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Critical Role for Activation of Antigen-Presenting Cells in Priming of Cytotoxic T Cell Responses After Vaccination with Virus-Like Particles[1]

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Virus-like particles (VLPs) are known to induce strong Ab responses in the absence of adjuvants. In addition, VLPs are able to prime CTL responses in vivo. To study the efficiency of this latter process, we fused peptide p33 derived from lymphocytic choriomeningitis virus to the hepatitis B core Ag, which spontaneously assembles into VLPs (p33-VLPs). These p33-VLPs were efficiently processed in vitro and in vivo for MHC class I presentation. Nevertheless, p33-VLPs induced weak CTL responses that failed to mediate effective protection from viral challenge. However, if APCs were activated concomitantly in vivo using either anti-CD40 Abs or CpG oligonucleotides, the CTL responses induced were fully protective against infection with lymphocytic choriomeningitis virus or recombinant vaccinia virus. Moreover, these CTL responses were comparable to responses generally induced by live vaccines, because they could be measured in primary ex vivo ^{51}Cr release assays. Thus, while VLPs alone are inefficient at inducing CTL responses, they become very powerful vaccines if applied together with substances that activate APCs. *The Journal of Immunology,* **2002, 168: 2880–2886.**

Major histocompatibility complex class I- and MHC class II-restricted Ag presentations are driven by two independent pathways. Endogenously generated Ags are presented to $CD8^+$ T cells by APCs in association with MHC class I molecules. This pathway limits the induction of CTL responses to intracellular replicating pathogens. Exogenous Ags are instead internalized by professional APCs, such as dendritic cells (DCs)[3] or macrophages (Mϕ), hydrolyzed by proteases in the endosomal vesicles and the MIIC compartments, and presented on MHC class II molecules for the priming of Th cells (1, 2). Therefore, immunization with soluble proteins is generally not effective in CTL priming. However, in particular circumstances it is observed that these two pathways are not completely separated, and exogenous Ags, when associated with macromolecular structures, are cross-presented to $CD8^+$ T cells (3–9). This pathway may be exploited for the development of nonreplicating, safe vaccines that nevertheless induce strong CTL responses. A variety of exogenous Ags have been shown to induce effective CTL responses in vivo, including Ags associated with apoptotic cells (10, 11), cell debris (7, 12, 13), heat shock proteins (14), Sephadex beads (5), and virus-like particles (VLPs) (6, 15–17).

A variety of viral proteins spontaneously assemble into structures that closely resemble virions, usually of icosahedral structure. Due to the highly repetitive structure of such VLPs they are very immunogenic for B cells and induce strong and long-lasting IgG responses in the absence of adjuvants (18, 19). However, probably due to their particulate nature, they also efficiently reach the MHC class I pathway in vivo. Proteins that assemble into VLPs derive from a variety of viruses, including HIV1 (16, 20, 21), rubella virus (22), human papillomavirus (15, 23), Semliki Forest virus (24), RNA phages (25), and hepatitis B virus (6, 25, 26).

The presentation of peptides in association with MHC class I molecules is essential for the generation of CTL responses. However, in the absence of additional costimulatory signals, T cell responses usually remain abortive. The best-characterized costimulatory molecule is CD28 expressed by T cells, interacting with B7 family members expressed by APCs (27). Additional costimulatory and accessory molecules expressed by T cells include LFA-1, CD2, HSA, ICOS, and OX40, to name only a few (for reviews, see Refs. 28–30). The respective ligands are expressed by professional APCs. Interestingly, these ligands are generally expressed in an inducible fashion; resting APCs express low levels of costimulatory ligands, and it is only upon activation and maturation that their expression is up-regulated. Thus, activation of APCs is a key step for efficient T cell priming. T cell-produced factors, including CD40 ligand (31), TRANCE/RankL (32), and molecules recognized by the innate immune system, such as DNA rich in nonmethylated CG motifs (33, 34) (CpGs), are most efficient at activating APCs. Administration of Ags together with factors that trigger activation of APCs has been shown to significantly augment Ag-specific T cell responses (35–38). In fact, stimulation of APCs together with administration of self-Ag may even be able to trigger autoimmunity (39, 40).

In this study we tested the ability of anti-CD40 Abs or CpGs to enhance CTL responses primed by VLPs. The hepatitis B core Ag (HBcAg) exhibiting a C-terminal fusion of the MHC class I-restricted peptide p33 derived from lymphocytic choriomeningitis virus (LCMV) glycoprotein was used as a model Ag (p33-VLP).

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[3] Abbreviations used in this paper: DC, dendritic cell; HBcAg, hepatitis B core Ag; LCMV, lymphocytic choriomeningitis virus; Mϕ, macrophage; Vacc-GP, recombinant vaccinia virus expressing the LCMV glycoprotein; VLP, virus-like particle.

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The 183-aa-long wild-type HBc protein assembles into highly structured particles composed of 180 subunits assuming icosahedral geometry (41). The flexibility of the HBcAg in accepting relatively large insertions of foreign sequences at different positions while retaining the capacity to form structured capsides is well documented in the literature (19). This makes the HBc VLPs attractive candidates for the design of nonreplicating vaccines.

