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Liposome-encapsulated antigens induce a protective CTL response against *Listeria monocytogenes* independent of $CD4^+$ T cell help

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Abstract

Protection against intracellular pathogens is usually mediated by cytotoxic T lymphocytes (CTL). Induction of a protective CTL response for vaccination purposes has proven difficult because of the limited access of protein antigens or attenuated pathogens to the MHC class I presentation pathway. We show here that pH-sensitive PE/CHEMS liposomes can be used as a vehicle to efficiently deliver intact proteins for presentation by MHC class I. Mice immunized with listerial proteins encapsulated in such liposomes launched a strong CTL response and were protected against a subsequent challenge with *L. monocytogenes*. Remarkably, the CTL response was induced independently of detectable $CD4^+$ T cell help.

Introduction

Protective immunity against intracellular pathogens is usually mediated by cytotoxic T cells. However, the generally used method of immunization using inactivated pathogens or purified proteins mostly fails to elicit a protective CTL response since these antigens only have limited access to the MHC class I presentation pathway. Several experimental approaches have been described that circumvent this problem. For example, attenuated intracellular bacteria like *Salmonella* and *Listeria* have been used to deliver heterologous antigens into the cytosol of host cells [1-5] although living vaccine carriers might be considered problematic in terms of safety and general applicability. Alternatively, peptides, either in adjuvant or loaded onto dendritic cells, could be used to induce a CTL response [6, 7]. However, peptide vaccines would have to comprise a large number of different epitopes to ensure presentation by the excessive number of HLA alleles. Bacterial toxins have also been used to deliver intact proteins or peptides into the cytosol for MHC I-restricted presentation [8]. Nevertheless, questions remain with regard to the safety of bacterial toxins for the use in humans.

A safe alternative to deliver intact proteins to the cytosol of professional APC would be to entrap subunit vaccines into liposomes that have the property to target the MHC class I presentation pathway. Liposomes are artificial membrane vesicles with an enclosed aqueous compartment. Many different forms of liposomes are known depending on their chemical formulation and some are commercially available [9, 10]. Using liposome-encapsulated antigens, effective induction of antibody production, T cell proliferation and

subsequent protection have been demonstrated in different infection models, including viral [11-13], protozoal [14-17] and bacterial [18-21] pathogens. In these cases the immune response was driven by $CD4^+$ T cells. On the other hand, efficient induction of CTL responses *in vitro* and *in vivo* has been achieved by the use of pH-sensitive liposomes [22-27]. These vesicles are stable at neutral pH but disintegrate under mild acidic conditions e.g. in endosomes and lysosomes [28]. Such liposomes have been demonstrated to deliver encapsulated antigens into the cytosol of host cells, thus targeting them into the MHC class I presentation pathway [29, 30]. Immunization with tumor associated antigens encapsulated in acid labile liposomes partially protected mice against subsequent tumor challenge [31]. However, it remained to be shown that immunization with liposome-encapsulated proteins can effectively protect the host against intracellular pathogens. Therefore, we used *Listeria monocytogenes* as a model for an intracellular pathogen. Protection against *L. monocytogenes* is normally conferred by $CD8^+$ T cells. $CD4^+$ T cells provide only limited protection [32-34], and a protective role for antibodies is controversial [35-37]. Thus, efficient vaccination against this pathogen requires the induction of a strong CTL response.

We immunized mice with listeriolysin O (LLO), p60, or a combination of both entrapped in PE/CHEMS liposomes. Both antigens elicited strong CTL responses. Mice immunized this way were protected against a subsequent lethal challenge with *L. monocytogenes*. Interestingly, immunization with PE/CHEMS liposomes did not induce a detectable $CD4^+$ T cell response. In addition, a CTL response could also be induced in mice deficient for MHC class II. Consistent with findings described earlier no CTL memory response was

generated under these conditions.

Material and methods

Mice.

