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Phenotypic and Functional Analysis of Immune CD8⁺ T Cell Responses Induced by a Single Injection of a HIV DNA Vaccine in Mice¹

Geraldine Arrode,²* Ramakrishna Hegde,∗ Arunmani Mani,∗ Yuhuai Jin,∗ Yahia Chebloune,† and Opendra Narayan²*

HIV DNA vaccines are potent inducers of cell-mediated immune (CMI) response in mice but elicit poor HIV-specific IFN-γ-producing T cells in monkeys and humans. In this study, we performed kinetic analyses on splenocytes of BALB/c mice that were immunized by a single injection with a unique DNA vaccine. Using IFN-γ-ELISPOT and multiparametric FACS analysis, we characterized the induced CMI response. We found that the response was detectable for at least 63 wk. ELISPOT detection of IFN-γ-producing T cells showed a profile with two waves separated by a long period of minimal response. Multiparametric FACS analysis showed two populations of CD3⁺CD8⁺ T cells that were specific for all HIV Ags. These cells had similar robust proliferation abilities and contained granzyme B. However, only a few produced IFN-γ. Both IFN-γ-producing and non-IFN-γ-producing HIV-specific CD8⁺ T cells were detected in the early stage (week (W)1 and W2 postimmunization (PI)), in the prolonged intermediate period of minimal response (W4-W26 PI), and in the final late phase of increased response (W30-W63 PI). Our longitudinal characterization showed that both subsets of cells underwent expansion, contraction, and memory generation/main-tenance phases throughout the lifespan of the animal. Altogether, these findings bring insight to the heterogeneity of the immune T cell response induced by a single immunization with this DNA and strengthen the concept that used of the IFN-γ-ELISPOT assay alone may be insufficient to detect critical T cell responses to candidate HIV vaccines. The Journal of Immunology, 2007, 178: 2318–2327.

Despite the pressing need for development of a vaccine or vaccines against HIV, none has been identified as yet. One of the challenges facing development of such vac-ccines is that there are no correlates of immunity that are known to be definitely associated with protection against HIV-induced disease. Only few infected individuals termed long-term nonprogres-sors (LTNPs)³ ever succeed in controlling HIV infection. Among LTNPs, elite suppressors, strongly associated with the MHC class I subtype HLA-B*57, maintain normal CD4 counts and viral RNA loads below the limit of standard detection (<50 copies/ml) in absence of antiviral treatment. The control of replication of the virus in LTNPs correlates with the development of multifaceted cell-mediated immune (CMI) responses. CD8⁺ T cells of these individuals have been characterized by a superior capacity to prolif-erate and this was linked to enhanced effector functions. These functions are exemplified by the ability of the cells to produce perforin (1), IL-2 (2, 3), IFN-γ, MIP-1β, TNF-α, and CD107-a simultaneously (4). In addition, it was demonstrated recently that elite suppressor develop de novo partially functional (IFN-γ⁺) CD8⁺ T cell responses against mutated HLA-B*57-restricted Gag epitopes that emerge during their chronic and extremely low viremia. These individuals also maintain highly functional (IFN-γ⁺ and IL-2⁺) CD8⁺ T cell responses to unmutated epitopes present in either archived cellular provirus or plasma virus (5). Collectively, these qualitative differences in the virus-specific responses define the best immunological correlates of protection (6, 7).

Since protective immunity seemed to correlate with CMI rather than humoral immune responses (8), a major effort was focused on examination of the potential of DNA vaccines to induce this type of immune response because plasmid DNA-expressing viral genes together with the CpG motifs are known to be potent inducers of CMI responses (9). Current HIV-1 DNA vaccines consist of one or more HIV genes whose expression is regulated by the CMV pro-moter. This type of DNA has been used for priming immunizations that are followed by boosts with viral vectors expressing HIV pro-teins (10, 11). We have developed another type of DNA vaccine that was derived from the genome of a highly pathogenic simian HIV (SHIV), SHIVKU2, from which the rt, int, vif, and the 3’ long terminal repeat (LTR) were deleted. This construct, named Δ4SHV_KU2, consists of the 5’ LTR of SIV driving expression of gag, env, tat, rev, vpu, and nef genes. The DNA was shown to be highly immunogenic in mice in which IFN-γ-ELISPOT responses were detected as long as 12 wk after immunization with the DNA (12).
Macques injected with the DNA were protected against disease caused by SHIV89.6P, but no prominent IFN-γ-ELISPOT responses were induced by the vaccine (13).

