Class I-restricted presentation of exogenous antigen acquired by Fcγ receptor-mediated endocytosis is regulated in dendritic cells

Patrick Machy, Karine Serre and Lee Leserman

Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, Marseille, France

We evaluated MHC class I- and II-restricted presentation of exogenous antigen by mouse bone marrow-derived dendritic cells (DC) and splenic B cells. DC presented to class I-restricted transgenic T cells femtomolar concentrations of antigens from liposomes targeted to the IgG Fc receptor. Targeting these liposomes to surface immunoglobulin did not permit B cells to stimulate class I-restricted responses. Nevertheless, both DC and B cells presented antigen from liposomes targeted to these same receptors with equivalent efficiency to class II-restricted T cells. Acquisition of the capacity to present class II-restricted antigens required shorter periods of differentiation of DC than presentation of exogenous class I-restricted antigens. The latent period for class I-restricted presentation of exogenous antigen by DC could not be shortened by exposing them to lipopolysaccharide, double-stranded RNA or antibody to CD40. Class I presentation depended on expression of the TAP1 transporter. Our data are consistent with the existence of a regulated transport process present in DC which can convey exogenous antigen from endocytic vesicles to the cytosol.

Received	9/9/99
Revised	29/11/99
Accepted	30/11/99

Key words: Antigen presentation / Dendritic cell / B cell / Fc receptor / Liposome

1 Introduction

$CD8^+$ CTL play a major role in protection against intracellular infection. Infected cells are killed by CTL specific for peptides from the infectious agent. Presentation of peptides requires both their production by the target cell and their association with MHC class I molecules in the endoplasmic reticulum in a manner dependent on proteasomes and the transporter associated with Ag presentation (TAP) [1]. Induction of CTL usually requires interaction with professional APC in lymphoid tissues. These APC present Ag in the context of both MHC class II molecules, to stimulate $CD4^+$ T helper cells, and class I to stimulate $CD8^+$ precursors of the CTL [2]. These APC need not be infected, but may acquire exogenous Ag in the form of dead cells, cellular debris or defective infectious particles. Uptake of exogenous Ag is well understood for class II presentation but there are gaps in our knowledge of the mechanisms of recognition and passage into the cytosol of exogenous Ag which become class I restricted, a process called "cross-priming" (reviewed in [3–5]). Ag acquired from non-hematopoietic cells by cross-priming also associated with class I molecules in a proteasome- and TAP-dependent manner after uptake by APC [6]. Since it is important for the immune system to be able to respond as quickly as possible to an infectious threat, characteristics expected for APC responsible for initiating both class II- and class I-associated responses include an efficient means for recognizing Ag to be taken up for presentation, and an efficient means for transferring this Ag to the cytosol for proteolysis and TAP transport.

B cells efficiently take up and induced responses of naive $CD4^+$ T cells to soluble [7] as well as particulate [8] Ag expressing determinants for which their sIg are specific. One report indicates that, in addition to augmenting class II presentation, binding of Ag to specific sIg permitted B cells to augment presentation of Ag in the context of class I and to stimulate $CD8^+$ T hybridoma cells [9]. However, specific B cells must survive in order to proliferate and synthesize Ab. Since efficient presentation by B cells to CTL precursors is likely to result in their elimi-

[I 20099]

Abbreviations: ICAM: Intercellular adhesion molecule **CF:** Carboxyfluorescein **DC:** Dendritic cell(s) **TAP:** Transporter associated with antigen presentation **HEL:** Hen egg lysozyme **PA-anti-DNP:** Protein A-anti-DNP Fab

0014-2980/00/0303-848$17.50+.50/0

nation by the CTL they induce, their behavior as APC would appear to be in conflict with the requirements of their function as B cells. We therefore re-evaluate in this study the capacity of B cells to cross-present exogenous Ag taken up by their sIg. Alternatively, B cells can act indirectly in the process of Ag uptake, by virtue of their capacity to secrete IgG after receiving T cell help. This Ab becomes widely distributed in the body and can combine with Ag to form immune complexes. Dendritic cells (DC), another population of APC, can recognize immune complexes by virtue of their receptors for the Fc portion of IgG (FcγR). These immune complexes may be efficiently taken up by the FcγR and initiate T cell responses to both class II-associated [8, 10–13] and class I-associated Ag [13].

In this study we compare presentation of a model exogenous Ag, liposome-entrapped ovalbumin (OVA), for its ability to stimulate class I- and II-restricted responses following targeting to sIg of B cells and the FcγR of bone marrow-derived DC. We show that this Ag is efficiently presented in the context of class II, but not in the context of class I by B cells. In contrast, it is presented both in the context of class II and class I by DC. Acquisition of the capacity to present exogenous Ag in the context of class I by DC required a longer period in culture than the presentation of the same Ag in the context of class II. This lag period for class I presentation appears due to the time necessary for the development in these bone marrow-derived DC of a system to transfer exogenous Ag to the cytosol.

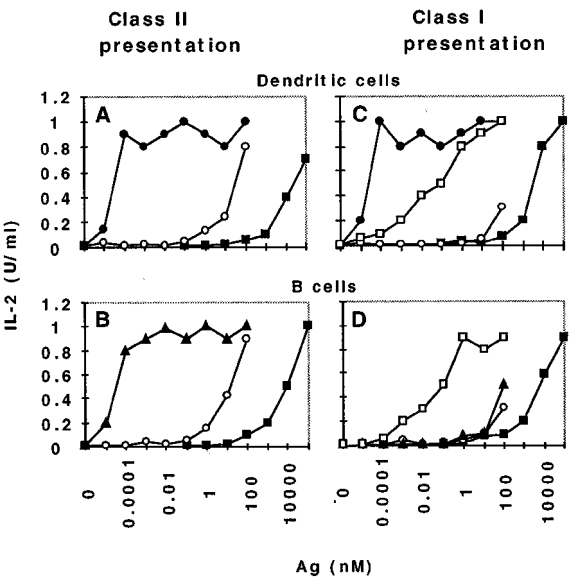

Fig. 1. DC and B cells efficiently present peptides derived from targeted OVA to class II-restricted T cells, but only DC present peptides derived from targeted OVA to class I-restricted T cells. B cells (B, D) or bone marrow-derived DC cultured for 12 days (A, C) were incubated with either free SIINFEKL peptide (□), free OVA (■), or with OVA-containing liposomes alone (○) (untargeted), or in the presence of anti-DNP mAb (●) (FcR targeted), or rabbit anti-mouse Ig Ab and a PA-anti-DNP conjugate (▲) (sIg targeted). After overnight incubation, APC were washed and either OVA peptide plus I-A^b-restricted T hybridoma cells (1D5) (A, B), or SIINFEKL plus K^b-restricted transgenic T cells (OT-I) (C, D) were added. IL-2 production after 48 h is shown. These are data from an experiment representative of more than ten performed.

