

APPLICATION NOTE

Detecting Intracellular Cytokines in Activated Monocytes

Scope

Originating in bone marrow, blood monocytes are incompletely differentiated, but mature to become macrophages/histiocytes on settling in tissues.¹ There they can become activated by various stimuli to assume differing forms having accessory or effector function, and whose principal activities are phagocytic and microbicidal.²

In natural immunity, effector cytokines are mainly produced by mononuclear phagocytes, and are termed monokines. Like cytokines, they are soluble protein hormones with both effector and immunoregulatory roles in normal and pathological responses. Monokines are responsible for the recruitment of other inflammatory cells, and the systemic effects of inflammation such as fever. Monocyte production of growth factors for fibroblasts and vascular endothelium indicates their involvement in tissue repair.

A high proportion of studies on monokine responses and production have utilized body fluids or tissue culture supernatants and have adopted bioassays or immunoassays. Evaluation of monokines by time consuming and cumbersome bioassays requires cautious interpretation of complex data which may not be specific to a single cytokine. Immunoassays are of limited sensitivity where a minor population may be producing the monokine under investigation.

A direct cellular approach to lymphokine and monokine assessment is of great advantage and could indicate the anatomical site of production.³⁻⁶ In situ hybridization does provide such a technique, which is very sensitive though time consuming, and the detection of mRNA is not a guarantee that it will be translated.⁷

Flow cytometric detection of intracellular or cell associated cytokines provides for the examination of multiple cytokines within individual cells; discernment of discrete populations expressing particular monokines and estimation of their frequency; and allows monitoring of responses to specific stimuli.⁸⁻¹² Additionally the method is sensitive and specific, and is relatively rapid and convenient. In combination with phenotyping for cell surface or intracellular markers,^{10,11} this provides a powerful investigative approach to cytokine/monokine studies, complementing some existing methods, and having advantages over others.^{9,13-15} Flow cytometric re-evaluation of earlier studies using humoral substances might yield further insights.

The aims of cytokine investigation of inflammatory diseases* such as rheumatoid arthritis¹⁶⁻¹⁹ and multiple sclerosis,^{20,21} or the role of cytokines in sepsis²² and inflammation,²³ are to determine which cytokines are involved in the disease process so that they may be distinguished from those which merely correlate with immune activation. Key cytokines involved in the disease process provide a focus for attempts at immune intervention,^{24,25} whereas those reflecting activation could provide diagnostic or prognostic information,^{4,26} and may be useful in monitoring the course of a disease during treatment.

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London, 1997

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Materials and Methods

Cells

Whole blood or peripheral blood mononuclear cells (PBMCs). Collect blood for whole blood activation assays into sodium heparin VACUTAINER® tubes. FastImmune™ assays are incompatible with lithium heparin, EDTA, and ACD anticoagulants.

Reagents

1. Brefeldin A (BFA, Sigma Catalog No. B7651), or equivalent: Reconstitute in DMSO at 5 mg/mL; store in small aliquots at -20°C.
2. Lipopolysaccharide (LPS, Sigma Catalog No. L2654): Reconstitute in DMSO (or PBS) at 0.5 mg/mL; store in small aliquots at -20°C.
3. BD Biosciences fluorescent-conjugated monoclonal antibodies (mAbs) for lymphocyte and monocyte phenotyping. CD33 or CD14 can be used for monocyte identification.

NOTE: CD14 is an LPS receptor and can be downregulated by LPS treatment in some donors.

