Susceptibility of rhabdomyosarcoma cells to macrophage-mediated cytotoxicity

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The prognosis of advanced stage rhabdomyosarcoma (RMS) is still sobering. In recent years, outcome has not been further improved by conventional therapy. Therefore, novel treatment options such as macrophage-directed immunotherapy have to be investigated. The aim of this study was to analyze the phagocytosis of RMS cells by macrophages and to modulate the susceptibility using monoclonal antibodies and cytotoxic drugs.

Expression of the macrophage activating ligand calreticulin and CD47, the counterpart of the inhibitory receptor SIRPα, was analyzed with Affymetrix mRNA expression arrays and immunohistochemistry on 11 primary RMS samples. Results were verified in two RMS cell lines using flow cytometry and immunocytochemistry. Macrophage cytotoxic activity was quantified by a MTT colorimetric assay in co-culture experiments of RMS cells with monocyte-derived, GM-CSF stimulated macrophages.

Gene expression analysis and immunohistochemistry revealed a high expression of CD47 and calreticulin in alveolar and embryonal RMS tissue specimens. Extracellular expression of CD47 on RMS cell lines was confirmed by flow cytometry, whereas calreticulin was exclusively detected in the endoplasmatic reticulum. After co-culturing of RMS cells with macrophages, viability dropped to 50–60%. Macrophage-mediated cytotoxicity was not influenced by a blocking antibody against CD47. However, susceptibility was significantly enhanced after pre-treatment of RMS cells with the anthracycline drug doxorubicin. Furthermore, translocation of calreticulin onto the cell surface was detected by flow cytometry.

The immunologic effect of doxorubicin may improve the efficacy of adoptive cellular immunotherapy and chemotherapy of childhood RMS.

Introduction

Rhabdomyosarcoma (RMS) is the most frequent pediatric soft tissue sarcoma. It represents 3–4% of all pediatric cancers[1] and 7–8% of all solid malignant tumors in children.[2] There exist two main histopathological subtypes of this malignancy, embryonal RMS (RME) and alveolar RMS (RMA) with completely different tumor biology.[3] RMS tumors are currently treated by multimodal therapies, including surgery, radiotherapy and systemic chemotherapy.[4] Major treatment problems are metastatic invasion, local tumor recurrence, and multidrug resistance.[5] Therefore, it is not only necessary to develop novel strategies to destroy cancer cells efficiently, but also to attempt a stimulation of the immune system in order to control residual tumor cells.

Macrophages play an important role in the defense against tumors.[6,7] They have the capacity to recognize and destroy tumor cells through several different mechanisms, including secretion of tumor necrosis factor-α,[8] nitric oxide,[9] interleukin-1β,[10] and reactive oxygen intermediates.[11] Furthermore, macrophages are involved in the antibody-dependent cellular cytotoxicity in therapeutic approaches with recombinant antibodies.[12] Macrophage cytotoxicity relies on the balance between activating stimuli and suppressive signals.

One well known signal for engulfment by phagocyting cells is a change in the composition of phospholipids on the target cells.[13] Furthermore, the interaction of calreticulin with the low-density lipoprotein-related protein (LRP) plays also an important role in apoptotic cell removal, resulting in an activation of the macrophages.[14] Calreticulin is an intracellular calcium-binding protein and anthracyclines are able to elicit it's translocation onto the cell surface.[15] On the other hand, the interaction between CD47 and the signal regulatory protein α (SIRPα) is a key function to protect viable cells from phagocytosis.[16] CD47, a widely distributed inhibitory receptor on macrophages that can

trigger a signal transduction cascade resulting in inhibition of phagocytosis, serves as the ligand for SIRPα.[17] Overexpression of inhibitory molecules like CD47 is a common mechanism of tumor cells to escape phagocytosis. A high expression of calreticulin or blocking of CD47 by monoclonal antibodies may shift the balance between activating and inhibiting signals in favor of phagocytosis.[18]

The aim of this study was to describe the distribution of CD47 and calreticulin in human RMS and to analyze the cytotoxic activity of GM-CSF activated macrophages in combination with a CD47-blocking monoclonal antibody (mAb). Furthermore, we evaluated the effect of doxorubicin on the interaction between RMS cells and macrophages.

