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Immunization with HIV-1 Gag Protein Conjugated to a TLR7/8 Agonist Results in the Generation of HIV-1 Gag-Specific Th1 and CD8$^+$ T Cell Responses

Ulrike Wille-Reece,* Chang-you Wu,[1]* Barbara J. Flynn,* Ross M. Kedl,[2†] and Robert A. Seder[3]*

One strategy to induce optimal cellular and humoral immune responses following immunization is to use vaccines or adjuvants that target dendritic cells and B cells. Activation of both cell types can be achieved using specific TLR ligands or agonists directed against their cognate receptor. In this study, we compared the ability of the TLR7/8 agonist R-848, which signals only via TLR7 in mice, with CpG oligodeoxynucleotides for their capacity to induce HIV-1 Gag-specific T cell and Ab responses when used as vaccine adjuvants with HIV-1 Gag protein in mice. Injection of R-848 and CpG oligodeoxynucleotides alone enhanced the innate immune responses in vivo as demonstrated by high serum levels of inflammatory cytokines, including IL-12p70 and IFN-α, and increased expression of CD80, CD86, and CD40 on CD11c$^+$ dendritic cells. By contrast, R-848 was a relatively poor adjuvant for inducing primary Th1 or CD8$^+$ T cell responses when administered with HIV-1 Gag protein. However, when a TLR7/8 agonist structurally and functionally similar to R-848 was conjugated to HIV-1 Gag protein both Th1 and CD8$^+$ T cells responses were elicited as determined by intracellular cytokine and tetramer staining. Moreover, within the population of HIV-1 Gag-specific CD8$^+$ CD62^{low} cells, ~50% of cells expressed CD127, a marker shown to correlate with the capacity to develop into long-term memory cells. Overall, these data provide evidence that TLR7/8 agonists can be effective vaccine adjuvants for eliciting strong primary immune responses with a viral protein in vivo, provided vaccine delivery is optimized. ***The Journal of Immunology,*** **2005, 174: 7676–7683.**

All currently approved vaccines directed against viral or bacterial infections elicit strong humoral immune responses, which successfully limit primary infection. In terms of infection with HIV-1, Abs are critical for blocking attachment to and subsequent entry of virus into cells. Moreover, high titers of neutralizing Abs are sufficient to prevent infection in nonhuman primates (1, 2). A major issue, however, with respect to current vaccine development against HIV is the inability to generate broadly neutralizing Abs. In this regard, cytotoxic CD8$^+$ T cells have been shown to play an important role in controlling HIV infection (3–7), and a preventive or therapeutic vaccine that induces or enhances CD8$^+$ T cell responses, respectively, could have an impact on limiting the viral set point or controlling viral load, thereby prolonging disease onset or progression.

Efficient generation of CD8$^+$ T cell responses in humans requires processing of Ag through the endogenous MHC class I-presenting pathway. Traditional approaches for inducing such responses include vaccination with live attenuated pathogen, which is currently precluded as a clinically applicable HIV vaccine due to potential safety constraints. In recent years, prime-boost immunization using plasmid DNA as a prime and replication defective pox or adenoviral vectors as a boost has been shown to be effective for inducing potent cellular immune responses in rodents and primate models of HIV (8–13). Potential limitations of prime-boost immunization include the limited potency of DNA immunization in humans, and whether pre-existing immunity against specific adenoviral serotypes will limit the immunogenicity of replication-deficient adenoviral vectors of the same serotype. Thus, alternative vaccine regimens that are capable of inducing strong cellular immune responses in people, and that can be safely administered and given repeatedly, could have important applications for infections requiring cellular immunity.

One recent strategy for inducing potent humoral and cellular immune responses is the use of adjuvants that specifically target dendritic cells (DCs)[4] and B cells. In this regard, DCs and other APCs can be activated through various TLRs (14–17), resulting in production of inflammatory cytokines, up-regulation of costimulatory molecules (CD80, CD86, CD40), and enhanced expression of MHC class II (18, 19). Previously, our group and others have shown that immunization with different proteins, including HIV-1 Gag, administered together with CpG oligodeoxynucleotides (ODN), induces Ab, Th1, and CD8$^+$ T cell responses (20–22). These studies provide a strong basis for using protein and CpG ODN immunization in humans for eliciting broad-based humoral and cellular immune responses. Importantly, however, the cellular

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[4] Abbreviations used in this paper: DC, dendritic cell; ODN, oligodeoxynucleotide; pDC, plasmacytoid DC; mDC, myeloid DC; Tg, transgenic; p.i., postinjection; LN, lymph node; WT, wild type.

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distribution of TLR9, the receptor for CpG ODN, differs substantially between rodents and humans. In mice, TLR9 is broadly expressed on all major DC subtypes (plasmacytoid (pDC), myeloid (mDC), and lymphoid) (23), whereas in humans, TLR9 is expressed on B cells and pDCs only (24–28). Thus, the induction of primary adaptive immune responses in humans using CpG ODN will depend on the efficiency of direct and/or indirect Ag presentation by pDCs and B cells. In contrast to TLR9, TLR7 is broadly expressed on DCs and other APCs in both mice and humans (on pDCs and $CD8^-$ mDCs in mice, and on pDCs and mDCs in humans) (23, 26, 28). Of note however, is that Jarrossay et al. (27) did not detect TLR7 on mDCs. Moreover, although TLR8 does not appear to be functional in mice, it is expressed on mDCs and macrophages in humans. Taken together, TLR7/8 agonists could potentially be more potent than CpG ODN as immune adjuvants in humans based on the capacity of the agonists to target both mDCs and pDCs.

