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J Immunol published online 22 April 2013
http://www.jimmunol.org/content/early/2013/04/21/jimmunol.1300220

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/04/23/jimmunol.1300220.DC1

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Induction of Protective Antitumor Immunity through Attenuation of ERAAP Function

Edward James,*† Ian Bailey,*† Gessa Sugiyarto,*† and Tim Elliott*†

The endoplasmic reticulum aminopeptidase associated with Ag processing, ERAAP, plays an important role in the trimming of antigenic peptides for presentation at the cell surface complexed with MHC class I molecules. Tumors express varying levels of ERAAP, highlighting a possible mechanism of immune-evasion through alteration of the peptide repertoire. Using the CT26 tumor model, we investigated the effects of ERAAP modulation on peptide presentation and the use of ERAAP inhibition as an antitumor therapy. We show that generation of the cross-protective tumor Ag GSW11 in the colorectal carcinoma CT26 is increased when ERAAP expression is reduced. BALB/c mice with reduced ERAAP expression challenged with CT26 induced protective immunity that was mediated by CD8⁺ T cells. This antitumor immunity also protected mice when rechallenged with wild-type CT26 tumor; strong CD8⁺ T cell responses to GSW11 were observed, despite its presentation being considerably lower. Furthermore, boosting the tumor immunogenicity through inhibition of ERAAP function with the small molecule inhibitor leucinethiol in vitro, or in established tumors in vivo, abrogated tumor growth and prolonged survival. Thus, our results highlight the promising possibility of using modulation of ERAAP to generate protective antitumor responses as a strategy for cancer immunotherapy. The Journal of Immunology, 2013, 190: 000–000.

The repertoire of antigenic peptides derived from intracellular proteins consists of thousands of different 8–10-aa peptides, many of which are generated as N-terminally extended precursors following partial hydrolysis of substrates by cytosolic proteases, including the proteasome (1, 2). These are then transported into the endoplasmic reticulum by the TAP. A key final step of the pathway involves aminopeptidases, which trim the extra N-terminal residues to generate peptides (1) that are an optimal length for loading onto MHC class I (MHC I) molecules. This completes MHC I biogenesis and permits exit from the endoplasmic reticulum to the cell surface (3–6), where they present the processed peptides to CTL. In humans, two aminopeptidases perform this function, ERAP1 and ERAP2 (7–9); in mice, only one aminopeptidase exists, ERAAP (10). ERAAP is critical for the generation of many antigenic epitopes in vivo and can influence the generation of the antigenic peptide repertoire (11–14). ERAAP can also destroy antigenic epitopes by trimming them to lengths too small for MHC binding, thus its characterization as an antigenic peptide editor (12).

In tumors, expression of ERAP1 and ERAP2 was shown to vary considerably, with high/medium levels in skin cancer and melanoma and low levels in breast and lung cancer (15–17). Varying expression of ERAP1/2 in tumors could indicate a mechanism by which the tumors evade detection by CTL through alteration of the peptide repertoire presented by MHC I, perhaps reducing the generation of dominant tumor-derived peptide epitopes either by reduced trimming activity or by destroying them by overtrimming.

To investigate the contribution of ERAAP to immunogenicity and immunological control of tumor growth, we used the transplantable murine colon carcinoma model CT26 and modulated ERAAP levels through small interfering RNA (siRNA). This model allowed us to investigate the correlation among ERAAP expression, the generation of well-characterized tumor epitopes, and protective and durable antitumor immunity. We found that reducing ERAAP levels enhanced tumor immunogenicity, thereby opening a window of opportunity for the potential use of small molecule ERAAP inhibitors to enhance antitumor immunity. Accordingly, treatment of CT26 with the ERAAP inhibitor leucinethiol (leuSH) prior to injection or following tumor establishment in vivo led to prolonged survival.