Results

Expression of CD47 and calreticulin on RMS tissues and cells. In a first attempt to evaluate the role of CD47 and calreticulin in RMS, expression of these genes was examined in RMS tissue samples by microarray analysis. CD47 inhibits phagocytosis and its gene was expressed in the 11 RMS tissue samples analyzed (**Fig. 1A**). With a fold change of 1.2, a significant higher expression was observed in RMA compared with RME tissues (p = 0.02; Student's t-test) and skeletal muscle biopsy samples (p = 0.002; Student's t-test). The macrophage activating gene calreticulin showed a very high expression in the array analysis of both RMA and RME, independent of the histological subtype (median, 0.7). Compared with muscle control tissues, a significant higher expression was found in RMS (p < 0.0001; Student's t-test). Accordingly, we detected high expression levels of the proteins CD47 and calreticulin on RMS tissue slices by immunofluorescence analysis (**Fig. 1B**). In immunofluorescence staining, fewer differences between RMA and RME samples were observed with regard to the expression of CD47. When examining CD47 on RMS cell lines by flow cytometry, the protein was observed on the cell surface of Rh30 and RD. Both showed a high expression of CD47 as revealed by staining with an anti-human CD47 mAb (**Fig. 2A**). Calreticulin could not be detected on the cell membrane. However, intracellular flow cytometry analysis revealed a low expression of calreticulin

both in Rh30 and RD cells. In these cell lines calreticulin seems to be compartmentalized to the endoplasmatic reticulum, as revealed by immunocytochemistry. In contrast to flow cytometry analysis, the expression of calreticulin was high, which is in turn consistent
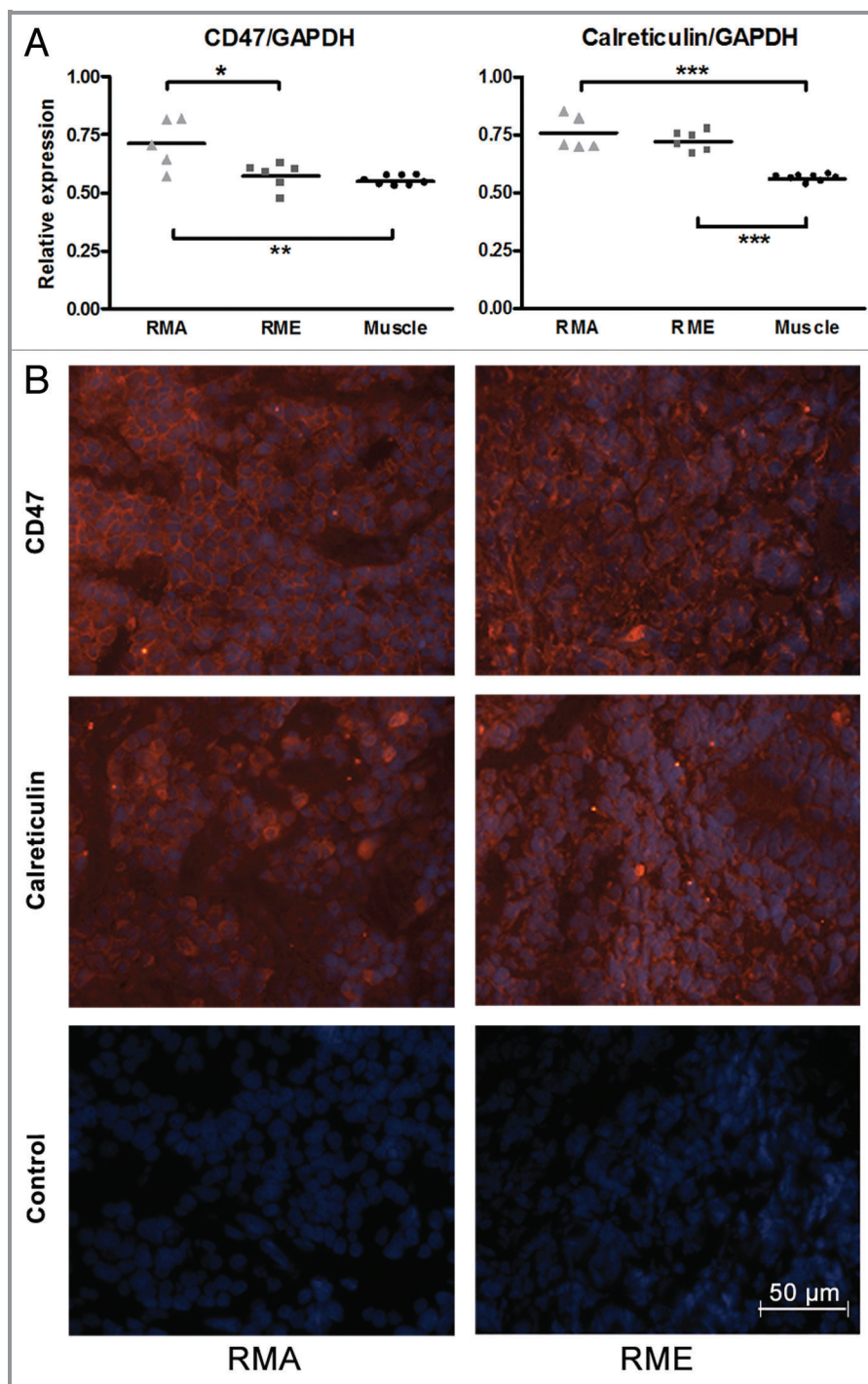

