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Optimisation of flow cytometric detection of intracellular SLAMF receptor-associated adaptor proteins SAP and EAT-2

Short communication

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Abstract SAP and EAT-2 are intracellular adaptor proteins that link SLAMF receptors to downstream intracellular signalling pathways. Their expression and functions vary among different types of hematopoietic cells. The roles of SAP and EAT-2 in haematological malignancies are poorly understood. Here, we attempted to optimise a method for the detection of intracellular SAP and EAT-2 by flow cytometry. We found that using PerFix EXPOSE kit for cell fixation and permeabilization ensures optimal results in terms of staining intensity and signal-to-noise ratio.

Keywords *SLAMF receptors – SAP – EAT-2 –l ymphocytes – flow cytometry*

INTRODUCTION

The SLAM (Signalling Lymphocyte Activation Molecule) family is a group of nine surface receptors (SLAMF1-9) expressed mainly on hematopoietic cells, that regulate the activation and differentiation of a wide range of immune cells involved in both innate and adaptive immune responses (Calpe et al., 2008; Cannons et al., 2011; Detre et al., 2012). SLAMF receptors interact with intracellular SLAM-associated protein (SAP)-related molecules that link SLAMF receptors to downstream intracellular signalling pathways. In T, NK, and NKT cells, SLAMF receptors interact with SAP. In mature B cells, SLAMF receptors induce a downstream cascade through EAT-2 (Tangye et al., 2002; Veillette, 2006; Radomir et al., 2017). This work's aim is the optimisation of flow cytometry method for the detection of intracellular SAP and EAT-2.

MATERIAL AND METHODS

Peripheral blood was obtained from a healthy donor (M.S., corresponding author). The expression of intracellular/cytoplasmic SAP (cySAP) and EAT-2 (cyEAT-2) was studied with multiparametric flow cytometry. Samples were stained with combinations of monoclonal antibodies as shown in Table 1. Rat anti-SAP-PE monoclonal antibody (Invitrogen, USA) and rabbit anti-EAT-2 antibody (Proteintech, UK) with secondary donkey anti-rabbit IgG-PE antibody (BioLegend, USA) were used for the detection of cySAP and cyEAT-2, respectively. Four different commercial kits were employed for cell fixation and permeabilization: Fix & Perm Cell Permeabilization Reagents (Invitrogen, USA), Transcription Factor Buffer Set (BD Pharmingen, USA), IntraPrep Permeabilization Reagent and PerFix EXPOSE Kit (both Beckman Coulter, USA). Manufacturers' instructions were closely followed. Samples were acquired using Navios EX flow cytometer (Beckman Coulter, USA). Data were analysed using Infinicyt software (Cytognos, Spain). The intensity of cySAP and cyEAT-2 expression was expressed as MFI (mean fluorescence intensity) value.

RESULTS AND DISCUSSION

Flow cytometric detection of intracellular (cytoplasmic or nuclear) versus cell surface antigens is significantly more laborious and time-consuming. It requires cell fixation, usually

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Table 1. Panel of antibodies.

	Antibodies and fluorochromes									
Tube No.	**PB**	**KO**	**FITC**	**PE**	**ECD**	**PC5.5**	**PC7**	**APC**	**APC-A700**	**APC-A750**
1	CD4 + CD20	CD45	-	cyCTRL	-	CD5	CD10	CD3	CD19	CD8
2	CD4 + CD20	CD45	-	cySAP	-	CD5	CD10	CD3	CD19	CD8
3	CD4 + CD20	CD45	-	cyCTRL +sec.Ab	-	CD5	CD10	CD3	CD19	CD8
4	CD4 + CD20	CD45	-	cyEAT-2 +sec.Ab	-	CD5	CD10	CD3	CD19	CD8

Table 2. Flow cytometric analysis of SAP and EAT-2 adaptor proteins in lymphocytes.

