Brief Definitive Report

Exogenous Antigens Gain Access to the Major Histocompatibility Complex Class I Processing Pathway in B Cells by Receptor-mediated Uptake

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Summary

Professional antigen-presenting cells, such as macrophages, dendritic cells, or B cells, take up soluble, exogenous antigens (Ags) and process them through the class II pathway. Several reports have shown that phagocytic macrophages also process particulate or soluble forms of exogenous Ag via the class I pathway. By contrast, B cells normally do not process soluble, exogenous Ag by way of the class I pathway unless Ags are directly introduced into the cytoplasm. Here we report that B cells present exogenous Ag via the class I pathway when Ags are taken up by receptor-mediated endocytosis. Thus, specialized methods of Ag uptake such as phagocytosis or receptor-mediated endocytosis deliver exogenous Ag into the class I pathway of Ag processing and presentation.

Exogenous Ags are taken up nonspecifically by fluid-phase pinocytosis or endocytosis, processed, and presented via class II pathway by professional APCs, such as macrophages, dendritic cells, or B cells. Exogenous Ags do not enter the class I pathway of most cells (1–3). However, a small population of phagocytic macrophages can process such Ags via the class I pathway when stimulated with high concentrations (4, 5) or particulate forms (6–8) of Ag. B cells normally do not appear to process soluble (4) or particulate forms (9) of exogenous Ags via the class I pathway.

The Ag-specific Ig receptors may have an important role not only in the activation of B cells but also in the efficient presentation of exogenous Ags to T cells. For example, hapten-specific B cells present hapten–carrier conjugates to carrier-specific $CD4^+$ T cells much more efficiently than unmodified protein Ags (10).

The possibility that Ig receptors might facilitate uptake and processing of exogenous Ags via the class I pathway has not been thoroughly investigated. However, Yefenof et al. reported that $CD8^+$ T cells from LNs of KLH-primed mice lysed hapten-specific virgin B cells after incubation with hapten–KLH (11). More recently, we found that adoptive transfer of $CD8^+$ CTLs suppressed humoral responses in recipient mice (12). These observations raise the possibility that B cells might process exogenous Ag via the class I pathway if Ags are taken up by receptor-mediated endocytosis. The current studies explored this possibility by using a hapten–carrier system to study Ag processing and presentation by B cells.

Materials and Methods

Antigens and Reagents. Chicken egg OVA, TNP, chloroquine, brefeldin A, and phenazine methosulfate (PMS) were purchased from Sigma Chemical Co. (St. Louis, MO). Crystallized beef insulin was purchased from Lilly Research Labs (Indianapolis, IN). TNP was conjugated to OVA or insulin as described (13). Tetrazolium salt XTT was obtained from Diagnostics Chemicals Ltd. (Oxford, CT). *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LLnL) and *N*-acetyl-L-leucinyl-L-leucunyl-methional (LLM) were provided by Dr. K.L. Rock (Harvard University, Boston, MA).

Antibodies. Culture supernatants from B cell hybridomas were used as sources of mAb. Anti-SP6 is a mAb specific for the idiotype of IgM_{TNP} (provided by Dr. N. Hozumi, University of Toronto, Toronto, Ontaro, Canada). MK-D6 (anti-$I\text{-}A^d$) was obtained from the American Type Culture Collection (Rockville, MD). DO4 (anti-K^dD^d), 25-9-35 (anti-$I\text{-}A^b$), and 270-JS (anti-K^b) were provided by Dr. V. Hauptfeld (St. Louis University, St. Louis, MO).

Cells and Cell Cultures. A HAT-sensitive variant of M12.4.1 ($H\text{-}2^d$), a B cell lymphoma producing surface IgG of unknown specificity (14), was provided by Dr. R. Asofsky (National Institutes of Health, Bethesda, MD). F8.11 is a B cell hybridoma produced by fusion of M12.4.1 and spleen cells from C57BL/6 ($H\text{-}2^b$) mice (15). SP6/HL is a B cell hybridoma provided by Dr. Hozumi. It secretes IgM, κ specific for TNP, and it reacts with the anti-SP6 mAb directed toward idiotypic determinants (16). E.G7–OVA (provided by Dr. M.J. Bevan, University of Washington, Seattle, WA) was produced by transfection of EL4 ($H\text{-}2^b$) with the OVA cDNA gene (17). EL4-INS was produced in our laboratory by transfecting EL4 with the genomic human insulin gene (18). Two T cell hybridomas, 4B10 ($CD8^+$, specific for K^b-

