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Comparison of Sample Fixation and the use of LDS-751 or anti-CD45 for Leukocyte Identification in Mouse Whole Blood for Flow Cytometry.

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Abstract

Flow cytometry methods used to measure leukocyte function often entail sample preparation procedures that cause artifactual cell activation. To avoid leukocyte activation by isolation techniques, some preparation methods use fluorescent markers to discriminate leukocytes from erythrocytes in whole blood. One of these markers, laser dye styryl-751(LDS-751), has been used to distinguish leukocytes by staining nucleic acid, but has been found to stain other blood cells and dead cells indiscriminately. Thus, LDS-751 may not be an appropriate reagent for leukocyte identification in whole blood. Fixing samples with formaldehydes increases cell permeability and causes surface protein cross-linking that may alter staining of both intra- and extracellular markers. The degree of this sample alteration by formaldehyde fixation, however, remains in question. In addition, little is known about flow cytometry and sample preparation methods in mouse whole blood. The purpose of this study was to determine if labeling leukocytes with a monoclonal antibody specific to leukocyte common antigen (CD45) was superior to labeling with LDS-751 and to determine the effect of sample fixation on a mouse whole blood preparation for flow cytometry. Samples were incubated with CD16/CD32 Fc receptor blocker, and either 10 µg/ml LDS-751 or phosphate buffered saline (PBS). The samples were then fixed with paraformaldehyde or diluted with PBS followed by incubation with 5µg/ml PerCP-conjugated anti-CD45, 5µg/ml FITC-conjugated anti-CD11b, or 80 µM dichlorofluorescein diacetate. We found that samples labeled with LDS-751 demonstrated decreased fluorescence intensity for granulocyte CD11b expression and ROS production compared to samples labeled with anti-CD45. In addition, sample fixation decreased mean fluorescence intensity in samples labeled with either LDS-751 or anti-CD45. We conclude that labeling leukocytes with monoclonal antibody CD45 in a mouse whole blood preparation is preferable, as it provides improved measurement of leukocyte indices compared to LDS-751. Also, while sample fixation prior

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to antibody staining caused a decrease in overall fluorescence; it can be used to successfully identify extracellular markers.

Keywords

LDS-751; paraformaldehyde; mouse; flow cytometry; CD11b; reactive oxidative species; neutrophil

Background

Flow cytometry is a widely used and important method for both research and clinical applications (Marti, Stetler-Stevenson, Bleesing, and Fleisher, 2001), but few studies describe the effects of sample preparation on results. Erythrocyte lysing, sample fixation, and other sample preparation techniques used in flow cytometry are known to activate blood cells, particularly leukocytes, leading to an alteration in surface glycoprotein expression and intracellular processes (Macey, et al., 1995; Hageberg, and Lyberg, 2000; Alvarez, Toll, Rivas, and Estella, 2005).

Erythrocyte lysing is a commonly used sample preparation technique for cytometry, because it allows for rapid identification of leukocytes by the flow cytometer. However, studies indicate that erythrocyte lysing reagents cause increased leukocyte expression of glycoprotein CD11b and shedding of granulocyte glycoprotein L-selectin (McCarthy, Macey, Cahill, and Newland, 1994; Macey, et al., 1995; Macey, et al., 1999; Alvarez-Larran, Toll, Rivas, and Estella, 2005). Erythrocyte lysing also causes other blood cells, including leukocytes and platelets, to lyse (Terstappen, Meiners, and Loken, 1989; Alvarez-Larran, et al., 2005), creating cell microparticles and debris that interact with and activate leukocytes (Repo, Jansson, and Leirisalo-Repo, 1993). Therefore, an improved sample preparation method is needed to discern leukocytes from other cells in whole blood samples for flow cytometry and minimize experimental artifact.

Without erythrocyte lysing, discrimination of leukocytes from other cells in whole blood samples requires labeling leukocytes with a fluorescent marker, either by nuclear staining or labeling a leukocyte specific protein. An example of a nuclear stain used historically for flow cytometry to label leukocytes is Laser dye styryl-751 (LDS-751) (Hokama, et al., 2000; McDonagh, Hokama, Copeland, and Reynolds, 1997; McCarthy, Macey, Cahill, and Newland, 1994; Macey, et al., 1999; Simon, Chambers, and Sklar, 1990; Terstappen, et al., 1991; Terstappen, Meiners, and Loken, 1989; Repo, Jansson, and Leirisalo-Repo, 1993; Himmelfarb, Hakim, Holbrook, Loeber, and Ault, 1992). Although LDS-751 has been used extensively to discriminate leukocytes in whole blood samples, it has been reported to indiscriminately stain both live and dead cells (O'Brien, and Bolton, 1995) and has been used to identify platelets and nucleated erythrocytes in whole blood (Terstappen, 1991). Consequently, the use of LDS-751 may not be the optimal method for discriminating leukocytes in whole blood samples for flow cytometry.