Materials and Methods

Cell lines and hybridomas

All CT26 and P815 tumor cells, COS-7 cells, and the GSW11-specific lacZ-inducible CD8⁺ T cell hybridoma CCD2Z were maintained as previously described (18). In all experiments, mice were injected s.c. with 10⁵ CT26 tumor cells in PBS. All cell lines were tested and were mycoplasma free.

Mice, Abs, and in vivo depletion

BALB/c mice were bred locally under specific pathogen-free conditions and were used at 6–8 wk of age. Mice were housed in conventional facilities during experimental procedures. All experiments were carried out under license according to local ethical committee approval under United Kingdom Home Office license guidelines. Hybridomas secreting CD8⁺ (YTS 169.4.2.1, YTS 156.7.7, both rat IgG2b) and CD4⁺ (YTS 191.1.2, YTA 3.1.2, both rat IgG2b)-specific mAb were described previously (19) and were grown in culture and purified by precipitation in saturated ammonium sulfate. For depletion, mice were injected with 100 μg each mAb twice with a 1-d interval. For intratumoral administration of leuSH, mice were challenged with CT26 tumor, and 100 μM leuSH in 0.1 M DTT in 50
μl was injected into the tumor at days 7 and 10. Controls received DTT-only injections.

**T cell–activation assays**

The GSW11-specific lacZ-inducible T cell hybridoma CCD2Z was co-cultured with CT26 (wild-type, ERAAP knockdown or inhibitor treated) or APCs that were transfected with Ag cDNAs. The lacZ activity was measured as described previously (20). For inhibitor treatment of CT26, cells were incubated with 10 μM MG132 (Calbiochem), 100 μM AAF-CMK (Calbiochem), or 30 μM leuSH (Sigma) for 16 h. Following incubation, the treated CT26 were assayed for stimulation of CDD2Z.

**siRNA and short hairpin RNA knockdown**

For stable ERAAP knockdown, ERAAP-specific siRNA (CT26-ERAAPmixed) or scrambled siRNA (ERAAPsh CT26) was cloned into the short hairpin RNA (shRNA) expression vector pSilencer 4.1 CMV/hygro (Life Technologies) and transfected into CT26 using FuGENE6 (Roche), according to the manufacturer’s instructions. After 24 h, transfected cells were selected by the addition of 0.5 μg hygromycin. CT26-ERAAPmixed cells were subcloned and screened for ERAAP protein knockdown using Western blot of CT26 cell lysates.

**Extraction and HPLC analysis of endogenously processed peptides**

Total acid-soluble peptide pools from tumor cells CT26, CT26-ERAAPmixed, ERAAPsh CT26, and ERAAPsh CT26 were extracted as described (12). Briefly, 10^7 cells were washed with PBS and extracted with 400 μl 10% boiling formic acid in the presence of a 10 μM "martyr" peptide ASNNEMETM for 10 min. Cellular debris was removed by centrifugation and fractionated by HPLC after filtration through a 10-kDa filter (Millipore, Billerica, MA). Reverse-phase C18 column (Grace Vydac, Hesperia, CA; 2.1 x 250 mm, 5 μM, 300 Å) was run in 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). The gradient was from 17 to 45% (solvent B) at a rate of 1%/min, with fractions collected and dried in a vacuum centrifuge. Mock injections with sample buffer alone were performed prior to each extracted sample, using the same column and identical run conditions. The collected fractions were assayed in the same experiment, using the same APCs and T cells, in parallel.

**CD8+ T cell responses and MHC I expression**

Spleen and draining lymph node cells from CT26-challenged mice (wild-type or ERAAP knockdown) were harvested between days 10 and 12. The CD8+ T cell responses to GSW11 and AH1 Ags were measured by production of IFN-γ by those cells in response to incubation with peptide. CD8+ T cells, APCs, and peptides were cultured together for 4 h in the presence of brefeldin A before being stained for surface CD8 and intracellular IFN-γ (BD Biosciences). For assessment of cell surface MHC I expression, H-2D^d-FITC (34-2-12) and H-2L^d-FITC (B22) Abs were incubated with CT26 for 30 min on ice and washed. Cells were then analyzed on a FACScanto (BD Biosciences), and data were analyzed with FlowJo software (TreeStar). CD8+ lymphocytes were gated, and the percentage of IFN-γ-producing cells was calculated from total CD8+ T cells.

