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Differential requirements for CTL generation by novel immunostimulants: APC tropism, use of the TAP-independent processing pathway, and dependency on CD80/CD86 costimulation

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Abstract

A major drawback of subunit vaccines is their inability to generate cytolytic T lymphocytes (CTL), a deficit attributed to segregation of the class I and class II antigen-processing pathways. We sought to understand processes involved in CTL induction by three proprietary adjuvants: Tomatine, PROVAX^{TM}, and a synthesized glycolipid (Glc-N-(8/16), Glycolipid). We used in vivo models to investigate antigen uptake, macrophage involvement, TAP-independent processing, and costimulatory molecule dependencies. Glycolipid required splenic and lymph node macrophages, whereas Tomatine generated CTL independently of either macrophage population. In contrast, PROVAX^{TM} showed partial macrophage requirements. Immunized TAP knockout mice revealed that ovalbumin (OVA)–Tomatine and OVA–PROVAX^{TM}, but not OVA–Glycolipid, generate class I–peptide complexes. All three immunostimulants also elicited CD86-dependent T_H1 cytokine responses.

Keywords: Adjuvant; Costimulation; Tomatine

1. Introduction

Control of infectious diseases requires pathogen-specific immune responses comprising multiple effector mechanisms. However, many subunit or recombinant protein-based vaccines are capable of stimulating humoral immunity but not cytolytic T lymphocytes (CTL). Conventional wisdom attributes this failure to the fact that soluble proteins are not processed in the MHC class I antigen-processing pathway but are channeled into the class II pathway for peptide loading onto MHC class II molecules and subsequent presentation to $CD4^+$ cells. The MHC class I pathway serves to target infected, damaged, or neoplastic cells for immune elimination [1]. Induction of CTL responses requires the generation of peptides that associate with and are presented by MHC class I molecules to $CD8^+$ T cells. Peptides are generated when endogenous protein is processed by the proteosome. They are then sorted by the transporter associated with antigen processing (TAP), which preferentially translocates 9–13 amino acid-sized peptides across the membrane and into the lumen of the endoplasmic reticulum [1]. Here peptides are further trimmed to a length of 8–11 amino acids and loaded onto empty MHC class I molecules [2]. Peptide-loaded molecules are then transported to the surface of the cell via the Golgi transport mechanism. On the cell surface, class I molecules present peptides to the T cell response of $CD8^+$ T cells and thereby deliver a primary signal to activate antigen-specific CTL. In contrast, the class II-processing pathway exists to prime $CD4^+$ T cells against extracellular threats by providing help to B cells engaged in antibody production. Both T cell subsets require a second

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signal, termed costimulation, for effective activation and prevention of anergy. It is thought that the two processing pathways are segregated, although they overlap at certain steps in the degradation of protein. Until recently, these observations implied that soluble protein/subunit vaccines are incapable of eliciting CTL responses because they do not access the class I-processing pathway.

To date adjuvants have been a somewhat overlooked component of vaccine design, and the only product licensed for clinical use is Alum, whereas incomplete Freund's adjuvant (IFA) is the most commonly used delivery vehicle for experimental studies. These two preparations have been in use since their discovery over half a century ago and are the accepted standards against which all new adjuvants should be compared [3]. However, although they have been used extensively with a broad range of antigen systems, neither stimulates cellular immune responses, specifically CTL. Over the last decade, a number of pivotal studies have shown that formulation of soluble protein with certain adjuvants, better termed immunostimulants or delivery vehicles, will result in the generation of CTL responses after parenteral administration into animals [4–14]. However, the manner in which adjuvants facilitate CTL induction has not been addressed. Although the role that antigen-presenting cells (APC) play in stimulating the immune system has been investigated extensively, there is little or no information on the interaction between APC populations and adjuvant-formulated protein, especially in the context of CTL induction. Both macrophages and dendritic cells (DC) are considered the major APC populations responsible for priming CTL, with DC perceived as the more potent of the two cell types. However as DC are somewhat difficult to isolate for functional analysis one approach to study the relative contribution of DC in CTL generation is to assess the capacity to generate CTL in the absence of competing macrophage APC. We therefore used three novel delivery systems described previously [8,15–17] to (i) understand how adjuvant-formulated antigen is assimilated for CTL induction; (ii) characterize the role phagocytic APC, such as macrophages, play in CTL induction; (iii) investigate whether non-conventional class I-processing pathways are utilized by the delivery systems; and (iv) determine whether there is a preference for CD80 or CD86 for CTL induction.

2. Materials and methods

2.1. Animals

Six to eight weeks old female C57Bl/6 mice (H-2K^b) (Harlan, Oxford, UK) were used in this study in accordance with United Kingdom Home Office guidelines and standards. For antigen-processing studies, TAP knockout (TAP$^{-/-}$) mice, Trp BMr1, were bred from breeding pairs obtained from Jackson Laboratories (Bar Harbor, ME USA) and used at ages similar to the C57Bl/6 mice.

2.2. Adjuvants, antigen and immunizations

Three adjuvant systems were used in this study: a synthesized glycolipid (Glc-N-(8/16), Glycolipid), Tomatine, and PROVAX^{TM} (IDEC Pharmaceuticals, San Diego, CA). The biochemical characteristics and processes used to formulate each of these reagents with antigen have been described previously in detail [4,12,15–17]. For all experiments we used the established and characterized ovalbumin (OVA, grade VI, Sigma, Dorset, UK) model antigen system [18]. Briefly, animals were immunized subcutaneously with 50 μg OVA formulated in a total volume of 100 μl with each of the delivery vehicles. The reference control adjuvants [8] were Alum (a kind gift of Dr. Eric Lindblad, Superfos, Denmark) and IFA (Sigma) as described previously [15–17].

2.3. In vivo inhibition of phagocytosis

Phagocytic activity was inhibited by intravenous injections of particulate silica (S-5631; Sigma). Mice were injected intravenously daily for two consecutive days with 100 μl of a 10 mg/ml silica suspension, which had been previously autoclaved [19,20]. Phagocytic activity was gauged by measuring in vitro uptake of latex beads (Molecular Probes, Eugene, OR). Splenocytes were plated onto sterile tissue culture six-well plates (Becton Dickinson, UK) in RPMI supplemented with 10% FCS, 40 μM glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (hereafter referred to as RF10) at 1×10^7 cells/ml, with 2 ml plated into each well. Plates were incubated at 37 °C, 5% CO_2 for 2 h. We removed non-adherent cells and retrieved adherent cells by incubating with ice cold RF10 accompanied by gentle agitation. Adherent cells were then washed and resuspended in RF10; 5×10^5 cells were aliquoted in a total volume of 1 ml with a final concentration of 1/10,000 1 μm latex beads (Molecular Probes), which had been subjected to two rounds of washing with sterile saline to remove any residual sodium azide. Cells were incubated at 37 °C, 5% CO_2 for varying lengths of time after which they were washed twice with PBS/2% BSA (Sigma, Dorset, UK) and then fixed with 1% paraformaldehyde. Samples were then analyzed through a FACScan (Becton Dickinson) flow cytometer with 10,000 events recorded for analysis using Lysis II software.

2.4. In vivo macrophage depletion

Macrophage depletion was achieved by treatment with liposome-encapsulated clodronate (dichloromethylene-diphosphonate; Cl_2MDP; kindly donated by Roche Diagnostics GmbH, Germany). We gave 200 μl clodronate liposome intravenously to mice to deplete splenic marginal macrophages or 50 μl subcutaneously to deplete draining lymph node macrophages. The preparation and depleting role of clodronate have been described previously [21,22]. In the case of the intravenously treated mice,

immunizations were carried out the following day, whereas subcutaneously treated mice were immunized two to 3 days later.