2 Results

2.1 Presentation of targeted liposome-encapsulated Ag by class I and II molecules

We have reported efficient class II-restricted presentation of APC-associated, liposome-encapsulated hen egg lysozyme (HEL) to HEL-specific transgenic $CD4^+$ T cells from nonimmunized mice [8]. DNP-bearing liposomes were targeted to the FcγR of bone marrow-derived DC cultured for 12 days following opsonization with anti-DNP Ab. The same liposomes were targeted to sIg of B cells by mixing them together with protein A-anti-DNP Fab (PA-anti-DNP) conjugates in the presence of protein A-binding anti-Ig [8]. In the present study we encapsulated OVA in liposomes of identical composition, using as readout IL-2 production by the I-A^b plus OVA peptide-restricted hybridoma ID5 [14]. Data from a representative experiment are shown in Fig. 1 A and B. As for our results for HEL, OVA was not presented by either B cells or DC at concentrations lower than about 1 μM when free in solution or 10 nM when encapsulated in non-targeted liposomes, but liposome-encapsulated OVA was presented at 100 000-fold lower concentrations (100 fM) when targeted to the DC FcγR (Fig. 1 A) or sIg of B cells (Fig. 1 B). B cells did not present FcγR-targeted liposomes containing OVA (data not shown), since the FcγR they express is not endocytosed [15].

To evaluate presentation of class I-restricted determinants from the same Ag, we used this system of liposome-encapsulated OVA to study presentation by the same APC of a peptide (SIINFEKL) derived from OVA to K^b plus SIINFEKL-specific T cells from OT-I mice [16] (Fig. 1 C and D). As for class II-restricted presentation, targeting of liposomes to the DC FcγR permitted efficient presentation of OVA to OT-I cells, while free OVA and OVA in non-targeted liposomes were not presented at concentrations below 100 nM (Fig. 1 C). However, in contrast to the equivalent class II-associated presentation, when liposomes were targeted to the sIg of B cells or the FcγR of DC, only the DC presented the class I-associated Ag (Fig. 1 D). B cells were not intrinsically less

efficient at stimulating these transgenic T cells than DC, since both APC presented the SIINFEKL peptide free in solution with equivalent efficiency.

We asked whether liposomes could deliver their contents into the class I presentation pathway in a manner independent of the cell surface determinant to which they bound. Presentation to OT-I cells was inhibited by a K^b-specific mAb when DC from B6 or (CBA × B6) F1 mice were used as APC, but neither K^k-nor I-A^k-specific Ab had any effect on K^b-restricted presentation in the case of F1 mice (data not shown). We were thus able to use these latter Ab to target DNP-bearing liposomes to DC from F1 mice in the presence of PA-anti-DNP conjugates. As shown in Fig. 2 A, targeting to either of these determinants permitted presentation of OVA in the context of class II to 1D5 cells with practically the same efficiency as targeting the FcγR, but only targeting to the FcγR permitted stimulation of class I-restricted OT-1 cells by the same APC (Fig. 2 B).

2.2 Presentation of liposome-encapsulated OVA is TAP1 dependent

We asked whether the liposome contents passed into the cytosol prior to their association with class I molecules. When we used DC derived from bone marrow from $TAP1^{-/-}$ mice [17] as APC, FcγR-targeted presentation to the class II-restricted hybridoma was not impaired relative to wild-type mice (Fig. 3 A; compare to Fig. 1). The ability of the same cells to present exogenous SIINFEKL was enhanced relative to wild-type cells (Fig. 3 B; compare to Fig. 1 C), despite their low expression of class I molecules observed by FACS® (data not shown). This probably reflects the lack of competition from peptides stably bound to the reduced number of class I molecules that are expressed by these cells. Despite this sensitivity to SIINFEKL, presentation of FcγR-targeted liposome-encapsulated OVA was poor (Fig. 3 B). Thus, as reported for most forms of class I-restricted presentation of exogenous Ag, liposomes or their contents probably must pass into the cytosol for proteolysis by the proteasome before transport by TAP in order to associate with class I molecules in the endoplasmic reticulum.

2.3 SIINFEKL from FcγR-targeted liposomes is not efficiently presented to OT-I cells

SIINFEKL efficiently associates with class I molecules at the cell surface or in cellular compartments accessible to fluid phase endocytosis. Thus, SIINFEKL would be expected to be efficiently presented if introduced into the cytosol by microinjection or if generated in the cytosol, as has been demonstrated when SIINFEKL is expressed from a minigene [18]. FcγR-targeted liposome-en-

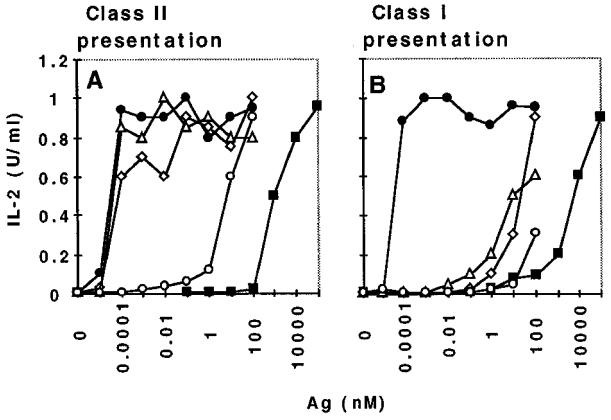

Fig. 2. Targeting the FcγR but not other tested DC surface determinants permits class I-restricted antigen presentation. Bone marrow-derived DC (cultured for 12 days) from (B6xCBA) F1 mice were incubated with free OVA (■), with OVA containing liposomes alone (○) (untargeted), in the presence of anti-DNP mAb (●) (FcR targeted), or with 5 μg/ml PA-anti-DNP conjugate and anti-I-A^k (◇) (class II targeted) or anti H2-K^k (△) (class I targeted) mAb. After overnight incubation, cells were washed and T cells 1D5 (A), or OT-1 (B) were added. Results shown are representative of three experiments.

