Coimmunization with an Optimized IL-15 Plasmid Results in Enhanced Function and Longevity of CD8 T Cells That Are Partially Independent of CD4 T Cell Help

Michele A. Kutzler, Tara M. Robinson, Michael A. Chattergoon, Daniel K. Choo, Andrew Y. Choo, Philip Y. Choe, Mathura P. Ramanathan, Rose Parkinson, Sagar Kudchodkar, Yutaka Tamura, Maninder Sidhu, Vidia Roopchand, J. Joseph Kim, George N. Pavlakis, Barbara K. Felber, Thomas A. Waldmann, Jean D. Boyer and David B. Weiner

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Coimmunization with an Optimized IL-15 Plasmid Results in Enhanced Function and Longevity of CD8 T Cells That Are Partially Independent of CD4 T Cell Help


DNA vaccines are a promising technology for the induction of Ag-specific immune responses, and much recent attention has gone into improving their immune potency. In this study we test the feasibility of delivering a plasmid encoding IL-15 as a DNA vaccine adjuvant for the induction of improved Ag-specific CD8⁺ T cell immune responses. Because native IL-15 is poorly expressed, we used PCR-based strategies to develop an optimized construct that expresses 80-fold higher than the native IL-15 construct. Using a DNA vaccination model, we determined that immunization with optimized IL-15 in combination with HIV-1 gag DNA constructs resulted in a significant enhancement of Ag-specific CD8⁺ T cell proliferation and IFN-γ secretion, and strong induction of long-lived CD8⁺ T cell responses. In an influenza DNA vaccine model, coimmunization with plasmid expressing influenza A PR8/34 hemagglutinin with the optimized IL-15 plasmid generated improved long term CD8⁺ T cell immunity and protected the mice against a lethal mucosal challenge with influenza virus. Because we observed that IL-15 appeared to mostly adjuvant CD8⁺ T cell function, we show that in the partial, but not total, absence of CD4⁺ T cell help, plasmid-delivered IL-15 could restore CD8 secondary immune responses to an antigenic DNA plasmid, supporting the idea that the effects of IL-15 on CD8⁺ T cell expansion require the presence of low levels of CD4 T cells. These data suggest a role for enhanced plasmid IL-15 as a candidate adjuvant for vaccine or immunotherapeutic studies. The Journal of Immunology, 2005, 175: 112–123.

The generation of functional cellular immunity that results in a long-lasting memory protective immune response upon pathogen rechallenge is the goal of vaccination. In a model of acute infection, the quality of memory CD8⁺ T cell response depends on help from CD4⁺ T cells (1–14). In the presence of CD4⁺ T cells, memory CD8⁺ T cells rapidly begin to proliferate and undergo further differentiation after a second encounter with Ag, resulting in enhanced effector responses, including secretion of cytotoxic molecules and the antiviral cytokines IFN-γ and TNF-α. A recent study by Sun et al. (15) investigated at what stage during the immune response to pathogen are CD8⁺ T cells essential in the promotion of functional CD8⁺ T cell memory in a model of acute infection. Adoptive transfer of effector or memory CD8⁺ T cells into wild-type or CD4⁺ T cell-deficient mice demonstrated that the presence of CD4⁺ T cells was important only after, not during, the early CD8⁺ T cell-programming phase. In the absence of CD4⁺ T cells, memory CD8⁺ T cells became functionally impaired and decreased in quantity over time (15). This study demonstrates that in the context of an acute infection, CD4⁺ T cells are required only during the maintenance phase of long-lived memory CD8⁺ T cells. However, unlike acute infection, the role of CD4 T cells in the priming of functional CD8⁺ T cell effector and memory cells in a model of DNA vaccination is unknown.

It is believed that CD8⁺ T cell responses are important for controlling HIV-1 infection and slowing disease progression. Although the exact function of HIV-1-specific CD8⁺ T cells in this process has not been completely elucidated, a correlation has been established between long term nonprogression of HIV-1 in seropositive individuals and HIV-1-specific CD8⁺ T cell-mediated cellular responses (4, 16–19). The loss of CD4⁺ T cell function during chronic HIV infection can have deleterious consequences on the ability of HIV-1-specific CD8⁺ T cells to maintain effector T cell activity and control virus. This suggests that understanding the factors that supplement CD4⁺ T cell function and that support CD8 T cell function could be important for HIV immune therapy strategies where CD4 help is compromised. Accordingly, generation of vaccines, including DNA vaccines, that encode their own forms of T cell help would be worthy of investigation.

*Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104; †Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322; ‡Program for Biological and Biomedical Science, Harvard Medical School, Boston, MA 02115; §National Institute of Advanced Industrial Science and Technology, Sapporo, Japan; #Vaccine Discovery, Wyeth Lederle, Pearl River, NY 10965; ¶Viral Genomix, Bluebell, PA 19422; and ‖Basic Research Laboratory and **Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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2 Address correspondence and reprint requests to Dr. David B. Weiner, University of Pennsylvania, 422 Curie Boulevard, Room 505 Stellar Chance Laboratories, Philadelphia, PA 19104-6100. E-mail address: dbweiner@mail.med.upenn.edu

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produced cytokines (20–22). One such cytokine that appears to have a significant impact on CD8+ T cells is IL-15 (23–26). In the context of immune therapy, genetic vaccines, IL-15 has been shown to be effective against tumors as well as some disease models (24, 27–37). IL-15 has been reported to support memory CD8+ T cell proliferation (38) and specifically induce the proliferation of CD44high or CD122high memory CD8+ T cells (27, 39, 40). Moreover, it has been shown that there is a lack of memory phenotype CD8+ T cells in IL-15- and IL-15-deficient mice (41). In addition, Ab-mediated blockade of IL-2/IL-15Rβ plays the inhibitory role in the proliferation of memory phenotype CD8+ T cells in vivo (22). Taken together, these data suggest that IL-15 plays an essential role in the proliferation of memory CD8+ T cells. We hypothesize that IL-15 may act to enhance the productive CD8+ T cell memory pool induced in the context of an HIV DNA vaccine.

