Microenvironment and Immunology

Natural Killer Cells Efficiently Reject Lymphoma Silenced for the Endoplasmic Reticulum Aminopeptidase Associated with Antigen Processing

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Abstract

The endoplasmic reticulum aminopeptidase ERAAP is involved in the final trimming of peptides for presentation by MHC class I (MHC-I) molecules. Herein, we show that ERAAP silencing results in MHC-I peptide-loading defects eliciting rejection of the murine T-cell lymphoma RMA in syngeneic mice. Although CD4 and CD8 T cells are also involved, rejection is mainly due to an immediate natural killer (NK) cell response and depends on the MHC-I-peptide repertoire because replacement of endogenous peptides with correctly trimmed, high-affinity peptides is sufficient to restore an NK-protective effect of MHC-I molecules through the Ly49C/I NK inhibitory receptors. At the crossroad between innate and adaptive immunity, ERAAP is therefore unique in its two-tiered ability to control tumor immunogenicity. Because a large fraction of human tumors express high levels of the homologous ERAP1 and/or ERAP2, the present findings highlight a convenient, novel target for cancer immunotherapy.

Introduction

Antigenic peptides are generated in the cytoplasm as proteolytic intermediates by degradation of endogenous proteins through the multicatalytic proteasome and other proteases (1). Proteolytic intermediates are then translocated into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) and further processed by ER aminopeptidases, that is ERAAP in mice (2) and ERAP1 and ERAP2 in humans (3–5), before being loaded onto MHC class I (MHC-I) molecules.

Inhibition or loss of cytoplasmic proteases, the TAP transporter and components of the peptide-loading complex results in the marked downregulation of MHC-I expression on the cell surface (6–10). In contrast, inhibition of ER aminopeptidases by siRNA or in ERAAP-knockout mice causes a partial suppression not exceeding 60% on MHC-I surface expression (2, 5). Yet, immunization of ERAAP-knockout mice with cells from wild-type mice, or vice versa, results in potent CD8+ T-cell responses (11, 12), suggesting qualitative and quantitative alterations in the MHC-I-peptide (pMHC) repertoire. Consistent with these findings, mass spectrometric analysis revealed a marked increase in the length of endogenous peptides presented by MHC-I in mice lacking ERAAP (13), but whether or not ERAAP-dependent pMHC alterations affect innate immune responses is presently unknown.

In agreement with these studies, we have previously observed that an altered expression of ERAP1 and ERAP2 in human tumors as compared with their normal counterparts (14) results in an abnormal MHC-I expression (15), but how this might affect host immune responses against tumors (either adaptive or innate) is presently unknown.

To address this issue, ERAAP expression was stably reduced in the murine T-cell lymphoma RMA by transfection of ERAAP-targeted microRNA. Taking advantage of in vivo and ex vivo molecular and functional studies, we show herein that ERAAP-silenced RMA cells are readily rejected by syngeneic mice mainly through a tumor-specific natural killer (NK) cell response, due to poor pMHC-I engagement of Ly49C/I NK inhibitory receptors.

Materials and Methods

Mice and cell lines

Six- to 8-week-old inbred female C57BL/6 (Charles Rivers Laboratories) and C57BL/6 nude (Taconic) mice were maintained at the Animal Resource Facilities in accordance with the experimental ethics committee guidelines. RMA (16), RMA-S (16–19), and YAC-1 (20) T-lymphoma cell lines (courtesy of R.
Kiessling and A. Santoni) were authenticated by staining with H-2 allele-specific monoclonal antibodies (mAb).

**RNA interference**

Micro RNAs (miRNA) to mouse ERAAP (NM_030711) were cloned into the pcDNA6.2-GW/EmGFP-miR vector, transfected into RMA cells by electroporation (21), selected in blasticidin (Invitrogen), sorted for green fluorescent protein (GFP) expression, and cloned. For further details see Supplementary Material.

