Plasmid-Encoded Interleukin-15 Receptor α Enhances Specific Immune Responses Induced by a DNA Vaccine *In Vivo*

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Abstract

Plasmid-encoded DNA vaccines appear to be a safe and effective method for delivering antigen; however, the immunogenicity of such vaccines is often suboptimal. Cytokine adjuvants including interleukin (IL)-12, RANTES, granulocyte-macrophage colony-stimulating factor, IL-15, and others have been used to augment the immune response against DNA vaccines. In particular, IL-15 binds to a unique high-affinity receptor, IL-15R α ; is *trans*-presented to CD8⁺ T cells expressing the common $\beta\gamma$ chain; and has been shown to play a role in the generation, maintenance, and proliferation of antigen-specific CD8⁺ T cells. In this study, we took the unique approach of using both a cytokine and its receptor as an adjuvant in an HIV-1 vaccine strategy. To study IL-15R α expression, a unique monoclonal antibody (KK1.23) was generated to confirm receptor expression *in vitro*. Coimmunization of IL-15 and IL-15R α plasmids with HIV-1 antigenic plasmids in mice enhanced the antigen-specific immune response 2-fold over IL-15 immunoadjuvant alone. Furthermore, plasmid-encoded IL-15R α augments immune responses in the absence of IL-15, suggesting its role as a novel adjuvant. Moreover, pIL-15R α enhanced the cellular, but not the humoral, immune response as measured by antigen-specific IgG antibody. This is the first report describing that IL-15R α itself can act as an adjuvant by enhancing an antigen-specific T cell response. Uniquely, pIL-15 and pIL-15R α adjuvants combined, but not the receptor α chain alone, may be useful as a strategy for generating and maintaining memory CD8⁺ T cells in a DNA vaccine.

Introduction

THE GENERATION OF POTENT CD8⁺ T cell responses by DNA vaccine technology is an important goal for this platform. The contribution of CD8⁺ T cells for controlling viral replication in both human (Koup *et al.*, 1994; Cao *et al.*, 1995; Musey *et al.*, 1997; Ogg *et al.*, 1998; Betts *et al.*, 1999) and nonhuman primate models (Jin *et al.*, 1999; Schmitz *et al.*, 1999; Barouch *et al.*, 2000; Amara *et al.*, 2001; Shiver *et al.*, 2002) in the HIV model as well as other viral infections has underscored the importance of improving the T cell responses induced by this technology. Many unique strategies including improved delivery techniques, enhanced construct design, heterologous prime-boost strategies, and the use of molecular adjuvants have been reported (Gao *et al.*, 2003; Leifert *et al.*, 2004; Fuller *et al.*, 2006; Hokey and Weiner, 2006; Schoenly and Weiner, 2008). Molecular adjuvants including chemokines and cytokines can be incorporated into a vaccine strat-

egy to skew the immune response toward cellular or humoral immunity. Cytokines such as interleukin (IL)-12 and IL-15 have been effective in enhancing the immune response in both murine and nonhuman primate models (Morrow and Weiner, 2008). In particular, IL-15 has been shown to be involved in the expansion of cellular immunity when used in this context.

IL-15 has been shown to play a prominent role in the generation and maintenance of CD8⁺ T cells (Zhang *et al.*, 1998; Ku *et al.*, 2000; Becker *et al.*, 2002; Moore *et al.*, 2002; Waldmann, 2002; Yajima *et al.*, 2002; Sprent, 2003; Oh *et al.*, 2004, 2008) as it signals through the common $\beta\gamma$ chain, which is also used by IL-2 (Giri *et al.*, 1994). IL-15 has a unique high-affinity receptor (K_a , $\sim 10^{-11}$ M⁻¹) chain, termed IL-15R α (Anderson *et al.*, 1995b). Systemically soluble IL-15 protein is virtually undetectable despite its constitutive mRNA expression by many cell types (Giri *et al.*, 1995) and its expression is tightly controlled at various levels (Tagaya *et al.*, 1996; Onu *et al.*, 1997; Bamford *et al.*, 1998; Waldmann and

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Tagaya, 1999). Interestingly, IL-15 has been shown to be *trans*-presented on the surface of antigen-presenting cells via IL-15R α during the priming of natural killer and CD8 $^{+}$ T cells (Dubois *et al.*, 2002; Koka *et al.*, 2004; Lucas *et al.*, 2007; Sato *et al.*, 2007). IL-15R α has also been shown to play a prominent role in the regulation of IL-15 secretion (Duitman *et al.*, 2008). This cell surface complex allows IL-15 to signal through the $\beta\gamma$ receptor on memory CD8 $^{+}$ T cells, promoting cell division and survival of these cells (Lodolce *et al.*, 1998, 2001; Kennedy *et al.*, 2000; Burkett *et al.*, 2003, 2004; Sandau *et al.*, 2004; Schluns *et al.*, 2004a,b). IL-15 and IL-15R α together as a complex exhibit enhanced stability and secretion compared with either molecule alone (Bergamaschi *et al.*, 2008).

