Detection of Intracellular Antigen-Specific Cytokines in Human T Cell Populations

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Determination of antigen-specific cytokine responses of T lymphocytes after vaccination is made difficult by the low frequency of responder cells. In order to detect these responses, the profile of intracellular cytokines was analyzed using flow cytometry after antigenic expansion. Peripheral blood mononuclear cells were stimulated with antigens for 5 days, further expanded with interleukin (IL)-2, and then restimulated on day 10. Cytokine production was detected by intracellular staining with monoclonal antibodies after saponin-based permeabilization. Influenza expansion resulted in specific interferon-γ (IFN-γ) production of 6%-20%, with less IL-4 production (0%-2%). Tetanus toxoid resulted in even greater production. IL-4 and IFN-γ were produced mainly by memory cells of the CD45RO+ phenotype. IFN-γ production was contributed by both CD4 and CD8 populations. These methods were then applied to a clinical trial of a candidate human immunodeficiency virus type 1 vaccine. Antigen-specific increases in IFN-γ were measured, which corresponded to antibody production, lymphoproliferation, and skin testing.

Cellular and humoral responses to vaccinations or to infections recently have been characterized by the cytokine production of activated peripheral blood lymphocytes or monocytes [1–3]. Profiles of cytokine production after infection or vaccination in animal models are often interpreted by a TH1/TH2 paradigm in which protection can be associated with either response [4–8]. In general, the TH1 profile is characterized by production of antigen-specific interferon (IFN)-γ, interleukin (IL)-12, and complement-fixing antibodies, while the TH2 phenotype is characterized by production of IL-4, -5, and -10 and an increase in IgE and isotype switching [9, 10]. This cytokine production by human peripheral blood mononuclear cells (PBMC) or murine lymph node lymphocytes can be measured directly by intracellular protein staining [11–13]. This technique allows the analysis of cytokine production at the single cell level and the determination of whether cells are pluripotent for production of more than one cytokine. In almost all studies performed to date, these measurements have been performed after mitogenic stimulation and therefore reflect the general, global potential of the PBMC. In settings of profound ongoing antigenic stimulation, such as human immunodeficiency virus type 1 (HIV-1) infection, schistosomiasis, or cytomegalovirus (CMV) viremia, quantification of the TH1/TH2 effector response may be possible by antigenic or mitogenic stimulation of the circulating effector PBMC. However, after most immunizations, the number of circulating antigen-specific effector cells is relatively small, and the low frequency of the antigen-specific T cell memory response may be lost in the background signal when the bulk response to mitogen is measured.

Although recent studies have shown the ability to detect circulating CD8-specific cytokines with minimal stimulation when the major histocompatibility complex class I epitope is known [14], no direct method for the detection of cytokines in PBMC after vaccination has been published. An example of the difficulty in measuring TH1/TH2 responses after disease or vaccination is the failure to detect antigen-specific IL-4 after vaccination, even when large amounts of antibody are produced. IL-4 has not been detected in human PBMC after brief antigenic stimulation, even in situations in which animal models would predict large amounts, such as in human visceral leishmaniasis [15]. In fact, the majority of antigen-specific TH2 responses can be measured in human PBMC only by using restimulation strategies that allow for expansion of the memory T cell population [16–18]. We and others have shown that the TH1/TH2 responses measured by these restimulation techniques have correlated with clinical responses to both immunotherapy and immune intervention [19–21]. For these reasons, we combined the methods of antigen expansion, restimulation, and intracellular staining to measure antigen-specific PBMC cytokine responses by flow cytometry in the setting of a phase 1 vaccine trial.
Materials and Methods

Volunteers. PBMC were obtained from healthy young adults enrolled in a randomized, double-blind, placebo-controlled phase I trial of a trivalent intranasal cold-adapted, live attenuated influenza virus vaccine (Aviron, Mountain View, CA). PBMC were also obtained from participants enrolled in a double-blind phase I trial (AVEG 016B) of recombinant MN gp120 (rgp120; VaxGen, South San Francisco), combined with either 600 μg of alum or 100 μg of the novel adjuvant QS21 (Aquilla, Worcester, MA). The trial design was based on the hypothesis that the QS21 group would have a more rapid and greater antibody response than the alum adjuvant group. Twenty volunteers received 3 μg and 10 received 30 μg of rgp120 intramuscularly on a 0-, 1-, and 6-month schedule. Half of each group received the immunization in either alum or QS21. Six volunteers received adjuvant alone. Only the participants from the University of Rochester and selected volunteers from St. Louis University were included in the cytokine studies. PBMC were obtained on day 0, 14 days after the first immunization, and 14 days after the second immunization. Skin testing was performed at 12 months, using a purified gp160 protein (MicroGeneSys, Meriden, CT) without adjuvant and with candida, mumps, and tetanus toxoid as controls.

