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Research Article

Optimization of Stimulation and Staining Conditions for Intracellular Cytokine Staining (ICS) for Determination of Cytokine-Producing T Cells and Monocyte

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ABSTRACT

Cell-mediated responses to immunological stimuli are often localized in inflammatory sites and involve a number of cell types. These responses can be functionally characterized at the single-cell level on the basis of the types of cytokines expressed. The ability to measure antigen-specific cell responses at the single cell level is an important tool with a wide range of potential applications ranging from studies of disease pathogenesis to the evaluation of vaccines.

A number of experiments were performed in this study in order to establish the optimal conditions for stimulating in vitro cytokine production by lymphocytes and monocytes in blood samples collected from healthy adult Malawian participants and the optimal staining conditions for various cytokines and cell types. Different stimulation methods and conditions, different culture tubes and incubators and different antibody labelling conditions were assessed in order to establish optimal conditions.

The use PMA plus Ionomycin produced highest cytokine-producing T cells whereas LPS was a better stimulant for cytokine producing monocytes. Stimulation of whole blood for five hours was optimal for cytokine detection in T cells whereas four hours was optimal for monocytes. BFA was found to be a better Golgi blocker than Monensin and that the use of 15ml Falcon-type polypropylene tubes while stationary resulted in the detection of the highest proportion of cytokine-producing cells. T cells were found to be producers of mainly TNF-α, IFN-□ and IL-2 whereas Monocytes were mainly producing TNF-α and IL-6. 2µl of anti-CD3-PerCP, 2µl of anti-CD14-APC and 4µl of anti-cytokine-PE resulted in the best results. The highest cytokine production monocytes were detected when 2ml of FACS Lysing solution was used compared to the other volumes. These optimal conditions are essential in determination of proportion of cytokine-producing cells using ICS.

.**Keywords:** Intracellular cytokine staining; Stimulating conditions; Staining conditions

INTRODUCTION

Cell-mediated responses to immunological stimuli are often localized in inflammatory sites and involve a number of cell types. These responses can be functionally characterized at the single-cell level on the basis of the types of cytokines expressed [1]. The ability to measure antigen-specific T cell responses at the single cell level is an important tool with a wide range of potential applications ranging from studies of disease pathogenesis to the evaluation of vaccines [2]. Several methods have been developed that allow cytokine expression to be measured and these include enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunospot (ELISPOT), limiting dilution assay (LDA), real-time polymerase chain reaction (RT-PCR) and intracellular cytokine staining (ICS) [3].

Performing flow cytometric analysis on ICS cells allows individual characterization of large numbers of cells and can fully display the heterogeneity of cell populations.

A great advantage of ICS over the other methods especially ELISA is that multicolor staining can demonstrate exclusive or mutual co-expression of different cytokines in individual cells. This allows the characterization of T cell subsets on the basis of cytokine production rather than just surface markers [4]. Thus it is possible to analyze simultaneously CD4+ and CD8+ T cell responses in the same sample and to assess expression of other phenotypic markers on the cells of interest [2]. ICS by flow cytometry not only allows detection of single cell expression of cytokines but facilitates simultaneous detection, quantification, and phenotypic characterization of antigen-specific T cell subset

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in either whole blood or peripheral blood mononuclear cells (PBMCs). Through this method, antigen-specific T cells are identified based on their intracellular accumulation of a cytokine in conjunction with the activation markers such as CD69 following stimulation with specific antigens [5-6].

ICS involves incubation of whole blood or PBMC with a stimulant while disrupting intracellular Golgi-mediated transport using Brefeldin A (BFA) or Monensin which allows cytokines to accumulate yielding an enhanced cytokine signal that can be detected by flow cytometry. The cells are then permeabilised with a reagent such as FACS Permeabilising solution (FACS Perm) which allows antibodies to detect the intracellular cytokine. This method can detect multiple cytokines per cell and discrete cellular populations that express a particular cytokine. This is crucial especially when studying cytokine response to specific stimuli [7].The objective of this work therefore was to determine the optimal conditions for stimulating cytokine production by lymphocytes and monocytes in vitro using whole blood or PBMCs from Malawian adults and the optimal staining conditions for various cytokines and cell types. Different stimulation methods and conditions, different culture tubes and incubators were assessed in order to establish optimal conditions. Antibody labelling conditions were assessed using blood stimulated in loosely capped 15 ml Falcon-type tubes in a 5% CO_2 at 37ºC, for different durations. T cell stimulations were performed using 50% whole blood/ 50% RPMI with PMA+IO and monocyte stimulations were performed using undiluted whole blood with LPS. Whole blood was diluted when stimulated with PMA+IO because some had previously shown that stimulation of undiluted whole blood resulted in decreased cell viability [8]. Each test the results of which are being reported was conducted either in duplicate or in triplicate and data are presented as arithmetic means ± standard deviation.

MATERIALS AND METHODS

Study Participants, Ethics Clearance and Blood Samples

Blood samples used in this study were collected from healthy adult study participants. Ethical approval for the study was obtained from College of Medicine Research and Ethics Committee (COMREC) and written informed consent was obtained from each participant before taking part in the study. A 10ml venous blood sample was taken at the time of recruitment.

Preparation of Various Reagents

This being a study mainly aimed at investigating various stimulating and staining conditions in ICS various reagents were used at different volumes and concentrations.

