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CD4 T cell help is required for primary CD8 T cell responses to vesicular antigen delivered to dendritic cells *in vivo*

Karine Serre[*1,2,3], *Laurent Giraudo*[1,2,3], *Carole Siret*[1,2,3], *Lee Leserman*[1,2,3,4] *and Patrick Machy*[1,2,3]

[1] Centre d'Immunologie de Marseille-Luminy, Université de la Méditerranée, Marseille, France
[2] INSERM, U631, Marseille, France
[3] CNRS, UMR, 6102, Marseille, France
[4] CNRS Groupement de Recherche 2352 "Immunociblage des Tumeurs", Marseille, France

Insight into the mechanisms by which dendritic cells (DC) present exogenous antigen to T cells is of major importance in the design of vaccines. We examined the effectiveness of free antigen as well as antigen with lipopolysaccharide, emulsified in complete Freund's adjuvant, and antigen encapsulated in liposomes in activating adoptively transferred antigen-specific CD4 and CD8 T cells. When contained in liposomes, 100- to 1000-fold lower antigen amounts were as efficient in inducing proliferation and effector functions of CD4 and CD8 T cells in draining lymph nodes as other antigen forms. $CD11c^+$/$CD11b^+$/$CD205^{mod}$/$CD8\alpha^-$ DC that captured liposomes were activated and presented this form of antigen in an MHC class I- and class II-restricted manner. CD4 T cells differentiated into Th1 and Th2 effector cells. Primary expansion and cytotoxic activity of CD8 T cells were CD4 T cell-dependent and required the transporter associated with antigen processing (TAP). Finally, adoptively transferred CD4 and CD8 T cells were not deleted after primary immunization and rapidly responded to a secondary immunization with antigen-containing liposomes. In conclusion, encapsulation of antigen in liposomes is an efficient way of delivering antigen to DC for priming of both CD4 and CD8 T cell responses. Importantly, primary CD8 T cell responses were CD4 T cell-dependent.

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Introduction

To develop better vaccines, it is important to understand how dendritic cells (DC) recognize Ag and present Ag-derived peptide/MHC complexes to CD4 [1] and CD8 [2, 3] T cells. In some instances targeting Ag to DC augments the immune response to the Ag [4, 5], while in other circumstances tolerance is induced [4, 6, 7]. Productive responses to Ag depend on appropriate activation of DC, since constitutive presentation of self Ag by DC to T cells in the absence of activating signals is thought to induce tolerance [8]. An important consideration with regard to responses to exogenous Ag includes the efficiency with which Ag is taken up and presented to T cells by DC. Liposomes are useful agents

Correspondence: Dr. Patrick Machy, CIML, Université de la Méditerranée, Case 906, 13288 Marseille Cedex 09, France
Fax: +33-491269430
E-mail: machy@ciml.univ-mrs.fr
Abbreviation: **CF:** carboxyfluorescein

* **Current address:** The Medical Research Council Centre for Immune Regulation, The Medical School IBR, Immunity and Infection Division, University of Birmingham, Wolfson Drive, Edgbaston, Birmingham B15 2TT, UK

for studying Ag acquisition and presentation by DC, as they are inert and may be generated to contain a high concentration of Ag that is protected from rapid degradation and dispersion. Liposomal formulations of protein or peptide vaccines are effective in inducing anti-viral and anti-tumor immune responses [9–11].

CD4 T cells play a major role in CD8 T cell responses, "licensing" DC to be effective CD8 T cell stimulators [12–14]. Many models using cell-based Ag [15], viruses [16] and peptides [17] have revealed that CD4 T cells are necessary for the induction of primary effector CD8 T cell responses *in vivo*. Despite these studies, the role of CD4 help in driving primary CTL immune responses has been challenged recently. Reports have dissociated their capacity to activate DC from CD4 help that provokes CD8 T cells to differentiate into memory cells and CD4 help that maintains CD8 memory T cells [18–21]. Even so, detailed information on the CD4 T cell dependence of CTL responses to different forms of exogenous Ag and how CD8 T cells proliferate and acquire cytotoxic activity *in vivo* is still lacking.

Here we have compared *in vivo* immune responses to exogenous free Ag, alone or associated with agents known for their inflammatory activity (the endotoxin LPS or CFA) or their carrier activity (liposomes). We have investigated whether these formulations, when injected subcutaneously (s.c.), induce DC in draining LN to stimulate adoptively transferred Ag-specific CD4 and CD8 T cells. At low doses of Ag, liposomes were much more efficient than the other forms of Ag in activating both CD4 and CD8 T cells. $CD11c^+/CD11b^+$ DC captured Ag-containing liposomes and were most likely responsible for priming of CD4 T cells, leading to the generation of Th1 and Th2 effector cells. Moreover, we found that CD4 T cells that recognize their cognate Ag play an early and major role in inducing proliferation of and acquisition of cytotoxic functions by CD8 T cells. Finally, capture of liposomes by DC did not lead to a state of tolerance, as transferred CD4 and CD8 T cells were able to respond to a second Ag injection. Our results show that Ag-containing liposomes efficiently deliver Ag to DC *in vivo*. These cells are then activated and, as a consequence, drive expansion of CD4 and CD8 effector T cells in primary and secondary immune responses. Importantly, primary expansion of CD8 T cells was CD4 T cell-dependent.

Results

Ag encapsulated in liposomes is very efficient in activating specific CD4 T cells *in vivo*

To assess the efficiency of different forms of Ag in activating T cells *in vivo*, CFSE-labeled HEL-specific (3A9) or OVA-specific (OT-II) CD4 T cells were adoptively transferred into mice. Different forms of HEL or OVA were injected into the hind footpads of recipient mice, and T cell proliferation was monitored 3 days later in the draining popliteal LN. The extent of cell proliferation was directly related to the amount of Ag administered (Fig. 1A), as revealed by the percentage of specific T cells that were in division (Fig. 1B, C). Ag contained in liposomes induced 3A9 and OT-II T cell proliferation at nanogram doses, which is 100- to 1000-fold lower than the amount of Ag required to induce an equivalent response when administered in other forms (free or in the presence of LPS or CFA). Empty liposomes or irrelevant Ag (BSA)-containing liposomes did not drive 3A9 or OT-II cell proliferation (not shown). These results show that vesicular Ag is very effective in inducing CD4 T cell proliferation.

Fc receptors do not play a role in acquisition and presentation of liposome-encapsulated Ag *in vivo*

Fc receptors have been implicated in the efficient uptake and presentation of IgG-opsonized liposomes by DC *in vitro* [22] and also target presentation of immune complexes *in vivo* [23]. To investigate whether FcR are involved in liposome uptake, we examined proliferation of adoptively transferred OT-II T cells in $IgM^{-/-}$ mice or WT mice immunized with OVA-containing liposomes. $IgM^{-/-}$ mice are deficient for the μ chain of the B cell receptor and have no B cells and thus no circulating Ab [24]. Liposome-encapsulated OVA induced OT-II cell proliferation in $IgM^{-/-}$ animals as efficiently as in WT animals (Fig. 1D). Therefore, IgG FcR play a negligible role in the acquisition of s.c. injected liposomes for Ag presentation to CD4 T cells in draining LN. In addition, these results show that B cells are not responsible for the induction of primary CD4 T cell proliferation after immunization with Ag encapsulated in liposomes.

