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Loading of MHC Class I and II Presentation Pathways by Exogenous Antigens: A Quantitative In Vivo Comparison

Tazio Storni and Martin F. Bachmann¹

The MHC class I pathway is usually fueled by endogenous Ags, while exogenous Ags reach the MHC class II pathway. Although exogenous epitopes may also enter the MHC class I pathway, quantification of the efficiency of the process has remained a difficult task. In an attempt of such a quantification, we directly compared the amount of exogenous virus-like particles required for induction of cytotoxic T cell responses by cross-priming with the amount of virus-like particles required for induction of Th cell responses by the conventional route of MHC class II loading as an internal standard. Surprisingly, we found that cross-presentation of peptides derived from exogenous Ags on MHC class I molecules is of only marginally lower efficiency (~1- to 10-fold) than the classical MHC class II pathway in vitro and in vivo. Thus, Ag quantities required for cross-presentation and cross-priming are similar to those required for fueling the MHC class II pathway. *The Journal of Immunology*, 2004, 172: 6129–6135.

T cell immunity is induced when Ag is presented to specific T cells together with costimulatory signals in secondary lymphoid organs (for review, see Refs. 1–3). CD4⁺ T (Th) cell activation is triggered when the specific antigenic peptide is presented on MHC class II molecules. The main source of Ag entering the MHC class II pathway is exogenous protein, which is endocytosed/phagocytosed by professional APCs. Upon endosomal processing, protein-derived peptides load MHC class II molecules, which travel to the cell surface. In contrast, CD8⁺ CTL activation is driven by peptide/MHC class I complexes. The classical source of Ag for the MHC class I pathway is protein synthesized inside the cell (4). A sizeable fraction of newly synthesized proteins misfolds and is degraded by cytosolic proteasomes (5). Peptides generated during this process are subsequently transported by TAP molecules into the endoplasmic reticulum, where they load MHC class I molecules before they are exposed on the cell surface. However, there are additional MHC class I-associated Ag-presentation pathways (6–14). In particular, exogenous Ags may also reach the MHC class I pathway under special conditions. This so-called cross-presentation seems to be an exclusive feature of dendritic cells (DCs)² and macrophages (Mφ) and may occur via various pathways (12, 15, 16). In the endosome to cytosol pathway, the Ag is taken up by APCs into endosomes, from where it then leaks into the cytosol. The process is therefore TAP dependent (11, 15). In contrast, Ags may also reach MHC class I molecules directly inside endosomes, because peptides may be exchanged on MHC class I molecules under acidic conditions (17, 18). This process is TAP independent and may be distinguished as direct endosomal loading pathway (19–24). Additional pathways have been described for heat shock-associated proteins and regurgitated peptides (25–28).

Since the first reports on cross-priming (i.e., priming across the MHC barrier (29, 30)), an impressive body of evidence has supported the importance of these original findings. Meanwhile, cross-priming has been documented for necrotic/apoptotic cells, tumor cells, DNA immunization, Ag-loaded latex beads, virus-like particles (VLPs), and viruses (6, 9–14, 16, 31), and various studies have demonstrated that the antigenic form and concentrations are playing a critical role (9, 24, 32).

Nevertheless, a quantification of the efficiency of cross-priming and cross-presentation has remained a difficult task, and a direct comparison between the efficiency of direct priming (i.e., priming through MHC class I molecules charged with peptides derived from endogenous protein) vs cross-priming has proven difficult to achieve. In fact, to create conditions in which only one Ag-presentation pathway is active and the other silent requires laborious experimental setups or particular transgenic animal hosts (33, 34). In addition, total blockade of one particular pathway is usually a complex mission and often results in altered replication of the viral vectors used to study cross-priming. Therefore, the elegant observations indicating a critical role for cross-priming made during polio virus, HSV, or murine CMV infections (33, 35–37) may be difficult to generalize and differ from results obtained with vaccinia virus, in which direct presentation seems to be the preferred route (38, 39).

An alternative strategy to compare direct priming with cross-priming would be to study the presentation of one and the same Ag given exogenously or produced endogenously. Under these conditions, it is, however, largely impossible to quantitate and/or compare amounts of Ag present in the host. To avoid these problems, we followed a different strategy to estimate the efficiency of cross-priming under physiological conditions. Rather than comparing priming of CTLs by endogenous vs exogenous Ags, we compared the efficiency of MHC class I-associated presentation of exogenous Ags with MHC class II-associated presentation of the same antigenic form. Thus, the classical MHC class II pathway of Ag presentation served as an internal standard to benchmark the efficiency of cross-presentation. Using exogenous VLPs containing the immunodominant MHC class I- and II-associated epitopes of lymphocytic choriomeningitis virus (LCMV), we determined amounts of Ag required for MHC class I- and II-associated Ag presentation, respectively. Furthermore, minimal amounts of Ag required in vivo for priming of CTL vs Th cell responses were also

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² Abbreviations used in this paper: DC, dendritic cell; HBcAg, hepatitis B core Ag; LCMV, lymphocytic choriomeningitis virus; Mφ, macrophage; VLP, virus-like particle.

assessed. Dependent on the readout, we found that cross-presentation was between equally efficient and maximally 10-fold less efficient than MHC class II-associated presentation of VLP-derived peptides. Thus, exogenous viral particles load the MHC class I and II pathway with similar efficiency.