Study PMA (Sigma Chemical Co., St. Louis, Mo)

A total weight of 0.1mg of PMA was reconstituted in 1ml of dimethyl sulfoxide (DMSO) (Aldrich) to give a concentration of 100µg/ml. This was stored in 50 µl aliquots at -20ºC until required. On the day of use, 2 µl of the stored solution was diluted with 198 µl PBS to provide a concentration of 1,000ng/ml. The recommended concentration for use is 10ng/ml and to achieve this 10 µl of 1,000 ng/ml was added to 1ml mixture of blood and RPMI-1640, which is a further 1:100 dilution. PMA was used with Ionomycin.

Ionomycin (Sigma)

A 0.5 mg sample of Ionomycin was reconstituted in 1ml of sterile ethanol in a sterile hood to make a 500µg/ml solution. This was stored in 50 µl aliquots at -20ºC until when required. At the time of setting up stimulation, a 1:5 dilution of the stock solution was made by mixing 2 µl of 500 µg/ml solution with 8 µl of PBS and this provided Ionomycin of a concentration of 100 µg/ml. The remaining stock solution was stored at -20ºC for use with subsequent stimulations. The recommended concentration for use is 1 µg/ml and to achieve this 10 µl of 100 µg/ml was added to 1ml mixture of blood and RPMI-1640.

Brefeldin A (BFA) (Sigma)

A 0.5mg portion of BFA was reconstituted in 1 ml of DMSO (Aldrich) in a sterile environment to make a 500 µg/ml solution. This was stored in 20 µl aliquots at -20ºC until required. At the time of setting up stimulation, a 1:5 dilution of the stock solution was made by mixing 2 µl of 500µg/ml solution with 8 µl of PBS and this provided a BFA solution of a concentration of 100 µg/ml. BFA was used at a final concentration of 1 µg/ml by adding 10 µl of 100 µg/ml to 1 ml mixture of blood and RPMI.

Lipopolysaccharide (LPS) (Sigma)

A total of 500 µg of LPS was reconstituted in 1 ml of DMSO (Aldrich) in a sterile environment and stored in 20 µl aliquots at -20ºC until required. At the time of setting up stimulation, a 1:5 dilution of the stock solution was made by mixing 2 µl of 500 µg/ml solutions with 8 µl of PBS and this provided an LPS solution of a concentration of 100 µg/ml. The recommended concentration for use is 1µg/ml and was attained by mixing 10 µl of 100 µg/ml LPS with 1ml of whole blood. 10 µl of diluted BFA was added as a Golgi blocker. The mixture was then vortexed and incubated at 37ºC, 5% CO_2 for 4hrs with caps loosely closed. A tube with 500µl heparinised blood, 5 µl BFA and 5 µl CD28 but without stimulant was also incubated under the same conditions as a negative control.

Staphylococcal enterotoxin B (SEB) (Sigma) and anti-CD28 antibody (BD)

500 µg of SEB was reconstituted in 1ml of sterile PBS. This was then stored in 20µl aliquots at 4ºC until required. A 1:5 dilution of the stock solution was made by mixing 2 µl of 500 µg/ml solution with 8 µl of PBS providing a SEB solution of a concentration of 100 µg/ml. The recommended final concentration for use is 1 µg/ml and to achieve this 10 µl of 100µg/ml was added to a 1ml mixture of whole blood and RPMI (or 4 µl to 400 µl), which is a 1: 100 dilution. In addition, 5 µl of anti-CD28 antibody, a co-stimulator for T cells, was also added at the start of the stimulation process.

Cytokine Stimulation Procedure

Two 1.5 ml polypropylene tubes were appropriately labeled; in one tube 200 µl of heparinised whole blood (WB) was mixed with 200 µl of RPMI-1640, 2 ml of PMA and 4ml of Ionomycin and stimulated at 37ºC for 4hrs. In the second tube, 200 ml of heparinised whole blood was mixed with 4ml LPS and incubated under the same conditions.

Labelling (Staining) Procedure of stimulated whole blood

Eight FACS tubes were used as per numbered in Table 1. Aliquots of 2µl of anti-CD3-PerCP (for T cells) and 1µl of anti-

CD14-APC (for monocytes) monoclonal antibody (mAb) were pipetted into each tube. 50µl of stimulated or unstimulated (negative control) whole blood was pippeted into each tube and the contents were mixed before incubating at room temperature for 15 minutes in the dark.

Where needed, monoclonal antibodies against lymphocyte surface markers were used to differentiate lymphocyte subpopulations. 50 µl of the specific stimulated or unstimulated blood sample was used per tube and mixed with the surface antibodies. The tubes were incubated in the dark at room temperature for 15 minutes.

After conducting various experiments on the ideal volume to use, 2 ml of 1x FACS lysis solution (Becton Dickinson) was added to each tube. The tubes were vortexed briefly and incubated again in the dark for 10 minutes. The tubes were vortexed again and centrifuge at 1,600 rpm and 4ºC for 5 minutes. The supernatant was aspirated and tubes were vortexed before 500 µl of 1x FACS Perm (Becton Dickinson) was added to each tube and incubated in the dark for 10 minutes. 1.5 ml of PBS + 0.5% Bovine Serum Albumin (BSA) (Aldrich) was added to each tube, briefly vortexed and centrifuged at 1,600 rpm at 4ºC for 5 minutes. The supernatant was aspirated and the tubes vortexed. 4µl of PE-conjugated anti-cytokine antibodies were pipetted to each tube as specified in Table 1.

