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# The Repertoire of Cytokines Produced by Human Leukocyte Subsets

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## ABSTRACT

We defined the cytokine repertoire of leukocyte subsets after activation with phorbol myristate acetate (PMA) and ionomycin (IONO) or lipopolysaccaride (LPS). We found that granulocytes produced only IL-8. IL-3, 11-13, IL-15, IL-18, TNFß and FLT3 were not produced by any subset. B cells exhibited no intracellular cytokines nor did T cells and NK cells when stimulated by LPS.

When stimulated by PMA + IONO, T cells and NK cells did not produce IL-4, IL-6 or IL-10, but produced INFγ, TNFα and MDP-1ß. T cell subsets also produced IL-la, IL-lα, IL-2, IL-8, IL-12, GM-CSF, G-CSF and M-CSF, except the CD4-CD8- subset, which did not produce IL-1α. NK cells produced the same cytokines except IL-2. Monocytes constitutively produced IL-8, but when stimulated by PMA + IONO, they produced IL-lα, IL-lß, IL-6, IL-8, IL-12, TNFα, M-CSF and MIP-lß. LPS induced additional production of IL-10 and G-CSF and enhanced the production of IL-6 by monocytes.

***Key Words:*** *Activation - Characterization - Flow cytometry.*

*Abbreviations used:*

PB : Peripheral blood.
TH1 : T-helper cell type 1.
TH2 : Helper cell type 2.
IL-X : Interleukin X.
TNF : Tumor necrosis factor.
PMA : Phorbal-13-myristate acetate.
IONO : Ionomycin.
LPS : Lypopolysaccharide.
FSC : Forward scatter.
SSC : Side scatter.
FITC : Fluoresce in isothiocyanate.
PE : Phycoerythrin.
PC-PE : Cyanin 5.
PcP : Peridinin chlorophyll protein.
APC : Allophycocyanin.
PBS : Phosphate buffered saline.
PAB : PBS supplemented with 0.5% bovine serum albumin (BSA) and 0.1% sodium azide.

## INTRODUCTION

Cytokines and chemokines are the molecules that provide communication among cells. A single cytokine is but one word, and it is the repertoire of them all when simultaneously presented to the target cell that results in the function elicited. The cytokines come from many different cell subsets, each of which is itself a cytokine target. Within this milieu occur the functions of life.

While there is a wealth of data on the characterization of the cytokines themselves extensively summarized **[1]**, nearly all the information about cellular production is from cell lines, predominantly murine, and to a lesser extent, normal murine hematopoietic cells. In human, most of our understanding about cytokines has focused on the TH1 and TH2 responses, and the characterization of the cells that produce the TH1 cytokines, IL-2, IL-4 and INFγ, or the TH2 cytokines) **[2-4]**. More recently, production of cytokines by dendritic cells has been evaluated because of their recognized importance in antigen presentation **[5-8]**. Furthermore, most of the research in our current understanding of their action has been on a very limited number of cytokines for the chosen subset for study.

There are currently over 30 cytokines and 11 chemokines that have been associated with human leukocyte subsets **[1]**. No comprehensive study was found that evaluated the identity of human leukocyte subsets from healthy donors that produce these cytokines. Defining all the conditions under which they are produced would certainly be an enormous task and is currently being addressed by many investigators. We hypothesize that the repertoire of cytokine pro-

duction by resting human leukocyte subsets and those stimulated with PMA + IONO, as an example of a general stimulus, and LPS, as an example of an important specific activation molecule, will differ by both Subset and stimulus. Results clearly show that not all cytokines are produced by leukocyte subsets. Our hypothesis was confirmed, as the results also show the repertoire of cytokines that are produced is the result of the kind of stimulus presented to the responding cells. They also show that there is a great deal of heterogeneity in the frequency of cells within a subset that produce any given cytokine.

## MATERIAL AND METHODS

A total of 11 peripheral blood (PB) samples from healthy adult subjects were analyzed under a protocol approved by the RPCI Institutional Review Board. All specimens were obtained as excess material used for other clinical tests. All samples were collected in heparinized green top vacationers (Becton Dickinson, San Jose, CA) as anticoagulant (10 units/ml).