Monoclonal antibodies (MAbs). CD45RO-fluorescein isothiocyanate (FITC) (clone UCHL1) and CD69-FITC (clone FN50), both from PharMingen (San Diego), and CD4-FITC (clone SK3), CD8-peridin chlorophyll protein (PerCP) (clone SK1), and CD3-PerCP (clone SK7), from Becton Dickinson Immunocytometry Systems (San Jose, CA), were used for cell-surface staining. Intracellular staining for cytokines was performed using phycoerythrin (PE) mouse anti-human IL-4 (IgG1, clone 8D4-8) or anti-human IFN-γ (IgG1, 4S.B3) (PharMingen). Isotype controls used were irrelevant IgG1, monoclonal MOPC-21 conjugated to PE, FITC, and PerCP.

Cell preparation and stimulation. PBMC were isolated from volunteers by density-gradient sedimentation over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). The isolated PBMC were then washed three times in Dulbecco’s PBS (Gibco, Grand Island, NY). Cells were cryopreserved in RPMI 1640 (Gibco) supplemented with 20% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT), 50 U/mL penicillin (Gibco), 50 μg/mL streptomycin (Gibco), 10 mM glutamine (Gibco), and 7.5% DMSO (Sigma, St. Louis). Cryopreserved cells were stored in liquid nitrogen until used in the assays. At the time of the assay, PBMC were rapidly thawed in a 37°C water bath and washed in RPMI 1640 supplemented with 20% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 20 mM glutamine. Cells were counted, checked for viability, and resuspended in serum-free AIM-V (Gibco) at 10⁶ cells/mL. PBMC were cultured in 24-well plates (1 mL/well) (Costar, Cambridge, MA) with the following antigens in different wells: medium (control), 100 μL of 5 × 10⁶ pfu of heat-inactivated influenza virus (strain A/Texas H1N1) or a 5-Lf dose of tetanus toxoid (Connaught Laboratories, Toronto, Canada) at 37°C and 5% CO₂. In the AVEG 016B trial, recombinant baculovirus–produced gp160 was used at 10 μg/mL. After 5 days in culture, 500 μL of culture supernatant was removed and frozen at −70°C and replaced with 500 μL of AIM-V supplemented with 40 U/mL IL-2 (Genzyme, Cambridge, MA). Cells were incubated for 5 additional days. On day 10, the contents of each well were transferred to 5-mL sterile tubes and supplemented with 1 mL of AIM-V. Cells were then stimulated with 1 μM ionomycin (Sigma) and 20 ng/mL phorbol 12-myristate (PMA) (Sigma), in the presence of 2 μM monensin (Sigma), for 5 h.

Control of lymphocyte phenotype and stimulation. The lymphocyte phenotype and degree of stimulation were assessed by experiments with surface staining using CD69-FITC and CD3-PerCP. Control experiments without stimulation with ionomycin and PMA were conducted on 6 samples.

Surface and intracellular staining with MAbs. After stimulation, cells were centrifuged at 1000 rpm for 15 min; an aliquot of the cell-free supernatant was saved and frozen at −70°C. The cells were resuspended in 100 μL of staining buffer (PBS supplemented with 0.1% sodium azide [Sigma] and 1% FBS, pH 7.4–7.6) and the MAbs CD45RO-FITC and CD8-PerCP and were incubated at 4°C in darkness for 15 min. After being stained, cells were washed with 2 mL of staining buffer and resuspended in 1 mL of fixation buffer (4% paraformaldehyde [Polysciences, Warrington, PA] in PBS at pH 7.4–7.6). Cells were fixed for 30 min at 4°C in darkness, centrifuged at 1000 rpm for 15 min, and resuspended in 3 mL of staining buffer. Each tube was split into 3 aliquots and centrifuged at 1000 rpm for 15 min. The cells were incubated for 30 min at 4°C in the dark with either no antibody (unstained tube), anti–IL-4–PE, or anti–IFN-γ–PE in the presence of 50 μL of permeabilization buffer (PBS supplemented with 0.1% sodium azide, 1% FBS, and 0.1% saponin [Sigma]). Cells were washed with 2 mL of permeabilization buffer and resuspended in 300 μL of staining buffer for flow cytometric analysis.

