CANCER

Calreticulin Is the Dominant Pro-Phagocytic Signal on Multiple Human Cancers and Is Counterbalanced by CD47

Mark P. Chao,[1]* Siddhartha Jaiswal,[1,2] Rachel Weissman-Tsukamoto,[1] Ash A. Alizadeh,[1,3,4] Andrew J. Gentles,[5] Jens Volkmer,[1] Kipp Weiskopf,[1] Stephen B. Willingham,[1] Tal Raveh,[1] Christopher Y. Park,[6] Ravindra Majeti,[1,3†] Irving L. Weissman[1†]

(Published 22 December 2010; Volume 2 Issue 63 63ra94)

Under normal physiological conditions, cellular homeostasis is partly regulated by a balance of pro- and anti-phagocytic signals. CD47, which prevents cancer cell phagocytosis by the innate immune system, is highly expressed on several human cancers including acute myeloid leukemia, non-Hodgkin's lymphoma, and bladder cancer. Blocking CD47 with a monoclonal antibody results in phagocytosis of cancer cells and leads to in vivo tumor elimination, yet normal cells remain mostly unaffected. Thus, we postulated that cancer cells must also display a potent pro-phagocytic signal. Here, we identified calreticulin as a pro-phagocytic signal that was highly expressed on the surface of several human cancers, but was minimally expressed on most normal cells. Increased CD47 expression correlated with high amounts of calreticulin on cancer cells and was necessary for protection from calreticulin-mediated phagocytosis. Blocking the interaction of target cell calreticulin with its receptor, low-density lipoprotein receptor–related protein, on phagocytic cells prevented anti-CD47 antibody–mediated phagocytosis. Furthermore, increased *calreticulin* expression was an adverse prognostic factor in diverse tumors including neuroblastoma, bladder cancer, and non-Hodgkin's lymphoma. These findings identify calreticulin as the dominant pro-phagocytic signal on several human cancers, provide an explanation for the selective targeting of tumor cells by anti-CD47 antibody, and highlight the balance between pro- and anti-phagocytic signals in the immune evasion of cancer.

INTRODUCTION

Malignant cellular transformation occurs through a progression of genetic mutations and epigenetic reprogramming that activates oncogenes and inactivates tumor suppressor pathways. Several hallmarks are shared by most cancer cells, including self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, poorly regulated replicative potential, sustained angiogenesis, and evasion of cell death by a variety of pathways, including apoptosis (*1*). In addition to these cell-intrinsic properties, recent evidence suggests that many cancers are also able to evade the immune system through several distinct mechanisms (*2*–*4*).

Recently, we showed that evasion of phagocytosis through up-regulation of the anti-phagocytic signal CD47 is another mechanism by which tumor cells escape immunosurveillance (*5*–*9*). CD47 is a pentaspanin cell surface protein that serves as a signal inhibiting phagocytosis through ligation of its receptor SIRPα (signal regulatory protein α) on phagocytic cells (*10*–*12*). The CD47-SIRPα interaction can be therapeutically targeted with a monoclonal blocking antibody against CD47, which enables phagocytosis of acute myeloid leukemia (AML), bladder cancer, and non-Hodgkin's lymphoma (NHL) cells in vitro and in vivo (*6*, *8*, *9*). In contrast, administration of anti–mouse CD47 antibody causes minimal toxicity (*6*, *9*), despite broad expression of CD47 on normal tissues (*13*).

In order for target cells to be phagocytosed upon blockade of an anti-phagocytic signal, these cells must also display a potent pro-phagocytic signal. CD47 has also been implicated in the regulation of phagocytosis of apoptotic cells; these cells become phagocytosed due to loss of CD47 expression and coordinate up-regulation of cell surface calreticulin (CRT) (*14*). During apoptosis, cell surface CRT serves as a pro-phagocytic signal by binding to its macrophage receptor, low-density lipoprotein–related protein (LRP), which leads to engulfment of the target cell (*14*, *15*). We hypothesized that the selective targeting of tumor cells with anti-CD47 antibody was due to the presence of a pro-phagocytic stimulus on tumor cells, but not on most normal cells, that becomes unopposed after CD47 blockade. Here, we identify cell surface CRT as this pro-phagocytic stimulus, whose differential expression helps to explain the lack of anti-CD47 antibody–mediated toxicity against most normal cells. We propose that CRT expression on newly arising neoplasms may be an early event and that only those tumor clones that up-regulate CD47 can escape the phagocytic consequences of cell surface CRT expression.

RESULTS

Cell surface CRT is expressed on cancer, but not most normal, stem and progenitor cells

Cell surface CRT expression was determined on a variety of primary human cancer cells and their normal cell counterparts by flow cytometry. In hematologic malignancies, cell surface CRT was expressed on a greater percentage of bulk cells in AML (average = 23.9%), acute lymphocytic leukemia (ALL; 17.6%), chronic-phase chronic myeloid leukemia (CML; 47.6%), and NHL (18.3%) when compared to normal bone marrow (2.6%) and normal peripheral blood cells (2.6%) (Fig.

[1]Institute for Stem Cell Biology and Regenerative Medicine, Stanford Cancer Center, and Ludwig Center at Stanford, Stanford, CA 94305, USA. [2]Department of Pathology, Massachusetts General Hospital, Boston, MA 02114, USA. [3]Department of Internal Medicine, Division of Hematology, Stanford University, Palo Alto, CA 94304, USA. [4]Division of Oncology, Stanford University, Palo Alto, CA 94304, USA. [5]Department of Radiology, Stanford University, Palo Alto, CA 94304, USA. [6]Departments of Pathology and Clinical Laboratories and Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA.
*To whom correspondence should be addressed. E-mail: mpchao@stanford.edu
†These authors contributed equally to this work.

1A). In solid tumors, cell surface CRT was also expressed on a greater percentage of bulk cells in glioblastoma (31.7%), bladder cancer (23.7%), and ovarian cancer (average = 20.5%) when compared to normal fetal neurons (0.3%), astrocytes (2.5%), and normal fetal bladder cells (1.41%) (Fig. 1B). In this analysis, annexin V–positive cells were excluded, indicating that CRT-positive cancer cells were not part of the apoptotic cell population. In addition, CRT-positive cancer cells (from AML and bladder cancer patients) formed tumors when engrafted into immunodeficient mice similarly to CRT-negative cancer cells, indicating that CRT-positive cancer cells were functionally viable and have tumorigenic potential in vivo (fig. S1).