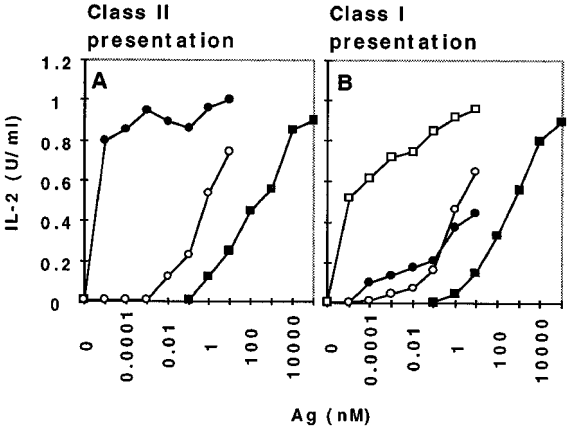

Fig. 3. Presentation of liposome-encapsulated OVA is TAP1 dependent. This experiment was performed using DC under the same conditions as in Fig. 1 except that the cells were from $TAP1^{-/-}$ B6 mice. Cells were incubated with free SIINFEKL (□), free OVA (■), with OVA-containing liposomes alone (○) (untargeted), or the presence of anti-DNP mAb (●) (FcR targeted). After overnight incubation, cells were washed and 1D5 (A) or OT-I (B) T cells were added. Results shown are representative of three experiments.

capsulated OVA was efficiently presented by DC after receptor-mediated uptake. However, no presentation was seen when liposomes containing equimolar concentrations of SIINFEKL, rather than OVA, were used (Fig. 4). This indicates that liposomes do not enter the cytosol as such, nor do their membranes fuse with the membrane of endocytic compartments, since these processes would be equally efficient for cytoplasmic delivery of OVA or SIINFEKL. Thus, liposome-encapsulated molecules are released in some endocytic compartment prior to their movement into the cytosol.

2.4 Class I-associated presentation depends on DC differentiation

In our earlier studies on class II-restricted response to HEL we routinely used DC from CBA mice cultured for 7 days [8]. We evaluated the effect of the age of the B6 DC cultures on class I- and II- restricted presentation of liposome-encapsulated OVA. In agreement with the results for the presentation of HEL by CBA cells, presentation to class II-restricted T cells of peptides derived from OVA in liposomes targeted to the FcγR was already as efficient at 4 to 6 days (Fig. 5 A) as at later times (Fig. 5 B and C). Surprisingly, we found little or no presentation in the context of class I of liposome-encapsulated OVA targeted to the FcγR for DC cultured for the same period (Fig. 5 D). The capacity to present OVA in the context of class I developed over the next 2 days and was fully established by day 10 (Fig. 5 E and F).

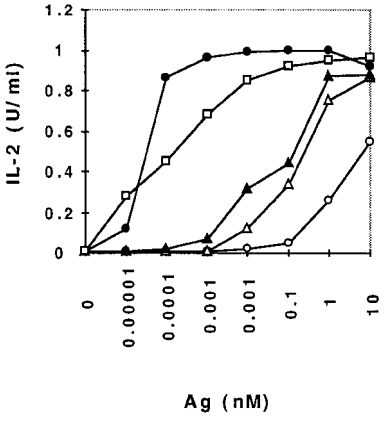

Fig. 4. SIINFEKL contained in FcγR-targeted liposomes is inefficient in inducing OT-I T cell stimulation. DC cultured for 12 days were incubated with free SIINFEKL (□), with SIINFEKL-containing liposomes alone (△) (untargeted), or in the presence of anti-DNP mAb (▲) (FcR targeted), or with OVA-containing liposomes alone (○) (untargeted), or in the presence of anti-DNP mAb (●) (FcR targeted). Results shown are representative of three experiments.

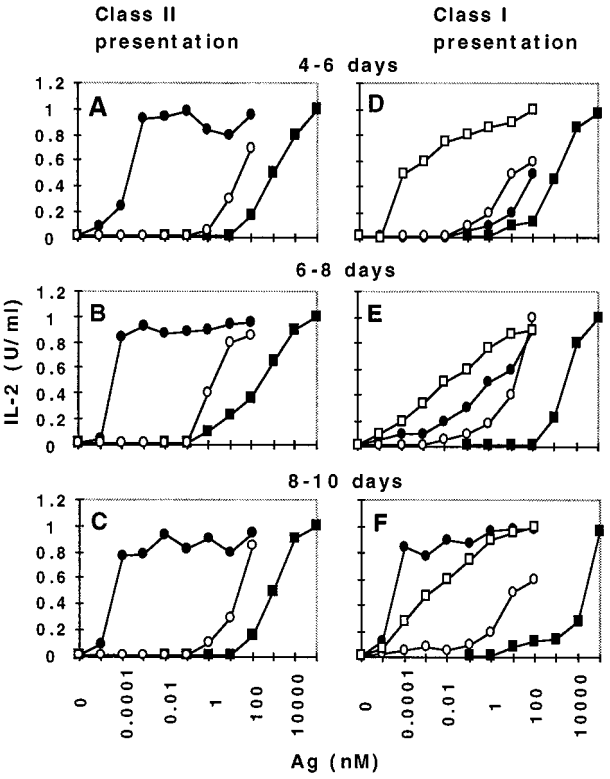

Fig. 5. Acquisition of the capacity to present Ag as a function of duration of culture of DC. After 4 (A, D), 6 (B, E) or 8 (C, F) days of culture, DC were incubated with free or liposome-encapsulated OVA overnight and then with class II- (A–C) or class I- (D–F) restricted T cells for 2 days. Symbols: free SIINFEKL peptide (□), free OVA (■), OVA-containing liposomes alone (○) (untargeted), or in the presence of anti-DNP mAb (●) (FcR targeted). Results shown are representative of more than five experiments.

Despite the differences for the presentation of endocytosed OVA associated with class I, FACS measurements (Fig. 6 A), indicated that the levels of expression of class I and II molecules, FcγR (as measured by IgG anti-DNP-opsonized liposome binding), co-stimulatory and myeloid cell markers examined on DC taken after 5 days are similar to those expressed by DC cultured for 12 days. In addition, presentation of a fixed concentration (1 nM) of SIINFEKL peptide in solution to OT-I T cells varied to the same extent as a function of the numbers DC cultured for either 5 or 12 days used as APC (Fig. 7 A). We also compared the number of DC required for presentation of SIINFEKL as the free peptide with the number of DC necessary to stimulate OT-I T cells when the DC acquired the Ag from targeted liposomes containing OVA. DC cultured for 5 and 12 days were incubated in the presence of

