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In Vivo Cross-Priming of MHC Class I–Restricted Antigens Requires the TAP Transporter

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Summary

Recent in vitro evidence suggests two alternative mechanisms by which bone marrow–derived APCs may process exogenous antigens for presentation to CTL in vivo, a phenomenon termed cross-priming. Although in vitro studies have suggested that both TAP-dependent and TAP-independent pathways exist, we have now demonstrated an absolute requirement for a functional TAP for cross-priming to occur in vivo. Bone marrow chimeras reconstituted with marrow from TAP-defective donors develop functional $CD8^+$ CTL, but have APCs with disrupted TAP function. In such chimeras, in vivo priming of naive CTL was observed when antigen was targeted to the ER in a TAP-independent fashion, but cross-priming could not be demonstrated. These results support the TAP-dependent mechanism of cross-priming.

Introduction

Induction of cytotoxic T lymphocyte (CTL) responses requires the processing of protein antigens into peptides that bind to major histocompatibility complex (MHC) class I molecules (Townsend and Bodmer, 1989). In vitro, most cell types only present endogenously expressed antigens, utilizing the transporter associated with antigen processing (TAP) to transport peptides generated in the cytoplasm into the endoplasmic reticulum (ER) (Bennink et al., 1993; Bijlmakers and Ploegh, 1993; Deverson et al., 1990; Goldberg and Rock, 1992; Kelly et al., 1992; Monaco et al., 1990; Rammensee et al., 1993; Spies et al., 1992; Trowsdale et al., 1990; Yewdell and Bennink, 1993). In contrast, CTL responses initiated in vivo can also involve processing and presentation of exogenous antigens by host bone marrow–derived antigen-presenting cells (APCs), a phenomenon termed cross-priming (Bevan, 1976a, 1976b; Gooding and Edwards, 1980; Huang et al., 1994).

The primary mechanism by which peptides derived from exogenous antigens combine with MHC class I molecules is currently unresolved. In vitro experiments with purified macrophage populations suggest alternative models that differ in their dependence on the TAP transporter complex. In one model of the TAP-independent pathway, ingested antigens are processed in the phagosome followed by regurgitation of the resulting peptides, which bind to empty MHC class I molecules on the surface of the same or neighboring cells (Harding and Song, 1994; Pfeifer et al., 1993). More recently, a TAP-independent pathway not involving extracellular peptide transfer has been demonstrated (Bachmann et al., 1995). In both models, ingested antigens never gain direct access to the cytoplasm of the APCs. Alternatively, the TAP-dependent pathway involves transfer of antigens from the phagosome to the cytoplasm for processing, followed by transport of resulting peptides into the ER and binding to nascent MHC class I molecules (Kovacsovics-Bankowski et al., 1993; Kovacsovics-Bankowski and Rock, 1995). This mechanism assumes a pathway by which phagocytosed antigens can efficiently enter the cytoplasm from the phagosomal compartment. Such a pathway has been described previously for some intracellular pathogens (Brunt et al., 1990; Campbell, 1994).

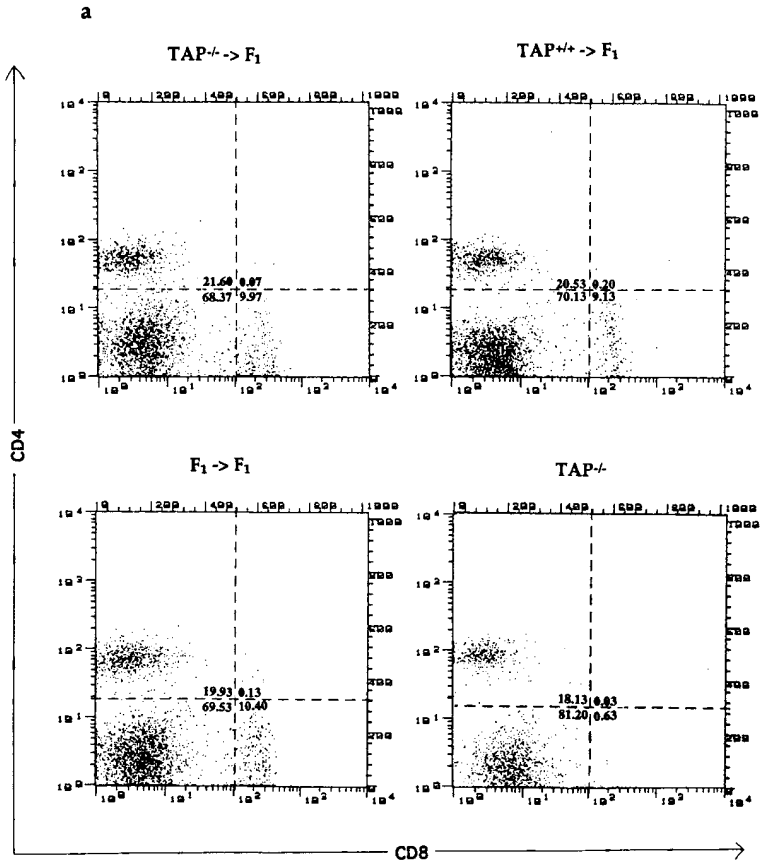
Using a bone marrow chimera system to study initiation of CTL responses in vivo, we demonstrate an absolute requirement for transport of antigenic peptides from the cytoplasm to the ER, even when the antigen originates from outside of the APCs. Mice with TAP-deficient ($TAP^{-/-}$) bone marrow–derived APCs are unable to initiate CTL responses against exogenous antigens, whereas mice with normal APC ($TAP^{+/+}$) generate a strong cross-priming activity in vivo. These results provides definitive in vivo evidence that cross-priming utilizes a phagosome to cytoplasm TAP-dependent pathway for antigen processing.

