The Journal of Immunology

RESEARCH ARTICLE | NOVEMBER 01 2004

Vaccination in Humans Generates Broad T Cell Cytokine Responses[1] FREE

Stephen C. De Rosa; ... et. al

J Immunol (2004) 173 (9): 5372–5380.
https://doi.org/10.4049/jimmunol.173.9.5372

Vaccination in Humans Generates Broad T Cell Cytokine Responses[1]

Stephen C. De Rosa,[2]* Fabien X. Lu,[‡] Joanne Yu,* Stephen P. Perfetto,* Judith Falloon,[†] Susan Moser,[§] Thomas G. Evans,[3§] Richard Koup,* Christopher J. Miller,[‡] and Mario Roederer[4]*

In recent years, the quantification of T cell responses to pathogens or immunogens has become a common tool in the evaluation of disease pathogenesis or vaccine immunogenicity. Such measurements are usually limited to enumerating IFN-γ-producing cells after ex vivo stimulation with Ag, but little is known about the phenotype or complete functional repertoire of the Ag-specific cells. We used 12-color flow cytometry to characterize Ag-specific T cells elicited by vaccines or natural infection to determine lineage and differentiation status as well as the capacity to produce four cytokines (IFN-γ, TNF-α, IL-2, and IL-4) and a chemokine (MIP1β). As expected, responding cells had a typical memory phenotype; however, the cytokine profiles associated with the responses were highly complex. The pattern of cytokine coexpression in response to specific Ags was a skewed subset of the complete repertoire (revealed by polyclonal stimulation). We found significant differences in the patterns of cytokines elicited by vaccination (where IFN-γ was by far a subdominant response) vs natural infection; in addition, there was fairly significant intersubject variation. Our findings illustrate the limitation of the evaluation of immune responses using single functional measurements (such as IFN-γ); in fact, it is likely that sensitive evaluation of Ag-specific T cells will require the coordinate measurement of several cytokines. The presence and variability of these complex response profiles introduce the possibility that selective functional expression patterns may provide correlates for vaccine efficacy or disease progression. *The Journal of Immunology,* **2004, 173: 5372–5280.**

Full evaluation of a vaccine regimen can take 10–15 years, including safety, dosing, and large scale efficacy trials. Because such a timescale is incompatible with rapid development of new regimens and immunogens, the paradigm of vaccine development has shifted focus to the characterization of ex vivo cellular immune responses in addition to in vivo humoral responses during the early (safety and dose escalation) clinical trial phases. This process has become important during the early stages of vaccine development to identify candidate vaccines that are likely to prove beneficial in longer term efficacy trials; furthermore, to optimize vaccine regimens, it is paramount to detect immunogen responses with the greatest sensitivity possible.

Many new vaccine development efforts now focus on T cells as well as Ab responses. Characterizing the quality of these responses, for example, Th1 vs Th2, or central vs effector memory, may become important as we begin to understand the range of different possible responses (1).

The last 5 years has seen an explosion in the ability to identify, characterize, and quantify Ag-specific T cells. Two commonly used assays for this purpose are the ELISPOT assay (2, 3) and the flow cytometric-based intracellular cytokine staining assay (4, 5). To date, both of these assays have relied nearly exclusively on the quantification of the number of T cells that produce the effector cytokine IFN-γ in response to in vitro stimulation with immunogen. The immunogen may be either the holo-Ag (e.g., tetanus toxoid) or pools of peptides that span the entire protein.

Peptide pools have become the most commonly used in vitro stimulus for several reasons. First, the responsiveness of the T cells is nearly as great in cryopreserved samples as it is in fresh blood. This is not the case for Ags requiring processing, because Ag processing cell function is significantly lost upon cryopreservation. Second, both CD4 and CD8 T cells can be stimulated, which is not the case for assays (such as class I MHC tetramers) that interrogate only CD8 T cells (6). Finally, the assay is independent of the MHC haplotype of the subject and of the potential haplotype-specific immunodominance that may steer responses to peptides other than commonly used immunodominant peptides used for the construction of class I or class II MHC tetramers.

We hypothesized that the breadth of T cell responses functionally is far greater than represented by the prototypical effector cytokine, IFN-γ. To test this, we studied the immune responses to immunization with two well-established licensed vaccines, hepatitis B virus (HBV)[5] and tetanus. We compared this to the responses induced by a candidate HIV vaccine and to the responses

*Vaccine Research Center and [†]National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; [‡]California National Primate Research Center, Center for Comparative Medicine and Division of Infectious Diseases, and [§]Department of Internal Medicine, University of California School of Medicine, Davis, CA 95616

Received for publication June 7, 2004. Accepted for publication August 4, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants to C.J.M.: RO133169 and U5429125 from the National Institute of Child Health and Human Development and Grants P51AG00169 and RR14555 from the National Center for Research Resources.

[2] Current address: Department of Laboratory Medicine, University of Washington/Fred Hutchinson Cancer Research Center, Seattle, WA 98109.

[3] Current address: Infectious Diseases Clinical Research, Vical Corporation, San Diego, CA 92121.

[4] Address correspondence and reprint requests to Dr. Mario Roederer, ImmunoTechnology Section, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 40 Convent Drive, Room 5509, Bethesda, MD 20892-3015. E-mail address: roederer@nih.gov

[5] Abbreviations used in this paper: HBV, hepatitis B virus; ART, antiretroviral treatment; Ax, Alexa; EMA, ethidium monoazide; SEB, staphylococcus enterotoxin B; TRPE, Texas Red-PE.

0022-1767/04/$02.00

induced by natural infection with HBV and HIV. In particular, we analyzed the breadth of the immune response as measured by a number of different cytokines in addition to IFN-γ; additionally, we characterized the type and number of responding CD4 and CD8 T cells on a weekly basis after immunization. Our results show that examination of only one cytokine alone (such as IFN-γ) is not sufficient to identify the extent of the immune response. In fact, for HBV, the dominant response among CD4 T cells consisted of cells making IL-2 and not IFN-γ. Examination of five cytokines simultaneously reveals functionally complex responses. We conclude that the most sensitive and complete description of immunization responses requires the quantification of several cytokines, a process that will be critical to the evaluation and discrimination of different vaccine regimens.

Materials and Methods

Subjects

Secondary immunization for HBV and tetanus. The portion of the study examining secondary immune responses to HBV and tetanus was initiated to evaluate the variation in immune function in relation to menstrual cycles; therefore, only menstruating women were enrolled in this part of the study (7). Subjects were required to have been immunized against HBV and/or tetanus at least 2 years before the start of the study. Seven subjects started the study on the same day; three received a booster immunization for HBV, and four received a booster immunization for tetanus. Blood was drawn at the University of California-Davis at baseline and twice weekly for the next 3 months. PBMC were isolated by Ficoll density centrifugation and frozen. Additional blood was drawn at baseline and 3 and 12 wk and was shipped to the National Institutes of Health for analysis without cryopreservation. Two additional subjects, both boosted for tetanus, started the study 3 wk after the first group began; the fresh blood samples for these subjects were excluded from analysis because, for unknown reasons, they exhibited very high background. Subject 2 was withdrawn after the 3 wk point due to protocol violation (the subject left the area and was unavailable for weekly blood draws).