Liposomes are efficient at inducing a Th1- and Th2-type mixed immune response

We next analyzed the effectiveness of liposomes in generating effector CD4 T cells, defined as cells having up-regulated mRNA specific for cytokines such as IFN-γ (Th1) and IL-4 (Th2). OT-II cells were transferred into mice that were immunized with OVA-containing liposomes. At day 6, popliteal LN cells were separated into two cell fractions, $CD45.1^-/CD4$ T cells (host CD4 T cells) and $CD45.1^+/CD4$ T cells (OT-II) (Fig. 2A), and expression of cytokines was evaluated with cDNA extracted from each of these cell fractions (Fig. 2B). IFN-γ and IL-4 mRNA were expressed in OT-II cells at levels that were on average 6- and 3-fold higher, respectively, than the levels in host CD4 T cells. IFN-γ

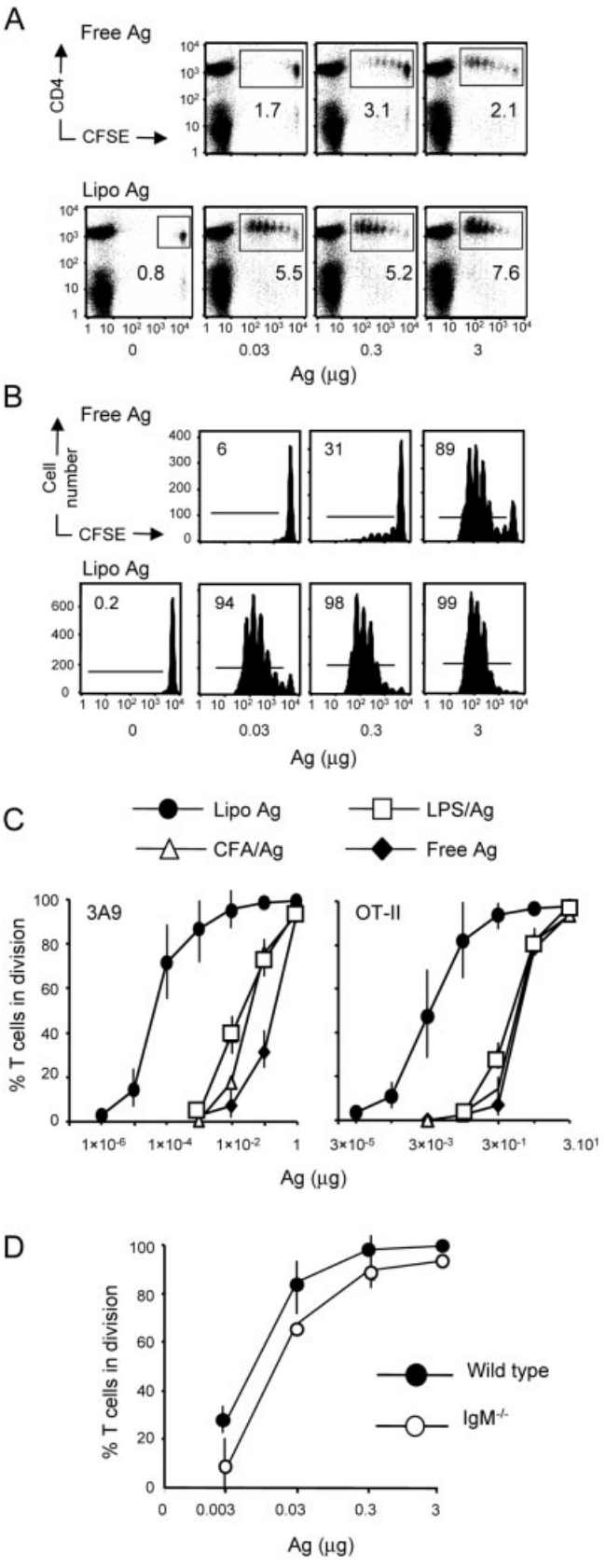

Figure 1. Proliferation of CD4 T cells in draining LN after injection of different forms of the same Ag: in PBS (free Ag), together with LPS or CFA, or encapsulated in liposomes (Lipo Ag). CBA/J or B6 mice received 2×10^6 CFSE-labeled 3A9 or OT-II cells, respectively. Various concentrations of different forms of HEL or OVA were then injected into footpads. 3A9 or OT-II cell proliferation was analyzed 3 days later. (A) Dot plots from LN cell suspensions. The percentage of 3A9 among total cells is indicated. (B) Histograms of CFSE dilution gated on 3A9 cells. The percentage of 3A9 cells that have undergone at least one division is indicated. (C) 3A9 or OT-II cell responses are plotted as a function of the Ag dose and form. The percentages are calculated as in (B). Results are representative of three experiments. (D) OT-II proliferative responses, as a function of the Ag dose administered in liposomes, in WT or $IgM^{-/-}$ mice. Results are representative of two experiments.

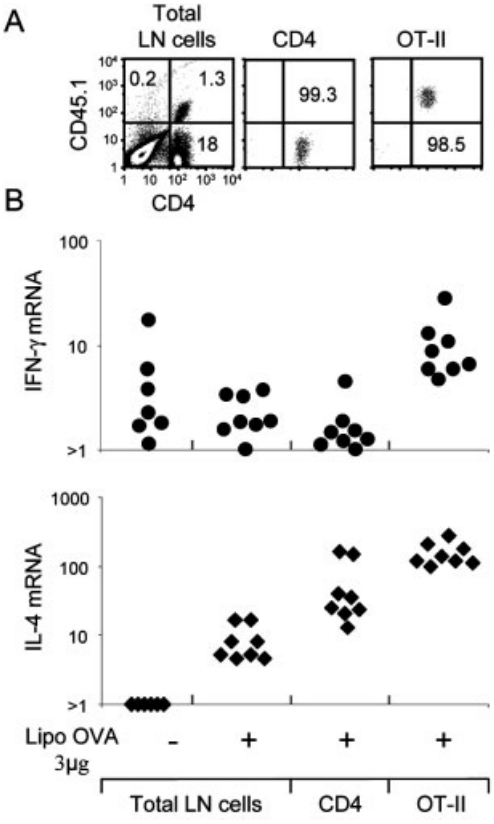

Figure 2. Liposomes induce a mixed Th1 and Th2 immune response. Mice received 2×10^6 $CD45.1^+$ OT-II cells and were immunized (or not) with OVA-containing liposomes (equivalent to 3 μg Ag). (A) Popliteal LN were harvested 6 days later, and cells were stained against CD4 and CD45.1 and sorted (or not, Total LN cells) into endogenous CD4 ($CD45.1^-/CD4^+$) and OT-II ($CD45.1^+/CD4^+$) cell populations. (B) Real-time PCR against IFN-γ (top panel) and IL-4 (bottom panel) was performed on cDNA extracted from each of these total or FACS-sorted cell fractions. Cytokine mRNA levels are represented relative to β-actin. Each symbol represents total or sorted cells from two LN derived from one mouse.