Cell surface antigen staining is another approach for leukocyte discrimination in whole blood samples. A leukocyte specific, surface protein antibody that has achieved wide use for flow cytometry in recent years is the monoclonal antibody to cell surface glycoprotein CD45 (leukocyte common antigen) (Hageberg, and Lyberg, 2000; Fugimoto, et al., 2000). CD45 is a receptor-like protein tyrosine phosphatase, and is a critical regulator of leukocyte signaling (Hermiston, Xu, and Weiss, 2003; Takeda, Matsuda, Paul, and Yaseen, 2004). To date, no studies have described an artifactual change in leukocyte function with the use of a monoclonal antibody to CD45 to identify leukocytes in whole blood.

In addition to erythrocyte lysing, the sample preparation process of cell fixation changes cell function and structure, making implementation of this method controversial for flow cytometry. Sample fixation increases cell permeability, causes cell surface protein cross-linking, (Shapiro, 2003), and produces cell surface aldehyde groups that can bind antibodies. Studies that have examined fixation on sample preparation have produced conflicting results. A number of studies determined that cell fixation decreases the mean fluorescence of several surface antigens, including leukocyte CD11b and CD18, regardless of erythrocyte lysing agents, anticoagulant, and time of fixation (McCarthy, Macey, Cahill, and Newland, 1994; Macey, McCarthy, Milne, Cavanaugh, and Newland, 1999). Conversely, other groups demonstrate that granulocyte CD11b expression and platelet-leukocyte conjugate formation are increased with sample fixation after antibody staining (Repo, Jansson, and Leirisalo-Repo, 1993). Because of this, Hageberg and Lyberg (2000), fixed samples before antibody staining and demonstrated a reduction in platelet-leukocyte conjugation, which they determined was closer to in vivo levels. Even though the use of sample fixation is controversial, it is often necessary, because cell interactions and surface antigen expression change over time (Macey, McCarthy, Vordermeier, Newland, and Brown, 1995; Hageberg, and Lyberg, 2000). Therefore, understanding the degree of cellular change caused by fixation during sample preparation and its effect on results is imperative to the development of flow cytometric methods with the least amount of experimental artifact possible.

Mice are commonly used to examine immune function in disease models, however, little is known about the effects of various flow cytometry methods on mouse blood. In order to address the methodologic issues of leukocyte identification and cell fixation in mouse blood, the purpose of these experiments was to compare the effects of 1) LDS-751 and anti-CD45 monoclonal antibody for identification of leukocytes and 2) fixed and unfixed samples in a mouse whole blood preparation on intra- and extracellular staining methods for flow cytometry. Two protocols were compared: whole blood samples stained with 10 µg/ml LDS-751 or a fluorescently labeled monoclonal antibody to CD45 specific for mouse leukocyte common antigen. Both protocols were then compared with and without cell fixation with paraformaldehyde prior to antibody staining. Granulocyte CD11b glycoprotein expression and reactive oxidative species production were measured to evaluate changes on cell function (artificial activation) and the effects on extra- and intracellular staining by use of LDS-751 and/or paraformaldehyde fixation. We found that samples labeled with LDS-751 demonstrated decreased fluorescence intensity for granulocyte CD11b expression and ROS production in fixed and unfixed samples compared to anti-CD45. In addition, sample fixation decreased mean fluorescence in samples labeled with the extracellular marker CD11b, but not with the intracellular marker for ROS.

Materials and Methods

Animal Model

All animal experiments were conducted in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals (OLAW, 2002) after IACUC approval of all researchers. C57BKS/J mice (Jackson Laboratories, Inc., Bar Harbor, MA), age 12–16 weeks, 5–7 mice per group, were transported to the laboratory the day prior to blood acquisition in order to decrease stress-induced changes in leukocyte protein expression. Mice were allowed free access to food and water and were housed in a temperature controlled, quiet environment.