			MFI control (unstained) cells			**MFI stained cells**		
			Mean	**SD**	**CV**	**Mean**	**SD**	**CV**
cySAP	T cells	Fix&Perm	3.1	1.2	39.6	4.8	1.6	32.7
		TFBS	2.7	1.3	47.9	17.1	11.5	67.5
		IntraPrep	3.8	0.9	22.6	23.9	10.3	43.1
		PerFix	4.9	2.0	41.3	73.9	54.2	73.3
	B cells	Fix&Perm	1.6	0.6	36.9	4.0	1.9	47.3
		TFBS	1.1	0.2	15.2	10.0	7.0	70.3
		IntraPrep	3.5	1.0	26.8	15.7	12.2	77.9
		PerFix	2.7	0.7	27.1	28.9	27.3	94.8
	NK cells	Fix&Perm	1.8	0.6	30.9	4.5	2.4	54.0
		TFBS	1.2	0.4	32.3	18.9	9.1	48.1
		IntraPrep	4.0	0.8	19.5	22.6	9.5	42.1
		PerFix	2.4	0.6	23.4	49.9	22.6	45.4
cyEAT-2	T cells	Fix&Perm	ND	ND	ND	ND	ND	ND
		TFBS	ND	ND	ND	ND	ND	ND
		IntraPrep	4.0	0.9	21.7	83.9	46.3	55.1
		PerFix	5.8	2.1	36.8	192.5	64.6	33.6
	B cells	Fix&Perm	ND	ND	ND	ND	ND	ND
		TFBS	ND	ND	ND	ND	ND	ND
		IntraPrep	3.7	0.9	24.2	63.1	37.3	59.1
		PerFix	3.7	1.3	35.2	189.0	60.9	32.2
	NK cells	Fix&Perm	ND	ND	ND	ND	ND	ND
		TFBS	ND	ND	ND	ND	ND	ND
		IntraPrep	4.2	1.0	24.0	211.2	178.6	84.6
		PerFix	3.1	0.7	23.1	537.7	227.3	42.3

with paraformaldehyde, followed by permeabilization with Triton-X or saponin. Unfortunately, these procedures lead to significant cell damage, changes in forward and side scatter characteristics of cells and increased autofluorescence, which may complicate subsequent data analysis. Here, we compared four different commercial fixation and permeabilization kits with the aim to optimise methodology for the detection of intracellular/cytoplasmic SAP and EAT-2 proteins by flow cytometry.

In clinical flow cytometry laboratories, Fix & Perm and IntraPrep reagents belong to the most widely used kits for the detection of diagnostically important intracellular antigens. In our study, Fix & Perm showed unsatisfactory performance when used for cySAP staining as reflected by the very low MFI of stained cells (Figure 1, Table 2). For that reason, it was not further evaluated for cyEAT-2 detection. In contrast, the usage of IntraPrep resulted in a significantly stronger cySAP and cyEAT-2 staining in terms of MFI and a good signal-to-noise