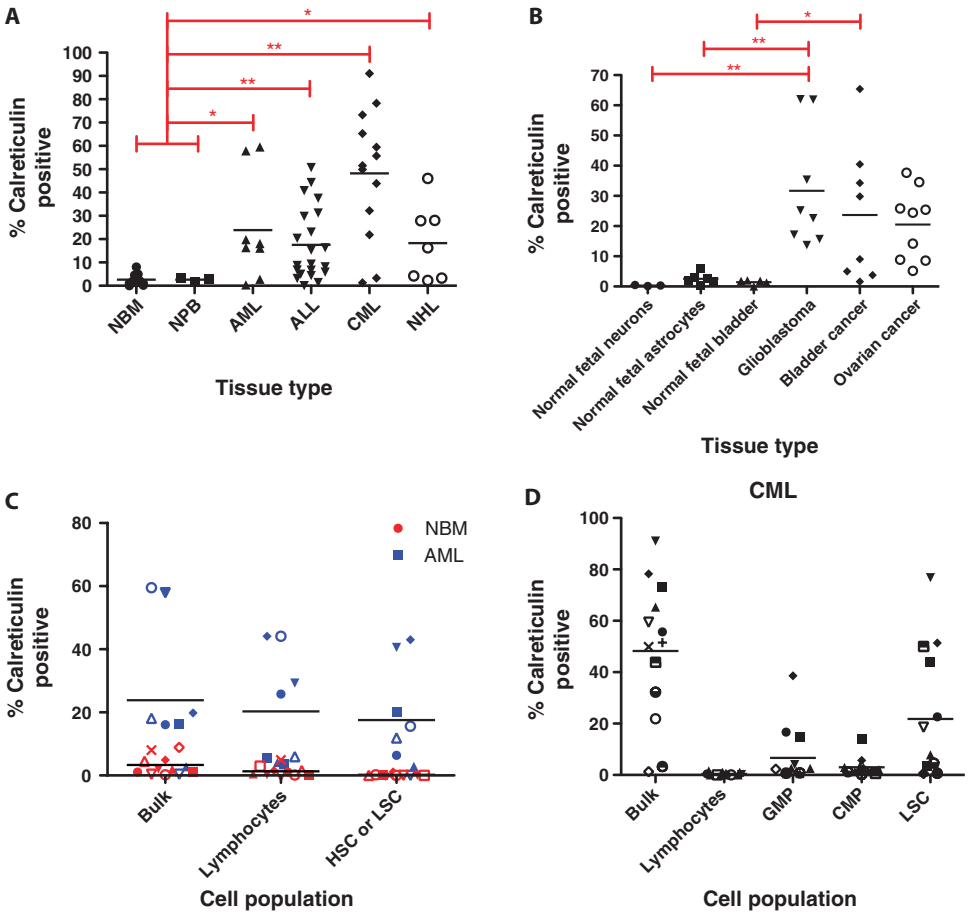

Fig. 1. Cell surface calreticulin (CRT) is expressed on cancer, but not normal, stem and progenitor cells. (**A**) Cell surface CRT expression was determined by flow cytometry on primary human patient samples from several hematologic cancer types and normal cell counterparts, including normal bone marrow (NBM; $n = 9$), normal peripheral blood (NPB; $n = 3$), acute myeloid leukemia (AML; $n = 8$), acute lymphoblastic leukemia (ALL; $n = 21$), chronic myeloid leukemia (CML; $n = 13$), and non-Hodgkin's lymphoma (NHL; $n = 7$). (**B**) A similar analysis as in (A) was performed for solid tumors (glioblastoma, $n = 9$; transitional cell bladder carcinoma, $n = 8$; serous papillary ovarian carcinoma, $n = 9$) and normal human fetal tissues (neurons, $n = 3$; astrocytes, $n = 6$; bladder cells, $n = 6$). ESA (epithelial-specific antigen)–positive urothelium was analyzed for normal fetal bladder. Primary human bladder cancer patient samples and samples that had been passaged once in mice were used for profiling. (**C** and **D**) Cell surface CRT expression was determined on normal stem and progenitor cells, lymphocytes, and cancer stem and progenitor cells. Each symbol represents a different patient sample. Patient samples tested: NBM = 10, AML = 8, CML = 13, bladder cancer = 8, glioblastoma = 8. NBM hematopoietic stem cell (HSC) = $CD34^+CD38^-CD90^+Lin^-$, AML LSC = $CD34^+CD38^-CD90^-Lin^-$, GMP = $CD34^+CD38^+IL3r\alpha^+CD45RA^+$, CMP = $CD34^+CD38^+IL3r\alpha^+CD45RA^-$. (**E**) CRT expression did not differ between bulk and cancer stem cell populations for either bladder cancer ($P = 0.54$) or glioblastoma ($P = 0.14$). Bladder cancer CSC = $CD44^+Lin^-$ (*8*), glioblastoma CSC = $CD133^+Lin^-$ (*22*, *23*). Annexin V–positive cells were excluded in the analysis of all samples.

Previous studies have identified that the endoplasmic reticulum (ER) protein ERp57 co-translocates with CRT to the cell surface and is required for CRT cell surface exposure under conditions of apoptosis (*16*, *17*). Accordingly, we assessed the relationship between cell surface CRT and ERp57 expression on tumor cells. On nonapoptotic (annexin V–negative) tumor cells, cell surface ERp57 expression was associated with cell surface CRT expression (fig. S2A). Furthermore, across several different tumor types (including primary human tumor samples and cancer cell lines), ERp57 was expressed on a higher percentage of CRT^+ cells compared to CRT^- counterparts (fig. S2, B and C).

Given that primary human tumors are heterogeneous and contain a subpopulation of tumor-initiating cells [reviewed in (*18*)], we next investigated whether cell surface CRT was present on the cancer stem cell (CSC) population of each tumor type in which the immunophenotype of functional CSCs is known. In AML and chronic-phase CML, cell surface CRT was expressed on $CD34^+CD38^-CD90^-Lin^-$ AML (*19*, *20*) and $CD34^+CD38^-CD90^+$ chronic-phase CML (*21*) leukemia stem cells (LSCs), as well as downstream progenitor populations, whereas normal bone marrow hematopoietic stem and progenitor populations expressed minimal cell surface CRT (Fig. 1, C and D). For AML, similar amounts of cell surface CRT were observed for LSC compared to other cellular subsets (Fig. 1C). In contrast, CML LSC expressed higher amounts of cell surface CRT compared to downstream common myeloid progenitor (CMP) and granulocyte macrophage progenitor (GMP) populations (Fig. 1D). Cell surface CRT was also present on CSC of solid tumors, including $CD44^+Lin^-$ bladder CSC (*8*) and $CD133^+Lin^-$ glioblastoma CSC (*22*, *23*) (Fig. 1E).

We next determined whether there was a correlation between CRT and CD47 expression in human tissues, postulating that

a balance between pro-phagocytic (CRT) and anti-phagocytic (CD47) signals may be maintained as a homeostatic mechanism. CRT and CD47 cell surface expression were profiled in a variety of human cancer cell lines, primary cancers, and normal cells. CD47 expression correlated with CRT expression in a variety of hematologic and solid tumor cell lines as well as in primary human AML, CML, and ALL patient samples (Fig. 2A). Notably, normal cells expressed minimal levels of both CRT and CD47 (Fig. 2A, top panels). In normal human bone marrow and fetal bladder, those cells that were CRT-positive expressed higher levels of CD47 compared to CRT-negative cellular counterparts (fig. S3). Thus, in both normal and cancer cells, there is a strong positive correlation between CRT and CD47 expression.

Increased CD47 on cancer cells protects them from CRT-mediated phagocytosis

We observed increased cell surface CRT and CD47 on human cancer cells, leading us to hypothesize that increased CD47 protects these cells from CRT-mediated phagocytosis. To investigate this hypothesis, we performed in vitro phagocytosis assays on two different CRT-expressing cancer cell lines: one expressing high CD47 levels (Raji) and one deficient in CD47 expression (MOLM13). First, Raji cells, a Burkitt's NHL cell line that expresses high levels of CD47 and CRT (Fig. 2B and fig. S4), were incubated with human macrophages under conditions where CD47 expression was knocked down to various levels by lentiviral transduction of short hairpin RNAs (shRNAs) (Fig. 2, B and C). Cell surface CRT expression was unaffected by shRNA-mediated CD47 knockdown (fig. S4). Upon incubation with human macrophages, Raji cells that expressed half as much CD47 (shCD47-1 and shCD47-2) were more robustly phagocytosed by human macrophages than were wild-type and glyceraldehyde-3-phosphate dehydrogenase (GAPD) control–transduced Raji cells, which were minimally phagocytosed (Fig. 2D). Phagocytosis of

