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Major histocompatibility class I presentation of soluble antigen facilitated by *Mycobacterium tuberculosis* infection

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ABSTRACT **Cell-mediated immune responses are essential for protection against many intracellular pathogens. For *Mycobacterium tuberculosis* (MTB), protection requires the activity of T cells that recognize antigens presented in the context of both major histocompatibility complex (MHC) class II and I molecules. Since MHC class I presentation generally requires antigen to be localized to the cytoplasmic compartment of antigen-presenting cells, it remains unclear how pathogens that reside primarily within endocytic vesicles of infected macrophages, such as MTB, can elicit specific MHC class I-restricted T cells. A mechanism is described for virulent MTB that allows soluble antigens ordinarily unable to enter the cytoplasm, such as ovalbumin, to be presented through the MHC class I pathway to T cells. The mechanism is selective for MHC class I presentation, since MTB infection inhibited MHC class II presentation of ovalbumin. The MHC class I presentation requires the tubercle bacilli to be viable, and it is dependent upon the transporter associated with antigen processing (TAP), which translocates antigenic peptides from the cytoplasm into the endoplasmic reticulum. The process is mimicked by *Listeria monocytogenes* and soluble listeriolysin, a pore-forming hemolysin derived from it, suggesting that virulent MTB may have evolved a comparable mechanism that allows molecules in a vacuolar compartment to enter the cytoplasmic presentation pathway for the generation of protective MHC class I-restricted T cells.**

Tuberculosis remains the largest cause of death from a single infectious disease, with over 7 million new cases and 2.0 million deaths each year (1). The molecular basis for pathogenesis of *Mycobacterium tuberculosis* (MTB) and the immunological mechanisms required for protection remain largely undefined. Many studies in experimental models have demonstrated that cell-mediated immunity is necessary for protection against tuberculosis. Cell transfer experiments have implicated both $CD4^+$ and $CD8^+$ T cells in protection (2), and the lymphokines interferon γ and tumor necrosis factor α appear to be required (3–5). In addition, T cells restricted by major histocompatibility complex (MHC) class I or class I-like molecules are necessary for resistance to MTB infection, as mice deficient in MHC class I expression due to a targeted disruption of the β_2-microglobulin gene are unable to control MTB growth and eventually succumb to infection (6).

Following uptake by macrophages, soluble protein antigens and intracellular bacteria are initially localized in endocytic vesicles, where protein antigens are cleaved to peptides that associate with MHC class II molecules for presentation to $CD4^+$ T cells. The mechanism by which MTB elicits class I-restricted T cells remains unclear, since the bacilli are thought to reside and grow primarily within these membrane-bound vesicles (7, 8), which generally do not generate antigenic peptides for presentation on MHC class I molecules. While there is some controversy as to whether a fraction of viable MTB escape into the cytoplasm of chronically infected macrophages (9, 10), ultrastructural studies are consistent in that early after infection (24–48 hr), all MTB are localized within membrane-bound vesicles (7–9, 11). If the development of vaccines against MTB and the delineation of surrogate endpoints for protection to assess future vaccine trials remain high priorities, then precisely how MTB provides access to the cytoplasmic compartment to engender protective MHC class I-restricted T cells remains a fundamentally important issue to be resolved.

MATERIALS AND METHODS

Bacterial Strains. MTB (strain Erdman; Trudeau Institute, Lake Saranac, NY) was harvested from the spleens of infected C57BL/6 mice and passaged twice in Middlebrook 7H9 broth (Difco) supplemented with glycerol, 0.05% Tween-80 (Sigma), and 10% Middlebrook OADC Enrichment (BBL). Aliquots from logarithmically growing cultures were frozen in 10% glycerol in phosphate-buffered saline (PBS) containing 10% glycerol, and representative vials were thawed and enumerated for viable colony-forming units on Middlebrook 7H10 agar (Difco). Bacille Calmette-Guérin (BCG; strain Pasteur; Trudeau Institute) was grown and frozen as above.