To test an effective IL-15 plasmid for vaccination that secretes enhanced levels of IL-15, several obstacles had to be overcome. The regulation of IL-15 is complex, with multifaceted controls at the levels of message transcription, message translation, and protein translocation and secretion (42, 43). IL-15 contains two alternative leader peptides, one with 48 aa (long signal peptide (LSP)3) and one with 21 aa (short signal peptide (SSP)) (44, 45). The classical LSP form of IL-15 is associated with all secreted IL-15, whereas the SSP form of IL-15 is not secreted, but, rather, is stored intracellularly. IL-15 mRNA includes a number of elements that impede its translation into protein. Bamford et al. (46) showed that by replacing the leader with the signal peptide-coding sequence from IL-2, the sum of IL-15 retained and secreted increased 17- to 20-fold with the IL-2 leader. Conversely, by replacing the leader on IL-2 with that of IL-15 SSP, the quantity of IL-2 secreted was reduced 40- to 50-fold. Furthermore, in a similar study conducted by Onu et al. (45), replacing the SSP of IL-15 with the signal peptide from CD33 resulted in increased levels of translation and secretion of IL-15 protein. It has yet to be determined whether an optimized form of IL-15, modified through PCR strategies to remove negative regulatory features resulting in enhanced protein secretion, can influence immunogenicity in vivo in a model of DNA vaccination.

In this report, through PCR-based strategies, we have successfully optimized and enhanced the production and secretion of a biologically active IL-15 in a DNA plasmid (pIL-15-Opt). The optimized pIL-15-Opt acts as a potent adjuvant for HIV-1gag DNA vaccines by significantly enhancing Ag-specific CD8+ T cell effector function and proliferation. To test the physiological importance of the enhanced CD8+ T cell immune responses using pIL-15-Opt, we used an influenza mucosal challenge model in rodents (47). The cloning of the human IL-15 construct (sequence from GenBank, accession no. NM005855) into pVAX1 cloning vector (Invitrogen Life Technologies) was conducted by PCR amplification with the following 5’ to 3’ primers: pIL-15-SSP sense, GCCCCGTGACGCGCCGCCACCATGGAATTCACTTTGGT; pIL-15-SSP antisense, ATCCGGGCTCGATCAAGAATGTTGAGAATTTGG; pIL-15-Opt sense, GCCCCCGTGACGCGCCGACCATGGAATTCACTTTGGT; and pIL-15-Opt antisense, GCACCATGCCGCCGACCATGGAATTCACTTTGGT. PCR conditions for all reactions were one cycle at 94°C for 1 min; 30 cycles at 94°C for 1 min, 55°C for 1 min and 15 s, and 72°C for 1 min and 15 s; followed by a final extension at 72°C for 10 min. The 405 bp (SSP), 486 bp (LSP), and 396 bp (Opt) PCR product was ligated into pVAX1 cloning vector after a restriction enzyme digestion using EcoRI (New England Biolabs) and XhoI (New England Biolabs), which were designed into the PCR primers and are in the multiple cloning region of the vectors. All positive clones were verified by sequence analysis.

**Materials and Methods**

**DNA plasmids**

DNA vaccine constructs expressing HIV-1gag were prepared as described previously (47). The cloning of the human IL-15 construct (sequence from GenBank, accession no. NM005855) into pVAX1 cloning vector (Invitrogen Life Technologies) was conducted by PCR amplification with the following 5’ to 3’ primers: pIL-15-SSP sense, GCCCCGTGACGCGCCGCCACCATGGAATTCACTTTGGT; pIL-15-SSP antisense, ATCCGGGCTCGATCAAGAATGTTGAGAATTTGG; pIL-15-Opt sense, GCCCCCGTGACGCGCCGACCATGGAATTCACTTTGGT; and pIL-15-Opt antisense, GCACCATGCCGCCGACCATGGAATTCACTTTGGT. PCR conditions for all reactions were one cycle at 94°C for 1 min; 30 cycles at 94°C for 1 min, 55°C for 1 min and 15 s, and 72°C for 1 min and 15 s; followed by a final extension at 72°C for 10 min. The 405 bp (SSP), 486 bp (LSP), and 396 bp (Opt) PCR product was ligated into pVAX1 cloning vector after a restriction enzyme digestion using EcoRI (New England Biolabs) and XhoI (New England Biolabs), which were designed into the PCR primers and are in the multiple cloning region of the vectors. All positive clones were verified by sequence analysis.

**In vitro transfection**

Expression levels of the plasmid construct were tested after transient transfection of RD cells (American Type Culture Collection). Cells were plated in six-well tissue culture dishes at a density of 2 × 105 cells/well in complete DMEM plus 10% FBS (Invitrogen Life Technologies) and allowed to adhere overnight. The next day cells were transfected with IL-15 plasmid (1 μg/well) using FuGene 6 transfection reagent (Roche) according to the manufacturer’s protocol. After 48 h, cell supernatants were harvested and analyzed for the presence of human IL-15 protein by commercial ELISA kits (R&D Systems).