**Statistical analysis**

Statistical significance was assessed by the two-tailed paired Student t test or one-way ANOVA, using Bonferroni multiple-comparison posttest. Survival data are presented as Kaplan–Meier plots.

**Results**

**Destructive processing of tumor Ag GSW11 by ERAAP**

In the absence of vaccination, protective immunity against the transplanted CT26 colon carcinoma requires the induction of a strong CTL response against the GSW11 epitope (18). Anti-GSW11 CTLs are preferentially suppressed by regulatory T cells (Tregs) and are only allowed to expand when Tregs are depleted prior to tumor challenge (18). Because GSW11-specific CD8+ T cell responses are important for tumor rejection, we were interested to know how the GSW11 epitope (GGPESFYCASW) was generated by proteases. Because GSW11 is relatively “long” for a CTL epitope, does not conform to the canonical H-2D^d binding motif (XGPXXXXX[I,L, F]) (21), and has a very low affinity for MHC I (t1/2 = 20 min) (18), we wondered whether it would be unusually susceptible to over- trimming by ERAAP, as observed for other CTL epitopes (12).

We incubated CT26 tumor cells with MG132 (a proteasome inhibitor), AAF-CMK (an inhibitor of tripeptidyl peptidease II [TPPII]), and leuSH (an ERAAP inhibitor). We also transfected COS-7 cells with the gp70 cDNA construct C8 (which contains the GSW11 epitope) and/or a TAP inhibitor derived from human herpesvirus 1, ICP47 (22, 23). The effect of the inhibitors on the generation of GSW11 and its cell surface presentation by H-2D^d were assessed using the GSW11-specific T cell hybridoma CDD2Z, which is able to detect nanomolar concentrations of presented peptide (Supplemental Fig. 1) (18). CDD2Z is a fusion between the GSW11-specific CTL, CDD2, and BWZ.36 CD8ε fusion partner that contains an inducible reporter gene encoding β-galactosidase (lacZ) (24). Activation of the CDD2Z T cell hybridoma is assayed by measuring lacZ activity. MG132-treated CT26 and C8 and ICP47 cotransfection resulted in complete abrogation of CDD2Z stimulation (Fig. 1A, 1B). AAF-CMK treatment had no effect on CDD2Z stimulation, indicating that TPPII has no role in the generation of GSW11 (Fig. 1A). In contrast, incubation of CT26 with leuSH resulted in an increase in CDD2Z stimulation (Fig. 1A). Therefore, we concluded that, although generation of GSW11 requires both the proteasome and TAP for presentation, it is over- trimmed and destroyed by ERAAP, resulting in a steady-state level of presentation that is detectable but low.

To confirm the role of ERAAP in destroying GSW11, CT26 tumor cells were transfected with ERAAP-specific siRNA. LaminB1-specific siRNA knockdown was used as a control. ERAAP siRNA knockdown resulted in an increase in CDD2Z stimulation (Fig. 1C), consistent with the results following leuSH treatment (Fig. 1A). As expected, laminB1 knockdown of CT26 did not affect CDD2Z stimulation (Fig. 1C). The increase in GSW11 generation occurred despite an overall decrease in MHC I levels; MHC I downregulation is typically observed in ERAAP-knockout cells (Fig. 1D) (11–14). This confirmed that the generation of GSW11 is ERAAP sensitive.