Two different activation regimens were used. In the first, PMA at 1.0µg/ml (Sigma, St. Louis, MO) and IONO at 50µg/ml, from streptomycines conglobatus, (Sigma) were used. In the second, LPS at 100ng/ml from Escherichia Coli Serotype 026-B6 (Sigma) was used. These were all final concentrations.

Cytokine production was analyzed after six hours of incubation of PB supplemented with an equal volume of RPMI 1640 (GIBCO Life Technology, Grand Island, NY) in the presence of 10µg/ml Brefeldin A (Sigma). The incubation was performed at 37°C in a 5% $CO_2$ humid atmosphere. Three conditions were evaluated. First, no activation was provided so basal endogenous cytokine levels could be evaluated. Second, cells were incubated after adding PMA and IONO in a final volume of 1ml. Third, 1ul of LPS (Sigma) was added also in a final volume of 1ml. Cells were at a final concentration of $10^6$/ml. The medium also contained 15% fetal bovine serum.

Immediately after the incubation period, both stimulated and unstipulated samples were transferred to an ice bath. To block Fc receptors, 34ul of a 3mg/ml solution of normal mouse IgG (Caltag/Burlingame, CA) was added to each tube and incubated on ice for 10 minutes.

Samples were then liquated into 20 tubes of 100ul each and stained with appropriate combination of monoclonal antibodies to membrane markers, as previously described in detail **[9]**. The combinations and amount added to tubes was as follows: 5ul CD3-FITC (Becton Dickinson), 5ul CD56-PE (Beckman-Coulter, Hialeah, FL) and 5ul CD33-PC (Immunotech-Beckman-Coulter, Hialeah, FL). The second and third combinations were evaluated on only eight donors: CD3-FITC CD8-PE (Caltag) CD4-PcP (BD) CD41-FITC (Caltag) (CD3CD56CD33)-PE CD19-PC (Immunotech-Beckman Coulter). We purchase our reagents in bulk at higher concentration than when they are delivered in 100 test vials. All reagents are titered prior to use to verify the saturating concentration as described in detail elsewhere **[5]**.

After gentle mixing, cells were incubated for 15 minutes on ice. Erythrocytes were lysed using three ml of pre-warmed (25°C) lysing reagent (8.26gm $NH_4C1$ + 1.00gm $KCO_3$ + 0.037gm tetra Na-EDTA in one liter distilled water) into each tube, inverted twice and centrifuged at 1500 x G for three minutes at room temperature. After discarding the supernatant and re-suspending the cells in residual buffer the cells were fixed in 200ul of 2% ultra-pure formaldehyde (Polysciences, Malvern, PA). Three ml of PAB (PBS + 0.5% BSA + 0.1% $NaN_3$) was added to each tube and centrifuged at 1500 x G for three minutes at room temperature. After discarding the supernatant, the cells were permeabilized with 100 ul of Permeabilization reagent (Caltag) followed immediately with five ul of appropriate biotinylated antibody to the appropriate cytokine. The cytokine antibodies used in this study were IL-lα, IL-1ß, IL-2, IL-3, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-18, INFγ, TNFα, TNFß, GM-CSF, G-CSF, M-CSF, MIP-1ß and FLT3. They were all from R&D Systems except IL-4, which was from (BD PharMingen, San Diego, CA). After incubation for 30 minutes at room temperature, 3ml of PAB was added to each tube for 15 minutes. This incubation step provides time for unbound antibody to diffuse out of the cell. They were then centrifuged at 1500 x G for three minutes at room temperature; decanted and blotted; the cells were permeabilized again with 100ul of Permeabilization reagent followed immediately with 5ul of APC streptavidin (Caltag) and incubated for 30 minutes at room tem-

perature. Three ml of PAB was added for 15 minutes, the cells were centrifuge as above, the supernatant discarded and the cells fixed in 250ul of 2% ultra-pure formaldehyde.

Data was acquired using a FACSCalibur flow cytometer. The instrument performance was verified daily using microspheres. PB stained with CD45-FITC, CD4-PE, CD8-PC or CD45- APC as single color reagents in separate tubes. A tube creating cells co-stained with CD45-APC, CD4-PE and CD8-PC was also prepared. After verifying instrument timing according to instructions supplied by the manufacturer, microspheres and stained cells were acquired. (They all must appear in a specific region for the instrument to be verified). These important evaluation processes have been described in more detail elsewhere **[9]**. Color compensation was performed using the single color reagent stained cells and verified using the co-stained cells, as previously described in detail **[11,12]**. Twenty thousand ungated events were acquired on every sample.