**IL-15 functional assay**

The MTT cell proliferation assay, according to the manufacturer’s recommendation (American Type Culture Collection), was used to assess the functional activity of secreted IL-15. CTL2-2 cells obtained from American Type Culture Collection were grown and maintained in RPMI 1640 (Invitrogen Life Technologies) complete medium containing 5% supernatant from Con A-stimulated rat spleen cells (BD Biosciences) to supplement IL-2, which is required for cell growth. At the time of the assay, cells were centrifuged and washed five times with RPMI 1640 complete without any Con A supernatant to remove residual IL-2. Cells were plated at a concentration of 5 × 104 cells/well in 96-well, flat-bottom polystyrene dishes. Standard human IL-15 (R&D Systems) as well as the supernatants from transfected cells to be assessed for the capacity to induce CTL2-2 proliferation were added to each well, and incubation was continued for 48 h at 37°C. At this time, 10 μl of MTT reagent was added, and incubation was continued for another 2 h. Readings at 570 nm absorbance from the wells containing the standard IL-15 sample (picograms per milliliter of standard IL-15) were used to make a standard curve, which was then used to estimate the activity of the unknown samples and depicted as bioactivity (picograms per milliliter).

**Indirect immunofluorescent assay**

The indirect immunofluorescent assay for monitoring IL-15 expression constructs was conducted by the following protocol (48). HeLa cells grown in slide chambers (BD Biosciences) were transfected with IL-15 constructs using DOTAP transfection agent (Roche). Forty-eight hours after transfection, the cells were washed twice with cold PBS and fixed on slides using methanol for 15 min. Upon removal of the residual solvents from the slides, the cells were incubated with anti-mouse human IL-15 (BD Pharmingen) for 90 min. The slides were incubated with PE-conjugated secondary Ab (Roche) for 45 min. 4’,6-Diamido-2-phenylindole hydrochloride (Sigma-Aldrich) was added to the solution of secondary Ab to counterstain the nuclei to show the nuclei of the total number of cells available in the given field. The slides were incubated containing antifading reagent (Molecular Probes). The images were analyzed using the Phase 3 Image Pro program for fluorescent microscopy (Media Cybernetics).

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3 Abbreviations used in this paper: LSP, long signal peptide; HA, hemagglutinin; KO, knockout; Opt, optimized leader sequence; pHA, plasmid HA; mae, macrophages; p.i., postinfection; SFC, spot-forming cell; SI, stimulation index; SSP, short signal peptide.
Plasmid immunization and mice

The quadriceps muscle of 6- to 8-wk-old female BALB/c (The Jackson Laboratory) were injected two or three times, each separated by 2 wk (47). We obtained the CD4 knockout (CD4KO) mice as a gift from Dr. R. Eisenberg (University of Pennsylvania, Philadelphia, PA; B6.129S6-Cd4tm1Knw/J obtained from The Jackson Laboratory). CD4KO (B6.129S6-Cd4tm1Knw/J) mice have a significant block in CD4 T cell development, >90% circulating T cells are CD8\(^+\), and manifest a class II-restricted deficit in Th cell activity. We obtained the inbred parental strain C57BL/6J mice from The Jackson Laboratory. For all experiments in mice, the animals were immunized with either 50 or 100 \(\mu\)g of HIV-1 antigenic plasmids, IL-15 constructs, or a vector control in 0.25% bupivicaine-HCl (Sigma-Aldrich) in isotonic citrate buffer as designated in the figures (47). Coadministration of various gene plasmids involved mixing the designated DNA plasmids before injection in a final volume of 100 \(\mu\)l (47). All DNA was made using Qiagen columns (endotoxin free) (47). All animals were housed in a temperature-controlled, light-cycled facility at the University of Pennsylvania, and they were cared for under the guidelines of the National Institutes of Health and the University of Pennsylvania.

Splenocyte purification

Spleens from each mouse were harvested and pooled in a 15-ml conical containing RPMI 1640 (one tube for each experimental group) (47). In a sterile tissue culture hood, the pooled spleens from each experimental group were placed in a petri dish and crushed using the plunger from a 3-ml syringe. The cells were then incubated for 5–10 min at room temperature in RBC lysing solution. The splenocytes were washed and put through a 70-\(\mu\)m cell strainer to remove any remaining spleen organ stroma. The splenocytes were washed in RPMI 1640 twice, resuspended in R10 medium (RPMI 1640 plus 10% FBS), and counted (cell viability is determined using trypan blue stain) using a hemocytometer.

**T cell proliferation assay**

A T cell proliferation assay was conducted as previously described (47). Briefly, splenocytes (5 \(\times\) 10\(^5\) cell) were added to each well of a 96-well, microtiter, flat-bottom plate and stimulated in the presence of recombinant p24 protein (Immunodiagnostics) in triplicate, resulting in final concentrations of 5 and 1 \(\mu\)g/ml. The cells were incubated at 37°C in 5% CO\(_2\) for 3 days. One microcurie of tritiated thymidine was added to each well, and the cells were incubated for an additional 12–18 h at 37°C. Plates were harvested, and the amount of incorporated tritiated thymidine was measured in a \(\beta\) Plate reader (Wallac). The stimulation index (SI) was determined from the formula: SI = (experimental count/spontaneous count). Spontaneous count wells included 10% FCS, which served as an irrelevant protein control. Similarly, splenocytes from pHIV-1gag- or control-immunized mice routinely have an SI of 1 against an irrelevant protein target (47). To assure that cells were healthy, PHA (Sigma-Aldrich) or Con A (Sigma-Aldrich) was used as a polyclonal stimulator positive control.

