Calreticulin promotes immunity and type I interferon-dependent survival in mice with acute myeloid leukemia

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ABSTRACT
Exposure of cancer cells to particular chemotherapeutic agents or γ-irradiation induces a form of cell death that stimulates an immune response in mice. This “immunogenic cell death” requires calreticulin (CRT) translocation to the plasma membrane, which has been shown to promote cancer cell phagocytosis. However, it remains unclear whether the effect of CRT on cancer cell phagocytosis is alone sufficient to affect tumor immunity. Acute myeloid leukemia (AML) cells expressing cell-surface CRT were generated in order to characterize the mechanism(s) through which CRT activates tumor immune responses. Potent immune-mediated control or rejection of AML was observed in mice with CRT-expressing leukemia. The “CRT effect” was ultimately T-cell dependent, but dendritic cells (DCs), and CD8α+ DCs in particular, were also necessary, indicating that CRT might act directly on these DCs. CRT-expressing AML cells were slightly more susceptible to phagocytosis by DCs in vivo, but this effect was unlikely to explain the potent immunity observed. CRT did not affect classical DC maturation markers, but induced expression of type I interferon (IFN), which was critical for its positive effect on survival. In conclusion, CRT functions as a “danger signal” that promotes a host type I IFN response associated with the induction of potent leukemia-specific T-cell immunity.

Introduction
Exposure of cancer cells to particular chemotherapeutic drugs or γ-irradiation induces a form of apoptosis that stimulates an immune response in mice.1,2 This “immunogenic cell death” requires translocation of a chaperone protein called calreticulin (CRT) from the endoplasmic reticulum (ER), where it normally resides, to the plasma membrane.3 Under homeostatic conditions, calreticulin is involved in major histocompatibility complex (MHC) class I assembly, and in the maintenance of ER calcium homeostasis.4 Cell surface CRT translocation requires activation of pancreatic ER kinase (PERK), which phosphorylates eukaryotic translation initiation factor (eIF2α). eIF2α-mediated triggering of ER stress pathways culminates in anterograde transport of CRT through the Golgi to the cell surface.5 Following translocation, CRT functions as an “eat me” signal to promote efferocytosis by APCs through a receptor called low-density lipoprotein-related protein (LRP; CD91).6,7 CRT translocation also occurs in viable malignant cells,5 suggesting that apoptosis may not be required for CRT translocation, and that activation of “ER stress” programs can be sufficient to promote its cell surface expression.

A major unanswered question regarding CRT is whether its effect on tumor immunity depends exclusively on its ability to enhance cancer cell engulfment. By inducing phagocytosis of apoptotic tumor cells by APCs, CRT might promote cross-presentation of tumor antigens to T cells. However, antigen cross-presentation by immature dendritic cells (DCs) results in T-cell tolerance, rather than activation.9 We therefore hypothesized that CRT might also directly stimulate APCs, leading to increased cross-priming of tumor-specific T cells. In support of this hypothesis, soluble CRT was recently shown to stimulate production of NF-kB-related cytokines by peritoneal macrophages in a CD91-dependent manner.9

Increased cell-surface CRT exposure on malignant cells has been reported in a number of human cancers,7,10-12 including acute myeloid leukemia.7,13 In a recent study, the degree of cell-surface CRT expression on human acute myeloid leukemia (AML) cells was found to correlate with enhanced T-cell immunity to AML-associated antigens and improved clinical outcomes.14 In addition, our laboratory is interested in characterizing pathways that promote innate immune sensing of AML.15 With these notions in mind, we sought to investigate mechanisms through which CRT translocation on AML cells might promote anti-leukemia immunity. Thus, AML cells with stable cell-surface CRT expression were generated in order to investigate how CRT, as an isolated variable (i.e., without the requirement for chemotherapy or radiation exposure), affects anti-leukemia immunity. In a systemic disease model known to induce a potent T-cell tolerant state,16 CRT expression on AML cells was associated with prolonged survival in wild-type, but not in T-cell-deficient mice. Moreover, leukemia-specific CD8+...
T-cell responses were significantly augmented in mice harboring CRT-expressing AML, indicating that T cells were required to mediate the CRT effect on anti-leukemia immunity. CD8a+ DCs were also necessary for the “CRT effect,” which suggested that CRT acts upstream of adaptive immunity. A modest increase in engulfment of CRT-expressing AML cells by DCs was observed, but did not appear to explain its potent effect on immunity. Rather, CRT-induced expression of type I interferon (IFN) in leukemia-bearing mice, and host type I IFN signaling, was required for the effect of CRT on AML survival. In conclusion, CRT functions as a “danger signal” to promote innate immune sensing of cancer through type I IFN.

