CORRESPONDENCE

Targeted tumor coating with antigenic, CTL-recognizable peptides via Annexin A5 chimeric constructs following chemotherapy redirects adaptive CD8⁺ T cell immunity for tumor clearance

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A common drawback of many cancer immunotherapies, including immune checkpoint blockade, is their reliance on the expression of immunogenic tumor antigens by cancer cells for immune recognition and clearance, which limits their efficacy against cancers with weak antigenicity.¹ To overcome this obstacle, we and others have explored strategies to harness the immune responses against nontumor antigens and redirect those immune responses to target tumor cells.^{2–5} In particular, we have pioneered a therapeutic strategy fusing tumor-targeting proteins with known antigenic peptide epitopes not naturally expressed by tumor cells, which are flanked by peptide sequences that can be recognized and cleaved by proteases overexpressed in most cancers.^{2,3} This strategy facilitates the targeted delivery of immunogenic peptides into tumor foci, the release of immunogenic peptides into the tumor microenvironment (TME) via tumor protease cleavage, and subsequent loading of these peptides onto major histocompatibility complex class 1 (MHC-I) on the tumor cell surface, allowing the recognition of coated tumor cells by the respective peptide-specific cytotoxic CD8⁺ T lymphocytes (CTLs) for elimination.

We recently reported that Annexin A5 (AnxA5), a phospholipid-binding protein with high binding affinity for phosphatidylserines expressed on apoptotic cells,⁶ can be utilized to alleviate the immune-suppressive effects of apoptotic tumor cells and for the targeted delivery of tumor antigens into the TME following chemotherapy.⁷ We therefore hypothesized that AnxA5 may be a promising candidate for antitumor immune redirection. In this report, we explored the potential of AnxA5 as a tumor-homing molecule to target and deliver antigenic peptides into the TME following chemotherapy for antigenic coating of tumors and direction of non-tumor-specific immune responses for tumor control.

We first created chimeric proteins consisting of AnxA5 fused to the ovalbumin_{257–264} (SIINFEKL) antigenic peptide with or without the furin cleavage site (AnxA5-R-O and AnxA5-O, respectively). We tested the ability of AnxA5-R-O to coat and display the OVA peptide antigen on MHC-I-expressing tumor cells by treating TC-1 murine cervical cancer cells that have no intrinsic ovalbumin expression with cisplatin and various concentrations of

AnxA5-R-O. Flow cytometry analysis confirmed the loading of the OVA-peptide onto the MHC-I of treated tumor cells in a dose-dependent manner (Fig. 1a).

We examined whether OVA loading onto tumor MHC-I renders tumor cells susceptible to OVA-specific CTL killing by treating luciferase-expressing TC-1 (TC-1-luc) cells with cisplatin, AnxA5, AnxA5-O, AnxA5-R-O, and/or PBS control in vitro. We then cocultured treated TC-1-luc cells with OVA-specific OT-1 CTLs and assessed OT1-mediated tumor killing by examining the reduction in luminescence signal. Incubation with AnxA5-R-O, but not AnxA5-O, following cisplatin treatment resulted in significant OT-1-mediated tumor killing (Fig. 1b), demonstrating the importance of protein cleavage by the furin protease secreted by tumor cells in facilitating the loading of OVA peptides onto tumor MHC-I for OT-1 CTL recognition.

We then evaluated the antitumor effects of AnxA5-R-O in vivo by treating TC-1 tumor-bearing C57BL/6 mice with cisplatin, AnxA5-O, and/or AnxA5-R-O. We observed a significantly stronger OVA-specific CTL response within TC-1 tumor loci in the group treated with cisplatin and AnxA5-R-O compared to the other treatment groups (Fig. 1c), which corresponded to more prolonged mouse survival (Fig. 1d). A prolonged mouse survival effect was also observed when we tested AnxA5-R-O in C57BL/6 mice bearing PancO2 (a murine pancreatic cancer cell line) tumors (Fig. 1e). Notably, no mouse survival benefit was observed in PancO2 tumor-bearing mice treated with cisplatin and a green fluorescent protein–OVA (GFP-R-O) fusion peptide, suggesting the importance of AnxA5-mediated tumor targeting in the generation of therapeutic antitumor effects following chemotherapy. These data demonstrate that AnxA5-R-O treatment following chemotherapy leads to the generation and direction of an OVA-specific CTL response against OVA-negative tumors for effective tumor control in vivo.