4. FACS™ Lysing Solution* (10X), dilute 1:10 in deionized water. Refer to package insert for details.
5. FACS Permeabilizing Solution (10X), dilute 1:10 in deionized water. Refer to package insert for details.
6. Wash buffer: PBS containing 0.5% bovine serum albumin (BSA) and 0.1% NaN₃.
7. FastImmune anti-cytokine and control mAbs
8. 1% paraformaldehyde

Equipment

1. Disposable 12 x 75-mm capped Falcon® polypropylene test tubes (BD Labware Catalog No. 2063), or equivalent
2. Disposable 12 x 75-mm capped Falcon polystyrene test tubes (BD Labware Catalog No. 2058), or equivalent
3. 37°C incubator with 7% CO₂
4. Vortex mixer
5. FACS brand flow cytometer
6. Centrifuge
7. Vacuum cell aspirator
8. Micropipettor with tips (Pipetman®, Rainin Instrument Co. Inc., or equivalent)

* US Patent Nos. 4,654,312; 4,902,613; and 5,098,849

Procedure

Activation

Activation is done in the presence of BFA which inhibits intracellular transport so antigens and cytokines produced during the activation will be retained inside the cell. The unstimulated control sample should also contain BFA.

1. Label each of two 12 x 75-mm polypropylene tubes (with caps): Unstimulated and Activated.
2. Add 10 µg BFA to the Unstimulated tube.
3. Add 10 µg BFA and 1 µg LPS to the Activated tube.
4. Add 1 mL whole blood (sodium heparin), 1 mL PBMCs in autologous plasma (prepare PBMCs using BD VACUTAINER Cell Preparation Tubes (CPTs) [BD VACUTAINER Catalog No. 362753] containing sodium heparin), or 1 mL PBMCs in tissue culture medium (2×10^6 cells/mL) to each tube.

NOTE: This procedure provides enough cells for staining 20 samples, based on 50 µL/test.

5. Cap tubes loosely and vortex.
6. Incubate at 37°C, 7% CO₂ for 4 hours.

Staining

1. Label 12 x 75-mm polystyrene tubes.
2. Add surface antigen-specific fluorescent-conjugated mAbs to the appropriate tubes.
3. Add 50 µL activated or unstimulated blood to the tubes. Mix well. Incubate for 15 to 30 minutes at room temperature in the dark.
4. Add 2 mL of 1X FACS Lysing Solution to lyse the red cells (and fix white cells). Vortex gently. Incubate 10 minutes at room temperature in the dark.

NOTE: At this point, samples can be frozen in PBS with 1% BSA and 10% DMSO.
5. Centrifuge at 500 x *g* for 5 minutes. Remove the supernatant; avoid disturbing the pellet.
6. Add 500 µL of 1X FACS Permeabilizing Solution. Mix well to resuspend the pellet. Incubate 10 minutes at room temperature in the dark.
7. Add 2 to 4 mL wash buffer. Centrifuge at 500 x *g* for 5 minutes. Remove the supernatant; avoid disturbing the pellet.
8. Add intracellular antigen-specific fluorescent-conjugated mAbs. Mix well. Incubate for 30 minutes at room temperature in the dark.
9. Repeat step 7.
10. Resuspend cells in 500 µL of 1% paraformaldehyde.
11. Analyze on a FACS brand flow cytometer. Samples can be stored at 4°C in the dark for up to 24 hours prior to analysis.

Data Acquisition and Analysis

1. Use CaliBRITE™ beads and the appropriate software, such as FACSComp™, version 1.1 or later, set on Lyse/NoWash, or AutoCOMP™, version 3.0.2, for setting the photomultiplier tube (PMT) voltages and the fluorescence compensation, and for checking instrument sensitivity prior to use. Refer to the appropriate TriTEST™ three-color application note for flow cytometric setup, acquisition, and analysis.

NOTE: Proper instrument setup with the correct version of FACSComp or AutoCOMP is important for obtaining accurate results with the FastImmune assay. Please contact your BD Biosciences representative if you have an older version.

2. Analyze prepared samples on a FACS brand flow cytometer. Acquire data with CellQuest™ or LYSYS™ II software, using a fluorescence or forward scatter (FSC) threshold. Acquire all events with at least 1,000 monocytes by setting an acquisition gate on CD14⁺ or CD33⁺ monocytes in SSC-monocyte marker dot plot.
3. Display data as two-color dot plots to determine cytokine expression. Analyze data using CellQuest, LYSYS II, PAINT-A-GATE^{PRO}™, or Attractors™ software.