Data was analyzed using WinList (Verity Software House, Topsham, ME). The gating strategies are described in the results. Data from WinList was transferred to EXCEL where the mean and standard deviation were computed.

## RESULTS

We initially screened three donors for endogenous production, and for production after PMA + IONO or LPS stimulation. IL-lα, IL-1ß, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, INFγ, TNFα, GM-CSF, G-CSF, M-CSF and MIP-1ß were the 14 cytokines found to be expressed in >2% of cells within at least one subset and these were further evaluated in eight additional healthy donors.

The leukocyte subsets, shown in Fig. (1), were resolved into monocytes (CD33 high), granulocytes (CD33 1ow), T cells (CD3+), Helper T cells (CD4+), Suppressor T cells (CD8+), cytotoxic T cells (CD3+ CD56+), NK cells (CD3- CD56+), B cells (CD19+) and platelets (CD41+). The frequency of each subset for 11 healthy donors used in this study is shown in Table (1). The values are consistent with our clinical reference ranges. The strategy for resolving each leukocyte subset is shown in Fig. (1). The forward scatter (FSC) versus side scatter (SSC) bivariate histogram, shown in A, was not used for gating. The first antibody combination, which contains CD3-FITC, CD56-PE and CD33-PC) is shown in Fig. (1B-C), and it can be divided into CD33 high fluorescent cells that are monocytes and CD33 low fluorescent cells that are predominantly granulocytes. Dendritic cells were less than 1% of these cells and were not studied. After gating on the CD33 negative population, cytotoxic T cells and NK cells are resolved as shown in Fig. (1C). The combination CD3-FITC CD4-PE and CD8-PC, shown in Figure ID, after gating on CD3+ cells, produced the four T cell subsets based on CD4 and CD8 expression. The combination CD41-FITC, (CD3, CD56, CD33)-PE and CD19-PC was used to resolve platelets and B cells. This was accomplished by producing the Boolean gate: NOT (CD3 or CD56 or CD33)-PE and gating the bivariate histogram (CD41 versus CD19), as shown in Fig. (1E).

PMA + IONO had an extremely toxic effect on granulocytes and not all CD33 1ow cells were found to be granulocytes. The kinetics of granulocyte killing is shown in Fig. (2). By five hours only about 5% resistant cells (CD33dim) were found. We identified this resistant subset by immunophenotyping and cell sorting (for morphological assessment), The resistant cells were found to be highly granular monocytes (data not shown). The CD33 1ow subset of monocytes found in the blood may be newly released and less differentiated monocytes from bone marrow that also exhibit a lower expression of CD33. These cells are negative or express CD14 at a lower intensity and can be completely resolved as monocytes because their CD4 expression is identical to that found on mature monocytes. (Data not shown).

Since cytokine expression by granulocytes stimulated with PMA + IONO could not be evaluated; only LPS stimulation was studied. IL-8 was the only cytokine produced by a significant fraction (40%) of granulocytes after LPS. No endogenous cytokine was produced by granulocytes.

Cytokine production by monocytes is shown in Fig. (3). Unstimulated monocytes produced only IL-8 and no other cytokines. For PMA + IONO, monocytes could be divided into CD33 high and CD33 1ow subsets. IL-lα and ß, IL-

6, IL-8, TNFα, M-CSF and MIP-1ß, were produced by both CD33 high and low monocytes. The CD33 high monocyte subset had a greater frequency producing IL-lα and ß, IL-8, TNFα, M-CSF and MIP-1ß while the CD33 low subset produced more IL-6 as well as some IL-4 and IL-12. Some of this production could be associated with dendritic cells that were not specifically resolved. LPS induced the highest frequency of cytokine positive monocytes and they did not produce IL-2, IL-4, INFα, GM-CSF and M-CSF.