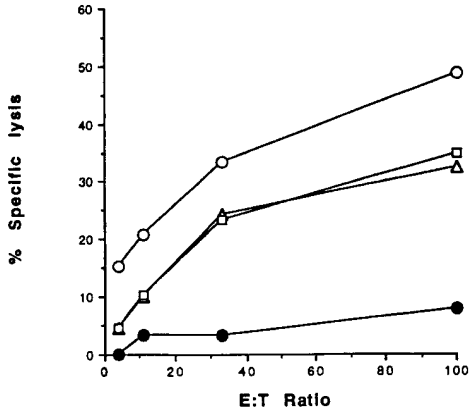
Results

Characterization of the $TAP^{-/-}$ → F1 Bone Marrow Chimera

To study cross-priming phenomenon in vivo, we have utilized a model system in which influenza virus nucleoprotein (NP), which has distinct dominant H-$2K^d$ and H-$2D^b$ restricted epitopes, is expressed in immunizing tumor cells, CT26-NP. We have previously shown that vaccination of parent → F1 chimeras (H-2^b→H-$2^{b\times d}$), in which the MHC haplotype of bone marrow–derived cells does not match the MHC haplotype of the tumor cell (H-2^d), generates CTL with exclusive specificity for NP epitopes restricted to the MHC class I allele present on the bone marrow–derived cells (Huang et al., 1994). Because there is no detectable direct priming of CTL by the tumor in this system, it is well suited to determine whether or not cross-priming utilizes a TAP-dependent pathway.

Two parallel sets of bone marrow chimeric mice were constructed. Lethally irradiated (B6 × BALB/c)F1 (H-$2^{b\times d}$) mice were rescued with bone marrow from either wild-type H-2^b mice ($TAP^{+/+}$) or H-2^b mice expressing a disrupted *TAP1* gene ($TAP^{-/-}$). The bone marrow chimeras were tested 4 months later for complete graft reconstitution using flow cytometric analysis with haplotype-specific anti-MHC class II antibodies (Huang et al., 1994; data not shown). Peripheral T cells were also tested for the presence of both mature $CD4^-CD8^+$ and $CD4^+CD8^-$ T cell subsets (Figure 1a). While the $TAP^{-/-}$ donor mice have markedly reduced numbers of $CD4^-CD8^+$ T cells





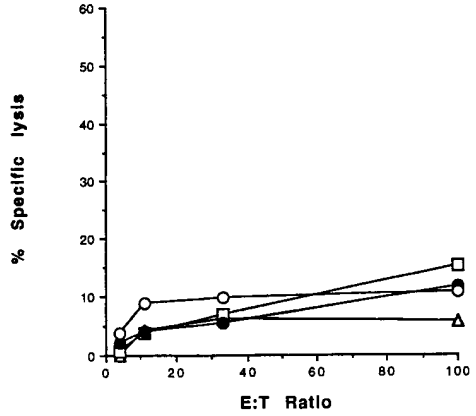


Figure 1. Characterization of TAP$^{-/-}$ (H-2^{b}) → F1 (H-2$^{b\times d}$) Bone Marrow Chimera Mice

(a) Mature peripheral CD4^{+}CD8^{-} and CD4^{-} CD8^{+} cells developed in the TAP$^{-/-}$ bone marrow chimeras (upper left). As controls, splenocytes from the TAP$^{+/+}$ (H-2^{b}) → F1 (H-2$^{b\times d}$) chimera (upper right), the F1 (H-2$^{b\times d}$) → F1 (H-2$^{b\times d}$) chimeras (lower left), and the TAP$^{-/-}$ (H-2^{b}) donor mice were also analyzed. Percent of cells was displayed in each of the four quadrants. X axis: CD8–fluorescein isothiocyanate. Y axis: CD4–phycoerythrin.