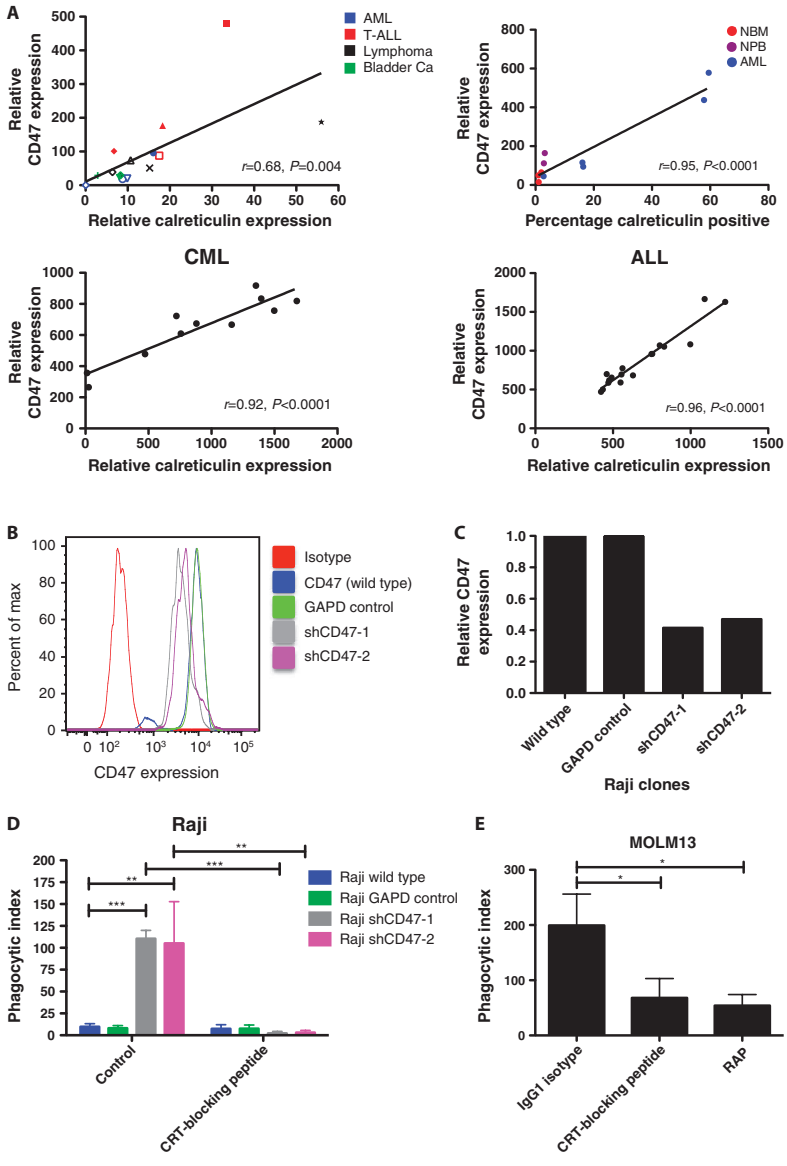

Fig. 2. Increased CD47 expression on cancer cells protects them from CRT-mediated phagocytosis. (**A**) Correlation between cell surface CRT and CD47 expression was determined for human cancer cell lines (top left) and primary human normal and cancer samples (top right, bottom panels). Expression was calculated as mean fluorescence intensity (MFI) normalized over isotype control and for cell size. Pearson correlation (*r*) and *P* value are shown for each correlation. Top left panel: blue solid circle, HL60; blue open circle, Kasumi1; blue open inverted triangle, MOLM13; blue open diamond, KG-1; red triangle, Jurkat; red solid square, CCRF-CEM; red open square, CCRF-HSB2; red diamond, MOLT4; black star, Raji; black open diamond, SUDHL6; black open triangle, Daudi; black ×, U937; green plus, 639V; green open diamond, HT1197; green inverted triangle, UMUC3. (**B**) CD47 protein expression was determined by flow cytometry on Raji cells transduced with lentiviruses encoding shRNA CD47 knockdown constructs (shCD47) or controls. (**C**) Relative CD47 expression levels were quantified by comparing MFI to wild-type Raji cells. (**D**) Raji cell clones were incubated with human macrophages in media alone or with CRT-blocking peptide for 2 hours, after which phagocytosis was analyzed by fluorescence microscopy. Knockdown of CD47 in Raji cells (shCD47-1 and shCD47-2) resulted in increased phagocytosis compared to untransduced Raji cells. No difference in phagocytosis was observed between untransduced and GAPD control–transduced Raji cells ($P = 0.45$) Blockade of CRT on CD47 knockdown Raji cells completely abrogated phagocytosis. (**E**) MOLM13 cells, a CD47-deficient human AML cell line, were incubated with human macrophages for 2 hours with the indicated peptides and monitored for phagocytosis as above. High levels of phagocytosis were observed with IgG1 isotype control, whereas blockade of CRT or LRP reduced levels of phagocytosis ($P = 0.03$ and $P = 0.01$, respectively). Conditions were performed in triplicate; data are presented as means ± SD. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$ (two-tailed Student's *t* test).

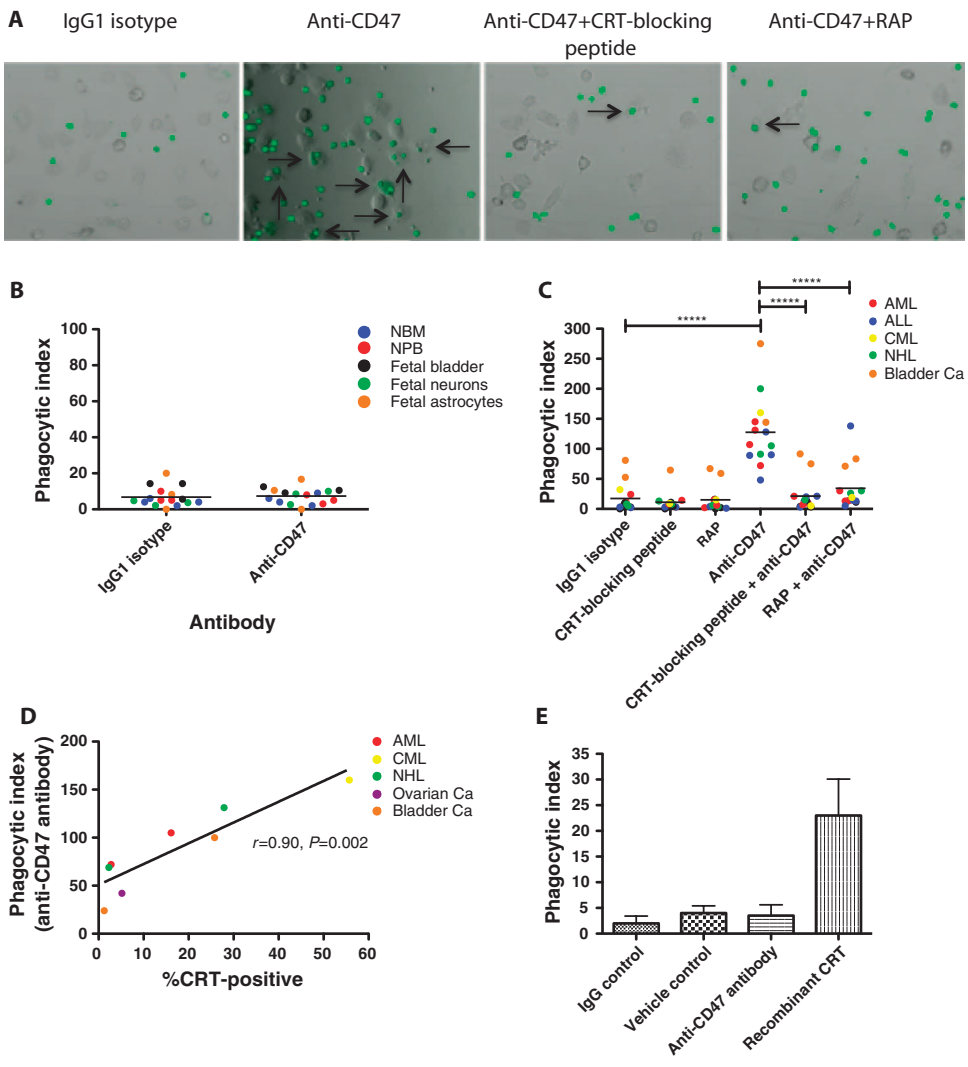

Fig. 3. Cell surface CRT is the dominant pro-phagocytic signal on several human cancers and is required for anti-CD47 antibody–mediated phagocytosis. (**A**) Primary human AML cells were fluorescently labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and incubated with human macrophages in the presence of the indicated antibodies/peptides for 2 hours, after which phagocytosis was analyzed by fluorescence microscopy. Arrows indicate phagocytosis. (**B**) Cells from several normal human tissue types were incubated with human macrophages in the presence of the indicated antibodies and monitored for phagocytosis. No difference in phagocytosis was detected between IgG1 isotype control and anti-CD47 antibody incubation ($P = 0.77$). (**C**) Primary human cancer cells were incubated with human macrophages in the presence of the indicated antibodies/peptides for 2 hours and monitored for phagocytosis. Each data point represents a different patient sample. Compared to IgG1 isotype control, incubation with anti-CD47 antibody enabled phagocytosis of cancer cells ($P < 0.0001$), whereas incubation with CRT-blocking peptide ($P = 0.37$) or RAP, an LRP inhibitor ($P = 0.67$), did not enable phagocytosis. In the presence of anti-CD47 antibody, incubation of cancer cells with either CRT-blocking peptide or RAP completely abrogated anti-CD47 antibody–mediated phagocytosis ($P = 0.77$ and $P = 0.16$, respectively, compared to IgG1 isotype control). *****$P < 0.00001$ (two-sided Student's t test). (**D**) A positive correlation was observed between cell surface CRT expression and degree of anti-CD47 antibody–mediated phagocytosis (Pearson's correlation coefficient is shown). Each point represents a distinct patient sample that was incubated in the same in vitro phagocytosis assay. (**E**) Human NBM cells were incubated with human macrophages in the presence of the indicated antibodies or protein. Exogenous CRT enabled increased phagocytosis of NBM cells compared to vehicle control ($P = 0.05$). No difference in phagocytosis was observed between IgG1 isotype control and anti-CD47 antibody ($P = 0.49$). Conditions were performed in triplicate; data are presented as means ± SD.

shCD47-1 and shCD47-2 Raji cells was dependent on the CRT-LRP interaction, because the observed phagocytosis was completely abrogated in the presence of a CRT-blocking peptide (Fig. 2D). In the second experiment, MOLM13 cells, a human AML cell line that is deficient in CD47 expression (*5*) but expresses CRT (fig. S4), were incubated with human macrophages. As expected, MOLM13 cells were robustly phagocytosed at baseline, whereas phagocytosis was significantly reduced when the CRT-LRP interaction was blocked (Fig. 2E). These findings demonstrate that overexpression of CD47 in cancers counterbalances CRT-mediated phagocytosis.

