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Direct Demonstration of Cytokine Synthesis Heterogeneity Among Human Memory/Effector T Cells by Flow Cytometry

By Louis J. Picker, Manoj K. Singh, Zoran Zdraveski, John R. Treer, Sharr L. Waldrop, Paul R. Bergstresser, and Vernon C. Maino

The array of cytokines produced by T cells in effector sites is a primary means by which these cells mediate host defense. It is well recognized that cloned T cells are heterogeneous with regard to cytokine synthesis and, thus, in their ability to mediate specific immune responses, but the extent to which the patterns of cytokine secretion observed in cloned cells reflect actual populations of memory/effector T cells existing in vivo is largely unknown. Here, we report our findings using a multiparameter flow cytometric assay that allows simultaneous determination of an individual T-cell's ability to produce multiple cytokines and its phenotype after only short (4 to 8 hours) in vitro incubation with an activating stimulus and the secretion inhibitor Brefeldin A. This assay shows a rapid accumulation of interleukin-2 (IL-2), IL-4, and γ-interferon (γ-IFN) in the cytoplasm of CD4$^+$ cells after stimulation with either accessory cell-independent (phorbol 12-myristate 13-acetate [PMA] + ionomycin [I]) or accessory cell-dependent (staphylococcal enterotoxins [SE] A and B) T-cell–activating stimuli. Further analysis showed that production of γ-IFN and IL-4 is predominantly, if not exclusively, restricted to the CD45RO^{high} memory/effector T-cell subset, whereas IL-2 may be produced by both the CD45RO^{high} and CD45RO^{low} subsets. Simultaneous determination of IL-2 and γ-IFN production among CD45RO^{high}/CD4$^+$ T cells showed distinct subsets that produce each of these cytokines alone (an average of 30% for IL-2 alone, 8% for γ-IFN alone), both (16%), or neither (46%). Similar analyses with the small IL-4–producing memory/effector T-cell subset (only 4.3% of total CD4$^+$/CD45RO^{high} T cells) showed that an average of 51% of these IL-4–producing cells also synthesize IL-2, 23% synthesize only IL-4, 16% synthesize all three cytokines, and 9.6% synthesize IL-4 and γ-IFN. These patterns of cytokine synthesis were found to be similar with both PMA + I and SEA/SEB stimulation and were observed in both peripheral blood memory/effector CD4$^+$ T cells and in T cells of similar phenotype obtained from cutaneous delayed-type hypersensitivity sites. Taken together, these data strongly support the in vivo existence of human memory/effector T-cell subsets with "preprogrammed" cytokine synthesis potential, although they suggest that these subsets may be more complex than originally proposed in the TH1/TH2 hypothesis.

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THE MANIFESTATION OF immunity, either protective or pathologic, critically depends on the functional activity of the so-called memory/effector T-cell subset, characterized in the human by its CD45RA^{low}/RO^{high} phenotype. Indeed, only these previously activated T cells appear to have the capability of efficiently homing to and localizing within sites of T-cell effector function, including all extralymphoid sites of inflammation (eg, lung, skin, synovium, and so on) and the unique lymphoid microenvironment of the germinal center.[1,2] However, memory/effector T cells are not uniform in their functional potential. Similar to the immune reactions they initiate, these cells manifest remarkable heterogeneity, both in terms of their ability to extravasate in a particular site and to manifest effector function therein.[1-9] For example, we have described and characterized a subset of CD45RA^{low}/RO^{high} T cells that are selectively targeted to skin,[3,4] and subsets thought to be targeted to other tissues have been described as well.[1-4] However, perhaps the most investigated functional heterogeneity shown by memory/effector T cells is their cytokine synthesis potential.[1,5-10] Detailed studies of cloned CD4$^+$ T cells in both mice and humans have suggested that the ability to synthesize and secrete many key effector cytokines, including interleukin-2 (IL-2), IL-4, IL-5, and γ-interferon (γ-IFN), is restricted to a fraction of the overall memory/effector population. Indeed, the finding of predominant patterns of cytokine secretion among T-cell clones has led to the proposal that CD4$^+$ effector T cells may be generally separated into TH1 (IL-2– and γ-IFN–producing) and TH2 (IL-4– and IL-5–producing) subsets.[5-10]

Even though the cytokine synthesis potential of individual, genuine (ie, noncloned) effector T cells is largely unknown, the TH1/TH2 terminology has become so well accepted that it is routinely extended to complex in vivo immune responses (so that responses with a predominance of γ-IFN– or IL-4–producing T cells are termed "TH1" or "TH2" responses, respectively). Because recent evidence has strongly suggested that the ability of memory/effector T cells to produce a given cytokine is heavily influenced by the microenvironmental conditions present at the time of T-cell activation,[8-12] it is plausible that the long-term in vitro culture required for cloning T cells may shape the cytokine synthesis phenotype of the resulting clones. Thus, the patterns of cytokine secretion observed in such clones may not be representative of all physiologically relevant memory/effector T-cell subsets. Given the clear importance of understanding the entire physiologic range of T-cell functional heterogeneity, we sought to assess the cytokine (γ-IFN, IL-2, and IL-

From the Laboratories of Experimental Pathology and Clinical Immunology, Department of Pathology, and the Department of Dermatology, University of Texas Southwestern Medical Center, Dallas, TX; and Becton Dickinson Immunocytometry Systems, San Jose, CA.

Submitted December 22, 1994; accepted April 3, 1995.

Supported by National Institutes of Health Grants No. AI31545 and AR41940. L.J.P. is an awardee of the President's Research Council of the University of Texas Southwestern Medical Center and of the Advanced Technology Program of the Texas Higher Education Coordinating Board. M.K.S. was supported by a fellowship grant from the World Health Organization.

Address reprint requests to Louis J. Picker, MD, Department of Pathology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75235-9072.

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4) synthesis capabilites of normal, freshly isolated, human memory/effector T cells on a single-cell basis. These studies were based on the pioneering methodological report of Jung et al,[13] who first showed the feasibility of using intracytoplasmic staining and flow cytometry to assess T-cell cytokine production. Here, we improve on these methods and combine them with sophisticated multiparameter analysis techniques to simultaneously determine the cytokine production capabilities of precisely defined subsets of human peripheral blood (PB) and inflammatory site T cells after only short periods (4 to 8 hours) of in vitro activation with mitogen or superantigen. Our results confirm an extensive heterogeneity in the ability of memory/effector T cells to produce these three cytokines in response to both accessory cell-dependent and accessory cell-independent stimuli and show reproducible patterns of cytokine secretion by these cells. The direct visualization of T-cell effector responses afforded by this flow cytometric approach allows an unparalleled ability to determine the functional potential of phenotypically distinct T-cell subsets and, thus, the opportunity to evaluate the participation of these subsets in human immune responses.

MATERIALS AND METHODS

Monoclonal antibodies (MoAbs). MoAbs Leu4 (CD3; fluorescein isothiocyanate [FITC], phycoerythrin [PE], peridinin chlorophyll protein [PerCP]), Leu3a (CD4; FITC, PE, PerCP), Leu2a (CD8; FITC, PE, PerCP), Leu45RO (CD45RO; unconjugated, PE), Leu18 (CD45RA; unconjugated), Leu23 (CD69; FITC, PE), G1CL (mouse IgG1 control; FITC, PE, PerCP), and G2GL (mouse IgG2 control; FITC, PE, PerCP) were obtained from Becton Dickinson Immunocytometry Systems (BDIS; San Jose, CA). Purified MoAbs B-G5 (antihuman IL-2; mouse IgG1) and B-B1 (antihuman γ-IFN; IgG1) were purchased from Biosource International (Camarillo, CA) and were conjugated to PE or FITC by the reagent laboratories at BDIS. The antihuman IL-4 MoAb IL-4I (mouse IgG1) and a mouse IgG1 control reagent were purchased from Pharmingen (San Diego, CA). A series of mouse antihuman cytokine MoAbs (all IgG1) from the research labs of R & D Systems (Minneapolis, MN) were also used in this study (largely to confirm the staining specificity of the above-listed reagents), including clones no. 3010.2-4 and 3011.1 (both anti–IL-4), no. 25723.11 (anti–γ-IFN), and no. 5302.111, 5338.111, and 5344.111 (all anti–IL-2). The unconjugated anticytokine MoAbs were visualized with a Tricolor-conjugated goat antimouse IgG1 second stage obtained from Caltag (South San Francisco, CA).