Table 1: Type and amount of monoclonal antibodies used to label the whole blood samples.

Labelling details	PE	PerCP	APC
Whole blood isotype	Isotype	CD3	CD14
Whole blood unstimulated	TNF-a	CD3	CD14
Whole blood stimulated	TNF-a	CD3	-
Whole blood stimulated	TNF-a	-	CD14

Each tube was vortexed and incubated for 30 minutes in the dark at room temperature. 2ml of PBS + 0.5% BSA was added to each tube, vortexed and centrifuged at 1,600 rpm at 4ºC for 5 minutes. The supernatant was aspirated and the tubes vortexed before the cells were fixed with 200µl of PBS/1% formaldehyde solution. Samples were acquired on Flow Cytometer (Four Colour Becton Coulter FacsCalibur) within an hour of being stained and fixed.

Type and amount (volume) of Different stimulants used

The volumes of different reagents indicated in Table 2 were used for the stimulation of either T cells or monocytes. Following the stimulation stage, surface and intracellular labelling for T cells and monocytes was performed as explained already using anti-CD3-PerCP and anti-CD14-APC and anti-TNF-α before the permeabilisation stage.

Types of Cytokines detected in T cells and Monocytes

The ability of the study's assay to detect intracellular cytokines in T cells and monocytes in whole blood samples was assessed to establish which cytokines to study in clinical specimens as indicated in Table 3. Stimulation was performed as described earlier and the antibody labelling was performed as already described. 2µl of anti-CD3-PerCP, 1µl of anti-CD14-APC and 4µl anti-cytokine-PE were used.

Table 2: Type and amount of stimulant used

	Blood	RPMI	PMA	Iono-mycin	LPS	SEB	CD 28	BFA
Stimulated	200 ml	200 ml	4 ml	8 ml	-	-	-	8 ml
Unstimulated	200 ml	200 ml	-	-	-	-	-	8 ml
Stimulated	400 ml	-	-	-	8 ml	-	-	8 ml
Unstimulated	400 ml	-	-	-	-	-	-	8 ml
Stimulated	400 ml	-	-	-	-	8 ml	2 ml	8 ml
Stimulated	400 ml	-	-	-	-	-	2 ml	8 ml

Table 3: Names of cytokines and monoclonal antibodies used.

	PE	PerCP	APC
Isotype control	Isotype	CD3	CD14
Unstimulated control	TNF-a	CD3	CD14
Stimulated	TNF-a	CD3	CD14
Stimulated	IFN-g	CD3	CD14
Stimulated	IL-10	CD3	CD14
Stimulated	IL-6	CD3	CD14
Stimulated	IL-4	CD3	CD14
Stimulated	IL-2	CD3	CD14
Stimulated	TGF-b	CD3	CD14
Stimulated	IL-10 p40	CD3	CD14
Stimulated	IL-10 p70	CD3	CD14

Volume of Monoclonal antibodies to use for labelling T cells

50µl of stimulated and unstimulated blood (with all stimulations conducted according to the already explained procedure) were labelled with antibodies in volumes as indicated in Table 4 using the procedure that has already been explained.

Table 4: Volumes of different cytokine and antibodies used.

	CD4-FITC	Isotype or TNF-a-PE	CD3-PerCP	CD69-APC
1	1 ml	1 ml Isotype	1 ml	0.5 ml
2	1 ml	1 ml TNF-a	1 ml	0.5 ml
3	1 ml	1 ml Isotype	1 ml	0.5 ml
4	1 ml	1 ml TNF-a	1 ml	0.5 ml
5	2 ml	2 ml Isotype	2 ml	1 ml
6	2 ml	2 ml TNF-a	2 ml	1 ml
7	4 ml	4 ml Isotype	4 ml	2 ml
8	4 ml	4 ml TNF-a	4 ml	2 ml
9	8 ml	8 ml Isotype	8 ml	4 ml
10	8 ml	8 ml TNF-a	8 ml	4 ml

Effect of Volume of 1X FACS Lysing Solution on cytokines detected

Biosciences) or ammonium chloride solution is required when staining whole blood. In addition to lysing RBCs, FACS lysing solution also fixes cells. The effect of the volume of 1 X FACS lysing solution on cytokine detection was assessed.

Whole blood samples were stimulated using LPS for monocytes and PMA + Ionomycin for T cells. Stimulation was conducted in 15ml Falcon tubes (once it was established that the type of tubes used did not have any effect on the proportion of cytokine-producing cells) which were incubated in a 5% CO2 incubator for 4 hours. 50µl of stimulated blood from each tube was labelled with 2µl of anti-CD3-PerCP or 1µl of anti-CD14-APC as surface antibody and anti-TNF-α-PE as presented in Table 5.

Table 5: Volume of 1X FACS lysing solution used.