(b) Mature splenic T cells in the TAP$^{-/-}$ → F1 chimera mice (open triangle) developed and responded functionally to minor histocompatibility antigens presented on the APCs from the DBA/2 mice. BALB/c (open circle) and TAP$^{+/+}$ (H-2^{b}) → F1 (H-2$^{b\times d}$) (open square) mice also developed CTL responses against ConA blasts from DBA/2 mice after immunization with DBA/2 splenocytes. As a negative control, DBA/2 (closed circle) mice failed to generate CTL against self-antigens when immunized with syngeneic APC.

(c) None of the immunized mice in (b) developed CTL activity against ConA blasts from BALB/c mice. The experiments were repeated twice with similar results.

in the periphery, both TAP$^{-/-}$ → F1 and TAP$^{+/+}$ → F1 chimeras have normal numbers and ratios of peripheral mature T cells similar to that of the control F1 → F1 mice. The presence of normal numbers of CD4^{-}CD8^{+} T cells in the chimeras suggests that positive selection occurs normally on the TAP$^{+/+}$ thymic epithelium of the recipient.

T cells from the TAP$^{-/-}$ → F1 chimeric mice were next characterized functionally for their ability to respond to minor histocompatibility antigens in vivo after immunization with DBA/2 (H-2^{d}) splenocytes. Professional APCs in the immunizing splenocyte population are capable of directly priming host T cells to minor antigens, enabling an assessment of their function independent of the endogenous APC function of the chimeras. Both TAP$^{+/+}$ → F1 and TAP$^{-/-}$→ F1 bone marrow chimeras developed CTL responses against DBA/2 minor antigens that were similar to those generated by BALB/c mice (Figure 1b). The CTL responses were specific to the DBA/2 minor antigens, since no CTL activities can be detected against syngeneic BALB/c minor antigens (Figure 1c). Thus, T cell development in the TAP$^{-/-}$ → F1 chimera seemed to be both phenotypically and functionally similar to that seen in the control TAP$^{+/+}$ → F1 chimera.

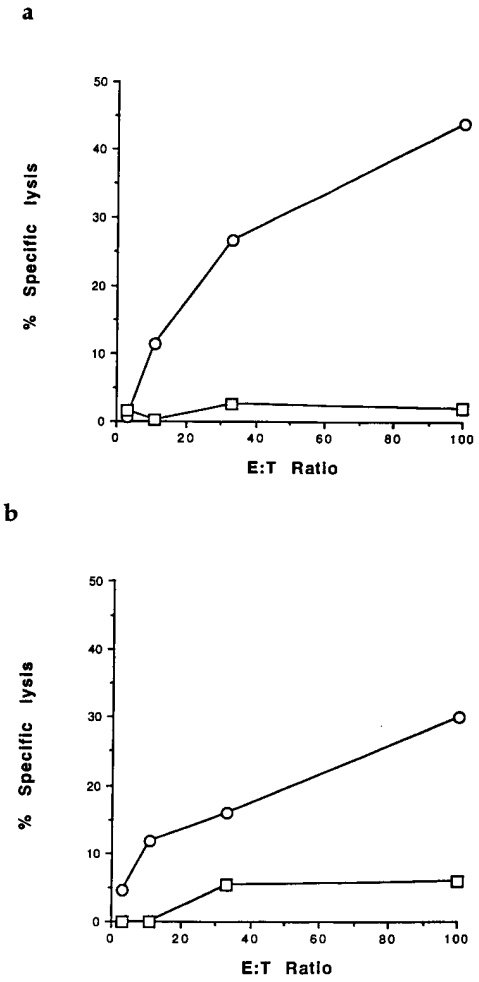


Figure 2. TAP$^{-/-}$ → F1 Chimeras Fail to Generate H-2^b-Restricted NP-Specific CTL after Immunization with CT26-NP

Both TAP$^{-/-}$ → F1 (open square) and TAP$^{+/+}$ → F1 (open circle) chimeras were immunized with irradiated CT26-NP either once (a) or twice 2 weeks apart (b). Splenocytes were analyzed in vitro for the presence of NP-specific CTL by lysis against NP–peptide pulsed surrogate (H-2^b) target cells. Lysis of target cells pulsed with an inappropriate peptide (NP 147–155) was less than 5%.