Previous studies have shown that small synthetic imidazoquinoline compounds such as R-848 activate DCs through TLR7 in mice and TLR7 and TLR8 in humans. Furthermore, using OVA as an Ag, R-848 has been shown to increase production of IFN-γ and to alter the specific pattern of Ab isotypes toward a Th1 type response (29), suggesting that R-848 could be used as an immune adjuvant. To test the adjuvanticity of TLR7/8 agonists using a viral protein vaccine regimen, we performed a series of studies in which the ability of R-848 and another TLR7/8 agonist to induce Th1 and $CD8^+$ T cell responses in vivo with HIV-1 Gag protein was monitored in mice. We show that although R-848 enhanced innate cytokine production and DC maturation in vivo, it was a poor adjuvant for eliciting T cell responses against HIV-1 Gag protein. Importantly, Th1 and $CD8^+$ T cell responses were generated when mice were immunized with HIV-1 Gag protein that had been conjugated to the TLR7/8 agonist. Collectively, these data suggest that improving vaccine delivery by direct linkage of the protein Ag to the TLR7/8 agonists represents a promising approach for inducing such responses in humans.

Materials and Methods

Mice

Female BALB/c mice and C57BL/6 mice were obtained from The Jackson Laboratory. Mice weighed ~20 g each. For adoptive transfer studies, female DO11.10 TCR transgenic (Tg) mice specific for OVA and wild-type (WT) BALB/c mice were purchased from Taconic Farms. Animals were maintained in the Vaccine Research Center Animal Care Facility (Bethesda, MD) under pathogen-free conditions and tested negative for pathogens in routine screening. All experiments were conducted following the guidelines of the Vaccine Research Center Animal Care and Use committee.

Reagents

Complete RPMI 1640 (BioSource International) medium was supplemented with 10% heat-inactivated FCS, 25 mM HEPES buffer, 1% sodium pyruvate, 1% nonessential amino acids, 0.1% 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. Purified recombinant HIV-1 Gag p24 Ag (SF-2 isolate) produced in yeast cells was purchased from Austral Biologicals. CpG ODN (1826) was purchased from Coley Pharmaceutical Group, the imidazoquinoline R-848 was obtained from GLSynthesis, and 3M-012 (TLR7/8 agonist) and the HIV-1 Gag-3M-012 conjugate (Gag-TLR7/8 conjugate) were provided by 3M Pharmaceuticals. R-848 and 3M-012 are structurally similar differing only in conversion of a hydroxyl to an amino group, allowing for photoactive conjugation to the HIV-1 Gag protein. R-848 and 3M-012 are also functionally similar in eliciting innate cytokine production in vitro. PMA, ionomycin, and brefeldin A were purchased from Sigma-Aldrich. Mixtures of 15-mer peptides overlapping by 11 aa spanning the entire HIV-1 Gag protein were prepared as previously described (30). OVA, IL-12, and IL-18 used in adoptive transfer studies were purchased from Worthington Biochemical, Wyeth-Ayerst Pharmaceuticals, and RhiGene, respectively.

Analysis of serum cytokines and expression levels of costimulatory molecules on DCs

For detection of innate cytokine production in vivo, mice received PBS, CpG ODN (25 μg), or R-848 (17.5 μg) i.p. in a volume of 100 μl. At 1, 2, 4, 8, and 24 h postinjection (p.i.), mice were bled and serum levels of IL-12p40 and IFN-α were analyzed by ELISA (OptEIA ELISA; BD Pharmingen and PBL Biomedical Laboratories, respectively). Serum levels of IL-6, IL-12p70, IL-10, and TNF-α were measured by BioSource International using its Multiplex Ab kit for the Luminex LABMAP100 system and by Pierce Biotechnology using its SearchLight Multiplex technology. Maturation of splenic DCs was assessed following i.p. injection of PBS, CpG ODN (25 μg), or R-848 (17.5 μg). Spleens were harvested at 16, 40, and 90 h p.i., dissociated into single-cell suspensions, and prepared for FACS staining using anti-CD11c PE plus anti-CD80 FITC, anti-CD86 FITC, or anti-CD40 FITC. Cells were washed twice with FACS buffer, fixed in 1% paraformaldehyde-PBS, and analyzed by flow cytometry.

Ab resources

Anti-CD16/CD32, anti-CD28, anti-CD4 FITC, anti-CD8 PerCp, anti-IFN-γ allophycocyanin, anti-IL-2 PE, anti-CD62 ligand (CD62L) FITC, anti-CD4 PerCp, anti-CD127 PE, anti-CD80 FITC, anti-CD86 FITC, anti-CD40 FITC, anti-CD11c PE, and isotype-matched control Abs were obtained from BD Biosciences. PE-conjugated anti-DO11.10 TCR (KJ1-26) was purchased from Caltag Laboratories. Allophycocyanin-conjugated H-$2K^d$ tetramer loaded with the Gag peptide AMQMLKETI was obtained from the National Institute of Allergy and Infectious Diseases Tetramer Facility at Emory University Vaccine Center (Atlanta, GA) or from Beckman Coulter.

Vaccinations

HIV-1 Gag protein (10 μg/mouse) with or without CpG ODN (25 μg/mouse), R-848 (4–100 μg/mouse), 3M-012 (TLR7/8 agonist, 17.5 μg/mouse), or HIV-1 Gag-3M-012 conjugate (Gag-TLR7/8 conjugate, 10 μg/mouse) were mixed in PBS and injected s.c. three times into the rear footpads in a volume of 40 μl/foot at 3-wk intervals.