CRT is the dominant pro-phagocytic signal on several human cancers and is required for anti-CD47 antibody–mediated phagocytosis

Given the ability of the pro-phagocytic function of CRT to be counteracted by CD47 (Fig. 2), we investigated whether the expression of cell surface CRT on cancer but not normal cells could explain the selective targeting of tumor cells by a blocking anti-CD47 antibody. In vitro phagocytosis assays were performed by incubating primary human normal cells or cancer cells with human macrophages in the presence of antibodies to CD47. CD47 was expressed on all normal and cancer cells profiled (Fig. 2A and figs. S3 and S5), but expression of CRT was primarily restricted to tumor cells (Fig. 1, A and B). No phagocytosis of cells from a variety of normal human tissue types was observed with anti-CD47 antibody treatment (Fig. 3B), whereas primary cancer cells from a variety of tumor types were robustly phagocytosed (Fig. 3, A and C). Phagocytosis of cancer cells mediated by antibodies to CD47 was completely abrogated in most cases when cells were simultaneously incubated with peptides that inhibit the CRT-LRP interaction, including a CRT-blocking peptide and receptor-associated protein (RAP), an inhibitor of LRP (*14*) (Fig. 3C). Increasing concentrations of a CRT-blocking peptide led to a dose-dependent reduction in anti-CD47 antibody–mediated phagocytosis (fig. S6). Notably, additional blockade of other pro-phagocytic signals was not required to abolish anti-CD47 antibody–mediated phagocytosis, because cells in-

cubated with anti-CD47 antibody under CRT-LRP blockade were phagocytosed at levels similar to that of baseline controls (Fig. 3C). However, two bladder cancer samples exhibited higher baseline levels of phagocytosis with immunoglobulin G1 (IgG1) isotype control compared to other cancer cell types, which may be due to expression of other pro-phagocytic signals on these specific cells. Nevertheless, blockade of CRT or LRP in the presence of anti-CD47 antibody abrogated phagocytosis of these bladder cancer cells to levels similar to those of IgG1 isotype controls. Moreover, blockade of the CRT-LRP interaction alone by CRT-blocking peptide or LRP had no effect on phagocytosis when compared to IgG control (Fig. 3C).

Next, the relationship between the level of tumor cell surface CRT expression and the level of phagocytosis by anti-CD47 antibody was investigated. Cell surface CRT expression on tumor cells positively correlated with the degree of anti-CD47 antibody–mediated phagocytosis, regardless of tumor cell type (Fig. 3D). Finally, given that normal cells express minimal levels of cell surface CRT, we investigated whether the addition of CRT to the surface of these cells could enable phagocytosis. An in vitro phagocytosis assay was performed on NBM cells incubated with exogenous recombinant CRT protein, which was previously demonstrated to adsorb onto the cellular surface (*14*), allowing NBM cells to bind to macrophage LRP. In contrast to vehicle control, incubation with exogenous CRT enabled phagocytosis of NBM cells, whereas anti-CD47 antibody did not (Fig. 3E). Collectively, these results demonstrate that anti-CD47 antibody–mediated phagocytosis requires the presence of cell surface CRT.

Increased CRT expression confers a worse clinical prognosis in multiple human malignancies

Last, we sought to investigate the clinical relevance of these findings by investigating the association between CRT expression and clinical outcomes. We analyzed *CRT* messenger RNA (mRNA) levels in patients with human malignancies of distinct tumor types and investigated their correlation with tumor progression and clinical outcome. Using previously published gene profiling data sets with associated clinical outcome data, we determined *CRT* expression in both hematologic and solid tumor malignancies, including NHL [mantle cell lymphoma (MCL)], superficial and invasive bladder cancer, and neuroblastoma. Patients were stratified into *CRT* high- and low-expressing cohorts relative to the median value and analyzed for clinical outcomes. For each tumor type, correlations between *CRT* expression and event-free, disease-specific, or overall survival were measured in two independent data sets to test and validate significant associations. Regardless of tumor type, higher *CRT* expression predicted a worse clinical outcome in all malignancies analyzed: neuro-

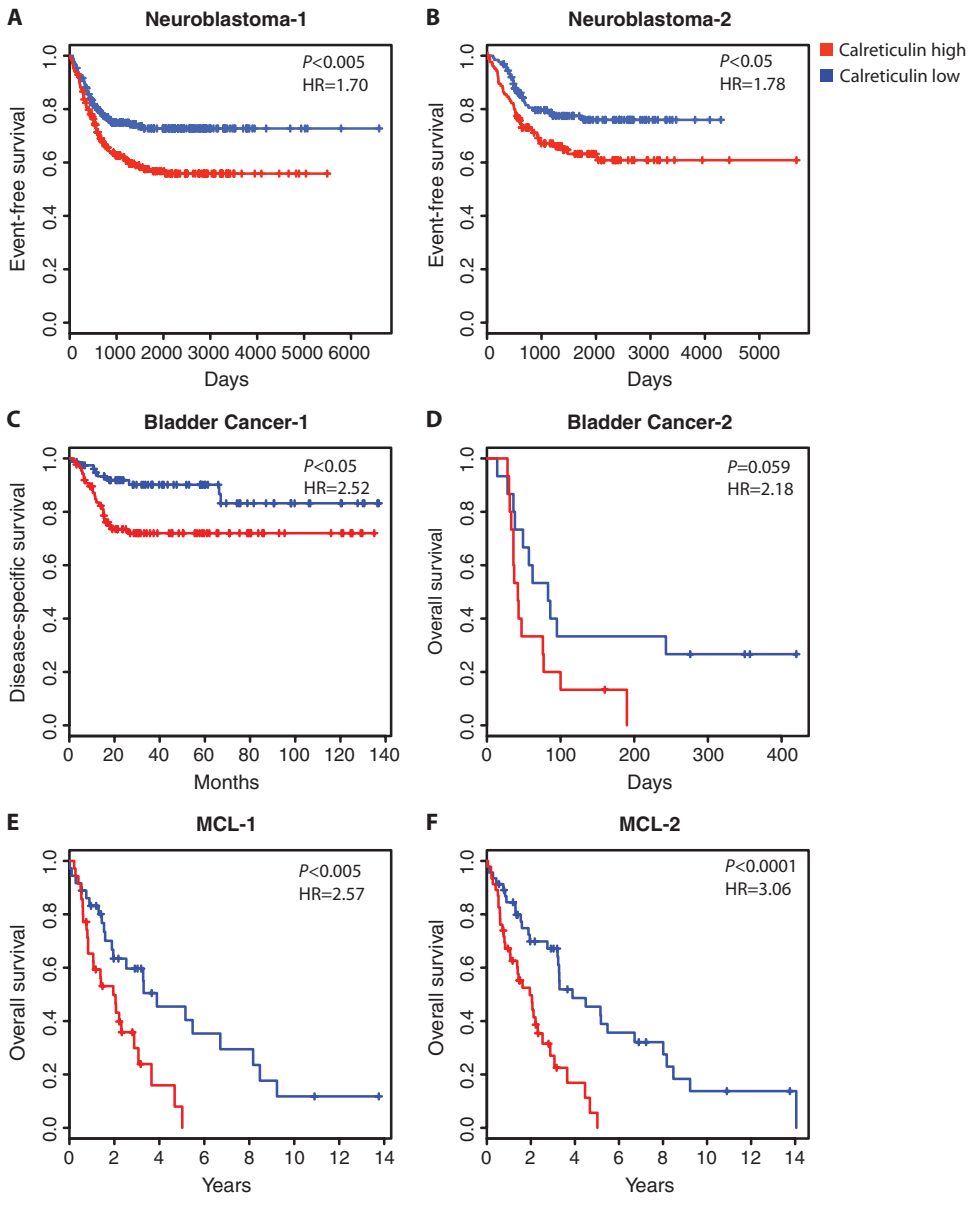

Fig. 4. Increased CRT expression confers a worse clinical prognosis in multiple human malignancies. (**A** to **F**) Stratification of clinical outcomes based on the level of expression of *CRT* mRNA is shown in previously described cohorts (*50*–*55*) of patients with diverse malignancies including neuroblastoma (A and B), superficial or invasive bladder cancer (C and D), and mantle cell lymphoma (E and F). Patients were divided into *CRT* high- and low-expression groups based on median *CRT* expression, with Kaplan-Meier analyses of patient outcome shown. Hazard ratios (HR) and log-rank *P* values are shown for the relationship of outcomes to dichotomous expression of *CRT*. HR and log-rank *P* values for *CRT* expression as a continuous variable using a univariate Cox regression model are shown in table S1. All *P* values were <0.05 when *CRT* was considered as a continuous variable. Description of clinical data sets is shown in table S1.

blastoma (Fig. 4, A and B), bladder cancer (Fig. 4, C and D), and NHL (MCL; Fig. 4, E and F). These associations were significant when *CRT* expression was considered either as a dichotomous variable (relative to the median) or as a continuous variable (table S1). The prognostic power of CRT was independent from type of therapy, because patients with the various tumors received disparate treatments including observation, surgery, or chemotherapy (table S1). Additionally, the prognostic power of CRT was preserved in both early- and late-stage tumors, because increased *CRT* levels correlated with worse survival in both superficial and invasive bladder cancer (Fig. 4, C and D, and table S1). Thus, *CRT* expression is associated with tumor progression and worse clinical outcome across several tumor types. It should, however, be cautioned that only *CRT* mRNA was analyzed here, and thus, the specific role of cell surface CRT in tumor progression cannot be inferred from this study.