**Level of anti-GSW11 CTL stimulation corresponds to the level of ERAAP expression in CT26**

To investigate the effect of GSW11 presentation on antitumor immunity, we created stable ERAAP knockdown lines of CT26 using an ERAAP-specific shRNA vector. A polyclonal line was established (CT26-ERAAPmixed) from which two clones were generated that showed greater stimulation of CCD2Z than control-transfected CT26 (Fig. 2A). Analysis of ERAAP expression revealed that these showed an ~80% (ERAAP^low CT26) and 65% (ERAAP^med CT26) reduction in protein levels (Fig. 2B), thus establishing a correlation between the level of ERAAP expression and the level of CDD2Z stimulation.

**ERAAP expression is directly correlated with the quantity of GSW11 presented**

To confirm that the increase in CDD2Z stimulation observed with reduced ERAAP protein was due to an increase in the generation of GSW11, we generated peptide extracts from tumor cells with different ERAAP expression levels. These were fractionated by reverse-phase HPLC, and the amount of GSW11 was quantified in the fractionated mixture using CDD2Z as a biological detector. The profile obtained from CT26 peptide extracts revealed one significant peak that coeluted with synthetic GSW11 (Fig. 3A). Analysis of the polyclonal line CT26-ERAAPmixed revealed a 3-fold increase in GSW11 compared with CT26 (Fig. 3B). Greater increases in GSW11 were observed for ERAAP^med and ERAAP^low CT26 (8- and 30-fold increases, respectively; Fig. 3C, 3D). Importantly, the amount...
of GSW11 generated correlated with the amount of ERAAP protein present within the cells. The presence of an additional peak in ERAAP-knockdown CT26 extracts eluting ahead of GSW11 is also seen in CT26 cells when developed for a longer period of time; this was shown to be an oxidized adduct (or dimer) that probably forms during the extraction process (18). Therefore, we concluded that the increased response of CCD2Z observed after ERAAP knockdown is due to an increase in the amount of GSW11 generated in these cells.

Silencing ERAAP causes tumor rejection

Having shown that ERAAP-knockdown CT26 generated and presented significantly more GSW11 compared with CT26, we wanted to determine whether the increase in GSW11 presentation was able to overcome Treg suppression and induce GSW11-specific T cells in vivo. We inoculated BALB/c mice with ERAAP lo, ERAAP med, or untreated CT26 and assessed tumor growth and survival. Mice inoculated with CT26 had detectable tumors from day 8 that continued to grow until reaching the humane end point, with a median survival time of 20 d (Fig. 4A, 4C). All mice challenged with ERAAP lo and ERAAP med CT26 had tumor growth at days 8–12 that was subsequently cleared in the majority of cases (8/12), indicating a role for an effective adaptive antitumor response in these mice. Mice challenged with ERAAP lo CT26 showed a significant increase in survival time compared with CT26, with >90% of mice rejecting the tumor (p < 0.001; Fig. 4A, 4C). Inoculation with ERAAP med CT26 induced tumor protection in 50% of mice (Fig. 4A), with a median survival time of 64.5 d compared with 20 d for CT26 (p < 0.005; Fig. 4A, 4C). These results showed that the increased generation of GSW11 resulting from ERAAP knockdown produced a protective antitumor response whose intensity correlated negatively with the level of ERAAP expression. To investigate the cells responsible for this tumor protection, we depleted mice of either CD4 or CD8 T cells prior to challenge with ERAAP lo CT26. Depletion of CD8 T cells completely abrogated the ability to clear tumor (Fig. 4B). Depletion of CD4 T cells also resulted in a small decrease in tumor rejection, although the majority of mice survived tumor challenge (Fig. 4B), confirming that the rejection of ERAAP lo CT26 tumor was primarily mediated by CD8+ T cells.
ERAAP<sub>lo</sub> CT26 induces CD8<sup>+</sup> T cell responses to CT26 Ags GSW11 and AH1