Cell preparation. PB mononuclear cells (PBMCs) obtained from normal donors were isolated from heparinized venous blood by density-gradient sedimentation over Ficoll-Hypaque (Histopaque; Sigma Chemical Co, St Louis, MO). Cells were then washed 3 times in Hank's balanced salt solution (HBSS; GIBCO, Grand Island, NY) and resuspended in medium appropriately for cell culture, immunofluorescence staining of freshly isolated cells, or futher purification. Purified T cells (>95% CD3+) were isolated from the PBMC preparations by negative selection using R & D Systems T-cell purification columns, as described by the manufacturer. Purified whole virgin ($\geq$95% CD45RO$^-$), whole memory/effector ($\geq$95% CD45RO$^+$), or CD4$^+$ memory/effector ($\geq$95% CD4$^+$ and $\geq$95% CD45RO$^+$) T cells were isolated from T-cell preparations via 2 to 3 rounds of negative panning using the Leu45RO, Leu 18, and Leu2a + Leu18 MoAbs, respectively, as previously described.[14] In some experiments, accessory cell (monocyte) reconstitution was required to allow purified T-cell preparations to respond to superantigens (see below). For these experiments, the culture wells to be used with the purified populations were preseeded with PBMCs at 0.5×10^6 cells/mL in complete media and allowed to incubate for 1 hour at 37°C, after which the culture wells were extensively washed to remove nonadherent cells (leaving >95% monocytes) and then were seeded with purified T-cell populations (see below).

Leukocyte preparations from delayed-type hypersensitivity (DTH) sites in skin were obtained as previously described.[15] Briefly, common skin-test antigens (Ags; eg, mumps Ag) were injected intradermally into the normal forearm skin of sensitized volunteers before the placement of suction blisters 18 to 24 hours later. (Clinical evidence of a delayed hypersensitivity reaction usually appeared at about 24 hours, peaking at 40 to 50 hours.) Leukocyte-containing blister fluid was withdrawn at successive 24-hour intervals after blister placement for up to 92 hours. (Fluids were completely evacuated at each time point, with natural refilling occurring between samplings.) Collected leukocytes were washed by centrifugation and processed immediately for the immunofluorescent cytokine production assays. The results presented in this study represent the characterization of T cells taken from the blister fluid sample correlating with the peak of the observed DTH response.

Flow cytometric cytokine production assays. Flow cytometric assessment of T-cell cytokine production is based on the stimulation of T cells in the presence of a pharmacologic inhibitor of secretion, followed by cell fixation and permeabilization, and then intracytoplasmic staining of acccumulated cytokines. This technique was originally described by Jung et al[13] but has been significantly modified in these studies. These changes have both simplified the staining procedure and considerably enhanced the sensitivity and reproducibility of the cytokine detection (data not shown). In our protocol, Brefeldin A (BFA), a relatively nontoxic but potent inhibitor of intracellular transport,[16] was used to block the cytokine secretion stimulated by both accessory cell-independent (phorbol 12-myristate 13-acetate [PMA] + ionomycin [I]) and accessory cell-dependent (the bacterial superantigens, staphylococcal enterotoxin [SE] A and SEB)[17,18] T-cell agonists. In preliminary experiments, BFA (10 μg/mL final concentration) completely blocked the surface appearance of the activation Ag CD69[19] on 4-hour PMA + I-stimulated T cells without any loss of viability (viability maintained for for up to 18 hours). Importantly, BFA did not interfere with early activation events, because the level of intracytoplasmic CD69 staining (see below) shown by PMA + I-activated, BFA-treated T cells was almost identical to the level of surface CD69 staining on non–BFA-treated, similarly activated T cells (data not shown). Preliminary experiments also indicated that maximal accumulation of intracellular cytokine occurred after 4 hours of BFA-inhibited PMA + I stimulation and after 6 to 8 hours of BFA-inhibited superantigen stimulation. (Cytokine accumulation plateaued and then decreased with longer periods of BFA exposure.) Thus, T cells (without accessory cells for PMA + I stimulation; with accessory cells for superantigen stimulation) were cultured in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (Hyclone Sterile Systems, Inc, Logan UT), 20 mmol/L HEPES buffer (GIBCO), 100 U/mL penicillin (GIBCO), 100 μg/mL streptomycin (GIBCO), and 2 mmol/L L-glutamine (GIBCO) with or without the T-cell stimulants PMA + I (5 ng/mL and 1 μmol/L, respectively; Sigma), or the superantigens SEA and SEB (100 ng/mL of each; Toxin Technology, Sarasota, FL). BFA was added 4 hours (PMA + I) or 6 to 8 hours (SEA + SEB) before harvest so that, in kinetic experiments, the period of secretion inhibition and, thus, cytokine accumulation was for 4- or 6-hour windows of time (eg, 0 to 4 hours, 14 to 18 hours, 40 to 44 hours, and so on).

After harvesting, cells (0.1 to 0.5×10^6/test) were washed with cold HBSS, stained (if appropriate) on the cell surface with an MoAb-fluorochrome conjugate (IgG2 MoAbs only, in this case Leu45RO), and then were simultaneously fixed and permeabilized

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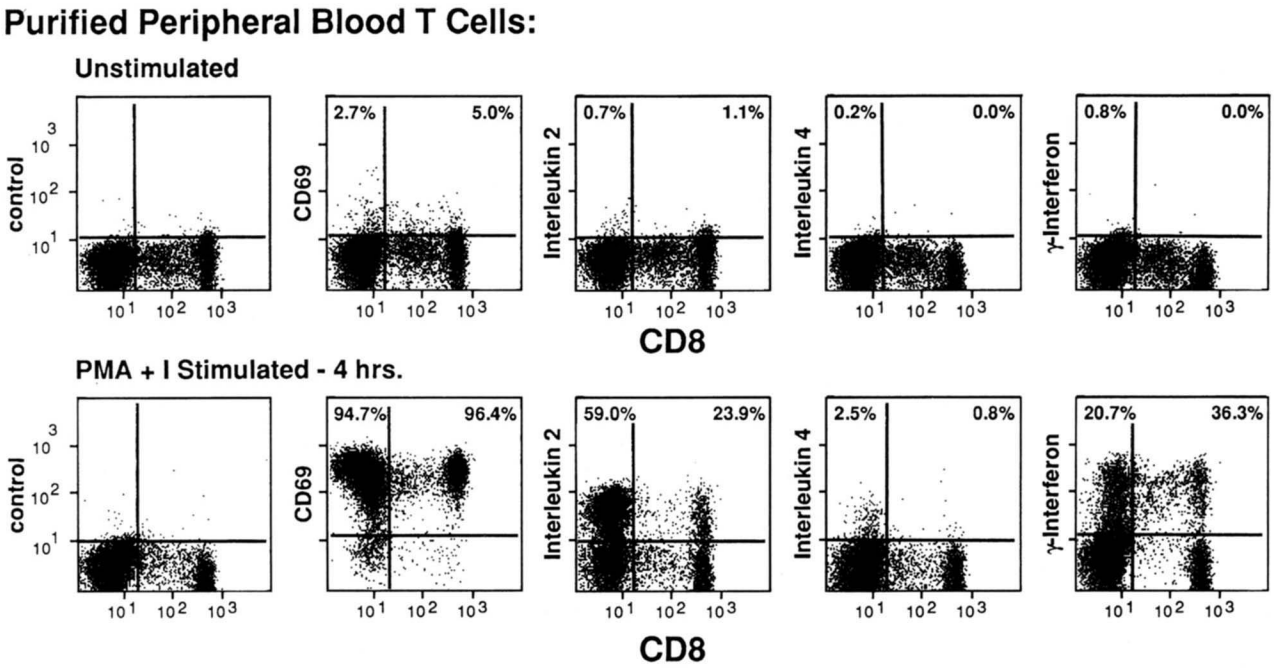

