On cross-priming of MHC class I-specific CTL: rule or exception?

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Introduction

It has been generally accepted as a rule that peptides binding to MHC class I molecules are derived from proteins synthesized within the cell, including peptides of genuine cellular origin as well as those encoded by viruses or other intracellular infectious agents. In contrast, MHC class II molecules, in general, bind peptides derived from exogenous proteins that have been processed via phago-lysozomes and replace the invariant chain clip fragment associated with class II molecules [1, 2]. MHC class I restriction correlates with CD8+ cytotoxic T cells (CTL) and class II restriction with CD4+ T (Th) cells. However, soon after the initial formulation of these general rules, differential MHC restriction as well as the division of labor between endogenous versus exogenous loading of MHC class I and class II molecules were questioned [3–13]. The subject of this editorial is so-called “cross-presentation” and “cross-priming” as well as “cross-tolerance”, which may be defined as the presentation of exogenous antigen via MHC class I for generating stimulatory or tolerogenic responses in CD8+ T cells, respectively (Table 1) [10]; accordingly, exogenous antigen has “crossed” over to the endogenous pathway to gain access to MHC class I. The use of the term “cross-priming” for peptides presented on MHC class II molecules is not warranted because this is the conventional pathway for class II presentation (e.g. [14]). Recently, MHC class I-specific presentation of exogenous antigen to induce cross-priming, cross-presentation, or cross-tolerance has become viewed by many as a general and important phenomenon [5, 9, 10, 13, 15]. This emerging view is based on the concept that activation of CD4+ Th cells or CD8+ T CTL requires antigen (signal 1) plus costimulation (signal 2), whereas encounters with signal 1 alone result in anergy or deletion [16–19]. This was the basis for the proposal: Unless cross-presentation plays an essential role, self-encoded antigens, which are extralymphatic, could not render T cells tolerant, and viruses, which do not infect professional antigen-presenting cells (APC, expressing both antigen as signal 1 and signal 2) or tumor cells (that do not express signal 2) could not elicit MHC class I-restricted CTL [5, 9, 10, 13].

In biology, particularly in immunology, nothing is impossible. However, as a result of co-evolution of pathogens and their hosts, there has been selection of frequent or likely pathways and effector mechanisms against unlikely rare forms and exceptions. Therefore, the key question here is: Is cross-priming via MHC class I a general and essential characteristic of physiologically important CD8+ CTL cytolytic or tolerogenic responses or, alternatively, is this process an exception that, perhaps under special conditions, can be exploited therapeutically to induce responses that otherwise would be difficult to generate (Table 1)?

Experimental evidence documenting “cross-priming”

The first evidence for this concept was obtained in early experiments by Bevan [5, 6] and subsequently by Simpson and Gordon [20] and von Boehmer (unpublished observations) using so-called minor histocompatibility antigens (miH) as targets for cytotoxic T cells. miH represent allelic differences of usually numerous host protein-derived peptides that are presented by MHC class I (also by MHC class II, but those are rarely studied [21, 22]). In some cases, miH differences may include antigens encoded by endogenous retroviruses [23, 24]. The original experiments and postulates were not based upon 2-signal theories or the exclusive inductive capacity of so-called professional APC, but were based on empirical findings involving skin graft rejection or CTL responses. For example, if an H-2b BALB.B mouse was immunized with H-2b C57BL/6 (B6) splenocytes, a minor histocompatibility-specific CTL response was measur-
Table 1. A summary on reasoning and views about “cross-presentation”, “cross-priming” and “cross-tolerance” of cytotoxic CD8+ T cell responses

Cross-presentation via MHC class I?
1. Two signal hypothesis demands obligatory “professional” (i.e. signal 2 positive, upregulated, “mature”) antigen-presenting cells (APC) for T cell induction to become effector T cells.
2. Negative selection is mandatory for all T cells specific for self-encoded peptides.
3. T cell induction to become effector T cells and T cell deletion- anergy-tolerization are two distinct processes; absence of signal 2 on APC is a negative signal (i.e. immune regulation by suppression).

