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Detection of Plasma Cells and their Precursors in Bone Marrow and Peripheral Blood

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Abstract

Plasma cells are antibody-producing B cells that play a major role in conferring immune protection but can also contribute to the pathogenesis of autoimmune diseases like multiple sclerosis, and hematologic cancers like multiple myeloma. These cells are terminally differentiated, long-lived, quiescent cells with the capacity to secrete large numbers of antibodies. In contrast, their precursors, plasmablasts, are dividing short-lived cells with migratory capabilities. While plasma cells typically reside in the bone marrow (BM), circulating plasma cells have been detected in the peripheral blood (PB) of multiple myeloma patients. An increased number of circulating plasma cells is often correlated with poor prognosis. As both plasmablasts and plasma cells are present at low frequencies in BM and PB, flow cytometry is the method of choice to detect and study them.

Here, we present a method for the detection of both cell types in the BM and PB of cynomolgus monkeys dosed with a test article (an antibody) developed for the treatment of multiple myeloma. PB, as well as BM aspirates from the humerus or femur head of sedated, dosed, and control animals, was collected into CPT tubes. Mononuclear cells isolated from the centrifugation of CPT tubes were stained with antibody cocktails: BM (CD19, CD20, CD27, CD20, and CD138) and PB (CD3, CD20, CD14, CD38, and CD138). In the BM, plasmablasts were defined as CD19+CD20-CD27+, with plasma cells defined as a subset of plasmablasts that expressed CD138. In the PB, circulating plasma cells and plasmablasts were gated on CD3-CD20-CD14- cells, followed by CD38+CD138+ of the residual cells.

Analysis of BM aspirates collected from eight control animals indicated a frequency of plasmablasts out of CD20+CD19+/- from 0.4%−6.1%. Plasma cells out of plasmablasts from the same animals ranged from 1.5%−13.5%. In the PB, the frequency of plasma cells and plasmablasts in 51 control animals was lower than noted in BM, ranging from 0.0%−0.2% of its parent population (CD14- gated on CD3-CD20-). An increase in the percentage of plasma B cells was noted in several TA-dosed animals ranging from 0.1%−0.9%, providing further confirmation that our method can detect not only these rare cells, but also TA-induced changes.

In conclusion, our method provides a valuable tool for the detection of plasma cells and plasmablasts in the BM and PB of cynomolgus monkeys, and further expands the versatility of flow cytometry as a technique to interrogate TA-related effects on the immune system.

Introduction

Plasma cells are antibody-producing B cells that play a major role in conferring immune protection but can also contribute to the pathogenesis of autoimmune diseases like multiple sclerosis, and hematologic cancers like multiple myeloma.

Here, we present a method for the detection of plasma cells in the BM and PB of cynomolgus monkeys dosed with a test article (an antibody) developed for the treatment of multiple myeloma. Harnessing the information gathered from control animals, as well as collections prior to TA-dosing, we sought out to establish the range of plasma cells in both BM and PB. We leveraged the range obtained to establish the number of events needed to achieve our desired level of sensitivity in future studies. Lastly, we also determined the % coefficient variation (%CV) on repeated measures performed on twelve controls animals throughout the course of the study.

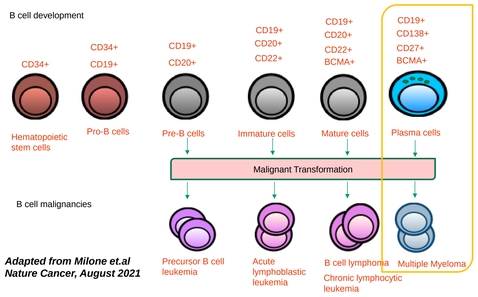

Figure 1: Overview of B cell development and B cell malignancies that resulted at various stages of development. Plasma cells are terminally differentiated, long-lived, quiescent B cells with the capacity to secrete large numbers of antibodies. Multiple myeloma cells are abnormal plasma cells that can accumulate in the bone marrow and crowd out other healthy immune cells.

Materials and Methods

Sample Collection

Flow Cytometry Analysis on Bone Marrow Aspirates (BMA)	
Species	Cynomolgus (*Macaca fascicularis*) monkey
Matrix	2 mL of humerus or femur head aspirates collected in 4 mL BD Vacutainer CPT tubes with sodium citrate
Time points	Day 95, Day 183
Storage conditions	Ambient temperature
Flow Cytometry Analysis on Peripheral Blood (PB)	
Species	Cynomolgus (*Macaca fascicularis*) monkey
Matrix	3 mL of whole blood collected in 4 mL BD Vacutainer CPT tubes with sodium citrate
Time points	Day -8, Day 8, Day 36, Day 85
Storage conditions	Ambient temperature

Isolation of Mononuclear cells with BD Vacutainer CPT tubes

Bone marrow (BM) Mononuclear cells and peripheral blood mononuclear cells (PBMC) were isolated with CPT tubes by centrifuging collection tubes at 1,800 × g for 30 minutes at AT with no brakes. Mononuclear cell layer was collected and washed with 1X-calcium and magnesium-free phosphate buffered saline (1X-CMF-PBS) and centrifuged at 500 × g for 5 minutes with maximum brake. Cell pellet was resuspended in 100 µL of 1X PBS.