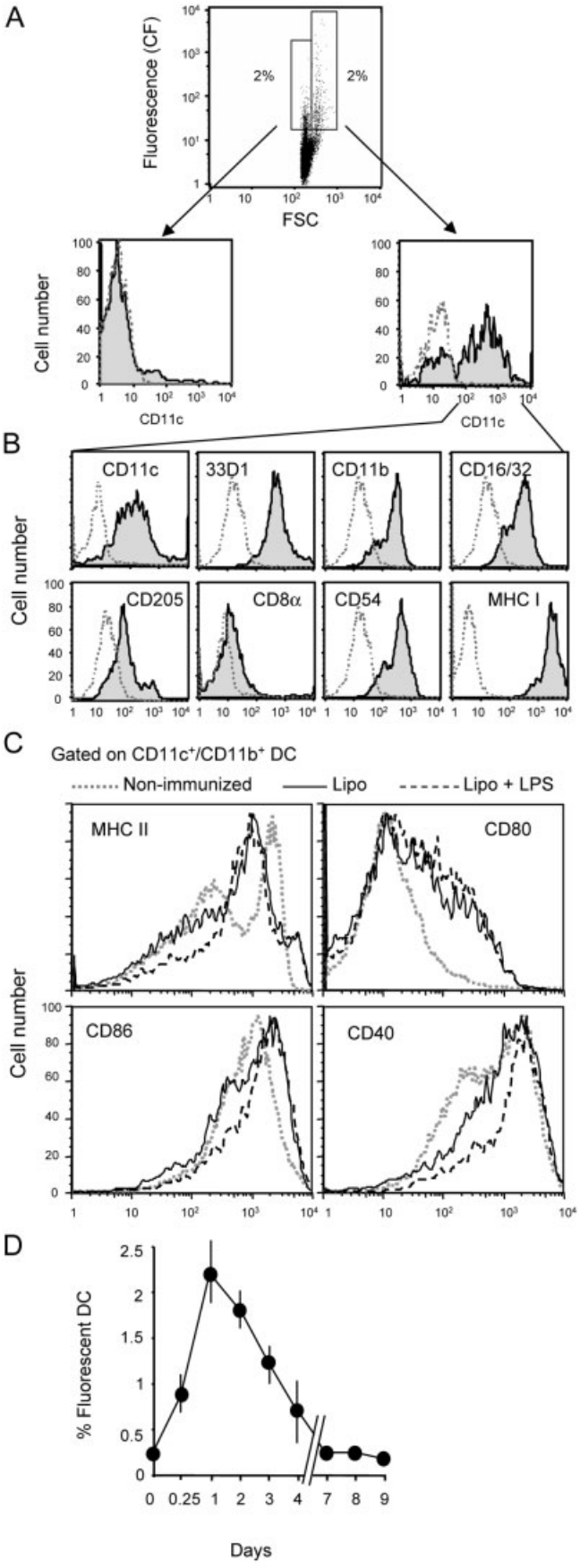

Figure 3. DC are the major population that capture liposomes, and they exhibit an activated phenotype. Fluorescent liposomes (equivalent to 3 μg Ag) were injected into footpads of ten mice. Popliteal LN were pooled for FACS analysis 24 h later. (A) Total cells were analyzed for size, CF fluorescence and expression of CD11c. (B) Fluorescent $CD11c^+$ cells were enriched by negative selection and stained with various Ab (indicated in each histogram). Open histograms indicate background levels of control Ab or streptavidin. (C) Mice were immunized with liposomes in the absence or presence of LPS, and the phenotype of the $CD11c^+/CD11b^+$ DC was compared to total $CD11c^+/CD11b^+$ resident DC from non-immunized mice (dotted line, non-immunized; solid line, liposomes alone; dashed line, liposomes + LPS). (D) Kinetics of the appearance of fluorescent DC in the draining LN. Results are representative of two to three experiments.

mRNA in the LN was almost exclusively associated with the responding OT-II cells, but this was less apparent for IL-4 mRNA. Importantly, OT-II cells have previously been shown to selectively produce IL-4 mRNA, without up-regulating IFN-γ mRNA, in response to alum-precipitated OVA, a well-characterized Th2 inducer [25]. These results show that Ag-containing liposomes promote the generation of both Th1 and Th2 effector CD4 T cells.

DC are the major cell population responsible for the capture of liposomes *in vivo*

Carboxyfluorescein (CF)-containing liposomes were injected into footpads, and fluorescent cells (that had captured the liposomes) in the popliteal LN were analyzed. At 24 h we observed two major fluorescent cell populations that represented 4% of the total cells (Fig. 3A). The first population was positive for DC markers (CD11c, 33D1), with the majority of these cells expressing high levels of CD11b and CD16/32, intermediate levels of CD205 and very low levels of or no CD8α (Fig. 3B). The second population of fluorescent cells was most likely B cells, as they were $CD11c^-$ (Fig. 3A) but expressed B220 and MHC class II or costimulatory molecules at low levels (not shown). In order to determine the maturation stage of the DC, we compared the phenotype of the $CD11c^+/CD11b^+$ DC after immunization with the liposomes, in the absence or presence of LPS, with steady-state LN-resident $CD11c^+/CD11b^+$ DC (Fig. 3C). Total $CD11c^+/CD11b^+$ DC, containing the DC subset that captured the liposomes, up-regulated MHC class II molecules as well as CD80 and CD86 and were among the DC that expressed the highest level of CD40 as compared with $CD11c^+/CD11b^+$ steady-state LN-resident DC. Moreover, the presence of LPS induced a further stage of activation, as demonstrated by a reduction in the frequency of DC that expressed low levels of MHC class

II, CD86 and CD40 molecules. The number of DC that had captured liposomes peaked at 24 h and decreased thereafter (Fig. 3D). No fluorescent cells were detected on day 5 or later. These results show that the cells in the draining LN that capture liposomes after s.c. injection *in vivo* are CD11c$^+$/CD11b$^+$/CD205^{mod}/CD8α^-, a phenotype described for dermal DC [26]. Although the mechanism involved in liposome uptake *in vivo* is still unclear, these DC exhibit an activated phenotype as compared to steady-state LN-resident DC, which can be further increased in the presence of LPS.