**Antibodies and peptides**

mAbs to H-2Kb and H-2Db (K204), H-2Kb (Y-3), H-2Db (B22.249), NK1.1 (PK136), CD4 (GK1.5), and CD8 (53-6-72) were used. Peptides SIINFEKL (22), ASNENMETM (23), and SLYNTVATL (24) and SIINFEAL (25) were used. A detailed list of commercial fluorescent antibodies for flow cytometry and peptide features used is provided in Supplementary Materials.

**Flow cytometry, immunofluorescence, protein biochemistry, and aminopeptidase activity**

Flow cytometry was done on FASCalibur and FASCantoII (BD-PharMingen). Acetone-fixed tumor sections were stained with specific mAbs and analyzed by confocal microscope. Western blotting, microsomal aminopeptidase activity tests, pulse-chase, and *in vitro* assembly were performed as described (15, 26, 27). For further details see Supplementary Materials.

**Surface stability**

The rate of generation of newly assembled pMHC-I complexes, the dissociation of preformed pMHC-I complexes, and the replacement of the endogenous MHC-I-binding peptides were detected as described (11, 25, 28). To detect the OVA257–264 peptide replacement, cells were stained with 25-D1.16 mAb (29).

**In vivo tumor challenge, immunodepletion, and peritoneal clearance assay**

Tumor cells (10⁴) were injected subcutaneously into the right flank of 6- to 8-week-old syngeneic C57BL/6 and C57BL/6 nude mice. Tumor growth was monitored every 2 days. For ethical reasons, animals were sacrificed when tumor reached a volume greater than 5 cm³. NK1.1⁺, CD4⁺, and CD8⁺ cells were depleted *in vivo* by intraperitoneal administration of 200 μg of PK136, GK1.5, or 53.6-72 at day −1, 0, 1, 3, 7, and 15 relative to tumor inoculation (30). Alternatively, tumor cells (10⁶) were injected intraperitoneally. Apoptosis of tumor (GFP⁺) cells was evaluated with APC-AnnexinV (BD-PharMingen) and propidium iodide (PI; Sigma-Aldrich), whereas peritoneal exudate (PE) leukocytes (GFP/C0) were stained with specific antibodies. For further details see Supplementary Materials.

**Interleukin 2-activated splenocytes**

Single-cell suspensions from C57BL/6 spleens were cultured (15 × 10⁶ cells/mL) with 100 ng/mL recombinant human interleukin 2.
interleukin 2 (IL-2; PeproTech) for 7 days. The cells were sorted on FACS Aria by gating on NK1.1<sup>+</sup>CD3<sup>−</sup>Ly49C/I<sup>+</sup> and NK1.1<sup>+</sup>CD3<sup>−</sup>Ly49C/I<sup>−</sup> and then cultured (2 x 10<sup>6</sup>/mL) for 40 hours with IL-2 and used as cytotoxicity effectors (25).

**NK functional assays**

NK cell cytotoxic activity was evaluated by a standard 5-hour <sup>51</sup>Cr release assay (31). The degranulation assay was performed by coculturing target and effector cells at a 1:1 ratio for 6 hours in the presence of GolgiStop (BD) and anti-CD107a (32–35), as described in detail in Supplementary Materials.

**Statistical analysis**

Statistical significance was assessed by the 2-tailed unpaired Student's t test. Survival data are presented as Kaplan–Meyer plots.