Escherichia coli DH5α (GIBCO/BRL) and hemolytic *Listeria monocytogenes* (strain 10403S; a gift from J. Miller, University of California School of Medicine) were also used in these experiments. The *L. monocytogenes* hemolysin, termed listeriolysin (LLO), is encoded by the *hly* gene. An LLO null mutant was created by allelic exchange of the wild-type *L. monocytogenes hly* locus with a 77% in-frame deletion of *hly*. Amino acids 52 through 460 of the LLO protein were deleted from a plasmid-borne 6.8-kb *Mlu*I–*Sac*I genomic fragment of the *hly* locus by digestion with *Bsa*HI and *Nhe*I followed by blunting with the Klenow fragment of DNA polymerase and ligation. The fusion junction was confirmed by DNA sequencing. An erythromycin resistance gene and a temperature-sensitive origin of replication for *L. monocytogenes* were inserted into the targeting vector containing the *hly* with deletion, and allelic exchange was performed as described (12). Nonhemolytic mutants were selected on blood agar plates, and

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Abbreviations: BMM, bone marrow-derived macrophage; BCG, bacille Calmette-Guérin; CTL, cytotoxic T lymphocytes; ER, endoplasmic reticulum; IL-2, interleukin 2; LLO, listeriolysin; MHC, major histocompatibility complex; moi, multiplicity of infection; MTB, *Mycobacterium tuberculosis*; sOVA, soluble ovalbumin; TAP, transporter associated with antigen processing.

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the deletion of the *hly* gene and loss of LLO were confirmed by Southern and Western blotting, respectively.

Where indicated, bacteria were rendered nonviable by heat killing at 80°C for 30 min or by formalin fixation for 2 min in 10% buffered formalin followed by extensive washes with PBS.

Cell Lines. The C57BL/6-derived murine bone marrow macrophage (BMM) clone BM A3.1A7 has been described (13). The cytotoxic T lymphocyte (CTL) hybridoma RF33.70 secretes interleukin 2 (IL-2) upon recognition of the ovalbumin-derived peptide SIINFEKL when presented in association with the MHC class I K^b molecule (13). MF2.2D9 hybridoma cells secrete IL-2 when ovalbumin is presented by the MHC class II I-A^b molecule (14). All cell lines were maintained at 37°C in 6% CO_2 in air in DME-C, which is Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO/BRL), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM Hepes buffer, 100 units/ml penicillin, and 100 μg/ml streptomycin. Primary BMM were harvested from the femurs of TAP −/− (15) and C57BL/6 (TAP +/+) mice (Jackson Laboratory) and cultured in DME-C containing 30% L929 cell supernatant for at least 7 days prior to use. IL-2-dependent CTLL-2 cells were obtained from the American Type Culture Collection and maintained in DME-C supplemented with recombinant murine IL-2 (Boehringer Mannheim) at 20 units/ml.

Antigen Presentation Assays. The standard assay for presentation of exogenously added antigen consisted of 5×10^4 macrophages and 10^5 RF33.70 or MF2.2D9 hybridoma cells per well of 96-well plates. Soluble hen egg ovalbumin (sOVA; Sigma) or the SIINFEKL peptide was added at the indicated concentrations. For experiments measuring MHC class II-directed presentation of ovalbumin, murine interferon γ (Boehringer Mannheim) was added to the wells at a final concentration of 100 units/ml to enhance processing. In experiments using MTB or BCG, fresh vials were thawed and diluted in tissue culture medium before being added to the wells containing macrophages, T-cell hybridomas, and antigen in a final volume of 200 μl of DME-C. All cultures were incubated for a total of 24 hr at 37°C in 6% CO_2 in air, at which time stimulation of the hybridomas was quantitated by assaying supernatants from each well for the presence of IL-2, using CTLL-2 cells. All data represent incorporation of [*methyl*-^{3}H]thymidine (1 μCi per well; Amersham) as counts per minute (cpm) in proliferating CTLL-2 cells after an 8-hr pulse ± the standard error of duplicate samples.

For experiments using *E. coli* or *L. monocytogenes*, bacteria were harvested from cultures in the logarithmic phase of growth in tryptic soy broth (Difco) buffered with 100 mM Mops (Sigma) and washed with PBS. Bacteria were enumerated spectrophotometrically prior to infection. Input multiplicity of infection (moi) was confirmed by plating serial dilutions on agar. Macrophages were infected with bacteria in the presence of 50 μg/ml sOVA and RF33.70 hybridoma cells in DME-C lacking antibiotics in a volume of 200 μl. After 45 min of incubation at 37°C, gentamicin was added to the wells at a final concentration of 10 μg/ml to inhibit extracellular bacterial growth. Two hours later, additional gentamicin (40 μg/ml), tetracycline (4 μg/ml), penicillin (25 units/ml), and streptomycin (25 μg/ml) were added to inhibit growth of intracellular bacteria. Supernatants were harvested after a total of 24 hr at 37°C and assayed for IL-2 as above.

Soluble LLO was partially purified (16) and diluted in DME-C prior to being added to macrophages and CTL hybridoma cells in the presence of sOVA at the indicated concentrations for 24 hr. Supernatants were then assayed for IL-2.