To evaluate the specificity of anti–cytokine antibody stains, blocking experiments were performed. Stimulated PBMC were surface-stained and incubated with a 5-fold excess concentration of unlabeled purified mouse anti–human IL-4 or IFN-γ in the presence of permeabilization buffer, followed by incubation with the PE-labeled mouse anti–human IL-4 or IFN-γ. Surface staining by the anti–cytokine antibody was assessed by staining the cells 5 h after PMA and ionomycin stimulation using no permeabilization.

Flow cytometric analysis. Cell samples were analyzed on a FACScan (Becton Dickinson Immunocytometry Systems). Acquisition and analysis was performed using CellQuest software (Becton Dickinson Immunocytometry Systems). Fluorescence voltages and compensation values were determined using cells (from the same blood sample of a healthy volunteer) single-stained with CD45RO-FITC or CD8-PerCP and triple-stained with CD45RO-FITC, anti–IFN-γ–PE, and CD8-PerCP. For each tube, 10,000 events were acquired in a stored live lymphocyte gate.

To analyze the results, a template was set up for each sample collected, which contained the plots and histograms of each specific antigen. A gate on the lymphocytes was drawn using the side- and forward-scatter plot. The quadrant markers used to determine the percentage of lymphocytes producing IL-4 or IFN-γ were set from the unstained tube dot plot of the unstained sample. There was no difference between isotype controls and unstained samples in setting gates. The percent antigen-specific cytokine response was controlled for by subtracting the medium-stimulated cytokine, based on the following formula (figures 2–4): [(antigen-expanded % anti–cytokine-stained) – (antigen-expanded unstained control)] – [me-
medium-expanded % anti-cytokine-stained) – (medium-expanded unstained control).

Control of CD8 proportion during the experiments. To address the variability in stimulation and staining on different days, a blood sample from a healthy unvaccinated volunteer was obtained; PBMC were separated as described above and cryopreserved in 9 aliquots. On the day of each experiment, 1 aliquot was thawed, and a control and tetanus toxoid stimulation were set up in parallel with the cryopreserved PBMC from the participants of the study. At day 10, the control cells were subjected to the same surface staining, fixing, and intracellular staining steps as the samples from the volunteers.

Statistical analysis. The pre- and postvaccination cytokine responses were compared by analyzing the gp160-induced IFN-γ by a Wilcoxon matched pairs test using Statistica/w software (version 5.1E; StatSoft, Tulsa, OK). Tetanus responses were used as an internal control for each volunteer.

Results

Mitogens enhanced cytokine production in vitro. In repeated experiments, the 5-h mitogenic stimulation of freshly isolated PBMC with the combination of PMA (3–40 ng/mL) and a calcium ionophore (1 μM) in the presence of monensin (2 μM) resulted in percentages of total cells stained intracellularly for IFN-γ (4%–20%) and IL-4 (0%–3%) similar to values previously reported [11–13]. With the use of directly conjugated PE-labeled anti-cytokine antibodies, little background staining was found. Attempts to lower the background staining (0.1%–0.6% for IFN-γ) in nonstimulated cells with either dry milk or excess irrelevant isotype antibody did not improve on the background staining resulting from direct staining alone (data not shown).

After long-term culture of PBMC, CD4 and CD8 can be separated by CD8<sup>+</sup> gating. When the cells were further surface-stained with either CD4 or CD8, along with CD45RO, the relative contribution of these two subsets could be delineated. Because the use of PMA and ionomycin resulted in a much larger down-regulation of CD4 than of CD8, we chose to use the CD8 as a marker in our experiments. In three separate experiments, the percentage of cells that were IL-4<sup>+</sup>-positive and CD4<sup>-</sup> (down-regulated)–positive was similar to the percentage of cells that were CD8 negative (which is down-regulated to a lesser extent by the mitogen stimulation) and IL-4<sup>+</sup>-positive. Similar results were found for IFN-γ. We therefore chose to use CD8, CD45RO as the standard surface-staining procedure in the cytokine experiments.

