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Single-cell cytokine profiles in normal humans: comparison of flow cytometric reagents and stimulation protocols

J. Jason *, J. Larned

Immunology Branch, Division of AIDS, Sexually Transmitted, Diseases, and Tuberculosis Laboratory Research (DASTLR), National Center for Infectious Diseases, Centers for Disease, Control and Prevention (CDC), U.S. Department of Health and Human Services (DHHS), U.S. Public Health Service (PHS), Atlanta, GA 30333, USA

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Abstract

Cytokines are produced and function at a micro environmental level; intracellular assessment has only recently become practically feasible. We used 3-color flow cytometry to examine surface and cytoplasmic antigens on peripheral blood lymphocytes of 18 normal donors, assessing the applicability/comparability of various directly conjugated anti-human cytokine reagents and stimulation protocols using separated cells or whole blood preparations. Interdonor variability far exceeded variability due to reagent or stimulation and separation techniques. Based on all results with various reagents, post 4–5.5 h stimulation with PHA/PMA/ionomycin, the range of the percents of T lymphocytes producing various cytokines included: γ-IFN-13.2–65.0%, IL-2-10.0–56.7%, and TNF-α-17.1–79.2%. Compared to CD8+ cells, CD4+ cells more often expressed IL-2 (mean 45.7% of CD4+ vs. 21.4% of CD8+, $p < 0.0001$), less often expressed γ-IFN (18.5% vs. 55.3%, $p < 0.0001$), and did not differ in TNF-α expression (52.9% vs. 59.4%). Of T cells producing γ-IFN, 64.8–100.0% also produced TNF-α and 3.5–100.0%, IL-2. Of T cells producing IL-2, 6.0–63.9% also produced γ-IFN and 37.6–100.0%, TNF-α. These results demonstrate the broad spectrum of cytokine patterns in normal human adults, as well as the usefulness and limitations of various currently available cytokine products. © 1997 Elsevier Science B.V.

Keywords: Flow cytometry; Cytokine; Intracellular immunofluorescence; T lymphocyte

1. Introduction

Cytokines regulate intercellular immune interactions, are produced in the cytoplasm of mononuclear cells, and function at a micro environmental level. Cellular level cytokine assessment has been problematic, relying on in situ hybridization, limiting dilution, plaque/ELISPOT, or T cell cloning techniques (Lewis, 1991). All these approaches are laborious and complex; some are fraught with measure-

Abbreviations: BD, Becton Dickinson; BFA, brefeldin A; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; MoAb, murine monoclonal antibody; PE, phycoerythrin; PMA, 12-myristate 13-acetate; PHA, phytohaemagglutinin; PhM, PharMingen; RT, room temperature; rh, recombinant human; SC, separated mononuclear cells; TNF, tumor necrosis factor; WB, whole blood assay

* Corresponding author. Tel.: +1-404-6393919; fax: +1-404-6392108.

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ment difficulties. Thus, for practical reasons, investigators have usually measured plasma or culture supernatant cytokine titers, which reflect the contributions of many cells and/or a physiologic macro environment. Alternatively, T cell clones have been produced from a variety of sources and cultured under a variety of conditions. Clonal characteristics, e.g., surface antigenicities, have been examined and supernatant cytokine levels determined. It has been assumed that cytokine production and responsiveness are identical for all cells of a given clonotype.

Research using these approaches led to creation and refinement of T cell network concepts, including type I, type II, and type 0 responsiveness (Mosmann et al., 1986; Seder and LeGros, 1995). However, a more direct — and perhaps more physiologically relevant — approach remained desirable. Recent technical advances now make it possible to investigate cytokine profiles on a cellular level and concurrently examine other cell characteristics.

A number of obstacles had to be addressed before this goal was achievable. First, cell permeabilization, necessary for examination of cytoplasmic molecules, usually damaged cell surface antigens; caused autofluorescence, nonspecific staining, and/or cell aggregation; and affected cell size and density. Initial success in minimizing these problems was achieved with the use of saponin, which is thought to act by reversibly solubilizing cholesterol in the cell membrane (Andersson et al., 1990; Sander et al., 1991; Jacob et al., 1991; Assenmacher et al., 1994), or fixative/detergent combinations (Henter et al., 1988; Andersson et al., 1988). Greater refinement has now led to commercially available products, the composition of which are proprietary (Pizzolo et al., 1994; Van Zaanen et al., 1995; Tiirikainen, 1995; Ferrick et al., 1995; Francis and Connelly, 1996). These commercial reagents irreversibly permeabilize cell membranes while minimizing autofluorescence and nonspecific staining. In addition, several reagents lyse red blood cells and contain fixatives, permitting rapid and simple assessment of peripheral blood specimens without cell separation. Cell scatter properties are altered, but not greatly and in a manageable and predictable fashion. Staining of most surface antigens is minimally altered if done before permeabilization (Pizzolo et al., 1994; Van Zaanen et al., 1995; Tiirikainen, 1995; Ferrick et al., 1995; Francis and Connelly, 1996).