RESULTS

***M. tuberculosis* Enables Presentation of sOVA Through the MHC Class I Pathway.** Using a model *in vitro* system, we have attempted to determine whether virulent MTB can facilitate entry of a classical soluble antigen, ovalbumin, into the MHC class I processing pathway. Here, we tested for the ability of the ovalbumin-specific CTL hybridoma, RF33.70, to recognize its peptide epitope, SIINFEKL, presented on MHC class I by a murine BMM clone, BM A3.1A7, exposed to sOVA and infected with MTB at various moi values. Under the conditions studied, macrophages exposed to sOVA alone do not present antigenic peptides in association with MHC class I molecules (Fig. 1*A*), and macrophages infected with MTB in the absence of sOVA do not stimulate IL-2 production by the CTL hybridoma (data not shown). However, infection of macrophages with virulent MTB in the presence of sOVA results in recognition of the sOVA epitope by the MHC class I-restricted hybridoma that is dependent on both the dose of sOVA and the moi of the bacteria (Fig. 1*A*). As in previous studies, no MTB were detected by electron microscopy outside of membrane-bound vesicles during the 24-hr period of our assays (data not shown). Nonetheless, within this period, MTB provides access of soluble antigen from the endocytic compartment to the macrophage MHC class I pathway.

To ascertain whether presentation of sOVA through the MHC class I pathway by MTB is dependent on metabolically active bacteria, MTB were rendered nonviable by heat killing or formalin fixation. Bacilli killed by either method lost the ability to facilitate MHC class I-restricted recognition by CTL (Fig. 1*B*), from which we infer that access of soluble antigen to the class I pathway is mediated by a product of viable MTB. A comparison of the relative abilities of the attenuated BCG vaccine and virulent MTB strains to stimulate presentation of sOVA through the MHC class I pathway indicated that although both bacteria were able to do so, the BCG vaccine strain was less effective than MTB (Fig. 1*C*).

It has been documented that macrophages infected with mycobacteria are deficient in their ability to present mycobacteria-derived or exogenously added antigens on MHC class II molecules to $CD4^+$ T cells (17, 18). We therefore sought to determine if virulent MTB, while promoting presentation on MHC class I molecules, affected presentation of MHC class II-directed antigens. At the same time that MTB facilitated presentation of sOVA through the MHC class I pathway of infected macrophages (Fig. 2*A*), MTB infection inhibited MHC class II-directed presentation of sOVA (Fig. 2*B*), indicating that the enhancement of antigen processing was selective for the MHC class I pathway.

TAP Dependence of MHC Class I Presentation of sOVA by MTB-Infected Macrophages. In general, MHC class I-associated peptides are generated by the degradation of cytoplasmic antigens into peptide fragments through the proteolytic activities of the proteosome complex (reviewed in ref. 19). After translocation by TAP, the peptides gain access to nascent MHC class I heavy chains and β_2-microglobulin in the lumen of the ER (19). The heterodimeric transporter encoded by the TAP-1 and TAP-2 genes is localized to the ER membrane, and it translocates cytosolic oligopeptides into the ER in an ATP-dependent manner. Loss of one or both the TAP subunits results in the inability of cells to present cytoplasmic antigens to MHC class I-restricted T cells. Surface expression of MHC class I molecules is also diminished, although empty molecules on the surface are competent to bind exogenously added peptide.

While soluble antigens are generally processed in the endocytic compartment and presented by MHC class II molecules, antigens that are coupled to large, inert matrices, such as iron or latex beads, or soluble antigens taken up by macrophages in the presence of such particulate carriers can also be processed through the MHC class I pathway (20–22). This alternate pathway allows antigens access to the cytoplasm in an intact and functional form for processing into peptides by the proteosome complex, and, like cytoplasmically localized