Regarding its employment in vaccination models, the use of plasmid-encoded IL-15 as an HIV-1 vaccine adjuvant has been previously reported to enhance cytolytic and memory CD8 $^{+}$ T cell responses in mice (Oh *et al.*, 2003; Kutzler *et al.*, 2005; Zhang *et al.*, 2006; Calarota *et al.*, 2008; Li *et al.*, 2008). Studies in rhesus macaques have also shown the ability of IL-15 to enhance effector functions of CD4 $^{+}$ T cells (Picker *et al.*, 2006) and rescue dual interferon (IFN)- γ /tumor necrosis factor (TNF) responses in both effector CD4 $^{+}$ and CD8 $^{+}$ T cells (Halwani *et al.*, 2008). Importantly, addition of pIL-15 with SIV/HIV antigens in rhesus macaques resulted in enhanced protection after SHIV89.6p challenge (Boyer *et al.*, 2007).

However, to our knowledge, no previous vaccination study has attempted to take advantage of the increased stability of the IL-15/IL-15R α complex as a DNA vaccine adjuvant. To that end, we coimmunized mice with pIL-15 and pIL-15R α to examine whether we could further enhance the immune response generated by IL-15 in a model using HIV-1 DNA vaccine antigens. Our data show that although the IL-15 and IL-15R α combination indeed enhanced the overall cellular immune response, surprisingly the IL-15R α plasmid augmented immune responses in an IL-15-independent manner. Importantly, the induced memory response was maintained only in mice covaccinated with pIL-15 as well as pIL-15R α , but not with IL-15R α alone. These studies demonstrate for the first time that the IL-15R α protein alone can function as an adjuvant with a limited immune expansion phenotype.

Materials and Methods

Recombinant human IL-15R α protein production

Recombinant human IL-15R α protein was generated by Abgent (San Diego, CA). Briefly, the open reading frame of human IL-15R α (a generous gift from T. Waldmann, National Cancer Institute, National Institutes of Health, Bethesda, MD) was cloned into the high-expressing bacterial vector pET21a (EMD Biosciences, Gibbstown, NJ). Competent cells were transformed and amplified in *Escherichia coli*, and recombinant protein was purified with a nickel-nitrilotriacetic acid (Ni-NTA) column. The accuracy of the purified protein was confirmed by direct enzyme-linked immunosorbent assay (ELISA) using anti-human IL-15R α antibody (R&D Systems, Minneapolis, MN).

Generation of monoclonal KK1.23 antibody

Recombinant human IL-15R α protein was injected into BALB/c mice ($n=4$) for monoclonal antibody generation.

Five micrograms of total protein emulsified in complete Freund's adjuvant (first immunization only) or incomplete Freund's adjuvant (subsequent immunizations) was given per injection (Sigma-Aldrich, St. Louis, MO). Fifty microliters was injected subcutaneously into each flank and 100 μ l was injected into the peritoneum. Mice were given a final boost of 35 μ g of protein in sterile phosphate-buffered saline (PBS) intravenously 3 days before fusion. Antibody levels in the sera were determined by direct ELISA using recombinant IL-15R α protein and horseradish peroxidase-conjugated anti-mouse IgG (IgG-HRP) (Zymed, San Francisco, CA). One mouse with a 1:8000 titer of antibody against IL-15R α was sacrificed and its spleen removed for fusion with a myeloma cell line. A total of 1500 hybridoma supernatants were screened by ELISA, 8 positive clones were expanded, and 1 was purified by passage through an ammonium sulfate column, yielding antibody KK1.23. Monoclonal antibodies were generated and purified by J. Conicello (Wistar Institute Hybridoma Facility, Philadelphia, PA).

Western blot analysis

Western blotting analysis was performed according to standard protocols. Three micrograms per well of recombinant IL-15R α or Vpr protein (Abgent) was run on a sodium dodecyl sulfate (SDS)-polyacrylamide gel (Cambrex, Rockland, ME), blotted on nitrocellulose membrane, and probed with either the R&D Systems or KK1.23 anti-human IL-15R α antibody. The signal was amplified with anti-mouse IgG-HRP (Zymed) and detected by enhanced chemiluminescence (ECL) (GE Healthcare, Chalfont St. Giles, UK).

DNA plasmids

DNA vaccine constructs expressing HIV-1 Gag and HIV-1 Pol (Kim *et al.*, 1998) and human IL-15 (Kutzler *et al.*, 2005) were prepared as previously described. The open reading frame of human IL-15R α was moved into pVAX1 and pTRACER vectors (Invitrogen, Carlsbad, CA). Restriction enzyme digestion using, respectively, *Eco*RI and *Bam*HI or *Nhe*I and *Eco*RI (New England BioLabs, Beverly, MA) was performed. Positive clones were verified by sequence analysis.

In vitro translation assay

The TNT-T7 quick coupled transcription/translation reticulocyte lysate system (Promega, Madison, WI) and [35 S]methionine were used to create labeled IL-15R α protein product. pVAX vector alone (negative control) or pVAX vector carrying IL-15R α and [35 S]methionine was added to the reaction mix according to the instructions supplied by the manufacturer. The reaction was carried out at 30°C for 1 hr. Labeled proteins were immunoprecipitated with 5 μ g of purified monoclonal anti-IL-15R α antibody (R&D Systems) or clone KK1.23 at 4°C with rotation overnight in radio-immunoprecipitation assay (RIPA) buffer. Approximately 5 mg of protein G-Sepharose beads (GE Healthcare) (50 μ l of 100-mg/ml stock) was added to each immunoprecipitation reaction, and the samples were incubated at 4°C with rotation for 2 hr. The beads were washed three times with binding buffer containing high salt and bovine serum albumin and finally resuspended in 2 $\times$ sample buffer.