Fig 1. Flow cytometric detection of cytokine synthesis among purified PB T cells stimulated with PMA + I. Purified PB T cells were stimulated with PMA + I for 4 hours in the presence of the secretion inhibitor BFA and then compared with unstimulated T cells for their correlated expression of intracellular cytokine (IL-2, IL-4, γ-IFN) or the activation Ag CD69 versus CD8. A total of 10,000 events, gated on viable cells using light scatter criteria, are shown in each plot. The percentages in the upper left and right corners of each plot represent the net percentage (%) of positive (+) cells (ie, after subtracting background) among the CD8⁻ and CD8⁺ subsets, respectively. As discussed in the Materials and Methods section, the CD8⁻ population is essentially equivalent to the CD4⁺ subset in these experiments. Note that the vast majority of the PMA + I-treated T cells are highly positive for the activation Ag CD69, indicating that the lack of production of a particular cytokine by a given cell was not likely due to incomplete activation.

in HBSS with 10 mmol/L HEPES buffer, 4% paraformaldehyde, and 0.1% saponin (all from Sigma) for 10 to 15 minutes at 4°C. After washing 2 times with cold Dulbecco's phosphate-buffered saline (dPBS) containing 0.1% bovine serum albumin, cells were stained as previously described[15,20] for surface Ag immunofluorescence. Continued or additional permeabilization was not necessary for optimal staining. Briefly, cells were incubated successively with the following reagents (all at 0 to 4°C) with washing in dPBS/bovine serum albumin in between: (1) an appropriately titred unconjugated anticytokine MoAb (usually 0.1 to 0.4 μg/test for 30 minutes); (2) the Tricolor-conjugated goat antimouse IgG1 second stage in dPBS with 5% normal human serum (30 minutes); (3) 0.4 mL/test of 10% normal mouse serum (10 minutes; for blocking); and (4) 1 to 2 conjugated MoAbs against additional cytokines or other Ags (CD4, CD8, and/or CD69).

The CD4 and CD8 MoAbs delineated identical populations whether they were used before or after permeabilization. However, because of the previously documented rapid and complete downmodulation of CD4 in response to phorbol esters,[21] CD4 MoAbs could not be used to delineate the CD4⁺ T-cell subset after more than 4 hours of PMA + I stimuluation. Because CD8 expression diminishes only slightly after this treatment, and because multiparameter staining of the unstimulated purified T-cell preparations used in our study showed that the vast majority of CD8⁻ cells were indeed CD4⁺ (not shown), CD8 MoAbs alone could effectively differentiate the CD4⁺ and CD8⁺ subsets. Therefore, in the experiments examining PMA + I-stimulated purified T cells, the CD8⁻ subset was considered equivalent to the CD4⁺ subset. CD4 downmodulation does not occur with superantigen stimulation; therefore, the CD4 subset could be directly identified in these experiments. CD69 expression was used in these studies as an indicator of activated cells.[19] In BFA-treated cells, CD69 upregulation is confined to the cytoplasm, requiring that CD69 staining be performed after permeabilization (see above). In contrast, BFA protected the cell surface from activation-induced changes in CD45RO expression, allowing delineation of the original virgin and memory/effector subsets only if staining was performed before permabilization. Because continuous BFA was required for this effect and because continuous BFA for longer than 8 hours results in diminshing cytokine accumulation, this technique could only be used for the 0- to 4-hour or 0- to 6-hour time points.

Five-parameter analysis was performed on a FACSort flow cytometer (BDIS) using FITC, PE, and PerCP or Tricolor (PE/Cy5 tandem) as the three fluorescent parameters. Methods of cytometer setup and data acquisition have been described previously.[20] List-mode multiparameter data files (each file with forward scatter, side scatter, and three fluorescent parameters) were analyzed using the PAINT-A-GATE[Plus] program (BDIS). Negative control reagents were used to verify the staining specificity of experimental antibodies and as a guide for setting markers to delineate positive and negative populations. All percentages listed in the figures and text represent net percent positive, after subtraction of background. All analyses were performed using a light scatter gate designed to include only viable lymphocytes. In some analyses, additional live gating based on CD4 or CD8 reactivity or CD69 reactivity was performed to enhance the sampling of small populations. Preliminary experiments using this flow cytometric cytokine assay on two well-characterized human TH1 clones and on IL-4 (versus control) transfectants showed an excellent qualitative correlation between the flow cytometric results and the level of overall cytokines measured by conventional techniques (immunoassay, analysis of mRNA; data not shown), similar

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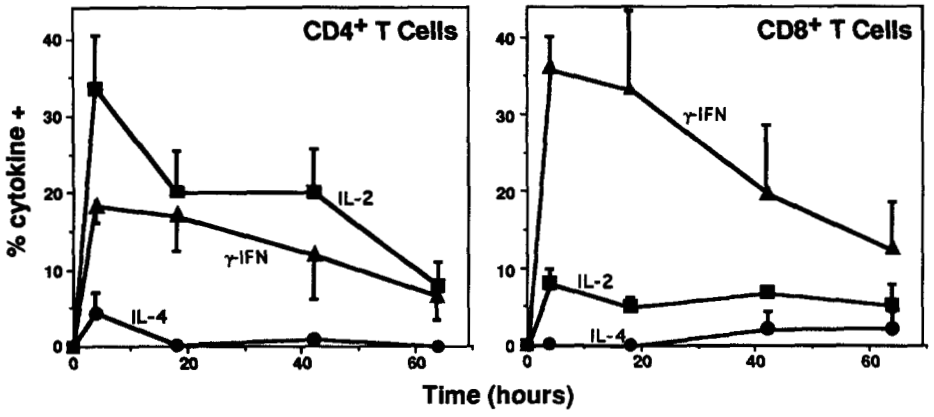

Fig 2. Flow cytometric characterization of the kinetics of cytokine synthesis among purified PB T cells stimulated with PMA + I. Purified PB T cells were stimulated with PMA + I for 4, 18, 42, and 66 hours (with BFA added during the final 4 hours of culture to inhibit cytokine secretion) and then analyzed for their correlated expression of intracellular cytokine (IL-2, IL-4, γ-IFN) versus CD8. The results presented are the mean percentage (%) of cytokine positives (± SEM) for the CD4⁺ (CD8⁻) and CD8⁺ T-cell subsets in 3 independent experiments. In these experiments, essentially 100% of T cells manifested CD69 expression by the 18-hour time point, indicating complete activation.

to previously reported results.[13] Moreover, identical patterns of reactivity were observed with at least two independent MoAb clones for each cytokine. The data presented in the figures are representative of 3 to greater than 10 separate experiments.

RESULTS

Initial studies focused on the flow cytometric characterization of the cytokine production capabilities of purified PB human T cells activated by the accessory cell-independent stimulus of PMA + I in the presence of BFA. Significant cytokine reactivity was not detected within fresh, unstimulated T cells but was dramatically upregulated (along with the activation Ag CD69) after as little as 4 hours of incubation with this potent activating stimulus (Fig 1). This response was not apparent or was markedly diminished with (1) in vitro incubation in the absence of an activating stimulus (with or without BFA), (2) appropriate activation in the absence of secretion inhibition (ie, the inclusion of BFA with the PMA + I), or (3) the lack of cell permeabilization before staining (data not shown). The absolute requirement for permeabilization (ie, the lack of specific signal after cell surface staining), along with the inability of exogenously added cytokine to produce a signal using our staining protocols (data not shown), indicates that T-cell binding to soluble cytokine in the culture medium did not contribute to the observed responses. Cytokine synthesis clearly preceded blastogenesis, because, at the 4-hour time point, the vast majority of cytokine-producing cells still manifested the light scatter properties of small lymphocytes (not shown). Significantly, despite the fact that essentially all PMA + I-treated T cells showed marked upregulation of CD69 (see Fig 1), which is consistent with universal activation, only a subset of T cells produced detectable cytokine at any given time point (Figs 1 and 2). Both the CD4⁺ (CD8⁻) and CD8⁺ subsets participated in this cytokine response, with more CD4⁺ T cells producing IL-2 than CD8⁺ T cells, and vice versa for γ-IFN. Among CD4⁺ cells, IL-2 production usually peaked in the 0- to 4-hour time period, with a sharp partial decrease at the 14- to 18-hour time points and a slow decrease thereafter. Peak levels of γ-IFN by CD4⁺ T cells and of both γ-IFN and IL-2 by CD8⁺ T cells were achieved in the first 4 hours of stimulation, with a very slow decrease in the numbers of producing cells over the next 3 days. Among CD4⁺ T cells, IL-4 production was transient (usually identified in 0- to 4-hour time period only) and was only observed in a small subset of these cells (overall, mean ± SEM = 2.7% ± 0.6% for 18 different experiments). IL-4 was usually detected in even fewer (usually <1%) CD8⁺ T cells at the 4-hour time point but, in some experiments, reappeared in a slightly larger subset of CD8⁺ cells after 40 hours of stimulation (Fig 2).

