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ORIGINAL RESEARCH

The "don't eat me" signal CD47 is a novel diagnostic biomarker and potential therapeutic target for diffuse malignant mesothelioma

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ABSTRACT

Diffuse malignant mesothelioma (DMM) is one of the prognostically most discouraging cancers with median survivals of only 12–22 months. Due to its insidious onset and delayed detection, DMM is often at an advanced stage at diagnosis and is considered incurable. Combined chemo- and radiotherapy followed by surgery only marginally affect outcome at the cost of significant morbidity. Because of the long time period between exposure to asbestos and disease onset, the incidence of DMM is still rising and predicted to peak around 2020. Novel markers for the reliable diagnosis of DMM in body cavity effusion specimens as well as more effective, targeted therapies are urgently needed. Here, we show that the "don't eat me" signalling molecule CD47, which inhibits phagocytosis by binding to signal regulatory protein α on macrophages, is overexpressed in DMM cells. A two-marker panel of high CD47 expression and BRCA1-associated protein 1 (BAP-1) deficiency had a sensitivity of 78% and specificity of 100% in discriminating DMM tumour cells from reactive mesothelial cells in effusions, which is superior to the currently used four-marker combination of BAP-1, glucose transporter type 1, epithelial membrane antigen and desmin. In addition, blocking CD47 inhibited growth and promoted phagocytosis of DMM cell lines by macrophages *in vitro*. Furthermore, DMM tumours in surgical specimens from patients as well as in a mouse DMM model expressed high levels of CD47 and were heavily infiltrated by macrophages. Our study demonstrates that CD47 is an accurate novel diagnostic DMM biomarker and that blocking CD47 may represent a promising therapeutic strategy for DMM.

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Introduction

Diffuse malignant mesothelioma (DMM), a cancer that originates from mesothelial cells lining the pleural, pericardial, and abdominal cavities including the testis, is mainly caused by asbestos exposure.[1] The annual incidence of DMM varies between countries and is estimated to be 0.8 – 2.9 per 100'000 people. Depending on the stage and the modality of treatment, median survival is between 12 and 22 months, making DMM one of the prognostically most discouraging human malignancies.[2] This is in part related to diagnostic delay associated with its typically insidious onset and the difficulties of early diagnosis. DMM commonly presents with pleural, pericardial or peritoneal effusion, but diagnosis of DMM is notoriously difficult by effusion cytology or histology at an early stage given that benign reactive mesothelial cells may demonstrate degrees of cytological atypia that overlap with that of DMM.[3] Furthermore, DMM shows limited sensitivity to chemotherapy and irradiation, which makes surgery the mainstay of therapy for the minority of patients with tumours amenable to resection – and results in a need of targeted therapies for those with advanced disease.[4]

A large variety of biomarkers have been studied for their ability to discriminate between benign and malignant mesothelial cells in cytological effusion specimens. Until recently, the best studied immunophenotypic aberrations in malignant as opposed to reactive mesothelial cells were overexpression of epithelial membrane antigen (EMA) or glucose transporter type 1 (GLUT-1) and loss of desmin expression, all of which suffer, however, from suboptimal sensitivity and specificity.[3,5,6] Loss of *CDKN2A*, as detected by fluorescence in-situ hybridisation (FISH), has reasonable sensitivity (around 70%) and is, according to several studies, 100% specific for DMM in contradistinction from reactive mesothelial cells, thereby allowing for a definite diagnosis of malignancy in cytological samples.[7-9] However, longer turnaround times and higher costs limit the widespread applicability of FISH, especially in cases with a lower level of suspicion for DMM. Recently, loss of expression of BRCA1-associated protein 1 (BAP-1) due to inactivating mutations and/or chromosomal loss[10] has emerged as a marker of malignancy in mesothelial cells that has a sensitivity similar to that of FISH for

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CDKN2A and an identical 100% specificity, but can be reliably assessed by immunostaining.[8,11]

In recent years, cancer immunotherapies that interfere with immune-inhibitory receptors mainly on T cells (so-called checkpoint inhibitors), such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or programmed cell death 1 (PD-1), have shown promising results in clinical trials and were approved as routine therapeutic drugs for defined advanced malignancies.[12] The cell surface protein CD47, also known as integrin-associated protein (IAP), is a member of the immunoglobulin superfamily. In addition to its function as a receptor for integrins and thrombospondin 1 (TSP-1), CD47 plays an important role in various cellular processes such as immune cell activation, cell migration and neuronal development.[13-17] Recent research activities, however, were focussed on the role of CD47 in the inhibition of phagocytosis. CD47 binds to signal regulatory protein α (SIRPα) expressed on macrophages, resulting in the dephosphorylation of myosin and inhibition of the phagocytic contractile engulfment machinery.[18] Normal cells present the "don't eat me" signal CD47 as a marker of self to macrophages, and loss of CD47 expression in senescent or apoptotic cells results in phagocytosis.[19-22] CD47 is highly expressed in multiple human cancer types, such as acute and chronic myeloid leukaemia, non-Hodgkin's lymphomas and multiple myeloma, leiomyosarcoma, glioblastoma and various carcinomas including bladder, ovarian, hepatocellular, prostate, breast and colon.[23-29] Blocking of CD47 on cancer cells results in enhanced macrophage phagocytic activity and elimination of tumours in various murine models.[23,24,26-29] In the present study, we addressed the question as to whether CD47 is upregulated in DMM cells as compared to benign mesothelial cells and might serve as a diagnostic biomarker and potential therapeutic target for DMM.