The current standard for assessing the immunogenicity of candidate HIV-1 DNA vaccines has been the IFN-γ responses induced by the vaccine (13, 15). While this technical approach is of value in quantifying vaccine-elicited T cell responses, it does not assess the functional and phenotypic T cell heterogeneity associated with induction of an immune response. Indeed, using tetramer recognition, Seaman et al. (16) identified Env-specific CD8+ T cells with both central memory (CD62L+), and effector memory (CD62L-) phenotypes 18 wk following a single injection of DNA in mice with a plasmid-expressing HIV-1 gp120 protein under the CMV promoter. Results from this study strongly stress the importance of monitoring the phenotype and functionality of the vaccine-induced Ag-specific primary T cell response before the design of any boost strategies (16).

Today, the advent of multicolor flow cytometry (17), which allows for finer characterization of these responses, gives us the opportunity to better identify the phenotypes and functions of vaccine-induced Ag-specific CTL.

In this study, we performed a longitudinal characterization of the T cell immune responses elicited by a single immunization of BALB/c mice with the Δ4SHIVKU2 DNA vaccine. We used polychromatic flow cytometric assays to dissect the phenotypes (central memory T cell (Tem), CD127+, CD62L+/effector memory T cell (Tem), CD127+, and CD62L-) and functions (IFN-γ and IL-2 secretion, proliferation, and granzyne B expression) of the elicited T cells. We focused our study on detailed examination of the HIV-specific CD8+ T cell response because that of CD4+ T cell was too weak or below the threshold of our detection system (0.01%).

### Materials and Methods

#### HIV peptides

Overlapping 15-mer peptides, with 11-aa overlaps, spanning the entire molecules of HIV Gag, Env, Tat, Rev, and Nef, proteins were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (catalog nos. 8117, 6451, 5138, 6445, and 5189, respectively). These peptides are based on consensus sequences from clade B HIV viruses. Our HIV DNA vaccine encodes Gag and Nef from the SF2 HIV strain and Tat, Rev, and Env from the HXB2 HIV strain (12). These two strains are both clade B viruses.

#### Animals

BALB/c mice were purchased from Harlan Laboratories and housed in the Laboratory Animal Resources of the University of Kansas Medical Center. All mice were used in accordance with the National Institutes of Health and the University of Kansas Medical Center Institutional Animal Care and Use Committee guidelines.

#### Vaccine Δ4SHIVKU2 plasmid DNA

The construction procedures of SHIVKU2 plasmid DNA have been described earlier (18). The inserted sequences were derived from SHIVKU2 (GenBank accession no. AY751799) and the HIV-1 SF2. The strategy of construction of Δ4SHIVKU2 plasmid DNA has been described recently (12). This construct encodes vpx and vpr genes of SIVmac239 and gag, provo, and a portion of rt, vpu, tat, rev, env, and nef genes of HIV-1 under the transcriptional control of the 5’ LTR promoter and the poly(A) sequences of SV40. The reverse transcriptase gene was truncated, and integrase, vif, and the 3’ LTR were deleted from SHIVKU2.

#### Inoculation of mice

BALB/c mice were inoculated once with 200 μg of vaccine DNA at 2 μg/μl concentration. DNA solution was prepared in PBS (0.1 M pH 7.4). Each mouse was injected with a total of 100 μl of DNA solution, 50 μl in each gastrocnemius muscle.

#### Detection of IFN-γ-producing cells by ELISPOT assay

Spleens were aseptically collected from mice and squashed between glass slides to dissociate and harvest splenocytes. Cells were collected in Hank’s solution, treated with a lysis solution (BD Biosciences) to remove the erythrocytes, and mononuclear cells were counted in a hemocytometer. A portion of the splenocytes from each individual mouse was used for ELISPOT assay, and a second portion was used to produce a single pool of splenocytes from the six mice of each group. Cells were used for intracellular and surface staining for flow cytometry analysis.

Quantitative ELISPOT assay, previously described in Ref. 12, was used to measure IFN-γ-producing splenocytes by response to groups of overlapping peptides. The cutoff for positivity in this assay was determined at 12 spots/million splenocytes. This corresponds to the average of spots obtained in cultured medium controls +3 SD (three times the value of SD).