Figure 1. Expression of CD47 and calreticulin in rhabdomyosarcoma and muscle tissue samples. (A) Gene expression is shown for each tissue sample enrolled in the microarray expression analysis relative to GAPDH. (***Student's t-test p < 0.0001). (B) Immunofluorescence analysis of representative RMS tissue sections using CD47 and calreticulin mAbs followed by the red fluorescent secondary antibody and Hoechst 33342 for nuclear blue staining. As control served RMS tissues incubated without primary mAb.
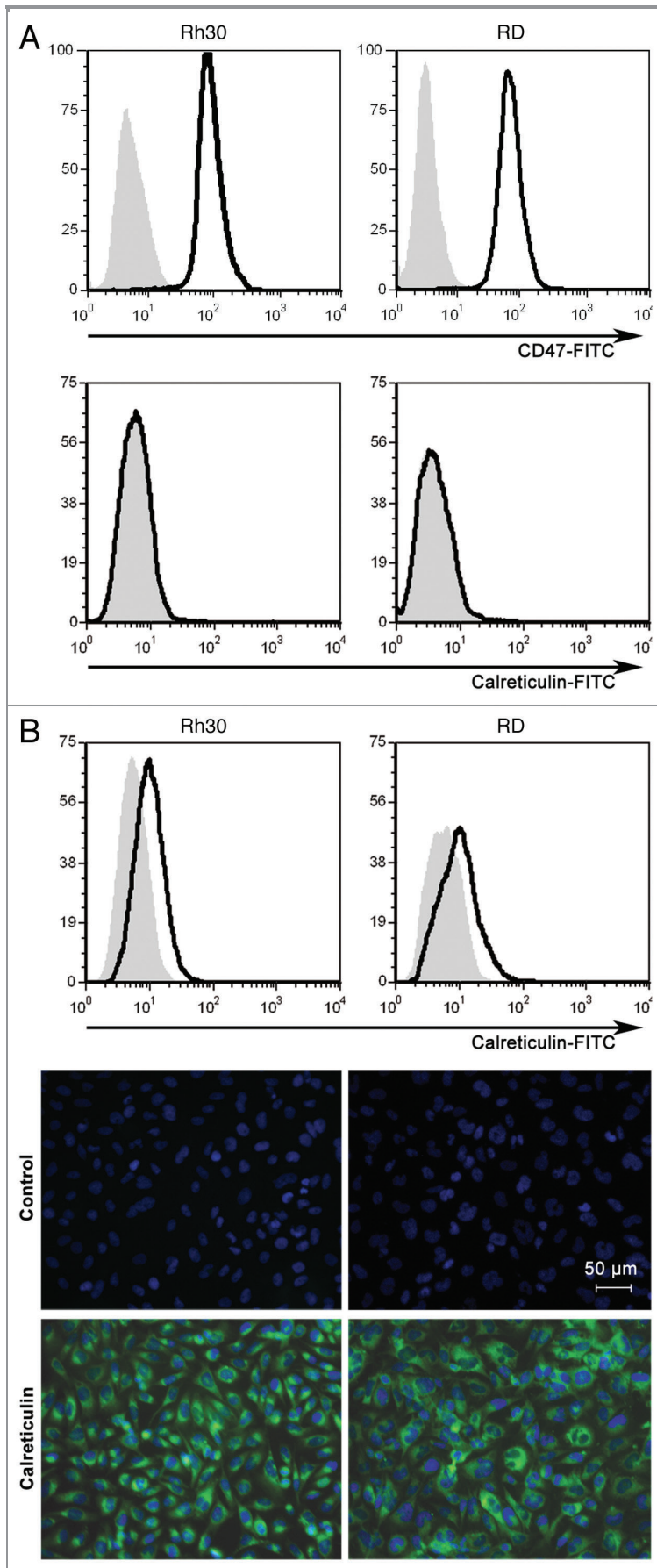
Figure 2. CD47 and calreticulin expression in rhabdomyosarcoma cell lines. (A) High expression of CD47 (black lines) but no extracellular expression of calreticulin was detected on both Rh30 and RD cells by flow cytometry. The control staining without primary antibody is plotted on each panel as gray histogram. Dead cells were gated out by their position in forward and site scatter. (B) Expression of calreticulin shown by intracellular flow cytometry analysis and immunocytochemistry in the endoplasmatic reticulum of RMS cells. Secondary FITC-labeled antibody and Hoechst 33342 for nuclear staining were used. Controls were incubated without primary antibody.

with the array analysis obtained previously (**Fig. 2B**). The low signal in flow cytometry might be a result of an ineffective permeabilization of the endoplasmatic reticulum. The "don't eat me" signal CD47 was strongly expressed in RMS tissue samples of both histological subtypes and on the cell surface of RMS cells. Therefore, we next modulated the macrophage cytotoxic activity in vitro using inhibitory antibodies.

