Signal regulatory protein alpha in phagocyte function

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Chapter 5
CD47-SIRPα interactions form an intrinsic limitation for antibody-mediated tumor cell clearance by macrophages

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Abstract

Monoclonal antibodies (mAb) are among the most promising therapeutic agents for treating cancer. They generally act by binding to tumor cells turning them into targets for killing by immune cells. We have discovered that this antibody-mediated killing of tumor cells is limited by an intrinsic mechanism involving interactions between CD47, expressed on tumor cells, and the inhibitory receptor SIRPα present on macrophages and granulocytes. SIRPα-mutant mice that lack the cytoplasmic tail, and hence its signaling capacity displayed an increased capacity for antibody-mediated elimination of B16 melanoma cells *in vivo*. Moreover, interference with CD47-SIRPα interactions employing antagonistic antibodies significantly enhances the destruction of tumor cells *in vitro*. These findings suggest that CD47-SIRPα interactions are part of a homeostatic mechanism that limits antibody-mediated killing of tumor cells. Importantly, the blocking of this interaction may enhance the therapeutic effects of cancer therapeutic antibodies.

Introduction

In recent years, therapeutic monoclonal antibodies (mAb) have become a valuable addition to current cancer treatment modalities, such as surgical resection, chemotherapy, and radiotherapy. Therapeutic antibodies directed against tumor cells act by binding to tumor-associated antigens on the tumors cells turning them into targets for antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent cellular cytotoxicity (ADCC) by immune cells[1]. For instance, the monoclonal antibodies Rituximab, directed against CD20 on both normal B cells and on most low-grade and some higher grade B-cell lymphomas, and Trastuzumab, that recognizes the HER-2/neu growth factor receptor on metastatic breast cancer, have demonstrated to be well tolerated and capable of initiating tumor regression in a significant percentage of patients[2]. Nevertheless, the current number of FDA-approved anti-tumor antibodies is limited to four, which is certainly disappointing[1].

ADCC activity mediated by NK cells are regulated to an important extent by 'self' signals, such as MHC class I molecules (MHCI) that are broadly expressed on host cells. This involves killer inhibitory receptors (KIR), expressed on NK cells, which provide inhibitory cytoplasmic signals that prevent the ADCC and killing of healthy (i.e. MHCI-expressing) host cells[3]. However, it has become clear that such homeostatic regulation of immune cells activity is not only limited to NK cells. For instance, it has become clear that myeloid cells, including macrophages and granulocytes, also express inhibitory receptors that regulate their effector functions.

SIRPα is a major inhibitory receptor on myeloid cells and the best characterized member of the signal regulatory protein family. SIRPα is predominantly expressed by neuronal and myeloid cells, including macrophages, granulocytes, myeloid dendritic cells, mast cells and their precursors, including hematopoietic stem cells[4-6]. The extracellular region of SIRPα is composed of a single V-set and two C1-set IgSF domains which shares a close structural similarity to that of TcR and BcR chains, even when compared to other non-rearranging Ig-superfamily members. The cytoplasmic region of SIRPα contains immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that recruit and activate the src homology 2 (SH2) domain-containing tyrosine phosphatases SHP-1 and SHP-2[7-9] that regulate proximal signal transduction cascades and cellular functions, generally in a negative fashion.

CD47, a broadly expressed molecule, has been identified as a cellular ligand for SIRPα[10]. This protein, which was originally identified in association with αvβ3 integrin, is also a member of the Ig superfamily, possessing an Ig-V-like extracellular domain, five putative membrane-spanning segments, and a short cytoplasmic tail[5;10]. The binding of CD47 is mediated through the NH_2 terminal V-like domain of SIRPα.

