Dissection of spontaneous and therapy-induced T cell immunity in mice
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Chapter 5

Antigen bias in cross-priming

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Antigen bias in cross-priming

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Effector CD8+ T cells detect virus-infected or tumor cells through recognition of MHC-bound peptide fragments that are produced upon degradation of endogenous disease-associated proteins. In contrast, the activation of naïve CD8+ T cells occurs in many cases through interaction with migratory antigen-presenting cells displaying MHC-bound peptides that are derived from exogenous sources, thereby allowing T cell detection of extra-lymphatic antigen production. To reveal whether these two pathways for antigen presentation generate a similar set of MHC bound peptides, we have compared endogenous and exogenous presentation of CTL epitopes in model antigens. Here we show a marked inefficiency of the exogenous pathway in the presentation of signal peptide-derived epitopes. These results reveal a main dichotomy between the endogenous and exogenous MHC class I antigen presentation pathway and suggest that whereas endogenous presentation correlates with protein synthesis rate, exogenous presentation correlates with accumulation of the protein from which the epitope is derived.

Circulation of naïve CD8+ T cells is confined to peripheral blood and the secondary lymphoid organs. When virus-infected or tumor cells are present in secondary lymphoid organs, these cells can directly activate naïve CD8+ T cells through presentation of endogenously produced MHC class I-restricted antigens1. However, to allow T cell access to antigens that are exclusively produced in peripheral tissues, an alternative pathway is required. In this pathway, professional antigen presenting cells (APCs) take up antigens and migrate to the lymphoid organs where they present these exogenous antigens to naïve CD8+ T cells, a process referred to as cross-presentation2. The activation of antigen-specific T cells through cross-presentation is readily apparent for a number of antigens3-5. However, cross-presentation does not occur for all peripheral antigens, including antigens that are well presented through the endogenous pathway6-7.

Recent studies have provided evidence that a substantial fraction of MHC class I-bound peptides produced through the endogenous pathway are derived from proteins that are degraded shortly after synthesis8. This pathway should allow MHC class I molecules to monitor protein synthesis rates rather than protein concentrations, enabling the rapid display of pathogen-derived epitopes upon infection9. To address whether the parameters that govern the efficiency of exogenous antigen presentation are identical to those that determine the efficiency of endogenous presentation, we created GFP fusion genes that encode two MHC class I D8-bind epitopes at separate locations. In a first construct (sNP36-GFP-E749), the influenza A-derived antigen NP36 was inserted into the NH2-terminus of the signal peptide of a secreted GFP molecule, and the Human Papilloma Virus (HPV) 16-derived antigen E749 was inserted near the COOH-terminus of the same molecule (Fig. 1A).
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Figure 1: Direct antigen presentation of T cell epitopes from signal peptides and mature protein parts. a: Structure of the sNP_{56}-GFP-E7_{49} and sE7_{49}-GFP-NP_{56} fusion gene products (for details see Supplementary data). b: Indicated RMA and (TAP-deficient) RMA-S transfectants were analysed for NP_{56} antigen presentation. Spleen cells from NP_{56}-immune mice were restimulated in vitro with the NP_{56} peptide and were subsequently incubated with the indicated number of tumor cells. After 4hr incubation in the presence of Brefeldin A (PharMingen), the percentage of IFNγ-producing CD8+ T cells was determined. c: Alternatively, peptides were stripped from the indicated tumor cells by boiling in 10% acetic acid that contained 10μM of an irrelevant peptide, passed through a 10 kDa cut-off filter, lyophilized and resuspended in culture medium. Serial dilutions were loaded on 5x10^4 D1 cells as target cells, and the percentage of IFNγ-producing CD8+ T cells was determined as in b.