Cytotoxic activity of macrophages modulated by anti-CD47. The ability of human macrophages to phagocyte allogeneic RMS cells in co-cultures at an E:T ratio of 2.5:1 was monitored for up to 96 h. After 24 h, a few poly-nucleated cells were observed in the confluent grown tumor cell culture (pictures not shown). Distinct holes in the cell layer caused by phagocytosis were also observed and photos were taken after 96 h (**Fig. 3A**). No cell free areas were detected in the control samples. The most and largest holes in the tumor cell monolayer were present in the cultures treated with the anti-CD47 mAb (**Fig. 3B**). When treated without antibody, Rh30 cells were more efficiently phagocyted than RD cells with a cell free area of 4600 μm^2 compared with 2500 μm^2. Blocking CD47 with an anti-CD47 mAb increased the phagocytosis of both RD cells and Rh30 cell cultures (17900 μm^2 and 12700 μm^2, respectively). In relation to the whole area of 151810 μm^2, a 5% increase in phagocytosis of Rh30 was detected and the increment for RD was 10%.

By measuring cytotoxicity in co-cultures, we observed a specific phagocytosis of both RMS cell lines again (**Fig. 4**). After co-culturing with macrophages at an E:T ratio of 2:1 for 24 h, 70% of Rh30 and 45% of RD cells remained viable. When additionally treated with the anti-CD47 mAb, cell viability did not change significantly (Rh30: 80%; RD: 60%). As a control, exclusive antibody treatment had no effect on Rh30 cell viability. In contrast, cell viability of RD cells dropped to 85%. Taken together, the phagocytosis of RMS cells could not be increased by blocking of the inhibitory ligand CD47 using mAbs in this short period of time. Therefore, we next investigated other mechanisms to shift the balance between activating and inhibiting signals in favor of phagocytosis.

Retrieving calreticulin to enhance phagocytosis. Macrophages can be activated by the interaction of calreticulin and LRP. Preliminary data has shown that RMS cells do not express calreticulin on their surface
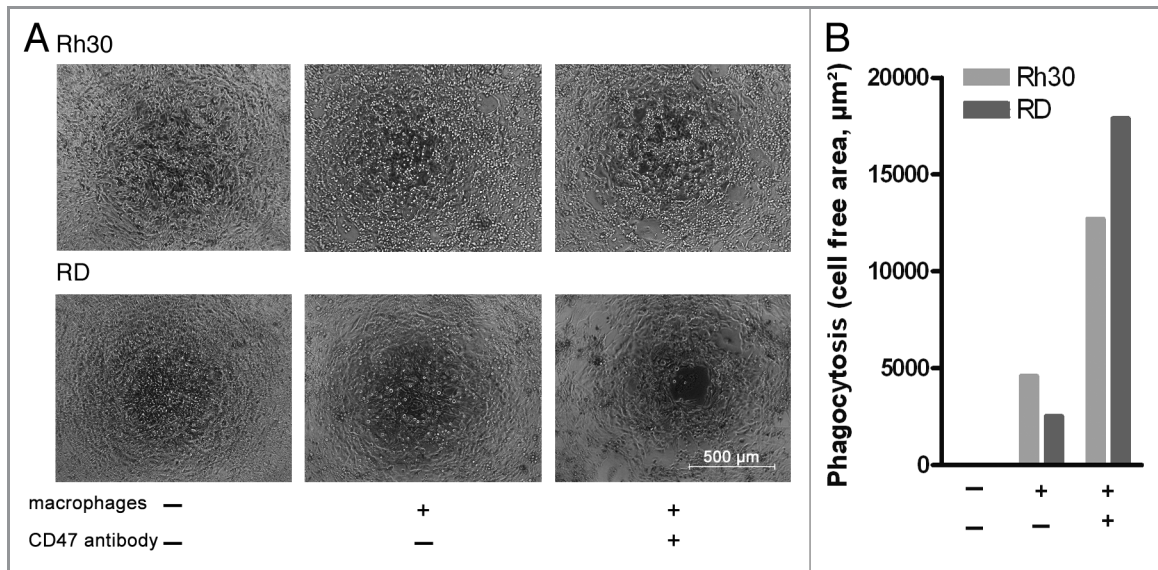
Figure 3. Susceptibility of rhabdomyosarcoma cell lines to cytotoxicity by macrophages. (A) Rh30 and RD cells were co-cultured with macrophages for 96 h at an E:T ratio of 2.5:1. Indicated wells were pre-incubated with 10 μg/ml blocking antibody to CD47 for 30 min. Pictures show tumor cell free areas in cultures with macrophages. (B) Cell lysis was quantified by measuring the cell free area in the tumor cell layer by an image analyzer system.

(**Fig. 2A**). Therefore, we intended to induce the transport of calreticulin to the RMS cell surface by using doxorubicin. The IC_{50} for both RMS cell lines was determined to be 0.1 μg/ml (**Fig. 5A**). A lower concentration (0.01 μg/ml) was also tested in flow cytometry and co-culture experiments, which on the one hand had an effect on RMS cell viability (80% viability in an 96 h assay), but on the other hand was low enough, to avoid massive drug dependent cell death and to assess phagocytosis effects.