SIRPα has been implicated in a variety of cellular functions including growth, differentiation, adhesion, transendothelial migration of monocytes and granulocytes, migration of Langerhans cells, the formation of multinucleated giant cells, the phagocytosis of host cells and the production of inflammatory mediators[11-14]. Clearly, the best documented function of SIRPα is its inhibitory role in the phagocytosis of host cells, including red blood cells, platelets and hematopoietic stem cells by

macrophages[15-17]. In particular, the ligation of SIRPα on macrophages by CD47 expressed on host target cells generates an inhibitory signal, predominantly mediated by SHP-1, that negatively regulates target cell phagocytosis. The role of SIRPα in host cell phagocytosis is supported by *in vivo* studies either with macrophages from SIRPα-mutant mice[16;18] or with target cells from CD47 deficient mice[18;19]. Consistent with a negative role of CD47-SIRPα interactions in platelet and red blood cell clearance SIRPα-mutant mice that lack the SIRPα cytoplasmic domain are mildly thrombocytopenic and anemic[16;18;20]. SIRPα also acts as a negative regulator of osteoclast bone resorption[21]. Together, these findings support the idea that CD47-SIRPα interactions control the immune effector function of phagocytes. This is analogous to the 'self' signals provided by MHCI molecules to NK cells via KIR or Ly49 receptors.

In the present study, we have investigated the effect of the CD47-SIRPα interactions on the Ab-dependent elimination of tumor cells. Our results clearly show that blocking of CD47-SIRPα interactions by suitable antagonists enhances Ab-mediated phagocytosis and destruction of tumor cells *in vitro*. Furthermore, we observed a dramatic enhancement of antibody-mediated elimination of metastatic melanoma cells in SIRPα-mutant mice. This identifies CD47-SIRPα interactions as an intrinsic homeostatic mechanism that limits the therapeutic effects of Ab against tumor cells.

Materials and methods

Cell lines

The rat colon carcinoma cell line CC531s was originally derived from Wag/Rij rats after induction with 1,2-dimethylhydrazine[22]. B16F10 mouse melanoma cell line and the rat NR8383 macrophage cell line have been described before[23;24]. All cells were cultured in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C 5% CO_2. CC531s and B16F10 cells were harvested by enzymatic detachment using trypsin-EDTA solution and resuspended in RPMI 1640 medium or in Hanks' balanced salt solution (HBSS) when used for *in vivo* experiments. Viability, as determined with trypan blue exclusion, always exceeded 95%.

Mice

C57BL/6 mice with a targeted deletion of the SIRPα cytoplasmic region have been described previously[18]. The mice that were originally generated onto the 129/Sv background have been backcrossed onto the C57BL/6 mice for ten generations. Wild type C57BL/6 mice of the same genetic background were maintained together with the SIRPα-mutant mice in the breeding facility of the Vrije Universiteit Medical Center. Mice were kept in specifically pathogen free conditions according to FELASA recommendations and used at 8 to 12 weeks of age. All experiments had been approved by the Animal Welfare committee of the University.

Antibodies

The following monoclonal antibodies were used: unlabeled or biotin labeled ED9 (anti-rat-SIRPα, mouse IgG1, Serotec, Oxford, UK)[4], OX101 (anti-rat-CD47, mouse IgG1, Serotec, Oxford, UK)[25], ED1 (pan-monocyte/macrophage marker, mouse IgG1, Serotec, oxford, UK)[26], ED2 (tissue macrophages, mouse IgG1, Serotec, oxford, UK)[26], CC52 (mouse IgG1 Ab directed against CC531 tumor cells)[27;28] HIS48 (granulocytes marker, mouse IgM, Serotec, Oxford, UK)[29], anti-rat CD11a ([alpha]L integrin subunit, clone WT.1, IgG2a, Serotec, Oxford, UK), MIAP301 (anti-mouse-CD47, IgG2a, BD Pharmingen, San Diego, CA, USA)[30]. The monoclonal antibody TA99 (mouse IgG2a) was a kind gift of Dr J.G. van de Winkel. TA99 is directed against the gp75 antigen present on B16F10 melanoma cells. The secondary antibodies used included biotinylated anti-mouse IgG1 (Zymed, San Francisco, CA, USA), biotinylated anti-mouse IgM (Zymed, San Francisco, CA, USA), Alexa 488-labeled anti-mouse IgG (Molecular Probes, Eugen, OR, USA), Alexa 594-streptavidin conjugate (Molecular Probes, Eugen, OR, USA), Alexa 633-labeled anti-rat IgG (Molecular Probes, Eugen, OR, USA), phycoerythrin-labeled anti-mouse IgG (Jakson ImmunoResearch, West Grove, PA, USA) and FITC-labeled anti-rat Ig (Dako, Heverlee, Belgium) were used.