To investigate the specificity of the anti-ERAAP<sub>lo</sub> CT26 response in more detail, we inoculated mice with either ERAAP<sub>lo</sub> CT26 or CT26 and assessed the anti-AH1 and anti-GSW11 CD8<sup>+</sup> T cell responses in spleens and draining lymph nodes using intracellular cytokine staining. As shown previously (18), anti-AH1 CD8<sup>+</sup> T cell responses were observed following inoculation with CT26 (~0.4%), with no GSW11-specific responses detected above that seen in unstimulated controls (Fig. 5A). Interestingly, despite detectable anti-AH1 responses in tumor and draining lymph nodes, these mice succumb to tumor by day 25. In contrast, all mice challenged with ERAAP<sub>lo</sub> CT26 had GSW11-specific CD8<sup>+</sup> T cell responses that were much greater than those observed in CT26-challenged mice (~0.75%, p < 0.001; Fig. 5A), similar to those observed in mice depleted of Tregs (18). The anti-GSW11 response dominated over AH1-specific responses (~0.75% versus ~0.6%; p < 0.05) in these mice, even though the latter were also increased compared with CT26 challenge (~0.6% versus ~0.4%; Fig. 5A). Because >90% of mice challenged with ERAAP<sub>lo</sub> CT26 reject the tumor, the correlation of protection and the appearance of GSW11-specific CD8<sup>+</sup> T cells suggests the importance of these cells in clearance of the tumor. Although AH1-specific CD8<sup>+</sup> T cells were also increased, the modest difference suggests that these responses are unlikely to play a major role in tumor clearance.

Having shown that challenge with ERAAP<sub>lo</sub> CT26 induces robust protective antitumor immunity that is CD8 dependent, we went on to show that tumor challenge also induced immunological memory.

FIGURE 3. Knockdown of ERAAP expression increases the amount of GSW11 generated. Peptides were extracted from CT26 (A), CT26-ERAAPmixed (B), ERAAP<sub>med</sub> CT26 (C), or ERAAP<sub>lo</sub> CT26 (D) cells and fractionated on a reverse-phase C18 column by HPLC. Each fraction was dried, resuspended in PBS, and tested for its ability to stimulate lacZ activity in CCD2Z hybridoma with P815 cells as APCs. The arrow indicates the peak activity of synthetic GSW11 peptide run under identical conditions. Data shown are representative of four experiments.

FIGURE 4. ERAAP<sub>lo</sub> CT26 induces CD8<sup>+</sup> T cells capable of mediating tumor rejection. BALB/c mice were challenged with wild-type (n = 6), ERAAP<sub>med</sub> (n = 6), or ERAAP<sub>lo</sub> (n = 7) CT26, and tumor survival (A) and tumor growth (C) were assessed. **p < 0.01, ***p < 0.001, versus CT26. (B) Untreated CD4<sup>+</sup> or CD8<sup>+</sup> T cell–depleted ERAAP<sub>lo</sub> CT26–challenged mice were assessed for tumor survival. Control mice were challenged with wild-type CT26 (n = 5/group). *p < 0.05, versus ERAAP<sub>lo</sub> CT26. Data represent two independent experiments with at least five mice/group.
We rechallenged ERAAPlo CT26 immune mice with ERAAPlo CT26 50 d after the primary challenge. All mice that rejected the first tumor challenge also rejected the second (Fig. 5C). Examination of CD8+ T cell responses in mice 10 d after rechallenge revealed increased anti-GSW11 and anti-AH1 T cell responses compared with those observed in the primary challenge with dominant anti-GSW11 responses (~2.25% versus ~1%, p < 0.01; Fig. 5D).