We found that p33-VLPs were well processed for MHC class I presentation in vitro and in vivo. Nevertheless, they triggered rather weak CTL responses in mice if administered alone. Moreover, protection from infection with LCMV or recombinant vaccinia virus expressing the LCMV glycoprotein (Vacc-GP) was only partial or even absent. By contrast, p33-VLP injected together with agonistic anti-CD40 Abs or CpGs triggered strong CTL responses that were able to fully control infection with LCMV or Vacc-GP. Surprisingly, p33-VLP was able to induce CTL responses that were strong enough to be measured in primary ex vivo ^{51}Cr release assays. In contrast, free peptide p33 given in IFA or together with CpGs or anti-CD40 Abs induced CTL responses inferior to the responses obtained with the recombinant VLP. Thus, VLPs applied together with factors that trigger APC activation induce CTL responses as strong as those usually only observed after infection with replicating viruses such as live LCMV or vaccinia virus.

Materials and Methods

Mice

C57BL/6 mice were purchased from Harlan (Horst, The Netherlands) at the age of 8–14 wk. 318 TCR-transgenic mice (42) were obtained from the breeding colony at the Institut für Labortierkunde (University of Zurich, Zurich, Switzerland). 318 mice express transgenic Vα2 and Vβ8.1 TCR chains specific for the LCMV glycoprotein epitope p33 on 50–60% of the CD8$^+$ CTL. Animals were kept under specific pathogen-free conditions.

Viruses and cell lines

The LCMV isolate WE was originally obtained from Dr. R. Zinkeragee (Institute of Experimental Immunology, University Hospital, Zurich, Switzerland) and propagated on L929 cells (43). Virus titers were determined using a focus-forming assay on MC57 fibroblasts (44).

Vacc-GP (described previously in Ref. 7) was grown and titrated on BSC40 cells (7).

EL-4 thymoma cells (H-2^b) were used for the in the in vitro ^{51}Cr release experiments. Cells were grown in MDM (Life Technologies, Gaithersburg, MD) containing 10% FCS and supplemented with glutamine and penicillin/streptomycin.

Peptides, oligonucleotides, and rat anti-mouse CD40 Abs

LCMV glycoprotein peptide p33–41 (KAVYNFATM) (45) was synthesized by a solid phase method and was purchased from Neosystem Laboratoire (Strasbourg, France). The original cysteine at anchor position 41 in the LCMV glycoprotein peptide was replaced by methionine to avoid dimer formation.

Phosphorothioate-modified CpG-ODN was synthesized by Microsynth (Balgach, Switzerland). The following oligonucleotide sequence was used: 1668pt (5′-TCC ATG ACG TTC CTG AAT AAT-3′) (46).

Anti-CD40 mAbs (clone FGK45) were provided by T. Rolink (Basel Institute for Immunology, Basel, Switzerland).

Generation and purification of recombinant p33-VLP

The p33-VLPs were generated as follows. Hepatitis B clone pEco63 containing the complete viral genome of hepatitis B virus was purchased from American Type Culture Collection (Manassas, VA). The gene encoding HBcAg was introduced into the *Eco*RI/*Hin*dIII restriction sites of expression vector pKK223.3 (Amersham Pharmacia Biotech, Piscataway, NJ) under the control of a tac promotor. The p33 peptide (KAVYNFATM) derived from LCMV was fused to the C terminus of HBcAg (aa 1–183) via a three-leucine linker by standard PCR methods. *Escherichia coli* K802d were transfected with the plasmid and grown in 2-L cultures until an OD of 1 (at 600 nm wavelength). Cells were induced by adding isopropyl β-D-thiogalactoside (Sigma, Buchs, Switzerland) to a final concentration of 1 mM for 4 h. Bacteria were then collected by centrifugation and were resuspended in 5 ml lysis buffer (10 mM Na_2HPO_4, 30 mM NaCl, 10 mM EDTA, and 0.25% Tween 20, pH 7). Two hundred microliters of lysozyme solution (20 mg/ml) was added. After sonication 4 μl benzonase (Merck, Darmstadt, Germany) and 10 mM $MgCl_2$ were added to the cell lysate. The suspension was then incubated for 30 min at room temperature and centrifuged for 15 min at 27,000 $\times$ *g*. The retained supernatant was complemented with 20% (w/v) ammonium sulfate. After incubation for 30 min on ice and centrifugation for 15 min at 48,000 $\times$ *g*, the supernatant was discarded, and the pellet was resuspended in 2–3 ml PBS. The preparation was loaded onto a Sephacryl S-400 gel filtration column (Amersham Pharmacia Biotech) for purification. Fractions were analyzed for protein content in an SDS-PAGE gel, and samples containing pure HBc capsids were pooled.