Intracellular cytokine staining

Seven days after the final immunization, spleens, draining lymph nodes (LNs), and lungs were harvested and dissociated into single cell suspensions. Erythrocytes were depleted using ACK lysing buffer (10 min, 4°C; BioSource International), cells were washed twice in complete medium, and a total of 3×10^6 cells were pulsed with 1 μg/ml anti-CD28 and a single pool of HIV-1 Gag peptides (15-mer peptides overlapping by 11 aa spanning the entire protein) (30). The final concentration of any single peptide was 400 ng/3×10^6 cells. In every experiment, as a negative control, 1 μg/ml anti-CD28 was added in the absence of Gag peptides. As a positive control, 20 ng/ml PMA plus 1 μM ionomycin were added to ensure that cells from all immunized groups were capable of responding. Cells were stimulated for 1 h at 37°C followed by an additional 4 h of stimulation in the presence of 10 μg/ml brefeldin A, and then stained with FITC-conjugated anti-CD4, PerCP-labeled anti-CD8, allophycocyanin-conjugated anti-IFN-γ, and PE-conjugated anti-IL-2 as previously described (20). For analysis, 150,000–200,000 cells were acquired on a FACSCalibur flow cytometer (BD Biosciences) and FACS data were analyzed using FlowJo software (Tree Star).

Adoptive transfer and CFSE labeling

Naive $CD4^+$ T cells from DO11.10 TCR-Tg mice were isolated from spleens and mesenteric LNs using anti-CD4 microbeads (>95% $CD4^+$ purity) according to the manufacturer's instruction (Miltenyi Biotec). Cells were labeled with 0.25 μM CFSE (Molecular Probes) for 8 min at 4°C, labeling was quenched for 1 min with FCS, and cells were washed twice with PBS. A total of 5×10^6 DO11.10 TCR-Tg $CD4^+$ T cells were injected (i.v.) into WT BALB/c mice. One and 7 days after adoptive transfer, mice were injected s.c. in both rear footpads with PBS, 100 μg of OVA (OVA protein) alone, or in combination with CpG ODN (25 μg), R-848 (25 μg), or IL-12 (100 μg) plus IL-18 (100 μg). Draining LNs from these mice were harvested 3 days after second injection, dissociated into single cell suspensions, pooled, and 3×10^6 cells were pulsed with 1 μg/ml OVA-specific peptides (American Peptide Company) and 1 μg/ml anti-CD28 for 1 h at 37°C under CO_2 saturating conditions followed by an additional 4 h of stimulation in the presence of 10 μg/ml brefeldin A. Intracellular cytokine staining was performed using PE-conjugated anti-KJ1-26 specific for the DO11.10 TCR, PerCp-conjugated anti-CD4 for surface staining, and allophycocyanin-conjugated IFN-γ or the respective isotype control mAb for intracellular staining.

Detection of HIV-1 Gag-specific Abs

Serum was obtained from mice 7 days after the third immunization injection. Immulon 4HBX 96-well plates (Thermo Labsystems) were coated overnight with 1 μg/ml HIV-1 Gag protein, washed with PBS/Tween 20, and blocked with PBS/10% FCS for 2 h. Plates were washed, serum samples were added in serial dilutions and incubated for 1 h at room temperature. After washing plates, HRP-conjugated anti-IgG1 or anti-IgG2a (BD Pharmingen) was added for 1 h at room temperature. Plates were washed, developed with *o*-phenylenediamine dihydrochloride (SigmaFast; Sigma-Aldrich), and read using a SpectraMax Plus machine (Molecular Devices).

Tetramer staining

Spleens were harvested 7–8 days after the third immunization and dissociated into single-cell suspensions. A total of 3×10^6 cells per sample were washed with FACS buffer, blocked in 100 μl of PBS-5% milk buffer containing anti-CD16/CD32 for 15 min at 4°C and stained for 30 min (4°C) with allophycocyanin-conjugated H-2K^d tetramer loaded with the Gag peptide (AMQMLKETI), PerCP-conjugated anti-CD8, PE-conjugated anti-CD127, and FITC-conjugated anti-CD62L. Cells were washed three times with FACS buffer, fixed in 1% paraformaldehyde-PBS, and analyzed by flow cytometry as described.

Conjugation of a TLR7/8 agonist to HIV-1 Gag protein

TLR7/8 agonist and HIV-1 Gag protein were mixed at an ~40:1 drug to protein ratio in 96-well polypropylene plates and exposed for 2–5 min to UV light. To remove any nonconjugated TLR7/8 agonist as well as peptides of <100 amino acids, the resulting conjugate was extensively dialyzed in PBS using a 10-kD membrane. The remaining conjugate was restored at pH 7.4 and tested for protein concentration by Bio-Rad assay. The conjugation procedure was performed under endotoxin-free conditions.

Statistics

Statistical analysis was performed using INSTAT software (GraphPad). Unpaired Student's *t* test was used to determine the significance of differences in cytokine production. Levels of $p < 0.05$ were considered significant.

Results

R-848 and CpG ODN are potent inducers of innate cytokines in vivo

Before assessing the ability of R-848 to induce adaptive immune responses, we determined its capacity to enhance innate immunity in vivo. For comparative purposes, a group of mice treated with CpG ODN was included. As shown in Fig. 1*A*, CpG ODN and R-848 induced high serum levels of IL-12p40, which peaked at 4 h p.i. Bioactive IL-12p70 also peaked at 4 h p.i., but the amount induced by R-848 was five times higher than IL-12p70 induced by CpG ODN (Fig. 1*B*). Both stimuli also elicited production of TNF-α and IL-6, which peaked at 1 and 2 h, respectively (Fig. 1, *C* and *D*). Production of IFN-α was detected at 60 and 90 min p.i. from mice treated with R-848 (180 pg/ml) and CpG ODN (46 pg/ml), respectively. Finally, we were not able to detect production of IL-10 in serum from mice treated with R-848 or CpG ODN at any time point (data not shown). At all time points tested, cytokine levels induced by R-848 were similar or greater than production of cytokines induced by CpG ODN. Moreover, different routes of injection (s.c. vs i.p.) or using mice on a different background (C57BL/6) gave comparable results (data not shown). These data demonstrate that R-848 effectively stimulates production of various inflammatory cytokines important for induction of Th1 type and CD8$^+$ T cell responses in vivo.