To consider ERAAP silencing as a potential antitumor therapy, the induced immunity following primary challenge with ERAAPlo CT26 would also need to be effective against tumor cells with normal levels of ERAAP. ERAAP silencing may not be effective in all tumor cells or those at additional sites in the body. The presence of anti-AH1 and anti-GSW11 CD8+ T cell responses in ERAAP lo CT26–challenged mice suggests that ERAAPlo CT26 immune mice would be able to clear wild-type CT26, despite a lower level of epitope production. To assess this, ERAAP lo CT26 immune mice were immunized with CT26 50 d after primary tumor challenge. All ERAAP lo CT26–immune mice were protected from CT26 tumor challenge, whereas control naive mice succumbed to the tumor by day 25 (Fig. 5C). Assessment of CD8+ T cell responses 10 d following CT26 challenge revealed strong anti-GSW11 and anti-AH1 responses greater than those observed after primary ERAAPlo CT26 challenge (2 and 1.25% versus 0.8 and 0.6%; Fig. 5A, 5E). This suggests that the dominant protective CD8+ T cell responses in these mice were directed toward GSW11 and AH1 peptide epitopes. Therefore, these memory CTL responses to epitopes shared between ERAAPlo CT26 and CT26 (GSW11 and AH1) allow rejection of CT26 tumor following rechallenge, even though the presentation of these epitopes is considerably lower in the latter. The results also highlight the validity of attenuating ERAAP function as a strategy for the induction of protective antitumor immunity.

Chemical inhibition of ERAAP prolongs tumor survival in vivo

We investigated whether attenuation of ERAAP activity in CT26 with leuSH treatment either prior to injection or in situ would trigger tumor rejection. Treatment of CT26 with LeuSH in vitro results in a transient inhibition of ERAAP that persists for 24–48 h (Supplemental Fig. 2). Mice challenged with CT26 treated in vitro with leuSH showed prolonged survival in four of seven recipients (Fig. 6A; p < 0.05), with two mice surviving beyond day 50. Tumor was present in these two mice but was stable (Fig. 6C), indicating that a protective anti-CT26 response was induced that favored tumoristasis over rejection.

We next investigated whether inhibition of ERAAP by topical delivery of leuSH could prolong survival in mice where tumor was established. CT26-challenged mice with a palpable tumor (~0.5 cm³) were injected intratumorally with leuSH at day 7, with a repeat dose given at day 10. Intratumoral administration of leuSH resulted in the attenuation of tumor growth, leading to prolonged survival in five of seven mice (p < 0.05; Fig. 6B, 6D). Tumor growth was arrested in three mice, with two showing a dramatic reduction in tumor size (Fig. 6D). In all instances of tumor growth arrest, the tumor size remained stable throughout the remainder of the experiment, again indicating the presence of a tumoristatic T cell response. Control mice injected with DTT alone showed no retardation of tumor growth (Fig. 6D). Attenuation of tumor growth in most mice after inhibition of ERAAP function provides proof of principle that small molecule inhibitors of ERAAP might have therapeutic value, particularly for tumors in which high expression correlates with poorer prognosis.

Discussion

We used ERAAP silencing to induce a robust protective antitumor immunity to CT26 and show that this immunity is CD8+ T cell mediated, which also protects against challenge with wild-type CT26. Although it is not known whether modulation of ERAAP expression or activity leads to immune-evasion by human tumors, it is interesting to note that ERAIP1/2 expression in human cancers is variable; one study (15) showed that, unlike ovarian, breast, and lung cancers, which tended to downregulate ERAAP expression, colorectal adenocarcinoma showed enhanced expression relative
to normal gut tissue. This observation is consistent with the model that we investigated in which a potent tumor epitope is over-processed by ERAAP, rendering it cryptic. Downregulation of ERAAP in CT26 led to the increased generation of protective epitopes and, in particular, the cryptic epitope GSW11, leading to strong antitumor CTL and tumor rejection. Importantly, ERAAPlo CT26–immune mice were also able to reject CT26 when challenged 50 d after rejection of the primary tumor. We also showed that transient ERAAP inhibition with leuSH attenuated tumor growth in established tumors, demonstrating the potential of this strategy as a therapy.