Long-term antigen stimulation of lymphocytes with mitogen restimulation elicits cytokine expression. We performed initial studies in an attempt to delineate antigen-specific responses without mitogenic restimulation. PBMC were cultured with tetanus toxoid, and IL-2 was added at 5 days to promote the further expansion of the relevant population. When the cells were cultured for 7–12 days and monensin was added for the last 6–8 h of growth, we were unable to detect antigen-specific IFN-γ responses. Since an antigen expansion with mitogen re-stimulation strategy [16] was successful for detecting soluble cytokine production by ELISA [21], this strategy was also adapted for flow methodology. Cells were expanded for 10 days in the presence of antigen, and IL-2 was added for the last 5 days. They were then washed, mitogenically restimulated with PMA and ionomycin in the presence of monensin, surfaced-stained, fixed, and intracellularly stained.

High-level cytokine production was then easily detected in mitogen-restimulated cells. Parallel experiments in PBMC after 10 days of exposure in vitro to either tetanus toxoid or influenza antigen revealed a significant increase in the percentage of both CD4 and CD8 cells expressing IFN-γ when stimulated versus unstimulated cells were compared (figure 1). There was a small, but nonstatistically significant, difference in the percentage of cells staining for IL-4 after initial stimulation with tetanus or influenza followed by restimulation with medium or PMA and ionomycin (data not shown). However, in experiments using either herpes simplex virus or CMV antigen we were easily able to measure antigen-specific IL-4 by flow cytometry, which was confirmed at the mRNA level [22]. In these experiments, no specific IFN-γ production was measured in herpes simplex virus– or CMV-seronegative persons.

PBMC were also frozen in 9 aliquots from a single donor, and 1 sample was thawed for each experimental set-up during the influenza trial. This allowed monitoring of the variation in culture techniques or in stimulation intensity from experiment to experiment. To control for the number of contaminating B

Figure 1. Peripheral blood mononuclear cells from healthy controls (n = 22) were cultured in AIM-V for 10 days with medium alone, influenza antigen, or tetanus toxoid (as per Materials and Methods) and then stimulated with either medium control or phorbol myristate acetate (PMA) and ionomycin in presence of 2 μM monensin for 6 h. Cells were then surface-stained for CD8 and intracellularly stained. Graphic shows % interferon-γ production in CD8<sup>+</sup> population for either unstimulated or PMA and ionomycin-stimulated cells. For both tetanus and influenza, medium control (either unstimulated or PMA- and ionomycin-stimulated) has been subtracted from same sample.
cells, NK cells, or monocytes in the lymphocyte gate, the CD3 population percent was assessed in the 10-day cultures. In four separate experiments, the nongated, nonadherent cells comprised at least 92% CD3+ cells. Therefore, the CD8+ population is essentially CD4+ T cells (after a lymphocyte gate is applied). The percentage of CD8+ and CD8- cells in the 10-day cultures remained replicable in eight separate experiments on aliquoted cells from the single donor (CD8+ percent: mean = 38.6, SE = ±1.9).

The extent of mitogen stimulation after antigen expansion was assessed by examining the expression of CD69 in the stimulated versus nonstimulated population. Essentially all of the stimulated cells expressed this activation marker after 6 h. Thus, the percentage of cytokine-producing cells after 10 days of culture in these experiments represents the true overall potential for cytokine production in this expanded cell population.

Detection of cytokine production in naive and memory subsets. These techniques were then used for volunteers who were enrolling in an influenza vaccine trial. As can be seen in figure 2, antigen-specific expansion to all of the antigens was found. The number of IFN-γ-positive cells in the medium control that were restimulated with PMA and ionomycin ranged from 0% to 10% and averaged ~1%–2%. These values were subtracted from those for the antigen-stimulated cells for each sample. The percentage of cells expressing IFN-γ ranged up to 21% in the CD8+ memory population to almost none in the CD8- naive population. The percentage of antigen-expanded cells that stained positive for IL-4 was far less, ~2%, in the CD8+ memory population and was essentially nonexistent in the naive CD8- or CD8+ populations.

We have previously documented by magnetic-bead CD8- and CD4-depletion experiments using these restimulation techniques that ~80%–90% of the soluble IFN-γ measured in the supernatants is produced by the CD4+ population. The flow-based experiments here confirm those observations and also show that the IFN-γ-producing CD8 population is only ~20%–30% the size of the CD8+ pool. In addition, the naive population remained relatively constant over the 10 days of expansion, as assessed by CD45RO- [21], and has only about one-fifth as many cells producing IFN-γ after PMA and ionomycin stimulation as does the CD45RO+ memory cell population.

