± 6.7 | 15.6 ± 4.2 | 23.9 ± 6.4 | 6.2 ± 2.0 | 83.4 ± 7.3 |

**B.**

| | CD3+ | CD3+CD4+ | CD3+CD8+ | CD3-CD20+ | CD3-CD16+ | CD3-CD14+ | Lymphocytes | Monocytes |
|---|---|---|---|---|---|---|---|---|
| Adolescents | 3.1 ± 1.3 | 1.9 ± 0.8 | 1.1 ± 0.6 | 1.4 ± 0.8 | 0.8 ± 0.5 | 0.3 ± 0.2 | 5.4 ± 2.0 | 0.4 ± 0.2 |
| Male Adolescents | 3.3 ± 1.4 | 2 ± 0.9 | 1.1 ± 0.6 | 1.5 ± 0.9 | 0.9 ± 0.5 | 0.3 ± 0.2 | 5.8 ± 2.2 | 0.4 ± 0.2 |
| Female Adolescents | 2.9 ± 1.2 | 1.7 ± 0.7 | 1.0 ± 0.6 | 1.3 ± 0.6 | 0.7 ± 0.4 | 0.3 ± 0.1 | 5.0 ± 1.8 | 0.4 ± 0.2 |
| Infants | 5.0 ± 1.6 | 3.6 ± 1.2 | 1.2 ± 0.5 | 1.9 ± 1.1 | 0.5 ± 0.2 | 0.5 ± 0.3 | 7.5 ± 2.5 | 0.6 ± 0.3 |

## CONCLUSIONS

We report relative percentage and absolute numbers of helper T cell, cytotoxic T cell, NK cell, B cell, and monocyte populations present in peripheral blood from a large cohort of cynomolgus monkeys, to assist in establishing a normal baseline reading. Our analysis also identifies differences in peripheral blood immune cell populations based on gender and age. We believe our analysis will assist in improved understanding of the toxicology changes introduced by therapeutic treatments during drug development.