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TAP1-independent loading of class I molecules by exogenous viral proteins

Presentation of peptides derived from endogenous proteins on class I molecules needs functional TAP peptide transporters. To reveal whether class I-associated presentation of exogenous proteins also required the presence of TAP transporters, we assessed *in vitro* the ability of spleen cells and macrophages from TAP1-deficient mice (TAP1−/−) to present peptides derived from exogenous recombinant viral proteins on their class I molecules. We found that recombinant glyco- and nucleoprotein from lymphocytic choriomeningitis virus and nucleoprotein of vesicular stomatitis virus were presented as efficiently by TAP1−/− cells as by control cells. Peptide regurgitation was not involved. Since particulate, non-replicating antigens can efficiently prime anti-viral cytotoxic T cells *in vivo*, this new, TAP-independent pathway of class I-associated antigen presentation may be applicable for vaccine strategies.

1 Introduction

Peptides derived from endogenous antigens are usually presented on class I molecules, whereas exogenous proteins are degraded in endosomes and are presented on class II molecules. However, it has become increasingly evident that this class discrimination by antigen-presenting cells (APC) [1] is not absolute: endogenous antigens can be presented in association with class II molecules and exogenous antigens can reach the class I pathway [2–8]. Endogenous proteins are thought to be degraded in the cytosol by proteasome complexes and to be transported into the endoplasmic reticulum (ER) by the TAP transporter molecules. Mutant cell lines deficient for TAP molecules virtually lack surface expression of class I molecules, since peptides are necessary to stabilize the class I/β_2 microglobulin heterodimer in the ER [9–11]. Most, though not all [12–15] endogenously produced antigens cannot be presented in association with class I on these cells. Furthermore, gene-targeted mice lacking TAP1 expression [16] and humans expressing mutant TAP2 molecules [17] have drastically reduced class I expression and virtually no $CD8^+$ T cells which need class I molecules for positive selection [18].

It has recently been shown that recombinant viral proteins such as the glycoprotein (GP) and nucleoprotein (NP) of LCMV and the nucleoprotein of vesicular stomatitis virus (VSVN) can efficiently induce cytotoxic T cell responses *in vivo* [7, 8]. Therefore, these proteins represent an ideal tool to reveal whether class I loading by exogenous proteins requires the presence of TAP. We found that these three different exogenous viral proteins were presented in association with class I as efficiently in the absence as in the presence of TAP by professional antigen presenting cells, but not by EL-4 thymoma cells. This new pathway of antigen presentation is apparently efficient as suggested by the induction of antiviral CTL *in vivo*.

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Abbreviation: **VSV:** Vesicular stomatitis virus

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2 Materials and methods

2.1 Mice

TAP1−/− mice have been described in detail elsewhere [16]. C57BL/6 control mice were obtained from the breeding colony of the Institut für Zuchthygiene, Zürich. Transgenic mice expressing the P14 TCR have been described previously [19]. Mice exhibiting a mutation in their D^b molecule (B6.C-H-2^{bm13}) were originally kindly provided by Dr. C. J. Melief; Academisch Ziekenhus, Leiden.

2.2 Viruses

Lymphocytic choriomeningitis virus isolate WE (LCMV-WE) was originally obtained from Dr. F. Lehmann-Grube, Hamburg [20]. VSV Indiana (VSV-IND) (Mudd-Summers isolate) seeds had been originally obtained from Dr. D. Kolakofsky, University of Geneva, and were grown on BHK 21 cells infected with low multiplicity of infection and plaqued on Vero cells. Recombinant baculovirus expressing the NP or GP of LCMV or the N of VSV have been described [21, 22]. All recombinant baculoviruses were derived from nuclear polyhedrosis virus and were grown at 28 °C in *Spodoptera frugiperda* cells in spinner cultures in TC-100 medium [21]. All recombinant baculoviruses were generously provided by Dr. D. Bishop, Institute of Virology and Environmental Microbiology, GB. Recombinant protein was produced as described [21, 22] and used at 10 μg/ml for spleen cell and macrophage loading.