Results

Generation of CRT-expressing C1498 AML cells

To investigate how CRT, as an isolated variable, regulates antitumor immune responses, its cell surface expression was engineered on C1498 and C1498.SIY AML cells through a GPI anchor (Fig. 1A). GPI-mediated protein anchoring to the plasma membrane is sensitive to enzymatic cleavage by phospholipase C. Treatment of CRT-expressing C1498 cells with phospholipase C resulted in significantly decreased cell surface CRT (Fig. S1A and B), clearly demonstrating its GPI-dependent anchoring to the plasma membrane. Cell surface CRT expression on engineered C1498 AML cells was only slightly higher than that on control C1498 cells undergoing doxorubicin-induced apoptosis (Fig. S1C). Thus, we have generated an AML cell line that expresses cell surface CRT at a similar level to that observed following treatment with a chemotherapeutic agent known to induce CRT translocation.

CRT is a member of a multi-protein peptide-loading complex (PLC), which is involved in MHC class I folding and peptide loading. Thus, engineered CRT expression could conceivably affect the MHC class I presentation pathway in C1498 cells. However, equivalent cell surface Kb levels were observed on CRT-expressing and control C1498 cells (Fig. 1B). Also, SIY-specific CD8+ T cells proliferated similarly when cultured with C1498.SIY or C1498.SIY.CRT cells, indicating that MHC class I presentation of the SIY peptide antigen was not influenced by induced CRT expression (Fig. 1C). Last, the in vitro growth of C1498 and C1498.CRT cells was identical.

Figure 1. Generation of CRT-expressing AML cells. Representative FACS plots depicting expression of (A) CRT and (B) H-2Kb on control C1498 and C1498.CRT cells. (C) CTV-labeled 2C T cells were cultured in vitro for 3 d with parental C1498 cells (left), C1498.SIY cells (center) or C1498.SIY.CRT cells (right). Representative FACS plots demonstrating CTV dilution of 2C T cells are shown after gating on live CD8+ T cells. Numbers indicate the percentage of divided 2C T cells. Quantified data are shown to the right. (D) MTS assay of in vitro cultured C1498 and C1498.CRT cells. n.s.: not significant. (C, D) Data are representative of 2–4 experiments, each performed in triplicate.
demonstrating that engineered CRT expression did not affect AML cell viability or proliferation (Fig. 1D).

**CRT expression on AML cells is associated with impaired tumor development**

To determine whether CRT expression on C1498 cells affected their capacity to develop and progress as localized tumors, C1498 or C1498.CRT cells were inoculated subcutaneously (SC) into recipient hosts. Control C1498 tumors progressed rapidly in C57BL/6 mice. However, following SC C1498.CRT inoculation, 70% of mice remained tumor-free (Fig. 2A). Conversely, both control and C1498.CRT tumors progressed similarly in *Rag2*−/− hosts (Fig. 2B), which indicated that the adaptive immune system was necessary to prevent localized growth of CRT-expressing C1498 tumors. Further, wild-type mice that rejected a SC C1498.CRT challenge were resistant to re-challenge with parental C1498 cells, suggesting that CRT expression on AML cells was sufficient to promote immunological memory against native leukemia antigens (Fig. 2C). This result also indicates that CRT itself is not a direct antigenic target of adaptive immune cells in mice harboring CRT-expressing tumors. Collectively, these results demonstrate that CRT expression on cancer cells negatively impacts localized tumor progression through a mechanism which requires adaptive immunity.