Immunohistochemistry

CC531s colon carcinoma cells were injected into WAG/Rij rats as described in detail before[31]. Metastatic liver tumors were dissected, snap frozen in liquid nitrogen and stored until further use. Serial cryostat sections (7 μm) were cut and mounted on glass slides. Sections were fixed in acetone for 10 minutes, followed by incubation in PBS containing 1% H_2O_2 for 10 minutes, to block endogenous peroxidase activity. After washing twice with PBS, sections preincubated for 30 minutes with PBS containing 1% Bovine Serum Albumin (BSA, Boehringer, Mannheim, Germany) and 10% animal serum, the source of which was determined by the specific secondary antibody used. Next, sections were washed and treated with avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, USA).

For immunohistochemical stainings, tissue sections were subsequently incubated for 1 h at RT with (mixture of) primary antibodies in PBS-1%BSA. After washing three times with PBS-1%BSA, sections were further incubated for 1 hour at RT with secondary antibodies and 1% normal serum. Sections incubated with biotin-labeled secondary antibodies were treated with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was demonstrated by incubation with 0.5 mg/ml 3.3'-diaminobenzidine (DAB) in Tris buffer containing 0.03% H_2O_2. All sections were counterstained in Mayer's haematocylin, and mounted in Entellan (Merck, Darmstadt, Germany).

For colocalization studies, sections were incubated with for 1 hour at RT with (mixture of) primary antibodies in PBS-1%BSA. After washing with PBS-1%BSA, sections were further incubated for 1 hour at RT with secondary antibodies labeled with Alexa 594 or Alexa 488 and 1% animal serum in which conjugated was raised. After washing, sections were dried, and mounted in Vectamount (Vector, Laboratories, Burlingame, CA, USA). Immunohistochemical stainings and colocalization studies were

examined with a Nikon Eclipse E800 microscope and recordings were made with a digital Nikon DX1200 camera.

ADCP assay

CC531s tumor cells were labeled with DiI (20 μg/ml, Invitrogen, Breda, The Netherlands) for 30 minutes. NR8383 macrophages were stained with mouse anti-rat CD11a for 1 hour on ice, followed by FITC labeled anti-rat Ig for 30 minutes on ice. CC52 (10 μg/ml), ED9 (10 μg/ml), ED9Fab (20 μg/ml), OX101 (10 μg/ml) and control antibodies (10 μg/ml) were added to the labeled CC531 cells, which were immediately added to NR8383 macrophages at a ratio of 10:1, and phagocytosis was allowed to proceed for 24 hours at 37℃. Cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Data were processed with CellQuest software (BD Biosciences, San Jose, CA, USA). The percentage of ingested tumor cells was calculated by dividing the percentage of ingested DiI-FITC double positive cells with the percentage of ingested plus non-ingested DiI-labeled cells and multiplying this number with 100.

Flow cytometry

The surface expression of CD47 on B16F10 and CC531 cells was examined by flow cytometry. Cells were harvested by treatment with trypsin-EDTA solution and washed with ice-cold PBS-0.01%BSA. They were incubated with anti-mouse CD47 antibody for mouse B16F10 cells (clone MIAP301, IgG2a)[30] or with anti-rat CD47 for the rat CC531 cells (clone OX101, IgG1)[25] for 1 hour on ice. After washing with ice-cold PBS-0.01%BSA, B16F10 cells were incubated with Alexa 633-labeled anti-rat IgG antibody and CC531 cells with phycoerythrin-labeled anti-rat IgG for 30 minutes on ice. Cells are washed and analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Data were processed with CellQuest software (BD Biosciences, San Jose, CA, USA).

Melanoma model

Wild type and SIRPα-mutant mice were injected intravenously with 1.5 X 10^5 B16F10 tumor cells (in 100 μl HBSS) on day 0. Mice were injected intraperitoneally with the indicated suboptimal dose of 10 μg of TA99 antibody (or PBS as control) on day 0, 2, and 4 as determined in pilot experiments. At day 21, the mice were sacrificed, and lungs were scored for numbers of metastases and tumor load. Tumor load was defined as detailed in[32] as the sum of the following scores: metastases less than 1 mm were scored as 1; metastases between 1 and 2 mm were scored as 3; and metastases larger than 2 mm were scored as 10.