DISCUSSION

In this report, we identify CRT as a pro-phagocytic signal highly expressed on the surface of several human cancers, but minimally expressed on normal cell counterparts, and demonstrate that CRT expression is required for anti-CD47 antibody–mediated phagocytosis.

Anti-CD47 antibody preferentially eliminates tumor cells because of differential expression of cell surface CRT

We recently demonstrated that several cancers overexpress CD47 and that a blocking anti-CD47 monoclonal antibody can eliminate tumor cells in vitro and in vivo (*6*, *8*, *9*). These preclinical findings provide a strong rationale for the use of an anti-CD47 antibody in the treatment of human cancers. However, given the broad low-level expression of CD47 on both hematopoietic and most other normal tissues, antibody toxicity could be a considerable barrier to clinical translation. To investigate this issue, we previously injected a blocking anti–mouse CD47 antibody into wild-type mice at a dose that coated >98% of bone marrow cells but observed no overt toxicity, with the exception of isolated neutropenia (*6*). Moreover, a recent report demonstrated that inhibition of CD47 with either an antibody or morpholino could confer radioprotective effects to normal tissues (*24*). Here, we demonstrate that despite low-level CD47 expression, normal human cells from several tissues are not phagocytosed by human macrophages when coated with anti-CD47 antibody (Fig. 3B).

We speculate that the selective phagocytosis of tumor cells is not simply dictated by CD47 expression level, but is also governed by the presence of the pro-phagocytic signal CRT, which is present on tumor cells but not on normal cells. Several lines of evidence support this hypothesis. First, normal cells that express CD47 but not CRT are not phagocytosed with an anti-CD47 antibody despite being coated with the antibody (Fig. 3B). Second, tumor cells that express CD47 and CRT are phagocytosed when coated with anti-CD47 antibody (Fig. 3, A and C). Third, phagocytosis of tumor cells with anti-CD47 antibody is completely abrogated when the CRT-LRP interaction is blocked (Fig. 3, A and C). Fourth, adsorption of exogenous CRT onto the surface of NBM cells, which express minimal CRT (Fig. 1A), enabled increased phagocytosis compared to vehicle control or anti-CD47 antibody administration (Fig. 3E). Collectively, these findings demonstrate that CRT is necessary for anti-CD47 antibody–mediated phagocytosis and that surface expression of this protein is primarily restricted to tumor cells.

This study indicates that the therapeutic window for anti-CD47 antibody therapy is not just a consequence of CD47 level on target cells, but that it also depends on the surface expression of pro-phagocytic CRT. On the basis of our findings, we propose a model in which the overall contribution of pro-phagocytic (CRT) and anti-phagocytic (CD47) signals determines whether normal or tumor cells are phagocytosed at steady state, or by anti-CD47 antibody therapy (fig. S7). At steady state, tumor cells express CRT but evade phagocytosis through overexpression of CD47, indicating the dominance of the "don't eat me" anti-phagocytic signal (fig. S7, A and B). Normal cells express low levels of CD47 and avoid phagocytosis because of a lack of CRT expression. In contrast, cells undergoing DNA damage or apoptosis express CRT on their cell surface (*14*, *25*), which is dominant over low CD47 expression and leads to phagocytosis. In the context of anti-CD47 antibody therapy, the anti-phagocytic signal (CD47) is blocked, unmasking the pro-phagocytic signal (CRT) on tumor cells, leading to phagocytosis (fig. S7, C and D). In contrast, blockade of CD47 on normal cells does not lead to phagocytosis because the pro-phagocytic "eat me" signal (CRT) is absent.

Although CRT appears to be primarily expressed on the surface of apoptotic or malignant cells, previous reports detected surface CRT on some human normal cells including activated peripheral blood T cells (*26*) and circulating neutrophils (*27*). In addition, a blocking monoclonal anti-CD47 antibody enhances phagocytosis of apoptotic neutrophils (*28*, *29*). In our mouse toxicity studies, administration of a blocking anti–mouse CD47 antibody led to selective depletion of neutrophils, whereas other hematopoietic cells were unaffected (*6*). Similar to tumor cells, this selective neutropenic toxicity may be due to unmasking of CRT on neutrophils when the "don't eat me" signal (CD47) is blocked by anti-CD47 antibody. Although most normal cells do not express cell surface CRT, normal cells may up-regulate CRT under certain conditions, including radiation and anthracycline-based chemotherapy, as has been shown in some tumor types (*25*, *30*). Our findings provide a cautionary note that normal cells might up-regulate CRT as a consequence of radiation and chemotherapy-based cancer therapy, and thus, combination of chemoradiation and anti-CD47 antibody therapy must be tested for potential increased toxicity to normal cells.

CRT is the dominant pro-phagocytic signal on several human cancers

We demonstrate that several human cancers, including both hematopoietic and solid tumor malignancies, express the pro-phagocytic signal CRT. Known physiologic pro-phagocytic signals have previously been identified in several cancers including phosphatidylserine (*31*–*35*) and annexin-1 [reviewed in (*36*)]. However, most of these studies were not performed on primary human patient samples as in this study. Additionally, ligand expression appears to be mixed across tumor types (*36*), with the functional role of these ligands in cancer not known. A complete survey of human tumors for cell surface CRT expression will be required to determine whether the regulation of the CD47-CRT phagocytic axis is a universal trait of cancers. Although CRT appears to be the dominant pro-phagocytic signal on the cancer samples profiled, it is possible that these and other tumor types might express other pro-phagocytic signals that similarly regulate phagocytosis. A number of pro-phagocytic signals have been identified on apoptotic cells [reviewed in (*37*)], thus warranting an investigation of the potential pro-phagocytic roles of these ligands in cancer.

One key question is raised by these studies: Why do cancers express cell surface CRT, a pro-phagocytic signal? We have demonstrated that certain cancers evade the innate immune system by up-regulating anti-phagocytic signals, specifically CD47 (*5*, *6*, *8*). One might expect cancers to simultaneously down-regulate pro-phagocytic signals to further increase their ability to evade macrophage phagocytosis. We propose two possible explanations. First, expression of cell surface CRT may be an unwanted consequence of cellular stress, whereby CD47 expression is up-regulated to compensate and enable phagocytic evasion. In normal physiology, cell surface CRT is induced on cells undergoing DNA damage (*14*, *25*, *30*), marking these damaged cells for homeostatic phagocytosis. It is possible that a small fraction of these cells may selectively avoid phagocytic clearance due to higher levels or up-regulation of CD47, which allows these damaged cells to survive and acquire additional mutations, eventually transforming into fully malignant cells. Several lines of evidence support this hypothesis. First, CD47 and CRT expression are highly correlated in several human tumors (Fig. 2A). Second, the small percentage of live cells that are CRT-positive in some normal human tissue types (bone marrow and bladder) express higher CD47 levels than their CRT-negative counterparts (fig. S3). Third, this increase in CD47 expression appears to protect against CRT-mediated phagocytosis, because knockdown of CD47 to 50% of wild-type levels enabled CRT-dependent phagocytosis (Fig. 2).

In a second hypothesis, expression of cell surface CRT may confer some unknown protumorigenic phenotype to cancer cells that is independent of phagocytosis. This hypothesis is supported by the finding that increased *CRT* expression in human tumors confers a worse clinical outcome across disparate tumor types, tumor stage, and tumor-specific therapies (Fig. 4). One possibility is that cell surface CRT may allow more invasion and angiogenesis, because its ligand, LRP, is expressed on several vascular cell types [reviewed in (*38*)]. In two reports, overexpression of CRT or CRT fragments in tumor cell lines enhanced in vitro migration and invasion (*39*, *40*); however, other studies have reported alternative roles for CRT (*41*–*43*). In all of these studies, the function of cell surface CRT was not distinguished from its intracellular roles. Other possible tumorigenic roles include cell adhesion (*44*) and immune escape through reduction of major histocompatibility complex (MHC) class I antigen presentation (*45*).

In summary, we have identified cell surface CRT as the dominant pro-phagocytic signal on several human cancers, which is absent on normal cell counterparts and is required for anti-CD47 antibody–mediated phagocytosis. These findings support the rationale for the development of an anti-CD47 antibody therapy for human malignancies and highlight the dynamic relationship between pro- and anti-phagocytic signals in human cancer.

MATERIALS AND METHODS

Cell lines and human samples

MOLT4 and Daudi cell lines were obtained from the lab of R. Levy. 639V was obtained from the German Resource Centre for Biological Material. All other cell lines were obtained from the American Type Culture Collection (ATCC). Normal human bone marrow mononuclear cells were purchased from AllCells Inc. Normal peripheral blood and human cancer samples were obtained from patients at the Stanford Medical Center with informed consent according to Institutional Review Board (IRB)–approved protocols: AML, ALL, and NHL human samples from Stanford IRB #76935, #6453, and #13500; bladder cancer samples from Stanford IRB #1512; glioblastoma samples from Stanford IRB #9363; and ovarian cancer samples from Stanford IRB #13939. Normal fetal bladder and brain cells were purchased from ScienCell Research Laboratories.