#### Flow cytometry assays for HIV-specific immune T cells

Polychromatic (six- to seven-color) flow cytometry analysis was performed on a three-laser BD LSRII instrument with standard set up. Data files were collected and analyzed using the FACSDiva software program (version 4.1.2; BD Biosciences). Each analysis included lineage-defining markers (CD4, CD8, and CD3). Briefly, to identify HIV-responsive T cells, 2 × 10^6 spleen cells were stimulated with different HIV pools of peptides (2 μg/ml) or medium alone in the presence of 1 μg/ml costimulatory CD28 mAb (clone 37.51; BD Biosciences) and brefeldin A (Sigma-Aldrich) for 6 h to accumulate intracellular cytokines. Alternatively, during this restimulation process, TAPI-2 (Calbiochem) was added at 35 μg/ml for the last 4 h to prevent activation-induced cleavage of CD62L (19). Splenocytes costimulated with CD3 (clone 145-2C11) and CD28 mAbs at 2 μg/ml concentrations were used as positive controls. Cells were washed, incubated 15 min at 4°C with anti-mouse CD16/32 mAb (eBioscience) to block FcRs, and surface stained with Alexa Fluor 405-conjugated anti-CD3 (clone KT3; Serotec), allophycocyanin-Cy7-conjugated anti-CD8 (clone 53-6.7; BD Biosciences), and FITC-conjugated CD262L (clone MEL-14) mAbs for 20 min at 4°C. Additionally, ethidium monoazide (EMA; Molecular Probes) was added at 0.5 μg/ml during the surface labeling step to allow exclusion of dead cells in samples that have been cultured for 4 days and restimulated for 6 h (17). In such a case, all samples were exposed to light for 15 min at room temperature to allow EMA to accumulate intracellularly. The dead cells were then fixed/permeabilized (Cytofix/Cytoperm Plus; BD Biosciences) and stained with PE-Cy7-conjugated anti-CD4 (clone RM4-5), PE-Cy5-conjugated anti-CD127 (clone A7R34; eBioscience), and anti-CD69 mAbs for 30 min at room temperature. The cells were then washed (Perm/Wash; BD Biosciences), fixed in 1% paraformaldehyde in PBS, and stored at 4°C until flow cytometry analysis. All Abs were purchased from BD Biosciences unless specified. For each experiment, unstained and all single-color controls were processed to allow proper compensation as well as all fluorescence-minus-one controls to determine proper population gates. Each analysis was gated on forward and side scatter splenocytes (FSC/SSC), EMA- (when specified), CD3+, and high CD8+ population to allow the collection of 25,000–50,000 CD8+ events (>10^4 total events). Data were displayed as two-color dot plots to measure the proportion of the single-positive or double-positive cells in the highly CD3+ CD8+ population (orange color). Bioexponential display was also used to show each population in its entirety.

To monitor the expansion and proliferation of Ag-specific T cells, CFSE (Molecular Probes)-labeled splenocytes were seeded in 96-deep well tissue culture plates (Nunc) at a density of 2 × 10^5 cells/well in 1 ml of medium only or supplemented with 2 μg/ml of indicated pools of HIV peptides and incubated for 4 days at 37°C. After 4 days of incubation, cells were re-stimulated, stained, and analyzed following the same procedure described above. CFSE labeling of cells was performed according to the manufacturer’s protocol (10^7 cells/ml in 1 μM CFSE for 10 min at 37°C).

#### Statistical analysis

We used the two-sample t test to assess statistical significance of the CMI responses induced 63 wk PI. Calculations were performed with Statistix 8 (Analytical Software).
ELISPOT assay to detect IFN-γ/H11569). This indicates that the increase of IFN-γ was statistically significant.

To confirm this hypothesis, we repeated and extended the single Δ4SHIVKU2 DNA vaccine immunization of mice and followed groups of them until W63 PI. The ELISPOT responses were kinetically analyzed by IFN-γ-ELISPOT in addition to multiparametric flow cytometry assays to specifically identify the phenotype and function of the induced T cells.