Immunoprecipitated protein complexes were eluted from the Sepharose beads by boiling for 5 min and were run on an SDS-12% polyacrylamide gel (Cambrex). The gel was fixed and treated with amplifying solution (GE Healthcare) and dried for 2 hr in a gel drier (Bio-Rad, Hercules, CA). The dried gel was exposed to X-ray film at -80°C and developed with a Kodak automatic developer (Eastman Kodak, Rochester, NY).

Indirect immunofluorescence assay

The indirect immunofluorescence assay for confirmation of pIL-15R α plasmid expression was conducted according to the following protocol as previously described (Ramanathan *et al.*, 2002). HeLa cells (ATCC, Manassas, VA) grown in slide chambers (BD Biosciences, San Jose, CA) at a density of 100,000 cells per chamber in complete Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and antibiotic-antimycotic (GIBCO; Invitrogen) were allowed to adhere overnight. Cells were transfected with pIL-15R α pTRACER or pVAX-1 (1 μg /well), using FuGENE 6 transfection reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. Twenty-four hours after transfection, cells were washed with PBS and fixed on slides, using 2% paraformaldehyde (PFA)-PBS, for 1 hr at room temperature. Slides were incubated with 5 μg of clone KK1.23 mouse anti-human IL-15R α made in our laboratory or with IgG₁ isotype control (R&D Systems) for 90 min at 37°C . Rhodamine-conjugated anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added at 1:200 and the slides were incubated for 45 min at room temperature. After 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes Invitrogen, Eugene, OR) staining for 10 min at room temperature, slides were mounted in Fluoromount G medium (Electron Microscopy Sciences, Hatfield, PA) and analyzed with the phase 3 Image-Pro program for fluorescence microscopy (MediaCybernetics, Bethesda, MD).

Plasmid immunization and mice

The tibialis anterior muscle of 6- to 8-week-old female BALB/c (Jackson Laboratory, Bar Harbor, ME), C57BL/6 (Taconic, Germantown, NY), or IL-15 knockout (Taconic) (Kennedy *et al.*, 2000) mice was injected three times, 2 weeks apart, and electroporated as previously described (Khan *et al.*, 2003; Laddy *et al.*, 2008) with the CELLECTRA adaptive constant current device (VGX Pharmaceuticals, The Woodlands, TX). For all experiments in mice, the animals were immunized with either 35 μg of pVAX1, 5 μg of HIV-1 antigenic plasmid (Gag, Pol), 10 μg of pIL-15, and/or 7.5, 10, or 15 μg of pIL-15R α ($n = 3-7$ per group). Coadministration of various gene plasmids involved mixing the designated DNA plasmids before injection in 0.25% bupivacaine-HCl (Sigma-Aldrich) in isotonic citrate buffer (Kim *et al.*, 1998; Kutzler *et al.*, 2005) to a final volume of 40 μl . All animals were housed in a temperature-controlled, light-cycled facility at the University of Pennsylvania, and their care was performed under the guidelines of the National Institutes of Health and the University of Pennsylvania (Philadelphia, PA).

Method for mouse sacrifice, sample collection, and tissue harvest

At time points designated in the immunization schedule, the animals were sedated with an analgesic and blood was taken before the animals were sacrificed by cervical dislocation. The spleen from each mouse was harvested and spleens were pooled (per experimental group) into a 15-ml conical tube containing R10 medium (RPMI 1640 plus 10% fetal bovine serum, antibiotic-antimycotic, and 2-mercaptoethanol). In a sterile tissue culture hood, the pooled spleen-medium mixture from each experimental group was crushed into a single-cell suspension, using a stomacher apparatus (Seward 80; Metrohm, Riverview, FL). The cell/tissue stroma were put through a 40- μm cell strainer and washed with R10, pelleted, and incubated for 5 min at room temperature in ACK lysing buffer (Lonza, Basel, Switzerland) to lyse red blood cells. The splenocytes were then counted and used in immune assays described subsequently.

IFN- γ enzyme-linked immunospot assay

An IFN- γ enzyme-linked immunospot assay (ELISpot) was performed as previously described (Kutzler *et al.*, 2005) to determine antigen-specific cytokine secretion from immunized mice. Briefly, ELISpot 96-well plates were coated with anti-mouse IFN- γ capture antibody and incubated for 24 hr at 4°C (R&D Systems). Splenocytes (2×10^5) from immunized mice were added to each well of an ELISpot plate and stimulated overnight at 37°C , 5% CO_2 , in the presence of R10 (negative control), concanavalin A (positive control), or specific peptide (HIV-1 Gag or Pol) antigens (10 $\mu\text{g}/\text{ml}$). HIV-1 consensus clade B subtype HIV-1 Gag and Pol 15-mer peptides spanning the entire respective protein, overlapping by 11 amino acids, were acquired from by the AIDS Reagent and Reference Repository (Frederick, MD). For CD8 $^{+}$ cell depletion experiments, CD8 $^{+}$ T cells were removed from total splenocytes by positive magnetic selection using an anti-CD8a (Ly-2) antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. After 24 hr of stimulation, the plates were washed and incubated at 4°C overnight with biotinylated anti-mouse IFN- γ antibody (R&D Systems). The plates were washed and incubated with streptavidin-alkaline phosphatase (R&D Systems) for 2 hr at room temperature. The plates were washed, and 5-bromo-4-chloro-3'-indolyl phosphate *p*-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT) (the chromogen color reagent; R&D Systems) were added. The plates were rinsed with distilled water, and dried at room temperature. Spots were counted with an automated ELISpot reader (Cellular Technology Limited [CTL], Cleveland, OH). Raw values were determined and multiplied by a factor of 5 so that data are represented as spot-forming cells per million splenocytes. Background values in the R10 wells of each group were subtracted from peptide-stimulated wells before graphing.