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restricted $OVA_{257-264}$) and 2D5 ($CD4^+$, specific for $I\text{-}A^b$–restricted $OVA_{265-280}$) (15), were used as reporter systems for the class I and class II presentation of OVA, respectively. An insulin-specific, $CD8^+$, MHC class I–restricted CTL line was generated by priming C57BL/6 mice with beef insulin in CFA and stimulating with EL4-INS as we described previously (18). All cells were cultured in RPMI 1640 containing 10% FCS, 1 mM L-glutamine, 1 mM Na-pyruvate, 50 μM 2-ME at 37°C in 6% CO_2 in air.

Vector and Gene Transfer. F8.11 cells were transfected with $pR\text{-}HL_{TNP}$ vector containing the structural genes for the μ and κ chains of TNP-specific IgM from SP6/HL and the neomycin-resistance gene (19). G418-resistant transfectants, designated as F8.11HL, were selected and cloned by limiting dilution.

Measurement of Abs. Secretion of TNP-specific Ab by various cell lines was tested by ELISA. In brief, microtiter plates were coated with OVA or TNP–OVA and then blocked with PBS plus 1% BSA. Serially diluted supernatants were added and incubated at 37°C for 1 h. Alkaline phosphatase–conjugated goat anti–mouse IgM was then added and incubated at 37°C for 1 h. Finally, *p*-nitrophenyl phosphate substrate was added, and colorimetric reaction was read as absorbance at 405 nm using an automatic microplate reader (Molecular Devices, Menlo Park, CA).

Ag Presentation Assay. In most experiments, IL-2 production by T cell hybridomas was used as a measure of Ag-induced activation. T cells were cocultured with irradiated (10,000 rads) B cell hybridomas in 96-well plates with or without Ag. In some cases, B cell hybridomas were pretreated with mAbs or inhibitors before incubation with Ag and T cells. Supernatants were harvested after a 24-h incubation at 37°C, and IL-2 production was tested with HT-2 cells (20) by using XTT plus PMS (21). Absorbance was read at 450 nm, and data were averages of triplicates ± SD. Representative experiments that have been repeated at least three times are shown.

Cytotoxicity Assay. Cytotoxicity assays were performed with ^{51}Cr-labeled B cell hybridomas as targets and insulin-specific CTLs as effectors (18). Labeled target cells were plated in 96-well round-bottomed plates at 10^4 cells per well and incubated with various concentrations of Ag at 37°C for 1 h. CTLs were then added to the plates at an E/T ratio of 10/1. After a 4-h incubation at 37°C, supernatants were harvested, and radioactivity was detected in a gamma counter (Wallac, Turku, Finland). The percentage specific lysis equals 100 × ([release by CTLs − spontaneous release]/[maximal release − spontaneous release]). Maximal release was determined by addition of 1% Triton X-100. Spontaneous release in the absence of CTLs was <15% of maximal release. Representative experiments that have been repeated at least three times are shown.

Results and Discussion

Characterization of B Cells Expressing Anti-TNP mIg Receptors. B cells, like most other somatic cells, do not efficiently take up and process soluble, exogenous Ag into the class I pathway (1). The purpose of these studies was to determine definitively whether B cells can take up and process exogenous Ag via the class I pathway if Ag uptake is facilitated by receptor-mediated endocytosis. For this purpose, we wanted to use a B cell line that expresses high levels of membrane Ig (mIg) of a known specificity. We thought it was also important that the mIg-expressing B cells should not secrete significant amounts of Ab, because formation of Ag–Ab complexes would introduce ambiguities into the interpretation of these experiments. For example, such complexes might decrease the amount of available Ag or alternatively increase the amount of Ag taken up if B cells expressed FcR. Therefore, we chose M12.4.1 B cell lymphoma for the starting cells. This tumor arose in BALB/c mice and expresses both MHC class I and MHC class II Ag (14). In addition, M12.4.1 expresses mIgG of an unknown specificity (J.A. Kapp, unpublished observations) but does not secrete Ig (14). A HAT-sensitive variant of M12.4.1 was fused with SCs from C57BL/6 mice injected with LPS. This fusion gave rise to a hybridoma (F8.11) that expresses MHC class I and class II Ag from both $H\text{-}2^b$ and $H\text{-}2^d$ haplotypes (15).