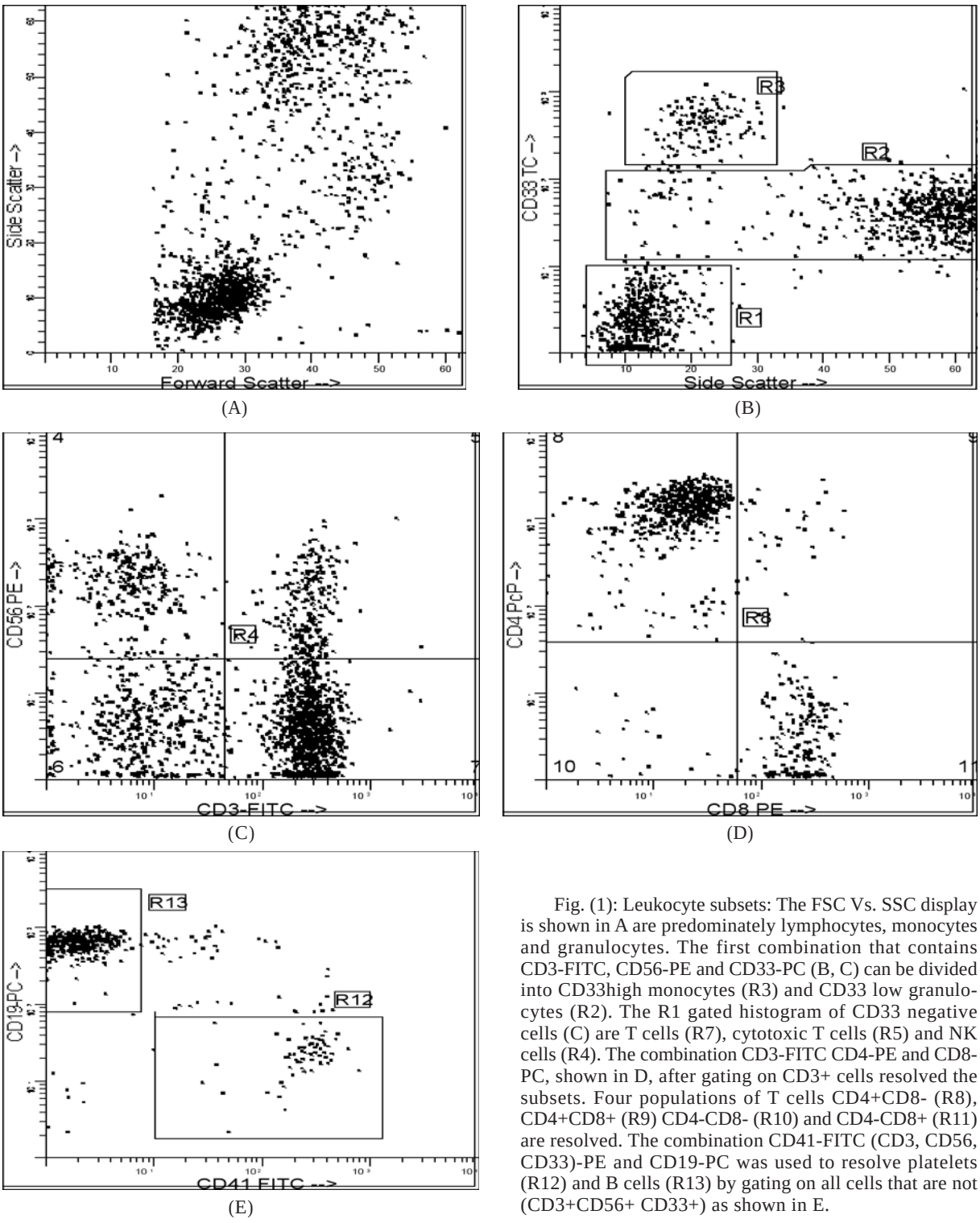


Fig. (1): Leukocyte subsets: The FSC Vs. SSC display is shown in A are predominately lymphocytes, monocytes and granulocytes. The first combination that contains CD3-FITC, CD56-PE and CD33-PC (B, C) can be divided into CD33high monocytes (R3) and CD33 low granulocytes (R2). The R1 gated histogram of CD33 negative cells (C) are T cells (R7), cytotoxic T cells (R5) and NK cells (R4). The combination CD3-FITC CD4-PE and CD8-PC, shown in D, after gating on CD3+ cells resolved the subsets. Four populations of T cells CD4+CD8- (R8), CD4+CD8+ (R9) CD4-CD8- (R10) and CD4-CD8+ (R11) are resolved. The combination CD41-FITC (CD3, CD56, CD33)-PE and CD19-PC was used to resolve platelets (R12) and B cells (R13) by gating on all cells that are not (CD3+CD56+ CD33+) as shown in E.