Vaccine-induced cytokine responses. In the protocol AVEG 016B, an increase in IFN-γ production by CD8+ lymphocytes is apparent in vaccines 14 days after the first and second immunizations. A representative example of the IFN-γ production seen at these time points is shown in figure 3. As can be seen, there is an increase in both the number of CD8+ cells producing IFN-γ and an increase in the intensity of the signal. This increase was not seen in the medium-stimulated control. This

![Figure 2](https://academic.oup.com/jid/article-abstract/179/5/1124/802141)

**Figure 2.** Relative contribution of memory (CD45RO+) and naive (CD45RO-) T cell subsets to cytokine staining is plotted for 2 antigen stimulations, tetanus toxoid and influenza, with control medium stimulation subtracted for each sample. Data are shown for 28 persons.
Representative volunteer was vaccinated with candidate gp120 immunogen, and cryopreserved peripheral blood mononuclear cells (PBMC) obtained 2 weeks after initial vaccination and 2 weeks after second vaccination (day 45) were stimulated with medium control, gp160, and tetanus antigens. After restimulation with phorbol myristate acetate and ionomycin on day 10, PBMC were surface-stained for CD45RO and CD8 and intracellularly stained for interferon (INF)-γ according to Materials and Methods. Lymphocytes are gated by light scattering characteristics, and the CD45RO⁺ population displayed in FL2 (INF-γ) vs. FL3 channels (CD8). Percents shown refer to CD8⁺ (CD4⁺) cells producing INF-γ.

In this trial, there was a statistically significant effect of QS21 compared with alum on lymphoproliferation (stimulation index of 39.7 vs. 11.1, respectively), neutralizing antibody (geometric mean titer of 416 vs. 28, respectively, on day 182), and delayed-type hypersensitivity testing (92% vs. 44% >5 mm to gp120 skin testing), which paralleled these IFN-γ responses. However, similar to observations in other studies, lymphocytes appear to be

Figure 3. Representative volunteer was vaccinated with candidate gp120 immunogen, and cryopreserved peripheral blood mononuclear cells (PBMC) obtained 2 weeks after initial vaccination and 2 weeks after second vaccination (day 45) were stimulated with medium control, gp160, and tetanus antigens. After restimulation with phorbol myristate acetate and ionomycin on day 10, PBMC were surface-stained for CD45RO and CD8 and intracellularly stained for interferon (INF)-γ according to Materials and Methods. Lymphocytes are gated by light scattering characteristics, and the CD45RO⁺ population displayed in FL2 (INF-γ) vs. FL3 channels (CD8). Percents shown refer to CD8⁺ (CD4⁺) cells producing INF-γ.

increase was significant after both the second and third vaccination for both the alum (P = .04) and QS21 (P = .03) groups, compared with prevaccination. In addition, there was greater IFN-γ production in the QS21 arm than in the alum arm (figure 4). There was no statistically significant increase in tetanus responses in the groups between the time points (P = .28).
Figure 4. Interferon (INF)-γ responses are shown before vaccination and after first and third vaccinations for recipients of rsgp120 plus QS21 ( ) and rsgp 120 plus alum ( ). Data reflect mean % of CD8+ T cells producing INF-γ in gp160 condition minus medium-only control.

more responsive to activation after nonspecific immunization [23]. Whether this reflects a nonspecific bystander effect is unclear. When the degree of gp120 stimulation for each individual is normalized for tetanus stimulation, there is still a significant increase in IFN-γ production by stimulated cells (P = .04).

Discussion

In these experiments, we have been able to show that intracellular cytokines specific for antigenic responses can be measured by intracellular staining and flow cytometric analysis. As the precursor frequency for a given antigen-specific cell is rare in the peripheral blood, we utilized a first-step antigenic expansion, followed by IL-2-supplemented medium to allow for a short-term antigen-expanded cell line to develop. Although TH1 cytokines can be measured in the supernatants of antigen-stimulated cells after 1–3 days, the number of responders as measured by flow cytometry 6 h after antigenic stimulation is quite small [24]. In our studies of antigens with lower precursor frequencies of T cells, we have been unable to determine specific responses without antigenic expansion of the population.

The cytokine responses seen with this technique of short-term cell line development may not reflect pure antigen-specific responses but may include a bystander effect of the cytokines on neighboring cells. Such a bystander amplification for IFN-γ has been shown both in vitro and in vivo [25]. However, the relevance of this model is most clearly shown in the correlation of IL-4 measured by these techniques with actual clinical outcomes. Marshall et al. [16], using a protocol similar to our own (PHA and PMA rather than PMA and ionomycin) were able to correlate IL-4 levels to outcomes in allergic patients. Bellinghausen et al. [26] used a restimulation with bound anti-CD3 and showed a close correlation between TH1/TH2 profiles and responses to bee venom therapy. In a separate study, we have also shown that the use of such culture techniques allows for the detection of TH2 responses after immunization with soluble protein HIV-1 candidate vaccines and that the timing of these responses correlates with the timing of antibody responses [21].