Materials and Methods

Mice

C57BL/6 mice were purchased from Harlan Netherlands B.V. (Horst, The Netherlands) at the age of 8–12 wk. Transgenic mice expressing a TCR specific for peptide p13 in association with H-2 I-A^b have been described previously (40), as well as mice expressing a TCR specific for peptide p33 in association with H-2 D^b (41). To allow for discrimination between endogenous and transgenic CD4⁺ and CD8⁺ T cells in the adoptive transfer experiments, the transgenic animals were additionally crossed with Ly-5.1⁺ mice. Mice were bred and kept in a specific pathogen-free facility at Cytos Biotechnology AG.

Viruses, peptides, VLPs, and oligonucleotides

LCMV isolate WE was originally obtained from R. Zinkernagel (Institute of Experimental Immunology, University Hospital, Zürich, Switzerland) and propagated on L929 cells (42). Vacc-G2, a recombinant vaccinia virus expressing the LCMV glycoprotein, was described elsewhere (14). Vacc-GP was grown and plaqued on BSC40 cells (14).

LCMV glycoprotein peptides p13 (sequence GLNGPDIYKGVYQFKS VEFD) and p33 (sequence KAVYNFATM) were synthesized by a solid-phase method and purchased from Eurogentec (Hestel, Belgium). Production and purification of recombinant p33-VLPs were previously described in detail (43). Production of p13-VLPs was performed with the same protocol. Note that the p33 peptide was fused to the C terminus of hepatitis B core Ag (HBcAg) (aa 1–185) via a three-leucine linker, whereas the p13 epitope was fused via the original p13 N-flanking region of the LCMV-glycoprotein (RSSGMV) by standard PCR methods. Phosphorothioate-modified CpG-containing oligonucleotide was synthesized by Microsynth (Balgach, Switzerland). The following oligonucleotide sequence was used: 1668pt (5'-TCC ATG ACG TTC CTG AAT AAT-3').

CFSE labeling and adoptive transfer of transgenic T cells

CFSE was purchased from Molecular Probes (Eugene, OR). Erythrocytes were removed from spleen and lymph node cell suspensions by Lympholyte-M gradient (Cedarlane Laboratories, Hornby, Ontario, Canada). Transgenic CD4⁺ and CD8⁺ T cells were obtained by positive MACS MicroBeads isolation, according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany), with a purity of at least 90%. Cells were labeled by diluting the 0.5 mM CFSE stock 1000-fold into the cell suspension (final concentration 0.5 μ M) and incubating them for 10 min at 37°C. After labeling, FCS was added to a final concentration of 10% and cells were subsequently washed with PBS at 4°C. A total of 5×10^6 labeled T cells was resuspended in 250 μ l of PBS and injected into the tail vein of sex-matched C57BL/6 recipients. After 16 h, recipients were s.c.

vaccinated with titrated amounts (100, 30, 10, 3, 1, 0.3, 0.1 μ g) of p13-VLPs or p33-VLPs, respectively.

Flow cytometry

To analyze cell proliferation of adoptively transferred transgenic T cells, single cell suspensions were prepared from draining lymph nodes of treated C57BL/6 mice. Cells were then incubated with PE-labeled anti-Ly-5.1, biotin-labeled anti-V α 2 TCR Abs (followed by streptavidin-allophycocyanin staining), in combination with CyChrome-labeled anti-CD4 or anti-CD8 Abs for discrimination of transgenic T cells (all Abs were purchased from BD PharMingen, San Diego, CA). Cells were then acquired in a FACSCalibur device and analyzed using CellQuest software (BD Biosciences, Mountain View, CA).

ELISA

A total of 5 μ g/ml p33-VLPs in coating buffer (0.1 M NaHCO₃, pH 9.6) was coated onto ELISA plates (Nunc Immuno MaxiSorp, Rochester, NY), and ELISAs were performed, according to standard protocols using HRP-conjugated secondary Abs (Sigma-Aldrich, St. Louis, MO). Plates were developed with *o*-phenylenediamine substrate buffer (0.5 mg/ml *o*-phenylenediamine, 0.01% H₂O₂, 0.066 M Na₂HPO₄, 0.038 M citric acid, pH 5.0; 100 μ l each well) and were read at 450 nm. All Ab titers are presented as $-\log_4$ of 40-fold prediluted sera. Titers represent half-maximal OD.