Intracellular cytokine staining

HIV-1-specific T cell responses were also determined by intracellular cytokine staining, using a Cytotfix/Cytoperm kit (BD Biosciences) according to the standard protocol. Splenocytes from immunized mice were stimulated for 5 hr in

the presence of GolgiPlug (1 μ l/ml; BD Biosciences) with R10 and dimethyl sulfoxide (DMSO; negative control), phorbol myristate acetate (PMA, 10 ng/ml) and ionomycin (250 ng/ml; positive control), or HIV-1 consensus clade B Gag or Pol 15-mer peptides. Before surface staining, cells were stained, using a LIVE/DEAD fixable violet dead cell stain kit (Molecular Probes Invitrogen), at 37°C for 10 min and Fc Block (BD Biosciences) was added for 15 min at 4°C to block Fc receptors. All antibodies were purchased from BD Biosciences and used at 1 μ l/test. Before permeabilization/fixation cells were stained with CD4–Alexa 700 and CD8–PerCP (peridinin chlorophyll protein) for 30 min at 4°C. CD3–PECy5 (phycoerythrin, cyanine 5) and IFN- γ –PECy7 (phycoerythrin, cyanine 7) were included in the intracellular stain for 45 min at 4°C. Data from 50,000 live CD3⁺ lymphocyte-gated events were acquired with an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Treestar, Ashland, OR). Responses from the negative control wells were subtracted from the antigenic stimulations before graphing.

Analysis of HIV-1 Gag-binding antibodies

ELISA was used to determine HIV-1 Gag-specific IgG antibodies in mouse sera as described (Ogawa *et al.*, 1989; Mestecky *et al.*, 2004). EIA/RIA plates (Corning Costar, Cambridge, MA) were coated with a 1- μ g/ml concentration of recombinant HIV-1IIIB Gag p24 (Immunodiagnostics, Woburn, MA) diluted in PBS (Mediatech, Herndon, VA) at a final volume of 100 μ l/well and incubated overnight at 4°C. Plates were washed with PBS–Tween (0.05% Tween 20) and blocked against nonspecific binding with 200 μ l of blocking buffer–diluent (3% BSA in PBS) for 2 hr at room temperature. The plates were washed and dilutions of pooled sera from immunized mice were added in triplicate (100 μ l/well), at dilutions ranging from 1 to 10 to 1 to 1600 and incubated at room temperature for 2 hr. Bound antibodies were detected with horseradish peroxidase-labeled goat anti-mouse IgG(H+L) (Zymed) and developed with substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma-Aldrich). The color reaction was stopped with 2 N H₂SO₄, and the absorbance at 450 nm was determined with an EL312 Bio-Kinetics microplate reader (Bio-Tek Instruments, Winooski, VT).

Results

Generation of anti-human IL-15R α antibody

To detect expression of the IL-15R α plasmid (pIL-15R α) on cells, we first set out to generate a monoclonal antibody against human IL-15R α as commercially available antibodies are deficient in this activity. Recombinant human IL-15R α was generated as described in Materials and Methods. To confirm the size of the newly generated IL-15R α protein, decreasing dilutions of purified protein were run on an SDS–polyacrylamide gel and stained with Coomassie blue dye (Fig. 1A). As shown in Fig. 1A, the generated protein runs at approximately 30 kDa, the expected size. We next tested the ability of this protein to bind to commercially available antibody as an indication of its correct integrity. Figure 1B shows an ELISA with plates captured with recombinant IL-15R α or Vpr protein, a negative control. Vpr was used as it was produced by a similar method as the IL-15R α protein.

Figure 1B shows that the commercially available anti-human IL-15R α antibody can detect the generated recombinant protein. To generate an antibody against IL-15R α , recombinant human IL-15R α protein was injected into BALB/c mice as described in Materials and Methods and in Fig. 1C. After screening approximately 1500 hybridoma supernatants by ELISA, one hybridoma KK1.23 exhibited titers of antibody (≥ 1 to 12,800) as shown in Fig. 1D. This hybridoma was subsequently cloned, expanded, and purified. Purified antibody KK1.23 is specific for human IL-15R α as shown by Western blot analysis in Fig. 1E. In addition, KK1.23 appears to bind to human IL-15R α with higher affinity than does the commercially available antibody (Fig. 1E).