Are such restimulation steps really necessary for the detection of accurate TH1/TH2 profiles? Most previously published studies of intracellular staining have used either mitogens or superantigens to characterize the cytokine phenotypes of PBMC or lymph node cells. Conditions that result in a large, ongoing antigenic stimulation, such as severe viral infections, HIV-1 infection, schistosomiasis, filariasis, or allergic diatheses, might lend themselves to cytokine phenotyping by flow cytometry without the need for an expansion step. For example, Waldrop et al. [24] were able to measure CMV-specific responses in HIV-1 infected persons by stimulating PBMC with CMV antigen and measuring responses by flow cytometry 4–6 h later. By using a gating strategy that employed intracellular staining of activated cells using CD69, they were able to show that up to 0.5% of such cells produced IFN-γ. However, since surface expression of CD69 does not occur after antigen stimulation until ~48 h [27], these investigations were likely measuring ongoing effector responses in contrast to the overall T cell memory pool. In our case, in which we wish to show the potential memory immune response after vaccination, the level of ongoing stimulation is likely too small to measure directly in effector cell populations. Other techniques, such as ELISPOT or polymerase chain reaction, may be able to determine these responses with greater sensitivity but suffer in the lack of ability to accurately quantitate such responses. It is, however, possible that our techniques allow stimulated effector cells to undergo apoptosis and that this population was missed by our techniques.

IFN-γ and IL-4 were chosen to measure the TH1/TH2 profile induced by antigenic expansion. Although other cytokines could have been used to represent the TH1/TH2 profile, in most studies, these two lymphokines have been the clearest and most representative markers of the two phenotypes. In addition, the mutually exclusive production of cytokines by a single cell has been most clearly shown for these two markers [28]. In contrast, simultaneous staining for IL-2 and IL-4, IL-10 and IFN-γ, or IL-5 and IFN-γ, which have also been used to represent the TH1/TH2 paradigm, have been shown to occur with high frequency [22, 28, 29].
Our gating strategy of using CD8 and CD45 as the surface markers served two purposes. It first allowed us to separate our cytokine responses by the T cell subset responsible for producing cytokines and allowed for potential correlation of these subsets independently with other markers after vaccination, such as CD8⁺ cytotoxic T lymphocyte responses or CD4⁺ proliferative responses. This also allows for the detection of TH2 predominance in a CD4 population when TH1 may predominate in the CD8 subset. This response, which may be needed to elicit high levels of antibodies simultaneous with an activated CTL population, may be obscured when measuring the CD4 and CD8 responses as total PBMC. The use of the CD45 gate was used to increase the probability of detecting changes in IL-4 production by the CD4 memory subset. As previous studies have shown that the majority of IL-4 is produced by memory cells, gating on CD45RO⁺ cells should increase the percentage of IL-4-producing cells [30]. However, more recent reports have documented large production of IL-4 by a CD45RA⁺ cell subset that are memory cells, as determined by the loss of the CD62L (L-selectin) surface marker. This may eventually prove to be a more useful gate for determining the IL-4 production in some study populations.

These techniques were applied to a trial in which the novel adjuvant QS21 was used along with a low dose of a well-studied candidate HIV-1 vaccine (recombinant soluble MN gp120). The dose of gp120 was specifically chosen to be suboptimal for antibody production in the alum arm. Consistent with results in mouse and primate studies, the QS21 led to at least a 1.5 log dose sparing effect as measured by either neutralizing or binding antibody, improved lymphoproliferation, and greater responses on delayed type hypersensitivity skin testing. When we analyzed the CD4⁺ responses, we found that the group receiving QS21 had greater INF-γ production at early time points. This contrasted with the inability to measure significant INF-γ using supernatants collected 5 days after stimulation. Thus, these techniques may have advantages over other cytokine-measurement methods. They allow the specific cell populations to be phenotyped, they do not require precise counting of the readout cells (as this is performed by the cytometer), and they allow the measurement of CD4- or CD8-specific responses without having to physically remove one of these populations during the in vitro culture stage. Further applications of these techniques to the measurement of CD4 and CD8 cytokine responses in the context of HIV-1 vaccine trials is ongoing.

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