F8.11 was subsequently transfected by electroporation with a plasmid ($pR\text{-}HL_{TNP}$) containing the rearranged μ and κ genes cloned from genomic DNA of SP6/HL (19). Hozumi and colleagues have previously shown that transfection of B cell hybridomas with this plasmid yielded cells that secreted TNP-specific IgM (16). By contrast, transfection of A20-2J, a B lymphoma line, with this construct produced cells expressing TNP-specific mIgM (19). Thus, these results illustrate that the phenotype of the recipient cell dictates differential mRNA processing of the μ chain (22, 23). Neomycin-resistant transfectants were identified by rosette formation with TNP-conjugated sheep RBCs and binding of fluoresceinated anti-SP6, an idiotype-spe-

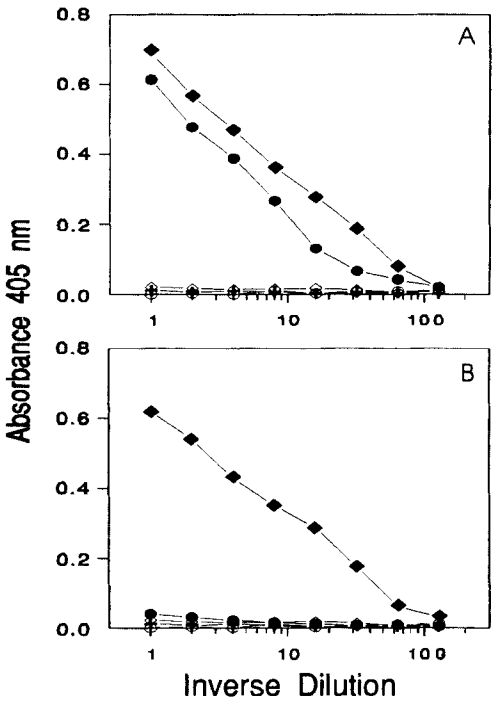

Figure 1. Production of $sIgM_{TNP}$ by SP6/HL but not F8.11HL. Supernatants from SP6/HL (●), F8.11 (+), or F8.11HL (◇) were serially diluted and tested for Abs against TNP–OVA (*A*) or OVA (*B*) by ELISA. Normal mouse IgM (○) and serum from mice primed with OVA in CFA (◆) were used at a starting dilution of 1/50. Bound Ab was detected by alkaline phosphatase–conjugated goat anti–mouse IgM and *p*-nitrophenyl phosphate substrate. Absorbance was read at 405 nm.

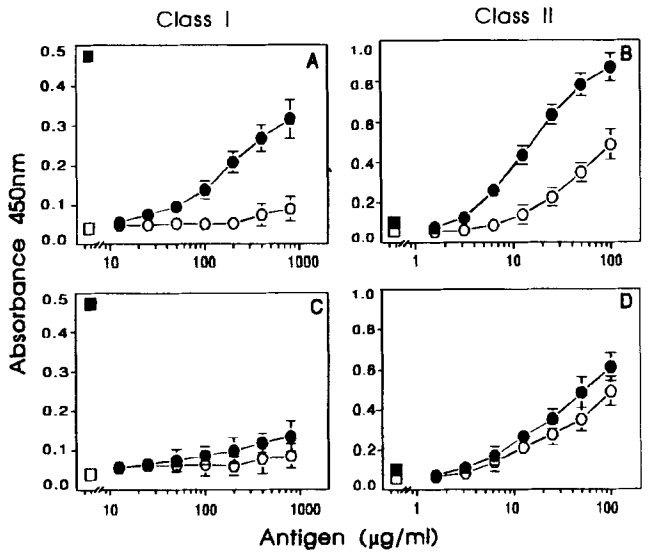

Figure 2. Ag presentation by B cells. In microtiter plates, 5×10^4 of 4B10 (*A* and *C*) or 2D5 (*B* and *D*) were incubated with 10^5 irradiated (10,000 rads) F8.11HL (*A* and *B*) or F8.11 (*C* and *D*) cells in the presence of serially diluted TNP–OVA (●) or native OVA (○). Irradiated EL4 (□) and E.G7–OVA (■), added at 5×10^4/well to T cell hybridomas without exogenous Ag, were used as negative and positive controls for class I–restricted responses and as negative controls for class II–restricted responses. After a 24-h incubation at 37°C, supernatants were tested for IL-2 production by using HT-2 cells in a colorimetric assay. Absorbance was read at 450 nm. The results shown are averages of triplicates ± SD.