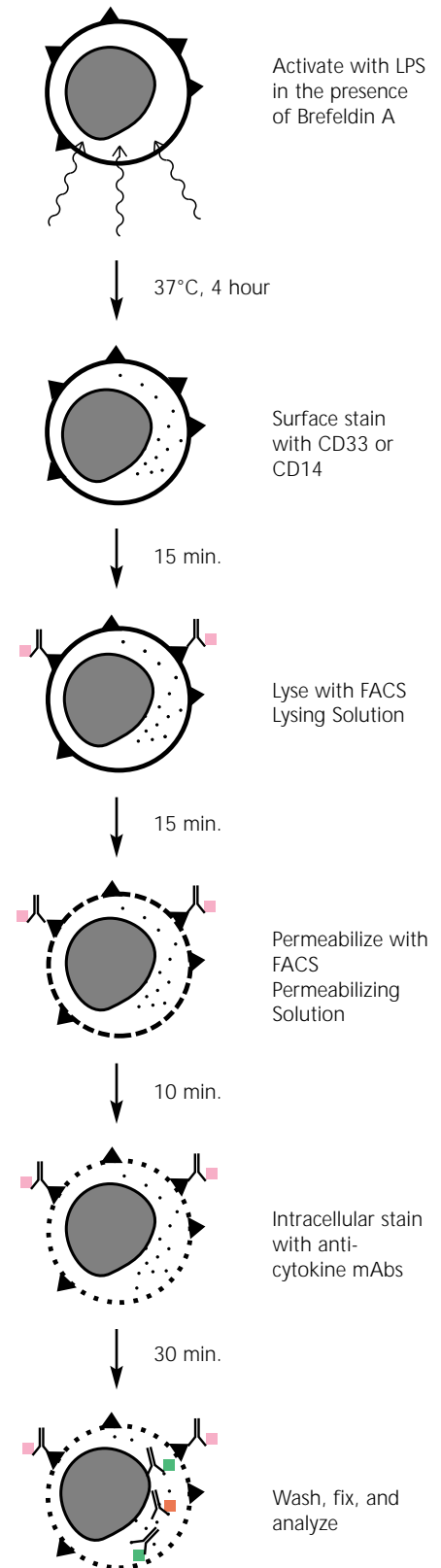


Figure 1 FastImmune procedure

Controls

The specificity of immunofluorescence detected in monocytes can be demonstrated by several methods. Standard techniques using isotype-matched irrelevant control antibodies are useful methods to control for Fc-mediated binding or other kinds of nonspecific interactions general to fluorochrome-conjugated antibodies and fixed, permeabilized cells. The isotype-matched controls are not, in fact, the same reagent, so that confidence in the similarity of concentration, reagent chemistry, purity, and solubility of protein needs to be assumed and is not always valid, especially when using reagents from several vendors.

When possible, use experimental approaches to examine the performance of the reagents themselves. For example, monocytes detected in blood treated with EDTA as an anticoagulant are not responsive to LPS and should show normal expression of surface markers but no upregulation of cytokines. Note that this can be very different from the unstimulated controls which may show upregulation of cytokines, even without the addition of LPS. This is especially true of IL-1 and TNF. Thus EDTA-treated blood can serve as a useful control for the specificity of the anti-cytokine antibodies.

Another strategy that is perhaps more useful procedurally is to simply omit the BFA from some samples. BFA is a potent inhibitor of the trafficking of consensus signal sequence bearing secreted proteins, including TNF- α , IL-6, and IL-8. Antibodies against these proteins bound to cells in the absence of BFA should be carefully evaluated. IL-1 α and IL-1 β are not secreted by this classical pathway and are unaffected by BFA treatment.

