Quantitative Assessment of Myeloid Nuclear Differentiation Antigen using Flow Cytometry

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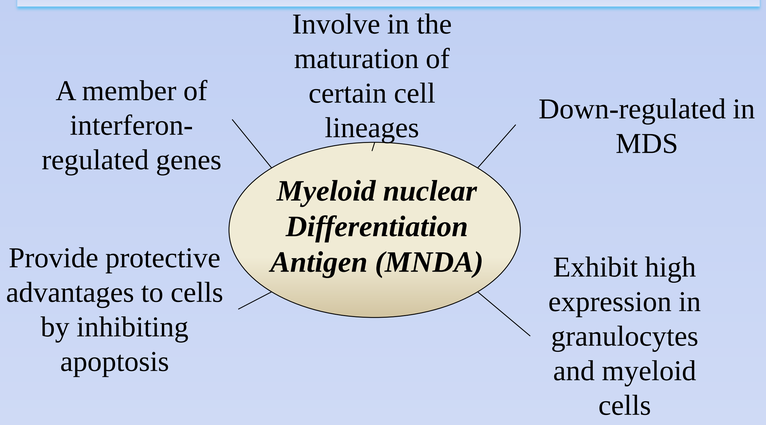
Abstract

Myelodysplastic Syndrome (MDS) is a set of clonal marrow failure disorder that are difficult to diagnose due to the lack of standard diagnostic parameters, admixture of normal bone marrow in MDS sample and large differential diagnosis in MDS. A combination of markers must be used due to the absence of single marker that can reliably distinguish MDS from non-MDS patient. The ultimate goal of this study is to design the best cocktail that could be applied to diagnose the disease with high confidence, sensitivity and specificity. Cell were lysed and permeabilized for both intercellular and intracellular staining and the intensity of the signals were assessed using flow cytometry. A standard protocol based on formaldehyde & saponin (Caltag) and one developed by *Chow et al.*, which based on formaldehye & Triton X-100 were compared to determine which methodology provides better permeabilization as it is crucial to our intracellular staining. *Chows et al.* based methodology was deemed to be superior in permeabilizing cell; both THP1 and U937 cells lines produced positive signals. However, cell lines K562 did not provide enough information, of whether the signals came from intracellular staining, or from the remains of anti-MNDA stain that stayed in the cells. An isotype was used and it was found that K562 were stained positive by anti-MNDA, even though the signal was approximately 3 to 5 times weaker compared to THP-1 and U937. The exclusion of methanol from the washing step did not weaken our fluorescence signals.

Introduction



In this study, we are trying to:

- Access the staining pattern of positive and negative control in order to determine the reproducibility and reliability of MNDA to detect MDS before we move on using patient samples and adding more CD markers. Cell lines that were used include:
  a) Positive cell lines : THP1, U937
  b) Negative cell lines : K562
- Determine if the inclusion of methanol in the washing steps will produce non-specific signals or noise.
- According to our past experience, methanol could possibly unmask phosphate group and promotes non-specific binding to CD markers.

Materials and Methods

Treatment with and without Methanol

Tubes	Permeabilizing Agent	Wash with Methanol?
Set 1	CalTag	No
Set 2	10% Formaldehyde	No
Set 3	10% Formaldehyde	yes

Staining, Fixation, and Permeabilization

- 100 µL of cells were transferred into tubes. Tubes labeled with CD45 were stained with CD45 – APC
- Mouse anti-IgG was added to the tube. Incubation was done on ice for 30 minutes followed by washing steps.
- For tube set 2 and set 3, 65µL10% formaldehyde was added, followed by 1 mL of Triton X-100. For set 1, 2 mL of CalTag was added. Samples were incubated for 30 minutes at room temperature
- 1mL cold buffer consist of 4% fetal bovine serum diluted in PBS was added into each tube followed by centrifugation. Tube 1&2 and tube 3 were resuspended using 1mL PBS and 1mL 50% methanol respectively
- Tube labeled with MNDA were stained with anti-MNDA and incubated at room temperature for 30 minutes. Centrifugation was performed and the cell pellets were resuspended to 2mL
- Emission spectrum was acquired using LSR-II B flow cytometer. Data analysis was performed using WinList 7.0