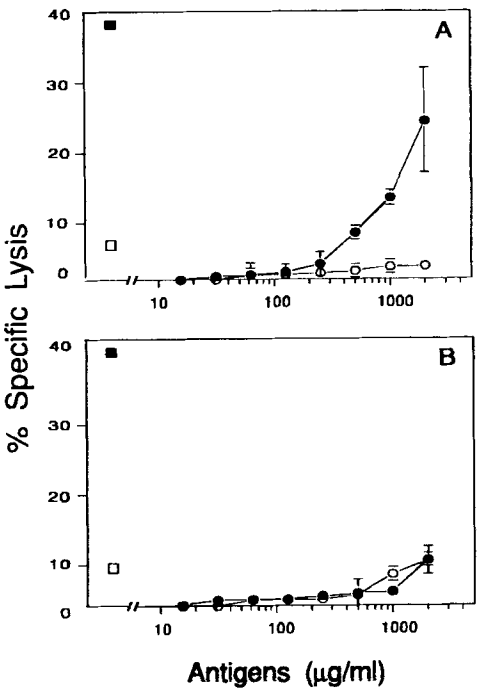

Figure 3. Class I presentation of insulin by B cells. In microtiter plates, 10^4 per well of ^{51}Cr-labeled F8.11HL (*A*) or F8.11 (*B*) targets were first incubated with serially titrated TNP–insulin (●) or native insulin (○) at 37°C for 1 h. Insulin-specific, CD8$^+$ CTLs were then added at an E/T ratio of 10/1. ^{51}Cr-labeled EL4–INS (■) and EL4 (□) targets, added at 10^4 per well to insulin CTLs without exogenous Ag, were used as positive and negative controls for class I–restricted responses. The results shown are the % specific lysis representing averages of triplicates ± SD.

cific Ab (not shown). The transfectant (F8.11HL) that was selected for cell surface expression of anti-TNP Ig was tested for secretion of IgM_{TNP} (sIg). Supernatants were tested for TNP-specific Abs by ELISA. SP6/HL secreted anti-TNP Abs, whereas neither F8.11 nor F8.11HL did so (Fig. 1), indicating that F8.11HL cells produce $mIgM_{TNP}$ but not $sIgM_{TNP}$. Thus, F8.11HL could be used to provide APCs without concern that secreted Ab might interfere with the interpretation of the results.

B Cells Expressing mIg Receptors Process Exogenous Ag via The Class I Pathway. The ability of mIg-bearing B cells to present exogenous Ag via the class I pathway was first tested by using the $OVA_{257-264}$-specific, CD8$^+$, K^b-restricted T-cell hybridoma 4B10. Stimulation of 4B10 by F8.11HL was dose dependent when incubated with TNP–OVA but not native OVA (Fig. 2 *A*), whereas B cells lacking $mIgM_{TNP}$ receptors (F8.11) did not stimulate 4B10 with either OVA or TNP–OVA (Fig. 2 *C*). These data suggest that uptake of TNP–OVA into the class I pathway was mediated by $mIgM_{TNP}$ receptors. For comparison, the ability of F8.11 and F8.11HL to process Ag via the class II pathway was tested with the $OVA_{265-280}$-specific, CD4$^+$, I-A^b–restricted T-cell hybridoma 2D5. As expected, both F8.11HL (Fig. 2 *B*) and F8.11 (Fig. 2 *D*) presented TNP–OVA and OVA to 2D5. As shown by others (10, 19, 24), B cells bearing hapten-specific receptors presented haptenated Ag to CD4$^+$ T cells much more efficiently than unmodified Ag (Fig. 2 *B*). B cells lacking $mIgM_{TNP}$ receptors presented haptenated and unmodified OVA to 2D5 equally well (Fig. 2 *D*). It should be noted that presentation of TNP–OVA by F8.11HL to CD4$^+$ T cells (2D5) was ~100-fold more efficient than presentation to CD8$^+$ T cells (4B10).

We wanted to determine whether presentation of exogenous Ag via the class I pathway by $mIgM_{TNP}$-bearing B cells was unique for OVA or generalizable. Thus, these experiments were repeated with an insulin-specific CTL line (18). As shown in Fig. 3 *A*, F8.11HL cells pulsed with TNP–insulin, but not unmodified insulin, were killed by the CD8$^+$ CTL. F8.11HL cells pulsed with TNP–OVA were not killed by the insulin-specific CTLs (not shown). By contrast, F8.11 cells pulsed with either TNP–insulin or insulin were not killed by these CTLs (Fig. 3 *B*). These results demonstrate that processing via the class I pathway is not unique to OVA. Moreover, these data show that processing via the class I pathway renders B cells susceptible to lysis by CTLs.