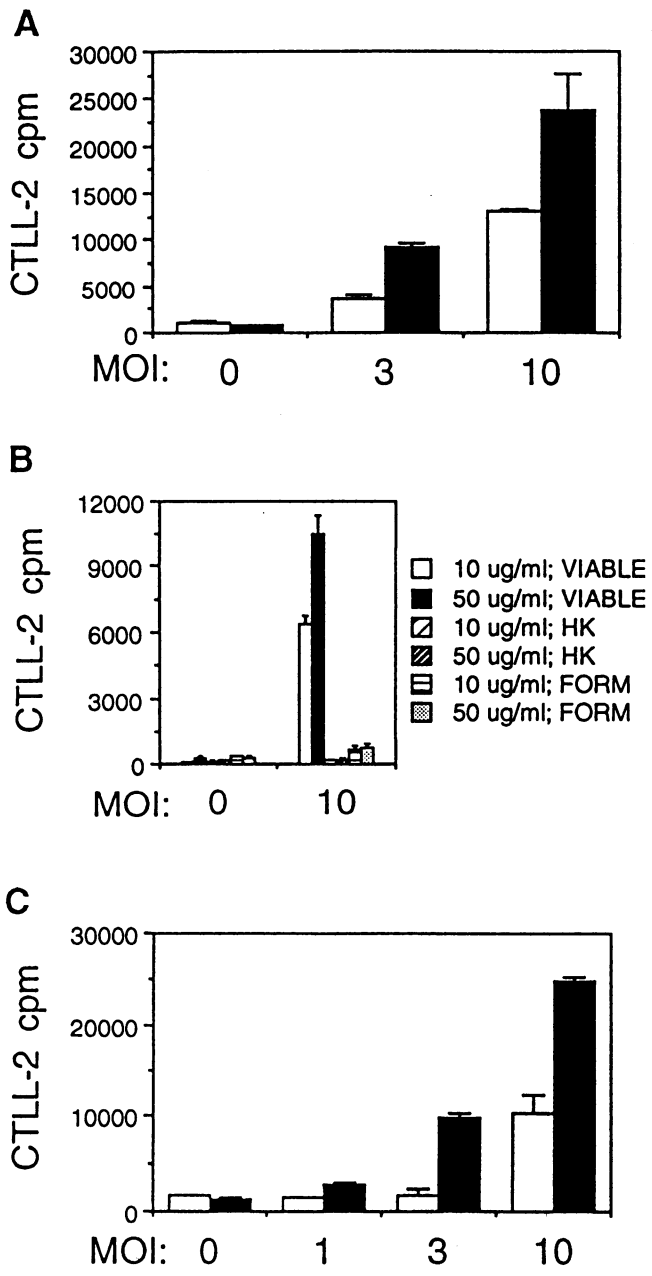

FIG. 1. sOVA is presented to T cells through the MHC class I pathway in macrophages infected with live virulent MTB, but not dead MTB, and less so with attenuated mycobacteria. (*A*) BM A3.17 macrophages were infected with virulent MTB at moi of 0, 3, and 10 in the presence of 10 μg/ml (white bars) and 50 μg/ml (black bars) sOVA and MHC class I-restricted RF33.70 CTL hybridoma cells for 24 hr before supernatants were assayed for the presence of IL-2. (*B*) BM A3.17 macrophages were infected with viable or nonviable MTB in the presence of sOVA (10 μg/ml and 50 μg/ml) and RF33.70 CTL hybridoma cells for 24 hr. MTB were rendered nonviable by heat killing (HK) or formalin fixation (FORM). (*C*) BM A3.17 macrophages were infected with virulent MTB (black bars) or attenuated BCG (white bars) at moi of 0, 1, 3, and 10 in the presence of 50 μg/ml sOVA and RF33.70 hybridoma cells.

antigens, it is dependent on the TAP complex for MHC class I assembly and presentation to CTL (20). This mechanism, however, is unlikely to account for MHC class I presentation stimulated by virulent MTB since only viable bacilli, not dead bacillary particles, are effective in facilitating MHC class I presentation.

It has recently been reported that bacterially derived proteins can be degraded within phagosomes and antigenic peptides can be regurgitated into the medium for exogenous loading onto MHC class I molecules (22, 23). This mechanism bypasses the cytoplasm of the infected cell and can be inferred to be TAP independent. Thus, TAP-deficient mice can provide insight into the cellular pathway of MTB-induced MHC class I presentation. To determine if regurgitation is responsible for the MHC class I presentation stimulated by MTB, primary BMM from wild-type C57BL/6 mice and mice made deficient in the TAP-1 gene by targeted disruption (15) were infected with MTB in the presence of sOVA. TAP-deficient macrophages were totally ineffective at presenting sOVA on MHC class I molecules when infected with MTB (Fig. 3*A*). TAP-deficient cells have been shown to possess fewer surface MHC class I molecules available to bind exogenously added peptide. This was evident when cells were exposed to peptide for short periods of time at low temperatures (24) and during bacteria-induced regurgitation (25). Nevertheless, we have confirmed (25) that upon longer antigen exposures, corresponding more closely to the circumstance of bacteria-infected macrophages *in vivo*, both wild-type and TAP-deficient macrophages were equally competent to load exogenously added SIINFEKL peptide for presentation to the CTL hybridoma (Fig. 3*B*). Further, we confirmed that regurgitation of sOVA peptides released from macrophages infected with *E. coli* does occur and permits exogenous binding to MHC class I for presentation to CTL, but does so in a TAP-independent manner (Fig. 3*C*). Our results indicate that presentation through the class I pathway facilitated by MTB is entirely TAP dependent, therefore excluding peptide regurgitation as the responsible mechanism.