Results

The "don't eat me" signalling molecule CD47 is expressed in diffuse malignant mesothelioma

CD47 overexpression has been found in multiple malignancies, such as various carcinomas, glioblastoma and leiomyosarcoma.[23,24] To test if CD47 is expressed in DMM as well, we analysed a publicly available microarray dataset (GSE2549) that includes surgical samples of 40 patients suffering from pleural DMM as well as 5 normal pleura specimens.[30] *CD47* mRNA expression was significantly higher in DMM as compared to normal pleura (Fig. 1A). In addition, we observed a trend towards higher mRNA expression of *calreticulin* (*CALR*), the dominant pro-phagocytic molecule on cancer cells.[31] Furthermore, we analysed the expression of markers commonly used in cytology and surgical pathology to discriminate DMM from reactive mesothelial cells.[3,5] As expected, DMM showed significantly higher *mucin 1* (*MUC1*/*EMA*) mRNA expression than normal pleura, which is in line with higher EMA protein expression in DMM.[5] In contrast, no differences were found for *desmin* (*DES*), *solute carrier family 2 member 1* (*SCL2A1*/*GLUT1*) and *BAP1* mRNAs (Fig. 1A).

FACS analysis of different DMM cell lines showed strong surface CD47 protein expression in 5/5 cell lines tested, and calreticulin was detected on the cell surface in 3/5 cell lines (Fig. 1B-C). In addition, CD47 was detectable by IHC of cell blocks generated from the different cell lines (Fig. 1D and data not shown).

These data indicate that DMM cells express the "don't eat me" signalling molecule CD47 and the pro-phagocytic molecule calreticulin.

High CD47 expression discriminates DMM from reactive mesothelial cells in effusion cytology specimens

To investigate whether CD47 can be used as a biomarker for DMM, we constructed tissue TMAs of cell blocks from pleural and peritoneal effusions collected from 2010–2014 at the division of cytopathology of our institute. CD47 expression levels by IHC were significantly higher in DMM than in benign mesothelial cells ($p < 0.0001$, Chi-square test). DMM tumour cells were CD47 high (2+, 3+) in 24/38 (63%) DMM samples, whereas reactive mesothelial cells were CD47 low (0, 1+) in all of the 87 benign samples tested (Fig. 2A-B and Table 1). BAP-1 was deficient in 21/37 (57%) of DMM samples, while 0/103 (0%) of non-neoplastic samples were BAP-1 deficient. Representative examples of stainings for EMA, GLUT-1, desmin and calreticulin are displayed in Fig. 2A-B and data are summarized in Tables 1 and 2. Importantly, the combination of high CD47 expression and/or BAP-1 deficiency was 78% sensitive (28/36 DMM samples) and 100% specific (0/87 reactive samples). This two-marker panel was easier to perform and showed greater sensitivity and specificity than combination of 4 established markers BAP-1, EMA, GLUT-1 and desmin (Table 1).

Calreticulin was expressed at significantly higher levels in DMM than in benign mesothelial cells ($p < 0.0001$, Chi-square test). In DMM, calreticulin expression was not statistically associated with CD47 expression levels (Table 2).

In summary, these data indicate that high CD47 expression on mesothelial cells can be used as a biomarker for DMM and that a two-marker combination of CD47 and BAP-1 is a sensitive, specific and easy to perform assay to discriminate DMM from reactive mesothelial cells in body cavity effusions.

Targeting CD47 inhibits growth and promotes phagocytosis of DMM cell lines

In addition to inhibit phagocytosis of tumour cells by binding to SIRPα on macrophages[22,26,27,32], CD47 signalling has also been shown to stimulate tumour cell proliferation directly by activating the PI3K/Akt pathway.[33] To address whether targeting CD47 could represent a therapeutic strategy for DMM, we cultured DMM cell lines in the presence of IgG isotype control or blocking anti-CD47 monoclonal antibody (mAb). Numbers of DMM cells per well were up to two-fold higher in IgG-treated conditions than in the presence of anti-CD47 mAb after three days of culture (Fig. 3A), indicating that blocking CD47 directly inhibits DMM cell growth. In addition, to investigate if blocking CD47 also increases phagocytosis of DMM cells, we performed a well-established *in vitro* phagocytosis assay by co-culturing human macrophages with fluorescently labelled ACC-MESO-1 cells (Fig. 3B).[24,27] Blocking of CD47 resulted in a two-fold increase in the phagocytosis of ACC-MESO-1 cells by macrophages compared to control IgG treatment, as analysed by FACS (Fig. 3C-D).