An alternative view
1. Any antigen-expressing cell reaching secondary lymphatic organs (or equivalents for some, such as intraepithelial micropatches, etc.) induces effector T cells.
2. No negative selection (equivalent to exhaustive induction) necessary for T cells specific for self or non-self encoded peptides that stay extralymphatic (ignorance).
3. T cells are inducible only: induction of <100% of precursor T cells to become effector T cells causes elimination or control down to very low levels of antigen (“immunity” = maintenance of activated T cells by low level persistence or re-encountered antigen). Induction of 100% of precursor T cells deletes/exhaustively induces all precursor T cells due to the 3–4 d half-life of effector T cells.

Questions and unresolved problems of experiments on cross-priming
1. Why are necessary conditions for cross-priming special, i.e.: great quantities of proteins, or lipids, or lipoproteins, or aggregates, and/or corpuscular materials (local concentrations high, mostly non-physiological in the sense that conditions cannot be easily reached by apoptotic or necrotic cells \textit{in vivo}).
2. Inactivation of virus materials often poorly controlled \textit{in vitro} and \textit{in vivo}.
3. Very sensitive cytotoxicity read out (lymphoblast targets, high spontaneous $^{51}$Cr released, shallow slopes of Cr-release, high E:T ratios), or T cell proliferation.
4. Multiple (very many mH or other) targets, often undefined at peptide level.
5. Protective capacity of effector T cells often not tested \textit{in vivo}, or exclusive involvement of CTL unclear.
6. Whenever a single defined cellular antigen with defined T epitope peptides is analyzed, cross-priming is “difficult” to show, or not demonstrable in the classical F1 experiment.
7. Role of T help (using class II MHC external “normal” pathway) is often unclear, T help can be limiting for CTL responses.
8. High frequencies of tg TCR+ CTL, or T hybridomas show enhanced responses via bystander activation.

able on Con A- or LPS-treated lymphoblast cells derived from the donor B6 H-2$^b$. If the same experiment was performed by immunization of (H-2$^d$ BALB/c×H-2$^b$ BALB.B)F1 recipients with H-2$^b$ B6 spleen cells, followed by restimulation \textit{in vitro} with either H-2$^b$ B6 or H-2$^d$ B10.D2 lymphocytes from H-2 congenic mouse strains, CTL activity specifically restricted to the H-2$^d$ haploptype was also observed. This “cross-primed” CTL activity of the F1-host was detectable \textit{in vitro} using LPS or Con A blasts as target cells, but apparently was not readily found against non-mitogen-treated lymphocytes or fibroblast targets. The role of Th in skin graft rejection or CTL responses \textit{in vitro} was subsequently studied in detail and positively demonstrated [21, 25]. Interestingly, even in these very early experiments, the use of tumor cells instead of spleen cells revealed no such cross-priming under similar experimental conditions [5, 6]. This finding was interpreted as reflecting a “narrow” spectrum of specific responses whereby the injected tumor cells had expanded to large numbers and perhaps selected one dominant CTL subspecificity [26–29].

Subsequent experiments used latex beads coated with a protein antigen [30, 31], protein antigens forced into the cytosol of cells via hypotonic shock [32], or, more recently, immune complexes [15, 33] (or exosomes [34]).
These approaches avoided the uncertainties of undefined miH, for which neither the precise epitope nor the relative distribution and expression was known or controllable (even in so-called miH-congenic mouse strains). These more recent experiments formally showed that antigens entering cells from the outside, under special experimental conditions, could enter the MHC class I presentation pathway. Thus, cross-presentation and cross-priming are possible; but are they phenomena of general importance or an exception?