Female 6-8 weeks old BALB/c, C57Bl/6 and C3H/HeJ mice were obtained from Harlan (Borchen, Germany). MHC class II deficient mice on the C57Bl/6 genetic background [38] were kindly provided by Dr. Blüthmann (Hoffmann-La Roche, Basel, Switzerland).

Cell lines, bacterial strains, and antigens.

P815 (H-2^{d}), EL-4 (H-2b) and CH27 (H-2$^{d/k}$) cells were used as target cells in killer assays. *L. monocytogenes* EGDe was used for the challenge of mice. Bacteria derived from a culture at logarithmic growth phase were used. Bacterial number was determined by optical absorbance at 600 nm and confirmed by plating after each infection experiment. β-Gal was purchased from Boehringer Mannheim, Germany. LLO and p60 were purified as described elsewhere [39, 40], except that for purification of p60 a hyperexpression mutant was constructed. To this end, a hyperexpression vector containing *prf*A and the *act*A promoter driving *p60* was used to transform a variant strain of *L. monocytogenes* in which the complete *prf*A containing virulence gene cluster including *inlA* and *inlB* was deleted. The mutant LLO92A bears an amino acid exchange in the T cell epitope p91-99 preventing it from binding to H-2 K^{d}. The mutant was purified following the same protocol used for wild-type LLO [41].

Inclusion of antigens into pH-sensitive PE/CHEMS liposomes and immunization.

Phosphatidylethanolamine (PE; Sigma) and cholesterylhemi-succinate (CHEMS; Sigma) in

chloroform were mixed at a molar ratio of 2:1. Approximately 1 mg of the lipid mixture was dried under a stream of N_2 gas. Residual chloroform was removed in a vacuum dessicator for 1 hr. Each antigen was dissolved at a concentration of 100 µg/ml in PBS and added to the dried lipid film. All mixtures were vortexed for 1 min and incubated for 1 h at room temperature. After overnight hydration on a roller table at 4°C samples were sonicated using a micro probe. Free antigen was removed by gel filtration (G-50, Pharmacia). Usually, approx. 30 % of the initially used antigen was found entrapped in the liposomes. Liposomes were used immediately for immunization. Mice were injected i.p. with 100 µl of the liposome preparation.

Functional T cell assays.

For proliferation assays spleen cells from immunized mice were isolated and seeded in 96 well plates at $5x10^5$ cells /well. Antigen concentrations were as described in Figure legends. After 48 hours, 1 µCi ^{3}H-Thymidine/well was added and proliferation was measured after 72 hours. Cytotoxicity was measured using the JAM killer assay [42]. Briefly, $3x10^5$ target cells were labeled for 4 hours with 5 µCi ^{3}H-Thymidine. Cells were washed and incubated with LLO92A or LLO (0,5 µg/ml) plus passenger antigen (p60 or ß-Gal at 5 µg/ml) for 10 minutes and washed again. EL4 cells were sensitized using 1 µg/ml SIINFEKL peptide. For measurement of LLO-specific killing LLO p91-99 peptide was directly included in the killer assay. Targets were seeded at 10,000 cells/well and incubated with different amounts of effector cells for 4 hours. Killing of target cells results in DNA fragmentation. This leads to a decreased number of counts when the cells are harvested

onto glass fibre filters and measured in a standard β-scintillation counter, and can be used to determine the specific killing according to the following formula: % killing = (1-cpm/cpm_{max}) x 100, where cpm_{max} represents counts of labeled target without effector cells.