2.5. Assessment of CTL

CTL were measured by the method established by Moore et al. [18]. After 10–12 days of immunization, spleens were removed, passed through a 40 μm cell strainer (Becton Dickinson, San Diego, CA), and contaminating erythrocytes were lysed by treating with 0.17 M Tris–ammonium chloride. The resultant single cell suspension was then washed twice and resuspended in RF10. A total of 3×10^7 splenocytes in a final volume of 10 ml were stimulated with 1.5×10^6 EG7–OVA, which had been irradiated with 20,000 rad before being subjected to two washes. Splenocyte/EG7–OVA cultures were placed in 25 cm^2 tissue culture flasks (Boehringer Mannheim, UK) in an upright position with 10 U/ml IL-2 (R&D Systems Europe, Oxford, UK). Cultures were incubated for 5 days at 37 °C at 5% CO_2, after which non-adherent cells were retrieved and washed once with RPMI and tested for CTL activity against 51-chromium ([^{51}Cr], Amersham, UK)-labeled EG7–OVA and [^{51}Cr]-labeled EL4 cells. Both EG7–OVA and EL4 cells were grown overnight in RPMI supplemented with 20% FCS, and 10^6 cells were labeled with 1 mCi chromium in 200 μl medium for 1 h before being washed twice with 10 ml of medium. A total of 5×10^3-labeled cells were used as targets in a total volume of 150 μl in V-bottom microtiter plates (Boehringer Mannheim) with effector cells. Plates were spun for 2 min at 200 × *g* before being incubated for 4–6 h at 37 °C in 5% CO_2. After this period, plates were again spun for 2 min, 100 μl supernatant was taken, and [^{51}Cr] activity was measured on a γ counter (Canberra Packard, UK).

2.6. Detection of H-2K^b/SIINFEKL complexes by flow cytometry

Spleens isolated from either C57Bl/6 or TAP$^{-/-}$ mice were processed as described. H-2K^b/SIINFEKL peptide complexes were detected on the surface of splenocytes using the 25D1.16 rat monoclonal antibody [23] (a kind gift of Dr. R.N. Germain, NIAID, USA). Splenocytes were then washed and incubated with goat anti-rat (Cy3 conjugated) antibody (Sigma Aldrich), washed again, and fixed in 2% paraformaldehyde. Stained cells were analyzed on a FACScan (Becton Dickinson, CA), and 10,000-gated events were collected and compared against isotype control-stained cells.

2.7. Detection of functional H-2K^b/SIINFEKL complexes

In addition to the flow cytometry-based detection system, a chromogenic technique utilizing B3Z cells was used to detect for presence of functional MHC class I–peptide complexes. B3Z cells are a T–T hybridoma specific for the H-2K^b/SIINFEKL complex. They were established by fusion of a OVA H-2K^b restricted CTL clone with a *lacZ* inducible derivative BW5147 fusion partner [24]. The *lacZ* operon is under the control of the IL-2 promoter and engagement of peptide–class I complex results in activation of this promoter and subsequently enhanced LacZ activity. Varying numbers of splenocytes were added to 4×10^4 B3Z cells (a kind gift of Dr. N. Shastri, University of California, Berkeley), in a total volume of 200 μl in the presence of 1 nM SIINFEKL peptide [24] in flat bottom 96-well plates. Plates were incubated overnight, after which they were centrifuged for 5 min at 400 × *g*. Supernatants were discarded and 100 μl substrate mixture (PBS/0.25% NP-40 with 5 mM *o*-nitrophenyl β-D-galactopyranoside) (Sigma–Aldrich, Dorset, UK) was added to the remaining cells. The plates were further incubated at 37 °C and B3Z activation was measured by reading plates on a Dynatech MR5500 (Dynatech, Sussex, UK) plate reader at a wavelength of 405 nm. B3Z cells and splenocytes alone were included as negative controls.

2.8. In vivo inhibition of ACE activity

We treated TAP$^{-/-}$ mice with captopril (Sigma) in drinking water [25] (250 μg/ml drinking water) to inhibit angiotensin-converting enzyme (ACE) activity. The drug was freshly formulated in water every day, and treatment was initiated at least 3 days prior to immunization. Water bottles were covered in aluminum foil to prevent photodegradation of the drug.

2.9. Assessment of in vivo inhibition of ACE activity

Mice were cardiac bled with heparin-free needles and syringes; blood was allowed to clot and then was centrifuged. Plasma was collected and used in the ACE assay [26]; 140 μl plasma was added to 50 μl distilled water and 500 μl substrate (2 mM *N*-(3-[2-furyl]acryloyl)-Phe-Gly-Gly, 50 mM HEPES 0.4 M NaCl) and incubated at 37 °C. OD was measured at 340 nm 30 min after incubation with substrate. The relative amount of ACE activity in test plasma was calculated using 0.1 U/ml ACE reference control.

2.10. In vivo CD80 and CD86 blocking

CD80 and CD86 were blocked by administration of monoclonal antibodies. Specifically, anti-CD80 (hamster derived, clone 1610A1) [27], and anti-CD86 (rat derived, clone GL1) [28] were used. Fifty micrograms of antibody were administered in 200 μl sterile saline, intraperitoneally, 1 day prior to, on the day of, and 1 day post-immunization. The antibody clones were a kind gift of Dr. Hans Reiser (ICSTM, London).

2.11. In vitro detection of cytokine production after antigenic stimulation

Splenocytes were prepared as previously described; 4×10^5 cells were dispensed in triplicate in 100 μl RF10 into the wells of sterile 96-well round bottom plates. Hundred microliters of 100 μg/ml OVA were then added and plates incubated at 37 °C. After 24, 48, and 72 h, 150 μl tissue culture fluid was retrieved and assayed for IL-4 and IFNγ using an ELISA capture system. IFNγ was captured using clone R46A2 (obtained from ECACC, Porton Down, UK) and detected using biotin-conjugated XMG1.2 (Pharmingen, San Diego, CA). IL-4 was captured using clone 11B11 (Pharmingen) and detected using biotin-conjugated BVD6-24G2 (Pharmingen).

3. Results

3.1. Inhibition of phagocytosis results in abrogation of both cytolytic and proliferative responses

Phagocytosis was abolished by treating mice intravenously with particulate silica, rendering phagocytic cells incapable of engaging in phagocytic activity. We assessed the effectiveness of inhibition by measuring the in vitro uptake of fluorescent latex beads by adherent splenocytes. Mice treated with particulate silica were incapable of phagocytosing the latex beads (data not presented), whereas mice treated with saline were unaffected. Differences in splenocyte yields from treated and untreated mice were not observed, nor was there a difference in viability of splenocytes, indicating that silica treatment was not toxic. To determine whether cells had been depleted by silica treatment, splenocytes were stained for a number of cell markers. There were no differences observed between silica- or saline-treated mice in either the staining pattern or numbers of cells bearing F4/80, CD11c and MHC class I and class II (data not shown). Splenocytes from silica-treated mice showed no CTL activity, regardless of the adjuvant system used (Fig. 1). Proliferative responses against soluble OVA were not completely abolished after treatment with silica but were reduced for all three preparations (data not shown).

3.2. Depletion of splenic and draining lymph node macrophages has differing effects on CTL induction

Having established that phagocytosis was a requirement for CTL responses, we studied the role of macrophages in CTL induction, namely, whether they are the principal APC population targeted by the delivery vehicles tested. When given intravenously, liposome-encapsulated clodronate depleted splenic red pulp macrophages, and when given subcutaneously, it depleted lymph node macrophages. Analyses of $CD11b^+$, $F4/80^+$ cells, in comparison with numbers of $CD11c^+$ cells, were not altered (data not shown).