Analytical method for detection of plasma cells in BMA

Antibodies	Clone
CD20-PerCP-Cy5.5	2H7
CD19-PE-Cy7	J3119
CD27-APC	M-T271
CD138-PE	MI15

Isolated mononuclear cells (0.1 mL) were incubated with immunophenotyping antibodies and incubated in the dark at ambient temperature (AT) for 15 to 20 minutes. Cells were incubated with 1x BD FACS Lysing solution at AT for 5 to 12 minutes followed by centrifugation (500 × g). Cells were washed once by adding 1X-CMF-PBS, resuspended in 1X-CMF-PBS, and analyzed by flow cytometry. The cytometer was set to acquire 200,000 events in the lymphocyte+monocyte gate limited to a total of 3 minutes for each sample acquired.

Analytical method for detection of plasma cells in PB

Antibodies	Clone
CD14-FITC	M5E2
CD20-PerCP-Cy5.5	2H7
CD3-APC-Cy7	SP34-2
CD38-APC	AT1
CD138-PE	MI15

Isolated PBMCs (0.1 mL) were transferred to appropriate tubes and incubated with 10 µL of Fc block for 10 minutes at AT. Without additional washing, antibody cocktail was added to appropriate tubes and samples were incubated for 20 to 30 minutes at AT, protected from direct bright light. Samples were washed with 2 mL of stain buffer followed by centrifugation (500 × g for 5 minutes with maximum brake). Cells were fixed by adding 2 mL of 4% formaldehyde to each tube and incubated for 10 minutes at AT, protected from direct bight light. Cell pellet was resuspended with 500 µL of stain buffer and samples were maintained at 2 to 8 °C or on ice until data acquisition when not immediately analyzed. The cytometer was set to acquire 1,000,000 events in the P2 gate and limited to a total of 5 minutes for each sample acquired.

Flow Cytometry Data Acquisition and Analysis

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 and # of events within each gate of interest. Single cells were identified out of total cells using the following sequential gate: FSC-H vs. FSC-W followed by SSC-H vs. SSC-W. Lymphocytes and Monocytes were identified from single cells via FSC-A vs. SSC-A profile. Subsequent gating strategies to identify population of interests are described in Results section.

Results

Detection of Plasma Cells in Bone Marrow

Plasma cells in the bone marrow were detected using the gating strategy and cell surface markers described in Figure 2. The frequency of plasma cells in 8 naïve control animals examined were low and biological variations are observed across animals (Table 1). Despite the variability, the assay was able to detect potential TA-related increases in plasma cells in several TA-administered animals (one shown in Figure 2).

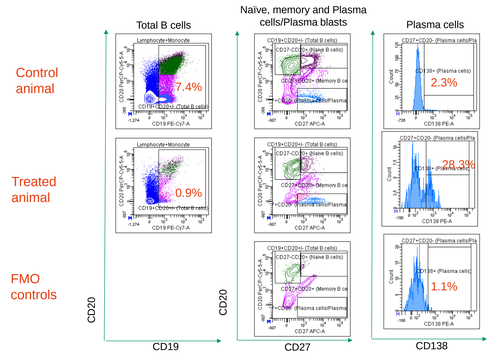

Figure 2: Detection of Plasma cells in BMA of Control and TA-treated animals. Bone marrow aspirates from a control and TA-treated animals were collected on Day 95 and were processed per analytical method described. To identify plasma cells, Total B cells were first gated based on CD19+CD20+/- expression. Total B cells were further divided into Naïve B cells (CD20+CD27-), Memory B cells (CD20+CD27+) and Plasma cells/Plasma blasts (CD20-CD27+). Plasma cells were identified as CD138+ cells within its parent gate of Plasma cells/Plasmablasts.