2.3 Restimulation of CTL

CTL restimulation and ^{51}Cr release: spleen cells from LCMV-infected mice were restimulated for 5 days in the presence of irradiated (2000 cGy) macrophages (10^5 cells/well) or spleen cells (10^6 cells/well). Spleen cells were har-

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vested and tested in a 5 h ^{51}Cr-release assay as described [7]. Effector: target ratio is based on input cell numbers before restimulation.

2.4 Proliferation assay

Spleen cells or EL-4 cells were seeded into flat-bottom 96-well plates at the indicated cell numbers and used to stimulate TCR H-2^{bm13} cells (3×10^5 cells/well) for 48 h. Proliferation was assessed by incorporation of [^{3}H]thymidine (25 μCi/ml). The ultrafiltration device was purchased from Amicon (Centricon-30, No. 4306) (Beverly, MA).

3 Results and discussion

Peritoneal macrophages from TAP1−/− mice [16] or control mice were incubated for 2 h at 37 °C with recombinant LCMV-GP protein (10 μg/ml). The cells were washed three times and used for restimulation of LCMV-specific cytotoxic T cells (CTL) from mice immunized 10 weeks previously with LCMV; cytolytic activity of cultures was determined 5 days later (Fig. 1). Cells loaded with the relevant peptide derived from LCMV-GP protein (p33, aa33–41 [23]) or left untreated were used as controls for restimulation. As expected, both peptide-loaded TAP1−/− cells and control cells restimulated LCMV GP-specific CTL, whereas unloaded cells did not. Interestingly, protein-loaded TAP1−/− cells restimulated CTL as efficiently as control cells, demonstrating that the LCMV GP protein reached the class I pathway in the absence of TAP. The same results were obtained with spleen cells from TAP1−/− mice as stimulator cells (not shown).

To exclude the possibility that protein bound by TAP1−/− cells was released, taken up and presented by the TAP1+/+ effector cell population during the 5 days of restimulation, the following control experiment was performed.

Transgenic mice expressing a TCR (P14) specific for the LCMV GP-derived peptide p33 presented on H-2D^b were bred into mice exhibiting a mutation in their D^b molecule (B6.C-H-2^{bm13}). This mutant D^b molecule cannot present the relevant peptide p33 but can positively select the T cell receptor (Fig. 2A) [24, 25]. Spleen cells from TAP1−/− and from control mice were loaded with LCMV GP protein and used to stimulate TCR H-2^{bm13} spleen cells,

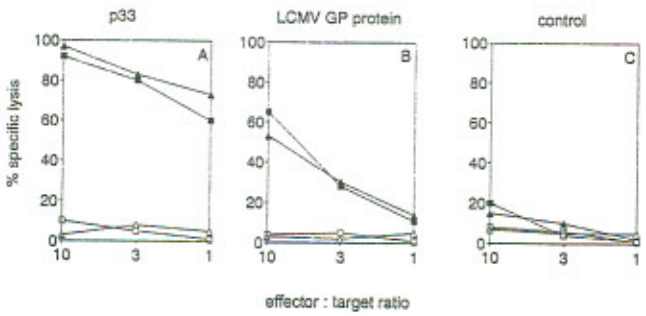

Figure 1. Recombinant LCMV GP protein reaches the class I pathway in the absensce of TAP. TAP1−/− (squares) and control (triangles) peritoneal macrophages were incubated with p33 (aa 33–41) (1 μg/ml) (A), recombinant LCMV GP protein (10 μg/ml) (B), or left untreated (C) for 2 h and used for restimulation of spleen cells from mice infected 8–10 weeks previously with LCMV (200 plaque-forming units, pfu). After 5 days, cytotoxicity was assessed in a standard ^{51}Cr release assay, using EL-4 cells loaded with p33 (filled symbols) or left untreated (open symbols) as target cells. One representative experiment of three is shown. The same results were obtained with TAP1−/− spleen cells.