CpG ODN and R-848 induce maturation of DCS

To further characterize the ability of R-848 to activate innate immunity in vivo, mice were treated i.p. with PBS, CpG ODN, or R-848, and expression of CD80, CD86, and CD40 on splenic DCs was determined. As shown in Fig. 2, both R-848 and CpG ODN enhanced expression of CD80, CD86, and CD40 with the highest level detected at 40 h p.i. By 90 h p.i., expression of all costimulatory molecules decreased to levels determined on splenocytes from control mice treated with PBS only.

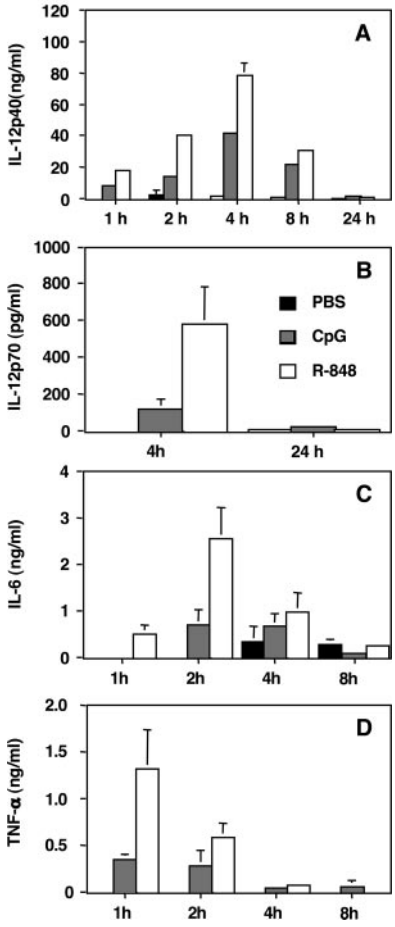

FIGURE 1. Production of serum cytokines after administration of CpG ODN or R-848. PBS, 25 μg of CpG ODN, or 17.5 μg of R-848 were injected (i.p.). Mice were eye bled and serum levels were measured by ELISA (IL-12p40), Multiplex Ab kit (IL-6, TNF-α), or SearchLight Multiplex technology (IL-12p70) at various times p.i. Results shown represent the mean ± SD of $n = 4–6$ mice in each group per time point.

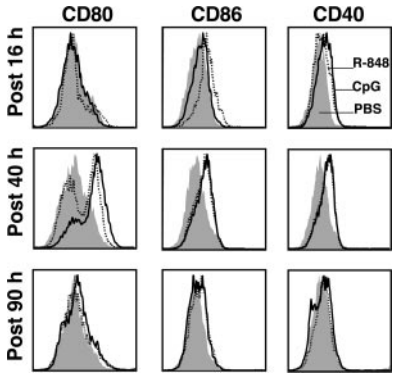

FIGURE 2. Maturation of DCs after administration of CpG ODN or R-848. PBS, 25 μg of CpG ODN, or 17.5 μg of R-848 were injected (i.p.). At various times p.i., splenocytes were harvested, stained with anti-CD11c PE plus anti-CD80 FITC, anti-CD86 FITC, or anti-CD40 FITC, and analyzed by FACS. Results shown are representative of $n = 4$ mice in each group per time point. R-848 (dotted line), CpG ODN (solid line), and PBS (shaded histogram) are shown.

CpG ODN but not R-848 induce potent Th1 and CD8$^+$ T cell responses when administered with HIV-1 Gag protein

Based on the potent immunogenicity of R-848 on innate immunity in vivo, we determined its ability to promote Th1 and CD8$^+$ T cells responses when administered with HIV-1 Gag protein. As a positive control, a group of mice was immunized with HIV-1 Gag protein plus CpG ODN (20). Mice were immunized three times at 3-wk intervals with PBS, CpG ODN, R-848, or HIV-1 Gag protein with or without CpG ODN or R-848. Seven days after the third immunization injection, the frequency of HIV-1 Gag-specific IFN-γ- and IL-2-producing CD4$^+$ and CD8$^+$ T cells was determined in secondary lymphoid (spleen and LNs) and nonlymphoid (lungs) organs by intracellular cytokine staining. As shown in Fig. 3, CD4$^+$ T cells from mice immunized with HIV-1 Gag protein plus CpG ODN responded to ex vivo stimulation with pooled HIV-1 Gag peptides by production of intracellular IFN-γ and/or IL-2 consistent with our previous study (20). In contrast, splenocytes (lymphoid) or lung cells (nonlymphoid) from mice immunized with HIV-1 Gag protein plus R-848 did not induce appreciable numbers of Gag-specific Th1 cells above those induced by the control groups (PBS, HIV-1 Gag protein, or the TLR agonists/ligands alone).