Primary immunization for HBV. Subjects completing the primary immunization series for HBV were recruited from the Employee Health Clinic at the National Institutes of Health, and blood was drawn at the clinical office for the Vaccine Research Center at the National Institutes of Health. Blood was drawn within the first week after the 6 mo immunization and also between 2 and 3 wk after immunization. PBMC were isolated by Ficoll centrifugation and analyzed without freezing.

Primary immunization for HIV. Frozen PBMC from subjects receiving the Vaccine Research Center VRC004 DNA HIV vaccine were analyzed. The vaccine contained DNA for clade B gag/pol/nef and clades A, B, and C envelope. The vaccine was administered at 0, 1, and 2 mo. PBMC were drawn at 0, 2, 6, 8, 10, and 12 wk, but not all time points were available for all subjects. The multicolor FACS studies reported in this study were ancillary studies performed on extra thawed PBMC that were not used for the primary end-point analyses. The samples were analyzed before unblinding, and the subjects shown in this study are four individuals who had detectable responses.

HBV infection. Frozen PBMC samples from subjects with chronic HBV infection who were enrolled in National Institute of Allergy and Infectious Diseases studies were analyzed. These subjects had high HBV viral loads (at least 1 million copies/ml, by the COBAS Amplicor HBV Monitor Test version 2.0) despite at least 1 year of treatment with lamivudine. Five of the 11 subjects tested were coinfected with HIV. Of the three subjects who had detectable responses to surface or core peptides, two were coinfected with HIV.

HIV-1 infection. Frozen PBMC samples from subjects chronically infected with HIV-1 who volunteered to take part in an HIV pathogenesis study at the Vaccine Research Center at the National Institutes of Health were used. Data for each of the five individuals in Fig. 6 are shown in different colors. CD4 count (cells per microliter), HIV-1 viral load (copies per milliliter), and antiretroviral treatment (ART) status for these five individuals are as follows: red, 490, <500, on ART; dark blue, 1273, 1310, treatment naive; light blue, 233, <500, on ART; green, 62, 465,000, on ART; purple, 305, 221,000, off ART. These studies were approved by the institutional review boards at University of California-Davis and/or at the National Institutes of Health/National Institute of Allergy and Infectious Diseases.

In vitro stimulation

Secondary immunization for HBV and tetanus. For the whole blood samples shipped to the National Institutes of Health from University of California-Davis, PBMC were isolated and were placed immediately into culture. Cells were cultured in 96-well, V-bottom plates at a density of 1 million cells/200 μl well. For each subject at each time point, cells were divided and stimulated either with tetanus toxoid (10 μg/ml for all fresh PBMC analyses (List Biological Laboratories, Campbell, CA); 20 μg/ml for all frozen PBMC analyses (University of Massachusetts Biologic Laboratories, Jamaica Plain, MA)), a pool of hepatitis peptides, staphylococcus enterotoxin B (SEB; 1 μg/ml; Sigma-Aldrich, St. Louis, MO), or costimulation alone. Costimulation consisted of anti-CD28 and anti-CD49d (BD Pharmingen, La Jolla, CA), both at 1 μg/ml, and was included in all the stimulations. Monensin (2 μM; BD Pharmingen) was also included in all stimulations. The HBV peptide pool consisted of 22 peptides of 20 aa overlapping by 10 made from the HBV surface Ag (final concentration for each peptide, 4 μg/ml). Depending on the number of cells available, between 3 and 6 million cells were cultured for each stimulation condition for a total of 6 h. For previously frozen PBMC, cells were thawed into medium containing DNase (20 μg/ml; Sigma-Aldrich), washed, and placed into culture overnight. Cells were then stimulated as described above.

Primary immunization for HBV. These samples were processed similarly to the fresh samples above, except that brefeldin A (1 μg/ml; BD Pharmingen) was used instead of monensin, and a HBV peptide pool consisting of 15 mer overlapping by 11 was used (final concentration for each peptide, 2 μg/ml).

Primary immunization for HIV. These previously frozen samples were processed as described above using brefeldin A and stimulated with a peptide pool to envelope clade A (15 mer overlapping by 11).

HBV infection. These samples were previously frozen and were processed as the frozen samples above, using brefeldin A and the HBV surface Ag 15-mer peptide pool. An additional stimulation was included using a peptide pool to the HBV core protein (15 mer overlapping by 11).

HIV-1 infection. These previously frozen samples were processed as described above using brefeldin A and stimulated with a peptide pool to gag clade B (15 mer overlapping by 11).

Flow cytometric analysis

After stimulation, cells for each stimulation condition were harvested and pooled. Cells were surface-stained for CD4 and CD8. The phenotyping panels included surface staining for CD11a, CD62L, and CD45RA. In addition, during this staining, ethidium monoazide (EMA; 1 μg/ml; Molecular Probes, Eugene, OR) was included to label dead cells (8), and CD14 and CD163, detected in the same channel as EMA, were included to label and exclude monocytes. Cells were stained for 10 min at room temperature in the dark, then placed under an intense fluorescent light source for 10 min to photolink the EMA. After washing, fixation, and permeabilization (Cytofix/Cytoperm kit; BD Pharmingen), cells were stained intracellularly for cytokines and CD3 (which detects both intracellular and surface CD3).

All mAb reagents, either purified or preconjugated, except for Texas Red-PE (TRPE) CD3, were obtained from BD Pharmingen. TRPE CD3 was obtained from Immunotech/Beckman Coulter (Marseille, France). Abs that were not obtained preconjugated were conjugated in our laboratory using standard protocols (Ref. 8 and http://drmr.com/abcon/index.html). All reagents manufactured in our laboratory were validated by titration and comparison to commercial conjugates of the same clone.

The fluorescent dyes used in each staining combination include the following: Cascade Blue, Alexa 430 (Ax430), FITC, PE, TRPE, Cy5PE, Cy5.5PE, Cy7PE, Ax594, allophycocyanin, Cy5.5-allophycocyanin, Ax680, and Cy7-allophycocyanin. PE and allophycocyanin were obtained from ProZyme (San Leandro, CA). Cy5, Cy5.5, and Cy7 were obtained from Amersham Biosciences (Pittsburgh, PA). The Alexa dyes were obtained from Molecular Probes.

Table I lists the staining combinations. Stain 1 was used for the fresh HBV and tetanus samples. Stain 2 was used for the frozen samples for HBV subject 1. Stain 3 was used for the frozen samples for HBV subject 7. Stain 4 or stain 5 was used for all remaining samples.