Table (1): Leukocyte subset differential for healthy donors.

| Control | Percent of total cells | Absolute count | Range (Mean ± 2 Std) |
|---|---|---|---|
| CD33 high | 5.6 | 336 | 264-408 |
| CD33 1ow | 50.8 | 3048 | 2160-3936 |
| T cells (CD3+) | 20.9 | 1254 | 1026-1482 |
| CD4+CD8- | 13.8 | 828 | 588-1068 |
| CD8+CD4- | 5.6 | 336 | 180-492 |
| CD4+CD8+ | 0.51 | 31 | 22-40 |
| CD4-CD8- | 0.71 | 43 | 20-66 |
| CD56+ | 1.5 | 90 | 6-174 |
| NK cells (CD3-CD56+) | 3.1 | 186 | 42-330 |
| B cells (CD19+) | 2.9 | 174 | 90-258 |
| Platelets (CD41+ D45-) | 1.7 | 102 | 6-204 |

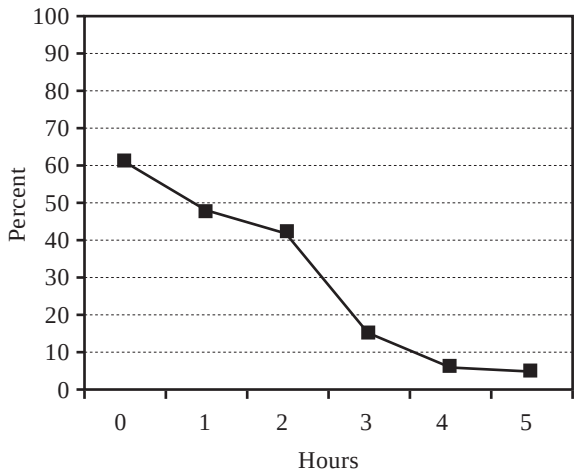


Fig. (2): The kinetics of granulocyte killing by PMA. Blood was incubated for five hours with PMA + IONO. At one-hour intervals an aliquot was removed and the percentage of CD33 1ow cells remaining was determined.