In Vivo Cross-Priming of NP-Specific CTL Does Not Occur in the TAP$^{-/-}$ → F1 Bone Marrow Chimera

The TAP$^{-/-}$ → F1 and TAP$^{+/+}$ → F1 chimeras were next immunized with irradiated colorectal tumor, CT26-NP (H-2^d), expressing the influenza virus NP antigen (Figure 2). As we found previously (Huang et al., 1994), cross-priming occurred readily in the TAP$^{+/+}$ → F1 mice, which developed a strong NP-specific H-2D^b-restricted CTL response after one (Figure 2a, circle) or multiple rounds (Figure 2b, circle) of immunizations. None of the immunized chimeric mice developed a H-2K^d-restricted NP-specific CTL response (data not shown), reconfirming that the immunizing tumor cells, CT26-NP, cannot directly prime naive CTL in vivo. In contrast with the cross-priming demonstrated in the TAP$^{+/+}$→F1 mice, no NP-specific H-2D^b-restricted CTL were detected in the chimeras reconstituted with TAP$^{-/-}$ bone marrow, reflecting an absence of cross-priming in vivo (Figure 2a, square). The lack of NP-specific CTL persisted even after a secondary boosting in vivo (Figure 2b, square).

NP-Specific CTL Can Be Primed in the TAP$^{-/-}$ → F1 Chimera Immunized with Targeted Vaccinia Minigene Constructs

While these results suggest that in vivo cross-priming is a TAP-dependent process, it is also possible that the absent NP 366–374 + D^b response was due to a repertoire defect within the CD8 T cell subset of the TAP$^{-/-}$ → F1 chimera related to abnormal T cell development. Although their response to minor histocompatibility antigens appeared normal (see Figure 1b), subtle repertoire perturbations might specifically affect CD8 responses to NP 366–374 + D^b. Another possibility is that the APCs derived from TAP$^{-/-}$ bone marrow progenitors have a partial defect in providing costimulatory signals to naive T cells.

To address these possibilities, we utilized a vaccine approach that should introduce NP 366–374 peptide into the ER of APCs through a TAP-independent pathway. TAP$^{-/-}$ → F1 chimeras were immunized with a recombinant vaccinia virus expressing a minigene encoding NP 366–374 linked to a ER signal sequence (Vac–sig-NP 366–374) (Minev et al., 1994; Restifo et al., 1995). This peptide can use the signal-recognition particle complex to translocate to the ER followed by cleavage of the signal sequence. Figure 3 shows that TAP$^{-/-}$ → F1 chimeras generated strong NP 366–374 + D^b CTL responses after immunization with Vac–sig-NP 366–374 (circle) but not with Vac–NP 366–374 (which expresses NP 366–374 without a signal sequence) (square) or Vac–NP (encoding the full-length NP protein) (triangle), whereas TAP$^{+/+}$ → F1 chimeras developed NP 366–374 + D^b CTL responses to all three recombinant vaccinia constructs. These results indicate that the TAP$^{-/-}$ → F1 chimeras are perfectly capable of responding to this antigenic peptide, NP 366–374, as long as it can get into the ER.

The TAP dependence of cross-priming seen in this chimera system may reflect inefficient exogenous loading of MHC class I molecules on TAP$^{-/-}$ APCs compared with normal APCs. To address this possibility, we compared the ability of TAP$^{-/-}$ versus TAP$^{+/+}$ splenocytes to activate NP-specific CTLs in vitro. CTL from influenza-primed mice were used to determine the NP peptide dose response curve (Figure 4a). Subsequently, two subsaturating concentrations of NP peptide were used to compare the relative efficiency of peptide loading for the two APC populations. An equivalent level of NP-specific CTL activation was observed when stimulated by either TAP$^{-/-}$ or TAP$^{+/+}$ splenocytes pulsed with either 1 μM (Figure 4b) or 10 μM (data not shown) NP 366–374 peptide for as little as 5 min, suggesting that differences in the ability to load MHC class I on two APC populations are not likely to account for the TAP dependence of cross-priming seen in the chimera system.