A second barrier to single cell assessment was the low signal-to-noise ratio prevalent in these systems. This problem has been addressed through production (Henter et al., 1988; Andersson et al., 1990) and direct conjugation of monoclonal anticytokine antibodies reactant to cytokines in their intracellular conformation and through the use of blocking techniques (Prussin and Metcalfe, 1995; Picker et al., 1995). These permeabilizing and staining reagents provide a facile means of combining surface and intracellular fluorescent staining (Jacob et al., 1991; Ferrick et al., 1995; Picker et al., 1995). A third barrier to intracellular cytokine measurement was the rapid transport of cytokines to and through the cell membrane, leading to inadequate cytoplasmic cytokine detection. This problem was creatively resolved by using short term cell stimulations (Andersson et al., 1990; Jung et al., 1993; Picker et al., 1995), combined with agents blocking cytoplasmic transport. The latter agents include monensin, a lipophilic metabolite of *Streptomyces cinnamonensis* that disrupts ion gradients in cell membranes, causing transport arrest at the Golgi complex level (Tartakoff, 1983; Henter et al., 1988; Jung et al., 1993; Prussin and Metcalfe, 1995; Lee et al., 1990), and brefeldin A (BFA), a carboxylic ionophore that blocks transport in a pre-Golgi compartment (Klausner et al., 1992; Openshaw et al., 1995; Picker et al., 1995). The stimulation/blockade approach provides results comparable to supernatant cytokine levels determined by enzyme-linked immunosorbent assays (Jung et al., 1993).

Monoclonal anti-cytokine antibodies directly conjugated to fluorochromes are now commercially available but have not been widely assessed. We examined directly conjugated anti-human cytokine reagents available in the United States to determine: their practical applicability and comparability; the ranges in values for CD4 + and CD8 + peripheral blood lymphocytes (PBLs) from normal donors; the proportion of cells producing various combinations of cytokines; and differences seen when varying the stimulation protocol or cell preparation technique. These results provide information on the practical usefulness and limitations of these products.

2. Materials and methods

2.1. Donors

For each cytokine, between 15 and 18 donors were evaluated (Table 1). Of these donors, five had blood samples split; half of each sample was applied to a gradient and half was used in a whole blood assay (see below). Both aliquots were stimulated using protocol B (see below). These split samples will be referred to herein as those from '*matched donors*'.

2.2. Reagents

Fluorescein isothiocyanate-conjugated (FITC) or phycoerythrin-conjugated (PE) murine monoclonal antibodies (MoAb) to human cytokines were obtained from Becton Dickinson Immunocytometry Systems (BD), San Jose, CA (γ-interferon [γ-IFN]-FITC [clone 25723.11], γ-IFN-PE [clone 25723.11], interleukin-2 [IL-2]-FITC [clone 5344.111], and IL-2-PE [clone 5344.111] and PharMingen (PhM), San Diego, CA (γ-IFN-FITC [clone 4S.B3], γ-IFN-PE [clone 4S.B3], tumor necrosis factor-α [TNF-α]-PE [clone MAb11], and IL-2-PE [clone MQ1-17H12] [1]. We also evaluated interleukin-4 (IL-4)-PE (clone 3010.211, BD and clone 8D4-8, PhM), tumor necrosis factor-β (TNF-β)-PE (clone 359-81-11, PhM), and interleukin-10 [IL-10]-PE (clone JES3-9D7, PhM) reagents. However, with the protocols described here, as well as those recommended by the manufacturers, we obtained signal-to-noise ratios that were unacceptably low in relation to the rates of positivity; therefore, results will not be provided. CD3-PECy5, CD4-PECy5, and CD8-PECy5, used at 20 ng/ml) were obtained from PhM. Isotype controls were obtained from BD and PhM. CD4-FITC, CD4-PE, and CD3 MoAb were obtained from BD.