Results

Proteins encapsulated in PE/CHEMS liposomes induce a specific CTL response in vivo. Sterile eradication of an infection by *L. monocytogenes* requires the induction of $CD8^+$ T cells specific for secreted listerial proteins [34, 43]. To test whether PE/CHEMS liposomes can be used as antigen delivery vehicles for the induction of a protective CTL response, BALB/c mice were injected intraperitoneally (i.p.) with 100 µl liposomes containing approximately 3 µg of either LLO, the major virulence factor of *L. monocytogenes*, or p60, a housekeeping murein-hydrolase for which homologues exist in all listerial species. Both proteins are known to be protective antigens [44, 45]. Spleen cells from immunized animals were isolated on day 9 and used as effectors in an *ex vivo* killer assay with antigen-sensitized P815 target cells. A strong, antigen-specific CTL activity could be readily detected in the spleen of mice after immunization with LLO, p60, or the control antigen β-Galactosidase (β-Gal) (Figure 1A). Similar results were found when mesenteric lymph node cells were used as effector cells (data not shown).

Due to its pore-forming activity, LLO itself is able to enter the cytosol and even shuttle passenger proteins to the MHC class I presentation pathway [46]. It has also been shown that LLO enclosed in pH-sensitive liposomes can facilitate delivery of co-encapsulated proteins into the cytosol [47-49]. To test whether the CTL response against p60 could be enhanced by co-encapsulation with LLO, we immunized BALB/c mice with liposomes loaded with p60, LLO, or a mixture of both. As shown in Figure 1B, the mixture of LLO

and p60 induced CTL specific for either protein. However, spleen cells from animals that had been immunized with p60 alone lysed p60-sensitized target cells to a similar degree. This demonstrates that PE/CHEMS liposomes are highly efficient antigen delivery vehicles for MHC class I-restricted presentation. Addition of pore-forming molecules such as LLO does not further enhance the induction of the CTL response under these conditions.

The lipids used for vesicle formulation might be responsible for the cytosolic delivery of the encapsulated antigen or simply act as strong adjuvants that result in the induction of CTL responses against soluble proteins. Therefore, to investigate whether encapsulation of the antigen was in fact necessary for delivery into the MHC class I presentation pathway we injected soluble β-Gal alone or together with empty liposomes intraperitoneally. As shown in Figure 1C, only injection of β-Gal encapsulated into liposomes elicited a CTL response against this protein while the other forms of delivery did not.

Immunization with liposome-encapsulated antigen confers protective immunity.

To test whether immunization with listerial proteins encapsulated in PE/CHEMS liposomes conferred protection against a subsequent challenge with *L. monocytogenes*, BALB/c mice were immunized with encapsulated LLO or p60 and challenged intravenously on day 9 after immunization with $5x10^3$ ($1xLD_{50}$) *L. monocytogenes*. Three days later, animals were sacrificed and the numbers of bacteria in liver and spleen were determined. Mice, immunized with liposome-encapsulated LLO or p60 displayed an approximately 200- to 600-fold reduction of bacterial numbers in the spleen and a 5000- to 10000-fold reduction

in the liver (Figure 2). Immunization with a mixture of LLO and p60 did not further reduce the bacterial counts.

In addition, we used a mutated form of LLO that bears an amino acid exchange in the dominant T cell epitope (LLO92A). Due to this mutation LLO92A does not induce a CTL response, but it retains its hemolytic activity and the ability to shuttle antigens into the cytosol [41]. As expected, no protection, as measured by bacterial counts, was conferred by immunization with LLO92A (Figure 2). In contrast, when mice were immunized with a mixture of LLO92A and p60, protection was restored. Protection was not increased compared to immunizing with encapsulated p60 alone. This confirms that LLO is not required to deliver the encapsulated antigen into the MHC class I presentation pathway *in vivo*, nor does it enhance the protective CTL response by acting as an adjuvant.

Immunization with liposome-encapsulated LLO or p60 also protected mice against a lethal challenge (5xLD_{50}) with *L. monocytogenes* (Figure 3A). While all control mice immunized with liposomes containing ß-Gal died within 3 days, 60 % or 80 % of mice immunized with liposome encapsulated LLO or p60, respectively, survived.