To evaluate the translational potential of the AnxA5-R-O treatment strategy, we constructed a chimeric protein with AnxA5 fused to the influenza A virus M1 (GILGFVFTL) peptide, a known peptide epitope for human MHC-I HLA-A*0201, flanked by a furin cleavage site (AnxA5-R-M1). We tested the treatment effect of AnxA5-R-M1 in vitro by treating luciferase-expressing OVCAR3

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Fig. 1 Redirection of the antitumor immune response by AnxA5-R-Ag chimeric proteins. **a** Generation of the AnxA5-R-O chimeric fusion protein. Top: Illustration of various AnxA5 protein constructs. Bottom: The binding of OVA antigen by AnxA5-R-O and its loading onto TC-1 tumor cells following cisplatin treatment were evaluated via flow cytometry. **b** TC-1-Luc cells were treated with cisplatin, AnxA5, AnxA5-O, AnxA5-R-O, and/or PBS control and then cocultured with OVA-specific OT-1 cells, and the degree of CTL-mediated tumor cell killing was determined by analysis of luminescence activity. **c**, **d** TC-1 tumor-bearing C57BL/6 mice ($n = 10$ per group) were treated with cisplatin, AnxA5-O, and/or AnxA5-R-O. **c** Tumor tissues were excised 1 week after the last treatment, and the OVA-specific CD8⁺ T cell response in the tumor microenvironment was analyzed via flow cytometry. **d** Kaplan–Meier survival analysis of mice. **e** PancO2 tumor-bearing C57BL/6 mice ($n = 10$ per group) were treated with cisplatin, AnxA5-R-O, and/or GFP-R-O. Top: Schematic diagram. Bottom: Kaplan–Meier survival analysis of mice. **f** Top: Illustration of the generation of the AnxA5-R-M1 chimeric protein construct. Bottom: OVCAR3-Luc cells were treated with cisplatin, AnxA5, AnxA5-M1, AnxA5-R-M1, and/or PBS control and then cocultured with M1-specific CTLs, and the degree of CTL-mediated tumor cell killing was determined by analysis of luminescence activity. Significance was determined by Student's *t* test (**b**, **c**, **f**) or ANOVA (**d**, **e**). The data are presented as the mean $\pm$ SD. * $P < 0.01$, ** $P < 0.001$

(OVCAR3-Luc) human ovarian tumor cells with cisplatin, AnxA5, AnxA5-M1, and/or AnxA5-R-M1, followed by coculture with M1-specific CTLs. Similar to that observed in the OVA model, incubation with AnxA5-R-M1 following cisplatin treatment resulted in significant OVCAR3 tumor killing by M1-specific CTLs (Fig. 1f), demonstrating that the AnxA5-R chimeric peptide treatment strategy is also useful for human-relevant antigens.

Our study showed that AnxA5-R-Ag chimeric proteins effectively target antigenic peptides into tumor foci and coat tumor cell MHC-I molecules with immunogenic peptides following chemotherapy, rendering tumor cells susceptible to removal by peptide-specific immune responses. A major advantage of this strategy is the tremendous flexibility of the chimeric protein construct design. In addition to AnxA5 utilized in the current study, similar antitumor effects have been achieved using other tumor-binding molecules, such as the NKG2D ligand,³ as well as antibodies targeting molecules expressed on the surface of various cancers, including mesothelin,² CD20, and EGFR.⁴ Furthermore, while the current study utilized OVA and M1 peptides as antigenic targets, we expect that other identified immunogenic peptide epitopes, such as those derived from common viral pathogens, including cytomegalovirus (CMV) and Epstein-Barr virus (EBV),⁸ may also be utilized to redirect viral-specific immune responses against tumors. Furthermore, the strategy of incorporating multiple CTL epitopes flanked by furin-sensitive linkers has previously been demonstrated to be feasible, with the antigens capable of being presented through different MHC-I molecules.⁸ By creating a chimeric protein containing multiple CTL epitopes

restricted to different HLA alleles, a single therapeutic chimeric protein construct may be applicable in a variety of patients.

In addition to serving as a tumor-homing molecule for the delivery of peptide antigens into the phosphatidylserine-enriched TME, we have previously shown that AnxA5 may also act as an immune checkpoint inhibitor against phosphatidylserine-expressing apoptotic tumor cells.⁷ While the immune modulatory effect of AnxA5 was not examined in the current study, it is perceivable that the AnxA5-based chimeric protein would be able to both coat the tumor cells with antigenic peptide for immune redirection and alleviate the immunosuppressive properties of the TME, making AnxA5 a particularly attractive molecule for the purpose of antitumor immune redirection.

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AUTHOR CONTRIBUTIONS

T.H.K. and C.F.H. designed and developed the assays and experiments. T.H.K. and Y.T. performed the experiments. T.H.K., A.Y., and C.F.H. analyzed the data. T.H.K., A.Y., and C.F.H. interpreted the data. T.H.K., A.Y., L.F., and C.F.H. wrote the manuscript. All authors read and approved the final manuscript.

ADDITIONAL INFORMATION

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