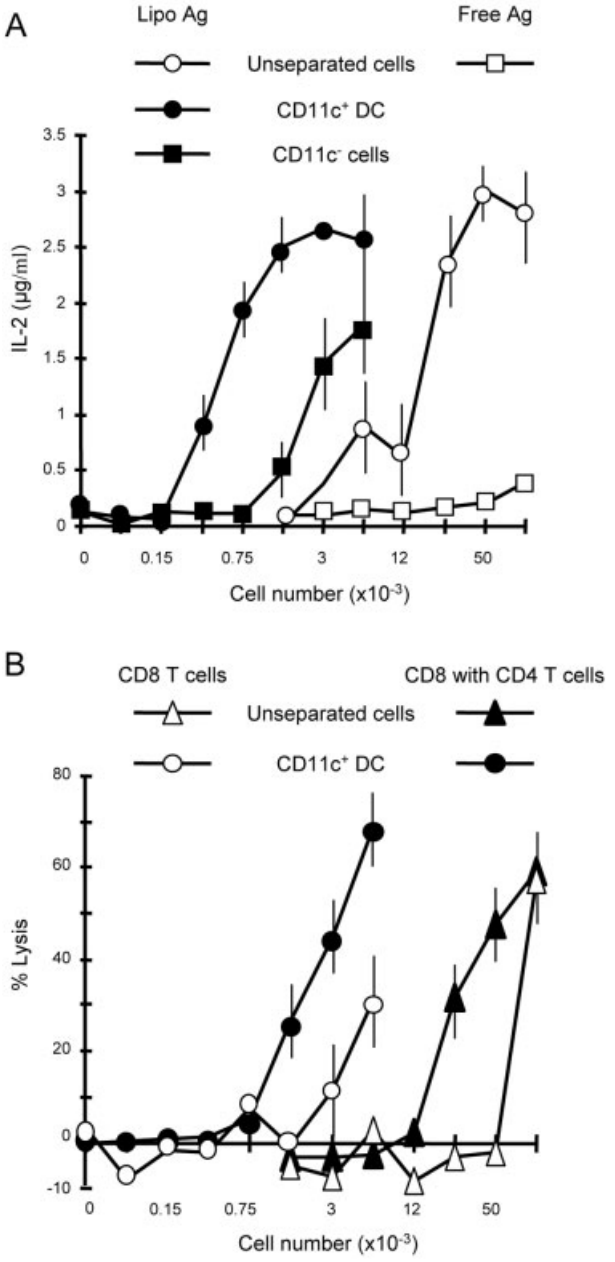

Figure 4. DC capture Ag from liposomes and are responsible for *ex vivo* MHC class II- and class I-restricted Ag presentation. Free HEL Ag or (HEL + OVA + CF)-containing fluorescent liposomes (equivalent to 10 µg of Ag) were injected into the footpads of ten (CBA × B6) F1 mice. (A) One day later, various numbers of unseparated cells or FACS-sorted CF$^+$ CD11c$^+$ DC or CD11c$^-$ cells (as in Fig. 3A) from the popliteal LN were used as APC to stimulate 3A9 cells. Curves show IL-2 secretion. (B) Unseparated cells or FACS-sorted CD11c$^+$ DC were mixed with OT-I cells, with or without 3A9 cells, and SIINFEKL-specific cytotoxicity was evaluated.

DC are the major cell population in draining LN initiating *ex vivo* CD4 and CD8 T cell responses

We next tested the capacity of CD11c$^+$/CD11b$^+$ DC that had captured liposomes to stimulate CD4 and CD8 T cells *ex vivo*. (CBA × B6) F1 mice were immunized with fluorescent liposomes containing HEL and OVA. At 24 h popliteal LN were removed and fluorescent cells FACS-sorted (based on the staining shown in Fig. 3A). In order to investigate MHC class II-restricted presentation, various numbers of either unseparated cells or cells enriched for CD11c$^+$ (DC) or CD11c$^-$ (B cells) were incubated with HEL-specific (3A9) CD4 T cells (Fig. 4A). DC were the most efficient cells for MHC class II-restricted presentation; as few as 750 enriched DC were as efficient as 25 000 unseparated cells or 6000 CD11c$^-$ cells. Unseparated cells taken from mice injected with free proteins were ineffective in stimulating T cells. This Ag presentation was also observed for cells harvested at 48 h but not at day 4 after immunization (not shown). In order to investigate MHC class I-restricted Ag presentation, various numbers of unseparated cells or enriched CD11c$^+$ cells (DC) were incubated with OVA-specific (OT-I) CD8 T cells in the absence or presence of 3A9 cells (Fig. 4B). OT-I cell activation was measured by the induction of specific cytolysis of target cells. Enriched DC were more efficient (6000 cells) in OT-I cell priming than unseparated LN cells (100 000 cells). Addition of 3A9 CD4 T cells that recognized their cognate Ag presented by DC enhanced CD8 T cell activation; in this instance, only 1500 enriched DC were required. There was no cytolysis of target cells in the absence of OT-I cells (not shown). These results suggest that CD4 T cells help MHC class I-restricted exogenous Ag presentation or CD8-priming activities by CD11c$^+$/CD11b$^+$ DC derived from the LN.

Ag-specific CD4 T cells play a role in primary Ag-specific CD8 T cell activation *in vivo* when Ag is delivered by liposomes

We investigated further the role of CD4 T cells in CD8 T cell priming *in vivo*. We transferred OT-I cells, with or without OT-II cells, into mice that were then immunized with OVA-containing liposomes. OT-I proliferation in the draining LN (detected with fluorescent H-2K^b/SIINFEKL tetramers) had increased 5 days later in an Ag dose-dependent manner (Fig. 5A) in mice that had received