pIL-15R α expresses bioactive protein

We next set out to create an IL-15R α expression vector suitable for use in vaccination studies. The human IL-15R α open reading frame (ORF) was cloned into the pVAX1 expression vector as shown in Fig. 2A, under the control of the cytomegalovirus (CMV) promoter. To assess appropriate expression of the IL-15R α plasmid, an *in vitro* translation assay was carried out. The ³⁵S-radiolabeled protein is shown in Fig. 2B and C migrating at roughly 30.0 kDa, whereas the control plasmid, pVAX, did not yield any detectable protein product as expected. The commercial R&D Systems (Fig. 2B) or the KK1.23 (Fig. 2C) antibody against human IL-15R α was used to immunoprecipitate the radiolabeled protein. To confirm expression of the plasmid IL-15R α , an immunofluorescence assay was also performed with the KK1.23 antibody. For this assay, the ORF of human IL-15R α was cloned into the pTRACER expression vector, which also encodes the green fluorescent protein (GFP) reporter. Therefore, cells fluorescing green (Fig. 2E–G) also express pIL-15R α , and the KK1.23 anti-human IL-15R α is detected with anti-mouse IgG–PE (red). The untransfected control is shown in Fig. 2D, and the isotype control in Fig. 2E. The data illustrate both the ability of the pIL-15R α plasmid to express as well as the ability of the anti-human pIL-15R α antibody to detect the translated protein product. Clearly, the pIL-15R α plasmid encodes a conformationally accurate and surface-localized protein.

Combining pIL-15 and pIL-15R α elicits potent immune responses

To examine the ability of pIL-15R α to enhance immune responses as compared with pIL-15, BALB/c mice were immunized intramuscularly in the tibialis anterior muscle accompanied by *in vivo* electroporation, according to the schedule shown in Fig. 3A. Mice were immunized with either pVAX control vector or with 5 μ g of antigenic construct (HIV-1 Gag, HIV-1 Pol) with 10 μ g of pIL-15, 15 μ g of pIL-15R α , or both pIL-15 and pIL-15R α in a final volume of 40 μ l. These doses were predetermined to give optimal responses in preliminary studies (data not shown). As shown in Fig. 3B, immunization with antigenic constructs alone resulted in 2300 spot-forming cells (SFCs) per 10⁶ splenocytes as measured by IFN- γ ELISpot. The addition of pIL-15 enhanced the response to 3800 SFCs, whereas coimmunization with pIL-15R α and pIL-15 exhibited the most dramatic increase relative to the antigenic group alone, resulting in 5900 SFCs. These results support the idea that formation of the IL-15/IL-15R α immune complex can serve as a more potent adjuvant than IL-15 alone.

FIG. 1. The generation of an interleukin-15 receptor α (IL-15R α) monoclonal antibody. **(A)** Coomassie staining of the recombinant human IL-15R α protein in 2-fold dilutions from 12.0 to 0.16 μ g of protein. **(B)** A commercial anti-human IL-15R α antibody can detect recombinant IL-15R α protein in a direct ELISA. **(C)** Immunization schedule for anti-human IL-15R α monoclonal antibody generation in BALB/c mice. **(D)** Hybridoma supernatants from clone KK1.23 have high titers of antibody against recombinant IL-15R α protein by ELISA. **(E)** Purified monoclonal antibody KK1.23 binds recombinant IL-15R α in ELISA and specifically as shown by Western blot analysis. CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant; IV, intravenous.

To determine whether this immune complex was truly being formed *in vivo*, we added another immunization group in which pIL-15 and pIL-15R α were injected (with antigen) in separate legs. In this split delivery method, plasmid-delivered IL-15 and IL-15R α would be unable to

form an immune complex. We found that coimmunization of pIL-15 and IL-15R α in separate legs elicited levels of IFN- γ similar to those observed with the same combination delivered in the same leg (4562 vs. 4072 SFCs, respectively).

FIG. 2. Construction and expression of the IL-15R α DNA plasmid. (A) Human IL-15R α cDNA was inserted into a pVAX1 expression vector. Plasmid IL-15R α expresses the appropriate size protein (~ 30 kDa) as detected by radioactive *in vitro* translation with the R&D (B) or the KK1.23 monoclonal (C) anti-human IL-15R α antibody. (D) Monoclonal KK1.23 antibody (IgG1 isotype) does not bind nontransfected HeLa cells (original magnification, $\times 20$). (E) pTRACER-IL-15R α -transfected cells (green) stained with a mouse IgG1 isotype control (original magnification, $\times 20$). (F and G) The KK1.23 anti-human IL-15R α antibody (red) binds pIL-15R α -pTRACER-transfected cells. Original magnification: (F) $\times 20$; (G) $\times 60$.

Somewhat unexpectedly, the immunization group with antigenic construct and pIL-15R α also augmented antigen-specific IFN- γ secretion to approximately 3500 SFCs (Fig. 3B). To confirm these results, we immunized a new set of mice with increasing doses of pIL-15R α plasmid in conjunction with antigenic constructs to determine whether pIL-15R α would induce responses in a dose-dependent fashion. As

shown in Fig. 4, the inclusion of pIL-15R α did enhance the induced IFN- γ secretion in a dose-dependent fashion, in measured responses against pGag (Fig. 4A) or pPol (Fig. 4B). Regardless of the HIV-1 antigenic construct used, coimmunization with pIL-15R α augmented cellular immune responses by 1.5- to 2-fold at the highest dose used. These results reveal an interesting phenomenon whereby IL-15R α

FIG. 3. The combination of pIL-15 and pIL-15R α augments immune responses compared with either plasmid delivered alone. **(A)** Immunization schedule for groups of BALB/c mice that were injected with DNA formulations containing combinations of vector, antigenic plasmid (HIV-1 Gag and Pol), pIL-15, and/or pIL-15R α . The combination of pIL-15/pIL-15R α was given either in the same leg or split between two different legs (pIL-15 in one, pIL-15R α in another). Intramuscular immunizations were given with electroporation three times, and mice were killed 1 week after the final boost. **(B and C)** To determine cellular responses, splenocytes from immunized mice were used in an IFN- γ ELISpot assay. Splenocytes were stimulated overnight with medium (negative control), concanavalin A (positive control), or antigenic peptide (HIV-1 Gag and Pol pools) and IFN- γ spot-forming units were counted.

may enhance antigen-specific immune responses even in the absence of IL-15, which advances its role as a novel adjuvant.