We next assessed the ability of the accessory cell-dependent bacterial superantigens SEA and SEB to stimulate detectable T-cell cytokine responses. Because these superantigens are known to stimulate T cells in a Vβ-specific manner (eg, Vβ1.1, Vβ5, Vβ6, Vβ7.3, and Vβ9.1 for SEA; Vβ3, Vβ12, and Vβ17 for SEB)[22] and, thus, only activate a subset of the overall T-cell population, we simultaneously evaluated CD69 expression along with cytokine synthesis and CD4/8 phenotype so as to determine the percentage of cytokine-producing cells within the activated CD4⁺ or CD8⁺ T-cell populations. As shown in Fig 3, only the CD69⁺ T cells synthesized and accumulated significant intracellular cytokine, allowing us to gate on the CD69⁺ cells to determine the extent to which SEA-/SEB-responsive cells produce these cytokines. A representative experiment comparing the participation of (CD69-gated) CD4⁺ versus CD8⁺ T cells in an SEA-/SEB-induced cytokine response is shown in Fig 4. Similar to what was observed with PMA + I, more CD4⁺ T cells produce IL-2 than CD8⁺ T cells, whereas the reverse was true for γ-IFN. However, the overall number of cells manifesting an IL-2 or γ-IFN response to SEA/SEB was less than that observed for PMA + I (even with analysis restricted to the activated population). This was not apparent for IL-2–producing CD4⁺ cells, which were twofold to threefold fewer in number (see below). In contrast, the percentage

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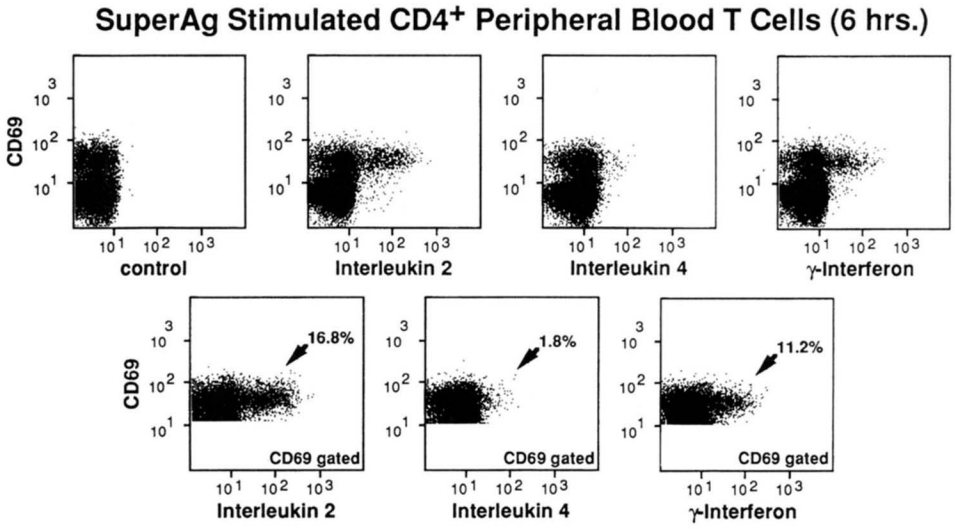

Fig 3. Flow cytometric characterization of cytokine synthesis by superantigen-stimulated PB T cells; evaluation per total activated T cells. PBMCs were stimulated with the bacterial superantigens SEA + SEB for 6 hours in the presence of BFA and then stained intracellularly for (1) IL-2, IL-4, γ-IFN, or an isotype-matched control; (2) CD4; and (3) the rapid activation Ag CD69. A total of 15,000 events gated on CD4 expression (top panel) or 7,500 to 8,000 events gated on CD4 and CD69 expression (bottom panel) are shown. Note that cytokine synthesis is restricted to the activated CD69⁺ subset (which in this experiment, comprise 20.9% of CD4⁺ cells at 6 hours). Thus, gating on CD69 allows determination of the cytokine-producing cells as a fraction of the activated population only (16.8%, 1.8%, and 11.2% for IL-2, IL-4, and γ-IFN, respectively, in this experiment). Such gating greatly increases the accuracy and reproducibility of cytokine-producing cell quantitation after stimulation with subset-restricted activating agents (such as superantigen), because it (1) corrects for differences in the number of T cells responding to these agents and (2) allows collection of more positive events (ie, cytokine-producing cells).

of IL-4–producing T cells induced by SEA/SEB (after CD69 gating) was as large or larger than that induced by PMA + I (note particularly the relatively increased IL-4 response in the $CD8^+$ subset). SEA/SEB-stimulated responses for all three cytokines were transient (peak responses always in the 0- to 6-hour time period), despite the fact that the mean percentage (± SEM; n = 4) of $CD69^+$ cells increased from 17.6% ± 1.7% after 6 hours of SEA/SEB stimulation to 51.1% ± 5.5% after 18 hours of stimulation.

Previous studies have established that the so-called virgin and memory/effector subsets of $CD4^+$ T cells, delineated by differential expression of the CD45RA and RO isoforms,

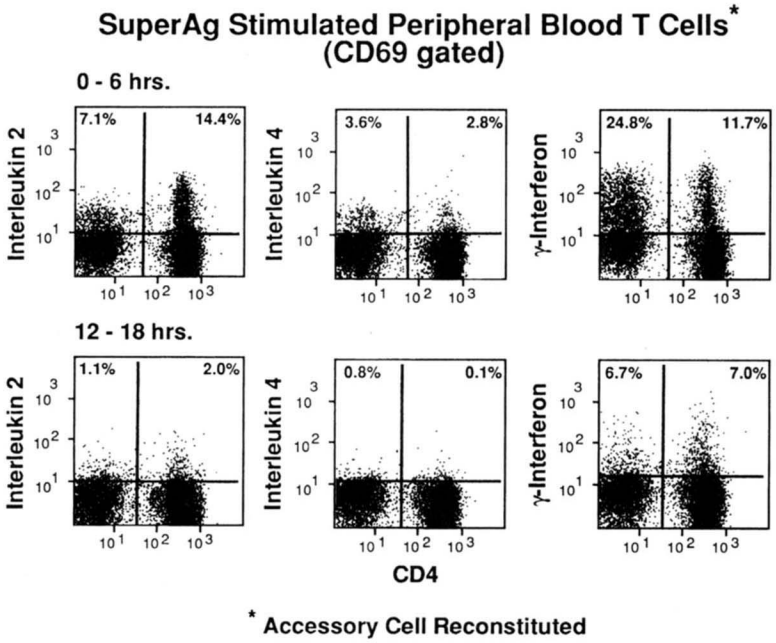

Fig 4. Flow cytometric characterization of cytokine synthesis patterns among superantigen-stimulated PB T cells. Purified PB T cells were reconstituted with monocytes and stimulated with the bacterial superantigens SEA + SEB for 6 and 18 hours (with BFA added during the final 6 hours of culture). These cells were then assessed for their correlated expression of intracellular cytokine (IL-2, IL-4, γ-IFN), CD4, and CD69 and analyzed after gating on the $CD69^+$ T-cell subset, as shown in Fig 3 (10,000 events in each plot). The percentages in the upper left and right corners of each plot represent the net percentage (%) of positive (+) cells among the $CD4^-$ and $CD4^+$ subsets, respectively. Because the original T cells are highly purified, the $CD4^-$ population is essentially equivalent to the $CD8^+$ subset in these experiments (the few nonadherent monocytes present at the time of harvest are eliminated by appropriate gating on light scatter parameters).