Flow cytometry analysis

For analysis of normal peripheral blood cells, normal bone marrow cells, AML, CML, ALL, bladder cancer, ovarian cancer, and brain cancer, the following antibodies were used: CD34, CD38, CD90, CD45, CD31, CD3, CD4, CD7, CD11b, CD14, CD19, CD20, CD56, and Glycophorin A (Invitrogen and BD Biosciences). Lineage-negative (Lin^-) was defined as $CD3^-CD19^-CD20^-$ for AML LSC and $CD45^-CD31^-$ for glioblastoma multiforme (GBM) and bladder cancer CSC. Lin^- was defined as $CD3^-CD4^-CD7^-CD8^-CD11b^-CD14^-CD19^-CD20^-CD56^-$Glycophorin A^- for NBM hematopoietic stem cell (HSC), chronic-phase CML GMP, CML CMP, and CML LSC. Analysis of CD47 expression was performed with an anti-human CD47 fluorescein isothiocyanate (FITC) antibody (clone B6H12.2, BD Biosciences). Analysis of human cell surface CRT expression was performed with mouse anti-human CRT conjugated to phycoerythrin (PE) or FITC (clone FMC 75, Abcam). Human ERp57 expression was performed with a polyclonal rabbit anti-ERp57 antibody (Abcam) and then staining with a donkey anti-rabbit secondary antibody conjugated to PE (Ebioscience).

In vitro phagocytosis assay

Generation of human macrophages and in vitro phagocytosis assays were performed as previously described (*6*). Primary human samples or cell lines were incubated with IgG1 isotype control (10 μg/ml) (Ebioscience), anti-CD47 antibody (10 μg/ml) (clone B6H12.2, ATCC), CRT-blocking peptide (4 μg/ml) (MBL International Corporation), RAP (10 μg/ml) (Fitzgerald Industries International), or recombinant CRT human protein (125 μg/ml) (Thermo Scientific). Per MBL, confirmation of blocking activity was performed by Western blot analysis and verified by incubation of an anti-CRT antibody with five times higher concentration of the peptide and performing a Western blot analysis to determine whether the specific band had been diminished. Characterization of the LRP-antagonizing effects of RAP is detailed in (*46*–*48*). Cells were then analyzed by fluorescence microscopy to determine the phagocytic index (number of cells ingested per 100 macrophages).

shRNA knockdown of Raji cells

shRNA constructs targeting knockdown of human CD47 or a GAPD control packaged in the SMARTvector 2.0 lentiviral vector containing a turbo green fluorescent protein (GFP) reporter were purchased from Dharmacon Inc. Viral titers for each shRNA construct were greater than 10^8 transducing units/ml. Raji cells were transduced with these lentiviral constructs, analyzed, and sorted for GFP expression, expanded, and sorted again for GFP expression for stable propagation of lentivirally transduced cells. Knockdown of CD47 protein levels was assessed by flow cytometry with anti-CD47 antibody (B6H12.2), with fold knockdown calculated by reduction in mean fluorescence intensity (MFI) normalized over isotype control.

Xenotransplantation of primary human cancer cells into mice

For engraftment of human AML cells, AML LSCs ($CD34^+CD38^-CD90^-Lin^-$) were sorted by fluorescence-activated cell sorting (FACS) and transplanted

into the facial vein of newborn NOD.Cg-*Prkdcscid*Il2rgtm1Wjl/SzJ (NSG) mice, sublethally irradiated with 2 gray (Gy) (1 Gy = 100 rads). Leukemic engraftment was analyzed 8 weeks later in the bone marrow of transplanted mice. For engraftment of human bladder cancer, bulk bladder cancer cells were resuspended in 25% Matrigel (BD Biosciences) and transplanted subcutaneously into the flanks of adult NSG mice. Tumor volume was serially monitored after transplantation by analyzing weights of excised tumors.

Analysis of the prognostic value of CRT expression in human malignancies

Gene expression and clinical data were analyzed for six previously described cohorts of neuroblastoma, superficial and invasive urothelial carcinoma of the bladder, and mantle cell lymphoma (see table S1 for data set descriptions). Patients were stratified into high– and low–*CRT* expression groups based on the median expression level within each cohort and analyzed for event-free, disease-specific, or overall survival by Kaplan-Meier analysis. Subsequent dichotomous hazard ratios, 95% confidence intervals, and log-rank *P* values were analyzed reflecting estimates within Kaplan-Meier analyses (table S1). Additionally, analyses were performed based on continuous expression of *CRT* and clinical outcome as measured by log-likelihood *P* values within a univariate Cox regression model (table S1). Affymetrix microarray data were processed starting with CEL files, with Entrez Gene probe set summarization using CustomCDF version 12 (*49*), and normalization using MAS 5.0 linear scaling method. Overlapping samples from related studies (Fig. 4, A, B, E, and F) have not been removed.

SUPPLEMENTARY MATERIAL

www.sciencetranslationalmedicine.org/cgi/content/full/2/63/63ra94/DC1
Fig. S1. Live calreticulin-positive cancer cells form tumors in vivo.
Fig. S2. Cell surface CRT correlates with ERp57 expression on tumor cells.
Fig. S3. Live calreticulin-positive cells from normal human tissues have higher levels of CD47 compared to calreticulin-negative cells.
Fig. S4. Calreticulin expression is unaffected by CD47 shRNA knockdown in Raji cells.
Fig. S5. CD47 is expressed on normal human cells.
Fig. S6. Abrogation of anti-CD47 antibody–mediated phagocytosis is dose-dependent on calreticulin blockade.
Fig. S7. Model for the integration of pro-phagocytic (CRT) and anti-phagocytic (CD47) signals on normal and tumor cells at steady state and during anti-CD47 antibody therapy.
Table S1. Analysis of the prognostic value of calreticulin in human malignancies.