Cells for the samples for the secondary immune responses (stains 1–3) were analyzed on a FACS DiVa (BD Biosciences, San Jose, CA) equipped for detection of 12 fluorescences. All other samples (stains 4 and 5) were collected on an LSR-II (BD Biosciences) equipped for detection of 17 fluorescences. Between 0.5 and 2.5 million events were collected for each sample. Data were analyzed using FlowJo (TreeStar, San Carlos, CA). The FACS plots in Figs. 1 and 2 are displayed as 5% probability contours with outliers. The FACS plots in Figs. 4 and 5 are displayed as pseudocolor density plots.

Table I. *FACS staining panels*

	Staining Combinations				
	1	2	3	4	5
Cascade Blue	CD62L	CCR7	CD4	CD4	CD4
Alexa 430		CD57			
Fluorescein	IFN-γ	IL-2	IFN-γ	TNF-α	IFN-γ
PE	IL-2	IL-4	IL-4	MIP-1β	MIP-1β
TRPE		CD3	CD3	CD3	CD45RO
EMA	EMA	EMA	EMA	EMA	EMA
Cy5PE		CD14	CD14	CD14	CD14
		CD19	CD64	CD19	CD19
		CD163			
Cy5.5PE	CD4	CD4	CD8		CD8
Cy7PE	CD8	CD11a		IFN-γ	TNF-α
Alexa 594	CD45RA	TNF-α	MIP-1β		
Allophycocyanin	CD3	IFN-γ	TNF-α	IL-4	IL-4
Cy5.5 allophycocyanin	CD57	CD8			
Alexa 680			IL-2	IL-2	IL-2
Cy7 allophycocyanin	CD11a	CD45RO		CD8	CD3

Statistical analysis

For each stimulation condition, the percentages of either CD4 or CD8 cells staining for each combination of cytokines were determined. Data for stimulation with anti-CD28/anti-CD49d alone was subtracted from the percentages for the Ag-specific stimulations. Data were analyzed using JMP software (version 5; SAS Institute, Cary, NC).

Results

After secondary immunization to HBV or tetanus, the Ag-specific cells have a typical effector/memory phenotype

At the beginning of this study, subjects who had been previously immunized received booster immunizations for either HBV (n = 3) or tetanus (n = 6). Blood samples were collected twice weekly for the next 3 mo, and PBMC were cryopreserved. In addition, fresh PBMC were analyzed at 0, 3, and 12 wk for most subjects.

To identify Ag-specific T cells, the fresh or viably cryopreserved PBMC were stimulated in vitro for 6 h, either with a pool of HBV surface Ag peptides or with whole tetanus toxoid, in the presence of Abs to CD28 and CD49d for costimulation and monensin to inhibit cytokine secretion. The cells were then surface-stained for several lineage and differentiation markers, stained intracellularly for the cytokines IFN-γ and IL-2, and analyzed by flow cytometry. Fig. 1 shows data at the 3 wk point for two subjects immunized for HBV.

The Ag-specific cells have a typical (and fairly homogeneous) effector/memory phenotype. The cells express high levels of CD11a; most do not express CD45RA. Note, however, that the IL-2 response for subject 1 includes cells that express CD45RA. These cells are potentially newly recruited naive cells because the $CD45RA^+IL\text{-}2^+$ cells express lower levels of CD11a (data not shown). The tetanus-specific cells in the subjects immunized to tetanus show a memory phenotype similar to the majority of the HBV-specific CD4 T cells (data not shown).

Ag-specific cells produce IFN-γ, IL-2, or both after secondary immunization against HBV or tetanus

Examination of two cytokines simultaneously identifies cells producing each cytokine individually, cells producing both cytokines, and cells not producing either cytokine. At the 3 wk point after immunization, we observed cells making IFN-γ or IL-2 individually or in combination in response to the respective in vitro stimulations (Fig. 2). In comparison, the PBMC samples treated with costimulation alone had few, if any, cytokine-producing cells. As another specificity control, PBMC from subjects boosted for tetanus were stimulated in vitro with the HBV surface Ag peptide pool. These subjects had never been immunized for HBV and show little staining for IFN-γ or IL-2.

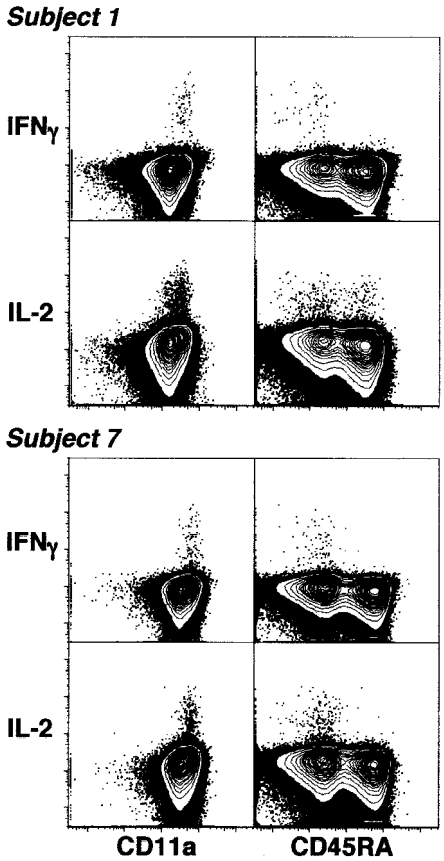

FIGURE 1. HBV-specific T cells have a typical effector/memory phenotype. Shown are data from the analysis of fresh PBMC at the 3 wk point for two subjects boosted for HBV. PBMC were stimulated in vitro with the HBV peptide pool and anti-CD28/anti-CD49d costimulation. $CD3^+CD4^+$ T cells are shown after gating for EMA^- (live), scatter-gated lymphocytes. The expression of CD11a and CD45RA are shown for cells expressing IFN-γ (*upper panels*) or IL-2 (*lower panels*).