In vitro proliferation of specific T cells

Purified DCs obtained from spleens (24) were pulsed for 3 h with the indicated amounts of p13-VLPs or p33-VLPs at 37°C. After three washing steps, presenter cells (10^5 cells/well) were cocultured with Ag-specific transgenic CD4⁺ and CD8⁺ T cells (10^5 cells/well). After 2 days, T cell proliferation was measured by [³H]thymidine incorporation for 16 h (1 μ Ci/well).

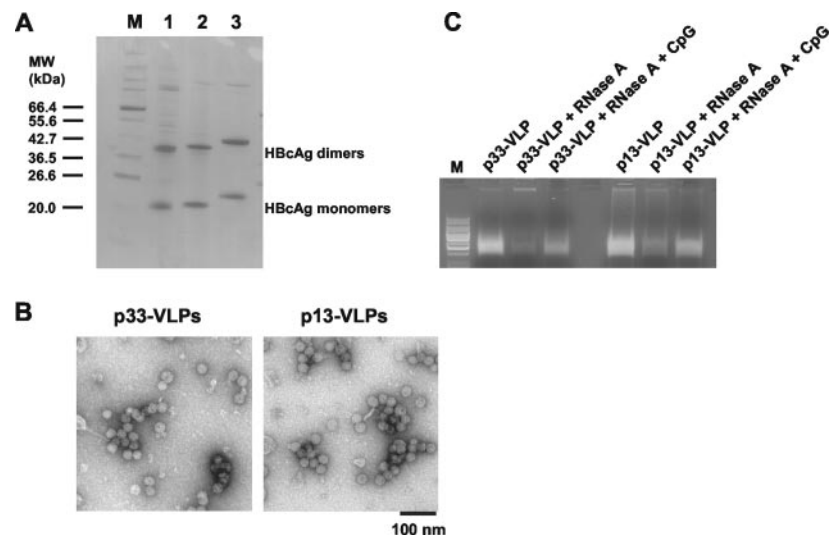
Assessment of antiviral immunity

C57BL/6 mice were immunized with titrated amounts of p33-VLPs packaged with CpGs. To examine antiviral immunity, vaccinated mice were infected i.v. 12 days after priming with 200 PFU LCMV strain WE. Five days later, spleens were isolated and LCMV titers were determined by a LCMV focus-forming assay, as described (42).

Quantification of p13/p33-specific IFN- γ -secreting T cells

Specific CD4⁺ and CD8⁺ T cells were quantified in IFN- γ ELISPOT assays by using whole splenocytes from immunized mice. Briefly, Multi-screen plates (Millipore, Billerica, MA) were coated with 10 μ g/ml anti-IFN- γ Ab (BD PharMingen), and single cell suspensions were diluted into plates starting from concentration of 5×10^5 cells/well. Cells were then incubated for 18 h at 37°C in the presence of the specific peptides (2.5×10^{-6} M). For the detection of IFN- γ spots, cells were removed (PBS, 0.05% Tween) and plates were incubated first with a biotinylated anti-IFN- γ Ab (BD PharMingen) (4 μ g/ml) and subsequently with streptavidin-conjugated alkaline phosphatase (Roche, Basel, Switzerland). Alkaline phosphatase substrate was finally added to obtain coloration of the spots (AP substrate kit; Bio-Rad, Hercules, CA).

FIGURE 1. Characterization of rHBcAg p33-VLPs and p13-VLPs. **A**, SDS-PAGE analysis (12% gel without antioxidants) of wild-type VLP (lane 1), mutated p33-VLP (lane 2), and p13-VLP (lane 3) monomers and dimers after gel filtration purification. The NEB Broad Range protein marker (NEB, Beverly, MA) was used as reference (lane M). **B**, Electron microscopy image of the VLPs. The magnification is indicated by the 100-nm bar. **C**, p33-VLPs and p13-VLPs were left untreated or treated first with RNase A for 3 h at 37°C and subsequently incubated with CpG-containing DNA oligonucleotides (ODN1668pt) for another 3 h. Analysis was performed by running the preparations on an agarose gel and by staining it with ethidium bromide. The gene ruler, 1-kb DNA ladder was used as reference (lane M).



Results

Recombinant p33-VLPs and p13-VLPs form structured capsid particles

The MHC class I-restricted p33 epitope and the MHC class II-restricted p13 epitope of the LCMV glycoprotein were genetically fused to the C terminus of the hepatitis B core Ag. The p33 was attached to the HBcAg sequence via a three-leucine linking sequence and the p13 via its original LCMV glycoprotein N-flanking region (sequence RSSGMV). The chimeric proteins were produced in *Escherichia coli* and purified by ammonium sulfate precipitation and subsequently by gel-filtration chromatography. The SDS-PAGE gel analysis of the purified preparations showed that the p33-VLP and p13-VLPs monomers and dimers had, as expected, a higher molecular mass (molecular mass of the monomeric forms: 22.7 and 24.0 kDa, respectively) than the wild-type HBcAg (molecular mass of the monomer: 21.4 kDa) (Fig. 1A). As confirmed by electron microscopy, the recombinant p33- and p13-containing HBcAg maintained the capacity to correctly fold and self-assemble into structured capsid particles with a diameter of ~ 30 nm (Fig. 1B).