**CRT promotes enhanced survival in animals with systemic AML**

It was next of interest to determine whether a similar result would occur in a systemic AML setting known to induce a T-cell tolerant state, and which more accurately recapitulates human AML progression. To that end, survival of C57BL/6 mice was assessed following an intravenous (IV) challenge with C1498 or C1498.CRT cells. As shown in Fig. 2D, survival of mice harboring disseminated C1498.CRT AML was significantly prolonged compared to those with control C1498 leukemia. The effect on survival was much more striking when CRT-expressing C1498.SIY cells were introduced IV, where 90% of mice survived long-term (Fig. 2E), and were consistently able to reject a secondary C1498.SIY cell challenge (Fig. 2F). Thus, CRT expression on AML cells is sufficient to generate effective immunological memory responses, even in a disease setting associated with a profound T-cell tolerant state.

Adaptive immunity was also required to promote CRT-mediated survival in mice with systemic AML, as the survival
benefit associated with CRT expression on leukemia cells was abrogated in Rag2<sup>−/−</sup> mice (Fig. S2A), in TCRβ<sup>−/−</sup> hosts (Fig. 2G and H), and also in C57BL/6 mice depleted of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. S2B). In conclusion, the enhanced survival of mice with systemic CRT-expressing AML requires the adaptive immune system, and T cells in particular.

**Augmented leukemia-specific T-cell responses in mice with CRT-expressing AML**

Because T cells were found to be necessary for the CRT-mediated effect on systemic leukemia progression, their ability to promote leukemia-specific T-cell responses was investigated. Six days following AML induction, SIY/K<sup>b</sup> pentamer staining revealed a significantly higher frequency (Fig. 3A and B) and absolute number (Fig. 3B) of SIY antigen-specific CD8<sup>+</sup> T cells in the spleens of mice with C1498.SIY.CRT versus control C1498.SIY AML. More impressive was the capacity for SIY-specific CD8<sup>+</sup> T cells from C1498.SIY.CRT-challenged mice to produce IFNγ upon in vitro re-stimulation, which was approximately 6-fold higher compared to mice challenged with control C1498.SIY (Fig. 3C).

To track the proliferation and accumulation of leukemia antigen-specific CD8<sup>+</sup> T cells in leukemia-bearing mice, a T-cell adoptive transfer model was employed. CTV-labeled, SIY antigen-specific, T-cell receptor (TCR) transgenic 2C T cells were transferred, and 1 d later, animals received C1498.SIY or C1498.SIY.CRT cells IV. 2C T cells accumulated poorly in mice with C1498.SIY leukemia as we have previously demonstrated (Fig. 3D and E). However, 2C T-cell accumulation was...
enhanced 2-fold in C1498.SIY.CRT-bearing mice (Fig. 3D and E; Fig. S3A and B). CRT expression on AML cells also led to an improved capacity of 2C T cells to produce IFNγ (Fig. S3C), and to lyse SIY antigen-pulsed targets in vivo (Fig. 3F and G). Taken together, these data reveal that CRT-expression on leukemia cells significantly enhances the priming of highly functional antigen-specific CD8\(^+\) T cells with the capacity to control, and even eradicate leukemia.

**CRT modestly enhances the uptake of AML cells by splenic DCs in vivo**

To determine whether cell surface CRT would enhance leukemia cell engulfment in vivo, C1498 and C1498.CRT cells were labeled with unique fluorescent dyes (CFSE and CTV, respectively) and were co-injected IV into mice in equal numbers. Three hours later, spleens of recipient animals were analyzed by flow cytometry for the frequency of fluorescence-containing CD11b\(^-\)CD11c\(^+\) and CD11c\(^+\) cells as a readout for phagocytosis. Very few CD11b\(^+\) cells from mice challenged with either C1498 or C1498.CRT cells contained a CFSE or CTV signal, suggesting that macrophages were not adept at AML cell uptake (Fig. 4A and B). However, a small population of CD11c\(^+\) cells was also CTV\(^+\) or CFSE\(^+\), indicating that splenic DCs engulf AML cells in vivo (Fig. 4A and B). Moreover, the frequency of CD11c\(^+\) cells that were CTV\(^+\) was significantly higher than for CFSE (Fig. 4A and B), demonstrating in a competitive setting that CRT modestly enhances DC-mediated phagocytosis of AML cells in vivo. To control for the possibility that the difference in observed uptake of cellular material from control and CRT-expressing AML cells was dependent on the particular fluorescent dye utilized, control C1498 cells were labeled either with CFSE or with CTV and a similar experiment was performed. Here, identical frequencies of CD11c\(^+\) cells contained CTV versus CFSE fluorescence (Fig. S4A and B), indicating that the enhanced uptake of C1498.CRT cells by CD11c\(^+\) cells was not an experimental artifact.