Perhaps the most convenient strategy using LPS-stimulated whole blood is to capitalize on the fact that LPS stimulates monocytes but not lymphocytes. Consequently, by using light-scatter properties or surface markers, unstimulated and, therefore, cytokine-negative lymphocytes can be identified in each sample and used to measure nonspecific binding of the anti-cytokine antibodies. It is true that autofluorescence, Fc-mediated binding, and other biological properties render lymphocytes as less-than-perfect controls for monocytes. However, gross problems with nonspecific behavior of antibodies can certainly be recognized by re-gating data files, even retrospectively, with no added sample preparation time or reagent expense.

LPS Stimulation of Whole Blood

LPS is a monocyte-specific stimulus. Figure 2 illustrates 4-hour LPS-stimulated whole blood gated on CD14⁻ lymphocytes (R1) and CD14⁺ monocytes (R2). Only the monocyte population is activated and producing the cytokine TNF- α .

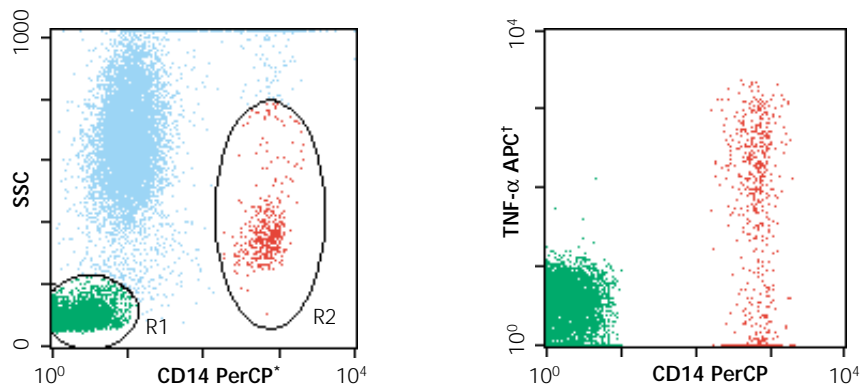


Figure 2 LPS-stimulated whole blood

CD33 Staining on Intact vs Permeabilized Cells

Surface markers can be stained before or simultaneously with intracellular cytokines. Surface staining reagents should be titrated if used in the intracellular staining step to ensure optimal results. The data below compares the two methods for the monocyte marker, CD33, on 4-hour LPS-stimulated whole blood. CD33 PE⁺ surface staining was performed before lysing and permeabilization of the cells (Figure 3a). Figure 3b demonstrates staining of CD33 PE in permeabilized cells.

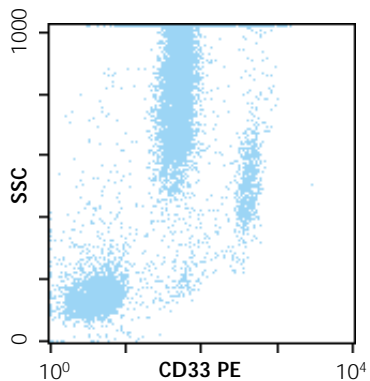


Figure 3a Intact cells

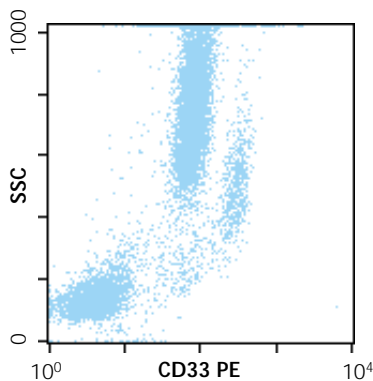


Figure 3b Permeabilized cells

* US Patent No. 4,876,190

† US Patent No. 4,520,110; European Patent No. 76,695; Canadian Patent No. 1,179,942

Whole Blood vs PBMCs (CPT)

Whole blood or PBMCs can be used in the FastImmune Cytokine Assay. The data shown in Figure 4 compares 4-hour LPS-stimulated whole blood with 4-hour LPS-stimulated PBMCs (in autologous plasma) gated on CD33⁺ monocytes (R2) and CD33⁻ lymphocytes (R1). Cytokine staining is similar for the two sample preparations.

The reagents used are TNF- α FITC/CD33 PE/CD45 PerCP.