**Results**

**Downregulation of ERAAP affects MHC-I surface expression in RMA cells**

To assess the relevance of ER peptide trimming on tumorigenicity, we stably suppressed the expression of ERAAP in the murine T-cell lymphoma RMA of C57BL/6 origin by transfection of vectors encoding GFP in combination with microRNAs to either ERAAP (RMA-siERAAP) or a control, scrambled sequence (RMA-scramble). ERAAP mRNA levels were similar in parental cells and 10 RMA-scramble clones but were decreased, although to a different extent, in 31 distinct RMA-siERAAP clones (Supplementary Fig. S1). Eight representative RMA-siERAAP clones and 1 RMA-scramble clone selected at random (hitherto RMA-sc) were selected and tested by Western blotting to confirm specific ERAAP downregulation at the protein level. RMA-siERAAP clone 22 (RMA-siERAAP-22) showed the lowest ERAAP levels, corresponding to a relative decrease of 90% compared with the parental RMA cells and RMA-sc, whereas RMA-siERAAP clones 5, 6, 28, and 50 showed a less pronounced downregulation (Fig. 1A). Aminopeptidase activity in isolated microsomes from 3 representative RMA-siERAAP clones and control cells, RMA-sc and RMA cells, was proportional to the amount of the aminopeptidase expressed, whereas essentially no substrate cleavage was seen in microsomes from RMA cells treated with Leu-SH, a specific aminopeptidase inhibitor (Fig. 1B). MHC-I surface expression was decreased in these RMA-siERAAP clones by 48.5% to 56%, as assessed by K204 mAb, whereas no change in
the expression of control surface markers CD5 and CD45 was detected (Fig. 1C). MHC-I expression was similarly reduced (52%) in RMA cells treated with Leu-SH (Fig. 1C). Similar results were obtained using Y3 and B22.249 antibodies specific to H-2K\(^b\) and H-2D\(^\beta\), respectively (Supplementary Table SI).

Thus, downregulation of ERAAP affects both microsomal aminopeptidase activity and MHC-I surface expression in RMA cells.

**Instability of pMHC-I complexes in RMA-siERAAP cells**

ERAAP deficiency has been associated with alterations in MHC-I assembly and/or stability (11, 36). To verify whether reduced MHC-I surface expression in RMA-siERAAP clones is a consequence of the limited generation of stable pMHC-I complexes, the biochemical features of MHC-I expressed by RMA-siERAAP-22 cells were compared with those of RMA, RMA-sc, and TAP-defective RMA-S cells (37). In pulse-chase experiments (Fig. 2A), the conformational antibody Y-3 (37, 38) detected 20% less K\(^\beta\) in RMA-siERAAP-22 than in RMA and RMA-sc cells at the 0 \(^\circ\) chase point, and a further 20% reduction was evident at the 2-hour point despite complete maturation of endoglycosidase Endo H-\(^s\)-sensitive (s) into Endo H-resistant (r) K\(^\beta\) glycoforms (Fig. 2A). Much lower K\(^\beta\) levels and more than 85% decrease in the mature glycan component were instead detected in RMA-S cells, as expected (37, 38).

To investigate heavy chain (HC) stability and association with β\(_2m\), detergent-soluble H2-K\(^\beta\) molecules were tested by *in vitro* assembly in the absence and presence of a large excess of the high-affinity H-2K\(^\beta\)-binding peptide SIINFEKL at 2 different temperatures (Fig. 2B). Lesser amounts of K\(^\beta\) and associated β\(_2m\) were detected in RMA-siERAAP cells than in parental RMA and RMA-sc cells, although these 3 cells were much more similar to one another than to deeply defective RMA-S cells, which was confirmed (38) to be composed of low levels of thermally unstable, mostly immature, K\(^\beta\) glycoforms (Fig. 2B). Similar results were obtained using K204 mAb (Supplementary Fig. S2A). Thus, ERAAP silencing affects slightly, if at all, thermal stability and peptide receptivity *in vitro* but detectably impairs the spontaneous assembly and intracellular transport of K\(^\beta\) *in vivo*.

To further document MHC-I instability in RMA-siERAAP clones, the generation of newly assembled pMHC-I complexes and the dissociation of preformed pMHC-I complexes were evaluated in RMA-siERAAP clones. In the former approach, pMHC-I complexes were denatured on the cell surface with isotonic, low pH buffer and then allowed to regenerate by returning the cells to culture conditions (37), whereas in the latter approach, the surface expression of newly assembled MHC-I molecules was inhibited by treatment with brefeldin A. The reconstitution of MHC-I surface expression (monitored over time by flow cytometry with K204 mAb) was slightly