Other reagents and concentrations used included: 12-myristate 13-acetate (PMA) (Sigma Chemical, St. Louis, MO) (50 ng/ml), phytohaemagglutinin (PHA) (Difco Laboratories, Detroit, MI) (0.005 μg/ml), purified IL-2 (PhM) (10 ng/ml), BFA (Sigma) (10 μg/ml), ionomycin (Sigma) (1 μg/ml), and ORTHO PermeaFix™ (ORTHO Diagnostics, Raritan, NJ).

2.3. Methods

Venous blood was obtained from volunteer blood donors screened and negative for serology to hepatitis A, B, and C viruses, human T-cell lymphotropic viruses, and human immunodeficiency virus. The blood was collected in heparinized vacutainers and either used directly in a whole blood assay (WB) or placed on a gradient to separate mononuclear cells (SC). WB or SC were stimulated for 4–5.5 h with PMA and ionomycin, in the presence of BFA and RPMI-1640 with 2 mM L-glutamine, with PHA (protocol A) or without PHA (protocol B). *Unstimulated control* cells remained in only BFA and media, also for 4–5.5 h. Surface staining was then done at room temperature (RT) for 15 min in the dark. ORTHO PermeaFix™ was added, followed by a wash. Intracellular staining was next done, at RT for 30 min in the dark, followed by a wash.

2.4. Flow cytofluorometry

Three-color cytofluorometry was done using a FACSort and Lysis II or CellQuest software (BD). 20,000 ungated events were collected from each sample.

2.5. Analytic and statistical techniques

All comparative analyses were done by a single individual. Analyses are reported for CD4 +, CD8 +, and/or CD3 + cells, using a lymphocyte scatter gate including both resting and stimulated cells. Measurements of intrasample variability for T lymphocyte values (variability among multiple measurements of the same sample) for each cytokine reagent are provided as a weighted mean standard deviation, i.e., within-sample standard deviations weighted by the number of observations per donor and then averaged. Interdonor variabilities are presented as means and standard deviations (SD); for triplicate samples, the mean of triplicate samples was used in this calculation.

[1] Use of any and all trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

Table 1
Variability in repeated measurements for T lymphocytes of same sample, by reagent

Cytokine Reagent [a]	# Donors	Measurements/Donor		Weighted Mean Standard Deviation
		Mean	Range	
γ-IFN-Fitc				
PhM	18	3.9	2–6	1.6
BD	17	4.2	3–7	1.6
γ-IFN-PE				
PhM	15	2.7	2–3	1.9
BD	17	2.6	2–3	1.6
IL-2-Fitc				
BD	18	4.6	3–7	1.5
IL-2-PE				
PhM	17	2.9	2–3	2.3
BD	18	2.9	2–3	1.1
TNF-α-PE PhM	18	3.4	3–6	2.3

[a] Fluorescein (Fitc), Phycoerythrin (PE),PharMingen (PhM), Becton Dickinson (BD).

Table 2
Means and standard deviations for percent of cells producing indicated cytokine, by reagent and stimulation protocol used [a]

Cytokine Reagent [b]	Whole Blood Assay		Separated Cells	
	Protocol A	Protocol B	Protocol A	Protocol B
CD4 + Cells:				
γ-IFN-Fitc				
PhM	21.6 (12.1)	20.2 (9.3)	11.2 (4.5)	13.4 (5.9)
BD	26.2 (10.2)	22.0 (10.4)	11.6 (4.6)	14.2 (6.3)
γ-IFN-PE				
PhM	21.7 (13.9)	22.9 (8.9)	12.9 (4.9)	15.4 (7.2)
BD	25.9 (10.0)	22.3 (8.1)	12.9 (5.2)	15.4 (6.6)
IL-2-Fitc BD	46.6 (18.6)	70.6 (11.7)	41.9 (23.0)	52.8 (12.3)
IL-2-PE				
PhM	45.2 (16.0)	68.1 (9.6)	42.2 (17.6)	50.5 (12.7)
BD	52.2 (17.2)	72.0 (10.8)	45.1 (17.2)	54.4 (11.5)
TNF-α-PE PhM	58.4 (13.4)	44.8 (12.0)	47.6 (16.6)	43.1 (14.9)
CD8 + Cells:				
γ-IFN-Fitc				
PhM	56.3 (21.1)	41.2 (18.2)	53.4 (15.6)	37.1 (18.1)
BD	49.8 (26.0)	42.7 (18.7)	52.7 (14.8)	38.7 (19.7)
γ-IFN-PE				
PhM	46.2 (31.7)	44.4 (16.1)	56.5 (14.0)	40.8 (19.5)
BD	75.6 (11.0)	42.8 (17.4)	56.1 (14.4)	40.5 (18.7)
IL-2-Fitc BD	20.3 (14.9)	33.9 (11.1)	20.2 (16.6)	26.0 (9.2)
IL-2-PE				
PhM	9.2 (4.6)	35.1 (8.5)	23.6 (11.2)	25.4 (9.9)
BD	26.5 (19.0)	43.2 (13.0)	28.8 (14.2)	32.1 (8.1)
TNF-α-PE PhM	64.8(14.8)	38.9 (18.4)	54.1 (19.9)	40.8 (17.4)