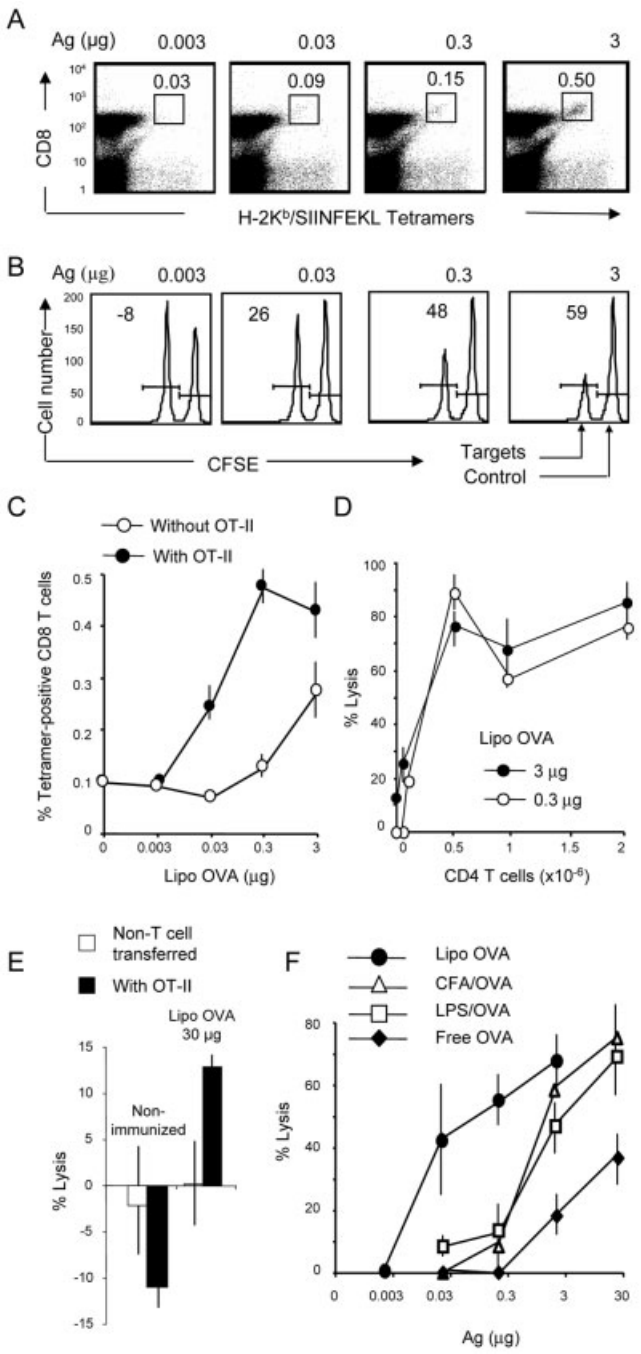

Figure 5. Activation of CD8 T cells *in vivo* is CD4-dependent after injection of Ag contained in liposomes. Mice received 10^5 OT-I and 5×10^5 OT-II cells. Various concentrations of OVA-containing liposomes were then injected. (A) Dot plots represent OT-I cells from draining LN labeled with H-2K^b/SIINFEKL tetramers 5 days after immunization. The quantity of OVA contained in the injected liposomes is shown above each dot plot. The percentage of the OT-I cells among total CD8 T cells is indicated. (B) *In vivo* cytotoxic activity of OT-I cells 1 week after immunization as determined by the lysis of SIINFEKL-pulsed CFSE^{low} target cells. The percentages within the histograms refer to the specific cytolysis. (C) In a separate series of experiments, tetramer binding was determined as in (A). (D) Mice received 10^5 OT-I cells with different numbers of OT-II cells, and CTL function was assessed 1 week after immunization. The percent of cytolysis *in vivo* is plotted as a function of the CD4 T cell number. (E) Normal mice (non-T cell transferred) and mice that received 2×10^6 OT-II cells were immunized with OVA-containing liposomes (30 μg), and *in vivo* cytotoxicity was evaluated 1 week later. (F) Mice received 10^5 OT-I cells with 5×10^5 OT-II cells, and *in vivo* cytotoxicity was evaluated 4 days after immunization as a function of the concentration of different forms of OVA. Results are from three different experiments.

both OT-I and OT-II cells (see below). OT-I cell proliferation was closely related to the induction of specific cytotoxicity *in vivo* (Fig. 5B).

Both the proliferation (Fig. 5C) and the induction of cytotoxicity (Fig. 5D) of OT-I cells were profoundly dependent upon adoptively transferred OT-II cells. The small number of endogenous OVA-specific CD4 T cells [27] was presumably not sufficient to provide help to induce activation of transferred OT-I cells at low Ag doses (0.3 or 3 μg). OVA-specific OT-II CD4 T cells are thus required to induce optimal primary expansion and cytotoxic activities of OT-I cells after immunization with Ag-containing liposomes. Moreover, OT-II cells were also capable of delivering help to induce primary endogenous CD8 T cell cytotoxic responses *in vivo* (Fig. 5E). In this set of experiments, while no cytotoxicity could be detected in normal mice, OT-II cells were able to provide help to endogenous CD8 T cells for low but reproducible responses. Importantly, efficient endogenous CTL responses were induced in normal mice immunized s.c. into footpads with much higher doses of Ag-containing liposomes (not shown). In conclusion, both endogenous OVA-specific CD8 T cells and Tg OVA-specific OT-I cells generated CTL effectors in primary immune responses after immunization with OVA-containing liposome in a CD4 T cell-dependent manner. These findings reveal that our model of adoptively transferred CD4 and CD8 T cells provides biological data that are relevant *in vivo* and offers a good tool to investigate the requirements for the induction of primary CTL responses.

Having defined optimal conditions (Fig. 5D), 10^5 OT-I cells with 5×10^5 OT-II cells were adoptively transferred into recipient mice, and different formulations of Ag were compared for their capacity to induce CD8 T cell activation. Compared to free Ag or Ag injected with LPS or emulsified in CFA, a 100- to 1000-fold lower concentration Ag encapsulated in liposome was sufficient (Fig. 5F). Thus, of the Ag formulations tested, Ag-containing liposomes were the most efficient in inducing CD8 T cell primary activation in a CD4-dependent manner. This finding highlights the importance of Ag delivery into DC, along with CD4 T cell help, in triggering efficient primary CTL responses.

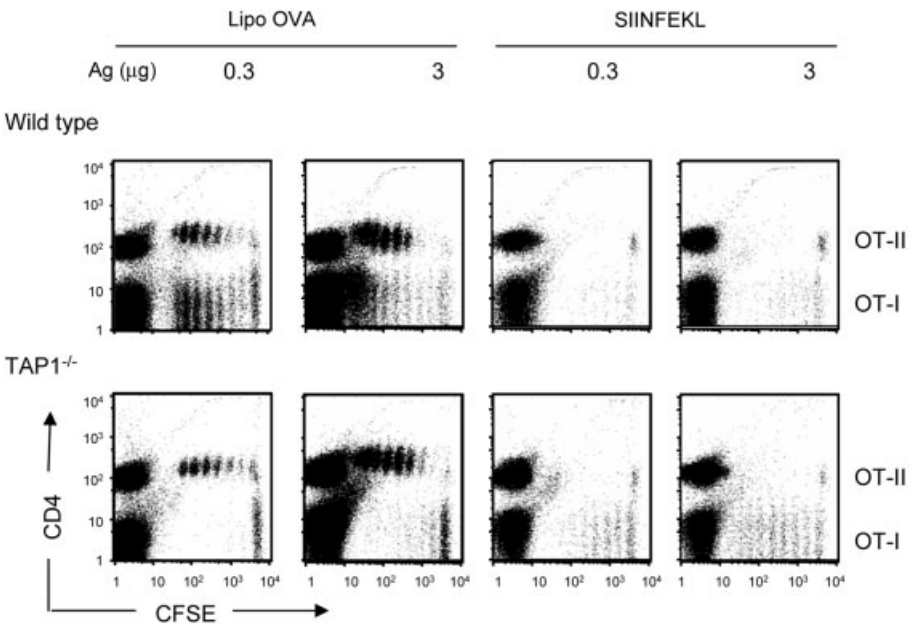

Figure 6. Activation of specific CD8 T cells *in vivo* is TAP1-dependent after injection of Ag in liposomes. WT or $TAP1^{-/-}$ mice received 2×10^6 CFSE-labeled OT-II cells and 2×10^6 CFSE-labeled OT-I cells and were immunized with OVA-containing liposomes or free SIINFEKL peptide. Proliferation of OT-II and OT-I cells was evaluated in LN 3 days later. OT-II cells are $CD4^+/CFSE^+$, while OT-I cells are $CD4^-/CFSE^+$.