Discussion

Taken together, our experiments support a TAP-dependent pathway for in vivo cross-priming. As noted, in vitro

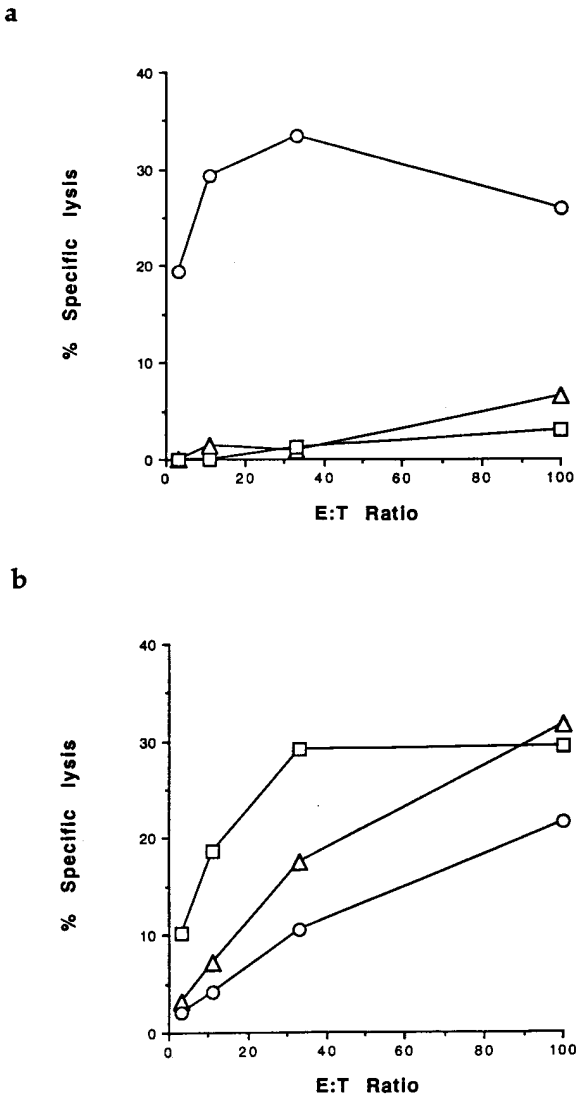


Figure 3. TAP$^{-/-}$ → F1 Chimeric Mice Have the Potential of Mounting an NP-Specific H-2D^{b}-Restricted CTL Response

(a) The chimeric mice were infected with recombinant vaccinia virus encoding the whole NP protein (Vac–NP, open triangle), the minigene encoding the nonameric NP 366–374 sequence (Vac–NP 366–374, open square), or the minigene downstream of an ER insertional sequence (Vac–sig-NP 366–374, open circle). Splenocytes from these chimeras were analyzed for their ability to lyse a MC57G (H-2^{b}) pulsed with the synthetic NP 366–374 peptide.

(b) As a control, TAP$^{+/+}$ → F1 chimeras were also infected with the same recombinant viruses. This experiment has been repeated three times with similar results. Lysis of target cells pulsed with NP 147–155 was less than 5% in all cases.