**DCs are required for the CRT effect on survival in leukemia-bearing hosts**

The role of DCs in regulating the effect CRT expression on AML survival was next examined. To ablate DCs from leukemia-bearing hosts, CD11c\(^{DTR/GFP}\) BMC mice were generated. DT-mediated elimination of CD11c-expressing cells was associated with a rapid demise of mice harboring both control and...
CRT-expressing C1498.SIY leukemia (Fig. 4C), indicating that DCs are critical to control the progression of disseminated AML in general. The extended survival observed in mice with CRT-expressing leukemia was lost following CD11c+ cell depletion (Fig. 4C), revealing that the CRT-mediated effect on anti-leukemia immunity requires DCs.

Our laboratory has recently identified that splenic CD8α+ DCs, uniquely capable of engulfing and cross-presenting leukemia-derived antigens to CD8+ T cells in vivo, mediate a form of deletional T-cell tolerance in mice with AML (D. Kline, unpublished observation). To investigate the role of CD8α+ DCs in mediating the CRT effect, Batf3−/− mice, which lack CD8α+ DCs,18 were utilized. As shown in Fig. 4D, the survival benefit associated with a CRT-expressing AML cell challenge was completely abolished in the absence of CD8α+ DCs. Collectively, these data suggest that, although T cells are ultimately essential as effectors in leukemia cell killing, CRT likely acts upstream of adaptive immunity—possibly through CD8α+ DCs.

**CRT does not induce classical measures of DC activation in vivo**

The requirement for DCs, and specifically for CD8α+ DCs, to mediate the powerful CRT effect in vivo suggested that its expression on leukemia cells might directly activate DCs, enhancing their ability to cross-prime leukemia-specific CD8+ T cells. First, the phenotypes of DCs from leukemia-bearing mice were analyzed. Surprisingly, expression of co-stimulatory B7 and MHC class I and II molecules was identical among mice were analyzed. Surprisingly, expression of co-stimulatory B7 and MHC class I and II molecules was identical among mice challenged with C1498.CRT or control C1498.AML cells in vivo (Fig. 5A). IL-12 production by CD8α+CD11c+ cells that had acquired AML cells in vivo was higher than that of those that had not (Fig. 5B and C), suggesting either that phagocytosis of AML cells induced IL-12 production by DCs, or that a subpopulation of IL-12-producing DCs was more efficient at AML cell uptake. Regardless, there was no difference between the frequencies of IL-12-producing CD8α+CD11c+ cells that had acquired C1498 versus C1498. CRT cells in vivo (Fig. 5B and C). Furthermore, purified CD8α+ DCs from C1498.SIY or C1498.SIY.CRT-bearing animals equivalently induced the proliferation of 2C T cells directly ex vivo (Fig. 5D and E), demonstrating that CRT expression on AML cells did not appear to enhance cross-presentation of leukemia cell-derived antigens by DCs to T cells. Taken together, these results indicate, at least at the time points chosen for analysis, that CRT does not appear to activate classical pathways associated with DC maturation.