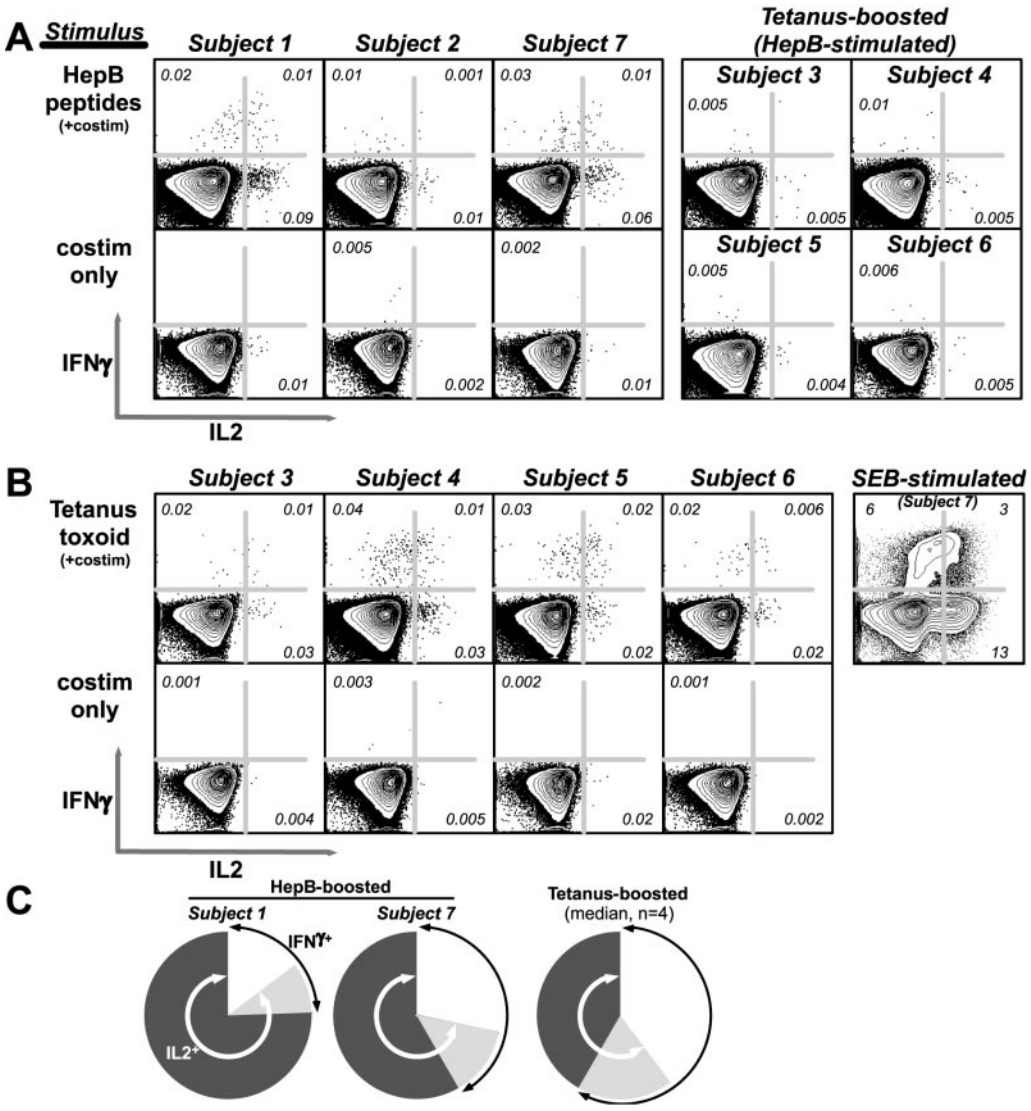

FIGURE 2. Ag-specific T cells producing IFN-γ and/or IL-2 are detectable by FACS. Cells producing IL-2 only and not IFN-γ represent a large proportion of the immune response. Shown are data from the analysis of fresh PBMC at the 3 wk point. $CD3^+CD4^+$ T cells are shown after gating for EMA^- (live), scatter-gated lymphocytes. Subjects 1, 2, and 7 were immunized to HBV (*A*), and subjects 3–6 were immunized to tetanus (*B*). For each part of the figure, data after Ag-specific stimulation (including costimulation) are shown in the *upper panels*, and data for costimulation alone (background) are shown in the *lower panels*. The percentage of CD4 T cells producing each or both of the cytokines is shown. Percentages <0.001 are not shown. The four panels in the *upper right* show data for the four subjects boosted for tetanus and stimulated in vitro with the HBV peptide pool. These subjects had not been previously immunized for HBV. SEB-stimulated cells for subject 7 (gated on CD4) are shown to the *right* of the panels in *B*. *C*, Data are shown as pie graphs, indicating the proportion of the responding CD4 T cells at wk 3 that produce only IL-2 (dark gray), IFN-γ (white), or both (light gray). The two plots on the *left* are for two of the subjects immunized to HBV. The plot on the *right* shows the median proportions for four subjects immunized to tetanus. The percentage for costimulation alone was subtracted from the percentage for Ag-specific stimulation.

Many responding cells make only IL-2, but not IFN-γ

The cytokine profiles in Fig. 2 demonstrate that cells producing only IL-2 are a large proportion of the Ag-specific response. In fact, although IFN-γ is thought of as the prototypical effector cytokine, the dominant CD4 T cell response for HBV was to produce IL-2 without IFN-γ. This is more clearly shown in Fig. 2*C*, where the proportion of the response due to each cytokine is plotted. Cells producing only IL-2 also represent a large proportion of the response to tetanus. The median values for the four subjects boosted for tetanus are plotted in Fig. 2*C*.

Because the Ag-specific stimulations include costimulation with anti-CD28/anti-CD49d, to quantify the responses, the frequency of cells responding to costimulation alone was subtracted from the frequency of cells responding to Ag. In general, the response to costimulation alone was low. For the overall CD4 T cell response, including either IFN-γ or IL-2, the median response was 0.01% (interquartile range, 0.006–0.05%; $n = 19$).

Using the cyropreserved samples (collected twice weekly after immunization), we were able to determine the kinetics of the dominant cytokine response to HBV. In subject 1, there was a detectable CD4 T cell response as early as 1 wk after immunization, and this response declined over time (Fig. 3), with an approximate half-life of 6–8 wk.

Simultaneous examination of five cytokines reveals complex cytokine profiles

Given our observation that IL-2 was dominant to IFN-γ for both HBV and tetanus responses, we wondered whether there were more Ag-specific T cells in these individuals that produce neither

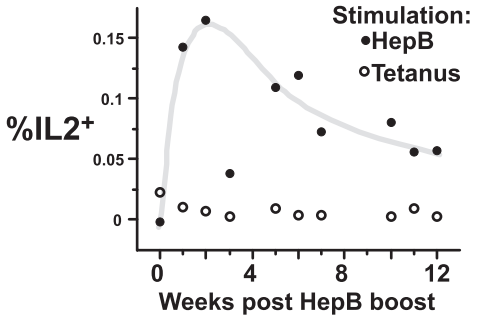

FIGURE 3. Time course of a secondary immune response to HBV booster immunization. Shown are the results of the analysis of the frozen PBMC samples for subject 1. The percentage of CD4 T cells producing IL-2 is shown after in vitro stimulation with the HBV peptide pool (●) or with tetanus toxoid (○). The $CD3^+CD4^+$ T cells have been gated as EMA^- (live), scatter-gated lymphocytes. The percentage of CD4 T cells producing IL-2 in response to anti-CD28/anti-CD49d costimulation alone has been subtracted from each time point.

IL-2 nor IFN-γ. Thus, we developed panels to simultaneously and independently quantify the responses to IL-2, IL-4, IFN-γ, TNF-α, and MIP-1β, produced by either CD4 or CD8 T cells.

As shown in Fig. 4, subject 7, who was boosted for HBV, had detectable IL-2 and MIP-1β responses in both CD4 and CD8 T cells. In addition, there was a small IL-4 response among CD4 T cells. Compared with subject 1, the response had a slower onset, reaching a peak by wk 2, and had a shorter half-life of <4 wk.