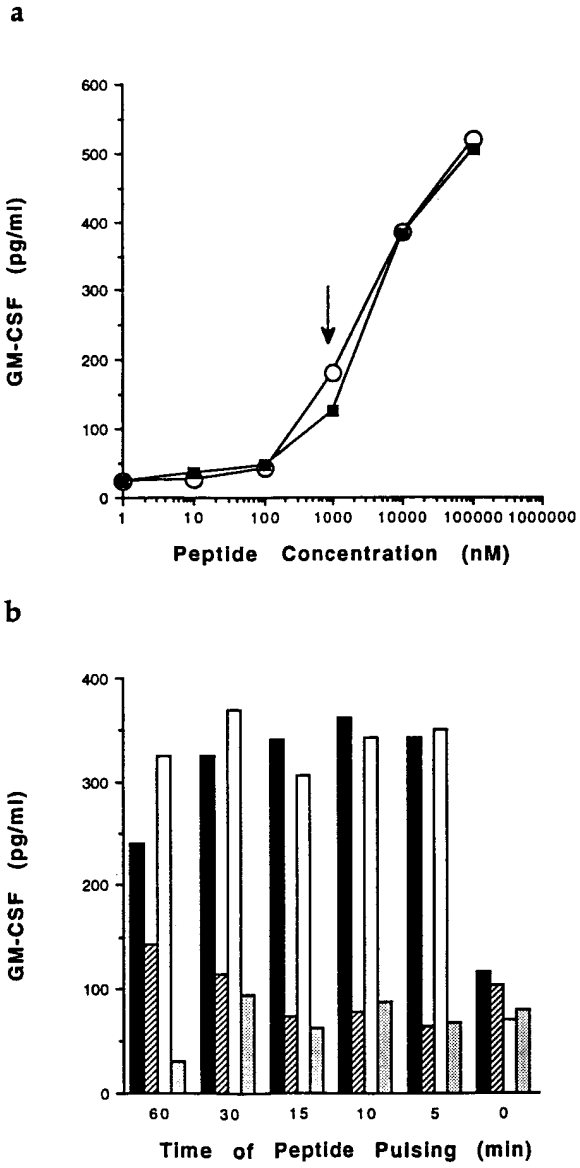


Figure 4. TAP$^{-/-}$ APCs Can Efficiently Capture and Present NP 366–374 to CTLs In Vitro

(a) Splenocytes from TAP$^{-/-}$ and TAP$^{+/+}$ mice were pulsed with various concentrations of NP 366–374 peptide at 37°C for 1 hr. We analyzed 24 hour supernatants for GM-CSF after incubating the peptide-pulsed splenocytes with CTLs specific for the NP 366–374 peptide.

(b) TAP$^{-/-}$ splenocytes were pulsed at 37°C at various time intervals with a subsaturating amount (1 μM, arrow in [a]) of NP 366–374 peptide (solid bars) or NP 147–155 peptide (hatched bars). As a comparison, TAP$^{+/+}$ APCs were also pulsed with either NP 366–374 peptide (open bars) or NP 147–155 peptide (dotted bars). The pulsed APCs were washed after the indicated time interval and mixed with NP 366–374-specific CTLs for 24 hr. The supernatants were assayed for GM-CSF in an ELISA asay.

studies have yielded conflicting results that have led to alternate models to account for how exogenous antigen gains access to class I MHC. It has been proposed that antigen can be regurgitated onto the APC surface from the phagosome, delivered to a specialized endosomal compartment where both class II and class I molecules are loaded, or transported from the endosomal compartment to the cytoplasmic space for further processing. While these studies demonstrate that each of these pathways may be operative under certain experimental conditions, it is difficult to predict which of these conditions reflect those that exist in vivo. In spite of the alternate routes of antigen trafficking demonstrated in vitro, we have shown a strict dependence of in vivo cross-priming on APCs with a functional TAP transporter.

Several important factors are likely to influence cross-priming in vivo, including the local antigen density, the form in which antigen is encountered for processing, the migration of APCs to the appropriate lymphoid microenvironment, and the type(s) of APCs involved. Rock and colleagues (Kovacsovics-Bankowski and Rock, 1994; Rock et al., 1993) have argued that such a pathway may only be operative in a subset of phagocytic macrophages. In contrast, nonmacrophage APCs have recently been shown to have the capacity to process antigens in this fashion in vitro (Reis e Sousa and Germain, 1995). In the present study, the outcome is not biased by assumptions relating to the relevant APC population mediating cross-priming in vivo.

It could be argued that this model system lacks the sensitivity to detect exogenous peptide loading. We feel this is unlikely due to the fact that splenocytes from $TAP^{-/-}$ mice, just as with TAP^{-} cell lines, express very low levels of peptide-filled MHC class I molecules (as measured by a conformation-dependent antibody). (Van Kaer et al., 1992; data not shown). Despite their reduced MHC class I molecule expression at the cell surface, TAP^{-} cells required 100-fold less peptide epitope than the wild-type cell to sensitize them for lysis by CTLs (Cerundolo et al., 1990). Furthermore, we have shown that when peptide was added to $TAP^{-/-}$ APCs in vitro at a concentration where no increase in the level of stable class I molecules was detected by flow cytometry, $TAP^{-/-}$ APCs were still able to present peptides to CTLs. While the in vitro peptide loading experiments were performed at 37°C, and resulted in equivalent levels of CTL activation by the two APC populations exposed to peptide for as little as 5 min, we recognize that this experiment cannot precisely mimick the conditions encountered in vivo, and cannot formally rule out a subtle bias against exogenous loading. Nevertheless, we feel it is likely that, if the regurgitation pathway of MHC class I presentation were operative in vivo, $TAP^{-/-}$ bone marrow–derived APCs should be capable of efficiently capturing and presenting the NP 366–374 epitope.