Activation of specific CD8 T cells *in vivo* after injection of Ag in liposomes is TAP1-dependent

$TAP1^{-/-}$ mice were used to gain insight into the mechanisms by which OVA-containing liposomes elicit OT-I cell proliferation. $TAP1^{-/-}$ or WT mice received CFSE-labeled OT-I and OT-II cells. Both OT-II ($CD4^+$) and OT-I ($CD4^-$) cells were induced to divide in WT mice immunized with Ag-containing liposomes (Fig. 6). In contrast, OT-I cells transferred into $TAP1^{-/-}$ mice were not activated by OVA-containing liposomes but proliferated when mice received free SIINFEKL peptide, which binds directly to MHC class I molecules at the cell surface. OT-II cells responded similarly in $TAP1^{-/-}$ mice and WT mice, indicating that $TAP1^{-/-}$ DC had endocytosed OVA-containing liposomes. These results confirm that Ag delivered by liposomes to DC requires release into the cytosol to be loaded, via TAP1 molecules, into the classical pathway for class I-restricted presentation.

Induction of a secondary response to liposome-encapsulated Ag

Finally, it has been reported that in some circumstances targeting Ag to DC induces an expansion phase that is followed by drastic contraction of the pool of responding T cells. This leads to peripheral T cell unresponsiveness by the deletion of the T cells and the loss of effector functions in recall challenges [6, 7]. Although liposomes induce DC maturation (Fig. 3C), we next determined whether DC that capture liposomes have tolerogenic activities. The fate of Tg T cells responding to liposomal Ag was therefore addressed in secondary responses. Mice received $CD45.1^+$ OT-I and OT-II cells. If these mice were not immunized, few of the transferred T cells were recovered in the draining LN, and no *in vivo* cytolysis was observed (Fig. 7A, Group 1). When the mice were immunized once, both OT-II and OT-I cells could be readily detected in draining LN 3 to 5 days later, and cytotoxic responses were obtained (Fig. 7A, Group 2). Without a second immunization, the number of $CD45.1^+$ T cells observed in LN 1 month after immunization was as for non-immunized animals, and no cytotoxicity was observed (Fig. 7A, Group 4). When these mice were immunized a second time, both OT-II and OT-I cells were recovered, and cytotoxicity was identical to the primary immune response (Fig. 7A, Group 3). Finally, a kinetic analysis of OT-II cell proliferation and OT-I cell cytotoxic responses after two immunizations (Fig. 7B) showed that both CD4 and CD8 T cell responses were faster and of a shorter duration, as reported for CD8 T cell proliferation [28]. Thus, adoptively transferred T cells were not tolerized or deleted and responded well when mice were immunized a second time with liposome-encapsulated Ag, suggesting that DC that capture Ag-containing liposomes are immunogenic.

Discussion

Immunization of mice with Ag-containing liposomes resulted in a mixed Th1 and Th2 response, along with IgG Ab production (not shown) and the generation of cytotoxic T cells. The majority of the cells taking up the liposomes appear to be dermal DC ($CD11c^+/CD11b^+/CD205^{mod}/CD8\alpha^-$) [26]. These results are consistent

with reports indicating the importance of skin-derived $CD11c^+/CD11b^+$ DC in CD4 T cell activation after s.c. immunization [29]. Although $CD8\alpha^+$ DC are reported to be important for cross-presentation of Ag injected i.v., or following viral skin infection [30–32], in our system $CD11c^+/CD11b^+$ DC that were mostly $CD8\alpha^-$ were able