In vitro and in vivo proliferation of CD4⁺ T cells compared with CD8⁺ T cells upon stimulation with VLPs

We have previously shown that DCs efficiently cross-present the LCMV-derived p33 peptide fused to the hepatitis B core Ag (24). To compare the efficiency of this cross-presentation with the classical MHC class II presentation pathway, p33- and p13-VLPs were purified and used to pulse CD11c⁺ DCs in vitro. T cells derived from TCR-transgenic mice expressing a TCR specific for the H-2 D^b-restricted peptide p33 (327 mouse (41)) and the H-2 I-A^b-restricted peptide p13 (SMARTA mouse (40)) were subsequently stimulated with the VLP-pulsed DCs, and proliferation of T lymphocytes was measured by a classical [³H]thymidine incorporation assay (Fig. 2A). Both types of VLPs efficiently stimulated proliferation of specific T cells even if CD8⁺ T cells needed ~ 4 – 5 times more p33-VLPs to reach the same extent of proliferation as CD4⁺ T cells upon stimulation with p13-VLPs. The ability of the VLPs to induce T cell proliferation in vivo was assessed next. P33- and p13-specific T cells were isolated from Ly-5.1⁺ TCR-transgenic mice, and after labeling with CFSE, 5×10^6 cells were adoptively transferred into Ly-5.2⁺ C57BL/6 mice, which were then immunized with titrated amounts of p33- and p13-VLPs (ranging from 100 to 0.1 μ g VLPs). Five days later, cells from draining lymph nodes were isolated and stained for the expression of CD8 or CD4 and Ly-5.1, allowing to specifically follow the transferred T cells (Fig. 2B). As observed in vitro, only 10 times more VLPs were required for T cell proliferation induced by cross-presentation vs regular MHC class II-associated presentation. Specifically, 10 μ g of p33-VLPs was required to drive significant proliferation (only 19% of undivided cells left), whereas a similar proliferation extent was observed with ~ 1 μ g of p13-VLPs for the CD4⁺ T cells (22% of undivided cells left). Thus, using these particular TCR-transgenic T cells, the in vivo Ag requirements for efficient CD4⁺ and CD8⁺ T cell proliferations differ so maximally by a factor of ~ 10 .

Minimal amounts of VLPs required for induction of T cell effector function

The efficiency of the peptide-stimulated T cell proliferation in the TCR-transgenic systems described above is dependent both on the extent of peptide presentation as well as on the affinity of the TCR for the peptide. Thus, it was possible that the affinity of the TCR-peptide/MHC class I interaction may bias the result. To exclude this possibility and use a less biologically artificial setup, we re-

peated the experiments in a nontransgenic system. Because both p33 and p13 peptides are immunodominant during the course of an LCMV infection, they may be viewed as immunologically representative MHC class I- and II-associated peptides, respectively. Although an exact repetition of the experiments in a nontransgenic system was not possible for practical reasons, we set out to determine the minimal amount of Ag required to induce a measurable CD8⁺ or CD4⁺ T cell response by vaccination with VLPs. However, the in vivo function of CD4⁺ and CD8⁺ T cells is different, and the chosen readout to measure T cell function may therefore be essential. A well-described function of CD8⁺ T cells is to protect