While the TAP molecule was clearly involved in the processing of the exogenous NP antigens in our system, it is certainly possible that the TAP-independent peptide regurgitation pathway defined by Pfeifer and colleagues (Pfeifer et al., 1993) in vitro may be operative for some antigens in vivo. However, if peptides generated in the phagosome do not perfectly fit the MHC class I–binding groove, it is not clear that the cell membrane has the peptide trimming apparatus that exists in the ER (Snyder et al., 1994) to modify peptides for effective binding to the empty MHC class I molecules on the surface of the APCs.

Because TAP localizes exclusively to the ER membrane and transports peptides from the cytoplasm to the ER (Kelly et al., 1992; Kleijmeer et al., 1992; Shepherd et al., 1993; Spies et al., 1992; Van Kaer et al., 1992), the TAP dependence of cross-priming implies that phagocytosed antigens must somehow be released from the phagosome into the cytoplasm. Transfer of phagocytosed antigens to the cytoplasm can either occur via a specialized protein transporter or by disruption of the phagosome. Precedent for the later mechanism can be found in certain intracellular bacteria, such as Listeria monocytogenes, which transfer from phagosome to cytoplasm by secreting a chaotropic phagosome destabilizing protein (Brunt et al., 1990; Campbell, 1994). In addition, recent morphological evidence in vitro has shown that soluble proteins, such as ovalbumin, can be taken up by bone marrow macrophages via macropinocytosis and gain access to the cytosol for conventional MHC class I antigen processing pathway (Norbury et al., 1995).

Bevan and others (Bevan, 1987; Huang et al., 1994; Rock et al., 1993) have proposed that the necessity for cross-priming stems from the requirement that bone marrow–derived APCs, which express appropriate costimulatory molecules and migrate to the appropriate lymphoid microenvironment, present MHC class I–restricted antigens in order to initiate CD8 responses. Thus, if an antigen is expressed exclusively in non-APCs (such as tumor antigens in an epithelial tumor or a virus with epithelial tropism), it must be transferred to a bone marrow–derived cell for MHC class I processing. Recent evidence suggests that a specialized peptide-carrier, gp96, may be capable of mediating such transfer (Arnold et al., 1995; Suto and Srivastava, 1995). However, how such proteins deliver peptide antigens into the cytoplasm of the APCs is currently unknown. That the process of cross-priming also requires TAP, as shown in the current study, suggests that the immune system processes all MHC class I antigens through a common final pathway.

Experimental Procedures

Mice

F1 (BALB/c × B6, H-$2^{d\times b}$), $TAP^{+/+}$ (B6, H-2^{b}), BALB/c (H-2^{d}), and DBA/2 (H-2^{d}) mice were obtained from the National Institutes of Health (Frederick, Maryland). The $TAP^{-/-}$ (H-2^{b}) donor mice were gifts of Dr. L. Van Kaer (Nashville, Tennessee). The $TAP^{-/-}$ donor mice were on a B6x.129 background.

Cell Line

The CT26-NP line was produced by infection of CT26, a colorectal tumor line derived from BALB/c mice (H-2^{d}), with a Moloney-based defective recombinant retrovirus containing both the *NP* gene from the PR8 influenza strain and a neomycin-resistance gene, as described previously (Huang et al., 1994). The expression of *NP* by the CT26-NP was confirmed by intracellular staining with anti-NP monoclonal antibody (MAb) (HB65) and by in vitro lysis by H-$2K^{d}$-restricted NP-specific CTL.

Construction of the Bone Marrow Chimeras

The $TAP^{-/-} \rightarrow$ F1, $TAP^{+/+} \rightarrow$ F1, and F1 → F1 bone marrow chimeras were constructed as described previously (Huang et al., 1994). From each of the constructs one mouse was sacrificed 4 months after the bone marrow reconstitution. Splenocytes were stained for MHC class II molecules I-E^{d} and I-A^{b} with MAb 14.4.4 (IgG2a) and MAb Y-3P (IgG2a), respectively, to confirm complete reconstitution with donor bone marrow (data not shown). The splenocytes were also stained with MAb 2.43 (anti-CD8) directly conjugated to fluorescein isothiocyanate (X axis) and MAb GK1.5 (anti-CD4) conjugated to phycoerythrin (Y axis) to access the relative levels of peripheral $CD4^{+}$ and $CD8^{+}$ T cell subtypes.