REFERENCES AND NOTES

1. D. Hanahan, R. A. Weinberg, The hallmarks of cancer. *Cell* **100**, 57–70 (2000).
2. J. Stagg, R. W. Johnstone, M. J. Smyth, From cancer immunosurveillance to cancer immunotherapy. *Immunol. Rev.* **220**, 82–101 (2007).
3. J. B. Swann, M. J. Smyth, Immune surveillance of tumors. *J. Clin. Invest.* **117**, 1137–1146 (2007).
4. L. Zitvogel, A. Tesniere, G. Kroemer, Cancer despite immunosurveillance: Immunoselection and immunosubversion. *Nat. Rev. Immunol.* **6**, 715–727 (2006).
5. S. Jaiswal, C. H. Jamieson, W. W. Pang, C. Y. Park, M. P. Chao, R. Majeti, D. Traver, N. van Rooijen, I. L. Weissman, CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. *Cell* **138**, 271–285 (2009).
6. R. Majeti, M. P. Chao, A. A. Alizadeh, W. W. Pang, S. Jaiswal, K. D. Gibbs Jr., N. van Rooijen, I. L. Weissman, CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. *Cell* **138**, 286–299 (2009).
7. S. Jaiswal, M. P. Chao, R. Majeti, I. L. Weissman, Macrophages as mediators of tumor immunosurveillance. *Trends Immunol.* **31**, 212–219 (2010).
8. K. S. Chan, I. Espinosa, M. Chao, D. Wong, L. Ailles, M. Diehn, H. Gill, J. Presti Jr., H. Y. Chang, M. van de Rijn, L. Shortliffe, I. L. Weissman, Identification, molecular characterization, clinical prognosis, and therapeutic targeting of human bladder tumor-initiating cells. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 14016–14021 (2009).
9. M. P. Chao, A. A. Alizadeh, C. Tang, J. H. Myklebust, B. Varghese, S. Gill, M. Jan, A. C. Cha, C. K. Chan, B. T. Tan, C. Y. Park, F. Zhao, H. E. Kohrt, R. Malumbres, J. Briones, R. D. Gascoyne, I. S. Lossos, R. Levy, I. L. Weissman, R. Majeti, Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. *Cell* **142**, 699–713 (2010).
10. R. K. Tsai, P. L. Rodriguez, D. E. Discher, Self inhibition of phagocytosis: The affinity of 'marker of self' CD47 for SIRPα dictates potency of inhibition but only at low expression levels. *Blood Cells Mol. Dis.* **45**, 67–74 (2010).
11. A. N. Barclay, M. H. Brown, The SIRP family of receptors and immune regulation. *Nat. Rev. Immunol.* **6**, 457–464 (2006).
12. P. Jiang, C. F. Lagenaur, V. Narayanan, Integrin-associated protein is a ligand for the P84 neural adhesion molecule. *J. Biol. Chem.* **274**, 559–562 (1999).
13. M. I. Reinhold, F. P. Lindberg, D. Plas, S. Reynolds, M. G. Peters, E. J. Brown, In vivo expression of alternatively spliced forms of integrin-associated protein (CD47). *J. Cell Sci.* **108**, 3419–3425 (1995).
14. S. J. Gardai, K. A. McPhillips, S. C. Frasch, W. J. Janssen, A. Starefeldt, J. E. Murphy-Ullrich, D. L. Bratton, P. A. Oldenborg, M. Michalak, P. M. Henson, Cell-surface calreticulin initiates clearance of viable or apoptotic cells through *trans*-activation of LRP on the phagocyte. *Cell* **123**, 321–334 (2005).
15. A. W. Orr, C. E. Pedraza, M. A. Pallero, C. A. Elzie, S. Goicoechea, D. K. Strickland, J. E. Murphy-Ullrich, Low density lipoprotein receptor–related protein is a calreticulin coreceptor that signals focal adhesion disassembly. *J. Cell Biol.* **161**, 1179–1189 (2003).
16. T. Panaretakis, N. Joza, N. Modjtahedi, A. Tesniere, I. Vitale, M. Durchschlag, G. M. Fimia, O. Kepp, M. Piacentini, K. U. Froehlich, P. van Endert, L. Zitvogel, F. Madeo, G. Kroemer, The co-translocation of ERp57 and calreticulin determines the immunogenicity of cell death. *Cell Death Differ.* **15**, 1499–1509 (2008).
17. M. Obeid, ERP57 membrane translocation dictates the immunogenicity of tumor cell death by controlling the membrane translocation of calreticulin. *J. Immunol.* **181**, 2533–2543 (2008).
18. T. Reya, S. J. Morrison, M. F. Clarke, I. L. Weissman, Stem cells, cancer, and cancer stem cells. *Nature* **414**, 105–111 (2001).
19. T. Lapidot, C. Sirard, J. Vormoor, B. Murdoch, T. Hoang, J. Caceres-Cortes, M. Minden, B. Paterson, M. A. Caligiuri, J. E. Dick, A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367**, 645–648 (1994).
20. A. Blair, D. E. Hogge, L. E. Ailles, P. M. Lansdorp, H. J. Sutherland, Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo. *Blood* **89**, 3104–3112 (1997).
21. C. H. Jamieson, L. E. Ailles, S. J. Dylla, M. Muijtjens, C. Jones, J. L. Zehnder, J. Gotlib, K. Li, M. G. Manz, A. Keating, C. L. Sawyers, I. L. Weissman, Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N. Engl. J. Med.* **351**, 657–667 (2004).
22. S. K. Singh, I. D. Clarke, M. Terasaki, V. E. Bonn, C. Hawkins, J. Squire, P. B. Dirks, Identification of a cancer stem cell in human brain tumors. *Cancer Res.* **63**, 5821–5828 (2003).
23. S. K. Singh, C. Hawkins, I. D. Clarke, J. A. Squire, J. Bayani, T. Hide, R. M. Henkelman, M. D. Cusimano, P. B. Dirks, Identification of human brain tumour initiating cells. *Nature* **432**, 396–401 (2004).
24. J. B. Maxhimer, D. R. Soto-Pantoja, L. A. Ridnour, H. B. Shih, W. G. Degraff, M. Tsokos, D. A. Wink, J. S. Isenberg, D. D. Roberts, Radioprotection in normal tissue and delayed tumor growth by blockade of CD47 signaling. *Sci. Transl. Med.* **1**, 3ra7 (2009).
25. M. Obeid, T. Panaretakis, N. Joza, R. Tufi, A. Tesniere, P. van Endert, L. Zitvogel, G. Kroemer, Calreticulin exposure is required for the immunogenicity of γ-irradiation and UVC light-induced apoptosis. *Cell Death Differ.* **14**, 1848–1850 (2007).
26. F. A. Arosa, O. de Jesus, G. Porto, A. M. Carmo, M. de Sousa, Calreticulin is expressed on the cell surface of activated human peripheral blood T lymphocytes in association with major histocompatibility complex class I molecules. *J. Biol. Chem.* **274**, 16917–16922 (1999).
27. I. Ghiran, L. B. Klickstein, A. Nicholson-Weller, Calreticulin is at the surface of circulating neutrophils and uses CD59 as an adaptor molecule. *J. Biol. Chem.* **278**, 21024–21031 (2003).
28. Y. J. Park, G. Liu, E. F. Lorne, X. Zhao, J. Wang, Y. Tsuruta, J. Zmijewski, E. Abraham, PAI-1 inhibits neutrophil efferocytosis. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 11784–11789 (2008).
29. D. W. Lawrence, S. B. King, W. A. Frazier, J. M. Koenig, Decreased CD47 expression during spontaneous apoptosis targets neutrophils for phagocytosis by monocyte-derived macrophages. *Early Hum. Dev.* **85**, 659–663 (2009).
30. M. Obeid, A. Tesniere, F. Ghiringhelli, G. M. Fimia, L. Apetoh, J. L. Perfettini, M. Castedo, G. Mignot, T. Panaretakis, N. Casares, D. Métivier, N. Larochette, P. van Endert, F. Ciccosanti, M. Piacentini, L. Zitvogel, G. Kroemer, Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat. Med.* **13**, 54–61 (2007).
31. J. Connor, C. Bucana, I. J. Fidler, A. J. Schroit, Differentiation-dependent expression of phosphatidylserine in mammalian plasma membranes: Quantitative assessment of outer-leaflet lipid by prothrombinase complex formation. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3184–3188 (1989).