It was possible that immunization with more than one antigen might result in enhanced protection, as had been described [50], but could not be detected under the experimental conditions used so far. We therefore repeated the immunization with p60 and p60 plus LLO, and challenged the immunized mice with a high dose of *L. monocytogenes*

($25xLD_{50}$). As shown in Figure 3B, at this high dose the survival of animals immunized with p60 was lower as compared to mice that had been challenged with lower numbers of Listeria (Figure 3A). However, co-immunization with both LLO and p60 did not improve the survival rate. This confirmed that a maximal protective CTL response was elicited under these conditions independent of whether one or two antigens were used.

PE/CHEMS liposomes induce a CTL response but no detectable CD4 T cells response

The induction of an efficient CTL response usually depends on CD4 T cell help and it has been shown that many liposome formulations induce T helper cell responses. To test whether a $CD4^+$ T cell response was elicited with the liposome formulation used in this study, we immunized mice with p60, a mixture of p60 plus LLO or β-Gal, either encapsulated in liposomes or emulsified in incomplete Freund's adjuvant (IFA). On day 9 after immunization, spleen cells were isolated and used as effector cells in a killer assay to measure the CTL response. In addition, to assess the T helper response, spleen cells were stimulated with 1 μg/ml of purified p60 and β-Gal or 0.5μg/ml of LLO, respectively, and the proliferation was measured. As expected, immunization with antigen in IFA induced a strong proliferative response (over 125.000 cpm) suggesting an expansion of CD4 T cells in all cases (Figure 4, upper left panel). CTL induction could only be detected when p60 was injected together with LLO (Figure 4, lower left panel). This is caused by the ability of LLO to obtain access to the cytosol and to shuttle other proteins into this compartment [46]. The CTL induced in this way recognized both LLO and p60. In contrast, immunization with liposome-encapsulated antigens resulted in a strong CTL response against all antigens or antigen mixtures used (Figure 4, lower right panel). Interestingly, no proliferative

response against the proteins could be detected upon restimulation with 0.5 or 1µg/ml of antigen *in vitro* when mice were immunized with antigens enclosed in PE/CHEMS liposomes. This indicates that antigen delivered by such liposomes induced no or only a very weak $CD4^+$ T cell response (Figure 4, upper right panel).

These results suggested that immunization with PE/CHEMS liposomes induced a protective CTL response independently of a detectable CD4 T cell help. To confirm that PE/CHEMS liposomes can indeed induce a CTL response in the absence of CD4 T cell help, we used MHC class II-deficient mice, which have greatly reduced numbers of peripheral $CD4^+$ T cells [38]. The animals were immunized with liposome-encapsulated ovalbumin (OVA), and OVA-specific lysis of EL-4 target cells was measured. As shown in Figure 5A, OVA-specific killing was equal in immunized wild type and MHC class II-deficient mice. The absence of appropriate $CD4^+$ T help in such MHC class II deficient mice could be demonstrated by immunization with OVA in complete Freund's adjuvant. No OVA-specific antibodies were detected in the sera of immunized MHC class II-deficient mice while WT mice readily responded (Figure 5B).

A possible explanation for the highly efficient CTL induction by our liposomes could be an adjuvant effect by bacterial endotoxins possibly contaminating the liposome formulation. To exclude this possibility, we used C3H/HeJ mice that have a defect in the Toll like receptor 4 [51] and therefore should be unresponsive to such a contamination. Immunization of these mice with β-Gal encapsulated in PE/CHEMS liposomes resulted in

a strong and specific CTL response (Figure 6). Thus, CTL induction is intrinsic to the liposomes and not due to contaminating endotoxin. Taken together, these data firmly demonstrate that pH-sensitive liposomes are able to induce a protective CTL response in absence of $CD4^+$ T cell help.

The CTL response generated in the absence of $CD4^+$ T cell help is short-lived.