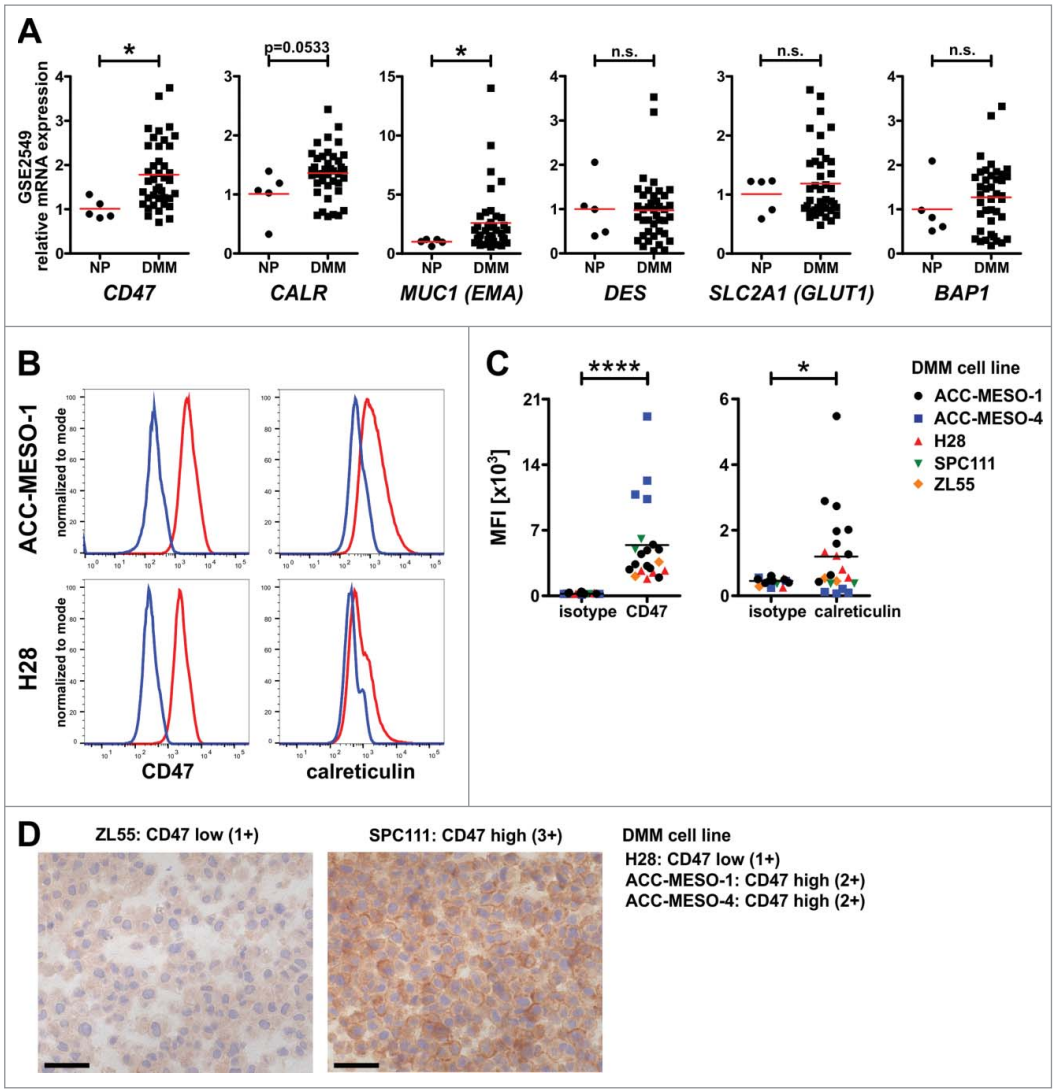

Figure 1. The "don't eat me" signal CD47 and the pro-phagocytic molecule calreticulin are expressed in diffuse malignant mesothelioma. (A) Relative expression of *CD47*, *CALR*, *MUC1 (EMA)*, *DES*, *SLC2A1 (GLUT1)* and *BAP1* mRNA in normal pleura (NP; n = 5) vs. pleural DMM (n = 40) were analysed in a publicly available microarray dataset (GSE2549) using the Gene Expression Omnibus GEO2R online tool. (B) CD47 and calreticulin expression on mesothelioma cell lines ACC-MESO-1 and H28, respectively, as analysed by FACS. One representative histogram of 9 (ACC-MESO-1) and 4 (H28) per staining is shown. Red lines, CD47 and calreticulin stainings; blue lines, respective isotype controls. (C) Mean fluorescence intensities (MFI) of CD47 vs. isotype (left panel) and calreticulin vs. isotype (right panel) in 5 different DMM cell lines. (D) Immunohistochemistry for CD47 on FFPE cell blocks from DMM cell lines. Examples of low (1+) and high (3+) CD47 expression are shown. Scale bars, 50μm. Statistics: (A) Mann-Whitney test; (C) paired t-test. $^{*}p < 0.05$; $^{****}p < 0.0001$

These data indicate that blocking CD47 directly inhibits the growth and increases macrophage-mediated phagocytosis of DMM tumour cells.

Surgical specimens from patients as well as mouse DMM tumours express CD47 and are heavily infiltrated by macrophages

To address if CD47 is expressed in DMM tissue sections from patients and whether this correlates with the expression in corresponding cytological effusion cell blocks, we performed IHC on surgical DMM resection specimens. Fig. 4 shows the comparison of the matched cytological effusion specimens and surgical resections for patients 2 (cytology CD47 2+) and 3 (cytology CD47 0), respectively. Interestingly, moderate to high levels of CD47 were expressed in tissue sections of all patients analysed, including patients that had low or negative CD47 expression in cytology. In contrast, sarcomatoid tumours tended to show faint cytoplasmic CD47 staining only (Figure S1 and data not shown). In addition, we performed stainings for the macrophage markers CD68 and CD163 that showed heavy infiltration by macrophages in all DMM tumours analysed (Figs. 4 and S1 and data not shown).

Furthermore, we investigated CD47 expression and tumour infiltration by macrophages in a well-established syngeneic mouse DMM model.[34] Mouse DMM tumours showed high CD47 expression and were heavily infiltrated by macrophages, as analysed by immunohistochemistry for CD68 and F4/80 (Fig. 5A). In line with this, *Cd47*, *Cd68* and *Adgre (F4/80)* mRNA expression was detectable in mouse DMM tumours (Fig. 5B).

These data indicate that in both humans and mice, CD47 is expressed in the united cell structures of solid DMM tumours and that these tumours are heavily infiltrated by macrophages.