Electron microscopy was performed according to standard protocols

In vitro proliferation of p33-specific CD8$^+$ T cells

For the in vitro analysis of VLPs, purified DCs obtained from spleens (47) and thioglycolate-stimulated peritoneal Mϕ were pulsed for 1 h with p33-VLPs (1 μg/ml) or p33 peptide as a positive control (10 ng/ml) at 37°C. After three washing steps, presenter cells (10^4 cells/well) were cocultured with Ag-specific transgenic CD8$^+$ T cells (10^5 cells/well). After 2 days T cell proliferation was measured by ^{3}[H]thymidine incorporation for 16 h (1 μCi/well).

In vivo activation of p33-specific CD8$^+$ T cells

318 TCR-transgenic mice were i.v. immunized with 100 μg p33-VLPs or wild-type HBcAgs as a negative control. Twenty-four hours later single-cell suspensions were prepared from spleens and incubated for 20 min at 37°C with PE-labeled p33-H-2^b tetrameric complexes and subsequently with anti-CD8 Tricolor-conjugated Abs (BD PharMingen, San Diego, CA) to detect CD8$^+$ p33-specific T cells. To analyze cell surface expression of the activation marker CD69, the cell suspensions were incubated with FITC-coupled anti-mouse CD69 mAbs (BD PharMingen). Live cells (5×10^4) were acquired in a FACSCalibur device and analyzed using CellQuest software (BD Biosciences, Mountain View, CA).

Cytotoxicity assay

For detection of primary ex vivo cytotoxicity, effector cell suspensions were prepared from spleens of vaccinated mice 9 days after priming. EL-4 cells were pulsed with p33 peptide (10^{-6} M, 2 h at 37°C in 2% FCS/MEM) and used in a 5-h ^{51}Cr release assay (7). Unlabeled EL-4 cells were used as a control. Radioactivity in cell culture supernatants was measured in a Cobra II Counter (Canberra Packard, Downers Grove, IL). Spontaneous release was always <10%.

To detect specific cytotoxicity after restimulation in vitro, splenocytes from primed mice (4×10^6/well) were cultured for 5 days in 24-well tissue culture plates with 2×10^6 p33-labeled syngenic spleen cells in IMDM medium supplemented with 10% FCS, L-glutamine, penicillin/streptomycin, and 10^{-5} M 2-ME. Where indicated, recombinant mouse IL-2 (2 ng/well; R&D Systems, Abingdon, U.K.) was added to cultures. Restimulated effector cells were resuspended in 300 μl MEM/2% FCS; 3-fold dilutions were subsequently made (indicated as dilution of standard culture) and tested in a ^{51}Cr release assay.

Assessment of antiviral immunity in vivo

To examine systemic antiviral immunity, vaccinated C57BL/6 mice were infected i.v. 12 days after priming with 200 PFU LCMV WE. Four days later spleens were isolated, and LCMV titers were determined by an LCMV focus-forming assay as described previously (44).

To assess antiviral immunity in peripheral tissues, female C57BL/6 mice were infected i.p. with 1.5×10^6 PFU Vacc-GP. Five days later ovaries were collected, and the vaccinia titers were determined on BSC 40 cells as previously described (7).

Results

In vitro and in vivo cross-presentation of the p33 epitope fused to the recombinant HBcAg VLPs

The MHC class I-restricted p33 epitope of the LCMV glycoprotein was genetically fused to the C terminus of the HBcAg via a three-leucine linking sequence. The chimeric protein was produced in *E. coli* and purified by ammonium sulfate precipitation and gel filtration chromatography. As confirmed by electron microscopy, the recombinant p33-containing HBcAg maintained the capacity to correctly fold and self-assemble into structured capside particles

with a diameter of ~30 nm (Fig. 1*A*). The SDS-PAGE gel analysis of the purified preparations showed that the p33-VLP monomer had, as expected, a higher molecular mass (22.7 kDa) than the wild-type HBcAg (21.4 kDa; Fig. 1*B*).

To examine the capacity of DCs and peritoneal Mϕ to cross-present VLPs, cells were isolated and pulsed in vitro with p33-VLP or, as controls, with wild-type HBcAg or free p33 peptide and subsequently used to stimulate p33-specific TCR-transgenic T cells in culture (Fig. 2). p33-VLP-pulsed DCs and, to a minor extent, Mϕ were able to efficiently stimulate the proliferation of specific $CD8^+$ T cells. Cells pulsed with wild-type HBcAg showed that unspecific stimulation was negligible. As expected, free p33 peptide was also presented by both cell types. Thus, p33 derived from recombinant VLPs is efficiently presented by DCs in vitro.

To study VLP processing in vivo, transgenic mice expressing a TCR specific for p33 were i.v. immunized with 100 μg p33-VLP, and T cell activation in the spleen was assessed 24 h later by analyzing the expression level of the surface molecule CD69 on specific $CD8^+$ T cells (Fig. 3). The p33-VLP priming elicited CD69 expression in $CD8^+$ T cells, but not in $CD8^-$ splenocytes (Fig. 3*A*). Furthermore, p33-tetramer staining of the $CD8^+$ T cells demonstrated that all cells expressing CD69 were specific for p33 (Fig. 3*B*). Thus, bystander activation did not occur. Immunization with wild-type HBcAg served as a negative control to exclude unspecific activation of CTLs (Fig. 3, *C* and *D*). These in vivo data confirm that p33-VLPs can be taken up by APCs and that the p33 epitope is presented to specific $CD8^+$ T cells. Moreover, the data indicate that such cross-presentation is a rapid and efficient process. In fact, the majority of the transgenic p33-specific $CD8^+$ T cells present in the spleen were activated within 24 h after immunization (78% of the p33-tetramer$^+$$CD8^+$ T cells were expressing CD69; Fig. 3*B*).