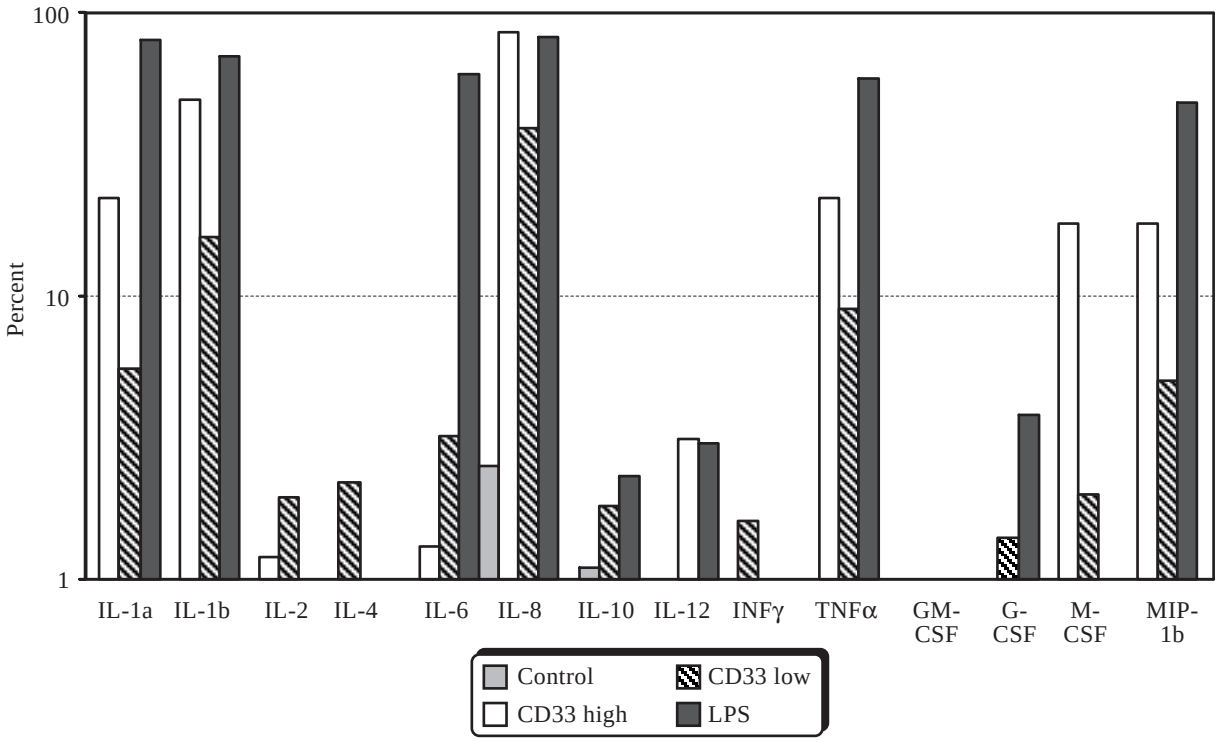


Fig. (3): Cytokine Production by Monocytes. The percentage of monocytes that produce cytokines constitutively (control) and after PMA + IONO or LPS stimulation for six hours is shown. Monocytes stimulated with PMA + IONO could be further resolved into CD33 high and CD33 low populations.

CD3+ T cells can be divided into five subsets. CD4+CD8-, CD4+CD8+, CD4-CD8-, CD4-CD8+ and CD56+ (Figure 1). The latter is also a subset of CD8+ T cells and is the large granular cytotoxic T cell fraction. The CD3-CD56+ cells are NK cells. As shown in Fig. (4), PMA + IONO was the most potent stimulus for inducing cytokine production by T cells. Using an arbitrary threshold of 2% positive cells for cytokine production, all the cytokines were evaluated except IL-3, IL-4, IL-6, and IL-10 and were produced by at least one subset of

T cells. The CD4+CD8+ subsets produced IL-lα, IL-lß, IL-2, IL-8, IL-12, INFγ, TNFα, GM-CSF, G-CSF, M-GSF and MIP-lß. This immature subset represented only 0.51% of cells. The CD4-CD8- subset represented 0.71% of cells and they are mainly delta gamma TCR expressing T cells and they produced IL-1ß, IL-2, IL-8, INFγ, TNFα, M-CSF and MIP-1ß. The CD4+CD8- and CD4-CD8+subsets produced IL-2, IL-8, INFγ and TNFα. The CD4+CD8- cells produced GM-CSF, while MIP-lß was produced by the CD4-CD8+ subset. The T cell subset (CD3+CD4-CD8+CD56+) produced IL-2, INFγ, TNFα, GM-CSF and MIP-1ß. Except for IL-2, NK cells (CD3-CD56+) also produced these cytokines. No lymphocytes were found to produce detectable cytokines if they were not stimulated or if they were stimulated with LPS. Neither B cells nor platelets produced any cytokines with any regimen used in this study.