	Volume of FACS lysis	Cell type	PE	PerCP	APC
1	1 ml	Isotype control	Isotype	CD3	CD14
2	1.5 ml	Isotype control	Isotype	CD3	CD14
3	2 ml	Isotype control	Isotype	CD3	CD14
4	1 ml	CD3+TNF-a+	TNF-a	CD3	-
5	1.5 ml	CD3+TNF-a+	TNF-a	CD3	-
6	2 ml	CD3+TNF-a+	TNF-a	CD3	-
7	1 ml	CD14+TNF-a+	TNF-a	-	CD14
8	1.5 ml	CD14+TNF-a+	TNF-a	-	CD14
9	2 ml	CD14+TNF-a+	TNF-a	-	CD14

Effect of tube type and contribution of agitation on the amount of cytokine produced

Three types of polypropylene tubes were used for stimulations: 8ml Bijou-type tubes, 15ml Falcon-type tubes and 1.5ml Eppendorf-type tubes. To each tube, whole blood in RPMI and stimulant were added according to Table 6.

Tubes were vortexed and incubated at 37ºC for 4hrs. During the incubation, some tubes were placed on a rocker-plate (Bibi-Sterilin, 20 rpm) while others were left stationary in the incubator. 50µl of stimulated blood was labelled with 2µl of anti-CD3-PerCP as surface antibody and anti-TNF-α-PE.

Effect of Type of Incubator used on the amount of cytokine produced

To a series of 15ml Falcon tubes, whole blood (+/- RPMI) and stimulant were added in volumes indicated in Table 7. After mixing the contents of the different tubes, each tube was vortexed and their caps left loose and for the 1.5ml Eppendorf-type tubes, their covers were perforated to allow gas exchange. Half of the tubes were placed in a normally aerated incubator set at 37ºC and the other half were placed in an incubator supplied with 5% CO_2 at 37ºC.

Table 6: Type of tubes used, volume of blood and different reagents (µl) and stimulation conditions used.

Tube type and conditions	Blood	RPMI	PMA	Ionomycin
Bijou on rocker	500	500	5	10
Bijou stationary	500	500	5	10
15 ml Falcon tube on rocker	500	500	5	10
15 ml Falcon tube stationary	500	500	5	10
Eppendorf tube on rocker	200	200	2	4
Eppendorf tube stationary	200	200	2	4
Bijou on rocker	1,000	-	-	-
Bijou stationary	1,000	-	-	-
15 ml Falcon tube on rocker	1,000	-	-	-

50µl of stimulated blood mixture from each tube was labelled with 2µl of anti-CD3-PerCP or 1µl of anti-CD14-APC as surface antibody and anti-TNF-α-PE.

Table 7: Types of tube, volume of blood and different reagents (µl) and the incubation conditions used.

Stimulation conditions	Blood	RPMI	PMA	Ionomycin	LPS	BFA
15 ml Falcon in normal incubator	500	500	5	10	-	10
15 ml Falcon in CO_2 incubator	500	500	5	10	-	10
15 ml Falcon in normal incubator	1 ml	-	-	-	10	10
15 ml Falcon in CO_2 incubator	1 ml	-	-	-	10	10

Effect of the type of Golgi Blocker used on the amount of cytokine detected

Cytokines are produced in vitro when cells are activated by the use of an appropriate stimulant. In order to accumulate the cytokines within the cells as they are produced, protein secretion needs to be blocked by the addition of reagents that inhibit Golgi apparatus/endoplasmic reticulum function. The most widely used secretion blocking reagents are Monensin and BFA. These two reagents differ slightly in their mode of action. Monensin is an inhibitor of trans-Golgi function, whereas BFA inhibits protein transport between the endoplasmic reticulum (ER) and the Golgi [9]. In order to determine which of these two Golgi blockers would result in detection of more cytokines, stimulations were performed in eight 1.5ml polypropylene tubes. To each tube, either whole blood or whole blood diluted with an equal volume of RPMI-1640 was used together with stimulants using quantities indicated in Table 8.

Table 8: Volumes (µl) of blood and reagents and types of stimulants and Golgi- blockers used.

	Blood	RPMI	PMA	Ionomycin	LPS	BFA	Monensin
1	200	200	4	8	-	8	-
2	200	200	4	8	-	-	8
3	200	200	4	8	-	4	4
4	200	200	4	8	-	-	-

The tubes were incubated for 4 hours at 37ºC with 5% CO_2. PMA, Ionomycin, LPS and BFA were all prepared and used at concentrations already provided. For Monensin, 0.5mg was reconstituted in 1ml of sterile ethanol to give a 500µg/ml solution. 20µl aliquots were stored at 4ºC until required. A 1:5 dilution of the stock solution was made by mixing 2µl of 500µg/ml solution with 8µl of PBS and this provided a Monensin solution of a concentration of 100µg/ml. Monensin was used at a final concentration of 1µg/ml (10µl of 100µg/ml in 1ml). Surface and intracellular staining for T cells and monocytes were performed as already explained using anti-CD3-PerCP, anti-CD14-APC, anti-TNF-α.

Time Course Experiments for cytokine stimulation

Stimulations were performed in three 15ml polypropylene tubes. To each tube either whole blood or whole blood diluted with an equal volume of RPMI-1640 was used and stimulants were added in the quantities indicated in Table 9.

Table 9: Volumes of stimulants, RPMI and blood used in each tube.