32. T. Utsugi, A. J. Schroit, J. Connor, C. D. Bucana, I. J. Fidler, Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes. *Cancer Res.* **51**, 3062–3066 (1991).
33. L. V. Rao, J. F. Tait, A. D. Hoang, Binding of annexin V to a human ovarian carcinoma cell line (OC-2008). Contrasting effects on cell surface factor VIIa/tissue factor activity and prothrombinase activity. *Thromb. Res.* **67**, 517–531 (1992).
34. H. Woehlecke, A. Pohl, N. Alder-Baerens, H. Lage, A. Herrmann, Enhanced exposure of phosphatidylserine in human gastric carcinoma cells overexpressing the half-size ABC transporter BCRP (ABCG2). *Biochem. J.* **376**, 489–495 (2003).
35. R. F. Zwaal, P. Comfurius, E. M. Bevers, Surface exposure of phosphatidylserine in pathological cells. *Cell. Mol. Life Sci.* **62**, 971–988 (2005).
36. L. H. Lim, S. Pervaiz, Annexin 1: The new face of an old molecule. *FASEB J.* **21**, 968–975 (2007).
37. J. Savill, I. Dransfield, C. Gregory, C. Haslett, A blast from the past: Clearance of apoptotic cells regulates immune responses. *Nat. Rev. Immunol.* **2**, 965–975 (2002).
38. D. K. Strickland, M. Z. Kounnas, W. S. Argraves, LDL receptor-related protein: A multiligand receptor for lipoprotein and proteinase catabolism. *FASEB J.* **9**, 890–898 (1995).
39. M. Liu, H. Imam, K. Oberg, Y. Zhou, Gene transfer of vasostatin, a calreticulin fragment, into neuroendocrine tumor cells results in enhanced malignant behavior. *Neuroendocrinology* **82**, 1–10 (2005).
40. C. N. Chen, C. C. Chang, T. E. Su, W. M. Hsu, Y. M. Jeng, M. C. Ho, F. J. Hsieh, P. H. Lee, M. L. Kuo, H. Lee, K. J. Chang, Identification of calreticulin as a prognosis marker and angiogenic regulator in human gastric cancer. *Ann. Surg. Oncol.* **16**, 524–533 (2009).
41. S. E. Pike, L. Yao, J. Setsuda, K. D. Jones, B. Cherney, E. Appella, K. Sakaguchi, H. Nakhasi, C. D. Atreya, J. Teruya-Feldstein, P. Wirth, G. Gupta, G. Tosato, Calreticulin and calreticulin fragments are endothelial cell inhibitors that suppress tumor growth. *Blood* **94**, 2461–2468 (1999).
42. S. E. Pike, L. Yao, K. D. Jones, B. Cherney, E. Appella, K. Sakaguchi, H. Nakhasi, J. Teruya-Feldstein, P. Wirth, G. Gupta, G. Tosato, Vasostatin, a calreticulin fragment, inhibits angiogenesis and suppresses tumor growth. *J. Exp. Med.* **188**, 2349–2356 (1998).
43. W. M. Hsu, F. J. Hsieh, Y. M. Jeng, M. L. Kuo, C. N. Chen, D. M. Lai, L. J. Hsieh, B. T. Wang, P. N. Tsao, H. Lee, M. T. Lin, H. S. Lai, W. J. Chen, Calreticulin expression in neuroblastoma—a novel independent prognostic factor. *Ann. Oncol.* **16**, 314–321 (2005).
44. M. G. Coppolino, M. J. Woodside, N. Demaurex, S. Grinstein, R. St-Arnaud, S. Dedhar, Calreticulin is essential for integrin-mediated calcium signalling and cell adhesion. *Nature* **386**, 843–847 (1997).
45. C. Howe, M. Garstka, M. Al-Balushi, E. Ghanem, A. N. Antoniou, S. Fritzsche, G. Jankevicius, N. Kontouli, C. Schneeweiss, A. Williams, T. Elliott, S. Springer, Calreticulin-dependent recycling in the early secretory pathway mediates optimal peptide loading of MHC class I molecules. *EMBO J.* **28**, 3730–3744 (2009).
46. G. Bu, The roles of receptor-associated protein (RAP) as a molecular chaperone for members of the LDL receptor family. *Int. Rev. Cytol.* **209**, 79–116 (2001).
47. L. M. Obermoeller-McCormick, Y. Li, H. Osaka, D. J. FitzGerald, A. L. Schwartz, G. Bu, Dissection of receptor folding and ligand-binding property with functional minireceptors of LDL receptor-related protein. *J. Cell Sci.* **114**, 899–908 (2001).
48. A. W. Orr, C. A. Elzie, D. F. Kucik, J. E. Murphy-Ullrich, Thrombospondin signaling through the calreticulin/LDL receptor-related protein co-complex stimulates random and directed cell migration. *J. Cell Sci.* **116**, 2917–2927 (2003).
49. M. Dai, P. Wang, A. D. Boyd, G. Kostov, B. Athey, E. G. Jones, W. E. Bunney, R. M. Myers, T. P. Speed, H. Akil, S. J. Watson, F. Meng, Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. *Nucleic Acids Res.* **33**, e175 (2005).
50. A. Oberthuer, D. Juraeva, L. Li, Y. Kahlert, F. Westermann, R. Eils, F. Berthold, L. Shi, R. D. Wolfinger, M. Fischer, B. Brors, Comparison of performance of one-color and two-color gene-expression analyses in predicting clinical endpoints of neuroblastoma patients. *Pharmacogenomics J.* **10**, 258–266 (2010).
51. K. De Preter, J. Vermeulen, B. Brors, O. Delattre, A. Eggert, M. Fischer, I. Janoueix-Lerosey, C. Lavarino, J. M. Maris, J. Mora, A. Nakagawara, A. Oberthuer, M. Ohira, G. Schleiermacher, A. Schramm, J. H. Schulte, Q. Wang, F. Westermann, F. Speleman, J. Vandesompele, Accurate outcome prediction in neuroblastoma across independent data sets using a multigene signature. *Clin. Cancer Res.* **16**, 1532–1541 (2010).
52. W. J. Kim, E. J. Kim, S. K. Kim, Y. J. Kim, Y. S. Ha, P. Jeong, M. J. Kim, S. J. Yun, K. M. Lee, S. K. Moon, S. C. Lee, E. J. Cha, S. C. Bae, Predictive value of progression-related gene classifier in primary non-muscle invasive bladder cancer. *Mol. Cancer* **9**, 3 (2010).
53. A. B. Als, L. Dyrskjøt, H. von der Maase, K. Koed, F. Mansilla, H. E. Toldbod, J. L. Jensen, B. P. Ulhøi, L. Sengeløv, K. M. Jensen, T. F. Orntoft, Emmprin and survivin predict response and survival following cisplatin-containing chemotherapy in patients with advanced bladder cancer. *Clin. Cancer Res.* **13**, 4407–4414 (2007).
54. S. Blenk, J. C. Engelmann, S. Pinkert, M. Weniger, J. Schultz, A. Rosenwald, H. K. Müller-Hermelink, T. Müller, T. Dandekar, Explorative data analysis of MCL reveals gene expression networks implicated in survival and prognosis supported by explorative CGH analysis. *BMC Cancer* **8**, 106 (2008).
55. A. Rosenwald, G. Wright, A. Wiestner, W. C. Chan, J. M. Connors, E. Campo, R. D. Gascoyne, T. M. Grogan, H. K. Muller-Hermelink, E. B. Smeland, M. Chiorazzi, J. M. Giltnane, E. M. Hurt, H. Zhao, L. Averett, S. Henrickson, L. Yang, J. Powell, W. H. Wilson, E. S. Jaffe, R. Simon, R. D. Klausner, E. Montserrat, F. Bosch, T. C. Greiner, D. D. Weisenburger, W. G. Sanger, B. J. Dave, J. C. Lynch, J. Vose, J. O. Armitage, R. I. Fisher, T. P. Miller, M. LeBlanc, G. Ott, S. Kvaloy, H. Holte, J. Delabie, L. M. Staudt, The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. *Cancer Cell* **3**, 185–197 (2003).

56. **Acknowledgments:** We thank R. Levy for providing clinical samples and support; A. Mosley for animal husbandry; and L. Jerabek, T. Storm, and F. Zhao for excellent lab management. **Funding:** M.P.C. is supported by a medical fellowship from the Howard Hughes Medical Institute and the Stanford Cancer Biology Program by PHS grant number CA09302, awarded by the National Cancer Institute, DHHS. R.M. holds a Career Award for Medical Scientists from the Burroughs Wellcome Fund. C.Y.P. is supported by National Cancer Institute (NCI)/NIH (KO8). A.J.G. is supported by an NCI Integrative Cancer Biology Program grant 1U54CA149145-01. This work was supported in part by a grant from the American Association for Cancer Research to R.M., NIH grant P01CA139490 to I.L.W., and funding from the Ludwig Foundation. **Author contributions:** M.P.C., R.M., and I.L.W. wrote the manuscript. M.P.C., R.M., and I.L.W. designed the experiments. M.P.C., R.W.-T., A.A.A., A.J.G., S.J., J.V., and K.W. performed the experiments and analyzed the data. A.A.A., J.V., S.B.W., T.R., and C.Y.P. provided clinical samples. **Competing interests:** S.J., M.P.C., R.M., and I.L.W. filed U.S. Patent Application Serial No. 12/321,215 entitled "Methods for Manipulating Phagocytosis Mediated by CD47." M.P.C., R.M., and I.L.W. have filed a patent application regarding therapeutic and diagnostic methods for manipulating phagocytosis through calreticulin and low density lipoprotein-related receptors. The other authors declare that they have no competing interests.

Submitted 11 June 2010
Accepted 3 December 2010
Published 22 December 2010
10.1126/scitranslmed.3001375

Citation: M. P. Chao, S. Jaiswal, R. Weissman-Tsukamoto, A. A. Alizadeh, A. J. Gentles, J. Volkmer, K. Weiskopf, S. B. Willingham, T. Raveh, C. Y. Park, R. Majeti, I. L. Weissman, Calreticulin is the dominant pro-phagocytic signal on multiple human cancers and is counterbalanced by CD47. *Sci. Transl. Med.* **2**, 63ra94 (2010).

Science Translational Medicine

Calreticulin Is the Dominant Pro-Phagocytic Signal on Multiple Human Cancers and Is Counterbalanced by CD47

Mark P. Chao, Siddhartha Jaiswal, Rachel Weissman-Tsukamoto, Ash A. Alizadeh, Andrew J. Gentles, Jens Volkmer, Kipp Weiskopf, Stephen B. Willingham, Tal Raveh, Christopher Y. Park, Ravindra Majeti and Irving L. Weissman

Sci Transl Med **2**, 63ra9463ra94.
DOI: 10.1126/scitranslmed.3001375

Eat Up!

Immune cells constantly patrol the body on a search and destroy campaign against foreign invaders. Designed to detect differential molecular signals, cells of the immune system can distinguish healthy from infected tissue by the types of proteins produced: Infected cells, for example, often produce unfamiliar proteins, which then activate innate immune cells to "eat" (phagocytose) the infected ones. Cancer cells also carry aberrant cargo such as unfamiliar proteins or normal proteins at abnormal levels, yet these cells frequently escape immune attack because they express a "don't eat me" signal, the cell surface protein CD47. Blocking this signal on a cancer cell makes them targets for phagocytosis, but surprisingly does not do the same for normal cells that express CD47. Chao *et al*. have now identified calreticulin as the "eat me" signal on cancer cells that leads to phagocytosis when the counterbalancing "don't eat me" signal CD47 is blocked.

Calreticulin is a pro-phagocytic molecule that is highly expressed on the surface of several types of human cancer cells, including acute myeloid and lymphoblastic leukemias, chronic myeloid leukemia, non-Hodgkin's lymphoma, bladder cancer, glioblastoma, and ovarian cancer. However, calreticulin is expressed only at very low levels on normal cells. Chao *et al*. found a correlation between calreticulin and CD47 expression levels on cancer cells and showed that blocking the interaction between calreticulin and its ligand prevented phagocytosis initiated by blocking the "don't eat me" signal CD47. Moreover, high calreticulin expression on cancer cells was a poor prognostic indicator in human patients with neuroblastoma, bladder cancer, and non-Hodgkin's lymphoma. Therefore, a balance between calreticulin and CD47 expression in cancer cells may be a double-edged sword: In the absence of a CD47 blocker, this equilibrium may support tumor cell survival, but when CD47 function is inhibited, the presence of calreticulin tells immune cells to "eat up!" This information provides a key insight for the therapeutic development of CD47-inhibitory agents.

Science Translational Medicine (ISSN 1946-6242) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. The title *Science Translational Medicine* is a registered trademark of AAAS.