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Antigen presentation by naive macrophages, dendritic cells and B cells to primed T lymphocytes and their cytokine production following exposure to immunostimulating complexes

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SUMMARY

Influenza virus envelope proteins incorporated into immunostimulating complexes (iscoms) are taken up and processed by various kinds of antigen-presenting cells (APC), encompassing peritoneal cells (PEC), unfractionated splenocytes, splenic dendritic cells (DC) or B cells. The iscom-pulsed naive APC stimulated primed T cells to proliferate and produce cytokine *in vitro*. In contrast, only DC and B cells pulsed with the same antigen (Ag) in the micelle form functioned as accessory cells stimulating the primed T cells to proliferate and produce cytokine. In general, iscoms were better inducers of cell proliferation than micelles. Iscoms stimulated more secretion of IL-2 and interferon-gamma (IFN-γ) than the micelles, but both antigenic forms stimulated secretion of IL-4. DC and B cells pulsed with iscoms stimulated most efficiently the secretion of IL-2 and IFN-γ. DC were superior to the other APC in stimulating primed T cells to secrete IFN-γ. On the other hand, micelles stimulated more efficiently than iscoms splenic T cells from micelle-primed as well as iscom-primed mice to secrete IL-10. These data indicate that influenza virus envelope proteins incorporated in iscoms stimulate a broad T cell response, possibly emphasizing a Th1 type of response. The same Ag in a micelle form induce a more prominent Th2 type of T cell response. The results indicate that the administration of an Ag in an adjuvant formulation can superimpose a different cytokine profile on the immune response than that induced by the protein Ag alone.

Keywords iscoms APC antigen-presentation cytokines adjuvants

INTRODUCTION

Several studies have revealed that protective immunity to pathogenic microorganisms is not primarily determined by the level but rather by the type of immune response. The preferential activation of $CD4^+$ T helper cell subsets [1] secreting certain cytokines determines whether a protective or even a detrimental immune response will develop. For example, in human leprosy as well as in murine leishmaniasis, progression of disease is associated with the development of the Th2 type of T cell response [2,3]. In HIV infection, healthy virus positive individuals display a Th1 type of response, while progression to immunodeficiency may be linked to a development of a Th0 [4] or Th2 cytokine response [5]. Additional support for this thesis has been provided by the survival of IL-4 deficient mice infected with MAIDS virus [6]. While the reasons for evolving the one or the other type of immune response are not yet understood, it is conceivable that the cells taking up, processing and presenting antigen (Ag) to immune competent cells are crucial for the initiation and outcome of the response. For instance, dendritic cells (DC) are required as antigen-presenting cells (APC) for the production of interferon-gamma (IFN-γ) in allogenic reactions [7] and cytotoxic T lymphocyte (CTL) response both *in vivo* and *in vitro* [8,9]. Therefore, knowledge of the mechanism of APC activation and their role in the induction of immune response is of a primary interest for the rational design and development of efficient vaccines.

Immunostimulating complex (iscom) is a 40 nm cage-like particle made up of Quil A triterpenoids and lipids, in which soluble amphipathic proteins can be incorporated by hydrophobic interactions [10,11]. In the iscom, a multimeric form of the Ag is combined with a built-in adjuvant activity. Several studies have shown that iscom-borne Ag induces immune responses under MHC class I and II restriction [12–16]. These studies complemented with recent evidence [17] strongly

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Table 1. The conditions and detection limit of the immunoassays for cytokines

Cytokine	Coating	Blocking	Second Ab	Detection limit (pg/ml)
IL-2	1 μg/ml pH 9·6	1% BSA	1:3000	50
IL-4	3 μg/ml pH 9·6	5% skimmed milk	1:1000	35
IL-10	4 μg/ml pH 8·2	1% BSA	1:1500	60
IFN-γ	12 μg/ml pH 7·4	1% BSA	1:2000	750