In the same experiment, the frequency of Gag-specific CD8$^+$ T cells increased from LN to spleen to lungs following immunization with HIV-1 Gag protein plus CpG ODN (Fig. 4), consistent with enrichment of effector cells from secondary lymphoid to nonlymphoid organs. Similar to the data described above, we were unable to detect Gag-specific CD8$^+$ T cells in mice immunized with HIV-1 Gag plus R-848 above any of the control groups. To determine whether varying the dose of R-848 elicited Th1 or CD8$^+$ T cell responses, mice were immunized three times at 3-wk intervals with doses of R-848 ranging from 4–100 μg in combination with HIV-1 Gag protein. As shown in Fig. 5, irrespective of the amount of R-848 used, there was no enhancement in the frequency of Gag-specific T cells. Furthermore, increasing amounts of HIV-1 Gag protein up to 50 μg during vaccination did not affect the ability of R-848 to induce T cell responses (data not shown). Together, these data suggest that despite its potent ability to induce innate immune responses, the capacity of R-848 to elicit cellular responses in vivo with HIV-1 Gag protein is limited when compared with CpG ODN.

CpG ODN are more efficient than R-848 for inducing Th1 cells in vivo

To further assess the capacity of R-848 to elicit Th1 responses in vivo with a more sensitive assay, we used a well-defined adoptive

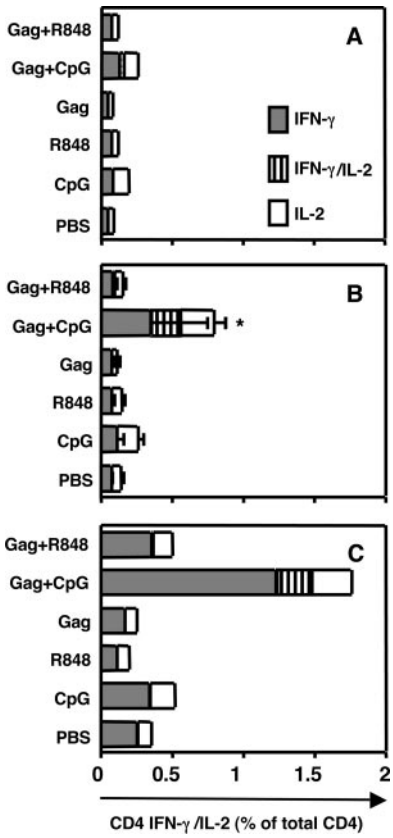

FIGURE 3. Cytokine production by Ag-specific CD4$^+$ T cells after immunization with HIV-1 Gag protein plus CpG ODN or R-848. Mice were immunized with three injections of 10 μg of HIV-1 Gag protein, 25 μg of CpG ODN, 17.5 μg of R-848, or the combination of HIV-1 Gag protein and CpG ODN or R-848 at intervals of 3 wk. Cells from LN (*A*), spleens (*B*), and lungs (*C*) were harvested 7 days after the third immunization injection, incubated ex vivo for 5 h with Gag pooled peptides, anti-CD28, and brefeldin A, and analyzed by FACS for production of IFN-γ and IL-2. Results shown represent the mean ± SD of two independent experiments with $n = 6$ mice per group. In each experiment, LNs and lungs were pooled from $n = 3$ mice per group. $p < 0.05$ when compared with all groups.

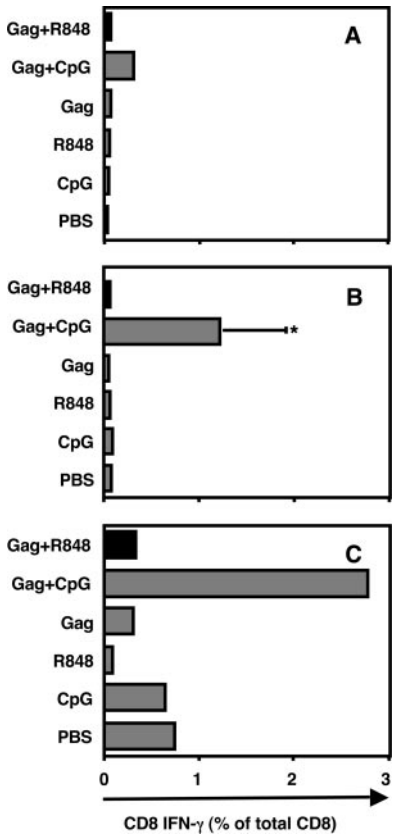

FIGURE 4. Cytokine production by Ag-specific CD8$^+$ T cells after immunization with HIV-1 Gag protein plus CpG ODN or R-848. Mice were immunized with three injections of 10 μg of HIV-1 Gag protein, 25 μg of CpG ODN, 17.5 μg of R-848, or the combination of HIV-1 Gag protein and CpG ODN or R-848 at intervals of 3 wk, and cells were prepared for intracellular FACS analysis as described in Fig. 3. Results shown represent the mean ± SD of two independent experiments with $n = 6$ mice per group. In each experiment, LNs and lungs were pooled from $n = 3$ mice per group. LN (*A*), spleen (*B*), and lung (*C*) are represented. $p < 0.05$ when compared with all groups.