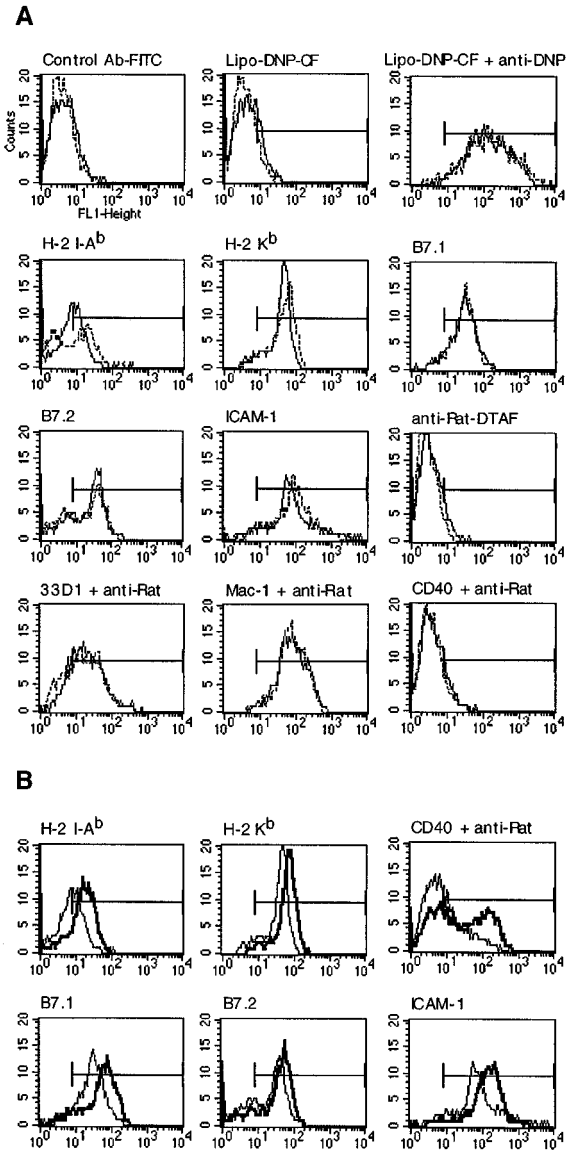

Fig. 6. (A) FACS profiles of DC 5 (continuous line) or 12 (dotted line) days after initiation of bone marrow cultures in conditioned medium containing GM-CSF. (B) FACS profiles of DC following 5 days of culture in conditioned medium containing GM-CSF and subsequent overnight incubation without (fine line) or with 10 μg/ml LPS (bold line).

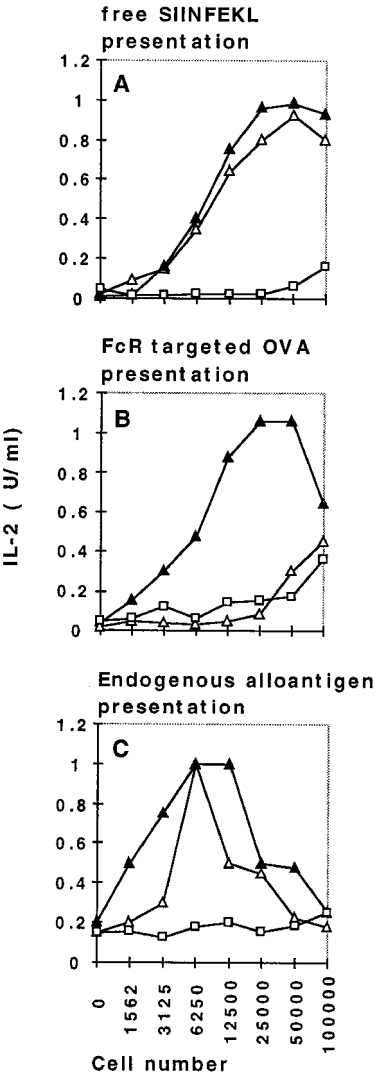

Fig. 7. Acquisition of the capacity to present SIINFEKL or an endogenous antigen as a function of duration of culture of DC. After 5 (△) or 12 (▲) days of culture various numbers of DC from H-2b mice were incubated with 1 μM SIINFEKL (A) or with 10 pM OVA in liposomes in the presence of anti-DNP mAb (B), washed, and incubated with OT-I cells. In (C) cells were incubated for 2 days without Ag in the presence of class I-restricted alloreactive transgenic T cells (KB5C20) specific for an K^b-restricted peptide derived from an endogenous mouse protein. The control DC were bone marrow-derived cells from CBA/J mice cultured for 12 days (H-2k) (□). Results shown are representative of two experiments.

anti-DNP Ab and DNP-bearing liposomes at 10 pM OVA concentration in culture. The results, presented in Fig. 7 B, show that the number of DC cultured for 12 days required for presentation of SIINFEKL derived from processing of FcγR-targeted liposome-encapsulated OVA corresponded well to the number of DC cultured for either 5 or 12 days which presented SIINFEKL as the free peptide (Fig. 7 A), while any number of cells cultured for 5 days failed to present the Ag from opsonized liposomes. This suggests that the capacity of processing of the exogenous OVA was a property, acquired over time, of DC that were nevertheless already capable of presenting SIINFEKL as the free peptide after 5 days.

The lack of major phenotypic differences between DC cultured for 5 and 12 days in Fig. 6 A did not rule out a

developmental delay in some element required for class I-peptide presentation by the DC cultured for 5 days, which could occur at the level of the proteasome, TAP or other molecules necessary for class I presentation [1]. To evaluate this question, we analyzed the presentation of an endogenous Ag as a function of the age of the DC. We used B6 DC cultured for 5 and 12 days to stimulate T cells from B10.BR mice transgenic for a $CD8^+$ TCR (KB5C20), specific for K^b associated with peptide sequences derived from one or more endogenous mouse proteins [19]. Presentation to KB5C20 of these determinants is TAP dependent [20] but CD4 independent [21]. As shown in Fig. 7 C, DC cultured for 5 days presented endogenous K^b-restricted determinants practically as well, on a per cell basis, as those cultured for 12 days ruling out an obvious defect in any pathway required for class I-restricted presentation of endogenous proteins by early DC. Taken together, these results show that the failure to present exogenous OVA by DC cultured for 5 days appears related to their inability to transport FcγR-targeted OVA into the cytosol.

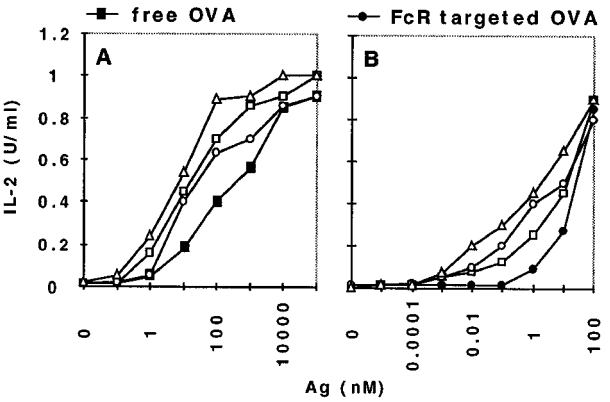

Fig. 8. Agents inducing maturity of DC cultured for 5 days have little influence on their capacity to present free (A) or FcγR-targeted OVA (B) in association with class I molecules. After 5 days of culture DC were incubated with 20 μg/ml anti-CD40 mAb (□), 10 μg/ml LPS (○), or 10 μg/ml poly (I) poly (C) (△). After overnight incubation, free OVA (■) or liposome-encapsulated OVA in the presence of anti-DNP Ab (●) were added overnight. The following day cells were washed, OT-I cells added, and IL-2 measured 2 days later. The results were the same when these agents were added to the DC at the same time as the OVA (data not shown). Results shown are representative of two experiments.