In this new experiment, six BALB/c mice were examined at each sampling time point. Each animal was examined individually by ELISPOT. As shown in the Fig. 1C, the ELISPOT data obtained with Gag peptides in this new experiment confirmed those of the previous experiments showing two waves separated by a phase of minimum or absence of IFN-γ-producing cells from W14 to W26 until their re-emergence at W30-W32 PI until W63 PI. Responses with similar profiles but weaker intensities were obtained in response to Env and Tat+Rev+Nef (TRN) pools of peptides (data not shown).

Results

ELISPOT analyses of IFN-γ-producing cells induced by Δ4SHIVKU2 DNA vaccine

Recently, we reported that 200 μg of Δ4SHIVKU2 DNA vaccine injected into the gastrocnemius muscles of mice resulted in expression of viral p24 Ag in myocytes and cells in lymph nodes and spleen for at least 10 days. This was followed by the development of specific IFN-γ-ELISPOT responses in splenocytes (12).

These experiments showed that 2.0, 1.7, and 0.7% of the IFN-γ-producing cells directed against Env and Tat+Rev+Nef Ag-specific CD8+ T cells persisted for at least 32 wk PI, in absence of any boost or persistence of viral Ag.

Δ4SHIVKU2 DNA vaccine induced broad primary and secondary HIV Ag-specific CD8+ effector T cell responses

For the flow cytometric analyses, the specificity of the induced immune responses was examined by using separated (Gag and Env) or combined (TRN) pools of peptides on pools of splenocytes from six mice. Only a small number of cells producing IFN-γ-ELISPOT response was detected at early time points. Therefore, we expanded the Ag-specific T cells by culturing total splenocytes, in vitro, in the presence of indicated peptides (Gag (4d)) or medium used as control (medium (4d)) for 4 days. On day 4, we detected the expanded HIV Ag-responsive T cells by their capacity to accumulate intracellular IFN-γ and/or IL-2 after stimulation for 6 h with indicated peptide mixtures (Gag (6h)). Following 6 h of restimulation, the cells were surface stained with anti-CD3, -CD8, and -CD4 in the presence of EMA (to allow the detection of dead cells) and subsequently permeabilized and stained with anti-IFN-γ and -IL-2 mAbs, as described in Materials and Methods.

These experiments showed that 2.0, 1.7, and 0.7% of the IFN-γ-producing CD8+ T cells responded to a combined pool of TRN and to Env and Gag peptides, respectively, 1 wk following immunization (Fig. 2A, upper row).

At 2 wk, the frequency of the IFN-γ-producing CD8+ T cells responding to TRN and Env decreased to 0.3 and 1.0%, respectively, whereas those specific for Gag became dominant with 1.1% of the CD8+ T cell population (Fig. 2A, lower row). The massive expansion of the primary effector CD8+ T cells directed against TRN and their dramatic reduction by W2 strongly suggested that a contraction phase, averaging 6-fold, in the immune response had taken place very early. A similar contraction phase, averaging 5-fold, for the Gag- and Env-specific responders developed between W4 and W18 (Fig. 2B). These successive events were then
followed by a phase of minimal to absent response from W18 to W26.

Remarkably, starting at W30 with a maximum at W63, there was a re-emergence of HIV-specific IFN-γ-producer CD8⁺ T cells, ranging from 0.1 to 0.4% for TRN, 0.5 to 0.9% for Env, and 0.3 to 2.5% for Gag (Fig. 2B). These cells were identified as secondary effector CD8⁺ T cells. The total response to HIV Ags of the secondary effector T cells reached a similar level to that of the primary effector T cells (Fig. 2B).

Overall, these results indicated that the single injection of the DNA successfully induced effector CD8⁺ T cells (IFN-γ-only producer) specific to all viral proteins encoded by the vaccine DNA.
These flow cytometry based data identified CD8$^+$ T cells as the main effectors of the CMI responses and confirmed the kinetics of the IFN-γ-ELISPOT responses measured previously. These data also gave evidence that the Ag-specific CD8$^+$ T cells, after successful expansion and contraction phases, had become programmed into memory cells.

**Δ4SHIVKu2 DNA vaccine induced long-term memory CD8$^+$ T cells**

To determine whether the single DNA immunization had induced specific memory T cells that could be phenotypically identified during the 2- to 63-wk period PI, we examined splenocytes after 6 h of stimulation with HIV-specific peptides or with medium in the presence of costimulatory Abs and TAP1-2. Cells were then surface stained with anti-CD3, -CD8, -CD4, -CD127, and -CD62L and subsequently permeabilized and stained with anti-IFN-γ and -IL-2 mAbs. Because of the low frequency of the Ag-specific CD8$^+$ T cells expanded in response to TRN mix of peptides after 2 wk of immunization (see Fig. 2), we focused on Gag and Env Ag responses for this phenotypic characterization.