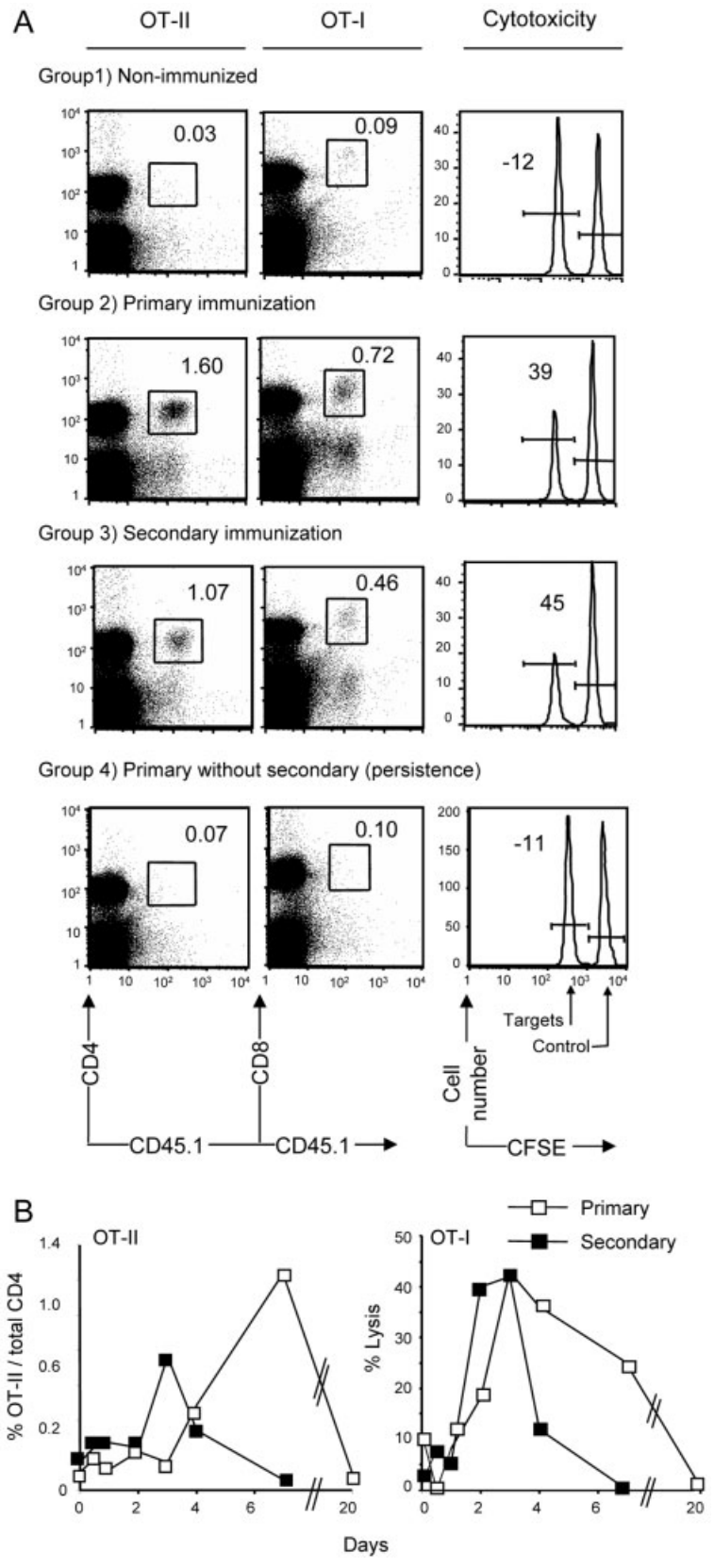

Figure 7. Adoptively transferred CD4 and CD8 T cells are responsive after a second Ag challenge *in vivo*. (A) Mice received $CD45.1^+$ OT-I cells (10^5) and OT-II cells (5×10^5). Groups of mice were not immunized (group 1) or were immunized with 3 μg OVA in liposomes once (groups 2 and 4) or twice (30 days after the first immunization) (group 3). OT-I and OT-II cell proliferation and CTL activity were monitored 3 days after T cells were transferred (group 1), 3–5 days after primary immunization (group 2), 2–4 days after secondary immunization (group 3), or 30 days after primary immunization (group 4). T cells from popliteal LN were probed with CD45.1, CD4 and CD8 Ab. The percentages of OT-II or OT-I cells among total CD4 or CD8 T cells, respectively, in the LN are indicated. In the other half of the mice, *in vivo* cytolysis was assessed 3 days after immunization. The percentages above the histograms refer to the specific cytotoxicity. Data are representative of results obtained in three separate experiments. B) Kinetics of presence of OT-II cells and cytotoxicity of OT-I cells in LN of mice tested at various times after immunization. Combined results of two experiments are shown.

to present Ag to both CD4 and CD8 T cells *ex vivo*. However, we have not determined whether DC captured liposomes at the site of s.c. injection or in the draining LN [33]. We also do not know whether LN-resident DC capture Ag from trafficking DC [34]. Nevertheless, Ag-presenting DC expressed an activated phenotype, as shown by the levels of MHC class II and costimulatory molecules, and were immunogenic.

The requirement for CD4 T cell help in the priming of cytotoxic CD8 T cell responses remains insufficiently explained. "Licensing" of DC by CD4 T cells is required to induce their ability to stimulate naive CD8 T cells [12–14]. In addition, CD4 T cell help has been reported to be involved in the development of functional CD8 T cell memory [18–20] and in the maintenance of these cells [21]. However, much of these data have focused on CTL responses induced by infections such as *Listeria monocytogenes*, LCMV or vaccinia virus. Whether CD8 T cell responses (primary expansion, acquisition of functional cytotoxicity, maintenance of memory) against exogenous protein Ag are CD4-dependent is still an open question. Studies using large numbers of adoptively transferred CD8 T cells specific for exogenous Ag, provided by injection of protein-pulsed DC, have reported that CD8 T cell responses are CD4-independent [35]. Here we adoptively transferred low numbers of OT-I cells and demonstrated that after immunization with low doses of Ag encapsulated in liposomes, CD4 T cells were required early during the primary CTL response to induce the expansion of CD8 T cells (Fig. 5C). This correlated with the generation of cytotoxic effectors (Fig. 5D).

Our results confirm and extend data reported by Smith *et al.* [36], who suggested that CD4 T cell help may be especially important in the absence of pathogen-derived signals, and may reconcile the recent controversy as to whether CD4 T cells "license" DC for the induction of primary CTL responses. After immunization, Ag is usually highly concentrated in a limited number of DC [37]. An even higher concentration of Ag in a small cohort of DC is likely to be efficiently achieved by the uptake of liposomes 200 nm in diameter, each containing about 1000 molecules of Ag, as were used in this study. Given the very low amount of liposome-encapsulated Ag that was sufficient for presentation, it is reasonable to believe that the same DC that acquired the liposomes also presented Ag to both CD4 and CD8 T cells.

Although our knowledge of how liposomes interact with cells and the parameters that influence this *in vivo* is limited, numerous serum proteins bind to the liposomal surface [38]. We believe that cell-surface receptors recognizing these opsonins might be involved in liposome uptake and may be responsible for the DC activation triggering efficient Ag processing and presentation to CD4 T cells. However, contrary to *in vitro* experiments [22] and although all $CD11c^+$ DC populations express FcR (Fig. 3B), presentation of Ag delivered by liposomes *in vivo* did not require the participation of circulating Ab, consistent with absence of an implication for FcR involvement in the uptake process (Fig. 1D). Nevertheless, in this context, as liposomes lack intrinsic "danger" signals that might "license" DC directly, CD4 T cell help is fundamental for the triggering of effective primary CTL responses. Inflammatory signals cannot be solely responsible for effective CD8 T cell priming, since CFA or LPS were not as efficient as Ag-containing liposomes, even in the presence of CD4 T cell help (Fig. 5F). The manner by which Ag is delivered into DC and the presence of CD4 T cell help are parameters to consider in designing vaccines.

As yet, little is known as to how DC acquire CD8 T cell-priming functions. DC require activation by stimuli such as immune complexes [23], CD4 T cell help mediated via CD40-CD40L [12], TLR ligands [39] or other undefined signals [40]. We previously proposed that CD4 T cells recognizing their cognate MHC class II/peptide complexes concentrated in cholesterol-rich microdomains stimulate bone marrow-derived DC to release exogenous Ag from endocytic vesicles into their cytosol for class I presentation [41]. In support of this hypothesis, the presentation of liposome-encapsulated Ag in the context of MHC class I molecules is TAP-dependent (Fig. 6) [42]. Moreover, we show that the generation of endogenous CTL effectors during the primary immune response after immunization with OVA-containing liposome is CD4 T cell-dependent. Thus, the CTL response is likely to be controlled by the probability of interaction between Ag-presenting DC and Ag-specific CD4 T cells. CD4 T cells may be dispensable *in vitro* if DC are directly loaded with peptides [42] or if exogenous Ag enters directly into the cytosol [43]. In addition to the DC activation signals mentioned above, CD4 T cells regulating the access of Ag into the classical pathway for class I-restricted presentation might also augment the cross-presentation of exogenous Ag *in vivo*.

Materials and methods

Mice

C57BL/6J (B6) (H-2^b), CBA/J (H-2^k) or (CBA × B6) F1 mice were from IFFA-CREDO (L'Arbresle, France). 3A9 mice have TCR specific for the HEL 46–61 peptide/H-2 I-A^k complex [44]. OT-II mice have TCR specific for the OVA 323–339 peptide/H-2 I-A^b complex [35]. OT-I mice have TCR specific for the OVA 257–264 peptide SIINFEKL/H-2K^b complex [35]. B6 TAP1$^{-/-}$ mice and IgM$^{-/-}$ mice were from Jackson Laboratories (Bar Harbor, ME). For some experiments, OT-I and OT-II mice were

crossed to B6 mice congenic for CD45.1 (CDTA, Orléans, France).