Depletion of macrophages had differing results in inducing CTL activity; there was complete elimination of CTL activity in the OVA–Glycolipid-immunized group when either spleen- or draining lymph node-resident macrophages were eliminated. In contrast, the OVA–Tomatine-immunized group showed no reduction in CTL activity, regardless of whether the effector cells were splenic or draining lymph node in origin. In animals immunized with OVA–PROVAX™, there was a partial reduction in CTL activity after either macrophage population was depleted (Fig. 2). Control mice from all three groups displayed enlarged spleens and draining lymph nodes, whereas in the clodronate-treated groups only OVA–Tomatine-immunized mice showed enlarged spleens.

3.3. Antigen-formulated adjuvants generate SIINFEKL/MHC class I complexes in $TAP^{-/-}$ mice

To study whether CTL induction by the delivery vehicles was mediated by the conventional class I processing pathway, we used $TAP^{-/-}$ knockout mice. Using the protocol established by Sandberg et al. [29] and Bachmann et al. [30], $TAP^{-/-}$ splenocytes were used as source of T cells and were stimulated at a ratio of 4:1 of splenocytes (responder) to EG7–OVA (stimulator) cells as opposed to the 20:1 ratio conventionally used. However, even this higher ratio did not elicit any specific killing (data not presented). This result is not surprising because $TAP^{-/-}$ mice have drastically reduced numbers of $CD8^+$ T cells [31].

Therefore, we studied whether TAP-independent processing had occurred by measuring surface expression of SIINFEKL/H-$2K^b$ complexes ex vivo from immunized animals using the monoclonal antibody 25D1.16. Immunization of C57Bl/6 or $TAP^{-/-}$ mice with OVA formulated in either Alum or IFA did not result in positive staining when compared with an isotype-matched control antibody. However both C57Bl/6 and $TAP^{-/-}$ mice immunized with OVA–PROVAX™ showed the presence of the peptide complex on the surface of splenocytes (data not shown). Having observed that PROVAX™ was capable of generating class I–peptide complexes, we next sought to establish whether Glycolipid or Tomatine was capable of generating class I–peptide complexes in $TAP^{-/-}$ mice. This objective was achieved using the B3Z T–T hybridoma system. We observed that all three adjuvants were able to generate SIINFEKL/MHC class I complexes on splenocytes of C57Bl/6 mice (Table 1). Only Tomatine and PROVAX™ were able to generate functional SIINFEKL/class I complexes on splenocytes of $TAP^{-/-}$ mice (Fig. 3).

3.4. Endoplasmic reticulum resident ACE is not responsible for TAP-independent processing

Having established that Tomatine and PROVAX™ were capable of generating class I peptides for CTL induction

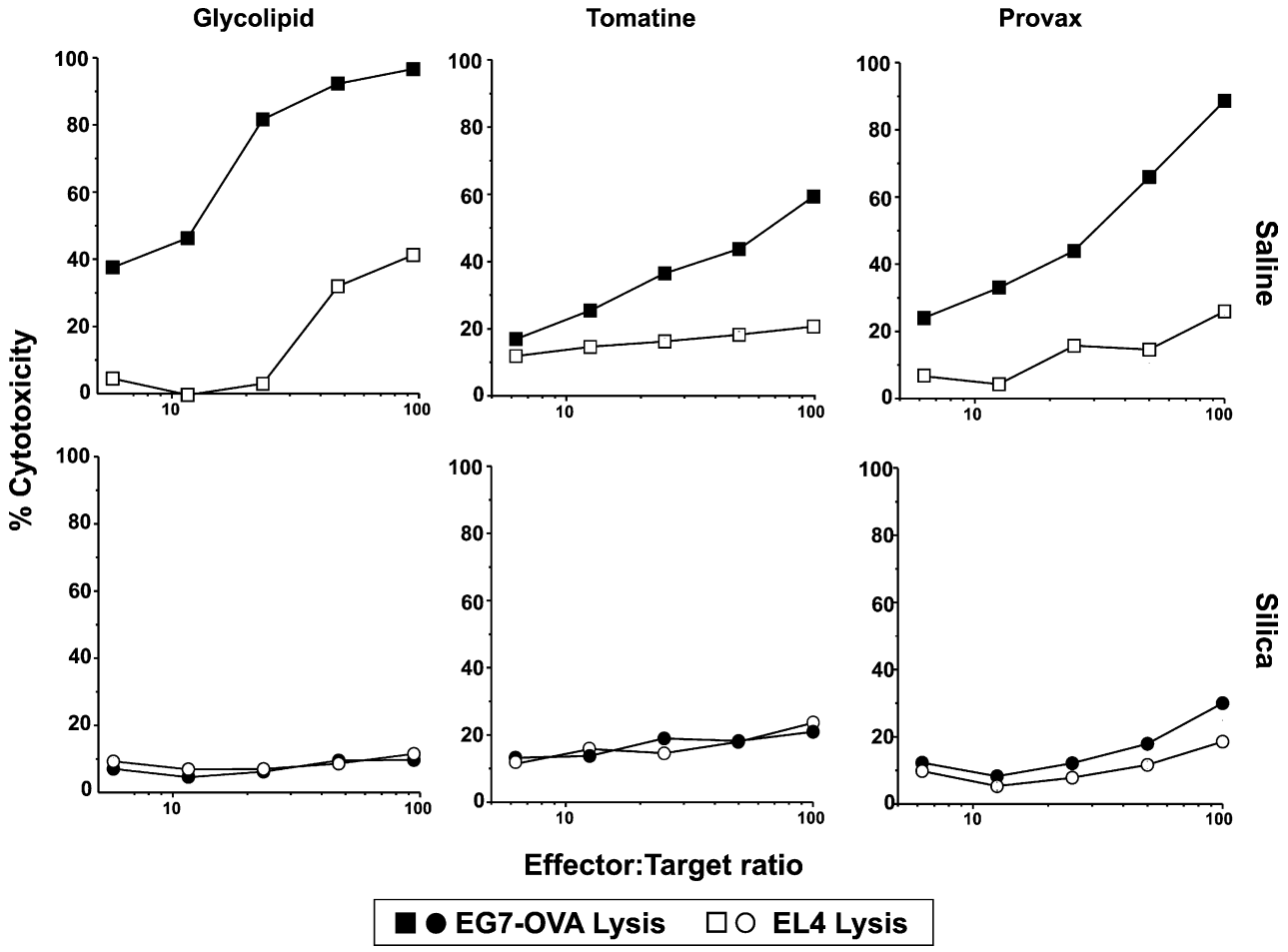

Fig. 1. Inhibition of phagocytosis results in abrogation of CTL induction after immunization with soluble protein formulated with either Glycolipid, Tomatine, or PROVAX^{TM}. Mice were injected with 1 mg silica for two consecutive days and then immunized with 50 μg OVA formulated with either Glycolipid, Tomatine, or PROVAX^{TM}. CTL responses were assessed 10–12 days after immunization as described in Section 2. Silica treatment abrogated CTL responses regardless of the delivery system used to administer OVA. Data consist of two pooled spleens from one experiment of five where six mice were used in each group. In all experiments performed the same trend was observed. Non-OVA-expressing EL4 cells were used as negative control targets in all chromium release assays.

in a TAP-independent manner, we next sought to determine whether the ER resident protease ACE was responsible for TAP-independent class I-peptide generation. We inhibited ACE activity in vivo by treating animals with captopril prior to immunizing with OVA-formulated adjuvants. We saw no difference in either the display of peptide-MHC class I molecules or the ability to stimulate B3Z cells between treated and non-treated TAP$^{-/-}$ mice after immunization with OVA formulated with either PROVAX^{TM} or Tomatine (data not shown).