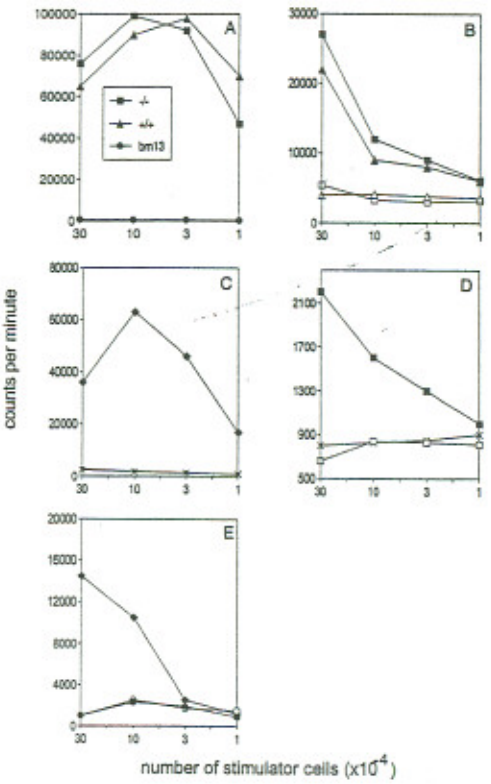

Figure 2. TAP-independent protein-derived peptide present on class I molecules is not due to protein transfer to effector or peptide contamination of the protein preparation. (A) S cells from H-2^{bm13} mice do not present the LCMV GP-derive tide p33. Spleen cells from H-2^{bm13} mice transgenic for the P14 recognizing p33 (TCR H-2^{bm13}) were stimulated with loaded spleen cells (10 ng/ml) from TAP1−/− mice (squ control C57BL/6 mice (triangles) or H-2^{bm13} mice (circles) proliferation was assessed 48 h later by incorporation of [^{3}H midine. (B) Spleen cells from TAP1−/− (squares) or control (triangles) were incubated for 2 h with LCMV GP protein (1 ml) (closed symbols) or left untreated (open symbols) and us stimulate TCR H-2^{bm13} spleen cells. (C) EL-4 cells do not pr peptides derived from LCMV GP. EL-4 cells were incubated peptide p33 (diamonds), LCMV GP protein (crosses), o untreated (circles), washed and irradiated (3000 cGy) and us stimulate TCR H-2^{bm13} cells. (D) Low molecular-weight con nation of the protein preparation is not responsible for stimul of TCR H-2^{bm13} cells. The LCMV GP protein preparation ultrafiltered and the low molecular-weight flowthr (< 30 kDa) (crosses) was used to load TAP1−/− spleen cell controls, untreated (open squares) or LCMV GP protein-lo (closed squares) spleen cells were also used for stimulatio TCR H-2^{bm13} spleen cells. One representative experiment of is shown. The same results were obtained with macroph (E) Peptide regurgitation is not responsible for stimulatio TCR H-2^{bm13} cells. H-2^{bm13} (circles) or TAP1−/− (diamo spleen cells were incubated with LCMV GP (10 μg/ml) for 2 h washed three times. Protein-loaded H-2^{bm13} spleen cells were either mixed with TAP1−/− spleen cells (open circles) o (closed circles). The different cell preparations were used the restimulation of TCR H-2^{bm13} cells.

thereby excluding presentation of p33 by the effector cell population. APC from TAP1−/− mice loaded with LCMV GP protein induced p33-specific proliferation of TCR H-2^{bm13} spleen cells with similar efficiency as control cells (Fig. 2B). Although the p33 peptide is located in the signal sequence of LCMV GP and is, therefore, not present on mature LCMV GP molecules, there is apparently sufficient LCMV GP containing the signal sequence and thus the p33 epitope present in the protein preparation. This is also demonstrated by the strong p33-specific CTL response induced *in vivo* with LCMV GP.

To exclude peptide contamination of the LCMV GP protein preparation, two additional control experiments were performed. First, EL-4 cells, which efficiently present peptide p33, were loaded with LCMV GP and used for stimulation of TCR H-2^{bm13} cells. No proliferation was measurable (Fig. 2C). Second, ultrafiltration of the protein preparation through a filter with a cutoff of 30 kDa removed the restimulatory capacity in the flowthrough, excluding a contamination of the protein preparation with small peptides (Fig. 2D). Peptide regurgitation was excluded by incubating H-2^{bm13} spleen cells with LCM GP and mixing them after extensive washing with TAP1−/− cells. No stimulation of TCR H-2^{bm13} spleen cells occurred, excluding the possibility that specific peptide was released from the stimulator cells that could exogenously load class I molecules of TAP1−/− cells (Fig. 2E).