After 2 and 3 wk PI, only 0.1% of total CD3$^+$ CD8$^+$ T cell population produced IFN-γ in response to Gag and Env Ags. No IL-2-secreting cells were detected. Further examination of these Ag-specific IFN-γ-producing CD8$^+$ T cells showed that they were predominantly CD127$^+$,CD62L$^+$ (Tcm phenotype). Minimal fractions of these CD8$^+$ T cells showed different phenotypes (CD127$^+$, CD62L$^-$: Tem; CD127$^+$,CD62L$^-$: effector; CD127$^+$,CD62L$^-$: intermediate) (Fig. 3). These data, demonstrating the distribution of the HIV-specific splenocytes within effector and memory CD8$^+$ T cells, suggested that the maturation/differentiation processes took place very early between W2 and W3 following immunization. We then assessed the evolution and distribution of these cells following mid (W12)- and long-term (W20) PI.

Phenotypic data obtained with W6, W8, and W10 splenocytes were all similar to those obtained with W3 cells, showing 0.1% of total CD3$^+$ CD8$^+$ T cells producing IFN-γ in response to Gag or Env Ags and no IL-2-secreting cells. In addition, these cells were predominantly CD127$^+$,CD62L$^+$ (Tcm) and CD127$^+$,CD62L$^-$ (Tem) (data not shown). Interestingly, and in contrast to the data obtained between W6 and W10, at W12, we observed that 0.4 and 0.03% of CD3$^+$ CD8$^+$ T cell population had produced IL-2 in response to Env and Gag peptides, respectively (Fig. 3). These cells were associated with central memory phenotype (CD127$^+$,CD62L$^+$). In addition, the low frequency of 0.1% of total CD3$^+$ CD8$^+$ T cells producing IFN-γ in response to Gag or Env Ags was maintained. These data suggested that true memory T cells had developed at these early time points. Therefore, we sought to determine whether these cells were also present at W63 PI. Similar to earlier time points, 0.1% (Env) and 0.2% (Gag) were identified as effector memory T cells (CD127$^+$,CD62L$^-$), i.e., CD8$^+$ T cells making IFN-γ within 6 h of Ag stimulation. In addition, 0.1% (Env) and 0.05% (Gag) of central memory cells (CD127$^+$,CD62L$^+$), i.e., CD8$^+$ T cells produced IL-2 within 6 h of Ag stimulation (Fig. 3). Therefore, the broad Ag-specific CD8$^+$ T cells response initiated at early time points (W1 and W2) after a...
A single injection of the DNA was followed by generation of HIV Ag-specific memory CD8⁺ T cells that persisted more than the half-life of the mice.

Δ4SHIVKU2 DNA vaccine induced CD8⁺ T cells capable of extensive proliferation in response to HIV Ags

To further characterize the quality of the CD8⁺ T cell immune response induced by the DNA, we investigated the ability of these cells to proliferate in response to HIV Ags by using a CFSE dilution approach. This proliferative response was thought to be optimal for measuring the recall response mediated by memory T cells. Total splenocytes were labeled with CFSE and then cultured with or without appropriate mixes of peptides for 4 days. On day 4, the cells were harvested to assess T cell function (i.e., IFN-γ and IL-2 secretion after 6 h of restimulation with relevant peptides). The cells were then surface stained with anti-CD3, -CD8, and -CD4.
CD3+ T cell responses measured against Env showed that 9.7% of total CD3+ T cells were IFN-γ producers. The remaining 80% of the proliferating cells did not produce detectable IFN-γ.

Thus, from W1 to W18, we observed that in the presence of Tat, Rev, and Nef peptides, 7.5% to undetectable level of total CD3+ T cells proliferated and 2.0% to undetectable levels produced IFN-γ. The response measured against Env showed that 8.1-0.4% of total CD3+ T cells proliferated and 1.7% to undetectable level produced IFN-γ. Finally, the response measured against Gag showed that 3.8-0.8% of the CD8+ T cell population proliferated and 1.1-0.1% produced IFN-γ. None of the Ag-specific CD8+ T cells produced IL-2.