Table 1
Immunization with OVA formulated in Tomatine or PROVAX^{TM}, but not Glycolipid, results in expression of SIINFEKL/H-2K^{b} complexes on the surface of splenocytes from both C57Bl/6 and TAP$^{-/-}$ mice

	C57Bl/6		TAP$^{-/-}$	
	Staining (%)	Staining above isotype (%)	Staining (%)	Staining above isotype (%)
Isotype control	50.5	–	31.7	–
OVA in saline	51.8	01.3	31.9	00.2
OVA in Glycolipid	75.1	24.6	34.5	02.8
OVA in Tomatine	66.1	15.6	48.3	16.6
OVA in PROVAX^{TM}	77.2	26.7	50.2	18.5

This finding was observed many times after immunization with OVA formulated in any of the delivery systems. In all cases, immunization with OVA formulated in PROVAX^{TM} resulted in the highest expression of the OVA CTL epitope in both C57Bl/6 and TAP$^{-/-}$ mice. Data are of two pooled spleens from one experiment of five where six mice were used in each group.

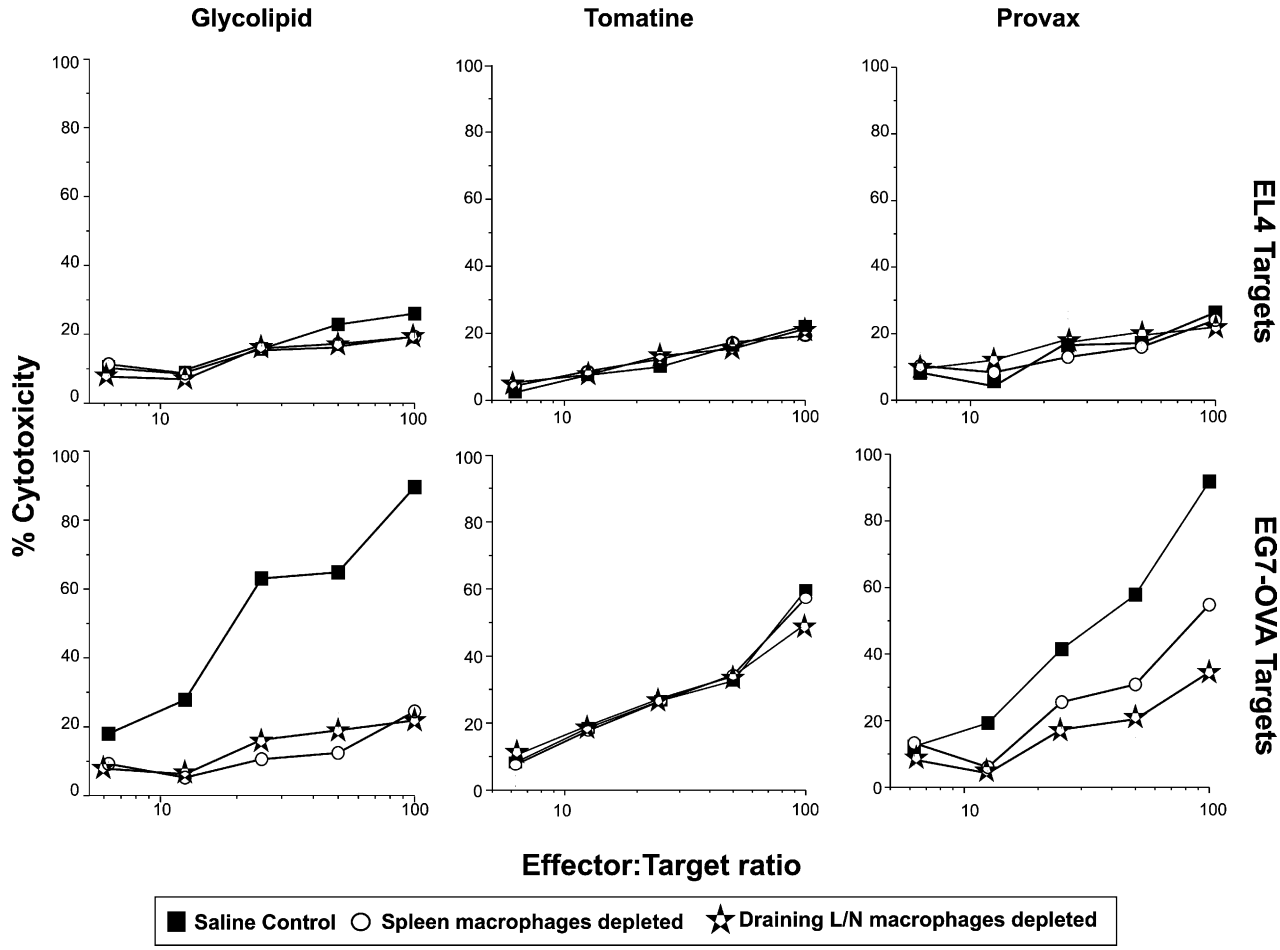

Fig. 2. CTL responses after depletion of either splenic or draining lymph node (L/N) macrophages as a consequence of clodronate treatment. Animals were given clodronate either intravenously or subcutaneously to deplete splenic or L/N macrophages, respectively, while leaving dendritic cells and other cell populations unaffected. Depletion revealed that OVA–Glycolipid-immunized animals were entirely dependent upon splenic and draining lymph node macrophages for CTL induction. In contrast, OVA–Tomatine-immunized mice did not show any requirement for the presence of macrophages for CTL induction. OVA–PROVAX^{TM}-immunized animals displayed a greater dependency for L/N macrophages than splenic macrophages for CTL induction. Data are of two pooled spleens from one experiment of five where six mice were used in each group.

3.5. Antigen-formulated adjuvants upregulate CD80 and CD86 expression

Effective T cell priming requires the presence of both an antigenic signal and a second signal, termed costimulation [32]. We noted that OVA formulated with either Glycolipid, Tomatine, or PROVAX^{TM} caused the upregulation of CD80 and CD86 on splenocytes (Table 2).

3.6. In vivo CD80 and CD86 blockade reveals differential requirements for costimulation to generate CTL and to induce T_H1 cytokine production

We addressed the role of CD80 and CD86 in facilitating adjuvant-mediated CTL generation by blocking each molecule in vivo and then immunizing with OVA formulated in the three adjuvant preparations. Blockade of either CD80 or CD86 in vivo at the time of immunization revealed that all three adjuvants required CD86 to generate CTL; however, only Tomatine required both CD80 and CD86 (Fig. 4, panel A). To test whether there was a different requirement for either CD80 or CD86 for CTL induction in primed mice, antibody-treated immunized mice were reimmunized 10–12 days after the first immunizing dose. Blocking CD80 again had no effect on CTL induction when mice were immunized a second time with either OVA–Glycolipid or OVA–PROVAX^{TM}, whereas CD86 blockade did prevent CTL generation (Fig. 4, panel B). Again, Tomatine showed a dependency for both CD80 and CD86 for CTL induction.