The five cytokines were quantified simultaneously; therefore, we were able to determine the extent of coexpression, or coordinate regulation, of the five cytokines. As shown in Fig. 4, there were six dominant patterns of cytokine expression at the 3 wk (maximal) time point for CD4 T cells. By far, MIP-1β represented the most common response (in this subject; see below), and this response overlapped somewhat with that of IL-2. Together, IL-2 and MIP-1β made up nearly 90% of the responding population. This is shown in Fig. 4*B*, where the proportion of the response due to each cytokine is plotted.

Surprisingly, we detected a significant HBV response among CD8 T cells in this individual at all time points tested; the frequency of this response rose and declined with similar kinetics as the CD4 T cell response. The cytokine profile for the CD8 T cells (Fig. 4) was also dominated by MIP-1β and IL-2. This expression pattern was not significantly different from the other time points (not shown), but was significantly different from that obtained with SEB, a nonspecific mitogenic stimulation (Fig. 4*B*; see below for a discussion of intra- and interindividual variability for SEB responses).

Indeed, the CD4 and CD8 Ag-specific T cell responses were a significantly skewed subset of the overall potential repertoire revealed by SEB. This indicates that the Ag response is selected from (or directed to) a specific profile of cytokine responses in both the CD4 and CD8 compartments. The specific profile selected during immunization or natural infection may be an important correlate to predict protection or progression.

CD4 T cell response to primary immunization for HBV

The data in Fig. 4 illustrate the secondary (memory) response to HBV immunization from a long-lived resting memory population; we wanted to determine whether this was different than an early response after primary immunization. We analyzed three subjects shortly after completing the primary immunization series for HBV. Fig. 5 shows the CD4 response at 2 wk after the 6 mo HBV immunization for freshly isolated PBMC. CD8 responses were not detected. Similar to the responses to booster immunization for HBV, the majority of responding cells produce IL-2. Some of these cells coproduce TNF-α or IL-4. Simultaneous staining with multiple cytokines identifies previously unknown functional subpopulations, e.g., IL-4-producing cells that also produce IL-2 and TNF-α. Unlike the subject in Fig. 4, HBV-specific T cells producing MIP-1β were not detected.

CD4 responses for this subject and for two other subjects are summarized in Fig. 6. In this figure, each cytokine profile is listed along the abscissa, and the percentage of the CD4 cells with each cytokine profile is plotted on the ordinate. As an alternate means of viewing the data, the graphs to the *right* in the figure show the percentage of the responding CD4 cells that are making each cytokine alone or in combination with other cytokines. Note that unlike the cytokine profile display on the *left*, in this data format a cell can be included in more than one column if it makes more than one cytokine (this would be the form of the data were cytokine responses measured one at a time, in separate tubes).

For comparison with the Ag-specific responses, CD4 and CD8 responses to polyclonal stimulation with the SEB superantigen in healthy HIV-uninfected individuals are shown at the *top* of Fig. 6. Although there is variability between individuals in the exact frequency of each subset, there are certain cytokine profiles that are consistently represented at higher frequencies in most individuals. When PBMC from the same individual drawn at different times are analyzed, the variability is low. When we analyzed samples from repeated draws for several individuals, the variability for the significant responses ($>0.1\%$) was, on the average, 17% (data not shown).

T cell responses to chronic HBV infection differ from the responses to immunization

For comparison with the responses to immunization, we analyzed 11 subjects chronically infected with HBV. T cell responses were detected in three of these subjects, and the cytokine profiles for these subjects are shown in Fig. 6. For one of these subjects (shown in red), the CD4 response was dominant, and it differed from the responses to immunization because of the larger number of cells producing IFN-γ and/or MIP-1β. Although one subject boosted for HBV (Fig. 4) produced MIP-1β in response to immunization, the particular cytokine profiles in this individual differ from those seen in infection. Except for the subject in Fig. 4, CD8 responses were not seen after immunization for HBV. CD8 responses were observed for HBV infection. The particular CD8 cytokine profiles differed between individuals. One subject had a dominant IFN-γ/TNF-α/MIP-1β response. For two other subjects, the dominant response was MIP-1β only.

T cell responses to HIV DNA vaccination and HIV-1 infection

Immunizations for HBV surface Ag and tetanus are both protein immunizations using alum as the adjuvant. We determined whether the patterns of immune responses (particularly the differences between vaccination and natural infection) were selective to the use of this adjuvant or are representative of a more general occurrence. We thus compared T cell cytokine profiles in response to HIV Ags after either unadjuvanted DNA immunization or to natural infection. The HIV-1 candidate vaccine consists of DNA encoding clade B gag, pol, and nef and clades A, B, and C envelope. HIV-uninfected subjects were immunized at 0, 1, and 2 mo and T cell responses to clade A envelope were determined at several time points after the second and third immunizations.

