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Modulation of T Cell Cytokine Profiles and Peptide-MHC Complex Availability In Vivo by Delivery to Scavenger Receptors via Antigen Maleylation¹

Nagendra Singh,² Sumeena Bhatia, Roshini Abraham,³ Sandip K. Basu, Anna George, Vineeta Bal, and Satyajit Rath⁴

We have previously shown that conversion of proteins to scavenger receptor (SR) ligands by maleylation increases their immunogenicity. We now show that maleyl-Ag-immune spleen cells make relatively more IFN- γ and less IL-4 or IL-10 than native Ag-immune cells. This is also reflected in the IgG1:IgG2a ratios in Abs generated in vivo. SR engagement on macrophages does not alter their surface levels of the adhesive/costimulatory molecules CD11a/CD18, CD11b/CD18, CD24, CD54, or CD40, nor does it enhance their ability to support anti-CD3-driven proliferation of naive T cells in vitro. Costimulatory molecules implicated in differential Th1/Th2 commitment—CD80, CD86, and IL-12—are not inducible by SR ligation. In addition to macrophages and dendritic cells, B cells also show receptor-mediated uptake and enhanced presentation of maleyl-Ags. Using a monoclonal T cell line to detect peptide-MHC complexes expressed on spleen cells in Ag-injected mice, we find that higher levels of these complexes are generated *in vivo* from maleyl-proteins and they persist longer than those generated from the native protein. Together, these data suggest that in certain situations, the levels of cognate ligand available and/or the time course of their availability may play a major role in determining the cytokine profiles of the responding T cells in addition to the costimulatory signals implicated so far. *The Journal of Immunology*, 1998, 160: 4869–4880.

Generation of a successful T cell response involves cognate interaction of TCR with appropriate peptide-MHC complexes on the APC surface as well as the delivery of costimulatory signals from APCs. Differing immune effector pathways of T cells may be optimally useful for different kinds of infections. Data from many experimental systems suggest that CD4 T cells can enter one of two effector pathways, commonly called the Th1 and the Th2 cell pathways, characterized by differing cytokine packages (1, 2). Th1 T cells appear to generate mainly IFN- γ and TNF- β , contribute to the generation of inflammation (3), and are needed to eliminate facultative intracellular parasites such as *Leishmania* (4), while Th2 T cells make IL-4, IL-5, and IL-10 and help in the generation of IgG1 and IgE Ab responses (2, 5).

The complex mechanisms regulating triggering of Th1 or Th2 responses are the subject of much interest, and many investigations have focused on the differential roles played by different APC types (6, 7). Th1 T cell clones are better stimulated in vitro by macrophages as APCs rather than by B cells, while the reverse is true for Th2 clones (8). Targeting Ags to small B cells in vivo, on

the other hand, may lead to T cell tolerance rather than activation (9), although recent reports suggest that the involvement of B cells in Ag presentation during T cell priming may bias the T cells toward a Th2 phenotype (10). There are data linking differential production of cytokines such as IL-12 (11) and IL-4 (12) early in the course of the immune response to the qualitative outcome of the immune response, and cell types differ in their abilities to contribute such cytokines (13, 14). Differential expression of costimulatory molecules such as CD80 and CD86 has also been implicated in the preferential induction of Th1 vs Th2 responses (15, 16). The other factor argued to be involved in regulating commitment to Th1/Th2 phenotypes is the ligand density of the cognate peptide-MHC signal. Peptides with a high affinity for MHC class II molecules generate a Th1 response, while mutant variants that bind weakly to MHC class II generate a Th2-dominated response (17), suggesting that cognate ligand density may also regulate the Th1/Th2 choice.

Scavenger receptors (SRs)⁵ bind to a variety of polyanionic ligands and deliver them into the endolysosomal pathway (18), and their expression levels are not altered by ligand binding (19, 20). They were initially thought to be present uniquely on cells of the monocytic lineage, although the more recently defined class B SRs and related receptors are found on B cells as well (21). Many proteins are known to become SR ligands when chemically modified to enhance their negative charge by alteration of the ϵ amino groups of their lysine residues with acetic or maleic anhydrides, or more physiologically, malondialdehyde (22). We have shown previously that maleylating protein immunogens so that they become SR ligands leads to better presentation to T cells in vitro and to greater immune responses in vivo (23). We have now investigated

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⁵ Abbreviations used in this paper: SR, scavenger receptor; CA, conalbumin; DT, diphtheria toxoid; EIAs, enzyme-linked immunoassays; PECs, peritoneal exudate cells; poly(I:C), poly inosinic-poly cytidylic acid; TT, tetanus toxoid; DC, dendritic cells; PE, phycoerythrin.

whether there is also a shift in the Th1/Th2 balance generated by maleylated Ags, and we have gone on to examine if alteration of costimulatory capabilities, dominance of APC lineages, and/or cognate signal modulation is likely to be responsible for the quantitative and qualitative modulation of the immune response by these SR-targeted Ags.

Materials and Methods

Mice

BALB/c and C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME), bred in the Small Animal Facility of the National Institute of Immunology, New Delhi, India were used in all experiments. Mice were immunized i.p. with varying weekly doses of Ag, either in PBS or with alum (Alhydrogel, Superfos, Vedback, Denmark) for 1 to 3 wk and used 4 to 6 days after the last dose. They were bled from the retro-orbital venous plexus under inhalation anesthesia and sera separated for Ab assays. For measurement of T cell responses, mice were killed by cervical dislocation, spleens were dissected, and single cell suspensions were prepared for use. Group sizes of five to eight mice each were used for the serologic experiments, while two to three mice each were used for the T cell proliferation and cytokine analysis experiments.

Chemical modification of proteins

BSA (Sigma, St. Louis, MO), diphtheria toxoid (DT) (Serum Institute, Pune, India), tetanus toxoid (TT) (Serum Institute), and chicken conalbumin (CA) (Sigma) were maleylated at alkaline pH as described earlier (23). The maleylated proteins were subjected to extensive dialysis against PBS, and the degree of maleylation was estimated from the loss of active ϵ -amino groups (24). Only proteins with over 90% substitution were used for experiments.

Ag-specific Ab assays

Polyvinyl chloride microtiter plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) were coated with the appropriate Ag (10 μ g/ml). Abs in immune sera were detected in enzyme immunoassays (EIAs) using goat-anti mouse Ig peroxidase followed by *o*-phenylenediamine (Sigma) and H₂O₂. Concentrations were calculated on the basis of standard curves of affinity-purified mouse Ig run in parallel EIAs. Ag-specific IgG1 and IgG2a levels were determined by using goat anti-mouse isotype-specific Abs (Sigma) for IgG1 and IgG2a and rabbit anti-goat Ig peroxidase for detection. Reciprocal log titers of Ag-specific IgG1 and IgG2a levels were estimated for calculating IgG1:IgG2a ratios.

T cell activation assays

Spleen cells from immune mice were cultured with graded doses of Ags in 96-well flat-bottom microtiter plates (Corning Glass, Corning, NY) at 2 to 3 \times 10⁶ cells/ml in 200 μ l of L-glutamine-fortified Click's EHAA medium (Irvine Scientific, Irvine, CA) containing 5 \times 10⁻⁵ M 2-ME (Life Technologies, Grand Island, NY), antibiotics, and 10% FCS (Hyclone, Logan, UT). The wells were pulsed with 0.5 to 1.0 μ Ci of [³H]thymidine (Amersham, Amersham, U.K.) for 12 to 16 h in an 84- to 100-h assay, and harvested onto glass fiber filters for scintillation counting (Betaplate; Wallac, Finland). Data are expressed as mean cpm of triplicate cultures. SEM were less than 20% of the mean values. For cytokine analyses, spleen cells from immune mice were cultured in parallel with graded doses of Ag at 5 \times 10⁶ cells/ml as above. At 48 to 72 h, culture supernatants were collected, freeze-thawed, and assayed for IL-4, IL-10, and IFN- γ using EIAs.

In order to examine the ability of SR ligands to modify costimulatory abilities of APCs for naive T cells, plastic-adherent peritoneal cells were cultured in the presence or absence of 100 μ g/ml maleyl-BSA. After 36 to 40 h, the cells were harvested, washed, and graded numbers of these cells were used as APCs with or without the further addition of 100 μ g/ml of maleyl-BSA as an SR ligand during T cell stimulation. They were cultured at various doses with normal syngeneic thymocytes (2.5 \times 10⁶/ml) and anti-CD3- ϵ Ab (500-A2 (25); gift of Dr. C. A. Janeway, Yale University, New Haven, CT) as a 1:10 dilution of culture supernatant. After 48 h of incubation, plates were pulsed with 0.5 μ Ci/well of [³H]thymidine for 12 h, harvested, and counted as above.