indicate that iscom-borne Ag has access to the cytosol of APC. Iscoms as well as its matrix, i.e. the iscom structure devoid of protein, stimulate the secretion of IL-1 by murine splenocytes and peritoneal cells [18]. Increased expression of MHC class II molecules, considered to be a requirement for efficient Ag presentation, has been observed in primary stimulation of peritoneal cells and also in primed splenocytes upon restimulation with iscoms, both *in vivo* and *in vitro* [19,20]. Some of these data indicate that macrophages have a relevant role in the enhanced immune response to iscom-borne Ag [18,19]. We and others have previously demonstrated that immunization with influenza virus iscoms induces the development of T cells secreting IL-2 and IFN-γ [21–23]. In this study we designed experiments to compare the effect of presenting an Ag in iscoms with that in micelles by evaluating their capacity to induce APC to stimulate primed T cells. The T cell response was evaluated by measuring Ag-driven cell proliferation and the profile of cytokines produced. In this case we consider the profile determined by the production of IL-2 and IFN-γ as a Th1 type of response and the production of IL-4 and IL-10 as a Th2 profile.

MATERIALS AND METHODS

Experimental design

Unfractionated splenocyte populations, resident peritoneal cells (PEC), splenic B cells and splenic DC from naive mice were used as APC for the experiments on the induction of IL-2 and IFN-γ. Splenic cell suspensions enriched for T lymphocytes from iscom-primed mice were used as responders.

Mice

Gnotobiotic BALB/c female mice, 10–12 weeks old, were obtained from the National Veterinary Institute, Uppsala, Sweden.

Immunizations

Mice were primed subcutaneously with 1 μg influenza virus envelope proteins in iscoms or as micelles, and boosted s.c. 4 weeks later with the same dose.

Iscoms and micelles

Iscoms and micelles containing the envelope proteins from the influenza virus A/PR/8/34 (H1N1) were prepared by the dialysis method as described before [24]. The preparations were sterile filtered and stored at −70°C.

Preparation of APC

Resident PEC were recovered from the peritoneal cavity of mice by washing with sterile PBS under aseptic conditions. Single cell suspensions from spleens were either used unfractionated as APC or as a source of DC or B cells. Splenocyte suspensions contained 24–25% Thy 1.2^+ cells, 48–50% Ig^+ cells and 1% DC as determined by immunofluorescence. For separation of DC, 4×10^7 magnetic beads coated with sheep anti-rat antibodies (Dynabeads, Oslo, Norway) were reacted with 2 ml supernatant from the 33D1 hybridoma cell line (ATCC TIB 227, MD), which recognize splenic DC [25]. Beads were mixed with the splenocyte suspensions ($100–200 \times 10^6$ in 500 μl culture medium) and incubated for 30 min at 4°C. Detachment of the bound cells was done by incubation with 10 μl Detach-a-Bead Ab (Dynabeads) according to the supplier's instructions. The yield of DC was routinely $6–10 \times 10^5$ per spleen, and contained >98% $33D1^+$ cells.