To reveal whether the TAP-independent, class I-restricted presentation of LCMV GP protein was an exception or the rule, two additional proteins were tested for their capacity to be presented on class I molecules in the absence of TAP: the nucleoproteins of LCMV and VSV. Both proteins are cytosolic and, therefore, do not contain signal sequences. They have no known special physical properties to gain access to class I molecules in the absence of TAP; although peptide-derived VSV N can be presented in RMA/S cells under some circumstances, this seems to be due to a leaky phenotype of the RMA/S cells and does not reflect true TAP-independent presentation of VSV-N [12]. Peritoneal macrophages and spleen cells from TAP1-/- and from control mice were incubated for 2 h with recombinant LCMV NP or VSV N protein (10 μg/ml) and used to restimulate spleen cells from mice immunized 8 to 10 weeks previously with LCMV or VSV, respectively. As shown for LCMV GP, LCMV NP and VSV N protein also restimulated specific CTL (Figs. 3 and 4). Peptide contamination of the protein preparations was unlikely, since they failed to sensitize EL-4 cells for lysis ([7] and not shown). Thus, exogenous proteins entering the class I pathway of APC seem not to require functional TAP transporter molecules.

Access to the class I pathway by proteins is facilitated *in vivo* by the presence of cellular debris [1, 7], by coupling the proteins to beads [26] or by denaturing them by boiling or SDS treatment [7, 8, 27], suggesting that macrophages and possibly dendritic cells play an important role for the priming of CTL with protein *in vivo*. *In vitro*, macrophages seem to be efficient in presenting exogenous proteins on their class I molecules [28]. It is important to note that the proteins used in this study for loading macrophages and spleen cells was not soluble, but were associated with insect cell debris [7]. The proteins were thus in a form able to facilitate phagocytosis.

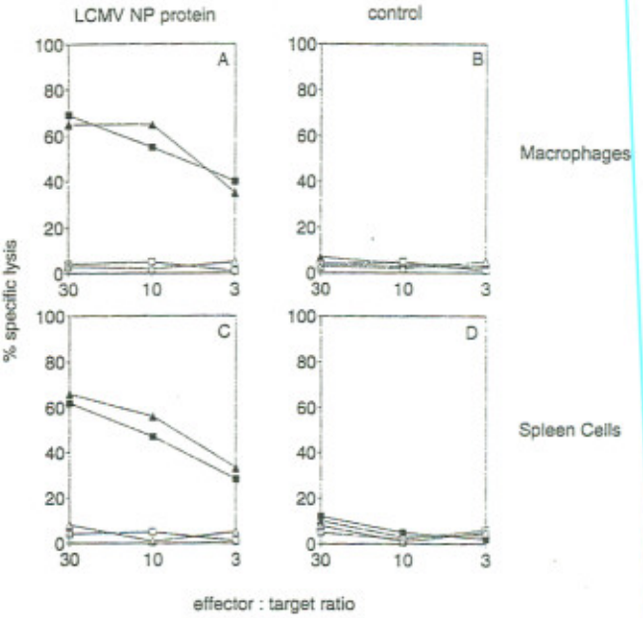

Figure 3. Recombinant LCMV NP protein reaches the class I pathway in the absence of TAP. TAP1−/− (squares) and control (triangles) peritoneal macrophages (A, B) or spleen cells (C, D) were incubated with recombinant LCMV NP protein (10 μg/ml) (A, C) or left untreated (B, D) for 2 h and used for restimulation of spleen cells from mice infected 8–10 weeks previously with LCMV (200 pfu). After 5 days, cytotoxicity was assessed in a standard ^{51}Cr release assay, using EL-4 cells loaded with the LCMV NP-derived peptide aa 118–132 [36, 37] (closed symbols) or left untreated (open symbols) as target cells. One representative experiment of three is shown.

With bacteria expressing recombinant ovalbumin, it was shown that class I presentation of peptides from phagocytosed bacteria was not inhibitable by brefeldin A [29]. The major mechanism responsible for the class I loading in these experiments was found to be peptide regurgitation – a mechanism that does not operate under the conditions used in our experiments.