These results clearly identified two populations of Ag-specific CD3+ T cells that have similar ability to proliferate but have different cytokine (IFN-γ) secretion pattern. In addition, the expansion and contraction phases were also seen among the non-IFN-γ-producing Ag-specific CD8+ T cells. This contraction reached ~4-fold reduction for Gag, 20-fold for Env, and >75-fold for TRN between W1 and W18 (Fig. 4B). Importantly, these cells would not have been identified in the IFN-γ-ELISPOT assay. Thus, the measurement of proliferation in response to Ag, as well as the IFN-γ secretion, may be a more sensitive and complete indicator of effective CMI than the direct ELISPOT assay usually performed.

Since the progression of the non-IFN-γ-producing CD8+ T cell population paralleled the effector responses during the expansion and contraction phases, we hypothesized that memory should also have been present in this population of T cells. We identified a phase during W18-W26 in which the proliferative response to HIV Ags was minimal or absent. At this time point, the proliferative response to Env (1.2%) started to emerge while no IFN-γ-producing Ag-specific CD8+ T cell response were detected (Fig. 4B and data not shown). Then, by W30 PI, we detected 0.6% of total CD3+ T cells that were proliferating and 0.1% were producing IFN-γ in the presence of TRN peptides. The response measured against Env showed that 5.0% of total CD3+ T cells had proliferated and 0.5% had produced IFN-γ. Finally, the response measured against Gag showed that 1.7% of total CD3+ T cells had proliferated and 0.3% had produced IFN-γ (Fig. 4B and data not shown). Remarkably, these long-term responses were even more pronounced at W63 when we detected 4.0% of total CD3+ T cells were proliferating and 0.4% were producing IFN-γ in the presence of TRN peptides. The response measured against Env showed that 9.7% of total CD3+ T cells had proliferated and 0.9% had produced IFN-γ. Finally, the response measured against Gag showed that 10.1% of total CD3+ T cells had proliferated and 2.5% only produced IFN-γ (Fig. 4A, W63).

Of particular interest, at the W63 time point, we also found that when the cells were cultured in the absence of Ag, HIV-specific CD8+ T cells produced both IFN-γ and IFN-γ plus IL-2 (0.3 and 0.2%) (Fig. 4A). This is the only other time point that showed the evidence of IL-2 secretion within the expanded CD8+ T cells. This peculiar phenotype might have signify a central memory response because similar results were obtained in other studies (20, 21).

These results demonstrated the ability of the long-term memory Ag-specific T cells to set up a strong, functional recall response composed of secondary effector CD8+ T cells (IFN-γ only producers) and non-IFN-γ-producing CD8+ T both with vigorous proliferative capacities.

Overall, it is remarkable that, more than 1 year following the initial DNA immunization, Ag-specific CD8+ T cells were still detectable. These cells constituted a pool of memory stem cells that were able to give rise to secondary CD8+ T cell response with similar features (broad response, strong proliferative capacity, and IFN-γ secretion) than the primary response.

Evidence of cytotoxic machinery within the HIV Ag-specific and proliferating CD8+ T cells

To further characterize the cells that proliferated in response to HIV Ags, we examined their content of lytic proteins. Splenocytes from W2-immunized mice were labeled with CFSE and stimulated with HIV peptides for 4 days. Cells were harvested, restimulated with the peptides before and then surface stained with anti-CD3 and -CD8 Abs in the presence of EMA, and subsequently permeabilized and stained with granzyme B mAb. For each sample, we collected 25,000 events within low FSC/SSC, EMA+CD3+, and high CD8+ T cell population (colored in orange). We then displayed and measured as two-color dot plots the proportion of total live EMA+CD8+ T cells proliferating (CFSE dilution) and producing granzyme B under specific restimulation. Frequencies of proliferating Ag-specific CD8+ T cells expressing or not granzyme B are reported. Two of four representative experiments are shown.
Discussion

DNA immunization is a simple and inexpensive vaccine strategy that has been shown to induce sustained specific cellular immunity against viruses, bacteria, and parasites (22). Extensive studies have been conducted to develop DNA based vaccines against the devastating human lentivirus HIV-1, the main assay for quantification of induced CMI response being the standard IFN-γ-ELISPOT assay. Even though DNA-vaccinated chimpanzees were protected against HIV-1 challenge (23), DNA vaccines alone, compared with viral vectors, are thought to be relatively weak immunogens in humans (11, 24, 25). Therefore, with only few exceptions (13, 26, 27), mainly used anti-HIV DNA vaccine strategies are based on DNA prime or multimerise and boost either with protein or recombinant viruses.