The T cell clone D10.G4.1 (26), which recognizes peptide 134-146 from chicken conalbumin on I-A^k (27), was maintained by stimulation with irradiated H-2^k (C3H/HeJ) spleen cell APCs and 100 μ g/ml of conalbumin at 7- to 10-day intervals. For use, D10.G4.1 cells were separated from residual APCs 10 to 15 days after restimulation by density centrifugation

(lympholyte M; Cedarlane, Hornby, Ontario, Canada). Such D10.G4.1 cells did not show any proliferation when Ag or APCs were added alone without the other, and were used at 1 \times 10⁵/ml in assays along with varying numbers of irradiated or paraformaldehyde-fixed APCs and varying doses of Ags as shown. Cultures were pulsed with [³H]thymidine 36 h later for 12 h, harvested, and counted as above.

For uptake and subsequent presentation of CA and maleyl-CA, C3H/HeJ dendritic cells (DCs) (1.5 \times 10⁴/ml), plastic-adherent peritoneal cells (3-5 \times 10⁵/ml) or plastic adherence-depleted spleen cells (5 \times 10⁵/ml) were cultured with titrated Ag doses and 10⁵/ml D10.G4.1 cells. These assays were also set up with and without 300 μ g/ml of maleyl-BSA in order to examine the effects of a "cold" SR ligand on the presentation of native and maleyl-CA.

For estimating the relative peptide-MHC ligand densities generated in vivo by native vs maleylated Ags, native or maleylated CA (1 mg/mouse) was injected i.p. in PBS at various times prior to sacrifice. Spleen cells from these mice were fixed with 1% paraformaldehyde (Sigma) in PBS for 45 s at ambient temperature, washed extensively with PBS to remove excess paraformaldehyde, and graded numbers of such fixed spleen cells were used to stimulate 1 \times 10⁵ cells/ml of D10.G4.1 in 96-well plates as above.

Cytokine analyses

EIAs were done on culture supernatants using appropriate purified and biotinylated Ab pairs for IL-4, IFN- γ , IL-12 (Genzyme, Boston, MA), and IL-10 (PharMingen, San Diego, CA). Purified anti-mouse IL-4/IFN- γ /IL-10/IL-12 mAbs were adsorbed for capture on polystyrene microtiter plates (Nunc, Roskilde, Denmark). The culture supernatants were followed by biotinylated anti-mouse IL-4/IFN- γ /IL-12/IL-10. Streptavidin-peroxidase (Genzyme), followed by hydrogen peroxide and tetramethylbenzidine (Sigma) were used for detection. Titration curves of rIL-4, IFN- γ , IL-12 (Genzyme), and IL-10 (PharMingen) were used as standards for calculating cytokine concentrations in the culture supernatants tested. The limits of detection for IL-4 were routinely in the range of 5 to 15 pg/ml, and for IFN- γ , IL-10, and IL-12 in the range of 15 to 30 pg/ml.

Preparation of cellular subpopulations

Splenic adherence-depleted cells were prepared from C3H/HeJ mice by plastic adherence for 1 h. Peritoneal adherent cells were prepared from C3H/HeJ mice by taking the plastic-adherent fraction of peritoneal exudate cells (PECs) from thioglycollate broth-primed C3H/HeJ mice after 1 h of adherence. Peritoneal cells similarly harvested from mice not primed with thioglycollate broth are referred to as peritoneal washout cells.

For preparing splenic DCs for functional assays, a method described previously was used with minor modifications (28). Briefly, low density splenic cells isolated on a Percoll gradient (40-50% interface) were adhered to plastic and the nonadherent cells discarded. The weakly adherent cells were then passed through a column of glass beads coated with mouse Ig-anti-Ig immune complexes to remove FcR-bearing macrophages.

In order to obtain partially purified DC-enriched splenic cells in sufficient numbers for flow cytometric analyses, plastic-adherent splenic cells were cultured overnight in the presence of 5 ng/ml granulocyte-macrophage-CSF (Peprotech, Rocky Hills, NJ), the weakly adherent cells were loaded on a Percoll density gradient, and the cells from the 40 to 50% interface were used as a DC-enriched population.

Flow cytometry

For flow cytometry, cells (1 \times 10⁵ to 1 \times 10⁶/well) were incubated with the primary staining reagents in 50 μ l for 45 min on ice. Heat-aggregated rabbit serum was used at 1% in the staining buffer (PBS containing 0.1% NaN₃ and 2% FCS) to block FcRs. After washing in staining buffer, similar incubations were used for secondary and tertiary reagents as needed. Stained cells were fixed in 0.2% paraformaldehyde and stored at 4°C until analyzed. Samples were analyzed on a Bryte-HS flow cytometer (Bio-Rad, Hemel Hempstead, U.K.), and data analysis was carried out using WinMDI shareware.

Monoclonal antibodies for CD11a/CD18 (M17/4.2, TIB217, American Type Culture Collection (ATCC); CD11b/CD18 (M1/70.16, TIB128, ATCC); and CD54 (YN/1.7.4, CRL 1878, ATCC) were used as culture supernatants and were detected by using donkey anti-rat IgG-phycocerythrin (PE) (Jackson ImmunoResearch, West Grove, PA). CD40 and CD86 were detected by using fluorescein-coupled Abs (PharMingen), while CD80 was detected by using anti-CD80-PE (PharMingen). mAbs for MHC class I (34-4-20S; HB75; (ATCC), and MHC class II (212.A1) (29) were used as culture supernatants and were detected by using biotinylated rabbit anti-mouse IgG(Fc) (Jackson ImmunoResearch) followed by avidin-fluorescein

(Vector, Burlingame, CA). The binding of the mAb to heat-stable Ag (CD24) (J11d.2; TIB183; ATCC) was detected by biotinylated anti-murine IgM (Jackson ImmunoResearch) followed by avidin-fluorescein.

Anti-maleyl-protein antisera were generated by immunizing Wistar rats with 100 μ g of maleyl-CA in CFA (Difco, Detroit, MI). Three weeks later, they were boosted with 100 μ g maleyl-CA in IFA (Difco) and bled from the retro-orbital plexus under inhalation anesthesia 1 wk later. As we have demonstrated previously, most maleyl-proteins are serologically cross-reactive with each other (23), and thus these rat anti-maleyl-CA antisera bind well to maleyl-BSA (data not shown). Pooled sera were used for detection of cell-bound maleyl-BSA in flow cytometric assays. SR binding on cells was detected by maleyl-BSA at 25 μ g/ml, followed by this rat anti-maleyl-protein antiserum at a 1:200 dilution, and revealed by species-specific polyclonal donkey anti-rat IgG-PE (Jackson ImmunoResearch). A culture supernatant containing the hamster mAb N418 (HB 224; ATCC; kind gift of Prof. C. A. Janeway, Yale University, New Haven, CT) recognizing mouse DCs (30) was used to identify splenic DCs, and binding was detected by a polyclonal species-specific anti-hamster IgG-fluorescein (Jackson ImmunoResearch). Anti-mouse IgM-fluorescein (Jackson ImmunoResearch) was used to stain B cells, while a monoclonal hamster anti-mouse CD3- ϵ (500-A2) followed by anti-hamster IgG-fluorescein (Jackson ImmunoResearch) was used to detect T cells.

Results

Effect of maleylation of immunogen on immune T cell cytokine profiles

BALB/c mice were immunized with 100 μ g of either DT or maleyl-DT in PBS i.p. at weekly intervals for 2 or 3 wk, and splenic T cell stimulation assays were set up with graded doses of maleyl-DT or DT 4 or 5 days after the last dose. Culture supernatants were collected 72 h later and analyzed for the presence of IFN- γ , IL-4, and IL-10. Maleyl-DT-immune cells clearly make more IFN- γ and less IL-4 and IL-10 than DT-immune cells do in response to maleyl-DT (Fig. 1, A, B, and C). This relative dominance of IFN- γ in maleyl-DT-immune mice does not depend on delivery of Ag to SRs during recall in vitro, since using DT instead of maleyl-DT gives similar results (Fig. 1, D, E, and F). The results of the proliferation assay on these samples (Fig. 1G) confirm that, as we have shown earlier (23), maleyl-DT-immune mice show higher responses to both forms of DT and that maleyl-DT recalls higher responses better than DT does in both groups of mice.

To ensure that this shift to an IFN- γ -dominated response induced by maleylation of Ag was not peculiar to DT, C3H/HeJ mice were immunized with either CA or maleyl-CA (single dose of 1 mg/mouse i.p. in PBS), their spleen cells were stimulated 10 days later with either Ag in vitro, and IL-4, IL-10, and IFN- γ were assayed in the culture supernatants. Again, maleyl-CA-immune mice generate more IFN- γ as compared with IL-4 or IL-10 than CA-immune mice do whether CA or maleyl-CA is used as the recall Ag in vitro (Fig. 2).

