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Measurement of Cytokine Secretion, Intracellular Protein Expression, and mRNA in Resting and Stimulated Peripheral Blood Mononuclear Cells

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Quantitation of cytokine production is a valuable adjunct to standard immunologic assays in defining several pathologic processes. Nevertheless, there is little agreement about which tissues should be assayed, which type of assay should be performed, and which stimulation protocol should be used. As these types of assays enter the clinical arena, there is need for standardization. There is also a need to maximize the amount of information which may be derived from a single sample. We compared secreted interleukin 4 (IL-4), IL-2, IL-6, tumor necrosis factor alpha (TNF-α), and gamma interferon proteins as measured by enzyme-linked immunosorbent assay with intracellular cytokine production (IL-2 and gamma interferon) as detected by flow cytometry and quantitative competitive PCR for IL-2, IL-4, TNF-α, and gamma interferon mRNA and cDNA. Results from unstimulated cells and cells stimulated with phorbol myristate acetate, phytohemagglutinin, and phorbol myristate acetate plus phytohemagglutin were compared. All three methodologies detected significant stimulation of cytokine production. The combination of phytohemagglutinin and phorbol myristate acetate was overall the most-potent stimulus.

Measurement of cytokine levels has yielded useful information on the pathologic process in different disease states such as Crohn's disease and rheumatoid arthritis (9, 18). It may also be of use in the monitoring of disease progression and/or inflammation. For example, studies suggest that changes in cytokine production in sepsis and human immunodeficiency virus (HIV) can predict outcome (11, 15, 20, 22, 25). Cytokines may be measured in the serum or plasma, in diseased tissues, or in peripheral blood mononuclear cell (PBMC) preparations (4, 10, 17). Each of these strategies has advantages. Detection of cytokines in the serum of patients with sepsis, burns, or HIV has led to a better understanding of the inflammatory component of the host response in these settings (7, 11, 20, 22, 26). While obtaining and storing serum is technically simple, the detection of cytokines in serum has two major limitations. Cytokines often circulate as proteins bound to soluble receptors, carrier proteins, or inhibitors, which may mask their easy detection by enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (5, 23). Many cytokines are undetectable in serum because they are produced locally and have a very short half-life. In order to circumvent these significant limitations, cytokine production is often measured in affected tissues or PBMCs. Affected tissue often requires a biopsy and may be difficult to obtain, and controls are generally not available. Therefore, while affected tissue cytokine detection is theoretically optimal, there are significant practical limitations. This study utilized PBMCs and measured cytokine production by three different strategies. As cytokine detection becomes increasingly popular, it will be important to define the relative limitations and strengths of these different methodologies. This study explores the potential of using a single stimulation protocol for PBMCs from which multiple assays could be run. This is an important first step in the process of standardizing these assays so that data from different laboratories may be compared.

Three common cytokine detection strategies were utilized in this study. Each offers its own advantages and disadvantages. The detection of secreted cytokine protein is by far the most widely used type of analysis. Because secreted protein is the biologically relevant moiety, its detection is the closest possible representation of what the cells are responding to. Secreted protein is typically measured by ELISA or radioimmunoassay due to the simplicity and sensitivity of these methods. Bioassays are available for some cytokines (16), and they have the advantage of being able to distinguish active cytokines from inactive cytokines, such as is seen with transforming growth factor β or when some cytokines are complexed with soluble receptors.

The detection of intracellular cytokines is a relatively new technique in which cells are permeabilized and antibodies are used to detect cytokine protein within cells by flow cytometry (17, 19, 20). This methodology does not provide exact quantitation of cytokines but has the advantage of being able to identify which specific cells are producing the cytokine of interest.

Finally, the detection of cytokine messenger RNA has been used in a number of different settings (3, 6, 8, 12, 21). The use of PCR greatly increases the sensitivity of detection and has been used much more widely than Northern blots or RNase protection assays. Unfortunately PCR is not inherently quantitative. Two main strategies have been used to try to circumvent this problem. In the first strategy, the cytokine cDNA is amplified simultaneously with a cDNA target that is thought to be relatively constant in all cells. Typically this is actin or ribosomal cDNA. When proper controls have been performed and both targets are measured during log-phase amplification, this technique allows relative quantitation of the cytokine mes-

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sage (3). Generally, fivefold or greater changes between a test sample and a control can be detected. Because this is a relative quantitation technique, this methodology is generally not comparable from laboratory to laboratory. A more quantitative method is to spike each cytokine amplification mixture with a mimic that is coamplified by the same primers but generates an amplimer of a slightly different length. Because the amount of mimic spiked into each tube is known and the primers are in direct competition for both targets, one can directly quantitate the amount of cytokine target in each amplification by comparison with the amount of mimic (3, 21, 24). This technique has the advantage of producing true quantitation but is hampered by technical difficulty. The mimics for the cytokine targets are not widely available, and all PCR methodologies are hindered by the inefficiency of reverse transcription, RNA degradation, and PCR cross-contamination. Nevertheless, the use of mimics would seem to offer significant advantages, and the sensitivity of PCR over other methodologies represents an attractive option.