Most of the current HIV DNA vaccines are based on constructs in which HIV genes are expressed constitutively by the early promoter of CMV (reviewed in Ref. 28). In our HIV DNA vaccine construct, all HIV genes were expressed by the SIV 5′ LTR that is transactivated by the HIV Tat. The unique RNA transcribed by this LTR is efficiently translated to produce all six HIV proteins (Gag, Env, Tat, Rev, Nef, and Vpu), as well as the two SIV, Vpx, and Vpr proteins (12). In this study, we showed that mice immunized with this DNA developed a HIV-specific CMI response that was directed against all tested HIV Ags (Gag, Env, and TRN). Interestingly, however, the magnitude of specific CD8+ T cell expansion was found to correlate with the dynamic of expression of viral Ags. Indeed, the initial response was found to be mainly against TRN and Env but to a lesser extent against Gag Ags. In contrast, the response against Gag became persistently dominant at later time points. This sequence of response specific to different HIV proteins mimics the chronology of protein expression and their accessibility during replication of the virus. Indeed, Tat, Rev, and Nef are early proteins compared with Env and Gag. Furthermore, Env is expressed at the surface of cells, whereas Gag is assembled in virus particles in the cytoplasm of the cells prior release of the virus particle. Interestingly, this sequential type of response has been reported during natural HIV infection with early strong responses often seen toward Nef and Env epitopes (29, 30). Later, most HIV-infected patients, particularly LTPN patients, develop and maintain a broad response directed to multiple HIV Ags with a vast majority of cells responding to Gag and Pol (30, 31). Our results with Δ4SHIV-KU2 DNA vaccine in mice demonstrated that an anti-HIV CD8+ T cell response expanded the magnitude and breadth of T cell epitopes in a manner similar to natural HIV infection. It is unknown whether anti-HIV DNA vaccine strategies using the constitutive CMV promoter for expression of HIV Ags induce a similar or different type of CD8+ T cell. It is tempting to speculate that the regulation of protein expression during the priming events may imprint particular quality to the immune T cell response.

Since there are minimal data (16) with respect to the properties rather than the quantity (32) of the T cell immune responses induced by a single HIV DNA immunization, using the mouse model system, we have focused on such baseline characterization for our HIV-1 DNA construct. This type of analysis revealed two important findings. First, single Δ4SHIV-KU2 DNA immunization in mice induced a biphasic pattern of CD8+ T cells response with initial periods of expansion and contraction that were followed by a late period of re-emergence independent of any Ag boost. This pattern was specific to the HIV DNA vaccine because the coimmunization of GM-CSF expressing plasmid with our HIV DNA vaccine strongly reduced the contraction phase. The persistent detection of HIV-specific IFN-γ-producing CD8+ T cells indicates that GM-CSF may increase the survival of these cells during the contraction period. Whether GM-CSF act preferentially to cause increase of effector CD8+ T cell population oravor the development of memory CD8+ T cells remains unknown.

The expansion and contraction phases observed in our study are classically associated with development of Ag-specific CD8+ T cell responses. However, the late re-emergence of HIV-specific CD8+ T cells observed after 30 wk PI is intriguing. This indicated an increase in the number of HIV-specific memory CD8+ T cells in the spleens of immunized mice at this time. One can speculate that: 1) following a slow process of maturation, these cells may have increased the expression of specific receptors (e.g., IL-15R and IL-7R) that allow their expansion in response to micro-environmental immuneostatic signals. Therefore, vaccinated animals inoculated with IL-15 or IL-7 cytokine, at the time of minimal to absent response (W18-W26), should experience earlier re-emergence of HIV-specific CD8+ T cells. 2) Subsets of HIV-specific memory CD8+ T cells with strong recall function may early reside in particular anatomic compartment (e.g., bone marrow) as reported recently (33). After an extensive absence of Ag and possibly unknown signals, these cells may progressively relocate in secondary lymphoid tissues like the spleen.