Cells were collected using a CD45 PerCP (FL3) threshold.

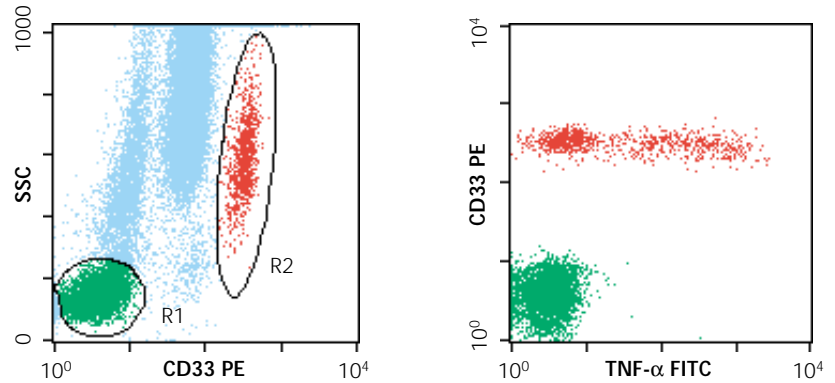


Figure 4a Whole blood

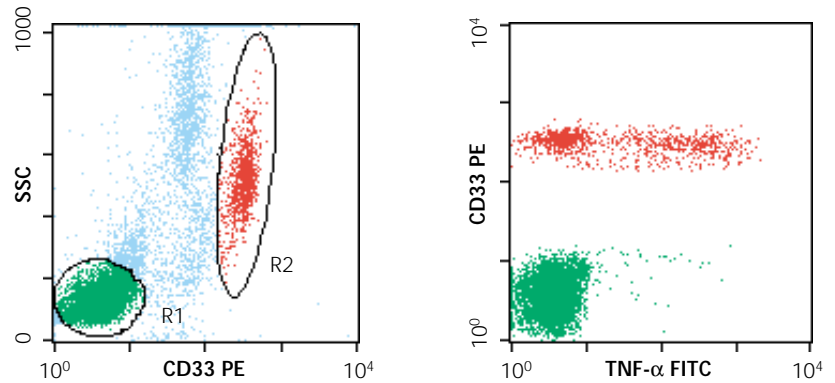


Figure 4b PBMCs (CPT prep)

Monocyte Cytokine Production Diversity

LPS-stimulated monocytes are able to produce a variety of cytokines. Figure 5 illustrates the use of four-color immunofluorescence staining. Four-hour LPS-stimulated whole blood was stained with CD14 PerCP (to identify monocytes) and three anti-cytokine antibodies. The three pair-wise combinations of anti-cytokine antibodies show CD14⁺ events.