Studies with melanoma or tumor virus antigen-associated CTL responses are often quoted as the basis for the in vivo relevance of cross-presentation and cross-priming. The experiments performed in Pardoll’s laboratory used melanoma B16 mutant cells that did or did not express MHC class I antigens to immunize syngeneic H-2b mice [35]. Specific CTL responses showing about 25–30% cytolysis were observed in vitro at high effector to target cell ratios (100:1), with 10% of control targets being lysed. Protection against tumor take was also documented in vivo, but the studies could not attribute the effect exclusively to CTL effector mechanisms. In fact, later studies suggested that alternative effector mechanisms were also important [36]. In another study, colon carcinoma H-2b tumors expressing the influenza nucleoprotein (NP) were used to immunize F1 (H-2b × H-2d) → H-2b or H-2d chimeras; chimeras were then tested for cross-primed CTL after restimulation in vivo. Peptide-specific lysis assays (measuring the difference between lysis of peptide-loaded versus unloaded target cells) tested at a 100:1 ratio yielded 10–20% specific lysis by cross-primed CTL [35].

The experiments by Melief and colleagues [37, 38] on adenovirus 5E1A or B + D6 (Ad5E1A or B) specific responses are based on AdE1A or B adenovirus-transformed tumor cells of BALB/c (H-2d) origin. C57BL/6 (H-2b) mice immunized with these tumor cells were restimulated in vitro with irradiated E1A or B + H-2b tumor cells for 5–6 days. They were then tested against Europium-labeled targets in a microassay. AdE1A or B expressing targets were lysed about ten times better than control targets, indicating that allogeneic AdE1A+ or B+ tumor cells had induced a CTL response. In vivo protection against tumor take was tested by similar vaccination protocols. By 14 days after immunization, up to 75% of the animals were protected against a challenge with 5×10⁶ Ad5E1+ ras transformed tumor cells. The role of mechanisms other than CTL killing, for example protective Th responses, was initially not determined, but addressed in later studies [39]. While the studies demonstrated that alloreponses as such are (alone) not responsible for the peptide specificity seen, they discussed the inherent problems of a widely cross-reactive alloreponse and formulated carefully “that cross-priming is most likely sufficient”. Nevertheless, they conclude “that completely allogeneic tumor cells can be successfully used for the induction of protective anti-tumor immunity and hold promise for the implementation of allogeneic tumor cell based vaccines”.

More recently, Heath and collaborators [10, 40–42] used transgenic mice expressing ovalbumin (OVA) under the rat-insulin promotor (RIP-OVA) to show that transgenic (tg) OVA-D8-specific T cell receptor (TCR) expressing CD8+ T lymphocytes were activated in draining lymph nodes of OVA-overexpressing pancreatic islets. This was interpreted as cross-presentation of a peripheral “self” antigen in the draining lymph nodes based on the assumption that only APC could pick up apoptotic or necrotic islet cells, cross-process antigen, and induce TgTCR+ CD8 T cells in the draining lymph node. A potential role of Th was not considered in the original studies, but was subsequently confirmed [43, 44]. The response was also dependent on antigen dose and on very high numbers of TgTCR+ CTL to achieve the described phenotype [10]. These experiments caused great enthusiasm for cross-presentation/cross-priming/cross-tolerance as a general and important mechanism.

Shortly following this report, two independent experiments were published, with many positive commentaries, which demonstrated in vitro that viral antigens (seemingly independent of an infectious process) could be taken up by APC and cross-presented via class I in the cases of influenza viruses [45] and polio virus [46]. Similar evidence had come earlier from experiments using hepatitis B surface (HBs) (but not HB core) particles [47] or undefined preparations of recombinant viral protein mixed with debris [48] (but, interestingly, not without debris) from a baculovirus expression system [49]. In contrast to the earlier miH model systems, the later experiments during the 1990s using latex beads, hypotonic shock or viral particles defined CTL responses at the peptide level. Interestingly, and surprisingly, none of these later investigations actually demonstrated cross-priming using classical F1 experiments to show cross-presentation via F1 APC.

Experiments not showing cross-priming or studies that falsify predictions of the general concept.