MATERIALS AND METHODS

Subject recruitment. Healthy adult controls (20 to 52 years old) were recruited locally. Informed consent was obtained. The Institutional Review Board of our institution has approved this study. Blood was obtained and used within 24 h. Sixteen donors were used for the detection of secreted protein, 6 donors were used for the detection of intracellular protein, and 18 donors were used for the detection of cytokine cDNA. The same assays were not always performed using the same donor, and therefore, comparative analyses are not possible.

Stimulation. Peripheral blood mononuclear cells were prepared using Ficoll-Paque (Amersham Pharmacia Biotech, Arlington Heights, Ill.) and resuspended in RPMI 1640 medium (Gibco BRL, Grand Island, N.Y.) with 10% fetal bovine serum. A total of 2×10^6 cells in 1 ml of medium were incubated for 48 h for each stimulation protocol for ELISA analysis, and 10^6 cells were incubated for either 4 or 24 h for each stimulation protocol for RNA analysis. Cells were incubated for 16 h with monensin for analysis of intracellular cytokines. A total of 10^6 cells in 1 ml of medium was used for each intracellular cytokine assay. Phorbol myristate acetate (PMA) (Sigma, St. Louis, Mo.) was used at a final concentration of 25 ng/ml for ELISA and RNA analysis and at 100 ng/ml for intracellular cytokine analysis. Phytohemagglutinin (PHA) (Sigma) was used at a final concentration of 2 μg/ml for ELISA and RNA analysis and 10 μg/ml for intracellular cytokine analysis. Monensin (PharMingen, San Diego, Calif.) was added to the cultures at the time of stimulation for the intracellular cytokine analysis at a final concentration of 2 μM.

Detection. Cell culture supernatants were harvested and stored frozen (−70°C). ELISAs for interleukin 2 (IL-2), IL-4, IL-6, tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) were performed on triplicate wells according to the manufacturer's instructions (Endogen, Woburn, Mass.). Studies have shown that ELISAs are generally reproducible, with a typical interassay variation of approximately 50% (1).

Staining and flow cytometry for intracellular IL-2 and IFN-γ were performed according to the manufacturer's protocol (PharMingen). Cells were simultaneously stained for cychrome-conjugated anti-CD3 and phycoerytherin-conjugated anti-CD45RO. Isotype controls were run with each set of samples and were used to define negative and positive cell populations. Once stained, the cells were permeabilized and fixed, and either fluorescein-conjugated anti-IL-2 or fluorescein-conjugated anti-IFN-γ antibodies (PharMingen) were added. The cells were stained according to the manufacturer's recommendations and were analyzed by flow cytometry. A Beckman-Coulter Epics ELITE flow cytometer was used. Gates on physical parameters and cychrome staining were integrated for the measurement of both cell surface CD45RO and intracellular cytokine (IL-2 or IFN-γ) staining. A total of 5,000 events were recorded for each assay.

RNA was prepared using TriReagent (Molecular Research Center, Cincinnati, Ohio), and cDNA was prepared by reverse transcription of RNA by avian myeloblastosis virus reverse transcriptase using oligo(dT) primers (Boehringer Mannheim, Indianapolis, Ind.). cDNA equivalent to 5×10^4 cells was used per amplification. Mimics and primers for IL-2, IL-4, TNF-α, and IFN-γ were obtained from Clontech (Palo Alto, Calif.) and used in a standard dilution series with 40 cycles of amplification according to the suggested protocol. Quantitation was performed by comparison of the cDNA target and mimic amplimers according to the manufacturer's recommendation. The lower limit of detection was generally 5×10^{-5} amol per 10^4 cells.

Statistics. The Wilcoxon test was used to compare results from unstimulated cells with the three stimulation groups. Data were treated as unpaired, and a two-tailed *P* value is reported. Data points which fell below the lower limit of detection for PCR were not included.