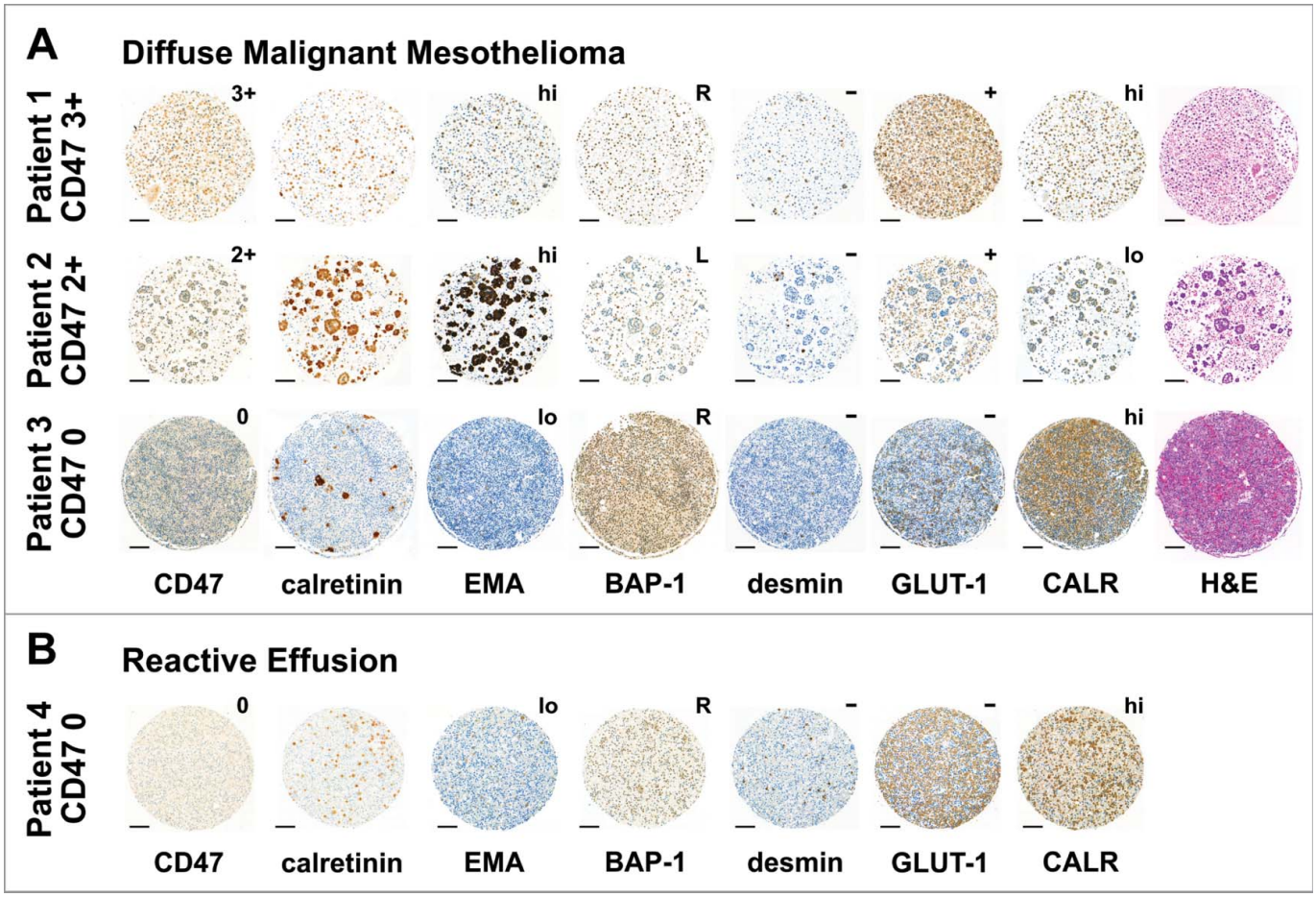

Figure 2. CD47 immunohistochemistry discriminates DMM from reactive mesothelial cells in effusion cytology. (A-B) Representative examples of different CD47 immunohistochemistry staining intensities in DMM and reactive effusions in TMAs constructed of cell blocks from effusion specimens. Each row represents one patient. The corresponding stainings for calretinin, EMA, BAP-1, desmin, GLUT-1, calreticulin and H&E morphology are shown. Staining intensity for the respective molecules was assessed in mesothelial cells identified using calretinin. The interpretation of the result is indicated at the upper right of each spot. (A) Diffuse malignant mesothelioma, CD47 staining intensity low (0) and high (2+, 3+). (B) Reactive effusion, CD47 staining intensity low (0). Scale bars, 100μm; original magnification 150x. Staining assessment: CD47 (membranous staining): 0, negative; 1+, incomplete or weak; 2+, complete, moderately strong; 3+, complete, strong. EMA (membranous staining, modified according to [5]): lo, negative or 1+ and <25% positive cells; hi, 2+ or 3+ or 4+ and ≥25% positive cells. BAP-1 (nuclear staining): L, lost; R, retained. Desmin (cytoplasmic staining): -, loss in any number of mesothelial cells; +, no loss. GLUT-1 (membranous staining): -, negative; +, positive. Calreticulin (cytoplasmic and membranous staining): lo, <95% positive cells; hi, ≥95% positive cells.

Discussion

DMM is one of the deadliest cancers and remains a diagnostic and therapeutic challenge. Based on the historical use of asbestos, the most important cause of DMM, and the decades-long delay of disease onset, the DMM pandemic is predicted to peak around 2020 with an estimated 43,000 annual deaths worldwide.[35] Current therapy for DMM is most often an individualized multimodality approach that includes, if possible, surgery with or without intraoperative chemotherapy, radiation therapy, and systemic chemotherapy.[2,36] The role of surgery, consisting of different more or less radical procedures including extended pleuropneumonectomy and decortication, remains controversial. 85–90% of patients are diagnosed in an advanced, inoperable disease stage, and radical surgical procedures are associated with high postoperative mortality rates up to 12% and significant morbidity.[36] Radiation therapy has traditionally most commonly been used for palliation of symptoms in advanced cases as well as an adjuvant therapy in

Table 1. Immunohistochemical findings in cell block TMAs.

Marker	DMM	Benign/reactive
CD47-high (2+, 3+)	24/38 (63%)	0/87 (0%)
BAP-1 deficient	21/37 (57%)	0/103 (0%)
CD47-high and/or BAP-1 deficient	28/36 (78%)	0/87 (0%)
Desmin loss	36/36 (100%)	19/65 (29%)
GLUT-1 positive	23/39 (59%)	7/87 (8%)
EMA positive	25/38 (66%)	17/80 (21%)
BAP-1 deficient and/or ≥1 out of desmin, GLUT-1, EMA abnormal	34/34 (100%)	28/53 (53%)
BAP-1 deficient and/or ≥2 out of desmin, GLUT-1, EMA abnormal	32/34 (94%)	8/53 (15%)
BAP-1 deficient and/or all 3 out of desmin, GLUT-1, EMA abnormal	26/34 (76%)	2/53 (4%)

Table 2. Calreticulin immunostaining on cell block TMAs.