At the time of assessment of CTL activity we also measured ex vivo production of IFNγ. In animals where CD80 and CD86 were not blocked, OVA–PROVAX^{TM}-immunized mice yielded the highest responses of this cytokine, closely followed by OVA–Tomatine-immunized animals. OVA–Glycolipid-immunized mice yielded the weakest responses. Blocking CD86 resulted in the abrogation of ex vivo IFNγ production after immunization with OVA when

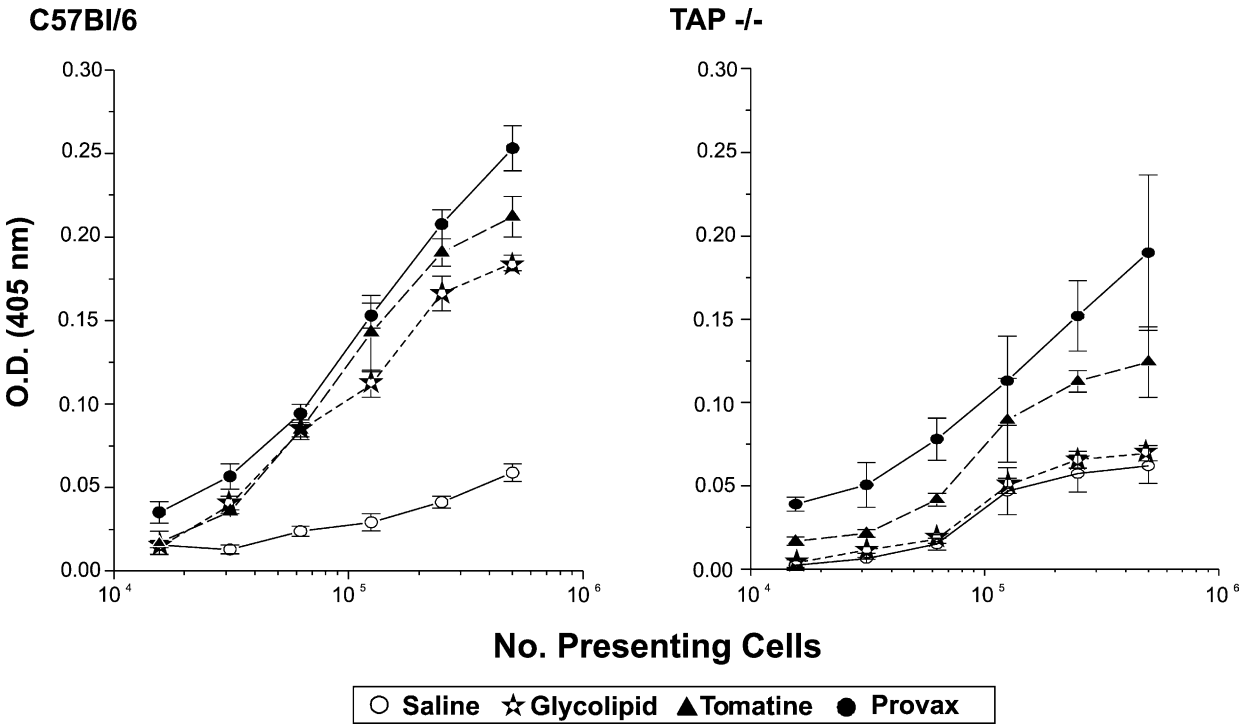

Fig. 3. Immunization with OVA formulated in Tomatine and PROVAX^{TM}, but not Glycolipid, results in the generation of functional SIINFEKL/H-2K^{b} complexes on the surface of C57Bl/6 and TAP$^{-/-}$ mice. Immunization of either C57Bl/6 or TAP$^{-/-}$ mice with OVA formulated in IFA or Alum did not result in activation of B3Z cells (data not presented), indicating that the OVA CTL epitope was not present on the surface of cells. However, immunizing C57Bl/6 mice with OVA formulated in either Glycolipid, Tomatine, or PROVAX^{TM} did result in the ability of splenocytes to activate B3Z cells. In contrast, when TAP$^{-/-}$ mice were immunized with OVA formulated in the three CTL-inducing delivery vehicles, only splenocytes from Tomatine- or PROVAX^{TM}-immunized animals were able to stimulate B3Z cells. This result indicated that functional OVA CTL motifs were present on APC, although the degree of stimulation was lower than in C57Bl/6 mice. Data are mean ± S.E. (of quadruplicate wells) of two pooled spleens from one experiment of five where six mice were used in each group.

formulated with any of the three preparations. However, there was no inhibition of IFNγ production after CD80 was blocked (Fig. 5, panel A). A second immunization resulted in increased IFNγ production, and all three delivery vehicles still showed CD86 dependency for the production of this cytokine (Fig. 5, panel B). In general, IL-4 production could not be detected in either control or CD80/CD86-blocked mice. This finding conforms with the fact that C57Bl/6 mice produce extremely low levels of IL-4 [33].

Table 2
OVA formulation with CTL and non-CTL delivery vehicles results in an increase in numbers of splenocytes bearing CD80 and CD86 and also in the upregulation of surface expression of both molecules

	CD80		CD86	
	Staining (%)	Relative fluorescence (Gm)[a]	Staining (%)	Relative fluorescence (Gm)[a]
Isotype control	23.6	6.06	23.6	6.06
OVA in saline	43.6	14.27	42.8	11.31
OVA in IFA	59.6	13.22	60.1	14.39
OVA in Alum	54.4	14.58	55.9	11.62
Isotype control	14.8	14.48	14.8	14.48
OVA in saline	30.31	27.81	36.7	22.1
OVA in Glycolipid	54.6	42.88	55.7	28.3
OVA in Tomatine	51.0	41.7	48.4	28.26
OVA in PROVAX^{TM}	65.5	41.25	63.5	24.39

All three CTL-inducing delivery vehicles (Glycolipid, Tomatine, and PROVAX^{TM}), as well as non-CTL-inducing adjuvants (IFA and Alum), facilitated the upregulation of CD80 and CD86, as assessed by staining with anti-CD80 and anti-CD86 antibodies. In all cases, there was an increase in numbers of cells bearing each molecule, as well as an increase in relative fluorescence, implying an upregulation of surface expression of CD80 and CD86. Data are of two pooled spleens from one experiment of five where six mice were used in each group.

[a] Gm: geometric mean of fluorescence.