TABLE 1. Secreted cytokine production[a]

Treatment	Mean cytokine production ± SD (pg/ml)				
	IL-2	IL-4	IL-6	IFN-γ	TNF-α
No stimulation	27 ± 22	2 ± 4	49 ± 56	12 ± 11	15 ± 18
PHA	457 ± 581	44 ± 46	1,002 ± 578	653 ± 371	470 ± 552
PMA	293 ± 366	14 ± 30	672 ± 526	462 ± 428	646 ± 557
PHA + PMA	1,325 ± 278	68 ± 64	1,100 ± 556	1,005 ± 687	896 ± 529

[a] Supernatants were harvested at 48 h from 2×10^6 cells. Data from 16 donors are shown.

RESULTS

Unstimulated cells produced secreted cytokine proteins at very low levels (Table 1). IL-4 production was the most difficult to induce, and maximal stimulation with PMA and PHA resulted in a mean concentration of only 68 pg/ml. For all four cytokines, the optimal stimulus was the combination of PMA and PHA. IL-4 production was induced 34-fold compared to unstimulated cells, IL-2 was induced 49-fold compared to unstimulated cells, IL-6 was induced 22-fold compared to unstimulated cells, IFN-γ was induced 91-fold compared to unstimulated cells, and TNF-α was induced 75-fold compared to unstimulated cells. PMA resulted in less stimulation than PHA for each cytokine with the exception of TNF-α. When data were analyzed as paired sets, there was no correlation between reduced production of one cytokine and any other, nor was there any association between reduced response to one stimulus and reduced response to another stimulus. The secreted protein production from the dual stimulated cells (PHA and PMA) was significantly different than the protein production by the unstimulated cells for all five cytokines, with *P* values of <0.001 in all cases. The differences between PHA stimulation and dual stimulation ranged from not significant (IL-6 and IL-4) to moderately significant, with $P = 0.05$ (IFN-γ) and $P = 0.01$ (TNF-α), to very significant with $P = 0.0001$ (IL-2).

Intracellular cytokine staining was used to define the percent of total T cells (detected by anti-CD3 staining) producing IL-2 and IFN-γ. IFN-γ was not significantly induced by PHA, but all other stimulation protocols resulted in increased numbers of cells producing both cytokines (Fig. 1). For both IL-2 and IFN-γ, the combination of PMA and PHA was markedly more effective than either one alone, and PMA was superior to PHA. This methodology can be coupled with membrane staining for cell surface molecules to define mononuclear cell subsets. Because the large variation between individuals may represent variations in the cellular constituents of the mononuclear cells, cytokine production within the $CD3^+$ $CD45RO^+$ and $CD3^+$ $CD45RO^-$ T-cell subsets (i.e., memory phenotype and naive phenotype) were defined. The results from six donors are shown in Fig. 2. Here, the induction of both cytokines was most pronounced with the combination of PHA and PMA. In fact, there is significant synergy when both PHA and PMA are used. For example, IL-2-expressing cells within the $CD3^+$ $CD45RO^-$ T-cell subset (these cells were independently shown to be >95% $CD3^+$ $CD45RA^+$) constituted 0.3% of the unstimulated cells, 1.3% of the PHA-stimulated cells, 1.6% of the PMA-stimulated cells, and 13.9% of the PHA- and PMA- (dually) stimulated cells. Comparing the PHA-stimulated and the PMA-stimulated with the dually stimulated cells gave *P* values of 0.002 and 0.002. IL-2 expressing cells within the $CD3^+$ $CD45RO^+$ T-cell subset constituted 0.8% of the unstimulated cells, 2.2% of the PHA-stimulated cells, 3.4% of the PMA-stimulated cells, and 17.2% of the dually stimulated cells. Comparing the PHA-stimulated and the PMA-stimulated with the