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differ markedly in cytokine gene expression and secretion. In general, the putative virgin T-cell subset expresses IL-2 only after appropriate stimulation, whereas the putative memory/effector subset expresses all three cytokines.[23-26] Our multiparameter flow cytometric analysis confirms these data on a single-cell basis. Figure 5 shows a representative experiment for PB $CD4^+$ T cells after 4 hours of stimulation with PMA + I. Note that $CD4^+$ T cells with a surface $CD45RO^{high}$ phenotype include the vast majority of T cells producing detectable levels of γ-IFN and IL-4. About half of this subset produces IL-2 as well, but IL-2 is also produced by about 25% of $CD45RO^{low}$ putative virgin T cells. Significantly, among SEA/SEB-stimulated $CD4^+$ T cells, production of all three of these cytokines was restricted to the $CD45RO^{high}$ subset (data not shown), likely accounting for the above noted pronounced decrease in IL-2–producing $CD4^+$ cells after this stimulus, as compared with PMA + I stimulation.

It is possible that the differences observed in PMA + I-induced γ-IFN production by the CD45RO-defined, $CD4^+$ T-cell subsets in Fig 5 are a function of delayed kinetics of γ-IFN production by the $CD45RO^{low}$ subset. (This possibility cannot explain the observed differential IL-4 production, because significant IL-4 production is only present in the 0- to 4-hour time period.) To address this issue, we determined γ-IFN and IL-2 production by CD45RA/RO-defined $CD4^+$ T-cells subsets during the 14- to 18-hour and 36- to 40-hour time periods. Because continuous BFA cannot be used for these longer incubation periods (see Materials and Methods section), we purified $CD45RA^+(RO^-)$ and $CD45(RA^-)RO^+$ $CD4^+$ T cells before in vitro stimulation for these studies. As shown in Fig 6, both the CD45RA/RO-defined subsets responded to the PMA + I at these later time points with a marked upregulation of CD69 and substantial production of IL-2, yet only the $CD45(RA^-)RO^+$ subset produced significant γ-IFN.

To examine the degree of overlap between the $CD4^+$ memory/effector T-cell populations synthesizing γ-IFN, IL-2, and IL-4 after short-term PMA + I stimulation, we used our flow cytometric procedure to simultaneously analyze pairs of these cytokines among purified memory/effector T cells, with gating on the $CD8^-$ ($CD4^+$) subset (Fig 7). The results indicate a strikingly complex, yet consistent, pattern of cytokine synthesis-defined, $CD4^+$ memory/effector T-cell subsets. In all experiments, the most common memory/effector phenotype was the production of IL-2 alone (30.4% ± 2.0% of cells; n = 5), followed by the IL-2 + γ-IFN-producing phenotype (16.4% ± 2.6%), the γ-IFN alone-producing phenotype (7.7% ± 1.5%), and, finally, the phenotype characterized by IL-4 production (4.3% ± 0.8%), either alone or in combination with other cytokines (see below). Kinetic studies (Fig 8) indicated the IL-2 + γ-IFN–producing subset was relatively short-lived, compared with the IL-2 alone- or γ-IFN alone-producing subsets, practically disappearing by the 22-hour time point. Interestingly, the IL-4– and γ-IFN–producing $CD4^+$ T subsets were predominantly nonoverlapping, whereas, in contrast, the majority of IL-4 producers simultaneously produced IL-2 (Fig 7). These results were confirmed and extended in experiments simultaneously analyzing all three cytokines on PMA + I-stimulated T-cell populations gated on the IL-4–producing subset (Fig 9). A total of 51.1% ± 1.9% (n = 3) of IL-4–producing T cells also produced IL-2; 23.0% ± 4.3% of these T cells produced IL-4 alone, 16.3% ± 3.5% produced IL-4 in combination with both IL-2 + γ-IFN, whereas only 9.6% ± 0.5% produced IL-4 in combination with γ-IFN but not IL-2.

To determine whether the ability of $CD4^+$ memory/effector T cells to heterogeneously produce the cytokines under study was a function of the nature of the activating stimulus, the presence or absence of accessory cells, or the site from which the T cells were obtained, we performed similar multiple intracellular cytokine analyses on superantigen-stimulated $CD4^+/CD45RO^+$ T cells from both PB and from skin blisters overlying cutaneous DTH sites. The PB $CD4^+/CD45RO^+$ T cells were prepurified by negative selection, reconstituted with monocytes, and, after SEA+SEB stimulation, analyzed concomitantly for intracellular cytokine and CD69 expression, with cytokine responses measured on the activated (CD69-gated) population. The skin

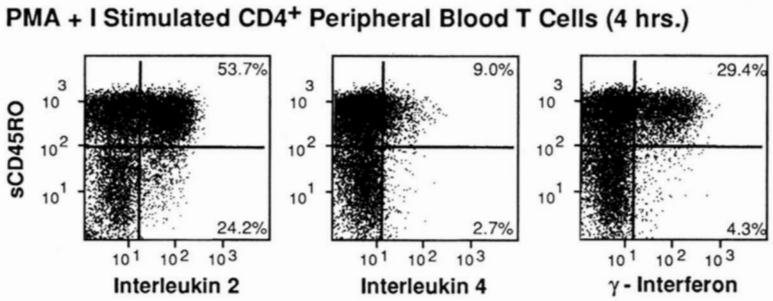

Fig 5. Characterization of the CD45RO phenotype of cytokine-producing $CD4^+$ PB T cells after short-term PMA + I stimulation. Purified PB T cells were stimulated with PMA + I for 4 hours in the presence of BFA and then assessed for their correlated expression of cell surface CD45RO versus intracellular cytokine (IL-2, IL-4, γ-IFN) and CD8. Representative profiles (10,000 events are shown in each plot) are shown, gated on the $CD8^-$ ($CD4^+$) subset. The percentages in the upper right and lower right corners of each plot represent the net percentage (%) of positive (+) cells among the $CD45RO^{high}$ and $CD45RO^{low}$ subsets, respectively. As discussed in the Materials and Methods section, BFA prevents activation-induced changes in surface CD45RO (sCD45RO) expression, leaving the sCD45RO expression pattern essentially identical to that observed among fresh T cells (data not shown).

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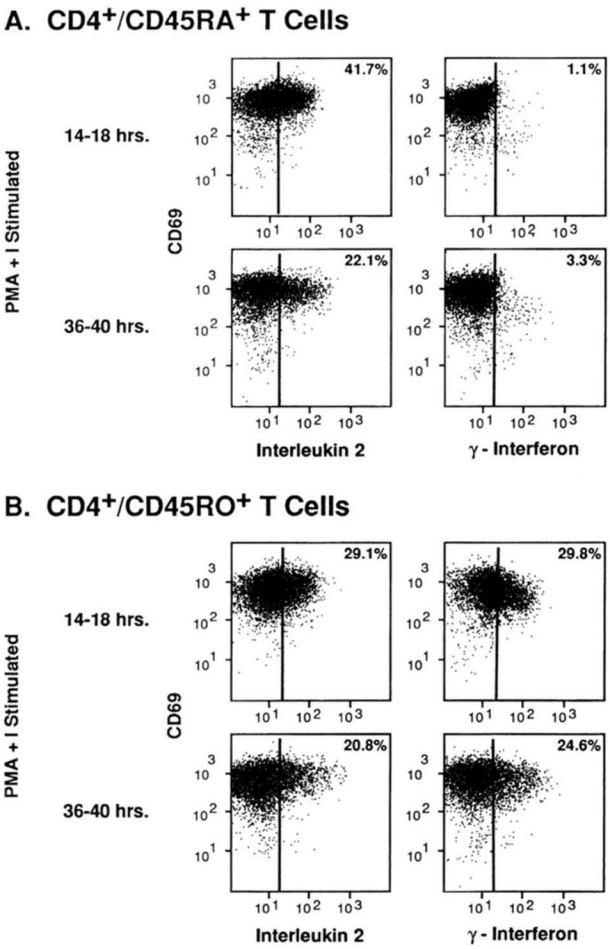

Fig 6. Characterization of the CD45RO phenotype of cytokine-producing CD4⁺ PB T cells after longer term PMA + I stimulation. CD45RA^{high}/RO^{low} and CD45RA^{low}/RO^{high} T cells were prepurified by negative selection, stimulated with PMA+ I for 18 and 40 hours (with BFA included the last 4 hours), and then analyzed for their correlated expression of intracellular cytokine (IL-2 and γ-IFN only; IL-4 is not significantly produced by CD4⁺ T cells at these time points), CD8, and CD69. Representative profiles of cytokine versus CD69 (5,000 events in each plot) are shown, gated on the CD8⁻ (CD4⁺) population. The percentages in the upper right corners of each plot represent the net percentage (%) of cytokine-positive (+) cells among the cells shown. Note the high CD69 expression by all populations at both time points, consistent with nearly universal activation, and the strong production of IL-2 by both the CD45RA^{high}/RO^{low} and CD45RA^{low}/RO^{high} subsets; yet, only the CD45RA^{low}/RO^{high} (CD4⁺) T cells produce significant γ-IFN.

blister T cells were simultaneously analyzed for two cytokines and CD4, with gating on the CD4⁺ population. These skin-derived T cells are essentially all CD45RO⁺ to begin with[1,15] and show an intrinsic twofold to fourfold higher response to SEA + SEB, obviating the need for CD69 gating (data not shown). As shown in Figs 10 and 11, the pattern of cytokine-defined T-cell subsets generated by superantigen stimulation of PB and cutaneous T cells are remarkably similar to each other and to the pattern observed with PMA + I, showing in each circumstance a similar relative proportion of cells synthesizing each of the three studied cytokines, alone and in combination.