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1 The fact that OVA is the most favored protein used for model experiments on cross-priming may have a specific reason. Perhaps it is linked to a very special property of OVA, which is, after all, a transport protein capable of binding to many other proteins, but this remains to be carefully analyzed.
Experimental evidence has accumulated, also on the negative side. Early H-Y-specific CTL experiments only occasionally showed cross-priming as predicted, and often the CTL activities obtained were low [20]. This was interpreted as an example of class I immune response (Ir) regulation implying additional limiting roles of Th cells or that no correct presentable peptides were generated [21]. Similar evidence came from a careful study of H43a versus H43b miH-congenic mice, which found specific CTL responses without cross-priming [50]. A third example was provided by Mx-congenic mouse strains, where no cross-priming could be demonstrated [51]. More recently, transformed fibroblast cells L929 (H-2b) were transfected with the NP of LCMV and tested with or without additional transfection with H-2Ld capable of presenting LCMV-NP118–126 [52] (Table 2). When BALB/c (H-2d) mice were immunized with a wide variety of protocols using the allogeneic transfected cells, no Ld+NP118–126-specific CTL were generated except when 10^6 living L929 (H-2d) NP118–126+Ld-positive fibroblasts were used. This indicated that CTL were induced directly by these fibroblasts, and not via cross-priming.

Comparable results were obtained with tumor cells of epithelial, mesenchymal or melanoma origin from H-2b B6 mice (Table 2). These tumors expressed tumor antigens plus the transfected glycoprotein (GP) of LCMV as an artificial tumor antigen. The tumor cells promptly induced D^b-GP33–41 CTL in B6 mice when 10^4 living cell were injected into the spleen; this efficiency is comparable to APC transgenic for the same GP_{LCMV}. In contrast, even after multiple injections into F1 mice of apoptotic, necrotic or living cells, the tumor cells all failed to reveal cross-priming. For each tumor example, the readout was specific for defined class I-presented peptides. As pointed out above, the lack of cross-priming with tumor cells might have been expected from earlier studies [6]. Therefore, the same experiments were repeated using spleen cells from H-2b or H-2d transgenic mice expressing the LCMV GP using an ubiquitous promoter. Again, no significant cross-priming was observed under conditions where direct priming was very efficient [53].

Taken together, these findings suggested two general conclusions (Table 1): (1) Whenever large quantities [15, 30, 31, 54] of particulate-lipid-containing materials [45–49, 55] are used, which allow antigen to gain access to the cytosol, “cross-priming” can be shown; but formal F1 experiments appear to be difficult or unfeasible. (2) Whenever a multitude of miH are used, cross-priming is readily shown with “physiological materials”, whereas if only a single miH or a defined viral antigen, exhibiting only very rare and few CTL epitopes, are used, cross-priming is not seen in a classical F1 experiment. How can these differing observations be explained? Do they reflect methodological/experimental differences? Or do they suggest separate pathways of efficient antigen processing that depend on the various antigens used?

### Table 2. Protective CD8+ T cell response induction in vivo with transfected fibroblasts

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination of mice i.v. or i.p. or s.c. with cells transfected with:</th>
<th>Protection: virus-titer reduction/ no lethal immunopathology</th>
<th>CTL response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10^4 GP-transgenic DC</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>10^4 live fibroblasts transfected with LCMV GP (no classical 2nd signal)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>LCMV GP-transfected fibroblasts (apoptotic, necrotic, or shortlived &lt;24 h, 10^7 injected 1x or 5x, over 5 days)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Experiments in BALB(H-2b) mice: CTL anti Ld+ NP_{LCMV118–126}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10^5+10^7 live completely allogeneic fibroblasts (H-2b) LCMV-NP (various protocols as for group 3)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>10^6 live allogeneic (H-2b) fibroblasts transfected with LCMV-NP plus Ld</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Critique of methods for assaying cross-priming responses