**CRT activates the host type I IFN pathway**

Type I IFNs are essential for bridging innate and adaptive immune responses against solid tumors.19,20 However, we recently observed that disseminated AML fails to induce a host type I IFN response,25 which may help to explain the dense T-cell tolerant state that exists in leukemia-bearing hosts. To determine whether CRT expression on AML cells would induce type I IFN transcription, levels of ifnb mRNA were measured in spleen cells from mice challenged with C1498.CRT or control C1498 AML. Interestingly, a 2-fold increase in ifnb mRNA expression was detected in spleens of mice as early as 24 h following IV challenge with C1498.CRT cells (Fig. 6A). Experiments were performed to identify the specific cell type(s) in which ifnb mRNA expression was induced in the spleen following exposure to CRT-expressing AML cells. Here, CD11b+ cells, but not CD11c+ or CD11b−CD11c− cells, were found to express increased ifnb mRNA following an IV C1498.CRT cell versus control C1498 cell challenge (data not shown). To test whether enhanced ifnb expression in mice harboring C1498. CRT AML cells correlated with improved outcome, wild-type and Ifnar−/− mice (defective in type I IFN signaling) were challenged with C1498.SIY or C1498.SIY.CRT, and survival was assessed. As previously demonstrated,15 the survival of wild-type and Ifnar−/− mice with control C1498.SIY cells was similar (Fig. 6B). Nearly all wild-type mice challenged with C1498.SIY.CRT cells survived long-term, as expected (Fig. 6B). Strikingly, the enhanced survival associated with CRT-expressing AML cells was nearly completely abrogated in Ifnar−/− mice (Fig. 6B). Collectively, these results reveal that CRT-expressing AML cells stimulate a type I IFN response in vivo, and that type I IFN signaling in host cells is essential for the CRT-mediated effect on survival. Thus, the type I IFN pathway is an important mechanism through which CRT promotes anti-leukemia immunity.

**Discussion**

"Danger signals" released by dying tumor cells, including tumor-derived nucleic acids and their byproducts, facilitate innate immune sensing of cancer.21 CRT translocation is also an important mechanism through which stressed or dying cancer cells place the immune system on alert,25 possibly through its ability to promote malignant cell phagocytosis.25 The role of CRT in activating antitumor immunity has been demonstrated in multiple murine tumor models,1,2 and in human studies including acute myeloid leukemia, non-Hodgkin lymphoma, bladder cancer, ovarian cancer and non-small cell lung cancer, where CRT exposure correlates with favorable clinical outcomes.7,10,11,14 Within tumor microenvironment, hyperploid cancer cells undergoing constitutive ER stress were shown to trigger the CRT translocation to promote early cancer immunosurveillance.22 Long non-coding RNA expressed from tumor suppressor retinoblastoma bidirectional promoter were reported as one mechanism that regulated CRT cell surface translocation in response to chemotherapy.23 However, the precise mechanism through which CRT stimulates antitumor immunity remains unknown, and the impact of CRT translocation on tumor immune responses has been described only after exposure of cancer cells to chemotherapy or radiation,1,2 which could result in CRT-independent effects. To circumvent this problem, AML cells exhibiting stable cell surface CRT expression were generated to directly examine its effect on anti-leukemia immunity.

Our results reveal that CRT expression on leukemia cells leads to potent immune-mediated control, and in some cases, eradication of disseminated AML. This observation is particularly interesting in light of our previous studies, which have revealed that a dense T-cell tolerant state is present in mice with AML.16 Furthermore, the finding that CRT expression on
AML cells enhanced functional leukemia-specific CD8+ T-cell responses, and generated effective immunological memory, indicated that adaptive immunity was crucial for its effect. However, the observation that DCs were also required to mediate enhanced survival in mice with CRT-expressing AML argued against a direct effect of CRT on T cells. The data presented in Fig. 3 reveal that a major effect of CRT is to promote the activation of leukemia-specific CD8+ T cells. Experiments in which CD8+ or CD4+ T cells are separately depleted could also be performed to examine whether one or both populations are required to mediate anti-leukemia immunity downstream of CRT.