To further confirm the adjuvant properties of pIL-15R α , we examined the effector functions of CD4 $^{+}$ and CD8 $^{+}$ T cells after vaccination. Accordingly, we depleted CD8 $^{+}$ T cells from splenocytes of mice immunized with each vaccine combination previously mentioned, before carrying out the IFN- γ ELISpot assay. As shown in Fig. 5A, depletion of CD8 $^{+}$ T cells from the splenocytes of mice immunized with either pIL-15, pIL-15R α , or the combination of both significantly decreased the amount of IFN- γ secretion detected. There was no difference between the CD8 $^{-}$ (presumably CD4 $^{+}$ T) cell

contribution (Fig. 5A, gray columns) in any of the immunized groups, compared with the total responses observed in whole splenocytes (Fig. 5A, solid columns). Taken together, the combination of pIL-15R α and pIL-15 in a vaccination strategy greatly enhances the immune response relative to either construct delivered alone. This additive effect acts primarily on CD8 $^{+}$ T cells, as the effect was lost with the depletion of this cell population.

To determine whether pIL-15R α would also have an effect on humoral immune responses, we also measured antibody responses elicited through each vaccination strategy by ELISA. Sera from immunized mice was assayed to measure

FIG. 4. IL-15R α DNA plasmid is immunogenic in a dose-dependent manner without pIL-15. (A) BALB/c mice were immunized as shown in Fig. 3A with DNA formulations containing combinations of vector, 5 μ g of antigenic plasmid (HIV-1 Gag and Pol), and increasing doses of IL-15R α (7.5, 10, or 15 μ g). IFN- γ ELISpot was carried out on splenocytes stimulated with R10 (negative control), concanavalin A (positive control), and (A) HIV-1 Gag peptide pools or (B) HIV-1 Pol peptide pools.

the levels of IgG antibodies against the HIV-1 Gag (p24) protein (Fig. 5B). Although the combination of pIL-15 and pIL-15R α was the best at eliciting cellular immunity, mice immunized with either pIL-15 or pIL-15R α alone had the highest titers of HIV-1-specific antibodies (1:1600) compared with mice immunized with pVAX (not detected), pGag alone, or the combination (1:800).

pIL-15R α adjuvant does not enhance CD8⁺ T cell memory

As IL-15 has been shown to play a role in the maintenance of CD8⁺ T cell memory, we were interested in examining the effect of IL-15R α adjuvant in a similar fashion. To study this, mice were immunized three times as previously mentioned; however, instead of sacrificing these animals 1 week after the third immunization, they were allowed to rest for approximately 30 weeks to be sure the responses observed would be contributed primarily by the memory population. As shown in Fig. 6A, the responses after a significant rest period were still quite robust. Mice immunized with the antigenic construct alone had responses of about 1700 SFCs. The highest responses were clearly in groups of mice coimmunized with pIL-15: ~2800 SFCs for both pIL-15 and the pIL-15/pIL-15R α combination. In mice coimmunized with pIL-15R α in the absence of pIL-15, an adjuvant effect was no longer observed (~1700 SFCs). The same trends were also observed by intracellular cytokine staining and flow cytometry (Fig.

6B), in which the level of IFN- γ production by CD8⁺ T cells was most pronounced in mice coimmunized with pIL-15. The inclusion of pIL-15R α in our vaccination strategy had little effect on memory responses, whereas pIL-15 was critical. These data support the idea that whereas pIL-15R α was a robust adjuvant early after vaccination, over time IL-15 is a better inducer of memory CD8⁺ T cells.

The memory antibody response was similar to that observed during the effector phase. As shown in Fig. 6C, mice immunized with pIL-15 had detectable antibodies against HIV-1 Gag (p24) at dilutions out to 1:1600, whereas all other groups, including the combination of pIL-15/pIL-15R α , diluted out at 1:400. These data suggest that pIL-15 is an effective adjuvant in the generation of humoral as well as cellular memory responses, whereas pIL-15R α is not, but instead likely plays a unique role in accelerating the acute immune response to antigen.

pIL-15R α is immunogenic in the absence of IL-15

We reasoned that it was possible that the transfected and subsequently translated human IL-15R α protein could be augmenting immune responses in vaccinated mice by forming complexes with endogenous murine IL-15, or independently of IL-15. We had previously studied the sera from IL-15R α -immunized mice for the induction of anti-IL-15R α antibodies and observed no induction of such antibody responses, ruling out a more trivial explanation of these