The MTB Effect on MHC Class I Presentation Is Mimicked by *L. monocytogenes* Infection and Soluble LLO. *L. monocytogenes* is a model pathogen that requires cytolytic activity of class I-restricted T cells for clearance and protection. Escape of the bacilli from the phagosome into the cytoplasm is mediated by the primary pore-forming hemolysin, LLO, and processing of cytoplasmically localized antigens proceeds through the class I pathway (26, 27). Loss of LLO function results in the inability of the bacteria to escape the phagosome and the failure to grow within macrophages or elicit protective immunity (27, 28). We therefore compared the ability of wild-type *L. monocytogenes* and an LLO null mutant to present sOVA through the class I pathway. Wild-type *L. monocytogenes* efficiently allows access of sOVA to the class I pathway for recognition by CTL (Fig. 4*A*). The activity is dependent on the presence of the pore-forming hemolysin, as LLO null mutants are totally unable to direct MHC class I presentation of soluble antigen (Fig. 4*A*). Like presentation by virulent MTB, presentation of soluble antigen by *L. monocytogenes* is dependent on TAP (Fig. 4*B*) and is not observed with heat-killed bacteria (data not shown). LLO is thought to create pores in vacuolar membranes, thereby allowing delivery of soluble macromolecules into the cytoplasm. Our data, confirming a previous report (29), indicate that soluble, purified LLO (16) can promote entry of sOVA to the MHC class I pathway in the absence of bacteria (Fig. 4*C*).

DISCUSSION

To develop and evaluate new vaccines against tuberculosis, it is important to understand the immunological mechanisms that are necessary and sufficient for protection. Several lines of evidence in humans suggest that lymphokines and macrophage activation alone are not sufficient to protect against tuberculosis. First, in large-scale clinical trials of BCG vaccines where >85% of recipients converted to tuberculin skin test positivity, which is a measure of the ability to produce lymphokines to mycobacterial antigens, protection has varied from 0% to 80% in different parts of the world (30, 31). Second, in a large vaccination trial against leprosy in Malawi,