![Figure 3. Downregulation of ERAAP renders mice resistant to tumor challenge. A, C57BL/6 mice (n = 10 per group) were challenged by subcutaneous inoculation of 10\(^4\) RMA, RMA-S, RMA-sc, or RMA-siERAAP-22 cells. Tumor growth (mean ± SD) was monitored every 2 days. Statistically significant differences are indicated (*, P = 0.007). B, overall survival (Kaplan–Meier). Results are representative of 4 independent experiments. C, frozen tumor sections from mice sacrificed 12 days after challenge with RMA-sc and RMA-siERAAP-22 were stained with APC-NK1.1 or PECy5-CD8, nuclear counterstained with Hoechst 33342, and analyzed by confocal microscopy (left). CD8\(^+\) and NK1.1\(^+\) cells (percentage ± SD of infiltrating lymphocytes) were averaged in 3 microscopic fields (right). Scale bar, 40 μm.](https://cancerres.aacrjournals.org/)
slower in RMA-siERAAP cell clones 5 and 22 than in RMA and RMA-sc cells (Fig. 2C). After 5 hours in culture, control cells recovered 80% of the MHC-I surface expression, whereas RMA-siERAAP cell clones 5 and 22 recovered only 40% (Fig. 2C and Supplementary Fig. S2B). During the same period, the MHC-I dissociation rate was faster in RMA-siERAAP clones 5 and 22 than in RMA and RMA-sc cells (Fig. 2D), suggesting that a greater fraction of surface MHC-I in RMA-siERAAP cells is unstable.

Altogether, these results demonstrate that the downregulation of surface MHC-I expression in RMA-siERAAP cells is due to both a stoichiometric loss of MHC-I molecules since early biosynthetic stages, and the instability of pMHC-I complexes on the cell surface.

In vivo evaluation of host immune responses against RMA-siERAAP cells

To investigate whether ERAAP deficiency affects RMA tumorigenicity, we subcutaneously inoculated RMA-siERAAP-22, RMA-sc, RMA, and RMA-S cells in syngeneic C57BL/6 mice. All mice injected with control RMA and RMA-sc cells developed progressively enlarging tumors and died within 32 days, whereas mice injected with RMA-siERAAP-22 and RMA-S did not develop palpable tumors in most cases (Fig. 3A and B). Only 3 of 10 mice injected with RMA-siERAAP-22 and 2 of 10 mice injected with RMA-S developed palpable tumors within 15 days after inoculation (Fig. 3A and B). Of these, one RMA-siERAAP-22 and both RMA-S tumors completely regressed in a few days whereas the remaining 2 RMA-siERAAP-22 tumors grew very slowly and did not kill their hosts by day 50.

Rejection of RMA-siERAAP-22 is likely mediated by a host immune response because the in vitro proliferation rates and apoptotic state of RMA-siERAAP-22, RMA, and RMA-sc cells were very similar (Supplementary Fig. S3A and B). In line with this interpretation, confocal microscopy revealed a significant increase of tumor-infiltrating NK1.1<sup>+</sup> and CD8<sup>+</sup> cells in RMA-siERAAP-22 tumors as compared with RMA-sc tumors.
The increased number of CD8⁺ and NK1.1⁺ lymphocytes in RMA-siERAAP-22 tumors was further confirmed by flow cytometry of cell suspensions from tumor lesions (Supplementary Fig. S3C).

Altogether, these data indicate an effective host immune response against ERAAP downregulated RMA cells.

Rejection of RMA-siERAAP cells requires intact NK cells and CD8 and CD4 T-cell responses

To identify the immune effectors rejecting RMA-siERAAP-22 tumors, C57BL/6 mice were antibody depleted of CD4⁺, CD8⁺, and NK1.1⁺ cell populations and challenged by subcutaneous injection with RMA-siERAAP-22. Depletion of the
different lymphocyte subset restored the growth of RMA-siERAAP-22 during the 17-day observation period but only partially and to a different extent (Fig. 4A). The different contribution of each lymphocyte subset was more evident in the 40-day Kaplan–Meyer curve (Fig. 4B). Similar to mice treated with an irrelevant IgG and challenged with control RMA-sc cells, depletion of NK1.1<sup>+</sup> cells resulted in palpable tumors by day 15 (Fig. 4A) that rapidly grew causing death of all mice by day 30 (Fig. 4B). In contrast, depletion of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells had much slighter effects, resulting in a slower tumor growth (Fig. 4A) and death of 40% and 20% of the mice, respectively, 5 weeks after challenge (Fig. 4B). Remarkably, combined NK1.1<sup>+</sup>/CD4<sup>+</sup> depletion, with or without CD8<sup>+</sup> depletion, as well as NK1.1<sup>+</sup>/CD8<sup>+</sup> depletion, completely restored RMA-siERAAP-22 tumor growth to RMA-sc levels. In contrast, the effect of double CD4<sup>+</sup>/CD8<sup>+</sup> depletion was marginal during the first 17 days (Fig. 4C) and resulted in delayed tumor growth and death of 60% of mice 29 days after challenge (Fig. 4D). Treatment with an irrelevant IgG did not affect tumor rejection (Fig. 4). Finally, C57BL/6 nude mice efficiently rejected RMA-siERAAP-22 and RMA-S cells as immunocompetent mice, emphasizing the minor contribution of CD4<sup>+</sup> and CD8<sup>+</sup> T cells to tumor control (Supplementary Fig. S4).