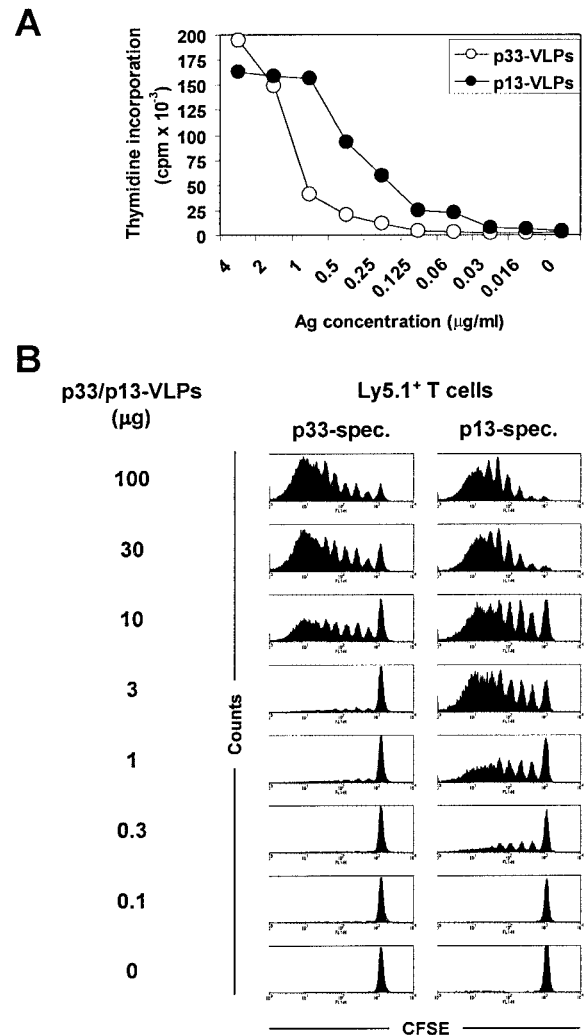


FIGURE 2. Detection of T cell proliferation after priming with titrated amounts of p33- and p13-VLPs. *A*, DCs isolated by collagenase D digestion and CD11c MACS beads purification were pulsed with 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.0312, and 0.0156 μ g/ml p33-VLPs or p13-VLPs for 4 h in IMDM medium (10% FCS). After extensive washing, presenter cells (10^5 cells/well) were cocultured with CD8⁺ and CD4⁺ Ag-specific T cells (10^5 cells/well) for 2 days, and proliferation was assessed by [³H]thymidine incorporation. Experiments were repeated by using different numbers of presenter cells. *B*, C57BL/6 recipient mice were transfused with CFSE-labeled Ly-5.1⁺ TCR-transgenic CD8⁺ or, alternatively, CD4⁺ T cells, and subsequently primed with Ag. Mice were s.c. primed with 100, 30, 10, 3, 1, 0.3, and 0.1 μ g of p33-VLPs or, alternatively, p13-VLPs. Draining lymph nodes were collected 5 days after Ag immunization, and flow cytometric analysis of adoptively transferred T cells was performed by gating on Ly-5.1⁺ cells. One representative experiment of three similar experiments is shown.

from LCMV infections by inducing cell lysis in a perforin-dependent fashion (44). Thus, protection from infection with LCMV is an excellent readout for CTL activity (45). In contrast, CD4⁺ T cells are inefficient at protecting from infection with LCMV (46), but are essential for Th cell-dependent IgG responses (47). Therefore, presence of VLP-specific IgG responses may serve as a readout for Th cell activity. These two readouts were used in a first set of experiments. Mice were s.c. immunized with p33-VLPs, and protection from LCMV infection was assessed. For this, 12 days after immunization, mice were i.v. challenged with LCMV (250 PFU) and after another 5 days, spleens were collected and assessed for viral titers (Fig. 3A). Despite efficient presentation of VLP-derived p33, no significant protection was induced by immunization with up to 100 μ g of p33-VLP if given alone, confirming earlier results (Fig. 3A) (43). These results indicate that efficient cross-presentation may not necessarily lead to cross-priming. Virus infection not only leads to endogenous production of viral proteins, but also to stimulation of the innate immune system, especially through cytokines and so-called pathogen-associated molecular patterns, which are recognized by Toll-like receptors (48). To mimic this situation, we activated DCs presenting the relevant peptide p33 in vivo. Under these conditions, protective immunity may be induced by vaccination with VLPs. Recombinantly produced HBcAg p33-VLPs carry RNA bound to arginine-rich repeats present between aa 149 and 183, which may be replaced by CpG-containing DNA oligomers (49). In contrast to VLPs containing RNA, VLPs containing CpGs are highly immunogenic for T cells, because CpGs trigger activation of DCs. Thus, p33- and p13-VLPs were packaged with CpG oligonucleotides by RNase A treatment, followed by incubation with chemically stabilized oligonucleotides containing phosphorothioate bonds (ODN1668pt) (Fig. 1C). Agarose gel analysis showed that the addition of oligonucleotides largely preserved the nucleic acid bands for both VLPs, indicating that the introduced mutations were not affecting the RNA/DNA-binding capacity of the rHBcAg.

C57BL/6 mice were vaccinated with titrated amounts of p33-VLPs packaged with CpGs (p33-VLP/CpG). Mice were immunized, as described above, and spleens were assessed for viral titers (Fig. 3B). Under these conditions, the minimal dose required for significant antiviral protection was 3 μ g of p33-VLPs. Note that wild-type VLPs given with CpGs (20 nmol) failed to induce protection, confirming that p33-VLP-induced protection is dependent on peptide p33 (Fig. 3C).