Statistical analyses

Statistical differences were determined using students t-test or ANOVA. P-values of 0.05 were considered significant.

Results

The expression of CD47 and SIRPα in tumor tissue

Previous studies have demonstrated that CD47-SIRPα interactions negatively regulate the phagocytosis of unopsonized- or antibody opsonized-host cells, such as red blood cells, platelets and hematopoietic stem cells, by macrophages[15-17], and this is supported by *in vivo* studies with target cells from CD47-deficient mice[18;19] as well as by employing macrophages from SIRPα-mutant mice[16;18]. Since CD47 is broadly expressed on the majority of normal host cells as well as transformed host cells we wanted to investigate whether CD47-SIRPα interactions also regulate the elimination of tumor cells by ADCC and/or ADCP by macrophages. In order to do so we initially employed the rat CC531 colon carcinoma model in which therapeutic antibody against CC531 cells has shown beneficial effects[33] and in which macrophages have previously been shown to play a prominent role[34]. We first studied the cellular expression and localization of SIRPα and CD47 in tumor tissue by immunohistochemistry. Sections of liver metastases of rat colon carcinoma CC531s cells were stained for SIRPα, CD47 and a variety of cellular markers. SIRPα expression was present on numerous cells both within as well as surrounding the tumor (Figure 1A), which were identified by double staining using appropriate markers as macrophages (ED1- and ED2-positive) and granulocytes (HIS48-positive) (Figure 1B). This is consistent with the generalized expression of SIRPα among cells of the myeloid lineage[4]. The CC531s tumor cells expressed CD47 *in vivo*, which was supported by flow cytometry (Figure 1C). As expected CD47 was not only detected on tumor cells, but also on the macrophages and granulocytes surrounding the tumor *in situ*. Taken together this demonstrates that, at least in the CC531 model, macrophages and granulocytes expressing SIRPα are closely associated with CD47-positive tumor cells *in vivo*.

CD47-SIRPα interactions inhibit tumor cell phagocytosis by macrophages

To investigate the role of CD47-SIRPα interactions in Ab-mediated phagocytosis and lysis of tumor cells, an *in vitro* ADCP assay was performed, using the rat colon carcinoma cell line CC531s as target, and the rat NR8383 macrophage cell line as effector cells. The tumor cells were labeled with the tumor specific antibody CC52, whereas the effects on CD47-SIRPα interactions were studied using the blocking antibodies against rat CD47 (OX101) and rat SIRPα (ED9). In the absence of any antibodies 8.5 ± 2.1% of the CC531s tumor cells were phagocytised by the NR8383 macrophages (Figure 2). With the CC52 antibody, that recognizes a surface antigen on the CC531s tumor cells, the phagocytosis was moderately enhanced to 11.6 ± 0.1%. Interference with the interaction between CD47 and SIRPα by the antagonistic CD47 and SIRPα antibodies in the presence of the CC52 antibody, dramatically enhanced phagocytosis of the CC531s tumor cells to 65.7 ± 2.4% (anti-CD47) or 61.5 ± 3.5% (anti-SIRPα). Also Fab-fragments of the SIRPα antibody enhanced ADCP of the tumor cells (24.8 ± 0.6%), thereby essentially excluding an involvement of Fc receptors on the NR8383 macrophages. The antagonistic antibodies had little or no effect on the