[a] Expressed as Mean (standard deviation).
For protocol A: whole blood, n = 4 for all, except *γ-IFN* Fitc BD for CD4 + cells and *γ-IFN* PE BD for CD8 + cells (n = 3); separated cells, n = 3 for all, except all IL-2 reagents and *TNF-α* (n = 4). For protocol B, n = 5 donors, triplicate samples for all, with same donors for whole blood and separated cell stimulations. Donors for protocol A were different from those for protocol B.
[b] Fluorescein (Fitc), Phycoerythrin (PE),PharMingen (PhM), Becton Dickinson (BD).

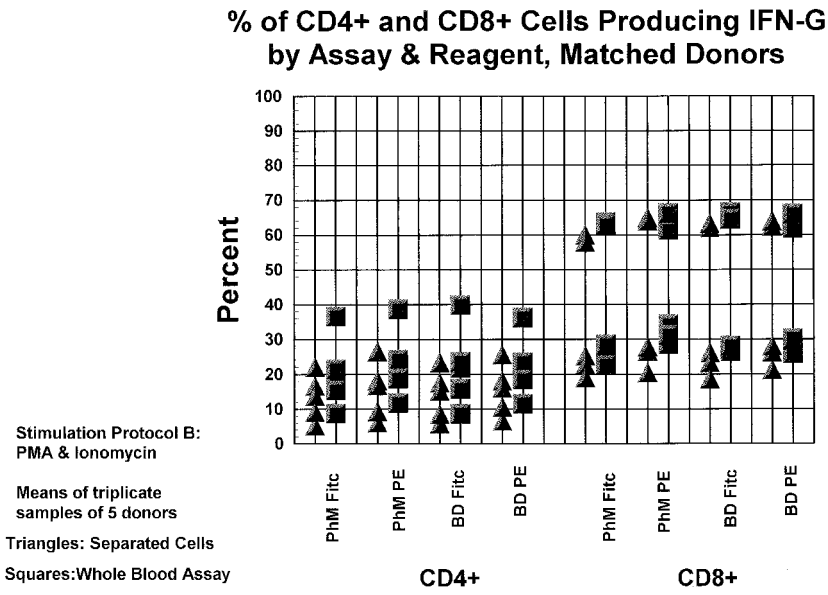

Fig. 1. Percent of CD4 + and CD8 + cells producing IFN-γ, by assay and reagent, matched donors. Footnotes: 1. Stimulation protocol B: PMA and Ionomycin. 2. Based on samples from 5 donors. Each sample was split and assessed post separation of mononuclear cells and by the whole blood technique. Each data point represents the mean of triplicate runs. 3. Triangles, stimulated, separated cells; squares, stimulated cells, whole blood assay.