Modulation of Ag-specific IgG1:IgG2a ratios in vivo by maleyl-protein immunization

In order to examine if the cytokine differences seen in culture were also reflected in immune responses in vivo, we analyzed the balance between the IL-4-dependent isotype IgG1 (31, 32) and the IFN- γ -dependent isotype IgG2a (33) in serum Abs in immunized mice. Maleylated and native Ags show no B cell cross-reactivity but demonstrate significant T cell cross-reactivity (23). We have therefore done "cross-priming" experiments, in which the first immunization or "priming" is with either DT or maleyl-DT as three weekly doses of 100 μ g each i.p., followed by the administration of native Ag in PBS for cross-priming 1 wk after the last priming dose. Serum Abs against various Ags were estimated 1 wk after the cross-priming. While the T cell cross-reactivity between native and maleyl-DT (23) (Fig. 1) permits generation of anti-DT Abs upon

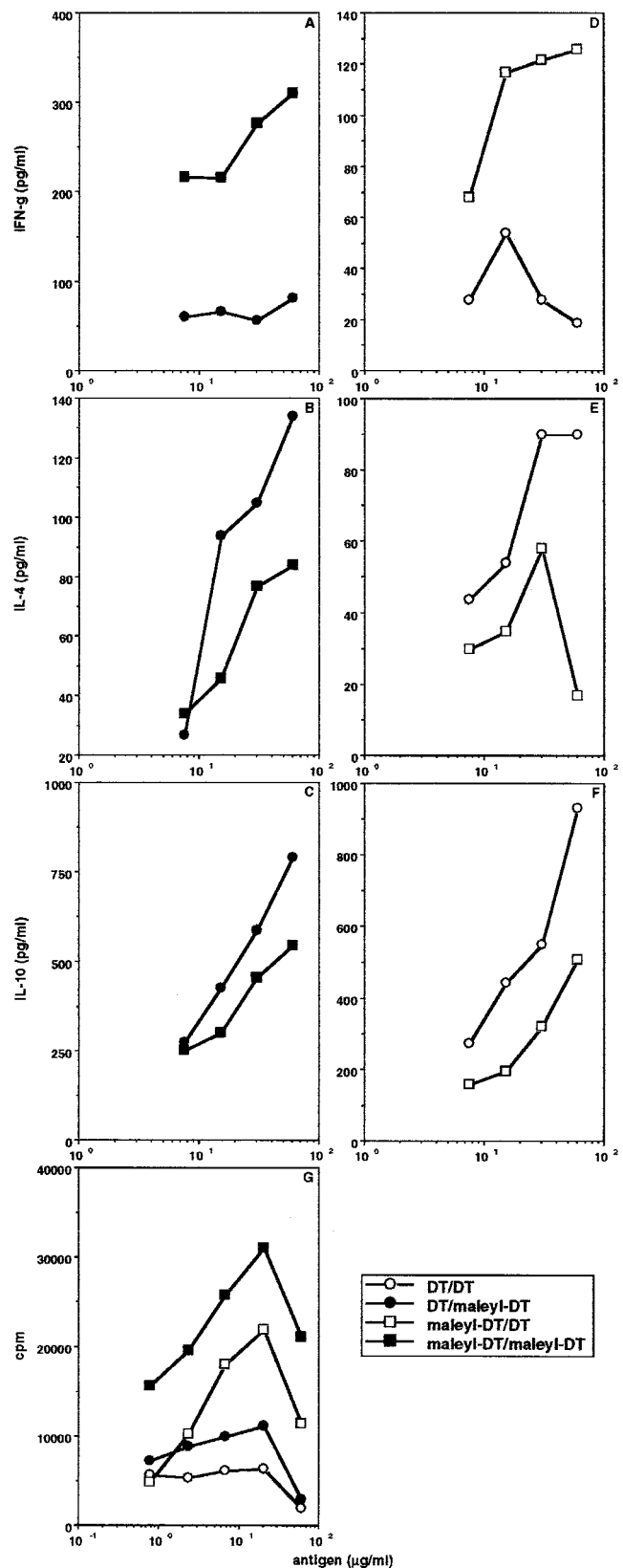


FIGURE 1. Immunization with maleyl-DT results in a relatively more Th1-dominant response than immunization with DT does. A and D show the IFN- γ levels, B and E show the IL-4 levels, and C and F show the IL-10 levels in stimulated spleen cell culture supernatants, while G shows the proliferation of stimulated spleen cells of mice immunized as shown in the key (immunogen/recall Ag). The experiment shown is representative of three independent experiments.

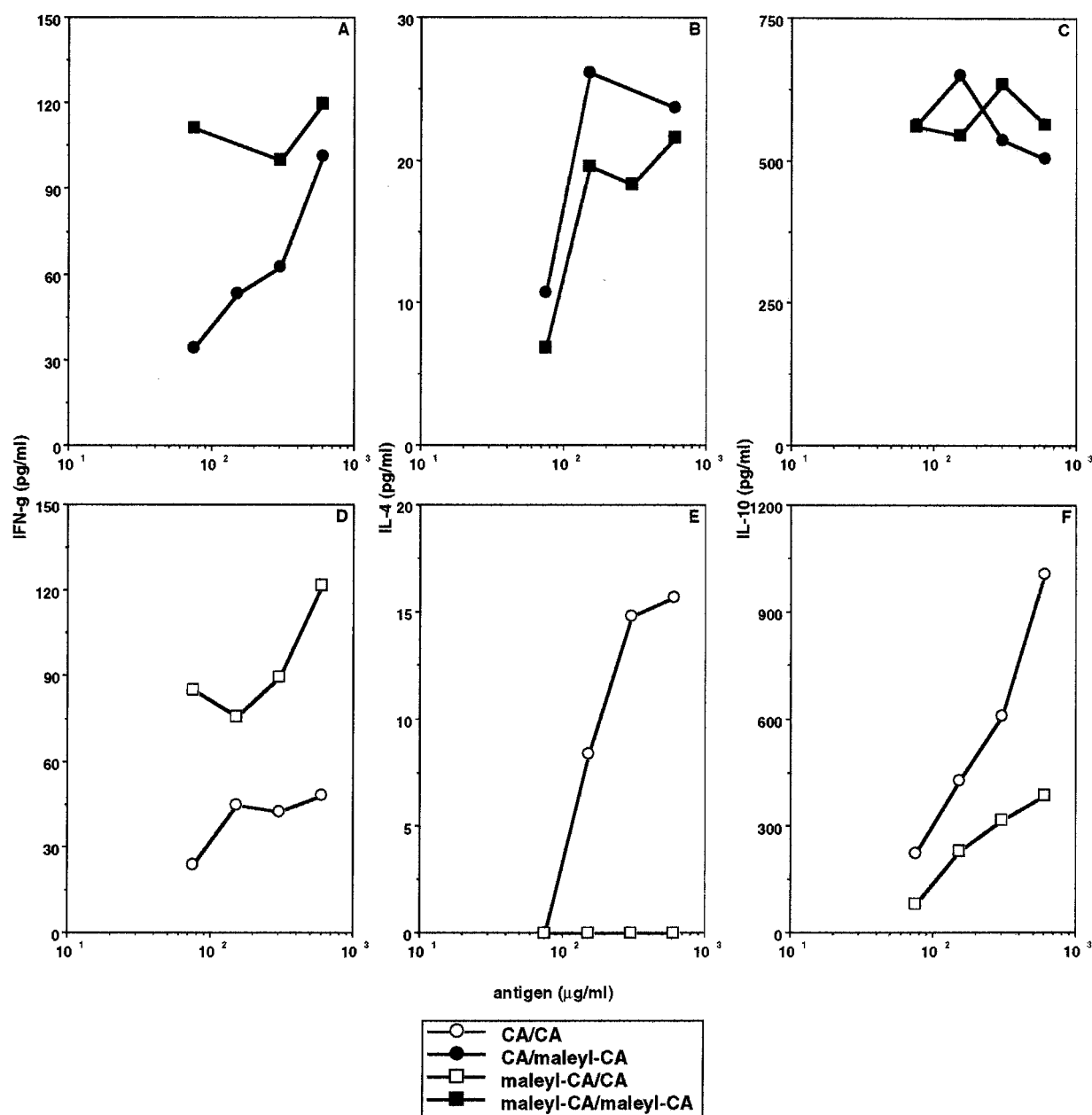


FIGURE 2. Immunization with maleyl-CA results in a relatively more Th1-dominant response than immunization with CA does. *A* and *D* show the IFN- γ levels, *B* and *E* show the IL-4 levels, and *C* and *F* show the IL-10 levels in culture supernatants from stimulated spleen cells of mice immunized as shown in the key (immunogen/recall Ag). The experiment shown is representative of five independent experiments.

initial priming with maleyl-DT-PBS, priming by DT-PBS did not generate detectable levels of serum Abs (data not shown), and therefore the IgG1:IgG2a ratios could not be compared. We therefore used alum as an adjuvant for the priming Ags. Mice were thus immunized i.p. with either DT-alum or maleyl-DT-alum. The cross-priming Ag was $100 \mu\text{g}$ DT-PBS, and mice were bled for determining serum Ab levels a week after the cross-priming dose. The IgG1:IgG2a ratio in serum anti-DT Abs after priming with DT-alum is significantly higher than in that following maleyl-DT-alum priming (Fig. 3). A similar modulation of the IgG1:IgG2a ratios can be observed using TT-PBS and maleyl-TT-PBS for priming, where the ratios can be compared without the need to invoke the help of alum as an adjuvant since TT-PBS triggers significant levels of serum anti-TT Abs (Fig. 3). These data support the probability that maleyl-immunogens skew the T cell response in favor of IFN- γ *in vivo*.