2.5 Lack of effect of induction of DC maturation by various agents on acquisition of class I presentation

DC have been reported to undergo maturation or acquire the capacity to stimulate $CD8^+$ T cells as a consequence of various stimuli derived from infectious agents, including LPS or double-stranded RNA in the case of viral infection [22]. DC will initially present Ag to $CD4^+$ T cells expressing the CD40 ligand. Activation of DC via CD40 has been shown to render them capable of stimulating $CD8^+$ T cells. We asked whether exposure to a ligand for CD40, to LPS, or to poly (I) poly (C), a potent inducer of type I IFN, would permit DC harvested at 5 days to present exogenous Ag in the context of class I molecules. FACS analysis showed that LPS stimulation resulted in marked up-regulation of class II, intercellular adhesion molecule (ICAM)-1, B7.1 and CD40, as well as smaller increases in B7.2 and H-$2K^b$ by DC cultured for 5 days (Fig. 6 B). Reflecting this up-regulation, data presented in Fig. 8 show that cells cultured for 5 days and incubated with Ab to CD40, LPS or double-stranded RNA [poly (I) poly (C)] all presented either free (Fig. 8 A) or FcγR-targeted OVA (Fig. 8 B) with 10- to 100-fold greater efficiency. However, cells treated with these agents still required 10 000-fold more FcγR-targeted liposome-encapsulated Ag for class I-restricted presentation than untreated DC obtained after 10 or more days of culture (compare to Fig. 1 C).

3 Discussion

Induction of cytotoxic T cell responses normally requires professional APC expressing the class I-peptide complexes for which the T cells are specific, as well as appropriate co-stimulatory molecules. DC are widely distributed in tissues and migrate to sites of inflammation. This increases their chance to contact infectious agents, enabling presentation of antigenic peptides derived from endogenous protein synthesis. These cells are also able to take up and present exogenous Ag in the form of debris from infected dead or dying cells in a TAP-dependent process [6] involving heat shock proteins [23] or particular membrane phospholipids for which they have specific receptors (reviewed in [24]). In the absence of such mechanisms, data presented here and other published results [25–28] show a requirement for relatively high (10^{-8} – 10^{-6}M) Ag concentrations for presentation of free Ag or Ag in non-targeted liposomes, for either class I- or II-restricted responses. However, when targeted in liposomes to the FcγR of DC, both class I- and II-restricted presentation occurred at Ag concentrations of 10^{-13}M. This quantity of Ag could be provided by the contents of a single 200-nm liposome, containing about 1600 molecules of OVA, per DC.

Ag presentation was blocked by an Ab to FcγRII/III for both class II- [8] and I (data not shown)-restricted responses. In addition, class I-restricted responses to immune complexes did not occur when DC from mice, in which the FcγRI- and RIII-associated γ chain was knocked out, were used [13]; thus, if immune complexes and IgG-opsonized liposomes are taken up by the same mechanism, the FcγRIII appears to be the relevant receptor. B cells also express an FcγR (FcγRIIb1) which does not mediate Ag uptake [15]. In the study of Regnault et al. [13] a chimeric FcR transfected in B lymphoma cells functioned for class II presentation of OVA-anti-OVA complexes by those cells but failed to confer class I-restricted Ag on the lymphoma. Thus, the capacity of an FcγR to permit translocation into the cytosol of the Ag to which it binds is not an intrinsic property of the receptor, but also depends on the cells expressing it. However, translocation of Ag into the cytosol is not the inevitable consequence of its internalization in general, since targeting of liposomes to other cell surface molecules than the FcγR on DC failed to result in $CD8^+$ T cell stimulation, although these molecules were excellent targets for endocytosis of Ag for presentation by class II for $CD4^+$ T cell stimulation (Fig. 2).

Like FcγRIII on DC, sIg is expressed at the cell surface of B cells in heterocomplexes with molecules containing immune receptor tyrosine-based activation motif (ITAM) sequences, which play a role in the activation of these APC [29]. Evidence for a role of sIg of Ag-specific B cells for priming naive $CD4^+$ T cells *in vitro* is well established [7, 8]. Transfection of B lymphoma cells with sIg specific for TNP was also reported to permit those cells to acquire and to present TNP-modified OVA in a class I-restricted manner more efficiently than Ag taken up by fluid-phase endocytosis [9]. Nevertheless, the concentrations of Ag used in the latter study were high (micromolar). Here we show that B cells which could use their sIg to present Ag for class II presentation at femtomolar concentrations failed to present these Ag in the class I presentation pathway even at concentrations many orders of magnitude higher (Fig. 1). Pulsing of B cells with SIINFEKL permitted efficient stimulation of OT-I cells from unimmunized mice, thus there are no elements of co-stimulation or of other elements required for Ag presentation lacking in B cells, provided that the class I-peptide complex is expressed at the cell surface.

It has been suggested that peptides derived from exogenous Ag taken up by DC may associate with class I molecules recycling through endocytic vesicles also rich in class II molecules [30, 31]. Co-localization of Ag internalized in coated pits and class I molecule has been recently demonstrated by electron microscopy [32]. However, the capacity of these complexes to be re-expressed at the cell surface or to stimulate T cells was not tested. In the present study, class I-restricted presentation of OVA taken up in FcγR-targeted liposomes was shown to be TAP1 dependent. In addition, SIINFEKL encapsulated in FcγR-targeted liposomes was not presented, while liposomes of identical composition but containing OVA released a sufficient fraction of their contents in these vesicles to result in efficient class II-restricted presentation. It is likely that the SIINFEKL peptide in liposomes targeted to the FcγR is more rapidly degraded when released in endocytic vesicles than the same sequence expressed within the OVA protein. Nevertheless, SIINFEKL peptide was capable of being presented in association with class I molecules on DC from $TAP1^{-/-}$ mice when present at concentrations of 10^{-14}M or less in the medium (Fig. 3 B). Thus either peptide association with class I molecules in endocytic vesicles is very inefficient, or molecules which bind peptides in endocytic vesicles accessible via the FcγR are not re-expressed at the cell surface. The lack of presentation of SIINFEKL from targeted vesicles also argues against fusion of liposomes with the membrane of endocytic vesicles, which would be expected to have permitted SIINFEKL to enter the cytosol to be efficiently transported by TAP [18]. Passage into the cytosol of peritoneal macrophages or DC *in vitro* was originally reported to be enhanced when "pH-sensitive" liposome formulations, which release their contents or which may fuse with endocytic vesicle membranes at acid pH, were used [25, 26, 33]. However, this dependence on pH-sensitive lipids was not observed for administration of liposomes *in vivo* [27]. The phospholipids we used for liposome formation are saturated and not known to be capable of fusion with cell membranes. This leaves the mechanism of the passage of OVA into the cytosol unexplained.