	DMM	Benign/reactive
Calreticulin-high	16	70
Calreticulin-low	22	18
	DMM, CD47 high (2+, 3+)	DMM, CD47 low (0, 1+)
Calreticulin-high	9	7
Calreticulin-low	15	5

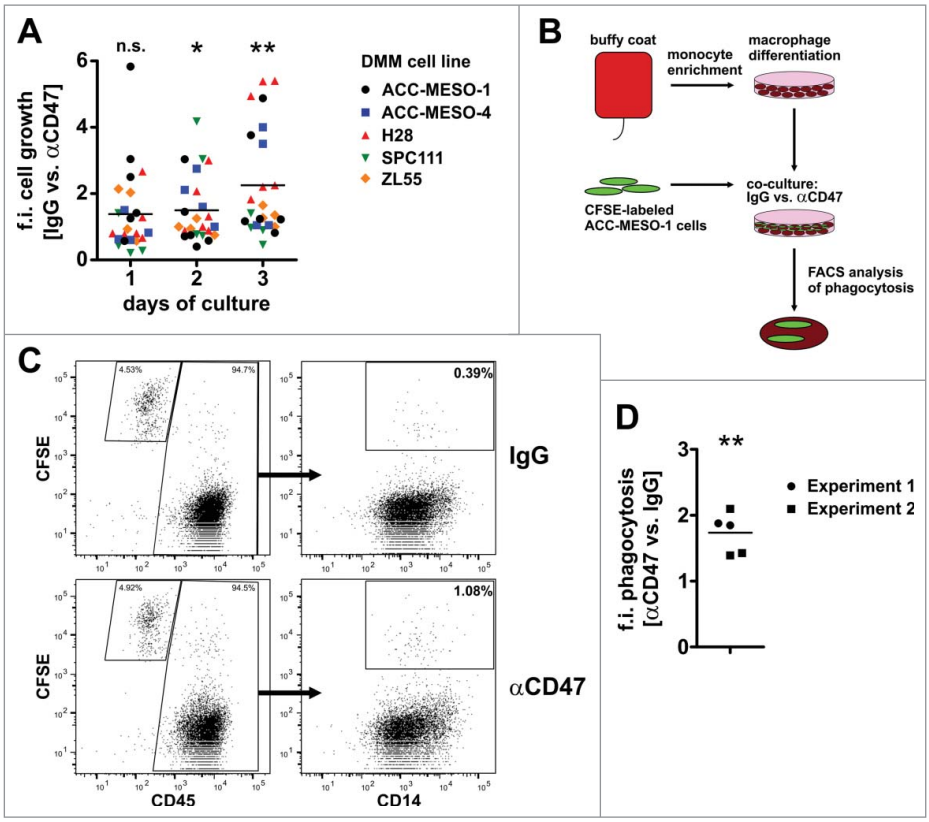

Figure 3. Blocking CD47 inhibits cell growth and promotes phagocytosis of malignant mesothelioma cell lines. (A) DMM cell lines were cultured in duplicates for three days in the presence of 5μg/ml isotype control (IgG) or anti-CD47 (αCD47) mAb and cell numbers were assessed daily in a Neubauer chamber using trypan blue staining. The fold increase in cell growth of IgG- vs. αCD47-treated DMM cell lines is shown for each day. Pooled data from 5 independent experiments. (B) Schematic of phagocytosis assay. Differentiated macrophages were serum starved for 2h and then co-cultured with CFSE-labelled ACC-MESO-1 cells for 2h in the presence of 10μg/ml IgG or αCD47 mAb, followed by FACS analysis. (C) Representative dot plots of $CD45^{+}CD14^{+}$ macrophages containing $CFSE^{+}$ ACC-MESO-1 cells. Non-phagocytized $CFSE^{+}$ tumour cells are gated in the left panels (top left). (D) Normalized fold increase in phagocytosis of αCD47 vs. IgG mAb-treated co-cultures (IgG = 1). Pooled data from two independent experiments run in duplicates or triplicates are shown. Results (mean±SEM), experiment 1: IgG, 18.7% ± 2.45%; αCD47 34.78% ± 4.28%; experiment 2: IgG 0.73% ± 0.12%; αCD47 1.15% ± 0.08%. f.i., fold increase. Statistics: (A, D) one-sample t-test (hypothetical value = 1). $^{*}p < 0.05$; $^{**}p < 0.01$.

patients undergoing surgery. In the advent of emerging immune checkpoint inhibitor therapies, however, radiation therapy may be increasingly used with the intent to induce immunogenic cell death.[2,37] A recent phase 2–3 clinical trial of anti-CTLA-4 mAb vs. placebo in advanced DMM (NCT01843374) did not show any benefit of the immune checkpoint inhibitor as a monotherapy.[38] Historically, the only treatment that has shown significant improvements in outcome is systemic chemotherapy[39,40]; however, clinical trials with different mAbs targeting the PD-1 pathway are ongoing and might show promising results in the future, especially if combined with radiotherapy or other targeted therapies.[38,41]

In the present study, we have identified the "don't eat me" signal CD47 as both a diagnostic marker and a potential therapeutic target in DMM. Targeting the CD47-SIRPα interaction to increase the phagocytosis of tumour cells is currently being investigated in several early-phase clinical trials in haematological malignancies (NCT02641002, NCT02663518, NCT02678338, NCT02953509) and solid tumours (NCT02216409, NCT0 2890368, NCT02953782). The expression of *CD47* mRNA in DMM was first identified in 2005 by Gordon et al. in a large-scale transcriptional profiling study[30]. In 2010, Melotti et al. reported the expression of CD47 protein as analysed by FACS of mesothelioma cell lines generated from four primary DMM samples.[42] However, a systematic analysis of the role of CD47 in DMM biology was not performed in these studies. To our knowledge, we present here the first study on the role of CD47 as a potential diagnostic marker for malignancy in body cavity effusions and as a promising therapeutic target for DMM.