It has been demonstrated that CRT translocation on malignant cells promotes their phagocytosis by macrophages and
DCs. This observation has led to the hypothesis that CRT-induced efferocytosis, particularly by DCs, augments cross-presentation of tumor-derived antigens to T cells, which ultimately drives the protective immunity that ensues. This model relies on the theory that tumor antigen cross-presentation by DCs is a limiting factor in generating functional antitumor T-cell responses. However, the activation status of DCs is also critical in their ability to either prime or tolerate antigen-specific T cells. For example, targeting antigen for cross-presentation by DCs leads to deletional T-cell tolerance, unless an activating signal is also provided. Thus, in addition to promoting engulfment of cancer cells, we hypothesized that CRT might also directly activate DCs, licensing them to stimulate superior antitumor T-cell responses.

In agreement with published studies, CRT expression on AML cells did result in their enhanced uptake by splenic DCs in vivo, although the effect was not striking (Fig. 4A and B). In our opinion, this finding did not likely explain the marked differences in functional CD8 T-cell responses generated in mice with CRT-expressing versus control AML (Fig. 3A, F and G). We therefore examined the phenotypes of DCs that had been exposed or not to CRT-expressing leukemia cells in vivo, and found no differences in expression of classical maturation markers or production of IL-12. On the surface, these results seemed to argue against a hypothesis supporting direct activation of antigen-presenting cells by CRT, until it was observed that splenocytes (and CD11b+ cells specifically) from mice with CRT-expressing AML expressed significantly higher levels of ifnβ mRNA, which is interesting in lieu of our recent finding that disseminated AML fails to induce a host type I IFN response.

A clear role for type I IFN in mediating the CRT effect was demonstrated in an experiment in which CRT-expressing or control AML cells were inoculated into wild-type or Ifnar−/− mice. As previously demonstrated, host type I IFN signaling played no role in regulating survival of mice with control AML. Strikingly, however, the survival benefit associated with CRT expression on AML cells was almost completely abolished in Ifnar−/− hosts. Together, these observations support a model in which CRT stimulates a host type I IFN response that may act through CD8α+ DCs to promote their ability to cross-prime leukemia-specific CD8 T-cell responses. Although speculative, our result demonstrating that the CRT effect on survival was completely eliminated in Batf3−/− mice strongly supports the conclusion that this DC subset is the target of type I IFN in vivo. Others have shown that type I IFN produced in solid tumor-bearing hosts acts directly on CD8α+ DCs, licensing them to prime antitumor CD8 T-cell responses.

Immunotherapeutic approaches aimed at enhancing CRT translocation with chemotherapeutic agents or radiation have been difficult to develop because these modalities can concomitantly induce apoptosis of the immune cells necessary to mediate the CRT effect in vivo. However, the observation that CRT stimulates type I IFN suggests that activating this pathway, for example, with stimulator of interferon genes (STING) agonists, may be an efficacious alternative immunotherapeutic strategy. Regardless, the identification of type I IFN as a mechanism through which CRT translocation stimulates host antitumor immunity represents an important step forward in our understanding of how cancers are sensed by the host immune system.

Material and methods

Mice

Mice were maintained in a specific pathogen-free environment. Animal experimentation was carried out under a protocol approved by an Institutional Animal Use and Care Committee. C57BL/6 mice (H-2b; CD45.2−/−) were purchased from Taconic. Rag2−/− and 2C TCR transgenic mice were bred in our facility. TCRβ−/− mice were provided by M. Alegre (University of Chicago). CD11cDTR/EGFP B6.FVB-Tg (Itgax-DTR/EGFP) mice and Ifnar−/− mice were purchased from X.Y. Fu (University of Texas Southwestern). YET40 B6.129-Il12b−/−Il6−/− IL-2−/− mice, B6.SJLPtprca Ptpε−/− Peprb/BoyJ mice (CD45.1−/−) and Batf3−/− mice were purchased from Jackson Labs and bred in our facility.
Cell lines