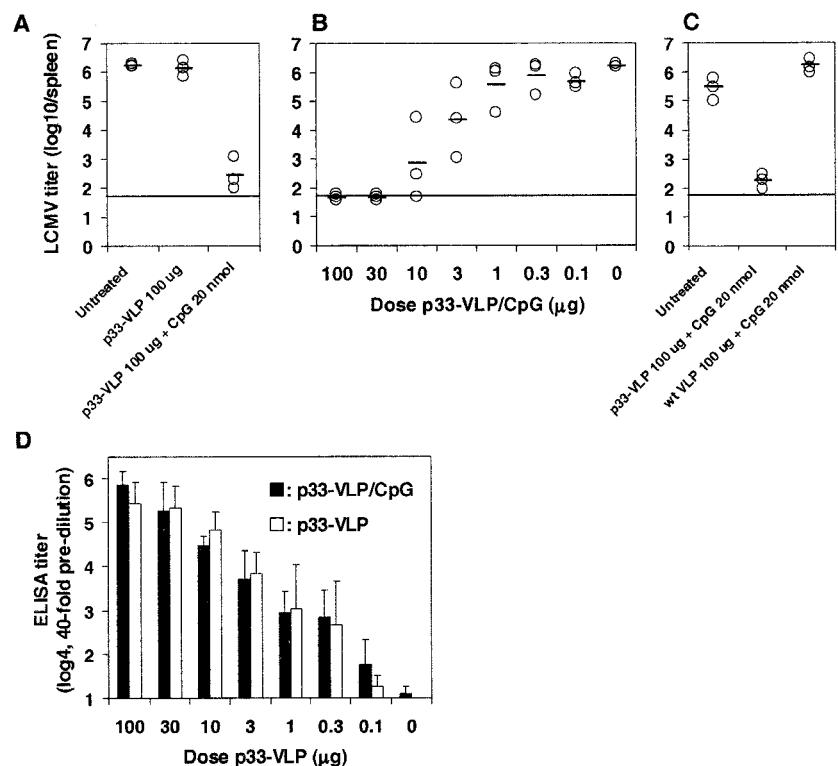
The ability of VLPs to induce Th cell-dependent IgG responses was assessed in comparison. Note that this particular assay is not specific for p13, because hepatitis B core particles already carry several Th epitopes in their sequence (for review, see Ref. 50). Mice were immunized with various doses of VLPs (with or without packaged CpGs), and Ab titers were measured 12 days later (Fig. 3D). Relevant IgG Ab titers were detected for 0.3 μ g of VLPs and higher doses, whereas titers induced by 0.1 μ g of VLPs were not significant. In striking contrast to the results obtained for CTLs, VLPs with or without CpGs were similarly effective at triggering Th cell-dependent IgG responses. Similar results were obtained with p13-VLPs (data not shown).

Taken together, the data suggest that CTLs need ~10 times more VLPs than Th cells to gain effector function in wild-type mice. Moreover, whereas strong CTL responses were induced only when VLPs were coadministered with CpGs, Th cell-dependent isotype switch was not enhanced by the presence of the adjuvant.

Minimal amounts of VLPs required to induce elevated frequencies of specific CD8⁺ T cells and CD4⁺ T cells

Although induction of antiviral protection and Th cell-dependent IgG responses may reflect the natural function of CD8⁺ and CD4⁺ T cells, the responses may nevertheless be difficult to compare. In particular, the sensitivity of the assays may not be the same. However, a common denominator of specific CD8⁺ and CD4⁺ T cells is their ability to expand and secrete cytokines upon immunization. Therefore, we assessed and compared side by side the frequencies

FIGURE 3. Induction of immune responses in wild-type mice with titrated amounts of p33-VLPs alone or packaged with CpGs. *A–C*, Protective CTL immunity against viral infection. Groups of C57BL/6 mice were primed s.c. with 100, 30, 10, 3, 1, 0.3, and 0.1 μ g of p33-VLPs alone or packaged with CpGs. Naive, untreated mice served as negative controls. As additional negative control, mice were immunized with 100 μ g of wild-type VLPs mixed with 20 nmol of CpGs (*C*). Twelve days after immunization, mice were i.v. challenged with LCMV strain WE (250 PFU). Five days later, spleens were isolated and viral titers were determined by a focus-forming assay. The detection limit of the assay is indicated by the continuous line in the diagrams. Dots represent individual mice; lines show median viral titers per group. One representative of two similar experiments is shown for *A* and *B*. *D*, Total IgG Ab responses against p33-VLPs. Groups of C57BL/6 mice were primed s.c. with 100, 30, 10, 3, 1, 0.3, and 0.1 μ g of p33-VLPs alone or packaged with CpGs. Naive, untreated mice served as negative controls. Twelve days after immunization, sera of mice were collected and assessed for IgG Ab titers against p33-VLPs. Values represent means and SDs obtained from three mice per group. One representative of two similar experiments is shown for p33-VLPs packaged with CpGs.



of IFN- γ -producing T cells induced by VLPs. Groups of C57BL/6 mice were immunized with titrated amounts of p33-VLPs or alternatively, p13-VLPs in the presence or absence of CpGs (Fig. 4, A–D). Additional mice i.v. infected with LCMV (250 PFU) or with recombinant vaccinia virus expressing the LCMV glycoprotein (1×10^6 PFU) served as control (Fig. 4, E and F). Ten days later, splenocytes were collected and restimulated in vitro with the specific peptides for 18 h, and frequencies of IFN- γ -producing cells were determined by ELISPOT assay. Interestingly, only when VLPs were coadministered with CpGs, significant numbers of IFN- γ -producing T cells could be detected, whereas stimulation with VLPs only did not generate detectable numbers of effector T cells (Fig. 4, A–D). Surprisingly, these results did not directly correlate with the previously analyzed Th-dependent anti-VLP Ab