Blood Acquisition

Mice were anesthetized in a chamber of 3.5% isoflurane (JD Medical Distributing Co., Inc) and oxygen (Medical Grade Oxygen) at 0.8 L/minute flow until unresponsive. The mice were then mask-ventilated with 1.5% isoflurane and 0.8L/minute flow oxygen for maintenance

anesthesia. Eight hundred to 900 μL of blood was withdrawn from the ascending vena cava with a 23-gauge needle/1 mL syringe containing 0.14 mL of undiluted citrate-phosphate-dextrose (Sigma, Cat #C7165) (Leino, and Sorvajarvi, 1992;Repo, Jansson, and Leirisalo-Repo, 1995;Macey, et al., 1995;Peter, et al., 1999).

Whole Blood Staining Procedure

Whole mouse blood was kept at room temperature in 1.5 mL amber tubes and covered with aluminum foil to protect it from light during all incubations. Whole blood (WB) samples were first incubated with 0.5 $\mu\text{g}/100\mu\text{L}$ WB/PBS purified rat anti-mouse CD16/CD32 Fc γ III/II receptor blocking monoclonal antibody (PharMingen, Clone 2.4G2) for 15 minutes at room temperature to decrease non-specific binding of antibodies to leukocyte Fc receptors (BD Biosciences, 2005). All incubations were performed at room temperature to diminish changes in leukocyte surface antigen expression with cooling and rewarming of samples (Forsyth and Levinsky, 1990;Repo, Jansson, and Leirisalo-Repo, 1995). During the 15 minute incubation with the Fc receptor blocker, samples were also stained with 10 $\mu\text{g}/\text{mL}$ LDS-751 at a 1:1 LDS-751:WB concentration, or with an equal volume of PBS (final volume-100 μL).

Granulocyte positive control samples were incubated for 30 minutes with either lipopolysaccharide (LPS, Sigma #2680, diluted in PBS, final concentration 10 $\mu\text{g}/0.1\text{mL}$) for CD11b expression (Repo, Jansson, and Leirisalo-Repo, 1995), or with phorbol myristate acetate (PMA, (Sigma # P-148) diluted in dimethyl sulfoxide (Sigma #D-5879), final concentration 16 μM) for ROS production (Himmelfarb, Hakim, Holbrook, Leeb, and Ault, 1992;Vowells, et al., 1995;Alvarez-Larran, Toll, Rives, and Estella, 2005). After incubation with agonists or PBS, samples were fixed with paraformaldehyde (Sigma, catalog # P6148, final concentration of 0.5%) or diluted with PBS for 20 minutes at room temperature.

Monoclonal antibodies or isotype controls (Becton-Dickenson, San Jose, CA) were added to each sample after fixation and incubated for 15 minutes. Ten μL diluted (1:9 concentration with filtered PBS) peridinin chlorophyll-*a* protein (PerCP)-conjugated rat anti-mouse CD45 (leukocyte common antigen, Ly-5) monoclonal antibody (catalog # 557235, clone 30-F11) were added to samples not labeled with LDS-751. For CD11b measurement, 6.25 μL (1:9 concentration with filtered PBS) fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD11b (integrin α_{M} chain, Mac-1 α chain) monoclonal antibody (catalog # 557396, clone M1/70) and FITC-conjugated rat IgG2b, κ monoclonal immunoglobulin isotype control (catalog # 553988, clone A95-1) were added to their respective samples. For granulocyte reactive oxidative species (ROS) analysis, cells were incubated for 15 minutes with 10 μL 2',7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Cat #D399) (diluted with filtered PBS, 80 μM final concentration, stored at -80°C) (Bass, et al., 1983;Himmelfarb, Hakim, Holbrook, Leeb, and Ault, 1992;McDonagh, Hokama, Copeland, and Reynolds, 1997). After incubation with the monoclonal antibodies, all samples were diluted with 100 μL 1% cold paraformaldehyde or PBS, and placed on ice (refer to Table 1 for a summary of whole blood staining procedure).