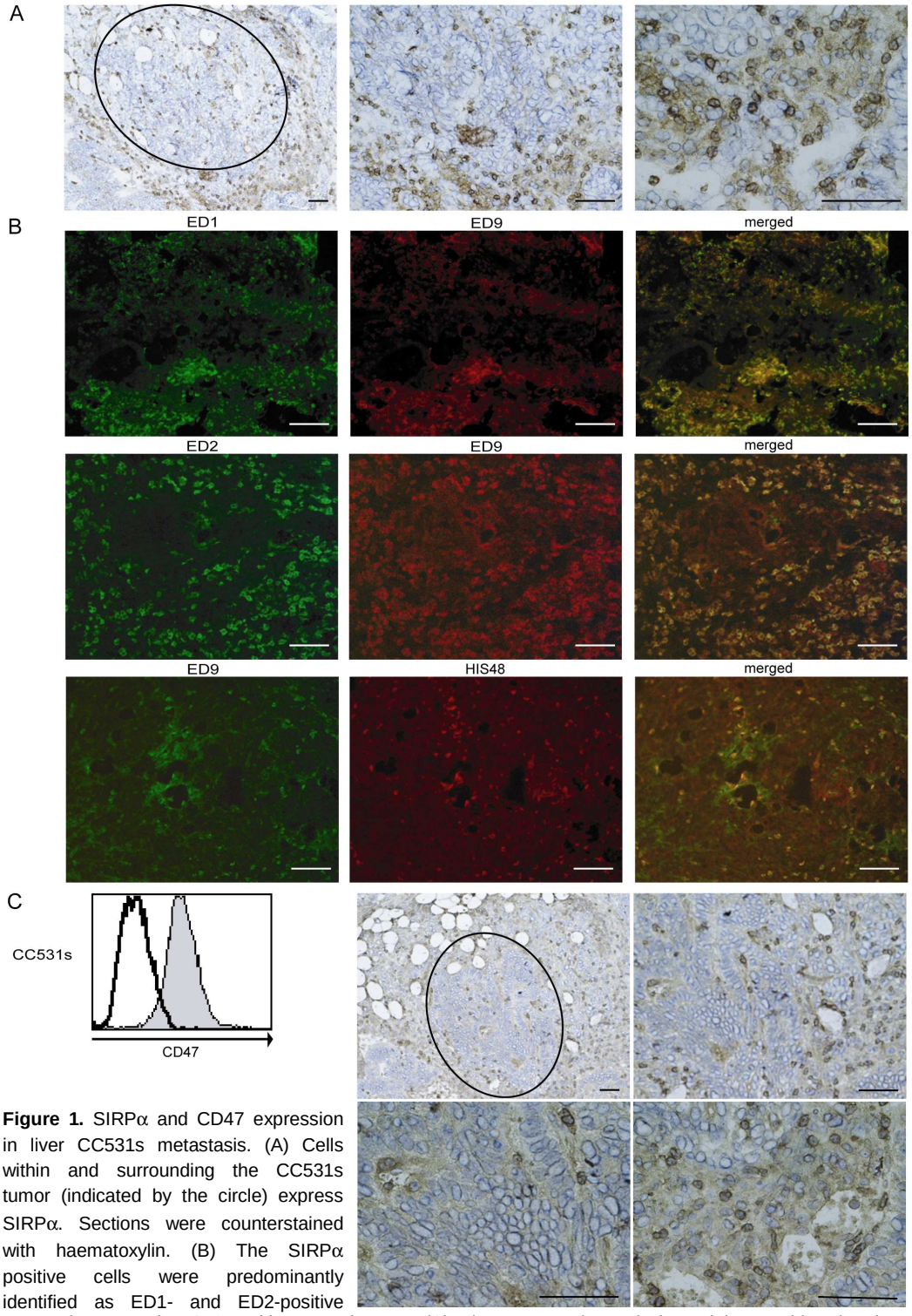

Figure 1. SIRPα and CD47 expression in liver CC531s metastasis. (A) Cells within and surrounding the CC531s tumor (indicated by the circle) express SIRPα. Sections were counterstained with haematoxylin. (B) The SIRPα positive cells were predominantly identified as ED1- and ED2-positive macrophages and HIS48-positive granulocytes. (C) Flow cytometric analysis and immunohistochemistry showed CD47 expression on the CC531s tumor cells. Stainings were counterstained with haematoxylin. Bar = 100 μm.