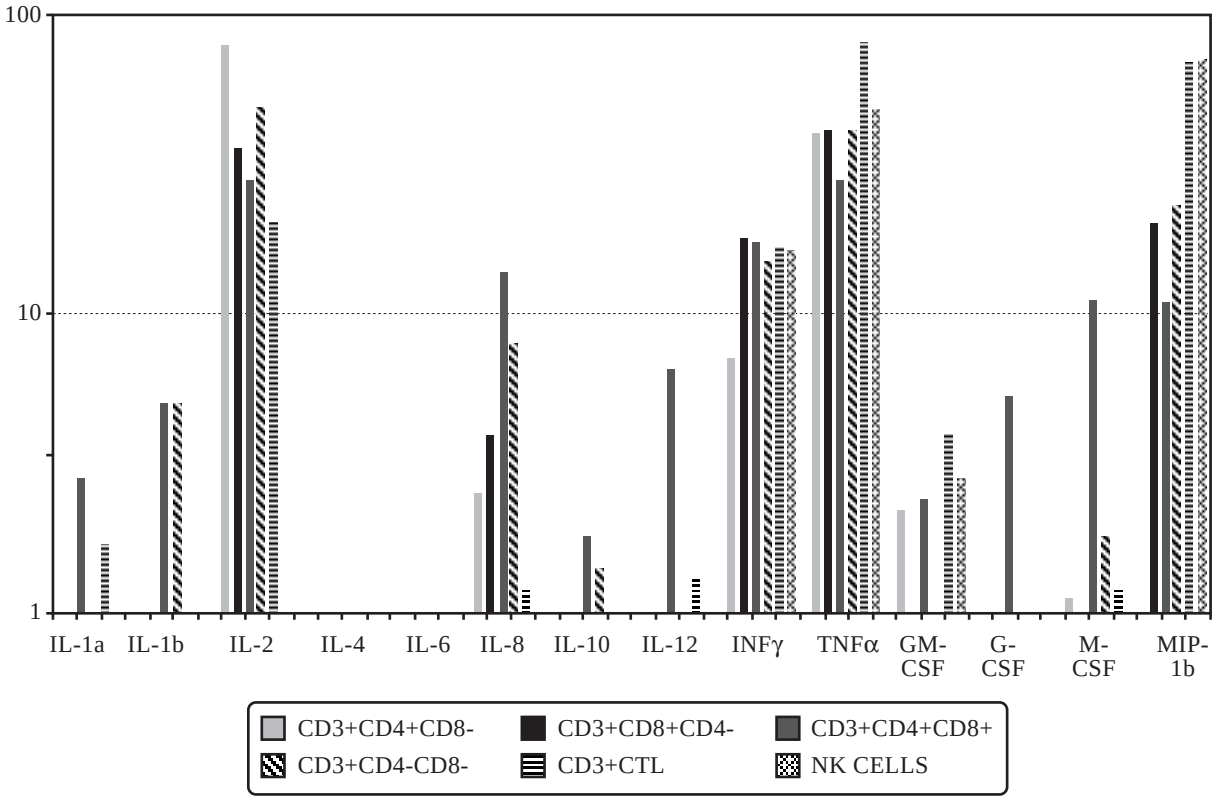


Fig. (4): Cytokine Production by Lymphocytes. Blood was incubated six hours with PMA + IONO. The frequency of positive lymphocytes for intracellular cytokines is shown for all subsets that produced cytokines. The four major subsets of T cells as well as the CD8+ cytotoxic T cell subset (CTL) and NK cells are shown. B cells did not produce any measurable cytokines.

## DISCUSSION

Our study represents the most comprehensive evaluation of cytokine production reported so far by leukocytes from healthy human donors, We compared our results to similar reports **[5,13-20]**, and with a few exceptions our results mirror those of others. Our study design, however, differs from others in that intracellular cytokine production was measured by phenotypically resolved subsets of all major leukocyte populations. Baran, et al. **[13]** reported production of IL-4 and TNFß by CD4 and CD8 T cells stimulated with PMA + IONO that differ somewhat from our Studies. Their placement of the marker for positive cells is considerably different from ours on the univariate histogram even though it was objectively set using an isotype control. This is the most likely reason for the difference and underscores the subjectivity of marker placement for determining positive and negative cells and the effect it can have on results when so few cells are actually positive. There appears to be a misprint in the report by Krampera, et

al. **[14]** in the results of TNFα and TNF*ß*. Their percentage positive CD4 and CD8 T ceils are reversed in the text from those reported in Table (1). Otherwise, their results are in agreement with those reported here.

Bueno, et al. **[5]** found 55±13% positive monocytes expressed IL-12. We did not find IL-12 expression by monocytes activated by LPS alone. This difference is likely due to the addition of TNFα to the stimulation protocol, demonstrating the profound influence of cytokines in the activation process. They and others have also shown that dendritic cells, which are present at a very low frequency (less than 1% in our specimens) and were not resolved by us, are a dominant source of this cytokine. While performed in separate studies, it is certainly likely that combinations of activators can produce profound differences in the repertoire of cytokines produced by any leukocyte subset. These are the words cells communicate with.

Because the frequency of cells producing most cytokines is low, and in most cases less than 5% of cells resolved by the antibody combination, it is clear that the antibodies we used to subset some populations are much too broad. Thus, those cells producing any given cytokine in low frequency are themselves a subset within the subset that might be resolvable using combinations of more specific differentiation markers. Dendritic cells are a prime example of the importance of this approach. We are focusing on the development of more specific differentiation markers associated with the subset of cells that produces the cytokine to better resolve them from those that do not.

When cytokines are combined with other agents the leukocyte response is likely to be specific for the combination. Because of the huge number of possible combinations, it will be a huge task to decode the language of cells. We believe it will be necessary to create a centralized database, similar to that found for the genome project. This database will then become the dictionary of words used by cells.