Using cell blocks from effusion samples, we found that CD47 protein expression was higher in DMM than in reactive mesothelial cells and – with complete membranous staining of at least intermediate intensity as cut-off – was 100% specific and 63% sensitive in discriminating DMM from reactive mesothelial cells. A two-marker panel of CD47 and BAP-1 outperformed our previous routine four-marker panel (BAP-1, EMA, GLUT-1 and desmin)[3,5,11] in terms of sensitivity and specificity. Of note, CD47 immunostaining was found easier to interpret than those for EMA, GLUT-1 and desmin. Even though calreticulin was expressed at significantly higher levels in DMM than in benign mesothelial cells, there was major overlap between both groups, limiting the diagnostic utility of this marker as a surrogate for malignancy. Given that our analysis focussed on cytological samples and that malignant cells are usually absent from effusions associated with sarcomatoid DMM[43], our findings mainly apply to the epithelioid subtype of DMM. Histological samples of selected sarcomatoid and biphasic DMM showed only weak cytoplasmic CD47 staining, indicating that CD47 may be less sensitive for a diagnosis of sarcomatoid DMM in histological samples, like the reported lower sensitivity of BAP-1 in this setting.[44]

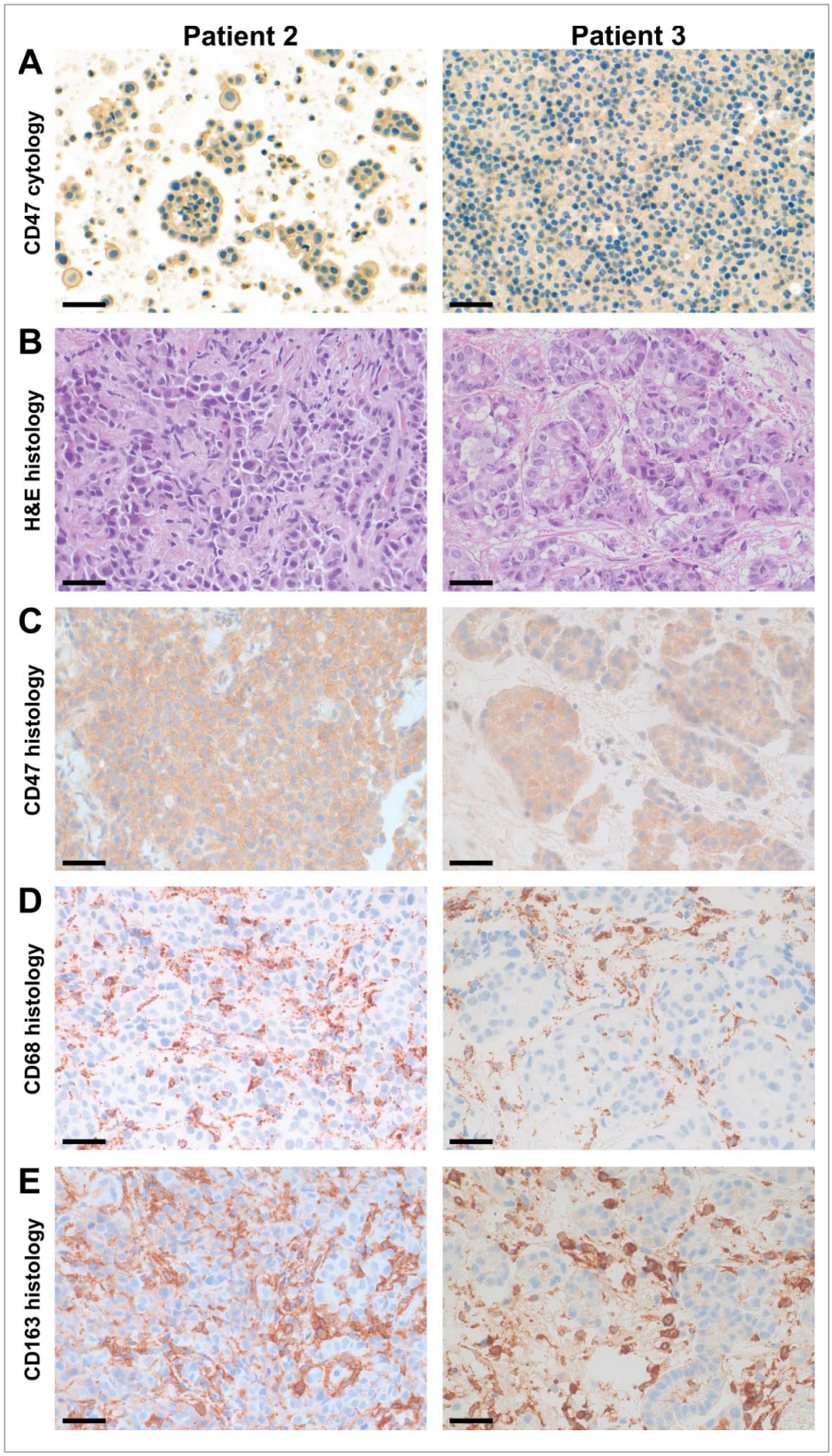

Figure 4. Surgical DMM specimens express CD47 and are heavily infiltrated by macrophages. Representative examples of CD47 high (Patient 2) and low (Patient 3) expressing pleural effusions and matched surgical resection specimens are shown. (A) CD47 immunohistochemistry of the effusion cell block (cf. Fig. 2). (B-E) H&E staining (B) and immunohistochemistry for CD47 (C), CD68 (D) and CD163 (E). Scale bars, 40μm; original magnification 400x.