Plasma cells in BM of control animals N=8	% CD138+ out of plasma cells/plasma blasts	% CD138+ out of CD19+CD20+/- cells	% CD138+ out of Lymphocytes + Monocytes	Events of Lymphocyte + Monocytes collected
Control 1, Day 85	8.4	0.03	0.003	235,871
Control 2, Day 85	2.3	0.05	0.006	223,112
Control 3, Day 85	13.5	0.09	0.002	225,280
Control 4, Day 85	1.5	0.06	0.005	222,068
Control 5, Day 183	*0.0*	*0.00*	*0.000*	*135,648**
Control 6, Day 183	*0.0*	*0.00*	*0.000*	*10,365**
Control 7, Day 183	64.3	0.32	0.037	170,427
Control 8, Day 183	17.4	0.48	0.058	207,389
Range	2.3 to 64.3%	0.03 to 0.48%	0.003 to 0.058%	
Estimated # events needed to achieve % CV of 30% **				**20,000 to 370,000 events within Lymphocyte + Monocyte gate *****
CD138 FMO, Day 85	1.1	0.00	0.000	
CD138 FMO, Day 183	0.0	0.00	0.000	

* Controls 5 and 6 were excluded from analysis as the # of events required per method were not achieved.
** % Coefficient variation (% CV)
*** Calculated based on range noted in Table 1 and per Sommer et.al. Cytometry 2021 100:42-51

Table 1: Frequency of Plasma cells in BMA of control animals. BMA from 8 control animals collected on Days 85 and 183 were analyzed using the gating strategy described in Figure 1. The frequency of plasma cells (CD19+CD20-CD27+CD138+) are expressed out of its immediate parent population (Plasma cells/Plasmablasts), its grandparent population, Total B cells (CD19+CD20+/- cells) and out of Lymphocyte + Monocyte.

Results

Detection of Plasma Cells in Peripheral Blood

Plasma cells in the peripheral blood were detected using the gating strategy and cell surface markers described in Figure 3. Plasma cells in PB of 50 naïve animals are present in very low frequencies and can be quite variable between animals. The reproducibility of the assay was investigated by examining three analysis performed on the same animals across time (Table 3).

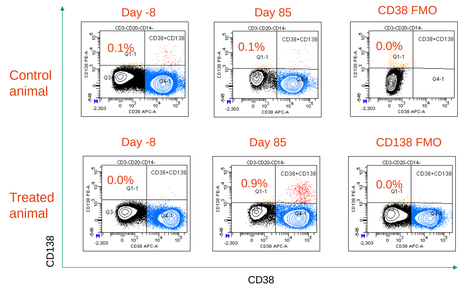

Figure 3: Detection of Plasma cells in PB of Control and TA-treated animals. Bone marrow aspirates from a control and TA-treated animals were collected on Day 85 and were processed as described per analytical method. To identify plasma cells, T cells, B cells and monocytes were removed by gating on CD3-C20-CD14- cells. The remaining cells were analyzed for expression of CD138 vs. CD38 and plasma cells (CD38+CD138+) cells were examined.

Plasma cells in PB of control animals	% CD38+138+ out of CD14-CD3-CD20- cells	% CD38+CD138+ out of Lymphocytes + Monocytes	Events of Lymphocyte + Monocytes collected
N=50, Range	0.010 to 0.188	0.003 to 0.034	259,951 to 1,181,129
Median	0.043	0.009	998,127
Estimated # events needed to achieve 30% CV			**33,000 to 370,000 events within Lymphocyte + Monocyte gate ***
CD38 FMO	0.001-0.002	0.000-0.001	
CD138 FMO	0.005-0.007	0.001-0.002	

* Calculated based on range in Table 2 and per Sommer et.al. Cytometry 2021 100:42-51

Table 2: Frequency of Plasma cells in PB of all study animals prior to dosing. PB from 51 study animals collected on Day -8 were analyzed using the gating strategy described in Figure 2. The frequency of plasma cells (CD38+CD138+) are expressed out its immediate parent population (CD14-, gated on CD3-CD20-) and out of Lymphocytes.

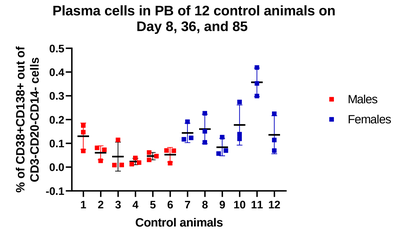

Figure 3: Detection of plasma cells in PB of twelve control animals on Days 8, 36 and 85. Mean and SD were plotted and % co-efficient variation (%CV) ranged from 16.9% (An4) to 138.2% (An3). Most animals had %CV under 50%.

Conclusion

Here we present analytical methods for the detection of plasma cells in bone marrow and peripheral blood. In both compartments, plasma cells were present at very low frequencies and variability across animals were observed. From this dataset, the range of frequencies on plasma cells were established and the estimated # of events needed to achieve 30% CV were calculated for both bone marrow and PB. For most animals, the number of events collected per the analytical method were within the events needed to achieve % CV of 30%. The reproducibility of plasma cells detection in the bone marrow of twelve animals were presented and while all animals and timepoints examined met the events needed to achieve 30% CV, the observed %CV varied across animals, with most animals ranging between 40%-50%. Despite these challenges, potential TA-related increases in frequency of plasma were observed in both bone marrow and peripheral blood. Taken together, the development of these assays highlighted the important considerations needed when establishing a high sensitivity assay for detection of rare events.