Our data demonstrate that immunization with PE/CHEMS liposomes efficiently induced a CTL response in the absence of any detectable $CD4^+$ T cell help. However, it has been shown that $CD4^+$ T cell help is essential in generating CTL memory [52-54]. We therefore tested whether immunization with PE/CHEMS liposomes would induce long-term protection against *L. monocytogenes*. Part of the groups of mice that were immunized with liposomes alone or with liposomes containing both LLO and p60 and tested for Figure 2 were kept for the long term experiment. Six months after immunization, these mice were challenged, and bacterial counts in spleen and liver were determined three days later. As shown in Figure 7, at this time-point, immunized mice were no longer protected against *L. monocytogenes* infection. Bacterial counts in spleens and livers of immunized mice were similar to those of controls. Similarly, mice were also no longer protected when the challenge was carried out three month after immunization. Therefore, consistent with earlier findings [52-54], the strong helper T cell independent CTL response induced by PE/CHEMS liposomes was short-lived.

Discussion

The design of vaccines requires controlled induction of the appropriate immune response. To date, commonly used vaccines rely on the induction of neutralizing antibodies along with a helper T cell response. However, protection against many intracellular viral, bacterial, or protozoal pathogens is conferred by cytolytic T cells. Therefore, vaccines against those pathogens should be able to induce an efficient CTL response.

Our results clearly demonstrate that pH-sensitive PE/CHEMS liposomes are a very efficient tool for inducing a protective CTL response. While certain pH-sensitive liposomes have been used before to induce CTL responses *in vitro* and *in vivo*, this is the first report demonstrating a protective effect in an infection model. The PE/CHEMS liposomes that we have used in this study induced strong CTL responses against all antigens tested. Thus, the liposome formulation might be particularly effective for delivery of proteins to the cytosol.

While inducing a strong CTL response, immunization with PE/CHEMS liposomes did not elicit any detectable $CD4^+$ T cell response, as judged by the lack of proliferation, nor did it induce antibody production (data not shown). Presumably, the loaded antigens were delivered to the MHC class I processing pathway of antigen-presenting cells in a highly efficient way. Cytological studies have revealed that proteins included in multilamellar liposomes were concentrated mainly in the trans-Golgi area of APC [55, 56], consistent with an enrichment in endosomes and lysosomes. Thus, the proteins encapsulated in acid-

sensitive liposomes might be released efficiently in compartments of APC i.e. dendritic cells that have been shown to possess the ability to transport proteins into the cytosol [57].

While pH-sensitive liposomes have been shown to deliver certain antigens for MHC class I-restricted presentation [22-27], in some cases delivery for the MHC class II-restricted presentation and subsequent stimulation of CD4 T cells have been described with pH-sensitive DOPE/PHC liposomes in vitro [58]. The same liposomes also delivered antigen for MHC class I-restricted presentation, but with relatively low efficiency [59]. At first it might appear surprising that we could not detect induction of a CD4 T cells response under our conditions. However, the composition of the liposomes used in our study differs from the one used by Harding et al. It is possible that PE/CHEMS liposomes release their cargo more efficiently into the cytosol than DOPE/PHC liposomes. More importantly, mounting an efficient CD4 T cell response *in vivo* might require a particular activation of the APC. This might not be achieved by our liposomes. Alternatively, the liposomes might target special APCs *in vivo* that favor MHC class I presentation. More elaborate studies will be needed to distinguish between these possibilities.

The question remains, how a CTL response could be raised without inducing a strong $CD4^+$ T cell helper response at the same time or even in the absence of MHC class II. Similar phenomena have been described in different experimental systems [60, 61]. According to the current model of APC "licensing", antigen-presenting cells are activated by helper T cells to subsequently prime CTL responses. However, activation of APC could also be

achieved by other signals rendering those cells capable of priming CTL responses without CD4$^+$ T cell help [62]. In preliminary experiments, we observed upregulation of the costimulatory molecules B7-1 and B7-2 on macrophages incubated *in vitro* with the liposomes used in this study (data not shown). Thus, activation of antigen-presenting cells by liposomal lipids might provide one explanation for CTL priming without the concomitant induction of a significant CD4 T cell response. On the other hand, we cannot exclude the possible involvement of other cell populations like NKT cells, which have been demonstrated to provide help in priming killer cells in the absence of conventional CD4$^+$ T cells [63].