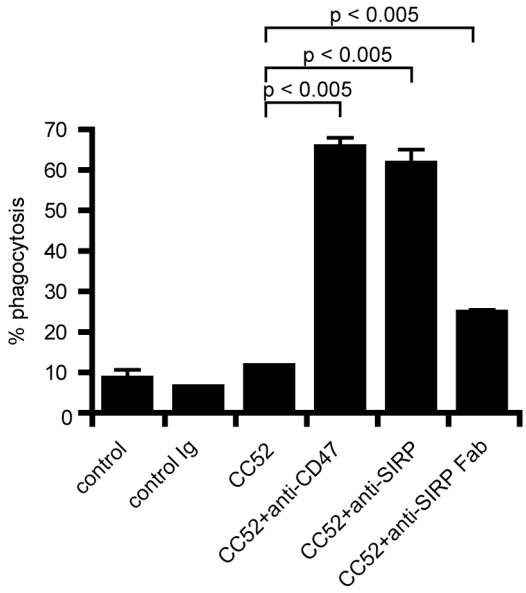

Figure 2. Interference of CD47-SIRPα interaction enhances Ab-mediated phagocytosis. Blocking mAbs against CD47 and SIRPα as well as anti-SIRPα Fab-fragments significantly enhanced the phagocytosis by NR8383 macrophages of the tumor CC531s cells induced by a tumor specific CC52 antibody. Data are expressed as the mean ± SEM and significant differences between groups were determined by the students t-test.

phagocytosis of tumor cells in absence of the CC52 antibody (data not shown). Taken together, these data strongly suggest that interactions between CD47 and SIRPα play a negative role in the antibody-dependent phagocytosis of tumor cells, and that blocking of CD47-SIRPα interactions by suitable antagonistic antibodies provides a method for enhancing Ab-dependent elimination of tumor cells.

CD47-SIRPα interactions inhibit tumor cell clearance in vivo

In order to investigate the role of CD47-SIRPα interactions in the destruction of tumor cells *in vivo*, we evaluated whether SIRPα-mutant mice, can potentiate Ab-mediated phagocytosis and thereby killing of tumor cells. The SIRPα-mutant mice, which were described previously[18], lack the cytoplasmic domain of SIRPα and thereby its capacity for intracellular signaling. The widely used B16F10 lung metastasis model was used, in conjunction with the mouse IgG2a TA99 monoclonal antibody against the gp75 melanoma tumor antigen[35;36], as tumor model for antibody immunotherapy. Flow cytometry analysis of the B16F10 cells showed that the vast majority (>90%) of cells have CD47 expression (Figure 3A). First, we tested whether there is an intrinsic difference in tumor formation between the SIRPα-mutant mice and wild type animals. Clearly, tumor outgrowth in the lung upon i.v. injection of B16F10 cells was unaffected by the defect in SIRPα signaling (tumor load wild type 34.7 ± 9.5 and SIRPα-mutant 35.9 ± 5.2) (Figure 3B) in the absence of therapeutic antibody. This demonstrates that, under these conditions, at least the net effects of metastasis, outgrowth and immune-mediated clearance are unaltered in the SIRPα-mutant mice. Next, wild type and SIRPα-mutant mice were challenged with tumor cells and treated with a suboptimal dose of TA99 antibody. As previously described[36], treatment with TA99 after tumor challenge reduced the formation of lung metastases *in vivo*. As can be seen in figure