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## REFERENCES

1- Stewart CC, Nicholson J. Immunophenotyping. Wiley-Liss, New York. 2000.

2- Picker LJ, Singh MK, Zdraveski Z, Treer JR, Waldrop SL, Bergstresser PR, Maino VC. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. Blood. 1995, 86: 1408-1419.

3- Rostaing L, Tkaczuk J, Durand M, Peres C, Durand D, de Preval C, Ohayon E, Abbal M. Kinetics of intracytoplasmic Thl and Th2 cytokine production assessed by flow cytometry following in vitro activation of peripheral blood mononuclear cells. Cytometry. 1999, 35: 318-328.

4- Romagnani S. The Thl / Th2 paradigm. Immunol. Today. 1997, 18 (6): 236-263.

5- Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998, 392: 245-252.

6- Bueno C, Almeida J, Alguero MC, Sanchez ML, Vaquero JM, Laso FJ, San Miguel JF, Escribano L, Orfao A. 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.

7- Willmann K, Dunne JF. A flow cytometric immune function assay for human peripheral blood dendritic cells. J. Leuko. Biol. 2000, 67: 536-544.

8- Olweus J, BitMansour A, Warnke R, Thompson PA, Carballido J, Picker LJ, Lund-Johansen F. Dendritic cell ontogeny: A human dendritic cell lineage of myeloid origin. Proc. Natl. Acad. Sci. USA. 1997, 94: 12551-12556.

9- Stewart CC, Stewart SJ. Cell preparation for the identification of leukocytes. In: Methods of Cell Biology, (Z Darzynkiewicz, H Crissman and JP Robinson eds.), Academic Press, Inc., New York. 2001, 64, Chapter 11: 218-270.

10- Stewart CC, Stewart SJ. Titering Antibodies. In Current Protocols in Cytometry, (JP Robinson, Z Darzynkiewicz, P Dean. L Dressier, P Rabinovitch, C Stewart, H Tanke, L Wheeless, eds.) J Wiley & Sons, Inc New York. 1997, 4.1.1-4.1.13.

11- Stewart CC, Stewart SJ. Four Color Compensation. Communications in Clinical Cytometry. 1999, 38: 161-175.

12- Stewart CC, Stewart SJ. A Software Method for Color Compensation. In Current Protocols in Cytometry, (JP Robinson, Z Darzynkiewicz, P Dean. L Dressier, P, Rabinovitch, C Stewart, H Tanke, L Wheeless, eds.) J Wiley & Sons, Inc New York, 10.12. in Press

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 and Diag Lab Immunology, Mar. 2001, 303-313.

14- Krampera M, Tavecchia L, Benedetti F, Nadali G, Pizzolo G. Intracellular cytokine profile of cord blood T-, and NK-cells and monocytes. Haematologica. 2000, 85: 675-679.

15- Sullivan KE, Cutilli J, Piliero LM, Ghavimi-Alagha D, Starr SE, Campbell DE, Douglas SD. Measurement of cytokine secretion, intracellular protein expression, and mRNA in resting and stimulated peripheral blood mononuclear cells, Clin and Diag Lab Immunol Nov. 2000, 920-924.

16- Tayebi H, Lienard A, Billot M, Tiberghien P, Herve P, Robinet E. Detection of intracellular cytokines in citrated whole blood or marrow samples by flow cytometry. J Immunol Meth. 1999, 229: 121-130.

17- Sewell WAC, North ME, Webster DB, Farrant J. Determination of intracellular cytokines by flow-cytometry following whole-blood culture. J Immunol Meth. 1997, 209: 67-74.

18- Collins DP. Cytokine and cytokine receptor expression as a biological indicator of immune activation: Important considerations in the development of in vitro model systems. J Immunol Meth. 2000, 243: 125-145.

19- Mendes R, Bromelow KV, Westby M, Galea-Lauri J, Smith IE., Brien MER, Souberbielle BE. Flow cytometric visualization of cytokine production by CD3-CD56+ NK cells and CD3+CD56+ NK-T cells in whole blood. Cytometry. 2000, 39: 72-78.

20- Pala P, Hussell T, Openshaw PJM. Flow cytometric measurement of intracellular cytokines. J Immunol Meth. 2000, 243: 107-124.