For the separation of B cells, suspensions depleted of DC

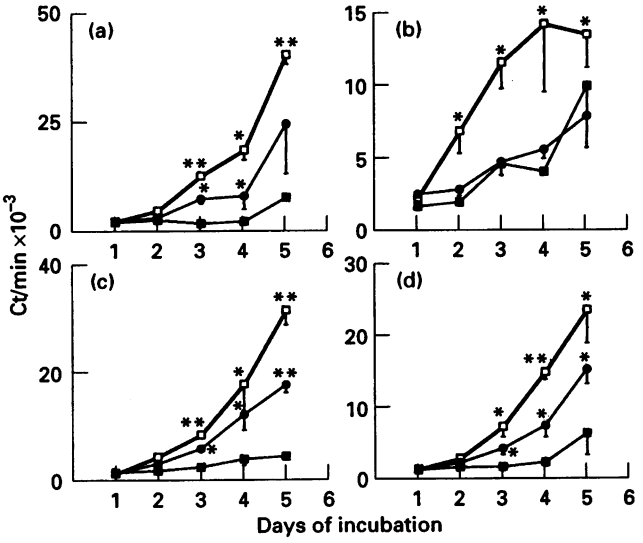

Fig. 1. The capacity of iscom- or micelle-pulsed APC to stimulate the proliferation of splenic T cells from iscom-primed mice. The APC used were: (a) unfractionated spleen cell suspension, (b) peritoneal cells, (c) dendritic cells, (d) B cells. The APC were pulsed with influenza virus envelope Ag in iscoms (□) or as micelles (●). Wells receiving medium only (■) were used as background controls. The results are expressed as mean ct/min ± s.d. of ^{3}H-TdR incorporation in triplicate wells, and are representative of four separate experiments. ^{3}H-TdR incorporation by the APC cultured in the absence of T lymphocytes was between 100 and 400 ct/min for the unfractionated spleen cell suspension, and 58 and 199 ct/min for B cells and DC. For peritoneal cells cultured with medium only the values were <200 ct/min at all time points, with micelles the values were 310, 382, 873, 2079 and 84 ct/min from day 1 to 5, and for iscoms the values were 130, 1012, 1663, 4848 and 2488 ct/min respectively. * $P < 0.05$; ** $P < 0.005$.

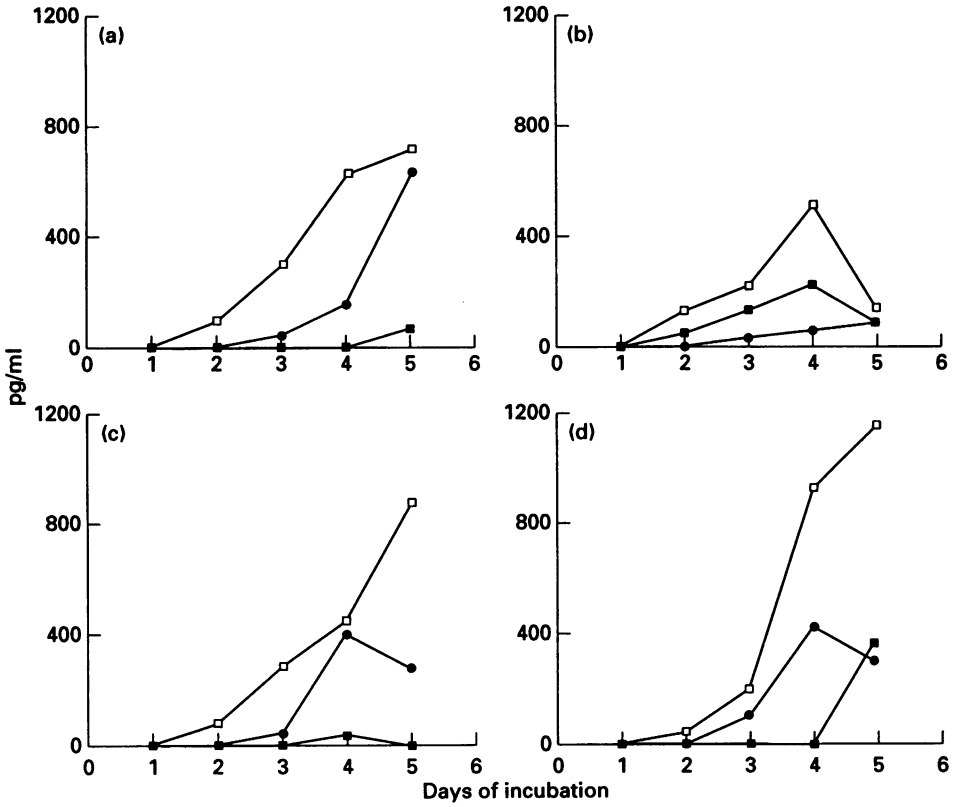

Fig. 2. The capacity of iscom- (□) or micelle-pulsed APC (●) to stimulate IL-2 secretion by iscom-primed T cells. Cultures receiving medium only (■) were used as background controls. The APC used were: (a) unfractionated spleen cell suspension, (b) peritoneal cells, (c) dendritic cells and (d) B cells. Unfractionated splenocytes, peritoneal cells or B cells (1×10^5 cells/well), or DC (2×10^4 cells/well) were pulsed with influenza virus envelope proteins in iscoms or as micelles and cultured with T cells from iscom-primed mice. Cell culture supernatants were harvested at the intervals shown and analysed for IL-2 by ELISA. Samples were tested in duplicates. The detection limit of the ELISA for IL-2 was 50 pg/ml.