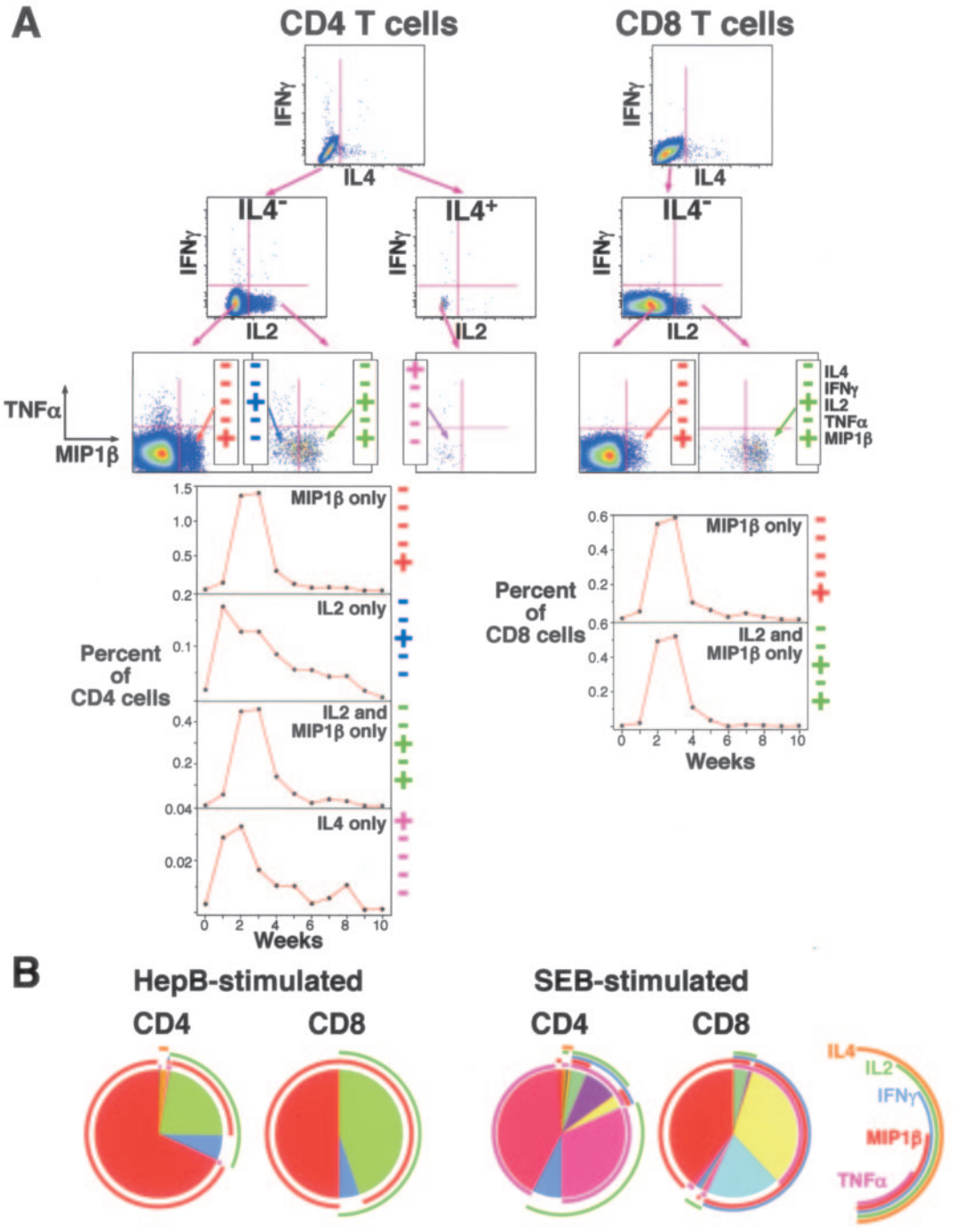

FIGURE 4. Analysis of multiple cytokines detects additional responding cells in response to HBV booster immunization. *A*, Shown are the results of the analysis of the frozen PBMC samples for subject 7 after 6-h in vitro stimulation with the HBV surface Ag peptide pool. The FACS plots show the 3 wk point. The $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells have been gated as $EMA^-/CD14^-/CD64^-$, scatter-gated lymphocytes. The CD4 and CD8 cells are further gated based on the expression of the cytokines shown in the figure. Only the gates or quadrants that include responding cells are shown. The cytokine profiles for the most frequent responses are labeled in the *third row* of FACS plots. These labels are listed in the order of the cytokines as indicated on the *far right* of this row of plots. The graphs below the FACS plots show the percentages of CD4 or CD8 T cells for each of these subsets for each of the weekly time points in the study. The labels are color-coded to match the example of the subset from the wk 3 point shown in the FACS plots. The percentages of CD4 or CD8 T cells producing the indicated cytokines in response to anti-CD28/anti-CD49d costimulation alone have been subtracted from each time point. *B*, Cells producing multiple cytokines alone or in combination are represented in the secondary immune response to HBV. Data are shown in a pie graph indicating the proportions of the HBV-responding CD4 or CD8 T cells that produce the indicated cytokines at the wk 3 point for the frozen PBMC sample for subject 7 (*left* plots). The plots on the *right* show the median proportions of the responding CD4 or CD8 T cells that produce the indicated cytokines after stimulation with SEB for eight healthy adults. Each segment of the pie graph represents a unique combination of cytokines; the color-coded arcs around the edges indicate those segments that include the indicated cytokine. The color key for these arcs is at the *far right*. For the Ag-specific responses, the cytokine pattern was much more restricted (fewer combinations of cytokines) than for the mitogenic stimulus SEB.

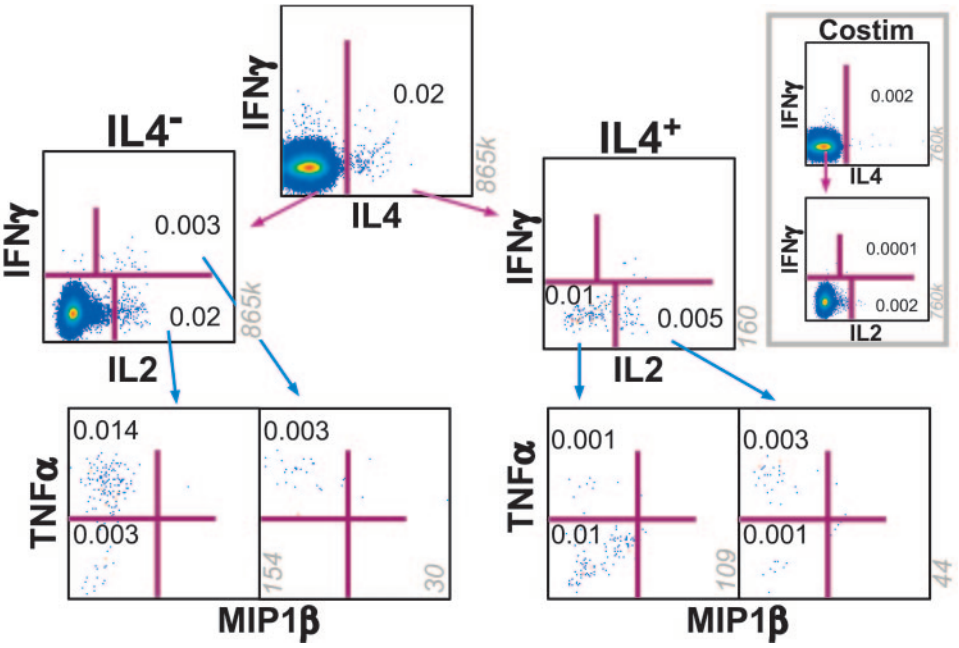

FIGURE 5. CD4 T cell responses to primary immunization for HBV. Shown are the CD4 T cell responses in one individual 2 wk after receiving the third (6 mo) immunization of the HBV primary immunization series. The CD4 cells are further gated based on the expression of the cytokines shown in the figure. Only the gates or quadrants that include responding cells are shown. The numbers within each gated region indicate the percentage of overall CD4 cells. The numbers to the *right* and outside of each plot indicate the number of events shown in that plot. The two plots at the *top right* show the responses for costimulation alone (background) for overall CD4 cells and for cells gated as IL-4 negative. The total number of PBMC collected by FACS for the Ag-specific and costimulation-only samples were 2×10^6 and 1.75×10^6, respectively.

After vaccination, cells producing multiple combinations of cytokines were observed (Fig. 6). As for HBV immunization, CD4 cells producing IL-2 alone or in combination with TNF-α were detected. Cells producing IFN-γ were observed in combination with IL-2/TNF-α and with IL-2/TNF-α/MIP-1β. For CD8 T cell responses, IFN-γ with TNF-α or IFN-γ with TNF-α/MIP-1β were most commonly detected.