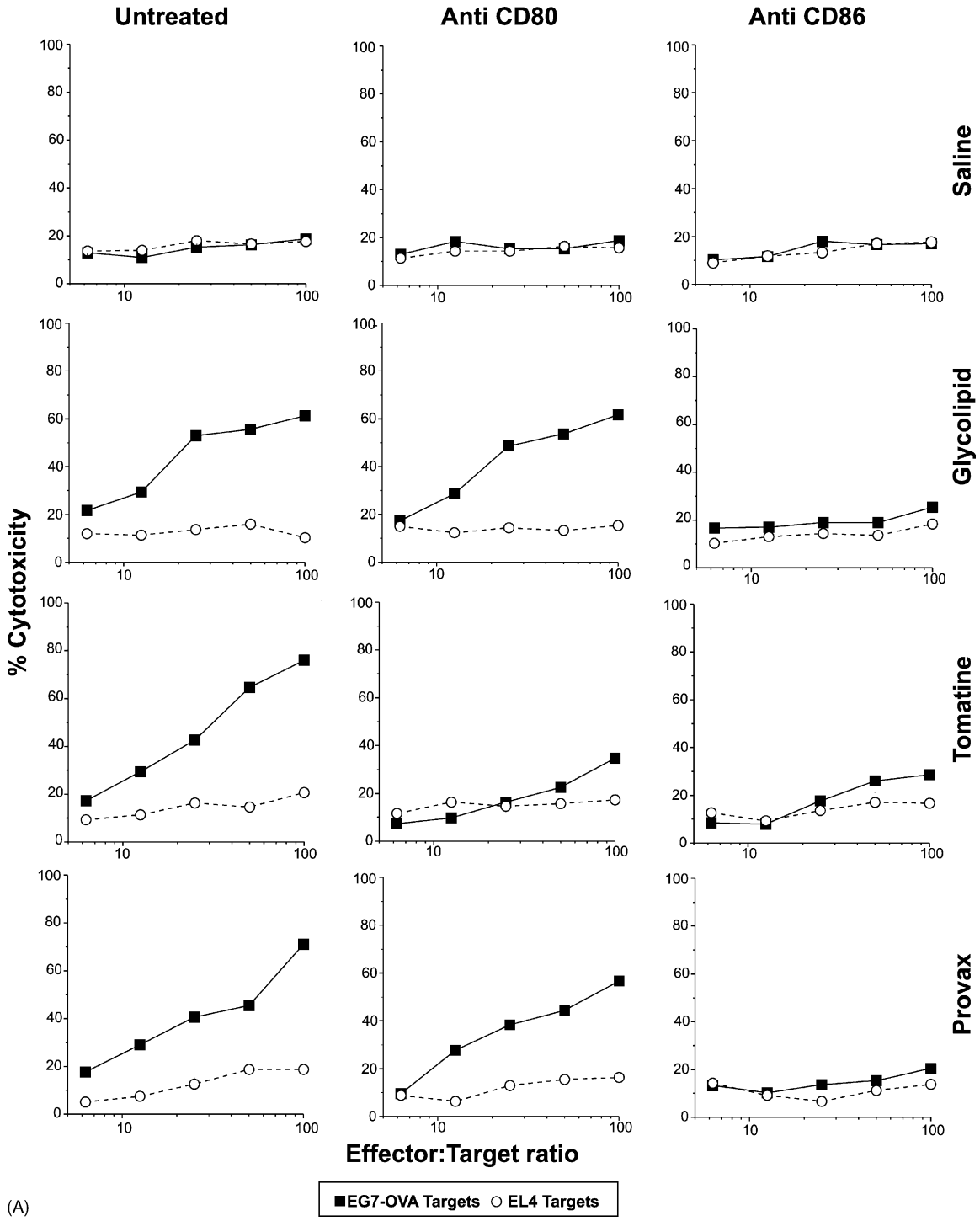

Fig. 4. Adjuvants have different costimulatory requirements for CTL induction. CD80 and CD86 were blocked with monoclonal antibodies in vivo before animals were immunized with formulated OVA. Animals were injected intraperitoneally with 50 μg of either 1610A1 (anti-CD80) or GL1 (anti-CD86) antibodies daily over a 3-day period. Animals were immunized on the second day of antibody treatment, spleens were removed 10–12 days later, and antigen-specific lytic activity was assayed as described in Section 2. Blocking CD80 abrogated CTL generation by OVA–Tomatine but had no effect upon CTL induction by OVA–Glycolipid or OVA–PROVAX™. However, blocking CD86 abrogated CTL generation from all three adjuvants (panel A). Reimmunizing animals (day 12 after the initial immunization), which had already been treated with blocking antibody and then given a second dose (days 11–13 after the initial immunization), resulted in a pattern similar to when they were immunized only once (panel B). Animals immunized with OVA–Tomatine were incapable of eliciting CTL when either CD80 or CD86 were blocked in vivo. However, both Glycolipid and PROVAX™ both showed a dependency for CD86 for CTL induction. Data presented here show one experiment of five; in each experiment, a similar pattern and magnitude of results were observed. To control for any effect secondary to inhibition by the antibodies used, we performed a control experiment using isotype-matched antibodies (hamster IgG for 1610A1 and rat IgG2a for GL1) where animals were immunized with OVA–PROVAX™ and CTL responses were compared to untreated, immunized mice, and we saw no difference in CTL responses.

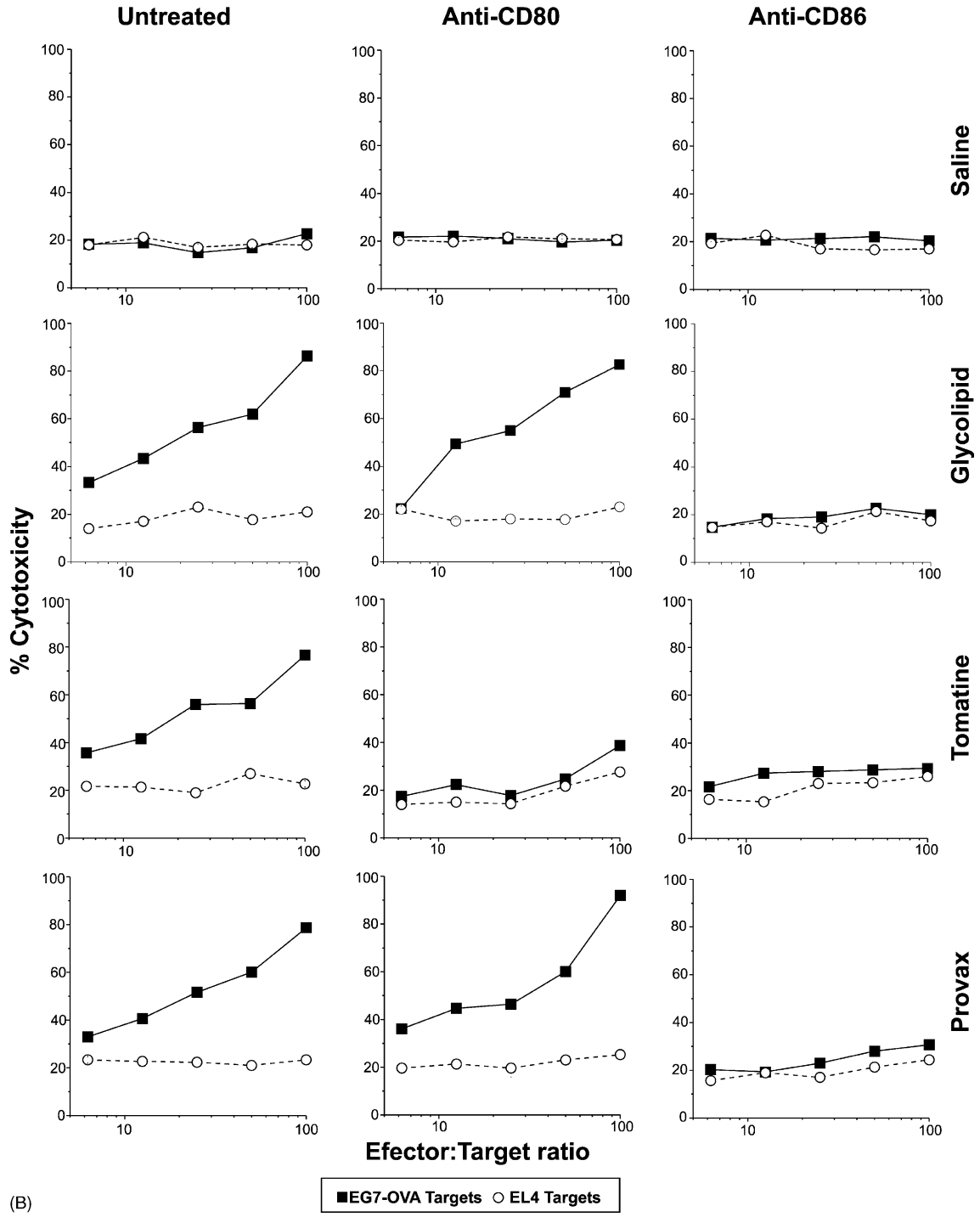

Fig. 4. (*Continued*).