Immunization of Bone-Marrow Chimeras with Minor Histocompatibility Antigens

$TAP^{-/-} \rightarrow$ F1 chimera, $TAP^{+/+} \rightarrow$ F1 chimera, BALB/c, and DBA/2 mice were immunized with 1.5×10^{7} live DBA/2 splenocytes intraperitoneally. The recipient mice were sacrificed 2 weeks later, and

the splenocytes from different responder mice were plated in 24-well plates in the presence of irradiated (30 Gy) DBA/2 splenocytes. Various dilution of the responders were analyzed for lysis 5 days later against ^{51}Cr-labeled DBA/2 and BALB/c concanavalin A (ConA) blasts (5 μg/ml, Calbiochem, La Jolla, California). Percent specific lysis = 100% × (total release − spontaneous release) / (maximum release − spontaneous release).

Synthetic NP Nonameric Peptides

The immunodominant nonameric H-2K^d-restricted NP 147–155 peptide (TYQRTRALV) and H-2D^b-restricted NP 366–374 peptide (ASNENMETM) were synthesized by the Protein/Peptide/DNA Facility at the Johns Hopkins University School of Medicine. The sequences were confirmed by amino acid analysis and mass spectrometry.

In Vivo Cross-Priming Experiment

CT26-NP cells (1×10^6) were irradiated (50 Gy) and injected subcutaneously in the left flank of the chimeras. After 2 weeks, one group of mice were sacrificed for analysis (one immunization), while the other group from each chimera line was reimmunized with a boost of irradiated (50 Gy) 1×10^6 CT26-NP cells (two immunizations). Spleens were removed from the mice 2 weeks after the final immunization, and splenocytes were cultured in vitro with either NP 147–154 peptide or NP 366–374 peptide in the presence of interleukin-2 (IL-2) and splenocytes from naive F1 mice. After a 7 day in vitro incubation, the splenocytes were harvested and plated in triplicate on a 96-well V-bottomed microtiter plate at various effector-to-target ratios. Surrogate target cells, P815 (H-2^d) (data not shown) and MC57G (H-2^b), were labeled with ^{51}Cr and added to the effector cells (3000 cells/well) in the presence of synthetic NP 147–155 peptide or NP 366–374 peptide (50 pg/ml). After a 4 hour incubation of the cell mixture at 37°C and in 5% CO_2, the supernatants were harvested and counted on a γ counter as described above.

Recombinant Virus

Recombinant vaccinia viruses containing a minigene encoding the nonameric peptide, NP 366–374, with or without an upstream ER insertional sequence of the adenovirus E6 protein (Vac–sig-NP 366–374 and Vac–NP 366–374, respectively) were gifts of Dr. N. Restifo (Bethesda, Maryland). A similar vaccinia virus was obtained that contains the gene encoding the entire NP protein of the PR8 strain (Vac–NP) (Huang et al., 1994).

In Vivo Virus Infection

TAP$^{-/-}$ → F1 chimeric mice were infected intraperitoneally with 1.5×10^7 pfu/mouse of various vaccinia recombinants. Splenocytes from these infected mice were restimulated in vitro 2–4 weeks later with 1 μg/ml of either synthetic NP 366–374 (H-2D^b) or NP 147–155 (H-2K^d) peptides in the presence of F1 splenocytes and 4 U/ml of IL-2. The responders were tested 6 days later for their ability to lyse ^{51}Cr-labeled MC57G (H-2^b) or P815 (H-2^d) surrogate targets pulsed with either synthetic peptides. The percent of peptide specific lysis was calculated as described above. Only the experiment with MC57G (H-2^b) targets is shown.

In Vitro Peptide Reconstitution and Cytokine Release Assay

Splenocytes from both TAP$^{-/-}$ and TAP$^{+/+}$ donor mice were removed and immediately pulsed either with 100 μM to 1 nM of NP 366–374 for 1 hr at 37°C (Figure 4a), or with 1 μM (Figure 4b) or 10 μM (data not shown) of NP 366–374 or NP 147–155 peptides for 60, 30, 15, 10, or 5 min. The peptide-pulsed APCs were washed three times in Hank's balanced salt solution and plated at 3×10^4 cells / well in a 96-well round-bottomed plate. CTLs specific for NP 366–374, generated by infecting F1 mice with a sublethal dose of PR8 influenza virus and stimulating the splenocytes in vitro with NP 366–374 peptide, were added to the 96-well plate at 1×10^5 cells / well. We collected and analyzed 24 hour supernatant samples for the amount of murine GM-CSF in an ELISA assay. The GM-CSF levels in wells containing T cells alone or APCs alone were below 50 pg/ml.

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