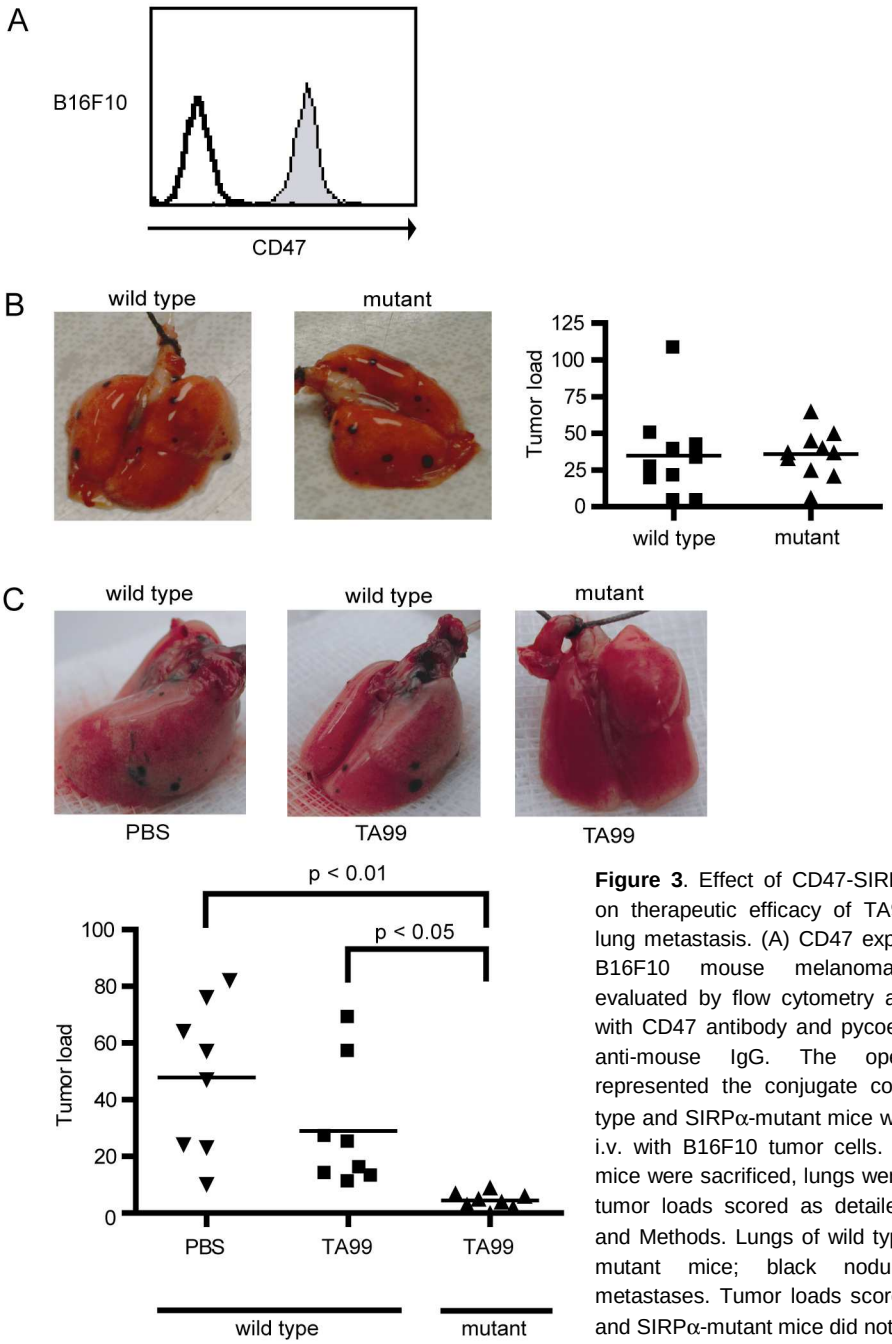

Figure 3. Effect of CD47-SIRPα interactions on therapeutic efficacy of TA99 antibody in lung metastasis. (A) CD47 expression on the B16F10 mouse melanoma cells was evaluated by flow cytometry after incubation with CD47 antibody and pycoerythrin labeled anti-mouse IgG. The open histogram represented the conjugate control. (B) Wild type and SIRPα-mutant mice were challenged i.v. with B16F10 tumor cells. After 21 days, mice were sacrificed, lungs were excised, and tumor loads scored as detailed in Materials and Methods. Lungs of wild type and SIRPα-mutant mice; black nodules represent metastases. Tumor loads scored in wild type and SIRPα-mutant mice did not differ between both mice strain. (C) Wild type and SIRPα-mutant mice were challenged i.v. with B16F10 tumor cells and treated with PBS or a suboptimal dose of mAb TA99 (10 μg). After 21 days, mice were sacrificed and tumor loads in the lungs were scored. In contrast to the TA99 treated wild type mice, almost no tumors were found in the TA99 treated SIRPα-mutant mice. Data are expressed as the mean ± SEM and significant differences between groups were determined by ANOVA. Each group represented 10 mice.

3C the tumor load in wild type mice treated with PBS was 47.9 ± 9.4 compared to 29.0 ± 7.8 in the wild type mice treated with TA99. Importantly, the tumor growth was

strongly reduced to 4.5 ± 1.0 ($p < 0.05$) in the SIRPα-mutant mice. In fact, some individual SIRPα-mutant mice did not show any metastases at all (Figure 3C). Taken together, these data provide evidence that signaling via SIRPα in macrophages or other relevant immune cells limits the Ab-mediated destruction of tumor cells.