It has recently been demonstrated that a primary CTL response against *L. monocytogenes* can be generated in the absence of CD4$^+$ T cells. However, the formation of a memory response was dependent on CD4$^+$ T cell help [52-54]. The CTL response induced in our system was short-lived and a protective effect could not be detected six months after immunization. This is in line with the above findings.

Our experiments also imply that an effective vaccine used in outbred populations (for human or veterinary use) should contain several different pathogen-derived proteins to enhance the probability that a subunit vaccine contains at least one epitope that can be presented to and recognized by host T cells. The number of possible T cell epitopes contained in a given protein sequence is strongly restricted by the MHC class I haplotype. This is demonstrated in our model system. LLO possesses only one epitope that binds to H-

2 K^d and in p60, three K^d epitopes are found [64]. The mutant LLO92A did not confer protection due to the mutated T cell epitope. However, if LLO92A was administered together with p60, the epitopes derived from the latter were sufficient to induce a protective CTL response. On the other hand, application of wild-type LLO plus p60 did not enhance protection, suggesting that protection can be fully induced already by a single epitope recognized.

Taken together, our data demonstrate that the use of PE/CHEMS liposomes as vaccine delivery system is a highly efficient way to induce CTL responses. Already a single application of encapsulated listerial antigens reduces the bacterial load by several orders of magnitude upon challenge with *L. monocytogenes*. The inability of the system to activate $CD4^+$ T helper cells does not hamper the efficient induction of CTL, although, it interferes with the induction or maintenance of memory of CTLs. To avoid this problem, liposomes that induce helper T cells could be admixed with our liposomes. Alternatively, additional adjuvants could be co-encapsulated into the PE/CHEMS liposomes. For instance poly I:C is known to induce cytokines that support the induction and maintenance of CTL memory [65]. Thus, we consider our findings as a step towards the development of subunit vaccines that can be used for the efficient induction of protective CTL responses against intracellular pathogens or tumors.

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Figure legends

Figure 1. Liposome-encapsulated antigens induce CTL responses in vivo.

BALB/c mice (2 mice/group) were immunized i.p. with 100 µl liposomes containing approximately 3 µg of each antigen shown. (A) CTL response in the spleen. Nine days after immunization, spleen cells were isolated and used as effector cells in a JAM killer assay. An effector:target ratio of 50:1 is shown. P815 target cells were sensitized for LLO-specific killing by incubation with the peptide LLO p91-99 (black bars). To measure p60-or β-Gal-specific killing, target cells were incubated with the mutant LLO92A + p60 (white bars) or LLO92A+β-Gal (grey bars). By this method p60 or β-Gal are shuttled into the MHC class I presentation pathway. Because of the mutation in the CTL epitope of LLO, only killing specific for the passenger protein is detected [41]. When LLO92A alone was used to label target cells, killing was always at background level (not shown). The assay was done in triplicates with the same results for both mice of each group (only one mouse/group is shown). Similar results were obtained with effector cells from mesenteric lymph nodes isolated 17 days after immunization (not shown). These experiments were performed three times with identical results. (B) LLO, p60, or mixtures of p60 with LLO, included in liposomes, were used for immunization. CTL activity of spleen cells against LLO and p60 was determined as in (A). (C) Mice were immunized with β-Gal included in liposomes, β-Gal in soluble form or β-Gal and liposomes injected into different sites of the peritoneum. β-Gal-specific killing of mesenteric lymph node cells was determined 17 days after immunization. Killing without antigen was below 10 % in all experiments. Immunization

with liposomes alone did not result in induction of CTL (not shown). All *in vitro* assays were carried out in triplicates. SD was below 10%. Repetitions of the experiment provided similar results.