Result

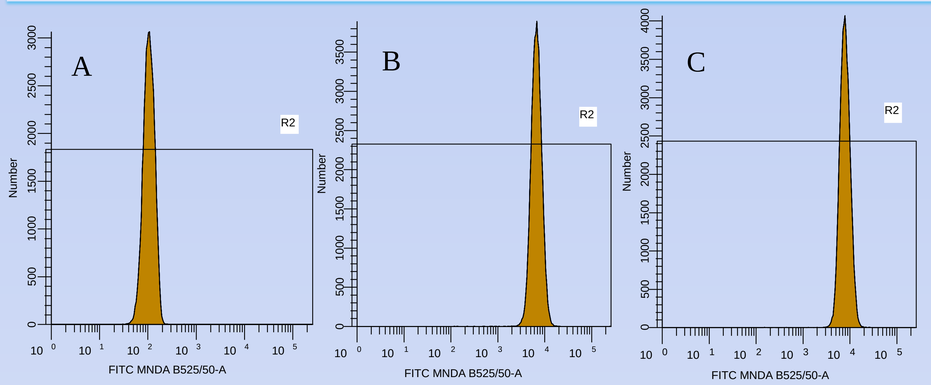


***Figure 1**: (A) auto fluorescent (B) and (C) Chow et al. with and without methanol treatment . Signal intensity of B and C did not show any significant difference.*

Cell Lines		APC (mean, std.)		FIT-C (mean, std.)	
U937	Control	3.82	0.22	3.39	0.80
	Stained	198.07	36.44	416.69	48.80
THP-1	Control	5.60	2.14	3.25	0.81
	stained	138.94	38.85	254.79	90.57

***Table 1**: Measurement of mean and standard deviation of triplicates (data not shown) using U937 and THP-1 cell lines. Both cell lines showed positive binding to surface marker and intracellular stain.*

Result

***Figure 2:** Control (A) compared to CD 45 and MNDA staining (B). Tests were done in triplicate (Table 1). Both U937 and THP-1 cell lines illustrate positive staining for MNDA and CD 45.*

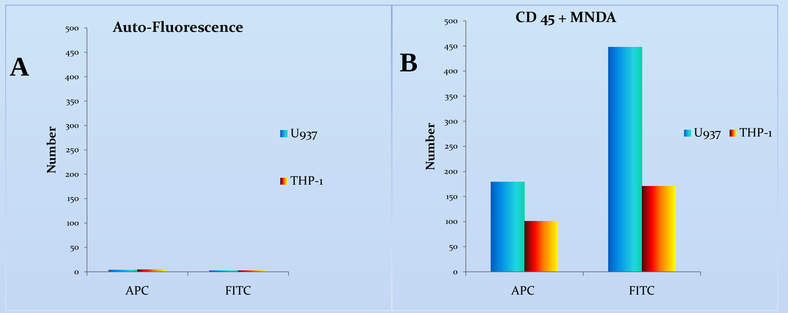


***Table 2**: Measurement of mean and standard deviation of triplicate using K562 cell lines. Isotype was detected as a negative signal. Therefore, the intracellular staining of K562 using anti-MNDA illustrated true positive.*

	APC			FIT-C		
	Control	Isotype	CD45+ MNDA	Control	Isotype	CD45+ MNDA
Mean	8.70	5.56	358.53	6.45	6.27	79.20
Std.	1.08	0.65	67.08	2.39	0.48	14.64

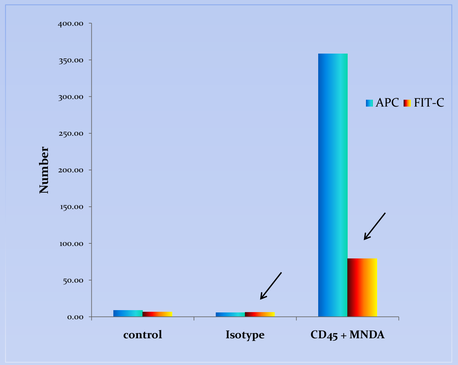


***Figure 3**: Staining pattern of K562 cell lines was observed. Compared to isotype, significant level of signal was detected in K562 cells stained with MNDA (arrow).*

Conclusion

- *Chow et al.* provided a better method to permeabilize cells.
- THP1 and U937 cell lines are positive to MNDA stain.
- K562 cell line is weakly stained by anti-MNDA, hence might not be a suitable negative control for further study.
- Methanol can be excluded from the assay since it did not interfere with the fluorescent intensity.

Citation of Literature

- Chow, S., Hedley, D., Grom, P., Magari, R., Jacobberger, J. W. and Shankey, T. V. (2005), Whole blood fixation and permeabilization protocol with red blood cell lysis for flow cytometry of intracellular phosphorylated epitopes in leukocyte subpopulations. Cytometry Part A, 67A: 4–17. doi: 10.1002/cyto.a.20167
- McClintock-Treep, Sara. "Quantitative Assessment of myeloid Nuclear Differentiation Antigen Distinguishes Myelodysplastic Syndrome from Normal Bone Marrow." *Hematophatology*. (2011): 380-85. Print.