Effect of maleyl-protein on levels of macrophage costimulatory molecules

One possibility to explain this shift in the Th1/Th2 balance by maleyl-immunogens is the modification of costimulatory properties of SR-bearing APCs by SR ligation via the maleyl-protein Ag. In order to begin examining if SR ligation could alter the costimulatory signaling capabilities of SR-bearing APCs, adherent PECs treated with maleyl-BSA were stained for various cell surface molecules involved in delivery of accessory signals to T cells. At 24, 48, and 72 h of incubation with various stimuli, adherent PECs were stained for MHC class I, MHC class II, CD11b/CD18, CD11a/CD18, CD54, CD24, CD40, CD80, and CD86. As Figure 4 shows, even by 72 h, there are no significant differences induced by maleyl-BSA ($500 \mu\text{g/ml}$) in the expression levels of any of these molecules. However, cells treated with LPS ($25 \mu\text{g/ml}$;

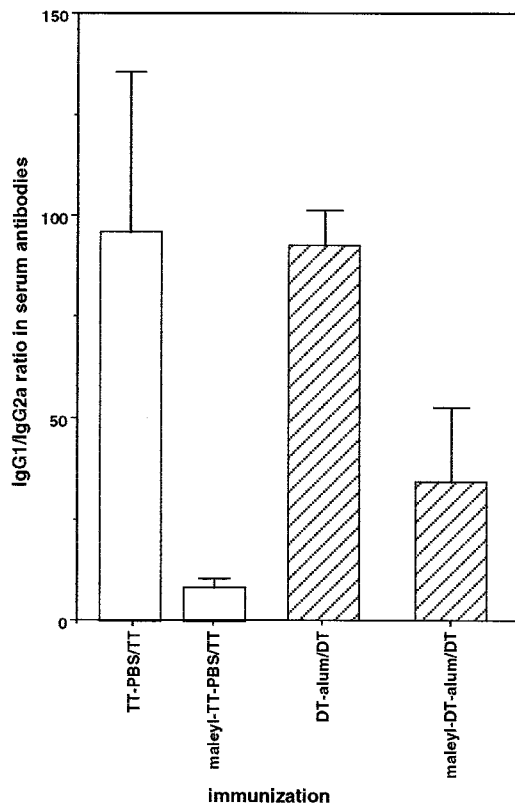


FIGURE 3. IgG1:IgG2a ratios in serum Abs depend on whether native or maleyl-Ag is used for priming. IgG1:IgG2a ratios in serum anti-DT (open bars) or anti-TT (hatched bars) Abs of various groups of mice (5–6 mice per group) are shown as mean ± SE. The immunization protocol for each group is shown as priming Ag/cross-priming Ag. The data are representative of three independent experiments.

Sigma) show higher levels of CD24, CD54, CD80, and CD86, while those treated with IFN- γ (0.3 μ g/ml; Genzyme) show increases in the levels of MHC class II, CD54, and CD86. Results at 24 and 48 h of incubation were similar (data not shown). Thus,

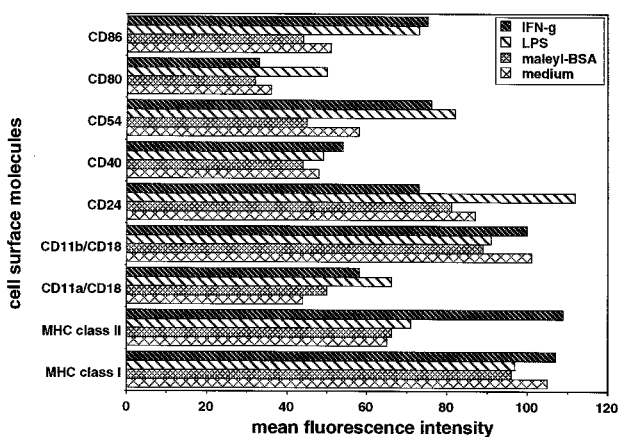


FIGURE 4. Ligation of SRs on macrophages does not cause any significant change in cell surface levels of the various molecules indicated. Flow cytometric analyses of adherent PECs cultured for 72 h in either medium alone, or LPS (25 μ g/ml), or IFN- γ (0.3 μ g/ml), or maleyl-BSA (500 μ g/ml) and stained for the detection of various molecules are shown as mean fluorescence intensity data. The data shown are representative of three independent experiments.

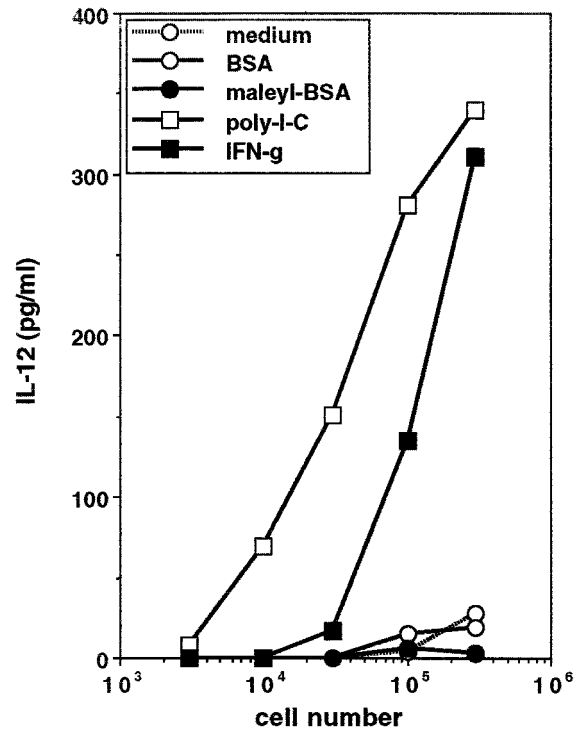


FIGURE 5. Maleyl-BSA does not induce the production of IL-12 from macrophages. C3H/HeJ-adherent PECs were incubated at various cell concentrations as shown with either 100 μ g/ml BSA, 100 μ g/ml maleyl-BSA, 50 μ g/ml poly(I:C), or 10 ng/ml IFN- γ . Culture supernatants were assayed for IL-12 after 48 h. The experiment shown is representative of four independent experiments.

none of the membrane-associated molecules involved in T cell-APC adhesion or costimulation that we have examined show any alteration in their expression upon SR ligation.

Since IL-12 has been shown to be critically involved in some instances of a preferential bias of the immune response toward Th1 cytokine production (6, 11, 13), we tested whether maleylated proteins could induce IL-12 production from macrophages. Adherent PECs were titrated at various doses from 3×10^6 /ml to 3×10^3 /ml in the presence or absence of 100 μ g/ml of BSA, maleyl-BSA, or 50 μ g/ml of polyinosinic-polycytidylic acid (poly(I:C)), or 10 ng/ml of IFN- γ , incubated at 37°C for 24 to 48 h, and the culture supernatants tested for the presence of IL-12 using the EIA as described. As shown in Figure 5, both poly(I:C) and IFN- γ induced IL-12 secretion from macrophages, while maleyl-BSA was unable to do so.

Inability of SR ligation to enhance T cell activation by macrophages

In addition to looking at the levels of costimulatory molecules on APCs stimulated with a maleyl protein, we also investigated the costimulatory properties of such APCs in a functional assay. D10.G4.1 cells were stimulated with titrated doses of CA or maleyl-CA in the presence or absence of 300 μ g/ml maleyl-BSA as a potential inducer of costimulation. The results (Fig. 6A) show that maleyl-BSA does not cause any increment in the response of D10.G4.1 to CA, and that it decreases the response to maleyl-CA down to levels comparable with that to CA. Thus, unless the specific Ag is delivered through the SRs, there is no enhancement of the T cell response.