Flow Cytometry Data Acquisition

Sample data was acquired by flow cytometry (FACScalibur, 488 nm argon laser, Becton Dickinson, San Jose, CA) within 3 hours of blood acquisition to decrease cellular changes that occur with time. CD11b expression is known to increase 3 hours from blood acquisition when unfixed samples are kept at 4°C (McCarthy and Macey, 1993) and ROS production increases after 3.5 hours when samples are kept on ice (Himmelfarb, et al., 1992). Calibration of the flow cytometer was performed daily prior to each experiment using Calibrite Beads and FACsComp software (Becton Dickinson). All samples were acquired on the low flow setting to avoid multi-

cell triggering of the flow cytometer (Himmelfarb, et al., 1992; Hageberg, and Lyberg, 2000) after adjusting PMT voltages to align negative control samples $<10^1$ on a log scale.

Flow Cytometry Analysis

FCS Express v2.0 (De Novo Software, Inc., Ontario, Canada) was used for all flow cytometry analyses. Spectral compensation was performed for each day's experiments by using negative and positive control samples. A dot plot of linear forward and side scatter properties of FL3 threshold events was used to identify and electronically gate the granulocyte population as demonstrated previously (Hageberg and Lyberg, 2000; Alvarez-Larran, Toll, Rivas, and Estella, 2005; Horn, et al., 2005). Each sample's fluorescence emission within the gated region was then analyzed with histograms for each fluorescent channel. The mean fluorescence intensity and number of positive events are reported for CD11b measurement of 5000 gated granulocytes, after background fluorescence subtraction ($<10^1$ on a log scale). Mean fluorescence intensity (MFI) of all 5000 gated granulocytes is reported ROS production.

Statistics

Three different group comparisons were made using t-test, or Mann-Whitney Rank Sum Test (non-parametric data) using SigmaStat 3.1 software (Systat Software, Inc., Point Richmond, CA). The group comparisons were 1) LDS-751 compared to anti-CD45 (i.e. unstimulated anti-CD45 v unstimulated LDS-751); statistically significant results denoted with #, 2) paraformaldehyde fixed compared to unfixed samples (i.e. anti-CD45 fixed v anti-CD45 unfixed); significant results denoted with +, and 3) unstimulated samples compared to samples stimulated with LPS (i.e. anti-CD45 unstimulated v anti-CD45 stimulated); significant results denoted with *. Results are represented as mean $\pm$ SEM. An a priori α of $p \leq 0.05$ was considered statistically significant.

Results

Granulocyte CD11b expression

There was no statistical difference in the number of CD11b positive events between samples labeled with anti-CD45 or LDS-751 (unfixed, unstimulated samples- anti-CD45 1273 ± 128 , LDS-751 1131 ± 83 , $p=0.419$; unfixed, stimulated samples- anti-CD45 2013 ± 253 , LDS-751 1329 ± 168 , $p=0.068$; fixed, unstimulated samples- anti-CD45 936 ± 86 , LDS-751 1425 ± 232 , $p=0.099$; fixed, stimulated samples- anti-CD45 1331 ± 114 , LDS-751 1606 ± 208 , $p=0.297$) (Figure 1). Additionally, fixation of the samples with paraformaldehyde did not significantly change the number of events in either samples labeled with anti-CD45 or LDS-751 (anti-CD45 unstimulated, $p=0.070$; stimulated, $p=0.056$; LDS-751 unstimulated, $p=0.300$; stimulated, $p=0.342$). The number of CD11b positive granulocytes increased with LPS stimulation in samples labeled with anti-CD45 (unfixed, $p=0.023$; fixed, $p=0.015$), but not with LDS-751 (unfixed, $p=0.321$; fixed, $p=0.574$).

Granulocyte CD11b mean fluorescence intensity was decreased in all samples labeled with LDS-751 compared to anti-CD45 (unfixed samples-unstimulated, anti-CD45 44.6 ± 3.5 , LDS-751 34.7 ± 1.3 , $p=0.073$, stimulated, anti-CD45 112.6 ± 26.3 , LDS-751 56.6 ± 9.0 , $p=0.048$; fixed samples-unstimulated anti-CD45 31.2 ± 3.6 , LDS-751 19.7 ± 0.8 , $p=0.009$, stimulated anti-CD45 65.9 ± 13.6 , LDS-751 29.3 ± 1.5 , $p=0.030$) (Figure 2). Sample fixation with paraformaldehyde also decreased CD11b mean fluorescence intensity in all samples (anti-CD45, unstimulated $p=0.021$, stimulated $p=0.189$; LDS-751, unstimulated $p<0.001$, stimulated $p=0.004$) (Figure 3). Despite the overall decrease in MFI, all fixed samples demonstrated a significant increase CD11b mean fluorescence with LPS stimulation (anti-CD45, unfixed $p=0.004$, fixed, $p=0.025$; LDS-751, unfixed $p=0.008$, fixed $p<0.001$).