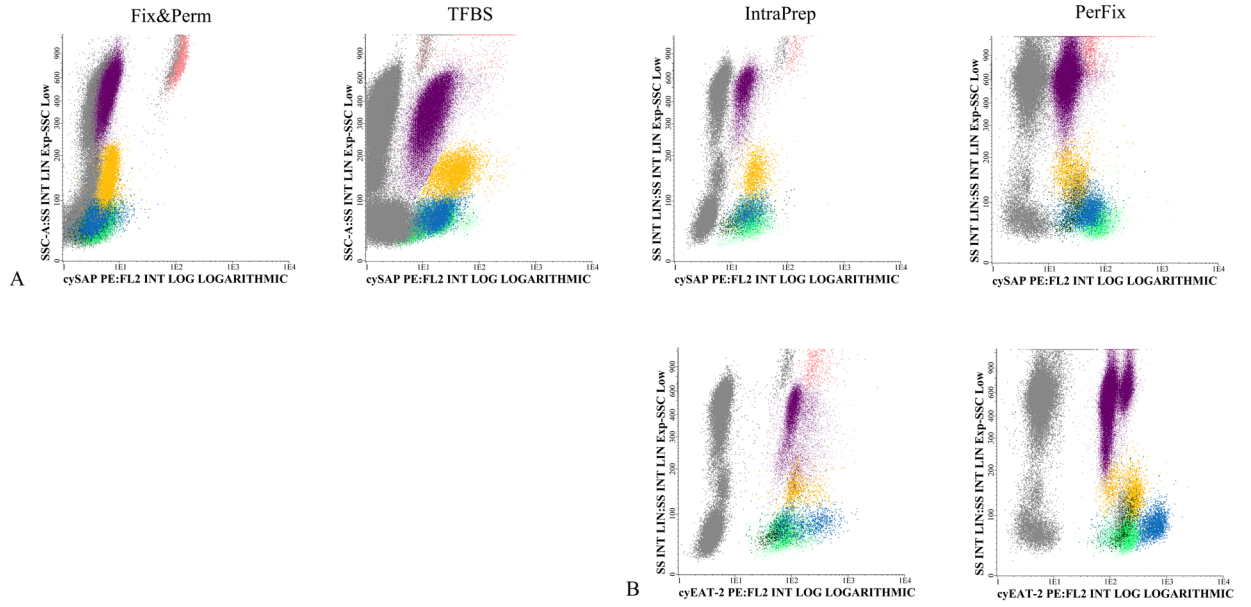


Figure 1. Illustrative bivariate dotplots showing the results of cySAP (A) and cyEAT-2 (B) analysis by flow cytometry: control (unstained) cells (grey), T cells (light green), B cells (dark green), NK cells (blue), monocytes (yellow), neutrophils (violet) and eosinophils (pink).

ratio. TFBS reagent led to a stronger cySAP staining than Fix & Perm but weaker than IntraPrep. Since the staining procedure using TFBS versus other reagents is significantly more time-consuming (approximately 3 hours versus approximately 1 hour), we decided not to evaluate it further. Finally, we used the PerFix kit. Originally developed for the detection of phosphorylated intracellular antigens, PerFix gave the best results in terms of cySAP and cyEAT-2 staining intensity and signal-to-noise ratio. Furthermore, it enabled separation of T cells, B cells, and NK cells based on the intensity of cySAP and cyEAT-2 expression. Previously, other authors who analysed cySAP and/or cyEAT-2 by flow cytometry reported only on the use of the traditional paraformaldehyde/Triton-X method (Schlums et al., 2015; Radomir et al., 2017; Gyurova et al., 2019). To conclude, we identified the staining procedure employing PerFix EXPOSE kit for cell fixation and permeabilization as the optimal method for cySAP and cyEAT-2 detection.

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References

[1] Calpe S, Wang N, Romero X, Berger SB, Lanyi A, Engel P, Terhorst C. The SLAM and SAP gene families control innate and adaptive immune responses. Adv Immunol. 2008;97:177-250.

[2] Cannons JL, Tangye SG, Schwartzberg PL. SLAM family receptors and SAP adaptors in immunity. Annu Rev Immunol. 2011;29:665-705.

[3] Detre C, Keszei M, Garrido-Mesa N, Kis-Toth K, Castro W, Agyemang AF, Veerapen N, Besra GS, Carroll MC, Tsokos GC, Wang N, Leadbetter EA, Terhorst C. SAP expression in invariant NKT cells is required for cognate help to support B-cell responses. Blood. 2012 Jul 5;120(1):122-129.

[4] Gyurova IE, Schlums H, Sucharew H, Ambroggio L, Ochayon DE, Win HT, Bryceson YT, Bernstein DI, Waggoner SN. Dynamic Changes in Natural Killer Cell Subset Frequencies in the Absence of Cytomegalovirus Infection. Front Immunol. 2019 Nov 22;10:2728.

[5] Radomir L, Cohen S, Kramer MP, Bakos E, Lewinsky H, Barak A, Porat Z, Bucala R, Stepensky P, Becker-Herman S, Shachar I. T Cells Regulate Peripheral Naive Mature B Cell Survival by Cell-Cell Contact Mediated through SLAMF6 and SAP. J Immunol. 2017 Oct 15;199(8):2745-2757.

[6] Schlums H, Cichocki F, Tesi B, Theorell J, Beziat V, Holmes TD, Han H, Chiang SC, Foley B, Mattsson K, Larsson S, Schaffer M, Malmberg KJ, Ljunggren HG, Miller JS, Bryceson YT. Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function. Immunity. 2015 Mar 17;42(3):443-456.

[7] Tangye SG, van de Weerdt BC, Avery DT, Hodgkin PD. CD84 is up-regulated on a major population of human memory B cells and recruits the SH2 domain containing proteins SAP and EAT-2. Eur J Immunol. 2002 Jun;32(6):1640-1649.

[8] Veillette A. Immune regulation by SLAM family receptors and SAP-related adaptors. Nat Rev Immunol. 2006 Jan;6(1):56-66.