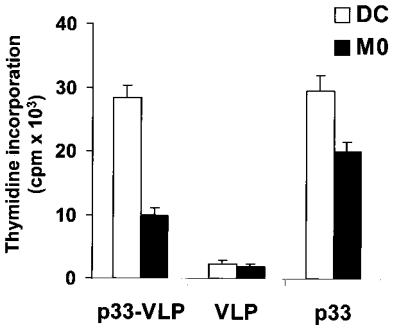

FIGURE 2. DCs and Mϕ process p33-VLPs and present the p33 epitope to $CD8^+$ Ag-specific T cells in vitro. DCs and Mϕ were pulsed with p33-VLPs, wild-type VLPs, and p33 peptide for 1 h. After three washing steps, presenter cells (10^4 cells/well) were cocultured with $CD8^+$ Ag-specific T cells (10^5 cells/well) for 2 days, and proliferation was assessed by [^{3}H]thymidine incorporation. Error bars represent the SD of triplicate measurements. One representative experiment of three is shown.

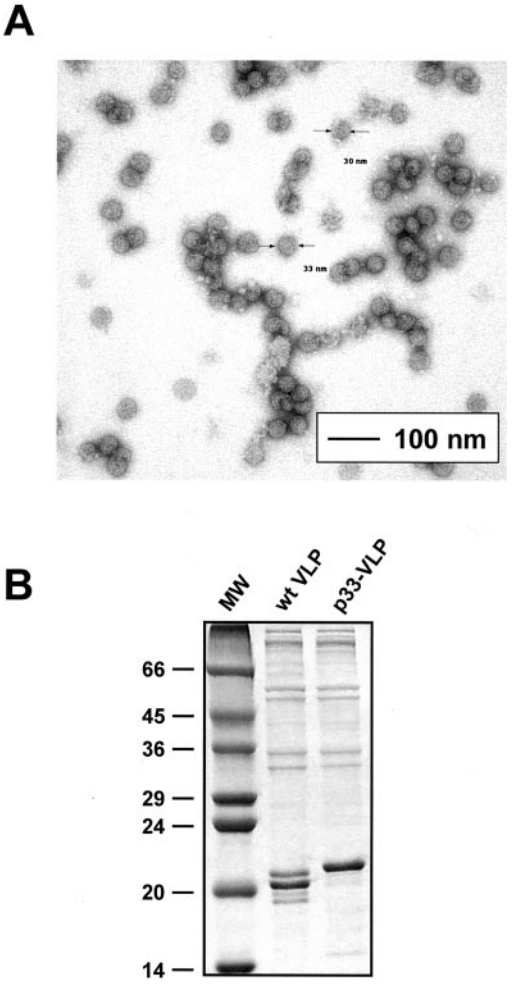

FIGURE 1. Characterization of recombinant VLPs. *A*, Electron microscopy image of recombinant VLPs. Original magnification, ×110,000. *B*, SDS-PAGE analysis of wild-type VLP and mutated p33-VLP monomers after gel filtration purification.

Induction of cytotoxic T cell responses in mice immunized with p33-VLP is enhanced by CpG-rich DNA or anti-CD40 Abs

To assess whether vaccination with p33-VLPs primes p33-specific CTL responses in normal mice, groups of C57BL/6 mice were immunized intradermally (Fig. 4*A*) or s.c. (Fig. 4*B*) with 100 μg p33-VLP. Alternatively, p33-VLPs were delivered in combination with 20 nmol CpGs (Fig. 4*C*) or 100 μg anti-CD40 Abs (Fig. 4*D*). Preliminary experiments had shown that CpGs enhanced the T cell

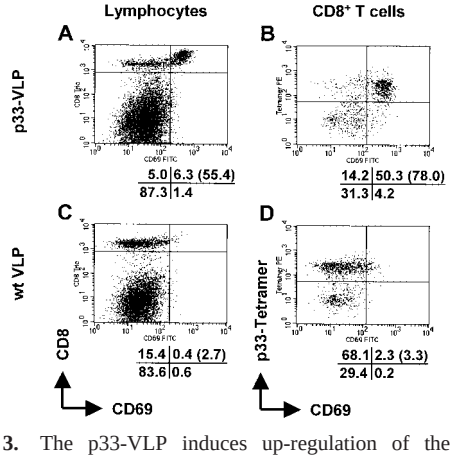

FIGURE 3. The p33-VLP induces up-regulation of the activation marker CD69 on $CD8^+$ p33-specific T cells. 318 TCR-transgenic mice were i.v. immunized with 100 μg p33-VLP (*A* and *B*) or with wild-type VLP as a negative control (*C* and *D*). One day after priming splenocytes were collected and stained with Tricolor-labeled anti-CD8 mAbs, FITC-labeled anti-CD69 mAbs, and PE-coupled p33-tetramers and analyzed by flow cytometry. *A* and *C*, Data are gated on total lymphocytes. *B* and *D*, Data are gated on $CD8^+$ lymphocytes. The percentages of the events falling into the individual quadrants are indicated below each dot plot. Values shown in parentheses for the *upper right quadrants* represent the percentages of CD69-expressing cells within the $CD8^+$ T cell population (*A* and *C*) or the p33-tetramer$^+$$CD8^+$ T cell population, respectively (*B* and *D*).