were incubated in complete medium, i.e. RPMI 1640 (GIBCO, Paisley, UK) supplemented with 5% FCS (National Veterinary Institute), 2 mM L-glutamine, 50 mM 2-mercaptoethanol, and 50 μg/ml gentamycin (Sigma, St Louis, MO) for 2 h in plastic tissue culture bottles to allow cells to adhere to the plastic. B cells were isolated from the non-adherent population by reaction with sheep anti-rat Ab-coated beads and magnetic separation. These preparations contained >98% Ig^+ B cells.

Preparation of T cell suspensions

For the determination of IL-2 and IFN-γ, spleen cell suspensions from iscom-primed mice ($n = 3$) were subjected to sequential depletion of adherent cells, DC and two rounds of B cell depletion, as described above for the preparation of APC. These preparations consisted of at least 80% Thy 1.2^+ lymphocytes.

Assay for Ag presentation

PEC (1×10^5/microtitre well) were preincubated for 3 days before the Ag pulse to allow for spontaneous release of membrane-bound IL-1 [26]. Unfractionated splenocytes and B cells (1×10^5/well) and DC (2×10^4/well) were used freshly prepared. The APC were pulsed for 1 h at 37°C with 0·2 μg/ml of influenza virus envelope proteins, in iscoms or as micelles, in a total volume of 0·1 ml/well. After the pulse, non-internalized Ag was removed and the APC washed once with complete medium. Subsequently, the T cell suspensions were added at a concentration of 2×10^5 cells/well. The cell mixtures were incubated for the periods of time shown in Results, in the presence of 2 μCi/well ^{3}H-TdR (5 Ci/mmol; Amersham, Aylesbury, UK) for the last 20 h of incubation. The supernatants were recovered for cytokine analysis and the cells were harvested for measurement of proliferation. Cell-bound radioactivity was measured in an automatic betaplate counter (LKB Wallac, Turku, Finland). All assays were run in triplicate or quadruplicate and the results are expressed as ct/min.

Assay for the determination of IL-4 and IL-10

Unfractionated splenocyte suspensions (4×10^5/well) from mice primed with iscoms or micelles ($n = 4$) were cultured with 0·2 μg/ml Ag, either in iscoms or as micelles. The cultures were monitored for 1 to 6 days, and supernatants were recovered every 24 h for measurement of cytokines. Control cultures consisted of primed cells cultured with medium only. Cells from non-primed mice cultured with medium only or with Ag were also included as background controls.

ELISA for the murine cytokines IL-2, IL-4, IL-10 and IFN-γ

The following pairs of Ab were used: MoAb JES6-1A12 for capture and MoAb JES6-5H4 for detection of IL-2 (Pharmingen, San Diego, CA); MoAb 11B11 (ATCC HB