First, let us evaluate the original miH experiments. In most cases, many, probably thousands, of miH differences were involved in the responses. In addition, these experiments all used LPS- or Con A-stimulated target cells to detect induced CTL responses. The characteristics of such lymphocyte blasts are that the MHC concentration is high; that cellular antigens including potential endogenous viruses are also expressed at relatively high levels; and that spontaneous \(^{51}\)Cr release is notoriously high (i.e. 20–40% for 4–5 h). In addition, data on target cell lysis is usually presented for antigen-positive and antigen-negative targets, sometimes as the difference between these values. Comparing lymphocyte blasts to quiescent or IFN-stimulated fibroblasts or other non-lymphohemopoietic cells, the difference in MHC antigen expression levels is 10–100-fold higher. The problems of target susceptibility are further documented by the usually rather shallow slopes of \(^{51}\)Cr release from lymphoblast targets. By comparison, \(10^{10}\) M peptide-loaded or virus-infected targets reveal an approximately linear and directly proportional relationship between dose of effectors and target cell lysis. Furthermore, as pointed out above, when investigators used a single defined miH, including H-Y antigens, cross-presentation and cross-priming cannot be easily demonstrated. One explanation for these discrepancies might therefore be that the numerous specificities of bulk anti-minor CTL together with the high sensitivity of the target cell population may reveal “cross-reactivities” of CTL that are demonstrable \textit{in vitro}, but are of no real consequence \textit{in vivo}. This may account for why cross-priming has not formally been documented unequivocally with peptide-specific CTL responses in properly controlled, classical F1 experiments.

In this context, it is relevant to note that acknowledged cross-reactive T cell clones have been documented with specificities for miH and for alloantigens [26, 28, 56] or miH x+H-2\(^a\) and z+H-2\(^b\) [28], but have not so far been observed for anti-virally protective T cells generated \textit{in vivo} against viral antigen-expressing fibroblasts or epithelial cells [57]. One conclusion from these comparisons is that the “cut-off” used to define so-called cross-primed CTL specificities is inadequately low in many, if not all, cases. When such cross-reactivities are tested for efficacy in anti-viral protection \textit{in vivo} (under conditions where only CD8\(^+\) CTL and neither neutralizing antibodies, T helper cells nor macrophage-dependent processes are responsible), cross-priming has not been documented convincingly under physiological conditions of strictly extralymphatic viral infections. Let me emphasize again that this statement does not negate the exceptional case that with “special” antigen and at non-physiological doses of antigen, cross-priming can be forced to occur.

The role of Th cells

The role of Th functions in generating CTL and cross-priming has been discussed repeatedly. Some experiments suggest that there is a more stringent requirement for Th in cross-priming than in direct priming [58, 59], probably because antigen doses are normally limiting. Experiments using H-Y [21], tumors, adenovirus or papilloma virus proteins [39], and miH [22, 36, 38] more recently also for OVA [33, 60], illustrate an important role for Th cells. In addition, a clear need for Th cells for CTL induction has also been documented for direct priming under conditions where antigen and cytokines may be limiting.

For example, infection of mice with several viruses that replicate well, induces CTL responses in the absence of Th cells. In other conditions where viruses do not replicate widely, or only abortively in mice (e.g. after influenza virus or vesicular stomatitis virus (VSV) infection), T help is an important factor for CTL induction [61]. Therefore, the question arises whether, in certain experimental conditions, limiting amounts of T help (induced via the conventional exogenous MHC class II pathway) were measured, instead of cross-presentation and cross-priming via MHC class I. This explanation has been discussed [12, 35–37, 39], though, in general, has not been favored.