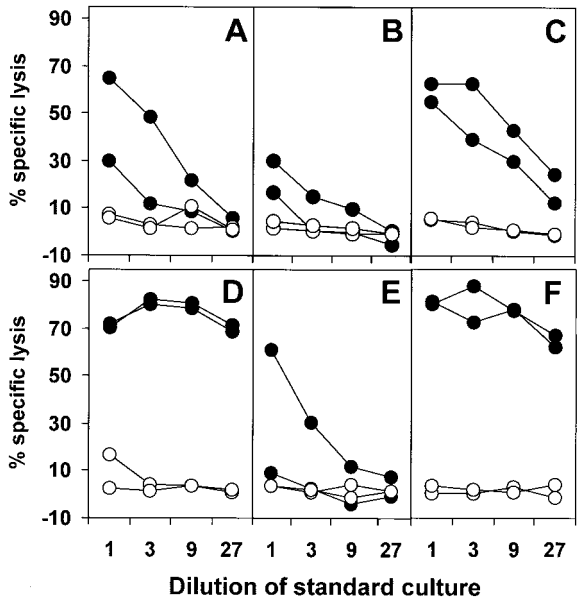

FIGURE 4. CTL Priming with recombinant VLPs. C57BL/6 mice were immunized with p33-VLP intradermally (*A*), s.c. without (*B*) or with 20 nmol CpGs (*C*), or i.v. in combination with anti-CD40 Abs (*D*). As controls, mice were immunized with 100 μg p33 peptide in IFA s.c. (*E*) or with 200 PFU LCMV i.v. (*F*). Twelve days later splenocytes were restimulated in vitro for 5 days with peptide-labeled naive spleen cells in the presence of rIL-2 (2 ng/well). Specific lysis was tested on p33-pulsed EL-4 target cells (●). Unspecific lysis was measured on unpulsed EL-4 cells (○). Data from individual mice are shown. Mice immunized with wild-type VLP did not induce any detectable response (data not shown). One of three comparable experiments is shown.

response best if administered s.c., while anti-CD40 treatment was most potent if applied i.v. Consequently, for vaccination in the presence of CpGs, the s.c. route was used, while anti-CD40 Abs were given i.v. Mice primed s.c. with 100 μg p33 peptide in IFA (Fig. 4*E*) or infected at least 30 days previously with LCMV (Fig. 4*F*) served as a positive control. Twelve days after immunization spleen cells were restimulated in vitro for 5 days with p33-pulsed APCs. Mice immunized with p33-VLP alone generated low, but detectable, p33-specific CTL responses (Fig. 4, *A* and *B*), especially when the VLPs were delivered intradermally. Similar results were obtained when lymphocytes from draining lymph nodes were used for restimulation (data not shown). The addition of substances that are known to induce maturation of DCs during vaccination strongly enhanced CTL immunity (Fig. 4, *C* and *D*). Surprisingly, the use of anti-CD40 Abs induced cytolytic responses comparable even to those observed in LCMV-infected mice, at least after in vitro restimulation.

These results show that p33-VLPs are efficiently cross-presented to specific T cells when injected into mice. However, the maturation and activation of professional APC seem to play a major role in the amplitude of the induced CTL responses.

p33-VLP immunization elicits protective responses against systemic infections with LCMV and peripheral infections with recombinant vaccinia virus

LCMV is cleared from the host by $CD8^+$ T cells in a perforin-dependent fashion (48, 49). To investigate whether the induced CTL responses were able to protect from viral infections, mice were challenged with LCMV. Mice primed with p33-VLPs in the presence or the absence of anti-CD40 Abs or CpGs were i.v. infected with 200 PFU LCMV 12 days after vaccination. Viral titers in the spleen were tested 5 days later (Fig. 5*A*). Intradermal or s.c. p33-VLP immunization elicited a median viral titer reduction of $\sim$1–2 $\log_{10}$ units compared with untreated, infected mice. By contrast, the p33-VLP formulations containing 20 nmol CpG DNA or 100 μg anti-CD40 Abs induced complete antiviral protection. Priming with 100 μg p33 peptide in IFA produced significant, but nevertheless incomplete, viral suppression. Thus, immunization of mice with p33-VLP induces full protection if APCs are activated at the same time. In contrast, immunization with VLPs alone is rather inefficient.