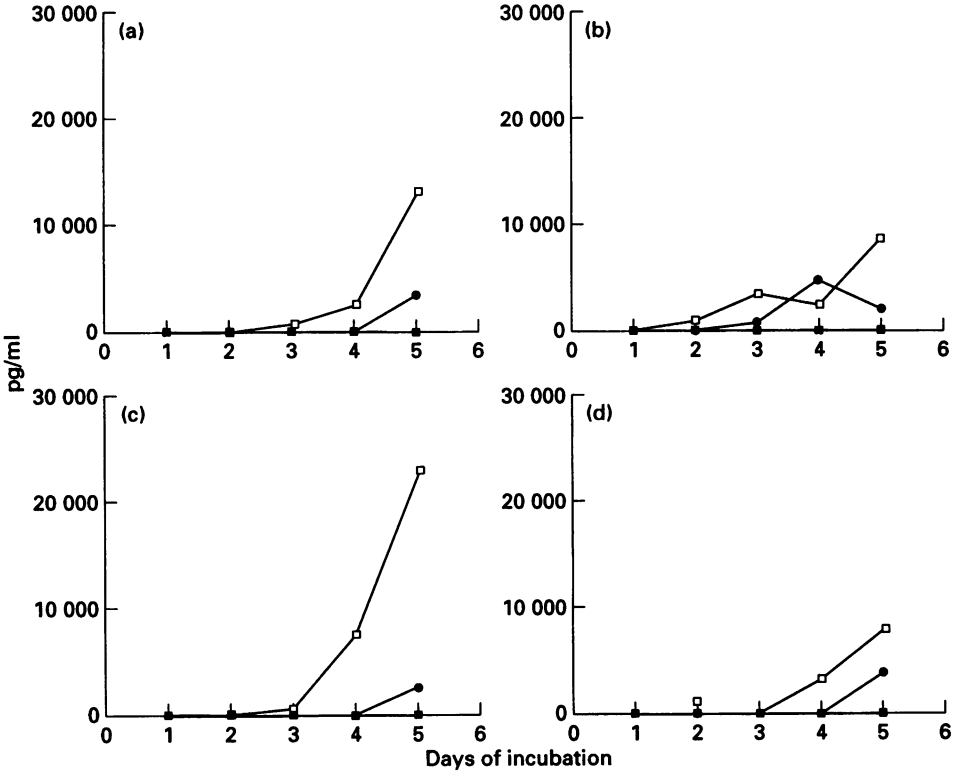

Fig. 3. The capacity of iscom- (□) or micelle-pulsed APC (●) to stimulate secretion of IFN-γ by iscom-primed T cells. Cultures receiving medium only (■) were used as background controls. The APC used were: (a) unfractionated spleen cell suspension, (b) peritoneal cells, (c) dendritic cells and (d) B cells. The cell cultures were set up as described in Fig. 2. The detection limit of the ELISA for IFN-γ was 750 pg/ml.

188) for capture and MoAb BVD6-24G2 (Pharmingen) for detection of IL-4; MoAb 18141D for capture and MoAb 18152D for detection of IL-10 (Pharmingen); MoAb R4-6A2 (ATCC HB 170) for capture and MoAb XMG1.2 (Pharmingen) for detection of IFN-γ. All Ab used for detection were biotinylated. Briefly, EIA/RIA plates (Costar, Cambridge, MA) were coated with the capturing Ab by overnight incubation at 4°C. The coating solution was carbonate buffer (pH 9·6) or PBS (pH 7·4), as indicated in Table 1. Empty binding sites were blocked by 1 h incubation at 37°C with blocking reagent (5% skimmed milk or 1% BSA, both diluted in PBS). Supernatants from proliferation assays were tested in duplicate by overnight incubation at 4°C under mild agitation. The plates were then incubated for 3 h at 20°C with the respective biotinylated Ab followed by a 2 h incubation at 20°C with streptavidin-peroxidase conjugate (Dako A/S, Glostrup, Denmark). Bound complexes were detected by reaction with tetramethylbenzidine (TMB) and H_2O_2. Absorbance was read at 450 nm. The cytokine concentration in the samples was calculated as pg/ml using recombinant murine cytokines (Genzyme, Cambridge, MA) for the preparation of standard curves.

Table 2. The concentration of IL-4 (pg/ml) in the supernatants from primed splenocytes stimulated *in vitro* with iscoms or micelles containing influenza virus envelope antigens

	Splenocytes cultured with	
Mice primed with*	Iscoms	Micelles
Iscoms	235 ± 188†	78 ± 10
Micelles	223 ± 87	153 ± 27

* BALB/c mice ($n = 4$) were immunized twice s.c. with 1 μg influenza virus envelope proteins in iscoms or as micelles. Spleen cell suspensions (2×10^6/ml) were restimulated with 0·2 μg/ml iscoms or micelles. Cell culture supernatants were harvested after 6 days incubation and analysed for IL-4 by ELISA.

† Samples were tested in triplicates. The values are pg/ml ± s.d.

Statistical analysis

Student's *t*-test was used for data evaluation.