Cell surface presentation of calreticulin, depending on the used doxorubicin concentration, was detected by flow cytometry (**Fig. 5B**). A higher expression was measured in RD cells compared with Rh30. Thereupon, after treatment with the higher
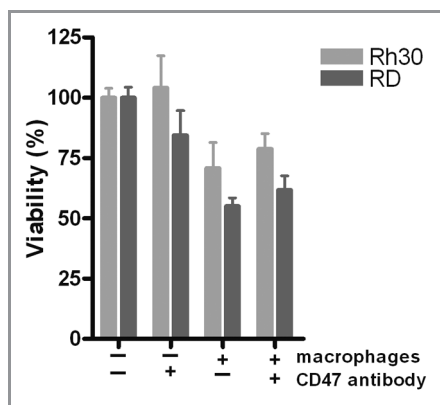

Figure 4. Effect of CD47 antibody on the cytotoxicity of macrophages against rhabdomyosarcoma cells. Co-cultures of Rh30 and RD cells with macrophages (E:T 2:1) were performed after incubating the tumor cells for 30 min with an anti-CD47 antibody (10 μg/ml). Cell viability was then determined by MTT assay 24 h later. Data represent means ± SD of relative cell viability from triplicates. Experiments without antibody were set at 100% viability.

concentration of doxorubicin (0.1 μg/ml) the co-culture experiments with macrophages revealed an efficient phagocytosis of RD cells up to 90% (**Fig. 6B**). When additionally treated with the anti-CD47 mAb (10 μg/ml), this effect could not be enhanced (viability RD 15%). In Rh30 cells, a lysis of 65% was achieved after treatment with the IC_{50} concentration of doxorubicin (0.1 μg/ml). After co-culture with macrophages, the Rh30 cell viability dropped without reference to the addition of the CD47 antibody to 35% and 40%, respectively (**Fig. 6A**). Viability of both RMS cell lines was half-reduced after treatment with the lower dose of doxorubicin (0.01 μg/ml) and co-culture with macrophages (viability of Rh30 55% and of RD 50%). These results were no further influenced by the addition of the anti-CD47 mAb (viability of Rh30 65% and of RD 55%). Four different treatment options served as controls: (A) Co-culturing with macrophages reduced the viability of Rh30 cells to 60% and RD cells to 55%. (B) After antibody treatment and co-culture with macrophages 65% of Rh30 and 60% of RD cells remained viable. (C) Exclusive treatment with the previously evaluated IC_{50} of doxorubicin (0.1 μg/ml) resulted in a drop of cell viability to 55% (**Fig. 5A**). (D) Treatment with the lower tested drug concentration (0.01 μg/ml) had no effect on the morphology and viability of Rh30 cells (95%). However, survival of RD cells dropped to 85% by doxorubicin alone. In summary, phagocytosis of RMS cells was significantly enhanced by retrieving calreticulin using the IC_{50} concentration of doxorubicin (two-way ANOVA, p < 0.001).

The cause-effect relationship between doxorubicin treatment, CRT exposure and enhanced susceptibility to cytotoxic effects mediated by macrophages was demonstrated using a blocking peptide to calreticulin. This peptide abrogated phagocytosis of lymphoblast cell lines by macrophages.[19] In co-cultures of RMS cells and macrophages, the enhanced phagocytosis achieved by
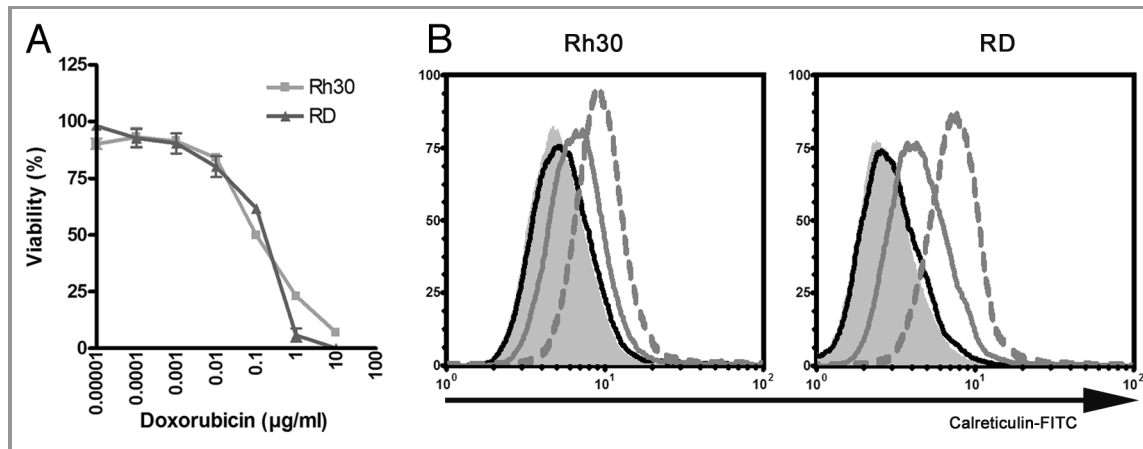
Figure 5. Viability and calreticulin expression of rhabdomyosarcoma cells pre-treated with doxorubicin. (A) Rh30 and RD cells were incubated for 96 h with increasing doses of doxorubicin. Data are means ± SD of triplicate viability measurements using MTT assay. Experiments without doxorubicin were set at 100% viability. (B) Extracellular calreticulin expression on Rh30 and RD cells was measured by flow cytometry after incubation for 24 h with 0.01 µg/ml (dark gray line) or 0.1 µg/ml doxorubicin (dashed line). No calreticulin on the cell surface was detected without doxorubicin (black line). The control staining without primary antibody is plotted on each panel as gray histogram.