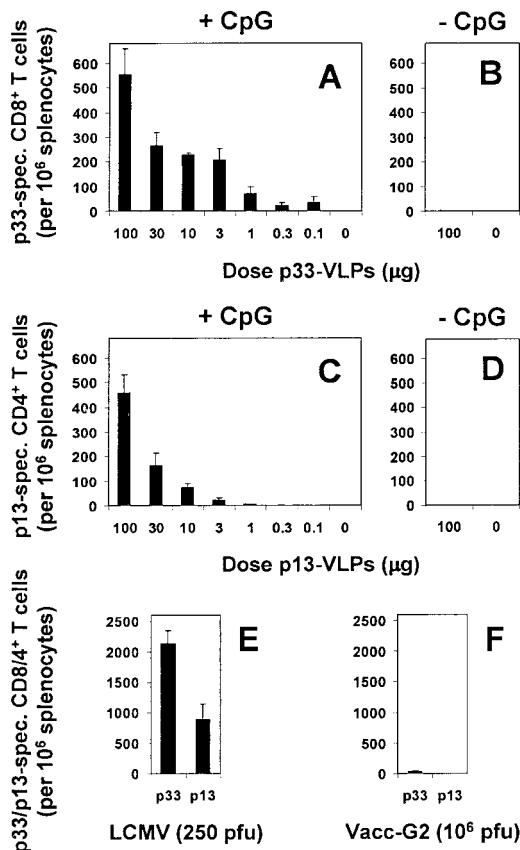


FIGURE 4. Frequencies of IFN- γ -producing CD8⁺ and CD4⁺ T cells in mice immunized with titrated amounts of p33-VLPs and p13-VLPs alone or in combination with CpGs. A–D, Groups of C57BL/6 mice were primed s.c. with 100, 30, 10, 3, 1, 0.3, and 0.1 μ g of p33-VLPs and p13-VLPs alone or mixed together with 10 nmol of CpGs. Naive, untreated mice served as negative controls. Ten days after immunization, splenocytes were collected and restimulated with the specific peptides (in Fig. 3, A and B, p33; in Fig. 3, C and D, p13) at a final concentration of 2.5×10^{-6} M for 18 h in vitro. Numbers of specific T cells expressing IFN- γ were determined by ELISPOT assays. Values represent means and SDs obtained from three mice per group. One representative of two similar experiments is shown for each panel. Note that numbers of IFN- γ -producing T cells were negligible in control experiments, in which T cells were stimulated in the absence of peptide. In addition, CpGs alone or wild-type VLPs did not induce measurable numbers of IFN- γ -producing T cells (data not shown). E and F, Mice were i.v. infected with 250 PFU LCMV virus (E) or with 1×10^6 PFU recombinant vaccinia virus expressing the LCMV glycoprotein (F), and frequencies of IFN- γ -producing T cells were assessed 10 days later by ELISPOT. One representative of two similar experiments is shown for each panel.

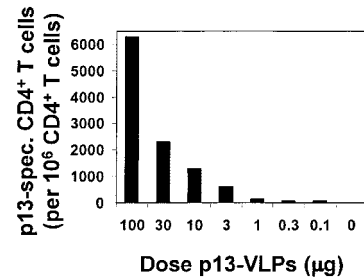


FIGURE 5. Frequencies of IFN- γ -producing CD4⁺ T cells in mice immunized with titrated amounts of p13-VLPs in combination with CpGs, upon in vitro restimulation with peptide-pulsed DCs. Groups of C57BL/6 mice were primed s.c. with 100, 30, 10, 3, 1, 0.3, and 0.1 μ g of p13-VLPs mixed together with 10 nmol of CpGs. Naive, untreated mice served as negative controls. Ten days after immunization, splenocytes obtained from three mice per group were pooled together and CD4⁺ T cells were purified by MACS beads separation and restimulated with p13 peptide-pulsed CD11c⁺ DCs (1×10^5 cells/well) for 18 h in vitro. Numbers of specific T cells expressing IFN- γ were determined by ELISPOT assays. Obtained values represent mean IFN- γ ⁺ CD4⁺ T lymphocyte frequencies in duplicate measurements.