4. Discussion

We have shown previously that Glycolipid, Tomatine, and PROVAX^{TM} are capable of generating potent antigen-specific CTL against soluble protein immunogens [15–17] and that they confer protection in infectious [34] and tumor model systems (unpublished observations). We sought to understand the mechanisms by which the aforementioned adjuvants generated antigen-specific CTL against a soluble protein immunogen. We demonstrated how adjuvant-formulated antigen was acquired by APC, the importance of macrophages in the generation of antigen-specific CTL,

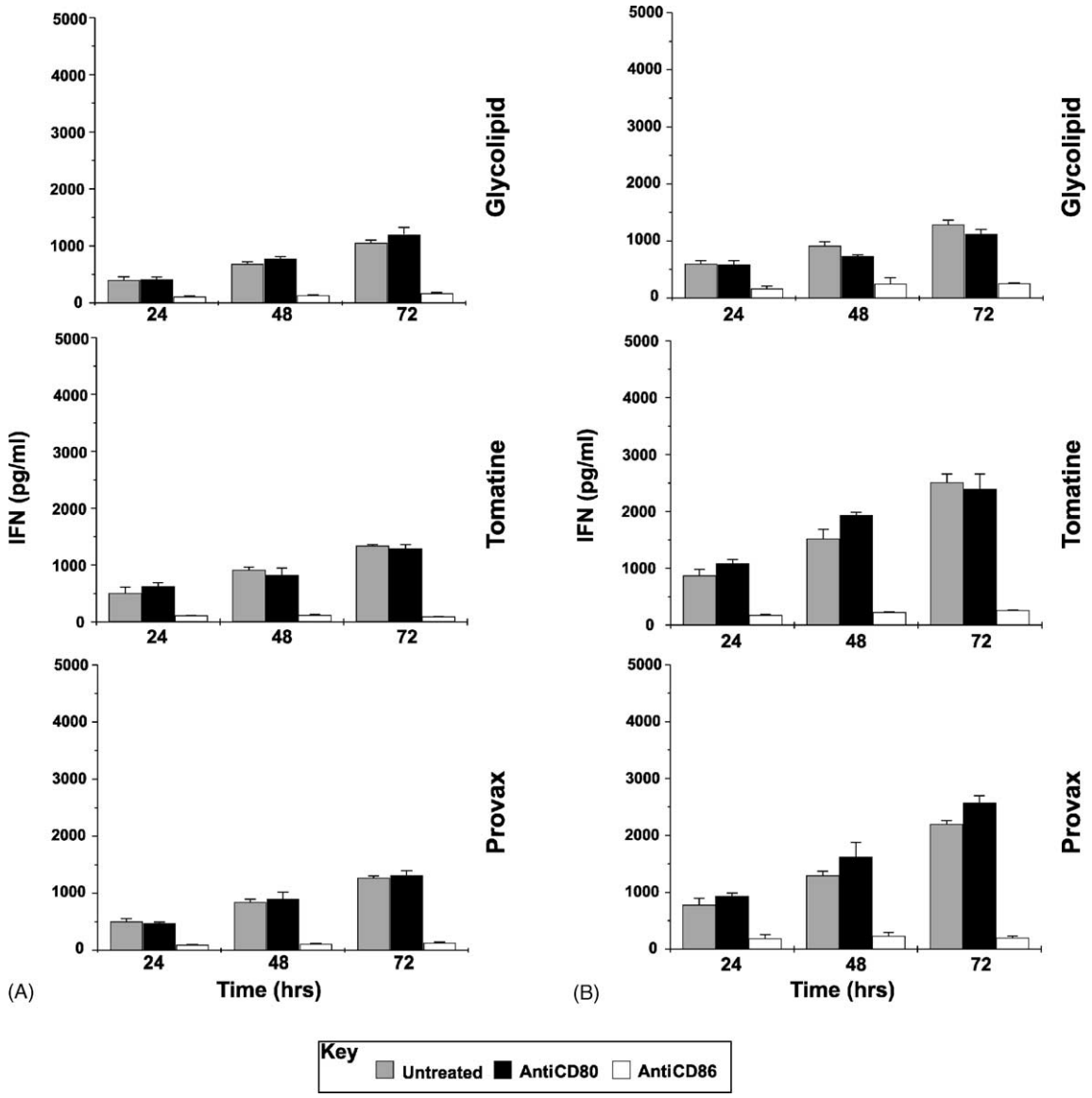

Fig. 5. Glycolipid, Tomatine, and PROVAX^{TM} induce antigen-specific T_H1 responses through CD86 and not through CD80. Animals were treated with antibody specific for either CD80 or CD86 as previously described. Spleens were retrieved and processed, and 4×10^5 splenocytes were dispensed in triplicate into 96-well round bottom plates and stimulated with 100 μg/ml OVA. Supernatants were then taken at 24, 48, and 72 h after ex vivo antigenic stimulation and quantified for IFNγ production using an ELISA capture system. Splenocytes were taken from animals that received a single immunization of OVA formulated with any of the three adjuvants produced IFNγ (panel A) and showed a requirement for CD86 at the time of immunization to produce IFNγ. Animals immunized with OVA–Tomatine displayed repeatedly a greater capacity to produce IFNγ than animals immunized with OVA–Glycolipid. Reimmunizing animals resulted in an increase in IFNγ production (panel B). However, all three adjuvants again showed a requirement for functional CD86 at the time of immunization. Results presented are means ± S.E. in one experiment of four.

the use in vivo of the TAP-independent processing pathway and finally the role of CD80 and CD86 costimulation.

Antigen uptake can take the form of fluid phase pinocytosis/macropinocytosis or phagocytosis. All cells perform the former function(s); however only specialized cells [9,19,22,35] are capable of phagocytosis, which involves the ingestion of particulate material. Many adjuvants make soluble antigen particulate; hence, it is conceivable that Glycolipid, Tomatine, and PROVAX^{TM}, because of their comparative sizes, undergo phagocytosis, thereby delivering antigen into phagocytes. Glycolipid forms particles in the size range of 200–300 nm (Attard, unpublished observations), whereas OVA–Tomatine ranges from 100 to 3000 nm, with the majority of particles being hollow tubular structures of 100–160 nm width and 2000–3000 nm length [36]. In comparison, PROVAX^{TM} forms particles of approximately 200–300 nm in diameter [4]. We hypothesized that phagocytic uptake of adjuvant-formulated antigen was a mechanism by which soluble protein was delivered into cells. To test this hypothesis we abolished in vivo phagocytic activity,

without affecting the viability of the cell population, by treating mice with silica particles [19,22,37]. The data presented here show that all three CTL-inducing preparations require the presence of functional phagocytes. This observation is consistent with a number of other studies [19,22].

We noticed that animals rendered incapable of phagocytosis showed in vitro proliferative responses (data not shown) when splenocytes were stimulated with soluble protein. This finding can be attributed to one of two explanations: (i) not all OVA was complexed with the adjuvant preparations. OVA is a tetrameric molecule comprising four polypeptide chains associated by hydrophobic means; it is therefore conceivable that OVA is not bound to components of either of the three adjuvants but forms free aggregates. Hence, non-associated OVA may have been taken up by non-phagocytic means, such as fluid phase pinocytosis, and therefore induced proliferative responses; or (ii) the adjuvant-formulated OVA is taken up by non-phagocytic cells to induce proliferative responses.

As we established that phagocytosis was essential for inducing adjuvant-mediated, antigen-specific CTL, we hypothesized that macrophages would be the main APC population responsible for eliciting this response. Originally, we tried to purify DC and macrophages from immunized animals to assess which APC population had been targeted by antigen/adjuvant mixtures after animals had been immunized. However, as the level of purity was not consistently high, we then sought to address the question of which population of APC was targeted by depleting macrophages in vivo using clodronate liposomes [21,22]. We used clodronate because it selectively depletes macrophages without affecting either non-phagocytic cells or having bystander cytotoxic effects [38].