doxorubicin could be partially blocked by the blocking peptide (**Fig.** 7). Therefore, the effect of doxorubicin on the phagocytosis of RMS seems to be mediated to some extent by calreticulin exposure.

Discussion

Effective antibody targeting of CD47 has been demonstrated for several human cancers including acute myeloid leukemia (ALL),[20] bladder cancer,[21] and non-Hodgkin lymphomas.[22] The therapeutic effect of blocking CD47 with antibodies is mediated primarily through macrophage phagocytosis[23] and was observed for ALL in vitro and in tumor engraftment in vivo.[24] Targeting of tumor cells with antibodies against CD47 has previously
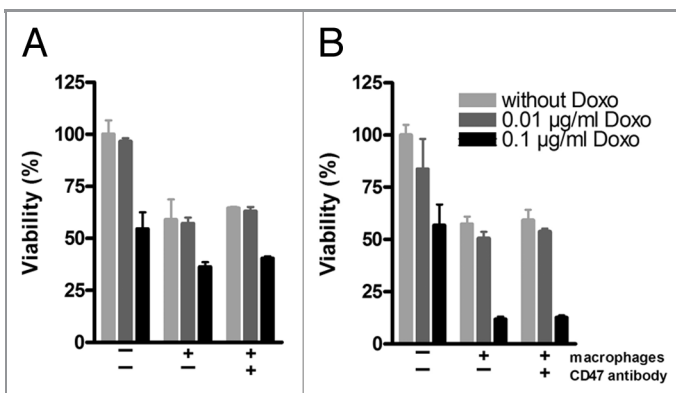

Figure 6. Phagocytosis of rhabdomyosarcoma cells after pre-treatment with doxorubicin. As indicated, Rh30 (A) and RD cells (B) were incubated with 0.01 µg/ml or 0.1 µg/ml doxorubicin (Doxo) for 24 h and CD47 antibody for 30 min. Macrophages were added at an E:T ratio of 2:1 and cell viability was assessed using MTT assay 24 h later. Data are means ± SD of relative viability from three experiments. Experiments without antibody and doxorubicin were set at 100% viability.

been reported to directly induce apoptosis.[25-28] Now the idea is supported, that targeting CD47 may lead to a disruption of the CD47-SIRPα interaction, thereby preventing a phagocytic inhibitory signal.[20] CD47 was found to be highly expressed not only on tumor cells but also on normal tissues.[29] However, potential toxic effects with a CD47 antibody therapy could be excluded.[24] Selective targeting of tumor cells is probably due to a high cell surface expression of calreticulin. Indeed, calreticulin is expressed in all cells, but only a low presentation was profiled on the cell surface of healthy tissues. By contrast, for several human cancers a high extracellular expression was demonstrated.[18]

This data led us to speculate, that targeting CD47 will be a promising tool against a wide range of human tumors, including RMS. Our data revealed a high expression of CD47 on RMS tissues and cells, which may play a role in the immune evasion of RMS. Furthermore, a significant association between the higher expression of CD47 and the more aggressive RMA subtype was found in gene expression analysis. Interaction of RMS cells with GM-CSF activated macrophages required more than four days of incubation to reveal a notable phagocytosis. Enhanced cytotoxicity was only obtainable by increasing the proportion of macrophages (data not shown). Usually, such assays with human acute myeloid leukemia stem cells revealed a fast engulfment of tumor cells within two hours of co-culture with stimulated macrophages.[20] We therefore conclude that RMS cells are less susceptible to phagocytosis by macrophages.