Thus, rejection of RMA-siERAAP mainly depends on NK1.1<sup>+</sup> cells, but complete tumor control is sustained by a synergy between innate (NK1.1) and adaptive (CD4 and CD8) immune subsets.

Specific recognition of RMA-siERAAP cells by NK cells

To demonstrate specific recognition by NK cells, RMA-siERAAP-22 cells were compared to the well-known NK-susceptible targets YAC-1 and RMA-S in their ability to induce the expression of CD107a (32–35), a marker of granule exocytosis, and the production of IFN-γ in splenic NK cells. Remarkably (Fig. 5), NK cell activation by RMA-siERAAP-22, YAC-1, and RMA-S was similar and much greater than that by RMA and RMA-sc cells.

Next, RMA-siERAAP-22 cell targets and NK cell effectors were studied as single-cell suspensions in a model of intra-peritoneal tumor growth (39, 40). RMA-siERAAP-22 cells and RMA-sc cells were injected intraperitoneally into C57BL/6 mice, either treated with an irrelevant IgG or were NK1.1 depleted. PE cells were harvested at different times and analyzed by flow cytometry for the presence of GFP<sup>+</sup> tumor cells and GFP<sup>+</sup> lymphocyte subsets (Fig. 6 and Supplementary Fig. S5).

Similar to subcutaneous injection experiments (Figs. 3 and 4), RMA-siERAAP-22 cells were rejected, but they grew in NK1.1-depleted mice as RMA-sc cells grow in control mice (Fig. 6A). Interestingly, GFP<sup>+</sup> RMA-siERAAP-22 cells began to decrease at 12 hours in the peritoneal cavity and disappeared almost completely at 48 hours (Fig. 6A). Their staining with AnnexinV and PI revealed very rapid killing kinetics, early apoptotic cells (AnnexinV<sup>+</sup>/PI<sup>−</sup>) appearing at 4 hours or earlier, and progressively turning into late apoptotic cells (AnnexinV<sup>+</sup>/PI<sup>+</sup>) at later times (Fig. 6B). Remarkably, apoptotic death was negligible when the same RMA-siERAAP-22 cells were injected into NK1.1<sup>−</sup>-depleted mice or RMA-sc cells were injected into C57BL/6 mice (Fig. 6B). The role of NK cells as mediators of this apoptotic death was strongly supported by the increased number of NK1.1<sup>−</sup>/CD3<sup>+</sup> cells in PE from mice inoculated with RMA-siERAAP-22 at 4 hours (Fig. 6C), that is, coinciding with the beginning of apoptotic decline. Likewise, the virtual absence of NK1.1<sup>−</sup>/CD3<sup>+</sup> PE cells ruled out an unlikely involvement of NK T cells in tumor rejection (Fig. 6C).

These results conclusively document the direct participation and major role of NK cells at early stages of RMA-siERAAP tumor control and demonstrate that 12 hours following tumor challenge are sufficient to provide PE cells with the ability to kill RMA-siERAAP cells.