Inhibition of Class I or Class II Presentation of Exogenous Ag by B Cells. To verify that $mIgM_{TNP}$ receptors were responsible for class I presentation of TNP–OVA, F8.11HL cells were pretreated with anti-Id mAb. Anti-SP6 inhibited presentation of TNP–OVA to both CD8$^+$ (Fig. 4 *A*) and CD4$^+$ T cells (Fig. 4 *B*). As expected, anti-SP6 did not inhibit presentation of native OVA to CD4$^+$ T cells, since uptake is by endocytosis and not facilitated by mIg recep-

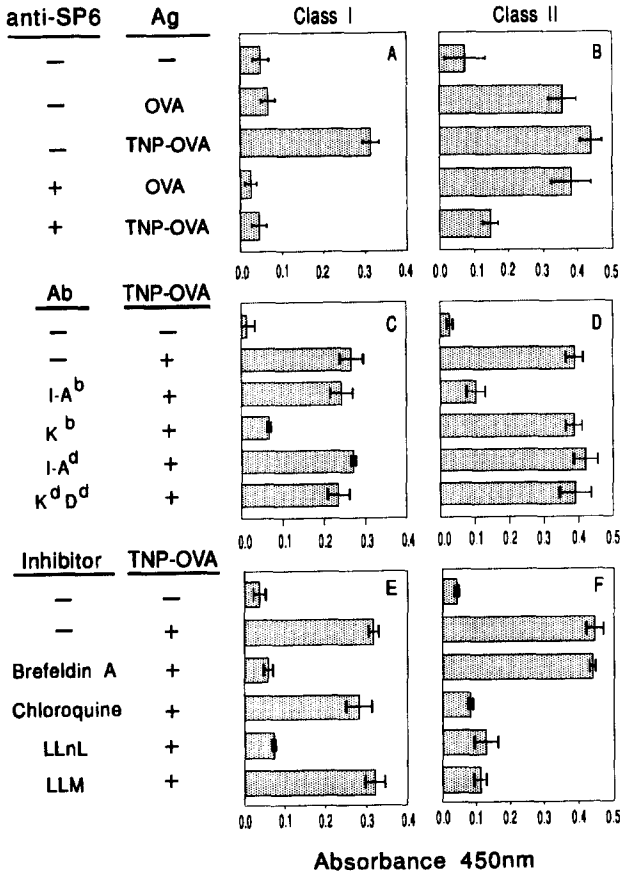

Figure 4. Inhibition of Ag presentation by B cells. In microtiter plates, 10^5 irradiated (10,000 rads) F8.11HL cells per well were either untreated or pretreated with the following: (*A* and *B*) anti-SP6 or (*C* and *D*) anti-MHC Ab at 4°C for 1 h, or (*E* and *F*) inhibitors (1 μg/ml brefeldin A, 12.5 μM chloroquine, 12.5 μM LLM or 12.5 μM LLnL) at 37°C for 1 h. Ag and 5×10^4 T cells per well were then added. On the basis of the titration curves in Fig. 2, TNP–OVA and OVA were used at 200 μg/ml for class I presentation (*A*, *C*, and E). TNP-OVA and OVA were used at 20 μg/ml and 200 μg/ml for class II presentation (*B*, *D*, and *F*), respectively. After a 24-h incubation at 37°C, supernatants were tested for IL-2 production, and the results are shown as the averages of triplicates ± SD.

tors (Fig. 4 *B*). The role of $mIgM_{TNP}$ receptors was also verified by the observation that TNP conjugated to other proteins, such as BSA, competitively inhibited presentation of TNP–OVA to both $CD4^+$ and $CD8^+$ T cells (not shown). Abs specific for MHC class I or II Ags were also used to determine whether stimulation of $CD4^+$ and $CD8^+$ T cells by B cells represented classically defined complexes of MHC and peptides. Presentation of TNP–OVA to $CD8^+$ T cells was inhibited by anti-K^b Abs but not by Ab specific for K^dD^d or class II Ag, both of which are also expressed by F8.11HL (Fig. 4 *C*). However, stimulation of $CD4^+$ T cells was inhibited by anti–$I\text{-}A^b$ Ab but not by Abs specific for $I\text{-}A^d$ or class I Ag (Fig. 4 *D*). These results verify that receptor-mediated endocytosis results in processing of OVA peptides that are complexed with both MHC class I and MHC class II molecules.