**ELISPOT assay**

An ELISPOT assay was conducted as previously described (49). Briefly, ELISPOT 96-well plates (Millipore) were coated with anti-mouse IFN-\(\gamma\) capture Ab and incubated for 24 h at 4°C (R&D Systems), and the following day, the plates were washed and blocked for 2 h with 1% BSA. Two hundred thousand splenocytes from the immunized mice were added to each well and stimulated overnight at 37°C in 5% CO\(_2\) in the presence of RPMI 1640 (negative control), Con A (positive control), or specific peptide (HIV-1gag, p55) Ags (10 \(\mu\)g/ml), HIV-1gag 15-mer peptides spanning the entire HIV-1gag consensus clade B subtype, overlapping by 11 aa, were provided by the AIDS Reagent and Reference Repository. For the ELISPOT assays, p55 peptide mix was used in which all 15-mer peptides were mixed together and added to splenocytes at a 10 \(\mu\)g/ml final concentration. For the epitope-mapping studies, a matrix format was used in which each
FIGURE 2. Comparison of two native isoforms and optimized IL-15 protein levels and functional activity after transfection with plasmid. A, Expression levels of plasmid constructs were tested using transiently transfected RD cells as described in Materials and Methods. Cells were transfected with pIL-15-SSP ( ), pIL-15-LSP ( ), or pIL-15-Opt ( ) using FuGene 6 transfection reagent. After 48 h, cell supernatants were harvested and analyzed for the presence of secreted IL-15 protein by ELISA. These data are representative of six independent experiments. B, Comparison of pIL-15-SSP ( ), pIL-15-LSP ( ), and pIL-15-Opt ( ) in an MTT cell proliferation assay. Standard human IL-15 as well as the supernatants from transfected cells to be assessed for the capacity to induce CTLL-2 proliferation were added to the CTLL-2 cells and incubated for 48 h at 37°C. Readings from the wells containing the standard IL-15 sample were used to obtain a standard curve, which was then used to determine the bioactivity of the unknown samples (shown as picograms per milliliters). These data are representative of three independent experiments.

CD8+ T cell depletion for lymphoproliferation and ELISPOT assays

Immunomagnetic cell separation using Dynabeads (Dynal Biotech) mouse CD8 (clone Lyt2) was used as the method for separation of Lyt2-positive cells, resulting in 90% depletion in 20 min using 1 × 107 beads/ml for 2.5 × 107 splenocytes. Depletion was conducted as described by the manufacturer. Briefly, the Dynabead mouse CD8 suspension was added directly to the cell suspension, mixed thoroughly, and incubated for 2 h at room temperature. The plate was washed, and 5-bromo-4-chloro-3′-indolyl phosphate p-toluidine salt and nitro blue tetrazolium chloride (chromogen color reagent; R&D Systems) were added to each well. The plate was then rinsed with distilled water and dried at room temperature. Spots were counted by an automated ELISPOT reader (CTL Limited).

CD4+ T cell depletion of BALB/c mice

Mice were given 0.1 mg of rat anti-mouse CD4 mAb i.p. (BD Pharmingen; clone GK1.5) diluted to 1 mg/ml in sterile PBS. The Ab was delivered by i.p. injection on days −1, 6, 13, and day 20 of the study. Flow cytometric analysis was performed to verify the percentage of CD4+ cells in the periphery on days 0, 1, 3, 5, 7, 8, 11, 18, and 21 postinjection (see Fig. 9B).

Statistical analysis

Data are presented as the mean ± SEM calculated from triplicate wells of pooled splenocytes from each experimental group. Where appropriate, the statistical difference between immunization groups was assessed by using
Student’s t test and yielded a specific p value for each experimental group. Comparisons between samples with a value of p < 0.05 were considered statistically different and therefore significant. A Kaplan-Meier survival analysis was used to depict results for the influenza challenge study, and statistical differences between vaccination groups were determined to be statistically different using a log-rank test.

Results

Cloning of IL-15 into pVAX1 vector and IL-15 expression analysis

Native IL-15 contains two alternative leader peptides that are not only involved in the regulation of IL-15 translation, but also direct its intracellular trafficking. An amino acid alignment showing the signal peptides and mature IL-15 for the two native IL-15 isoforms (SSP and LSP) as well as the optimized IL-15 isoform (Opt) is shown in Fig. 1A. The classical long (48-aa) signal peptide is associated with all secreted forms of IL-15, whereas IL-15, which contains the short 21-aa signal peptide, is not secreted, but, rather, is stored intracellularly (44, 45, 52, 53). Interestingly, the secreted form of IL-15, which contains the unusually long 48-aa signal peptide, was shown to act as a negative regulatory feature by Bamford et al. (46) and Onu et al. (45). Taken together, these data suggested that to design a plasmid form of IL-15 that secretes at high levels, part of our optimization strategy would require replacing the LSP with an optimized leader designed by our laboratory (54).

In addition to the negative regulatory feature of IL-15 signal peptides, IL-15 mRNA includes a number of other elements that impede its translation, including 5’-upstream AUGs, a start codon for the IL-15-coding sequence that has a weak Kozak context (GTAATGA), and the presence of a cis-acting negative element in the C terminus of the IL-15 mature protein-coding sequence (43). Because our vaccination strategy entails harnessing of the potent effects of IL-15 on immune function, we also optimized the IL-15 construct by targeting these other negative regulatory features through a PCR strategy. The strategy entailed the insertion/replacement of the existing Kozak context with a stronger Kozak context (CCTAAAG), and we selected positive clones that contained a 405-, 486-, or 396-bp insert for the SSP, LSP, or Opt construct, respectively, and confirmed the presence of constitutively active promoter (Fig. 1C). We selected positive clones that contained a 405-, 486-, or 396-bp insert for the SSP, LSP, or Opt construct, respectively, and confirmed the presence of the engineered forms of IL-15 by sequence analysis.