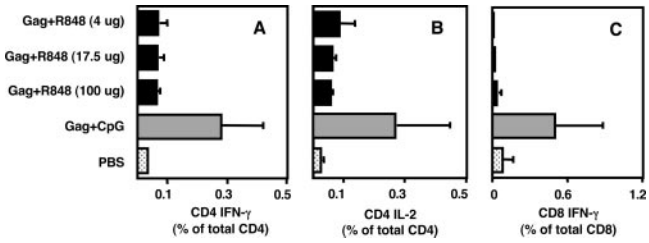

FIGURE 5. Cytokine production by Ag-specific $CD4^+$ or $CD8^+$ T cells after immunization with HIV-1 Gag protein plus various doses of R-848. Mice were immunized with three injections of 10 μg of HIV-1 Gag protein plus 25 μg of CpG ODN or various concentration of R-848 at intervals of 3 wk. Splenocytes were harvested 7 days after the third immunization injection and prepared for intracellular FACS analysis as described in Fig. 3. Results shown represent the mean $\pm$ SD from $n = 4$ mice per group.

transfer model (31), in which we could monitor its effects at the single cell level. In these studies, CFSE-labeled DO11.10 TCR-Tg $CD4^+$ T cells were adoptively transferred into naive WT BALB/c mice. At 24 h posttransfer, mice were treated with OVA protein alone or in combination with CpG ODN or R-848. Negative (PBS) and positive (OVA protein plus IL-12 and IL-18) control groups were also included. DO11.10 TCR-Tg $CD4^+$ T cells in all mice had dilution of CFSE in response to immunization with OVA (Fig. 6), indicating that these cells proliferated in response to the treatment. Although the frequency of $CD4^+$/IFN-γ-producing cells from mice treated with OVA protein plus R-848 was greater than in the OVA alone group, such responses were demonstrably less than in cells from mice immunized with OVA plus CpG ODN or IL-12 plus IL-18. Thus, in this model, R-848 did enhance Th1 responses in vivo, although it was less efficient than CpG ODN.

HIV-1 Gag protein plus R-848 immunization induces IgG1 and IgG2a responses

To further characterize the role of R-848 on adaptive immunity, we determined the Ab response following immunization with HIV-1 Gag protein plus R-848. As shown in Fig. 7, immunization with HIV-1 Gag protein plus R-848 induced production of both IgG1 and IgG2a. However, these levels were significantly lower than Ab responses induced with CpG ODN as an adjuvant. Mice vaccinated with HIV-1 Gag protein, R-848, or CpG ODN alone did not elicit

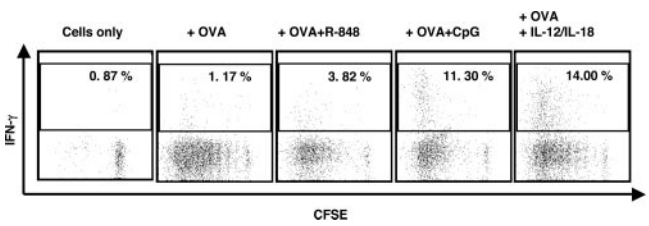

FIGURE 6. Proliferation and frequency of IFN-γ-producing cells of adoptively transferred DO11.10 TCR Tg $CD4^+$ T cells after in vivo administration of CpG ODN or R-848. A total of 5×10^6 DO11.10 TCR Tg $CD4^+$ T cells isolated from spleens and mesenteric LNs using anti-CD4 microbeads (>95% purity) were labeled with CFSE, and injected (i.v.) into naive BALB/c WT mice. At 1 and 7 days after adoptive transfer, mice were treated with PBS, 100 μg of OVA protein alone, OVA protein in combination with 25 μg of CpG ODN or 17.5 μg of R-848, or 100 μg each of IL-12 and IL-18. Draining LNs were harvested 3 days after the second injection, dissociated into single-cell suspensions, stimulated for 5 h with OVA-specific peptides, anti-CD28, and brefeldin A, and analyzed by FACS for proliferation and production of IFN-γ. Cells were gated on $KJ1.26^+$ $CD4^+$ T cells. Data shown are from pooled LNs ($n = 3$ mice per group).

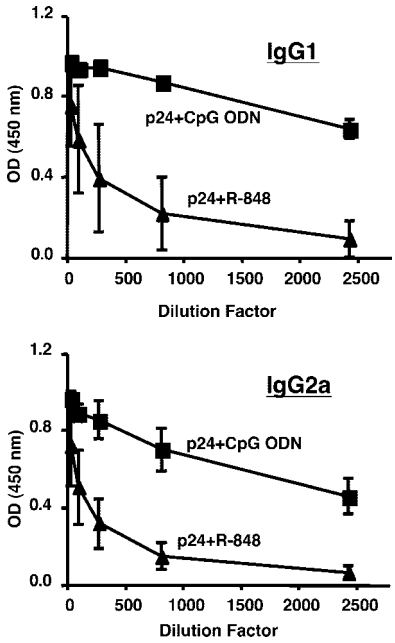

FIGURE 7. HIV-1 Gag-specific Ab responses following immunization with protein plus R-848. Mice were immunized with 10 μg of HIV-1 Gag protein, 25 μg of CpG ODN, 17.5 μg of R-848, or the combination of HIV-1 Gag protein and CpG ODN or R-848 at intervals of 3 wk. Seven days after the third immunization injection, mice were bled and Gag-specific IgG1 and IgG2a titers were determined by ELISA. Results shown are the mean $\pm$ SD of $n = 3$ mice per group. The data are representative of two independent experiments. Mice vaccinated with HIV-1 Gag protein, R-848, or CpG ODN alone did not elicit detectable Ab responses.

detectable Ab responses (data not shown). These data demonstrate that R-848 is able to induce humoral immune responses when administered together with HIV-1 Gag protein although it is less potent than CpG ODN.

HIV-1 Gag protein conjugated to a TLR7/8 agonist induces Th1 and CD8 responses

The discrepancy in the ability of R-848 to enhance innate cytokine production and DC maturation in vivo, but be limited in its capacity for inducing T cell responses, may be a delivery issue, in which timing of Ag presentation is not optimized with the activating effects of R-848. In this regard, it has previously been demonstrated that the efficiency of Ag presentation by DCs was strikingly enhanced when protein was covalently conjugated to CpG ODN (32–35). Thus, a TLR7/8 agonist that is structurally similar to R-848 was conjugated to HIV-1 Gag protein through a photoactive polylinker (Gag-TLR7/8 conjugate). Importantly, HIV-1 Gag-specific Th1 responses were detected in spleens from mice immunized with the Gag-TLR7/8 conjugate at comparable levels to mice vaccinated with HIV-1 Gag protein plus CpG ODN (Fig. 8*A*). In contrast, mice immunized with HIV-1 Gag protein plus the free TLR7/8 agonist used for conjugation or R-848 did not exhibit detectable Th1 responses.