	Blood	RPMI	PMA	Ionomycin	LPS	BFA
Unstimulated	500 ml	500 ml	-	-	-	10 ml
Stimulated T cells	500 ml	500 ml	10 ml	10 ml	-	10 ml
Stimulated monocytes	1 ml	-	-	-	10 ml	10 ml

The tubes were incubated for 4 hours for LPS stimulation and for 8 hours for PMA+IO. All stimulations were performed at 37ºC, 5% CO_2. PMA, Ionomycin, LPS and BFA were all prepared and used at concentrations already provided. At different time intervals, a 50µl aliquot was taken from each tube. Surface and intracellular staining for T cells and monocytes was performed with anti-CD3-PerCP and anti-CD14-APC added as surface antibodies before permeabilisation and anti-TNF-α added after the permeabilisation stage.

RESULTS

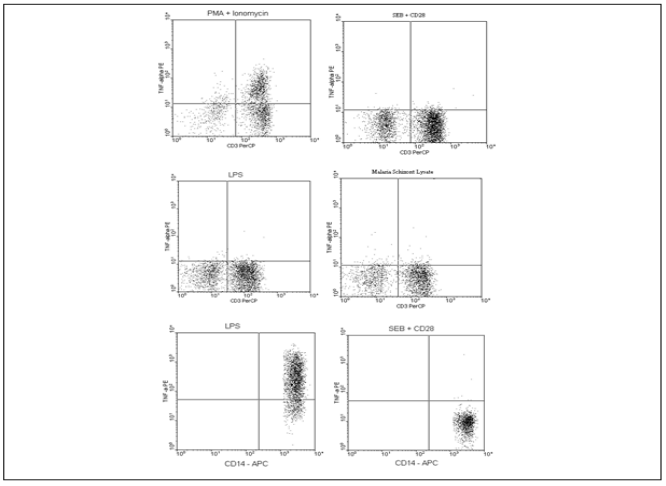
Type and Amount of Different Stimulants Used

Using PMA+IO as the stimulant resulted in a higher proportion of TNF-α producing T cells compared to the combination of SEB and CD28 (Table 10 and Figure S1). As expected, PMA+Ionomycin and SEB+CD28 did not work well as stimulants for cytokine production in monocytes. Instead, LPS produced the highest percentage of TNF-α- producing monocytes (Table 10).

Table 10: Means (± standard deviation) of percent of TNF-α expressing T cells and monocytes in whole blood samples using different stimulants.

	Type of stimulant	%CD3+TNF-a+	%CD14+TNF-a+
1	PMA+Ionomycin	48.86 ± 10.07	2.98 ± 0.63
2	SEB+CD28	6.36 ± 0.95	4.70 ± 0.86
3	LPS	1.74 ± 0.10	84.29 ± 2.74

Figure S1: Flow plots showing the percentage of TNF-producing T cells and monocytes after stimulation with PMA+IO, SEB+anti-CD28, LPS and malaria schizont lysate. Gating was set using unstimulated blood.



Type of Cytokines Detected in T cells and Monocytes

For monocytes, the two cytokines, TNF-α and IL-6 were detected in substantially higher levels compared to the other cytokines (Table 11). In contrast, higher percentages of TNF-α, IFN-α and IL-2 producing T cells were detected compared to the percentage T cells producing the other cytokines (Table 11). Only TNF-α was detected in both T cells and monocytes.

Table 11: Means (± standard deviation) of percentage of cytokine producing monocytes and T cells in blood samples from three donors

		% CD3+cytokine+	% $CD14^+$+cytokine+
1	Isotype	4.44 ± 0.27	3.16 ± 1.48
2	Unstimulated (TNF-a)	2.78 ± 0.43	2.58 ± 0.34
3	TNF-a	50.00 ± 7.30	88.33 ± 2.39
4	IFN-g	35.76 ± 2.13	2.48 ± 0.88
5	IL-10	4.81 ± 0.28	2.72 ± 0.66
6	IL-6	2.42 ± 0.99	88.82 ± 4.47
7	IL-4	7.96 ± 1.38	4.99 ± 0.48
8	IL-2	50.24 ± 3.35	1.41 ± 0.54
9	TGF-b	3.67 ± 2.15	2.24 ± 0.43
10	IL-12 p40	2.46 ± 1.14	2.98 ± 0.61
11	IL-12 p70	2.22 ± 0.60	3.69 ± 1.13

The Volume of Monoclonal Antibodies to use for labelling T cells

The highest percentage of TNF-α producing T cells was detected with 4µl of mAb (Table 12). For CD69-APC, percentages were similar for all volumes of antibody tested.

Table 12: Means (± standard deviation) of percent of TNF-α producing T cells and that of CD69+ T cells when different volumes of antibody were used.

Volume of TNF-a or CD69	CD3+TNF-a+	CD3+CD69+
1 ml	18.64 ± 0.10	49.48 ± 0.77
2 ml	23.80 ± 1.29	50.10 ± 1.08
4 ml	25.25 ± 0.71	49.46 ± 4.23
8 ml	24.79 ± 0.42	49.74 ± 1.82

Effects of volume of 1X FACS Lysing Solution on the amount of cytokine detected

For monocytes, as the volume of 1 X FACS lysing solution was increased from 1ml to 2ml, the amount of TNF-α detected also increased (Table 13 and Figure S2). However the percentage of TNF-α producing T cells was not affected by the volume of FACS Lysing solution (Table 13).