To address whether these constructs express IL-15 protein, we conducted a transfection assay in RD cells (Fig. 2). We observed a substantial increase in protein secreted from the optimized construct by specific ELISA analysis, in that pIL-15-Opt produced 13,955 pg/ml, whereas pIL-15-SSP and pIL-15-LSP secreted lower levels (Fig. 2A; 160 and 2436 pg/ml, respectively). These data illustrate that the pIL-15-Opt construct expresses 87-fold higher than the native IL-15, and 5.7-fold higher than the IL-15-LSP construct. Moreover, supernatants from pIL-15-SSP-, pIL-15-LSP-, or pIL-15-Opt-transfected RD cells were tested for the capacity to induce CTL2-2 proliferation (IL-15 bioactivity; picograms per milliliter) in a bioassay (Fig. 2B). Specifically, supernatants from the pIL-15-Opt-transfected RD cells induced 4.5-fold higher functional IL-15 than the native IL-15-LSP construct (bioactivity, 45,160 vs 10,035 pg/ml). To confirm reports that the
FIGURE 6. pIL-15-Opt augments the magnitude of Ag-specific effector CD8\(^+\) T cell epitopes, but not the breadth of the response. Splenocytes harvested from pIL-15-Opt/pHIV-1gag-vaccinated (□) and pHIV-1gag-vaccinated (●) animals were used in an IFN-γ ELISPOT assay. CD8\(^+\) T cell epitopes were mapped using a matrix format of 15-mer peptides overlapping by 11 aa spanning the HIV-1gag protein as described in Materials and Methods. A dominant epitope, AMQMLKETINEEAE, and a subdominant epitope, VDRFYKTLRAEQASQ, were identified.

IL-15-SSP isoform is not secreted, but, rather, is stored intracellularly, appearing in nuclear components, whereas the IL-15-LSP isoform and optimized IL-15 are associated with secreted IL-15, we conducted immunofluorescent staining of transfected cells using Abs specific for IL-15, as described in Materials and Methods (Fig. 3). As shown in Fig. 3, A, D, G, and J, nuclear regions of transfected cells can be visualized with 4',6-diamido-2-phenylindole hydrochloride stain. Using a PE-labeled IL-15 Ab, Fig. 3, B and C, show staining of the IL-15-LSP isoform that is retained in the nuclear region of the cell. Furthermore, we consistently observed staining in the cytoplasmic region of cells transfected with either IL-15-LSP (Fig. 3, E and F) or IL-15-Opt (Fig. 3, H and I). Control, mock-transfected cells did not show any IL-15 stain (Fig. 3, K and L). These data support in vitro expression studies and demonstrated the negative regulatory features of the IL-15-LSP isoform. Based on expression studies, we next tested the in vivo effects of optimized IL-15.

Optimized pIL-15 augments CD8\(^+\) T cell proliferation and Ag-specific IFN-γ secretion

We examined the effects of pIL-15-SSP, -LSP, and -Opt plasmid coimmunization on CD8\(^+\) T cell function. In the lymphocyte proliferation assay, pIL-15-SSP did not have a dramatic impact (2-fold increase) on the resulting proliferative responses, whereas splenocyte proliferation from mice coinjected with pIL-15-LSP resulted in stimulation indices that were at least 2.5-fold higher than those of mice immunized with pHIV-1gag alone (Fig. 4A). Interestingly, coinjection with pIL-15-Opt resulted in 6.5-fold higher SI (Fig. 4A). T cells from animals vaccinated with pHIV-1gag plasmid alone resulted in proliferation of CD4\(^+\) T cells (Fig. 4A), because after CD8\(^+\) T cell depletion, the SI was not statistically different from those of proliferating splenocytes before depletion (Fig. 4B). In addition, these data show that pIL-15 induces proliferation of CD8\(^+\) T cells, because depletion of these cells from the splenocyte culture results in abrogation of thymidine incorporation, particularly from the pIL-15-coimmunized groups (Fig. 4B).

We next examined Ag-specific IFN-γ secretion from CD8\(^+\) T cells by a peptide-based, quantitative, ELISPOT assay from immunized BALB/c mice that were rested 1 wk after their last coinjection of pIL-15 constructs and pHIV-1gag (Fig. 5). The ELISPOT assay measures the frequency of Ag-specific CD8\(^+\) T cells present in the spleen of vaccinated animals at the time of peptide (HIV-1p55 peptides are from a pool of 15-mer overlapping by 11 aa) Ag stimulation. Because restimulation with peptide Ag is for only 24 h, the IFN-γ secreted during the ELISPOT assay is assumed to be from existing memory and Ag-specific effector T cells, not naïve T cells. The strongest Ag-specific IFN-γ was detected in the pIL-15-Opt coimmunization group (656.3 spot-forming cells (SFC)), whereas modest increases in Ag-specific IFN-γ were observed after coimmunization with pIL-15-SSP (213.3 SFC) or pIL-15-LSP (360 SFC). Coinjection with the optimized IL-15 construct and HIV-1gag construct resulted in 3.3-fold higher IFN-γ SFC/million splenocytes than pHIV-1gag vaccination alone. Depletion of CD8\(^+\) T cells from the splenocytes before Ag-specific stimulation and ELISPOT analysis resulted in loss of IFN-γ activity, showing pIL-15-Opt results in significant enhancement of Ag-specific CD8\(^+\) T cell IFN-γ secretion (Fig. 5, □). Furthermore, in bulk CTL assays, coinjection of plasmids expressing HIV-1 envelope and IL-15 resulted in nearly 40% lysis of HIV-1 envelope-expressing targets at a 50:1 E:T cell ratio compared with 11% lysis observed with envelope plasmid and control vector (data not shown), illustrating the enhanced effector function of CD8\(^+\) T cells by this immunization strategy.