Electron microscopic studies of bone marrow-derived DC have revealed development of a tubulo-vesicular system, vesicles and multivesicular bodies over the period from 6 to 12–14 days in culture [34]. It remains to be determined whether these morphological changes are relevant to our finding that class I presentation of Ag targeted to the FcγR only becomes efficient for DC derived from bone marrow cells cultured for 10–12 days. Cells cultured for 5 or 12 days express nearly equivalent levels of class I molecules and bind equivalent numbers of opsonized liposomes (Fig. 6 A). DC cultured for 5 days also present endogenous peptides (Fig. 7 C) and exogenous SIINFEKL in the form of the free peptide (Figs. 5 D–F and 7 A) as efficiently as DC cultured for longer periods. These data are consistent with the existence of a regulated transport process present in DC which can convey exogenous Ag from endocytic vesicles to the cytosol. However, we cannot formally rule out other explanations for our results (for example, "pre-pro-

cessing" of OVA by an enzyme only active in endosomes of cells cultured for 12 days [35]).

In their study on presentation of Ag from Ag-Ab complexes, in addition to bone marrow-derived DC cultured for 3 weeks or more, Regnault et al. [13] successfully used the D1 long-term DC line for class I presentation of Ag from immune complexes [36]. This line is reported to have an "immature" phenotype [36, 37]. Interactions with immune complexes [13] and opsonized liposomes (data not shown) are themselves capable of increasing the expression of MHC and co-stimulatory molecules. Nevertheless, we did not observe presentation of OVA from OVA-anti-OVA immune complexes using DC cultured for 5 days, though we confirmed the results of Regnault et al. when 2-week-old cultures were used (data not shown).

We also exposed DC cultured for 5 days to agents that are known to up-regulate the expression of co-stimulatory molecules, such as LPS, to increase the half-life of class I molecules, such as double-stranded RNA in the form of poly (I) poly (C) [22], or to induce DC to activate class I-associated presentation of Ag by a CD40-dependent pathway [38, 39]. None of these treatments conferred the capacity of efficient class I-restricted presentation of exogenous Ag on these DC, which nonetheless became "mature" by the criteria of up-regulation of MHC, co-stimulatory and ICAM-1 molecules (Fig. 6 B).

The heterogeneity of DC pathways and their maturation *in vivo* are just beginning to be elucidated. It remains to be determined whether an *in vivo* population of DC of hematopoietic origin shares the antigen presentation characteristics with the *in vitro* derived cells used here. The ability to facilitate the induction of cytotoxic T cells by targeting DC via the FcR or other receptors is of evident interest for vaccine development.

4 Materials and methods

4.1 Animals, culture medium, cell lines and antibodies

OT-I mice are transgenic for a TCR $\alpha\beta$ specific for the chicken OVA 257–264 peptide SIINFEKL in the context of H-2K^b [16]. These mice were a kind gift of Matthias Merkenschlager (MRC, London). They were maintained on the C57BL/6 (B6) background and identified by FACS analysis as those mice in which a majority of peripheral blood CD8$^+$ cells express Vα2. B10.BR mice transgenic for a TCR recognizing the K^b molecule as an alloantigen (KB5C20) [19] were provided by Anne-Marie Schmitt-Verhulst (CIML, Marseille, France). T cells obtained from the spleens of transgenic mice 6–12 weeks of age were purified by passage over nylon wool columns.

T hybridoma cells OT4H.1D5 (1D5) [14] (provided by Yong Ke, Emory University, Atlanta, GA) are specific for an undefined I-A^b-restricted OVA peptide. These cells were cultured in RPMI medium supplemented with 5 % FCS, 50 μM 2-ME, 2 mM glutamine and antibiotics (supplemented RPMI).

DC were derived from bone marrow of B6, CBA/J or (CBAxB6)F1 mice (Iffa-Credo), or from TAP1$^{-/-}$ mice on a B6 background (JAX). Cells were cultured for 3 days in DMEM supplemented with 10 % FCS, antibiotics, 2 mM glutamine, 50 μM 2-ME and 30 % conditioned medium from NIH3T3 cells containing GM-CSF (provided by Jean Davoust, CIML), as described [36]. They were then diluted 1:1 in the same medium and, after an additional period of 1–12 days of culture, plastic-nonadherent cells were washed, resuspended in supplemented RPMI and used as APC. Splenic B cells were obtained from B6 mice by elimination of T cells using Ab to Thy-1, CD4 and CD8, and rabbit complement.

U7.27.7, a hybridoma producing an anti-DNP Ab, was provided by Zelig Eshbar (Weizmann Institute, Rehovot, Israel). 100.5.28 is specific for H-2K^k [40]. 10.2.16 [41] is specific for I-A^k. 5F1 is specific for H-2K^b [42]. The above mAb are mouse IgG2a. The CD40-specific rat mAb FGK45 [39] was provided by Rienk (Offringa, Leiden University, Leiden, The Netherlands). Rabbit anti-mouse IgG (H plus L) was from Jackson.

4.2 Liposomes and other reagents

Liposomes (80 μmoles lipids) were made from 65 % (mol/mol) dimyristoyl phosphatidylcholine, 34.5 % cholesterol (Sigma-Aldrich) and 0.5 % DNP-caproyl-phosphatidylethanolamine (DNP-cap PE) (Molecular Probes). Liposomes were formed by exposing lipids evaporated from chloroform/methanol (9:1 v/v) to an aqueous solution containing 30 mg/ml (680 μM) OVA (Grade VII, Sigma) in PBS, or 1 mg/ml (1 mM) SIINFEKL (CIML protein synthesis facility) in PBS containing 10 mM carboxyfluorescein (CF) (Molecular Probes). Following repeated cycles of freezing and thawing, liposomes were formed by extrusion (Extruder, Lipex Biomembranes, Vancouver, Canada) [43] through polycarbonate filters of 200 nm pore size at 40 °C, followed by gel filtration over Sepharose 4B columns to eliminate unencapsulated solute. Laser light scattering determinations indicate that these preparations are homogeneous with diameters closely corresponding to the pore size of the polycarbonate filters used (data not shown). Lipid vesicles were sterilized by filtration through 0.45-μm filters. ^{125}I-labeled OVA was used to determine % protein entrapment. SIINFEKL concentrations were determined by fluorescence of dilutions of free and liposome-entrapped SIINFEKL in 10 mM CF with reference to the stock solution of SIINFEKL used for liposome preparation. At the concentration of Ag used for encapsulation, 200-nm liposomes each contain

about 1600 molecules of protein or peptide. The amount of liposome-associated Ag used for presentation experiments was obtained by serial tenfold dilutions of these liposomes. We used anti-DNP mAb to target DNP-bearing liposomes to the FcγR. The preparation of PA-anti-DNP conjugated and their use to target DNP-bearing liposomes to various cell surface determinants other than the FcγR, in the presence of protein A-binding Ab specific for those determinants, has been described [8]. LPS (*Escherichia coli* 055:B5) and poly (I) poly (C) were from Sigma.

