A Method for Stabilizing CD69 Antigenic Epitopes on Activated Lymphocytes

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ABSTRACT

CD69, also known as activator inducer molecule (AIM), early activation antigen 1 (EA-1), Leu-23, and MLR3 is a type II integral membrane protein. Although not normally expressed on peripheral blood lymphocytes, surface expression is upregulated in response to a wide variety of stimuli and is identified as an early stage T-cell activation event. In addition to its involvement in T-cell mediated responses, it plays a pivotal role in the differentiation of the Th17 immune response via direct binding and inhibition of the Jak3/Stat5 transcription factor pathway. Because of CD69's role in immune responses, immune cell development, and potential antitumor effects, it is being evaluated as an important marker for the analysis of clinical patients in a variety of applications. Thus, the ability to measure CD69 expression for clinical or research flow cytometry is of significant value. We demonstrate the *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) followed by stabilization of CD69 expression for 7 days in Cyto-Chex® BCT. This is in contrast to stimulated cells maintained in K_2EDTA alone as cells were unstable and CD69 percent recoveries were overtly elevated. This discovery allows more flexibility in testing protocols which can benefit clinical sample analysis by contract research organizations and hospitals.

INTRODUCTION

CD69, also known as activation inducer molecule (AIM), early activation antigen 1 (EA-1), Leu-23, and MLR3, is a type II integral membrane protein with an extracellular c-type lectin domain. CD69 is not detectable on normal peripheral blood lymphocytes, but cell surface expression is upregulated in response to a wide variety of stimuli. Although CD69 null mice have no apparent developmental or T-cell function defects[1], these mice do display some degree of antitumor protection when challenged[2]. Additionally, it has recently been elucidated that CD69 plays a pivotal role in the differentiation of the Th17 immune response via direct binding and inhibition of the Jak3/Stat5 transcription factor pathway[3]. The Th17 immune response has been implicated in autoimmune and sensitivity reactions, including arthritis[4].

The ligand for CD69 has yet to be determined; however, CD69 expression is one of the earliest cellular changes noted in response to external stimuli and can be found on T, B, and NK cells[5]. RNA expression of CD69 reaches its peak 3-6 hours post-stimulation with cell surface expression by 12 hours. After a slight drop at 24 hours, expression remains steady through 48 hours[6]. This is in contrast to other activation markers, such as CD71 and HLA-DR, which are upregulated later in this process. Thus, the balance and regulation of CD69 expression with other extracellular signals is important in coordinating the regulation of the immune response.

Cyto-Chex® BCT is an FDA-approved blood collection tube for the preservation of lymphocytes tested during the evaluation of HIV disease progression[7]. In addition to HIV relevant CD markers, other leukocyte markers used for research purposes, including CD23, CD34, CD38, and HLA-DR, have shown stability for up to 7 days. With CD69's role in immune responses, immune cell development, and potential antitumor effects, it is being evaluated as an important marker for the analysis of disease patients in a variety of applications.

The data presented demonstrates stability of CD69 on activated lymphocytes for up to 7 days in Cyto-Chex BCT compared to samples maintained in K_2EDTA.

METHODS

Blood Collection and In Vitro Stimulation

Informed consent from healthy donors was obtained for whole blood collection on concurrent days. On the first day, whole blood was collected by venipuncture into K_2EDTA (BD Vacutainer®, Becton Dickinson, Franklin Lakes, NJ) tubes. The K_2EDTA whole blood was combined with Accuprep® (Accurate Chemical and Scientific Corporation, Westbury, NY) for isolation of peripheral blood mononuclear cells (PBMCs) according to the recommended separation procedure. The harvested fraction of cells at $1x10^6$/mL was diluted into RPMI Medium 1640 (Invitrogen, Grand Island, NY) containing 5% autologous human serum removed prior to PBMC isolation. Donor cells in RPMI with serum were split equally into 2 wells of a 6-well dish (BD Falcon®, Becton Dickinson, Franklin Lakes, NJ). One well from each donor was the unstimulated control while the other well received 2µg/mL purified phytohaemagglutinin (PHA) (Remel, Dartford, Kent, UK). Stimulated and control samples were maintained at 37˚C for 16-20 hours before further manipulation.

In Vitro PBMC and Whole Blood Combination

On the following day, whole blood was collected into K_2EDTA tubes and Cyto-Chex BCT from autologous donors (Streck, Omaha, NE). *In vitro* control or PHA-stimulated PBMC's were added to fresh K_2EDTA and Cyto-Chex BCT whole blood samples. Samples were maintained at 18-22˚C for the duration of the study.

Flow Cytometry and Evaluation

Flow cytometric analysis was performed on a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) and a FC 500 flow cytometer (Beckman Coulter®, Miami, FL). Whole blood/*in vitro* PBMC samples were processed and analyzed on days 1 and 7 with CD45 and CD69 antibodies.

Samples were evaluated on the BD FACSCalibur using BD CellQuest™ Pro Software v6.0 with instrument settings established with FACSComp. Samples were evaluated on the Beckman Coulter FC 500 using CXP SYSTEM Software v2.2 with instrument settings established during compensation.

Samples were classified stable if the Cyto-Chex BCT CD69 percent recovery had a percent difference of ≤ 20% from the K_2EDTA value collected at 6 hours. All samples were analyzed in duplicate and represent an average of the percent recovery values.