However, since the modulation we observe as a result of protein maleylation is in the immunogenicity, we also needed to test a

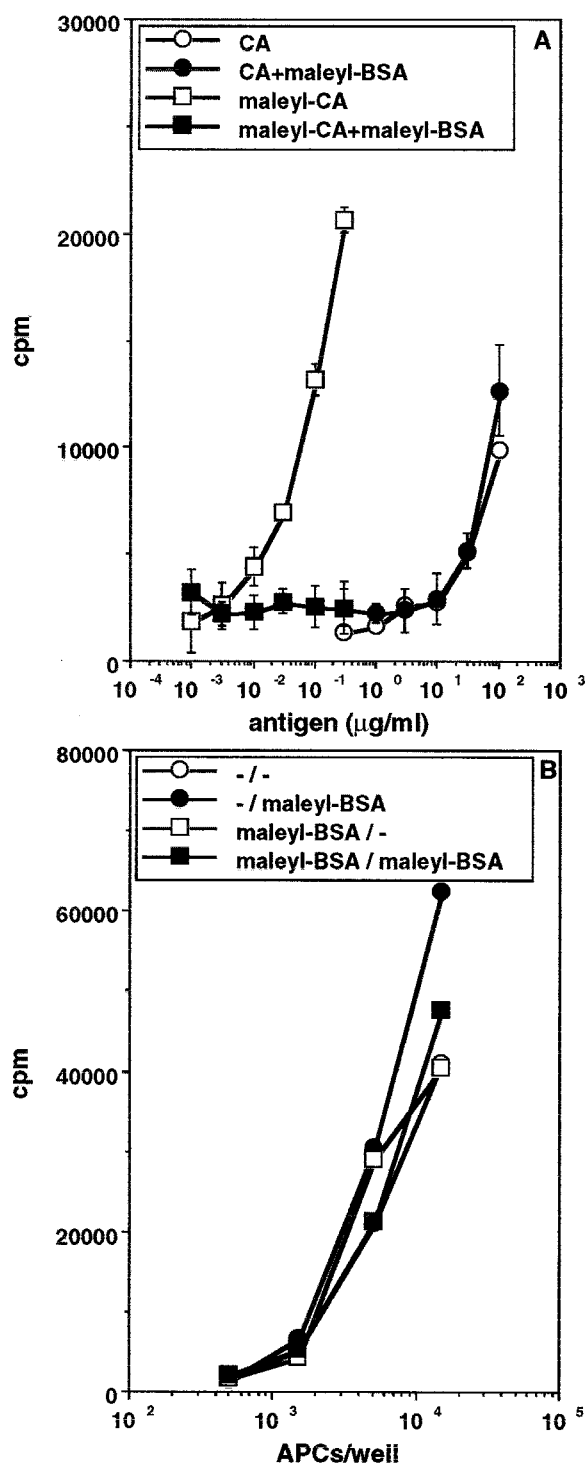


FIGURE 6. Engagement of SRs on macrophages does not alter their costimulatory capacities for T cell activation significantly. *A* shows the proliferation of D10.G4.1 cells stimulated with peritoneal APCs and titrated doses of CA or maleyl-CA in the presence or absence of 300 µg/ml of maleyl-BSA. The experiment shown is representative of three independent experiments. *B* shows the proliferation of thymocytes stimulated with anti-CD3 Ab and various doses of syngeneic adherent peritoneal cells treated with maleyl-BSA as shown (prior treatment/treatment during T cell assay). The experiment shown is representative of four independent experiments.

source of truly naive T cells so that the effects of the addition of a maleyl protein on the costimulatory properties of APCs could be examined in relevant fashion. We therefore used thymocytes as a

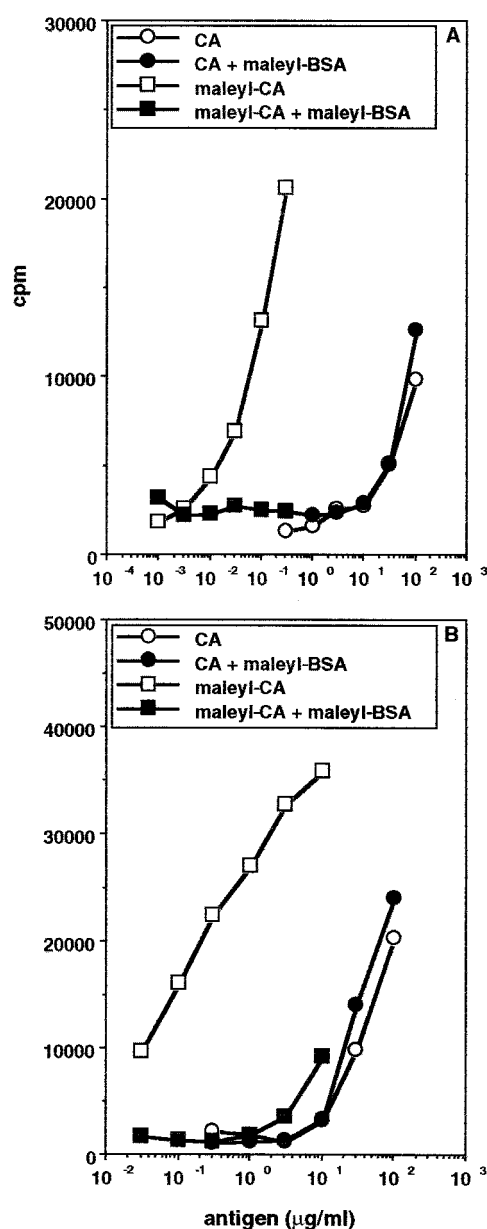


FIGURE 7. Like PECs, DCs present maleyl-protein better, and this presentation is blocked by an SR ligand. D10.G4.1 cells were stimulated with varying doses of either CA or maleyl-CA in the presence or absence of 300 µg/ml maleyl-BSA as shown. Adherent PECs as APCs were used in *A* and splenic DCs in *B*. The data are representative of three independent experiments.

source of predominantly naive T cells capable of responding to anti-CD3, and adherent peritoneal washout cells as a source of SR-bearing macrophages that would cross-link the anti-CD3 Ab via FcRs in addition to providing costimulation. Adherent peritoneal cells were cultured with or without maleyl-BSA for 40 h and then titrated as APCs for anti-CD3-mediated proliferation of syngeneic thymocytes, again in either the presence or absence of maleyl-BSA. It can be seen that the proliferation of thymocytes is not dramatically different whether or not maleyl-BSA is present, either as a prior stimulator of macrophages or during the T cell stimulation assay (Fig. 6*B*), suggesting that maleyl-BSA does not modify any costimulatory functions critically enough to change the resultant T cell activation achieved. Thus, maleyl proteins do not

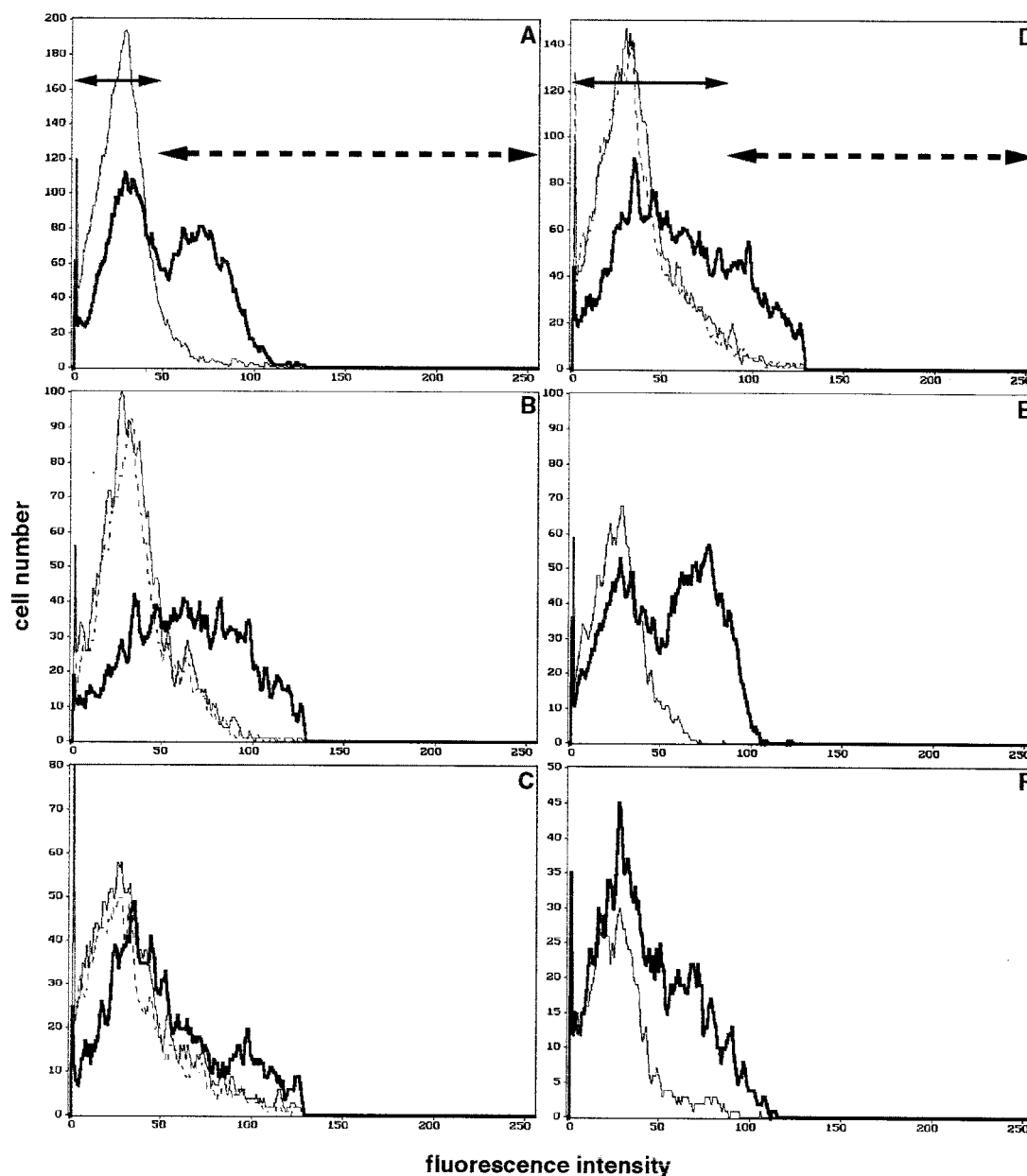


FIGURE 8. Some DCs bind to maleyl-BSA. Splenic low-density cells were stained in a two-color flow cytometric analysis with the anti-DC Ab N418 and maleyl-BSA. *A* shows the staining of these cells with N418 (thick line) over a control (thin line). *B* shows the staining of the N418-negative cells in *A* (unbroken arrow) with maleyl-BSA (thick line) or BSA (broken line) over a control (thin line). *C* shows the staining of the N418-binding cells in *A* (broken arrow) with maleyl-BSA (thick line) or BSA (broken line) over a control (thin line). *D* shows the staining of the total cell population with maleyl-BSA (thick line) or BSA (broken line) over a control (thin line). *E* shows the staining of the maleyl-BSA-negative cells in *D* (unbroken arrow) with N418 (thick line) over a control (thin line). *F* shows the staining of the maleyl-BSA-positive cells in *A* (broken arrow) with N418 (thick line) over a control (thin line). The data are representative of two independent experiments.