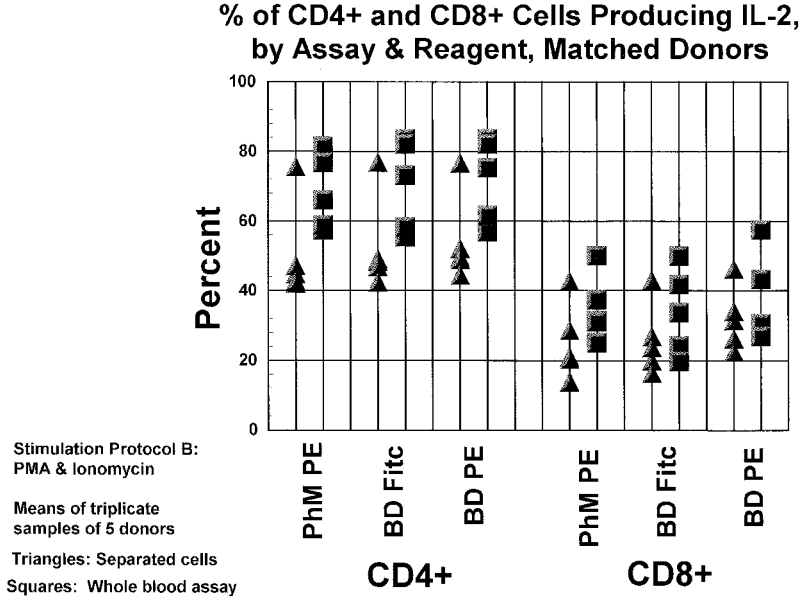

Fig. 2. Percent of CD4 + and CD8 + cells producing IL-2, by assay and reagent, matched donors. Footnotes: 1. Stimulation protocol B: PMA and Ionomycin. 2. Based on samples from 5 donors. Each sample was split and assessed post separation of mononuclear cells and by the whole blood technique. Each data point represents the mean of triplicate runs. 3. Triangles, stimulated, separated cells; squares, stimulated cells, whole blood assay.

3. Results

3.1. Controls

Cytokine staining was confirmed to be intracellular ($< 0.2\%$ of cells positive with surface staining, i.e., staining done prior to permeabilization) ($n = 2$ for each cytokine antibody). Blocking was effectively done by preincubation of the conjugated antibody with 1 μg of the appropriate rh cytokine (rhIL-2 or rhTNF-α) for 30 min at 4°C, followed by staining ($n = 2$), thus supporting specificity of the antibody reactivity. For γ-IFN, IL-2, and TNF-α, the percent of cells positive for intracellular staining in *unstimulated control* cultures was rarely $> 1\%$ and routinely $< 0.5\%$ (data not shown).

3.2. Effects of stimulation and varying stimulation protocol (Table 2)

Both protocols A and B induced cytokine production. Compared to CD4 + cells, proportionately more CD8 + cells produced γ-IFN (Table 2 and Fig. 1)

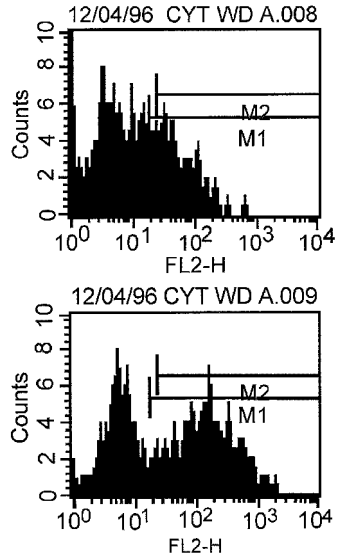

Fig. 4. A: Histogram of a representative donor's reactivity of CD8 + lymphocytes with anti-IL-2 PE, PharMingen reagent, whole blood assay. B: Histogram of a representative donor's reactivity of CD8 + lymphocytes with anti-IL-2 PE, Becton Dickinson reagent, whole blood assay.

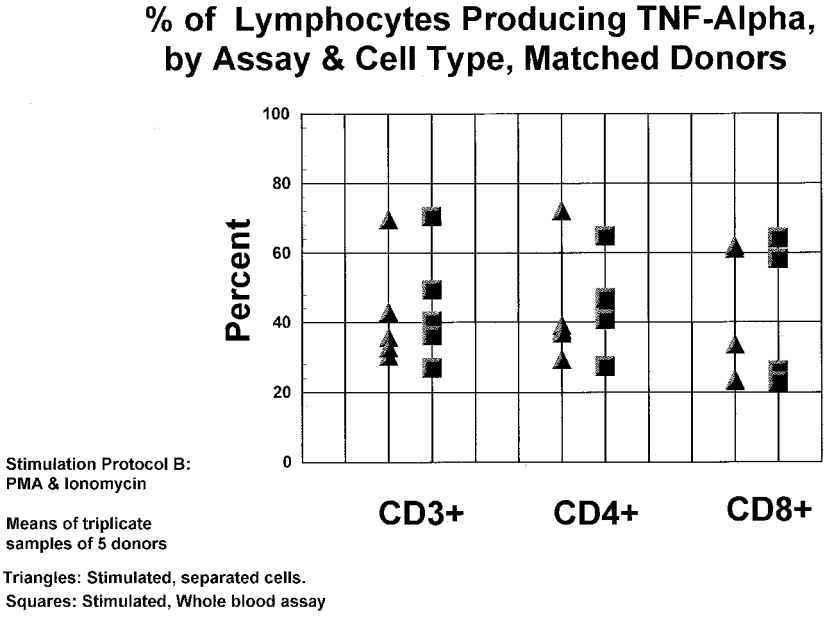

Fig. 3. Percent of lymphocytes producing TNF-α, by assay and cell type, matched donors. Footnotes: 1. Stimulation protocol B: PMA and Ionomycin. 2. Based on samples from 5 donors. Each sample was split and assessed post separation of mononuclear cells and by the whole blood technique. Each data point represents the mean of triplicate runs. 3. Triangles, stimulated, separated cells; squares, stimulated cells, whole blood assay.