Ag, reagents and liposomes

HEL, OVA Grade VII, BSA Grade V, CFA and LPS (*E. coli* 055:B5) were from Sigma-Aldrich (St. Louis, MO). SIINFEKL was synthesized at the CIML. Liposomes [80 μmoles with respect to lipids: 65% mol/mol dimyristoyl phosphatidylcholine, 35% cholesterol (both from Sigma-Aldrich)] were formed by exposing lipids evaporated from chloroform/methanol (9:1 v/v) to an aqueous solution containing HEL (20 mg/mL; 1.4 mM), OVA (60 mg/mL; 1.4 mM) or BSA (25 mg/mL; 0.7 mM) in PBS containing (or not) 100 mM carboxyfluorescein (CF; Molecular Probes) or containing only CF (empty liposomes). Liposomes were formed by extrusion (Extruder, Northern Lipids, Vancouver, Canada) as described [22]. Ag levels were determined by fluorescence of free and liposome-entrapped CF with reference to the stock solution of Ag and CF. The quantity of liposome-associated Ag used for immunization was obtained by dilution of these liposomes.

Immunization protocols

Mice were immunized s.c. (in 50 μL) in both hind footpads with Ag in free form dissolved in PBS, emulsified in CFA, with 1–5 μg LPS or encapsulated in liposomes. Control mice were not injected or received PBS, empty liposomes or BSA-containing liposomes. For secondary responses, mice were immunized twice with Ag-containing liposomes at 1-month intervals.

FACS analysis of DC that captured Ag-containing liposomes from LN

Mice were injected with CF-containing liposomes in the presence (or not) of 5 μg LPS. Draining popliteal LN were harvested 24 h after immunization, and cell suspensions were subjected to collagenase type I digestion (Sigma) at 37°C for 20 min. DC were also enriched by negative selection using magnetic beads. Cells were incubated with rat mAb specific for B220 (RA3-6B2), CD3 (KT-3) and Thy1 (H155.124.3) in Ca^{2+}- and Mg^{2+}-free HBSS medium (Sigma) at 4°C for 30 min. Cells not of DC cell lineage were removed with anti-rat Ig-coupled Dynabeads (Dynal, Oslo, Norway). Remaining cells were then incubated with CD11c-PE, CD8α-PE (PharMingen) or rat mAb (ATCC, Manassas, VA) against 33D1, CD11b (M1/70), CD80 (16-10A1), CD86 (GL-1), CD54 (BE29G1), CD16/32 (24G2), MHC class II (M5/114), CD205 (NLDC-145) or CD40 (FGK45), followed by anti-rat PE (Jackson). Biotinylated mouse anti-MHC class I (5F1) was used with streptavidin-PE (Jackson). Cells were then fixed in 2% formaldehyde and analyzed in a FACScan cytofluorimeter (Becton Dickinson, Mountain View, CA). For *ex vivo* analysis of functions of DC that had captured fluorescent liposomes, cell suspensions from LN were treated as described above and sorted on the basis of forward scatter as cells detected in the FACScan green fluorescent channel.

Ex vivo Ag presentation and cytotoxicity assay

Popliteal LN from (CBA × B6) F1 mice were harvested 24 h after immunization with fluorescent HEL- and OVA-containing liposomes. Cell suspensions were subjected to collagenase digestion at 37°C for 20 min. DC that had captured fluorescent liposomes were FACS sorted as described above. Dilutions of total or FACS-sorted cells were distributed in 96-well flat-bottom plates in RPMI complete medium. For *ex vivo* Ag presentation, 20 000 3A9 CD4 T cells were added for 48 h. IL-2 in supernatants was determined using the IL-2-dependent cell line CTLL [22]. For *ex vivo* cytotoxic assays, 20 000 OT-I T cells with or without 20 000 3A9 T cells were added for 5 days [41]. Target RMA cells were pulsed with the OVA peptide SIINFEKL (1 μM) and [^{3}H]-thymidine (0.25 μCi/mL) overnight and washed. RMA target cells (5000) were added for 4 h. Spontaneous cytolysis in the presence of cell suspensions of LN from non-immunized mice was indistinguishable from that of RMA cells incubated alone.

In vivo cytotoxicity assay

Target syngeneic spleen cells were divided into two populations. One population ($CFSE^{low}$) was pulsed with 10 μM SIINFEKL at 37°C for 2 h and then labeled with 0.5 μM CFSE (Molecular Probes) at 37°C for 10 min. The other population ($CFSE^{high}$) was not peptide-pulsed and was labeled with 5 μM CFSE. The two populations were mixed 1:1 and injected i.v. into mice (20×10^6 cells/mouse). The next day, popliteal LN were removed. Elimination of SIINFEKL-pulsed $CFSE^{low}$ target cells in the LN cell suspension was analyzed by flow cytometry based on the ratio between the percentage of not pulsed *versus* SIINFEKL-pulsed cells [($CFSE^{low}$ / $CFSE^{high}$) ×100].

Preparation of Ag-specific Tg T cells for adoptive transfer

Pooled LN were harvested from Tg mice. Cells were incubated with rat mAb against B220 (RA3-6B2), MHC class II (M5/114), CD11b (M1/70) and CD16/32 (2.4G2) plus CD8 (H59.101.02) for purification of 3A9 and OT-II cells or plus CD4 (H129.19.6) for purification of OT-I cells. Non-T cells were removed with anti-rat Ig-coupled Dynabeads. T cells were then labeled with 10 μM CFSE at 37°C for 10 min. Different numbers of T cells (as indicated) were injected i.v. in 200 μL PBS. Recipient mice were immunized 3 days after adoptive T cell transfer. To follow proliferation of CFSE-labeled T cells, popliteal LN were collected 72 h after immunization and digested with collagenase. Cells were stained with CD4-PE, CD8-PE or PE-labeled H-$2K^b$/SIINFEKL tetramers (Immunotech, Marseille, France).

Primary and secondary responses were analyzed following the co-transfer of $CD45.1^+$ OT-II and OT-I cells into $CD45.2^+$ (B6)mice. T cells were probed with CD45.1-PE, CD4-allophycocyanin (APC) and CD8-PerCPCy5.5 Ab (PharMingen), and 500 000 events were collected on FACScalibur cytofluorimeter (Becton Dickinson). Final analysis and graphical output were performed using FlowJo software (Treestar, Costa Mesa, CA).

FACS sorting of OT-II cells, reverse transcription of mRNA and relative quantification by PCR

Six days after immunization of OT-II cell adoptive transfer recipient mice, popliteal LN cells were harvested, stained with CD45.1-PE and CD4-PerCPCy5.5 and sorted by flow cytometry (MoFlow, DakoCytomation) into endogenous cell ($CD4^+$/$CD45.1^-$) and OT-II cell ($CD4^+$/$CD45.1^+$) fractions [25]. RNA was extracted from each fraction using RNAzol B (Biogenesis, Poole, UK), and cDNA was prepared as described [25, 45]. Relative quantification of specific cDNA species message was carried out in a multiplex PCR on the ABI 7700 (Applied Biosystems, Warrington, UK). Sequences for β-actin, IL-4 and IFN-γ have been described [45]. Relative quantification of signal per cell was achieved by setting thresholds within the logarithmic phase of the PCR for β-actin and the test gene to determine the cycle number at which the threshold was reached (C_T). The C_T for the target gene was subtracted from the C_T for β-actin, and the relative amount was calculated as $2^{-\Delta CT}$.

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