The C1498 AML cell line (H-2b) was purchased from ATCC. C1498 cells expressing the K\(^{\alpha}\)-restricted model SIY (SIYRYYGL) peptide antigen were previously generated to facilitate monitoring of endogenous antigen-specific CD8\(^{+}\) T-cell responses in leukemia-bearing animals. SIY is also the cognate antigen for the 2C transgenic TCR. C1498 and C1498.SIY cells were transduced by the retroviral pRetroX-IRES-DsRed Express vector containing full-length murine CRT cDNA inframed with the cDNA of the 5’ end of decay accelerating factor (DAF), which encodes a signal sequence for attachment of a glycosphatidylinositol (GPI) anchor to the C-terminus of the resulting CRT-DAF fusion protein to facilitate CRT anchoring in the plasma membrane (C1498.CRT and C1498.SIY.CRT). The CRT-GPI construct contains an IRES-DSRED; an empty IRES-DSRED construct was also transduced to generate control C1498 and C1498.SIY cells. All C1498 derivative cell lines were cultured in DMEM (Invitrogen) supplemented with 10% FBS, 2-mercaptoethanol, essential amino acids, penicillin and streptomycin.

C1498 AML model

To induce systemic AML, 10\(^{6}\) C1498 cells were injected IV through the lateral tail vein. For in vivo CD4\(^{+}\) and CD8\(^{+}\) T-cell depletion, anti-CD4 (L3T4) and anti-CD8\(\alpha\) (2.43) antibodies (BioXcell) were administered intra-peritoneal (IP) at a dose of 200 \(\mu\)g on day –1, and 100 \(\mu\)g on day 0, and every 3 d thereafter until day 14. To induce localized tumor growth, 10\(^{6}\) C1498 cells were inoculated subcutaneously (SC) in the lower lateral abdominal wall. Tumor growth was monitored 2–3 times per week with calipers, and mean tumor diameter was recorded.

Flow cytometry

Following red blood cell lysis and blockade of Fc receptors with anti-CD16/32 antibodies, spleen cells were stained with the following directly conjugated antibodies: CD11b (M1/70), CD11c (HL3), CD86 (GL1), I-A/I-E (M5/114.15.2) and H-2K\(^{\beta}\) (AF6-88.5) (BD Biosciences); CD8\(\alpha\) (53-6.7), CD80 (16-10A) and CD19 (eBio1D3) (eBiosciences); CD45.1 (A20), and CD3e (145-2C11) (BioLegend). Rabbit anti-calreticulin monoclonal antibody and fluorescence-labeled goat-anti-rabbit IgG polyclonal secondary antibody were purchased from Abcam. Fixable viability dyes (Invitrogen) were used to exclude dead cells. Flow cytometry was performed on LSRII or LSRFortessa cytometers (BD Biosciences). Analysis was performed using Flowjo software (Treestar). Fluorescence-activated cell sorting (FACS) was performed using a FACSARia (BD Biosciences).

For DC isolation, spleens were injected with 1 mg/mL collagenase IV (Sigma), 20 \(\mu\)g/mL DNAse I (Roche) were incubated at 37 °C for 15–20 min, and passed through a 70-\(\mu\)m filter to generate single cell suspensions. Cells were stained with CD3e (145-2C11) and CD19 (eBio1D3) biotinylated antibodies, followed by secondary streptavidin staining to eliminate T and B cells.

IFN\(\gamma\) ELISPOT

At the indicated time points, 10\(^{6}\) spleen cells from individual leukemia-bearing animals were re-stimulated with media alone or with SIY peptide (100 nM) in triplicate overnight in 96-well, flat-bottom plates using the mouse IFN\(\gamma\) ELISPOT kit (BD Biosciences). ELISPOT plates were read with an ImmunoSpot Series 3 Analyzer. Data were analyzed with ImmunoSpot software (Cellular Technology, Ltd.).

Adaptive transfer of 2C T cells into leukemia-bearing mice

2C CD8\(^{+}\) T-cells were isolated with a mouse CD8 microbead kit (Miltenyi), labeled with CellTrace Violet (CTV) (Invitrogen), and 10\(^{6}\) were injected IV. Twenty-four hours later, mice were challenged IV with 10\(^{6}\) C1498.SIY or C1498.SIY.CRT cells. Six days later, spleen cells of recipient mice were stained with anti-CD8\(\alpha\) and anti-1B2 antibodies (the 1B2 antibody specifically binds the 2C TCR). Flow cytometry was performed to assess the frequency and dilution of the CTV signal in 2C T cells (as a read-out of in vivo 2C T-cell proliferation).