pIL-15-Opt augments the magnitude of Ag-specific effector CD8\(^+\) T cell epitopes, but not the breadth of the response

To address whether the adjuvanting effect of IL-15 on the CD8\(^+\) T cell response was due to an increase in the number of responding CD8\(^+\) T cell epitopes or whether pIL-15-Opt augmented the response to the vaccine-induced epitopes, we mapped the CD8 responses induced by measuring IFN-γ using an ELISPOT assay with an HIV-1gag overlapping peptide matrix as described in Materials and Methods. From these experiments, two epitopes were identified. The dominant epitope was mapped to gag aa 197–211 (AMQMLKETINEEAE; Fig. 6). Mata et al. (55) have previously defined AMQMLKETI as the dominant CD8\(^+\) T cell epitope after immunization with a recombinant Listeria monocytogenes HIV-1 vaccine in the BALB/c H-2\(^d\) haplotype. We were able to further define a subdominant epitope, gag aa 293–307 (VDRFYKTLRAEQASQ; Fig. 6). We did not observe an increase in the breadth of the response, because both dominant and subdominant CD8\(^+\) T epitopes were observed in pHIV-1 gag and pIL-15-Opt/pHIV-1gag vaccination groups. However, IL-15 dramatically expanded the magnitude of the responses to these epitopes. In fact, only after coimmunization with pIL-15-Opt was the subdominant epitope clearly evident. These data suggest that plasmid-delivered IL-15 improved the expansion of effector CD8\(^+\) T cells and can bolster the response to weak epitopes, but is probably not effective at driving responses to previously unrecognized epitopes.

Coimmunization with pIL-15-Opt and pHIV-1gag constructs results in enhanced Ag-specific, long-lived T cell secretion of IFN-γ 5 wk after final injection

To analyze the effect of IL-15 on long-lived CD8\(^+\) T cells, we measured Ag-specific IFN-γ secretion by ELISPOT assay from
The immunization schedule is shown in A. Splenocytes from vaccinated animals were cultured overnight in the presence of medium (■; negative control) or 10 μg/ml HIV-1 p55 peptide mix (▲; HIV-1 gag-specific 15-mer, overlapping by 11 aa). IFN-γ SFC were quantified by an automated ELISPOT reader, and the raw values were normalized to SFC per million splenocytes. Values represent the mean (±SE) of the triplicate cultures and are representative of three independent experiments. * , p < 0.05.

**FIGURE 7.** pIL-15-Opt and pHIV-1gag coimmunization results in enhancement of CD8+ T cell longevity. The immunization schedule is shown in A. B. Splenocytes were harvested from naïve, vector control, and pHIV-1gag and pHIV-1gag/pIL-15-Opt and applied in an IFN-γ ELISPOT assay, as described in Materials and Methods. Splenocytes from vaccinated animals were cultured overnight in the presence of medium (■; negative control) or 10 μg/ml HIV-1 p55 peptide mix (▲; HIV-1 gag-specific 15-mer, overlapping by 11 aa). IFN-γ SFC were quantified by an automated ELISPOT reader, and the raw values were normalized to SFC per million splenocytes. Values represent the mean (±SE) of the triplicate cultures and are representative of three independent experiments. * , p < 0.05.

In vivo studies show Ag-specific IFN-γ secretion by CD8+ T cells after DNA vaccination requires CD4+ T cells during priming.

The loss of CD4+ T cell function during chronic HIV infection is well characterized. The generation of a CD8+ T cell immune response that can in part bypass the need for CD4+ T cell help may be useful in contributing to vaccine control of HIV-1 infection. We sought to determine the requirement for CD4+ T cell help during priming of a CD8+ T cell response to DNA-delivered antigenic plasmids in a model of CD4+ T cell depletion (Fig. 9C) as well as in a CD4KO model (Fig. 9D). As shown in Fig. 9A, BALB/c mice were depleted of CD4+ T cells by injecting an anti-CD4 Ab i.p. before coimmunization with pIL-15-Opt and pHIV-1gag. We monitored CD4+ T cell levels in the periphery of these mice by flow cytometry and observed that the CD4+ T cell levels were kept below 5% for the duration of the study (Fig. 9B). In control BALB/c mice that maintain CD4+ T cell help during the priming stage, immunization with a pHIV-1gag plasmid resulted in Ag-specific IFN-γ secretion. In contrast, CD4+ T cell-depleted mice that were immunized with pHIV-1gag alone resulted in no induction of IFN-γ secretion (Fig. 9C).

To study the requirement for CD4+ T cells in the generation of CD8+ T effectors in a model of DNA Ags, we repeated these studies in a CD4KO model and the parental strain controls (Fig. 9D). Immunization of the CD4KO parental strain, C57BL/6f mice, with pHIV-1gag plasmid consistently resulted in lower IFN-γ levels secreted by Ag-specific splenocytes compared with BALB/c mice (43 SFC; Fig. 9D). Although close to background levels, modest increases in Ag-specific IFN-γ protein after pHIV-1gag immunization were observed in the parental strain (43 SFC; Fig. 9D). However, our data show that in the complete absence of CD4+ T cells, splenocytes from CD4KO mice that were immunized with pHIV-1gag alone were unable to generate Ag-specific CD8+ T effector cells (Fig. 9D). These data support the need for CD4+ T cells during priming in the generation of functional CD8+ effector T cells for DNA vaccines.
pIL-15-Opt lowers the requirement and is partially able to replace CD4 T cell help in the generation of functional CD8 T cells