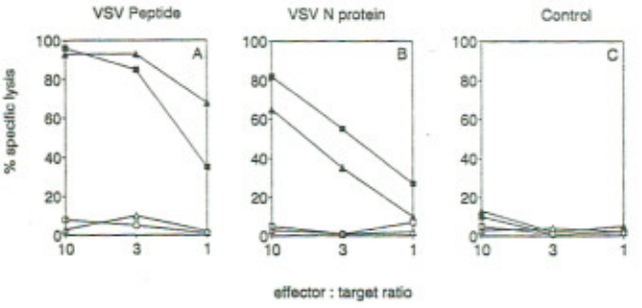

Figure 4. Recombinant VSV N protein reaches the class I pathway in the absence of TAP. TAP1−/− (squares) and control (triangles) peritoneal macrophages were incubated with peptide aa 49–62 [38] (1 μg/ml) (A), recombinant VSV N protein (10 μg/ml) (B), or left untreated (C) for 2 h and used for restimulation of spleen cells from mice infected 8–10 weeks previously with VSV (10^6 pfu). After 5 days, cytotoxicity was assessed in a standard ^{51}Cr release assay, using EL-4 cells loaded with peptide 49–62 (closed symbols) or left untreated (open symbols) as target cells. One representative experiment of three is shown.

Class I loading with ovalbumin coupled to beads has recently been shown strictly to require the presence of TAP [30]. On the other hand, the data presented here show with three different viral proteins and the unmanipulated, polyclonal TCR repertoire, that class I molecules can be loaded with peptides derived from exogenous proteins in the absence of TAP. The discrepancy may be explained either by the use of beads as a carrier for the protein or the use of a single hybridoma which may have a rather limited affinity. It is conceivable that the peptides generated by cytoplasmic proteasomes differ somewhat from those generated after the uptake of exogenous proteins; this may result in a failure of a particular hybridoma to recognize the latter. One possible way for proteins to enter the class I pathway is direct entry into the cytoplasm by osmotic shock [31]. This is not likely to operate under conditions as analyzed here, because such a mechanism depends upon the presence of TAP. Further arguments against the operation of such a mechanism in our study is the failure of EL-4 cells to present exogenous antigen (see Fig. 2C). Since it has recently been shown that professional APC may harbor a special endosomal compartment where class II molecules are loaded [32, 33], it is tempting to speculate that this class II loading compartment may be responsible for the observed TAP-independent loading of class I molecules by spleen cells and macrophages.

Collectively, the data and these considerations suggest a short circuit between the endocytic and the exocytic compartment in professional APC where the class I and the class II pathway interconnect. It should be pointed out that the class I-associated antigen presentation is, as a rule, much more efficient for endogenous than exogenous antigens; comparatively large amounts of antigen are needed to induce CTL with proteins as compared to infections viruses [7, 34]. Although relatively inefficient, this need for high concentrations of exogenous proteins for class I loading could be overcome for vaccine strategies by increasing local antigen concentrations. This apparently can be done by coupling the antigen to beads or if antigen is associated with cellular debris [7, 26].

Although this pathway seems to be considerably less effective than the classical pathway, from a biological point of view, and as pointed out previously [1], a special processing pathway allowing proteins to enter the class I pathway of professional APC may be advantageous for the following reason. It is of relevance for the immune system to separate exogenous proteins from endogenous (viral) proteins; this is particularly important for nonlymphohematopoietic somatic cells in the periphery which are the target cells for CTL, since only virus-infected and virus-producing host cells should be lysed. This rule may not apply to professional APC, since professional APC are (i) the most potent inducers of CTL, because they express costimulatory molecules and migrate to lymphoid organs and because they (ii) can be replaced easily, in contrast to terminally differentiated somatic cells such as neurons. Unless a professional APC takes up antigens from virus-infected, lysed cells and presents peptides by the described mechanism to induce CTL, any virus avoiding professional APC could also avoid efficient CTL responses (for exceptions, see *e.g.* papilloma viruses [35]). From this point of view, partial violation of the principle of class discrimination by professional APC may allow a more efficient CTL induction without widespread lysis of innocent bys der cells.

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