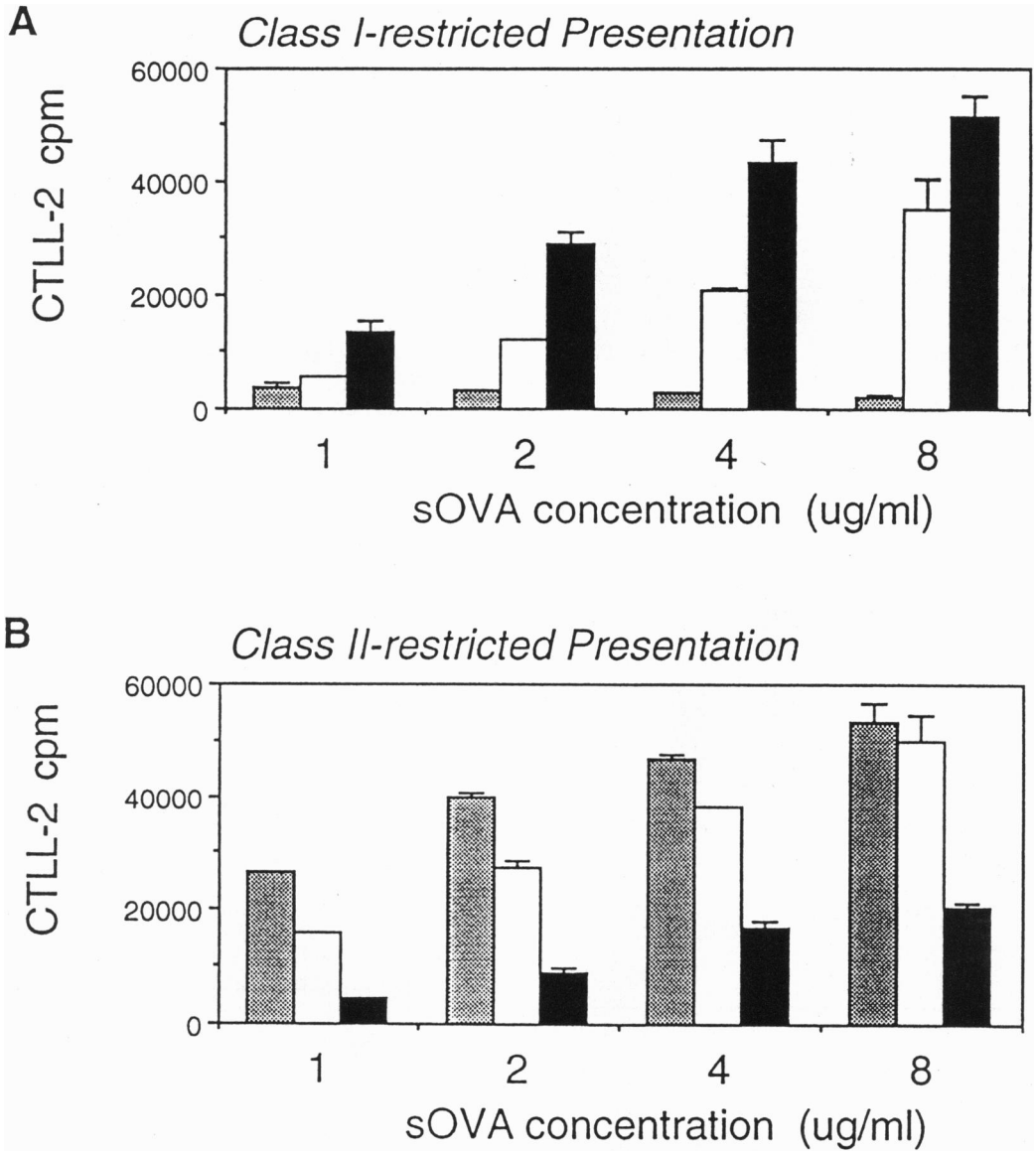

FIG. 2. Virulent MTB stimulates MHC class I-restricted presentation of sOVA while inhibiting MHC class II-restricted presentation. BM A3.1A7 macrophages were infected with virulent MTB at moi of 0 (shaded bars), 3 (white bars), and 10 (black bars) in the presence of sOVA at the indicated concentrations in 96-well plates. MHC class I-restricted presentation of sOVA was assessed by IL-2 production from RF33.70 CTL hybridoma cells (*A*), and MHC class II-restricted presentation of sOVA was assessed by IL-2 production from MF2.2D9 hybridoma cells (*B*).

BCG was found to be 50% protective against leprosy, caused by the related *Mycobacterium leprae*, but not at all protective in the same population against tuberculosis (32). These human data suggest that another mechanism beyond lymphokines and macrophage activation may be necessary for protection against tuberculosis, but perhaps not for leprosy. The studies on transgenic mice unable to present antigens on MHC class I or class I-like molecules suggest that MHC class I-restricted T cells may be a necessary condition for protection as well, although the nature of the protective antigens and the mechanism by which vacuolar antigens can gain access to the cytoplasm have remained problematic. The present work indicates that mycobacteria have evolved a mechanism that provides access of antigens to the cytoplasmic MHC class I pathway. Our data may explain the finding that $CD8^+$ CTL with specificity for mycobacterial antigens can lyse infected macrophages in an MHC class I-restricted manner (33, 34). Additionally, we demonstrate a mechanism by which BCG immunization can elicit MHC class I-restricted CTL (35).

Our experiments indicate that during the first 24 hr of infection, when all tubercle bacilli are found within membrane-bound vesicles of infected macrophages, sOVA can be presented to MHC class I-restricted T cells. Essentially all of the MHC class I presentation of sOVA induced in macrophages by MTB is TAP dependent. This distinguishes it from presentation by macrophages infected with *E. coli*, which is TAP independent and is likely to result from regurgitation of proteolytically cleaved antigenic peptides from the phagosome associating with MHC class I molecules on the cell surface. Our preliminary data on the resistance of TAP-1-null mutant mice to challenge with virulent MTB indicate that they succumb to a fatal infection (R.J.M., E. Figueroa, H.L.P., and B.R.B., unpublished observations). This finding demonstrates that the TAP transporter is essential for protection against virulent MTB infection *in vivo* and that regurgitation of antigenic mycobacterial peptides *in vivo* is not sufficient to engender protective immunity. Together, these results suggest that virulent MTB has evolved a mechanism distinct from that of a particulate, inert matrix, or of bacteria-induced regurgitation of peptides, that allows access of macromolecules from the MTB phagosome to the cytoplasmic compartment. More-