DISCUSSION

The concept of cytokine synthesis heterogeneity among memory/effector T-cell populations was initially suggested by data in the mouse indicating that otherwise similar, cloned CD4⁺ T cells differed in their cytokine production capabilities.[5,8-10] Two general patterns were observed: (1) the TH1 pattern characterized by IL-2, γ-IFN, and tumor necrosis factor β (TNF-β) production; and (2) the TH2 pattern characterized by IL-4, IL-5, IL-6, IL-10 and (later) IL-13 production. The physiologic relevance of these TH1 and TH2 T-cell clones was suggested by the demonstration of polarized TH1- and TH2-like cytokine responses in different in vivo situations that frequently correlated with immune protection against a variety of infectious agents.[9,27,28] More recently, analysis of T-cell clones in the human have shown an analogous, although not identical, cytokine synthesis heterogeneity.[6,7] In addition, bulk cytokine analysis techniques (measurement of secreted cytokines and cytokine mRNA from complex, nonclonal populations), as well as enzyme-linked immunospot assays that allow determination of the number of cells secreting a single cytokine, have indicated that in vivo-derived human T-cell effectors from different disease states or immune situations are often associated with different relative ratios of cytokine gene expression and/or secretion.[29-32]

However, despite the wealth of data on polarized cytokine responses in both mice and humans, methodological limitations have precluded a detailed understanding of the cytokine synthesis potential of single in vivo-derived T cells in either species. The vast majority of studies addressing the ability of individual cells to simultaneously produce two or more cytokines have relied on the study of T-cell clones that have, by necessity, spent weeks to months in vitro before analysis. Although it is unclear the extent to which bias is introduced by this long period of cell culture, it is possible that true in vivo T-cell phenotypes may be modified during T-cell cloning by both selection (only those cells capable of extensive cell division are evaluated) and the inadvertent regulation of cytokine synthesis phenotype. The latter would potentially be mediated by incidental cytokines that may have been added to the culture media to facilitate T-cell outgrowth or produced by the activated T cells themselves and/or their accessory cells during the cloning process. We reasoned that one feasible methodological approach to this problem was multiparameter immunofluorescent analysis of secretion-inhibited, short-term–activated, normal T cells. This technique allows simultaneous assessment of the synthesis of multiple cytokines by T cells that have been cultured in vitro as few as 4 hours. Both the limited time spent in vitro and the secretion inhibition (eg, BFA treatment) greatly diminishes the possibility that T-cell or accessory-cell cytokines could modulate the cytokine response patterns of the T cells. Moreover, because proliferation is not a requirement for a responding cell, and because in most experiments there is little or no cell death during the short-term activation period (L.J. Picker, unpublished observations), selection is unlikely to

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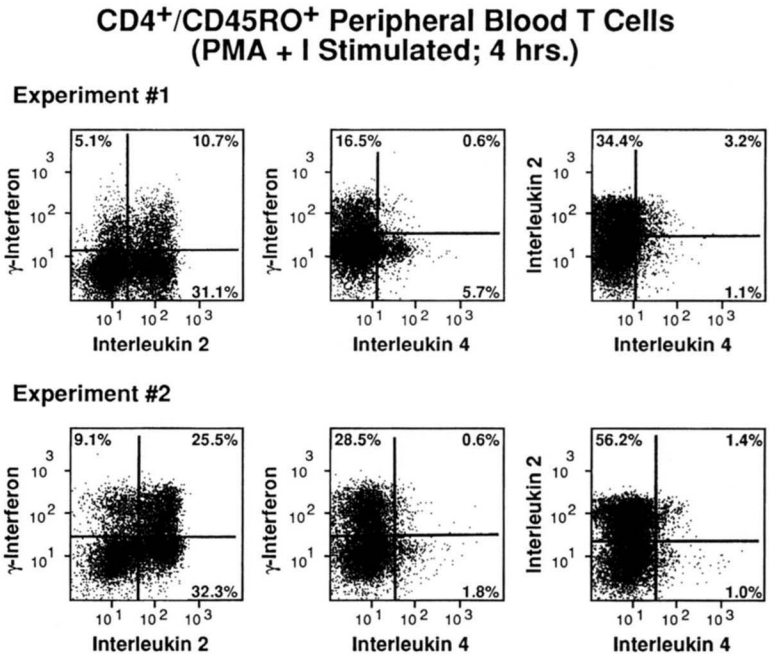

Fig 7. Characterization of patterns of multicytokine production by PMA + I-stimulated CD4⁺/CD45RO^{high} PB T cells. CD45RA^{low}/RO^{high} T cells were prepurified by negative selection, stimulated with PMA+ I for 4 hours in the presence of BFA, and then analyzed for their correlated expression of intracellular cytokines (γ-IFN versus IL-2; γ-IFN versus IL-4, and IL-2 versus IL-4) and CD8. Two representative experiments are shown with 10,000 events in each plot, gated on the CD8⁻ (CD4⁺) population. The percentages in the corners of each plot represent the net percentage (%) of positive (+) cells in the appropriate quadrants (ie, the +/−, +/+, −/+ subsets).

significantly bias the observed results. Finally, this technique allows simultaneous correlation of cytokine synthesis capabilities with other surface or cytoplasmic phenotypic markers, allowing determination of the cytokine synthesis potential of precisely defined T-cell subsets.

In the first part of this report, we showed the ability of multiparameter flow cytometric cytokine analysis to determine the cytokine synthesis capabilities of the CD4⁺, CD8⁺, CD4⁺/CD45RO^{low}, and CD4⁺/CD45RO^{high} T-cell subsets. Our results indicate that the relative cytokine production capabilities of CD4⁺ and CD8⁺ T cells are in the order of IL-2 > γ-IFN > IL-4 and γ-IFN > IL-2, respectively. In some experiments, we also identified a small IL-4–producing CD8⁺ T-cell subset, consistent with recent reports indicating the existence of such cells.[33,34] Both the absolute and relative percentages of IL-2–, IL-4–, and γ-IFN–producing CD4⁺ versus CD8⁺ T cells found in our study were remarkably similar to the percentages reported by Lewis et al[35] using in situ hybridization techniques. Also consistent with previous reports is our observation that, among CD4⁺ T cells, the CD45RO^{high} putative memory/effector subset accounts for the preponderance of IL-4 and γ-IFN production, whereas both the CD45RO^{high} and CD45RO^{low} virgin subset can produce IL-2.[23-26,36] Both our study and the few others in the literature using techniques capable of measuring cytokine expression/production on a per-cell basis[13,35-38] strongly suggest that these subset-based differences in cytokine production are largely attributable to differences in the frequency of cells capable of producing significant levels of these cytokines, as opposed to differences in either the amount of cytokine produced per cell or the kinetics of production. For example, the higher γ-IFN production by CD4⁺/CD45RO^{high} T cells as compared with that by CD4⁺/CD45RO^{low} T cells[23] was not due to homogeneous high production of γ-IFN by the former versus homogeneous low production by the latter subset, but rather to the ratio of γ-IFN–producing and nonproducing cells in each population. Indeed, for any given T-cell subset (CD4 versus CD8; CD45RO$^{high\ versus\ low}$), the number of IL-2–, γ-IFN–, and IL-4–producing cells generated in response to either PMA + I or superantigen was a consis-