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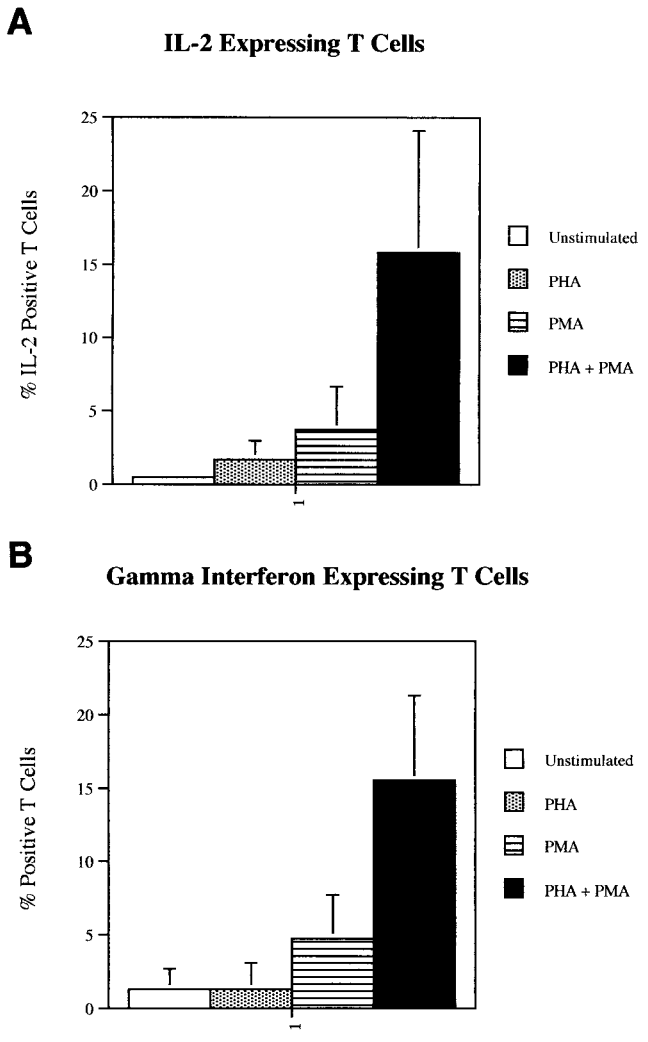

FIG. 1. Intracellular cytokine detection in total T cells. Comparison of IL-2 (A)- and IFN-γ (B)-producing cells from PBMC cultures stimulated with PHA, PMA, or PHA plus PMA. The total T-cell population was detected by anti-CD3 staining, and the percentage of total T cells producing either IL-2 or IFN-γ was determined ($n = 6$). Error bars, standard deviations.

dually stimulated cells gave P values of 0.004, and 0.009, respectively. IFN-γ expressing cells within the $CD3^+/CD45RO^-$ T cell subset constituted 0.9% of the unstimulated cells, 1.1% of the PHA stimulated cells, 3.4% of the PMA-stimulated cells, and 7.1% of the PHA and PMA dually stimulated cells. Comparing the PHA-stimulated and the PMA-stimulated with the dually stimulated cells gave P values of 0.009, and 0.485. IFN-γ-expressing cells within the $CD3^+$ $CD45RO^+$ T-cell subset constituted 1.8% of the unstimulated cells, 3.1% of the PHA-stimulated cells, 6.9% of the PMA-stimulated cells, and 28.4% of the dually stimulated cells. Comparing the PHA-stimulated and the PMA-stimulated with the dually stimulated cells gave P values of <0.001 and 0.002.

Cytokine cDNA was quantitated using a competitive quantitative PCR method. Two time points were analyzed and the three stimulation protocols were evaluated (Table 2). Again, IL-4 expression was the most difficult to induce. At 4 h, IL-2 message was increased 25-fold in the dually stimulated cells compared to controls, IL-4 was not induced, IFN-γ was induced 50-fold over in the dually stimulated cells compared to controls, and TNF-α was induced 350-fold in the dually stimulated cells compared to the unstimulated controls. At the 24-h time point, IL-2 message was 10-fold increased in the dually stimulated cells compared to controls, IL-4 was induced 1.5-fold over the unstimulated controls, IFN-γ was induced 10-fold over the dually stimulated cells compared to controls, and TNF-α was induced 40-fold in the dually stimulated cells compared to the unstimulated controls. These results are approximately in accordance with what was seen for secreted protein production. Direct comparison of results is not possible because different donors were used and there are significant interdonor differences in response to the stimuli. Also in accordance with the secreted protein results and the intracellular cytokine results, IL-2, IFN-γ and TNF-α expression was most strongly induced by the combination of PHA and PMA. PHA and PMA had generally modest effects on their own. Only the stimulation by PHA plus PMA resulted in a difference from unstimulated which was statistically significant for TNF-α. There were broad differences in expression between individual donors. Both the 4-h and 24-h time points resulted in increased cytokine message. Generally, the 4-h time point was superior to the 24-h time point, although the optimal stimulation protocol for TNF-α was the combination of PHA and PMA at 24 h.