Ex vivo evaluation of NK immune responses induced by RMA-siERAAP cells

To characterize NK cells infiltrated into the peritoneal cavity upon inoculation of tumor cells, PE cells from mice inoculated

![Figure 7. Peptide-specific protection of RMA-siERAAP cells against NK-mediated cytotoxicity.](image)
with RMA-siERAAP-22 cells, RMA-sc cells, or saline at different times were used as effectors on YAC-1, RMA-S, RMA-siERAAP-22, and RMA-sc cells in a standard 

\[ ^{51} \text{Cr} \] release assay (ref. 39; Fig. 6D). PE cells from mice inoculated with saline were poor effectors and failed to reveal any difference in NK susceptibility among YAC-1, RMA-S, RMA-sc, and RMA-siERAAP-22. In contrast, PE cells from mice inoculated with either tumor were much better effectors for all the 4 targets, particularly for YAC-1 and RMA-siERAAP-22 after 4 and 12 hours from injection, respectively. This increased lytic activity was further confirmed by a degranulation assay (Fig. 6E).

Next, we assessed the ability of PE cells from mice inoculated with RMA-siERAAP-22 to kill RMA-siERAAP-22 in which the endogenous MHC-I peptides were exchanged by incubation with a large excess of the high-affinity peptides ASNENMETM and SIINFEKL (22). As expected, the MHC-I–specific K204 mAb did not detect any significant difference regardless of the peptide added. In contrast, 25-D1.16 mAb (29) specific to the H-2Kb–bound peptides, but not the irrelevant SLYNTVATL ligand, totally protected RMA-siERAAP-22 from PE cell–mediated lysis (Fig. 7B). Thus, PE cells preferentially kill RMA-siERAAP-22. Moreover, replacement of preexisting endogenous peptides is necessary and sufficient to restore the NK-protective effect of MHC-I molecules, suggesting that MHC-I misfolding entirely depends on the peptide pool of RMA-siERAAP cells.

**RMA-siERAAP cells are lysed by NK cells, due to improper recognition of pMHC-I complexes by Ly49C/I**

To identify the NK cell receptor(s) involved in the rejection of RMA-siERAAP cells, we focused our attention on Ly49C/I, the main MHC-I–monitoring inhibitory receptors expressed in H-2\(^{b}\) mice (41). Ly49C/I receptor engagement has been shown to depend on the composition of MHC-I–bound peptides (25, 42–44). RMA-siERAAP and RMA-sc cells were tested as targets of NK1.1\(^+\)CD3\(^+\)Ly49C/I\(^+\) and NK1.1\(^+\)CD3\(^+\)Ly49C/I\(^-\) effector cells isolated from IL-2–activated splenocytes of C57BL/6 mice. NK1.1\(^+\)CD3\(^+\)Ly49C/I\(^+\) effector cells efficiently killed RMA-siERAAP-22 cells but not RMA-sc cells (Fig. 7C). Interestingly, as for PE-mediated lysis (Fig. 7B), loading with SIINFEKL peptide, but not with the irrelevant SLYNTVATL ligand, totally protected RMA-siERAAP-22 from lysis (Fig. 7C). As expected, loading with the K7A-substituted OVA-derived peptide SIINFEAL, known to support K\(^b\) assembly but contribute poor RMA-S protection from NK lysis (25), only partially protected RMA-siERAAP-22 (Fig. 7C). As previously described for RMA and RMA-S cells (25), NK1.1\(^+\)CD3\(^+\)Ly49C/I\(^-\) effector cells killed RMA-sc and RMA-siERAAP target cells with similar efficiency.

Thus, NK cells lyse RMA-siERAAP cells because pMHC-I complexes fail to engage the inhibitory Ly49C/I receptors.

**Discussion**

Herein, ERAAP downregulation in RMA lymphoma cells was shown to induce NK cell–mediated, and subsequently T-cell–mediated (CD4 and CD8), rejection in syngeneic mice. To our knowledge, this is the first demonstration that ERAAP regulates tumorigenicity. CD8 involvement might have been anticipated from previous studies, because in ERAAP-knockout mice a marked alteration in the pMHC-I repertoire (11, 12) resulted in potent CD8 T-cell responses. In contrast, a major NK cell triggering effect was particularly surprising and totally unanticipated, because ERAAP downregulation caused a modest (about 50%) suppression of MHC-I expression in all studies (2, 11, 12, 36) including the present one.