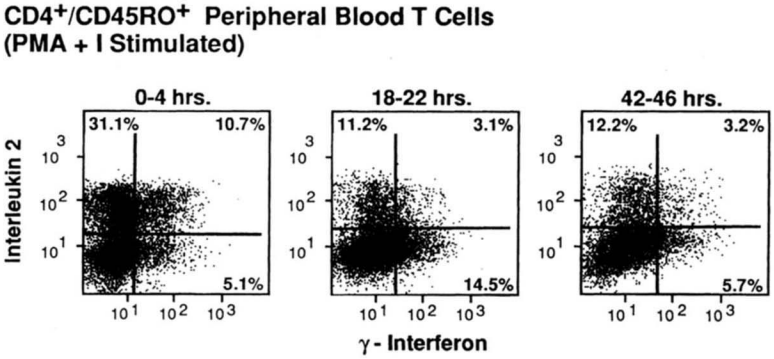

Fig 8. Kinetics of multicytokine-producing (γ-IFN versus IL-2) CD4⁺/CD45RO^{high} PB T cells after PMA + I stimulation. CD45RA^{low}/RO^{high} T cells were prepurified by negative selection, stimulated with PMA+ I for 4, 22, and 46 hours with BFA added during the final 4 hours of culture, and then analyzed for their correlated expression of γ-IFN, IL-2, and CD8. Representative profiles of γ-IFN versus IL-2 (10,000 events in each plot) are shown, gated on the CD8⁻ (CD4⁺) population. The percentages in the corners of each plot represent the net percentage (%) of positive (+) cells in the appropriate quadrants (ie, the +/−, +/+, −/+ subsets).

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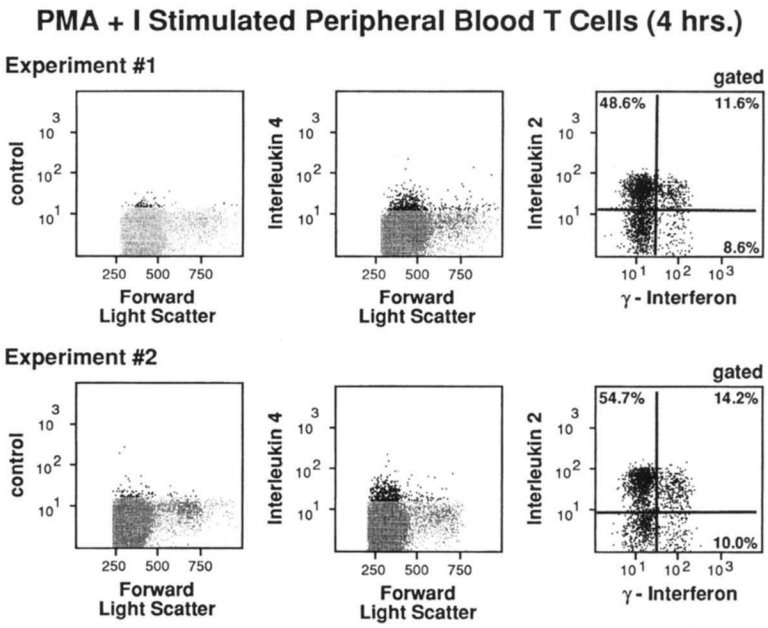

Fig 9. Characterization of γ-IFN and IL-2 production by IL-4 producing PB T cells. Purified PB T cells were stimulated with PMA + I for 4 hours in the presence of BFA and then analyzed for their correlated expression of γ-IFN, IL-2, and IL-4. Two representative experiments are shown. In the left and middle panels (forward light scatter versus IL-4 or isotype-matched control), the overall T-cell subset is shown with the positive cells colored black, and the remaining T cells gray (28,000 to 30,000 events shown). Specific IL-4 staining (after subtracting background) was 1.2% and 1.3% for experiments no. 1 and 2, respectively. The right panels show the γ-IFN versus IL-2 profiles of the IL-4$^+$ cells only (2,000 events shown; gated on the black-colored cells in the middle panel). As previously shown (Figs 1 and 5; and confirmed in parallel analyses in these experiments for CD4), under these conditions, a significant IL-4 response is only found in the CD4/CD45RO^{high} T-cell subset, obviating the need for subset prepurification or gating on these parameters in these experiments. The percentages in the corners of the gated plots represent the percentage (%) of positive (+) cells in the appropriate quadrants (ie, the +/−, +/+, −/+ subsets for IL-2 and γ-IFN, with the −/− population constituting the remaining cells). Note (as previously indicated in the text) that the vast majority of IL-4−producing cells have the light scatter characteristics of small lymphocytes (low forward light scatter), consistent with cytokine synthesis predominantly preceding blastogenesis during the activation response.

tent fraction of the overall activated (CD69$^+$) T-cell population. These cytokine-producing T cells manifested characteristic levels of intracellular cytokine, and they appeared and disappeared with predictable kinetics.

The differences in the overall number of CD4$^+$/CD45RO^{high} T cells producing IL-2 versus γ-IFN in these initial studies strongly suggested a divergence from the predicted IL-2$^+$/γ-IFN$^+$ phenotype of TH1 memory/effector T cells. Simultaneous evaluation of two to three cytokines within the same cells confirmed this divergence and showed the existence of distinct subsets of memory/effector T cells that manifested synthesis of all possible combinations of the three cytokines assessed. Most common were cells producing IL-2 alone (30%). Classic TH1 (IL-2$^+$/γ-IFN$^+$/IL-4$^-$) and TH2 (IL-2$^-$/γ-IFN$^-$/IL-4$^+$) patterns were displayed by only a fraction (16% and <1%, respectively) of the PMA + I-activated, memory/effector T cells. Interestingly, the TH1-like IL-2$^+$/γ-IFN$^+$ subset generated by PMA + I appeared only transiently at the 0- to 4-hour time point; by 18 hours, most of this subset either disappeared or converted to single IL-2− or γ-IFN−producing cells. Among the small, transiently-appearing IL-4−producing subset, 75% also produced IL-2, γ-IFN, or both. Taken together, these data clearly indicate that there is no obligate pairing of the ability to produce these three cytokines during the development of memory/effector T-cell function and strongly suggest that the expression potential of each of these three cytokines is independently regulated. Similar conclusions were drawn by Assenmacher et al[38] who used multiparameter flow cytometry to analyze cytokine expression patterns among murine splenic CD4+ T cells stimulated with SEB. Because cytokine synthesis phenotypes are thought to initially develop during the Ag-induced differentation of virgin to memory/effector T cells in secondary lymphoid tissues,[1,8-10] the myriad cytokine synthesis-defined T-cell subsets observed in this study are consistent with a highly complex spatial and/or temporal regulation of this differentiation process. The relative rarity of IL-4/γ-IFN double-producing memory/effector T cells suggests a relatively nonoverlapping, independent regulation of these two cytokines, as opposed to a likely more promiscuous regulation of IL-2.