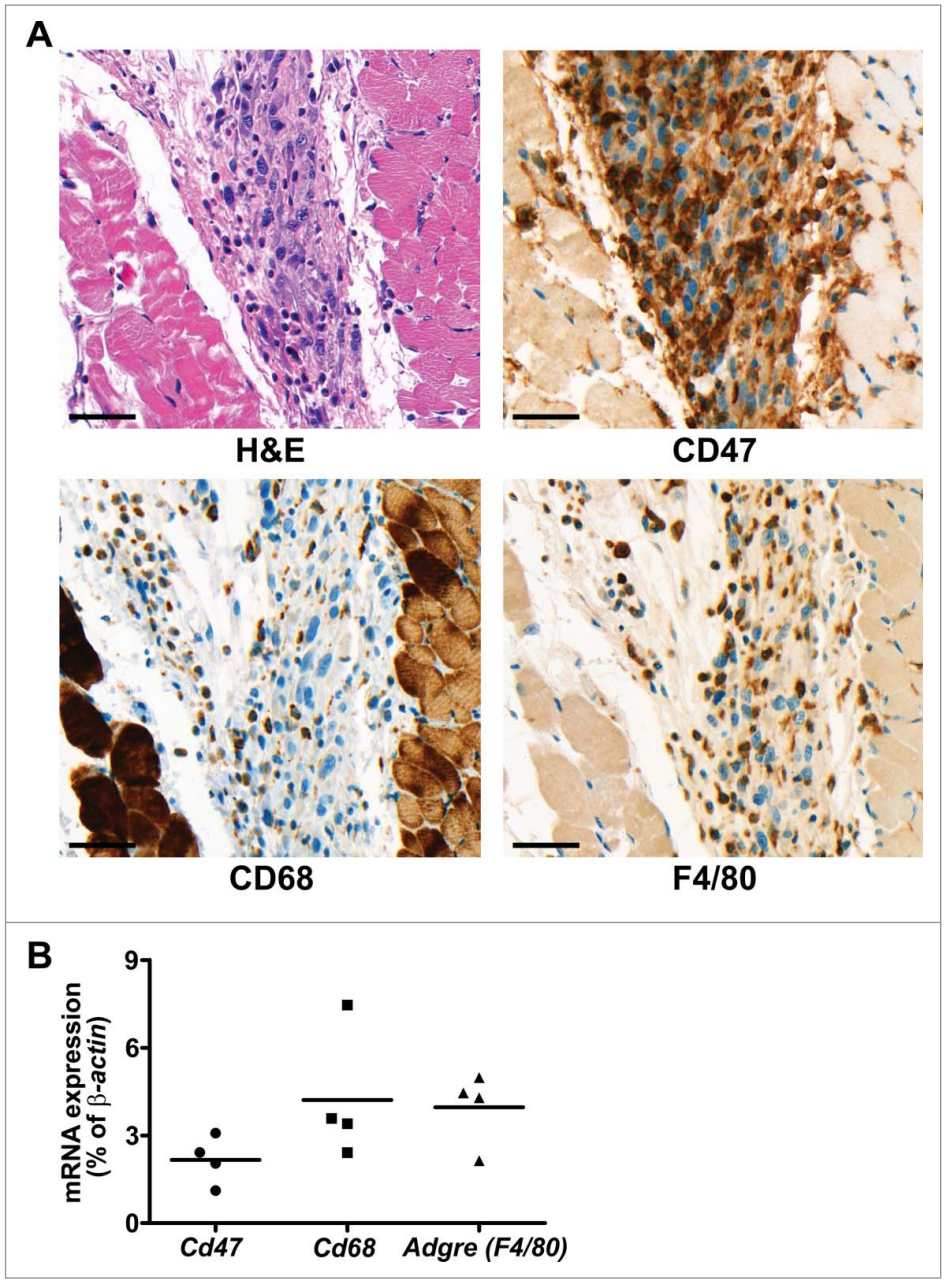

Figure 5. CD47 is expressed in a murine model of DMM. A) H&E staining and immunohistochemistry for CD47, CD68 and F4/80 in subcutaneously transplanted primary abdominal murine mesothelioma tumours. One representative example of n = 5 mice is shown. (B) Expression levels of *Cd47*, *Cd68* and *Adgre (F4/80)* mRNA in murine tumours from (A). Scale bars, 50μm; original magnification 400x.

Importantly, we could demonstrate that blocking CD47 *in vitro* directly inhibits the growth of DMM cell lines and increases their phagocytosis by macrophages. The mechanism by which CD47 interferes with macrophage phagocytosis has been well described and analysed in numerous studies.[18-29] On the other hand, how CD47 directly affects cell growth is less well understood. Sick et al. previously reported that CD47 promotes cell proliferation in astrocytoma cells in a PI3K/Akt-dependent manner.[33] TSP-1, which is overexpressed in malignant mesothelioma[45], is a well-known ligand for CD47[13,15], and ligation of CD47 by TSP-1 was shown to induce vascular smooth muscle cell proliferation.[46] Although further analysis of the exact mechanism by which CD47 directly promotes tumour cell proliferation in our *in vitro* cultures was out of the scope of this study, we propose TSP-1 secreted by mesothelioma cells to be a likely candidate mediating this effect in an autocrine manner.

In sum, this study identifies the "don't eat me" signal CD47 as a diagnostic marker and provides a foundation for further research into its use as a therapeutic target for DMM.

Materials and methods

Tissue microarrays (TMAs)

Archival cell blocks from body cavity effusions submitted for cytological analysis were retrieved and corresponding slides were assessed for the presence of mesothelial cells. Cell blocks were generated by clotting of sediments with plasma and

thrombin, fixation of clots in formalin and standard histological processing. Specimens were classified as DMM or reactive mesothelial cells based on the original diagnostic interpretation and clinical follow-up. For a classification as DMM, the presence of atypical mesothelial cells and confirmation by subsequent histological examination, by ancillary testing or clinical correlation was required. Cases were identified from a period from 2010 to June 2014. Out of 52 cases meeting the inclusion criteria, in 7 there was insufficient cell block material for TMA construction. 45 DMM cases were included in the TMA. For inclusion into the control ("reactive") group it was required that samples had ultimately been judged as benign during routine diagnostic work-up and that clinical follow-up did not indicate malignancy. TMAs were created from cell blocks using an automated tissue microarrayer (TMA Grand Master, 3DHistech) with a punch diameter of 600 μm.[47] The study was approved by the Ethics Committee of the Canton of Bern (KEK 200/14).