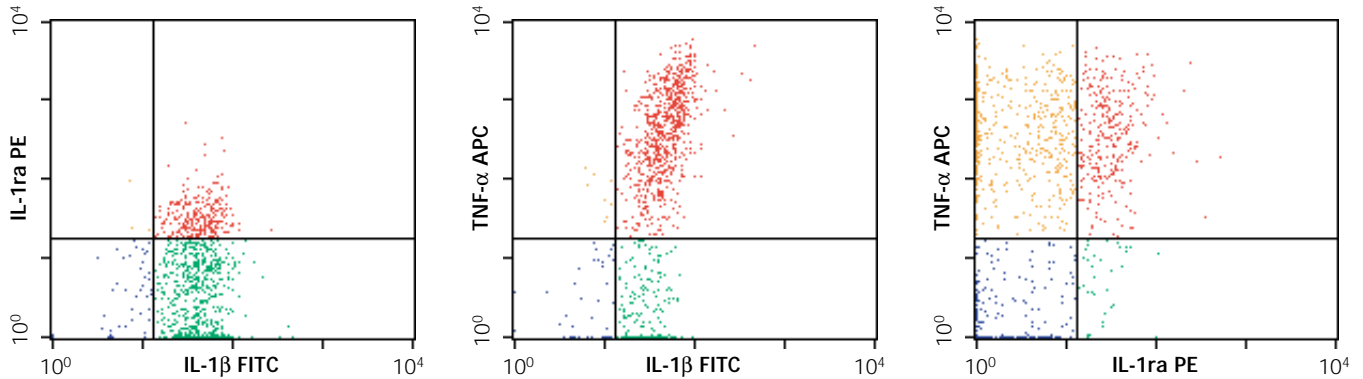


Figure 5a IL-1β FITC/IL-1ra PE/CD14 PerCP/TNF-α APC

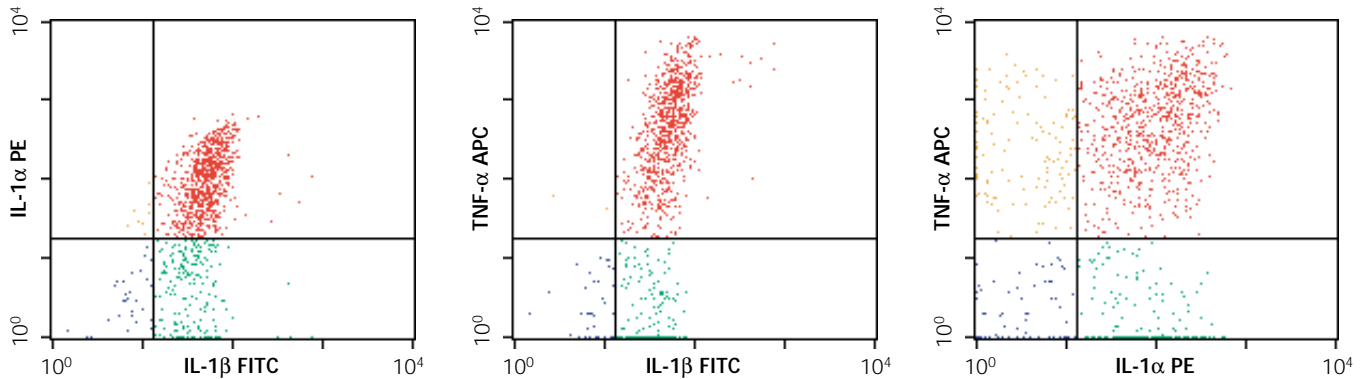


Figure 5b IL-1β FITC/IL-1α PE/CD14 PerCP/TNF-α APC

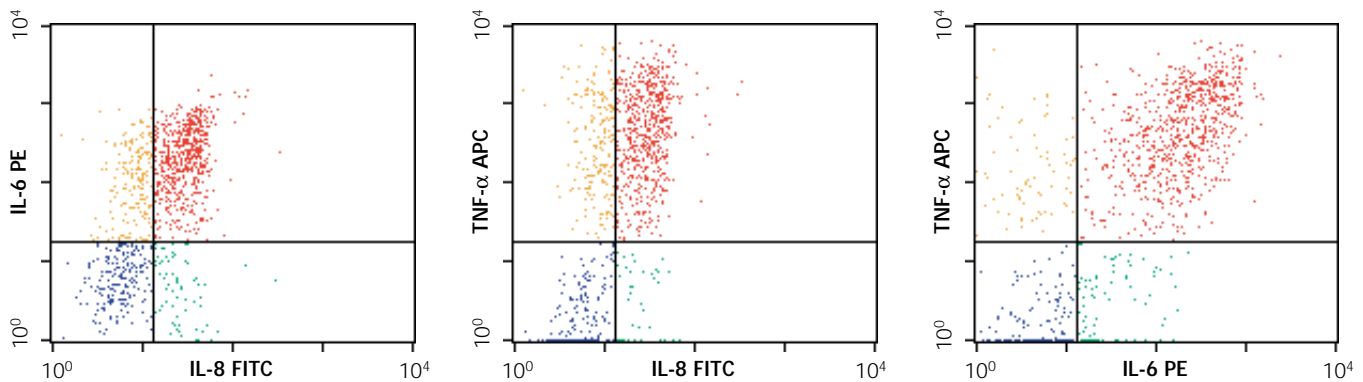


Figure 5c IL-8 FITC/IL-6 PE/CD14 PerCP/TNF-α APC

Troubleshooting

Problems	Possible Causes	Solutions	Comments
No intracellular staining of cytokines	Cells not activated	Lipopolysaccharides (LPS) – Check concentration (1 µg/mL of blood). – Do not freeze and thaw multiple times. – Dilute LPS in 1X PBS without azide. – Check expiration date on LPS.	BD Biosciences recommends freezing small aliquots at –20°C (20 µL at 0.5 mg/mL in DMSO). Reconstitute 1:10 in 1X PBS without azide. Add 20 µL of dilution to 1 mL of whole blood (final concentration is 1 µg/mL of blood). Do not refreeze aliquot.
	BFA inactive or incorrectly prepared	Brefeldin A – Check concentration (10 µg/mL of blood). – Do not freeze and thaw multiple times. – Dilute BFA in 1X PBS without azide. – Check expiration date on BFA.	BD Biosciences recommends freezing small aliquots at –20°C (20 µL at 5 mg/mL in DMSO). Reconstitute 1:10 in 1X PBS without azide. Add 20 µL of dilution to 1 mL of whole blood (final concentration is 10 µg/mL of blood). Do not refreeze aliquot.
	Wrong anticoagulant	Use sodium heparin as anticoagulant; do not use lithium heparin, EDTA, or ACD.	Ca ⁺⁺ is required for activation. Calcium chelating anticoagulants prevent activation. Monokine responses from freshly collected blood in EDTA can be partially recovered by incubation with normal Ca ⁺⁺ buffer.
	Cells not permeabilized	Treat cells with FACS Lysing Solution prior to treatment with FACS Permeabilizing Solution.	FACS Lysing Solution conditions cells for permeabilization.
Dim intracellular staining	Wrong concentration of anti-cytokine mAb	Use BD Biosciences antibodies at recommended concentrations.	This system was characterized using only BD FastImmune antibodies.
	Permeabilized cells not washed prior to stain	Wash permeabilized cells following protocol prior to staining.	