a second construct, the same two epitopes were introduced, but in reverse order (sE7_{49}-GFP-NP_{56}). The introduction of both CTL epitopes did not affect signal peptide cleavage (Suppl. data Fig. 1, lane 11 and 14, respectively), and the resulting GFP molecules were secreted as determined by flow cytometry and pulse chase analysis (Suppl. data Fig. 2). The introduction of NP_{56} and E7_{49} epitopes in this manner allowed the monitoring of antigen presentation of two epitopes that are derived from the same protein synthesis product but are located in two different protein fragments. Assessing presentation of the signal peptide-encoded epitopes seemed particularly useful because signal peptides are an important source of endogenously produced MHC class I-binding peptides (e.g. refs 10-13), and the half-life of signal peptides is generally thought to be limited[14]. To study NP_{56} and E7_{49} epitope presentation via MHC class I upon endogenous processing, we introduced the GFP fusion genes into the tumor cell line RMA. Tumor cells that contain the NP_{56} epitope located in either the signal peptide or mature protein were recognized efficiently and equally well by antigen-specific T cells (Fig.1b). Likewise, the E7_{49} epitope located in either the signal peptide or mature protein was presented efficiently to antigen-specific T cells (data not shown). However, saturation of T cell recognition at high antigen densities may mask differences in presentation efficiency. To provide a more quantitative measure of the antigen processing of T cell epitopes contained within the mature protein part or signal peptide, we isolated peptides from either cell line by acid elution and used these eluates to sensitize target cells (Fig 1c). These data document that T cell epitopes contained within mature protein parts and signal peptides can be presented with comparable efficiency through the endogenous pathway. Consistent with prior observations[10,15], this presentation, is in large part dependent on the transporter associated with antigen processing (TAP), as antigen presentation by TAP-deficient RMA-S cells that contain either gene construct was inefficient (Fig. 1b,c).
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Figure 2: In vivo cross-priming is biased towards epitopes located within the mature protein. a: C57/B110 mice were subcutaneously challenged with 2x10⁶ of the indicated cells. The percentage of NP₃₆₆- (top) and E7₄₉₉- (bottom) specific CD8⁺ T cells in peripheral blood was determined using PE-conjugated anti-CD8β (PharMingen), and APC-conjugated NP₃₆₆ or E7₄₉₉ tetramers. In 40 out of 40 mice (p < 0.0001), T cells specific for the epitope located in the mature protein outnumbered those specific for the signal peptide-encoded epitope. Antigen-specific T cell responses to the COOH-terminal antigens E7₄₉₉ and NP₃₆₆ were on average 30 and 16 fold stronger as compared to T cell responses against the same epitope when present in the signal peptide. b: Antigen-specific T cells induced by cross-priming are functional. 12 days post tumor inoculation, IFNγ production of CD8⁺ T cells was determined directly ex vivo upon a 4hr stimulation with 0.1µM NP₃₆₆ or E7₄₉₉ peptide in the presence of Brefeldin A (top) and was compared with tetramer staining (bottom). c-d: MHC tetramer staining of peripheral blood cells (c) or splenocytes (d) from H-2² x H-2² F1 mice that were challenged with 1x10⁶ of the indicated p815 transfectant, measured directly ex vivo (c), or after 7 day in vitro restimulation with the appropriate peptide (d).
To investigate whether antigen localization would influence T cell induction by cross-priming *in vivo*, naïve mice were challenged with RMA-S cells that contain the sNP<sub>366</sub>-GFP-E7<sub>49</sub> gene construct. Efficient induction of T cell immunity against the E7<sub>49</sub>-epitope located within the mature protein was detected, as determined by *ex vivo* tetramer staining of peripheral blood cells (Fig. 2a). In striking contrast, the NP<sub>366</sub> epitope contained in the signal peptide of the same fusion gene failed to induce substantial numbers of antigen-specific T cells. To examine whether this difference reflects a localization effect or is due to other factors, such as a difference in T cell precursor frequency, we challenged mice with RMA-S cells that contain the fusion gene with the two epitopes in reverse order (sE7<sub>49</sub>-GFP-NP<sub>366</sub>). In this setting, mice developed a pronounced NP<sub>366</sub>-specific T cell response, but the E7<sub>49</sub> epitope located within the signal peptide failed to induce detectable T cell immunity, as judged both by MHC tetramer staining and *ex vivo* IFNγ production by peripheral blood and spleen cells (Fig 2a,b; data not shown). Collectively, our findings indicate that T cell induction by cross-priming is much more efficient for epitopes derived from mature proteins than for epitopes that are contained within signal peptides (see legend Fig 2). To test whether this antigen bias is also observed in the classical F1 cross-priming setting<sup>2</sup>, we introduced the two GFP fusion genes into p815 cells (H-2<sup>b</sup> background). When C57/Bl6 x Balb/c F1 mice (H-2<sup>b</sup> x H-2<sup>d</sup>) were challenged with p815 sNP<sub>366</sub>-GFP- E7<sub>49</sub> or p815 sE7<sub>49</sub>-GFP-NP<sub>366</sub>, antigen-specific T cell induction to E7<sub>49</sub> occurred only when located within the mature protein (Fig 2c). T cell responses to NP<sub>366</sub> were undetectable directly *ex vivo* in this setting (data not shown), but a similar bias towards the epitope encoded within the mature protein was apparent after *in vitro* restimulation of spleen cells (Fig 2d). Identical results were obtained with H-2<sup>d</sup> mouse embryonic cells that contained either construct (data not shown). It seemed possible