cause any significant alteration in any of the costimulatory properties of macrophages that we have investigated.

Presentation of maleyl-CA by DCs

Since APC choice has been suggested to be a major factor in determining the Th1/Th2 balance in the immune responses generated (8, 10), it became necessary to test whether the other major “professional” APC types, DCs and B cells, could also bind and present maleyl-protein.

Splenic DCs were tested in comparison with peritoneal adherent cells for the ability to present maleyl-CA and CA to the CA-specific T cell clone D10.G4.1. Both the peritoneal macrophages and

the splenic DCs present maleyl-CA better than CA to D10.G4.1 (Fig. 7). The ability of splenic DCs to present maleyl-CA to D10.G4.1 was over 1000-fold better than for CA, similar to the ability of adherent PECs. Addition of an excess of maleyl-BSA in this assay does not affect the presentation of CA by either APC population at all, but it inhibits the presentation of maleyl-CA so that there is no longer any distinction between the presentation of maleyl-CA and CA (Fig. 7).

In order to ensure that possible contamination of these DC preparations by non-DC cells was not responsible for enhanced presentation of maleyl-CA, we next directly stained DC-enriched C3H/HeJ spleen cells in a two-color flow cytometric analysis for

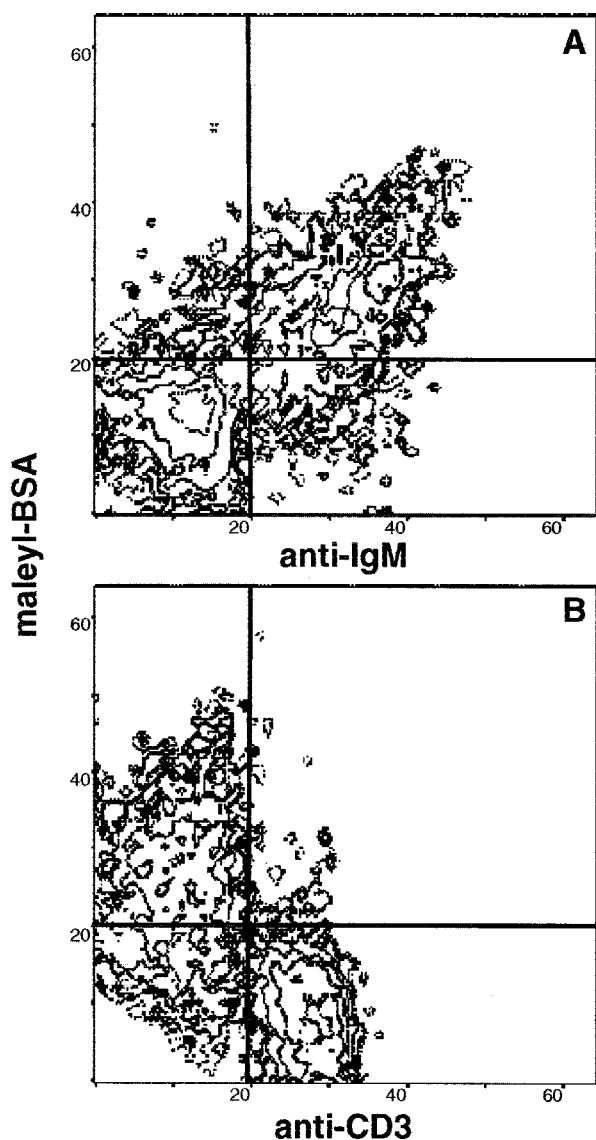


FIGURE 9. Splenic B cells bind to maleyI-BSA although T cells do not. Two-color flow cytometric analysis of plastic nonadherent spleen cells is shown for binding of maleyI-BSA vs either anti-IgM (A), or anti-CD3- ϵ (B). The data are representative of two independent experiments.

the ability of DCs to bind maleyI-proteins. Splenic DCs were detected by a DC-specific mAb, N418, and were tested for the binding of maleyI-BSA. These DC-enriched splenic cells show the presence of N418-binding DCs (Fig. 8A). When the N418-negative cells (shown by the solid arrow in Fig. 8A) are gated and analyzed, they show excellent staining with maleyI-BSA (Fig. 8B) as expected, since most of them are likely to be macrophages. Some of the N418-positive cells (from the gate shown by the interrupted arrow in Fig. 8A) also stain very well with maleyI-BSA (Fig. 8C), suggesting that at least some DCs can bind to SR ligands. Figure 8D shows the staining of the total DC-enriched population with maleyI-BSA. Figure 8E shows the staining of maleyI-BSA-dull cells (in the gate indicated by the solid arrow in Fig. 8D) with N418, while Figure 8F shows the staining of maleyI-BSA-bright cells (as gated in Fig. 8D by the interrupted arrow) with N418, establishing that a significant proportion of maleyI-BSA-binding cells bind N418 and are therefore DCs. These staining data, together with the earlier functional findings, show that enhanced