Table 13: Means (± standard deviation) for three donors of the percentage of TNF-α producing T cells and monocytes with different volumes of 1X FACS Lysing solution.

	Volume of 1X FACS lysing solution (ml)	CD3+TNF-a+
1	1	52.54 ± 2.79
2	1.5	52.43 ± 6.96
3	2	50.24 ± 2.14

Effect of Tube Type and Agitation on the amount of cytokine detected

The type of tubes in which the blood was stimulated made little difference to the percentage of TNF-α producing monocytes (with the exception of Eppendorf polypropylene tubes left stationary) (Table 14). The highest amount of TNF-α-producing T cells was observed when 15ml Falcon tubes were used.

When 15ml Falcon tubes were used, there were more TNF-α producing T cells when the tubes were left stationary compared with tubes placed on a rocker-plate, though the opposite was observed for monocytes (Table 14).

Table 14: Means (± standard deviation) for two donors of percentage of TNF-α producing T cells and monocytes when different stimulating tubes and conditions were investigated.

	Stimulation container and conditions	CD3+TNF-a+	CD14+TNF-a+
1	Bijou on rocker	50.75 ± 7.13	85.16 ± 1.12
2	Bijou stationary	43.15 ± 4.85	85.95 ± 1.97
3	15ml Falcon tube on rocker	52.97 ± 9.77	89.72 ± 0.68
4	15ml Falcon tube stationary	59.49 ± 3.60	85.32 ± 4.86
5	Eppendorf tube on rocker	41.71 ± 1.88	83.21 ± 3.04
6	Eppendorf tube stationary	41.58 ± 0.69	75.76 ± 8.03

Effect of Type of Incubator Used on the amount of cytokine detected

The percentages of TNF-α producing T cells (54.06%) and monocytes (86.58%) obtained when the stimulation was performed in an incubator supplied with 5% CO_2 were similar to those observed when stimulation was performed in a normally aerated incubator (55.29% for TNF-α producing T cells and 85.86% for TNF-α producing monocytes) (Table 15).

Table 15: Means (± standard deviation) for three donors of percentage of TNF-α producing T cells and monocytes when two types of incubators were used.

	Type of incubator	CD3+ TNF-a$^+$	CD14+TNF-a$^+$
1	Normal incubator	55.29 ± 3.07	85.86 ± 3.41
2	5% CO_2 incubator	54.06 ± 5.07	86.58 ± 2.40

Effect of the Type of Golgi Blocker on the amount of cytokine detected

Using a combination of BFA and Monensin produced similar levels of TNF-α-producing T cells as when BFA used on its own (Table 16 and Figure S3). Using Monensin on its own resulted in the detection of the lowest percentage of TNF-α producing T cells. The highest percentage of TNF-α producing monocytes was detected when BFA was used on its own and the least percentage was observed where Monensin was used on its own (Table 16).

Table 16: Mean (± standard deviation) for two donors of percent of TNF-α producing T cells and monocytes when BFA, Monensin or a combination of the two were used.

		% CD3+TNF-a+	% CD14+TNF-a+
1	BFA	52.36 ± 7.83	89.46 ± 1.05
2	Monensin	30.73 ± 2.77	60.17 ± 5.30
3	BFA+Monensin	59.70 ± 4.04	71.98 ± 4.95

Results of the Time Course Experiment

After 1hr of stimulation 53% of the monocytes were already producing TNF-α and only 15% of the monocytes were producing IL-6 (Figure 1), indicating that TNF-α is produced more rapidly by monocytes compared with IL-6. The peak proportions of cytokine-producing monocytes (about 85% for both TNF-α and IL-6) could be detected after the samples had been incubated for 3 hours and this was maintained at the 4-hour stimulation stage.

Production of TNF-α by T cells occurs at a faster rate compared to that of IL-2 and IFN-α with over 25% of T cells already producing TNF-α after 1 hour of stimulation while no IL-2 or IFN-α was detected at the same time point (Figure 2). Forty-five percent was the highest proportion of T cells producing TNF-α and this was observed after 4 hours of stimulation. The highest percentage of IL-2-producing T cells, about 48%, was observed after 6 hours of stimulation (Figure 2). The highest percentage of IFN-α-producing T cells was 28% and this was observed after 4 hours of stimulation (Figure 2).

DISCUSSION

The combination of PMA and Ionomycin (PMA+IO) has been used as an activating stimulus to induce cytokine expression in a number of studies with good results. Since the number of antigen-specific cytokine responding T cells is usually low and therefore can be difficult to detect, the PMA+IO combination is often used since it can potentially activate all T cells independent of their antigen-specific receptors [3]. One of the drawbacks of PMA+IO is that it induces a marked decrease in the levels of CD4 expression and as such the proportion of cytokine producing CD4+ T cells can be detected to be artificially lower than normal due to the effect of the PMA+IO.

This study also showed that when stimulating whole blood with PMA-IO, only TNF-α IFN-α and IL-2 producing T cells could be detected in substantial amounts compared with background numbers. In addition, only TNF-α and IL-6 producing monocytes were observed in substantial amounts in blood samples stimulated with LPS. Some researchers investigated the influence of the degree of dilution of whole blood and the incubation period on whole blood and PBMCs cultured with arious stimulants. The investigators assessed the expression of the cytokines IFN-α, TNF-α, IL-2, IL-4, IL-10, and IL-13 and found that the level of cells producing the cytokines other than IFN-αwere generally much lower, and those cells producing IL-4 and IL-13 were difficult to distinguish from background levels of unstimulated cultures [3].