Mice immunized with Gag-TLR7/8 conjugate also elicited detectable Gag-specific $CD8^+$ T cell responses as assessed by intracellular cytokine staining (Fig. 8*B*). Such responses, however, were less than in mice immunized with HIV-1 Gag protein and CpG ODN. To further show that HIV-1 Gag-specific $CD8^+$ T cell responses were induced by the Gag-TLR7/8 conjugate vaccine, we used an $H\text{-}2K^d$ tetramer loaded with the immune-dominant Gag peptide (AMQMLKETI) to characterize such responses. Mice immunized with HIV-1 Gag protein plus CpG ODN or Gag-TLR7/8

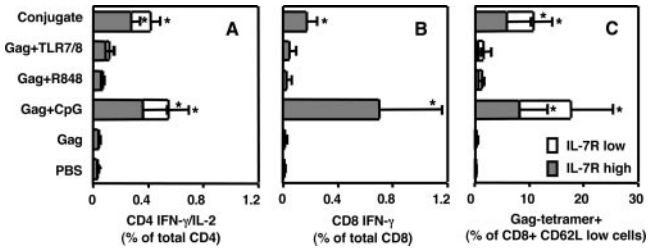

FIGURE 8. Generation of Ag-specific $CD4^+$ or $CD8^+$ T cells after immunization with HIV-1 Gag protein conjugated to a TLR-7/8 agonist (Gag-TLR-7/8 conjugate). Mice were immunized with three injections of HIV-1 Gag protein (10 μg), HIV-1 Gag protein (10 μg) in combination with CpG ODN (25 μg), R-848 (17.5 μg), or TLR7/8 agonist (17.5 μg) or received a novel Gag-TLR7/8 conjugate (10 μg) at intervals of 3 wk. Splenocytes were harvested 7 days after the third immunization injection, incubated ex vivo for 5 h with Gag pooled peptides, anti-CD28, and brefeldin A, and analyzed by FACS for production of IFN-γ and IL-2 (*A* and *B*), or were directly stained with Gag-tetramer, anti-CD62L, and anti-IL-7R without any further stimulation (*C*). Tetramer-IL-7R-positive cells were analyzed within the $CD8^+CD62L^{low}$ population (*C*). Results shown are the mean $\pm$ SD of one representative experiment with $n = 4$ mice per group. $p < 0.05$ when compared with Gag only, Gag+TLR7/8, or Gag+R-848.

conjugate generated a high frequency of AMQMLKETI peptide-specific $CD8^+$ T cells (Fig. 8*C*). Moreover, all tetramer-positive cells had low expression of the activation marker CD62L (data not shown). The relatively high number of tetramer-positive cells compared with the results seen using intracellular cytokine staining can be accounted for by the greater sensitivity of the tetramer assay and by the fact that all tetramer-positive cells were gated on $CD8^+CD62L^{low}$ cells, which were ~15–25% of the total CD8 T cells.

Within this population of tetramer-positive $CD8^+$ T cells, we also determined the frequency of cells expressing CD127, a marker shown to correlate with the capacity of effector cells to develop into memory cells (36). The ratio of the $CD127^{high}$ vs the $CD127^{low}$ population was similar in HIV-specific $CD8^+$ T cells from mice immunized with HIV-1 Gag plus CpG ODN or Gag-TLR7/8 conjugate (Fig. 8*C*), suggesting that the response induced by either vaccination regimen is qualitatively comparable at least by this marker. It was notable that ~50% of cells expressed CD127 within the $CD8^+CD62L^{low}$ population, which represents a considerably higher frequency of such cells when compared with a live infection (36). Taken together, these data provide strong evidence that indeed TLR7/8 agonists can mediate cross-priming in mice, provided they are conjugated to the Gag protein.

Discussion

In the current study, we compared the effects of R-848 and a structural TLR7/8 homologue to the TLR9 ligand CpG ODN on their ability to enhance innate and adaptive immunity in vivo. It should be reiterated that although R-848 and the TLR7/8 agonist signal through TLR7 and TLR8 in humans, their effects are limited to TLR7 in mice. Given that both R-848 and CpG ODN induced systemic innate cytokine production and maturation of DCs with similar kinetics (Figs. 1 and 2), it was surprising that R-848 was not an effective adjuvant for inducing primary T cell responses with HIV-1 Gag protein. It is notable, however, that when analyzed at the single cell level in adoptive transfer experiments using Tg mice expressing an OVA-specific TCR, immunization with OVA protein and R-848 enhanced the frequency of IFN-γ-producing TCR/Tg $CD4^+$ T cells compared with OVA protein alone (Fig. 6). Furthermore, in other reports, an increase in OVA-specific IFN-γ production was detected upon restimulation of splenocytes from mice immunized with OVA protein plus R-848 (29). In addition, using IgG2a and IγE as surrogates for Th1 and Th2 responses, respectively, Vasilakos et al. (29) showed that immunization of mice with OVA protein plus R-848 in alum enhanced IgG2a and decreased IgE serum levels. Together, these studies demonstrate that R-848 has some activity for eliciting Th1 type responses when administered with OVA protein, but is clearly more limited with HIV-1 Gag protein. Nevertheless, the immune enhancing effects of R-848 on T cell responses in vivo were markedly diminished when compared with CpG ODN (37), suggesting fundamental differences in the mechanisms by which CpG ODN and TLR7/8 agonists elicit adaptive immune responses in vivo in mice.