and fewer produced IL-2 (Table 2 and Fig. 2). The proportions producing TNF-α (Table 2 and Fig. 3) were similar for both lymphocyte types. Donors appeared to divide into two subgroups in regard to the proportion of cells producing γ-IFN and TNF-α (Fig. 1, Fig. 3, and unshown data); however, numbers were small. The proportion of CD4 + cells producing γ-IFN did not differ greatly for protocols A and B (Table 2). However, protocol A tended to induce a higher proportion of CD8 + cells to make

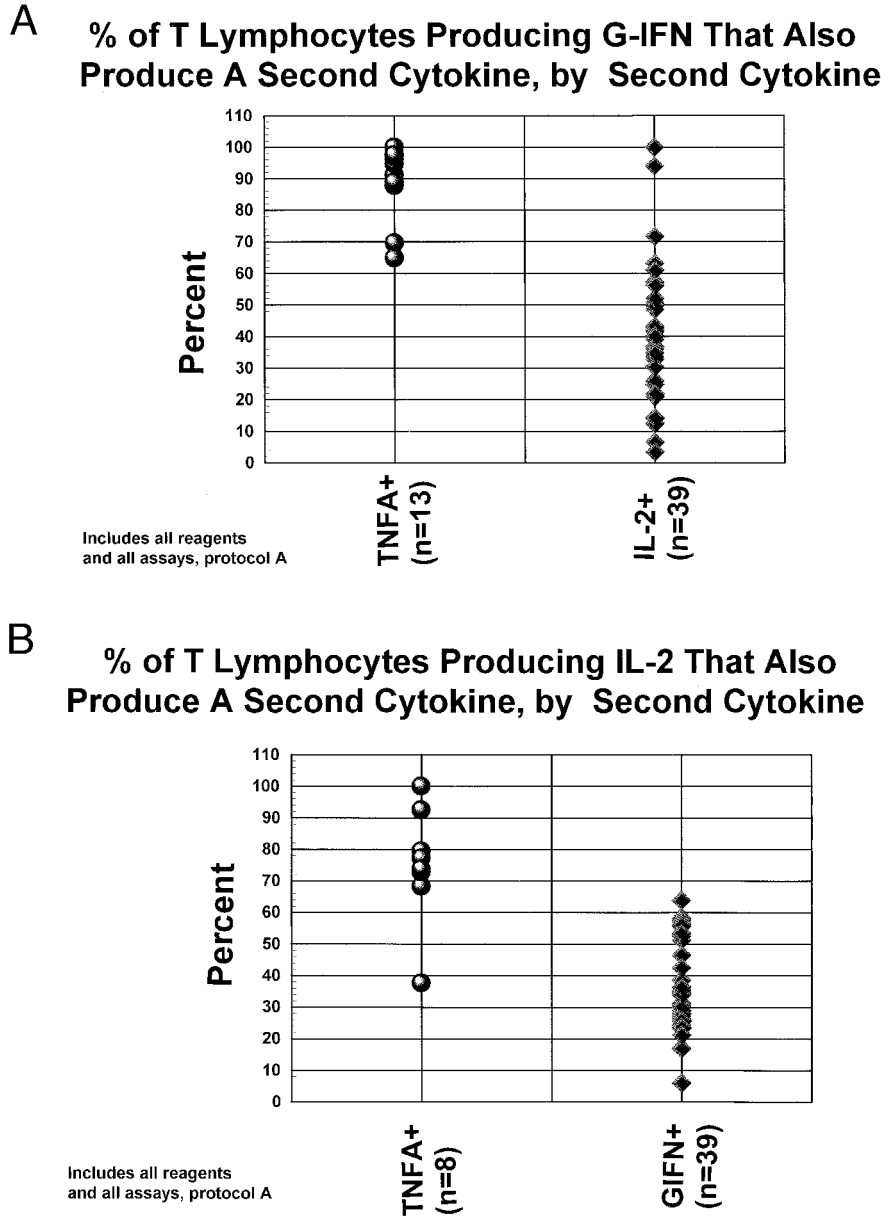

Fig. 5. A: Percent of T lymphocytes producing γ-IFN that also produce a second cytokine, by second cytokine. Footnotes: 1. Includes all reagents and all assays using protocol A. B: Percent of T lymphocytes producing IL-2 that also produce a second cytokine, by second cytokine. Footnotes: 1. Includes all reagents and all assays using protocol A.