To enhance phagocytosis, we tried to disrupt the CD47-SIRPα interaction with a mAb demonstrably able to specifically block CD47.[30] In our short-term assay of 24 h, no enhancement of phagocytosis was observed. This might be due to low activating signals for the phagocytes. Besides the exposure of GM-CSF and phosphatidylserine, which may interact with several phagocyte receptors, calreticulin functions on tumor cells as a ligand for the activating phagocyte receptor LRP/CD91.[13,16,31] Furthermore, annexin-1 is under active investigation.[26]
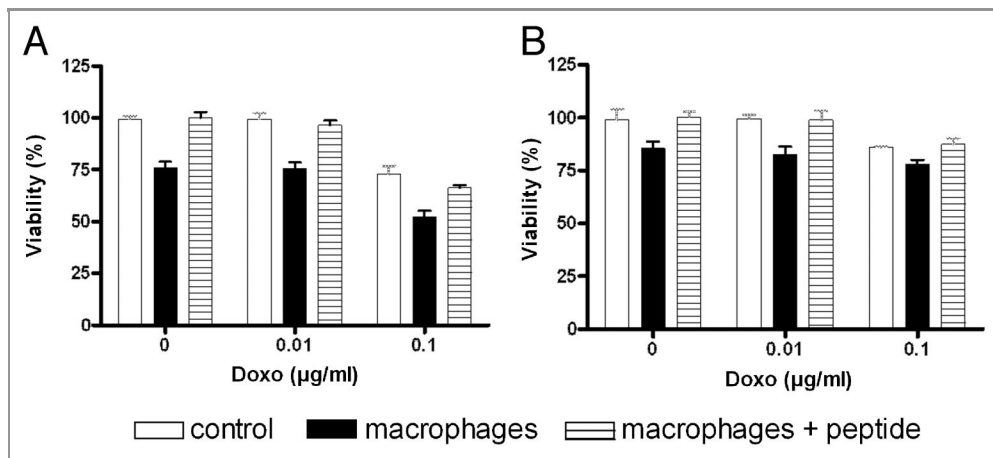
Figure 7. RMS cells Rh30 (A) and RD (B) were incubated with human macrophages in culture media alone or with calreticulin-blocking peptide (4 µg/ml) for 24 h, after which cell viability was analyzed by MTT assay. Pre-treatment of RMS cells with 0.01 and 0.1 ng/ml doxorubicin resulted in increased phagocytosis compared with control cells. Blockade of calreticulin on RMS cells partially abrogated phagocytosis.

Cellular stress, including apoptosis, induces the expression of many stress proteins, among them calreticulin.[32] This leads to increased amounts of calreticulin on the cell surface.[33] However, the exact transport mechanism of calreticulin remains unclear. Anthracyclines are able to induce the translocation of calreticulin from the endoplasmatic reticulum onto the cell surface. The presented results show a dose-dependent calreticulin presentation after doxorubicin treatment. For colon carcinoma cells, a concentration of 25 µM doxorubicin, converted 14.5 µg/ml, was described in the same incubation period.[15] For RMS cells, a measureable extracellular calreticulin expression and a significantly increased phagocytosis was demonstrated after incubation with 0.1 µg/ml doxorubicin. Furthermore, a different response of RME compared with RMA cells was observed. Under equal doxorubicin concentrations, the RME cells showed a higher extracellular calreticulin presentation and accordingly a more efficient phagocytosis compared with RMA. The role of calreticulin in the doxorubicin enhanced phagocytosis was demonstrated using a blocking peptide, which reversed the activity of macrophages, although CD47 was expressed at high density. A complete blockage can be expected in the absence of CD47 as demonstrated for mutant lymphomas.[19]

We could clearly demonstrate that macrophages are able to phagocyte allogeneic RMS cells under conditions of cell-to-cell contact. After GM-CSF activation of macrophages and co-culturing with RMS cells, viability dropped to 50–60% without any additional treatment. After activation of calreticulin, the efficient phagocytosis of RME cells was enhanced to 90%. This promising effect in vitro has to be verified in vivo. Doxorubicin may improve the efficacy of adoptive cellular immunotherapy and chemotherapy protocols. So far, doxorubicin is used in combination with ifosfamide and vincristine in the therapy protocol for the high risk soft tissue sarcoma group (CWS-2002 P).

Material and Methods

Gene expression analysis. The gene expression data used in this study were part of a microarray analysis described previously.[34] All patients were treated within the trials of the Cooperative Soft Tissue Sarcoma Study (CWS) Group of the Society of Pediatric Oncology and Hematology (GPOH). Mean age of patients was 6.4 y (range 1 to 15 y, 95% CI of mean 4.2 to 8.8). Histological analysis revealed 5 RMA and 6 RME. All cases received central pathological review and patients were treated according to the uniform protocols. Only samples with tumor cell content of at least 80% were included for analysis. Metastases were present at the time of surgery in all patients suffering from RMA and in none in RME patients. Analysis of tissue samples was approved by the local ethical committee (CWS 2002-P: 418/2004V). As controls, data of skeletal muscle biopsies from 8 patients acquired on the same array platform were used.[35] The expression of CD47 (probesets 211075_s_at and 213857_s_at on the Affymetrix HG-U133 Plus 2.0 microarray) and calreticulin (212953_x_at and 214315_x_at) was compared with the housekeeping gene GAPDH (212581_x_at and 213453_x_at) for each sample as fraction of the signal log ratio. Comparison of relative expression in RMA, RME, and control tissue was performed by Student's t-test.