In one study, high levels of TNF-α, IFN-α, IL-2 and TGF-β producing CD4+ and CD8+ T cells, IL1-α, IL-6 and IL-8 producing monocytes were observed but only minimal amounts producing IL-10 [10]. Other studies have also reported equally high levels of IL-12 and TNF-α producing monocytes in LPS stimulated cultures [8]. These variations in the type of cytokines observed in different studies suggest that the detection of each cytokine requires different stimulation and labelling conditions.

Although most, if not all, monoclonal antibodies come with instructions that include the volume of the antibody to be used per a specified volume of whole blood or PBMCs, it is always good practice that before one embarks on a major project, one performs a proper and systematic dilution exercise of the antibodies to determine the appropriate and ideal ratio of antibody to whole blood to use.

Although the FACS Lysing solution is mainly used for lysing the RBCs in the whole blood cells, it came as a surprise to us to observe that the volume of this solution used had a major bearing on the proportion of cytokine-producing monocytes. This study established that the highest percentage of TNF-α producing monocytes was obtained when 2ml of 1 X FACS Lysing solution was used. However, the amount of FACS Lysing solution did not affect the percentage of TNF-α producing T cells. The BD recommendation is to use 2ml of the 1 X FACS Lysing solutions and 500µl of 1 X FACS permeabilising solution when 50µl of activated cells are used [11]. In this study using 1 X FACS lysing solution in any lower ratio than 50µl whole blood to 2ml of 1 X FACS lysing solution resulted in lower percentages of TNF-α and IL-6 producing monocytes. Since lower volumes of 1 X FACS lysing solution had sufficed for conventional immunophenotyping with surface marker labelling, it is possible that this high volume of 1 X FACS lysing solution is necessary to fix intracellular cytokines within monocytes thereby preventing them diffusing out of these cells following permeabilisation.

The results of the study also showed that the type of tubes used during stimulation does not make much difference in the amount of cytokine produced. This might be because all three were made from polypropylene, but of different sizes and from different suppliers. Very few investigators have studied this aspect of cytokine stimulation. Various groups have used polyvinyl chloride (PVC) tubes [10], capped polystyrene round bottom tubes [3, 12], and Falcon-type 2063 non-stick polypropylene round-bottom tubes [13-14, 6]. None of these groups have investigated the effect of different tubes on cytokine stimulation although cells, especially monocytes, have been observed to adhere to the sides of polystyrene tubes [10].

The findings of this study also showed that shaking the tubes on a rocker during stimulation did not enhance cytokine production. [15] investigated the influence of shaking on the kinetics of TNF-α release induced by Cryptococcus neoformans. The investigators found that shaking resulted in a very rapid release of TNF-α and IFN-α which was then followed by a fast decrease in the levels of these cytokines for both C. neoformans-stimulated and LPS-stimulated PBMCs. The reduction in levels of the cytokines was attributed to them being broken down to fragments that could not be detected. Chaka et al also found that when tubes were left stationary the kinetics of TNF-α release appeared to be protracted, with detectable levels of TNF-α observed after 3 hours of stimulation and levels still increasing even after 18 hours of stimulation [15].

The study also found that using an incubator supplied with 5% CO_2 during stimulation does not result in the release of higher percentage of cytokines compared with a non-CO_2 incubator. In an attempt to determine the device that generated the most consistent values across two time points from the same donor, Ray et al. assessed the effect of performing stimulations in a Dubnoff water bath set at 37ºC, a humidified 5% CO_2 incubator and a dry heat incubator on the frequencies of TNF-α production [16]. The investigators found that incubation in a water bath resulted in higher TNF-α production compared to when humidified CO_2 incubator or the dry heat incubator were used [16].

The finding that the use of BFA results in higher detection of TNF-α compared to Monensin is consistent with the findings of other studies. In one study BFA, and not Monensin, was found to be capable of completely blocking extracellular CD69 expression after in vitro stimulation with PMA-IO [9]. Other investigators also found that BFA was a more potent, effective, and less toxic inhibitor of cytokine secretion than Monensin [17].

Bueno et al, working with human peripheral blood showed that BFA was superior to a combination of BFA and Monensin as a secretion-blocking agent [8]. Their results showed BFA being associated with a higher percentage of cytokine-positive cells and greater amounts of detectable cytokines per cell compared to BFA in combination with Monensin. Monensin is an inhibitor of trans-Golgi function, whereas BFA inhibits protein transport between the endoplasmic reticulum (ER) and the Golgi [9]. BFA mode of action seems to make it a better Golgi blocker compared to Monensin.

One variable that has a greater bearing on the amount of cytokines produced in vitro is the incubation period after the addition of stimulus. Some studies [18-19, 13] have shown that a-six hour incubation with the addition of BFA for the last 4 hours provided substantial levels of cytokine expression. Results of this study show that four hours was the optimal stimulation time for production of TNF-α and IL-6 in monocytes (using LPS as stimulant) and TNF-α and IL-2 in T cells (using PMA+IO as stimulant). However, this study found that six hours was the optimal stimulation time for the production of IFN-α in T cells.