There are several possible explanations for the discrepancy of R-848 being a strong inducer of innate but a limited adjuvant for adaptive immunity when compared with CpG ODN. The most likely mechanism relates to differences in the pharmacokinetics of R-848 and CpG ODN in vivo and the resultant effects on DCs. Thus, although the kinetics of splenic (Fig. 2) or LN DC maturation (38) was similar following immunization with R-848 or CpG ODN, the total number of DCs in the draining LNs is enhanced following CpG ODN injection compared with R-848 (data not shown). Accordingly, CpG ODN have been shown to have long-lasting effects in vivo on the ability of DCs to present Ag to T cells (39). In contrast, R-848 is a small synthetic molecule and might be metabolized and/or distributed systemically more rapidly from the injection site following s.c immunization. Furthermore, in modeling how Ag migrates from the site of immunization to the local draining LNs to elicit T cell responses, Itano et al. (40) demonstrated that presentation of Ag after s.c. injection occurs in two temporally distinct waves. First, resident skin-derived DCs in the draining LNs acquire Ag and present it to naive $CD4^+$ T cells. Second, DCs from the injection side migrate as a second wave and appear after 18 h in the LN, promoting sustained expression of the IL-2R and the generation of a delayed-type hypersensitivity response (40). Indeed, it is possible that R-848 may enhance maturation at the site of injection more quickly than CpG ODN, limiting the capacity of DCs to process the protein Ag. This premise suggests that optimizing vaccine delivery by ensuring that Ag and adjuvant are presented synchronously improves the capacity of an adjuvant such as R-848.

To improve the efficiency of protein and TLR adjuvant delivery, it has previously been demonstrated that covalent linkage of CpG ODN to OVA protein induces better immune responses than administering CpG ODN and protein together (32, 34, 35). In addition, studies by Shirota et al. (41) showed that once CpG ODN are directly conjugated to Ag, binding and uptake of Ag by DCs is improved, resulting in increased production of IL-12 and enhanced expression of CD86 and MHC class II molecules. Indeed in the present study, conjugation of HIV-1 Gag protein to the TLR7/8 agonist resulted in enhanced Th1 and $CD8^+$ T cell responses, demonstrating that the TLR7/8 agonist could be a useful adjuvant in vivo if administered as a protein conjugate. Increased efficiency of DC activation rather than prolonged duration of Ag presentation has been suggested to be the mechanism by which the protein-TLR7/8 conjugate enhances T cell responses in vitro (R. Kedl, manuscript in preparation). Finally, it was notable that the Gag-TLR7/8 conjugate was still less immunogenic than HIV-1 Gag protein plus CpG ODN.

A potential mechanism for why CpG ODN are a more potent adjuvant than the TLR7/8 agonist may relate to the cell-specific expression profile of TLR7 and TLR9 in mice. Although TLR9 is expressed on all three major DC subtypes in mice (pDC, $CD8^+$

DC, and mDC), TLR7 is expressed on pDCs and mDCs, but not on $CD8^+$ DCs. $CD8^+$ DCs have been shown to be important for IL-12p70 production in the context of protein and CpG ODN immunization (42), and cross-priming for $CD8^+$ T cells (43). Thus, with CpG ODN, Ag presentation and activation can occur directly in all subsets of murine DCs. In contrast, although the Gag-TLR7/8 conjugate might be taken up and processed by $CD8^+$ DCs, such cells would not be directly activated due to limited mRNA expression for TLR7. Nevertheless, because the TLR7/8 agonist induces production of IFN-α, which is required for cross-priming (37, 44), $CD8^+$ DCs could get activated and induce $CD8^+$ T cell responses in the presence of IFN-α secreted by pDCs. Alternatively, if the effectiveness of the protein-TLR7/8 conjugate for Th1 responses and cross-presentation requires activation and processing of Ag to be done within the same cell, it remains possible that mDCs and/or pDCs are mediating such responses (R. Kedl, manuscript in preparation). This latter explanation is potentially relevant for protein-based vaccines in humans because a $CD8^+$ DC subset has not yet been identified in humans. Future studies are underway to define which specific DC subsets are required for mediating cross-presentation.

In conclusion, although these data show the superiority of CpG ODN to TLR7/8 agonists as vaccine adjuvants in mice, this might be different in humans. As we noted, TLR7, TLR8, or both are broadly expressed on all APCs in humans, whereas TLR9 is limited to B cells and pDCs. Thus, TLR7/8 agonists could indeed be more effective in people than CpG ODN for eliciting humoral and cellular immune responses with a protein vaccine, provided there is optimization of vaccine delivery through conjugation or some other means. Current primate studies are underway to compare the TLR7/8 and TLR8 agonists with CpG ODN when administered with HIV-1 Gag protein to delineate the contributions of distinct DCs subsets for eliciting cellular immune responses. In addition, this concept of broadly targeting APCs, and in particular DC subsets based on their specific TLR expression, could be extended to other combinations of TLR agonists or ligands. It would be likely that combinations, which activate distinct TLR signaling pathways and cover the broadest distribution of DCs, would be optimal. Finally, the capacity of a protein-based vaccine to elicit broad-based immune responses may have clinical application for both preventive and therapeutic vaccines against diseases such as AIDS, malaria and tuberculosis, or cancer.

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Disclosures

The authors have no financial conflict of interest.

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