Granulocyte reactive oxidative species production

Granulocyte reactive oxidative species production was measured by the mean fluorescence intensity (MFI) of all 5000 granulocytes gated in each sample. The measurement of ROS in unfixed samples was significantly decreased with LDS-751 staining (unstimulated samples- anti-CD45 18.4 ± 2.5 , LDS-751 8.3 ± 1.4 , $p=0.009$; stimulated samples- anti-CD45 56.6 ± 8.4 , LDS-751 17.7 ± 3.4 , $p=0.004$), but not in samples fixed with paraformaldehyde (unstimulated samples- anti-CD45 18.3 ± 5.3 , LDS-751 17.8 ± 1.9 , $p=0.926$, stimulated samples- anti-CD45 35.3 ± 7.6 , LDS-751 30.3 ± 3.5 , $p=0.662$) (see Figure 4). Fixation did not change ROS MFI in anti-CD45 samples (unstimulated, $p=0.429$; stimulated, $p=0.138$), and increased MFI in samples labeled with LDS-751 (unstimulated, $p=0.002$, stimulated, $p=0.027$). Similar to CD11b MFI, all samples demonstrated an increase in ROS MFI with PMA stimulation (anti-CD45 unfixed, $p=0.009$, fixed, $p=0.073$; LDS-751 unfixed, $p=0.015$, fixed, $p=0.010$).

Discussion

Our experiments demonstrate that the use of LDS-751 to delineate leukocytes in mouse whole blood underestimates mean fluorescence measurement of granulocyte markers. Compared to labeling leukocytes with anti-CD45, leukocytes labeled with LDS-751 demonstrated a general decrease in mean fluorescence intensity in samples measuring PMN CD11b expression and ROS production. The reason for the decrease in fluorescence, though, is not clearly understood. The labeling of samples with the intracellular dye, LDS-751, may produce functional changes to the cell that inhibit cell surface marker expression (demonstrated by an inability of the LDS-751 group to increase the number of CD11b positive cells with stimulation) and intracellular production of oxidative species. Another potential explanation for the results is that LDS-751 altered the fluorescent properties of the cells labeled with these FITC stains. In other experiments using a lower dose of LDS-751 ($1 \mu\text{g/ml}$), we observed similar findings as the $10 \mu\text{g/ml}$ LDS-751 group, but to a lesser degree, indicating that the decrease in fluorescence is dose dependent (unpublished observation). In any case, our experiments demonstrate that LDS-751 caused an overall decrease in fluorescence of FITC labeled cells for both intra- and extracellular stains and should be used with caution when analyzing granulocytes from mice using flow cytometry.

The process of fixing samples with formaldehydes produces protein cross-linking, increases number of cell surface aldehyde groups, and increases cell permeability (Shapiro, 2003). Thus, its use has been controversial for flow cytometric cell analysis because it could potentially alter monoclonal antibody adherence and allow outward diffusion of intracellular probes. McCarthy, et al., (1994) compared the effects of formaldehyde fixation before and after antibody staining and found that overall, fixation underestimated the mean fluorescence intensity of each antigen stained. Our findings are consistent with theirs, as we found that samples fixed with paraformaldehyde before staining demonstrated lower mean fluorescence than unfixed samples. Interestingly, samples labeled with LDS-751 demonstrated a significant increase in ROS measurement when fixed, in both the unstimulated and stimulated groups.

The timing of fixation is an important consideration in sample preparation for flow cytometry. Fixation before antibody addition may inhibit binding of fluorescent monoclonal antibodies to surface antigens. In this study, we fixed prior to antibody staining, similar to the methods used in other studies (Hageberg, and Lyberg, 2000;Repo, Jansson, Leirisalo-Repo, 1993). McCarthy, et al., (1994) found an overall decrease in mean fluorescence intensity with fixation before or after antibody staining. Because of this finding, we did not include a group that was fixed after sample staining. This is a limitation to the study, as the addition of a group fixed after sample staining would have provided further information on the effects of fixation on sample characteristics.