Coimmunization of pIL-15-Opt and pHIV-1gag plasmids in BALB/c mice that maintain CD4⁺ T cell help during priming resulted in a 2-fold enhancement of Ag-specific IFN-γ over pHIV-1gag plasmid immunization alone (Fig. 10A). In contrast with the control group, after coimmunization of CD4 T cell-depleted mice with pIL-15-Opt and pHIV-1gag, we observed the presence of Ag-specific immune responses roughly equal to that after pHIV-1gag immunization in control, non-CD4 T cell-depleted animals (Fig. 10A). These data support the idea that pIL-15 can at least partially bypass the significant restriction of CD4 T cells in the generation of functional CD8⁺ T cell immunity in a model of DNA vaccination.

Immunization of the CD4 KO parental strain, C57BL/6J mice, with pHIV-1gag and pIL-15 enhanced (2.5-fold increase) IFN-γ secretion by control parental strain animals (Fig. 10B). CD4KO mice coimmunized with pHIV-1gag and pIL-15-Opt exhibited modest levels of CD8⁺ T effector cell responses (32 IFN-γ SFC/ million splenocytes), although coimmunization brought levels close to those observed in the pHIV-1gag group, parental strain (Fig. 10B). Taken together, these data support the need for CD4 T cells during priming of CD8⁺ T cell responses to DNA vaccine-delivered Ags, and our data indicate that in the complete absence of CD4⁺ T cells, plasmid-delivered IL-15 in vivo may restore CD8⁺ T cell function to low levels, but in the partial presence of CD4⁺ T cells, IL-15 can augment CD8⁺ T cell immunity to normal levels.

Discussion

Vaccines require the induction of functional Ag-specific cellular immunity that results in immunological T cell memory. For some pathogens, generation of Ag-specific CD8⁺ T cells is important, and several important studies of acute infection models have shown that the quality of the memory CD8⁺ T cell response depends on help from CD4⁺ T cells (9). CD4⁺ T cells are important for priming DCs to initiate CD8⁺ T cell (56) to help maintain memory T cells (57) and are important in the maturation of CD8⁺ T cells (58). In the context of HIV-1 infection, the loss of CD4⁺ T cell function during chronic HIV-1 infection may result in deficits of HIV-1-specific CD8⁺ T cells, which are necessary to maintain strong effector responses and productively impact virus. It is probably important to consider strategies to augment CD4⁺ T cell help in this context. This is the first report to show the requirement for CD4⁺ T cells during priming in the generation of functional CD8⁺ effector T cells in an in vivo DNA vaccination model, and the use of a cytokine immunomodulatory adjuvant to an HIV-1gag DNA vaccine that can provide helper support for the formation of memory CD8⁺ T cells in a model of partial CD4⁺ T cell depletion.

It has been determined that IL-15 plays an important role in the control of all phases of T cell differentiation into memory cells (initiation, clonal expansion, contraction, and maintenance). Moreover, IL-15 is involved in the activation of dendritic cells (59), the proliferation of Ag-specific T cells (38, 41, 60), the rescue of T cells from death during the contraction phase of the immune response (41, 61), and the proliferation/maintenance of memory T cells (41, 62). These data suggest that IL-15 may have an important role in the development of a long-lived, Ag-specific memory CD8⁺ T cell pool, which is probably important for both therapeutic and prophylactic vaccines. In a recent study, Oh et al. (31) evaluated coadministration of HIV vaccines with vaccinia viruses expressing either IL-2 or IL-15. Both cytokines enhanced long-lasting CD4⁺ T cell immunity. However, coadministration of HIV-1 vaccine vectors with vaccinia expressing IL-15 induced strong humoral and short term cytotoxic T cell responses as well as long term CD8⁺ T cell immunity that was Ag specific. The production of IL-15 is tightly controlled at the level of translation and secretion. Bamford et al. (46) showed that by replacing the leader with the signal peptide-coding sequence from IL-2, the sum of IL-15 retained and secreted increased 17- to 20-fold with the IL-2 leader. Conversely, by replacing the leader

![FIGURE 8. pIL-15-Opt induces a memory T cell response that protects mice from a lethal influenza challenge. The immunization schedule is shown in A. B. The total decrease in body weight was measured 0–9 days p.i. in naive ( ), pHAV ( ), and pHA/pIL-15-Opt ( ) mice. C. Survival was measured 0–12 days p.i. in naive ( ), pHAV ( ), and pHA/pIL-15-Opt ( ) mice and is shown as percentage survival.](http://www.jimmunol.org/content/119/3/1132/F1.large.jpg)
on IL-2 with that of IL-15 LSP, the quantity of IL-2 secreted was reduced 40- to 50-fold (46). Furthermore, in a similar study conducted by Onu et al. (45), replacing the LSP of IL-15 with the signal peptide from CD33 resulted in increased levels of translation and secretion of IL-15 protein. We successfully used several PCR-based strategies to develop an optimized construct that expresses higher than native IL-15 constructs. We demonstrated that the pIL-15-Opt construct expressed 87-fold more than the native IL-15, and 5.7-fold more than the IL-15-LSP construct.