Poor resolution between surface stain subsets	Wrong concentration of surface marker	Titrate the surface marker. The loss of resolution may be caused by nonspecific staining of the surface Ab, or lowered activity of the antibody. Stain cells with surface marker prior to lysing step.	Epitopes for some of the surface markers may be down regulated during activation by internalization or by shedding.
	Incubation time	Incubate cells for at least 30 minutes at room temperature in the dark.	
Poor monocyte recovery	Wrong concentration of monocyte marker	Titrate monocyte marker concentration.	A higher than normal monocyte antibody concentration will result in nonspecific binding of the Ab to lymphocytes and granulocytes. Monocyte markers stain granulocytes dimly.
	Monocytes are sticking to incubation vessel	Add ice cold EDTA (2 mM) to blood after activation.	EDTA will bind Ca ⁺⁺ , which is needed for adherence of monocytes to vessel.
		Wash cells with ice cold buffer. Use polypropylene tubes instead of polystyrene tubes.	The temperature shock causes monocytes to release from the vessel wall.
		Use high-speed vortex, vigorous pipetting, or scraping to mechanically remove monocytes from vessel wall.	These mechanical removal methods can damage the cells.
Monocytes not recovered during centrifugation washing steps or aspiration steps	Centrifuge cells at 500 x g for 5 minutes. Decant supernatant instead of aspirating with vacuum.	Fixed cells have lower density than live cells; therefore, they require higher centrifugal force to pellet and are easily lost.	
Resting sample shows cytokine expression	Monocytes are activated	To confirm the specificity of the staining and rule out ex-vivo stimulation, see the Controls section of this application note.	LPS is a common contaminant of laboratory reagents.

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BD Biosciences publishes this method

as a service to investigators.

Detailed support for non-flow

cytometric aspects of this

procedure may not be

available from BD Biosciences.

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