Vacc-GP is controlled by memory $CD8^+$ T cells in H-2^b mice (50). Eradication of Vacc-GP from peripheral tissues requires strong CTL responses, and the activated T cells have to be able to home to the site of infection, which is only a feature of recently activated CTLs (51). To test whether p33-VLP-primed mice are protected from recombinant vaccinia virus mice were i.p. challenged with 1.5×10^6 PFU Vacc-GP 12 days after immunization. Five days later viral titers were determined in the ovaries (Fig. 5*B*). The importance of the APC activation status for efficient CTL priming was also evident during Vacc-GP infection. Only mice immunized with p33-VLP in the presence of CpGs or anti-CD40 Abs were able to mount protective CTL responses, whereas p33-VLPs alone did not induce significant changes in the viral load compared with untreated infected mice.

p33-VLP, but not free p33 peptide, administered together with CpGs or anti-CD40 Abs elicits CTL responses detectable in primary ex vivo ^{51}Cr release assays

To compare CTL responses induced by recombinant VLPs with responses induced by free peptides, mice were primed with 100 μg p33-VLP or p33 alone or in combination with 20 nmol CpGs or 100 μg anti-CD40 Abs (Fig. 6, *A–E*). As a comparison, CTL responses elicited by replicating viruses were also analyzed. Mice were i.v. infected with 200 PFU LCMV or, alternatively, with 10^6

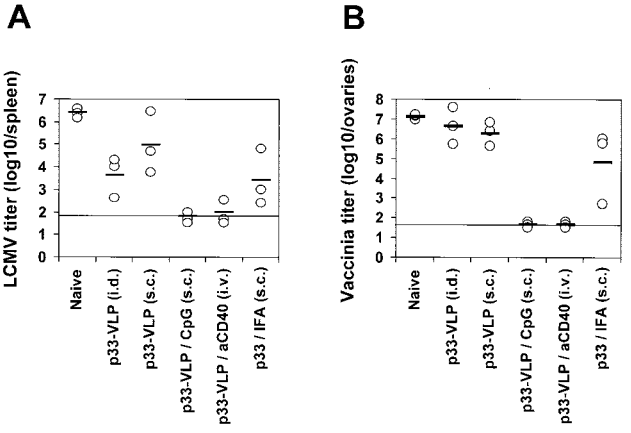

FIGURE 5. CpG-rich DNA or anti-CD40 Abs enhance p33-VLP immunogenicity for protection against challenge infection with LCMV or Vacc-GP. Groups of three C57BL/6 mice were immunized once with 100 μg p33-VLP given intradermally, s.c. with or without 20 nmol CpGs, or i.v. with 100 μg anti-CD40 Abs. Mice immunized s.c. with 100 μg p33 in IFA and naive, untreated mice served as controls. Twelve days after priming mice were challenged i.v. with 200 PFU LCMV (*A*) or i.p. with 1.5×10^6 PFU Vacc-GP (*B*). Five days later spleens or ovaries were isolated, and viral titers in the organs were determined. The detection limits of the assays are indicated by the continuous lines in the diagrams. ○, Individual mice. Lines show median viral titers per group. One of three similar experiments is shown. Mice that received p33-VLPs i.v. in the absence of anti-CD40 Abs were protected to a similar degree as mice immunized s.c. (data not shown).

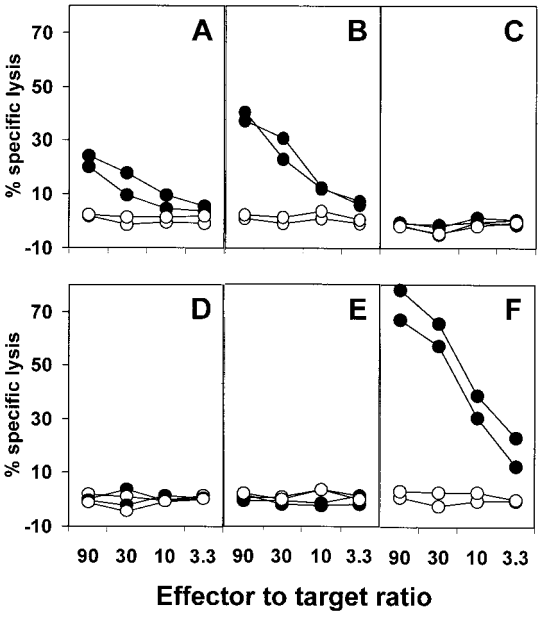

FIGURE 6. p33-VLP injected together with anti-CD40 Abs or CpG DNA induce strong CTL responses detectable in primary ex vivo ^{51}Cr release assays. Groups of two C57BL/6 mice were immunized once with 100 μg p33-VLP given s.c. together with 20 nmol CpGs (*A*) or i.v. with 100 μg anti-CD40 Abs (*B*) or alone (*C*). Alternatively, mice were immunized with 100 μg p33 peptide given s.c. with 20 nmol CpGs (*D*) or i.v. with 100 μg anti-CD40 Abs (*E*). As a positive control mice were i.v. infected with 200 PFU LCMV WE (*F*). Nine days later spleen cells were tested for direct ex vivo CTL activity in a 5-h ^{51}Cr release assay on p33-pulsed (●) or on unpulsed (○) EL-4 target cells at the indicated E:T cell ratios. Data from individual mice are shown. Mice immunized with 10^6 PFU Vacc-GP or wt VLP or, alternatively, p33 peptide alone did not induce any detectable response specific for p33 (data not shown).