Immunohistochemistry (IHC)

IHC stainings of TMAs and selected full slides of cell blocks and corresponding histological samples were performed on a Leica BOND RX automated immunostainer using Bond primary antibody diluent and Bond Polymer Refine DAB Detection kit according to the manufacturer's instructions (Leica Biosystems). Antibodies, clones and manufacturers are listed in Table S1. Analysis and interpretation of the staining results were conducted in accordance with the "reporting recommendations for tumour marker prognostic studies" (REMARK) guidelines.[48] CD47 staining was classified as low (0, absence of any membranous staining and 1+, weak or incomplete membranous staining) or high (2+, complete membranous staining of intermediate intensity and 3+, complete membranous staining of strong intensity) by two board-certified surgical pathologists (C.M.S and E.H.). BAP-1 was classified as proficient (presence of nuclear staining) or deficient (complete lack of any nuclear staining in the cell population of interest). EMA immunostaining was regarded positive if ≥25% of mesothelial cells were stained, corresponding to a score of ≥2+ according to Shen et al.[5] Loss of desmin staining was defined as presence of desmin-negative mesothelial cells. GLUT-1 was classified as positive when there was any membranous staining of mesothelial cells. While there was widespread calreticulin staining of mesothelial cells in essentially all samples, we observed two patterns of immunoreactivity: either diffuse staining of nearly all mesothelial cells (classified as high, ≥95%) or presence of a substantial fraction of negative mesothelial cells (classified as low, <95%). Staining results were required to be attributable to cells morphologically consistent with mesothelial cells. If attribution of findings to mesothelial cells was equivocal, we referred to stainings for calretinin (a marker of mesothelial differentiation) performed on consecutive slides from each TMA block.

Gene expression microarray data

Expression data were derived from the Gene Expression Omnibus (GEO) public repository for microarray data and are available under accession number GSE2549.[30]

Cell lines

DMM cell lines ACC-MESO-1[49], ACC-MESO-4[49], NCI-H28[50], ZL55[51] and SPC111[51] have been described before. Cell line identity was tested using a commercial cell line authentication service (Microsynth).

Flow cytometry / fluorescence activated cell sorting (FACS)

DMM cell lines were cultured to 70% confluence, harvested using non-enzymatic cell dissociation buffer (Sigma) and stained for CD47, calreticulin and the respective isotype controls (Table S1) in PBS for 30 min at 4°C. Samples were acquired on a BD LSR II flow cytometer (Becton Dickinson) and were analysed using FlowJo software (TreeStar).

Growth assay

DMM cell lines were plated in duplicates into 24-well tissue culture plates at a starting concentration of 10^4 cells per well and were cultured for three days in the presence of 5 μg/ml mouse anti-human CD47 (clone B6H12.2, ThermoFisher) or isotype control (mouse IgG1, ThermoFisher). Cell numbers per well were determined daily by trypan blue staining and a Neubauer chamber.

In vitro *phagocytosis assay*

The *in vitro* phagocytosis assay was performed as described before[24,27], with slight modifications. Buffy coats and human serum were obtained from the Swiss Blood Bank (Interregionale Blutspende SRK, Bern, Switzerland). PBMCs were enriched from buffy coats by density centrifugation using Lympho Spin Medium (pluriSelect). Monocytes were isolated from PBMCs using the EasySep Human CD14 Positive Selection Kit II (Stemcell Technologies) according to the manufacturer's instructions. 4–5 × 10^6 monocytes per well were differentiated into macrophages for 7 days in 6-well tissue culture plates in Iscove's modified Dulbecco's medium supplemented with 10% human serum, L-glutamine and penicillin/streptomycin. ACC-MESO-1 cells were harvested using non-enzymatic cell dissociation buffer (Sigma) and labelled with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) in PBS at a final concentration of 20 μM. Macrophages were starved in serum-free medium for 2 h and 1 × 10^6 CFSE-labelled ACC-MESO-1 cells were added. Cell were co-cultured for 2 h in the presence of 10 μg/ml mouse anti-human CD47 (clone B6H12.2, ThermoFisher) or isotype control (mouse IgG1, ThermoFisher), then harvested, stained for CD45 and CD14 (Table S1) and analysed by FACS.

Murine DMM model

The murine DMM model was established by s.c. injection of 2 × 10^6 RN5 syngeneic mesothelioma cells[34] into the dorsal flank of 6–8 weeks old C57Bl/6J mice. Tumours were measured with a calliper weekly and mice were euthanized and tumours harvested when reaching a volume of 0.25 cm^3. A part was fixed in 4% paraformaldehyde before paraffin embedding and a part was processed for RNA extraction as previously described.[52]

Murine mRNA analysis

The expression of selected genes was analysed as previously described and normalized to the housekeeping gene β-actin.[53,54] Primer sequences are listed in Table S2.

Statistical analysis

Statistical analysis was performed using GraphPad Prism® 5.0 (GraphPad Software). Data are represented as mean ± SEM.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

C.M.S. conceived the study, designed and performed most of the experiments, analysed and interpreted data and wrote the manuscript. S.F. and F.B. performed experiments and analysed data. S.H.Y. helped construct the TMA. E.F.-B. provided cell lines and mouse tissues, performed experiments, analysed data and wrote the manuscript. E.H. constructed the TMA, analysed and interpreted data and wrote the manuscript. All authors revised the manuscript and approved its final version.

Dual publication

The authors state that this manuscript contains original unpublished work that is not submitted, under consideration or published elsewhere, neither in full nor in part.

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