RESULTS

The proliferation of primed T cells mediated by influenza virus envelope proteins in iscoms or as micelles

The APC populations. PEC, unfractionated splenocytes, DC or B cells derived from non-primed mice were pulsed with Ag in iscoms or as micelles and cultured with splenic T cells from iscom-primed mice. The four APC populations pulsed with iscoms induced significantly increased cell proliferation (Fig. 1). Although unfractionated splenocytes, DC and

B cells pulsed with micelles stimulated the primed T lymphocytes to proliferate, the ct/min values were lower than those scored with iscoms. No significant stimulation was recorded in the system using micelle-pulsed PEC as APC. The proliferation of all cultures was maximal at day 5 and the ct/min values were significantly higher than those of control cultures.

The secretion of IL-2 and IFN-γ

Both iscoms and micelles stimulated primed T cells to secrete IL-2 *in vitro*. In all groups the iscoms induced higher levels of IL-2 than micelles, and the values were highest in cultures containing B cells as APC, although DC and unfractionated splenocytes also induced high levels of IL-2. The production of IL-2 was lowest in cultures containing PEC as APC (Fig. 2). The biological activity of IL-2 induced by iscoms or micelles has been previously demonstrated [21,22].

DC were the most efficient APC in inducing the production of IFN-γ (Fig. 3) and iscoms induced more secretion of this cytokine than micelles did. Primed T cells cultured with medium alone did not secrete IL-2 or IFN-γ, nor did T cells from non-primed mice cultured with iscoms, micelles or medium alone.

The secretion of IL-4 and IL-10

IL-4 was detected in supernatants of cultured splenocytes from iscom- as well as micelle-primed mice from day 4 of culture and was maximal at day 6. The amount of IL-4 secreted was not significantly different whether the cells originated from iscom- or micelle-primed mice, restimulated with iscoms or micelles (Table 2). Similar results were obtained in a bioassay using the D10.G4.1 cell line as indicator of IL-4 activity. The specificity of the test was verified by neutralizing the activity of IL-4 with the 11B11 MoAb [27] (data not shown).

IL-10 was detected in cell culture supernatants from day 2 to day 6 of incubation and was maximal at day 4. Splenocytes derived from micelle-primed mice secreted nearly twice as much IL-10 if restimulated with micelles compared with those restimulated with iscoms. Splenocytes from iscom-primed mice secreted less IL-10 than spleen cells from micelle-primed mice, regardless of whether the cells were restimulated with iscoms or micelles (Fig. 4). Primed cells cultured with medium alone did not secrete IL-4 or IL-10, nor did non-primed cells cultured with iscoms, micelles or medium alone.

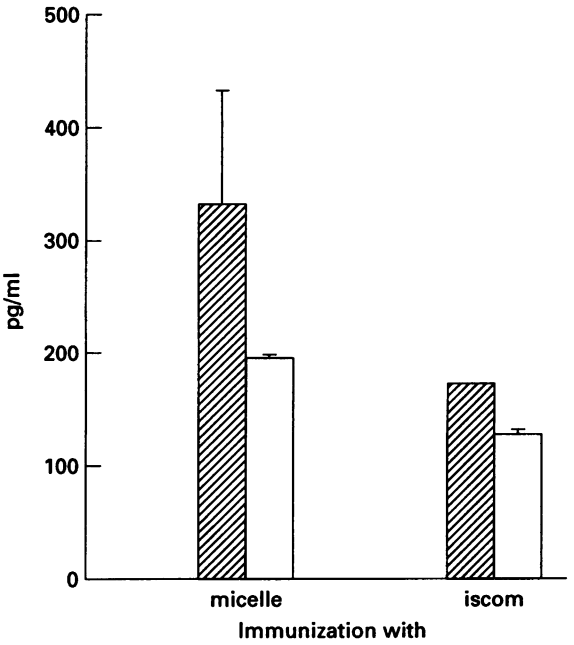

Fig. 4. The production of IL-10 by iscom- or micelle-primed splenocytes after *in vitro* restimulation with iscoms or micelles. Spleen cells (4×10^5 cells/well) from mice ($n = 4$) primed with influenza virus envelope Ag in iscoms or as micelles were cultured with 0·2 μg/ml Ag in iscoms or as micelles, for up to 6 days. The values represent pg/ml IL-10 in the supernatants of the tissue cultures after 4 days of incubation, when maximal values were scored. All samples were tested in duplicates. The detection limit for the IL-10 ELISA was 60 pg/ml. Restimulation with ▨, micelle; □, iscom.