Figure 2. Protection of immunized mice against challenge by Listeria monocytogenes.

BALB/c mice (4 mice/group) were immunized as described in Fig.1. On day 9 after immunization mice were challenged with $5x10^3$ ($1xLD_{50}$) *L. monocytogenes*. 3 days later, animals were sacrificed and the number of colony-forming units in liver and spleen was determined. Symbols indicate values obtained from individual mice; mean values are indicated by the crossbar. The experiment was performed three times with similar results.

Figure 3. Survival of immunized mice after challenge with L. monocytogenes.

(A) BALB/c mice (6/group) were immunized with liposomes containing p60, LLO, or ß-Gal as a control. After challenge on day 9 with $2,5x10^4$ *L. monocytogenes* ($5xLD_{50}$), the number of surviving mice was observed every day. (B) BALB/c mice (5/group) were immunized as shown and challenged on day 9 with $1,25x10^5$ *L. monocytogenes* ($25xLD_{50}$).

Figure 4. PE/CHEMS liposomes elicit a CTL, but no detectable helper T cell response.

BALB/c mice (2/group) were immunized with 10 µg of p60, p60 + LLO, or ß-Gal, either emulsified in IFA (left panels) or encapsulated in liposomes (right panels). On day 9, spleen cells were isolated and tested in proliferation and killer assays. For proliferation

(upper panels), spleen cells were incubated with LLO (0,5 µg/ml, black bars), 1 µg/ml p60 (white bars), 1 µg/ml ß-Gal (grey bars), or left without antigen. Proliferation was measured after 72 hours. Proliferation of spleen cells from mice injected with IFA only and cultured with antigen was always below 3000 cpm (not shown). Proliferation of immunized mice when antigen was left out was below 3000 cpm as well. For killer assays, P815 target cells were sensitized by incubation with LLO_{91-99} (black bars), LLO92A + p60 (white bars), or LLO92A + β-Gal (grey bars) to introduce p60 or β-Gal into the MHC class I presentation pathway. Concentration of passenger proteins was 5 µg/ml. The effector : target ratio was 50:1. Unspecific killing without antigen was always below 2 % (not shown). All conditions of killer and proliferation assay were done in triplicates. Results are shown for 1 mouse of each group but were the similar for both mice.

Figure 5. PE/CHEMS liposomes induce a CTL response in the absence of T cell help.

(A) Wild type or MHC II-deficient mice were immunized with liposome-encapsulated OVA. On day 9, mesenteric lymph nodes were isolated and the OVA-specific CTL response was measured in a JAM assay using EL-4 cells as targets and SIINFEKL as antigenic peptide. An effector:target ratio of 50:1 is displayed. (B) WT and MHC class II deficient mice were immunized with OVA in emulsified CFA and sera from immunized mice were tested by ELISA for the presence of OVA-specific antibodies. All assays were carried out in triplicates.

Figure 6. Induction of a CTL response in endotoxin-resistant C3H mice.

C3H/HeJ mice (3 mice/group) were immunized i.p. with liposomes containing β-Gal or empty liposomes (PBS) as a control. On day 9, mesenteric lymph nodes were harvested and β-Gal specific killing was measured in a JAM assay at different effector : target ratios using CH27 cells loaded with LLO plus β-Gal. The mean and standard error of 3 individual mice are shown. All *in vitro* assays were carried out in triplicates.

Figure 7. Lack of long term CTL-memory after immunization with PE/CHEMS liposomes.

BALB/c mice (3 mice/group) were immunized with liposomes alone (black bars) or with liposomes containing LLO and p60 as described in Figure 1. Actually, these mice were immunized as part of the groups shown in the short term protection experiment displayed in figure 2. Six months after immunization, mice were challenged with $5x10^3$ *L. monocytogenes*. Three days later, the mice were sacrificed and the number of colony-forming units in liver and spleen was determined.

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