Cell lines and culture conditions. The RMA cell line Rh30 (DSMZ, ACC-489) as well as the RME cell line RD (ATCC, CCL-136) were cultured in DMEM medium (Biochrom) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine (Biochrom) in a humidified atmosphere containing 5% CO_2 at 37°C. All cells were tested to be mycoplasma negative. Cell identity was proven by SLR analysis of the DNA profile using PowerPlex 16 (Promega).

Flow cytometry analysis. Trypsinized cells were incubated for 30 min in FACS-buffer (PBS with 2% FBS, 2 mM EDTA, 0.005% NaN_3; Sigma-Aldrich) containing anti-CD47 mAb (B6H12; BD Biosciences). Intracellular flow cytometry of calreticulin was performed with cells fixed in 3.7% formaldehyde solution (Merck), and permeabilized by 0.2% Tween 20 in PBS (Sigma-Aldrich). Primary antibody (anti-calreticulin, FMC 75; Enzo Life Sciences) was added for 30 min diluted in FACS-buffer. This was followed by a washing step with FACS-buffer and cells were labeled with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch). Data was acquired with FACSCalibur (Becton Dickinson) and analyzed by FCS Express (De Novo Software).

Immunofluorescence staining. RMS cells were first fixed with 3.7% formaldehyde solution to perform immunofluorescence staining with anti-CD47 or anti-calreticulin mAbs. Additionally, several sections from frozen specimens of RMS tumors were analyzed. Cells and tissue slides were fixed for 5 min in a 1:1 acetone and methanol mixture (Merck) and washed in PBST (PBS with 0.1% Tween 20, Sigma-Aldrich). Blocking was performed using 1% normal goat serum (Dako Cytomation) for 30 min followed by primary antibody diluted in PBST to 10 μg/ml. Slides were washed three times for 5 min in PBST. Secondary Alexa 546-conjugated goat anti-mouse antibody (1:500; Molecular Probes/Invitrogen) was added for 30 min at room temperature, followed by three times washing for 5 min. For counterstaining of the nucleus, Hoechst 33342 was used (1:2000; Sigma-Aldrich). Slides were mounted with fluorescence mounting medium (Dako Cytomation) and subsequently analyzed by fluorescence microscopy (AxioVision; Carl Zeiss).

Cell proliferation. RMS cells (1×10^4) were seeded out in a 96-well plate. After incubation over night, serial 10-fold dilutions of doxorubicin (Cell Pharm) ranging from 100 to 0.00001 μg/ml were added (as shown in **Fig. 5**). Cell viability was assessed 96 h later by an MTT assay. Therefore, 25 μl of 12 mM MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich) dissolved in PBS were added to each well containing 100 μl cell suspension. Incubation was stopped 3 h later with 100 μl of 10% SDS in acid water (Merck). Absorption was measured at 570 nm after incubation over night using a Milenia Kinetic Analyzer (DPC Biermann). All assays were performed in triplicates. The percentage of viability was calculated by normalization between background of cultures without cells and untreated cultures as control experiments.

Generation of macrophages. Peripheral blood mononuclear cells (PBMCs) isolated from buffy coats were plated into 25 ml cell culture flasks in serum-free X-VIVO 20 medium (Lonza).

After 1 h of incubation at 37°C, non-adherent cells were removed by washing twice in PBS. Adherent cells were then incubated in RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine, 1% non essential amino acids, and 1% sodium pyruvate (Biochrom) for 6 d with medium changes every 48 h. On day 6, 50 U/ml GM-CSF (BD Biosciences) were added to activate differentiated macrophages. On day 7, cells were incubated for 5 min in PBS, scraped off with a sterile cell scraper, collected by centrifugation, and re-suspended in culture medium.

Phagocytic activity of macrophages. RMS (2×10^4) cells were seeded out on a 96-well plate and incubated with 10 μg/ml anti-CD47 mAb for 30 min. Afterwards, macrophages were added at an effector to target (E:T) ratio of 2.5:1. Digital images were taken at a 6.3-fold magnification with AxioCam MRc (Carl Zeiss) on a Leitz Fluovert FS optical microscope (Leica) 24 and 96 h later. The cell free area in the cell layer was measured using an image analyzer system (AxioVision 3.1; Carl Zeiss).

RMS cells (1×10^4) were seeded out on a 96-well plate and incubated with either 0.01 μg/ml or 0.1 μg/ml doxorubicin (Cell Pharm) for 24 h. Cells were incubated for 30 min with 10 μg/ml anti-CD47 mAb, as indicated. A blocking peptide to calreticulin (MBL) at 4 μg/ml was added to the tumor cells.[19] Subsequently, macrophages were added at an E:T ratio of 2:1. Target phagocytosis was monitored after 24 h using MTT assay as described previously. Percent viability was calculated by normalization between background of cultures without cells and untreated cultures as control experiments. When macrophages were added, normalization was performed between background of macrophages and untreated cultures. All experiments were performed in triplicates.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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