Several inhibitors were used to determine whether presentation of TNP–OVA followed the classically defined pathway for class I or class II processing. The class I inhibitor brefeldin A (25) blocked presentation of TNP–OVA by F8.11HL to $CD8^+$ 4B10 (Fig. 4 *E*) but not to $CD4^+$ 2D5 (Fig. 4 *F*). By contrast, weak bases such as chloroquine inhibited presentation of TNP–OVA by F8.11HL to 2D5 (Fig. 4 *F*) but not to 4B10 (Fig. 4 *E*). Recently, Rock et al. showed that class I processing of electroporated OVA was inhibited by peptide aldehyde LLnL but not by LLM, a closely related analogue (26). Although both aldehydes are reported to inhibit lysosomal proteases, several lysosomal inhibitors failed to inhibit the class I pathway (26). Thus, these authors concluded that proteasomal processing generates the peptides presented by MHC class I molecules. Our data show that class I processing of TNP–OVA taken up by receptor-mediated endocytosis was similarly inhibited by LLnL but not by LLM (Fig. 4 *E*), whereas class II processing of TNP–OVA was inhibited by both LLnL and LLM (Fig. 4 *F*). The latter observation is not surprising, given that both aldehydes are potent inhibitors of lysosomal processing (26). Neither LLnL nor LLM inhibited presentation of $OVA_{257\text{-}264}$ or $OVA_{265\text{-}280}$ peptides to $CD8^+$ or $CD4^+$ T cells, respectively. Thus, these inhibitors are not nonspecifically toxic (not shown).

The present study suggests that exogenous Ags taken up by ligand-mediated endocytosis of mIg receptors are shuttled from the endosomal compartment into the cytosol for class I processing. Barnaba et al. reported that hepatitis B envelope (Hbenv)-specific B cells presented exogenous Ag to Hbenv-specific MHC-restricted $CD8^+$ T cells (27). However, normal B cells also presented Hbenv, unlike our findings for OVA. These investigators have shown that the transferrin receptor (TfR) is required for the uptake and processing of Hbenv into the class I and class II pathways by activated human T cells (28). Moreover, uptake via the TfR requires binding of Hbenv to transferrin (29). This pathway of peptide binding by transferrin and subsequent uptake by TfR is probably unique for Hbenv and not a common route for uptake of exogenous Ag by APCs. Nevertheless, these data and our studies provide evidence that uptake of exogenous Ag by receptor-mediated endocytosis supplies peptides to the class I and class II processing pathways.

By contrast to transferrin-mediated uptake, B cells lacking $mIgM_{TNP}$ receptors did not present TNP–OVA to $CD8^+$ T cells, nor did B cells expressing $mIgM_{TNP}$ receptors present unmodified OVA to $CD8^+$ T cells. Similar results were obtained with insulin-specific CTLs, demonstrating that this form of presentation is not limited to OVA or $CD8^+$, MHC class I–restricted T cells specific for OVA. Thus, Ags that have no known mechanisms for gaining access to the cytosol can be processed via the class I pathway if Ags are taken up by receptor-mediated endocytosis. How and where the Ags traverse from the endosomal compartment into the cytosol is unknown, but processing is independent of acidification; otherwise, chloroquine would have inhibited class I presentation. This mode of presentation

may be analogous to the transfer of Ag from phagosomes to the cytosol within macrophages, as demonstrated by other investigators (7, 9).

The physiological consequences of class I processing of exogenous Ag by specific B cells remain to be determined. Many investigators have reported that $CD8^+$ T cells, activated by exogenous Ags, specifically suppress humoral responses and cell-mediated immune responses. This phenomenon has been puzzling, in part, because exogenous Ags were not thought to be processed via the class I pathway. However, macrophages are now known to process exogenous Ag via the class I pathway and Ag bound to inert beads (6) or emulsified in CFA (12) prime MHC class I–restricted $CD8^+$ CTLs. Moreover, $CD8^+$ CTLs have been shown to inhibit responses to exogenous Ag by $CD4^+$ T cells in vitro (30) and Ab responses in vivo (12). Inhibition is likely caused by lysis of APCs. Taken together, our data demonstrate that Ag-specific B cells also can serve as targets for regulation by $CD8^+$ T cells.

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