We further showed that the pattern of cytokine-defined subsets was qualitatively similar after (1) PMA + I stimulation of accessory cell-depleted PB memory/effector T cells, (2) superantigen stimulation of monocyte-reconstituted PB memory/effector T cells, and (3) superantigen stimulation of skin blister-derived memory/effector T cells that were stimulated in the context of skin-derived accessory cells. Preliminary data also indicate that T-cell stimulation with anti-CD3 plus anti-CD28 yields analogous cytokine-produc-

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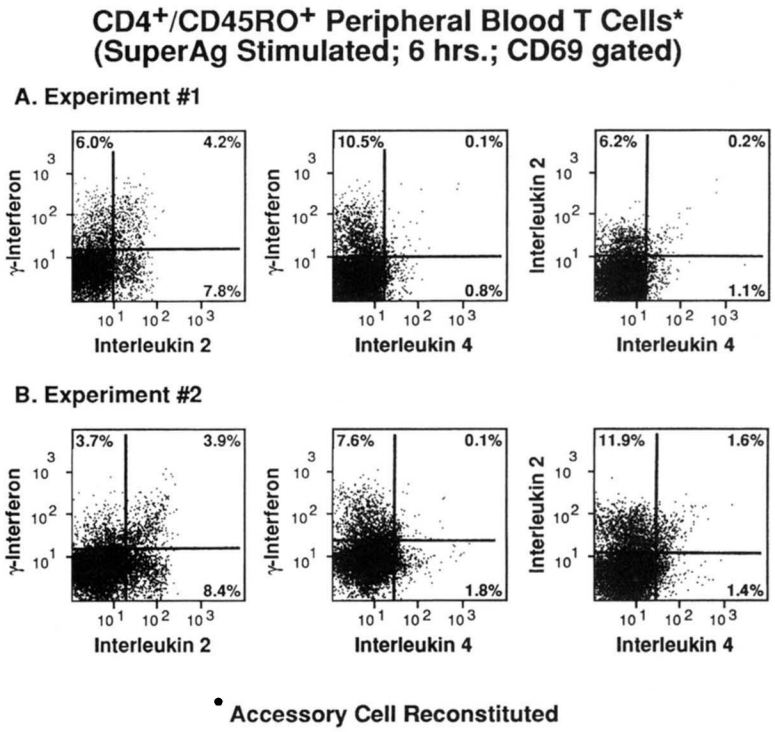

Fig 10. Characterization of patterns of multicytokine production by superantigen-stimulated CD4⁺/CD45RO^{high} PB T cells. CD4⁺/CD45RA^{low}/RO^{high} T cells were prepurifed by negative selection, reconstituted with monocytes, and then stimulated with the bacterial superantigens SEA + SEB for 6 hours in the presence of BFA. These cells were then assessed for their correlated expression of intracellular cytokines (γ-IFN versus IL-2, γ-IFN versus IL-4, and IL-2 versus IL-4) and CD69. Two representative experiments are shown with 10,000 events in each plot, gated on the CD69⁺ population, as shown in Fig 2. The percentages in the corners of each plot represent the net percentage (%) of positive (+) cells in the appropriate quadrants (ie, the +/−, +/+, −/+ subsets).

ing subsets (L. Picker and B. Ferguson-Darnell, unpublished observations). These findings strongly suggest that, in the setting of a fully activating stimulus, the observed heterogeneity of memory/effector T-cell cytokine production is an intrinsic characteristic of the T cell and not a function of the regulatory influences of the activating signal, or the activating microenvironment. (The caveat "fully activated" is important because cytokine synthesis-defined memory/effector T-cell subsets have been shown to differ in their activation requirements,[18,39] making it possible that some threshold stimuli may differentially activate a particular subset and not another, thus giving the false appearance of a short-term, functional plasticity among memory/effector T cells.) The stability of this memory/effector T-cell cytokine program-

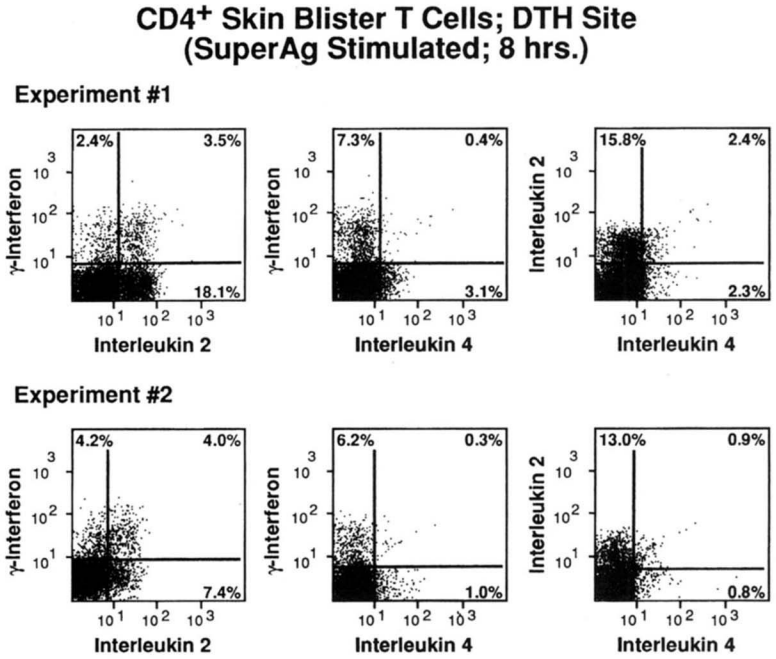

Fig 11. Characterization of patterns of multicytokine production by superantigen-stimulated CD4⁺/CD45RO^{high} cutaneous T cells. Leukocytes obtained from skin blisters overlying DTH reactions (against mumps virus Ag) were stimulated with the bacterial superantigens SEA + SEB for 8 hours in the presence of BFA. These cells were then assessed for their correlated expression of intracellular cytokines (γ-IFN versus IL-2, γ-IFN versus IL-4, and IL-2 versus IL-4) and CD4. Two representative experiments are shown with 10,000 and 5,600 events in each plot in experiments no. 1 and 2, respectively; both gated on the CD4⁺ T cells population. Because of the twofold to threefold increased reactivity to bacterial superantigens manifested by these skin-derived T cells versus that of PB T cells, it was not necessary to use CD69 gating to effectively visualize the cytokine response of this predominantly (>95%) CD45RO^{high} skin T-cell population. The percentages in the corners of each plot represent the net percentage (%) of positive (+) cells in the appropriate quadrants (ie, the +/−, +/+, −/+ subsets).

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ming has recently been attested to in experiments showing that the specific cytokine synthesis phenotype of adoptively transferred murine T-cell clones are retained for prolonged periods in vivo.[40]

It is important to note that, although we feel that our data suggest that the ability of memory/effector T cells to synthesize IL-2, IL-4 and γ-IFN is independently preprogrammed, they do not imply that all the cytokine-defined T-cell subsets identified in this report are independent of each other. Because it has been suggested that mouse TH1 and TH2 memory/effector T cells differentiate via TH0 (IL-2$^+$/IL-4$^+$/γ-IFN$^+$) intermediates[5,9] or, in some instances, via intermediates that produce IL-2 alone,[41] it is possible that the analogous human subsets reported here may represent intermediate forms that will terminally differentiate at some future encounter with Ag $\pm$ other relevant microenvironmental conditions. However, the presence of these putative memory/effector T-cell intermediates among circulating resting T cells and among T cells in inflammatory sites suggests that they can be fully contributory to T-cell mediated immune responses, and, thus, their putative intermediate status should not be confused with lack of functionality.

The technical simplicity and rapidity of the flow cytometric intracellular cytokine detection technique described in this report, as well as the widespread availability of appropriate flow cytometers and T-cell phenotyping antibodies in clinical laboratories, suggest the possiblity that this technique could be broadly applicable to the clinical evaluation of immune status. For example, previous studies (using more cumbersome immunologic assays) have suggested that alteration of T-cell effector function may be an earlier marker of progressing human immunodeficiency virus-associated immune dysfunction than the absolute loss of whole T-cell subsets (eg, the CD4$^+$ subset).[42] Our observation of qualitatively and quantitatively consistent patterns of memory/effector T-cell cytokine expression in normal subjects offers the possibility that changes in these patterns may precede and predict the clinical manifestations of progressive immunodeficiency. Therefore, flow cytometric quantitation of the cytokine-defined memory/effector T-cell subsets described in this report may prove to be an invaluable aid in both the diagnosis and monitoring of pathologic immunodeficiency and therapeutic immunosuppression.

ACKNOWLEDGMENT

The authors gratefully acknowledge the invaluable assistance of the following individuals: Drs A. Levine (Monsanto Co, St Louis, MO), H. Yssel (DNAX Research Institute, Palo Alto, CA), and H. Gaylord (R&D Systems, Minneapolis, MN) for providing IL-4 transfectants, T-cell clones, and anti-cytokine MoAbs, respectively; A. Yin, K. Teall, B. Ferguson-Darnell, and J. Bass for technical assistance, J. White, B. Reynolds, and Dr K. Houpt (UT Southwestern Medical Center, Dallas, TX) for assistance with the collection of skin blister leukocytes; and Dr K. Davis (BDIS, San Jose, CA) for the preparation of directly fluorochrome-conjugated anti-cytokine MoAbs.

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