DISCUSSION

The detection of cytokines provides valuable information about the nature of the host response to infection or the nature of an ongoing inflammatory response. Because of the great power of these types of studies, there has been interest in transplanting cytokine detection assays to a clinical arena. Before that can be accomplished, it would be desirable to define a type of stimulation protocol that would be broadly applicable and would maximize the amount of information that can be derived from a single patient sample. This study utilized three detection methods and three different stimulation protocols in an effort to identify one strategy from which several different types of information could be generated. In general, 24 h of stimulation with both PHA and PMA was the most potent stimulus. Extensive kinetic studies were not performed to determine the individual peaks of cytokine production using each cytokine and each methodology because the rationale for this study was to identify a common strategy to maximize the information from a single protocol. Nevertheless, others have found cytokine mRNA production to peak at 4 to 8 h and protein production to peak at 24 to 48 h (6, 8, 10, 11, 17, 22). We assayed a relatively small number of cytokines in this study although they were chosen to be representative of those most commonly implicated in various pathologic processes. Cytokines were selected to represent Th1 (IL-2 and IFN-γ production, Th2 (IL-4) production, and macrophage (TNF-α) production. Therefore, it is possible that this stimulation protocol may not be optimal for a different set of cytokines. Nevertheless, it is likely that one could use this stimulation protocol for most T-cell-derived cytokines.

One of the advantages of standardizing the stimulation protocol for multiple cytokines, is that a few million cells becomes sufficient for both secreted protein analysis, mRNA detection, and intracellular cytokine detection. The supernatant and cells from a single stimulation of 1 million cells are sufficient to detect five different cytokines in the supernatant and four different cytokines from the cellular mRNA. This has particular relevance in the pediatric population, where blood volume has been a significant constraint on this type of analysis. The flow cytometry requires the most cells, with 1 million cells required for each cytokine being assayed.

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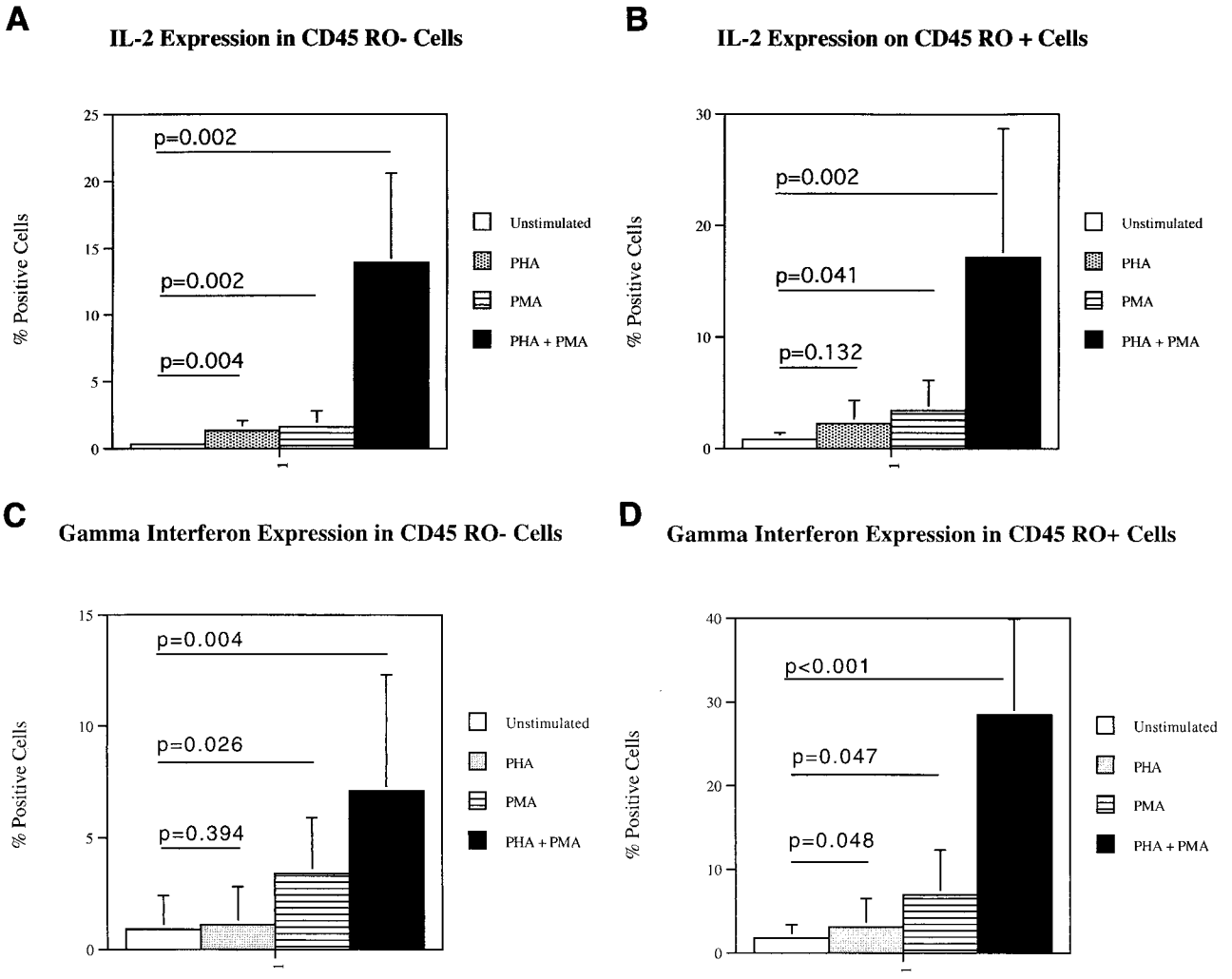