PFU Vacc-GP. Nine days after immunization primary ex vivo cytotoxicity was tested in a ^{51}Cr release assay. The results indicate that only p33-VLPs, but not free peptide, when administered with CpGs or Abs produced ex vivo detectable CTL responses (Fig. 6, *A–E*). Moreover, while the LCMV virus was generating extremely potent CTL immunity (Fig. 6*F*), Vacc-GP did not induce any detectable response specific for p33 (data not shown).

Table I. *Induction of p33-specific CD8$^+$ T cells after vaccination with p33-VLP or p33 peptide*

Immunization Protocol[a]	Frequencies of p33-Specific CD8$^+$ T Cells[b]
p33-VLP	<1
p33-VLP + CpGs	2.2 ± 0.5
p33-VLP + αCD40 Abs	4.6 ± 0.3
p33 + CpGs	1.3 ± 0.3
p33 + αCD40 Abs	1.4 ± 0.2
LCMV (5000 PFU)	25.7 ± 7.3
Memory LCMV[c]	7.6 ± 0.3
Vacc-GP (10^6 PFU)	1.7 ± 0.6
Untreated mice	<1

[a] C57BL/6 mice were immunized with 100 μg p33-VLP or, alternatively, 100 μg p33 peptide. Ags were administered alone or in combination with CpGs or anti-CD40 Abs.

[b] Six days after priming splenocytes were double-stained with PE-labeled p33 tetramers and CyChrome-coupled monoclonal anti-CD8 Abs for p33-specific CD8$^+$ T cell detection. Values represent percentages of p33-specific cells on the total CD8$^+$ T cell population. Numbers correspond to means and SDs derived from three individual mice per group.

[c] Mice infected with LCMV at least 3 mo before served as LCMV memory mice.

To further study the striking differences in the immunogenicities of the various vaccination protocols, we analyzed the frequencies of p33-specific T cells in immunized mice by tetramer staining (Table I and Fig. 7). When p33-VLPs were administered in combination with anti-CD40 Abs or CpGs 6 days after priming, 4.6 or 2.2%, respectively, of the CD8$^+$ T cells were specific for the p33 epitope. By contrast, p33-VLPs alone and free p33 peptide with adjuvants generated numbers of specific T cells barely above background. Importantly, the use of anti-CD40 Abs and CpG DNA induced spleen enlargements due to the accumulation of B cells and precursor cells in secondary lymph organs, confirming earlier data (52). This explains the drop in CD8$^+$ T cells from normal values of ~18 to 5–8% in mice treated with adjuvants (Fig. 7). Control mice infected with LCMV generated ~25% p33-specific CD8$^+$ T cells 6 days after infection, while memory mice immunized 3 mo previously had ~7% specific CD8$^+$ T cells. In contrast, mice immunized with recombinant vaccinia virus produced frequencies of only ~1.7% p33-specific CD8$^+$ T cells. These findings show that the p33-VLP formulations tested in this study are highly efficient at inducing strong CTL immunity if given together

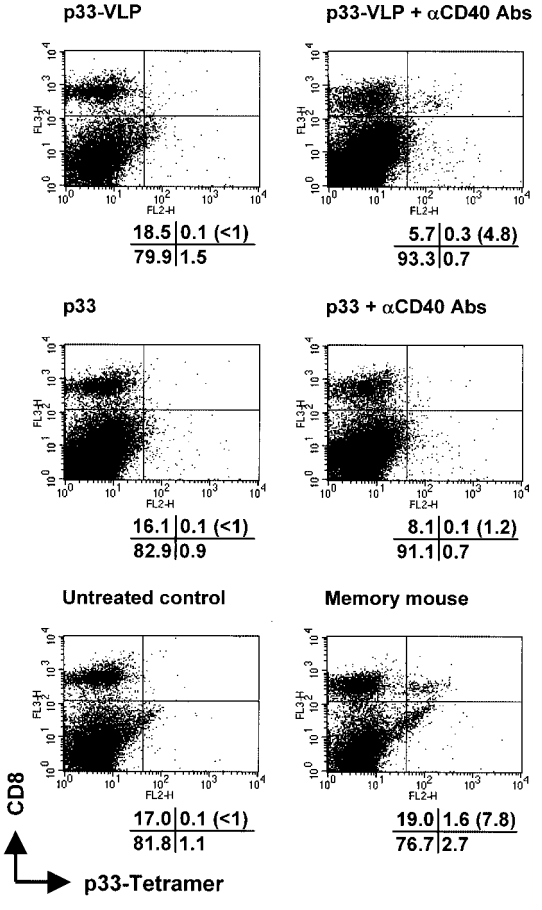

FIGURE 7. Induction of p33-specific CD8$^+$ T cells after vaccination with p33-VLPs or p33 peptide alone or combined with anti-CD40 Abs. C57BL/6 mice were immunized with 100 μg p33-VLP or p33 peptide. Ags were administered alone or in combination with 100 μg anti-CD40 Abs. As controls, untreated mice and "memory mice" (infected with LCMV 3 mo previously) were used. Six days after priming splenocytes were double-stained with PE-labeled p33-tetramers and CyChrome-coupled anti-CD8 mAbs for p33-specific CD8$^+$ T cell detection. The same number of CD8$^+$ T cells (2×10^3) are shown in each plot. The percentages of events falling into the various quadrants are indicated below each dot plot. Values shown in parentheses for the *upper right quadrants* represent percentages of p33-teramer$^+$ cells within the total CD8$^+$ T cell population.