immune responses, in which CpG did not seem to play a critical role for Th cell activation. These data suggest that for the induction of IFN- γ -producing inflammatory Th cells, CpG-dependent APC activation is required, while Th cell-dependent isotype switching was generated in the absence of DC activation. Moreover, the Ag titration curves indicated that similar, if not lower, VLP amounts were needed to generate significant CD8⁺ T cell responses compared with CD4⁺ T cells. To exclude that this was not due to inefficient stimulation of CD4⁺ T cells in vitro, we repeated the ELISPOT assay by using DCs as APCs. Splenic DCs were pulsed with p13 peptide present at high concentrations, i.e., 2.5×10^{-6} M, for at least 3 h and cocultured (1×10^5 CD11c⁺ DCs/well) with purified CD4⁺ Th cells (Fig. 5). Although frequencies of IFN- γ ⁺ CD4⁺ T cells were higher in absolute terms, the minimal amounts of p13-VLPs required to prime a measurable response were similar as observed when bulk splenocytes were used as APCs (Fig. 4C). Indeed, for both experimental setups, the limiting amount of Ag required to detect relevant effector Th cell responses was ~ 3 μ g/dose, a dose similar to the minimal amount of p33-VLPs required for CTL priming. Taken together, the results indicate that when mice are immunized with exogenous VLPs in combination with CpGs, comparable amounts of VLPs were required for the induction of a CTL response by cross-priming and a Th cell response by the classical exogenous Ag uptake and processing pathway.

Discussion

In this study, we estimated the efficiency of cross-presentation and cross-priming using VLPs. Rather than attempting to compare cross-presentation with the classical endogenous MHC class I pathway, we compared the efficiency of the two exogenous pathways of Ag presentation, namely cross-presentation and the MHC class II pathway. We found cross-presentation to be maximally 10-fold less efficient than the MHC class II pathway, indicating that peptides derived from exogenous viral particles are loaded on MHC class I and II molecules with similar efficiency in vivo.

Recombinantly produced VLPs have the typical structure of viruses, but do not carry genetic information and cannot infect target cells. VLPs are therefore typical exogenous Ags that have to be endocytosed/phagocytosed by DCs before processing. Thus, VLPs

behave like virions during a viral infection. Although such particles are known to be cross-presented, it has been a matter of debate whether such a process may be efficient enough to be biologically significant (51, 52). In marked contrast, it is generally appreciated that such exogenous Ags are the primary source of MHC class II-associated Ag presentation. Thus, for the generation of antiviral Th cell responses and protective IgG Ab titers, virions and perhaps material from lysed cells are feeding the MHC class II pathway. Consequently, MHC class II-associated presentation of exogenous virions or VLPs by DCs is certainly efficient enough to be biologically highly relevant. Consequently, the efficiency of the MHC class II pathway may be used as a benchmark for cross-presentation. Thus, our finding that both cross-presentation and cross-priming are maximally 10-fold less efficient than the MHC class II pathway supports the view that cross-presentation and cross-priming are highly efficient processes in vivo and may be expected to significantly contribute to the induction of antiviral CTL responses.

Moreover, the present study reveals a surprising discrepancy between cross-presentation and cross-priming. Specifically, p33-VLPs failed to induce sizeable CTL responses despite efficient presentation of p33 peptide in association with MHC class I. Only if APCs were concomitantly activated by CpGs, a potent T cell response was induced. These findings offer an explanation for the failure to detect cross-priming in some viral systems (4). In the past, in the absence of sensitive methods to determine presentation of peptides in association with MHC, cross-presentation could only be measured indirectly, by assessing induced T cell responses (i.e., cross-priming). Thus, a typical experiment was to compare CTL responses induced by live virus and inactivated virus (53–55). This almost always resulted in a failure to trigger a measurable CTL response by the inactivated virus. In contrast, inactivated virus preparations were still able to induce protective Th cell-dependent IgG responses. Therefore, inactivation of viruses resulted in absence of CTL responses, while Th cell responses were hardly affected. This led to the conclusion that exogenous Ags reach the MHC class II pathway, but fail to enter the MHC class I pathway. However, as shown in this work, presentation of relevant peptides is not sufficient for the induction of CTL responses, and activation of DCs via Toll-like receptors or cytokines is additionally required. During inactivation of viruses with β -propiolactone, formalin, or other cross-linking agents, the RNA/DNA of viruses is altered by the chemical agents. Thus, viruses lose the capacity to induce maturation of professional APCs because no dsRNA or viral DNA is produced upon immunization. Consequently, they may be cross-presented, but fail to generate functional CTLs. In contrast, the ability to induce Th cell-dependent IgG responses is shown in this work to be independent from stimuli triggering the innate immune system. Consequently, inactivation of viruses may not interfere with the generation of IgG responses (apart from the fact that the Ag load may be reduced due to absence of viral replication). Despite efficient induction of Th cell-dependent isotype switching, Th cell responses were dramatically reduced in the absence of CpGs if assessed by ELISPOT. This is consistent with the hypothesis that a distinct subset of Th cells may be responsible for isotype switching, which may not be measured easily by ELISPOT (56). In addition, these results are consistent with the observation that antiviral Th cell responses are usually not limiting for the generation of antiviral IgG responses (57).

Taken together, this study demonstrates that the exogenous pathway of MHC class I-associated Ag presentation is almost as efficient as the classical MHC class II pathway. This finding supports the conclusion that cross-presentation and cross-priming may be more important for the build-up of protective CTL responses during viral infections than thought by many.

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