It is important to note that the highest background responses (non-Ag-specific responses detected in the costimulation-only wells) were observed for CD4 T cells making solely TNF-α and for CD8 T cells making solely MIP-1β. The higher background for these two responses rendered the quantification less sensitive. Nonetheless, the power of multicytokine measurement was highlighted by this same phenomenon; because the background was restricted (in CD4 T cells) to TNF-α only, the other combinations that included TNF-α (for example, TNF-α and IL-2) still had very low background and thus could be quantified with high sensitivity. The use of TNF-α by itself would not allow this additional sensitivity, because the background from the TNF-α-only producing cells would contaminate the total TNF-α quantification.

For the small number of HIV-infected individuals we examined, the CD4 responses differed dramatically from the responses to vaccination; infection was accompanied by a much lower proportion of IL-2-producing cells. This difference was most evident in the *lower right graph* for CD4 T cells in Fig. 6*A*, which illustrates the percentage of the total responding CD4 cells making any given cytokine (alone or in combination with other cytokines). In contrast, the cytokine profiles of CD8 cells are more similar to the profiles induced by vaccination.

Discussion

We demonstrate in this study for the first time the simultaneous analysis of five cytokines at the single-cell level for Ag-specific T cells. Our results show that T cells are capable of expressing diverse cytokine profiles, and thereby demonstrate that classifying T cells into a small number of cytokine-based functional subsets is probably inadequate to characterize immune responses.

The measurement of five cytokines can potentially identify 32 cytokine profiles for each of CD4 or CD8 T cells. Although each of these profiles is not equally represented, the number of profiles observed in any individual is diverse. For the same individual measured at different time points, the variability for responses to SEB (in terms of each cytokine profile's representation among stimulated cells) is relatively small (average of 17%), demonstrating the reproducibility of this novel multicytokine assay (data not shown). The total potential cytokine repertoire and the pattern of those cytokine combinations represented at the highest frequencies, revealed by polyclonal stimulation with the superantigen SEB is remarkably consistent between individuals. In contrast, the particular cytokine profiles observed in response to immunization or infection are drawn from this potential repertoire and are comparatively skewed. Furthermore, the pattern of cytokine profiles differ based on the immunogen (and possibly adjuvant) or the infectious agent. Although responses among different individuals to a particular immunization or infection share characteristic features, there can be substantial differences from person to person. These results demonstrate that no single functional parameter adequately quantifies responses, and in fact, due to interindividual variability, limited combinations of functional parameters will not be adequate for all individuals. Therefore, optimal measurements of immunogenicity of a vaccine or of immune responses to pathogenesis will require the measurement of multiple distinct functional outcomes.

The methods introduced in this report provide a novel means for monitoring vaccine-induced immunogenicity and for monitoring T cell responses to infection. In terms of vaccine development, a method for the identification of those T cell responses induced by candidate vaccines that correlate with efficacy will help target the appropriate vaccines for further clinical testing. The numbers of individuals in this study are too small to draw statistically significant conclusions; however, our observations, demonstrating differences and similarities in the cytokine profiles induced by different vaccines and infections, provide pilot data for further study.

IL-2 was a dominant response for the HBV and tetanus immunizations. A study examining memory T cell responses in mice provides a potential explanation for the dominance of these cells (9). In this study, two types of Th1 cells were examined, those that do or do not produce IFN-γ; both cell types produced IL-2. The IFN-γ-producing Th1 cells were found to be short-lived in vitro and in vivo. In contrast, only the Th1 cells that did not produce IFN-γ efficiently developed into long term memory cells. The fact

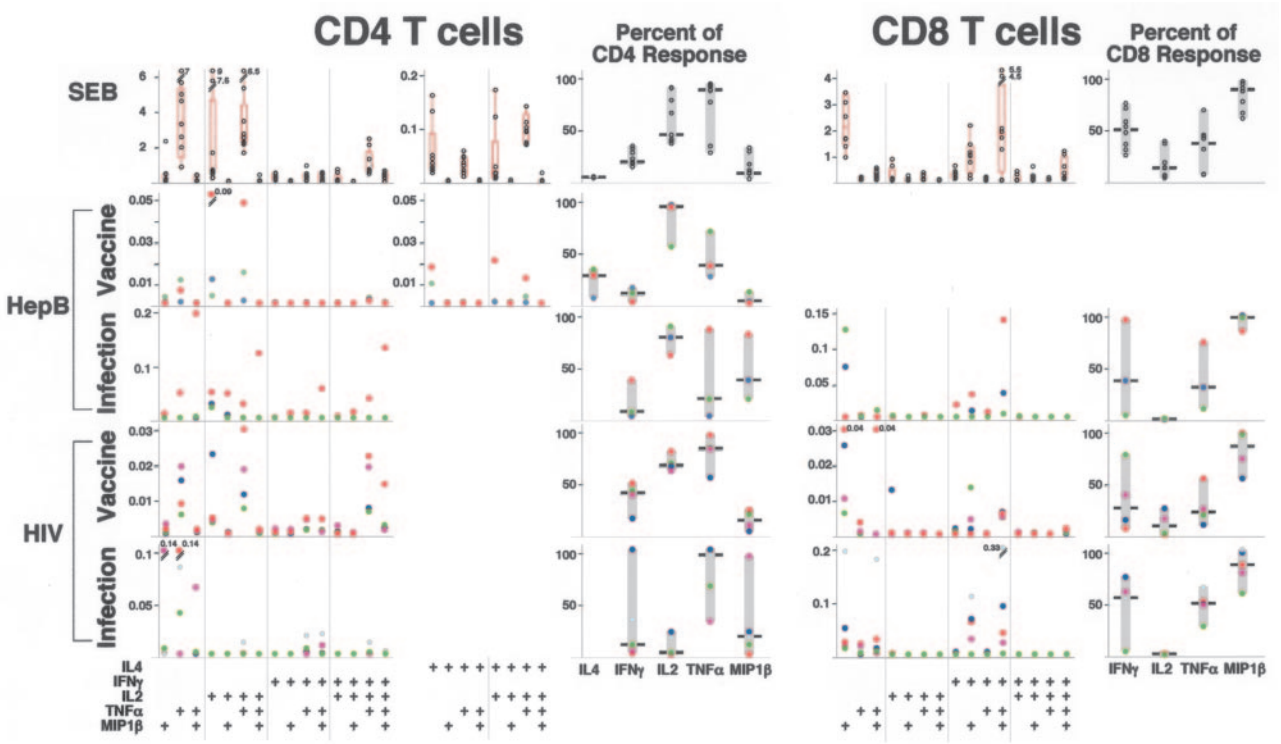

FIGURE 6. Cytokine profiles as determined by measurement of five cytokines are complex and differ based on type of immunization or infection. CD4 responses are shown on the *left*, and CD8 responses are shown on the *right*. For CD4 or CD8, the plots to the *left* show the percentage of CD4 or CD8 T cells with the cytokine profile as indicated at the *bottom* of the figure. For HBV vaccination, three subjects were examined 2 wk after receiving the 6 mo immunization in the primary immunization series (in vitro stimulation with surface Ag peptide pool). For HBV infection, data for three subjects chronically infected with HBV are shown (in vitro stimulation with surface Ag peptide pool for red and green subjects, with core protein peptide pool for blue subject; the red and blue subjects are coinfected with HIV). For HIV vaccination, data are shown for four subjects who received a candidate HIV DNA vaccine (in vitro stimulation with clade A envelope peptide pool). For each individual receiving the DNA vaccine, two to four time points between 6 and 12 wk are averaged. For HIV infection, data for five subjects chronically infected with HIV are shown (in vitro stimulation with gag peptide pool). For CD4 or CD8, the plots on the *right* show the percentage of the total responding CD4 or CD8 cells that make any individual cytokine (alone or in combination with other cytokines). The gray box highlights the range, and the black horizontal line indicates the median. For each type of immunization or infection, different individuals are shown in different colors. The responses to SEB for several HIV-uninfected individuals are shown in the plots at the *top* (for CD4, $n = 9$; for CD8, $n = 8$). For these plots, the red boxes show the interquartile range.