Few studies describe flow cytometry methods in whole mouse blood. It is important to note that in mouse blood, in contrast to humans, we found a consistently lower level of CD11b positive granulocytes. Human studies demonstrate constitutive expression of CD11b on granulocytes (80–90%) (Repo, Jansson, Leirsalo-Repo, 1993,1995; Alvarez-Larran, Toll, Rivas, and Estella, 2005; McCarthy, Macey, Cahill, and Newland; 1994). We obtained 15–30% positive CD11b granulocytes in unstimulated samples and 20–50% in stimulated samples. These results may be due to differences between species or other experimental artifacts. For example, mouse blood does not demonstrate well defined leukocyte populations on forward scatter/side scatter (FSC/SSC) dot plots as seen in human studies (unpublished observation). Thus, the granulocyte gate may be contaminated with lymphocytes that only express CD11b on a subset of cells. The choice of anticoagulant may also have underestimated the CD11b results. Citrate-phosphate-dextrose is a divalent cation chelator, and because CD11b is a Ca-dependent integrin (Leino, and Sorvajarvi, 1992), use of citrated anticoagulants may have reduced CD11b expression and its ability to bind with the fluorescent antibodies.

Development of reliable, valid methods for flow cytometry to analyze blood is challenging because blood cells interact and respond to their environment. There is a need to develop methods that closely reflect *in-vivo* conditions and that respond to positive controls. In addition, flow cytometry procedures in whole mouse blood have not been widely reported. The results of our experiments indicate that mouse leukocyte studies for flow cytometry should be conducted in whole blood with use of anti-CD45 for leukocyte identification. Also, while sample fixation prior to staining caused a decrease in overall fluorescence; it can still be used to successfully identify extracellular markers, but should be used with caution for measurement of ROS or other intracellular stains.

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Figure 1.

Number of granulocytes expressing CD11b with and without LPS stimulation in whole blood samples labeled with anti-CD45 or 10 μ g/ml LDS-751. Number of events expressing CD11b per 5000 PMNs in both unfixed samples and samples fixed with paraformaldehyde. Dark grey bars represent unstimulated samples, light grey bars are samples stimulated with LPS. Results are mean $\pm$ SEM. * $p < 0.05$, difference from unstimulated sample.

Figure 2.

Mean fluorescence intensity of granulocyte CD11b expression in whole blood samples labeled with 10 μ g/ml LDS-751 or anti-CD45. Mean fluorescence (arbitrary units) of 5000 PMNs in both unfixed samples and samples fixed with paraformaldehyde. Dark grey bars represent unstimulated samples, light grey bars are samples stimulated with LPS. Results are mean $\pm$ SEM. * p < 0.05, difference from unstimulated sample. # p < 0.05, difference from anti-CD45 sample. + p < 0.05, difference from unfixed samples.

3a.

3b.

Figure 3.

Histogram representation of granulocyte CD11b expression in fixed and unfixed samples. Shaded histograms are unfixed samples, open histograms are samples fixed with paraformaldehyde. (3a) samples labeled with anti-CD45, (3b) samples labeled with 10 µg/ml LDS-751.

Figure 4.

Mean fluorescence intensity of granulocyte reactive oxidative species production. Mean fluorescence (arbitrary units) of 5000 PMNs in both unfixed samples and samples fixed with paraformaldehyde. Dark grey bars represent unstimulated samples, light grey bars are samples stimulated with PMA. Results are mean $\pm$ SEM. * $p < 0.05$, difference from unstimulated samples. # $p < 0.05$, difference from anti-CD45 samples. + $p < 0.05$, difference from unfixed samples.

Table 1

Summary of Whole Blood Staining Procedure.

	LDS-751/Fc block stain × 15 min	Agonist addition × 30 min	Fixation × 15 min	Antibody/DCF-DA stain × 15 min	Final dilution
10 µg/ ml LDS-751	+ Fc receptor block + LDS-751	+ LPS, PMA, or PBS	+ PBS or PFA	+ anti-CD11b, or DCF-DA	+ PBS or PFA
anti-CD45	+ Fc receptor block	+ LPS, PMA, or PBS	+ PBS or PFA	+ anti-CD11b, anti- CD45, or DCF-DA	+ PBS or PFA

⁺ is the addition of reagent/solution, please refer to Methods section for amount and concentration

Times indicate incubation periods between reagent additions.