ERAAP-deficient cells were shown to present peptides with N-terminal extensions, as well as novel peptides uncovered in the absence of ERAAP (21, 25, 26). Interestingly, some peptide epitopes were overtrimmed by ERAAP, highlighting its role as a peptide editor (12). The increased T cell responses, together with the 30-fold increase in peptide generation when ERAAP is downregulated/inhibited, strongly indicate that the potent antitumor epitope GSW11 is overtrimmed. The precise mechanism for peptide trimming by ERAAP is not known; however, from other examples of aminopeptidases, it is likely that it trims a single amino acid at a time in a bind, trim, release mechanism. This suggests that ERAAP must compete for peptide binding with other molecules, such as MHC I, and this competition would ultimately determine whether a peptide is overtrimmed; GSW11 has a poor affinity for H-2Dd (its half-life for dissociation is only 20 min), which might bias competition toward ERAAP binding over MHC I, thereby providing more opportunity for ERAAP to bind and overtrim GSW11.

The induction of anti-GSW11 CD8+ T cell responses is fundamental to the clearance of CT26 tumor. In naive mice challenged with CT26, responses to GSW11 are absent, but we showed that GSW11-specific T cell responses are observed following Treg depletion prior to challenge, revealing that Tregs suppress these T cells (18). Downregulation of ERAAP in CT26 (ERAAPlo CT26) led to an increased abundance of GSW11 and the subsequent induction of GSW11-specific T cell responses similar to those observed in CT26-challenged Treg-depleted mice. This suggests that increasing the peptide abundance in CT26 tumors facilitates effective priming and activation of GSW11-specific T cells, thus overcoming Treg suppression. There are many mechanisms for Treg suppression, with strategies to overcome the effects of Tregs on protective T cell responses based on the depletion/blocking of important cells or interactions, such as Treg depletion (19), CTLA-4 (27), PD-L1 (28). These approaches, although showing promise, rely on the modulation of all immune responses, not just those restricted to the tumor, and were shown to cause undesirable autoimmune responses. Therefore, the use of ERAAP downregulation may provide a new approach by which Treg suppression of CTL responses can be overcome.

Alteration of ERAAP activity was shown to induce immune responses to tumors (mediated by NK cells) (29) and ERAAP-
deficient cells (mediated by CD8\(^+\) T cells restricted by MHC Ia and Iib) (25, 26), but a link between ERAAP expression/function and immune control of human tumors has yet to be made. There is growing evidence for the importance of tumor-infiltrating lymphocytes (including CTL) in controlling the progression of tumors, including colorectal cancer (30, 31). Furthermore, in human colon cancer there is a negative correlation between tumor survival and tumor-infiltrating Tregs, which adds further significance to our observation that ERAAP downregulation allows anti-GSW11 CTLs to overcome epitope-specific immunosuppression by Tregs (32). The same is likely to be true for other cryptic epitopes that arise as a result of ERAAP silencing, which, for other Ags, were shown to be highly immunogenic (21, 25). Therefore, it is probable that these Ags play a role in the rejection of ERAAP-silenced tumor cells (gene knockdown or leuSH treated). However, these Ags, like GSW11, would also have to be presented, albeit at a lower level, on ERAAP-competent tumor cells to account for the robust memory response against unimmunized CT26 cells in rechallenge experiments. CD8\(^+\) T cell responses directed against GSW11 are important for CT26 clearance in ERAAP\(^−/−\) CT26-immune mice; however, this may be indicative of other similar epitopes because anti-CT26 CD8\(^+\) T cells of other specificities (albeit subdominant) were identified (18).

We found that molecular inhibition of ERAAP in CT26 by preincubation with leuSH, which induces a transient loss of ERAAP activity lasting <48 h (Supplemental Fig. 2), also induced antitumor immunity. This indicated the possibility of using topical ERAAP inhibitors in vivo in a therapeutic setting, and we found that intratumoral injection of established and palpable tumors with preincubation with leuSH, which induces a transient loss of ERAAP activity in tailing peptides for presentation by MHC class Ia and class Ib molecules. J. Exp. Med. 203: 647–659.


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