Several pieces of data presented in this study support the idea that optimized plasmid-delivered IL-15 enhances CD8+ T cell function and proliferation as well as the notion that CD8+ T cells mediated protection observed with pIL15-Opt coinjection. These data demonstrate the ability of IL-15 to regulate CD8+ T cell function and suggest that pIL-15-Opt can be used as an immunoadjuvant for CD8+ T cell memory. The generation of a CD8+ T cell immune response that can in part bypass the need for CD4+ T cell help may be useful in contributing to vaccine control of HIV-1 infection. pHIV-1gag vaccination of animals depleted of 95% CD4+ T cells resulted in lower numbers of IFN-γ SFC; this supports the idea that the low numbers of CD4+ T cells were not able to provide a significant level of help to generate HIV-1gag-specific CD8+ effector T cells in a model in which Ags are delivered in the form of DNA plasmids in vivo. In contrast, coinmunization with
pIL-15 and pHIV-1gag was able to significantly rescue the function of CD8\(^+\) T effector cells in these two mouse models of CD4 depletion. The mechanism by which IL-15 provides help in the partial absence of CD4 T cells is still unknown, but we hypothesize that IL-15 may directly activate APCs, leading to the secretion of IL-12, which subsequently induces IFN-\(\gamma\). Perhaps, IFN-\(\gamma\) acts in an autocrine manner on dendritic cells and macrophages to further activate DCs and macrophages and provide costimulation of CD8\(^+\) T cells. It has been demonstrated by Ohteki et al. (59) that IL-15 knockout mice exhibit lower levels of IL-12, IFN-\(\gamma\), and NO by dendritic cells and macrophages, as well as a failure to up-regulate MHC class II and CD40. These data suggest that IL-15 is critical in the early activation of APCs and plays an important role in the immune system. Alternatively, our data suggest that IL-15 directly activates CD8\(^+\) T cells during the priming stage, leading to increased formation of memory CD8\(^+\) T cells. We are currently examining these possibilities.

There is a desperate global need for prophylactic and therapeutic HIV vaccines, and it has been difficult to find candidate vaccines that stimulate effective humoral and cellular responses, especially in a model of CD4 depletion. IL-15 has not been used systemically in any human study to date. It should be noted that recently, IL-15 has been implicated as a mediator of synovial inflammation (63), rheumatoid arthritis (64, 65), autoimmune thyroiditis (66), and autoimmune diabetes (67). McInnes et al. as well as other laboratories (63, 68) have reported high concentrations of IL-15 in the synovial fluid of rheumatoid arthritis joints and showed that IL-15 is expressed by cells of the synovial membrane lining. Rothe et al. (69) reported that development of early insulitis in NOD mice was associated with up-regulation of IL-15 gene expression that preceded lymphocyte invasion in islets and an increase in IFN-\(\gamma\) mRNA. IL-15 is also found to be elevated in the sera of patients suffering from T cell-mediated relapsing-remitting multiple sclerosis (70). However, taken together, these studies suggest only a correlation between high IL-15 expression and chronic disease progression, not a direct causal relationship. Furthermore, a study by Ruchatz et al. (71) showed treatment with a soluble, high affinity IL-15R\(\alpha\)-chain prevented collagen-induced arthritis in mice. To date, there have been no human studies that directly correlate a causal relationship of IL-15 to the induction of autoimmune disease. We have determined that injection of pIL-15-Opt results in undetectable levels of anti-human IL-15 Ab in the sera of mice,

**FIGURE 10.** pIL-15-Opt is required for CD4\(^+\) T help in the generation of a functional CD8\(^+\) T cell response in a model of CD4\(^+\) Th cell depletion and partially restores the response in a CD4 knockout model. A, Splenocytes from vector (first set of bars), pHIV-1gag (second set of bars), or pHIV-1gag/pIL-15 (third set of bars) in an IFN-\(\gamma\)-ELISPOT assay. Also shown in A, CD4-depleted BALB/c mice received vector (fourth set of bars), pHIV-1gag (fifth set of bars), or pHIV-1gag/pIL-15 (sixth set of bars), and an IFN-\(\gamma\)-ELISPOT assay was performed, as described in Materials and Methods. B, Splenocytes were harvested from immunized parental C57BL/6 mice that received vector (first set of bars), pHIV-1gag (second set of bars), or pHIV-1gag/pIL-15 (third set of bars). Also shown in B, splenocytes were obtained from immunized CD4KO mice that received vector (fourth set of bars), pHIV-1gag (fifth set of bars), or pHIV-1gag/pIL-15 (sixth set of bars), and an IFN-\(\gamma\)-ELISPOT assay was performed. Splenocytes were added to the ELISPOT plates and stimulated overnight at 37°C in 5% CO\(_2\) in the presence of R10 (negative control) or 10 \(\mu\)g/ml HIV-1 p55 peptide mix (HIV-1gag-specific 15-mer, overlapping by 11 aa). IFN-\(\gamma\) SFC were quantified by an automated ELISPOT reader, and the raw values were normalized to SFC per million splenocytes. Values represent the mean (\(\pm SE\)) of triplicate cultures and are representative of three independent experiments. * \(p < 0.05\).
and we are unable to detect human IL-15 in the sera of vaccinated mice (unpublished observations), suggesting that the levels of IL-15 produced after vaccination with pIL-15-Opt are very low, but locally sufficient to enhance the HIV-1-specific CD8+ T cell function, proliferation, and longevity observed in this study. It will be important to monitor this issue closely in non-human primates as a relevant model for human responses.

This report demonstrates a method by which an optimized cytokine plasmid-delivered IL-15 could enhance CD8+ T cell proliferation, function, and longevity. Taken together, these data suggest a role for IL-15 as a possible candidate adjuvant for HIV-1 DNA vaccine prophylactic or therapeutic studies. Additional study of this approach in non-human primate models of infection is warranted.

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Disclosures
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References
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IL-15 ENHANCES CD8+ T CELL FUNCTION INDEPENDENT OF T CELL HELP


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