Discussion

In the present study, we have investigated the involvement of the CD47-SIRPα interactions in the regulation of ADCP and ADCC of tumor cells. Our *in vitro* and *in vivo* results strongly suggest that the binding of CD47 present on the tumor cells to SIRPα on phagocytes generates an inhibitory signal that suppresses the killing of the tumor cells in the presence of therapeutic antibody directed against that tumor. This is in line with the well established idea that SIRPα acts as a so called 'inhibitory' receptor, which upon engagement with ligand (i.e. CD47) recruits and activates tyrosine phosphatases that negatively regulate cellular effector functions of phagocytes. Thus, similar to limiting ADCC of tumor cells CD47-SIRPα interactions have also been shown to restrict the phagocytosis of host cells, including erythrocytes, platelets and hematopoietic stem cells, by macrophages *in vitro* and *in vivo*[15-17;19;37;38]. Based on these results it has been proposed that CD47 acts as a signal of 'self' that limits the reactivity of immune cells against host cells, while allowing their actions against for instance microbes but perhaps also cells that lack CD47 expression[19;39]. This is analogous to the 'self' signals provided by MHCI molecules to NK cells via KIR or Ly49 receptors[3]. Clearly, this suggests that CD47 expressed on tumor cells may act as a protective mechanism against host immunity. This is in line with the results of a recent report by Kim et. al.[40] who showed that head-and-neck squamous cell carcinoma lines with high CD47 expression were better capable of escaping NK-cell mediated host immunity as compared to tumor cells expressing low levels of CD47. By employing blocking antibodies against CD47 and target cells overexpressing CD47 they also provided some direct evidence for a role of CD47 in NK-cell cytotoxicity *in vitro*. However, they did not clarify the role of SIRPα in their model further. Even if the NK92MI cell line that they employed expressed SIRPα and was subject to suppression of cytotoxicity via CD47-SIRPα interactions, the *in vivo* relevance of this clearly remains questionable since NK cells do not express detectable amounts of SIRPα *in vivo*. However, myeloid cells, including macrophages and granulocytes do and the current results do indeed support the idea that tumor cell clearance by these cells is limited to an important extend by CD47-SIRPα interactions.

Tumor cells have previously been shown to express other negative immune regulators to resist eradication by the immune system. For instance, there is evidence that tumor cells express complement control proteins that limit their complement-mediated killing in the presence of antibodies[41]. Based on our results that binding of CD47 on tumor cells by SIRPα suppresses killing, it seems feasible that during cancer progression the tumor cells which acquire higher CD47 expression levels have a selective advantage over the ones with relatively low CD47 levels. Indeed, the Cancer Genome Anatomy Project has shown that CD47 expression in, for example, renal and

breast cancers is considerably increased. It will be interesting to follow CD47 expression during the progression of such tumors.

By interference of molecular interaction between CD47 and SIRPα by antagonistic antibodies we have demonstrate that we are able to significantly enhance the destruction of tumor cells by immune cells in the presence of therapeutic antibody against the tumor. This indicates that antagonistic antibodies against CD47 and SIRPα could potentially be utilized to increase the therapeutic effect of the tumor specific antibodies and perhaps to overcome immune suppression in cancer patients. A limitation of the use of CD47 and SIRPα as a target to reverse immune evasion is the expression pattern of both proteins in healthy cells. CD47 is essential present on every cell, whereas SIRPα is expressed on myeloid and neuronal cells[4-6]. *In vivo* studies will have to prove whether treatment with antagonistic antibodies directed against CD47 and SIRPα have any side effects. At least, our initial experiments do not show such side effects of the blocking SIRPα antibody ED9 *in vivo*.

In conclusion, our data provide evidence for an intrinsic mechanism that relies on CD47-SIRPα interactions and that severely limit the effects of therapeutic antibodies against tumors. Importantly, interference with CD47-SIRPα interactions by antagonistic antibodies, in combination with therapeutic antibodies, is anticipated to enhance the effectiveness of antibody therapy and may therefore proof to function as general adjutants for antibody-mediated immunotherapy.

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