that it is the IFN-γ^- (yet IL-2^+) Th1 cells that develop into memory cells may explain the dominance of IL-2-producing cells we found in the secondary immune responses to HBV and tetanus and underscores the importance of measuring both cytokines in evaluating immune responses.

It is interesting to note that one HBV-immunized individual who had the significant MIP-1β dominance, also had a large CD8 response (>1%); the kinetics of this CD8 response after immunization was nearly identical with those of the CD4 response. Traditional Ag processing pathways should not be able to induce a CD8 response from soluble protein. Therefore, this represents an in vivo example of cross-presentation (10, 11). It is tempting to hypothesize that the MIP-1β dominance observed in the CD4 T cells may play a role in enhancing cross-presentation. MIP-1β, a CC chemokine, is chemotactic for several types of immune cells, including monocytes, macrophages, dendritic cells, and both T and B cells (12). MIP-1β might also serve to initiate a cytokine cascade that eventually recruits and rapidly differentiates monocytes into dendritic cells (13) to broaden the localized immune response. Because dendritic cells may be especially well suited for cross-presentation, there may be an association between the MIP-1β dominance in this subject and the large CD8 response.

This detailed analysis of functional responses to infection should also provide new insights into disease pathogenesis. Although the sample size was limited, we demonstrate that natural infection with HBV or HIV induced some cytokine profiles that overlapped with the vaccine-induced profiles as well as some unique profiles. HBV and HIV both induced CD4 and CD8 responses. For HIV, the CD4 response induced by infection included many fewer IL-2-producing cells compared with the vaccine-induced response. For all responses, there was variability between individuals; this variability introduces a novel set of parameters that can be considered for evaluation as correlates of vaccine protection, disease progression, or therapeutic efficacy.

In summary, we found that in the human, T cell responses to immunization are broad and complex, encompassing a far wider spectrum than can be measured solely by the production of a single cytokine. It is evident that sensitive quantification of immune responses requires the coordinate measurement of more than a single response, and that IFN-γ is a subdominant effector response in many immunizations. We propose that both the quantification as well as the pattern of the cytokine spectrum generated after immunization will become an important element of the iterative process of developing effective vaccine strategies.

Acknowledgments

We are especially grateful to all the subjects who volunteered for the various studies included in this report. We thank Pamela Edmonds and the entire vaccine clinical team directed by Barney Graham of the Vaccine Clinical Center for the organization and collection of many of the samples used in this study. We also thank James Schmitt, Lynn Stansbury, and the

staff of the Occupational Medical Service Clinic at National Institutes of Health for help in identifying individuals recently immunized for hepatitis B. We thank the Immunology Core Laboratory at the California National Primate Research Center and the clinical staff at the University of California School of Medicine (Davis, CA) for collection and processing of samples from the tetanus- and hepatitis B-immunized volunteers. Bob Bailor deserves special thanks for management of the HIV vaccine samples. We thank Michael Betts, Joseph Mattapallil, Jie Liu, and Della Berhanu for helpful discussions and for helping with the creation and validation of our fluorochrome-coupled Ab library. We also thank Drs. Robert Seder, Daniel Douek, Amos Panet, and Ronald Rabin for helpful discussions and critical reading of the manuscript.

References

1. Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature 383:787*.
2. Herr, W., J. Schneider, A. W. Lohse, K. H. Meyer zum Buschenfelde, and T. Wolfel. 1996. Detection and quantification of blood-derived $CD8^+$ T lymphocytes secreting tumor necrosis factor α in response to HLA-A2.1-binding melanoma and viral peptide antigens. *J. Immunol. Methods 191:131*.
3. Schmittel, A., U. Keilholz, and C. Scheibenbogen. 1997. Evaluation of the interferon-γ ELISPOT-assay for quantification of peptide specific T lymphocytes from peripheral blood. *J. Immunol. Methods 210:167*.
4. Waldrop, S. L., C. J. Pitcher, D. M. Peterson, V. C. Maino, and L. J. Picker. 1997. Determination of antigen-specific memory/effector $CD4^+$ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. *J. Clin. Invest. 99:1739*.
5. Pitcher, C. J., C. Quittner, D. M. Peterson, M. Connors, R. A. Koup, V. C. Maino, and L. J. Picker. 1999. HIV-1-specific $CD4^+$ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nat. Med. 5:518*.
6. Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science 274:94*.
7. Lu, F. X., Z. Ma, S. Moser, T. G. Evans, and C. J. Miller. 2003. Effects of ovarian steroids on immunoglobulin-secreting cell function in healthy women. *Clin. Diagn. Lab. Immunol. 10:944*.
8. De Rosa, S. C., L. A. Herzenberg, and M. Roederer. 2001. 11-Color, 13-parameter flow cytometry: identification of human naive T cells by phenotype, function, and T-cell receptor diversity. *Nat. Med. 7:245*.
9. Wu, C. Y., J. R. Kirman, M. J. Rotte, D. F. Davey, S. P. Perfetto, E. G. Rhee, B. L. Freidag, B. J. Hill, D. C. Douek, and R. A. Seder. 2002. Distinct lineages of Th1 cells have differential capacities for memory cell generation in vivo. *Nat. Immunol. 3:852*.
10. Belz, G. T., F. R. Carbone, and W. R. Heath. 2002. Cross-presentation of antigens by dendritic cells. *Crit. Rev. Immunol. 22:439*.
11. Heath, W. R., and F. R. Carbone. 2001. Cross-presentation, dendritic cells, tolerance and immunity. *Annu. Rev. Immunol. 19:47*.
12. Sherry, B., and G. Franchin. 2000. MIP-1α, MIP-1β. In *Cytokine Reference.* J. J. Oppenheim and M. Feldman, eds. Academic Press, San Diego, p. 1171.
13. Randolph, G. J., G. Sanchez-Schmitz, R. M. Liebman, and K. Schakel. 2002. The $CD16^+$ ($Fc\gamma RIII^+$) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting. *J. Exp. Med. 196:517*.