![Figure 3](image.png)

**Figure 3:** Differential cross-presentation of epitopes from signal peptides and mature proteins by DCs. **a:** *In vivo* T cell induction through cross-priming is independent of immunoglobulins. NP<sub>366</sub>- (top) and E7<sub>49</sub>- (bottom) specific CD8<sup>+</sup> T cell responses induced in µ-deficient mice upon tumor inoculation with RMA-S sNP<sub>366</sub>-GFP-E7<sub>49</sub> cells (left), or with RMA-S sE7<sub>49</sub>-GFP-NP<sub>366</sub> cells (right). **b:** Epitopes derived from mature antigens are more efficiently cross-presented than epitopes located within a signal peptide. The indicated tumor cells were irradiated with 80 Gray and incubated at 37°C for 24hrs. Subsequently, 1x10<sup>5</sup> D1 cells were incubated for 48 hrs with the indicated amount of tumor cells. For the analysis of *in vitro* cross presentation of the NP<sub>366</sub> epitope, 1x10<sup>5</sup> NP<sub>366</sub>-specific fluoZ hybridomas were added for 16 hrs and T cell activation was determined by monitoring the conversion of chlorophenol red-pyranoside to chlorophenol red at 595 nm<sup>27</sup>. In this assay, irradiation of tumor cells is required to avoid overgrowth of the cell cultures by the tumor cells but is not essential for antigen uptake by D1 cells (data not shown). Addition of tumor cell supernatant did not induce detectable cross-presentation by D1 cells, indicating that the source of the material that is cross-presented is cell-associated (data not shown).
that the selective priming of T cell responses against epitopes contained within mature antigens was a consequence of the formation of Ig-protein complexes and subsequent Fc receptor-mediated uptake by DCs\textsuperscript{16,17}. To assess whether Ig-protein complex formation is required to bias antigen-specific T cell immunity, we challenged \(\mu\)-deficient mice lacking B cells\textsuperscript{18} with RMA-S cells that contain either GFP fusion gene. Similar to T cell induction in wild type mice, T cell responses to the mature antigens were efficiently induced, whereas T cell immunity induced by signal peptide-encoded epitopes was marginal (Fig. 3A).

We subsequently examined whether the difference in the capacity of the two protein fragments to induce T cell immunity by cross-priming could be correlated to a difference in the ability of APCs to extract and present these antigens from cells or cellular remnants. To this purpose, immature murine dendritic D1 cells\textsuperscript{19} were incubated with irradiated RMA-S cells containing either GFP fusion gene, and the capacity of the APCs to process and cross-present NP\textsubscript{366} was determined. In this assay, the NP\textsubscript{366} epitope is cross-presented 10-30 times more efficiently to antigen-specific T cells when derived from the mature protein than when located within the signal peptide (Fig 3b). These results indicate that the superior T cell induction towards epitopes within mature proteins can at least in part be attributed to the ability of APCs to cross-present the two classes of antigens. This difference in ability to present these two types of exogenous antigens may conceivably lead to an even larger difference in T cell responses in vivo due to T cell competition for APC access\textsuperscript{20}.

Previous studies have looked at the contribution of direct presentation and cross-presentation in antigen-specific T cell induction by examining the effect of disruption of either pathway\textsuperscript{3-7}. Here we show that epitopes derived from signal peptides can efficiently be presented through the endogenous but not the exogenous pathway, thereby allowing the determination of the contribution of these two pathways in an unmanipulated system\textsuperscript{21}. To this purpose, we challenged naïve mice with RMA cells that contain either GFP fusion gene. Strikingly, also in this setting antigen-specific T cell responses were skewed towards the epitope contained in the mature antigen, be it either NP\textsubscript{366} or E7\textsubscript{49} (Fig. 4). This indicates that cross-presentation rather than direct presentation is the dominant mechanism for T cell induction in this type of tumor model.

Our data show a marked divergence in the efficiency of endogenous and exogenous antigen presentation. The fact that epitopes that are well-presented through the endogenous pathway are not in all cases well-presented...
upon cross-presentation may help to explain the lack of efficient cross-priming previously observed in several model systems\textsuperscript{6,7}. In this regard, it is interesting to note that the lymphocytic choriomeningitis virus (LCMV)-derived GP\textsubscript{x}, epitope that fails to induce T cell immunity via cross-priming is derived from the LCMV GP signal peptide\textsuperscript{10}.