For example, in the RIP-OVA-tg TCR model studying cross-tolerance, Th cells appear to be involved in an important way [43]. Therefore, some of the findings may actually reflect Th-mediated bystander activation of the large numbers of precursor transgenic TCR\(^{+}\) CTL rather than MHC class I cross-presentation and cross-priming by APC in the draining lymph node. This is not merely an esoteric consideration since the effects demonstrated in these experiments were dependent on both high OVA antigen expression in the islets (favoring their enhanced apoptosis [62]) as well as a high frequency of transferred transgenic TCR\(^{+}\) CTL. Both of these conditions favor bystander activation [42, 63, 64]. Similarly, a decisive role of Th in limiting CTL responses may explain the restimulation of multiple-miH-specific low-affinity CTL responses as well as the marginal cross-primed responses in melanoma or influenza-NP model systems.
Cross-priming against viruses

CTL induction against defined peptides have been documented for influenza NP [65, 66], HBs [47], crude preparations of viral antigens expressed in baculovirus [48], inactivated influenza virus [13, 45] and poliovirus [46]. The number of virus-like particles necessary per cell to induce such cross-presentation and cross-priming in vitro is still unclear, but probably is on the order of 10^5–10^7/cell [45, 55] [for example, 1 hemagglutinating unit (HA) represents about 10^6 plaque-forming units and about 10^8–10^9 particle equivalents]. Most of these experiments cannot exclude virus fusion with cell membranes. These and other experiments using hypotonic shock or latex beads show that it is possible to force antigens into the cytosolic pathways and onto MHC class I. However, independent experiments suggest that in vivo tumor cells or virus infections under physiological conditions (including apoptotic and necrotic cells), or antigen-expressing lymphohemopoietic cells from transgenic mice fail to exhibit the phenomenon of cross-presentation and cross-priming, particularly when tested in the classical F1 experiment.

From all this, one may conclude that, while it is possible to demonstrate cross-presentation and cross-priming under highly selected special conditions, this is not a generalizable phenomenon that reflects physiological conditions in vivo. Therefore, while cross-presentation and cross-priming may perhaps be applicable for novel types of vaccine conditions, this evidence cannot, and must not, be misused to argue that cross-presentation and cross-priming via MHC class I are essential and generalizable characteristics of CTL physiology and specificity.

Conclusion

It is worrying that there has been overemphasis of what may be exceptional cases of immunological phenomena. Recently, there has been an enthusiastic renaissance not only of signal theories, but also about regulatory T cells, negative (suppressiv e) T cell mechanisms, and idiotypic networks, as if they had not misled (or even ruined) entire generations of immunologists during the past 40 years. In fact, “cross-priming” resembles the historic debate on idiotypic networks. The fact that it was possible to induce anti-idiotypic antibodies that even in rare cases may control B cell tumors [67], never justified the broader concept [68, 69] of idio type-anti-idiotypic networks underlying the complex, interactive control of the immune system (for which there still exists no solid experimental evidence).

The difference between “what is possible under any experimental condition” versus “what happens physiologically” is a crucial distinction. The proposal that any tumor cell expressing melanoma antigens can effectively prime tumor-specific CTL in any host, independently of living tumor cells directly presenting MHC class I, is simply not proven in vivo; moreover, the reviewed evidence suggests that such therapy is extremely unlikely to be successful in vivo because it is too inefficient. While this can be achieved in an MHC-dependent fashion with the relevant peptides, completely inactivated extracts or apoptotic or necrotic tumor materials fail to generate an exclusively CTL-mediated cross-primed effector T cell mechanism. Of course, where the induction of T help or antibodies is important in controlling tumors or viruses, protection by other mechanisms than CTL – and not requiring “cross-processing” via MHC class I – may be effective. It is also important to re-emphasize that forcing exogenous antigens into the MHC class I pathway by special experimental tricks may promote cross-presentation via MHC class I. However, this is not a process that likely accounts for both conventional processes of induction of CTL against viruses (including those not infecting APC) or against strictly peripheral epithelial or mesenchymal tumors. Finally, evidence suggests that cross-presentation is unlikely to play an important role in tolerance of MHC class I-restricted CTL in vivo or in deletion or tolerance against self antigens expressed exclusively extralymphatically.

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