One study found that more diluted cells could be stimulated for a longer period without the integrity of the cells being affected. Activation with PMA+IO resulted in an increased frequency of CD4+IFN-α+ and CD8+IFN-α+ over time in cultures with whole blood diluted 1/5 or 1/10 in contrast to blood cultured at lower dilutions like 1/1 or ½ with the highest frequency observed in samples cultured for 72 hours [3]. We found that the main problem with 72-hours, or longer, stimulations is that cell integrity was affected and this makes gating for lymphocytes or T cells during data analysis very difficult.

CONCLUSION

The results of these sets of experiments could be useful for other researchers as they optimize stimulating and staining procedures for intracellular cytokine staining work just like our group did [20]. They provide a starting point for the optimal stimulation conditions for T cells and monocytes and on which cytokines to study as well as the optimal labelling conditions to use.

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REFERENCES

1. Maecker, HT. Cytokine flow cytometry. Methods Mol Biol.2004;263:95-108.
2. Gauduin MC, Kaur A, Ahmad S, Yilma T, Lifson JD, Johnson RP. Optimization of intracellular cytokine staining for the quantitation of antigen-specific CD4+ T cell responses in rhesus macaques. J Immunol Methods.2004;288:61-79.
3. Godoy-Ramirez K, Franck K, Mahdavifar S, Andersson L, Gaines H. Optimum culture conditions for specific and nonspecific activation of whole blood and PBMC for intracellular cytokine assessment by flow cytometry. J Immunol Methods.2004;292:1-15.
4. Pala P, Verhoef A, Lamb JR , Openshaw PJ . Single cell analysis of cytokine expression kinetics by human CD4+ T-cell clones during activation or tolerance induction. Immunology.2000;100:209-216.
5. Gauduin MC. Intracellular cytokine staining for the characterization and quantitation of antigen-specific T lymphocyte responses. Methods.2006;38:263-273.
6. Jung T, Schauer U, Heusser C, Neumann C, Rieger C. Detection of intracellular cytokines by flow cytometry. J Immunol Methods.1993;159:197-207.
7. Bueno C, Almeida J, Alguero MC, Sanchez ML, Vaquero JM, Laso FJ, et al. Flow cytometric analysis of cytokine production by normal human peripheral blood dendritic cells and monocytes: comparative analysis of different stimuli, secretion-blocking agents and incubation periods. Cytometry.2001;46:33-40.
8. Nylander S, Kalies I. Brefeldin A, but not monensin, completely blocks CD69 expression on mouse lymphocytes: Efficacy of inhibitors of protein secretion in protocols for intracellular cytokine staining by flow cytometry. J Immunol Methods.1999;224:69 76.
9. Hodge G, Markus C, Nairn J, Hodge S. Effect of blood storage conditions on leucocyte intracellular cytokine production. Cytokine.2005;32:7-11.
10. Becton Dickinson. Intracellular cytokine staining for flow cytometric analysis.1999.
11. Sewell WA, North ME, Webster AD, Farrant J. Determination of intracellular cytokines by flow-cytometry following whole-blood culture. J Immunol Methods.1997;209:67-74.
12. Nomura LE, Walker JM, Maecker HT. Optimization of whole blood antigen-specific cytokine assays for CD4+ T cells. Cytometry.2000;40:60-68.
13. Baran J, Kowalczyk D, Ozog M, Zembala M. Three-color flow cytometry detection of intracellular cytokines in peripheral blood mononuclear cells: comparative analysis of phorbol myristate acetate-ionomycin and phytohemagglutinin stimulation. Clin Diagn Lab Immunol.2001;8:303-313.
14. Chaka W, Verheul AF, Hoepelman AI. Influence of different conditions on kinetics of tumor necrosis factor alpha release by peripheral blood mononuclear cells after stimulation with Cryptococcus neoformans: a possible explanation for different results. Clin Diagn Lab Immunol.1997;4:792-794.
15. Ray CA, Dumaual C, Willey M, Fill J, O'Brien PJ, Gourley I, et al. Optimization of analytical and pre-analytical variables associated with an ex vivo cytokine secretion assay. J Pharm Biomed Anal.2006;41:189-195.
16. Schuerwegh AJ, Stevens WJ, Bridts CH, De Clerck LS. Evaluation of monensin and brefeldin A for flow cytometric determination of interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha in monocytes. Cytometry.2001;46:172-176.
17. Suni MA, Picker LJ, Maino VC. Detection of antigen-specific T cell cytokine expression in whole blood by flow cytometry. J Immunol Methods.1998;212:89-98.
18. Scheibenbogen C, Letsch A, Asemissen AM, Schmittal A, Thiel E, Keilholz U. Intracellular Cytokine Staining Cytokine Flow Cytometry for Characterization of Tumor Specific T cell Response. In: Nagorsen D., Marincola F. (eds) Analyzing T Cell Responses. Springer, Dordrecht.
19. Mandala WL, Msefula CL, Gondwe EN, Drayson MT, Molyneux ME, MacLennan CA. Monocyte activation and cytokine production in Malawian children presenting with P. falciparum malaria. Parasite Immunol. 2016; 38(5):317-25.