We expected all three adjuvants to be incapable of generating CTL in the absence of either draining lymph node or splenic macrophages. However, only OVA–Glycolipid-immunized animals display such a pattern; in contrast, there was no such dependency by OVA–Tomatine-immunized mice. OVA–PROVAX^{TM}-immunized animals showed a partial dependency on splenic or draining lymph node macrophages. These findings indicate that Glycolipid has an absolute requirement for macrophages to generate CTL; however, Tomatine functions by delivering antigen to a non-macrophage population of APC, probably DC. Clearly, Tomatine requires the presence of phagocytic cells for CTL generation, but it is not clear whether DC are capable of phagocytosis. It has been shown that human DC are capable of phagocytosing *Borrelia burgdorferi* [39], although the dermal and bone marrow-derived DC used were enriched by culturing and incubation with IL-4. This cytokine causes DC maturation [40] from bone marrow progenitors and hence different functionality from in situ, unprimed immature cells. Additionally, DC may be capable of phagocytosing antigen; however, this hypothesis again is based upon in vitro experimentation [41]. In contrast Steinman and colleagues showed that in vivo administration of colloidal carbon resulted in red pulp macrophages taking up the carbon particles, but DC could not phagocytose this material [42]. However, Leenan et al. [43] have shown that marginal splenic DC, but not interdigitating splenic DC, were capable of phagocytosis. In corroboration, Kamath et al. [44] have demonstrated that mouse spleens contain three distinct lineages of DC, which are all capable of phagocytosis of particulate matter. DC have been implicated in the generation of CTL by exogenous protein in a number of studies [44–46]. These cells are thought to possess superior antigen-processing and -presentation qualities in comparison with other APC. The ability to target DC is a desirable characteristic for any potential vaccine/delivery vehicle. However, to solely focus on this APC population may reduce the effectiveness of other adjuvants with tropisms for other APC.

Protein-based vaccines are generally seen as “exogenous” by the immune system, and the antigen is channelled into the class II or endocytic pathway to induce a humoral response. Thus, formulation with traditional adjuvants, such as Alum, does not result in induction of CTL activity. Glycolipid, Tomatine, and PROVAX^{TM} elicit potent CTL responses, which are CD8^{+} restricted and specific for the OVA H-2K^{b} peptide SIINFEKL. The manner in which these adjuvants deliver protein into the class I-processing machinery is key to understanding how this pathway is accessed by antigen. Therefore, we asked if phagocytosed soluble protein was processed by the conventional class I pathway or by an alternative pathway.

We addressed this question by immunizing TAP knockout mice. Only OVA–Tomatine and OVA–PROVAX^{TM} were capable of generating SIINFEKL/class I complexes, implying that OVA–Glycolipid requires intact, cytoplasmic-resident, class I pathway machinery for processing of exogenous protein. The mechanisms by which Tomatine and PROVAX^{TM} generate class I peptides by TAP-independent means can be seen as follows: either (i) the class II-processing pathway generates class I peptides, which are subsequently captured by empty surface-bound class I molecules, or (ii) class I-peptide generation occurs in a non-cytoplasmic compartment, such as the endoplasmic reticulum. We chose to explore the second possibility, as we had the means to address this issue. Localized in the endoplasmic reticulum [47], ACE is capable of generating class I peptides [48,49] and is responsible for peptide trimming of MHC class I-loaded peptides. We therefore postulated that Tomatine or PROVAX^{TM} may have delivered their antigenic loads directly into the endoplasmic reticulum where ACE could then process OVA to generate SIINFEKL. However, the presumption that ACE is capable of degrading a large tetrameric protein structure directly into class I peptides is unlikely, and it is more probable that a number of proteases would degrade OVA before ACE could generate SIINFEKL. Inhibition of ACE did not result in any difference in the generation of SIINFEKL/MHC class I complexes, implying that ACE was not involved in TAP-independent class I processing.

The display of MHC-associated peptide to the T cell receptor is essential to induce a T cell response. A second costimulatory signal is required to activate these T cells and prevent anergy [50]. Some of the best studied molecules involved in costimulation are CD28, CD80, CD86 and CTLA4 [51]. CD80 and CD86 are expressed upon activation of APC [52], interact with CD28/CTLA-4 [53] on T cells, and are equally capable of costimulating T cells. However, recent evidence shows that they may not deliver identical signals [54–56]. Both molecules bind to distinct determinants on CD28, suggesting that functional differences may exist, but they share only 25% homology, having marked differences in their cytoplasmic domains [54]. Thus they may have different qualitative effects on the induction of T cell responses. The ability of adjuvants to upregulate surface expression of costimulatory molecules has not been studied in detail. As Glycolipid, Tomatine, and PROVAX^{TM} were all capable of a general upregulation of CD80 and CD86 on splenocytes, we studied whether the adjuvant preparations had different requirements for either CD80 or CD86 to induce CTL responses.

It is clear from our data that CD86 is essential for effector CTL induction by Glycolipid, Tomatine, and PROVAX^{TM}, a finding that apparently contradicts previous studies. Sigal et al. showed that blocking both CD80 and CD86 together inhibited CTL induction when OVA conjugated to iron beads was used to induce CTL [27]. When CD80 or CD86 was blocked individually, CTL responses were unaffected, implying CTL induction required interaction of both costimulatory molecules. In class II-deficient mice, Sigal et al. [27] showed that CD86, but not CD80, was essential for CTL induction against exogenous antigen, implying that T_H functionality for CTL generation required different costimulation. In contrast to this latter study, Corr et al. [57] showed that CTL generation required CD80 but not CD86. In our investigation, CD86 was required for CTL induction by Glycolipid and PROVAX^{TM}, in accordance with the findings of Sigal et al. [27]. It has been proposed that CD80 and CD86 act synergistically for CTL induction [27]. This suggestion is not applicable to our findings because Tomatine depends separately on CD80 and CD86 for CTL induction. Furthermore, the absolute effect upon CTL induction is similar; blocking either molecule totally abrogates CTL induction. Lanier et al. [58] presented similar findings whereby naïve, resting human T cells were used as effectors in a redirected CTL assay against P815 tumor cells transfected with either CD80 or CD86. When either CD80 or CD86 was blocked, cytotoxicity against CD80- or CD86-transfected cells was abrogated but not against untransfected cells. In addition, La Motte et al. showed CD80 expression was essential for CTL responses against CD86-transfected P815 cells in vivo [59].

Despite these apparent discrepancies, it is likely that these results are all valid, although the technical differences between the studies lead to the conclusion that the field of costimulation relating to CTL induction is not fully understood or defined. Although Alum, IFA, and CFA were capable of enhancing the expression of both CD80 and CD86 (data not shown), only Glycolipid, Tomatine, and PROVAX^{TM} were capable of delivering signal 1, as assessed by the cell surface display of SIINFEKL.

Previously, we demonstrated that Glycolipid, Tomatine, and PROVAX^{TM} all select for T_H1 cytokine production based on detection of IFNγ production [17]. In our data, CD86, but not CD80, was necessary for IFNγ production. However, there is conflicting evidence for the role of CD86 in T_H1/T_H2 switching [60–62]. It is possible that the physiochemical properties of the three adjuvants may direct the utilization of CD86 in T_H1 cytokine production. The simplest parameter that could influence costimulatory molecule preference may be immunogen size. Indeed, it has been proposed that antigen size, in particular that of the vesicle-entrapped antigen, dictates the outcome of T_H cytokine secretion. Brewer et al. [63] showed that OVA vesicles of sizes greater than 225 nm induced T_H1 cytokine secretion and OVA-specific IgG2a antibody responses in BALB/c mice, whereas vesicles smaller than 155 nm in diameter resulted in T_H2 cytokine secretion and IgG1 antibody production. Glycolipid, Tomatine, and PROVAX^{TM} form particle sizes greater than 200 nm, and although they induce T_H1 cytokines [16,17] they also produce IgG1 [15,16], implying that a T_H2 stimulus is also induced. A speculative explanation may be that as each of the preparations does not form uniformly sized vesicles, then those below 155 nm may be inducing T_H2 responses and larger sized vesicles (above 255 nm) may be inducing T_H1 responses.

In conclusion, we have shown that three novel delivery vehicles capable of eliciting antigen-specific CTL against soluble protein do so by varying means, requiring different populations of APC, antigen-processing pathways and costimulatory molecules. The studies presented here will be extended to investigate how adjuvants facilitate cellular entry, activation, and processing compartment selection, particularly in relation to the biophysical nature of the vehicles. Elucidation of such mechanisms will contribute substantially to the design of subunit protein vaccines.

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