FIG. 5. pIL-15R α augments IFN- γ secretion primarily by CD8 $^{+}$ T cells. (A) The contribution of IFN- γ secretion by CD8 $^{+}$ T cells was measured by *ex vivo* depletion of CD8 $^{+}$ T cells from the splenocytes of immunized mice, using Miltenyi beads. The total antigenic response is shown for whole splenocytes (solid columns) and CD8 $^{+}$ cell-depleted splenocytes (gray columns) and was measured by IFN- γ ELISpot. (B) Antibody titers against HIV-1 Gag p24 antigenic protein were determined from serum samples of BALB/c mice immunized with the same constructs and timeline as described in Materials and Methods. Dilutions of sera taken at the time of death were run on an ELISA plate coated with p24 and detected with an HRP-conjugated anti-mouse IgG antibody to measure levels of antigen-specific IgG. Background responses in diluent wells only were subtracted from the sample optical density values before graphing.

results. To determine whether IL-15R α acts as an adjuvant together with endogenous IL-15 we studied vaccination in IL-15 knockout mice. As a control, we first tested whether the translated human IL-15R α protein could bind to mouse IL-15. As shown in Fig. 7A, 35 S-radiolabeled human IL-15R α protein incubated with murine IL-15 was able to be immunoprecipitated with an anti-mouse IL-15 antibody, suggesting the ability of murine IL-15 to bind to human IL-15R α .

We next examined the ability of pIL-15R α to act as an adjuvant in the absence of murine IL-15. Accordingly, we conducted the same vaccination studies in IL-15 knockout mice, which lack endogenous IL-15 and as a result have a deficiency in natural killer (NK) and memory CD8 $^{+}$ T cells (Kennedy *et al.*, 2000). Figure 7C shows that pIL-15R α enhances the immune responses in the absence of endogenous murine IL-15 in the knockout mice, as determined by IFN- γ ELISpot. Furthermore, the combination of pIL-15 and pIL-15R α fails to further enhance the immune response by either adjuvant administered alone as initially observed in BALB/c mice. The IL-15 $^{-/-}$ mice were generated on a C57/BL6 black-6 background from Taconic. Therefore, to verify that we would obtain similar responses in the appropriate back-

ground control mice as were observed in BALB/c mice, the same experiments were repeated. Figure 7B shows the results from the control mice immunized according to the identical schedule and shows the same trend as BALB/c immunized mice. From these data we conclude that pIL-15R α is able to act on CD8 $^{+}$ T cells independently of pIL-15, via an unknown and novel mechanism.

Discussion

Previous studies have shown the effectiveness of codelivering plasmid IL-15 in the DNA vaccine arena to enhance immune responses in both mice (Oh *et al.*, 2003; Kutzler *et al.*, 2005; Zhang *et al.*, 2006; Calarota *et al.*, 2008; Li *et al.*, 2008) and macaques (Picker *et al.*, 2006; Boyer *et al.*, 2007; Halwani *et al.*, 2008). These studies primarily show the effect of IL-15 on the enhanced function and survival of antigen-experienced CD8 $^{+}$ T cells. Because IL-15 has been shown to be a potent inducer of CD8 $^{+}$ T cell function we sought to further enhance these effects with the inclusion of the unique high-affinity receptor α chain, IL-15R α . Other reports have shown the increased function and stability of this complex (Bergamaschi *et al.*, 2008)

FIG. 6. The combination of pIL-15 and pIL-15R α does not enhance memory responses. Mice were immunized three times and rested for 30 weeks before being killed. (A) IFN- γ secretion was measured from splenocytes of immunized mice by IFN- γ ELISpot. (B) Intracellular cytokine staining was also used to determine the memory response from immunized mice. Splenocytes from immunized mice were stimulated for 5 hr with medium, PMA/ionomycin, or the dominant and subdominant HIV-1 Gag and Pol antigenic peptides in the presence of brefeldin A. (C) Sera was taken from immunized mice and run in an HIV-1 Gag p24 ELISA. Dilutions of sera were analyzed for antigen-specific IgG. Background responses in diluent wells only were subtracted from the sample optical density values before graphing.

and we further extended this concept by codelivering plasmid-encoded IL-15 and IL-15R α as a potential “super” adjuvant in the vaccine setting.

In this article, we describe the use of a plasmid-encoded human IL-15R α construct that was first tested for expression by *in vitro* translation and detectable in transfected cells by immunofluorescence with an antibody generated in our laboratory. Coimmunization of this IL-15R α construct together with our human IL-15 and HIV-1 antigenic DNA constructs resulted in levels of IFN- γ secretion that were 2.5-fold more potent than were achieved by immunization with the antigenic constructs alone (Fig. 3B). The IFN- γ secretion was attributable to CD8 $^{+}$ T cells, as the depletion of these cells before plating for the ELISpot assay resulted in 10-fold less IFN- γ secretion. The increased potency observed with

the combined delivery of IL-15 and IL-15R α is not likely due to the formation of a stable complex in transfected cells, as injecting pIL-15 and pIL-15R α into separate legs (with antigen) also elicited immune responses similar to delivery in the same leg. It is therefore more likely that the enhanced response observed with the codelivery of pIL-15 and pIL-15R α is an additive effect of two independent adjuvants. Codelivery of these two adjuvants did not appear to further enhance humoral immune responses as measured by IgG antibodies in the sera.