Our RMA-siERAAP clones display alterations in MHC-I surface expression and surface turnover similar to those seen in knockout mice (11, 36). In addition, these clones show a progressive stoichiometric loss of MHC-I molecules, evident since early biosynthetic stages, resulting in a reduced accumulation of β\(_2\)m-associated, conformed heavy chains. These alterations are much smaller than those seen in TAP-defective RMA-S cells (16), and yet they indirectly document a subtle conformational change that appears to be sufficient to completely derange MHC-I interactions with the prominent NK receptor and MHC-I folding sensor Ly49C/I. This effect is not due to a simple quantitative reduction in surface MHC-I, as in vitro assembly and in vivo pulse with canonical, trimmed peptides rescued peptide-specific conformational epitopes, as well as receptor engagement. It is also highly unlikely to result from a direct interference of an altered pMHC-I peptide pool with Ly49 binding, because the crystallographic Ly49C footprint on K\(^b\) molecules involves a broad cavity beneath the floor of the peptide-binding groove opposite to, and not including, bound peptides (44). To explain how different peptides might differently affect receptor engagement, it has been suggested that peptide-selective conformational changes are transmitted from the peptide-filled groove to the H-2K\(^b\)/Ly49C interface through a “domino” effect involving successive positional adjustments of not only heavy chain but also β\(_2\)m residues (25, 41). Altogether, in the context of the alterations to the pMHC-I pool previously described in ERAAP knockouts (11), the presently described decreases in MHC-I stability, β\(_2\)m association, and intracellular transport, as well as the peptide-reversible susceptibility to NK cell lysis of RMA-siERAAP cells, are fully consistent with crystallographic models of peptide-driven/conformation-dependent Ly49C engagement.

In summary, and quite strikingly, MHC-I molecules synthesized in cells impaired in N-terminal peptide trimming have been shown by others to be sufficiently conformed to present antigen to CD8 T cells (12) and by us to be insufficiently conformed to inhibit NK cells. Our immune subset depletion experiments provide in vivo evidence for both mechanisms, NK cell killing of tumor cells being clearly preponderant and occurring at an earlier stage than CD4-mediated and CD8-mediated rejection. Indeed, by inciting recognition of missing self and altered self at the same time, ERAAP appears to regulate tumor immunogenicity at a crucial crossroad between innate and adaptive immunity.

In the context of this synergistic effect, NK cells are clearly the major players not only because they contribute the major fraction of the phenotypically observed tumor control but also
because the immediate burst of NK-mediated apoptotic death leading to massive tumor clearance \textit{in vivo} begins just 4 hours following tumor challenge, for example, at a time that is inconsistent with any adaptive response. On the other hand, our \textit{in vivo} experiments show that depletion of CD4 and CD8 subsets results in late tumor growth, which in most instances does not kill mice for up to 50 days. This favors the possibility that CD4 and CD8 effectors are involved in the control of RMA-siERAAP tumors that have escaped NK attention and is reminiscent of the equilibrium phase of immune editing models of tumor immune surveillance (45).

In humans, we have previously shown that most tumor cells (15) and neoplastic tissues (14) express from intermediate to high levels of ERAp1 and ERAp2, tumor cell lines falling in the lowest 10\% range of expression for either aminopeptidase being a minority. In light of the dramatic enhancement on NK cell lysis presently observed by reducing ERAp expression to a residual 10\%, it is tempting to speculate that ERAp-low tumor phenotypes are subjected to immune counterselection in human patients. It may be of interest to determine whether in humans, as in mice, such a low expression in tumor lesions is associated with increased NK cell and T-cell infiltrates and a favorable outcome due to tumor cell killing by both innate and adaptive immunity.

Suppression of peptide trimming, using small drugs specifically inhibiting ERAp activity, may be easier than interfering with peptide transport by TAP. Pharmaceuticals mimicking the functional consequences of ERAp interference might unveil novel NK-mediated immunotherapeutic approaches for treatment of cancer.

Disclosure of Potential Conflicts of Interest

The authors have declared that no conflict of interest exists.

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