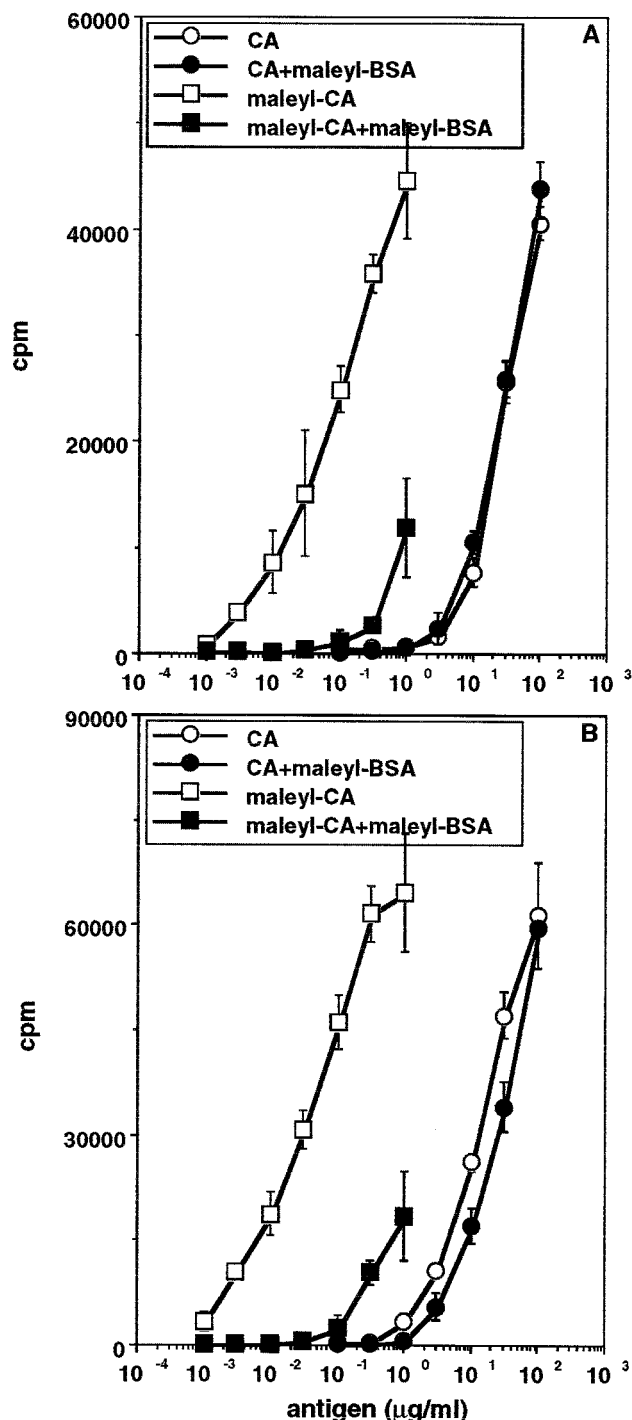


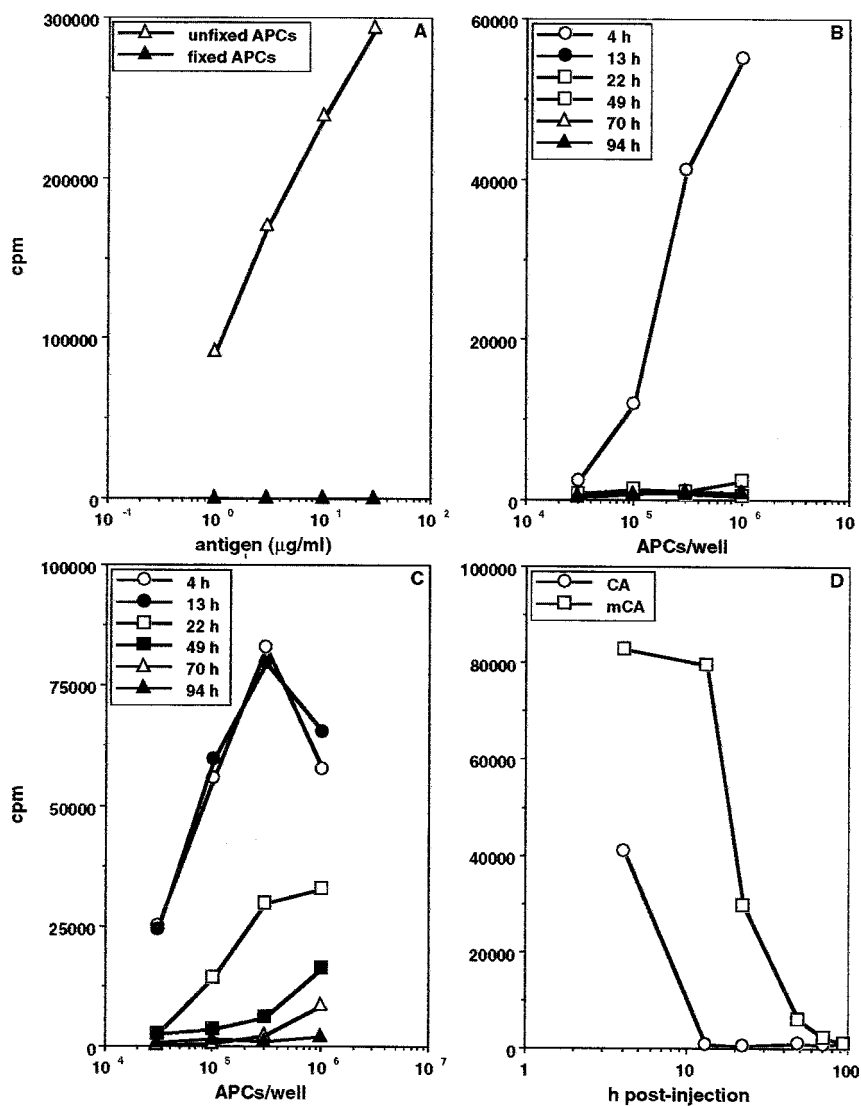
FIGURE 10. B cells present maleyI-CA better than CA, and this presentation is blocked by a SR ligand. Proliferative responses of D10.G4.1 to varying doses of CA or maleyI-CA either in the presence or absence of 500 $\mu\text{g/ml}$ of maleyI-BSA are shown. The APCs used were either total spleen cells (A), or macrophage- and DC-depleted spleen cells (B) from C3H/HeJ mice. The data are representative of three independent experiments.

presentation of maleyI-CA over CA by DCs occurs through receptor-mediated uptake of maleyI-CA.

Presentation of maleyI-CA by B cells

Although maleyI-BSA is indeed a ligand for class A SRs, it is also known to bind to other cell surface molecules such as the class B SRs and related receptors, some of which can be found on B cells

FIGURE 11. Peptide-MHC complex density declines differentially over time on spleen cells of mice given CA or maleyl-CA. *A* shows the proliferative response of D10.G4.1 cells to varying doses of CA presented by either unfixed gamma-irradiated (2000 rad) or fixed C3H/HeJ spleen cells (1.5×10^6 cells/ml). *B* and *C* show the responses of D10.G4.1 to varying doses of fixed spleen cells from mice previously injected with CA (*B*) or maleyl-CA (*C*) at the times shown. These data from *B* and *C* have been replotted in *D* to show responses of D10.G4.1 over a period of time to fixed spleen cells (1.5×10^6 cells/ml) from mice given either CA or maleyl-CA. The experiment shown is representative of four independent experiments.



(21). Two-color flow cytometric analysis of plastic nonadherent splenic cells from C3H/HeJ mice shows that maleyl-BSA binds well to IgM-bearing B cells but not to CD3- ϵ -bearing T cells (Fig. 9). In order to examine if this binding results in any improvement of presentation of maleylated protein over its native counterpart by B cells, splenic cells were depleted of macrophages and DCs by extensive plastic adherence until neither CD11b-bearing cells (macrophages) nor N418-binding cells (DCs) could be detected any longer by flow cytometry (data not shown). Such macrophage- and DC-depleted cell preparations could still present maleyl-CA about 100- to 300-fold better than CA to D10.G4.1 cells, and this enhanced presentation was blocked by the presence of competing maleyl-BSA (Fig. 10). Thus B cells also take up a maleyl-protein in receptor-mediated fashion and present it with greater efficiency than its non-maleylated counterpart. Maleyl-proteins therefore make no distinctions between the APC lineages through which they are presented to T cells.

Effect of Ag maleylation on T cell ligand densities generated in vivo

Another factor in the development of a T cell response dominated by IFN- γ is the possibility of higher densities of peptide-MHC ligands generated in situations in which such a dominance is seen (17). To test whether such a possibility could be invoked in our

experimental system, we have attempted to compare the ligand levels generated on APCs in vivo after immunization with either native or maleyl-CA.

C3H/HeJ mice were given 1 mg each of maleyl-CA or CA in PBS i.p. (as used in the experiment in Fig. 2) to look at the kinetics of appearance of specific CA peptide-MHC complexes on their splenic APCs by following the stimulatory capacity of the spleen cells for D10.G4.1 over a 4-day period post-Ag injection. Spleen cells were fixed with paraformaldehyde to inhibit any further Ag processing in vitro (34, 35). Figure 11A shows that the fixation protocol used does not permit any Ag processing to take place in vitro, since intact CA can no longer be processed by such fixed spleen cells from mice not given Ag. When spleen cells from mice given native or maleyl-CA 4 to 94 h earlier are tested, the maximal T cell stimulation, reflecting maximal availability of peptide-MHC complexes, occurs 4 h after immunization in the CA-treated group (Fig. 11B). From 13 h onward, the T cell response to splenic APCs from such CA-treated mice is practically undetectable. Spleen cells from mice given maleyl-CA also stimulate D10.G4.1 maximally as early as 4 h after injection; however, T cell stimulation remains comparable at 13 h, and although a decline is seen by 22 h, stimulation is detectable up to 70 h (Fig. 11C). No T cell stimulation is detected 94 h after injection of Ag in either group, but T cell

stimulation is greater with APCs from maleyl-CA-treated mice at all other time points (Fig. 11D).

Discussion

We have previously shown that immunization with maleyl-DT in PBS generates a better T cell proliferative response than does DT-PBS (23), indicating enhanced immunogenicity. We have now examined the possibility that immunization with maleyl-proteins may also modulate the cytokine profiles of the T cells triggered, by analyzing the cytokine secretion profiles of T cells primed with either native or maleyl-DT without adjuvant. Maleyl-DT-immune cells produce more IFN- γ than DT-immune T cells do when cultured in vitro either with DT or maleyl-DT, indicating that the recall Ag has little role in determining the cytokine response mounted (Fig. 1). This is not an isolated finding with just one Ag, since the findings are reproducible with CA/maleyl-CA (Fig. 2), and with yet another protein—OVA and maleyl-OVA (data not shown). These are not simply cytokine differences seen in the recall responses in vitro, since Abs generated in vivo using maleyl-proteins also show a relative dominance of the IFN- γ -dependent IgG2a isotype over the IL-4-dependent IgG1 isotype as compared with the Abs generated by native Ag priming (Fig. 3). Time course kinetics of the IFN- γ and IL-10 production in vitro show that the maximum level of these cytokines was reached in the culture supernatants between 72 and 96 h for both maleyl-CA-immune and CA-immune T cells (data not shown).

What is the basis of the observed differences (quantitative as well as qualitative) in the immune responses to maleylated Ags? Enhancement and modulation of T cell immunogenicity may be mediated through either cognate or costimulatory signals from APCs, and therefore we have tried to examine the effects of maleyl-proteins on both signals.