4.3 Antigen presentation assays

APC (20 000 B cells or DC) were incubated in 100 μl supplemented RPMI, in duplicate wells of 96-well flat-bottom tissue culture plates. Free or liposome-encapsulated Ag at the indicated concentrations were added overnight, in the presence or absence of targeting or control Ab (5 μg/ml). DC were then washed before addition of 10 000 T hybridoma or 20 000 T cells from transgenic mice for 48 h in supplemented RPMI, at which time undiluted supernatant fluids were harvested for determination of IL-2 secretion based on a bioassay using the IL-2-dependent cell line CTLL. IL-2 values are derived from a standard curve using CTLL in the presence of recombinant mouse IL-2 (Boehringer Mannheim).

4.4 FACS analysis

DC (3×10^5) cultured for 5 or 12 days were washed and incubated in PBS containing 1 % FCS, 1 mM EDTA and 0.1 % NaN_3. Incubation with FITC-conjugated Ab (anti-B7.1, -B7.2 and -ICAM-1 from Pharmingen; anti-I-A^b from Caltag) was performed at 4 °C for 1 h. For indirect labeling of Mac-1 (Hybritech, San Diego, CA), 33D1 (ATCC, Manassas, VA) and CD40 (FGK45), cells were incubated with 5 μg/ml rat mAb for 30 min and washed. Anti-rat fluorescein dichlorotriazine (DTAF) conjugates (Jackson, Bar Harbor, ME) were then added for an additional 30 min. Cells were washed and fixed in 2 % formaldehyde and analyzed in a FACScan (Becton Dickinson) cytofluorimeter. For FcγR-dependent binding of liposomes, DNP-bearing liposomes containing CF and OVA were used at a final OVA concentration of 50 nM. They were incubated with cells in the presence or absence of 5 μg/ml anti-DNP Ab for 1 h, washed and fixed as above.

Acknowledgements: We thank Delphine Palet for technical assistance and Jean Davoust, Sylvie Guerder, Bernard Malissen and Anne-Marie Schmitt-Verhulst for helpful discussions and criticism of the manuscript. This work was supported by institutional grants from the Institut National de la Santé et de la Recherche Médicale and the Centre National de la Recherche Scientifique and by a grant in the European Community Biomed II program. K.S. was supported by a fellowship from the Ministère de l'Education Nationale, de la Recherche et de la Technologie.

References

1 **Pamer, E. and Cresswell, P.,** Mechanisms of MHC class I-restricted antigen processing. *Annu. Rev. Immunol.* 1998. **16:** 323–358.

2 **Kurts, C., Heath, W. R., Carbone, F. R., Allison, J., Miller, J. F. and Kosaka, H.,** Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J. Exp. Med.* 1996. **184:** 923–930.

3 **Rock, K.,** A new foreign policy: MHC class I molecules monitor the outside world. *Immunol. Today* 1996. **17:** 131–137.

4 **Jondal, M., Schirmbeck, R. and Reimann, J.,** MHC class I-restricted CTL responses to exogenous antigens. *Immunity* 1996. **5:** 295–302.

5 **Carbone, F. R., Kurts, C., Bennett, S. R., Miller, J. F. and Heath, W. R.,** Cross-presentation: a general mechanism for CTL immunity and tolerance. *Immunol. Today* 1998. **19:** 368–373.

6 **Sigal, L. J., Crotty, S., Andino, R. and Rock, K. L.,** Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature* 1999. **398:** 77–80.

7 **Ho, W., Cooke, M., Goodnow, C. and Davis, M.,** Resting and anergic B cells are defective in CD28-dependent costimulation of naive CD4+ T cells. *J. Exp. Med.* 1994. **179:** 1539–1549.

8 **Serre, K., Machy, P., Grivel, J.-C., Jolly, G., Brun, N., Barbet, J. and Leserman, L.,** Efficient presentation of multivalent antigens targeted to various cell surface molecules of dendritic cells and surface immunoglobulin of antigen-specific B cells. *J. Immunol.* 1998. **161:** 6059–6067.

9 **Ke, Y. and Kapp, J. A.,** Exogenous antigens gain access to the major histocompatibility complex class I processing pathway in B cells by receptor-mediated uptake. *J. Exp. Med.* 1996. **184:** 1179–1184.

10 **Sallusto, F. and Lanzavecchia, A.,** Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med.* 1994. **179:** 1109–1118.

11 **Maurer, D., Fiebiger, E., Ebner, C., Reininger, B., Fischer, G. F., Wichlas, S., Jouvin, M.-H., Schmitt-Egenolf, M., Kraft, D., Kinet, J.-P. and Stingl, G.,** Peripheral blood dendritic cells express FcεRI as a complex composed of FcεRIα- and FcRIγ-chains and can use this receptor for IgE-mediated allergen presentation. *J. Immunol.* 1996. **157:** 607–616.

12 **Fanger, N. A., Voigtlaender, D., Liu, C., Swink, S., Wardwell, K., Fisher, J., Graziano, R. F., Pfefferkorn, L. C. and Guyre, P. M.,** Characterization of expression, cytokine regulation and effector function of the high affinity IgG receptor Fc gamma RI (CD64) expressed on human blood dendritic cells. *J. Immunol.* 1997. **158:** 3090–3098.

13 **Regnault, A., Lankar, D., Lacabanne, V., Rodriguez, A., Théry, C., Rescigno, M., Saito, T., Verbeek, S., Bonnerot, C., Ricciardi-Castagnoli, P. and Amigorena, S.,** Fcγ receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J. Exp. Med.* 1999. **189:** 371–380.

14 **Li, Y., Ke, Y., Gottlieb, P. D. and Kapp, J. A.,** Delivery of exogenous antigen into the major histocompatibiliy complex class I and class II pathways by electroporation. *J. Leukoc. Biol.* 1994. **56:** 616–624.

15 **Amigorena, S., Bonnerot, C., Drake, J., Choquet, D., Hunziker, W., Guillet, J.-G., Webster, P., Sautés C., Mellman, I. and Fridman, W. H.,** Cytoplasmic domain heterogeneity and functions of IgG Fc receptors in B lymphocytes. *Science* 1992. **256:** 1808–1812.

16 **Hogquist, K. A., Jameson, S. C., Heath, W. R., Howard, J. L., Bevan, M. J. and Carbone, F. R.,** T cell receptor antagonist peptides induce positive selection. *Cell* 1994. **76:** 17–27.