with CpGs or anti CD40 Abs. The CTL frequencies reached after immunization with p33-VLPs mixed with CpGs or anti-CD40 Abs were, in fact, at least as high as those observed after immunization with recombinant vaccinia virus.

Discussion

In this study we demonstrate that recombinant HBcAgs containing the H-2^b MHC class I-restricted epitope p33 of LCMV are efficiently processed by DCs and Mϕ and cross-presented to specific CTLs. Nevertheless, these VLPs alone were inefficient at priming protective CTL responses in vivo. However, the same VLPs applied together with CpGs or anti-CD40 Abs were able to induce CTL responses comparable to those observed after immunization with live viral vectors.

Mϕ and DCs are known to be able to cross-present VLPs and other particulate Ags (5, 15, 53, 54). This was confirmed in this study, because ex vivo-isolated DCs and Mϕ efficiently processed p33-VLPs for MHC class I-associated presentation. Surprisingly, p33-VLP processing occurred rapidly enough and with sufficient efficiency to mediate activation of essentially all splenic p33-specific T cells in a TCR-transgenic mouse model within 24 h. We therefore expected these p33-VLPs to be effective at priming protective CTL responses in normal mice. However, to our surprise, CTL responses induced by p33-VLPs turned out to be rather weak and failed to protect against viral infections. In striking contrast, full protection from both types of infections was observed in mice primed with p33-VLP in the presence of anti-CD40 Abs or CpGs. Thus, activation of APCs during vaccination drastically improved vaccination efficiency. Intriguingly, CpGs and anti-CD40 Abs also enhanced the immunogenicity of free peptide p33 (data not shown), but not to levels observed after vaccination with p33-VLPs, indicating that the combination of VLPs with CpGs or anti-CD40 Abs may be particularly powerful for the induction of protective CTLs. Thus, while VLPs encoding T cell epitopes may be ineffective tools if used on their own, they may be optimal for vaccination purposes if APCs are activated at the same time.

The mechanism responsible for these dramatically enhanced CTL responses remains to be further studied. It may be possible that anti-CD40 Abs or CpGs alter the processing capabilities of DCs and Mϕ and favor cross-presentation mechanisms. However, the observation that p33-VLPs injected into TCR-transgenic mice in the absence of CpGs or anti-CD40 Abs are processed at sufficient efficiency to activate virtually all specific T cells may argue against this possibility. Alternatively, activated APCs may exhibit an increased life span in vivo, facilitating the induction of strong CTL responses. This idea is supported by the fact that CD40 triggering prolongs DC survival (55). Also, it seems interesting that LPS, which activates DCs without prolonging their survival (56), does not seem very effective at enhancing p33-VLP-mediated CTL priming (not shown). Thus, the strong adjuvant effect of APC activation may be dependent on Toll-like receptor 9, which is activated by CpGs, rather than Toll-like receptor 2 or 4 (57–59).

Interestingly, the intradermal immunization route, even if not in a totally satisfactory manner, resulted in better CTL responses than the s.c. applications (Figs. 4 and 5). This difference may be due to the high frequencies of Langerhans cells present in the epidermis in particular, because this lineage of DC is very efficient in taking up skin-derived Ags for T cell stimulation (47). However, although for these reasons intradermal vaccinations may be considered attractive for vaccination, this technique may not represent a preferred immunization route because of the technical difficulties involved in its use in humans.

Surprisingly, p33-VLP-primed mice were partly protected from challenge infections with LCMV, while no protection was observed after challenge infection with recombinant vaccinia virus expressing LCMV-GP. In contrast, if priming occurred in the presence of CpGs or anti-CD40 Abs, full protection against both viruses was observed. This finding may indicate that activation of APCs during priming facilitates the generation of effector cells for the following reason. Resting virus-specific CTLs are able to mediate partial protection from infection with LCMV. In contrast, resting CTLs fail to mediate protection from peripheral vaccinia virus infection, and only recently activated, effector-like T cells are effective under these conditions (60, 61). Thus, protection against vaccinia virus infection indicates the presence of potent effector-like CTLs. Because it is most likely that such effector-like CTLs, rather than resting CTLs, are able to cope with tumors and chronic vial infections, vaccines based on VLPs combined with anti-CD40 Abs or CpGs may be optimal candidates for a new generation of therapeutic tumor or viral vaccines.

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