γ-IFN. With protocol A, a lower proportion of both CD4 + and CD8 + cells produced IL-2 and a higher proportion, TNF-α.

3.3. Stimulation of SC compared to WB

Stimulation was achieved in both SC and WB with protocols A and B (Table 2). However, proportionately, slightly fewer SC produced γ-IFN (Table 2 and Fig. 1) and IL-2 (Table 2 and Fig. 2) than did WB lymphocytes. TNF-α production did not differ by stimulation protocol (Table 2 and Fig. 3).

3.4. Reagent variability

Interdonor variation, variation between CD4 + and CD8 + cells, and variation between protocols A

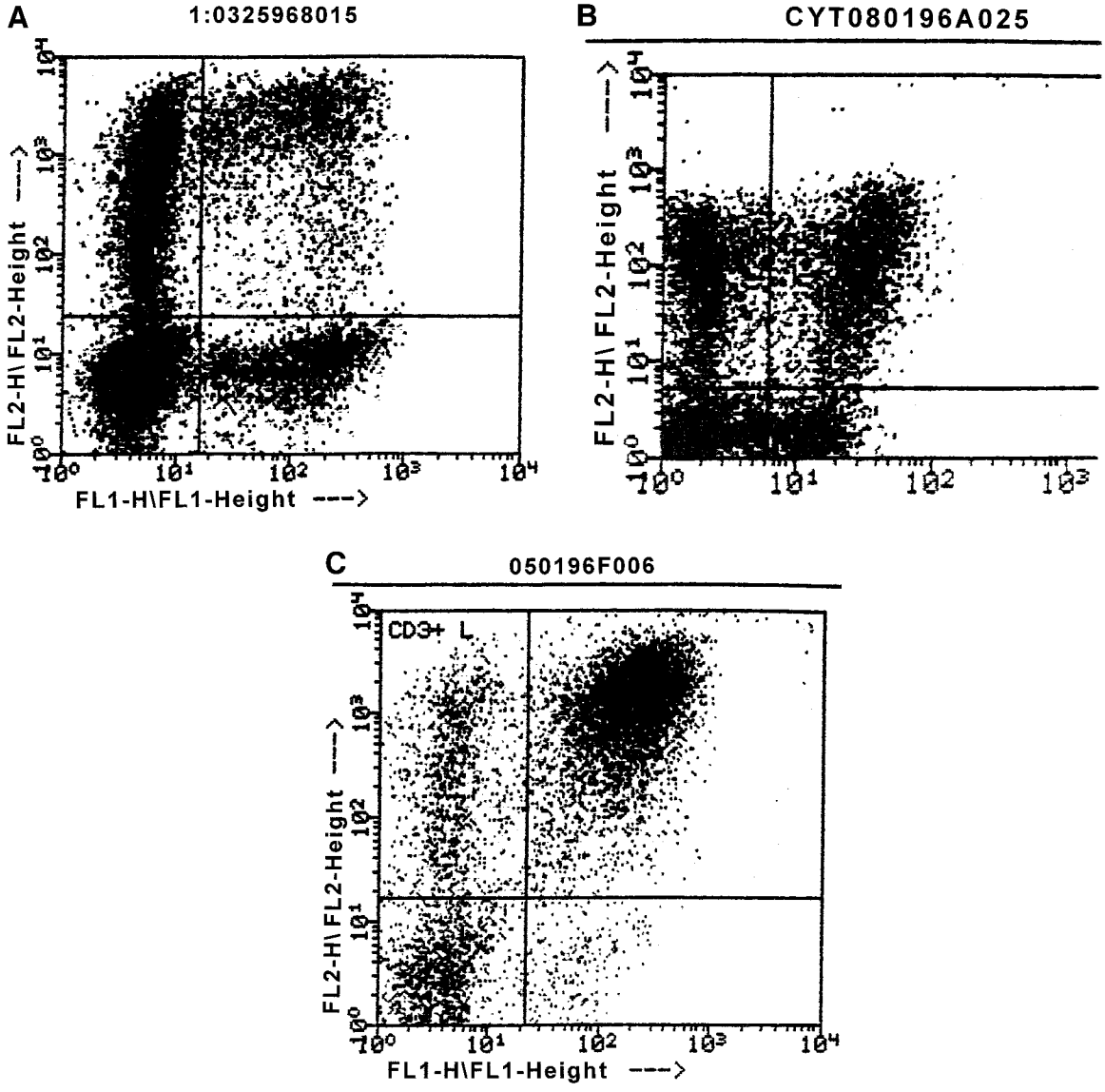

Fig. 6. A: Dot plot of IL-2-Fitc (Becton Dickinson) by IFN-γ-PE (Becton Dickinson), gated on CD3 + lymphocytes. B: Dot plot of IL-2-Fitc (Becton Dickinson) by TNF-α-PE (PharMingen), gated on CD3 + lymphocytes. C: Dot plot of IFN-γ-Fitc (PharMingen) by TNF-α-PE (PharMingen), gated on CD3 + lymphocytes.

and B all tended to be far greater than the variability related to fluorochrome or product. For IL-2 PE reagents, for both CD4+ and CD8+ cells, the separation between negative and positive reactivity tended to be poorer for the PhM product (Fig. 4A) than the BD product (Fig. 4B).

3.5. Intrasample variability (Table 1)

Weighted mean SDs for T cell values ranged from 0.6 to 2.3. Interdonor variability far exceeded intrasample variability (Table 2 vs. Table 1).

3.6. Dual cytokine production by individual cells (Figs. 5 and 6)

A large proportion of T lymphocytes producing γ-IFN also produced TNF-α (range 65–100%, Fig. 5A), as did a large proportion of those producing IL-2 (range 38–100%, Fig. 5B). The proportion of cells producing both γ-IFN and IL-2 was lower than for TNF-α, and interdonor variability was wide: 4–100% of cells producing γ-IFN also produced IL-2 and 6–64% of those producing IL-2 also produced γ-IFN. Representative results demonstrating dual staining are shown in Fig. 6A–C.

4. Discussion

Profiles of cytokine reactivity may be extremely important determinants of disease outcome for a large number of infections. These profiles may also play a role in the development of autoimmune diseases, presumably in response to self antigens. Until recently, investigations of cytokine responses to human disease states relied on cumbersome and/or crude assessment techniques. Due to a number of technical advances, it has recently become possible to determine cytokine profiles on a single cell level using flow cytometry. We addressed a number of issues related to the practical application of this technology in clinical and research settings. We also determined ranges of reactivity for 18 healthy blood donors from one location in the United States.