In vivo cytolyis assay

4\(\times\)10\(^{6}\) 2C T cells were transferred into C57BL/6 mice (CD45\(^{2/}\)) on day –1. On day 0, half of the mice were challenged with C1498.SIY or C1498.SIY.CRT cells IV. Six days later, spleen cells isolated from naive B6.SJL (CD45\(^{1/}\)) mice were separately labeled with different concentrations of CTV (2.5 \(\mu\)M, 50 nM), and were pulsed with SIYRYYGL or SIINFEKL (irrelevant) peptides, respectively. CTV-labeled, peptide-pulsed cells were mixed 1:1, and a total of 8\(\times\)10\(^{6}\) were injected IV into leukemia-bearing C57BL/6 mice. Six hours later, spleen cells were stained with an anti-CD45.1 antibody, and analyzed by flow cytometry. After gating on CD45.1\(^{+}\) cells, the ratio of CTV\(^{hi}\) to CTV\(^{lo}\) cells present was calculated using the following equations:

\[
\text{Ratio} = \frac{\text{% CTV}^{lo} (\text{OVA pulsed CD45.1}^{+}\text{cells})}{\% \text{ CTV}^{hi} (\text{SIY pulsed CD45.1}^{+}\text{cells})}
\]

\[
\text{% specific lysis} = [1 - (\text{ratio leukemia} - \text{free mice}) / \text{ratio leukemia} - \text{bearing mice}] \times 100.
\]

In vivo phagocytosis and cross-presentation assays

C1498 or C1498.CRT cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) or CTV, respectively, were mixed at a 1:1 ratio, and 8\(\times\)10\(^{6}\) cells were injected IV into C57BL/6 mice. Three hours later, spleen cells were stained with antibodies against CD11c, CD11b, CD8\(\alpha\), CD3e and CD19. The frequency of CD11c\(^{+}\) and CD11b\(^{+}\)CD11c\(^{-}\) cells that contained either a CFSE or CTV signal was analyzed by flow cytometry. For antigen cross-presentation assays, C1498.SIY or
C1498.SIY.CRT cells (4 × 10^6) were inoculated IV into C57BL/6 mice. Splenic CD8α^+ and CD8α^− DCs from these animals were isolated by FACS. Sorted DC populations were cultured 1:1 with purified CTV-labeled 2C T cells for 65–72 h, followed by analysis of CTV dilution by 2C T cells.

**Generation of bone marrow chimeric (BM) mice**

C57BL/6 mice were lethally irradiated (900 rads) and reconstituted 1 d later with 2.5 × 10^6 bone marrow cells from CD11c^DTR/GFP mice. Eight weeks later, mice were utilized experimentally. To deplete CD11c^+ cells from leukemia-bearing CD11c^DTR/GFP BM, mice, diphtheria toxin (DT) (500 ng) was administered IP 1 d prior to C1498 cell inoculation and continued every 48 h until day 11.

**Quantitative real-time PCR analysis**

C57BL/6 mice were challenged with 5 × 10^6 C1498.SIY or C1498.SIY.CRT cells IV. Twenty-four hours later, spleen cells from recipient mice were re-suspended in Trizol (Life Technologies). Total RNA was isolated via chloroform extraction. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Expression was measured by real-time quantitative PCR (RT-qPCR) using specific primers/probes, as previously reported.24

**Statistical analysis**

Grouped data were analyzed via two-way ANOVA with Bonferroni post-tests. Survival differences were analyzed with the Log-rank test. Statistics were performed using GraphPrism software. Data are presented as mean ± SD unless otherwise indicated. A p value of < 0.05 was considered statistically significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**Author contributions**

X.C. designed and executed experiments, analyzed data and drafted and reviewed the manuscript. D.F. designed and executed experiments, analyzed data and reviewed the manuscript. J.K. designed experiments, analyzed data, drafted and reviewed the manuscript.

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