17 **Van Kaer, L., Ashton-Rickardt, P. G., Ploegh, H. H. and Tonegawa, S.,** TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4-8+ T cells. *Cell* 1992. **71:** 1205–1214.

18 **Craiu, A., Akopian, T., Goldberg, A. and Rock, K. L.,** Two distinct proteolytic processes in the generation of a major histocompatibility complex class I-presented peptide. *Proc. Natl. Acad. Sci. USA* 1997. **94:** 10850–10855.

19 **Schönrich, G., Kalinke, U., Momburg, F., Malissen, M., Schmitt-Verhulst, A. M., Malissen, B., Hämmerling, G. J. and Arnold, B.,** Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. *Cell* 1991. **65:** 293–304.

20 **Guimezanes, A., Schumacher, T. N., Ploegh, H. L. and Schmitt-Verhulst, A. M.,** A viral peptide can mimic an endogenous peptide for allorecognition of a major histocompatibility complex class I product. *Eur. J. Immunol.* 1992. **22:** 1651–1654.

21 **Gaugler, B., Schmitt-Verhulst, A. M. and Guimezanes, A.,** Evaluation of functional heterogeneity in the CD8 subset with T cells from T cell receptor-transgenic mice. *Eur. J. Immunol.* 1993. **23:** 1851–1858.

22 **Cella, M., Salio, M., Sakakibara, Y., Langen, H., Julkunen, I. and Lanzavecchia, A.,** Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J. Exp. Med.* 1999. **189:** 821–829.

23 **Blachere, N. E., Li, Z., Chandawarkar, R. Y., Suto, R., Jaikaria, N. S., Basu, S., Udono, H. and Srivastava, P. K.,** Heat shock protein-peptide complexes, reconstituted in vitro, elicit peptide-specific cytotoxic T lymphocytes response and tumor immunity. *J. Exp. Med.* 1997. **186:** 1315–1322.

24 **Platt, N., da Silva, R. P. and Gordon, S.,** Recognizing death: the phagocytosis of apoptotic cells. *Trends Cell. Biol.* 1998. **8:** 365–372.

25 **Harding, C. V., Collins, D. S., Kanagawa, O. and Unanue, E. R.,** Liposome-encapsulated antigens engender lysosomal processing for class II MHC presentation and cytosolic processing for class I presentation. *J. Immunol.* 1991. **147:** 2860–2863.

26 **Nair, S., Zhou, F., Reddy, R., Huang, L. and Rouse, B. T.,** Soluble proteins delivered to dendritic cells via pH-sensitive liposomes induce primary cytotoxic T lymphocyte responses in vitro. *J. Exp. Med.* 1992. **175:** 609–612.

27 **Collins, D. S., Findlay, K. and Harding, C. V.,** Processing of exogenous liposome-encapsulated antigens in vivo generates class I MHC-restricted T cell responses. *J. Immunol.* 1992. **148:** 3336–3341.

28 **Nair, S., Babu, J., Dunham, R., Kanda, P., Burke, R. and Rouse, B.,** Induction of primary, antiviral cytotoxic, and proliferative responses with antigens administered via dendritic cells. *J. Virol.* 1993. **67:** 4062–4069.

29 **Cambier, J.,** Antigen and Fc receptor signalling: the awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). *J. Immunol.* 1996. **155:** 3281–3285.

30 **Albert, M. L., Pearce, S. F., Francisco, L. M., Sauter, B., Roy, P., Silverstein, R. L. and Bhardwaj, N.,** Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J. Exp. Med.* 1998. **188:** 1359–1368.

31 **De Bruijn, M., Peterson, P. and Jackson, M.,** Induction of heat-stable antigen expression by phagocytosis is involved in in vitro activation of unprimed CTL by macrophages. *J. Immunol.* 1996. **156:** 2686–2692.

32 **Arnold-Schild, D., Hanau, D., Spehner, D., Schmid, C., Rammensee, H. G., de la Salle, H. and Schild, H.,** Receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. *J. Immunol.* 1999. **162:** 3757–3760.

33 **Reddy, R., Zhou, F., Huang, L., Carbone, F., Bevan, M. and Rouse, B. T.,** pH sensitive liposomes provide an efficient means of sensitizing target cells to class I restricted CTL recognition of a soluble protein. *J. Immunol. Methods* 1991. **141:** 157–163.

34 **Zhang, Y., Harada, A., Wang, J. B., Zhang, Y. Y., Hashimoto, S., Naito, M. and Matsushima, K.,** Bifurcated dendritic cell differentiation in vitro from murine lineage phenotype-negative c-kit+ bone marrow hematopoietic progenitor cells. *Blood* 1998. **92:** 118–128.

35 **Pierre, P. and Mellman, I.,** Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. *Cell* 1998. **93:** 1135–1145.

36 **Winzler, C., Rovere, P., Rescigno, M., Granucci, F., Penna, G., Adorini, L., Zimmermann, V. S., Davoust, J. and Ricciardi-Castagnoli, P.,** Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J. Exp. Med.* 1997. **185:** 317–328.

37 **Rovere, P., Manfredi, A. A., Vallinoto, C., Zimmermann, V. S., Fascio, U., Balestrieri, G., Ricciardi-Castagnoli, P., Rugarli, C., Tincani, A. and Sabbadini, M. G.,** Dendritic cells preferentially internalize apoptotic cells opsonized by anti-beta2-glycoprotein I antibodies. *J. Autoimmun.* 1998. **11:** 403–411.

38 **Bennett, S. R., Carbone, F. R., Karamalis, F., Flavell, R. A., Miller, J. F. and Heath, W. R.,** Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 1998. **393:** 478–480.

39 **Schoenberger, S. P., Toes, R. E., van der Voort, E. I., Offringa, R. and Melief, C. J.,** T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 1998. **393:** 480–483.

40 **Lemke, H., Hämmerling, G. J. and Hämmerling, U.,** Fine specificity analysis with monoclonal antibodies of antigens controlled by the major histocompatibility complex and by the Qa/TL region in mice. *Immunol. Rev.* 1979. **47:** 175–206.

41 **Oi, V. T., Jones, P. P., Goding, J. W. and Herzenberg, L. A.,** Properties of monoclonal antibodies to mouse Ig allotypes, H-2, and Ia antigens. *Curr. Top. Microbiol. Immunol.* 1978. **81:** 115–120.

42 **Sherman, L. A. and Randolph, C. P.,** Monoclonal anti-H-2Kb antibodies detect serological differences between H-2Kb mutants. *Immunogenetics* 1981. **12:** 183–186.

43 **Hope, M. J., Bally, M. B., Webb, G. and Cullis, P. R.,** Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta* 1985. **812:** 55–65.

Correspondence: Lee Leserman, CIML, Case 906, F-13288 Marseille CEDEX 9, France
Fax: +33-491 269 430
e-mail: leserman@ciml.univ-mrs.fr