Interdonor variability in cytokine production was large relative to variabilities related to stimulation protocols, products, or fluorochromes. However, there was sufficient variability related to these latter parameters that for research — and probably also for clinical — applications one would still need standardized protocols specifying a single stimulation procedure, the use of SC or WB, and a stable reagent panel with a specific fluorochrome coupled to a specific reagent. Although one manufacturer recommends stimulation protocol B, those needing to use PHA for other reasons, e.g., stimulation of human immunodeficiency virus replication, should be able to perform adequate, concurrent cytokine assessments.

Intrasample variability was narrow for all products, protocols, and fluorochromes. For comparison, these weighted standard deviations were well within the variability ranges reported for CD4 values by the National Institute of Allergy and Infectious Diseases Division of AIDS Quality Assurance Program (interlaboratory) (Gelman et al., 1993) and the Centers for Disease Control and Prevention Model Performance Evaluation Program (CDC, 1994).

In general, we found all γ-IFN and IL-2 reagents comparable to one another, with the possible exceptions of the IL-2 PE reagent of PhM. In our experiments using the described reagents, proportionately few peripheral blood CD4+ or CD8+ cells from these healthy U.S. donors appeared to produce IL-4, IL-10, or TNF-β. Furthermore, differentiating from background noise, represented by pseudopositivity in unstimulated cells, was problematic, especially for CD8+ cells. We therefore did not present data on these reagents herein.

Previous reports employing in situ hybridization and cloning techniques have reported relatively greater production of γ-IFN by CD8+ cells and IL-2, by CD4+ cells (Morvan et al., 1994; Fitch et al., 1995). Those results are consistent with our own, supporting the validity of our findings.

To our knowledge, we provide the first data on normal ranges of single and dual cytokine profiles in stimulated peripheral blood lymphocytes of healthy adult blood donors. These data should prove useful to those performing flow cytometric cytokine assessment. Assessment of multiple cytokines being produced by individual cells, in conjunction with assessment of other surface and cytoplasmic antigens, should profoundly enrich investigations of human infectious disease pathogenesis.

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