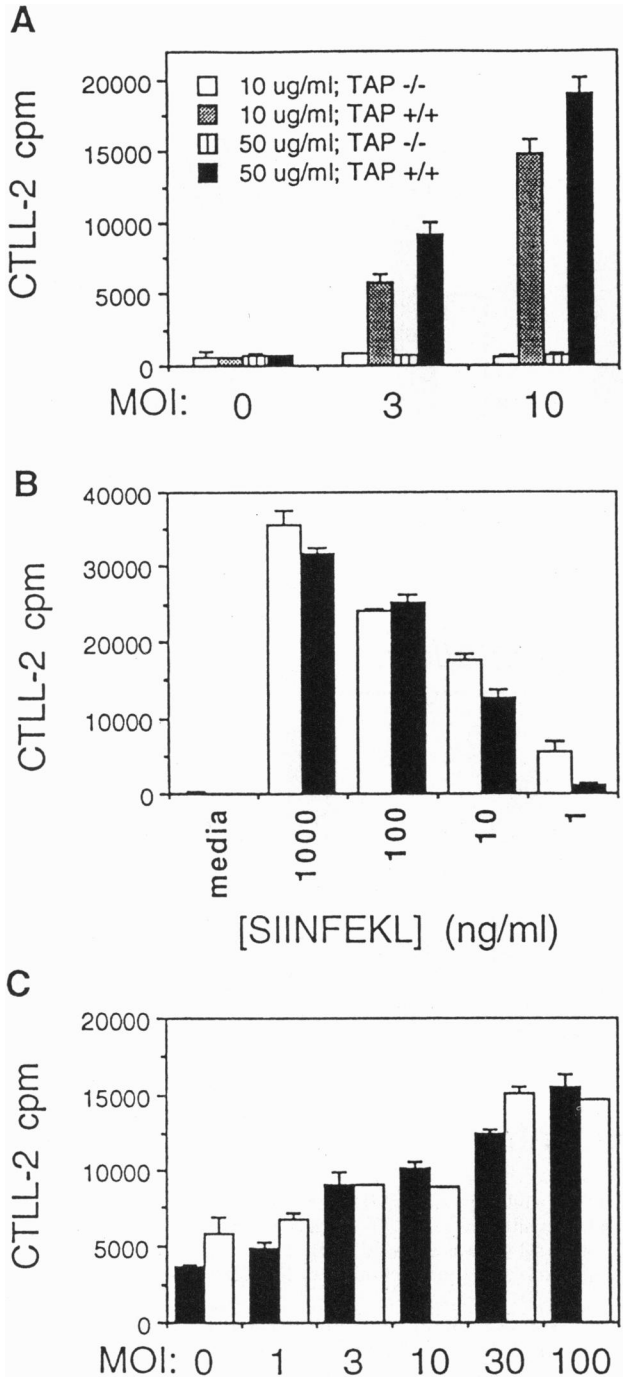

FIG. 3. MTB-mediated MHC class I presentation of soluble antigen is dependent on TAP function and is not the result of regurgitation. (*A*) Primary BMM harvested from TAP-1-deficient mice (TAP −/−) or wild-type C57BL/6 mice (TAP +/+) were infected with MTB at moi of 0, 3, and 10 in the presence of sOVA (10 μg/ml and 50 μg/ml) and MHC class I-restricted RF33.70 hybridoma cells for 24 hr. (*B*) TAP −/− (white bars) and TAP +/+ (black bars) BMM were pulsed continuously with the SIINFEKL peptide at the indicated concentrations in the presence of RF33.70 hybridoma cells for 24 hr. (*C*) TAP −/− (white bars) and TAP +/+ (black bars) BMM were infected with *E. coli* at various moi in the presence of 50 μg/ml sOVA and RF33.70 hybridoma cells in antibiotic-free medium. Antibiotics were added to the wells after 45 min to inhibit extracellular growth and at 2 hr to inhibit intracellular growth. Cultures were incubated for a total of 24 hr.