FIG. 2. Intracellular cytokine detection in T-cell subsets. Comparison of IL-2 (A and B)- and IFN-γ (C and D)-producing cells from $CD3^+$ $CD45RO^+$ (B and D) and $CD3^+$ $CD45RO^-$ (A and C) populations (n = 6). Error bars, standard deviations.

The regulation of expression of different cytokines depends on the rates of transcription, splicing, message turnover, translation, protein processing, export, and protein degradation. Each of these steps, in principle, is regulatable by different stimuli or inhibitors. It is generally believed that the most important sites of regulation are at the level of transcription, message turnover, and protein degradation. While cytokines share some general regulatory themes, the stimuli that maximally induce expression and minimize turnover and degradation are distinct for each cytokine. The use of PMA and PHA

TABLE 2. Cytokine message analysis[a]

Harvest time and treatment	Mean cytokine concn ± SD (amol/10^4 cells)			
	IL-2	IL-4	IFN-γ	TNF-α
4 h				
No stimulation	0.00012 ± 0.0001	0.0004 ± 0.000	0.00008 ± 0.00008	0.00002 ± 0.000
PHA	0.001 ± 0.0008	0.0002 ± 0.000	0.003 ± 0.004	0.0008 ± 0.001
PMA	0.002 ± 0.0008	0.0005 ± 0.0008	0.002 ± 0.004	0.0009 ± 0.002
PHA + PMA	0.003 ± 0.005	0.0004 ± 0.0008	0.004 ± 0.006	0.007 ± 0.0008
24 h				
No unstimulation	0.0002 ± 0.0001	0.0002 ± 0.000	0.00008 ± 0.00007	0.00005 ± 0.00007
PHA	0.0004 ± 0.0006	0.003 ± 0.0003	0.002 ± 0.003	0.0001 ± 0.00007
PMA	0.001 ± 0.001	0.0002 ± 0.0003	0.003 ± 0.004	0.0008 ± 0.0009
PHA + PMA	0.002 ± 0.005	0.0003 ± 0.0004	0.0008 ± 0.001	0.002 ± 0.003

[a] A total of 10^6 cells were harvested at the indicated time points. Data from 18 donors are shown.

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together activates multiple signalling pathways, which allows for broad stimulation of cytokines in mixed PBMC cultures (2, 13, 14).

A disadvantage of this method is that changes in the cellular constituents of the PBMC population, such as is seen in HIV infection, ongoing autoimmune disease, or advanced malignancies, may result in different patterns of cytokine production that reflect the cellular makeup of the PBMC population, not the inherent ability of the cells to produce certain cytokines. If flow cytometry is performed, it would be possible to characterize the cellular constituents and control for intersample variations in cellular makeup. While bulk stimulation of PBMC has the disadvantage of reflecting differences in cell distribution, it has the advantage of reflecting differences due to complex cell-cell interactions, which could be relevant for the individual disease processes being studied.

This study demonstrates the feasibility of using a single stimulation protocol on a single small sample of PBMCs to generate data on secreted cytokines, intracellular cytokines, and cytokine messages. This is an important first step in making these types of assays more applicable to a clinical setting. It is likely that defining cytokine expression will play an increasing role in the management of autoimmune disorders, for example. The use of a single stimulation protocol could also be of benefit in comparing results from different laboratories.

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