The current data document a bias against epitopes derived from signal peptides in cross-presentation. The observation that this bias can be mimicked in a simplified \textit{in vitro} co-culture system, coupled to the fact that signal peptides are considered to be degraded rapidly, suggests that a difference in the accumulation of the epitope precursor may form the mechanistic basis of this antigen bias. This view, that exogenous antigen presentation monitors protein accumulation rather than protein synthesis rates, is strongly supported by recent data from Yewdell and coworkers.

An interesting issue is whether these findings can be extended towards the efficiency of tolerance induction against peripheral antigens that are not expressed within the thymus. Epitopes derived from peripheral antigens that fail to efficiently enter the cross-presentation pathway may be less likely to induce peripheral tolerance\textsuperscript{22,23}. While this type of antigens may be ill-suited to study the cellular mechanisms of cross-presentation, such ignored peripheral antigens may form particularly interesting targets for the induction of tumor-specific T cell immunity\textsuperscript{24}. The current data document a fundamental difference between direct presentation and cross-presentation. Antigens that are well presented through the endogenous pathway need not to be presented effectively through the exogenous pathway. This depicts cross-presentation as an efficient but biased solution to a topological problem.

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Supplementary data

Methods:

Generation of the GFP fusion genes: The gene construct sNP\textsubscript{seq}-GFP-E\textsubscript{7\textsubscript{49}} was generated by adding the amino acids MGVQIA\textsubscript{SN}NMDAMVPTLLLLAAALAPTQTRAV to the NH\textsubscript{2}-terminus of the enhanced Green Fluorescent Protein (GFP). This sequence encodes the NP\textsubscript{seq} epitope (bold) preceded by four NH\textsubscript{2}-terminal influenza A NP-derived amino acids and followed by the H-2K\textsuperscript{b}-derived signal peptide (italic). The amino acids GVQIA\textsubscript{RAHIN}YVT\textsubscript{E}SEL\textsubscript{E}KD were introduced at the COOH-terminus of GFP. This sequence encodes the E\textsubscript{7\textsubscript{49}} epitope (bold) preceded by four NH\textsubscript{2}-terminal influenza A NP-derived amino acids and followed by a short spacer sequence (underlined) to ensure that antigen presentation required cytosolic processing. In the gene construct sE\textsubscript{7\textsubscript{49}}-GFP-NP\textsubscript{seq}, the two epitopes were introduced in reverse order, while the sequence of the flanking residues was maintained. The gene constructs were inserted into the retroviral vector pMX and were introduced into the indicated cell lines as described previously\textsuperscript{5}. Single cell clones were selected on the basis of identical GFP expression by flow cytometry.

Figures:

Suppl. Fig 1:

The introduction of NP or E7 epitope into the signal peptide does not hamper signal peptide cleavage. Cellular GFP products were obtained by immunoprecipitation from RMA cells that expressed cytosolic GFP-NP (cy), sGFP-NP (s), sE7-GFP-NP (sE), or sNP-GFP-E7 (sN). Cells were serum-starved with Met/Cys-free DMEM medium for 60 min prior to a 30 min pulse labeling with \textsuperscript{35}S-labeled Met/Cys. Labeled cells were washed with cold medium and GFP variants were precipitated with polyclonal rabbit anti-GFP. In vitro transcription/translation was performed with standard rabbit reticulocyte system (Promega) according to the Manufacturer’s protocol using pcDNA3.1 plasmids that encode the indicated GFP variant. Protein expression was analyzed by SDS-PAGE.
Suppl. Figure 2:

Secretion of GFP variants. RMA sE7-GFP-NP cells (a), RMA sNP-GFP-E7 cells (b), RMA cells (c) and RMA GFP-NP (d) (cytosolic location) were treated with Brefeldin A for 5 hrs (solid line) or left untreated (dotted line). GFP expression was determined by flow cytometry analysis. e: Indicated cell lines were serum-starved in Met/Cys-free DMEM medium for 60 min prior to a 30 min pulse labeling with 35S-labeled Met/Cys in DMEM medium containing 5% fetal bovine serum. Labeled cells were washed with cold medium and samples of cells and supernatant (sup) were harvested at indicated time points. GFP variants were immunoprecipitated with polyclonal rabbit anti-GFP. Samples were boiled and analyzed by SDS-PAGE.

Note: The supernatant of the cellular GFP-NP variant contains a substantial amount of GFP after 4 hrs of chase. This likely reflects GFP liberated from dying cells under these experimental conditions as the amount of GFP detected in the supernatant of continuously growing RMA GFP-NP is small, as revealed by Western blot analysis (data not shown).
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