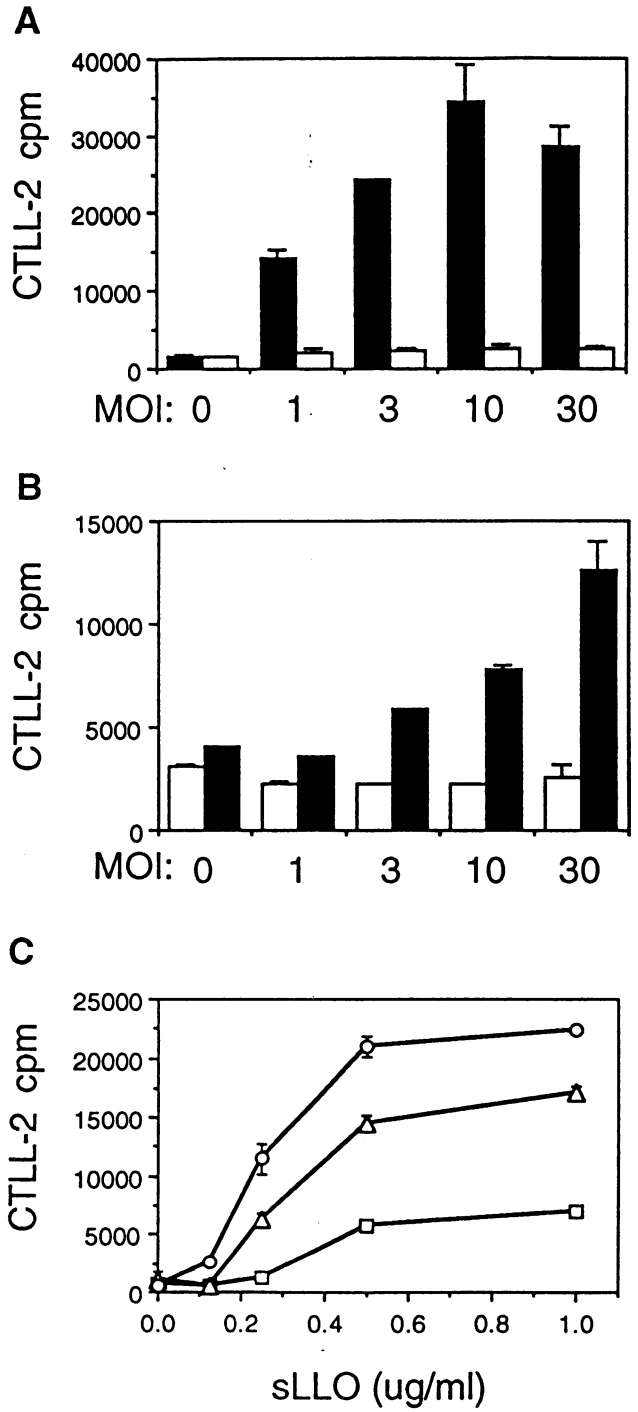

FIG. 4. sOVA presentation through the MHC class I pathway by *L. monocytogenes* is dependent on LLO and TAP and is mimicked by soluble LLO. (*A*) BM A3.1A7 macrophages were infected with wild-type *L. monocytogenes* (black bars) or an LLO-null mutant (white bars) at various mois in the presence of 50 μg/ml sOVA and RF33.70 hybridoma cells in antibiotic-free medium in 96-well plates for 24 hr. Antibiotics were added to the wells to inhibit extracellular and intracellular growth as described in the text. (*B*) TAP −/− (white bars) and TAP +/+ (black bars) primary BMM were infected with wild-type *L. monocytogenes* in the presence of 50 μg/ml sOVA and RF33.70 hybridoma cells in antibiotic-free medium. Antibiotics were added to the wells as in *A*. (*C*) BM A3.1A7 macrophages and RF33.70 hybridoma cells were incubated for 24 hr in the presence of 2 μg/ml (□), 10 μg/ml (△); and 50 μg/ml (○) sOVA and purified soluble LLO (sLLO) at the indicated concentrations (μg/ml).

over, the augmentation of presentation though the MHC class I pathway of infected macrophages was highly selective, in that MHC class II-directed presentation was found to be suppressed by MTB in parallel experiments. It is intriguing to speculate that high bacillary loads may suppress MHC class

II-restricted, $CD4^+$ T-cell responses, which could explain in part the skin test anergy seen in a proportion of tuberculosis patients.

The experiments indicating that only LLO^+, not LLO^-, *L. monocytogenes*, or purified LLO alone, allows presentation of sOVA through the MHC class I pathway suggest the possibility that viable MTB might produce a comparable pore-forming activity that could facilitate entry of sOVA into the cytoplasmic compartment for processing through the MHC class I pathway. It therefore remains important to establish what antigens and products of MTB itself gain access to the MHC class I pathway for presentation to T cells. Although their precise role in protection has not been established, these MHC class I-restricted T cells could contribute through the action of cytolytic mechanisms, cytokine production, or both.

Precisely why intracellular pathogens have acquired mechanisms for accessing the cytoplasm at the cost of generating protective CTL is not altogether clear. For some, such as *L. monocytogenes* (28) and *Shigella flexneri* (36), escape to the host cell cytoplasm is necessary for intracellular survival and cell-to-cell spread. For obligate intracellular parasites, such as *Trypanosoma cruzi* (37) and *Toxoplasma gondii* (38), access to cytoplasmic nutrients is essential for growth. One speculation would be that slow-growing MTB may require certain nutrients in the host cell cytoplasm that can enhance intracellular growth and survival. Alternatively, this process could enable access of toxic mycobacterial products to the cytoplasm, contributing to necrosis and tissue damage, particularly in the lung, which may be crucial for the survival and transmission of the pathogen.

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