Somewhat surprisingly, we found that delivery of the antigenic plasmid with pIL-15R α also augmented cellular immune responses, equal to those elicited by pIL-15. To be certain, we performed immunizations with increasing amounts of pIL-15R α and observed dose-dependent

FIG. 7. pIL-15R α can act as an adjuvant in the absence of endogenous IL-15. To explore the mechanism of IL-15R α as an adjuvant, we looked at the ability of human IL-15R α to bind murine IL-15. (A) Radiolabeled human IL-15R α binds mouse IL-15 and is coimmunoprecipitated with anti-mouse IL-15 antibody. Immunizations were also repeated in control C57BL/6 (B) and IL-15 knockout (C) mice, according to the schedule in Fig. 3A, and IFN- γ ELISpots were performed on splenocytes.

responses. We thought it may be possible that our human IL-15R α protein was able to bind to endogenous murine IL-15 and *trans*-present it in a similar fashion because murine IL-15 is approximately 73% identical to the human IL-15 (Anderson *et al.*, 1995a). To test this hypothesis, we immunized IL-15 knockout mice, which lack any endogenous IL-15. Interestingly, we observed an approximately 2-fold increase in the IL-15 knockout mice immunized with pIL-15R α relative to antigen alone. As IL-15^{-/-} female mice are notoriously difficult to acquire in large numbers between the ages of 6–8 weeks, we decided to exclude the pIL-15 group from these experiments. However, the enhanced effect of the combination of pIL-15 and pIL-15R α was no longer observed. It should be noted that control C57/BL6 mice exhibited the same trends that were seen in BALB/c mice (albeit lower total spot

counts and higher background in the pVAX group) and that the IL-15 knockout mice had overall lower responses even compared with the C57BL/6 mice. These mice have been previously described to have somewhat defective host defense responses including the inability to protect against a vaccinia challenge (Kennedy *et al.*, 2000). Regardless of the overall lower immune responses, the adjuvanting effect of pIL-15R α was still observed in the absence of any endogenous IL-15. Taken together, we propose that IL-15R α can serve as a novel adjuvant capable of eliciting responses independently of IL-15, which to our knowledge has not been previously reported. This amplification of the immune response appears particularly focused on immune expansion during the acute phase rather than the memory phase of the host T cell response.

We were also interested in examining the long-term effects of the combined pIL-15/pIL-15R α on the immune response. To look at memory responses we waited 30 weeks after the third immunization before carrying out immune analysis. We found that although the combination of these two adjuvants elicits potent CD8⁺ T cell responses in the early phase of an immune response, the effect on memory T cells is primarily observed only in mice immunized along with pIL-15. The inclusion of pIL-15 was absolutely necessary for an enhanced memory immune response relative to the antigen alone. Similarly, IL-15R α initially elicited immune responses equal to or greater than that of IL-15, but it did not help to sustain the memory response. Therefore, although IL-15R α expanded burst size, burst size in the absence of the IL-15 signal for memory was not enough to sustain a long-term response. This finding opens several questions and possibilities for immune modulation. For example, is it possible that IL-15R α binds a second molecule? Does the overexpression of IL-15R α increase formation and subsequent activation through the heterotrimeric complex including the β and γ chains? Alternatively, could overexpression of the IL-15R α molecule result in dimerization and signaling through the α chain in the absence of IL-15 and the other two receptor chains? As the cytoplasmic tail of the IL-15R α molecule is extremely short in length, it is typically not thought to play a role in direct signaling (Anderson *et al.*, 1995b). However, reports have shown that IL-15R α is indeed capable of signaling independently of the other two receptor subunits, specifically through the tyrosine kinase molecule Syk (Anderson *et al.*, 1995b; Bulanova *et al.*, 2001). In addition, Wu *et al.* (2008) performed an elegant study where they created a chimeric molecule of the IL-15R α extracellular region and the intracellular region of the IL-2 receptor α chain (248). They next created knock-in mice expressing this construct and observed diminished natural killer, natural killer T cells, and CD8⁺ T cell similar to IL-15 and IL-15R α knockout mice. These mice expressed wild-type levels of IL-15R α and were able to *trans*-present IL-15 to neighboring cells as well. These data show that the cytoplasmic domain of IL-15 α is clearly important for IL-15 function and may have a role in signaling. The exact mechanism by which IL-15R α enhances the immune response warrants further research, especially in the absence of IL-15.

In summary, we show here that the combination of cytokine IL-15 and its unique receptor α may be useful as a strategy for generating and maintaining memory CD8⁺ T cells in a model DNA vaccine. It would also be beneficial to test this combination in other vaccine models, such as cancer, in which immune responses against self tumor-associated antigens are often weak and enhancing CD8⁺ T cell responses is likely important. Further studies using IL-15R α and IL-15 in various prime-boost strategies to enhance both burst size and memory populations could provide important insight into this important immune expansion receptor system. This cytokine and receptor complex may even work better in other delivery strategies and warrants exploration.

Acknowledgments

The authors acknowledge helpful discussions with Jean Boyer, Ph.D. (University of Pennsylvania, Philadelphia, PA), and Julia Conicello of the Wistar Institute Hybridoma Facility (Philadelphia, PA) for help in generating and puri-

fying the KK1.23 monoclonal antibody. The authors also thank the Penn Pathology Flow Cytometry and Biomedical Imaging core facilities. K.A.K. acknowledges the support of NIH grants 1-T32-A107632. D.B.W. acknowledges support from the NIH, including HIVRAD funding.

Author Disclosure Statement

The laboratory notes possible commercial interests associated with this work, which may include the following: Wyeth, VGX, BMS, Virxsys, Ichor, Merck, Althea, and Aldeveron.

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Received for publication February 23, 2009;

accepted after revision June 16, 2009.

Published online: August 5, 2009.