DISCUSSION

The capacity of Ag internalization, processing and presentation by monocytic cells, DC and B cells has been the subject of numerous studies. However, information about the role of these cells in the processing of particulate forms of Ag is scarce. In this study we evaluated the capacity of APC to present two particulate formulations of Ag, iscoms and micelles containing influenza virus envelope proteins. While iscoms are rigid cage-like structures of 40 nm in diameter containing a built-in triterpenoid adjuvant, micelles are non-adjuvanted well-organized particles of similar size held together by hydrophobic interactions of the transmembrane part of the viral glycoproteins [28]. Both iscoms and micelles are taken up *in vitro* by PEC, DC and B cells, internalized, processed and presented to T cells, as demonstrated by the capacity of these APC pulsed with iscoms or micelles to induce proliferation of primed T cells and secretion of T cell products, such as IL-2, IL-4, IL-10 and IFN-γ. Monocytes and macrophages process and present both Ag formulations, which is expected due to the capacity of these cells to phagocyte particulate Ag. DC are efficient APC [7–9] and their capacity to take up and process soluble, i.e. monomeric, Ag has recently been elucidated [29]. Our data indicate that DC, as well as B cells, take up and process nanoparticles such as iscoms and micelles. Ag-specific B cells are also efficient APC, requiring minute amounts of Ag to activate cognate T cells [30]. In contrast to primed B cells, naive B cells are postulated to mediate Ag presentation leading to T cell anergy, explained by their lack of binding molecules such as B7 [31,32]. We could not confirm this postulation since the Ig^+ B cells used as APC in this study originated from non-primed mice, presented the Ag from both iscoms and micelles to primed T cells and induced these to proliferate. Possibly the reported anergy is restricted to monomeric forms of Ag and not to multimeric forms of Ag. The possibility that some of the B cells may have been in an activated state prior to the Ag pulse has not been ruled out.

Generally, iscoms were more efficient than micelles in eliciting a recall proliferative response of primed T cells. A higher degree of Ag internalization observed for iscoms compared with micelles [17] is probably a major factor in evoking this enhanced recall response. Another contributing factor is the capacity of iscoms to increase MHC class II expression and enhance the production of IL-1 [18–20]. A third enhancing factor is the secretion of the cytokines measured in this study

which was Ag-specific since they were only detected following restimulation with the relevant influenza virus Ag. The data demonstrate that iscoms enhance a T cell memory from which cells producing IL-2 and IFN-γ can readily be recalled *in vitro*. In previous studies we have shown that micelles of influenza virus glycoproteins poorly induce this kind of memory T cell in spite of the fact that the micelle is a particulate multimeric form of Ag presentation [21,22]. Instead, the micelles stimulate a Th2-like or possibly Th0-like response as suggested by the secretion of IL-4, the enhanced production of IL-10 and the low levels of IL-2 and IFN-γ. This strongly indicates that the adjuvant component of the iscom overrules the intrinsic immunological effect of the protein Ag, directing the immune system towards a Th1 type of response. Iscom-pulsed DC were the most efficient Ag-APC combination in recalling an IFN-γ response, which points to the importance of developing adjuvant formulations that target and recruit the proper APC.

Overall, the cytokine profile observed agrees with the profile of Ab response reported previously, e.g. iscoms stimulate an enhanced secretion of IgG2a [22,33], indicative of a Th1 response. Recently, the LTC component (now denominated QH-C) of *Quillaja saponaria* used as adjuvant to a commercial influenza vaccine preparation was shown to enhance *in vivo* the IL-2 and IFN-γ response in mice. The secretion of IL-4 was somewhat enhanced and IL-10 was not detected [34]. In this aspect, the effect of iscoms on the production of novel cytokines involved in the development of Th1 T cell response, e.g. IL-12 [35–37], deserves further investigation.

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