Macrophage SR ligation has already been reported to lead to phosphatidylinositol-4,5-bisphosphate (PIP₂) hydrolysis, NF- κ B induction, and TNF- α transcription in macrophages (36). It also induces the secretion of PGE₂ and IL-1 (37, 38), both of which have significant effects on T cell activation (39, 40). It is thus possible that ligation of SRs on such receptor-bearing APCs may lead to modifications in the costimulatory functions of these APCs, and such costimulation-associated changes may also affect the outcome of the resultant T cell priming. Thus, it was of interest to examine the outcome of SR ligation on costimulatory properties. One way to do this is to examine the levels of expression of various membrane molecules found to be important in adhesion and/or costimulatory signaling on SR-stimulated APCs. We have looked for modulation in the levels of CD11b/CD18, CD11a/CD18, CD54, CD24, CD40, CD80, and CD86, as well as in MHC class I and MHC class II, and we find no change in these levels on adherent PECs upon SR ligation (Fig. 4). Neither does maleyl-BSA induce any production of IL-12 from PECs (Fig. 5), in concordance with a recent report, which in fact shows that maleyl-BSA suppresses the induction of IL-12 production by bacterial LPS (41). Thus, neither of the two molecular interactions recently claimed to be critical in determining the Th1/Th2 balance of the resultant T cell commitment, CD80/CD86 (15) and IL-12 (6, 11, 13), is altered by maleyl-BSA. Maleyl-BSA does not induce the secretion of any IFN- γ from APCs either (data not shown). Thus, if any costimulatory property relevant to the differential control of Th1/Th2 pathways was indeed altered by maleyl-proteins, it would be a hitherto uncharacterized one.

Although the costimulatory signals assayed so far do not show any differences, the possibility always remains that some other, as yet unidentified, alterations are in fact altering costimulation. To

address this, we have directly tested the costimulatory properties in a functional fashion, with enhancement of T cell activation as the criterion.

We have shown previously that if DT-immune spleen cells are cultured in vitro with DT, the resultant levels of proliferation reached are not different either in the presence or absence of 100 μ g/ml of maleyl-BSA acting as a SR ligand (23). Clearly, SR-bearing APCs are present and important in these cell populations, since the presentation of maleyl-DT is enhanced, and the enhancement is blocked by the addition of maleyl-BSA (23). We have now also looked at the proliferation of D10.G4.1 cells when CA is presented in the presence or absence of maleyl-BSA, and no enhancement in stimulation can be observed in the presence of the SR ligand (Fig. 6A). Thus, it appears that SR ligation, by itself, does not affect the ability of SR-bearing PECs to present an Ag to secondary T cells if that Ag does not itself bind to the SR.

However, the costimulatory requirements of naive and primed T cells are significantly different (42–44). It is thus possible that, while SR ligation has no effects on the costimulation relevant to secondary T cells, the situation may be different with naive T cells, and that may contribute to the modulations in immunogenicity we have reported previously (23) as well as here. Therefore, it was necessary to test a population of naive T cells for such SR ligation-mediated modulation of costimulatory functions of APCs independent of SR-mediated delivery of Ag. Rather than attempting a CD45 isoform-based separation of phenotypically “naive” and “memory” CD4 T cells (42), we have used thymocytes as the responding population. Although thymocytes are a heterogeneous population of T lineage cells, the immature CD4⁺CD8⁺ thymocytes do not proliferate in response to anti-CD3, while the mature “single-positive” CD4⁺/CD8⁺ thymocytes do (45–48). The mature single-positive thymocytes consist predominantly of naive T cells, since circulating memory T cell reentry into the adult thymus is minimal (49, 50). Thus, the thymocytes responding to anti-CD3 by proliferation would be mainly naive mature T cells. We have used anti-CD3 as a stimulus that is independent of SRs, and have examined the effects of an SR ligand on presentation of this stimulus to thymocytes by FcR- and SR-bearing peritoneal adherent cells, mainly macrophages. Our data clearly show that such SR ligation makes little difference to the proliferative response of thymocytes (Fig. 6B). Thus, SR ligation is unlikely to modify the costimulatory properties of the relevant APCs to any major degree.

Maleyl-proteins have very short $t_{1/2}$ in vivo because of SR-mediated uptake by macrophages (51). They are therefore unlikely to reach non-receptor-bearing cells to any significant degree. Yet, they show enhanced immunogenicity in vivo, although DC-mediated Ag presentation is thought to be essential for immunogenicity (52, 53). It was therefore necessary to explore the possibility that DCs express SRs, especially since there are also suggestions that IL-12 from DCs plays a significant role in Th1 commitment (54). Macrophages and DCs are known to develop from common myeloid lineage precursors (55), and circulating monocytes can be induced to differentiate into DCs by certain combinations of cytokines (56). It did not therefore appear implausible that DCs may express SRs, and in fact, it has been previously reported that follicular DCs in germinal centers of human lymph nodes are recognized by anti-SR Abs (57). Our data show that maleyl-Ags are indeed presented better to T cells by murine DCs in addition to macrophages, and that this presentation is significantly inhibited by maleyl-BSA and is therefore receptor mediated (Fig. 7). We have also stained splenic low density cells for SRs and the DC-specific mAb N418 in a two-color analysis, and Fig. 8 shows that at least some DCs clearly bind well to maleyl-BSA. Somewhat

more surprisingly, the same is true for B cells—they do bind maleyl-BSA although T cells do not (Fig. 9), and they show some enhanced presentation of maleyl-CA to D10.G4.1 cells which is blocked by maleyl-BSA (Fig. 10). Similarly, transformed B cell lines also bind maleyl-BSA and present maleyl-CA better than CA to D10.G4.1 (data not shown). These data are consistent with the fact that molecules of the class B SR family are found on B cells in addition to cells of the monocytic lineage (21).

So it appears that all the three major MHC class II-bearing APC populations—macrophages, DCs, and B cells—can present maleyl-proteins better than their native counterparts due to receptor-mediated uptake. Thus, injected maleyl-Ags are quite probably delivered efficiently to DCs and B cells as well as to macrophages, and no selective differences are likely to exist between maleyl-immunogens and their native counterparts as far as APC choice is concerned. In fact, selective depletion of macrophages *in vivo* before immunization does not alter the cytokine balance generated by either native or maleyl-immunogens, although it does decrease the magnitude of the response triggered by the maleyl-immunogen (data not shown).

Thus, neither costimulatory modification nor preferential presentation by some APC types appears to be a viable possibility to explain the altered immunogenicity of maleyl-proteins. It remains possible that the enhanced T cell immunogenicity of maleylated proteins is due simply to greater quantities of peptide-MHC complexes becoming available on APC surfaces due to receptor-mediated delivery, and such comparatively higher ligand availability could also account for the relative skewing of T cell responses toward the Th1 cytokine profile as has been suggested earlier (17). With this possibility in mind, we have begun examining the relative ligand levels generated *in vivo* on splenic APCs in mice administered either native or maleyl-proteins. Such relative ligand levels have been read out using the ability of these APCs to stimulate a monoclonal T cell line, D10.G4.1, specific for CA—a method that has been used in other systems as well (58, 59). Although this is an indirect measurement of peptide-MHC complex levels, it appears to be valid since maleyl-CA and CA are equivalently presented to D10.G4.1 cells *in vitro* if any receptor-mediated delivery of maleyl-CA is blocked by maleyl-BSA (Figs. 7 and 10). Thus, the enhancement of D10.G4.1 stimulation is not due to Ag-nonspecific effects of SR ligation, nor is it due to altered processing of maleyl-CA *per se*. It is therefore reasonable to assume that variations in the stimulation of D10.G4.1 under these circumstances reflect real differences in ligand “doses.”

Using such a readout system, it can be seen that administration of maleylated Ags leads to the presentation of higher levels of stimulatory peptide-MHC complexes than injection of native Ags does (Fig. 11). We have confirmed that this pattern holds good for OVA as well, using OVA-specific T cell hybridomas (data not shown). This finding is consistent with arguments for a major role of ligand densities in determining the Th1/Th2 balance (17). In fact, the longer life of the peptide-MHC complexes *in vivo* may be more critical than the level available at any one time point. The $t_{1/2}$ of peptide-MHC complexes generated from native protein *in vivo* does not appear to be very long, in keeping with some reports (60–62) and contrary to other estimates (63, 64). In fact, our data show that the $t_{1/2}$ of such complexes generated from a targeted Ag, which is presumably taken up by receptor-mediated endocytosis over a short time span, can be considerably longer than those generated from an Ag taken up more slowly by pinocytotic uptake. It is not clear whether this indicates some differences in the trafficking of MHC class II molecules loaded with peptide in the two instances, or whether some differences between the processing com-

partments that SR ligation-initiated endosomes and nonspecific pinosomes enter are responsible.

There have been many indications that APC factors are principally responsible for the differential commitment of T cells to either the Th1 or the Th2 cytokine package. APCs may affect effector T cell commitment either by modulating the density of peptide-MHC complexes presented (17), and/or by providing differing combinations of costimulatory signals (10–12, 15, 16). Our data show that immunization with maleylated proteins tends to generate a relatively greater dominance of the Th1 response, and further suggest that, rather than any major modulation of costimulatory functions, it is the level of ligand availability generated over time that plays a major role in this differential commitment.

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