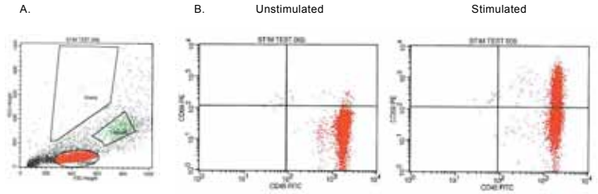

Figure 1: Representative Flow Cytometry Plots for PBMC isolation and CD69 Stimulation. **A.** FSC/SSC dot plot of peripheral blood mononuclear cells (PBMCs) isolated from a representative donor and incubated overnight at 37°C. Lymphocytes are >90%. **B.** Flow cytometry analysis of control (unstimulated) and stimulated (2µg/ml PHA) PBMCs stained with anti-CD45-FITC and anti-CD69-PE antibodies after 16-20 hours at 37°C. Quadrant settings were based on the unstimulated control.

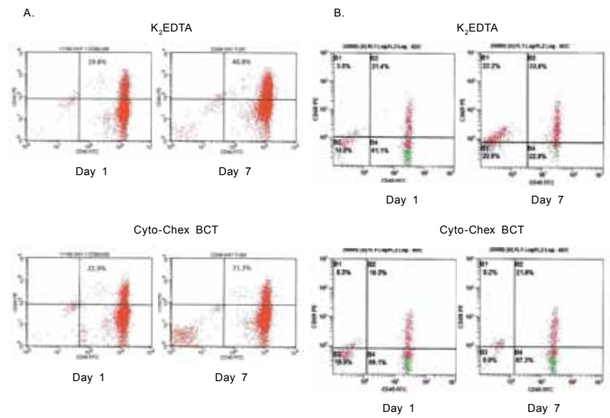

Figure 2: CD45+/CD69+ Flow Cytometry Evaluation of K_2EDTA vs. Cyto-Chex BCT Whole Blood Samples Containing Stimulated PBMCs. **A.** Flow cytometry analysis of *in vitro* stimulated PBMCs dual positive for CD45+/CD69+ on lymphocytes. K_2EDTA or Cyto-Chex BCT whole blood was tested after 6 hours (1 day) and 7 days. Samples were analyzed on the BD FACSCalibur. **B.** Flow cytometry analysis of *in vitro* stimulated PBMCs dual positive for CD45+/CD69+ on lymphocytes. K_2EDTA or Cyto-Chex BCT whole blood was tested after 6 hours (1 day) and 7 days. Samples were analyzed on the Beckman Coulter FC 500.

Table 1: CD45+/CD69+ Stimulated PBMCs are Stable in Cyto-Chex BCT. Ten-donor average of CD45+/CD69+ lymphocyte percent recovery after 6 hours (1 day) and 7 days tested in K_2EDTA and two lots of Cyto-Chex BCT. Averages represented were tested on the BD FACSCalibur and the Beckman Coulter FC 500. Each donor was tested in duplicate on each instrument on both testing days. Percent difference calculated from the 6-hour K_2EDTA average.

FACSCalibur

	K_2EDTA		Cyto-Chex BCT Lot A		Cyto-Chex BCT Lot B	
	Average	% Difference	Average	% Difference	Average	% Difference
6 hour	11.0	NA	11.1	1.4	11.0	0.4
Day 7	28.0	155	10.2	-6.8	9.9	-9.9

FC500

	K_2EDTA		Cyto-Chex BCT Lot A		Cyto-Chex BCT Lot B	
	Average	% Difference	Average	% Difference	Average	% Difference
6 hour	10.1	NA	9.1	-10.4	8.8	-12.8
Day 7	16.5	62.9	9.8	-3.4	9.6	-5.8

RESULTS

Stimulated lymphocytes and unstimulated control lymphoctyes were analyzed for CD69 percent recovery by flow cytometry prior to mixing with autologous donor K_2EDTA or Cyto-Chex BCT whole blood and after the 16-20 hour 37˚C stimulation period. In Figure 1, the BD FACSCalibur scans from a representative donor show isolation of peripheral blood mononuclear cells (PBMCs) and positivity for CD69. Only the PHA stimulated sample confirms a dual positive CD45+/CD69+ population (Figure 1B). Samples were prepared for flow cytometry analysis to assess CD45+/CD69+ populations. Initial testing on the BD FACSCalibur for both the K_2EDTA and Cyto-Chex BCT samples demonstrated CD45+/CD69+ cells in the stimulated samples with equivalent percent recoveries (Figure 2A). However, while the CD45+/CD69+ percent recovery is maintained for 7 days in stimulated samples stored in Cyto-Chex BCT, this value is overtly elevated in the K_2EDTA sample due to increasing amounts of dead cells (Figure 2A). These same characteristics can be seen with tests performed on the Beckman Coulter FC 500 (Figure 2B). Average CD45+/CD69+ percent recovery data from 10 healthy donors is summarized in Table 1 for samples analyzed on the BD FACSCalibur and the Beckman Coulter FC 500. Additionally, two Cyto-Chex BCT lots were used for testing; one current lot (Lot A) and one lot close to product expiration (Lot B). Both Cyto-Chex BCT lots show equivalent performance. Moreover, the CD45+/CD69+ dual positive PBMC population preserved in Cyto-Chex BCT is equivalent on both flow cytometry instruments.

CONCLUSION

The purpose of this study was to demonstrate stability of CD69 in Cyto-Chex BCT. In addition, we have provided a methodology for the *in vitro* stimulation of lymphocytes followed by marker preservation in Cyto-Chex BCT. The discovery of CD marker stability post-stimulation provides another level of flexibility allowing manipulation of samples prior to preservation and testing if required.

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