The second major finding that was also intriguing was that most of the CD8+ T cells induced by our DNA vaccination were made of fully proliferation-competent cells with poor or absent cytokine (IFN-γ and IL-2) production, but a superior recall response upon restimulation. Memory T cells are well known for their ability to secrete IL-2 (21). We were surprised to detect IL-2-producing HIV-specific CD8+ T cells only at W12 and W63, whereas the central memory phenotype markers, commonly associated with IL-2 production, were repeatedly depicted within the IFN-γ-producer CD8+ T cells. Nevertheless, this type of discrepancy between phenotype and function has been already observed in other studies (4).

We cannot exclude the possibility that these cells, known to recirculate preferentially through lymph nodes (34) may exist in higher proportion in other secondary lymphoid tissues. However, even though this type of cell has been mostly detected in transgenic mice following infection with recombinant Listeria monocytogenes (20), it is possible that our failure to detect more of these cells was due to their extreme rarity in the context of our single DNA immunization model. Further studies will be needed to determine which factors during the priming events (cytokine environment, amount of Ag, and nature of APCs) may help to imprint this particular quality on memory CD8+ T.

The high proportion of vaccine induced non-IFN-γ-producing CD8+ T cell led to several questions about the nature of these cells. Are these cells somehow defective? Are these cells found in other models of infection or immunization? Up to the present, there are no data on immunogenicity of a single HIV DNA immunization in which Ag-specific T cells proliferation and cytokine production properties were examined simultaneously. Therefore, we were unable to do any comparative examination with our data. In experimental infection studies in mice, the persistence of virus-specific proliferation competent CD8+ T cells without effector function (absence of IFN-γ secretion and cytotoxic activity) have been associated with chronic lymphocytic choriomeningitis virus infection. Remarkably, with CD4+ T cell help, the CD8 effector activity was maintained (35). Similar data were reported for HIV-1 infection showing that HIV-specific CD4+ T cells can sustain and restore HIV-1-specific CD8+ T cell function in HIV-1-infected patients (36, 37). These data clearly show the CD4+ T cell dependence of CD8+ T cells for the induction and maintenance of fully
functional CD8+ T cell response. Notably, the loss of IFN-γ-producing CD8+ T cells specific to HIV Ags has been observed in HIV Gag DNA-immunized CD8 knockout mice. These data support the need for CD4+ T cells during priming for generation of functional CD8+ effector cells (38). In our study, we found that the induced CD4+ T cell response in mice was below detectable level; this may explain in part the presence of a high proportion of CD8+ T cells that functionally were not in the immediate IFN-γ effector phenotype. However, the long-term persistence of this type of cell clearly illustrated that efficient effector functions but not programing into memory of CD8+ T cells may be affected by the absence of a sustained CD4+ T cell response. In others studies, addition of IL-2 (mimicking CD4 T cell functions) to naive Ag-specific CD8+ T cells was shown to promote the generation of cells with immediate effector functions. In contrast, a low concentration of IL-2 mostly promoted the development of memory-like cells with reduced in vitro cytotoxic effects (39). Consequently, effector differentiation is not a prerequisite for generation of memory CTL (40). Remarkably, in our study, we found that most of HIV-specific CD8+ T cells that proliferated were expressing lytic machinery (granocyte B), and therefore, they may have had cytotoxic activity. This result is similar to the observation in HIV-infected persons in whom the main qualitative difference between LTNPs and chronic HIV progressors was the presence of proliferating CD8+ T cells expressing perforin (1). A similar phenomenon of proliferating cytotoxic HIV-specific CD8+ T cells was observed in the single individual who controlled replication of HIV during postvaccinal exposure to the virus in an experimental HIV vaccine study group (41). Altogether, these observations point to a potentially important role for non-IFN-γ-producing CD8+ T cell for control of HIV. Whether these cells may produce other cytokines or noncytolytic antiviral factors that can block HIV-1 infection and possibly replication remains to be determined. In HIV-infected patients, it has been shown that Mip-1β and not IFN-γ dominates the HIV-specific CD8+ T cell response (4, 42). It would be interesting to determine whether non-IFN-γ-producing CD8+ T cell may decrease the susceptibility of activated CD4+ T cells to in vitro infection.

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Disclosures

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References


